CHAPTER ONE

1.INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Plasmodium falciparum is an apicomplexan parasitewhich causes devastating disease and death,constituting one of the most important groups of human and animal pathogens (Baum *et al*., 2009).Malaria, an important Protozoan parasitic disease, is caused by *Plasmodium*parasites transmitted to man through the bite of infected female *Anopheles* mosquitoes during blood meal.*Plasmodium* species associated with human infections are *P. falciparum,P. malariae,P. vivax,P. ovale* and *P. knowlesi* (Obare *et al*.,2013).*P.falciparum* is the most virulent species of *Plasmodium* and is responsible for most of the adverse consequences of malaria(Obare *et al*., 2013).

Malaria is still a leading global health concern with 3.4 billion people at risk, 214 million clinical cases and 438,000 deaths annually worldwide with sub-Saharan African countries having the highest burden (WHO, 2015a). Louise& McKenzie(2009) emphasized the significance of seasonal variations in the transmission of *P. falciparum* malaria in sub-*Saharan* Africa, including Nigeria, where the disease is highly endemic,Children under 5 years of age bear the brunt of the disease culminating in high mortality rate from cerebral malaria and anaemia.In Nigeria, malaria is most prevalent in the South West (NMIS, 2010) .Widespread drug resistance against commonly used anti-malaria drugs such as chloroquine and pyrimethamine-sulfadoxine (Fansidar) has been reported all over the world(WHO, 2001a).In Nigeria, Chloroquine and Sulfadoxine–Pyrimethamine were banned as first line drugs in malaria treatment in January 2005.

Currently, Artemether-Lumefantrine and Artesunate-Amodiaquine which are Artemisinin–based Combination therapies (ACTs) are now the approved first line drugs for the treatment of Malaria in Nigeria. However,in Thailand,Cambodia and South East Asia, resistance to ACTs have been reported (WHO,2001b).Similarly, control efforts through the use of insecticides and insecticide-

treated bed nets are becoming ineffective as themosquitoes that transmit the parasite are becoming resistant to the pyrethroid insecticides- the only class ofbed net insecticide approved for malaria control, in West and East Africa (Elissa *et al*., 1993; Awolola *et al*., 2003).The emergence of pyrethroid and DDT resistance in *Anopheles gambiae* s.s, the major Afro-tropical malaria vector would have considerable implications for the success of vector intervention and the monitoring of ongoing control programs.The gains of malaria control programmes of reduction inmorbidity,mortality and Disability Adjusted Life Years (DALY) could be attributed to the increased efforts to implement the classical methods of transmission reduction.

Due to limited number of drugs available for treatment of*Plasmodium falciparum* malaria as a result ofthe spread of multi-drug-resistance strains of the parasites, there is an urgent need for a malaria vaccine. Evidence exists from both animal models and human studies that antibodies to erythrocytic and exoerythrocytic stages antigens can induce protection against malaria. A combination of multiple antigens from the same or different parasite life cycle stages has been advocated as the ultimate vaccine (Reddy *et al*.,2014). Apical Membrane Antigen-1 (AMA1) and Reticulocyte-binding Protein Homolog 5 (RH5) are essential blood-stage vaccine candidate proteins while AMA1has also been recognized as sporozoite stage protein for invasion of liver cells by sporozoites.

AMA1protein is polymorphic with undesirable effects on vaccine development as a result of strain-specificity. Effective vaccine targets however, should show limited sequence diversity because antigenic variation presents a major hurdle in successful vaccine design. Several malaria antigens including merozoite surface Protein 1 and 2,display high levels of polymorphisms, whereas functional constraints limit the degree of variation in others, such as apical membrane antigen-1(AMA1) and circumsporozoite protein(CSP) (Farooq*et al*.,2009).One main limitation to malaria vaccine development is the antigenic diversity related to *P.falciparum*polymorphysims. Moreover,

the phenomenon of clonal fluctuation consisting of kinetic changes in the relative load of each parasite sub-population in the blood is important to consider when studying *P.falciparum* diversity (Aubouy *et al*.,2003).

In malaria endemic areas, infections due to multiple parasite clones are frequent and clone fluctuation may be a logical strategy to slow down the appearance of strain-specific response.Compared to several other blood stage antigens(MSP-,MSP-2,etc),AMA1 of *P.falciparum* shows limited inter-strain polymorphisms(Dutta*et al*.,2007);and it is expressed in two critical life-cycle forms,the sporozoite,which invades hepatocytes,and the merozoite,which invades red blood cells,and so offers a unique opportunity as a non-stage–specific vaccine target (Bai*et al.*,2005). Although AMA1 is less polymorphic than other sporozoite or merozoite proteins, some degree of variation is present across the entire AMA1 sequence but the rate of non-synonymous (dN) mutations at domain I (aa 138–308) has always been higher indicating that this domain is under strong diversifying selection (Garg*et al*.,2007). Knowledge of the distribution of the polymorphic sites on the surface of AMA1 is necessary to obtain a detailed understanding of their significance for vaccine development (Bai*et al*.,2005).

Erythrocyte invasion by merozoites involves multiple receptor-ligand interactions, a number of merozoite proteins have proposed or established roles in invasion and may be targets of inhibitory antibodies.Initial attachment is thought to involve antigens on the surface of merozoites, such as Merozoite Surface Protein -1(MSP1) and other GPI-anchored surface proteins and is followed by apical reorientation of the merozoite involving Apical Membrane Antigen-1(AMA1).

Indeed,AMA1is highly immunogenic, eliciting immune responses protective against *Plasmodium chabaudi* infection in mice (Crewther *et al*.,1996) and displaying significant antibody and T-cell

responses in endemic human populations(Dutta*et al*.,2002;Wickramarachchi *et al*., 2006).AMA1 protein is conserved in Plasmodium species and in the phylum of apicomplexan parasites and therefore offers the potential for the development of vaccine or therapeutics to a wide range of human and parasitic diseases (Healer *et al*., 2004). Further studies have also shown that AMA1 is immunogenic in malaria exposed individuals, and naturally acquired antibodies to AMA1 can inhibit merozoite invasion *in vitro*, but antibodies to strain 3D7 AMA1 were non–inhibitory for strain W2mef and this was shown to be due to AMA1 sequence differences in these two strains (Healer *et al*., 2004). Results from a study with 51 samples from Ibadan showed that Domain I and III of *Plasmodium falciparum*AMA1 nucleotide sequences are strongly polymorphic with high intra-specific non-synonymous polymorphisms (Polleyand Conway2001).Although antibodies are likely to be critical in immunity induced by AMA1, it is also clear that effector T-cells can mediate or contribute to immunity against malaria independent of B cells (Brake *et al*.,1998).

The family of *Plasmodium falciparum*reticulocyte-binding homolog proteins (*PfRh*) has attracted attention as a key determinant of erythrocyte invasion. PfRH comprises of 5 members: PfRH1, PfRH2a, PfRH2b, PfRH4 and PfRH5 of which PfRH5 plays the major role in eryhthrocyte invasion. PfRH5 has been mapped to chromosome 4 of *P. falciparum* and identified as an erythrocyte binding ligand. The specificity of PfRH5 binding has been shown to be affected even by a single nucleotide polymorphism (SNP)(Arevalo-Pinzon*et al.,* 2012). PfRH5 is essential as it cannot be genetically knocked out suggesting a crucial role in erythrocyte invasion(Patel *et al*., 2013). PfRH5 binds Basigin (CD147 on erythrocyte surface) and its expression is consistent among parasite clones.*Pf*RH5 is highly susceptible to cross-strain neutralizing vaccine-induced antibodies, out-performing all other antigens delivered by the same vaccine platform. These data challenge the widespread belief that any merozoite antigen that is highly susceptible to immune attack would be subject to significant levels of antigenic polymorphism, and that erythrocyte invasion by *P. falciparum* is a degenerate process involving a series of parallel redundant

pathways (Douglas*et al.,* 2011). Polymorphisms in *Pf*RH5 induce changes in receptor specificities and native structure (Arevalo-Pinzon *et al*., 2012). Currently, there is paucity of data from Nigeria on these antigens. Assessment of the complexity of genetic diversity and natural dynamics of polymorphisms in the genes coding these candidate antigenic proteins is essential for effective malaria vaccine development for Nigeria and in the diversity covering antigen (DiCOs) based vaccine (Remarque *et al.,* 2008) . Inaddition, understanding the genetic and biochemical basis of endemicity of *P.falciparum*infections, the morbidity and mortality will aid the deployment of appropriate malaria control strategies.

1.2 STATEMENT OF THE PROBLEM

The non-development of sterile immunity with the use of anti-malarial drugs (preventive and curative) and the decreasing efficacy of these drugs due to spread of drug resistance strains of the malaria parasite, demand a more reliable intervention. Malaria vaccines are the reliable alternative to elicit sterile immunity in malaria but are plagued with genetic sequence diversity resulting in polymorphisms in the vaccine targets. This therefore calls for determination of population-specific distribution of these polymorphisms and natural dynamics of polymorphisms in parasite antigenic proteins for effective vaccine design.

*Pf*AMA1 and *Pf*RH5 are less polymorphic vaccine candidate proteins required for erythrocyte invasion by *P. falciparum.* There is paucity of data about the extent of polymorphisms on these antigens in endemic African parasite populations. T-cell responses have been shown to be important in mediating immunity in malaria. Such immune responses might be associated with genetic variations observed in these vaccine candidate antigens. Many malaria vaccines have failed field trials including AMA1-based single antigen vaccine due to genetic variations resulting in strain specificity of vaccines, hence, the need for population-specific data on malaria vaccine candidate antigens for a multi-antigen vaccine.

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1.3AIM OF STUDY

The overall aim of the study was to determine polymorphisms in the *P. falciparum* reticulocyte-binding protein homolog-5 (PfRH5) and Domain I of *P. falciparum* apical membrane antigen-1 (*Pf*AMA1) genes in isolates from Lagos, Nigeria and their association with selected T-cell responses.

1.4 SPECIFIC OBJECTIVES OF STUDY

The specific objectives were to:

1. assess parasitaemia, body mass index (BMI) and haematological parameters of patients with malaria inselected centres in Lagos, Nigeria.
2. determine Multiplicity of Infection (MOI), serum levels of IL-12, IL-1β, TNF–αand their relationship in *P. falciparum* malaria.
3. determine Single Nucleotide Polymorphisms (SNPs), genetic structure and haplotypes of *Pf*RH5 and *Pf*AMA1 from selected centres in Lagos, Nigeria.
4. assess the effect of polymorphisms in *Pf*RH5 and *Pf*AMA1 on TNF-alpha, Interleukin-12, Interleukin-1β and multiplicity of infection (MOI).

1.5SIGNIFICANCE OF THE STUDY

This investigation will identify the SNPs and the circulating variants of two (2) leading *P. falciparum*blood stage vaccine candidate antigens (*AMA1* and *RH5*) in Lagos, Nigeria. This will contribute to solving the problem of strain specificities which causes loss of vaccine efficacy. The study will provide information on the association of cytokine responses in malaria with polymorphisms in the antigenic proteins.The study will also give insightinto the influence of haplotypic variations on multiplicity of *Plasmodium falciparum* infection. It will supply data from Nigeria useful for the design and development of effective malaria vaccine.

1.6DEFINITION OF TERMS

Alleles: Variant forms of the same gene sequence

Antibody: A protein produced by the body's immune system to neutralize harmful substances, called antigens

Antigenicity: Is the ability of a protein molecule to combine specifically with the secreted antibodies and/or surface receptors on T-cells.

Apical membrane antige I:A non-stage-specific *Plasmodium falciparum*antigenic micronemal transmembrane protein involved in erythrocyte invasion by the parasites.

Centres: Specific healthcare facilities offering healthcare services to specific populations where participants were recruited and included into the study.

Chemoprophylaxis: Prevention of diseases or infection by the use of a chemotherapeutic agent.

Clone: Genetically identical group of organisms

Cytokines: Small proteins which are secreted by some cells, acting as intercellular signalling molecules, modulating interactions and communications between cells.

Drug resistance: Parasite induced absence or reduction of therapeutic effect of a drug

Gene: A distinct sequence of nucleotides which codes for (and directs monomer order of) specific polypeptide or nucleic acid synthesized by a cell.

Genetic Variation: Difference in a gene sequence within an individual or organisms caused by mutation.

Genotypes: The genetic constitution of an organism which determines specific characteristics (phenotypes)

Haplotype: A contraction of the phrase “haploid genotype”, is a set of closely linked genetic markers present on one chromosome which tend to be inherited together (not easily separable by recombination)

Immunogenicity: Is the ability of a protein to induce humoral and/or cell-mediated immune response.

Mono-infections: Infections involving only one species of parasite.

Mutation: A change in the sequence of base pairs in the chromosomal molecule

Nested polymerase chain reaction: A modification of polymerase chain reactionintended to reduce non-specific binding using additional set of primers and previous PCR productsas templates.

Prevalence: The number of cases of a disease existing in a given population at a given time

Proteins: Large macromolecules made up of amino acids and play specific roles in an organism

Polymorphism: Genetic variation within a population, upon which natural selection can operate.

Polymerase chain reaction: Molecular process used to amplifyDNAsamples, via a temperature-mediatedDNA polymerase.

Reticulocyte-binding protein homolog 5: A rhoptry localised *Plasmodium falciparum*vaccine candidate antigenic protein involved in erythrocyte invasion by the parasite.

1.7 LIST OF ABBREVIATIONS AND ACRONYMS

ACT: Artemisinin-based Combination Therapy

AMA1: Apical Membrane Antigen 1

BMI: Body mass Index

C.I: Confidence Interval

DHFR: dihydrofolate reductase

DHPS: dihydropteroate synthase

DiCOs: diversity covering Antigens

FMOH: Federal Ministry of Health

HIV: Human Immunodeficiency Virus

IE: Infected erythrocyte

IgG: Immunoglobulin G

IFN-γ: Interferon gamma

IL-1β: Interleukin 1 beta

IL-12: Interleukin 12

MAbs: Monoclonal antibodies

NCBI: National Center for Biotechnology Information

PCR: Polymerase Chain Reaction

PCV: Packed Cell Volume

RH5: Reticulocyte-binding protein homolog 5

RON2: Ropthry Neck Protein II

SNPs: Single nucleotide polymorphisms

SP: Sulphadoxine-pyrimethamine

TNF-α: tumour Necrosis factor alpha

WHO: World Health Organization

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 MALARIA: TRANSMISSION AND BURDEN

Malaria is a life-threatening disease caused by *Plasmodium*parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. Malaria remains an important public health concern in countries where transmission occurs regularly, as well as in areas where transmission has been largely controlled or eliminated. In particular, young children, pregnant women, and non-immune visitors to malarious areas are at greatest risk of severe or fatal illness. Ninety percent of the global burden of malaria occurs in sub-SaharanAfrica, and with considerable efforts in the campaign against malaria, mortality rates have fallen by 47% globally since 2000, and by 54% in the WHO African Region (WHO, 2014a).Malaria is the most significant public health problem in Nigeria. Nigeria contributes about 32% of the global burden of malaria (WHO, 2015a) with a prevalence that varies by geopolitical zones and highest in the South- West (50%) followed by North –Central (49%) , North- West (48%), lowest in South-East (28%) and South-South (32%) (NMIS, 2010).The economic cost of malaria, arising from cost of treatment, loss of productivity and earning due to days lost from illness, may be as high as 1.3% of economic growth per annum (WHO,2014a).

Malaria is caused by *Plasmodium* parasites. The parasites are spread to people through the bites of infected *Anopheles* mosquitoes, called "malaria vectors", which bite mainly between dusk and dawn.There are four parasite species that cause malaria in humans:*Plasmodium falciparum,Plasmodium vivax,Plasmodium malariae,Plasmodium ovale. Plasmodium falciparum* and *Plasmodium vivax* are the most common while *Plasmodium falciparum* is the most deadly (. (In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi* – a

species that causes malaria among monkeys and occurs in certain forested areas of South-East Asia (WHO, 2014b).

2.1.1 MALARIA IN CHILDREN AND PREGNACY

Malaria is transmitted exclusively through the bites of infected *Anopheles* mosquitoes. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment. In Nigeria, malaria transmission is all year round. The main vectors belong to the *Anophelesgambiae* complex (*An. Gambiae ss* and *An. Arabiensis), An. funestus* is commonly encountered (FMOH,2005b). Specific populations at risk of malaria include young children (usually < 5 years) in stable transmission areas (Figure 1) who have not yet developed protective immunity against the most severe forms of the disease; non-immune pregnant women as malaria causes high rates of miscarriage and can lead to maternal death;semi-immune pregnant women in areas of high transmission (Malaria can result in miscarriage and low birth weight, especially during first and second pregnancies); semi-immune HIV-infected pregnant women in stable transmission areas during all pregnancies; people with HIV/AIDS and international travellers from non-endemic areas, because they lack immunity.

More than 90% of malaria cases occur in sub -Saharan Africa of which more than 90% are composed of children aged 6 months to 5 years (Rezai *et al*., 2013; WHO, 2014b).The epidemiology and management of malaria in school-age children have, until recently, received little attention (Brooker, 2009).

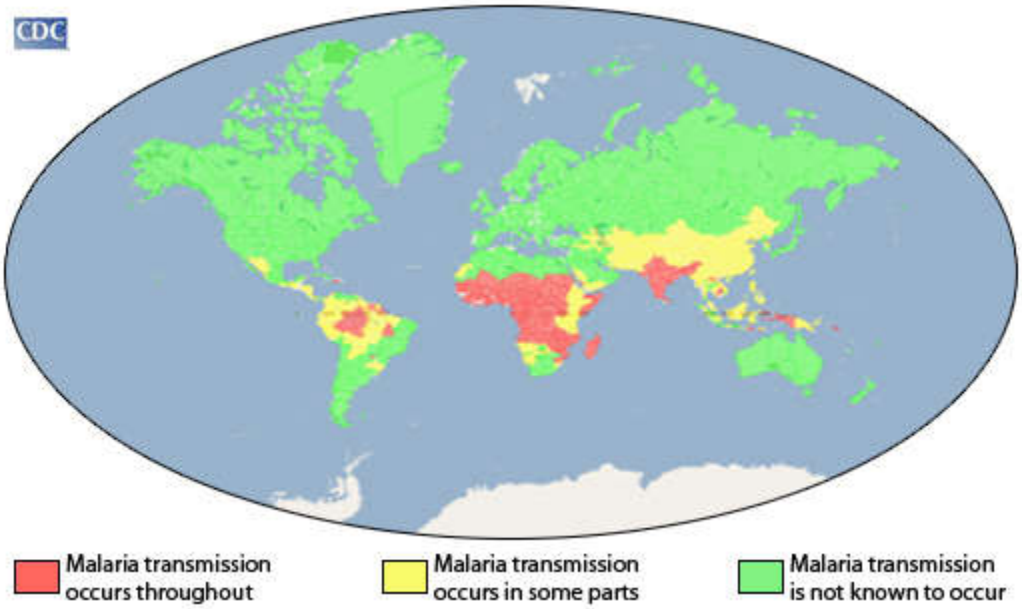

Figure 1: Geographical Distribution and Economic Importance of Malaria

Table 1:Geographical Prevalence of *Plasmodium* Parasites.

Region	*P. falciparum*	*P. malariae*	*P. ovale*	*P. vivax*
Central Africa	Predominant	Rare	Rare	Rare
East Africa	Predominant	Rare	Rare	Common
North Africa	Very rare	Very rare	Absent	Predominant
West Africa	Predominant	Rare	Common	Very rare
Central America	Common	Rare	Absent	Predominant
South America	Common	Common	Absent	Predominant
Central Asia	Common	Common	Absent	Predominant
South-west Asia	Common	Common	Absent	Predominant
South-east Europe	Very rare	Very rare	Absent	Predominant
Indian subcontinent	Common	Rare	Very rare	Predominant
Indochina	Predominant	Rare	Rare	Common
Indonesia	Predominant	Very rare	Very rare	Common
Madagascar	Predominant	Rare	Rare	Common
Indian Ocean	Predominant	Rare	Rare	Common
Pacific Islands	Predominant	Very rare	Rare	Common

Malaria transmission occurs in all six WHO regions. Globally, an estimated 3.3 billion people in 97 countries and territories are at risk of being infected with malaria and developing the disease, and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year) (WHO, 2014c). Nigeria is known for high prevalence of malaria and it is a leading cause of morbidity and mortality in the country (WHO, 2014c; Ademowo *et al*., 2006).

2.2 PARASITE LIFE CYCLE,BIOLOGY AND GENOMICS

2.2.1 Parasite Life Cycle

The malaria parasite has a complex, multistage life cycle involving two hosts namely an Arthropod (female Anopheline mosquito) and vertebrate (human) hosts (Figure 2) and can multiply in multiple cell types while avoiding clearance by the host immune system through expression of different surface proteins (Gazzinelli *et al*.,2014).

2.2.2 Parasite Biology

Sporozoites from the saliva of a biting female mosquito are transmitted to either the blood or the lymphatic system of the recipient while 10% of the parasites inoculated by the mosquitoes may remain in the skin where they develop into infective merozoites.

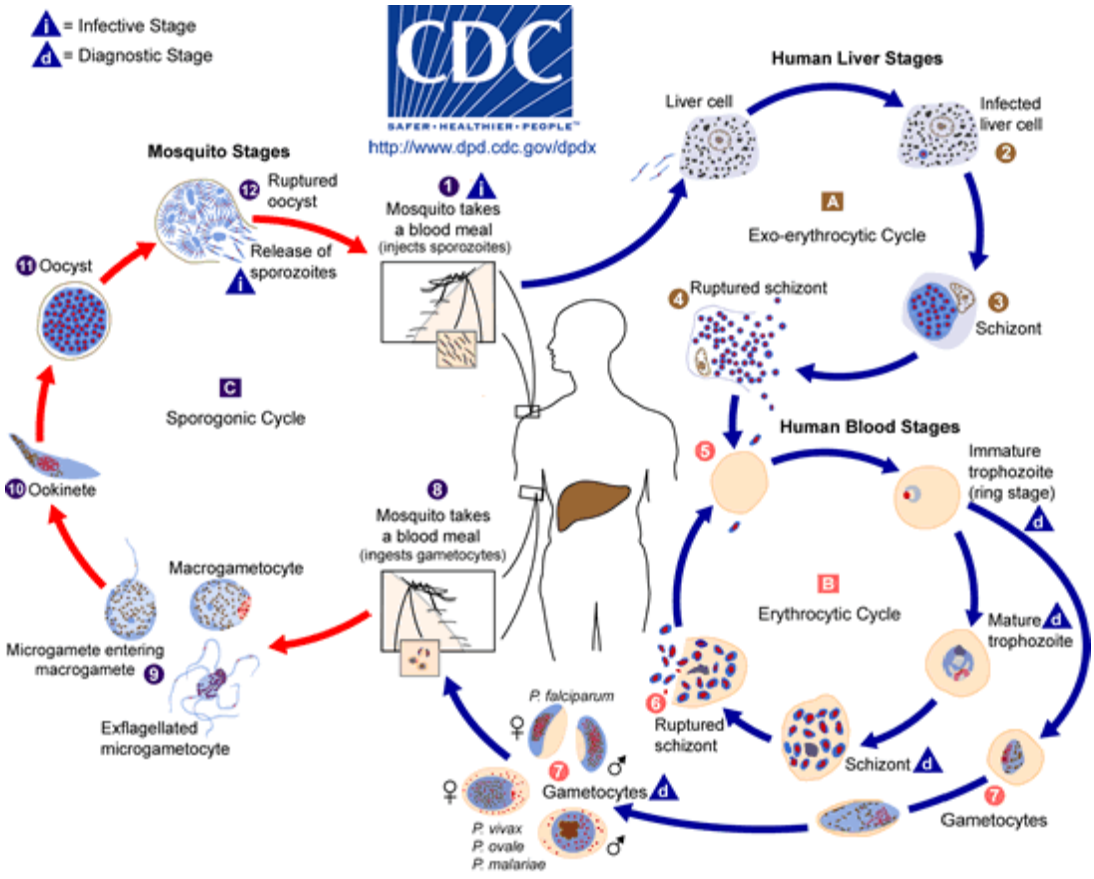

Figure 2: *Plasmodium falciparum Parasite* Life Cycle (CDC 2012)

The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷and mature into schizonts ❸, which rupture and release merozoites ❹. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease.The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites ❶into a new human host perpetuates the malaria life cycle.

2.2.2.1. The Hepatic Stage

Majority of sporozoites circulating in the peripheral blood migrate to the liver, invade hepatocytes and for reasons currently unclear, sporozoites typically penetrate several hepatocytes before choosing one to reside within (Igweh, 2012). At this point, the parasite loses its apical complex and surface coat, and transforms into a trophozoite in the parasitophorous vacuole of the hepatocyte where *P. falciparum* undergoes schizogonic development that culminates in the production of merozoites released into the blood stream for erythrocytic life cycle stage (Vaughan *et al.,* 2012). The released merozoites do not re-infect hepatocytes.

2.2.2.2 Erythrocytic Stage

After their release from the hepatocytes, the merozoites enter the bloodstream prior to infecting red blood cells. In the blood,the merozoites are roughly 1.5μm in length and 1μm in diameter, and use the apicomplexan invasion organelles (apical complex, pellicle and surface coat) to recognize and enter the host erythrocyte.After erythrocyte invasion, the parasite loses its specific invasion organelles (the apical complex and surface coat) and de-differentiates into a round trophozoite located within a parasitophorous vacuole in the RBC´s cytoplasm (Figure 2). The young trophozoite (or "ring" stage) grows substantially after which it starts to replicate its DNA multiple times without cellular segmentation, which occurs prior to undergoing schizogonic division (Ferreira,2010).

Trophozoite enlargement is accompanied by an active metabolism including the ingestion of host cytoplasm and the proteolysis of haemoglobin into amino acids. The end of the trophic period is manifested by multiple rounds of nuclear division without cytokinesis resulting in a schizont. Schizonts then undergo cellular segmentation and differentiation to form roughly 16-18

merozoite cells in the erythrocyte.The erythrocytic cycle results in the formation of 4 to 36 new parasites in each infected cell within a 44 to 72 hour period. At the end of the cycle, the infected red blood cells burst, releasing the merozoites. At this stage, merozoites can either infect new red blood cells to begin the erythrocytic cycle again, or, through the action of some unknown factor, the merozoites can develop into gametocytes. It is of note that blood stage parasites are responsible for the clinical symptoms of malaria. For example, lysis of the red blood cells is an important cause of malaria-associated anaemia. In addition, if a significant number of infected cells rupture simultaneously, the resulting material in the bloodstream is thought to induce a malarial paroxysm.

2.2.2.3 The sexual Stage

2.2.2.3aGametocytogenesis

A sub-population of intracellular parasites that forego mitosis undergoes gametocytogenesis(the production of gametocytes) in preparation for the sexual phase (Guttery *et al*., 2012). The master regulator(s) of this process are completely unknown but gametocytogenesis involves environmental stress responses and depends on parasite density and genetic variation, and is mediated by a number of signalling pathways (Baker, *2010).* At the molecular level, little is known of the mechanisms by which an individual parasite is triggered to differentiate into a gametocyte.Gender-specific markers have been observed in sibling progeny from single schizonts that have committed to gametocytogenesis, including*pfg377*, expressed only in females and*α-tubulin II,*expressed only in males(Baker, *2010)*.

2.2.2.3bMosquito Stage

These gametocytes take roughly 8-10 days to reach full maturity and remain within the erythrocytes until taken up by the mosquito host. The gametocytes of *P. falciparum* are taken up by the female *Anopheles* mosquito as it takes its blood meal from an infected human. Ingestion of the blood meal by the mosquito activates the gametocytes to produce gametes in the midgut (Billker *et al*., 2004). Activation of both male and female gametocytes is due to a drop in temperature, a rise in pH and calcium concentration, and the presence of the mosquito-derived metabolic intermediate xanthurenic acid. For the male gametocyte, activation results in three rounds of rapid DNA replication (within 15 min) and the assembly of flagella, leading to the formation of eight flagellated and highly motile microgametes on the surface of the male gametocyte, a process called exflagellation (Sinden *et al*., 2010).

As with the male, the female gametocyte is activated by exposure to mosquito factors. DNA replication does not occur but the gamete exits the erythrocyte, allowing the male gamete to attach and fuse. Nuclear fusion in the zygote is followed by DNA replication and meiosis, with the zygote developing into a motile ookinete that penetrates the gut wall (Guttery *et al*., 2012).

Once through the epithelium, the ookinete enters the basil lamina, and forms an oocyst. During the ookinete stage, genetic recombination can occur. This takes place if the ookinete was formed from male and femalegametes derived from different populations. This can occur if the human host contained multiple populations of the parasite, or if the mosquito fed on multiple infected individuals withina short time-frame (Bruce, 1985).

2.2.3.*Plasmodium falciparum* Parasite Genomics

The typical nuclear (haploid) genome of *Plasmodium* parasite consists of 14 chromosomes, has a size of 23-26 million base pairs (bp), essentially complete and encodes 5,500 protein-coding

genes which are about 2,200 bp long and contain only few or no introns (on average 1.5 introns per gene) and approximately 80 gaps (Gardner *et al*.,2002). One of the most noteworthy features of some *Plasmodium* genomes is their extremely biased nucleotide composition. *P. falciparum* has the lowest G+C content (19.4%) of all organism examined so far .*Plasmodium falciparum*chromosomes vary considerably in length,with most of the variation occurring in the subtelomeric regionsdue to recombination events between different parasite clonesduring meiosis in the mosquito(Gardner*et al*.,2002). Chromosome size variation isalso observed in cultures of erythrocytic parasites, but is due tochromosome breakage and healing events and not to meioticrecombination. Subtelomeric deletions often extend well intothe chromosome, and in some cases alter the cell adhesion propertiesof the parasite owing to the loss of the gene(s) encodingadhesion molecules. Because many genes involved in antigenicvariation are located in the subtelomeric regions, an understandingof subtelomere structure and functional properties is essential forthe elucidation of the mechanisms underlying the generation ofantigenic diversity.

Because many genes involved in antigenic variation are located in the subtelomeric regions, an understanding of subtelomere structure and functional properties is essential for the elucidation of the mechanisms underlying the generation of antigenic diversity(Gardner *et al*.,2002).Sub-telomeric regions of Plasmodium genomes are evolutionary melting pots of genomic diversity. Due to their genetic plasticity, subtelomeric regions harbor mostly large and species-specific gene families, some of which, like the *P. falciparum*gene family var, are important determinants of human virulence. Moreover, many subtelomeric gene families are expressed at the surface of the parasite or infected erythrocytes, where they are suspected to modulate important host-parasite interactions, including the parasite's ability to evade the host immune system.(Cunningham *et al*., 2010). The *P. falciparum* genome project has revealed the presence of several multicopy gene families which code for hypervariable antigens that are exported to the surface of the infected host erythrocyte and represent targets of naturally acquired immunity to

malaria. To date *var*, *rif* as well as *stevor* have been described as the major variant surface antigens (VSAs), multihgene families predominantly situated at the sub-telomeric ends of chromosomes where gene rearrangements are frequent leading to high rates of recombination, thus facilitating their rapid evolution and diversity ,even of gene families(Blythe *et al.,*2009).A gene family can be defined as a group of homologous genes; that is, a group of genes descending from a common ancestral gene(Dayhoff*et al.,* 1976). Gene families are the product of gene duplication, which occurs when gene-containing chromosomal segments are amplified through differentmolecular mechanisms, including segmental duplications, unequal crossing over, whole-genome duplications, or transposon-mediated amplification. Another intriguing peculiarity of Plasmodium genomes is that they seem to have lost transposons, which is probably because for large parts of its life cycle the parasite resides in a fairly protected environment inside other eukaryotic cells that shield them from viral infections. This might also explain why *Plasmodium* parasites have lost their RNA interference pathway (Baum *et al.,* 2009).

In addition to a nuclear genome, *Plasmodium* parasites harbor two organelle genomes. One is a tandemly repeated linear mitochondrial genome that is dramatically reduced in size (6 kb), making it one of smallest mitochondrial genomes known. It encodes for just three proteins—cytochrome C oxidase subunits I and III (Cox1 and Cox3) and cytochrome b (Cytb)—and for no tRNAs, thus all tRNAs for protein synthesis must be imported from the cytosol. Another noteworthy feature of the mitochondrial genome is that rRNA genes are fragmented and thus need to be assembled in trans. The genome structure of mitochondrial DNA is highly conserved in all examined Plasmodium species (Feagin *et al.,* 1997).The second organelle genome is the genome of the apicoplast. Like other Apicomplexan parasites, malaria parasites harbor a relict plastid, homologous to the chloroplasts of plants and algae. The apicoplast was once acquired by secondary endosymbiosis of a red algae and is a tell-tale hold-over from a more benign past in

the ocean as photosynthetic organism. The apicoplast genome is circular, 35 kb in size, and encodes for 30 proteins involved in essential pathways for fatty acids, isoprenoids, iron sulfur cluster assembly, and a segmentof the heme pathway (Gardener *et al*.,2002).

2.2.3.1 Homology, Orthology and Paralogy

Homology is an old concept expressing the relationship between parts of organisms. In 1843,Richard Owen introduced the term homology to refer to "the same organ in different animals under a variety of form and function". A character found in two species is homologous if it shares common decent, i.e. if this character is derived from a common ancestral species in which it might or might not have served a similar function or purpose. Mammalian hair would be an example of a homologous character, because all hairs in extant mammals derivefrom an ancestral hairy mammal. In contrast, an analogous character is one that serves a

similar purpose in two species but is not related by common decent, for example wings in birds and bats, which evolved independently.

In 1970, Walter M. Fitch applied the concept of homology to genes and proposed,to further distinguish between two kinds ofhomology: orthologous genes and paralogous genes. To use his own words, "where the homology is the result of gene duplication so that both copies have descended side by side during the history of an organism(forexample, in haemoglobin), the genes should be called paralogous (para = in parallel). Where the homology is the result of speciation so that the history ofthe gene reflects the history of the species (forexample haemoglobin in man andmouse) the genes should be called orthologous (ortho = exact)". In essence, orthologous genes are instances of 'the same gene'in different species that arose by speciation, whereas paralogous genes are genesin the same or different species that arose by gene duplication.Nowadays, orthologs and paralogs are often further differentiated into in-paralogs, out-paralogs, and co-orthologs. Out-paralogs are paralogous genes arising by duplication before

a given speciation event, whereas in-paralogs are paralogous genes arising by duplication after a given speciation event. Co-orthologs are groups of in-paralogous genes that are collectively, but not individually, orthologous to genes of another species(Kristensen*et al.,*2011). Thus, orthology is not necessarily a one-to-one affair, but can relate multiple genes per species, resulting in oftenuncertain functional correspondences between them. Homology, paralogy, and orthology are historical properties of genes and proteins that cannot be directly observed. Thus, one needs to infer these relationships from present data, which computationally is typically done by looking for sequence and structural similaritiesbetween genes and/or proteins.

2.3Malaria Diagnosis

Rational antimalarial drug usage is important in order to curtail resistance development and save costs for alternative therapies that are often more expensive. Accurate and reliable diagnosis is the key to rational treatment as it will be possible to distinguish malaria from other febrile illnesses such as viral (e.g. dengue fever and influenza), bacterial (e.g. typhoid, brucellosis, respiratory and urinary tract infections) and other acute septic syndromes. Often, in malaria endemic regions, co-morbidity may occur where malaria parasitaemia is observed in patients with febrile illness due to bacterial or viral infections.

The gold standard for thedefinitive diagnosis of malaria in nearly all settings is the directLight microscopic examination of intracellular parasites on stained blood films. However, several other approaches exist or are in development.

2.3.1Clinical/Presumptive Diagnosis and Treatment

Clinical diagnosis is the least expensive and the most commonly used method of facilitating treatment and involves examination based only on clinical algorithms (signs and symptoms). However, sole reliance on clinical diagnosis is problematic as misdiagnoses are frequent(Reyburn *et al.,* 2004) . Presumptive treatment is widely practiced in many malaria endemic regions as it offers the advantages of ease, speed, and low cost. However,due to overlapping symptoms between malaria with other non-malarial fevers, it often results in over - diagnosis and incorrect medication. In areas where malaria is prevalent, clinical diagnosis usually results in all patients with fever and no apparent other cause being treated for malaria. The Integrated Management of Childhood Illnesses (IMCI) programme defined an algorithm that has been developed in order to improve diagnosis and treatment of the mostcommon childhood illnesses in areas relying upon relatively unskilled health care workers working without access to

laboratories or special equipment. With this algorithm, every febrile child living in a “high-risk” area for malaria should be considered to have, and be treated for, malaria. “High risk” has been defined in IMCI Adaptation Guides as being any situation where as little as 5% of febrile children between the ages of 2 and 59 months are parasitaemic(WHO, 1997), a definition that will likely lead to significant over-diagnosis of malaria in areas with low to moderate malaria transmission.Furthermore, the recent WHO guidelines on malaria treatment reinforce the importance of parasitological confirmation of any clinical suspicion of malaria in order to rule out the possibility of other causes of febrile illnesses(WHO, 2014c).

2.3.2 The Quantitative Buffy Coat (QBC)is a laboratory test to detect infection with malaria,similar to theMicroscopy methodthe species of parasite can also be identified in > 90% of cases(However, this test is more sensitive than the conventional thick smear. The technique uses microhaematocrit tubes precoated with fluorescent acridine orange stain to highlight malaria parasites. With centrifugation, parasites are concentrated at a predictable location. The QBC test methods require less training to operate than for reading Giemsa-stained blood films and the test is typically quicker to perform than normal light microscopy(Kocharekar*et al*., 2014). Disadvantages are that electricity is always required, special equipment and supplies are needed, the per-test cost is higher than simple light microscopy.

2.3.3 Antigen Detection Tests (rapid or “dipstick” diagnostic tests (RDTs)

Rapid Diagnostic Tests (RDTs) assist in the diagnosis of malaria by detecting evidence of malaria parasites in human blood by rapid immuno-chromatographic techniques.Rapid diagnostic tests (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available---or is not available right away. This is the case in most of the malaria-endemic world. Malaria RDTs are being widely used in malaria-endemic countries,

but the use of an RDT does not completely eliminate the need for malaria microscopy(CDC, 2014). A number of commercially available kits (e.g. SD-biloine,ParaSight-F®, Becton-Dickinson, Malaquick®, ICT; fromKorea, Sydney, New South Wales, Australia) are based on the detection of the histidine-rich protein 2 (HRP-II) of *P. falciparum* while some others like OptiMAL-IT (Flow Inc. Portland, OR, USA),are based on *Plasmodium*-specific lactate dehydrogenase (pLDH) in patients' whole blood and detects viable parasites,which eliminates prolonged periods of false positivity post-treatment (Iqbal *et al.,* 2003). Monoclonal antibodies against pLDH are commercially available for the detection of *Plasmodium* spp. (pan-malaria), *P. falciparum*, and *P. vivax.* The *P. vivax*-specific assay is new and not yet adequately evaluated.Compared with light microscopy and QBC, this test yielded rapid and highly sensitive diagnosis of *P. falciparum* infection (WHO, 1996; Kocharekar *et al*., 2014). Advantages to this technology are that no special equipment is required, minimal training is needed, the test and reagents are stable at ambient temperatures and no electricity is needed. The principal disadvantages are a currently high per-test cost and an inability to quantify the density of infection. Furthermore, for tests based on HRP-II, detectable antigencan persist for days after adequate treatment and cure; therefore, the test cannot adequately distinguish a resolving infection from treatment failure due to drug resistance, especially early after treatment (Wongsrichanalai, 2007). As opposed to HRP-2, which often persists in the patient's blood for weeks after successful treatment, pLDH is a more appropriate target for treatment monitoring(Johanna*et al*., 2012). However, plasmodial gametocytes also produce pLDH and so a pLDH test may remain positive despite clearance of the asexual parasite forms (Nyunt*et al*., 2013).Persistent HRP-2, on the other hand, could be an advantage in detecting low-level, fluctuating parasitemia in chronic malaria.Newer generation antigen detection tests are able to distinguish between falciparum and non-falciparum infections, greatly expanding their usefulness in areas where non-falciparum malaria is transmitted frequently (Murray*et al*.,2008).

2.3.4 Serological Tests

Diagnosis of malaria using serological methods is usually based on the detection of antibodies against asexual blood stage malaria parasites. Serology is not useful for diagnosing acute infections because detectable levels of anti-malaria antibodies do not appear until weeks into infection and persist long after parasitaemia has resolved. Immunofluorescence antibody testing (IFA) has been a reliable serologic test for malaria in recent decades (She *et al*., 2007).Although IFA is time-consuming and subjective, it is highly sensitive and specific(Wang *et al*.,2009). The literature clearly illustrates the reliability of IFA, so that it was usually regarded as the gold standard for malarial serology testing (Doderer*et al.,*2007). IFA is useful in epidemiological surveys, for screening potential blood donors, and occasionally for providing evidence of recent infection in non-immunes.

2.3.5Molecular Diagnostic Methods

Traditional malaria diagnostic methods remain problematic. New laboratory diagnostic techniques that display high sensitivity andhigh specificity, without subjective variation, are urgently needed in various laboratories. Recent developments in molecular biological technologies, e.g. PCR, loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, have permitted extensive characterization of the malaria parasite and are generating new strategies for malaria diagnosis.

2.3.6 Polymerase Chain Reaction (PCR)Technique:

PCR-based techniques are a recent development in the molecular diagnosis of malaria, and have proven to be one of the most specific and sensitive diagnostic methods, particularly for malaria cases with low parasitemia or mixed infection (Noppadon*et al.,* 2009). The PCR technique continues to be used extensively to confirm malaria infection, follow-up therapeutic response, and identify drug resistance(Chotivanich *et al*., 2006). It was found to be more sensitive than QBC and some RDTs (Rakotonirina *et al*., 2008). Comparing with the gold standard method for malaria diagnosis, PCR has shown higher sensitivity and specificity than conventional microscopic examination of stained peripheral blood smears, and now seems the best method for malaria diagnosis (Morassin*et al*., 2002). PCR can detect as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT. Moreover, PCR can help detect drug-resistant parasites, mixed infections, and may be automated to process large numbers of samples (Hawkes and Kain, 2007, Swan *et al*., 2005). Some modified PCR methods are proving reliable, e.g., nested PCR, real-time PCR, and reverse transcription PCR. Recently, the PCR method has become widely accepted for identifying *P. knowlesi* infections (Cox-sign *et al*.,2008; Ng *et al*.,2008). Although PCR appears to have overcome the two major problems of malaria diagnosis-sensitivity and specificity- the utility of PCR is limited by complex methodologies, high cost, and the need for specially trained technicians. PCR, therefore, is not routinely implemented in developing countries because of the complexity of the testing and the lack of resources to perform these tests adequately and routinely (Mens *et al*.,2008). Quality control and equipment maintenance are also essential for the PCR technique, so that it may not be suitable for malaria diagnosis in remote rural areas or even in routine clinical diagnostic settings (Hanscheidand Grobusch, 2002).

2.3.7 Loop-mediated Isothermal Amplification (LAMP) Technique:

The LAMP technique is a simple and inexpensive molecular malaria-diagnostic test that detects the conserved 18S ribosomal RNA gene of *P. falciparum* (Poon*et al.,* 2006).It is a nucleic acid amplification method that relies on autocycling strand –displacement DNA synthesis using Bst DNA polymerase. Other studies have shown high sensitivity and specificity, not only for *P. falciparum*, but also *P. vivax*, *P. ovale* and *P. malariae* (Han *et al*., 2007,Noppadon*et al.,* 2009). These observations suggest that LAMP is more reliable and useful for routine screening for malaria parasites in regions where vector-borne diseases, such as malaria, are endemic.

2.3.8Microarrays

Microarrays may play an important role in the future diagnosis of infectious diseases. The principle of the microarrays technique parallels traditional Southern hybridization. Hybridization of labeled targets divided from nucleic acids in the test sample to probes on the array enables the probing of multiple gene targets in a single experiment. A pan-microbial oligonucleotide microarray has been developed for infectious disease diagnosis and has identified *P. falciparum* accurately in clinical specimens (Palacios *et al*., 2007). This diagnostic technique, however, is still in the early stages of development.

2.3.9 Flow Cytometry (FCM) assay

Flow cytometry has reportedly beenused for malaria diagnosis (Izumiyama*et al*., 2009). Briefly, the principle of this technique is based on detection of hemozoin, which is produced when the intra-erythrocytic malaria parasites digest host haemoglobin and crystallize the released toxic heme into hemozoin in the acidic food vacuole. Hemozoin within phagocytotes can be detected by depolarization of laser light, as cells pass through a flow-cytometer channel. It is potentially useful for diagnosing clinically unsuspected malaria. The disadvantages are its labour intensiveness, the need for trained technicians, costly diagnostic equipment, and that false-positives may occur with other bacterial or viral infections. Therefore, this method should be

considered a screening tool for malaria.Other methods which are yet strictly employed for researchpurposes include : Automated blood cell counters (ACC) and Mass spectrophotometry(de Langen *et al*.,2006)

2.4 Anti-malarial Drugs

Drugs represent the primary treatment available for human malaria, as caused by *Plasmodiumspp*. With primaquine as a notable exception, the available drugs work primarily against bloodstream parasite stages, which invade and replicate within human erythrocytes(Basore *et al*., 2015).Anti-malarials are used in three different ways: prophylaxis, treatment of falciparum malariaand treatment of non-falciparum malaria. Prophylactic anti-malarials are used almost exclusively by travellers from developed countries who are visiting malaria- endemic countries (Saifi*et al*.,2013); and by pregnant women for prevention of malaria during pregnancy.Treatment protocols for falciparum malaria vary, depending on the severity of the disease; fast-acting,parenteral drugs are best for severe, life-threatening disease. In addition, treatment protocols for falciparum malaria vary geographically and depend on the resistance profiles for strains in particular regions. Non-falciparummalarias, in contrast, rarely are drug – resistant while *P. vivax* and *P. ovale* have dormant liver stages that can cause relapses months to years after aninfections cleared, so they need to be treated with an additional agent that can clear this stage. The antimalarials in common use come from the following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artemisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones (atovaquine).

2.4.1 Quinine

Quinine is derived from the bark of the cinchona tree and was used for treating fevers as early as the 17th century, although not until 1820, it was the active ingredient of the bark, isolated and used in its purified form. Quinine is an alkaloid that acts as a blood schizonticidal and weak gametocide against *Plasmodium species.* As an alkaloid, it is accumulated in the food vacuoles of *Plasmodium*, especially *P. falciparum*.Quinine acts in a manner similar to that of chloroquine but with some differences; chloroquine causes clumping of the malaria pigment, whereas quinine

antagonizes this process (Peters, 1987). In addition, quinine is a weaker base than chloroquine and has less affinity for heme, implying that mechanisms other than ion transport into the food vacuole and heme-drug interactions are required for the action of these drugs (Foley and Tilley, 1998)

It is especially useful in areas where there is known to be a high level of resistance to Chloroquine, Mefloquine and sulfa drug combinations with pyrimethamine (Foley and Tilley, 1998). Quinine is used as a treatment for uncomplicated and severe malaria in many different therapeutic regimens. Quinimax and Quinidine are the two most commonly used alkaloids related to Quinine, in the treatment or prevention of Malaria. Quinimax is a combination of four alkaloids (namely Quinine, Quinidine, Cinchoine and Cinchonidine) (Mills and Bone, 2000).Quinidine is a direct derivative of Quinine. It is a distereoisomer, thus having similar anti-malarial properties to the parent compound. Quinidine is recommended only for the treatment or severe cases of malaria(Foley and Tilley, 1998). Although quinine treatment failure has been reported, many of these instances can be attributed to inadequate treatment.

2.4.2 Chloroquine (CQ)

Chloroquine is a 4-aminoquinolone derivative of quinine, first synthesized in 1934 and has since been the most widely used antimalarial drug until resistance developed and has more recently been explored for treating cancer and viral infections (WHO 2001b;Bijker *et al*., 2015). The emergence of drug resistant parasitic strains rapidly decreasing its effectiveness (Rieckmann *et al*., 1978; Wellems and Plowe, 2001); however it is still the first-line drug of choice in most sub-Saharan African countries. CQ is active against *P. falciparum* through interactions with heme in the digestive vacuole of the parasite. There is a large accumulation of CQ in the digestive vacuole in part because of a pH gradient between the cytoplasm of the red blood cells, the

cytoplasm of the parasite, and the digestive vacuole. The red blood cell and parasite cytoplasm are very similar in pH, about 7.1. At this pH, CQ has a +1 charge and is able to freely move through the cell membranes all the way into the digestive vacuole (Chinappi*et al*., 2010).The pH of the digestive vacuole is about 5,which makes CQ have a 2+ charge .Because of this large pH difference and formation of the doubly charged species, CQ is able to accumulate in the digestive vacoule much higher than in the cytoplasm (Kuhn *et al*., 2007).In the digestive vacuole, CQ interacts with heme, preventing the formation of hemozoin, eventually killing the parasite (Roepe,2009).

Despite the effectiveness of CQ,there is increasing worldwide resistanceto CQ as well as to many other antimalarial drugs. The digestive vacuole of the CQ-sensitive and -resistant *P. falciparum* has a membrane transporter called the *P. falciparum* chloroquine resistance transporter (PfCRT), which is partly responsible for the increasing resistance to chloroquine. Resistant parasites have been found to have a mutated version of the *pfcrt* gene, causing mutations in the protein sequence that allows the parasite to move CQ out of the digestive vacuole. With CQ at a reduced digestive vacoule concentration, hemozoin is once again formed allowing the parasite to survive (Chinappi, 2010).

CQ resistance isn't caused by just one mutation of a nucleic acid in the *pfcrt* gene, but by many mutations. It is important to note that the re isn't just one strain of CQ -resistant malaria that spread across the world, but rather resistance to CQ developed independently in different parts of the world, with different types of mutations leading to the resistance. A common mutation in all the strains is a change from lysine at the 76 position to threonine.The CQ-sensitive strain labelled 106/1 has many of the same mutations as the resistant strands, except it doesn't have the

mutation at the 76 position. This indicates that mutating the positively charged lysine to an uncharged threonine is a key mutation in resistance to CQ (Wellems and Plowe, 2001)

The increasing resistance of *P. falciparum* to CQ, leads to a need for more potent, cost effective drugs that are active against this parasite. A new drug, PL69, has been synthesized that is a "reversed chloroquine" (RCQ), which combines chloroquine with a reversal agent . Reversal agents are molecules that reverse resistance to drugs; in this case, reversing resistance to chloroquine. Reversal agents can be taken with a drug as a cocktail or can be combined with the drug molecule to make a new drug, which is what was done in the case of PL69. The reversal agent on PL69 reduces the efflux of the chloroquine-like drug from the digestive vacuole, reversing the parasite's resistance (Burgess*et al*., 2010).CQ is generally well tolerated, although it has a bitter taste. The most common adverse events are pruritus, headache, visual disturbances, nausea and gastrointestinal discomfort. Severe adverse events have rarely been reported e.g. neuromyopathy and retinopathy. CQ has low safety margin and doses as low as 30 mg/kg have caused fatal hypotension and cardiac arrhythmias. This is of special concern when CQ is given parenterally (Taylor and White, 2004; WHO, 2006).CQ is considered safe to give in pregnancy (Nosten *et al.,* 2006).It is now suggested that it is used in combination with other antimalarial drugs to extend its effective usage.

2.4.3 Amodiaquine

Amodiaquine is a 4-aminoquinolone anti-malarial drug which was widely used in the past for both prophylaxix and treatment of malaria. It was withdrawn from use because of fatal side effects, notably agranulocytosis and hepatitis, which occurred mainly in non-immune adults taking the drug for prophylaxis (Adjei *et al*., 2009) It is similar in structure and mechanism ofaction to Chloroquine,but thought to be more effective in clearing parasites in uncomplicated

malaria than Chloroquine (Olliaro and Taylor, 2003).It is readily absorbed after oral intake with a peak plasma concentration within 2 hours and a terminal plasma half -life of approximately 4 - 8 hours. AQ is rapidly and extensively metabolized by the hepatic enzyme system CYP2C8 to the active main metabolite desethylamodiaquine (DEAQ) with peak plasma concentration within 4 -6 hours. DEAQ has a total volume of distribution of about 20 –30 L/kg, more than 90% is protein bound in plasma and the terminal plasma half -life is 10 –14 days. However, the AQ/DEAQ pharmacokinetics appears to vary significantly (Lindegårdh *et al*., 2002). Due to the longer half -life, it is probable that DEAQ is mainly responsible for the antimalarial activity of AQ (Churchill *et al*., 1985)Available data suggest that AQ in standard dosageis not teratogenic and that the adverse events frequency is not elevatedin pregnancy. Thus, AQ in combination with other antimalarial drugs may be usefulfor malaria treatment in pregnancy, but inadequate data on its safety and pharmacokinetics in pregnancy limitits deployment for intermittent preventive treatment in pregnancy (Nosten *et al*., 2006)

2.4.4 Mefloquine

During the 1960s, United States Army synthesized and tested several drugs for protection of their soldiers against multi drug resistant falciparum malaria and hence, Mefloquine was born. Mefloquine is reasonably well absorbed from the gastrointestinal tract and administration together with food intake will enhance absorption. Mefloquine is about 98% bound to plasma proteins and is widely distributed throughout the body. Mefloquine is metabolized in the liver and is mainly excreted in the faeces. Mefloquine is administered as an oral dose with 150 mg base/tablet. For treatment, the recommended dose is 25 mg/kg body weight split into two doses, 15 mg/kg on the first day and 10 mg/kg on the second day. As a pro-phylaxis, the recommended dose is 5 mg/kg base once a week (equals 1 tab-let once weekly for an adult). Prophylaxis should start at least 2 – 3 weeks before departure (WHO,2006).

Parasites resistant to Mefloquine are found in South-East Asia, but are rare elsewhere in the world. Side effects associated with Mefloquine treatment include nausea, vomit-ing, abdominal pain, diarrhoea, headache, loss of balance and sleeping dis-orders like insomnia and abnormal dreams. Some more serious but rare side effects are neuropsychiatric disturbances like psychosis or hallucinations etc (WHO, 2006). Mefloquine is not recommended in the first trimester of pregnancy where it is associated with an increased risk of still births; and due to its long half-life, pregnancy should be avoided at least 3 months after completing chemoprophylaxis (WHO, 2006).

2.4.5 Atovaquone

Atovaquone (alternative spelling:**atavaquone**) is a chemical compound that belongs to the class ofnaphthoquinones. Atovaquone is a hydroxy-1,4-naphthoquinone, an analog ofubiquinone, with anti-pneumocystic activity (Glaxosmithkline, 2015).Atovaquone is the end product of half a century of research by many groups who researched the antiparasitic properties of numerous structurally related compounds(McKeage and Scott, 2003; Nakato *et al.*,2007).Currently, atovaquone is used as a fixed-dose combination with proguanil (Malarone) for the treatment of children and adults with uncomplicated malaria or as a chemoprophylactic agent for preventing malaria in travellers (Osei-Akoto*et al*., 2005).Atovaquone is a competitive inhibitor of ubiquinol, specifically inhibiting the mitochondrial electron transport chain at the bc1 complex. Inhibition of bc1 activity results in a loss of mitochondrial function.

During the intra-erythrocytic stage of infection, a key role of the parasite mitochondrion is to provide orotate for pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase (DHODH). Consistent with this, inhibition of the bc1 complex by atovaquone affects the concentrations of metabolites in the pyrimidine biosynthetic pathway. Indeed, transgenic*P. falciparum* parasites expressing ubiquinone-independent yeast DHODH have been shown to display an atovaquone-resistant phenotype (Biagini *et al.,* 2006).Absorption of atovaquone

shows dose limitations, with maximum absorption observed using 750 mg tablets.Poordrug solubility was suggested as the cause of this limit to absorption, and this led to the development of an atovaquone liquid suspension formulation that showed improved Pneumocystis pneumonia treatment success compared with the tablet formulation(Rosenberg *et al*.,2001).Elimination is primarily via the liver, with almost undetectable amounts (<0.6%) of drug being eliminated via the kidneys and is highly bound to plasma protein (99.5%) and shows a high affinity for human serum albumin(Roland *et al*., 1997; Zsila and Fitos,2010).Atovaquone has been found to be generally well tolerated and causes few side effects. Adverse events are generally mild and include rash, fever, vomiting, diarrhoea, abdominal pain and headache. Indeed, overdoses as large as 31 500 mg have been reported to cause little or no symptomatology (Nixon*et al*.,2013).

2.4.6 Primaquine

Primaquine is an antimalarial belonging to the 8-aminoquinolone group. It was used in treating all types of malaria .*Plasmodium falciparum* gametocytes are sensitive to 8-aminoquinolines (8AQ), and conseuently these drugs could prevent parasite transmission from infected people to mosquitoes (Graves *et al*.,2015). Primaquine is an antimalarial drug which does not cure malaria illness, but is known to kill the gametocyte stage of the malaria parasitewhich infects mosquitoes when they bite humans. Primaquine is also known to have potentially serious side effects in peoplewith glucose-6-phosphate dehydrogenase (G6PD) deficiency, an enzyme deficiency common in many malaria endemic settings. In these people, high doses of primaquine given over several days' damages red blood cells and causes anaemia, and sometimes these events maybe life-threatening(Graves *et al.,*2015).The World Health Organization (WHO) recommends adding a single dose of primaquine to falciparum malaria treatment with the intention of reducing malaria transmission and to contribute to malaria elimination. In 2013, the WHO amended their

guideline, reducing the PQ dose from 0.75 mg/kg to 0.25 mg/kg to reduce the risk of haemolysis, combined with indirect evidence suggesting this was as effective as the higher dose.

2.4.7 Halofantrine

Halofantrine (HF), a phenanthrenemethanol derivative of aminoalcohol, was first marketed in 1988. It was considered effective and safe for treating malaria, including multidrug resistant *P falciparum*strains until when ter Kuile*et al*(1993) reported the first death related to HF cardiotoxicity (Bouchou*et al*., 2009). It was developed by the Walter Reed Army Institute of Research in the 1960s (Mills and Bone, 2000). Itsmechanism of action is similar to other anti-malarials. Cytotoxic complexes are formed withferritoporphyrin XI that cause plasmodial membrane damage (Bloland, 2001). A popular drug based on halofantrine is Halfan. Halfan (halofantrine hydrochloride) is an antimalarial drug available as tablets containing 250 mg of halofantrine hydrochloride (equivalent to 233 mg of the free base) for oral administration

2.4.8 Antifolate drugs

Antifolates are drugs that antagonise (that is, block) the actions of folic acid (vitamin B_9).Folic acid's primary function in the body is as a cofactor to various methyltransferases involved in serine, methionine, thymidine and purine biosynthesis. Consequently antifolates inhibit cell division, DNA/RNA synthesis and repair and protein synthesis. Some such as proguanil, pyrimethamine and trimethoprim selectively inhibit folate's actions. Antifolate agents used for the treatment of malarial infection act on the folate metabolism of the parasite. With regard to the target enzyme they inhibit, the antifolates are subdivided into two classes: inhibitors of Dihydrofolate Reductase (DHFR), such as pyrimethamine, proguanil and chlorproguanil and inhibitors of Dihydropteroate Synthase (DHPS) such as sulfadoxine and dapsone. The

combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria (Mebrahtu, 2015). With rapidly growing Sulfadoxine-Pyrimethamine (SP) resistance, a new combination drug, Lapdap (chlorproguanil-dapsone), was tested in Africa in the early 2000s, but was withdrawn in 2008 because of hemolytic anaemia in patients with Glucose-6-Phosphate Dehydrogenase enzyme (G6PD) deficiency (Luzzatto, 2010).Antifolate combination drugs, such as sulfadoxine + pyrimethamine, act through sequential and synergistic blockade of two key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by Dihydrofolate Reductase (DHFR) whilesulfones and sulfonamides inhibit the step mediated by Dihydropteroate Synthase (DHPS) (Grimberg and Mehlotra, 2011). Specific gene mutations encoding for resistance to both DHPS and DHFR have been identified. Specific combinations of these mutations havebeen associated with varyingdegrees of resistance to antifolate combination drugs (Wang *et al*., 2005).

2.4.8.1Sulphadoxine Pyrimethamine (SP)

SP is part of the class of antifolate combination drugs that acts through sequential and synergistic mechanism to block two key enzymes involved in folate synthesis. Pyrimethamine inhibits the activity of dihydrofolate reductase (DHFR), while the sulfonamides and sulfones (in this case sulfadoxine) inhibit the activity of dihydopteroate synthase (DHPS). It is widely known as Fansidar® (Hoffman-La Roche) but is also sold in a variety of other commercial and generic form (Yaro, 2009).

The molecular targets of each of the drug component are the thymidylate synthase (TS) enzyme for pyrimethamine and 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK) enzyme for sulfadoxine (Yaro, 2009). These two enzymes are important for the folic acid biosynthesis pathway. Research has shown that resistances to SP are associated with distinct point mutations

in DHFR-TS and PPK-DHPS genes. When combined the two key stages in DNA synthesis in the plasmodia are prevented.

2.4.8.2.Proguanil

Proguanil marketed in combination with atovaquone is used for both the treatment of uncomplicated *P. falciparum*and prophylaxis of mild chloroquine-resistant malaria (Hussien,2007).Proguanil (Chloroguanadine) is a biguanide; a synthetic derivative of pyrimidine. It has many mechanisms of action but primarily is mediated through conversion to the active metabolite cycloguanil pamoate. This inhibits the malarial dihydrofolate reductase enzyme.Its most prominent effect is on the primary tissue stages of *P. falciparum,P. vivax* and *P.ovale*. It has no known effect against hypnozoites therefore is not used in the prevention of relapse. It has a weak blood schizonticidal activity when combined with Atovaquone (a hydroxynaphthoquinone).The most common Adverse Events (AEs) reported in >10% of patients taking atovaquone/proguanil for treatment of malaria are abdominal pain, nausea, vomiting, and head-ache in adults, and vomiting in children; for prophylaxis of malaria AEs include headache and abdominal pain and vomiting in children. It is well tolerated, and although oral aphthous ulcerations are not uncommon, they are rarely severe enough to warrant discontinuing this medication. Proguanil is considered safe during pregnancy and breastfeeding, but insufficient drug is excreted in the milk to protect a breastfed infant (Hussien,2007).

2.4.9Antibiotics

The impact of selected antibiotics on combating malaria infections was discovered in the middle of last century. Only recently, studies on their modes of action in malaria parasites have been initiated, prompted by the discovery of a prokaryotic organelle, the apicoplast. Due to its indispensability for parasite survival,apicomplast represents a promising target for the use of antibiotics in malaria therapy. Most antibiotics cause a delayed death phenotype, which manifests in the late onset of antimalarial activity during the second replication cycle of the pathogen (Pradel and Schlitzer,2010).

2.4.9.1Tetracycline

Tetracycline is a broad-spectrum antimicrobial which has a potent but slow action against the asexual blood stages of all plasmodial species. The tetracyclines were one of the earliest groups of antibiotics to be developed and are still used widely inmany types of infection. It is also active against the primary intrahepatic stages of *P. falciparum.* The closely related substances doxycycline and minocycline share its actions. Absorption of tetracycline from the gut is always incomplete and can be further impaired by alkaline substances and chelating agents and, particularly, by milk and milk products, and by aluminium, calcium, magnesium and iron salts (WHO,2015b). Peak plasma concentrations occur within 4 hours and decay with a half-life of about 8 hours. Excretion is effected primarily by glomerular filtration into the urine. Enterohepatic circulation gives rise to high concentrations in the liver and bile.Tetracyclines cross the placenta and are excreted into breast milk (WHO, 2015b).

Tetracyclines are active against a wide range of infectious pathogens. In limited studies, tetracycline and some tetracycline analogs were active against cultured malaria parasites and in murine malaria models. Despite limited experimental data, tetracycline and doxycycline were used for the treatment of malaria early after their introduction as antibacterial agents, and they

have become a component of some standard antimalarial regimens. In the United States, a standard therapy for falciparum malaria is quinine or intravenous quinidine plus doxycycline. Doxycycline is also recommended by the Centres for Disease Control and Prevention for chemoprophylaxis against malaria for travellers to regions where malaria is endemic, especially to areas where high levels of resistance to other agents are found (Draper*et al*., 2013).

Azithromycin (AZM) is a macrolide antibiotic that displays an excellent safety profile even in children and pregnant women and has been shown to have anti-malarial activity against blood stage *Plasmodium falciparum*. Takahashi*et al*., (2014) reported the transmission-blocking effect of AZM using a rodent malaria model

2.4.9.2Clindamycin (7-Chlorolincomycin)

Theparent compound in clindamycin is lincomycin,a water-soluble antibiotic that is very active against gram-positive microorganisms. It is available as clindamycin hydrochloride for oral administration in capsules, as clindamycin phosphate for intramuscular or intravenous injection, and as clindamycin palmitate for oral suspensions (Bertrand and Peter, 2002). Clindamycin monotherapy is an effective treatment which must however, be given for at least 5 days and twice daily. Analysis of all published studies that have used this regimen shows that clindamycin monotherapy has a mean efficacy of 98%, well tolerated with mild adverse events (Bertrand and Peter, 2002).

2.4.9.3Rifampicin

Rifampicin is an antitubercular drug with potent anti-malarial activity against *P. vivax* in humans, *P. chabaudi* in rodents and chloroquine resistant *P. falciparum in vitro*(Kwara*et al*., 2008). Combination of rifampicin with isoniazid and co-trimoxazole was found to be effective in patients with *P. falciparum* infections (George, 1999). Rifampicin appears to have a potential role to play in the management of malaria. However, multiple drug therapies increase the risk of pharmacokinetic drug-drug interactions which may be of clinical relevance. The availability of anti-malarial drugs over the counter (including the availability of artemether, amodiaquine or dihydroartemisinin tablets) especially in developing endemic countries promotes the concept of self-medication and this further confounds the challenges of drug-drug interaction. Rifampicin is a potent inducer of hepatic metabolism and can influence pharmacokinetics of other drugs (Kwara*et al.,* 2008).

2.4.9.4Artemisinin compounds

Artemisinin is a sesquiterpene lactone endoperoxide containing a structural feature called peroxide bridge which is believed to be the key to its mode of action (Figure 3). Artemisinin is a Chinese herb (Qinghaosu) that has been used in the treatment of fevers for over 1,000 years (WHO, 1996), thus predating the use of Quinine in the western world. It is derived from the plant *Artemisia annua*, with the first documentation as a successful therapeutic agent in the treatment of malaria in 340 AD (Mueller *et al.,*2000; 2004). In spite of increasing popularity in the use of artemisinin based therapies, the mechanism of action of these sesquiterpene lactone endoperoxides has eluded researchers due to its controversial nature(Klayman, 1985; Posner and O'Neill, 2004). According to one school of thought, the cleavage of the peroxide bridge in the presence of ferrous ion (Fe^{2+}) from haem forms highly reactive free radicals which rapidly rearrange to more stable carbon-centered radicals. It has been suggested that these artemisinin-

derived free radicals chemically modify and inhibit a variety of parasite molecules, resulting in parasite's death(Meshnick *et al.,* 1996;Posner, 1995;Meshnick*et al.,* 1991). A rich source of intracellular Fe^{2+} is haem an essential component of haemoglobin, the malarial parasite is rich in haem iron derived from a breakdown of the host cell haemoglobin. It has long been suspected that Fe^{2+}-haem is responsible for activating artemisinin inside the parasite(Ridley, 2003). Endoperoxides are known to be unstable, especially in the presence of iron. Theoretical studies on the cleavage of the peroxide bond suggest a thermodynamically favorable interaction between artemisinin and haem (Taranto *et al*., 2001;Araujo *et al*.,2008).

Artemesinin has a very rapid action and the vast majority of acute patients treated show significant improvement within 1-3 days of receiving treatment. It has demonstrated then fastest clearance of all anti-malarials currently used and acts primarily on the trophozoite phase, thus preventing progression of the disease. It is converted to active metabolite dihydroartemisinin which inhibits the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) encoded by *P. falciparum* (Eckstein-Ludwig *et al.,* 2003). A number of sesquiterpine lactone compounds have been synthesized from the plant *Artemisia annua* (artesunate, artemether, arteether) (Mills and Bone, 2000). These compounds are used for treatment of severe malaria and have shown very rapid parasite clearance times and faster fever resolution than occurs with quinine.

Combination therapy (an artemisinin compound given in combination with another antimalarial), typically a long half-life drug likemefloquine) has reportedly been responsible for inhibiting intensification of drug resistanceand for decreased malaria transmission levels in South-East Asia (Price *et al.,* 1996; White*,*1999*).*ACTs compensate for the poor pharmacokinetic properties of the artemisinins, increase treatment efficacy and are thought to reduce the emergence of drug-resistant parasites (Petersen *et al*., 2011) and are now central tothe first-line treatment of *P.*

*falciparum*malaria. The recent emergence of decreased sensitivity of the parasite to artemisinins in Cambodia is of grave concern and puts at risk the entire strategy for the treatment of malaria (Ward and Boulton, 2013).Initially an involvement of artemisinins with haemoglobin degradation was reported while another hypothesis postulates that the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) ortholog of *P. falciparum*, PfATP6, is a target of artemisinins. Artemisinins have been additionally considered to have an effect on the mitochondrial electron transport chain (Ariey *et al.,* 2014). Early studies linked artemisinin resistance to mutations in PfATP6, since artemisinins could inhibit *PfATPase6* activity in a heterologous system, and a single amino acid change (L263E) in *PfATP6* could abolish the inhibition (Eckstein-Ludwig *et al.,* 2003;Krishna *et al*., 2010).

Recent reports however suggest the association of mutations in the *PFATPASE6*(*PfATPase6*, is a target of artemisinins) and pfmdr1genes might be the main contributor to artemisinins resistance (Cui and Su, 2009; Ding *et al*., 2011). Recently, a molecular marker of artemisinin resistance was identified (WHO, 2014a). Mutations in the Kelch 13 (K13) -propeller domain were shown to be associated with delayed parasite clearance *in vitro* and *in vivo*. This new tool will help to improve the global surveillance of artemisinin resistance (WHO, 2014a).

Artemisinin is highly insoluble in oil and water and therefore can only be administered orally. Its empirical formula is $C_{15}H_{22}O_5$. No major side effects have been reported, but high parenteral doses of artemisinin derivatives in rats led to selective brain stem neuropathy, which fuelled debate over safety (Van *et al.,* 1999).Derivatives of Artemisinin include Artesunate (AS), Dihydroartemisinin and Artemether.

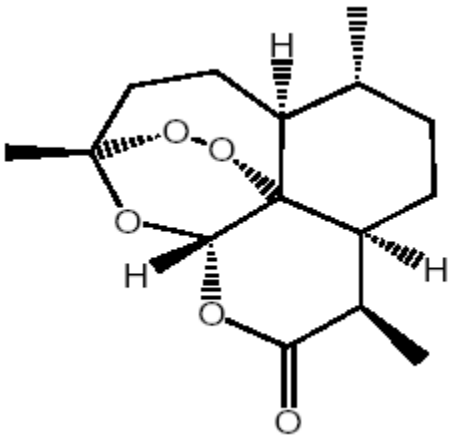

Figure 3: The chemical structure of artemisinin (Trinity Student Medical Journal, 2003)

2.5 OVERVIEW OF ANTIMALARIAL RESISTANCE

For decades, drug resistance has been one of the main obstacles in the fight against malaria. To date, drug resistance has been documented in three of the five malaria species known to affect humans in nature: *P. falciparum*, *P. vivax and P. malariae* (WHO, 2013). Over the past 60 to 70 years, since the introduction of synthetic antimalarials, only a small number of compounds, belonging to three broad classes, have been found suitable for clinical usage (Grimberg and Mehlotra, 2011).This limited antimalarial armament is now severely compromised because of the parasite's remarkable ability to develop resistance to these compounds. In many different malaria -endemic areas, low to high-level resistance in the predominant malaria parasites*, P. falciparum* and P. vivax, have been observed for CQ, amodiaquine, mefloquine, primaquine and SP. *P. falciparum* has developed resistance to nearly all antimalarial drugs in current use (White, 2004) although the geographic distribution of resistance to any one particular drug varies greatly.Antimalarial drug resistance is mediated by two processes:

(1) The rate that *de novo* mutations conferring resistance appear and are selected through drug use within an individual

(2) The spread of those resistant alleles to other individuals

High mutation rates at the cellular level, which provide a means of continually evading the immune system, offer a mechanism for selection of resistance within a host, while interactions with other parasites and their hosts due to variation in transmission and host susceptibility influence the probability of selection at the population level (Klein, 2012).

CQ, SP and more recently the artemisinin class of drugs have been widely adopted as first-line drugs because they are highly efficacious in eliminating *P. falciparum*-infected erythrocytes and they are well tolerated by almost all patients (Klein, 2013; Mehlotra and Zimmerman, 2006; Schlitzer, 2007). In addition, unlike other drugs such as atovaquone and pyrimethamine (when

not combined with sulfadoxine), the rate at which *de novo* mutations conferring resistance occur is low.

The biochemical mechanism of resistance has been well described for chloroquine, the antifolatecombination drugs and atovaquone.Acquired resistance usually arises in low transmission settings in people with lots of parasites (hyperparasitaemic) who for reasons of drug quality, inadequate dose, adherence, absorption or distribution kinetics, or vomiting have inadequate blood concentration levels of the drug (Beith, 2008).

Resistance mostly occurs through primary transmission of drug-resistant parasites.The molecular mechanism of the action of this conventional antimalarial drug, chloroquine, is becoming unveiled (Hyde, 2002). Chloroquine Resistance (CQR) in *P. falciparum* is now linked to point mutations in the chloroquine resistance transporter gene (PfCRT encoded by pfcrt, located on chromosome 7) (Van *et al.,* 2011). *Pfcrt*-K76T mutation confers resistance in vitroand is the most reliable molecular marker for CQR (Sidhu *et al*., 2002) although there were exceptions where there is the presence of K76T and the parasites responded to chloroquine.

The key elements of the strategy to prevent the emergence of drug resistance are: reducing overall drug pressure, monitoring antimalarial drug efficacy, improving the way drugs are used, ensuring a continuous pipeline of new antimalarial medicines and combination therapies.

Currently,the most effective treatments for malaria are artemisinin-based combination therapies (ACTs) that combine a semi-synthetic derivative of artemisinin, a chemical compound isolated from the plant Artemisia annua, with a partner drug of a distinct chemical class. ACTs compensate for the poor pharmacokinetic properties of the artemisinins, increase treatment

efficacy and are thought to reduce the emergence of drug -resistant parasites (Petersen *et al*., 2011) and are now central to the first-line treatment of *P. falciparum* malaria.

The recent emergence of decreased sensitivity of the parasite to artemisinins in Cambodia is of grave concern and puts at risk the entire strategy for the treatment of malaria (Ward and Boulton, 2013). WHO has established a strategy for dealing with antimalarial resistance, which has four key elements: preventing the emergence of antimalarial drug resistance, monitoring antimalarial drug efficacy and when necessary confirming drug resistance, ensuring a continuous pipeline of new antimalarial medicines and containing the spread of antimalarial drug resistance once it has emerged (WHO, 2009). Therefore as a result of increasing insecticide resistance by the vector and widespread drug resistant strains of the parasite there is an urgent need for a more reliable intervention,the malaria vaccine.

2.6 IMMUNOLOGIC RESPONSES IN MALARIA

These are bodily defense reactions that recognize invading substances (antigens such as viruses, bacteria or fungi or transplanted organ) and produce antibodies specific against the antigens. Human malaria has persisted through the development of miracle drugs and insecticides, a global eradication effort, and 30 years of intensive efforts to develop a practical vaccine. On 6 November 1880, Alphonse Laveran observed a male gametocyte exflagellating in a blood smear from an Algerian patient with malaria. This event marked the identification of plasmodia as the cause of malaria.Working in India in 1897, Ronald Ross identified plasmodial oocysts in the guts of mosquitoes fed on parasitemic birds, thereby implicating mosquitoes as the vector of malaria (Ross**,** 1899b).

Malaria immunity may be defined as the state of resistance to the infection brought about by all those processes which are involved in destroying the plasmodia or by limiting their multiplication. Natural (innate) immunity to malaria is an inherent property of the host, a refractory state or an immediate inhibitory response to the introduction of the parasite, not dependent on any previous infection with it (Doolan *et al.,* 2009). Acquired immunity may be either active or passive. Active (acquired) immunity is an enhancement of the defense mechanism of the host as a result of a previous encounter with the pathogen (or parts thereof). Passive (acquired) immunity is conferred by the prenatal or postnatal transfer of protective substances from mother to child or by the injection of such substances

The dominant factor driving protection from disease may be specific to effectors that diminish parasite numbers, but other effectors, e.g., responses that diminish proinflammatory cytokines, may also play a role.Sterilizing immunity against infection is never fully achieved, and an asymptomatic carrier status is the rule among adults. This phenomenon of a high degree of immune responsiveness together with the nearly permanent presence of relatively low densities of parasites was originally described by Koch in 1900 and is often termed “premunition”(Doolan *et al.,* 2009).

However, Th1- and Th2 – type immune responses as surrogates for any immune response that changes with age independent of exposure and plays a critical role in infection outcomes. Th1-driven effectors may dominate the immune response of children, whereas Th2-driven effectors may dominate the adult immune response. These age – and exposure –dependent responses cause harm or benefit to the host.

2.6.1. T-Cell and Humoral Responses in Malaria

Natural or innate immunityto malaria is an inherent refractoriness of the host that prevents the establishment of the infection or an immediate inhibitory response against the introduction of the parasite. The innate immunity is naturally present in the host and is not dependent on any previous infection. Alterations in the structure of haemoglobin or in certain enzymes have been found to confer protection against either the infection or its severe manifestations and these traits are often found in areas of high malaria transmission.

Duffy negativity in red cells protects against*P. vivax*infection. It is found to be widely prevalent in Africa and this may be responsible for the virtual elimination of this parasite from the continent. Certain thalassemias (50% reduction in infection), homozygoushaemoglobin C (90% reduction), haemoglobin E, and ovalocytosis carrier status hav ebeen reported to confer protection against*P. falciparum*or*P. vivax*. Glucose 6 phosphate dehydrogenase deficiency (50% protection) and sickle cell haemoglobin (90% protection) confer protection against severe malaria and related mortality (Carter *et al.,* 2002, Doolan *et al.,* 2009). Natural exposure to sporozoites does not induce complete (sterilizing) antiparasite and antidisease immunity but only limit the density of parasitemia and thereby decrease the malaria-associated morbidity and mortality. The acquired immunity is directed predominantly against the asexual erythrocytic stage, the primary targets being the extracellular merozoites in circulation. Although the preerythrocytic stage is also targeted by protective immune responses, it does not effectively block sporozoite invasion or intrahepatic development of the parasite (Doolan *et al.,* 2009).

The presence of genetically and antigenically distinct strains of the parasites in a given locality and the occurrence of clonal antigenic variation during the course of an infection force the host to mount immune response against these different strains and antigenic variants. The acquisition of immunity against malaria is, therefore, very slow and not very effective and remains species

specific and strain specific. However, in areas with stable endemic malaria and intense malaria transmission, such as sub-Saharan Africa and forest areas in the Indian states, acquired immunity develops at a very early age. In these areas, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies. (Carter *et al.,* 2002) This passive immunity is followed by 1 or 2 years of increased susceptibility before acquisition of active immunity. The risk of clinical disease increases from birth to about 6 months of age, depending on the transmission rate, and beginning at around 3 to 4 months of age, infants become susceptible to severe disease and death (Doolan *et al*., 2009). The risk of cerebral malaria increases with age in children 2 to 4 years old. At about 2 to 5 years of age, due to repeated and frequent infections, the frequency of clinical disease begins to diminish and the risk of mortality sharply decreases, and by adulthood, most inhabitants generally possess sterilizing immunity. On the other, people living in unstable endemic areas tend to acquire only partial immunity ((Perlmann and Troye-Blomgerg, 2002; Kumar *et al*., 2007)

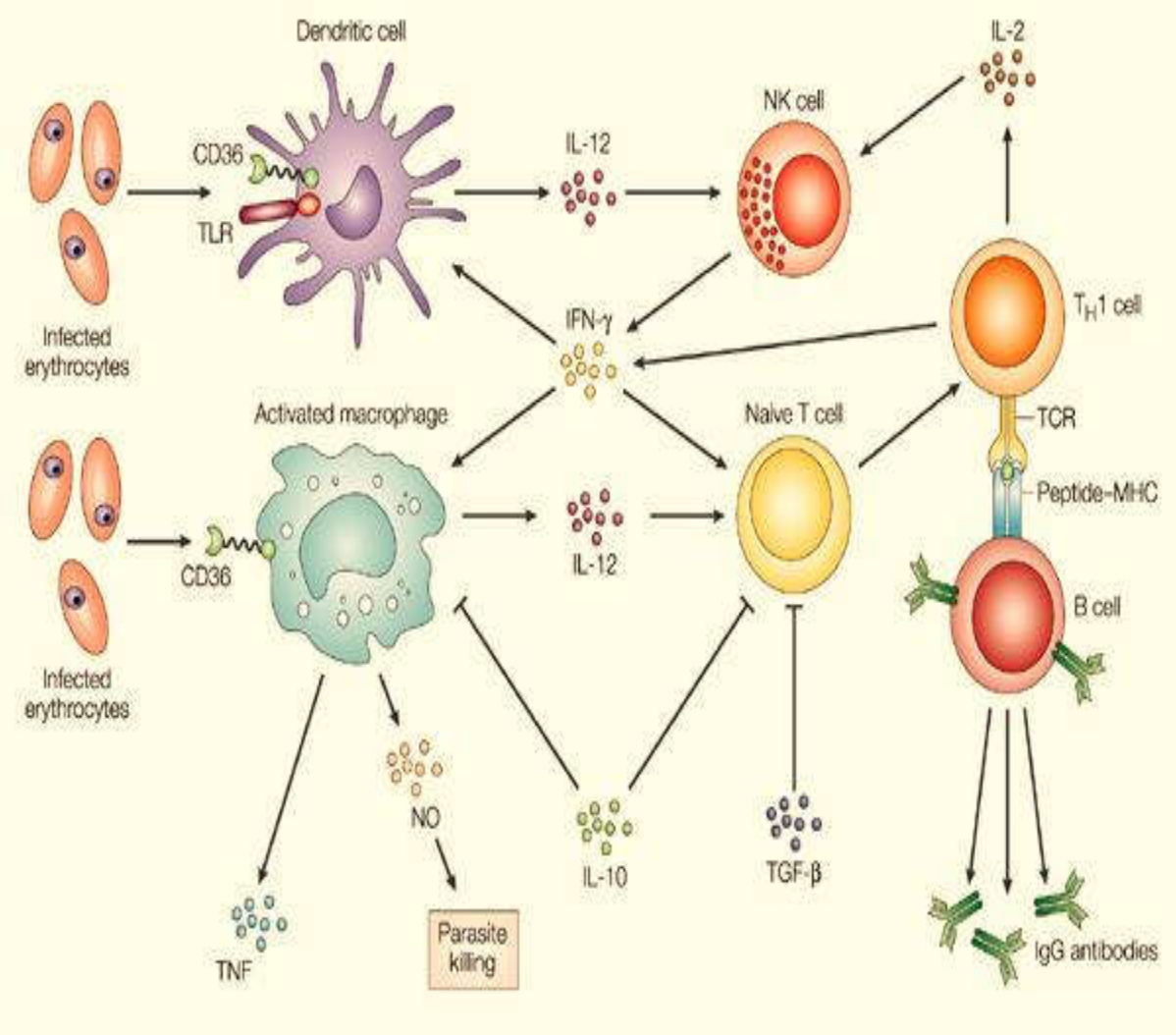

Figure 4: Regulation of Adaptive Immunity to Blood-stage Malaria by Cytokines Produced by Cells of the Innate Immune Response (Nature Reviews Immunology, 2004**)**

In response to parasite ligands recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, or inflammatory cytokines, such as interferon-gamma (IFN-γ), dendritic cells (DCs) mature and migrate to the spleen — the primary site of immune responses against blood-stage *Plasmodium*parasites. Maturation of DCs is associated with the upregulation of expression of MHC class II molecules, CD40, CD80, CD86 and adhesion molecules and the production of cytokines including interleukin-12 (IL-12) (Figure 4). IL-12 activates natural killer (NK) cells to produce IFN- γ and induces the differentiation of T helper 1 (TH1) cells. The production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive CD4+ T cells. IL-2 produced by antigen-specific TH1 cells further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response. Cytokines such as IL-10 and transforming growth factor-β (TGF-β) negatively regulate both innate and adaptive responses. NO, nitric oxide; TCR, T-cell receptor; TNF, tumour-necrosis factor. (Nature Reviews Immunology,2004).

Malaria infection induces both polyclonal and specific immunoglobulin production, predominantly IgM and IgG but also of other immunoglobulin isotypes. Of these, 5% or more represent species- as well as stage-specific antibodies reacting with a wide variety of parasite antigens. Passive transfer of IgG from immune donors may be protective by reducing parasitemia and clinical disease. Malaria infections of both humans and experimental animals are also associated with elevations in total IgE and IgE anti-malarial antibodies, reflecting a switch of regulatory T cell activities from Th1 to Th2 due to repeated exposure of the immune system to the parasites. IgE levels are significantly higher in patients with cerebral or other forms of severe disease than in those with uncomplicated malaria and the pathogenic effect of IgE is probably due to local overproduction in microvessels of tumor necrosis factor (TNF) and nitric oxide

(NO) caused by IgE-containing immune complexes (Perlmann and Troye-Blomgerg, 2002) (Figure 4).

Antibodies may protect against malaria by a variety of mechanisms. They may inhibit merozoite invasion of erythrocytes and intra-erythrocytic growth or enhance clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration in small vessels and promoting elimination by the spleen. Opsonization of infected erythrocytes significantly increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells such as neutrophils and monocytes/macrophages. Interaction of opsonized erythrocytes with these effector cells induces release of factors such as TNF which may cause tissue lesions but which are also toxic for the parasites. (Kumar *et al*., 2007, (Perlmann and Troye-Blomgerg, 2002).

Cell-mediated immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages. CD4 T cells are essential for immune protection against asexual blood stages in both murine and human malaria. However the role of CD8 T cells, which have important effector functions in pre-erythrocytic immunity and which contribute to protection against severe malaria, is less clear. It has been proposed that CD8 T cells may regulate immunosuppression in acute malaria and down-modulate inflammatory responses. As human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by CD8 cytotoxic T lymphocytes has a controversial role in the defense against blood-stage parasites (Imai *et al*., 2015).

Malaria parasites not only escape the host's immune response, owing to their antigenic diversity and clonal antigenic variation, but also modulate the immune response and cause significant immune suppression. The parasitized red cells, with the deposited hemozoin inside, have been

found to inhibit the maturation of antigen presenting dendritic cells, thereby reducing their interaction with T cells, resulting in immunosuppression. Immune suppression in malaria increases the risk of secondary infections (such as nontyphoidal Salmonella, herpes zoster virus, hepatitis B virus, Moloney leukemia virus and nematode infections and reactivation of Epstein-Barr virus) and may also reduce the immune response to certain vaccines. (Millington *et al*., 2006, Hisaedaa *et al*., 2005)

2.6.11. HOST-PARASITE RELATIONSHIP: ERYTHROCYTE INVASION BY *PLASMODIUM FALCIPARUM*

P. falciparum merozoites do not replicate outside of their host, they exist purely to find and invade erythrocytes by undergoing a series of complex manoeuvres which begins with initial contact with the erythrocytes (Glushakova *et al.,* 2005). Erythrocyte invasion is a complex process of which there are three invasive forms of the parasite (sporozoite, merozoite and ookinete). Merozoite invasion begins with the mature merozoites being propelled from the bursting schizont (the mature blood stage form) at egress (Glushakova *et al.,* 2005; Abkarian *et al.,* 2011), after which they associate with erythrocytes.Circumstantial evidence implicating Merozoite Surface Protein 1 (MSP-1) in erythrocyte invasion includes its uniform distribution over the merozoite surface and the observation that antibodies against MSP-1 inhibit invasion (Holder, 1994). Similarly, the circumsporozoite protein (CSP) probably plays a role in targeting sporozoites to hepatocytes by interacting with heparin sulfate proteoglycans (Sinnis and Sim 1997). The importance of MSP-1 and its processing are implied from the following observations:

- vaccination with the EGF-like modules can protect against malaria, and
- inhibition of the proteolytic processing blocks merozoite invasion.

The exact role(s) which MSP-1 and its processing play in the merozoite invasion process are not known. Other merozoite surface proteins are also involved in the interaction of the merozoite with the erythrocyte (Tham et al., 2012). Apical membrane antigen-1 (AMA-1) has been implicated in parasite reorientation on the erythrocyte (Mitchell, 2004). AMA-1 is a transmembrane protein localized at the apical end of the merozoite and binds erythrocytes. Antibodies against AMA-1 do not interfere with the initial contact between merozoite and erythrocyte thus suggesting that AMA-1 is not involved in merozoite attachment. But antibodies against AMA-1 prevent the reorientation of the merozoite and thereby block merozoite invasion. Following merozoite reorientation and microneme discharge a junction forms between the parasite and host cell.

Presumably, microneme proteins are important for junction formation. Proteins localized to the microneme include (Figure 5):

- EBA-175, a 175 kDa 'erythrocyte binding antigen' from *P. falciparum*
- DBP, Duffy-binding protein from *P. vivax* and *P. knowlesi*(including the RH family)
- SSP2, *Plasmodium* sporozoite surface protein-2.
  also known as TRAP (thrombospondin-related adhesive protein).
- Proteins with homology to SSP2/TRAP from *Toxoplasma* (MIC2), *Eimeria* (Etp100), and *Cryptosporidium*
- CTRP, circumsporozoite- and TRAP-related protein of *Plasmodium* found in the ookinete stage

In other words, these parasite proteins are probably involved inreceptor-ligand interactions with proteins exposed on the erythrocyte surface.Another family of adhesins involved in the binding of merozoites to erthrocytes are the reticulocyte-binding like homologues (Rh). These various adhesins bind to distinct receptors on the erythrocyte and provide redundancy in the ability of the

merozoite to form a junction with the erythrocyte (Tham, 2012). Another element of this junction involves a protein found in the neck of the rhopthry, in particular RON2. RON2 is released from the rhoptry and inserted into the membrane of the erythrocyte (Figure 6). RON2 then binds to AMA-1 which is localized to the surface of the merozoite (Tonkin, 2011). The RON2/AMA-1 complex then also contributes to this tight junction formed between the merozoite and erythrocyte.After a brief pause and major buckling of the erythrocyte surface, possibly as a result of parasite-induced reorganization of the erythrocyte cytoskeleton (Zuccala and Baum, 2011), the parasite enters the erythrocyte.

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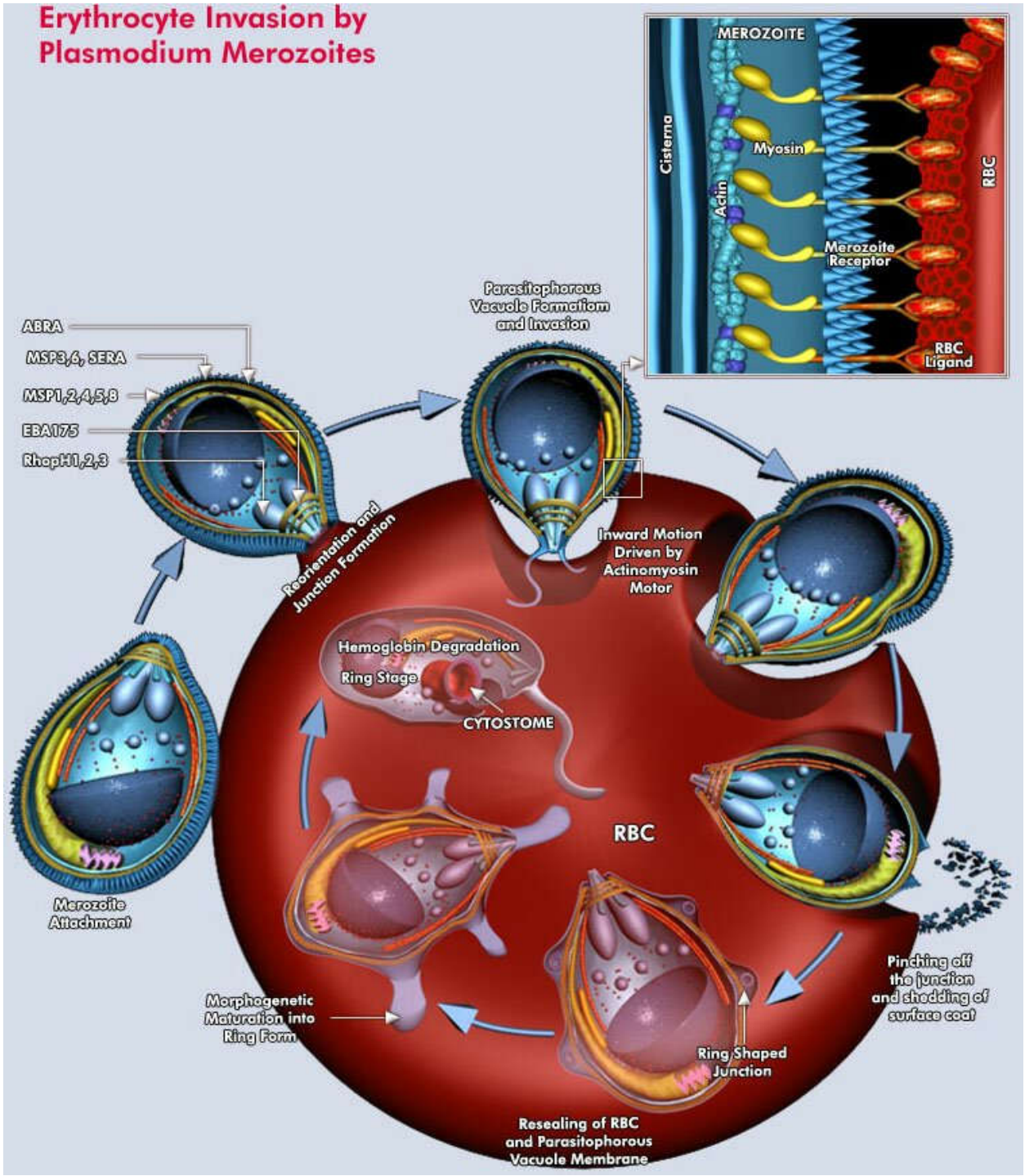

Figure 5: Schematic representation of Erythrocyte invasion by *Plasmodium falciparum* (http://www.malariasite.com/category/the-disease/)

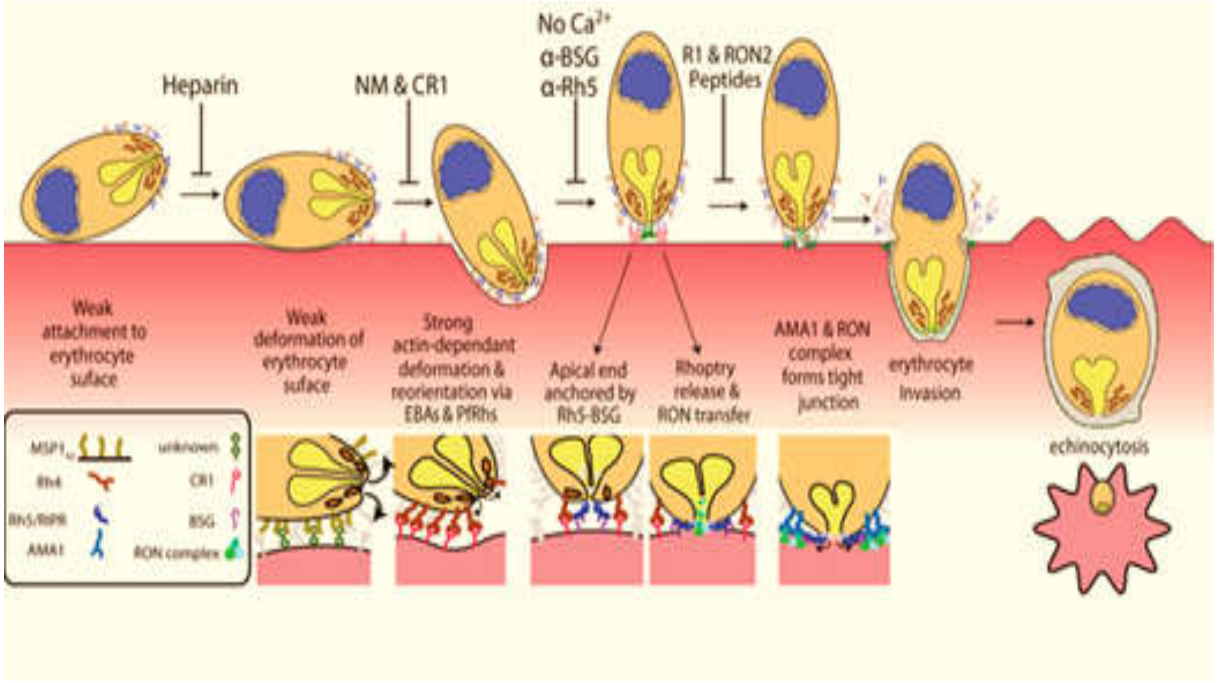

Figure 6: Model for the sequence of Interactions durin *P.falciparum* invasion of an Erythrocyte (Weiss *et al*., 2015)

2.6.2. *Plasmodium falciparum* Apical Membrane Antigen 1 (*Pf*AMA1)

The *P. falciparum* apical membrane antigen 1 (AMA1) is an 83 kDa membrane protein expressed in the late schizont stage of the parasite. Compared to several other blood stage antigens(MSP-1,MSP-2,etc), AMA1 of *P. falciparum* shows limited interstrain polymorphism (Dutta *et al.,* 2007);and is expressed in two critical life-cycle form,the sporozoite,which invades hepatocytes,and the merozoite,which invades red blood cells,and so offers a unique opportunity as a non-stage –specific vaccine target(Bai*et al.*, 2005).Although AMA1 is less polymorphic than other sporozoite or merozoite proteins, some extent of variation are present across the entire AMA1 sequence but the rate of non-synonymous (dN) mutations at domain I (aa 138–308) has always been higher indicating that this domain is under strong diversifying selection(Garg*et al.,* 2007). Knowledge of the distribution of the polymorphic sites on the surface of AMA1 is necessary to obtain a detailed understanding of their significance for vaccine development. (Bai*et al.,* 2005).

Studies show that AMA1 is immunogenic in malaria exposed individuals, and naturally acquired antibodies to AMA1 can inhibit merozoite invasion in vitro, but antibodies to strain 3D7 AMA1 were non–inhibitory for strain W2mef and this was shown to be due to AMA1 sequence differences of these two strains (Healer *et al.,*2004). Results from study with 51 samples from Ibadan showed that Domain I and III of *Plasmodium falciparum*AMA 1 nucleotide sequence are strongly polymorphic under a high intraspecific non-synonymous polymorphism.Recently, AMA-1-based adjuvanted protein vaccine was evaluated in clinical trials in Mali, based on AMA-1 derived from the 3D7 clone of *P.falciprum*, respectively and the FMP2.1/AS02A vaccine had a good safety profile and highly immunogenic (Thera *et al.,* 2010).It has been conclusively demonstrated, (Healer *et al.,* 2004), that sequence heterogeneity within AMA1 allows escape from inhibitory antibodies.

2.6.3*Plasmodium falciparum* Reticulocyte binding Protein Homolog 5(*Pf*RH5)

Recently, the family of *Plasmodium falciparum (Pf)* reticulocyte binding –like homolog proteins (PfRh) has attracted attention as a key determinant of erythrocyte invasion. PfRH comprises of 5 members: PfRH1, PfRH2a, PfRH2b, PfRH4 and PfRH5 of which PfRH5 plays the major role in invasion (Cowman and Crabb, 2006). Further, PfRH5 was found to be unique in being the only erythrocyte binding ligand among the EBA/PfRH families that essential for the parasite, as it cannot be genetically knocked out, suggesting a crucial role in erythrocyte invasion (Baum *et al*., 2009).

Of the PfRH families, PfRH5 is exceptional compared to other PfRH homologues, as it is smaller (63 kDa) and lacks a transmembrane domain (Baum *et al.,* 2009). PfRH5 has been shown to be localized on the merozoite surface in association with another parasite molecule, PfRipr (*P. falciparum*PfRH5-interacting protein) (Chen *et al.,* 2011). However, polymorphisms in PfRH5 as well as other*P. falciparum*adhesins have been shown to induce changes in their receptor specificities (Hayton *et al.,* 2013). It has been demonstrated that the specificity of PfRH5 binding is affected even by a single nucleotide polymorphism (SNP). PfRH5 is essential as it cannot be genetically knocked out suggesting a crucial role in erythrocyte invasion.

2.6.4 Merozoite Surface Protein 2 (MSP2)

Merozoite surface protein 2 (MSP2), is a blood-stage protein essential for viability and completion of the*Plasmodium*life cycle in humans. MSP2 is a glycosylphosphatidylinositol (GPI)-anchored protein present on the merozoite surface consisting of about 200–250 amino acids, encoded by a single exon on chromosome 2 (Carvalho *et al*., 2002). It contains conserved N- and C-terminal (C) regions flanking a highly polymorphic central repeat region. While its

function is not known, it induces specific antibodies that are active*in vitro*against parasite merozoites and are associated with protection in endemic areas (Anders and smythe 1989).

A non-repeat semi-conserved dimorphic (D) region defines the two allelic families of MSP2: 3D7 and FC27. It has been shown that specific semi-immune Ab against MSP2 protein is predominantly cytophilic IgG3, as in other blood stage proteins (Balam*et.al*., 1998). These cytophilic (IgG1 and IgG3) antibodies are thus thought to play an important role in antibody-mediated mechanisms of parasite clearance (Balam*et al,* .2014)

As part of immune evasion mechanism, *msp*-2 DNA sequences include variable block 3 which generates antigenically diverse forms that can be used to distinguish by size the different alleles after PCR amplification This gene is represented as a single copy on *P. falciparum* genome and high degree of polymorphism has been reported in the central variable region for *msp2* gene (Ibara-Okabande *et al.,* 2011). Typing of this polymorphic *P. falciparum* genome region has permitted the determination of malaria infection indicators e.g. diversity of *P. falciparum* strains and multiplicity of infection (MOI), which contribute to the description of malaria situation in a given location. Genotyping of the *msp*-2 gene therefore is a standard method for assessing MOI in *Plasmodium falciparum* studies, as it is highly polymorphic in length and sequence (Polley and Conway, 2001)

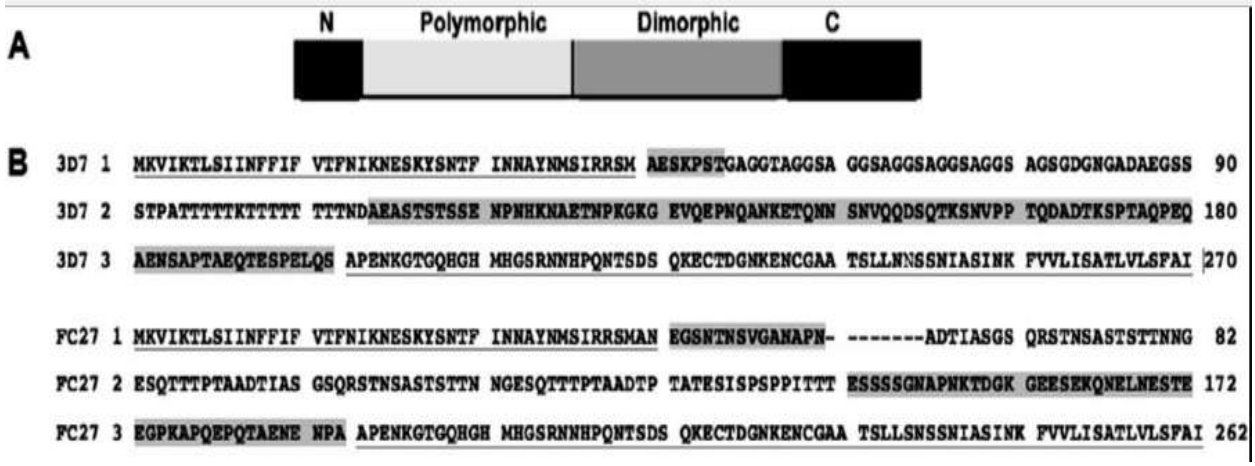

Figure 7: Schematic structure and full-length alignment sequence of MSP2 (Balam *et al*, 2014)

Schematic structures of merozoite surface protein 2 (MSP2) with different domains. Mature MSP2 consists about 200–250 amino acids and contains two conserved domains (black, N-and C-termini) flanking a polymorphic region that includes repetitive sequences and a non-repetitive dimorphic domain . The lengths of the non-conserved domains are strain-dependent

2.7.0 MULTIPLICITY OF INFECTION

Multiplicity of infection (MOI) is the number of different *P. falciparum* strains co-infecting a single host. In malaria endemic areas, MOI can be a useful indicator of the transmission level (Vafa *et al*., 2008). MOI can also be an indicator of the immune status. In areas with stable malaria transmission, MOI seems to increase as immunity develops, while in asymptomatic children, MOI is suggested to reflect acquired immunity or premunition and also to influence the risk of subsequent malaria attacks (Smith *et al*., 1999).

2.8.0 MALARIA PATHOGENESIS

Both parasite- and host-related factors contribute to the pathogenicity of the severe forms of malaria.All the clinical symptoms of malaria are the consequence of infection of human erythrocyte by merozoites.*P. falciparum*differs from other human malarial species in that infected red blood cells (IRBC) do not remain in the circulation for the entire life cycle. After 24–32 hours, when young parasites mature from the ring to the trophozoite stage, IRBC adhere to endothelial cells in the microcirculation of various organs. This phenomenon, termed “sequestration”, is believed to occur mainly to avoid splenic removal of IRBC. Sequestration causes microcirculatory obstruction, impaired tissue perfusion and inflammatory cells activation and it is linked to the severity of the disease (Autino *et al.,* 2012).

2.9.0MALARIA VACCINE: types and Status/Current Issues

A vaccine is an antigenic substance prepared from the causative agent of a disease or a synthetic substitute, used to provide immunity against one or several diseases.Despite many decades of intense research and development effort, there is no commercially available malaria vaccine at the present time. *RTS,S/AS01*is the most advanced vaccine candidate against the most deadly form of human malaria,*P. falciparum*. More than 20 other vaccine constructs are currently being evaluated in clinical trials or are in advanced preclinical development (WHO, 2016)

A Phase 3trial of *RTS,S /ASO1* began in May 2009 and completed enrolment in 2011, with 15,460 children in seven countries in sub-Saharan Africa. In July 2015, the European Medicines Agency issued a positive scientific opinion on the vaccine's risk-benefit balance. In October 2015, two independent WHO advisory groups recommended the pilot implementation of *RTS,S/ASO1* in parts of three to five sub-Saharan African countries. WHO has adopted these recommendations and is strongly supportive of the need to proceed with the pilots as the next step for the world's first malaria vaccine. *RTS,S/AS01* is being assessed as a complementary malaria control tool that could potentially be added to – and not replace – the core package of proven malaria preventive, diagnostic and treatment measures. (WHO, 2016)

2.9.1Major Vaccine Types

The difficulty of developing a highly effective malaria vaccine has led to the design and assessment of a very wide range of new approaches, arguably unparalleled in any other area of infectious disease vaccinology. Some of the vaccines types include (WHO, 2014b):

1. Recombinant vaccine e.g RTS,S Vaccine
2. DNA vaccine
3. Peptide-based vaccine e.g SPF66.
4. Prime-boost vector vaccine
5. Sub-unit vaccine
6. Recombinant virosome vaccines
7. Multiple antigenic peptide avccines
8. Transmission-blocking vaccines with sexual stage proteins
9. Pathogen challenge model vaccines
10. Whole parasite vaccines

2.10.0*PLASMODIUM FALCIPARUM* PARASITE ANTIGENIC VARIATION AND GENETIC DIVERSITY

Infectious agents have developed a range of mechanism allowing them to interact with their hosts through surface-expressed molecules while protecting themselves from the host immuneresponse (The persistence of the human malaria parasite *Plasmodium falciparum* during bloodstage proliferation in its host depends on the successive expression of variant molecules at the surface of infected erythrocytes This variation is mediated by the differential control of a family of surface molecules termed PfEMP1 encoded by approximately 60 *var* genes of which each individual parasite expresses a single *var* gene at a time maintaining all other members of the family in atranscriptionally sient state (Scherf *et al.*, 2008).

When antigens undergo variations randomly or programmed, randomly through DNA

alterations introduced by: (1) errors in the DNA or RNA replication and repair, (2)recombination between genes, or (3) re-assortment of gene segments.Programmed variations, also refered to as the Rue antigenic variation, is characterized by a family of genes called the paralogous genes, which encode proteins of similar structure and function and the ability to express only one of these genes at a time. This allows the parasite to alter the protein variants expressed from time to time (Borst, 2003).

It has been reported that diverse malaria isolates differ in their virulence pattern (James *et al.,* 1932). However, further work showed that immunity to malaria was strain-specific, emphasizing clonal antigenic variation in *Plasmodium falciparum* (Hommel *et al.,*1983). One likely hypothesis is that there is a frequent ongoing switching in a parasite population, expressing a dominant Variable Surface Antigen (VSA) repertoire on majority of the infected eryuthrocytes and one or few others on a small subpopulations of cells.Studies supporting this include *in nvitro* studies showing that switching of parasite populations occurs, and at rates as high as 20.4%, even in the absence of recognizing antibodies (Kaviratne *et al*., 2002).

When analysing sera from malaria patiens living in endemic regions, there is a strong correlation between the immunity of the individuals and the recognition of a broad range of VSAs (Kaviratne *et al*., 2002). This immunity is also associated with protection from severe disease symptoms, even though the patients carry parasites in their blood circulation (Treutiger *et al*., 1992).

Although PfEMP1 is the major ligand mediating rosette formation, detailed analysis of the surface of infected erythrocytes with radiolabelling, immunoprecipiation and trypsin cleavage revealed evidence for products of members of a second highly polymorphic multigene family

known as rif (repetitive interspersed family) translated into highly variable molecules, i.e RIFINS, inserted into the surface membrane and exposed on the surface (Fernandez *et al.,* 1999). REFINS are immunogenic and widely recognised by sera from semi-immune individuals living in malaria endemic areas (Abdel-Latif *et al*., 2002). Moreover, high titres of anti-RIFIN antibodies in patients correlate with rapid clearance of parasites upon treatment, and this reactivity is suggested to be the major ant-VSA response in natural malaria infection (Abdel-Latif *et al*., 2002).

Many studies on the parasite's polymorphisms have focused on variants exhibiting mutations that lead to amino acid substitutions (non-synonymous mutations) that are likely subjected to selection, such as immunogenic proteins and resistance phenotypes. Parasite genetic diversity determines the intensity of transmission, thus providing baseline data for antimalarial drug efficacy trial and the possibility of implementing control strategies based on vaccines.

2.11.0 PARASITE GENETIC STRUCTURE

Genetic diversity indices can be used as indicative of the adaptation and fitness of *Plasmodium* populations in a particular ecosystem (Schultz *et al.,* 2010). Genotyping of molecular markers is one of the most frequent approaches used to determine the structure of*P. falciparum* populations (Larranaga *et al.,* 2013). Genetic diversity parameters of parasite populations including the number of alleles expected heterozigosity (H_e), Linkage disequilibrium (LD), Fixation Index(Fst) and the number of haplotypes (*h*) are used inorder to determine parasite genetic structure (Larranaga *et al.,* 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 MATERIALS

All reagents used were of analytical grade while materials and consumables were sourced commercially.

3.1.1 METHODS

3.1.2 Description of Study Areas and Population

The study was cross-sectional, conducted in LagosState, Nigeria with an estimated 15 million people (NPC, 2017). The study population consisted of patients with symptoms of malaria (axillary temperature $\geq 37.5\ ^{o}C$, vomiting, etc) who voluntarily consented to participate in the study. Controls were Age-matched apparently healthy volunteers. The three study sites were**: (a)** Ijede General Hospital, Ijede, a secondary public health care facility **(b)** Ajeromi General Hospital, Ajegunle, a secondary public health care facility and **(c)** St. Kizito Primary Healthcare centre, Lekki a primary healthcare facility (Figure 8). Malaria transmission is reported to be high in these areas all year round while the selected facilities have above 500 outpatients per week. Sampling was done between March - September, 2013when the raining season was largely at its peak.

3.1.3 Site Description

Ijede

Ijede (IJE) is a rural settlement with ecological features favouring malaria transmission. It is an ancient town, under the Ikorodu Local Government Area, located along the Lagos Lagoon on Latitude 6^{0} 34' 00''N and Longitude 3^{0} 35' 18''; it shares boundary with Ogun State and takes a good percentage of the 535,619 enumerated population of Ikorodu according to the 2006 Census. IJGH was established in 1983 to provide healthcare facilities to residents of Ijede and environs. It is currently one of the centres characterised in 2011 as sentinel sites for malaria research in Nigeria.

Lekki

Lekki (LEK) is an urban outgrowth of highbrow Victoria Island Lagos into a setting of indigenes in the Ibeju/Lekki LGA (Latitude $4^0$15'North - $4^0$17'North and Longitude $13^0$15'East - $13^0$30'East). The population of dwellers was estimated to be 117,481 (NPC, 2006). St. Kizito Primary Healthcare centre is a Catholic mission hospital established to provide healthcare services to all the surrounding settlements. It averages about 500 outpatients per week.

Ajeromi

Ajeromi (AJE) is a low peri-urban settlement located in Ajegunle area of Lagos State. It is a district inhabited by about 57,276 people (2006 census) on Latitude 6^0 36' 22''N and Longitude 3^0 16' 57'' E. Ajeromi General Hospital, a secondary health care facility provides health care services to people within and around the district while others use the facility for other known reasons coming from distant parts of the state. It averages over 500 outpatients per week.

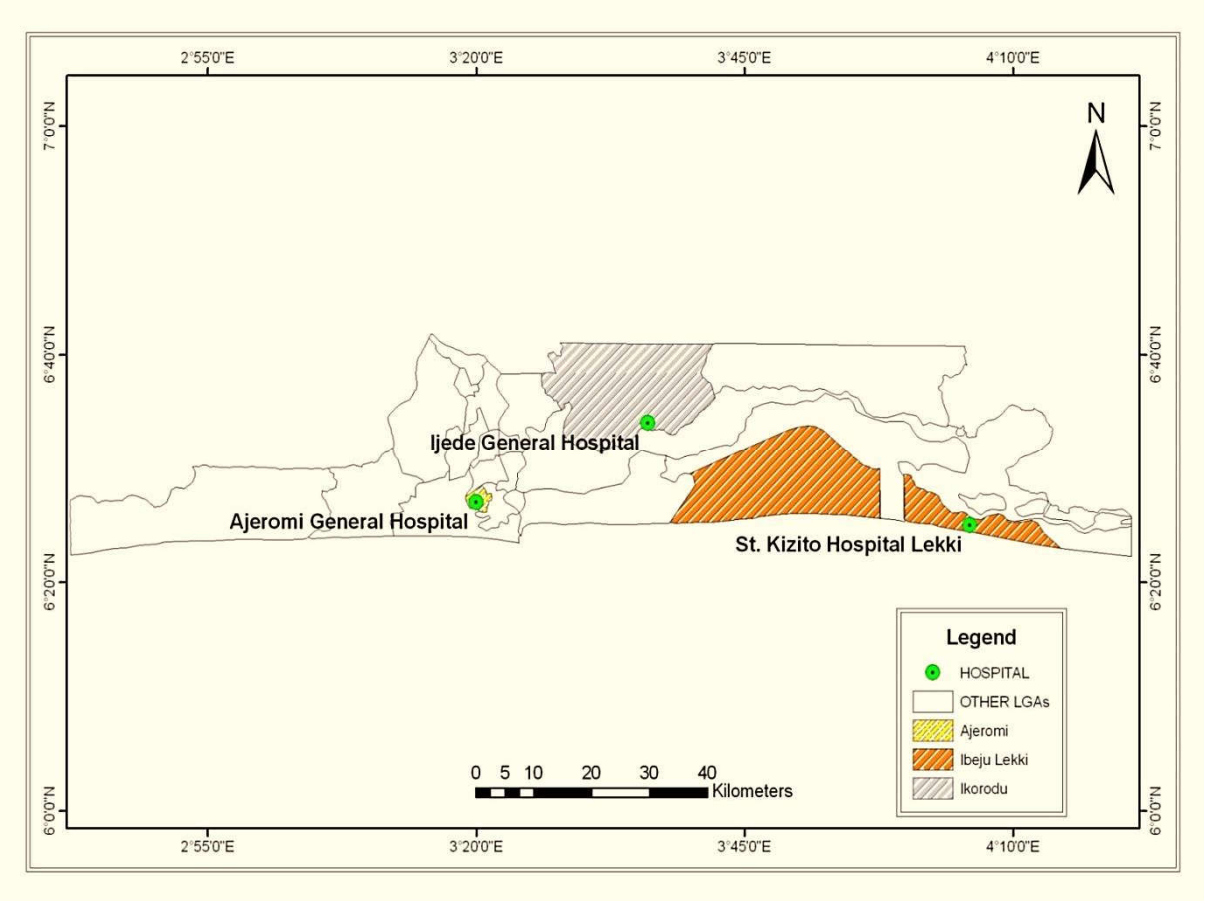

Figure 8: MAP OF LAGOS STATE SHOWING LOCATION OF THE THREE STUDY SITES

3.2ETHICAL CONSIDERATION

Ethical approval (Ref No: IRB/10/136) to conduct this study was obtained from the Institutional Review Board of the Nigerian Institute of Medical Research, Lagos (Appendix 1). Permissions were obtained from the different health facilities where the study was carried out. All the research participants gave written informed consent, guardian/parental consent and/or assent for participants less than 16 years (children). Participants who declined participation were not denied provided treatment. Generally, the study was conducted in line with the principle of Good Clinical Laboratory Practice (GCLP) and the 1994 Helsinki Declaration.

3.3. INCLUSION CRITERIA

(i) Age ≥12 months

(ii) Patients with *Plasmodium falciparum* mono-infection.

(iii) Signs and Symptomsof malaria (Axillary temperature ≥37.5^{o}C, fever or history of fever in the last 2-3 days, vomiting, general body weakness etc).

(iv) Consent of patients, parent or guardian and/or asssent in the case of children.

3.4.EXCLUSION CRITERIA

(i) Patients with mixed *Plasmodium* infection

(ii) Positive HIV result

(iii) Pregnancy

(iv) Presence of symptoms, findings on clinical examination, or additional diagnostic tests indicating other infections.

3.5SAMPLE SIZE DETERMINATION

The sample size was calculated using the World Health Organization's statistical method (WHO, 2003) based on the formula:

$$n = \frac{Z^2 pq}{d^2} \mathbf{1}$$

where: n=sample size; Z=1.96 (95% confidence level) ; d=Sample error (5%)=0.05

p=Prevalence = 14.7% (0.147) (Aina *et al*., 2013);

q = Complementary probability= 1-p

=1-0.147=0.853;

$$n = \frac{1.96^2 * 0.147 * 0.853}{0.05^2} = 194.18.$$

Three hundred study participants and 80 apparently healthy individuals were included in the study.

3.6SAMPLING

Finger-pricked blood samples were collected from each participant for malaria parasite detection and quantification. Using the finger-pricked blood samples, one thick and thin blood films were made on same slide per participant to be Giemsa stained for microscopy. Parasite densities were estimated in thick blood films, assuming an average white-blood-cell count of 8,000/µl. All slides were Giemsa stained and read by two certified Microscopists. Venous blood samples of positive patients (who were afterwards treated with artemether-lumefantrine (20-120mg) according to national policy (FMoH, 2005a) and 80 age-matched apparently healthy controls, were collected into appropriate bottles. All included samples were HIV negative, screened with DETERMINE^{R} (HIV RDT) kit. Approximately 4ml of venous blood sample was collected from each participant into ethylene diamine tetracetic acid (EDTA) –coated vacutainer and plain tubes for hematologic, biochemical and immunologic analysis while about two drops of blood were

spotted onto 3 mm Whatman® filter paper (Whatman International, England) for molecular analysis. Blood collected on the filter paper was air-dried and stored in a sealable nylon drug sachet, protected from sunlight and kept in a desiccator for DNA extraction and other molecular analyses.

3.6.1 MICROSCOPY

Malaria diagnosis by microscopy was carried out in accordance with WHO protocol (WHO, 1991). The thick and thin blood films were prepared on a single microscope slide per participant. The thick film was made with 12µl of blood taken with adjustable micropipette (Eppendorf 1-100 µl, Eppendorf AG) spread over a diameter of 15mm for parasite identification and quantification while 2µl of blood taken with adjustable micropipette (Eppendorf 0.5-5µl) was used for the thin film to differentiate the species. The thin film was properly fixed with absolute methanol and air-dried before staining. Malaria Blood Films (MBFs) made were stained with 10% (v/v) Giemsa stain (pH 7.2) for 10 minutes and examined for the presence and number of malaria parasites under the x100 objective (oil-immersed) of the microscope (Olympus CX21, UK). Examinations were made up to 2-200 high-power fields depending on the parasitaemia while the negative results for parasites were determined with 200 high-power fields in the thick blood films (Greenwood and Armstrong, 1991).The stained slides were read by two competent microscopists while discordant readings were resolved before final result was taken using the mean parasite counts of the two readers. The malaria parasite density (MPD) (parasite per µl of blood) was calculated using the formula:

$$\frac{\text{Number of parasites counted} \times \text{Total Leukocytes (8000)}}{200\ (\text{Number of Leukocytes (WBC)})} = \text{Parasite/µl of blood} \quad \mathbf{2}$$

3.6.2 ANTHROPOMETRIC MEASUREMENTS

Anthropometric data were generated in accordance with internationally recommended procedures (WHO, 1995). Weight measurements were taken using a scale (Téfal, Paris, France) precise to the nearest 100 g. Recumbent length measurements were taken for children under 2 years of age, while standing height was measured for older participants using a stadiometer (SECA, Hamburg, Germany). BMI (Kg/m^2) was calculated for the participants.

Theparticipants that werewithin the range 18.5 24.9 were grouped as Normal, while those less than or above this range were categorized Underweight (< 18.5) or Overweight (> 24.9), respectively.

For participants within the age of 1 and 20 years, anthropometric status was further determined using the International Reference Population defined by the U.S National Centre for Health Statistics (NCHS) as stunting, wasting orunder-weight when Z-score is less than -2.

$$Z \quad \text{score} = \frac{\text{Actual (Wt or Ht)} - \text{Average (Wt or Ht)}}{\text{Standard Deviation (SD)}} \mathbf{3}.$$

Where Ht=height; Wt= weight

3.6.3IMMUNOLOGICAL ASSAY

Levels of Tumour Necrosis Factor alpha (TNF-α), Interleukin 12 and 1-beta (IL-12 & IL-1ß) were assessed in sera of included participants using antigen capture ELISA kits (Enzyme Linked Immunosorbent Assay) (Kit Lot: WH-110) based on antigen-antibody reaction. Standards (ng/L) and serum samples were added into ELISA pre-coated plates (Maxisorb; NUNC Denmark) using appropriate diluents according to the manufacturer's manual. After incubation and washing step (WASHER: BioTeK Instrumentals, USA), goat anti-human IgG HRP conjugate reagent (Sigma A9544) was added followed by addition of chromogen solution A. The Optical Density (OD) was read within 15 mins after adding stop solution at 450 nm on an ELISA reader (BioTek EL

800, Biotek Instrumentals, USA) and the concentrations extrapolated from the standard curve obtained using values for the standards (Voller *et al*., 1978).

3.6.4 HAEMATOLOGY

EDTA anticoagulated blood samples were used for the determination of the haematological parameters by the ADVIA^{R} 60 Closed Tube (CT) Automated haematology system (TA9-216E01, Bayer,UK). Parameters determined included haemoglobin, platelets, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), lymphocytes, monocytes and granulocytes.

3.7 MOLECULAR DETERMINATION OF HAPLOTYPIC VARIATIONS IN *AMA*1 AND *PF*RH5

DNA samples of 195 MSP2 positive *P. falciparum* malariasamples from the recruited participants were used in molecular analysis for the determination of polymorphisms in *P. falciparum* Apical Membrane Antigen-1 (PfAMA1) and *P. falciparum* Reticulocyte Homologous Protein-5 (*Pf*RH5) in the isolates.

3.7.1 MALARIA PARASITE DNA EXTRACTION FROM DRIED BLOOD SPOTS (DBS) USING QIAGEN QIAAMP DNA EXTRACTION MINI-KIT

Two pieces of 3mm disk from the filter paper blood spots were punched out using a sterile hole Punch and placed into appropriately labelled 1.5 micro-centrifuge tube. The punch was cleaned and sterilized each time in sequence with 5% bleach followed by distilled water and then 70% ethanol. Animal tissue lysis buffer, 180 µl, was added ensuring the filter paper pieces were soaked before incubation at 85^{o}C for 10 minutes followed by addition of 20 µl of proteinase K stock solution. The mixture was vortexed and incubated at 56 ^{o}C for 1 hour after which buffer AL was added to the sample, thoroughly mixed by vortexing and incubated at 70 ^{o}C for 10 minutes. Absolute ethanol (200 µl) was added and mixed thoroughly by vortexing. The mixture

was then added to a QIAamp Mini kit spin column placed in a 2 ml collection tube and centrifuged at 6000 x g (8000 rpm) for 1 minute. The spin column was removed and placed in a clean 2ml well labelled collection tube while the filtrate was discarded with the tube. Buffer AW1 (500 µL) was added and the mixture centrifuged at 6000 x g (8000 rpm) for 1 minute. The collection tube containing the filtrate was discarded. Buffer Aw2 (500 µl) was added to the spin column and then centrifuged at full speed (20,000 x g or 14,000 rpm) for 3 minutes. Again, the filtrate was discarded and the spin column was placed in a 1.5 mL microcentrifuge tube. DNA was eluted with 150 µl elution buffer AE, incubated at room temperature for 1 minute and centrifuged at 6,000 x g (8000 rpm) for 1 minute. The extracted DNA was stored -20 ^{o}C until it was used for subsequent molecular studies (Ausubel *et al.,* 1991).

3.7.2 PARASITE DNA YIELD AND QUALITY DETERMINATION

The quality and quantity of extracted DNA were determined using the spectrophotometric method with NANODROP *1000*^{R} **(**Thermo Fisher Scientific, USA), which quantified the amount of extracted DNA in nanogramme per microlitre (ng/µL) and assessed the quality (purity) based on the ratio of absorbance at 260nm : 280nm for all the samples (Tiwari and Kumar*,* 2011).

3.7.3 PARASITE GENOTYPING BY PfMSP2

To differentiate the parasites based on clonal origin and distribution, oligonucleotide primers from published sequences, as listed in the nucleotide database of NCBI, were used to amplify the block 3 of MSP-2 (Table 2). The gene was amplified from each sample by nested-PCR, each amplification with conserved, or family-specific primer pair, being done separately as described by Snounou*et al*(2002). The genes were amplified in 50µl reaction mixtures containing: 1.25U BioTaq, TM DNA polymerase (Bioline, London, UK), 1x Taq polymerase reaction buffer, 2.5mM $MgCl_2$, 0.25 mM dNTPs and 1µM of each primer. The reactions included hot start at 95 ^oC for 5 mins, 30 cycles of denaturation at 94 ^oC for 30 seconds, annealing at 55 ^oC for 2 mins and extension at 72 ^oC for 2 mins followed by a final Extension at 72 ^oC for 5 mins, and finally hold at 10 ^oC. The secondary reactions were same as the primary PCR reactions using the nest-2 primers. PCR products were analysed by electrophoresis

Table 2: Merozoite Surface Protein-2 (MSP2) PCR Primer Sequences

Primer	Primer sequence	Description
M2-OF	ATGAAGGTAATTAAAACATTGTCTATTATA	Conserved
M2-OR	CTTTGTTACCATCGGTACATTCTT	Conserved
FC27M2-F	AATACTAAGAGTGTAGGTGCARATGCTCCA .	FC27 Family- specific
FC27M2-R	TTTTATTTGGTGCATTGCCAGAACTTGAAC	FC27 Family-specific
IC/3D7M2-F	AGAAGTATGGCAGAAAGTAAKCCTYCTACT	3D7 Family-specific
IC/3D7M2-R	GATTGTAATTCGGGGGATTTGTTCG	3D7 Family-specific

Primers for MSP2 amplification by family

3.7.4 AGAROSE GEL ELECTROPHORESIS

Amplification was confirmed by electrophoresing PCR amplicons on 1.2% w/v agarose gel(stained with ethidium bromide, 0.5 mg/mL) in Tris-borate EDTA. Electrophoresis was carried out at 120V, 50W, 300mA for 45 minutes. On completion of electrophoresis, bands were visualized with the gel documentation system (Infinity 3026, France). The sizes of the fragments obtained were estimated by comparison to the 100 bp (100bp - 1.3Kb) DNA ladder (Jena Bioscience GmbH, Germany) run alongside with standard purified genomic DNA from 3D7 laboratory strain similarly electrophoresed.

3.7.5 MULTIPLICITY OF INFECTION (MOI) OR NUMBER OF GENOTYPES PER INFECTION

The MOI was determined by dividing the total number of fragments detected by the number of samples positive for same marker.

$$\text{MOI} = \frac{\text{MOI=Total number of fragments (genotypes)}}{\text{Number of samples positive for the marker}} \quad \mathbf{4}$$

3.7.6 HETEROZYGOSITY

As a measure of parasite genetic diversity, the expected heterozygosity *(H_E)* which represents the probability of being infected by two parasites with different alleles at a given locus and ranging between 0 and 1 was calculated using the formula below:

$$H_E = [n/(n-1)]\ [(1-\sum P_i^2)] \quad \mathbf{5}$$

where n = sample size, Pi = allele frequency. H_E estimates the fraction of all parasites that would be heterozygous for msp-2 loci.

3.7.7 AMPLIFICATION OF *PfAMA1* GENE

The complete 2 kb AMA1 gene was amplified using the AMA1-F (5'-ACAAAAATGAGAAAATTATACTGC-3')andAMA1-R (5'-TTTTAATAGTATGGTTTTTCCATC-3')primers (Garg *et al.,* 2007). The cycling parameters for the PCR were as follows: 10 minutes initial denaturation at 94°C followed by 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 55°C, 2 minute extension at 72°C and a 10 minutes final extension at 72°C. The primary PCR product was diluted 10-times and 2 µl used in nested-PCR to amplify the 500 bp region encompassing domain I (or hyper-variable region-HVR) using the AHVR-F (5'-CTGGAACTCAATATAGACTTC-3') and AHVR-R (5'-TTCTTTCTAGGGCAAACTTTTTC-3') primers.The cycling parameters for the nested primers were the same as for primary PCR primers except that the extension at the 72^{o}C would be carried out for 1 minute. All PCR reactions were carried out in a total volume of 50 µl containing 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 µM of each primer, and 1 unit of AmpliTaq Polymerase (Perkin Elmer, England). In the first round reaction, 2 µl of genomic DNA was added as a template. In the nested reaction, 1 µl of the outer PCR product was used. Positive (3D7) and negative (water) controls were included in each set of reaction.

3.7.8 AMPLIFICATION OF *PF*RH5 GENE

*Plasmodium falciparum*Reticulocyte binding protein homolog 5 (PfRH5) gene was amplified from the genomic DNA (gDNA) collected as Dry Blood Spots (DBS) on filter paper Whatman 3 using the primer set sequences namely: RH5-F1 :5'-ACCCATGAGGAATTGAGTC-3' and RH5-R1: 5'-CGGTTTCATCATCTGTCTC-3' amplifying the region encoding HABPs 36718, 36727 and 36728 (Arevalo-Pinzon *et al*., 2012).

The cycling parameters for the PCR were as follows: 10 minutes initial denaturation at 94°C followed by 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 55°C, 2 minute

extension at 72°C and a 10 minutes final extension at 72°C. All PCR reactions were carried out in a total volume of 50 µl containing 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 µM of each primer, and 1 unit of AmpliTaq Polymerase (Perkin Elmer, England). In the first round reaction, 2 µl of genomic DNA was added as a template. In the nested reaction, 1 µl of the outer PCR product was used. Positive and negative controls were included in each set of reaction.

3.7.9.1GENE CLEAN-UP

Gel slices containing the excised DNA fragment of interest were cleaned with Qiagen QIAquick, DNA purification kit (Qiagen, UK) using the spin column chromatography method.

3.7.9.2NUCLEOTIDE SEQUENCING AND SEQUENCE ANALYSES

The complete 500 bp AMA1 Domain I and 810 bp RH5 HABPs region DNA bands excised from the 1.2% agarose gels after PCR amplification, were purified, cleaned and sequenced in both forward and reverse direction using an ABI3777 automatic DNA sequencer (Applied Biosystems).

3.8BIOINFORMATICS

The gene sequences were edited and the translated amino acids aligned amongst themselves and also with *P. falciparum*3D7 reference AMA-1 sequence [GenBank accession number XM_001347979.1] and RH5 sequences [XM_001351508.1] using the Bioedit software and CLUSTAL W in MEGA 6 (Tamura *et al*., 2013). BLAST searches were done to compare the AMA-1 and RH5 haplotypes with the corresponding sequences available in the Genbank database. The sequences were deposited in the GenBank database of NCBI (National Centre for Biotechnological Information).

3.9.STATISTICAL AND GENETIC ANALYSES

Parametric data generated from the study were analysed using One-way ANOVA and Tukey for multiple comparison while non-parametric data were analysed using Kruskal-wallis and Dunn's multiple comparison. Test for association were carried out by Chi-square analysis. Test of statistical significance was set at P value less than 0.05 at 95% confidence interval.

The numbers of segregating sites (S), observed nucleotide diversity per site between any two sequences assuming that the samples are random (∏), number of haplotype (H), number of mutations, haplotype diversity (Hd), Tajima D, Linkage disequilibrium and the rate of non-synonymous minus synonymous (dN-dS) were determined using DnaSP version 5.10.1 (Spain) while phylogenetic analysis was done by neighbour-joining (NJ) method with Kimura-2-parameter distance matrix.

CHAPTER FOUR

4.0RESULTS

4.1 DEMOGRAPHIC PROFILE OF PARTICIPANTS

Three hundred and eighty four febrile subjects comprising of 185 males and 199 females were positive for *P. falciparum* malaria out of the 1883 examined at the three different study areas in Lagos State. Of the 384 microscopically confirmed malaria patients, 41.93% were <5 years, 300 (100 per site) were included in this study. Overall, median age was 10 years and prevalence of *P. falciparum* malaria among the febrile participants was 20.39% ordered as IJE>AJE>LEK (Table 3). Approximately 36.25% of the uninfected healthy controls included were < 5 years (Table 3).

4.2 MALARIA PARASITE DENSITY DISTRIBUTION

There was no statistically significant difference in the geometric mean parasite density (GMPD) of samples from male and female patients. However, patients < 5 years had higher GMPD ($p< 0.05$) than those ≥ 5 years (Table 4).

4.3 BODYMASS INDEX (BMI) STATUS OF PARTICIPANTS

Based on participants BMI values, rate of underweight was highest in IJE (68%) while overall 136 (45.33%) participants were Underweight (Table 5). Table 6 shows status of all participants in the study with 50% of the uninfected controls being underweight. Among the participants within the age range 1-20 years, 23.75% were stunted of which 14 were severely stunted and 38 (47.5 %) were wasted of which 19 were severely wasted (Table 7).

Table 3: Demographic Profile of Study Population.

Profile		Ijede	Lekki	Ajeromi	Total
Number of subjects tested for *P. falciparum malaria*		480	616	787	1883
Number of subjects microscopically positive for malaria		114	117	153	384
Prevalence of Symptomatic Malaria (%)		22.05	18.99	19.44	20.39
Sex of participants (Infected)	Male	56(49.12%)'	58(49.57%)	72(47.06%)	185(48.18%)
	Female	58(50.88%)	59(50.43%)	81(52.94%)	199(51.82%
(Uninfected)	Male	11(50%)	14(50%)	15(50%)	40(50%)
	Female	11(50%)	14(50%)	15(50%)	40(50%)
Participants Age Groups					
Infected	< 5years	32	51	78	161
	≥ 5 years	82	66	75	223
Uninfected	< 5years	8	10	11	29
	≥ 5 years	14	18	19	51

Overall, prevalence of malaria among the febrile participants was 20.39%, ordered as Ijede> Ajegunle>Lekki

Table 4: Malaria Parasite Density of Participants

Group	% (No)	Geometric Mean Parasite density (GMPD) parasite/µL	p-value
Male	48.3 (145)	44,239 ± 2,985	0.43
Female	51.7(155)	37,203 ± 4,751	
< 5 years	32.7 (98)	136,267 ± 1,449	0.04
≥ 5years	67.3 (202)	32,912 ± 1,256	

Participants <5 years had GMPD higher than those ≥5 years, there was no significant difference in GMPD between male or female. Values are Mean ± S.E.M

Table 5: BMI Status of Infected Participants from the Three study Sites

Nutritional Status(kg/m^2)	**IJE (N)**	**LEK (N)**	**AJE (N)**	**Total Number (%)**
Underweight (BMI < 18.5)	68	33	35	136 (45.33)
Normal (BMI 18.5-24.9)	22	48	37	107 (35.67)
Overweight (BMI > 24.9)	10	19	28	57 (19)
TOTAL	100	100	100	300 (100)

Rate of underweight was observed as Ijede> Ajegunle>Lekki, overall 45.33% were underweight.
BMI: body mass index. N: number of participants.

Table 6: BMI Distribution among Participants

STATUS(kg/m^2)		**NUMBER**	**%**
Underweight (BMI<18.5)	Infected	136	45.33
Uninfected		40	50
Normal (BMI=18.5-24.9)	Infected	107	35.67
Uninfected		32	40
Overweight (BMI > 24.9)	Infected	57	19
Uninfected		8	10
Total	Infected	300	100
Uninfected		80	100

High rate of Underweight in the settings studied including the uninfected group

Table 7: Classification of Participants Under Twenty years by Nutritional Status

Measure		N (80^{n} : 26^{n1}) (%)	Z-score	Status
	Test	19 (23.75%)	< -2 =5	Stunted
HAZ			< -3 =14	Severe stunting
	Control	2 (7.69%)	< -2 =2	Stunted
	Test	23 (30%)	< -2 =14	Under-weight
WAZ			< -3 = 9	Severe underweigh
	Control	10 (38.4%)	< -2=10	Underweight
	Test	38 (47.5 %)	< -2 =19	Wasted
WHZ			< -3 = 19	Severe wasting
	Control	14 (53.8%)	< -3 = 14	SeverelyWasted

HAZ=Height-for-Age, WAZ=Weight-for-Age, WHZ=Weight-for-Height-Z-score. N=number of participants < 20 years underweight; n^{i} = number of uninfected controls < 20 years Underweight. Majority of the participants were wasted both infected and controls, 47.5 % and 53.8% respectively.

4.4 RELATIONSHIP BETWEEN BMI STATUS AND MALARIA PARASITAEMIA

At lower malaria parasitaemia, the infection rate was higher among participants with normal BMI status than the underweight and overweight groups which had higher number of participants having between 1000 – 100,000 parasites/µL. Malaria prevalence was significantly associated with BMI status of participants (χ^2= 14.45, df = 4, p =0.006). Malnourished participants were more heavily infected than those with normal nutritional status (Table 8)

4.5 EVALUATION OF CYTOKINE RESPONSE

TNF-α, IL-1β and IL-12 were significantly higher ($p < 0.05$) among participants from Lekki than those from Ijede and Ajeromi (Table 9).However, overall, TNF-α, IL-1β and IL-12 were significantly higher ($p< 0.05$) in infected participants than uninfected controls (Table 10**).** Although the study showed no significant difference in the levels of the pro-inflammatory cytokines studied ($p>0.05$) based on the parasite densities, however, IL-12 and IL-1β were relatively higher at lower parasitaemia while TNF-α was higher at parasitaemia well above 100,000 parasites/µL (Table 11).

Table 8: Relationship between BMI Status and Malaria Parasitaemia

Malaria Parasitaemia (parasites/µL)	N	BMI Status	
		Under- & overweight)	Normal
< 1,000	21 (7%)	9	12
≥ 1000 but < 100,000	218 (72.67%)	137	81
≥ 100,000	61 (20.33%)	47	14
TOTAL	300	193	107

$\chi^2 = 14.45$, $df = 4$, p=0.006

Table 9: Cytokine Response of Participants from Study Sites

Cytokine (ng/L)	Ijede	Lekki	Ajeromi
TNF-α	397.98±20.40[a,b]	446.67 ±19.3[a,c]	303 ±61[b,c]
TESTIL-1β	22.40±1.21[a]	30.95 ±1.39[a,b]	26.26 [b]± 04
IL-12	83.23 ± 1.21 [a]	92.92±2.05 [a,b]	86.67 ±1.81[b]
TNF- α	98**.5**± 2.2	100.0 ± 0.2	90.85 ± 7.2
ControlIL-1 β	11.10 ±0.5	13.3 ± 0.9	9.8 ± 0.41
IL-12	54.22 ±71	60.31 ± 10	58.42 ± 6.7

a,b,c: mean difference is significant ($p<0.05$); values are Mean ± S.E.M;N(test)=300, n(control)= 80

Table 10: Levels of Pro-inflammatory Cytokines of Participants

GROUP	TNF-α	IL-12	IL-1β
Infected (300)	390.0 ±11 49	87.62 ± 1.02	26.54 ± 0.74
Uninfected (80)	97.5 ± 10.8	57.25 ±2.8	10.47 ±0.3
p0.00016	0.003	0.02	

Pro-inflammatory cytokines were significantly ($p<0.05$) higher in malaria patients than controls. Values are Mean ± S.E.M

Table 11: Effect of Malaria Parasite Density on Cytokine Response of Participants

CYTOKINE (ng/L)	Malaria Parasite Density Groups (parasite/ ul)			
	<1,000 (n=21)	≥1,000 (n=218)	≥100,000 (n=61)	p-value
TNF-α	379.41± 50.60	395.16± 13.16	380± 25.46	0.85
IL-1β	28.88± 3.44	26.80± 0.89	24.11± 1.4	0.36
IL-12	94.12 ± 4.86	87.83 ± 1.20	85.17 ±1.80	0.52

No significant difference in cytokinemia with increase in parasitaemia ($p>0.05$);
Values are Mean ± S.E.M

4.6 EVALUATION OF HAEMATOLOGICAL PARAMETERS OF PARTICIPANTS

Analysis of participants' full blood counts (FBC) showed that haemoglobin (Hb), mean cell haemoglobin (MCH), platelet and mean cell volume (MCV) were significantly lower ($P < 0.05$) in test participants than uninfected controls. Further analysis (Pearson's correlation) revealed that IL-12 significantly correlated with MCH, granulocyte and platelet counts. Multiple regression analysis showed that MCH, granulocyte and platelet predicted IL-12 65%, 55% and 71% respectively (Tables 12,13 and 14).

4.7 DNA YIELD AND PURITY

Quantification of the extracted deoxyribonucleic acid (DNA) using Nanodrop *1000* Spectrophotometer gave average yield of 80 ng /µL and purity 0.86 (OD 260/280).

Table 12: Haematological Parameters of Participants

GROUP	Mean ± S.E.M								
	Haemoglobin (g/dL)	**Platelet (X 10^9/L)**	**Granulocyte (%))**	**Monocyte (X 10^9/L)**	**Lymphocyte (X 10^9/L)**	**MCHC (g/dL)**	**MCH (pg)**	**MCV (fl)**	**WBC (X10^9/L)**
Infected (300)	10.47 ± 0.3	123.12 ± 21	5.1 ± 0.6	0.6 ± 0.07	2.43 ± 0.3	55.4 ± 15	25.45 ± 0.41	77.88 ± 1.1	8.13 ± 0.8
Uninfected (80)	13.69 ± 0.4	175.8 ± 16	2.18 ± 0.2	0.4 ± 0.05	2.54 ± 0.21	3.44 ± 0.1	27.69 ± 0.34	85.39 ± 1.0	5.14 ±0.37
P	0.008	0.09	0.02	0.053	0.79	0.23	0.002	0.028	0.014

MCH: Mean cell haemoglobin concentration
MCH: mean cell haemoglobin
MCV: Mean cell volume
WBC: White Blood Cell

Table 13: Correlation of IL-12, IL-1β and TNF-α with Platelets, MCH, MCHC, WBC & Granulocytes

Haematological parameters		TNF-α (TEST/CTRL)	IL-12 (TEST/CTRL)	IL-1β (TEST/CTRL
WBC	r	0.26 / -0.28	0.25 / -0.31	0.13 / -0.02
	p	0.15 /0.3	0.07 / 0.24	0.39 / 0.95
MCH	r	-0.27 /-0.5	-0.57 / -0.25	-0.54 / -0.02
	p	0.19 /0.03	0.001[a] / 0.35	0.001/ 0.42
MCHC	r	0.22 /-0.47	0.25 / 0.15	0.25 / -0.28
	p	0.29 /0.05	0.09 / 0.54	0.18 / 0.27
GRANULOCYTES	r	0.23 /-0.18	0.48 / -0.29	0.34 / 0.16
	p	0.25 /0.47	0.003[a]/ 0.25	0.03 / 0.54
PLATELETS	r	-0.06 /-0.08	-0.327 / -0.04	-0.30 /0.04
	p	-0.30 /0.04	0.03[a]/ 0.89	0.056 / 0.08

MCH, Granulocytse and Platelet counts were significantly correlated with IL-12, (a= p< 0.05); r = correlation coefficient,

p = p-value.

Table 14: Multiple Regression Analysis on MCH, GRA & Platelet with IL-12

Model	R^2	Coefficient	P
MCH	0.65	-3.12	0.003
GRA	0.55	2.24	0.03
PLATELETS	0.71	-2.48	0.018

Both platelet and mean cell haemoglobin (MCH) significantly ($p< 0.05$) inversely predicted serum levels of IL-12. Granulocytes (GRA) directly significantly predicted IL-12 (dependent variable).$R^2$is coefficient of multiple determination for multiple regression.

4.8 PARASITE CLONAL DISTRIBUTION AND GENETIC DIVERSITY

Out of the 300 isolates included in this study, 195 were PCR positive for *msp2* (showing bands for one or both families). Nested PCR on *msp-2* confirmed samples revealed that both 3D7 and FC27 allele classes of *P. falciparum msp-2* were present in the study sites (Plates 1 & .2). Eighteen different alleles were observed for *msp*-2 locus, FC27 family being more polymorphic. Analysis showed that 61 (31.0%) of the isolates had only FC27-type alleles, 38 (19.7%) had only 3D7-type alleles and 96 (49.3%) had parasite lines with both alleles (Figure 9). The band sizes were 275-625bp for FC27 and 150-425bp for 3D7. Four alleles were observed from LEK, 2 (375 and 425bp) and 2 (275 and 325bp) of FC27-types and 3D7-types respectively; 12 alleles from AJE, 9 (275-625bp) and 3 (325-425bp) of FC27 –types and 3D7-types respectively while IJE had a total of 12 alleles 9 (275-625bp) and 3 (325-425bp) of FC27–types and 3D7-types respectively (Table 15). Analysis also showed that FC27 genotypes were more prevalent among participants< 5 years than 3D7 while the 3D7genotypes were more in participants ≥5years (Figure 10). The three sites recorded relatively high expected H_E values (0.77-0.87) which was highest for IJE (0.87).

4.8.1 MULTIPLICITY OF INFECTION

Mean multiplicity of infection (MOI) was 1.54, while in Ijede, Lekki and Ajeromi the MOI was 2.12, 0.86 and 1.33 respectively. MOI was significantly higher in IJE $p<0.05$ (Table 15) and was found to be strongly positively correlated with age ($r=0.79$, $p=0.003$). There was no significant correlation between MOI and any of sex, IL-12, IL-1β or TNF-α ($P>0.05$) (Table 16). Figure 11 shows the distribution of the two MSP2 families in the study sites. The 3D7 family was highest in the Ajeromi and Ijede while both 3D7 and FC27 were almost equally prevalent in Lekki among the populations studied although the difference across population was not statistically significant ($p=0.51$).

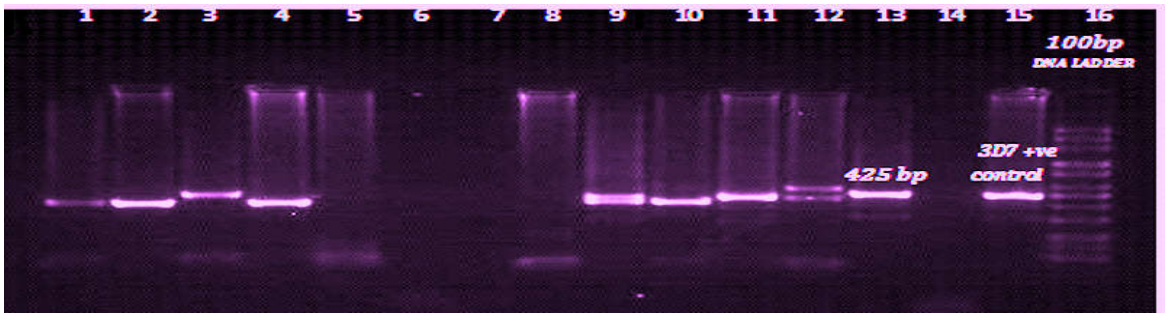

Plate 1: Agarose gel electrophoregram of MSP2/ FC27 family resolved on 1.2% gel
Lane 1,2,3, 4,10,11,13==infection with single FC27 clone of *P. falciparum*(425bp,475 & 500bp); Lane 12 ==infection with 2 FC27 clones of *P. falciparum*
Lane 14==No template control; Lane 15=*P. falciparum* 3D7 positive control, 500bp (FC27clone). Lane 16==100bp to 3kb DNA Ladder; Lane 5, 6,7 & 8==Negative for FC27

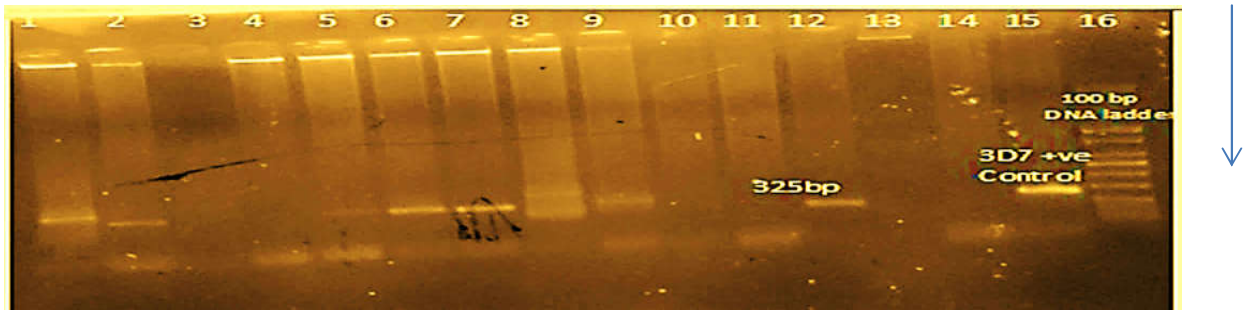

Plate2: Agarose gel electrophoregram of MSP2 / 3D7 family resolved on 1.2% gel
Lane 1,2,6,7,12 == infection with 1(one) 3D7 clone (275bp,325bp &325bp);
Lane 3 & 4 == negative for 3D7 clone, Lane 15 ==*P. falciparum* 3D7 positive control (375 bp 3D7 clone); Lane 16==100bp to 3kb DNA Ladder. Arrow= Direction of run

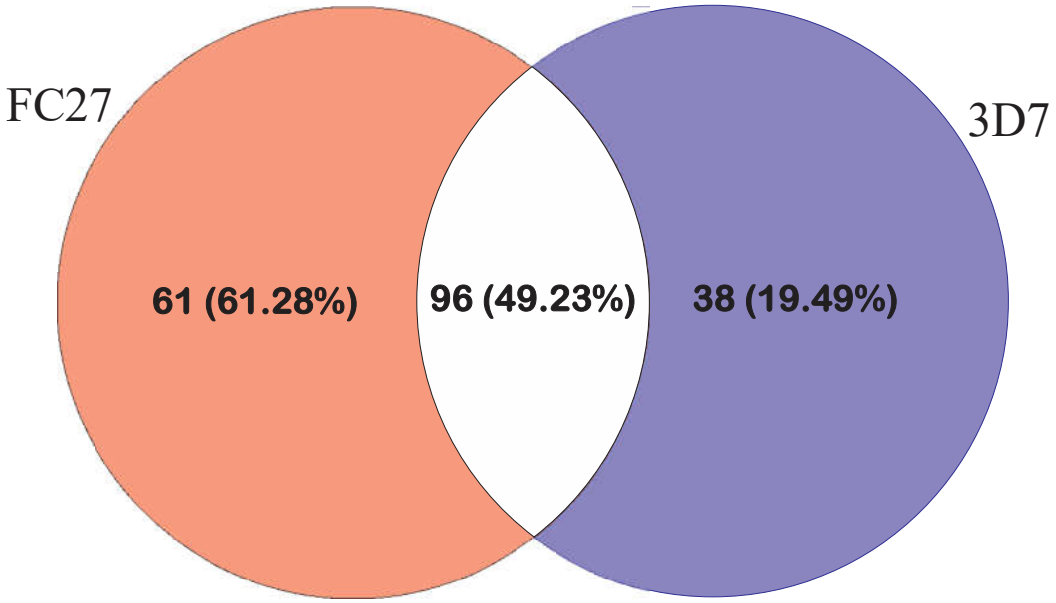

Figure9:Distribution of MSP2 Alleles in isolates from the sites studied

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Table 15: Distribution of Merozoite Surface Protein (msp)-2 in Isolates from 195 Nigerians Presenting *with P. falciparum* malaria

Marker	family	IJE	LEK	AJE	Total
MSP2	3D7	No of distinct bands	3	2	3	8
		No (%) of samples with corresponding alleles	63(96.92)	40(61.54)	31(47.69)	134(68.72%)
		% of fragments with Corresponding alleles	60(56.60)	46(41.07)	39(34.82)	145(43.94%)
		Size range (bp)	150-325	375-425	325-425	
	FC27	No of distinct bands	9	2	9	20
		No (%) of samples with Corresponding alleles	60 (92.31)	43(66.15)	54(83.08)	157(80.51%)
		% of fragments with Corresponding alleles	46(43.4)	66(58.93)	73(65.18)	185(56.06%)
		Size range (bp)	375-550	375-425	275-625	
		(*MOI) CI=95%	2.12	0.86	1.33	1.54
		H_E	*0.87*	*0.77*	*0.85*	

Fragments of *P.f* msp-2 established by family (FC27 or 3D7),*MOI= Multiplicity of Infection). Over-all MOI = 1.54.The three sites recorded relatively high expected (H_E) values (0.77-0.87) which was highest for Ijede (0.87). *FC27-t*ype alleles were more in circulation than 3D7-type

IJE: Ijede
LEK: Lekki
AJE: Ajeromi

4.8.2 Amplification of Apical Membrane Antigen-I *(*AMA1*)* and Reticulocyte Binding Protein Homology 5 (*RH5*)

Single clone 195 *P. falciparum* isolates were used to successfully amplify the 500bp hyper-variable (HVR) domain I of AMA1 from the entire 2Kb AMA1 gene in nested-PCR reactions while the 810 bp high affinity binding peptide (HABPs) 36718, 36727 and HABP 36728 sequence of RH5 was also amplified. The PCR products of both genes were separated on agarose gel as shown **o**n the electrophoregram (Plates 3and 4.).

Table 16: Relationship of MOI with Age, TNF, IL-1B and IL-12.

AGE	r=0.79, p=0.003
SEX	r= 0.11, p = 0.09
TNF-α	r= 0.44, p =0.44
IL-12	r= 0.57, p = 0.18
IL-1 β	r= 0.17, p =0.22

MOI was strongly positively correlated with age but not with any of Sex or cytokine response (Pearson's correlation).r= Pearson's correlation coefficient; p= p value

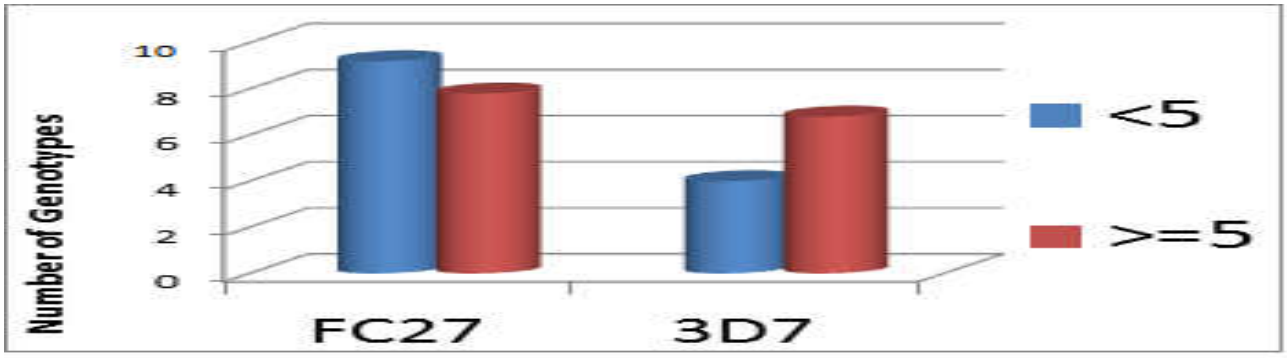

Figure10: Mean number of genotypes in merozoite surface protein -2 (MSP2) polymorphic families of *P. falciparum* from blood samples of malaria patients in Lagos, by age (Pearson's correlation r=0.79,(P< 0.05).

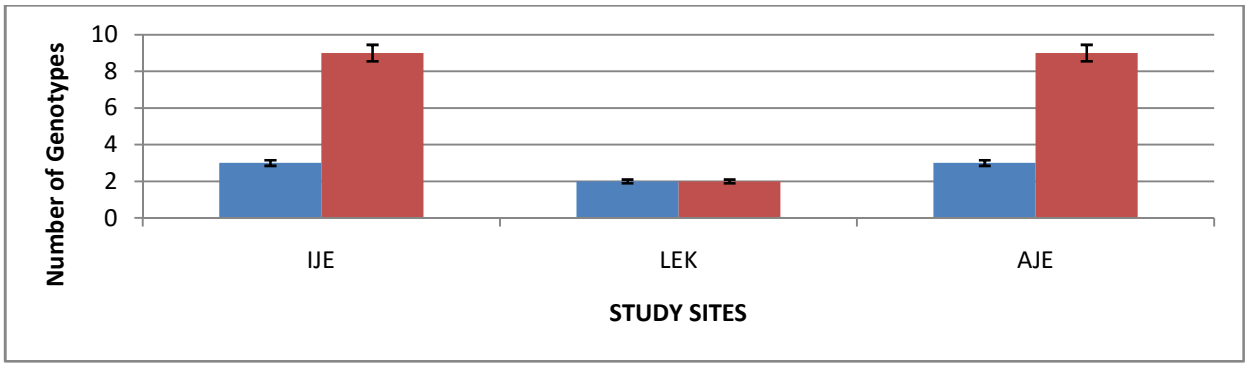

Figure 11: Distribution of msp-2 families during the cross-sectional sample collection at the three sites.

:3D7: One of the two allelic families of *Plasmodium falciparum* Merozoite surface protein-2 (*MSP2*).*MSP2* is a recommended marker of plasmodium parasite genetic diversity comprising of two allelic groups for genotyping in the block 3 of *msp2*.**: FC27:** The second allelic family of *Plasmodium falciparum* Merozoite surface protein-2

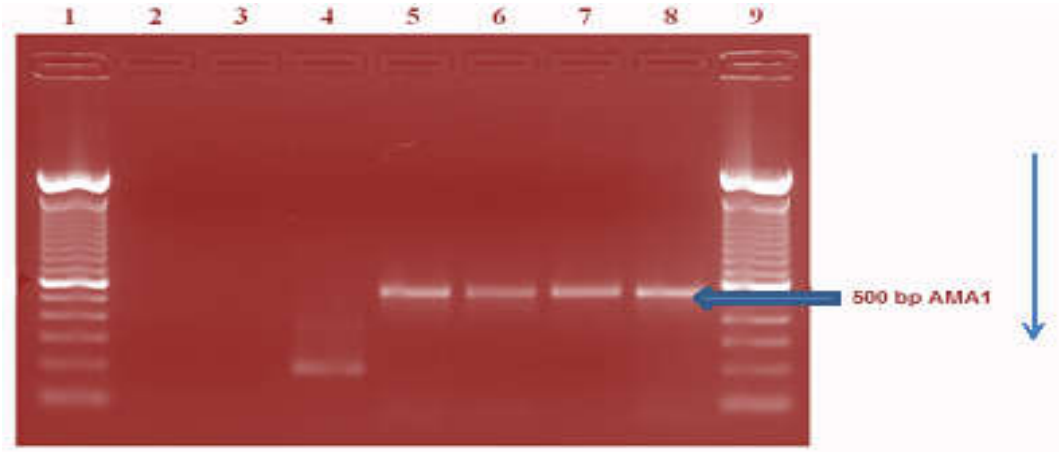

Plate 3: Agarose gel electrophoregram of *AMA1* resolved on 1.2% Agarose gel.

Lane 1& 9=100 bp DNA ladder,Lane 2 =no template control, Lane 8=3D7 positive control(Reference sequence),Lane 5,6,7= AMA1 500bp
Arrow = Direction of run

Plate4: RH5 Electrophoregram resolved on 1.2% Agarose gel.

Lane 1=100bp DNA ladder; Lane 2 =3D7 positive control (Reference sequence); Lanes 3,4,5,6,17,19 &20 = RH5 gene; Lane 7= no template control. Arrow= Direction of run

4.8.3 GENETIC DIFFERENTIATION,NUCLEOTIDE AND HAPLOTYPIC DIVERSITY OF *Pf*AMA1

The sequence encoding the domain I of AMA1 was determined from 195 clinical isolates from three sites in Lagos, Nigeria (Ijede, n=54; Lekki, n= 63; and Ajeromi, n=78) with relatively different malaria transmission intensities. The analysis of the 456 bp sequence obtained (corresponding to 448-903 bp region of the *ama1* gene encoding amino acid residues 150-301) revealed that 422 nucleotide positions were either monomorphic or invariable. The remaining 34(7.43%) sites were polymorphic (1 singleton variable and 33 parsimony informative) generating 93 different AMA1 Haplotypes (H1 to H93) among Nigerian *P. falciparum* populations (Table 17).BLAST searches against the *P. falciparum* AMA1 sequences available in the GenBank database confirmed that forty- eight (48) of these 93 haplotypes were new as they have not been reported earlier from any region of the world. The remaining 26 haplotypes showed 100% identity with reported AMA1 sequences as available in the GenBank (www.ncbi.nlm.nih.gov/). The H1 was the most abundant while none of the isolates had identical sequences to the 3D7, Dd2 or HB3 AMA1 alleles. The sequences reported in this study have been deposited in the database [GenBank: KU986915-KU987007].

Amino acid changes were observed at 30 of the 152 codons (456 bp) across the entire domain I of AMA1.Of these 30 polymorphic sites, 22 codons were di-morphic (150 (Pro/Ser),154 (Lys/Tyr), 173 (Asn/Lys), 175 (Tyr/Asp), 189 (Leu/Pro), 201 (Phe/Leu), 204 (Asp/Asn), 205 (Asn/Lys), 206 (Lys/Glu), 296 (Lys/Glu), 207 (Tyr/Asp), 225 (Ile/Asn), 228 (Asn/Lys),243 (Lys/Asn), 244 (Asp/Asn), 267 (Glu/Gln), 269 (Lys/Ile), 270 (Arg/Lys), 277 (Arg/Lys), 282 (Ser/Leu), 285 (Gln/Glu) and 296 (Asp/His)), five codons were tri-morphic (172 (Gly/Glu/Val), 187 (Glu/Asn/Lys), 196 (Asp/Asn/Tyr), 230 (Lys/Glu/Gln) and 242 (Asp/Tyr/Asn) while one (1) site [codon 197 (Glu/Asp/Gly/Gln/His) showed penta-morphic and one site [codon 200 (His/Arg/Asp/Val/Asn/Leu/Ser)] showed hepta-morphic alleles. The haplotype diversity (Hd), nucleotide diversity (π) and average number of pairwise nucleotide differences (K) within

population showed regional variation (Table18). Phylogenetic analysis of the haplotypes showed an evolutionary pattern with clustering revealing divergence and relatedness of the haplotypes with other AMA1 sequences from Guinea, Nigeria and the 3D7 global reference strain using *P. reichenowi*, the closest species to *P. falciparum* as the out-group (Figure 12).

4.8.4 GENETIC DIFFERENTIATION, NUCLEOTIDE AND HAPLOTYPE DIVERSITY OF PFRH5

However, analysis of the HABPs 36718, 36727 and 36728 of PfRH5 revealed 810 bp sequence (corresponding to 436-1246 bp region of *rh5* gene encoding amino acid residues 147-367) of which 795 sites were invariable or monomorphic and 15 (1.85%) were polymorphic (6 singleton and 9 parsimony informative) sites. Three different RH5 haplotypes (H1 to H3) were found among Nigerian *P. falciparum* populations studied (Table 17). A single nucleotide change was observed in the major polymorphic nucleotide of the RH5 HABP 36727, position 608 (according to the 3D7 reference strain numbering) as G → Aproducing amino acid change at residue 203 from cysteine (C) to tyrosine (Y). The haplotype diversity (Hd), nucleotide diversity (π) and average number of pairwise nucleotide differences within population showed no clear evidence of regional variation (Table 19). The haplotypes reported in this study have been deposited in the database [GenBank: KX418644-KX418646].The phylogenetic tree showing the evolutionary relationship of the haplotypes with reported PfRH5 around the world is shown in Figure 13using *P. reichenowi* as the out-group.

Table 17: Measures Of DNA Sequence Polymorphisms t Domain I of AMA-I and RH5 Among Nigerian *P. falciparum* parasite Isolates

GENE	Segregating sites (S)	Singleton variables sites	Parsimony informative sites	Total No .of mutation	K	H	Hd ± S.D	Π ± S.D	dN-dS ± S.E	Tajima's D
AMAI	34	1	33	52	11.30	93	0.992± 0.004	0.239± 0.00113	0.0463 ± 0.007	0.359
RH5	15	9	6	15	3.72	3	0.318±0.016	0.005±0.0001	-0.0171±0.005	-1.07169

S; Number of segregating (polymorphic/variable) sites, **K**; Average number of pairwise nucleotide differences, **H**; Number of haplotypes, Hd ; Haplotype diversity, π; Observed average pairwise nucleotide diversity, dN-dS ; rate of non-synonymous mutations minus rate of synonymous mutations, Tajima's D test statistics.

Table 18: Measures of DNA sequence polymorphisms at Domain I of AMA-I among Nigerian *P. falciparum* populations

Study Areas (n=195)	Segregating Sites (S)	Singleton sites	Parsimony Informative Variable sites	Total no. of Mutations	K	H	Hd ±S.D	Π ± S.D	dN-dS ± S.E	Tajima's D
IJEDE29 (n= 54)	1	28	30	10.22	21	0.966± 0.00112	0.0247±0.00078	0.026±0.002	0.358	
LEKKI (n=63)	32	3	32	44	12.037	25	0.989±0.011	0.0223±0.00119	0.017± 0.004	1.021
AJEROMI (n=78)	30	2	30	41	10.962	35	0.99± 0.007	0.0263± 0.00131	0.037± 0.05	0.257
TOTAL	34	1	33	52	11.303	93	0.992± 0.004	0.239± 0.00113	0.0463±0.007	0.359

(n=195)

S; Number of segregating (polymorphic/variable) sites, **K**; Average number of pairwise nucleotide differences, **H**; Number of haplotypes, **Hd ;** Haplotype diversity**, π**; Observed average pairwise nucleotide diversity, **dN-dS**; rate of non-synonymous mutations minus rate of synonymous mutations, **D; Tajima's D test statistics.**

Table 19: Measures of DNA Sequence Polymorphisms at The HAPBs 36718, 36727 and 36728 of Rh5 Among Nigerian *P. falciparum* parasite Populations

Study areas (n=195sites	Segregating Sites (S)	Singleton Variable Sites	Parsimony Informative Mutations	Total no. of	K	H	Hd ±S.D	Π ± S.D	dN-dS ± S.E	Tajima's D
IJEDE (n= 54)	13	13	0	13	5.20	3	0.40± 0.0237	0 .00642±0.0058	-0.0171±0.0048	-1.2104
LEKKI (n=63)	11	11	0	11	7.53	2	0.67± 0.314	0.0091 ±0.00119	-0.0126 ±0.314	-0.8374
AJEROMI (n=78)	9	8	1	10	7.33	2	0.509± 0.0265	0.00802± 0.0070	-0.0087± 0.015	-0.8431
TOTAL	15	6	9	15	3.727	3	0.318±0.016	0.005±0.00011	-0.0171±0.005	-1.07169

(n=195)

S; Number of segregating (polymorphic/variable) sites, **K**; Average number of pairwise nucleotide differences, **H**; Number of haplotypes, **Hd ;** Haplotype diversity**, π**; Observed average pairwise nucleotide diversity, **dN-dS**; rate of non-synonymous mutations minus rate of synonymous mutations, **D; Tajima's D test statistics.**

4.8.5 *P. FALCIPARUM* GENETIC STRUCTURE, EVIDENCE OF SELECTION AND RECOMBINATION

The average difference of dN-dS for all 195 AMA1 isolates was 0.0463 ± 0.007 SD, and the Tajima's D was 0.359 indicating that domain I of AMA1 is under positive natural selection which might be the cause for the high allelic diversity (Table 17). The dN-dS value was however highest in Ajeromi revealing higher pressure and selection. Also the Linkage Disequilibrium (LD) Index, R^2, plotted against the nucleotide distances showed a decline across the length of AMA1 sequences (Figures 14) depicting increase in recombination rate and its likely contribution to genetic diversity in AMA1.In addition, generally polymorphic nature of sites on AMA1 may have resulted from the recombination vents at high levels on this gene.Thus, the high number of haplotypes and segregating sites are resultant events of theses recombination events. On the contrary, LD and dN-dS values for PfRH5 showed purifying selection with negative values for dN-dS and Tajima's D (Figure 15 and Table 17). Inter-population nucleotide differences (Kxy) varied from 10.662 (between Ijede and Ajeromi) to 11.939 (between Lekki and Ajeromi).Similarly, the average number of nucleotide difference (Dxy) and net nucleotide substitution per site (Da) between populations ranged from 0.0233 to 0.0261 and 0.00015 to 0.00096 respectively (Table 20).

Table 20 Inter-Population Genetic Differentiation of *P. falciparum*

S/N	POPULATION 1	POPULATION 2	Kxy[a]	Dxy[b]	Da[c]	Fst[d]
1	IJEDE	LEKKI	11.535	0.0252	0.00089	0.035
2	IJEDE	AJEROMI	10.662	0.0233	0.00015	0.007
3	LEKKI	AJEROMI	11.939	0.0261	0.00096	0.037

a; Average number of nucleotide differences between populations, b; The average number of nucleotide substitutions per site between populations, c; The net nucleotide substitutions per site between populations, d; Fixation Index, a measure of genetic differentiation between population (range from 0 to + 1)

4.8.6 EFFECT OF POLYMORPHISMS ON MOI AND CYTOKINE RESPONSE

Analysis of the effect of the relationship between genetic variations in AMA1 and RH5 showed that there was no statistically significant relationship between variations in AMA1 and any of the cytokines studied nor MOI. However, polymorphisms in RH5 was strongly negatively correlated with TNF-α and positively correlated with MOI (Table 21).

Table 21: Relationship between pro-inflammatory cytokines and Polymorphisms in P. falciparum antigenic proteins

GENE	TNF-α	IL-1β	IL-12	MOI
AMA-1	r =0.025	r = - 0.016	r =0.24	r= 0.076
	P=0.66	P=0.22	p=0.684	P=0.522
RH5	r = -0.85	r = 0.02	r = -0.156	r= 0.68
	P=0.006	P=0.34	p=0.684	P=0.03

Pearson's correlation (r) showed no statistically significant association between antigenic variations in AMA1and TNF-α, IL-1β and MOI. RH5 polymorphisms was strongly negatively correlated with TNF-α and positively correlated with MOI. (n=195*)*

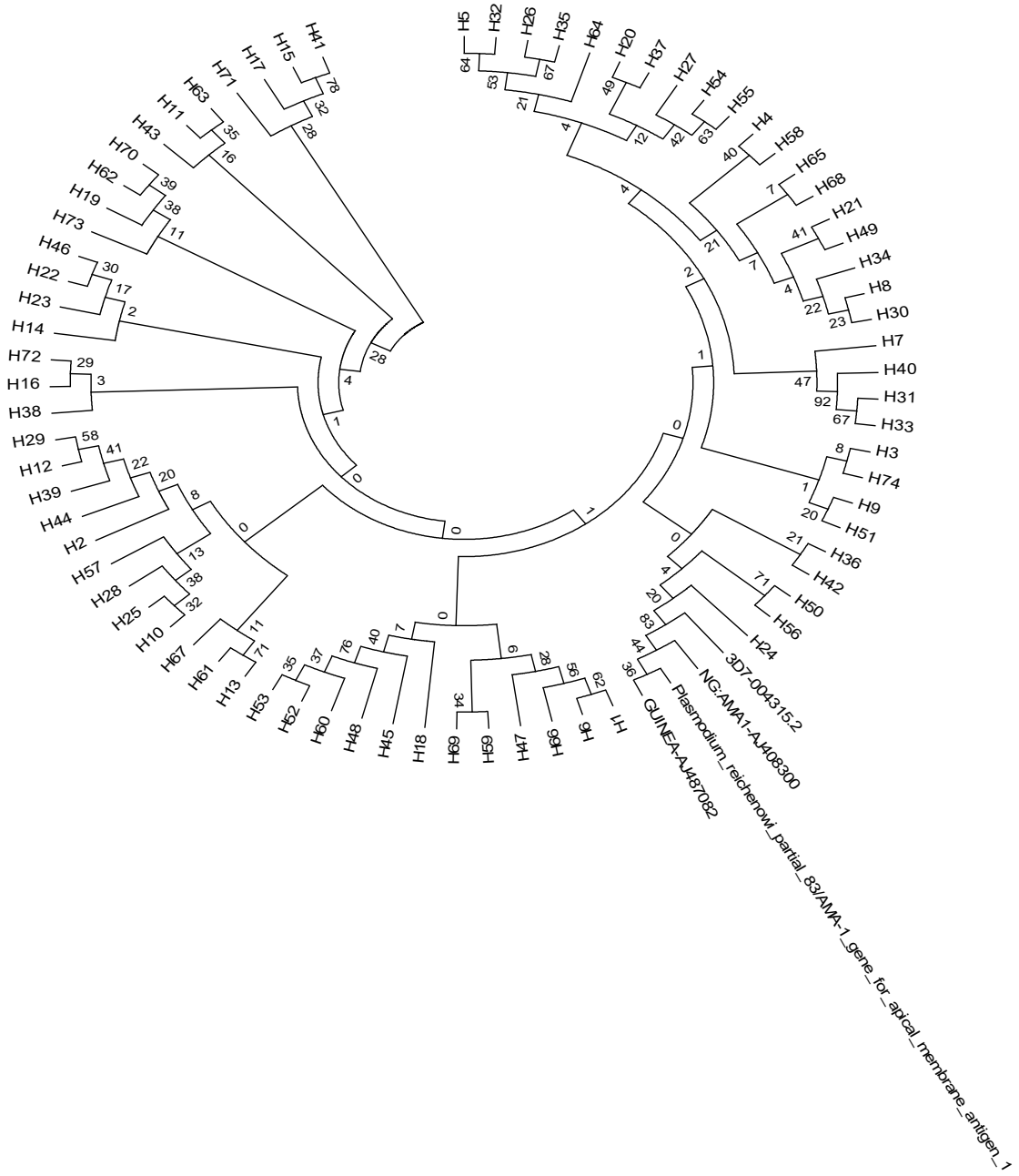

Figure 12: Molecular Phylogenetic Tree of AMA1 Haplotypes by Maximum Likelihood method

A neighbour-joining (NJ) tree depicting the relationship between different AMA1 haplotypes observed among Nigerian *P. falciparum* populations and with AMA1 sequences from Guinea (AJ487082), Nigeria,(AJ408300) and 3D reference strain (NC_004315.2).The evolutionary history was inferred by using the Maximum Likelihood method. The analysis involved 78 nucleotide sequences. Numbers below the lines indicate percentage bootstrap values for 1000 replications. All positions containing gaps and missing data were eliminated. There were a total of 456 positions in the dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).The partial AMA1 sequence of *P.reichenowei* [AJ252087] was used as an outgroup,

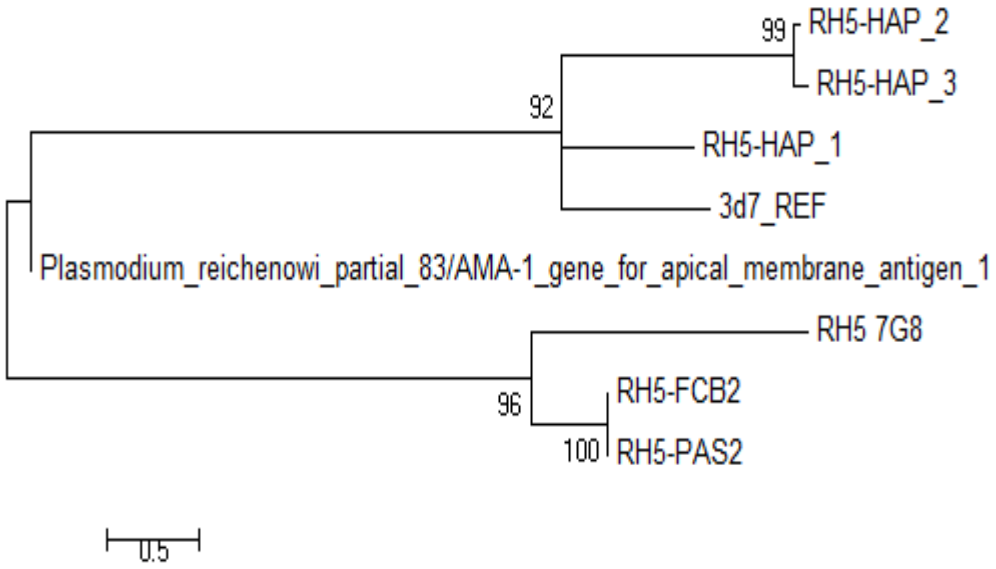

Figure 13: Molecular Phylogenetic Tree of RH5 Haplotypes by Maximum Likelihood Method

A neighbour-joining (NJ) tree depicting the relationship between different RH5 haplotypes observed among Nigerian *P. falciparum* populations and with other RH5 sequences including PAS-2, FCB-2, 7G8 and 3D reference strain RH5 .The evolutionary history was inferred by using the Maximum Likelihood method. The analysis involved 78 nucleotide sequences. Numbers below the lines indicate percentage bootstrap values for 1000 replications. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [Tamura *et al*., 2013].The RH5 sequence of *P. reichenowei* was used as an outgroup.

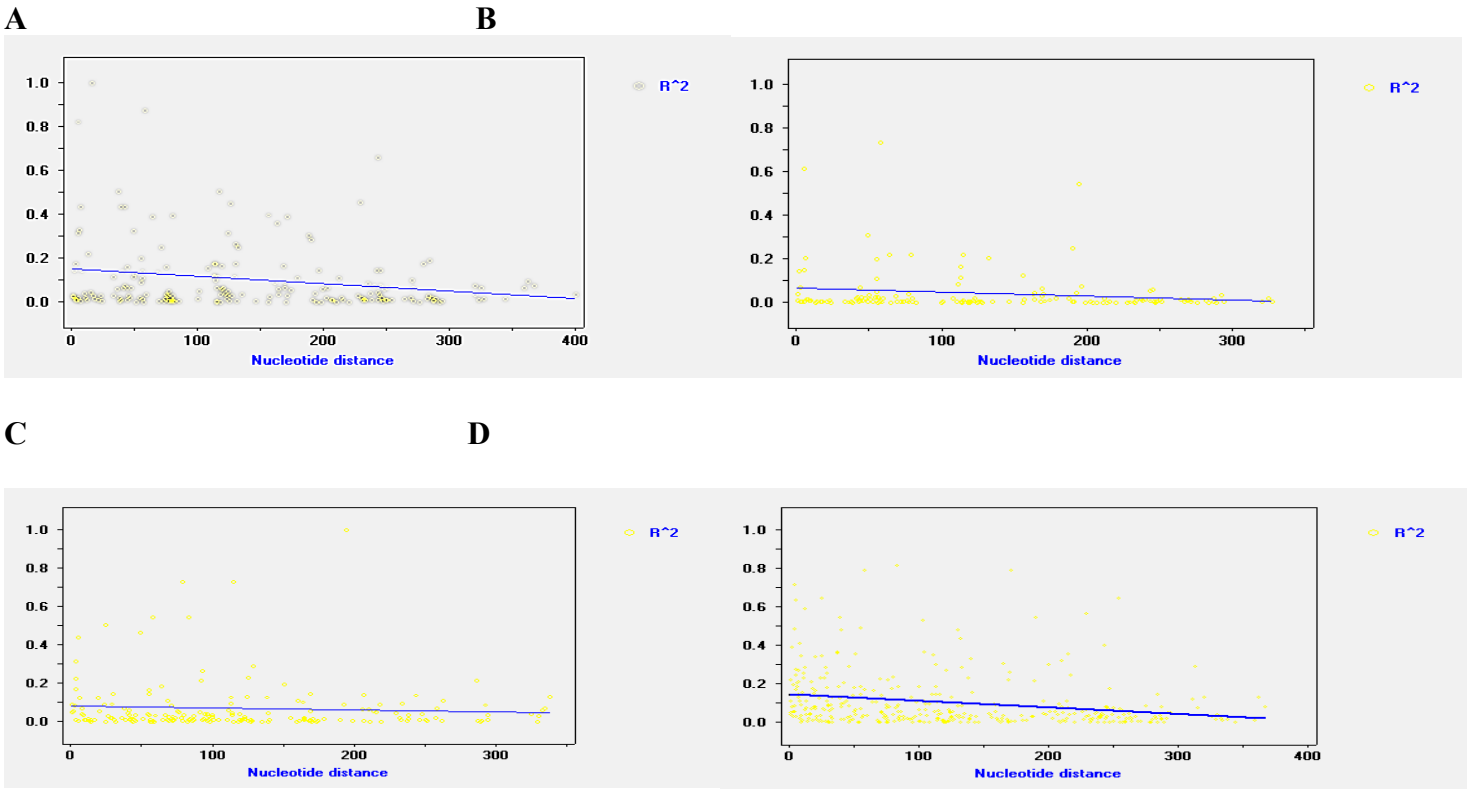

Figure 14: Linkage disequilibrium (LD) plot showing non-random association between nucleotide variants at different polymorphic sites on AMA1 Domain I

The R 2 values are plotted against the nucleotide distances with two-tailed Fisher's exact test of significance using DnaSP. The value of LD index (range from -1 to +1) declined with increasing distance ,indicating that recombination events are taking place.All polymorphic sites were considered in the analysis.(A) Ajeromi (B) Lekki (C) Ijede (D) Total .

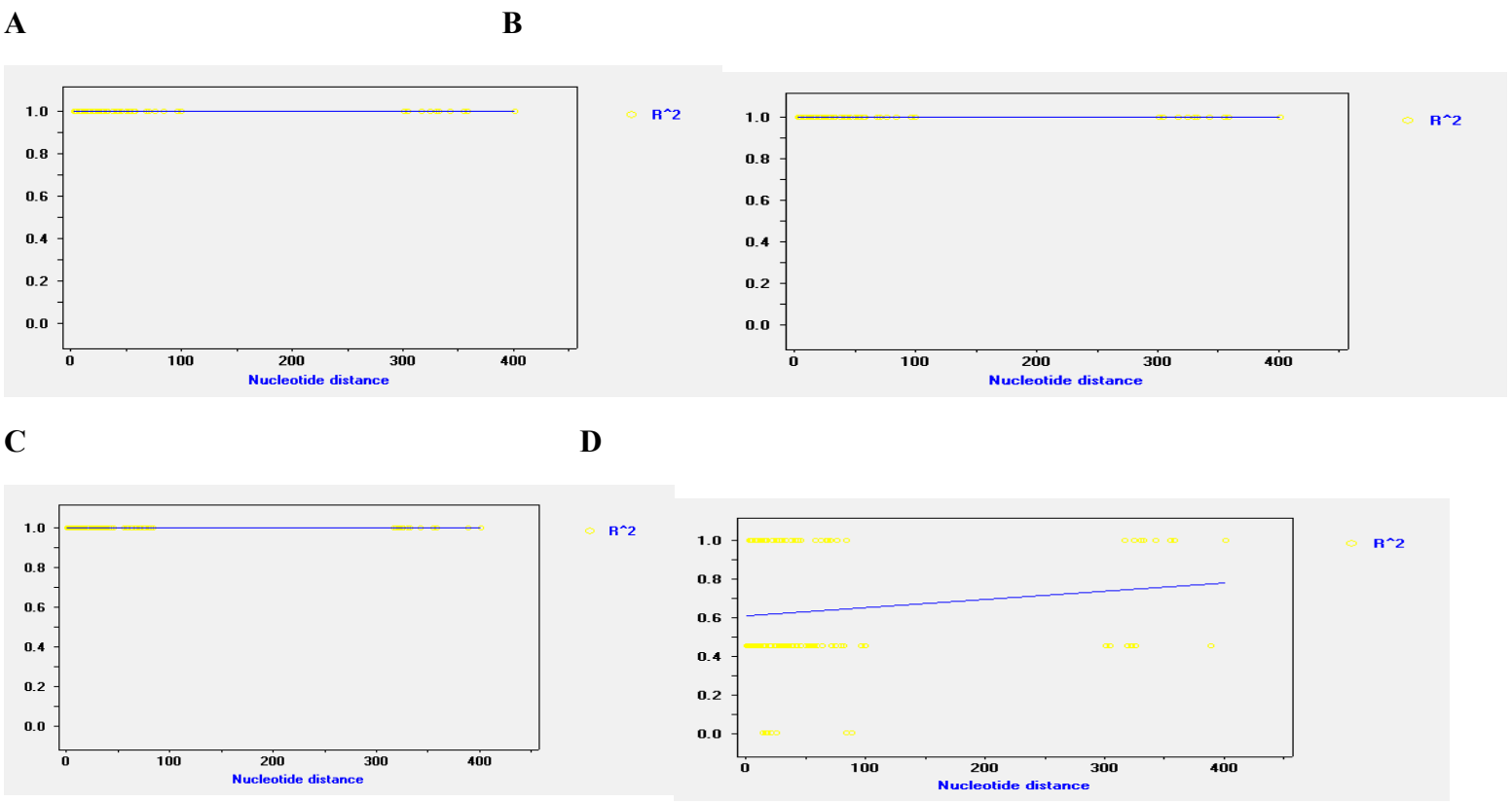

Figure 15: Linkage disequilibrium (LD) plot showing non-random association between nucleotide variants at different polymorphic sites on RH5.

The R 2 values are plotted against the nucleotide distances with two-tailed Fisher's exact test of significance using DnaSP. The value of LD index (range from -1 to +1) increased with increasing distance, indicating **no** recombination events taking place.All polymorphic sites were considered in the analysis.(A) Ajeromi (B) Lekki (C) Ijede (D) Total.

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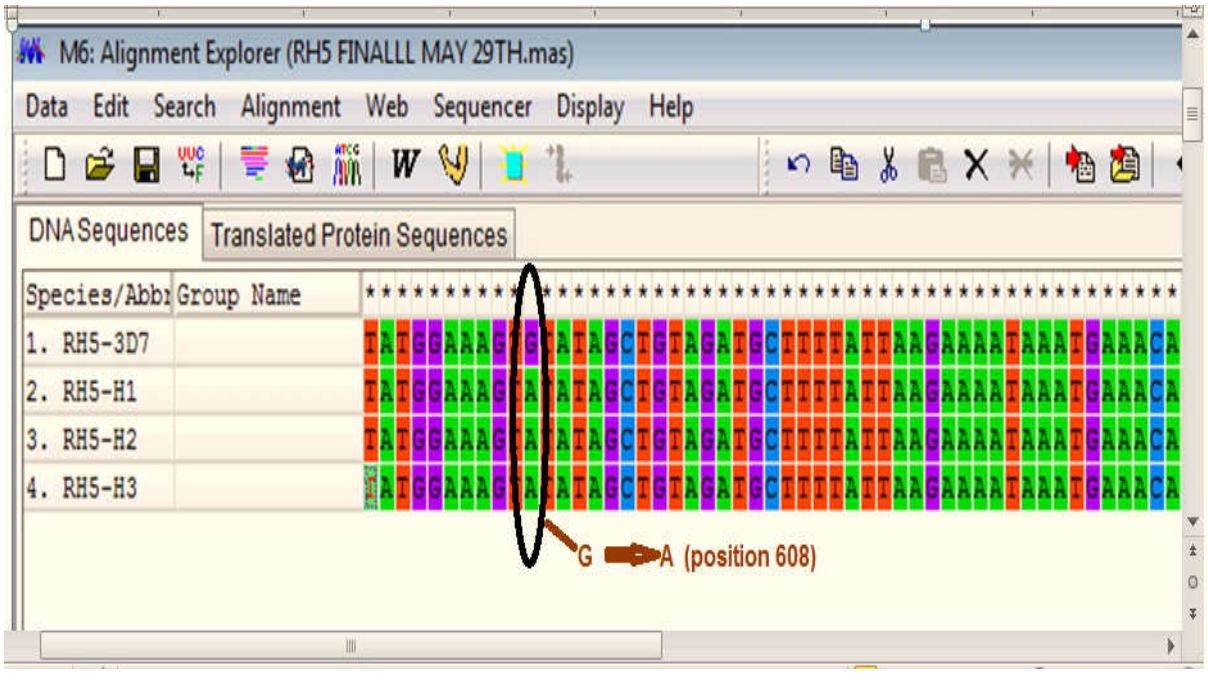

Figure16: Single nucleotide change on position 608 of RH5 (G ➡ A) based on the 3D7 RH5 sequence

CHAPTER FIVE

5.0 DISCUSSION

The development of an effective *Plasmodium falciparum* vaccine is the topic of intense research globally. Moreover, valid reasons have been given to support vaccines targeting erythrocytic stage in humans (Bustamante *et al*., 2013) Varying selection caused by differences in host immunity and antimalarial drug pressure results in evolutionary changes responsible for high levels of genetic variations in the parasite. Therefore, effective control strategy employing malaria vaccine must effectively protect the largest number of people against the widest possible range of *P. falciparum* genetic variants necessitating studies on the nature and dynamics of polymorphisms on the surface vaccine targets. Transcriptome analysis of the *P. falciparum* intra-erythrocyte cycle identified and characterized a new protein, *Pf*RH5, (Arevalo-pinzon*et al.*, 2012), presently in global spotlight alongside *Pf*AMA1 for the development of effective malaria vaccine. This study is therefore timely as the country intensifies efforts on malaria control. The study employed molecular methods to investigate polymorphisms in two antigenic vaccine candidate proteins among *P. falciparum* populations and described their genetic structure and possible selection pressure using genetic analyses.

A generally high genetic conformation and homology of *Pf*RH5 isolates from this study in comparison to major reference strains, is an indication of potential parasite susceptibility to any universally efficacious *Pf*RH5-based vaccine.

Inferentially, consequent upon non-adherence to National Malaria Elimination guidelines and ineffectiveness of the local herbs used against malaria in rural settings, the prevalence of *P. falciparum* malaria in the febrile populations studied was only a little different from what was reported by Afolabi *et al*(2001) and was highest (22.05%) in Ijede, a rural settlement in Ikorodu

axis of Lagos State with relatively high disease transmission, leaving the pregnant women and children under-five years to bear the brunt (WHO, 2015a).

High poverty level negatively affecting the standard of living, nutritional status and susceptibility to infections result in high rate of wasting in children especially of rural dwellers as found in this study. High rate of underweight was also highest in Ijede reflective of the low socio-economic status of the dwellers justifying low immunity and increased progression to disease severity.

In malaria, especially uncomplicated malaria, levels of the pro-inflammatory cytokines, IL-12, IL-1β and TNF-α areraised as revealed in this study.This is in accordance with the findings by Okocha *et al* (2015)from their study in South-East Nigeria. Cytokines play both protective and pathological roles in malaria, however, early and effective inflammatory response is crucial in the control of parasitaemia and resolution of malaria infection through the mechanism of tumor necrosis alpha (TNF-α) (Wroczyńska *et al*., 2005). Numerous studies have investigated the relationship between child malnutrition and either malaria morbidity or intensity of infection (Snow *et al*., 1991, Muller *et al*., 2003). In contrast, only a few studies have explored the interaction between child malnutrition and specific anti-*P. falciparum*immune responses however, Fillol*et al* (2009)demonstrated that nutritional status could modulate the immune response directed to malaria antigens.

Although, haematological changes in malariasuch as anaemia, thrombocytopaenia and leucocytosis or leucopaenia are well recognized. The extent of these alterations varies with level of malaria endemicity, background haemoglobinopathy, nutritional status, demographic factors, and malaria immunity.This investigation has revealed reduced platelet and anaemia which were predominant among the infected participants, consistent with reports by Maina *et al* (2010) and

Olutola and Mokuolu (2012) in Kenya and Nigeria repectivey, which might be consequence of cytokine inhibition of erythropoiesis with increased TNF-α in malaria.

Nonetheless, analysis of the haematological indices showed that IL-12 was negatively correlated with mean cell haemoglobin, granulocytes and platelets in the test participants as IL-12 plays a significant role in the induction of Th1 immunity while IL-1 β has an early protective role in malaria (Butler *et al*., 2012). Platelets play a major role as first-line inflammatory mediators. This is evident from their capability to recruit early inflammatory cells such as neutrophil granulocytes and monocytes and even to exhibit direct antimicrobial activity, hence might have delayed the immune functions of pro-inflammatory cytokines in malaria infected subjects. However, cytotoxic properties of blood platelets are induced by cytokines including interferon gamma (Inf-γ) and tumor necrosis factor alpha (TNF-α).

MOI and parasite genetic diversity are indicators of malaria infection with significant consideration for place and time in relation to immunity, including innate T-Cell immunity in malaria. MOI is a measure of transmission and infection intensity,it allows predictions to be made about the potential for re-assortment and recombination in malaria as MOI favours recombination between genotypes and the breakdown of linkage disequilibrium(Hill and Babiker, 1995). MOI was observed for the first time in Ajegunle and Ijede areas of Lagos State from this study and was highest in Ijede (2.12) among the three study sites.Over-all mean MOI was low compared to what was reported by Amodu *et al* .(2015) and Ojurongbe *et al.* (2007) .This could be expected in view of the myriad of intervention and control measures that must have played significant role in curbing malaria transmission regardless of seasonality and intensity of infection. Happi *et al* (2004), in their findings from a study in South-west Nigeria reported high MOI values in contrast to this present investigation. This is significant as an indication of reducing trend of malaria intensity because multiplicity of any infection obtained

by genotyping pertinent antigenic markers has a major influence on the differentiation of the infection types, wether re-crudescence of re-infection as the amount of DNA greatly affects the sensitivity of detecting multiple bands from the samples (Ranford-Catwright *et al*., 2002). With respect to the initial stages of spread of mutant alleles, low MOI vaues may depict slow spread of resistant profiles , however, this is strongly influenced by Fixation index. Happi *et a*l (2004) reported a non-age-dependent MOI contrary to findings from this study.

In addition to the previously documented shortcomings of antigen-coding genetic loci including immune selection, this study revealed the existence of few allelic variants of *P. falciparum*circulating in Nigeria suggesting minute clonal distribution. Thus there may be a need to use a more discriminatory technique other than MSP2 PCR genotyping for correction of *in vivo* drug trials and outcomesto reduce probability of misclassification of parasite infections (re-infection or recrudescence) and curb overestimation of failure rates in efficacy studies.The Heteroduplex tracking assay is a modification of the MSP genotyping technique, may be effective in tracking complex infections with high parasite polyclonality. However, this assay is relatively expensive and difficult to employ in many field settings as it depends on the application of a radioactive tracer (Zhon *et al*., 2007). Nonetheless,Understanding the genetic structure of malaria parasite is essential to predict how fast phenotypes of interest, such as novel antigenic variants or drug resistance, originate and spread in populations (Zhon *et al.,* 2007)

The most predominantly distributed alleles of MSP2 discovered in circulation in the study area belongs to the FC27 family. This is contrary to reports by Oyedeji *et al*(2013), from the northern region of Nigeria and Aubouy *et al* (2003) from Gabon, which found the 3D7-type to be in higher proportions. However, the finding from this study was consistent with findings by Kiwuwa *et al.* (2013) from their study in Uganda. These clearly underscore the significance of spatial dynamics in *P. falciparum* genetic diversity.

The genetic diversity of a parasite population is shaped by various influences, including genetic drift, mutation, natural selection, and gene flow.MSP2 is the second most abundant protein on the surface of the *Plasmodium falciparum*merozoites and a target of naturally acquired antibodies and therefore genetic diversity is influenced by immune selection (balancing) (Al-Yaman*et al*., 1995; Sanders*et al*., 2005; Stanisic *et al*., 2009).

Heterozygosity is an estimate of genetic diversity, of which high levels have been reported in several low transmission settings (Pumpaibool *et al*., 2009; Branch *et al*., 2011; Mobegi *et al.,* 2012) which may be a reflection of past human demographic processes as opposed to recent epidemiological factors. This suggests that further studies are needed in diverse parasite-host ecological settings to better understand the relationship between malaria transmission rates and *P. falciparum* genetic diversity and population structure. In general, parasites from human populations with low malaria transmission (1% infection rate) carry more genetic diversity, and lower levels of population structure.This is because individuals in high transmission populations are more likely to be infected by more than one *P. falciparum* parasite which increases the frequency of recombination and subsequently results in a highly diverse population with low linkage disequilibrium (Anderson *et al*., 2000)

The study revealed high Expected Heterozygousity, (H_E) with narrow range in the three populations studied connoting diverse genetic structure of *P. falciparum in* these populations taken together. One explanation for the high levels of diversity in all the populations studied is that parasite populations in the region are panmictic.Indeed, results suggest that parasite populations are polymorphic enough to adapt to their host and to counteract interventions, such as anti-malarial vaccination which is in accordance with Olasehinde *et a*l (2012) and Oyebola *et*

al(2014). Additionally, the panmictic parasite population structures imply that resistance traits may disseminate freely from one area to another, making control measures performed at a local level ineffective.The results are however comparable to findings from studies that employed use of neutral markers such as microsatellites or single nucleotide polymorphism (SNPs) for population genetics assessment.

This investigation also revealed that there was no dependence of genetic diversity or number of genotypes *of P. falciparum* on the T-cell responses regardless of their involvement in establishing natural immunity against malaria. IL-12, IL-1β and TNF-α were not significantly correlated with number of genotypes of *P. falciparum*although IL-1β was significantly correlated with number of 3D7alleles of *MSP-2.* The implication ofthe findings reported here is that the diversity of *P. falciparum* parasite populations and therefore the resilience to antimalarial interventions will remain high unless the MOI can be significantly reduced.

The genetic make-up of malaria parasite is useful in understanding the parasite virulence, designing antimalarial vaccine and evaluating the impact of malaria control measures. Antigenic variation in the natural *P. falciparum* populations is one of the major obstacles in the development of an effective vaccine against malaria; therefore a vaccine based on one allelic form of an antigen cannot provide fully protective immunity (Wright and rayner, 2014). Analysis of the AMA1 sequences indicated that the levels of diversity amongst Nigerian *P. falciparum* isolates was relatively high compared to previous data from African and South American populations (Escalante *et al*., 2001; Polley Conway 2001). An hepta-morphic codon, 200, was found in this study which has not been reported previously, in addition to other di-morphic, tri-morphic and penta-morphic codons in accordance with some other studies (Cortes *et al*., 2003). The over-all nucleotide difference (dN-dS) was positive along-side the positive values for Tajima's D. This is an indication that the domain I of AMA1 isolates from these sites is under natural selection which is possibly the reason for the

increased allelic diversity in AMA1 observed. Taken togethe, results from this study support the theory that the high allelic diversity at domain I of *Pf*AMA1 is due to strong selective pressure by host immune response (Polley and Conway 2001).

Balancing selection, as observed in AMA1 from this study, may be widespread, but its most common form may be difficult to detect.This indicates that there is a need for independent and consistent temporal and spatial investigations of selections on these vaccine candidate antigens of *P. falciparumi* population in the country.

A relatively new protein, PfRH5, recently identified and characterized is the fifth member of the *Pf*RH family (Arevalo-pinzon *et al.,* 2012). *Pf*RH5 is located in the tight junction together with AMA1 and RON2, probably in association with these proteins, due to lack of transmembrane proteins in its sequence. Data from experimental studies have shown the crucial role of *Pf*RH5 in erythrocyte invasion. Otsuki *et al* (2009) have demonstrated that a single amino acid substitution in the *P. yoelii* EBL protein affected protein virulence and localization.

The high activity binding polypeptides 36718, 36727 and 36728 of RH5 were genetically analysed from Nigerian isolates in this study. A single nucleotide polymorphism was observed generating three haplotypes similar to that reported by Arevalo-Pinzon *et al*(2012); genetic analysis of the 810 bp sequences revealed that the Tajima's D and dN-dS values for PfRH5 were negative suggesting a purifying selection on this gene. This is the first report on RH5 from Nigeria. Consequent upon the very low polymorphism in this gene, there is a high tendency for susceptibility of RH5 to universally effective vaccines based on PfRH5.Bustamante *et al* (2013) have reported a rationale to advance *Pf*RH5-based vaccine to the PhaseI/IIa human trials, which if successful, could be combined with vaccines targeting other stages of the *P. falciparum* life cycles, such as the

sporozoite stage in the *RTS,S* vaccine currently undergoing trials. This, consequently promises a good efficacy of such vaccines in Nigeria going by the results from this study.on RH5.

Phylogenetic analysis of the two genes also revealed a clustering of the haplotypes and evolutionary relationship of the haplotypes with the reference 3D7 and other reported strains including the PAS-2, FCB-2 RH5 sequences and AMA1 sequences including those from the only data available from Nigeria (Polley and Conway 2001). Sironi *et al*(2015*)* has shown that evolutionary analysis may help explain changes in tropism when information on the location and nature of functional genetic variation are provided.

The low Fst values also suggest high gene flow between two populations in the study sites. This implies that the introduction of any drug- or vaccine-resistant allele may be dangerous as it spreads fast among the *P. falciparum* isolates owing to low genetic differentiation between populations in the study sites with a resultant negative impact on control measures.

One limitation in applying genetic technique is the possible occurrence of ancillary causes of selection other than immunity which may operate on the parasite at other stages of development. These may include rapidly increased frequency of antigenic alleles at a locus which occasionally occurs per chance and may be indistinguishable from selection events. Alleles existing at a locus for a period time can acquire individual sets of neutral mutations that are unique unless recombination events occur causing linkage between the functionally different alleles.

No association between malaria transmission intensity and genetic variations on vaccine candidate antigenic proteins was found. The MOI which is considered a key indicator of malaria infection in humans, transmission and immunity (Smith *et al*., 1999), was not significantly associated with genetic variations in *Pf*AMA1.

In summary, the data generated from this study provide a molecular basis for the explanation of the pattern of natural dynamics of polymorphisms in two leading vaccine candidate antigenic proteins potentially useful in the development of effective *Pf*AMA1- and *Pf*RH5- based vaccines.

5.1 CONCLUSION

The study has established a genetic direction describing the molecular structure of Nigerian *P. falciparum* isolates having a complex population structure for polymorphic *Pf*AMA1 with new haplotypes reported. The study showed that Nigerian isolates of *P. falciparum* would be susceptible to any universally efficacious RH5-based vaccines. These suggest a need for a national survey of polymorphisms in these genes to facilitate the development of multi-component vaccine effective in Nigeria. Nutritional status and transmission intensity may modulate T-cell responses in *Plasmodium falciparum* malaria.susceptibility to malaria and clinical episodes are associated with elevated TNF-α.

5.2 SUMMARY OF FINDINGS

The following findings emanated from studies to determine polymorphisms in *Plasmodium falciparumPfAMA1* and *PfRH5* genes in isolates from selected centres in Lagos, Nigeria .

1. Malaria prevalence among febrile patients in the study populations was 20.39% and highest in Ijede in Ikorodu (22.05%), 19.44% in Ajeromi and lowest in Lekki (18.99%).

2. Anaemia, thrombocytopenia (< 150,000 x 10^9/L) and Leucocytosis (proliferation of WBC) are predictivehaemtological features in malaria.

3. In malaria, levels of pro-inflammatory immune responses are raised independent of host gender and parasite density

4.Overall multiplicity of infection (MOI) was 1.54 in the sites put together while ijede has the highest MOI of 2.12, Ajeromi 1.33 and lowest was Lekki (0.86) suggesting relatively low malaria transmission in Lagos. This study reported MOI for the fisrt time in Ijede and Ajegunle.

5. FC27 family of merozoite surface protein 2 (MSP2) was the most prevalent family of MSP2 circulating in Lagos.

6. Genetic diversity (H_e) values from the three sites ranged from 0.77-0.87 and was highest in Ijede and lowest in Lekki.

7. Ninety-three (93) different haplotypes of AMA1 were found in this study, forty-eight (48) of which have not been previously reported.

8. There was a strong selective pressure on AMA1 in Lagos, Nigeria with positive values for Tajima's D and dN-dS, LD value also showed recombination events on AMA1 in Nigeria.

9. Three haplotypes of PfRh5 were found from this study with a single nucleotide polymorphism (SNP, G608A) within the high activity binding polypeptide (HABP) 36727 similar to 7G8 *P. falciparum* strains.

10. The Linkage Disequilibrium index for *RH5* was increasing showing no recombination events taking place on RH5 in Lagos, Nigeria.

11. The genetic differentiation analysis showed little or no population sub-structuring of *P. falciparum* parasites in the studied populations with high rate of gene flow between populations (Fst values ranged from 0.007 to 0.037).

12. There was no significant association between polymorphisms in the *PfAMA1* and any of the pro-inflammatory cytokines studiedwhile RH5 was strongly negatively correlated with TNF-α and positively correlated with multiplicity of infection.

5.3CONTRIBUTIONS TO KNOWLEDGE

1.The study revealed that Nigerian isolates of *P. falciparum* would be susceptible to globally efficacious *Pf*RH5-based malaria vaccine

2.Three haplotypes (RH5-H1, RH5-H2 and RH5-H3) of *Pf*RH5 from Nigerian *P. falciparum* isolates were found and have been submitted to the data base [GenBank: KX418644-KX418646] with single nucleotide polymorphism (SNP) G → A at position 608 in Nigerian isolates of *Pf*RH5 which has **not** been reported before in Nigeria.

3. The study found forty-eight new *Pf*AMA1 haplotypes out of the total ninety-three haplotypes identified which have been submitted to the GenBank nucleotide data base (ww.ncbi.nlm.nih.gov/) with accession number [GenBank: KU986915-KU987007] as data to guide vaccine development.

4.The study found that T-cell responses are independent of polymorphisms in *Pf*AMA1 useful for any PFAMA1-based vaccine development efforts

5.4RECOMMENDATIONS

In view of the findings from this study on the determination of polymorphisms in *Pf*AMA1 and *Pf*RH5 genes in selected centres in Lagos, Nigeria, the following recommendations are made to assist the country in malaria control efforts especially effective vaccine development:

1. A relatively high malaria prevalence was reported calling for or review and scale-up of malaria control programmes in the state. This should include community enlightenment efforts to improve knowledge, attitude and awareness of the people on malaria especially in realtively less developed and rural areas.

2.The study revealed high rate of gene flow, low genetic differentiation and low genetic sub-structuring of *P.falciparum* parasites in Lagos, therefore effective surveillance strategy should be instituted to detect drug- or vaccine- resistance alleles early to prevent upsurge in morbidity and mortality due to malaria.

3.This investigation has shown that globally efficacious RH5-based vaccine will be protective in Lagos, strategies aimed at generating population- specific immunogenetic data useful for vaccine design should be implemented.

4. A national immunogenetic study should be conducted to ascertain the efficacyand potentialof globally efficacious RH5-based vaccine in Nigeria.

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APPENDICES

Appendix 1: Approval of the Institutional Ethics Review Board of the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria.

INSTITUTIONAL REVIEW BOARD

IRB-NIMR

NIGERIAN INSTITUTE OF MEDICAL RESEARCH

6, Edmond Crescent Off Murtala Muhammed Way, P. M. B. 2013 Yaba, Lagos.
Tel: 01-4823123, 01-7744723, 08050254484, 08033460947 Fax: 01-4823123, 234-1-3425171
E-mail: nimr_irb@yahoo.com *Website:* www.nimr-nig.org
Secretariat: Room 207, Biochemistry Division, Research Block, NIMR

03/09/2011

PROJECT TITLE:

POLYMORPHISMS IN PLASMODIUM FALCIPARUM APICAL MEMBRANE ANTIGEN-1(AMA1) IN RELATION TO MALARIA OUTCOMES IN LAGOS, NIGERIA.

PROJECT NO.IRB/10/136

APPROVAL LETTER

The above named proposal has been adequately reviewed; the protocol and safety guidelines satisfy the conditions of NIMR IRB, policies regarding experiments that use human subjects.

Therefore the study under its reviewed state is hereby approved by Institutional Review Board, NIMR.

INSTITUTIONAL REVIEW BOARD Room 207 Research Block Nigerian Institute of Medical Research Signature & Date

DR. P. U. AGOMO 03/09/11
Name of vice IRB chairman *Signature & Date of IRB vice Chairman*

DR. A.A ADEIGA 3/9/11
Name of IRB Member *Signature & Date of IRB Member*

This approval is given with the investigator's Declaration as stated below; By signing below I agree/certify that:

1. I have reviewed this protocol submission in its entirety and that I am fully cognizant of, and in agreement with, all submitted statements.
2. I will conduct this research study in strict accordance with all submitted statements except where a change may be necessary to eliminate an apparent immediate hazard to a given research subject.
   - I will notify the IRB promptly of any change in the research procedures necessitated in the interest of the safety of a given research subject.
   - I will request and obtain IRB approval of any proposed modification to the research protocol or informed consent document(s) prior to implementing such modifications.

Appendiix 1 Cont'd

3. I will ensure that all co-investigators and other personnel assisting in the conduct of this research study have been provided a copy of the entire current version of the research protocol and are fully informed of the current (a) study procedures (including procedure modifications); (b) informed consent requirements and process; (c) potential risks associated with the study participation and the steps to be taken to prevent or minimize these potential risks; (d) adverse event reporting requirements; (e) data and record-keeping; and (f) the current IRB approval status of the research study.

4. I will respond promptly to all requests for information or materials solicited by the IRB or IRB Office.

5. I will submit the research study in a timely manner for IRB renewal approval.

6. I will not enroll any individual into this research study until such time that I obtain his/her written informed consent, or, if applicable, the written informed consent of his /her authorized representative (i.e., unless the IRB has granted a waiver of the requirement to obtain written informed consent).

7. I will employ and oversee an informed consent process that ensures that potential research subjects understand fully the purpose of the research study, the nature of the research procedures they are being asked to undergo, the potential risks of these research procedures, and their rights as a research study volunteer.

8. I will ensure that research subjects are kept fully informed of any new information that may affect their willingness to continue to participate in the research study.

9. I will maintain adequate, current, and accurate records of research data, outcomes, and adverse events to permit an ongoing assessment of the risks/benefit ratio of research study participation.

10. I am cognizant of, and will comply with, current federal regulations and IRB requirements governing human subject research including adverse event reporting requirements.

11. I will make a reasonable effort to ensure that subjects who have suffered an adverse event associated with research participation receive adequate care to correct or alleviate the consequences of the adverse event to the extent possible.

12. I will ensure that the conduct of this research study adheres to Good Clinical Practice guidelines.

MR. AJIBAYE OLUSOLA
Principal Investigator Name

3/9/11
.....
Principal Investigator signature and Date

Appendix 2: Consent / Assent Form

CONSENT FORM

POLYMORPHISMS IN *PLASMODIUM FALCIPARUM* APICAL MEMBRANE ANTIGEN-I (*Pf*AMA1) AND RETICULOCYTE-BINDING PROTEIN HOMOLOG -5 (*Pf*RH5) GENES FROM SELECTED CENTRES IN LAGOS NIGERIA.

This study seeks to characterize the Malaria parasites in circulation in Lagos state towards the development of an effective malaria vaccine.

As volunteer(s) in this malaria study, a small portion of blood sample will be collected from patients that have been recommended by the doctor for malaria test at the hospital's blood collection point and the body Temperature will be examined for fever.

This document seeks your permission to also test your blood for parasitaemia and molecular study and other blood test necessary. Only a little amount of blood is needed for this purpose.

This is at no cost to you and I will not disclose confidential information that may be obtained in the process of your participation to anyone.

Your decision to participate is voluntary and is indicated in your signing bellow. If you decide not to participate, there will be no repercussion to it. In addition, you can ask questions on anything that is not clear to you about your participation in this study.

The above information has been read to me or I have read it. I have been able to ask questions about this study. I voluntarily consent to my/my child's participation in this study, and I understand that I can withdraw myself/my child anytime from the study.

Name of Participant:_____

Name of Parent/Guardian:_____

Signature (or finger print/ Assent):_____

Name and Signature of Researcher:_____ Phone: 08069032569

Date:_____

Appendix 3:*Plasmodium falciparum* Apical Membrane Antigen-I (*PF*AMA1) Amino Acid Sequences From The Study Sites

>Seq1

GIIIENSKTTFLTPVATENQDLKDGGFAFPPTNPLISPMTLNGMRDFYKNNEYVKNLDELTLCSRHAGNMNPDNDKNS
NYKYPAVYDYNDKKCHILYIAAQENNGPRYCNKDQSKRNSMFCFRPAKDKSFQNYTYLSKNVVDNWEKVCPRK

>Seq2

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>seq3

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>Seq4

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>Seq5

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>Seq8

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>Seq9

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>Seq10

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>Seq12

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>Seq21

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>Seq22

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>Seq23

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>Seq24

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>Seq25

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>Seq26

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>sEQ27

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>seq28

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>Seq29

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>SeQ30

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>Seq31

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>Seq34

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>Seq35

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>Seq36

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>seq38

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>Seq39

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>Seq42

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>Seq44

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>Seq45

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>Seq46

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>seq47

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>Seq48

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>Seq49

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>Seq50

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>seq51

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>seq52

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>Seq53

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>Seq54

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>Seq57

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>Seq58

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>seq59

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>Seq60

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>Seq61

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>Seq62

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>seq63

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>Seq64

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>Seq65

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>seq66

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>Seq67

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>Seq68

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>Seq69

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>Seq70

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>Seq71

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>Seq72

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KYPAVYDDKNKKCHILYIAAQENNGPRYCNKDESKKNSMFCFRPAKDRSFQNYTYLSKNVVDNWEKVCPRK

>seq73

GIIIENSKTTFLTPVATENQDLKDGGFAFPPTNPLMSPMTLNGMRDLYKNNEYVKNLDELTLCSRHAGNMNPDNDKN
SNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDKSFQNYTYLSKNVVDNWEKVCKKK

>Seq74

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>Seq75

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>Seq76

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SNYKYPAVYDYNDKKCHILYIAAQENNGPRYCNKDQSKRNSMFCFRPAKDKLFENYTYLSKNVVDNWEKVCPRK

>Seq77

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>Seq78

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>Seq79

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>seq80

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>Seq81

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NYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDQSKRNSMFCFRPAKDISFQNYTYLSKNVVDNWEKVCLKK

>Seq82

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>seq83

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>Seq85

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>seq86

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>Seq87

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>seq88

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>seq89

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>seq90

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>seq91

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>seq92

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>Seq93

GIIIENSNTTFLTPVATEKQDLKDGGFAFPPTKPLMSPMTLNDMRHFYKNNEYVKNLDELTLCSRHAGNMNPDNDKNS
NYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDQSKRNSMFCFRPAKDKSFQNYTYLSKNVVDNWEKVCPRK

Appendix 4: *Plasmodium falciparum* Reticulocyte-binding protein homologue-5 (*Pf*RH5) Nucleotide sequences (Haplotypes) from the study sites

Appendix

>Seq1 [organism=Plasmodium falciparum] Plasmodium falciparum isolate IJ07 RETICULOCYTE BINDING PROTEIN HOMOLOG 5 (PfRH5) gene partial CDS

ATTAAGTTTTGAAAATGCAATAAAAAAAACGAAGAATCAAGAAAATAATTTGACGTTACTAACAATACAGAGCACT
GAAAGAAGAAAAGATGATATAAAAAATGGAAAGAATATAAAGAAAGAAATTGATAATAATAAAGAGAATATAAA
AACAAATAATGCTAAAGATCATTCAACAAATATAAAATCATATTTGAATACAAATGTAAATGATGCTTTAAAATATT
TGTTTATTCCTTCTCATAATTCTTTTATAAAAAAATATTCTGTATTTAATCAAATAAATGATGGCATGTTATTAAATGA
AAAAAATGATGTGAAAAATAATGAAGACTATAAAAATGTGGATTATAAAAATGTTAATTTTTTACAATATCATTTTA
AAGAGTTATCAAATTATAACATTGCAAATTCTATTGATATTTTACAAGAAAAAGAAGGACATTTGGATTCTGTTATA
ATACCTCATTATACTTTTTTAGATTATTATAAACATTTATACTCTAATTGTATATATCCTAAGTCCTGTACATATGGCA
AGTACATAGCTGTAGATGCTTTTATTAAGAAAATAAATGAAACATATGACAAAGTGAAAAGTAAAACCAACGAGA
CTACCCATGACGCCCTTCCTATCCCCCGAGGAATACCCCGCGGTA

>Seq2 [organism=Plasmodium falciparum] Plasmodium falciparum isolate IJ08 RETICULOCYTE BINDING PROTEIN HOMOLOG 5 (PfRH5) gene partial CDS

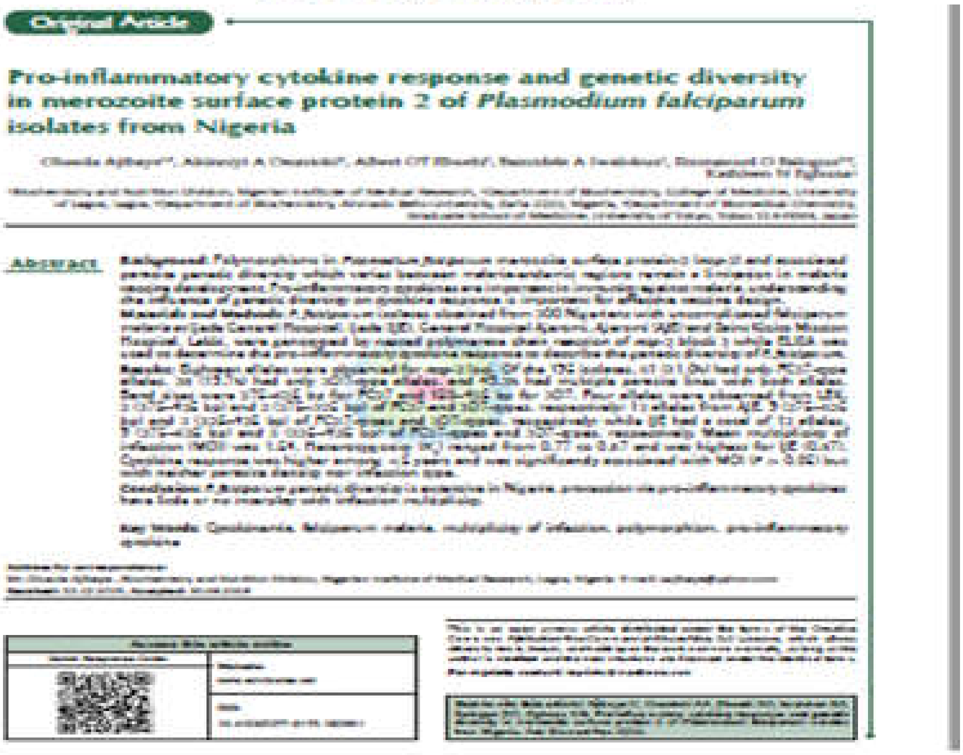
ATTAAGTTTTGAAAATGCAATAAAAAAAACGAAGAATCAAGAAAATAATCTGACGTTACTACCAATAAAGAGCACT
GAAGAAGAAAAAGATGATATAAAAAATGGAAAGGATATAAAAAAAGAAATTGATAATGATAAAGAGAATATAAA
AACAAATAATGCTAAAGATCATTCAACATATATAAAATCATATTTGAATACAAATGTAAATGATGGTTTAAAATATT
TGTTTATTCCTTCTCATAATTCTTTTATAAAAAAATATTCTGTATTTAATCAAATAAATGATGGCATGTTATTAAATGA
AAAAAATGATGTGAAAAATAATGAAGACTATAAAAATGTGGATTATAAAAATGTTAATTTTTTACAATATCATTTTA
AAGAGTTATCAAATTATAACATTGCAAATTCTATTGATATTTTACAAGAAAAAGAAGGACATTTGGATTTTGTTATA
ATACCTCATTATACTTTTTTAGATTATTATAAACATTTATCTTATAATTCTATATATCATAAGTCCTCTACATATGGAA
AGTATATAGCTGTAGATGCTTTTATTAAGAAAATAAATGAAACTCATGAGGAATTGAGTCATGAAACCAATGGGA
ATGAGTATAGCATAAAAGATATCCCCCAGAAAACAACCAGAGATA

>Seq3 [organism=Plasmodium falciparum] Plasmodium falciparum isolate IJ22 RETICULOCYTE BINDING PROTEIN HOMOLOG 5 (PfRH5) gene partial CDS

ATTAAGTTTTGAAAATGCAATAAAAAAAACGAAGAATCAAGAAAATAATCTGACGTTACTACCAATAAAGAGCACT
GAAGAAGAAAAAGATGATATAAAAAATGGAAAGGATATAAAAAAAGAAATTGATAATGATAAAGAGAATATAAA
AACAAATAATGCTAAAGATCATTCAACATATATAAAATCATATTTGAATACAAATGTAAATGATGGTTTAAAATATT
TGTTTATTCCTTCTCATAATTCTTTTATAAAAAAATATTCTGTATTTAATCAAATAAATGATGGCATGTTATTAAATGA
AAAAAATGATGTGAAAAATAATGAAGACTATAAAAATGTGGATTATAAAAATGTTAATTTTTTACAATATCATTTTA
AAGAGTTATCAAATTATAACATTGCAAATTCTATTGATATTTTACAAGAAAAAGAAGGACATTTGGATTTTGTTATA
ATACCTCATTATACTTTTTTAGATTATTATAAACATTTATCTTATAATTCTATATATCATAAGTCCTCTACATATGGAA
AGTGTATAGCTGTAGATGCTTTTATTAAGAAAATAAATGAAACATATGACAAAGTGAAAAGTAAATGTAATGATAT
AAAGAATGATTTAATTGCAACTATAAAAAAATTAGAGCATCCTT

Appendix 5: Published data on Pro-inflammatory cytokine response and genetic diversity in merozoite surface protein 2 of *Plasmodium falciparum* isolates from Nigeria

PUBLICATION



Original Article

Pro-inflammatory cytokine response and genetic diversity in merozoite surface protein 2 of *Plasmodium falciparum* isolates from Nigeria

Abstract

Appendix 6: EQUIPMENT AND REAGENTS

The following equipment and reagents were used in the study:

1. ALL Well-Being Digital Thermometer
2. Glycerol
3. 4mL EDTA vacutainer bottles (BD Vacutainer)
4. 5mL syringes with needles (Dana-Ject,Zheijiang kindly Medical Devices & Plastics Co.Ltd,China)
5. 96 well 1ml Plate
6. Beckmann Coulter Closed Tube Automated Hematology system
7. BIO-RAD^{R} DNA Engine, Peltier Thermal cycler ,Mexico
8. Consort Electrophoresis System (Power Pack & Tank)
9. CX21 Olympus Microscope (Olympus Corporation,Tokyo ,Japan)
10. DELE Absorbent cotton wool BP (Ayo –Ayodele Pharmaceutical Chemists Ltd)
11. Determine HIV Rapid Diagnostic kits
12. Disodium Hydrogen phosphate (BDH Chemicals Ltd, Poole, England)
13. ELISA Reader
14. ELISA Washer
15. Eppendorf adjustable autoclavable Micropipette (0.1-5.0µL)- Eppendorf AG,Germany
16. Eppendorf adjustable autoclavable Micropipette (100-1000 µL)- Eppendorf AG,Germany
17. Eppendorf adjustable autoclavable Micropipette (1-100 µL)- Eppendorf AG,Germany
18. Eppendorf Centrifuge 5810R (Eppendorf AG,Germany)
19. Equitron^{R} Semi-automatic Autoclave (Equitron, Mumbai)
20. Gel Documentation Machine
21. Gel Documentation System ,UV Gel Imager(Science Instrumentation Ltd)
22. Giemsa stain powder

23. Imersion Oil (QCA)
24. Jena Boscience, PCR Master mix
25. Kangaro^{R} hole Punch
26. Latex Hand gloves (Neogloves Latex Examination gloves, Neomedic Ltd,UK)
27. Methanol
28. Methylated Spirit
29. Microscope slides (Marienfeld Laboratory Glassware, Paul Marienfeld GmbH and Co.Germany)
30. Pioneer ^{TM} Ohaus Weighing Balance, Ohaus Corp.,Pine Brook,NJ USA.
31. Potassium dihydrogen Phosphate (BDH Chemicals Ltd, Poole ,England)
32. Qiagen DNA Extrction kit (QIAamp DNA mini kits,Qiagen group,GmbH,Hilden)
33. Standiometer (SECA, Hamburg, Germany)
34. Tourniquet (Bioscientia ,Germany)
35. Waterbath
36. Whatman No.3 Filter paper (for Dried blood Spots)