HOST CYTOKINE GENE POLYMORPHISMS AND PARASITE GENETIC VARIABILITY IN DETERMINING THE DISEASE OUTCOME OF *Plasmodium falciparum* INFECTION

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ABSTRACT

Malaria, the world's most important tropical parasitic disease is caused by *Plasmodium* species. Infection with *Plasmodium falciparum* can result in one of three possible disease outcomes: Asymptomatic (AS), Uncomplicated Malaria (UM) or Severe Malaria (SM). Information on host genetic factors and parasite genetic diversity can improve understanding of the disease pathogenesis. In this study, the genetic diversity of *P. falciparum* isolates as well as polymorphisms in host cytokine genes was investigated in relation to the outcome of *P. falciparum* infection.

Four hundred and thirty-seven children recruited from the Specialist Hospital, Lafia, Nasarawa State were assigned into UM or SM based on malaria severity, determined by clinical and laboratory diagnoses. Asymptomatic children recruited from primary schools within the study location constituted the control group (AS). *Plasmodium falciparum* infection was confirmed by PCR-based assay of SSUrRNA genes. Genetic diversity of *P. falciparum* was analyzed by genotyping the polymorphic domains of the Merozoite Surface Protein 2 *(MSP-2).* Host cytokine genes investigated included Interleukin-18 (IL-18), IL-18 receptor alpha (IL-18Rα) and Tumour Necrosis Factor alpha (TNF-α). Sequencing of *MSP*-2 gene and of the pro-inflammatory cytokines was carried out using ABI PRISM® 3100. Sequences were analyzed using the BioEdit Sequence Alignment software. Genotype and allelic frequencies were analyzed by Chi-square test. The level of significance was set at P=0.05.

All participants had *P. falciparum* infection. Polyclonality was significantly higher in the AS (61%) and UM (60%) groups compared with the SM (34%) group. Mean multiple infections was 2.1 ±1.0 in AS, 2.0 ±1.0 in UM and 1.3 ±0.6 in SM. A total of 32, 35 and 28 distinct *MSP-2* alleles were found in the AS, UM and SM groups respectively. Frequency of the 3D7 allele type was significantly higher in UM (51%) and SM (54%) groups compared to the AS group (38%). Sequence analysis of the central variable region of the *MSP-2* gene showed that the FC27-type sequence was characterised by two unique subtypes and hybrid sharing sequence with the two subtypes. The 3D7-type sequence was characterised by three subtypes of repetitive domains: a GSA-rich repeat unit, a TPA repeat motif and a poly-Threonine stretch. Three single nucleotide polymorphisms (SNPs): -656G/T, -607C/A and -137G/C were identified at the promoter region

of IL-18 gene. The -656G/T and -607C/A SNPs were found to be in complete linkage disequilibrium. The genotype frequency of -607AA was significantly higher in the AS group compared to SM cases (χ^2=4.26, P<0.05). Likewise, four SNPs were identified at the promoter and Exon 1 of the IL-18Rα: -661T/C, -175G/A, -93C/T and Ex1 +21C/G but none was associated with disease outcome based on statistical level of significance. Exons 2 to 11 of IL-18Rα gene were relatively conserved. Furthermore, two SNPs: -308G/A and -238G/A were identified at the promoter region of TNF-α but none was associated with disease outcome.

Plasmodium falciparum was found to be genetically heterogeneous. Higher carriage of *Plasmodium falciparum* 3D7 alleles indicates higher risk of developing symptomatic malaria. There was association between IL18 -607AA genotype and asymptomatic infection, probably indicating a protective role.

Keywords: *Plasmodium falciparum*, Merozoite, Cytokine, Polymorphism, Disease outcome.

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CERTIFICATION

I certify that this work was carried out by Mr. Segun I. OYEDEJI, Department of ZOOLOGY, University of Ibadan.

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DEDICATION

To the loving memory of my late Dad, Dn. Isaac B. Oyedeji and my late older sister, Mrs Grace Onwuasoanya, as well as my late father-in-law, Mr. Chris Michael Ekwuribe.

To my wife, Mrs Mayowa Ngozi Oyedeji and my sons, Ephraim and Obaloluwa for their patience, love, sacrifices, prayers and support all through the period of this study.

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ABBREVIATIONS

HWE:	Hardy-Weinberg equilibrium:
IL:	Interleukin
IL-18Rα:	Interleukin-18 receptor alpha
TNF-α:	Tumour necrosis factor alpha
IFN-γ:	Interferon gamma
LD:	Linkage disequilibrium
OR:	Odd ratio
AM:	Asymptomatic malaria
UM:	Uncomplicated malaria
SM:	Severe malaria
PCR:	Polymerase chain reaction
SNP:	Single nucleotide polymorphism
dNTPs:	2'-deoxynucleoside-5'-triphosphate
HLA:	Human Leukocyte Antigen
MHC:	Major Histocompatibility Complex
G6PD:	Glucose-6-phosphate dehydrogenase
Hb:	Haemoglobin
NK Cells:	Natural killer cells
JAK-STAT:	Janus-Kinase protein-signal transducers and activators of transcription factors
Ig:	Immunoglobulin
GPI:	Glycosyl-phosphatidyl-inositol
µl:	Microlitre
MOI:	Multiplicity of Infection
kDa:	Kilodalton
mg:	Milligram
PCV:	Packed cell volume (haematocrit)
WBC:	White blood cells
WHO:	World Health Organisation

DNA: Deoxyribonucleic acid

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Malaria is the world's most prevalent and by far, the world's most important tropical parasitic disease (Garcia *et al.*, 1995; WHO, 2000b; Garcia, 2010). It is a global health problem and a threat to about 40% of the world's population (WHO, 2003b). It has been estimated that about 300-500 million clinical cases occur each year and more than one million people die from the disease annually, mostly infants, young children and pregnant women (WHO, 2000b, 2009; Murray *et al.*, 2012).

Malaria exerts its heaviest toll in Africa, where around 90% of the more than a million deaths from malaria occur each year (WHO, 2003a; Snow *et al.*, 2005). The largest population at risk of the disease in Africa is in Nigeria, where malaria is a major public health problem (WHO, 2008b), accounting for about 65% of hospital cases and resulting in the death of close to 300,000 children annually (WHO/UNICEF, 2005; FMOH, 2008).

Human malaria is traditionally known to be caused by four species of plasmodial parasites: *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae.* A fifth species, *Plasmodium knowlesi*, previously known to infect rhesus monkeys has now been found to be widely distributed in human population in Malaysia with the potential to cause disease and deaths (Cox-Singh *et al.*, 2008, 2010). Malaria parasites are usually transmitted by the bites of infected female *Anopheles* mosquitoes. Africa is home to three major malaria vectors: *Anopheles gambiae*, *Anopheles funestus* and *Anopheles arabiensis* (Collins *et al.*, 2000; Rizzo *et al.*, 2011).

P. falciparum is the most virulent species of human malaria parasites (Gupta *et al.*, 1994; Chen *et al.*, 2000). It has been estimated that about 2.37 billion people worldwide, are at risk of *P. falciparum* infection alone (Guerra *et al.*, 2008). In sub-Saharan Africa, most malaria infections are caused by *P. falciparum* where it is one of the most common causes

of childhood morbidity and mortality (Snow *et al.*, 2005; WHO, 2005). Infections with *P. falciparum* can lead to different degrees of illness which initially starts as symptomless or asymptomatic infection; but may progress to acute uncomplicated disease, or to life-threatening severe forms (Snow *et al.*, 2005; WHO 2000). However, the vast majority of malaria cases still present as non-specific febrile illnesses that are relatively easily terminated by either antimalarial treatment or, eventually, by host responses. Only a small proportion of cases, approximately 1%, progress to severe life-threatening disease (Mackintosh *et al.*, 2004; WHO/UNICEF, 2005). The reasons for these differences are not fully understood. However, several studies have suggested that variability in the clinical outcome of *P. falciparum* infection may be a consequence of heterogeneity in parasite phenotypes and host factors (Craig *et al.*, 2000; Migot-Nabias *et al.*, 2000; Conway, 2007; Olotu *et al.*, 2012).

Several parasite factors have been shown to influence the clinical outcome of malaria. High propagation capacity of parasites, leading to high parasite loads and subsequent depletion/destruction of erythrocytes is a key factor that contributes to the clinical features of malaria (Haldar *et al.*, 2007). The unique ability of *P. falciparum* infected RBCs to adhere to vascular endothelium (cytoadherence) and to non-infected erythrocytes (rosetting) is also a major contributor to the clinical manifestations of malaria (Kyes *et al.*, 2001; Doumbo *et al.*, 2009; Fatih *et al.*, 2012). In addition, the malaria parasite has a notorious survival mechanism- the ability to undergo almost unlimited antigenic variation through changing the antigens on the infected red cell surface (Chen *et al.*, 2000; Moxon *et al.*, 2011; Merrick *et al.*, 2012; Witmer *et al.*, 2012).

Furthermore, there exists wide range of genetic polymorphisms in natural populations of malaria parasites. Specifically, studies have shown that infections with *P. falciparum* exhibit a wide range of genetic diversity (Ntoumi *et al.*, 1995; Smith *et al.*, 1999b; Amodu *et al.*, 2008; Takala and Plowe, 2009; Auburn *et al.*, 2012). Genetic diversity of *P. falciparum* infections have been found to be higher in areas of high transmission than in low transmission areas (Babiker *et al.*, 1997; Farnert *et al.*, 2001; Ghanchi *et al.*, 2010; Atroosh *et al.*, 2011) and, it is observed at any of the developmental stages of the parasite. Genetic diversity in the parasite population increases the likelihood of an individual being

infected with different parasite genotypes (Arnot, 2002). Such infections are generally described as multiple or complex infections (Bendixen *et al.*, 2001; Mayengue *et al.*, 2011). This is particularly indicated by the large pool of merozoite surface protein-1 (MSP-1) and MSP-2 alleles reported so far (Ntoumi *et al.*, 1996; Robert *et al.*, 1996; Beck *et al.*, 1999; Hussain *et al.*, 2011; Mayengue *et al.*, 2011; Koukouikila-Koussounda *et al.*, 2012). Genetic complexity of *P. falciparum* (Arnot, 2002) and especially polymorphism of its surface antigen (Kyes *et al.*, 2001) have been associated with clinical malaria (Al-Yaman *et al.*, 1997; Beck *et al.*, 1997; Farnert *et al.*, 1999) in disease endemic areas. In Nigeria however, only few studies have been conducted on the genetic diversity of *P. falciparum* infections and most were in Southern Nigeria (May *et al.*, 1999; Engelbrecht *et al.*, 2000; Happi *et al.*, 2004; Amodu *et al.*, 2008; Ngoundou-Landji *et al.*, 2010; Ojurongbe *et al.*, 2011). The only available data in Northern Nigeria assessed genetic diversity in children with asymptomatic *P. falciparum* infections (Engelbrecht *et al.*, 2000). There is therefore need to assess genetic diversity in clinical infections including uncomplicated malaria as well as severe malaria cases. Moreover, there is no available data on the sequence diversity of *P. falciparum* isolates in Nigeria. These gaps in information are part of what this study is set out to fill in order to generate data that will be relevant for malaria control interventions.

Apart from the role of genetic constitution of the parasite in determining disease outcome, there is evidence also that the genetic background of the host, influences the degree of protection an individual develops against malaria (Hill, 1996; Hill, 1999; Knight and Kwiatkowski, 1999; Kwiatkowski, 2005). Host genetic factors may determine an individual's initial resistance to malaria, their likelihood of developing severe complications once an infection has occurred, or their prospect of acquiring effective long-term immunity (Kwiatkowski, 2000). One of the best examples of host genetic resistance to malaria came from studies on genes encoding red blood cell proteins such as haemoglobin S (HbS), HbC, α-thalassaemia and glucose-6-phosphate dehydrogenase (G6PD), with the HbS allele regarded as the classic paradigm of balanced polymorphism (Haldane, 1949; Allison, 1954; Lederberg, 1999; Feng *et al.*, 2004; Kwiatkowski, 2005; Cappellini and Fiorelli, 2008; Taylor *et al.*, 2012).

Beside studies on variants of red cell genes, several other studies on immunity-related genes as well as genes encoding adhesion molecules have shown variation among individuals for disease resistance to malaria (Hill *et al.*, 1991; Jepson *et al.*, 1997). Analyses of the major histocompatibility complex (MHC) have shown strong evidence for associations between both HLA Class I and Class II alleles and susceptibility to *P. falciparum* malaria (Hill *et al.*, 1991; Weatherall and Clegg, 2002). Variants of genes encoding adhesion molecules such as ICAM-1 and CD36 have also been suggested to affect the outcome of *Plasmodium* infections (Fernandez-Reyes *et al.*, 1997; Aitman *et al.*, 2000).

It is well established that host immune responses to malaria include components of the innate and adaptive immune systems (Perlmann and Troye-Blomberg, 2002). These two arms of the immune defence mechanism are pivotal in controlling parasite replication and clearance from parasitized host. Furthermore, there are indications from both murine models and human studies suggesting that cytokines, along with T cells, NK cells and macrophages, contribute to the pathophysiology of either survival or fatal outcome in *P. falciparum* infections (Winkler *et al.*, 1999; Hensmann and Kwiatkowski, 2001; Torre *et al.*, 2002b; Wroczynska *et al.*, 2005). Cytokines are immuno-modulatory proteins produced by macrophages as well as lymphocytes or monocytes in the blood, to influence the function of other cells through specific receptor binding (Plebanski *et al.*, 2002). Although several studies have shown association between cytokine profile or concentration and disease pathology, data are also emerging showing that differences in susceptibility to, and severity of malaria might well have their roots in genetically-defined differences in the host's ability to produce vital cytokines (McGuire *et al.*, 1999; Kwiatkowski, 2000; Ubalee *et al.*, 2005; Tchinda *et al.*, 2007b; Israelsson *et al.*, 2011). Consequently, many attempts have been made to elucidate the contribution of polymorphisms in genes encoding proinflammatory mediators, amongst them the interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and Interleukin-18 (IL-18) genes (Giedraitis *et al.*, 2001; Claser *et al.*, 2011; Santovito *et al.*, 2012).

TNF-α is a proinflammatory cytokine involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, and coagulation (Beutler and Grau, 1993). It is a potent pyrogen, causing fever by direct action

or by stimulation of interleukin-1 secretion. TNF-α is secreted mainly by macrophages. Studies have suggested that serum levels of TNF-α may impact on individual susceptibility to disease (Clark and Rockett, 1994; Wilson *et al.*, 1997; Miller *et al.*, 2002). TNF levels have been shown to be elevated in the serum of malaria subjects who have a poor disease outcome (Kwiatkowski *et al.*, 1990). TNF is also raised in placental malaria and is associated with low birth weight (Fried *et al.*, 1998; Moormann *et al.*, 1999).

Genetic variations in the TNF-α gene have been suggested to influence TNF-α production (Abraham and Kroeger, 1999; Knight, 2005; Sohail *et al.*, 2008). A particular polymorphic form of the TNF promoter has been shown to be associated with susceptibility to cerebral malaria (McGuire *et al.*, 1994). A single nucleotide polymorphism from guanine (G) in the normal TNF 1 allele to adenine (A) in the TNF 2 allelic variant at the position -308 (relative to the transcription start site of the TNF-α gene), has been found to be associated with increased TNF-α production (Aidoo *et al.*, 2001). In addition, the TNF 2 allele was found to be associated with higher parasitaemia, severe anaemia, pre-term birth and early childhood mortality. The presence of another polymorphism in the TNF-α promoter region (TNF -376A) was found to be associated with a four-fold increased susceptibility to cerebral malaria (Knight and Kwiatkowski, 1999; Knight *et al.*, 1999). However, the pathophysiology of malaria appears to be more complex than the apparent centrality of TNF-α alone. So, there are propositions that other cytokines are potentially as important (Clark and Cowden, 2003).

Interleukin-18 (IL-18) is another proinflammatory mediator of innate and acquired immune responses which belongs to the IL-1 superfamily of cytokines (Okamura *et al.*, 1998; Gracie *et al.*, 2003; Maxwell *et al.*, 2006). IL-18 is an early inducer of the Th1 response, and together with IL-12 or IL-15, it is a potent enhancer of IFN-γ expression on T cells. In an inflammatory environment, it stimulates the production of TNF-α, granulocyte-macrophage colony stimulating factor and IL-2 (Okamura *et al.*, 1995a; Nakanishi *et al.*, 2001b; Singh *et al.*, 2002). IL-18 is secreted by antigen-presenting cells (APCs) and signals through the IL-18 receptor (IL-18R) complex which is a heterodimer consisting of IL-18Rα subunit, responsible for extracellular IL-18 binding, and IL-18Rβ, responsible for signal transduction.

Though IL-18 was described less than two decades ago (Okamura *et al.*, 1995a; Okamura *et al.*, 1998), ample data referring to IL-18 plasma/serum levels in various infectious diseases including malaria, as well as autoimmune disorders, neoplastic and cardiovascular diseases are available (Cebeci *et al.*, 2006; Corvino *et al.*, 2007). In patients with uncomplicated *P. falciparum* malaria, a significant increase in IL-18 concentrations was noted during the acute and recovery phase of the disease compared to healthy controls, reflecting a proinflammatory role of IL-18 in these patients (Torre *et al.*, 2001). Similarly, a significant increase in serum levels of IL-18 was found in children with uncomplicated *P. falciparum* malaria compared to children with severe malaria, in Burkina Faso (Malaguarnera *et al.*, 2002; Musumeci *et al.*, 2003), although this was not confirmed in studies conducted in Bangkok, Thailand where higher levels of IL-18 was observed among severe malaria patients (Nagamine *et al.*, 2003; Kojima *et al.*, 2004).

Owing to accruing evidence suggesting that serum levels of IL-18 may impact on individual susceptibility to disease, and that IL-18 variation may influence IL-18 production (Arimitsu *et al.*, 2006; Ueda *et al.*, 2006; Frayling *et al.*, 2007; Lotito *et al.*, 2007), a number of investigators have looked for associations between IL-18 single nucleotide polymorphisms (SNPs), or haplotypes and disease (Gracie *et al.*, 2005; Lee *et al.*, 2006; Bossu *et al.*, 2007; Dong *et al.*, 2007; Lin *et al.*, 2007; Thompson and Humphries, 2007). Indeed, there are observations that polymorphisms within the IL-18 promoter region may themselves affect the conformation of binding sites for transcription factors, resulting in its differential expression (Tiret *et al.*, 2005). Three single nucleotide polymorphisms in the promoter of IL-18 gene at the position -656G/T, -607C/A, and -137G/C have been identified with influence on the expression of IL-18 and potentially also of IFN-gamma (Giedraitis *et al.*, 2001; Tiret *et al.*, 2005). However, there is very little information on the genetic variability of IL-18 or IL-18Rα and its association with malaria (Anyona *et al.*, 2011).

This study therefore, aimed at determining the role of host cytokine gene polymorphisms of IL-18, IL-18Rα and TNF-α, as well as parasite genetic variability (using the *MSP-2* gene as molecular marker) in determining the disease outcome of *P. falciparum* infections in children in north-central Nigeria.

1.2 Justification for the Study

The interaction between the malaria parasites and the human host leading to disease and death is not fully understood. Given the role of malaria in morbidity and mortality, an understanding of the molecular mechanisms of malaria pathogenesis is fundamental to developing novel intervention strategies. This study therefore, aimed at determining the contribution of parasite genetic diversity as well as polymorphisms of host cytokine genes in determining the disease outcome of *P. falciparum* infection.

Data generated from this study will help in defining the parasite population structure in the study area as well as possible genotypes associated with disease outcome. It will also provide information on the association of genetic variants of some host cytokine genes with the outcome of *P. falciparum* infection.

This study is important because it could provide novel diagnostic markers of disease susceptibility as well as useful information for malaria vaccine development based on natural antigens in the study region. It may also help to identify potential immuno-genetic risk factors and consequently, facilitate the identification of individuals who are at high risk of developing severe disease or other complications as well as individuals who might need immuno-modulatory therapy; thus providing novel approach to disease management and prevention. Furthermore, it will improve our understanding of the molecular mechanisms of malaria pathogenesis.

1.3 Aims and Objectives

The aim of this study was to characterize the host cytokine gene polymorphisms of IL-18, IL-18Rα and TNF-α, as well as the parasite genetic diversity using the merozoite surface protein-2 as a molecular marker, and to determine the association between these host-parasite genetic factors and the disease outcome of *P. falciparum* infection.

Specific Objectives

1) To determine the parasite species population predominant in children with severe malaria, uncomplicated malaria and asymptomatic infection using the genus and species-specific PCR.

2) To characterize the *P. falciparum* parasite population in children with severe malaria, uncomplicated malaria and asymptomatic infection using the polymorphic MSP-2 antigen as gene marker.

3) To determine the pattern of sequence diversity in the *P. falciparum* MSP-2 gene in the study population.

4) To characterize and compare the host genetic polymorphisms and genotype frequencies of the interleukin-18 Receptor alpha (IL-18Rα) gene and of the promoter region of the IL-18 gene between groups of children with severe malaria, uncomplicated malaria and asymptomatic infection.

5) To characterize and compare the host genetic polymorphisms and genotype frequencies in the promoter region of the tumour necrosis factor-alpha (TNF-α) gene between groups of children with severe malaria, uncomplicated malaria and asymptomatic infection.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Background of Malaria

Human malaria has been recognized since the earliest period of man's recorded history, and occurrence of mosquitoes trapped in amber suggests its prevalence in prehistoric times (Garnham, 1952; Manson-Bahr, 1961; Bellomo, 1965). Recognizable descriptions of the disease were recorded in various Egyptian papyri. The Ebers papyrus (1550 BC) mentions fever, splenomegaly and the use of oil of the Balamites tree as a mosquito repellent (Griffith, 1893; Hallmann-Mikolajczak, 2004). Hieroglyphs on the walls of the ancient temple of Denderah in Egypt describe an intermittent fever following the flooding of the Nile (Halawani and Shawarby, 1957).

It was often thought that there was an aetiological relationship between swamps and fever. Italians referred to the bad air in fever-producing areas as mal'aria and it was commonly concluded that the disease was contacted by breathing "bad air", hence the name malaria, meaning bad area (Wahlgren and Bejarano, 1999). In 1717, Giovanni Lancisi (1654-1720), physician to the Pope and a professor at the Sapienzia in Rome, suggested that malaria is transmitted by the bite of mosquitoes, whilst at the same time accepting the miasmatic theory for transmission of disease. In 1716, Lancisi demonstrated 'grey-black pigment in malaria tissue (Gillespie and Pearson, 2001). Progress was made in 1847 about the aetiology of malaria when Meckel observed black pigment granules in the blood and spleen of a patient who died of the disease. It was not until 1880, that Alphonse Laveran (1845-1922), recipient of the Nobel Prize for medicine in 1907 discovered and described the parasite as bodies within the human erythrocyte while working in Algeria (Bruce-Chwatt, 1985; Cook, 1993; Cox, 2010). He witnessed one of the most dramatic events in protozoology: the formation of male gametes by the process of exflagellation (Schmidt and Robert, 1989; Cox, 2010). By 1890, several scientists in different parts of the world had verified his findings.

The mode of malaria transmission was however still unknown, until Sir Roland Ross in the late 1890s discovered that the vector of transmission of the disease was the female *Anopheles* mosquito and demonstrated that the parasites pass through a stage in mosquitoes and are then inoculated into man (Schmidt and Robert, 1989). However, it was Bignami, Bastianelli and Grassi in Italy, who experimentally transmitted the malaria parasite from mosquito to man in 1893 (Cox, 2010). Manson in 1900, by experiments with human volunteers in the Roman Campagna and in London, confirmed the mosquito-malaria transmission theory (Manson, 1901a; Manson, 1901b).

In 1938, James and Tate discovered the exoerythrocytic stages of *P. gallinaceum.* After this discovery, large-scale work began in order to find the exoerythrocytic stages of human malaria parasites. Finally, in 1948, Shortt and Garnham demonstrated the exoerythrocytic stages of *P. cynomolgi* in monkeys and *P. vivax* in humans (Shortt and Garnham, 2000). Worthy of mention however, is the continuous *in vitro* culture of *P. falciparum* developed by Trager and Jensen in the USA in 1976, which opened the gate to diverse fields of malaria studies and has brightened our understanding especially about the parasite (Trager and Jensen, 1976; Trager and Jensen, 2005).

Meanwhile, the first important event in the history of malaria was the discovery of the Peruvian fever tree, from which quinine and cinchonine were isolated. These were used to treat and cure fevers in the early 17th century by Jesuit missionaries in South America. Research in the twentieth century was devoted largely to malaria control with the discovery of various antimalarial agents such as chloroquine, proguanil and primaquine (Bruce-Chwatt, 1985). Similarly, the discovery of relatively low cost synthetic compounds in the 1940s such as dichloro-diphenyl-trichloroethane (DDT) and dieldrin among others introduced the concept of malaria eradication (Desowitz, 2005). Fifteen years after this period, malaria was eradicated in most parts of Europe, America, Middle East, parts of former Soviet Union and some countries in Asia. However, there are reports that malaria is gradually returning to some of the places where eradication had been successful due to human and environmental factors (Hay *et al.*, 2004).

2.2 Global Malaria Situation

Malaria is one of the most common and important parasitic diseases worldwide (Trigg and Kondrachine, 1998; WHO, 2003b). Malaria is distributed worldwide throughout the tropics and subtropics. Half of the world's population is said to be at risk of the disease (WHO, 2008a) and about 106 countries or territories in the world are considered malarious (Fig. 2.1), almost half of which are in Africa, south of the Sahara (WHO, 2011). Although this number is considerably less than it was in the mid-1950s (140 countries or territories), more than 2.4 billion of the world's population are still at risk of malaria (WHO, 2000b; Snow *et al.*, 2005). An estimated 3.3 billion people were at risk of malaria in 2010 (WHO, 2011). Of this total, 1.2 billion are at high risk (>1 case per 1000 population), living mostly in the WHO African region (49%) and South-East Asia region (37%).

There were an estimated 247 million cases of malaria as at 2006 (WHO, 2008a) although the figure reduced to 225 million in 2009 (WHO, 2010), owing to the impact of the global malaria control measures. Eighty-six percent, or 212 million (152-287 million) cases, were in sub-Saharan African and were mainly due to *P. falciparum* infections (Fig. 2.2). Eighty percent of the cases in Africa were reported in 13 countries, and over half of these were in Nigeria, Democratic Republic of the Congo, Ethiopia, United Republic of Tanzania and Kenya (WHO, 2008b). About 1% of clinical cases of *P. falciparum* infections that occur globally each year have been estimated to be complicated by severe manifestations leading to death. Available data showed an estimated 881 000 (610,000-1,212,000) malaria deaths as at 2006, of which 91% (801 000, range 520,000-1,126,000) were in Africa (Fig. 2.3) and 85% were of children under 5 years of age (WHO, 2008b). This figure has reduced to 781,000 by 2009 due to the concerted effort at malaria control (WHO, 2010). However, this figure corresponds to one death in nearly every 30 seconds (Greenwood *et al.*, 1987). Conversely to the above data generated by the World Health Organisation, a recent study now suggests that the global malaria mortality burden is larger than previously estimated, with about 1.24 million deaths in 2010 (Murray *et al.*, 2012).

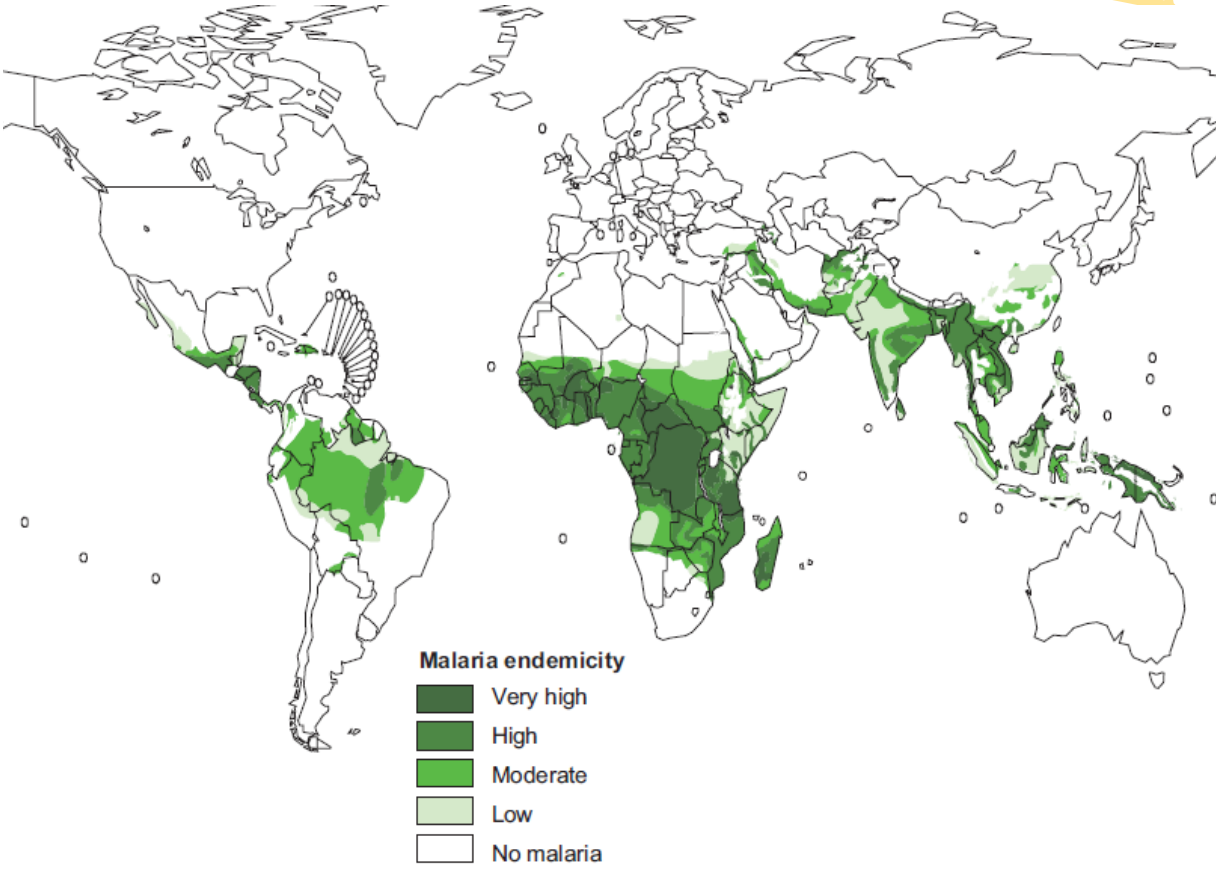


Figure 2.1: Global distribution of malaria showing risk of malaria transmission (WHO/UNICEF, 2005)

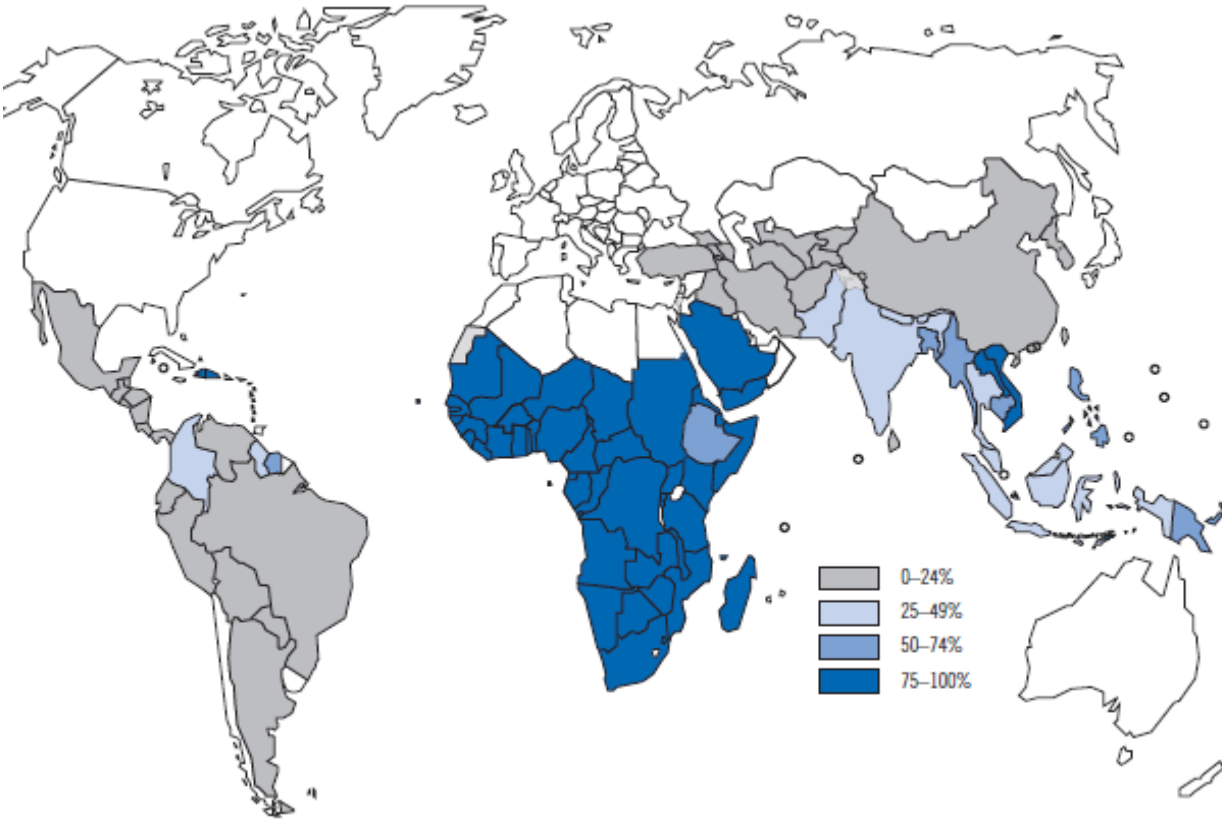


Figure 2.2: Estimated percentage of malaria cases due to *P. falciparum* (WHO, 2008b).

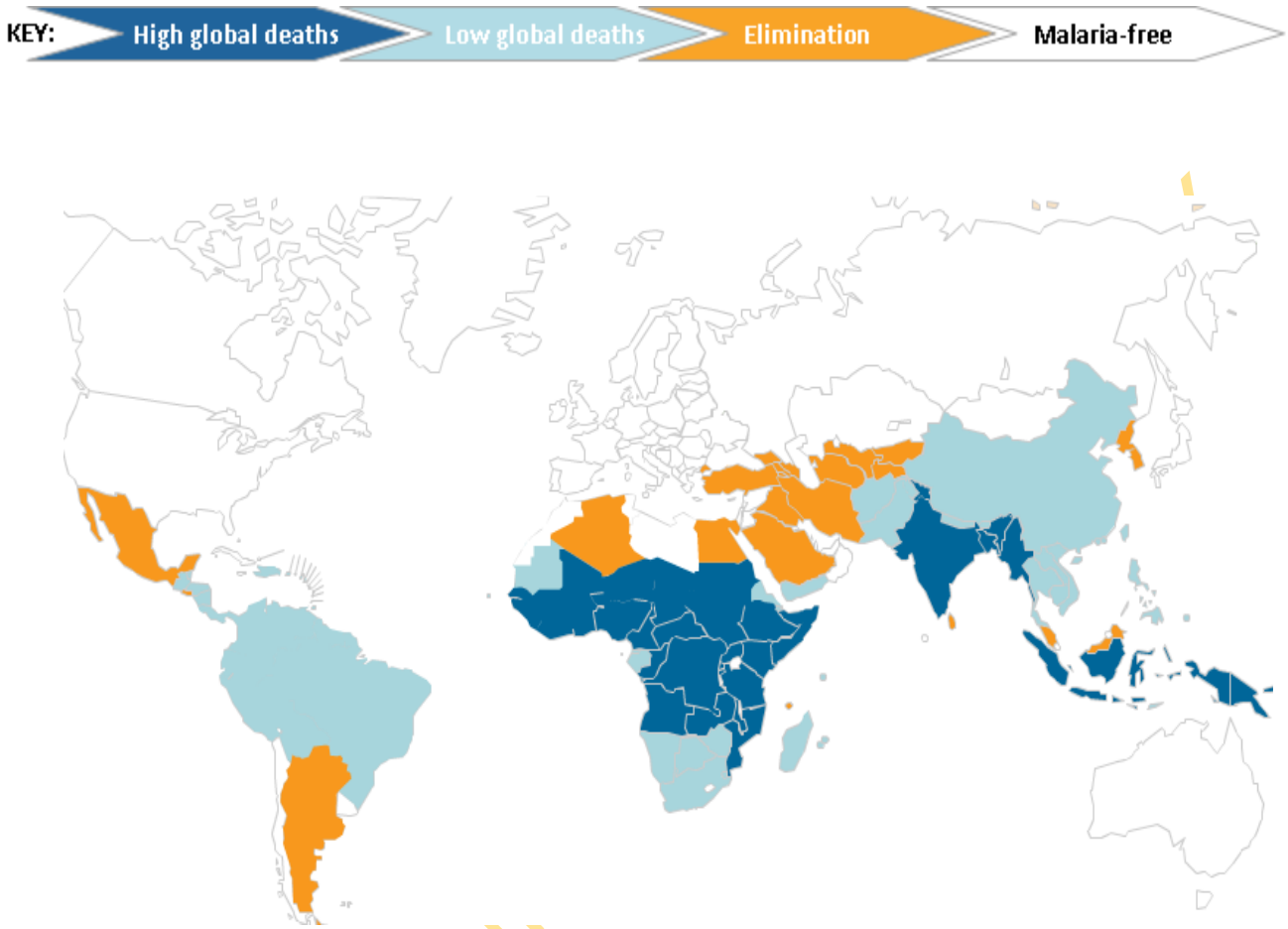


Figure 2.3: Global malaria burden showing regional relationship to morbidity and mortality. **(**http://www.rollbackmalaria.org/worldmalariaday/ Accessed 15 February, 2010).

Besides children, pregnant women (particularly primigravidae) and non-immune individuals such as travellers and foreign workers are at highest risk of severe disease. In addition to the overwhelming death toll, over 213 million malarial "attacks" lead to more than 800 million days of illness in Africa annually (Breman *et al.*, 2004).

In malarious areas, malaria transmission may be endemic, occurring predictably every year, or it may be epidemic, occurring sporadically when conditions are favourable. Endemic transmission of malaria may be year round or seasonal. However, all age groups may be at risk of severe disease during malaria epidemics, which occur either when changes in the physical environment (caused by climatic variation, agricultural projects or mining) increase the capacity of mosquitoes to transmit the disease or when population displacements through natural disasters or war expose non-immune populations to infection (Suh *et al.*, 2004). In some areas of Africa, 90 to 100 per cent of children less than 5 years old have malaria parasites circulating in their blood at most times. Because naturally acquired immunity develops with increasing exposure, in endemic areas, malaria disease is primarily found in children. In epidemic areas, on the other hand, naturally acquired immunity falls off between epidemics, and malaria therefore affects all age groups during epidemics (Gilles, 1993). Likewise, the epidemiological pattern of *P. falciparum* infection varies in different geographical locations. In regions of high malaria transmission, adults develop potent but non-sterile immunity against malaria (Giha *et al.*, 2000). In areas of low transmission, malaria infections are infrequent and no age group has significant acquired immunity to malaria.

Malaria is transmitted primarily by the bite of infected female *Anopheles* mosquito but congenital malaria and acquisition through infected blood transfusion have also been described in the literature (Zheng and Kafatos, 2005; Falade *et al.*, 2007; Lesi *et al.*, 2010; Poespoprodjo *et al.*, 2010). Anophelines feed at night and their breeding sites are primarily in rural areas. The greatest risk of malaria is therefore from dusk to dawn in rural areas. However, urban transmission is common in some parts of the world especially in Africa where the sanitary condition of the environment is generally poor. Temperature and humidity are the most important environmental factors favouring transmission of the

disease. The optimal conditions for the parasite and vector are a mean temperature of 20-30^{o}C and a relative humidity of 60% (Schmidt and Robert, 1989; Gilles, 1993).

In most parts of sub-Saharan Africa, there is a high endemicity of malaria in areas where transmission is stable; 80% of clinical malaria cases worldwide and 90% of mortality occur in these areas (Gilles, 1993; Snow *et al.*, 2005). In Nigeria, malaria is endemic and stable with minimal seasonal fluctuations, but peak transmissions occur during the rainy season (Ekanem *et al.*, 1990). Nigeria accounts for a quarter of all malaria cases in the WHO African Region (WHO, 2008b). Transmission especially in southern Nigeria occurs all-year round. There is no evidence of a systematic decline in malaria burden; estimated malaria cases in 2007 was in the range of 2.97-3.98 million (WHO, 2008b). The predominant species of the parasite in this region is *P. falciparum* and almost all cases are caused by this species of human parasite but most are unconfirmed (WHO, 2008). However, *P. malariae* accounts for about 5% of total infection (Salako *et al.*, 1990). Two main species of vectors that are predominant in Nigeria are *Anopheles gambiae* and *A. funestus* (Molineaux and Gramiccia, 1980; Awolola *et al.*, 2005) although *A. arabiensis* and *A. moucheti* are also found (Awolola *et al.*, 2002; Okwa *et al.*, 2009).

2.2.1 *Socio-economic impact of malaria*

Malaria is an exceptionally complex disease (WHO, 2003b). It is most serious in the poorest countries, among underprivileged populations living under the most difficult and impecunious conditions. The causes of malaria and the impediments to its control are rooted in the social, cultural, political, economic, and ecological conditions of endemic countries. The situation is so dire that malaria is now recognized as both a disease of poverty and a cause of poverty (Gallup and Sachs, 2001). Malaria strikes the most vulnerable and impoverished communities, ensuring that those most in need of treatment are those least able to afford it. The disease disproportionately affects the poor, in whom higher morbidity and mortality can be attributed to lack of access to effective treatment; 60% of malaria deaths worldwide occur in the poorest 20% of the population (Suh *et al.*, 2004). Malaria undermines the health and welfare of families, endangers the survival and education of

children, debilitates the active population, and strains the resources of countries and their inhabitants, thus limiting their ability to contribute to economic and social growth (Trigg and Kondrachine, 1998). Malaria is estimated to be responsible for an annual loss of 35.4 million Disability Adjusted Life Years (Meyrowitsch *et al.*, 2011). In some rural villages, a family may spend up to 25% or more of its annual income on malaria prevention and treatment (WHO, 2003b). Malaria thus anchors people in perpetual poverty.

Malaria causes substantial losses to households in the form of foregone income and decreased agricultural production (Kiszewski and Teklehaimanot, 2004). Able-bodied men, who are the economically more productive family members, are attacked by malaria during planting and harvesting seasons, thereby shrinking productive capacity when agricultural workers are in highest demand (Attanayake *et al.*, 2000). The concurrent infections of massive number of people usually overload health facilities; thereby degrading the effectiveness of health care. In addition to the immense human suffering it causes, malaria costs Africa more than US$ 12 billion annually. Malaria has slowed economic growth in African countries by 1.3% per year (WHO, 2003b). In Nigeria, the financial loss due to malaria annually is estimated to be about ₦132 billion in the form of treatment cost, prevention and loss of man-hours (FMOH, 2009). Consequently, funding for malaria control have been increased from US$ 17 million in 2005 to US$ 60 million in 2007, provided by the government, the Global Fund and the World Bank (WHO, 2008b). Recent report suggests that commitment towards malaria control from International sources has risen from US$200 million in 2004 to US$2 billion in 2011 (WHO, 2011).

2.3 The Malaria Parasite

2.3.1 *Classification*

Malaria parasites belong to the Genus *Plasmodium* which includes over 125 species infecting reptiles, birds and mammals (Bruce-Chwatt, 1985). *Plasmodium spp* are intracellular, blood-dwelling parasitic protozoa that cause the disease malaria. These species are restricted to the Family Plasmodiidae which includes parasites which undergo asexual division (schizogony) and a single sexual multiplication (sporogony) in an invertebrate host.

They belong to the Order Haemosporidia and to the Class Sporozoea (Table 2.1). Many members of Haemosporidia live in the red blood cells of vertebrates. These groups of parasites belong to the Phylum Apicomplexa which are spore-forming unicellular organisms and belong to the Sub-Kingdom Protozoa in the Kingdom Protista (Levine *et al.*, 1980). Important characteristic features of the Apicomplexans include the presence, at specific stages of the life-cycle of an apical complex consisting of a number of specialized organelles (Fig. 2.4), which may include a conoid, polar rings, rhoptries and micronemes (Gilles, 1993; Cowman and Crabb, 2006).

Of the four species of malaria parasites traditionally known to infect humans (Fig. 2.5), three (*Plasmodium vivax, P. malariae* and *P. ovale*) belong to the sub-genus *Plasmodium* , while the remaining one (*P. falciparum*) belongs to the sub-genus *Laverania* (Garnham, 1988).

2.3.2 *Life cycle of human malaria parasites*

Human malaria parasites undergo a complex cycle of development, alternating between two hosts: the mosquito vector (invertebrate host) and man (vertebrate host). The life cycle occurs in a sequence of four phases, which includes one sexual phase and three asexual phases.

Stages in vertebrate host

Exoerythrocytic cycle

Infection of the human host occurs when an infective female *Anopheles* mosquito injects saliva containing tiny elongate sporozoites into the bloodstream during blood meal

Table 2.1: Hierarchical Taxonomy of Human Malaria Parasites (Levine *et al.*, 1980; Gilles, 1993).

Taxonomic Group	Name
Domain	Eukaryota (Eukarya)
Kingdom	Protista
Sub-Kingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoae
Subclass	Eucoccidia
Order	Haemosporidia
Family	Plasmodiidae
Genus	*Plasmodium*
Species	*P. falciparum*
	P. vivax
	P. malariae
	P. ovale

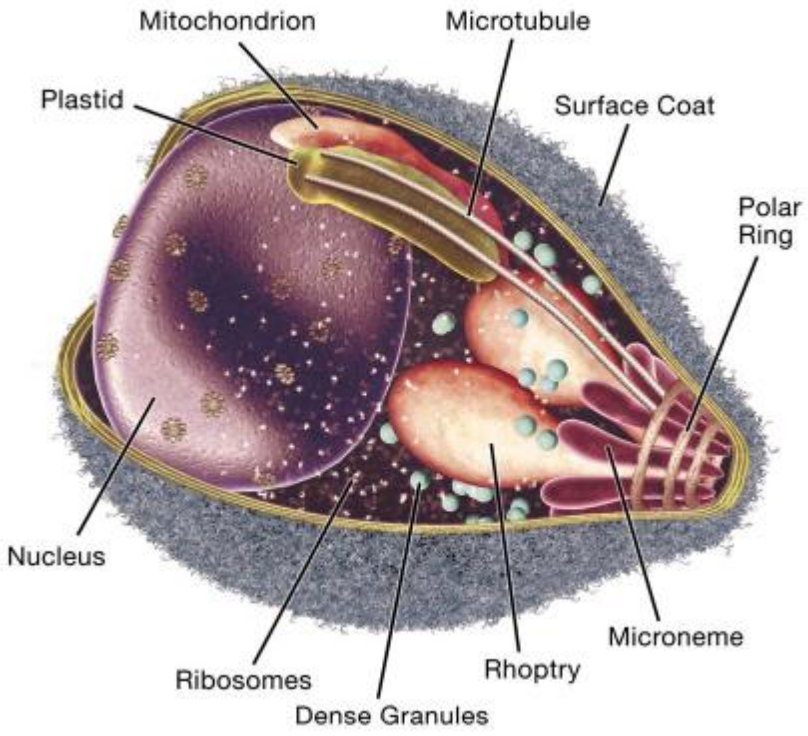


Figure 2.4: Diagram of a merozoite, highlighting major organelles and cellular structures (Cowman and Crabb, 2006).

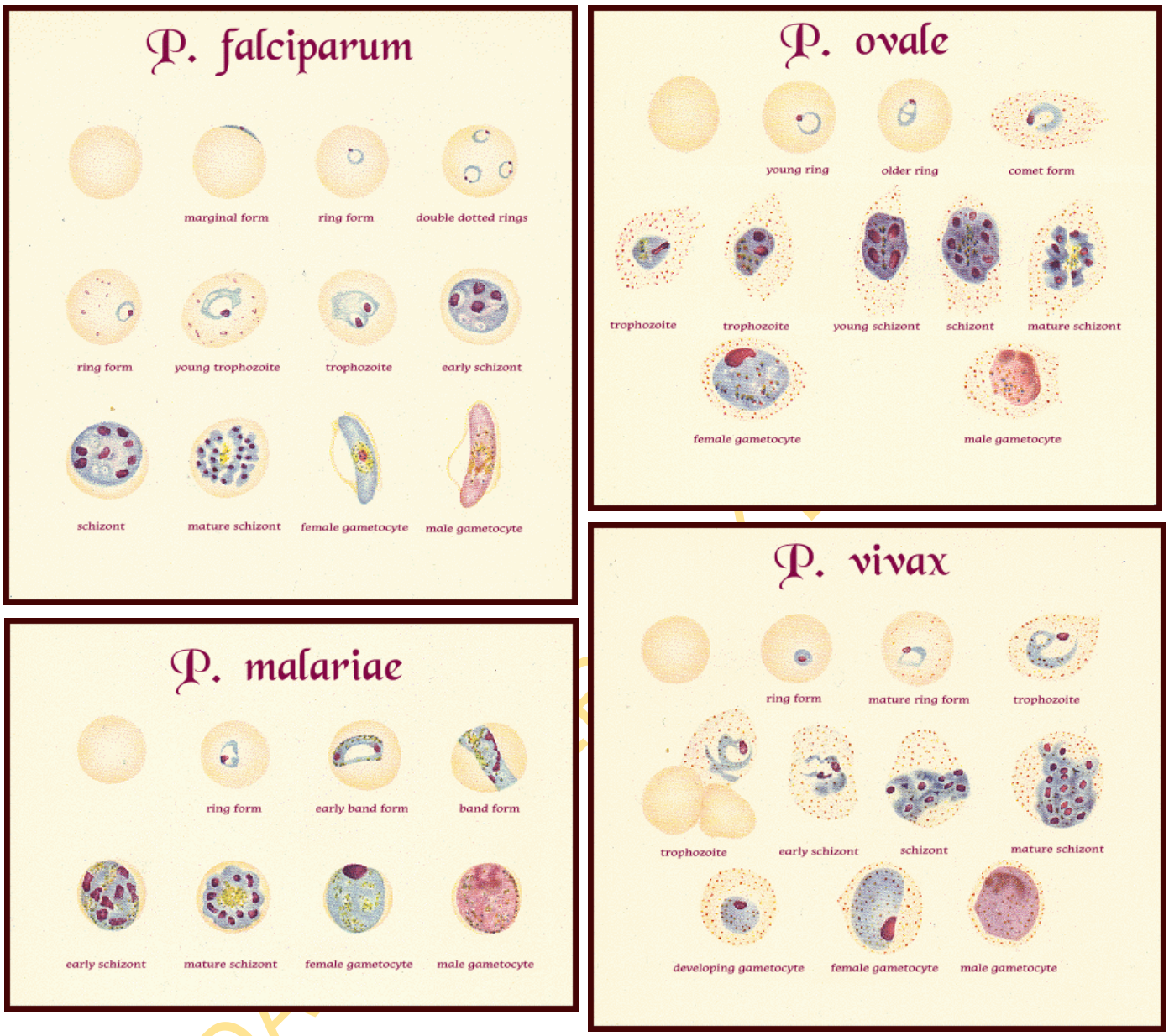


Figure 2.5: Four major species of the human malaria parasites.

(http://www.google.com/imgres?imgurl=http://www.bioon.com/Article/UploadFiles/200406/20040603215831151.gif&imgrefurl=http://www.bioon.com/Article/Class741/40125.shtml&usg=__on8oe6ywiW4PSCOVUnPH9g1Seoc=&h=334&w=488&sz=47&hl=en&start=6&zoom=1&tbnid=DHJJcHu_aKRXGM:&tbnh=89&tbnw=130&ei=Zx6sTuupM4mZOrWpvN8P&prev=/images%3Fq%3D*Plasmodium* %2Bmalariae%26hl%3Den%26sa%3DX%26rls%3Dcom.microsoft:*:IE-SearchBox%26rlz%3D1I7ACAW_en%26tbm%3Disch&itbs=1. Accessed 15 February, 2010).

(Figure 2.6). Sporozoites are deposited through the skin, an organ comprising three characteristic layers. The epidermis provides a physical barrier, the underlying highly vascularized dermis harbours immune cells, such as macrophages and dendritic cells, and the subcutis contains larger blood vessels and lymphatics. While acquired immunity could interfere with crossing this natural barrier, sporozoites migrate extensively in the skin of naive individuals and eventually leave the site of the mosquito bite (Frevert, 2004). The sporozoites circulate round the body via the blood and within 1-30 min penetrate the liver and then into the hepatocytes, initiating the pre-erythrocytic or exoerythrocytic cycle.

In the hepatocyte the sporozoite undergoes drastic changes in morphology, losing its apical complex and surface coat and transforming into a round or oval trophozoite (Shortt *et al.*, 1951). Once established within a hepatocyte, the sporozoite undergoes a multiplicative process known as exoerythrocytic schizogony. Development takes place within a parasitophorous vacuole in the hepatocyte. The trophozoite increases in size and finally develops into a tissue schizont (meront) containing several thousand merozoites. *P. vivax* sporozoites take 6-8 days to mature and produce about 10,000 merozoites; *P. ovale* takes 9 days to produce about 15,000 merozoites; *P. malariae* takes 12-16 days to produce 2,000 merozoites and *P. falciparum* 5-7 days to produce 40,000 merozoites, from a single sporozoite. In 2006, it was shown that the parasite buds off the hepatocytes in merosomes containing hundreds or thousands of merozoites (Sturm *et al.*, 2006). These merosomes lodge in the pulmonary capillaries and slowly disintegrate there over 48-72 hours releasing merozoites (Baer *et al.*, 2007). Similarly, when distended by the parasite meront, the cell ruptures, releasing the merozoites into the bloodstream (Murphy *et al.*, 1989). Erythrocyte invasion is enhanced when blood flow is slow and the cells are tightly packed: both of these conditions are found in the alveolar capillaries.

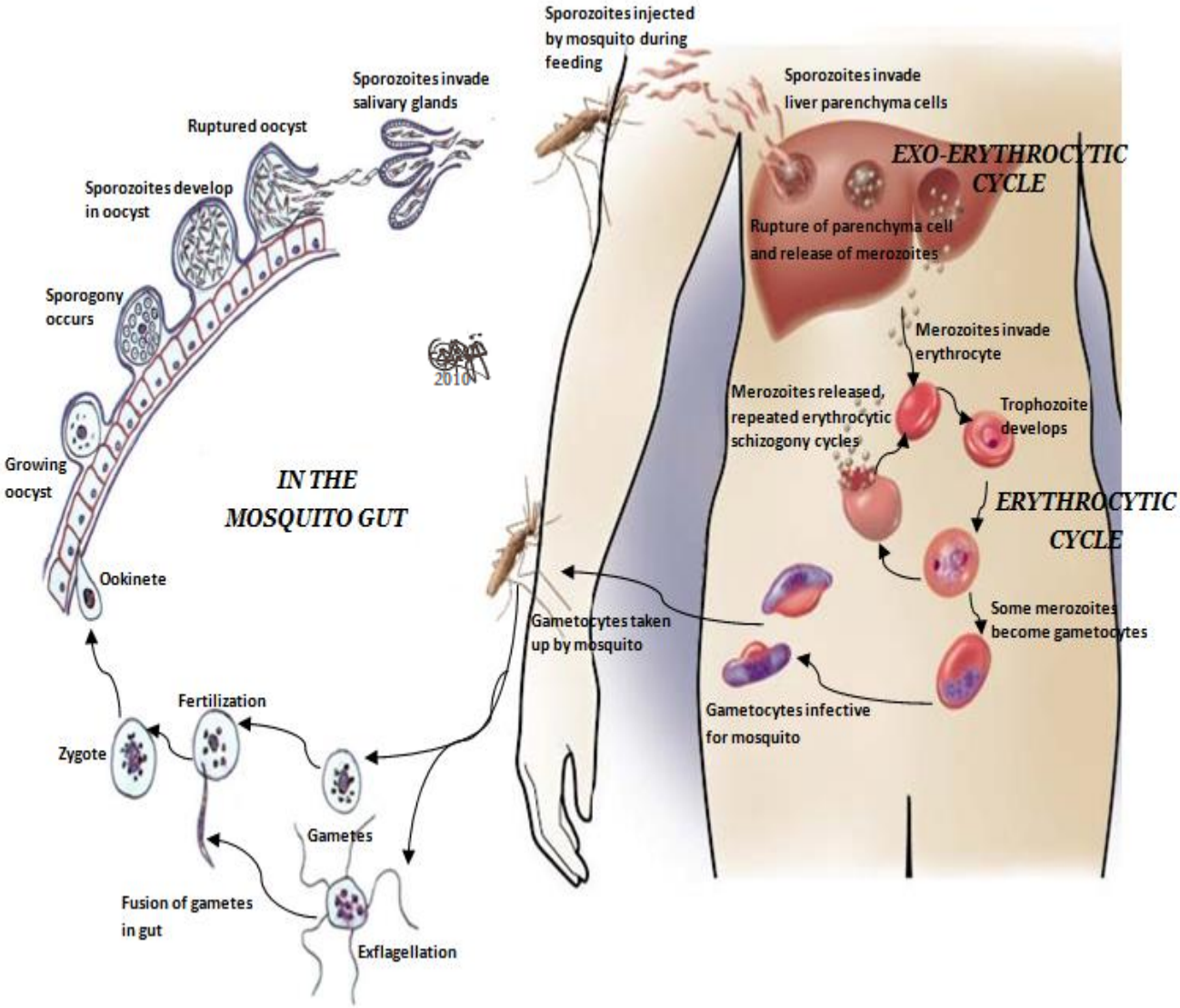


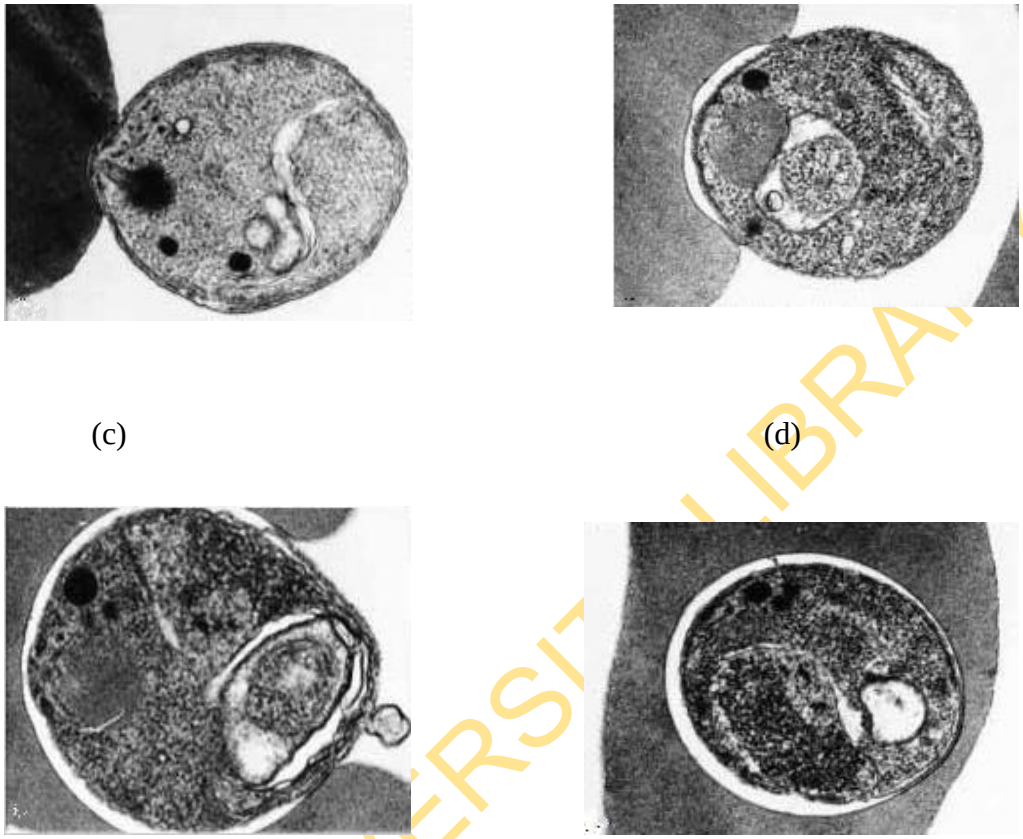
Figure 2.6: Life Cycle of the Human Malaria Parasite

Erythrocytic cycle

Merozoites rapidly attach to and invade erythrocytes to initiate the erythrocytic cycle. They attach to receptor sites located on red blood cells before invasion. They have distinctive features and are specialized to recognize and attach to specific molecules of the membrane of the erythrocyte for invasion (Fig. 2.4). The merozoite enters the cell by five stages: initial recognition and attachment, formation of a junction, creation of a vacuole membrane continuous with the red cell membrane, entry into the vacuole through the moving junction (Fig. 2.7), and sealing of the erythrocyte after entry (Aikawa *et al.*, 1978; Gilles, 1993; Gaur and Chitnis, 2011). After invading the erythrocyte, the parasite loses its specific invasion organelles and differentiates into a round trophozoite located within a parasitophorous vacuole in the red cell cytoplasm. Once inside an erythrocyte, the merozoite develops into a feeding trophozoite. A parasitophorous vacuole forms round the trophozoite and the trophozoite takes on a 'signet-ring' shape, more or less amoeboid and uninucleate.

The young trophozoite grows substantially before undergoing several nuclear divisions. This is soon followed by the division of cytoplasm forming a schizont, each containing merozoites in numbers characteristic of the parasite species (Bruce *et al.*, 1990). The merozoites then invade fresh erythrocytes and the cycle continues (Fig. 2.6). The erythrocyte eventually bursts releasing the merozoites into the circulation and these then invade non-infected red blood cells. The result is a dramatic increase in parasitaemia and, when the number of parasites reaches a certain level, the process of schizogony and the subsequent liberation of merozoites become synchronized and periodic. The length of the intraerythrocytic development is variable, being 48hr for *P. falciparum* , *P. vivax* and *P. ovale*, and 72hr for *P. malariae* (Eichner *et al.*, 2001). At the end of each cycle when the erythrocytes burst and release the merozoites into the bloodstream, 'toxins' are released into the host. Each time there is the liberation of parasites into the bloodstream it has a pyrogenic effect upon the host, making the host to feel feverish. After a few erythrocytic cycles, some merozoites develop into a male or a female gametocyte. Whether this development is pre-determined genetically or as a response to some specific stimulus is unknown.

(a) (b)



(c) (d)

Figure 2.7: Invasion of Red Blood Cell by Merozoite.

(a) Apical attachment and junction formation. A merozoite has begun to invade a red blood cell. The apical complex is attached to the RBC. One of the rhoptries is visible close to the apical complex. (b) The junctional complex is forming around the point where the erythrocyte membrane is invaginating. The junction is represented by the dense area beneath the erythrocyte membrane. (c) The junctional complex area has now moved to the rear of the merozoite and further invagination has taken place, so that the merozoite is now effectively inside the erythrocyte. (d) The red cell membrane surrounding the merozoite is now complete, forming the parasitophorous vacuole (Aikawa *et al.*, 1978).

Stages in invertebrate host

During a blood meal from an infected human, the female *Anopheles* mosquito takes up gametocytes, which transform into gametes (Fig. 2.6). The maturation of the female gametocyte into a macrogamete takes place without major morphological changes. In the extracellular male gamete, the nucleus divides three times and each of the eight nuclei formed combines with cytoplasm to form 6-8 thread-like microgametes in exflagellation (Day *et al.*, 1998).

Sporogony

Fertilization occurs in the stomach wall of the mosquito by the fusion of a microgamete with a macrogamete, resulting in the formation of a diploid zygote. This soon elongates to become a mobile ookinete. The slow motile ookinete crosses the peritrophic membrane of the midgut (probably by means of a chitinase), then passes through the single cell layer of the midgut epithelium and establishes itself beneath the basal lamina, forming an oocyst (Gilles, 1993). The oocyst gradually increases in size, enlarges and undergoes nuclear division forming sporoblasts. The sporoblasts in turn divide repeatedly to form thousands of sporozoites. Being motile, the sporozoites burst through the weakened or ruptured wall of the oocyst and invade the body cavity of the mosquito (Fig. 2.6). From the haemolymph, sporozoites migrate to the salivary glands of the female *Anopheles* mosquito, which becomes infective. When the mosquito feeds on blood meal, it then injects sporozoites into the bloodstream of the vertebrate host.

2.4 Genetic Diversity of the Malaria Parasite

Plasmodium falciparum exhibits great genetic variability which gives rise to a number of antigenically different parasite populations (Fenton *et al.*, 1991). The inherent variability of *P. falciparum* provides multiple effective immune evasion and drug resistance mechanisms for the parasite. Particularly, the extensive diversity of malaria surface antigens has been suggested as one of the main reasons why clinical immunity develops only after repeated infections with the same species over time (Day and Marsh, 1991). Genetic diversity is manifested in extensive allelic polymorphisms of many parasite genes, especially those encoding antigens, and in the occurrence of mixtures of genetically distinct parasite clones in individual patients. Antigenic diversity has a dual origin. One is the classical genetic mechanism of nucleotide replacement and recombination that creates allelic polymorphism or stable alternative forms of antigen-coding genes (Ferreira *et al.*, 2004). The second mechanism is antigenic variation, whereby a clonal lineage of parasite expresses successively alternative forms of an antigen without changes in genotype (Chen *et al.*, 2000; Hoffmann *et al.*, 2006).

Malaria endemic areas are generally characterized by extensive parasite diversity. Genetic diversity of *P. falciparum* parasites usually leads to complex infections where infected individuals often carry multiple parasite genotypes (Paul *et al.*, 1998; Arnot, 2002), and is an indicator of malaria transmission intensity. Knowledge of the genetic structure of the parasites in natural populations is critical to any intervention strategy in a particular region. Antigenic diversity of *MSP-2* genotypes in clinical malaria have been reported in south-west Nigeria (Happi *et al.*, 2004; Amodu *et al.*, 2008; Ojurongbe *et al.*, 2011), and some parts of northern Nigeria (Engelbrecht *et al.*, 2000). However, little information is currently available on the relationship between parasite genetic diversity and disease outcome of *P. falciparum* infection as well as sequence variations in naturally circulating strains of *P. falciparum* parasites in Nigeria. These gaps in information are part of what this study was designed to bridge.

2.4.1 *Merozoite surface protein-1*

Merozoite surface protein-1 (MSP-1) is the most abundant protein on the surface of the blood stage of the parasite and it is thought to play a role in erythrocyte invasion (Holder *et al.*, 1992). It is synthesized as a 190 kDa precursor, which undergoes proteolytic cleavage into four fragments that remain on the merozoite surface as a glycosylphosphatidylinositol (GPI)-anchored complex. Before erythrocyte invasion, the entire MSP-1 complex is shed, except for the C-terminal 19 kDa (MSP-1_{19}), which remains on the surface as the merozoite enters the erythrocyte (Blackman *et al.*, 1990). MSP-1_{19} contains two epidermal growth factor (EGF)-like domains, which are thought to have an important function in erythrocyte invasion (Holder *et al.*, 1992). Naturally acquired antibodies to MSP-1_{19} can inhibit erythrocyte invasion by preventing the secondary processing that releases this fragment from the rest of the MSP-1 complex (Blackman *et al.*, 1990; Guevara Patino *et al.*, 1997; Nwuba *et al.*, 2002), and are associated with protection from clinical malaria in field studies (Riley *et al.*, 1992; Egan *et al.*, 1996; Branch *et al.*, 2000; John *et al.*, 2004; Okech *et al.*, 2004; Bisseye *et al.*, 2011).

The sequence of the *MSP-1* gene can be organized into 17 blocks based on sequence variability (Tanabe *et al.*, 1987; Miller *et al.*, 1993). The Block 2 region includes repetitive motifs of three amino acids, grouped into three allele-families: K1, MAD-20, and RO33 with high variability when comparing the groups, but less variability within them. Alleles in K1 and MAD-20 contain antigenically unique, tripeptide repeats, with extensive diversity in the number of repeats (Miller *et al.*, 1993). RO33 lacks the tripeptide repeats observed in the other two families; however, outside Block 2, this allele is similar to the MAD-20 type (Hughes, 1992). Fragment size in the three Block 2 allele families has commonly been used as a molecular marker in studies of malaria transmission dynamics and host immunity in *P. falciparum* malaria (Konate *et al.*, 1999; Branch *et al.*, 2001; Ghanchi *et al.*, 2010; Atroosh *et al.*, 2011). Block 17 contains MSP-1_{19}, which has been the focus of malaria vaccine development because of its highly conserved sequence and hypothesized critical function. However, even this region contains at least six nonsynonymous single nucleotide polymorphisms (SNPs). Studies of populations naturally exposed to *P. falciparum* have

shown various degrees of association between anti-MSP-1_{19} antibodies and protection from clinical malaria (Egan *et al.*, 1996; Kitua *et al.*, 1999).

2.4.2 *Merozoite surface protein-2*

The merozoite surface protein-2 (MSP-2) is a 45- to 50-kDa glycoprotein anchored in the merozoite surface by a glycosylphosphatidylinositol anchor. MSP-2 is a very immunogenic antigen malaria antigen, and is a promising candidate for inclusion in a malaria subunit vaccine (Hill, 2011; McCarthy *et al.*, 2011), as both *in vitro* and *in vivo* studies have demonstrated the ability of immune responses to MSP-2 to inhibit parasite multiplication (Saul *et al.*, 1992; Saul *et al.*, 1999; Genton *et al.*, 2002). The corresponding *MSP-2* gene is characterized by highly conserved amino and carboxyl-termini flanking a variable central region (Figure 2.8). The central polymorphic region contains a central repetitive sequence flanked by non-repetitive regions that have been used to define two major allelic families: the FC27 family and the 3D7 family after the names of the strains from which they were first described (Smythe *et al.*, 1991; Snewin *et al.*, 1991).

Two features make MSP-2 of importance in the molecular genotyping of *P. falciparum*. First, it is a promising candidate for inclusion in a subunit vaccine against the asexual intraerythrocytic stages of *P. falciparum* (Saul *et al.*, 1992; Genton *et al.*, 2003), and second, *MSP-2* has been shown to be a good discriminatory marker for assessing the genetic profile of *P. falciparum* isolates (Prescott *et al.*, 1994; Snounou *et al.*, 1999; Basco and Ringwald, 2001). Due to its polymorphism, the *MSP-2* gene has been extensively used to type natural isolates of *P. falciparum* (Prescott *et al.*, 1994; Felger *et al.*, 1999; Franks *et al.*, 2001; Ojurongbe *et al.*, 2011; Koukouikila-Koussounda *et al.*, 2012). Most studies examining the distribution and frequency of different allelic forms of *MSP-2* have enumerated the presence of the allelic families (Felger *et al.*, 1994; Engelbrecht *et al.*, 1995; Kang *et al.*, 2010; Atroosh *et al.*, 2011).

By typing the gene for this protein, some symptom-specific molecular characteristics have been delineated for *P. falciparum* isolates collected from asymptomatic, uncomplicated and severe malaria cases in Senegal (Robert *et al.*, 1996), in Tanzania (Smith *et al.*, 1999a;

Bendixen *et al.*, 2001) and in Nigeria (Happi *et al.*, 2004; Amodu *et al.*, 2008) amongst other countries. This information has been useful in understanding the dynamics of transmission as well as in evaluating the impact of malaria-control interventions, such as the use of antimalarial drugs and prototype malaria vaccines, on parasite populations (Beck *et al.*, 1997; Beck *et al.*, 1999; Haywood *et al.*, 1999; McCarthy *et al.*, 2011).

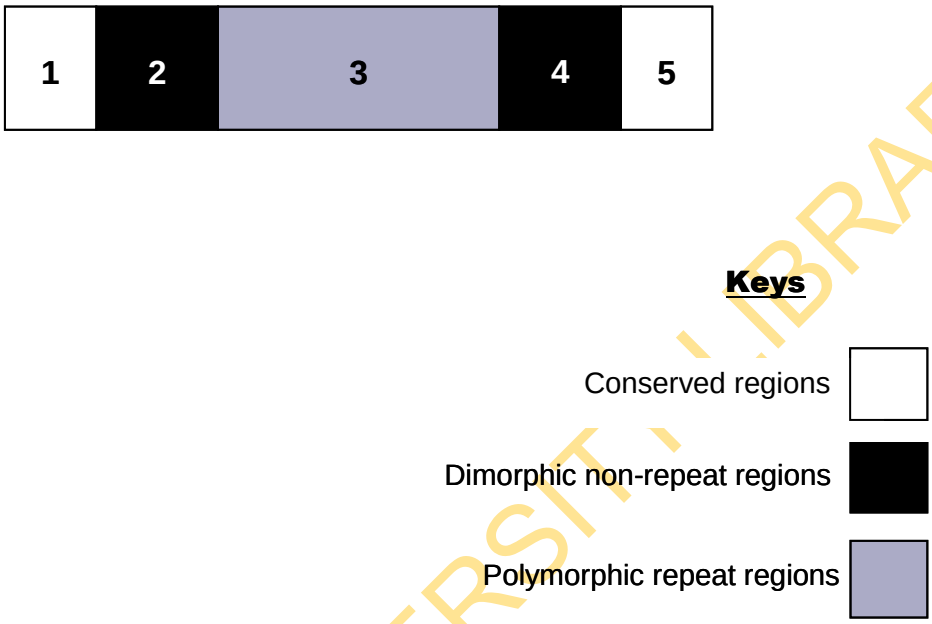


Figure 2.8: Schematic diagram of *MSP-2* gene

2.5 Pathogenesis of Malaria

Of all the *Plasmodium* species that cause malaria in humans, *P. falciparum* is the most deadly and the major cause of severe disease and death. Infection with *P. falciparum* produces a clinical outcome that depends on a combination of parasite, host and environmental factors, including the age of the patient and the pattern of prior exposure of that individual to malaria (Miller *et al.*, 2002). Infections range from being asymptomatic, through mild or uncomplicated malaria, to life-threatening severe forms of the disease involving multiple organ systems and varied pathological processes, often with case fatality. However, the most common clinical manifestation of malarial infection is a non-specific febrile illness with mild clinical symptoms such as fever, malaise, headache, and lethargy. Only a small proportion of cases often result in severe disease and in the worst case scenario, death.

Severe malaria is frequently described as complicated malaria. Severe and complicated malaria includes clinical features above and beyond fever and malaise. These include prostration, impaired consciousness (coma), hypoglycaemia, respiratory distress, acidosis, severe anaemia, hyperparasitaemia, hyperlactataemia (WHO, 2000a; Weatherall *et al.*, 2002; Perkins *et al.*, 2011). Severe malaria is a complex multi-system disorder that affects several tissues and organs (Gay *et al.*, 2012a; Renia *et al.*, 2012). There are important differences in the clinical spectrum of severe malaria with age. Inhabitants of malaria-endemic areas gradually acquire immunity to these signs of severe malaria illness, while remaining susceptible to malaria infection. For this reason, African children between the ages of six months and five years are the group at highest risk of developing severe and complicated malaria.

Efforts by the scientific community at understanding the pathogenesis of severe malaria have shown that development of severe malaria probably results from a combination of parasite-specific factors such as adhesion and sequestration to the vascular endothelium, the release of bioactive molecules, together with host inflammatory responses and metabolic acidosis (Manning *et al.*, 2012). Indeed, adhesion of parasitized erythrocytes to these cells

could drive their activation, which could participate in the trigger of an immune response and haemostatic derangements (Gay *et al.*, 2012a).

The most common presentations of severe malaria in African children include cerebral malaria, severe anaemia and respiratory distress (Fig. 2.9), but combinations of these, especially with other syndromes result in high mortality rates (Schellenberg *et al.*, 1999; Haldar *et al.*, 2007; Santos *et al.*, 2012; von Seidlein *et al.*, 2012). However, metabolic acidosis is now being widely recognised as a principal pathophysiological feature that cuts across the classical syndromes of cerebral malaria and severe malaria anaemia (Krishna *et al.*, 1994; Marsh *et al.*, 1995). Although the underlying pathophysiology of metabolic acidosis is likely to be complex, two factors seem to be of major importance: reduced blood circulating volume and reduced oxygen carrying capacity (Marsh, 2005). Undoubtedly, much remains to be found out about the pathogenesis of metabolic acidosis in severe malaria but the clinical implications of what is known are simple and important: acidotic children require immediate and rapid attention to circulating volume and oxygen delivery.

Anaemia is an inevitable consequence of *Plasmodium* infection, particularly in children. Anaemia develops rapidly in the course of severe malaria and blood haematocrit drops greatly especially when the parasite load is high (>100,000/µl). A haematocrit <13% (Hb <4g/dl) has been shown to be associated with a significant increase in mortality in children presenting with malaria (WHO, 2000a). In areas of high and stable transmission, the presentation of severe anaemia is the most important manifestation of severe malaria and occurs predominantly in children less than 3 years of age (WHO, 2000a). Severe malarial anaemia causes over half of all malaria-related morbidity and mortality in children under five years of age in Africa and carries a case fatality rate of ~10% in endemic regions (Murphy and Breman, 2001). There is increasing data showing that deaths associated with severe malarial anaemia can occur within the first 12hr of admission (Marsh *et al.*, 1995; English, 2000).

Severe malarial anaemia consists of a group of conditions with different causes, including direct destruction of parasitized red blood cells, indirect destruction of non-parasitized red blood cells by immune mechanisms, and bone-marrow suppression which is

associated with imbalances in cytokine concentrations (Weatherall and Abdalla, 1982; Ekvall, 2003). Clearance of uninfected erythrocytes from the periphery may be due to multiple factors, including deposition of parasite ligands on erythrocytes. Parasite antigens shed during invasion or released upon lyses of infected schizonts, may be present at high concentrations in plasma and may adhere to uninfected erythrocytes, possibly resulting in immunoglobulin G or complement binding and eventual clearance from the circulation (Waitumbi *et al.*, 2000; Goka *et al.*, 2001; Stoute *et al.*, 2003). In addition to adhesive parasite ligands, erythrocyte clearance may be linked to oxidative damage (Griffiths *et al.*, 2001), reduced deformability (Dondorp *et al.*, 2002), and/or phosphatidylserine externalization (Kiefer and Snyder, 2000).

In severe malarial anaemia, a major fall in haemoglobin correspondingly represents a radical fall in oxygen carrying capacity. One consequence of a net failure in oxygen delivery to areas of greatest need is a shift from aerobic to anaerobic metabolism in tissues with the production of lactate and associated acidosis (English, 2000). This invariably leads to respiratory distress characterised by sustained nasal flaring, indrawing of the bony structure of the lower chest wall on inspiration, and deep breathing (WHO, 2000a). The idea that a tissue oxygen debt plays an important role in the generation of metabolic acidosis is supported by the demonstration that total oxygen consumption of children with severe malarial anaemia rises markedly during the course of blood transfusion and in proportion to the lactate level on admission (English *et al.*, 1997; Narsaria *et al.*, 2012).

Cerebral malaria (CM) is the best-known presentation of severe malaria (Grau and Craig, 2012). The defining feature of the clinical syndrome of cerebral malaria is deep coma. This is defined by the inability to localise a painful stimulus or with a Blantyre Coma Score <2 in a patient with a *P. falciparum* parasitaemia in whom other causes of encephalopathy such as meningitis have been excluded (Molyneux *et al.*, 1989; Warrell, 1992; WHO, 2000a). In most research settings, the case fatality rate for children meeting this standard clinical case definition of CM ranges between 15% and 30% (Snow *et al.*, 1999; Murphy and Breman, 2001). Carefully undertaken post-mortem studies in children have shown that this disease is a heterogeneous syndrome in which sequestration probably has a major role in some cases but little in others (Crawley *et al.*, 2001; Taylor *et al.*, 2004; Gay *et al.*, 2012b;

Ponsford *et al.*, 2012). Metabolic derangement including hypoglycaemia and subclinical convulsions are important in many cases. Sequestration is probably more consistently the cause in adults than in children (Silamut and White, 1993). In around 70% of cases, the onset of coma is with a seizure (Marsh, 2005). Convulsions occur in around 80% of cases of cerebral malaria. Multiple or prolonged convulsions are associated with a worse outcome, particularly with neurological (Molyneux *et al.*, 1989; Steele and Baffoe-Bonnie, 1995; van Hensbroek *et al.*, 1997) and cognitive impairment. Around 40% of children with cerebral malaria have one or more of four distinct abnormalities of respiratory pattern which may be of prognostic significance (Crawley *et al.*, 1998). Deep breathing is a sign of metabolic acidosis and is an indication of the need for urgent fluid resuscitation. Some surviving patients have an increased risk of neurological and cognitive deficits, behavioural difficulties and epilepsy, making cerebral malaria a leading cause of childhood neuro-disability in malaria transmission areas (Gay *et al.*, 2012a).

Among malaria-exposed adults, pregnant women are particularly susceptible to malaria, despite substantial immunity prior to pregnancy, and the risk is highest in first pregnancies (Brabin, 1983; McGregor, 1984). The major complications of infection are maternal anaemia, which in turn increases maternal deaths, and reduced infant birth weight from a combination of intrauterine growth retardation and premature delivery leading to excess infant mortality (Brabin, 1983; Granja *et al.*, 1998; Desai *et al.*, 2007). In some settings, maternal malaria may also cause spontaneous abortion or stillbirth (McGregor, 1984).

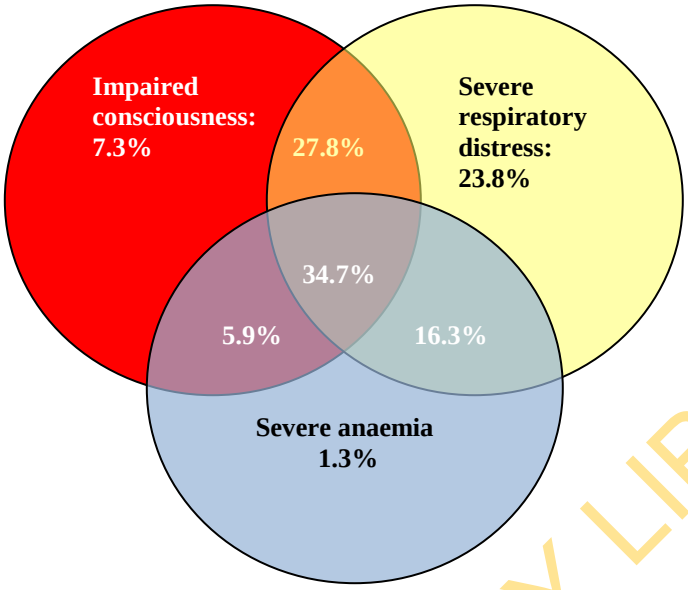


Figure 2.9: Case fatality of major clinical syndromes of severe malaria among African children (Marsh *et al*., 1995)

2.6 Host Immune Response to Malaria

Malaria infection gives rise to a series of host immune responses which can be divided into two general categories: innate immune responses and adaptive immune responses, that are regulated by the innate and the adaptive immune system respectively (Perlmann and Troye-Blomberg, 2002; Good *et al.*, 2004; Van Den Steen *et al.*, 2011). Innate immune responses are those the body mounts immediately, without requiring previous contact with the parasite and thus provides the body with a first line of defence. Effector agents of this response include: cells that kill or ingest infected or altered cells such as macrophages, dendritic cells, natural killer (NK) cells; and soluble proteins (complement and cytokines) that can neutralize, immobilize, agglutinate, kill or activate other effector cells (Karp, 2010; Teirlinck *et al.*, 2011). The adaptive immune responses on the other hand, require a lag period of several days and are highly specific. They are broadly categorized into two: humoral responses which involve the production of antibodies (proteins called immunoglobulins); and cell-mediated responses which involve the production of specialized cells (Stevenson *et al.*, 2011). However, both the innate and the adaptive immune system work closely together in order to destroy the parasite, since the same phagocytic cells and NK cells that carry out an immediate innate response are also involved in initiating the much slower, more specific adaptive immune response. Immunity to various stages of *P. falciparum* infection is thought to contribute to host protection (Plebanski and Hill, 2000).

2.6.1 *Innate (non-specific) immunity to malaria*

Innate mechanisms of parasite growth inhibition by the human host are probably the reason for the low parasitaemia seen in acute *P. falciparum* infection. Accumulating evidence support the concept that macrophages, dendritic cells, NK cells, γδ and NKT cells are important effectors of the innate immunity against malaria (Perlmann and Troye-Blomberg, 2002; Mauduit *et al.*, 2012). In addition, there are indications that dendritic cells (DCs) may play a critical role in amplifying the innate immune response, in particular, by stimulating the activation of NK cells (Ferlazzo *et al.*, 2002).

NK cells have been shown to be the first group of cells to respond to *P. falciparum* infection by increasing in number, and with the ability to lyse infected RBC *in vitro* (Orago and Facer, 1991). NK cells are lymphocytes of the innate immune system that are involved in the early defense against foreign cells and autologous cells infected with parasites. They are widespread throughout the body (Lanier *et al.*, 1986; Stevenson and Riley, 2004), being present in both lymphoid organs and non-lymphoid peripheral tissues (Cooper and Caligiuri, 2004; Cooper *et al.*, 2004; Ferlazzo *et al.*, 2004). NK cells are cytotoxic, inducing apoptosis of cells recognized as targets. Observations of rapid and marked increases in NK cell cytotoxicity levels during infection with a variety of protozoan parasites and *Plasmodium* suggest a role for NK cells in innate immunity against these pathogens (Korbel *et al.*, 2004).

NK cells are also able to modulate the immune response through the secretion of cytokines, including interferon-γ (IFN-γ), tumor necrosis factor, interleukin (IL)-13, granulocyte-macrophage colony-stimulating factor, and chemokines such as CCL3 (macrophage inflammatory protein 1α), CCL4 (macrophage inflammatory protein 1β), and RANTES (Roetynck *et al.*, 2006; McCall *et al.*, 2010; McCall and Sauerwein, 2010). NK cells identify their targets through a set of activating or inhibitory receptors that allow them to recognize pathogen-encoded molecules, self-proteins whose expression is up-regulated in transformed or infected cells, or self proteins that are expressed by normal cells but down-regulated by infected or transformed cells (Vivier and Biron, 2002; Moretta *et al.*, 2004; Moretta and Moretta, 2004; Raulet, 2004). NK cell activation is controlled by the dynamic balance between these activating and inhibitory signals (Vivier *et al.*, 2004). NK cells represent an important early source of IFN-γ during primary murine malaria infections and NK depletion leads to a more rapid increase in parasitemia and higher mortality (Roetynck *et al.*, 2006). Studies in mice have earlier indicated that during infection with *P. chabaudi*, innate responses, involving IFN-γ production by NK cells, and IFN-γ-producing Th1 cells predominate, whereas induction of Th2 responses with IL-4-secreting Th cells predominate after the peak of parasitaemia (Langhorne *et al.*, 1998; Stevenson and Riley, 2004).

Another group of lymphocytes, the Natural killer T (NKT) cells have also been suggested to exhibit capacity to inhibit the liver-stage parasite replication in a murine model *in vitro* (Pied *et al.*, 2000). NKT cells have been shown to determine the outcome of

experimental cerebral malaria caused by *P. berghei* ANKA (Engwerda and Good, 2005). These cells express NK and T cell receptors and recognize antigen presented by CD1 molecules on antigen-presenting cells. CD1-restricted NKT cells are known to recognize the glycosylphosphatidylinositol-anchor molecule of *P. falciparum*. NKT cells from susceptible C57BL/6 mice produce IFN-γ and promote pathology following *P. berghei* ANKA infection, whereas in BALB/c mice they promote Th2 polarization and resistance to experimental cerebral malaria (Hansen *et al.*, 2003). A possible role of the NKT cells in the human malaria could be speculated from their simultaneous production of high levels of both IFN-γ and IL-4 upon primary TCR stimulation (Harris *et al.*, 2005).

Besides, dendritic cells and macrophages have been suggested to be activated as one of the earliest events in the innate response to malaria (Engwerda and Good, 2005). DCs are a heterogeneous antigen-presenting cell population with a crucial role in both the initiation and regulation of cell-mediated immune responses as well as adaptive immune response (Mauduit *et al.*, 2012). DCs drive the differentiation of naïve T cells into IFN-γ-secreting Th1 cells, IL-4- secreting Th2 cells, or other subsets, such as IL-10-secreting regulatory T cells. Plasmacytoid dendritic cells (PDC), a unique subset of DC, have also been shown to have a key role in the innate immunity because of their ability to produce high levels of IFN-α in response to viral (Cella *et al.*, 1999) or microbial DNA or CpG DNA stimulation (Kadowaki *et al.*, 2001). Macrophages on the other hand, have been shown to have a role in innate immunity as evident in their ability to phagocytose infected erythrocytes (Gyan *et al.*, 1994; Serghides *et al.*, 2003), and thus contributing to the reduction of initial parasitaemia in malaria.

2.6.2 *Acquired (adaptive) immunity to malaria*

Acquired immunity to malaria requires both humoral and cellular components of the adaptive immune system. Both types of acquired immunity are mediated by a special class of white blood cells known as lymphocytes. These cells express cell surface molecules capable of recognizing a wide variety of proteins. Lymphocytes are also comprised of two subsets: T lymphocytes and B lymphocytes. Studies have suggested that during blood-stage infection with *Plasmodium* species in mice and humans, both cell-mediated and antibody-

dependent responses are critical for the control of parasitaemia and parasite-induced pathology (Bouharoun-Tayoun *et al.*, 1990; Day and Marsh, 1991; Marsh and Kinyanju, 2006). In malaria endemic areas, acquired immunity develops with repeated exposure to the malaria parasite and, as such, is neither sterile nor permanent. Likewise, infection by one strain of *P. falciparum* does not induce acquired immunity to all strains. Thus, immunity is species-, stage-, and strain-specific (Plebanski and Hill, 2000).

Humoral (Antibody-mediated) immune responses to the malaria parasites

Humoral immune response is mediated by B lymphocytes (B cells). B cells have antigen receptors (BCR) on their surfaces which consist of membrane-bound immunoglobulins or antibodies that bind selectively to a portion of an intact antigen. When B cells are appropriately activated, they differentiate into cells that secrete their receptors or antibodies into the blood. Within the first week of primary malaria infection, specific antibodies of several isotypes (IgG, IgA, and IgM) can be detected (Marsh and Kinyanju, 2006). Immunoglobulin G and IgM antibody levels may be long sustained and may form the basis of many serological reactions useful in diagnosis and epidemiological surveys, although they give little correlation with clinical immune status. It has been demonstrated that passive transfer of monoclonal antibodies against parasite antigens may confer protection in naive mice (Narum *et al.*, 2000). In humans, treatment of Thai *P. falciparum* - infected patients with IgG extracted from African immune adults resulted in reduction of parasitaemia and clinical symptoms in an antibody-dependent cellular inhibitory effect in cooperation with monocytes (Bouharoun-Tayoun *et al.*, 1990). The most direct evidence that antibodies are important mediators of immunity to malaria comes from passive transfer studies in which antibodies from malaria-immune adults were successfully used to treat patients with severe malaria (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991). Studies in mice deficient in Fc-γ receptors further support an important role for antibodies (Rotman *et al.*, 1998).

Initially after an infectious bite by the mosquito vector, sporozoites may either be eliminated by antibodies or may proceed to infect liver cells. Anti-sporozoite antibodies are specific to sporozoites and directed mostly to a repeat sequence within circumsporozoite

(CS) protein or sporozoite STARP protein (Doolan and Martinez-Alier, 2006). Antibodies that bind to the sporozoite are able to prevent hepatocyte infection (Plebanski and Hill, 2000), and numerous studies have correlated anti-CS protein antibodies with protection in humans.

Immunity to blood stages is thought to be mediated by antibodies that block merozoites from entering RBCs, antibodies that agglutinate infected RBCs or antibodies that opsonize infected RBCs (Engwerda and Good, 2005). Antibodies are directed either against a number of identified proteins on the parasite itself or against parasite-derived proteins expressed on the surface of the infected erythrocyte during intra-erythrocytic development of the parasite (Giha *et al.*, 2000; Stanisic *et al.*, 2009; Omosun *et al.*, 2010; Khosravi *et al.*, 2011). Although the potential of many antigens as targets for immune responses has been suggested, little is known about the mechanisms of protection *in vivo*. Several studies have suggested possible mechanisms by which antibody responses may mediate immunity to asexual blood stage of malaria including: inhibition of merozoite invasion of the RBC, or by blocking merozoite release from schizonts either by binding to surface exposed antigens (Green *et al.*, 1981; Wahlin *et al.*, 1984; Bull and Marsh, 2002); opsonization of parasites and infected RBCs for enhanced phagocytosis (Giribaldi *et al.*, 2001); prevention of cytoadherence of infected RBCs to the microvasculature endothelium, thereby allowing them to be removed by the spleen (David *et al.*, 1983); and inhibition of rosetting of infected RBCs to uninfected RBCs (Carlson *et al.*, 1990a; Carlson *et al.*, 1990b). In collaboration with other effector immune cells, parasite antigen-specific antibodies play an important role via antibody-dependent cellular inhibition (ADCI), whereby binding of antibodies to phagocytes via Fc receptors lead to inhibition of parasite growth (Aucan *et al.*, 2000; Tebo *et al.*, 2001).

Specific antibody isotypes and subclasses may play diverse roles in malaria. The polarization of antibody responses towards IgG1 and IgG3 subclasses, which bind to Fcγ receptors (FcγR) on the surface of monocytes, macrophages, and neutrophils, is believed to play a key role in immunity to blood-stage *Plasmodium falciparum* infection (Bouharoun-Tayoun *et al.*, 1990). Specifically, high levels of malaria-specific cytophilic antibodies, such

as IgG3, were found in malaria-exposed donors and suggested to be protective (Shi *et al.*, 2001; Leoratti *et al.*, 2008; Courtin *et al.*, 2009). Once merozoites have been released from the schizonts, cytophylic antibodies may mediate parasite elimination through complement-dependent lysis or by cellular effector mechanisms (Kumaratilake *et al.*, 1997; Ramasamy *et al.*, 2001). An almost exclusive restriction of the humoral immune response of IgG3 subclass to MSP-2 blocking has been observed by several investigators (Taylor *et al.*, 1995; Ferrante and Rzepczyk, 1997). Hence, these IgG3 antibodies are potentially effective mediators of protection due to their cytophylic nature. In another study however, IgG subclass distribution of naturally acquired antibodies to *P. falciparum* merozoite surface proteins in adults exposed to low to moderate levels of malaria transmission was found to be primarily epitope driven (Scopel *et al.*, 2006). IgG3 polarization was also found to be more evident for polymorphic domains of MSP-1 than those of MSP-2, being little affected by cumulative or current exposure to malaria and not affected by the subject's age and FcγRIIA. In contrast, high levels of IgE have been implicated in the pathology of malaria (Maeno *et al.*, 2000; Perlmann *et al.*, 2000).

Cellular (Cell-mediated) immune responses to the malaria parasites

Cell-mediated immune response is carried out by T lymphocytes (T cells) which, when activated, can specifically recognize and kill an infected or foreign cell. T cells have receptors on their surfaces, termed the T cell receptor or TCR (Marrack and Kappler, 1987). T cells may be classified into three based on the type of TCR that they express. These are: Cytotoxic T Lymphocytes (CTLs) distinguished by the presence of CD8 protein on their surfaces; Helper T Lymphocytes (T_H cells) distinguished by the presence of CD4 protein on their surfaces; and Regulatory T Lymphocytes (T_{Reg} Cells) which are characterized by the possession of $CD4^+$ $CD25^+$ surface markers (Spellberg and Edwards, 2001; Karp, 2010). Helper T cells may be further sub-divided into three (T_H1, T_H2 and T_H17), which can be distinguished by the cytokines they secrete (Hall, 2011). T_H1 cells secrete IFN-γ, T_H2 cells secrete IL-4, while T_H17 cells secrete IL-17. For T cells to be activated, the TCR must recognize and interact with foreign peptide on the surface of an antigen presenting cell

(APC) in association with a major histocompatibility complex (MHC) molecule. $CD4^+$ T cells are activated by a foreign peptide bound to a class II MHC molecule, whereas $CD8^+$ T cells recognize antigens presented to it by MHC class I molecules (Spellberg and Edwards, 2001). Cell-mediated immune responses induced by malaria infection have been suggested to be protective against both pre-erythrocytic and erythrocytic parasite stages and thus play a critical role in the host defence system against malaria (Horowitz *et al.*, 2010; Kimura *et al.*, 2010).

There are data showing that T cells play a major role in the acquisition and maintenance of protective immune responses to malaria infection (Li *et al.*, 2012). Mice with severe combined immunodeficiency (SCID) and reconstituted with T cells from immune donors suppress parasite growth, suggesting a protective role of T cells against malaria parasites, while B cell-deficient mice were able to suppress parasitaemia at the same rate as normal mice (van der Heyde *et al.*, 1993). A correlation between resistance to fever and high parasitaemia and in vitro T cell responses to *P. falciparum* blood stage antigens has also been reported (Riley *et al.*, 1992), although this was not found in previous studies (Hviid *et al.*, 1990; Riley *et al.*, 1990). In humans, direct studies of the responding T cells during malarial infection are difficult, as these cells may leave the peripheral circulation and sequester in the spleen or other tissues (Hviid *et al.*, 1991a; Hviid *et al.*, 1991b).

$CD4^+$ and $CD8^+$ T Cells

Of the major T-cell subpopulations, $CD4^+$ T cells are essential for immune protection against asexual blood stages in both murine and human malaria systems (Spellberg and Edwards, 2001). For experimental malaria, evidence for this was based on adoptive transfer of protection by such cells and on increased susceptibility to infection of $CD4^+$ T-cell-depleted mice. For *P. falciparum* malaria in humans, the existence of functionally different $CD4^+$ T cells in naturally exposed donors has also been established experimentally. $CD4^+$ T cells play a central role in regulating the immune responses to the asexual blood stages of *P. falciparum* via cytokine production and B-cell help (Claser *et al.*, 2011). These cells respond to malaria antigen by *in vitro* proliferation and/or secretion of cytokines, e.g. IFN-γ

or IL4 (Bouyou-Akotet and Mavoungou, 2009). In general, these *in vitro* responses are poorly correlated with protection. Nevertheless, in vitro stimulation of $CD4^+$ T cells from malaria-exposed donors may result in the production of IL4 in concordance with the serum concentrations of antibodies specific for the antigens used for lymphocyte stimulation. It has been shown that $CD4^+$ T cells from individuals naturally exposed to malaria respond to blood stage antigens of *P. falciparum* by proliferation, production of IFN-γ and is IL-2 dependent in animal model (Kimura *et al.*, 2010).

Similarly, $CD8^+$ T cells have been shown to play important role in the pre-erythrocytic immunity to malaria (Tse *et al.*, 2011), thereby contributing to protection against severe forms of malaria (Nardin and Nussenzweig, 1993; Aidoo and Udhayakumar, 2000). However, no available evidence for a protective role of $CD8^+$ T cells against *P. falciparum* blood stage has been reported (Schmidt *et al.*, 2009; Tse *et al.*, 2011). This is supported by the fact that RBC do not express classical MHC class I molecules, hence lacking the antigen processing machinery, suggesting that RBC do not represent a target for $CD8^+$ T cells. In any case, since human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by $CD8^+$ cytotoxic T lymphocytes has no role in the defence against blood-stage parasites.

2.7 Cytokines in Host Defence against Malaria

Cytokines are a diverse group of small, secreted proteins or glycoproteins which mediate, regulate and help direct many critical aspects of an immunity, inflammation, and hematopoiesis (Karp, 2010). They are rapidly produced in response to foreign antigen exposure and can promote the expansion, activation, recruitment, and differentiation of the responding cell types (Clark *et al.*, 2007; Bakir *et al.*, 2011). Cytokines generally (although not always) act over short distances and short time spans and at very low concentration. They may act on the cells that secrete them (autocrine action), or on nearby target cells which are in the immediate vicinity of the producer cells (paracrine action). However, some cytokines may also enter the blood stream and act on distant cells in an endocrine fashion (Hill and Sarvetnick, 2002; Vandenbroeck *et al.*, 2006).

Cytokines are key molecules that interact with other immune cells in the activation of immune response to malaria (Torre *et al.*, 2002b). They can have a profound effect on the balance between cellular and humoral response as well as the isotype of immunoglobin produced (Khaled and Durum, 2002; Borish and Steinke, 2003). Cytokines are the major inducers of Th1 and Th2 subset development, which are important for the eradication of malaria parasites. Development of Th1 response is mediated by pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-12 and IL-18 but can be antagonised by anti-inflammatory cytokines, including IL-4, IL-10 and transforming growth factor (TGF) β (Malaguarnera and Musumeci, 2002; Cabantous *et al.*, 2009). Cytokine release in malaria may be triggered by parasite antigens, pigments or toxins. The balance between Th1 and Th2 immune response and between pro-inflammatory and anti-inflammatory cytokines is important in determining the level of malaria parasitemia, disease outcome and rates of recovery (Winkler *et al.*, 1998; Riley, 1999; Sinha *et al.*, 2010; Bakir *et al.*, 2011), while the overproduction of both pro-inflammatory and anti-inflammatory cytokines can as well be responsible for disease severity and mortality (Day *et al.*, 1999; Cabantous *et al.*, 2009).

2.7.1 *General features of cytokines and gene expression*

Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages (Dong and Flavell, 2001). They have been referred to by a number of names depending on the cell types that produce them or their functional properties. For example, cytokines that are derived primarily from mononuclear cells such as monocytes or macrophages have been referred to as monokines while the cytokines produced by activated T lymphocytes are termed lymphokines. Cytokines with chemotactic activities, which specifically regulate the migration of other cells, are called chemokines. Historically however, cytokines are referred to as interleukins since in a general sense they are being produced by leukocytes and acting on other leukocytes (Khaled and Durum, 2002; Wurster *et al.*, 2002; Borish and Steinke, 2003; Mehta *et al.*, 2004).

The biology of cytokine is so complex. Most of them are pleiotropic, meaning that different cell types may secrete the same cytokine or for a single cytokine to act on several different cell types within the immune system as well as on cell types outside of the immune system. They are also redundant in nature: many biological properties originally described for one cytokine can also be ascribed to others, suggesting that the loss of a particular cytokine can be compensated for by the action of another (Hill and Sarvetnick, 2002). Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. They can also act synergistically, where two or more cytokines act together; or antagonistically, where cytokines cause opposing activities (Dong and Flavell, 2001).

Cytokines mediate their biological effect through the binding of specific receptors on the outer surface of target cells, generating cytoplasmic signals that act on various intracellular targets (Ihle, 1996; Karp, 2010). These signal transduction events usually result in gene expression, which occurs rapidly after an inducing stimulus. The rapid increase in cytokine gene mRNA results in an outburst of cytokine protein secretion into the surrounding environment and can act directly on the cell that produced it or on neighbouring cells. This rapid increase in transcription is usually transient and is turned off rapidly resulting in a self-limited event. Thus, cytokine expression and signalling is highly regulated

because dysregulated cytokine responses can lead to pathological conditions (Wurster *et al.*, 2002; Mehta *et al.*, 2004). Cytokine receptors are transmembrane proteins where binding of the cytokine occurs in the extracellular region and interaction with signalling proteins occurs in the cytoplasm (O'Shea *et al.*, 2002).

Cytokine receptors can be divided into two groups: those whose intracellular domains exhibit intrinsic protein-tyrosine kinase (PTK) activity and those whose intracellular domains are devoid of such activity. Many of the latter group of receptors, however, activate intracellular soluble PTKs upon ligand binding. Protein-tyrosine kinases (PTKs) are enzymes that phosphorylate specific tyrosine residues on protein substrates: a mechanism for signal transduction (Karp, 2010).

Cytokines utilize a novel signal transduction pathway referred to as Janus kinase-signal transducers and activators of transcription (*JAK-STAT*) pathway (Shuai and Liu, 2003; Hideshima *et al.*, 2005). JAKs represent a family of PTKs whose members become activated following the binding of a cytokine to a cell-surface receptor. They play a central role in mediating signal transduction of many cytokines (O'Shea *et al.*, 2002). JAKs harbour two potential active sites and were thus named after Janus, a two-faced Roman god. STAT is a family of transcription factors that become activated when one of their tyrosine residues is phosphorylated by a JAK (Ihle, 1996; Marrero, 2005). Once phosphorylated, STAT molecules interact to form dimmers that translocate from the cytoplasm to the nucleus. In the nucleus, the dimerized STAT proteins are able to bind to specific DNA sequences in the promoters of cytokine inducible genes and activate or repress the transcription of those genes directly. The JAK-STAT signalling pathway provides a direct link between cytokine binding at the cell surface to changes in gene expression at the level of new gene transcription (Marrero, 2005; Rakesh and Agrawal, 2005). Different cytokine receptors bind to unique JAK and STAT combinations resulting in specific outcomes to cytokine exposure.

2.7.2 *Pro-inflammatory and anti-inflammatory cytokines*

Interferon gamma (IFN-γ)

IFN-γ is a key Th1 cytokine involved in the innate immune response to malaria. It is mainly produced by NK cells, CD8+ and CD4+ T lymphocytes and antigen presenting cells (Frucht *et al.*, 2001; Bouyou-Akotet and Mavoungou, 2009; Horowitz *et al.*, 2010). T cell secretion of IFN-γ may help to induce cytophilic IgG blood-stage-specific antibodies and assist in antibody-dependent cellular inhibitory mechanisms (Bouharoun-Tayoun *et al.*, 1990). *P. falciparum* blood stage antigen can induce IFN-γ production by CD4+T cells and this has been shown to be associated with protection against malaria re-infection in Africa (Luty *et al.*, 1999). Likewise, sporozoites which are rapidly processed by the host cell and presented on the surface of infected hepatocytes in combination with MHC class I, leads to stimulation of NK and CD4+ T cells to produce IFN-γ, which can trigger a cascade of immune reactions and can lead to the death of intracellular parasites (Malaguarnera and Musumeci, 2002; McCall and Sauerwein, 2010).

IFN-γ activates macrophages to kill parasites, exerting its effects through its receptor IFNGR1. Animal models have shown a critical role of IFN-γ in immunity against malaria parasites (Favre *et al.*, 1997; Angulo and Fresno, 2002). High IFN-γ production as part of a Th1-driven immune response has been associated with a more favorable outcome in most animal models of malaria (Sedegah *et al.*, 1994). This effect has been attributed to the monocyte-macrophage activating capacity of IFN-γ, with rapid killing of the malarial blood-stage parasites by reactive oxygen and nitrogen intermediates (Taylor-Robinson and Looker, 1998). The role of IFN-γ as a key molecule in human antimalarial host defence has been demonstrated in patients with uncomplicated *P. falciparum* malaria (Winkler *et al.*, 1998).

However, IFN-γ is also part of an inflammatory response that, if exacerbated, can be associated with adverse pathology (Claser *et al.*, 2011). It can also contribute to the acute symptoms of malaria through the induction of TNF-α and IL-1 (Riley, 1999).

TNF-α

The first characterized parasite-induced cytokine was TNF-α, induced in macrophages by *Plasmodium* -infected erythrocytes, malarial pigment (Pichyangkul *et al.*, 1994), and certain glycolipids such as GPI moiety (Schofield and Hackett, 1993; Malaguarnera and Musumeci, 2002). TNF is one of the most important mediators of inflammation. It is produced primarily by macrophages and monocytes after activation by foreign antigens. Antibodies directed against GPI blocked the stimulatory function of lysates from different strains of plasmodia infected erythrocytes to induce TNF-α from mononuclear cells (Bate and Kwiatkowski, 1994). TNF-α can increase the phagocytic capacity of monocytes or macrophages due to an increased expression of Fc receptors on them, or to the modulation of Fc-receptor signalling pathways by signals originating from the binding of TNF-α to its receptors (Muniz-Junqueira *et al.*, 2001). However, different strains of *P falciparum* obtained from children with mild or cerebral malaria have been found to show marked variation in their ability to induce TNF-α from monocytes/macrophages (Allan *et al.*, 1995).

TNF-α has also been suggested to play a similar role to that of IFN-γ in early responses against *Plasmodium* (Randall and Engwerda, 2010). In animal models, treatment with an anti-TNF-α monoclonal antibody resulted in a tendency toward longer times for parasite clearance (Looareesuwan *et al.*, 1999), while treatment with recombinant human tumour necrosis factor-α reduced parasitaemia and prevented *Plasmodium berghei* K173-induced experimental cerebral malaria in mice (Postma *et al.*, 1999; Depinay *et al.*, 2011). Similarly, an association between the ability to produce high levels of TNF-α and an accelerated cure and improved prognosis has been reported in humans (Mordmuller *et al.*, 1997; Randall and Engwerda, 2010).

TNF-α also seems to have, in roughly 1% of individuals with malaria, detrimental properties such as fever, aches and pains correlated to acute illness, hypoglycaemia, shock, bleeding, and reversible coma (Beutler and Grau, 1993). Elevated concentrations of TNF alter the surface properties of vascular endothelial cells and results in the local accumulation of leukocytes. These sequestered leukocytes release more TNF, thus amplifying the

cytotoxic effect on endothelial cells, and resulting ultimately in vascular wall damage and haemorrhagic necrosis. In severe *falciparum* malaria, several studies have shown association between plasma concentrations of TNF-α as well as other pro-inflammatory cytokines and mortality (Grau *et al.*, 1989; Kern *et al.*, 1989; Kwiatkowski, 1990), although this relationship was not confirmed in other studies (Shaffer *et al.*, 1991). In murine models, immunopathological damage was found to be associated with elevated concentrations of TNF-α, which can be prevented by the administration of anti-TNF polyclonal antibodies (Grau *et al.*, 1987).

Several studies have suggested that TNF-α is the main mediator of secondary complications accompanying severe malaria caused by *P. falciparum* and that it could become fatal among people suffering from cerebral malaria, severe anaemia, lactic acidosis and hypoglycaemia (Odeh, 2001). Likewise, a close association between the presence of severe anaemia, high TNF-α concentrations, and large numbers of circulating haemozoin containing monocytes has been reported (Luty *et al.*, 2000), suggesting that haemozoin-induced TNF-α-production plays a part in either initiation or exacerbation of anaemia as a clinical outcome of chronic, uncontrolled parasitaemia. Likewise, several experimental studies have demonstrated this cytokine's role in pathogenic malarial anaemia, including severe disruption of erythropoiesis and erythroid cell suppression and proliferation (Tchinda *et al.*, 2007a). However, the production of TNF-α could be regulated by the anti-inflammatory effect of IL-10 (de Waal Malefyt *et al.*, 1991).

Interleukin (IL) 10

IL-10 was initially characterized as ‘‘cytokine synthesis inhibitory factor’’ produced by Th2 cell clones that inhibited the production of IFN-γ by Th1 cells (Fiorentino *et al.*, 1989; Niikura *et al.*, 2011). IL-10 is produced by B cells, Th2 cells, monocytes and macrophages after antigen stimulation (Vandenbroeck *et al.*, 2006). The major function of IL-10 is to serve as an anti-inflammatory and immunosuppressive cytokine (Sunder *et al.*, 2012). It inhibits cytokine production in Th1 and CD8+ cells as well as the production of

pro-inflammatory cytokines like TNF and IL-1. It however, does not affect the proliferation of Th1 and CD8+ T cells, but induces B-cell proliferation, and immunoglobulin production, which is essential for the development and maturation of antimalarial antibodies (Malaguarnera and Musumeci, 2002). However, evidence is accumulating that Th1 and CD4+ T cells provide a crucial source of IL-10 (Freitas do Rosario *et al.*, 2012; Freitas do Rosario and Langhorne, 2012). IL-10 down-regulates the expression of MHC class II molecules on macrophages and of co-stimulatory molecules such as CD80 and CD86, leading to decreased antigen presentation (Akdis and Blaser, 1999), inhibits ROI and NOI production, prevents Tcell priming and proliferation, and suppresses the production of interferon-γ, interleukin 6, TNF-α, and GM-CSF by T cells (Akdis and Blaser, 1999). It also inhibits the ability of malaria antigens to induce or release tumour necrosis factor (Ho *et al.*, 1995).

IL-10 is a key cytokine that has been shown to have important regulatory function in establishing a balance between pro- and anti-inflammatory responses in malaria (Freitas do Rosario and Langhorne, 2012). A prominent role in switching from Th1 to Th2 responses is attributed to IL-10 (Angulo and Fresno, 2002). Therefore, it is probably involved in controlling the adequate timing of antiparasitic responses (Li *et al.*, 1999). Plasma levels of IL-10 have been reported in patients with acute malaria (Wenisch *et al.*, 1995). Similarly, an anti-IL10 antibody used to treat C57BL/6 mice infected with *P. yoelii* 17XL *in vivo* was shown to increase survival times with no detectable changes in parasitemia (Kobayashi *et al.*, 2000). Early IL-10 production has been associated with susceptibility to infection, and it is thought that this cytokine has a prominent anti-inflammatory effect, limiting in some way the damage inflicted on normal tissues by an excessive Th1 response (Kobayashi *et al.*, 1996).

It has been shown that the increase of interleukin 10 is more pronounced and more specific than interleukin 6 and interleukin 8 in patients with malaria parasitaemia compared with other infections (Jason *et al.*, 2001). A study of malaria-infected children and adults in Gabon recorded many interleukin-10-producing CD4+ and CD8+ T cells co-expressing interferon-γ (Winkler *et al.*, 1999). These cells may provide a fertile ground for parasite-

driven immune modulation. However, it is not yet clear whether the increased concentrations of interleukin 10 have a beneficial role by reducing the parasite-induced inflammatory response, or a detrimental one by decreasing the cellular immune responses.

Similarly, in severe malaria, particularly in fatal cases, it has been suggested that there is a relative failure of IL-10 production, and thus of control of pro-inflammatory cytokine release (Ho *et al.*, 1998). Likewise, it has been shown that severe anaemia is associated with reduced concentrations of circulating interleukin 10 (Kurtzhals *et al.*, 1998) and, that an increased ratio between TNF-α and IL-10 contributes to the reversible bone-marrow suppression seen in malaria patients (Othoro *et al.*, 1999). The inhibition of interferon-γ and TNF-α secretion by IL-10 synthesis has been reported to be important to counteract the pathological role of macrophages in cerebral malaria (Kossodo *et al.*, 1997).

Interleukin-18

IL-18 is a pro-inflammatory cytokine that enhances innate and specific Th1 immune responses. It was originally discovered as a factor that induces IFN-γ production from Th1 cells (Okamura *et al.*, 1995b). Apart from Th1 cells, IL-18 can act on nonpolarized T cells, NK cells, B cells, and dendritic cells to produce IFN-γ in the presence of IL-12 (Okamura *et al.*, 1995b; Okamura *et al.*, 1998; Akira, 2000; Ajiki *et al.*, 2003). IL-18 has also been shown to have a role in inducing a proper Th2 response because of its capacity to directly induce IL-4 and IL-13 secretion, as well as high IgE expression by B cells (Hoshino *et al.*, 1999; Yoshimoto *et al.*, 2000; Nakanishi *et al.*, 2001a). The ability of IL-18 to induce a Th2 response depends on CD4+ T cells, NK-T cells, and IL-4. IL-18-induced IgE production has also been shown to be abrogated in CD4+ T cell-depleted mice (Yoshimoto *et al.*, 2000). Therefore, IL-18 has the capacity to stimulate innate immunity and both Th1- and Th2-mediated responses (Nakanishi *et al.*, 2001b). IL-18 is produced by monocytes, macrophages, dendritic cells, epithelial cells, Kupffer cells, astrocytes, kerotinocytes and osteoblasts when activated by antigenic products, or in response to microbial

lipopolisaccharides (Staak *et al.*, 1997; Stoll *et al.*, 1998; Dinarello, 1999; Nakanishi *et al.*, 2001a; Seki *et al.*, 2001; Swain *et al.*, 2001).

Analysis of the amino acid sequence and structural motifs of IL-18 shows that it belongs to the IL-1 family of cytokines (Okamura *et al.*, 1995b). Like IL-1, IL-18 is produced as an inactive precursor that is cleaved by the IL-1 converting enzyme (caspase 1) to generate an 18.3 kDa biologically active peptide (Ghayur *et al.*, 1997; Liu *et al.*, 2000) and the activity of mature IL-18 is closely related to that of IL-1β (Bazan *et al.*, 1996). Proteinase-3, caspase-3, and cathepsin proteases can also cleave the precursor polypeptide but this sometimes results in inactive forms of IL-18 (Su *et al.*, 1997; Sugawara *et al.*, 2001).

Mature IL-18 binds a heterodimeric surface receptor (IL-18R) which is comprised of an α chain (IL-18Rα) responsible for extracellular binding and a β chain (IL-18Rβ) which is a nonbinding, signal transducing chain (Kato *et al.*, 2003). Both chains are required for functional IL-18 signaling (Born *et al.*, 1998; Boasberg *et al.*, 2006). IL-18R is expressed on a variety of cells including macrophages, neutrophils, natural killer (NK) cells, endothelial, and smooth muscle cells (Leung *et al.*, 2001; Afkarian *et al.*, 2002; Gerdes *et al.*, 2002). The interaction of IL18 and IL18R results in the recruitment of myeloid differentiation 88 (MyD88), an adaptor molecule involved in IL-1 and Toll-like receptor signaling (Chandrasekar *et al.*, 2004). The high affinity IL18R complex also recruits the IL-1R-associated kinase (IRAK) to the receptor complex resulting in phosphorylation of NFκB-inducing kinase with subsequent translocation of NFκB to the nucleus (Sareneva *et al.*, 2000). Similar to IL-1, a soluble IL-18 binding protein which is constitutively secreted exists, and has high affinity for IL-18 and blocks its biological activity (Nakanishi *et al.*, 2001b).

In terms of its biological effects, IL-18 is closely related to and acts synergistically with IL-12 (Malaguarnera and Musumeci, 2002). The combination of IL-18 plus IL-12 has been suggested to be more effective at inducing interferon-γ production by macrophages than IL-18 alone (Dinarello, 1999). In synergy with IFN-α or IL-12, IL-18 induces IFN-γ production in T cells and enhances Th1 cell development (Okamura *et al.*, 1995b; Micallef *et al.*, 1996; Robinson *et al.*, 1997), but may also exert its effect independently of IL-12

(Kohno *et al.*, 1997). IL-18 can also induce IFN-*γ* production from T cells independently of TCR activation, a property unique to IL-18 (Dinarello and Fantuzzi, 2003).

IL-18 has been reported to play a protective role in malaria, particularly in early immunity against *Plasmodium* by enhancing IFN-γ production *in vivo* (Singh *et al.*, 2002). In human malaria elevated levels of IL-18 in serum/plasma of patients with *P. falciparum* malaria have been reported in several studies from endemic areas (Malaguarnera *et al.*, 2002; Chaisavaneeyakorn *et al.*, 2003; Nagamine *et al.*, 2003). Likewise, increased levels of IL-18 as well as IFN-γ were observed in symptomatic individuals compared with non-symptomatic or aparasitaemic individuals suggesting the induction of these cytokines in response to active *P. falciparum* infection (Torre *et al.*, 2001). As a result of the positive correlation between IL-18 levels and disease severity as well as parasitemia, IL-18 has been suggested to be a marker for disease severity in malaria (Torre *et al.*, 2001; Nagamine *et al.*, 2003). MyD88-dependent signalling of IL-18 is however important for early parasite control as suggested by studies using animal models (Cramer *et al.*, 2008).

2.8 Genetic Basis of Host Resistance to Malaria

The impression that variations in host response to infection might have a genetic basis is not new (Haldane, 1949; Allison, 1954; Cooke and Hill, 2001). Way back in 1949, Haldane proposed that genetic variation in globin genes gave a selective advantage for survival in malaria-endemic areas, and that similar forces from other pathogens could maintain great biochemical diversity (Haldane, 1949). Malaria has exerted an almost unparalleled selective pressure on humans, leading to the appearance of gene polymorphisms at high frequency (Kwiatkowski, 2000; Driss *et al.*, 2011). Malaria has been described as the strongest known selective pressure in the recent history of the human genome (Kwiatkowski, 2005). The greatest number of genes conferring differential susceptibility to any disease has been reported only for the various manifestations of malaria (Frodsham and Hill, 2004). Malaria is the evolutionary driving force behind sickle-cell disease, thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other erythrocyte defects.

A growing number of human genes have been related to malaria resistance or susceptibility and these can be broadly divided into: genes that are related to erythrocyte metabolism, such as Duffy antigen chemokine receptor (DARC), G6PD, and β-globin haemoglobin (HBB); genes that mediate cytoadherence by *P. falciparum* -infected erythrocytes, such as complement component receptor 1 (CR1), CD36 ligand, and intercellular adhesion molecule 1 (ICAM-1); and genes that are directly involved in immune responses, such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukins (IL), and HLA genes (Jenkins *et al.*, 2005; Mackinnon *et al.*, 2005; Barreiro and Quintana-Murci, 2010; Lyke *et al.*, 2011).

2.8.1 *Haemoglobin variants*

The most well-known examples of erythrocyte variants conferring resistance to malaria are the haemoglobin gene variants. Haemoglobin comprises of four globin chains: foetal haemoglobin (HbF) has two α and two γ chains ($\alpha_2\gamma_2$), while adult haemoglobin (HbA) has two α and two β chains ($\alpha_2\beta_2$). The α-globin and β-globin genes are located on chromosomes 16 and 11 respectively, and they control the production of globin chain (Ashley-Koch *et al.*, 2000; Voskaridou *et al.*, 2012). The most striking of the haemoglobinopathies is associated with the β-globin gene (HBB), of which three different amino acid changes are observed at polymorphic frequencies: HbS (β6Glu→Val), HbC (β6Glu→lys), HbE (β26Glu→lys) and they confer different levels of malaria protection (Flint *et al.*, 1998; Weatherall and Clegg, 2001; Verra *et al.*, 2007).

The term sickle-cell disease (SCD) or "sickle hemoglobin" refers to a group of symptomatic disorders associated with mutations on the HBB gene (Richer and Chudley, 2005), which produce the hemoglobin form known as Hemoglobin S (HbS). HbS homozygotes suffer from sickle-cell disease, but heterozygotes have a tenfold reduced risk of severe malaria (Allison, 1954; Ackerman *et al.*, 2005). The mechanism of protection in heterozygotes having the sickle cell trait (HBAS) has been suggested to be innate (Gong *et al.*, 2012). HbS is widespread all over malaria endemic areas and common in sub-Saharan Africa with carriers reaching 15-20% in some areas (Weatherall and Clegg, 2001). Within this region however, it has been shown that the HbS allele occurs in four different

haplotypes (Lapoumeroulie *et al.*, 1992; Flint *et al.*, 1998).

Distinct mechanisms conferring protection against severe and complicated malaria have been proposed for the different haemoglobinopathies such as sickle-cell trait and beta thalassemia trait (Ayi *et al.*, 2004; Williams *et al.*, 2005a; Ferreira *et al.*, 2011). Among the most relevant mechanisms, reduced erythrocyte invasion by the parasite, decreased intra-erythrocytic parasite growth (Pasvol *et al.*, 1992), enhanced phagocytosis of parasite-infected erythrocytes (Cappadoro *et al.*, 1998; Ayi *et al.*, 2004) and increased immune response against parasite-infected erythrocytes have all been described (Duffy and Fried, 2006). The HbS allele seems not to prevent the infection per se, but is protective against death or severe disease (profound anaemia and cerebral malaria), probably owing to impaired entry and growth of the parasites during the erythrocytic stage of development (Chippaux *et al.*, 1992a; Chippaux *et al.*, 1992b; Pasvol *et al.*, 1992; Shear, 1993; Shear *et al.*, 1993). Friedman (Friedman, 1978) had earlier described the mechanisms by which HbS-containing erythrocytes inhibit malaria parasite growth. He showed that HbS changes its nature in deoxygenating conditions and parasites become gravely affected, therefore showing that only erythrocyte mechanisms are sufficient for providing resistance "*in vivo*". He also showed that 90% of young parasites are eliminated in HbAS cell populations, of which only 60% are sickled or distorted and that the parasite contributes to conditions inducing sickling in its host cell.

Haemoglobin C (Hb C) results from a point mutation leading to the replacement of glutamate by lysine in the β-globin's sixth amino-acid position (Agarwal *et al.*, 2000; Fairhurst *et al.*, 2005). Hb C is restricted to parts of West and North Africa (Modiano *et al.*, 2001; Mockenhaupt *et al.*, 2004a). Although HbAC is asymptomatic, HbCC can produce mild haemolysis, splenomegally and gallstones (Mockenhaupt *et al.*, 2004a). Similarly, the relatively high frequencies of HbC have also been suggested to be maintained by resistance to *P. falciparum* malaria in West Africa (Modiano *et al.*, 2001). The study showed evidence both for heterozygote and homozygote resistance and suggested that, unlike the sickle cell mutation, HbC may be an example of transient polymorphism, based largely on the perceived lack of clinical disability or haematological changes of HbC homozygotes, although it is not absolutely clear whether homozygotes for this variant are completely

unaffected by the condition. However, there are indications that HbC reduces parasitaemia and confers protection against mild malaria attack (Rihet *et al.*, 2004).

Haemoglobin E (HbE) is produced when the glutamic acid in position 26 of the β-globin chain is replaced by a lysine (Nagel *et al.*, 1981; Chotivanich *et al.*, 2002; Ohashi *et al.*, 2004). It has been observed that erythrocytes from people having HbE show reduced plasticity and deformability "*in vitro*", thus impairing merozoite growth and release (Bunyaratvej *et al.*, 1992). Homozygous HbE erythrocytes (HbEE) are microcytic (having low mean corpuscular volume) at low haemoglobin concentration (Nagel *et al.*, 1981). Such haemoglobinopathy is very common in South-eastern Asia (Chotivanich *et al.*, 2002). HbE is found in the eastern half of the Indian sub-continent and throughout Southeast Asia, where, in some areas, carrier rates may exceed 60% of the population (Ohashi *et al.*, 2004). Homozygotes generally have symptoms of anaemia. It has been observed that erythrocytes from HbE-heterozygous individuals are relatively resistant to invasion by *P. falciparum* and presumable that HbE protects against severe malaria (Chotivanich *et al.*, 2002; Kwiatkowski, 2005).

The thalassaemias are the most common haemoglobin variant with high prevalence and distribution in malaria endemic areas and provide one of the most compelling evidence of genetic factors controlling disease susceptibility in humans (Haldane, 1949; Allison, 1954; Weatherall and Clegg, 2001; Verra *et al.*, 2007). The thalassaemias comprise a group of clinical disorders that result from defective production of α- or β-globin chains, which arise from deletions or other disruptions of the globin gene clusters on chromosomes 11 and 16 (Allen *et al.*, 1997; Vento *et al.*, 2006). The α-globin is produced by two identical (linked) HBA genes referred to as HBA1 and HBA2, with the genotype (αα/αα). Homozygous thalassaemia occurs when both genes are deleted, causing α^{o}-thalassaemia which results in severe disease or death, whereas heterozygotes only have mild anaemia (Kwiatkowski, 2005). However, if one of the pair of genes (HBA1 or HBA2) is deleted or inactivated such that some α-globin synthesis is possible (α^{+}-thalassaemia), the homozygotes are only mildly anaemic with hypochromic erythrocytes, whereas the heterozygotes are clinically normal. The distribution of α^{+}-thalassaemia appears to be highly correlated with

malaria endemicity (Flint *et al.*, 1998) and it is suggested to confer protection against severe malaria (Mockenhaupt *et al.*, 2004b; Williams *et al.*, 2005b).

2.8.2 *Variants of erythrocyte enzyme*

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide (Cappellini and Fiorelli, 2008). The geographical distribution of G6PD deficiency is consistent with evolutionary selection by malaria (Ganczakowski *et al.*, 1995; Sarkar *et al.*, 2010; Millimono *et al.*, 2012). G6PD is a cytoplasmic enzyme that catalyses the first step in the hexose monophosphate pathway leading to synthesis of pentose phosphate (Ruwende and Hill, 1998). It also catalyses conversion of nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form (NADPH), thus providing reducing power to all cells and protecting erythrocytes from oxidative damage (Frank, 2005). NADPH enables cells to counterbalance oxidative stress that can be triggered by toxic by-products that results from the digestion of haemoglobin by the malaria parasites after erythrocyte invasion (Cappellini and Fiorelli, 2008). G6PD is encoded by a 16.2kb gene located on chromosome Xq28, the telomeric region of the X chromosome's long arm, and hence one of the two G6PD alleles present in females is subject to inactivation. This gene comprises 13 exons (Ruwende and Hill, 1998) and displays different mutations varying among different populations (Mehta *et al.*, 2000).

The *G6PD* gene exhibits remarkable polymorphism in human populations and G6PD is known to have over 400 variants (Beutler, 1994; Frank, 2005; Hue *et al.*, 2009). Many mutations reducing G6PD activity have been associated with protection against malaria (Clark *et al.*, 2009) and might be beneficial by reducing parasite growth rate into the erythrocytes or by causing a more efficient phagocytosis of infected red cells at an early stage of parasite maturation (Friedman, 1978; Cappadoro *et al.*, 1998; Ruwende and Hill, 1998). In clinical field studies, fewer *P. falciparum* parasites were found in children heterozygous for G6PD deficiency than in children with normal copies of the gene (Bienzle *et al.*, 1972). Furthermore, G6PD-deficient children were found to have fewer episodes of life-threatening malaria than did children in whom G6PD activity was normal (Ruwende *et*

al., 1995; Orimadegun and Sodeinde, 2011; Luzzatto, 2012). *In vitro* studies showed that *P. falciparum* is able to invade G6PD-deficient red blood cells but does not mature normally (Usanga and Luzzatto, 1985). Parasitized G6PD-deficient red blood cells are phagocytosed more readily by macrophages than parasitized red cells with normal G6PD activity (Cappadoro *et al.*, 1998; Ayi *et al.*, 2004).

2.8.3 *Immunogenetic variants*

Major Histocompatibility Complex/Human Leukocyte Antigen

The human major histocompatibility complex (MHC) is one of the most important components of the immune system, which is located on chromosome 6 and encodes cell-surface antigen-presenting proteins and many other proteins related to immune system function (Matsumura *et al.*, 1992; Babbitt *et al.*, 2005; Falk *et al.*, 2006a; Ahn *et al.*, 2011). T cells recognize proteolytic fragments of antigens that are presented to them on major histocompatibility complex (MHC) molecules (Doytchinova *et al.*, 2011). MHC class I molecules present primarily products of proteasomal proteolysis to CD8(+) T cells, while MHC class II molecules display mainly degradation products of lysosomes for stimulation of CD4(+) T cells (Munz, 2012). MHC in humans is denoted as human leukocyte antigen (HLA) for its predominant expression in these cells (Costantino *et al.*, 2012).

Major histocompatibility complex is formed by multiple polymorphic genes which have been subdivided into three main groups: Class I (HLA-A, B, C); Class II (DRB1 and DQB1) and Class III genes. These genes encode proteins involved in the recognition of parasite-derived antigens (Radwan *et al.*, 2012). The first two classes are membrane-bound molecules able to activate T-lymphocytes to initiate or enhance an acquired immune response (Dausset, 1981; Bjorkman *et al.*, 1987; Stern and Wiley, 1994; Pieters, 1997), while class III genes encode soluble proteins such as the complement cascade proteins and some cytokines and heat shock proteins. Multiple different human class II loci have been identified. These have been named DP, DM, DO, DN, DQ and DR, and exhibit different degrees of allelic polymorphism (Bell *et al.*, 1986; Bjorkman and Parham, 1990; Mason and Parham, 1998).

Susceptibility to malaria has been shown in people having certain HLA class I and class II alleles (Weatherall *et al.*, 2002; Yamazaki *et al.*, 2011). Studies have suggested that the HLA-DR system could play an important role in protection against malaria (Mehta *et al.*, 2004). In other studies, carriers of the class I HLA antigen HLA-Bw53 (frequently occurring in sub-Saharan Africa) were shown to be protected against severe malaria and associated with 14.7% reduction in cases of severe anaemia as well as 16.1% reduction of cerebral malaria (Hill *et al.*, 1991; Hill *et al.*, 1992). These findings were also supported by data reported by other authors (Wilkinson and Pasvol, 1997; Gilbert *et al.*, 1998). Moreover, the class II HLA haplotype, DRB1*1302-DQB1*0501, is associated with reduced susceptibility to severe malaria in the population of Gambia in western Africa. Furthermore, a recent study in Mali identified the HLA-A*30:01 and A*33:01 as potential susceptibility factors for cerebral malaria (Lyke *et al.*, 2011), thus providing further evidence polymorphism of MHC genes results in altered malaria susceptibility.

Nitric oxide synthase 2 (NOS2) polymorphism

The nitric oxide synthase 2 (NOS2) enzyme produces nitric oxide (NO), free radical mediating several physiological processes in immune-regulation (Hobbs *et al.*, 2002), which is implicated in innate immunity against malaria. It has been shown that GPI moiety induces NOS in macrophages (Tachado *et al.*, 1996) and activates endothelial cells by tyrosine-kinase-mediated signal transduction (Schofield *et al.*, 1996). The NOS2 encoding gene is localized in chromosome 17 and consists of 26 exons, its transcription site beginning in exon 2 and its stop codon in exon 26 (Chartrain *et al.*, 1994; Coia *et al.*, 2005). Even though the molecular mechanisms responsible for protection against severe malaria is yet to be elucidated, some studies have suggested that polymorphism in the NOS2 gene promoter region does increase NO production and could thus be an antimalarial resistance mechanism (Lopez *et al.*, 2010). Perkins *et al*. (1999) measured NO production and NOS (NO synthase) activity in peripheral blood mononuclear cells (PBMCs) from Gabonese children having a history of prior mild malaria (PMM) or prior severe malaria (PSM) caused by *P. falciparum* (Perkins *et al.*, 1999). The study showed that the PMM group had significantly higher levels of NOS activity in isolated PBMCs, high NO production and NOS activity in cultured

PBMCs (Perkins *et al.*, 1999), contrary to earlier study (Kremsner *et al.*, 1996) showing increased NO levels in plasma from patients suffering from severe *P. falciparum* malaria, thus suggesting association of high levels of NO production with the disease's severity. In animal models, NO has been shown to be associated with protection against cerebral malaria through impaired brain microcirculatory haemodynamics and decreased vascular pathology (Cabrales *et al.*, 2011).

Promoter polymorphisms in the NOS2 gene has been suggested to be involved in antimalarial resistance, mainly in children, partly depending on their innate immune response in malaria-endemic areas (Kun *et al.*, 1998). The promoter variants were shown to protect heterozygous carriers against severe malaria (Kun *et al.*, 2001). Carriers of certain promoter variants were also found to have higher basal levels of nitric oxide (Coia *et al.*, 2005). The promoter mutation has likewise been shown to be associated with protection against cerebral malaria and severe malarial anaemia in Tanzania and Kenya (Hobbs *et al.*, 2002). Recently, it was shown that the NOS2 genotype protects against severe malaria by increasing NO production during episodes of uncomplicated malaria (Planche *et al.*, 2010). Moreover, promoter variants in the NOS2 gene have also been found to be associated with severe megaloblastic anaemia in malaria patients (Aggarwal *et al.*, 2011).

However, in another study assessing the relation between NOS2 promoter SNPs and haplotypes with malaria severity in Tanzanian children, no consistent associations were found and it was concluded that the cause-effect relationship might be more complex than previously thought (Levesque *et al.*, 2010).

Tumour necrosis factor-α promoter polymorphism

As described earlier, tumour necrosis factor-α (TNF-α) is a cytokine presenting a broad range of pro-inflammatory activities. It plays an important role in inflammation (Bayley *et al.*, 2004) and acts on several cell systems, regulating the expression of adhesion molecules (Tchinda *et al.*, 2007a). The TNF-α gene is located within the major histocompatibility complex and contains within its promoter region, several single nucleotide polymorphisms at positions -863, -857, -376, -308, -244 and -238 relative to the

transcription start site (Wilson and Duff, 1995). These polymorphisms could modify DNA conformation at the promoter and thereby, the binding of transcription factors that may ultimately alter TNF-α gene expression.

The polymorphism at position -308 has either a G in the TNF-α1 allele or an A in the TNF-α2 allele. TNF-α2 allele (TNFα -380A/A) has been found to be a stronger transcription factor activator than the TNF-α1 allele (TNFα -308G/G) and has been associated with malaria susceptibility (Wilson *et al.*, 1997; Baseggio *et al.*, 2004). Homozygousity at the TNF-308A has been found to be associated with increased risk of cerebral malaria in Gambian and Kenyan children (McGuire *et al.*, 1994) while a second polymorphism (TNF-238A) also showed association to severe anaemia in the Gambian patients (McGuire *et al.*, 1999). The frequency of the heterozygous genotype (TNFα -380A/G) has recently been found to be high in the malaria endemic area of Ivory Coast, suggesting a possible selective advantage of the heterozygote genomes (Santovito *et al.*, 2012). The TNF-376A polymorphism appears to recruit the transcription factor OCT-1 to the promoter, resulting in increasing gene expression in monocytes, and increased susceptibility to infection (Knight *et al.*, 1999). In The Gambia, the TNF-376A alleles were associated with increased susceptibility to developing cerebral malaria (Kwiatkowski, 2000).

IL-18 and IL-18Rα gene and promoter polymorphisms

The human IL-18 gene is located on chromosome 11q22.2-q22.3, and is composed of six exons and five introns (Kruse *et al.*, 2003). Its promoter is relatively unique in that it contains multiple transcription initiation sites. Studies have shown the presence of three SNPs at position -656G/T, -607C/A and -137G/C in the promoter of IL-18 gene (Giedraitis *et al.*, 2001; Sugiura *et al.*, 2002; Higa *et al.*, 2003) although there are very few reports on the -656G/T SNP. The -607C/A and -137G/C are believed to be within a transcription initiation site. These promoter regions are predicted to be the binding sites for cyclic (Adenosine 30, 50-cyclic monophosphate) AMP-responsive element-binding protein [cAMP] (Haus-Seuffert and Meisterernst, 2000) and human histone H4 gene-specific transcription factor-1 [H4TF-1] (Giedraitis *et al.*, 2001), respectively. A change from C to A

at position -607 disrupts a potential cAMP-responsive element-binding protein binding site and a change at position -137 from G to C changes the H4TF-1 nuclear factor binding site, thereby impacting on IL-18 gene activity and potentially also to IFN-γ (Giedraitis *et al.*, 2001).

Polymorphisms in the IL-18 gene promoter have been implicated in various diseases and disorders such as type I diabetes, Alzheimer's disease (Yu *et al.*, 2009), Cancer (Palmieri *et al.*, 2008; Khalili-Azad *et al.*, 2009), HIV (Castelar *et al.*, 2010; Sobti *et al.*, 2011), tuberculosis, asthma (Hollegaard and Bidwell, 2006), hepatitis B virus (Migita *et al.*, 2009), and rheumatoid arthritis (Thompson and Humphries, 2007). Meanwhile, no data was available to my knowledge, on the relationships between IL-18 gene variants as well as IL-18Rα gene variants and malaria disease outcome as at the time this study was designed. However, there is now evidence to show that IL-18 promoter variants are associated with severe malaria anaemia (Anyona *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was conducted in Lafia (Latitude 8° 30′ N and Longitude 8° 31′ E), a city in the middle belt region of Nigeria. Lafia is the capital city of Nasarawa state, which shares boundaries at the North-West with Abuja (the Federal Capital Territory), at the North-East with Plateau State, at the North with Kaduna State, at the South with Benue State, at the South-West with Kogi State and at the South East with Taraba State (Fig. 3.1). Lafia has an estimated population of 134,185 out of the estimated 1,863,275 people in Nasarawa state. Lafia is an agrarian town with a large percentage of its populace engaged in farming and agro-allied activities. The town has rich fertile soils, good for cultivating crops such as cassava, rice, yams, cashew, mangoes, oranges, groundnuts, beans, melon, maize, millet and guinea corn (*http://www.nasarawastate.org/data.htm*. *Accessed 4th January, 2008*).

Lafia lies within the Guinea savanna ecological zone in north-central Nigeria. In this region, malaria transmission is described as stable and intense through most of the year (Bruce-Chwatt 1951; Molineaux and Gramiccia 1980). In Lafia, malaria is endemic and perennial with 7-12 months of transmission season (Craig *et al*., 1999). *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles funestus* are the predominant vectors in this region (Bruce-Chwatt, 1951; Boreham *et al*., 1979; Molineaux and Gramiccia, 1980).

3.2 Study Populations

A total of four hundred and thirty seven unrelated children between 6 months and 8 years of age were enrolled into the study between November, 2005 and December, 2006, after satisfying the inclusion criteria. Children were enrolled into three groups: uncomplicated malaria, severe malaria and asymptomatic infections (control group) based on clinical and laboratory diagnoses. Children who present with clinical symptoms of

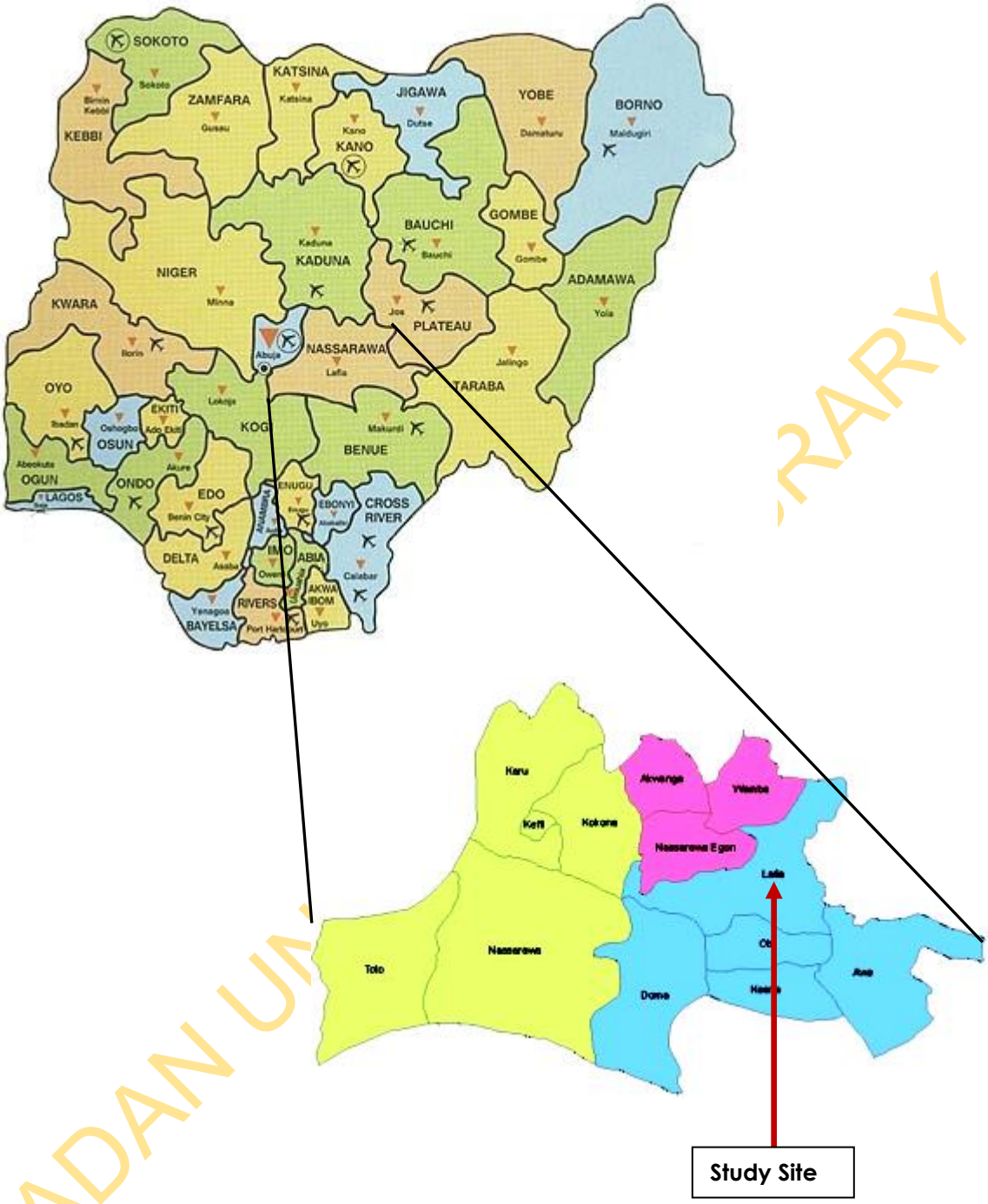


Figure 3.1: Map of Nigeria showing Lafia, Nasarawa State where the study was conducted (*indicated by the bold arrow*)

malaria were enrolled at the Dalhatu Araf Specialist Hospital (DASH) Lafia. The control group comprised of non-symptomatic children infected by *P. falciparum*, residing within 3km radius of the hospital.

Inclusion criteria for uncomplicated malaria was presentation with symptoms compatible with malaria which includes chills, history of fever within the preceding 48 hr or pyrexia at presentation (axillary temperature >37.5^{o}C), and the presence of asexual forms of *P. falciparum* in peripheral blood smears without any indication of severe malaria. Participants enrolled into the severe malaria group satisfied at least one of the following: impaired consciousness assessed using the Blantyre coma scale of ≤2 (unrousable coma), hyperparasitaemia corresponding to >5% infected cells (>250,000/µl), severe anaemia (haematocrit <15%), hypoglycaemia (serum glucose < 2.2 mmol/L or <40 mg/dL) and the presence of asexual forms of *P. falciparum* in peripheral blood smear according to WHO criteria (WHO, 2000). Non-symptomatic infection was defined as the presence of asexual forms of *P. falciparum* in peripheral blood smear with a measured axillary temperature <37.5^{o}C and no history of febrile illness or of antimalarial drug use in the preceding 2 weeks.

3.3 Ethical Considerations

Ethical approval for the study was granted by the Ethics Review Committees of the Nasarawa State Ministry of Health and the Dalhatu Araf Specialist Hospital, Lafia before the commencement of the study (Appendix 6 and 7). The details of the study were explained to parents/guardians of likely participants. Informed consent was then obtained from interested parent or guardian of each child prior to being included in the study.

3.4 Sample collection

Blood (1ml) was collected by venepuncture from each child into EDTA bottles for molecular, parasitological and haematological analysis. Three drops of blood were spotted on labelled filter paper, air dried, individually sealed in plastic bags and stored at room temperature until DNA extraction. Thick and thin blood smears were made for microscopic

examination. The slides were labelled, allowed to dry and stored in a slide rack until microscopy.

3.5 Microscopy

Slides were stained with freshly prepared Giemsa stain. Thick and thin blood films were examined for malaria parasites. Parasitaemia were quantified relative to 250 white blood cells (WBC) on thick films and estimated as parasites per µl assuming a mean WBC of 8,000 per µl of blood. Blood smears were labelled negative if no parasites were seen after examination of 200 oil immersion fields (x1,000) on a thick blood film. Thin films were used for screening and identification of species of malaria parasites other than *P. falciparum* .

3.6 Determination of Blood haemoglobin (PCV)

Blood haemoglobin levels were estimated by haematocrit measurement using the micro-haematocrit centrifuge. Briefly, blood sample in the EDTA bottle was gently mixed and a plain capillary tube was used to draw blood by capillarity to about 70% of the length of the tube. Excess blood was wiped off from the tip of the tube and the ends of the tube were sealed with plasticine. The tube was then placed in a microhaematocrit centrifuge and spun at 3,000rpm for 5 minutes. A microhaematocrit reader (Hawksley) was afterwards used to measure the packed cell volume. Centrifugation step was performed at room temperature.

3.7 Determination of Haemoglobin genotypes

Haemoglobin genotypes were determined by electrophoresis of blood lysate on cellulose acetate paper. Briefly, 200µl of blood was taken from the EDTA bottle and transferred to a 2 ml microfuge tube. Water, about 4 times the blood volume, was added to the blood and allowed to stand for 20 minutes to facilitate cell lysis. Meanwhile, the electrophoresis chamber was half-filled with Tris-EDTA/Borate buffer at pH 8.6 and chromatography papers (used as wicks) placed over the Perspex^{TM} chamber shoulder. One

end of the chromatography paper on the chamber shoulder was totally immersed in the cathodic compartment, while another end at the opposite side of the chamber shoulder was immersed in the anodic compartment of the buffer solution.

The cellulose acetate membrane was slowly impregnated with the buffer solution for 10 minutes prior to the run. The haemolysate was mixed and small volume of each diluted sample was transferred onto a tile. The buffer impregnated membrane was then blotted and an applicator used to transfer the haemolysate sample of test subjects and known standards (Hb A, S, C) onto the cellulose acetate membrane. The membrane was then positioned at the chamber shoulder with the chromatography paper. Current was supplied at 200-240 volts and allowed to run for 20 minutes or until adequate separation was obtained. Reading was made immediately after separation. The haemoglobin genotype of each sample was determined using the bands of the standards as references.

3.8 DNA Extraction

DNA was extracted from the dried blood spots on filter paper using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and stored at -20^{o}C until further analysis. Briefly, 3-5 pieces of approximately 3x5mm blood spot from the filter paper were cut-out using razor blades or disposable surgical blades, one for each sample (to avoid contamination). The cut-out parts were transferred into a 1.5 ml microcentrifuge tube and 180 µl of Buffer ATL added to it to enable cell lysis. This was incubated at 85°C for 10 min and briefly centrifuged to remove drops from inside the lid. For deproteination, 20 µl of proteinase K stock solution was added to the sample, mixed by vortexing, and incubated at 56°C for 1 hr. 200 µl of Buffer AL was afterwards added to the sample, mixed thoroughly by vortexing, and incubated at 70°C for 10 min. 200 µl of absolute ethanol (98-100%) was added to the sample, mixed thoroughly by vortexing and briefly centrifuged to remove drops from inside the lid. The mixture was then carefully applied to a QIAamp® Mini spin column and centrifuged at 8000 rpm for 1 min. The QIAamp® Mini spin column was then placed in a clean 2 ml collection tube and the filtrate discarded. 500 µl of Buffer AW1 was carefully added to the QIAamp® Mini spin column and centrifuged at 8000 rpm for 1 min. This step was repeated using Buffer AW2 and

centrifuged at full speed 14,000 rpm for 4 min. The QIAamp® Mini spin column was afterwards placed in a clean 1.5 ml microcentrifuge tube while the collection tube containing the filtrate was discarded. To elute DNA, 150µl of distilled water was carefully added to the QIAamp® Mini spin column, incubated at room temperature for 1 min, and then centrifuged at 8000 rpm for 1 min. All centrifugation steps were performed at room temperature.

DNA amplifications were performed using a BIOMETRA TB1 thermal cycler (Biotron, Göttingen Germany).

3.9 Design and Synthesis of Oligonucleotide Primers

With the exception of primers used for the genus and species PCR, Oligonucleotide primers were specifically designed for all PCR and sequencing reactions in this study. Primers were designed with the aid of two software programs that are freely available on the Internet, for use to the scientific community: Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) and ExonPrimer (http://ihg.gsf.de /ihg/ExonPrimer.html). With respect to the host cytokine gene study, gene sequences and mRNA sequences for the primer design were taken from both the Entrez Gene cytogenetic band and the Ensembl gene cytogenetic band. Sequences for the *P. falciparum* MSP-2 study were taken from the Entrez PubMed and PlasmoDB databases. Following design of primers, Oligonucleotide sequences were reconfirmed on the cytogenetic bands and sent for synthesis at Operon Biotechnologies GmbH, Cologne, Germany. Primers were shipped salt-free in dried state (lyophilized). Upon arrival, primers were spun at 1000 rpm for 5 min, resuspended in sterile TE buffer at pH 7.0, vortexed thoroughly and stored at -20^{o}C until use.

3.10 PCR Determination of *Plasmodium spp*

In addition to microscopy, the PCR technique was used for the identification of the *Plasmodium* species because of its high sensitivity and specificity. The genus and species-specific PCR-based assay used in this study was designed to amplify portions of the sequence coding for the small subunit ribosomal RNA (SSUrRNA) and has the added advantage of being able to detect all the four major species of human *Plasmodium*.

3.10.1 *SSUrRNA gene PCR*

2.0µl of DNA template were amplified in a final volume of 25µl containing 2.5µl x10 reaction buffer, 100µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.5pM of each primer (PLU5 / PLU6 for the primary reaction and FAL1 / FAL2; MAL1 / MAL2; OVA1 / OVA2 in the nested reaction for *P. falciparum, P. malariae and P. ovale* respectively) and 0.75 units of *Taq* DNA polymerase (Qiagen, Hilden, Germany). Primer sequences (Appendix 1) are based on the *SSUrRNA* sequences described by Snounou *et al*. (1993). The PCR programme used was: denaturation at 95 ^{o}C for 5 min followed by 25 cycles (30 cycles in nested) of 1 min at 94 ^{o}C, 2 min at 60 ^{o}C and 2 min at 72 ^{o}C and a final extension period of 5 min at 72 ^{o}C.

3.10.2 *Gel electrophoresis*

PCR products were subjected to electrophoresis on 1.5% and 2% agarose gels for *P. malariae/P. ovale and P. falciparum* respectively, and visualized by transillumination with ultraviolet light after staining with SYBR® Green. Fragment sizes were calculated relative to the standard size marker (100bp DNA ladder) using the BioDocAnalyze (Biometra, Göttingen, Germany) computer software package.

3.11 Molecular Characterization of *P. falciparum* MSP-2

The genetic diversity of *P. falciparum* infections was investigated by genotyping one of the major vaccine candidate antigens, the merozoite surface protein 2 (MSP-2), suggested to be the most informative single marker for assessing the mean number of parasite genotypes per infected individual, which is also known as the multiplicity of infection (Snounou *et al*., 1999; Farnert, 2001).

3.11.1 *MSP-2 gene PCR*

The primary reaction was designed to amplify the entire coding region of the MSA-2 gene using the MSA2-1 and MSA2-4 primer pairs (Appendix 2). The reaction mixture was

performed in a final volume of 25µl containing 5.0µl of DNA template, 2.5µl x10 reaction buffer, 100µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.75 units of *Taq* DNA polymerase and 0.5pM of each primer. The PCR programme was: denaturation at 94 ^{o}C for 5 min followed by 35 cycles of 10 sec at 94 ^{o}C, 30 sec at 57 ^{o}C and 40 sec at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C. This was followed by two sets of nested reactions using allelic family-specific primers (FC27 and 3D7). A third reaction was performed to amplify the entire central variable region with the primer pairs MSA2-2 and MSA2-3 (Appendix 2) in order to detect sequences that may not be allelic family-specific. All nested reactions were performed in a final volume of 25µl containing 2.0µl of PCR product from the primary reaction, 2.5µl x10 reaction buffer, 100µM of each dNTPs, 0.5pM of each primer and 0.75 units of *Taq* DNA polymerase. The PCR programme was: denaturation at 94 ^{o}C for 5 sec followed by 30 cycles of 10 sec at 94 ^{o}C, 30 sec at 57 ^{o}C and 40 sec at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C.

3.11.2 *Gel electrophoresis*

PCR products were subjected to electrophoresis on 2% agarose gels and visualized by transillumination with ultraviolet light after staining with SYBR® Green. Fragment sizes were calculated relative to the standard size marker (100bp DNA ladder) using the BioDocAnalyze (Biometra, Göttingen, Germany) computer software package.

3.11.3 *DNA purification*

PCR products that showed single band on gel electrophoresis were purified on a HiBind® DNA spin column using the E.Z.N.A.® Cycle Pure Kit according to the manufacturer's instructions. Briefly, 20µl of XP1 Buffer was added to the PCR product and mixed thoroughly by vortexing. The sample was then applied to a HiBind® DNA spin column which had been inserted into a 2 ml collection tube and centrifuged at 13,000 rpm for 1 min. 700µl of SPW Buffer diluted with absolute ethanol was added to the sample and centrifuged at 13,000 rpm for 1 min. The liquid in the collection tube was discarded and

another 700µl of SPW Buffer was added to wash the sample again. The liquid in the collection tube was again discarded and the empty column was centrifuged at 13,000 rpm for 1 min to dry the column matrix. The HiBind® DNA spin column was afterward placed in a clean 1.5 ml Eppendorf tube. 30µl of sterile deionised water was then added to the centre of the column matrix and incubated at room temperature for 1 min. This was then centrifuged at 13,000 rpm for 1 min to elute DNA. All centrifugation steps were performed at room temperature.

3.11.4 *MSP-2 sequencing PCR*

The sequence reactions were performed using the Big Dye terminator reaction mix (PE Biosystems, Weiterstadt, Germany). Each amplicon was sequenced in the forward direction. The forward primers of the family-specific primers (FC 27-1, 3D7-1) were used for the sequencing reactions of the PCR products earlier generated. Sequencing PCR was performed in a reaction volume of 10µl with 30ng of template DNA, 4µl sequencing buffer, 1µl BigDye™ Terminator reaction mix and 0.5µl of primer. Cycling conditions (Biometra, Göttingen, Germany) were as follows: 25 cycles of 96 ^{o}C for 30 seconds, 50 ^{o}C for 15 seconds and 60 ^{o}C for 4 minutes.

3.11.5 *Purification of sequencing products*

Sequencing products were cleaned by centrifugation through Sephadex™ G-50 DNA Grade Fine (GE Healthcare Bio-Sciences, Sweden) in 96-well Millipore plates. One gram of Sephadex was added to 15ml of sterile water and mixed gently for 30 minutes. 200µl of the mix was then added to each well of a 96-well Millipore purification column plate, spun at 3,000rpm for 2 minutes at 4^{o}C. Samples were then added to the gelatinized Sephadex for purification. A sterile microtitre plate was attached to the bottom of the purification column plate to collect the purified product. It was then spun at 3,000rpm for 2 minutes at 4^{o}C.

3.11.6 *MSP-2 Gene sequencing*

Samples were separated by capillary electrophoresis in ABI PRISM® 3100 sequencers using the default sequencing protocol in 50-cm capillary arrays.

3.11.7 *MSP-2 Gene sequence analysis*

The sequences were analyzed and compared with published sequences on the Entrez PubMed and PlasmoDB, using the BioEdit sequence alignment software (Hall, 1999) [http://www.mbio.ncsu.edu/BioEdit/BioEdit.html]. Sequences were cleaned up by manual adjustment. Multiple Alignments were carried out with the Clustal W programme (Thompson *et al*., 1994).

3.12 Determination of Genetic Polymorphisms in IL-18 Gene Promoter Region

The promoter region of IL-18 gene evaluated in this study is shown in Figure 3.2.

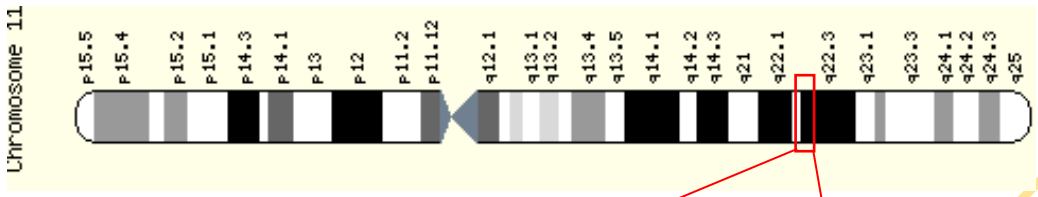
3.12.1 *IL-18 PCR*

PCR products were generated in a reaction volume of 25µl containing 2.0µl of DNA extract, 2.5µl x10 reaction buffer, 100µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.25 units of *Taq* DNA polymerase and 0.5pM each of the forward (IL-18Pro -F) and reverse (IL-18Pro -R) primers (Appendix 3). The cycling conditions consisted of 94 ^{o}C for 3 min followed by 35 cycles of 40 sec at 94 ^{o}C, 40 sec at 62 ^{o}C and 1 minute at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C.

3.12.2 *Gel electrophoresis*

PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized by transillumination with ultraviolet light after staining with SYBR® Green. Fragment sizes were calculated relative to the standard size marker (100bp DNA ladder) using the BioDocAnalyze (Biometra, Göttingen, Germany) computer software package.

(a)



(b)

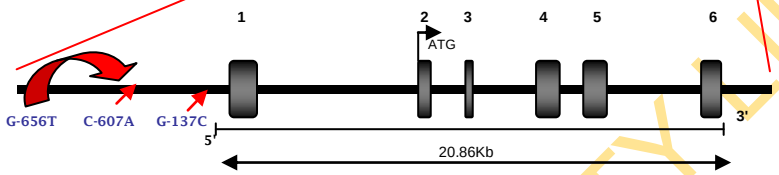


Figure 3.2: IL-18 gene showing location of promoter polymorphisms.

(a) The human IL-18 gene is located on chromosome 11q22.2-q22.3 on the *Entrez Gene Cytogenetic band*.

(b) The gene is composed of six exons and five introns. Promoter polymorphisms in the 5' region are numbered upstream of the transcription start site.

3.12.3 *DNA Purification*

DNA purification was done as described earlier in section 3.11.3.

3.12.4 *IL-18 Sequencing PCR*

The sequence reactions were performed using the Big Dye terminator reaction mix (PE Biosystems, Weiterstadt, Germany). Each amplicon was sequenced in the forward direction. A separate primer (IL-18Pro -Fb) was designed and used as internal primer for the PCR products of the promoter region (Appendix 3). Sequencing PCR was performed in a reaction volume of 10µl with 40ng of template DNA, 4µl sequencing buffer, 1µl BigDye™ Terminator reaction mix and 0.5µl of primer. Cycling conditions (Biometra, Göttingen, Germany) were as follows: 25 cycles of 96 ^{o}C for 30 seconds, 50 ^{o}C for 15 seconds and 60 ^{o}C for 4 minutes.

3.12.5 *Purification of sequencing products*

Purification of sequencing product was done as described earlier in section 3.11.5.

3.12.6 *IL-18 gene sequencing*

Samples were separated by capillary electrophoresis in ABI PRISM® 3100 sequencers using the default sequencing protocol in 50-cm capillary arrays.

3.12.7 *IL-18 gene sequence analysis*

The sequences were analyzed and compared with published sequences on the Entrez PubMed using the BioEdit sequence alignment software (Hall, 1999) [http://www.mbio.ncsu.edu/BioEdit/BioEdit.html]. Sequences were cleaned up by manual adjustment. Multiple Alignments were carried out with the Clustal W programme (Thompson *et al*., 1994).

3.13 Determination of Genetic Polymorphisms in IL-18 Receptor-α Gene

The entire gene for IL-18 Receptor-α as well as the promoter region was screened in this study (Figure 3.3). Molecular screening of the regulatory and coding regions of the gene were performed on 40 samples, drawn at random, from the non-symptomatic control group in order to determine common polymorphisms in this gene that could be of importance in this region. Subsequently, the promoter region, Exon 1 and Exon 7 were screened for single nucleotide polymorphisms in all the study participants.

3.13.1 *IL-18 Receptor-α promoter and Exon 1 PCR*

PCR products were generated in a reaction volume of 25µl containing 3.0µl of DNA template, 4.0µl x10 reaction buffer, 200µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.75 units of *Taq* DNA polymerase and 0.4pM each of the forward and reverse primers (Appendix 4). The cycling conditions consisted of 94 ^{o}C for 3 min followed by 35 cycles of 40 sec at 94 ^{o}C, 40 sec at 62 ^{o}C and 1 minute at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C.

3.13.2 *IL-18 Receptor-α Exons 2, 3, 6 and 11 PCR*

PCR products were generated in a reaction volume of 25µl containing 2.0µl of DNA template, 4.0µl x10 reaction buffer, 200µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.75 units of *Taq* DNA polymerase and 0.25pM each of the forward and reverse primers (Appendix 4). The cycling conditions consisted of 94 ^{o}C for 3 min followed by 35 cycles of 40 sec at 94 ^{o}C, 40 sec at 63 ^{o}C and 1 minute at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C.

3.13.3 *IL-18 Receptor-α Exons 4, 5, 7, 8, 9 and 10 PCR*

PCR products were generated in a reaction volume of 25µl containing 2.0µl of DNA template, 2.5µl x10 reaction buffer, 200µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.75 units of *Taq* DNA polymerase and 0.25pM each of the forward and reverse primers (Appendix 4). The cycling conditions consisted of 94 ^{o}C for 3 min followed by 35

cycles of 40 sec at 94 ^{o}C, 40 sec at 64 ^{o}C and 1 minute at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C.

3.13.4 *Gel electrophoresis*

PCR products were subjected to electrophoresis on 1.5-2% agarose gels and visualized by transillumination with ultraviolet light after staining with SYBR® Green. Fragment sizes were calculated relative to the standard size marker (100bp DNA ladder) using the BioDocAnalyze (Biometra, Göttingen, Germany) computer software package.

3.13.5 *DNA Purification*

DNA purification was done as described earlier in section 3.11.3.

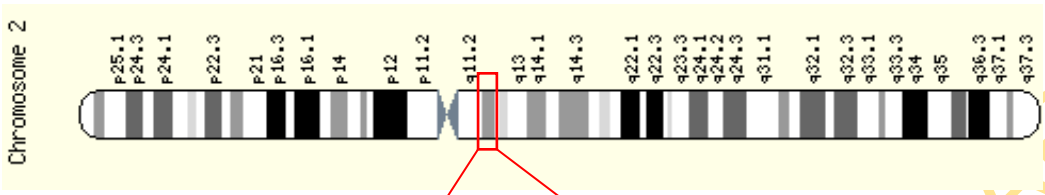
3.13.6 *IL-18 Receptor-α sequencing PCR*

The sequence reactions were performed using the Big Dye terminator reaction mix (PE Biosystems, Weiterstadt, Germany). Each amplicon was sequenced in the forward direction except for Exon 1 (which also included the promoter region), which was also sequenced in the reverse direction. Separate primers (IL18R1 Ex1-Fb, IL18R1 Ex1-Rb and IL18R1 Ex1-Rc) were designed and used as internal primer for the PCR product of Exon 1 and the promoter region (Appendix 4). Same primers used in the primary reaction were used for sequencing Exons 2-11. Sequencing PCR was performed in a reaction volume of 10µl with 40ng of template DNA, 4µl sequencing buffer, 1µl BigDye™ Terminator reaction mix and 0.5µl of primer. Cycling conditions (Biometra, Göttingen, Germany) were as follows: 25 cycles of 96 ^{o}C for 30 seconds, 50 ^{o}C for 15 seconds and 60 ^{o}C for 4 minutes.

3.13.7 *Purification of sequencing products*

Purification of sequencing product was done as described earlier in section 3.11.5.

(a)



(b)

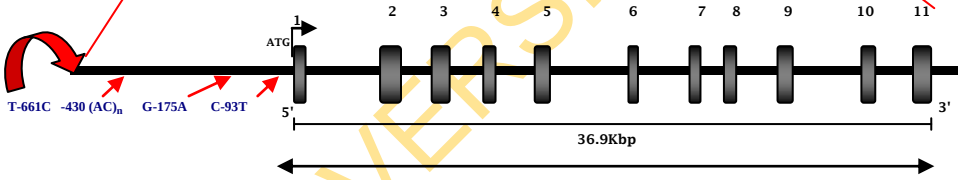


Figure 3.3: IL-18Rα gene showing location of promoter polymorphisms.

(a) The human IL-18Rα gene is located on chromosome 2q12 on the *Entrez Gene Cytogenetic band*.
(b) The gene is composed of 11 exons and 10 introns. Promoter polymorphisms in the 5' region are numbered upstream of the transcription start site.

3.13.8 *IL-18 Receptor-α gene sequencing*

Samples were separated by capillary electrophoresis in ABI PRISM® 3100 sequencers using the default sequencing protocol in 50-cm capillary arrays.

3.13.9 *IL-18 Receptor-α gene sequence analysis*

The sequences were analyzed and compared with published sequences on the Entrez PubMed using the BioEdit sequence alignment software (Hall, 1999) [http://www.mbio. ncsu.edu/BioEdit/BioEdit.html]. Sequences were cleaned up by manual adjustment. Multiple Alignments were carried out with the Clustal W programme (Thompson *et al*., 1994).

3.14 Determination of Genetic Polymorphisms in TNF-α Gene Promoter Region

The promoter region of the TNF-α gene evaluated in this study is shown on Figure 3.4.

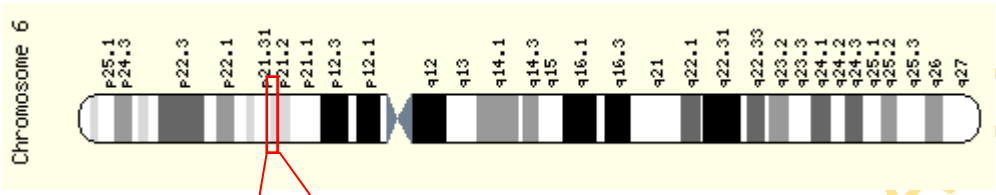
3.14.1 *TNF-α PCR*

PCR products were generated in a reaction volume of 25µl containing 2.0µl of DNA template, 2.5µl x10 reaction buffer, 100µM of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.25 units of *Taq* DNA polymerase and 0.5pM each of the forward (TNFPro-F) and reverse (TNFPro-R) primers (Appendix 5). The cycling conditions consisted of 94^{o}C for 3 min followed by 35 cycles of 40 sec at 94 ^{o}C, 40 sec at 62 ^{o}C and 1 minute at 72 ^{o}C and a final extension period of 3 min at 72 ^{o}C.

3.14.2 *Gel electrophoresis*

PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized by transillumination with ultraviolet light after staining with SYBR® Green. Fragment sizes were calculated relative to the standard size marker (100bp DNA ladder) using the BioDocAnalyze (Biometra, Göttingen, Germany) computer software package.

(a)



(b)

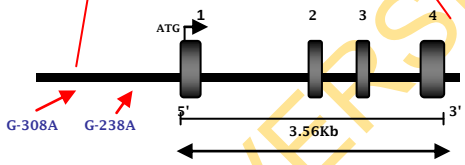


Figure 3.4: TNF-α gene showing location of promoter polymorphisms.

(a) The human TNF-α gene is located on chromosome 6p21.3 on the *Entrez Gene Cytogenetic band*.
(b) The gene is composed of four exons and three introns. Promoter polymorphisms in the 5' region are numbered upstream of the transcription start site.

3.14.3 *DNA purification*

DNA purification was done as described earlier in section 3.11.3.

3.14.4 *TNF-α sequencing PCR*

The sequence reactions were performed using the Big Dye terminator reaction mix (PE Biosystems, Weiterstadt, Germany). Each amplicon was sequenced in the forward direction. A separate primer (TNFPro-Fb) was designed and used as internal primer for the PCR products of the promoter region (Appendix 5). Sequencing PCR was performed in a reaction volume of 10µl with 40ng of template DNA, 4µl sequencing buffer, 1µl BigDye™ Terminator reaction mix and 0.5µl of primer. Cycling conditions (Biometra, Göttingen, Germany) were as follows: 25 cycles of 96 ^{o}C for 30 seconds, 50^{o}C for 15 seconds and 60 ^{o}C for 4 minutes.

3.14.5 *Purification of sequencing products*

Purification of sequencing product was done as described earlier in section 3.11.5.

3.14.6 *TNF-α gene sequencing*

Samples were separated by capillary electrophoresis in ABI PRISM® 3100 sequencers using the default sequencing protocol in 50-cm capillary arrays.

3.14.7 *Sequence analysis*

The sequences were analyzed and compared with published sequences on the Entrez PubMed using the BioEdit sequence alignment software (Hall, 1999) [http://www.mbio.ncsu.edu/BioEdit/BioEdit.html]. Sequences were cleaned up by manual adjustment. Multiple Alignments were carried out with the Clustal W programme (Thompson *et al*., 1994).

3.15 Statistical analysis

Data were entered into Microsoft® Excel, 2002 (Microsoft Corporation). A test for deviation from Hardy-Weinberg equilibrium was performed on all groups. Data were analyzed using JMP Statistical Discovery Software version 5.0.1.2 (SAS Institute Inc.) and Stata version 9.2 (StataCorp, College Station, Texas). Gene frequencies were obtained by simple gene counting and tested with chi-square test for comparing observed and expected values. For each locus, the chi-square statistic (χ^2) and Fisher's exact test (for small cell size, $n< 5$) were used to compare allele and genotype frequencies between cases and control. Odds ratios (OR) and 95% confidence intervals (CI) were estimated by logistic regression (Moore *et al*., 2002). Differences in clinical features between asymptomatic and uncomplicated malaria group as well as between asymptomatic and severe malaria group was compared using Student's *t*-test. The level of statistical significance was set at P=0.05.

CHAPTER FOUR

RESULTS

To determine host cytokine gene polymorphisms and parasite genetic factors that could contribute to disease outcome, a total of 437 children with microscopically confirmed *P. falciparum* infection were enrolled into this study. The baseline characteristics of the study population are shown in Table 4.1. On average, children in the severe malaria group were the youngest with mean age of 32.6 (±18.4) months. The ratios of male to female were not statistically different in all groups. The levels of parasitaemia were significantly higher in both the uncomplicated and severe malaria groups compared with the asymptomatic group (P<0.001). Children were of comparable height across the group, but there were decreases in mean weight with increasing disease severity (Table 4.1).

The distribution of haemoglobin genotype in the three study groups are shown in Table 4.2. The AS haemoglobin genotypes were distributed according to the Hardy-Weinberg equilibrium, although individuals who were known to carry the SS genotype were excluded from the study even if they had *Plasmodium falciparum* infection. A higher percentage of children (13.7%) who carried the sickle cell trait (AS) were in the asymptomatic control group compared to 10% and 6.9% in the uncomplicated and severe malaria groups respectively (Table 4.2). There was a statistically significant difference in the distribution of the AS genotype between the asymptomatic group and the severe malaria group (13.7% vs 6.9%; $P < 0.05$).

4.1 Microscopy and PCR-based diagnosis of mixed infections.

All the children who participated in this study tested positive for *P. falciparum* species by microscopy. Results of the PCR-based determination of the SSUrRNA gene showed that out of the 437 malaria positive participants, seven were mixed *Plasmodium* species infection (Figure 4.1-4.3; Table 4.3). Five of the seven cases were mixed infections

Table 4.1. Baseline characteristics of study participants (n = 437)

	AS (n=161)	**UM** (n=160)	***P*-value** (AS vs UM)	**SM** (n=116)	***P*-value** (AS vs SM)
Mean age (months)	36.3 (±16.9)*	38.5 (±18.8)	*P*=0.344	32.6(±18.4)	*P*=0.072
Sex (male/female)	83/78	77/83		60/56	
Mean temperature (°C)	36.6 (±0.6)	37.8 (±1.0)	*P*=0.001	38.1 (±1.0)	*P*=0.001
Mean haematocrit (%)	33 (±5.2)	30 (±5.7)	*P*=0.011	20 (±6.6)	*P*=0.001
Mean weight (Kg)	13.0 (±4.4)	12.8 (±4.5)	*P*=0.584	11.9 (±3.3)	*P*=0.018
Mean height (cm)	88.5 (±12.4)	90.2 (±16.4)	*P*=0.351	88.9(±15.6)	*P*=0.647
Parasite density (µl)	893§ (118-5720)	5,403 (120-160,000)	*P*=0.001	64,751 (640-1,825,500)	*P*=0.001

AS= Asymptomatic Group (Control)
UM= Uncomplicated Malaria Group
SM= Severe Malaria Group
*±Standard deviation in parentheses
§Geometric mean (range in parentheses)

Table 4.2: Distribution of haemoglobin (Hb) genotype among the study participants

Hb Genotype	Asymptomatic Group n= 161(%)	Uncomplicated Malaria n= 160(%)	Severe Malaria n= 116(%)
AA	130(80.75)	130(81.25)	102(87.93)
AC	8(4.97)	14(8.75)	6(5.17)
AS	22(13.66)	16(10.00)	8(6.90)
SC	1(0.62)	0(0)	0(0)
CC	0(0)	0(0)	0(0)
SS	0(0)	0(0)	0(0)

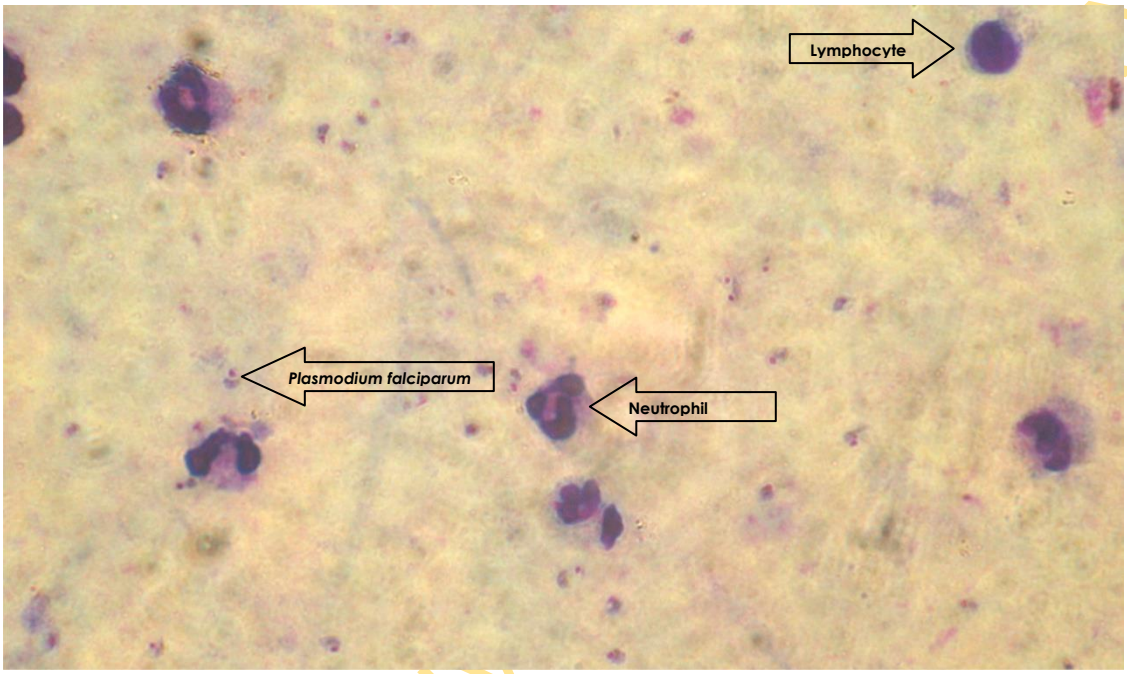


Figure 4.1: *Plasmodium falciparum* parasites on thick blood film of a study participant. Arrows show representative of cells present on the film at mg= x1000.

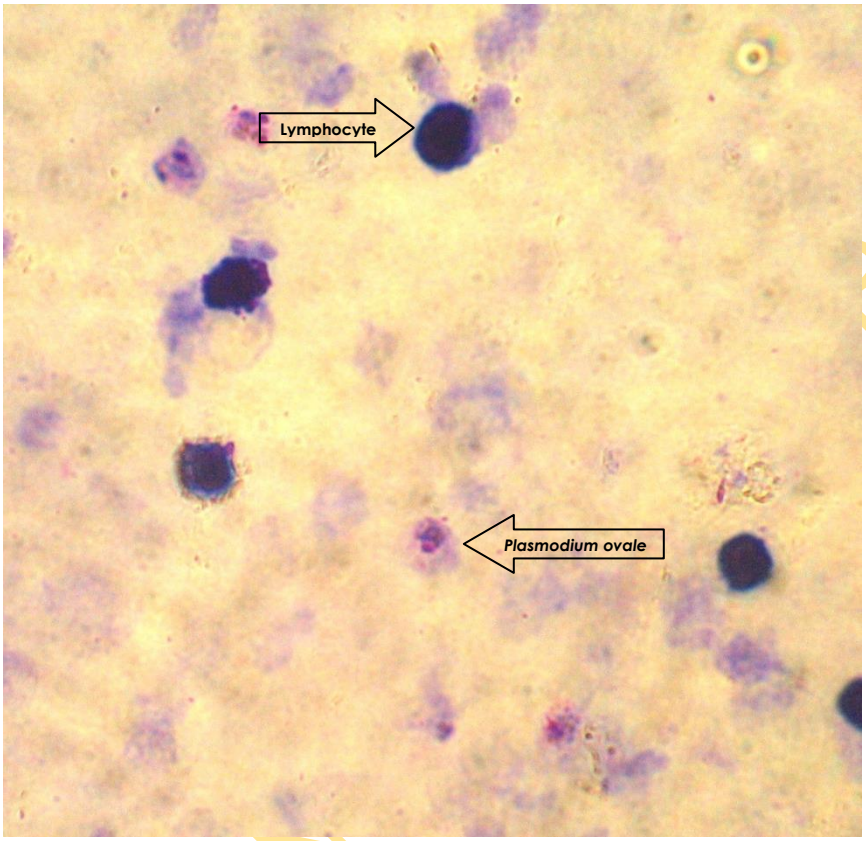


Figure 4.2: *Plasmodium ovale* parasites on thick blood film of a study participant. Arrows show representative of cells present on the film at mg= x1000.

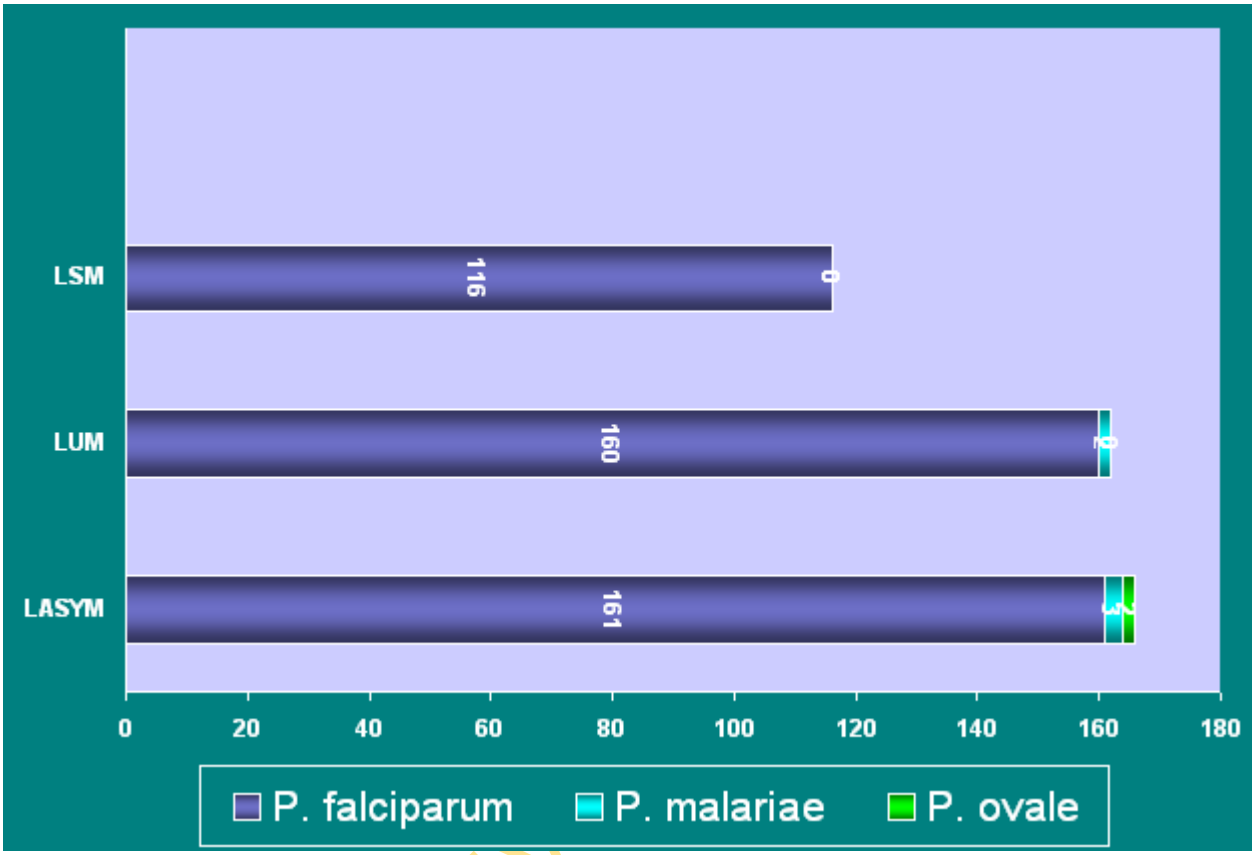


Figure 4.3: Distribution of *Plasmodium* species among the study groups

LASYM= Asymptomatic Group (Control)
LUM= Uncomplicated Malaria
LSM= Severe Malaria

Table 4.3: Frequency of *Plasmodium* species infections in the study population

Infection category	Number of Individuals infected
(Single) Mono-species infection	
Pf	430
Pm	0
Po	0
(Mixed) Multi-species infection	
Pf + Pm	5
Pf + Po	2
Pm + Po	0
Pf + Pm + Po	0

Pf= *Plasmodium falciparum*

Pm= *Plasmodium malariae*

Po= *Plasmodium ovale*

of *P. falciparum* and *P. malariae*, while the other two were mixed infections of *P. falciparum* and *P. ovale*. Infection with *P. vivax* was not determined in this study.

Participants with *P. malariae* infection included three from the asymptomatic group and two from the uncomplicated malaria group. The two participants that had *P. ovale* infection were from the asymptomatic group. No relationship was found between multi-species infection and disease outcome. In this study, multi-species infections were not found in the severe malaria group.

4.2 MSP-2 genotyping of parasite population

Isolates from all the 437 participants were genotyped for allelic polymorphisms at the MSP-2 locus. A total of 32, 35 and 28 distinct MSP-2 alleles were found in the asymptomatic group, uncomplicated malaria and severe malaria groups respectively (Table 4.4). The distribution of MSP-2 alleles in the different groups showed high genetic diversity of isolates in this study population (Table 4.4, Figure 4.4). The allelic frequency of FC27 type was higher in the asymptomatic group (59%) compared with the severe malaria group (43%), while 3D7 alleles, in the asymptomatic group had a lower frequency (37%) compared to the severe malaria group (54%). A significant difference was found in the distribution of FC27 alleles and 3D7 alleles between the asymptomatic controls and uncomplicated malaria ($P < 0.05$; $P < 0.05$) as well as between the asymptomatic controls and severe malaria group ($P < 0.05$; $P < 0.01$).

Clonality of infection (the distinct number of clones detected per child) ranged from 1 to 4 amongst the three study groups (Figure 4.5). Majority of children from the three clinical groups had one or two clonal infections (Figure 4.5). Most of the children with four distinguishable clonal infections were from the asymptomatic group. Polyclonality (the presence of more than one distinct clone) was found to be higher in the asymptomatic (61%) and uncomplicated malaria (60%) groups than in the severe malaria group where most infections were monoclonal and only 34% of the study participants had polyclonal infections.

The multiplicity of infection (MOI), defined as the average number of distinct genotype per infected subject, was calculated as the mean number of fragments (on gel electrophoresis) per infected subject in each group. The multiplicity of infection in children with asymptomatic infection, uncomplicated malaria and severe malaria groups were 2.1 (95% CI 1.9-2.3), 2.0 (95% CI 1.8-2.4), and 1.3 (95% CI 1.2-1.6) respectively. There was no statistically significant difference in the MOI between the asymptomatic malaria group and the uncomplicated malaria group ($P > 0.05$). However, the multiplicity of infection between the asymptomatic malaria group or uncomplicated malaria group and the severe malaria group were statistically significant ($P < 0.001$).

Table 4.4: Genetic diversity of isolates using the *P. falciparum* MSP-2 as molecular marker

	MSP-2 FC 27 Allelic family		MSP-2 3D7 Allelic family		Total No. of alleles in each group	Non-specific alleles
	No. of alleles	% frequency	No. of alleles	% frequency		
Asymptomatic group	19	59	12	38	32	1
Uncomplicated malaria	16	46	18	51	35	1
Severe malaria	12	43	15	54	28	1

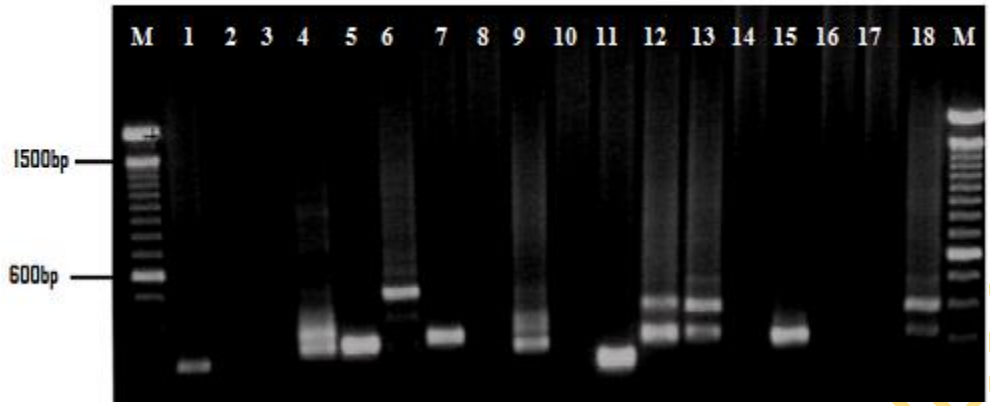


Figure. 4.4: Electrophoretic separation of PCR products showing intra-allelic diversity in one of the MSP-2 allele type, as reflected both in the number of distinct clones and in length polymorphisms.

Lane M: 100-bp ladder. Lanes 1-18: parasite DNA from infected individuals showing mono- (lanes 5,6,7,11 and 5) and multiple infections (lanes 4,9,12,13 and 18) as well as variation in number or repeat units.

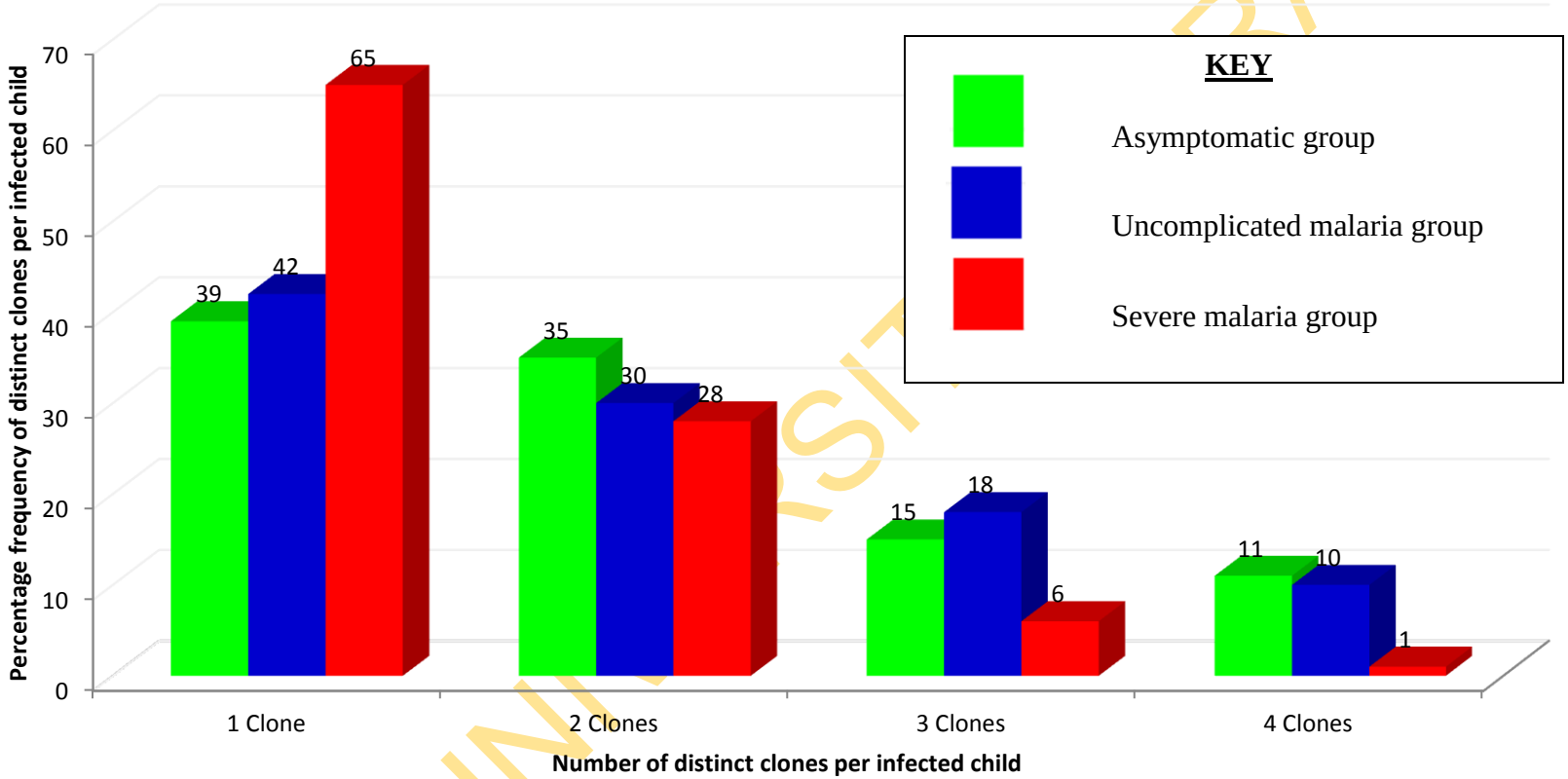


Figure 4.5: Clonality of *P. falciparum* infection amongst the three study groups.

4.3 MSP-2 sequence diversity

In order to explore the sequence diversity at the *MSP-2* locus in isolates from this population, a total of 97 monoclonal infections that showed single fragment on gel electrophoresis were investigated across the three study groups. Sequences for *MSP-2* gene were analyzed for FC27 allelic family from 46 isolates of *P. falciparum* which do not show multiclonal infection by gel electrophoresis of PCR product, as well as for 3D7 allelic family from 51 isolates of *P. falciparum* that do not show multiclonal infection by gel electrophoresis of PCR product. Sequence data from multiple sequence alignment using ClustalW programme showed high sequence diversity in the *MSP-2* gene characterized by single nucleotide substitutions, insertions and deletions (Fig. 4.6). In addition, there were both synonymous and non-synonymous single nucleotide polymorphisms and proliferations of repeat units along the DNA sequence at the *MSP-2* locus (Fig. 4.7).

4.3.1 *MSP-2 sequence diversity in the FC27 allelic type*

The FC27 allelic type in this population has two distinct subtypes and a hybrid sharing amino acid sequences from the two subtypes. Subtype 1 (S1) consists of a 32-amino acid motif (96-bp unit) SQSSTNSASTSTTNNGESQNTTPTAADTPTAT which is also present in subtype 2, followed by a 12-amino acid motif (36-bp unit) ESNSPSPPITTT that is present in 3-7 copies (Fig. 4.8). Subtype 2 (S2) consists of a slightly different 32-amino acid (96-bp) repeat unit SQRSTNSASTSTTNNGESQTTTPTAADTIASG that may be present in 2-3 copies followed by one copy of a 32-mer sequence that is identical to the one in subtype 1 (Fig. 4.8). The hybrid of these subtypes has one copy of the 32 amino acid repeat unit seen in subtype 2 followed by a copy of the 32-mer motif present in both subtypes 1 and 2 and then one copy of the 12 amino acid repeat unit that is present in subtype 1 (Fig. 4.8).

Analysis and comparison of the FC27 allelic type sequence at the nucleotide level showed several non-synonymous polymorphisms (Figure 4.7, 4.9). A G→A SNP at the first amino acid position of the 12-mer repeat motif seen in subtype 1 results in the synthesis of

Figure 4.6: Central region of the polymorphic *MSP-2* gene showing nucleotide sequence variations resulting from base substitution, deletions and differences in the copy number of repeat units.

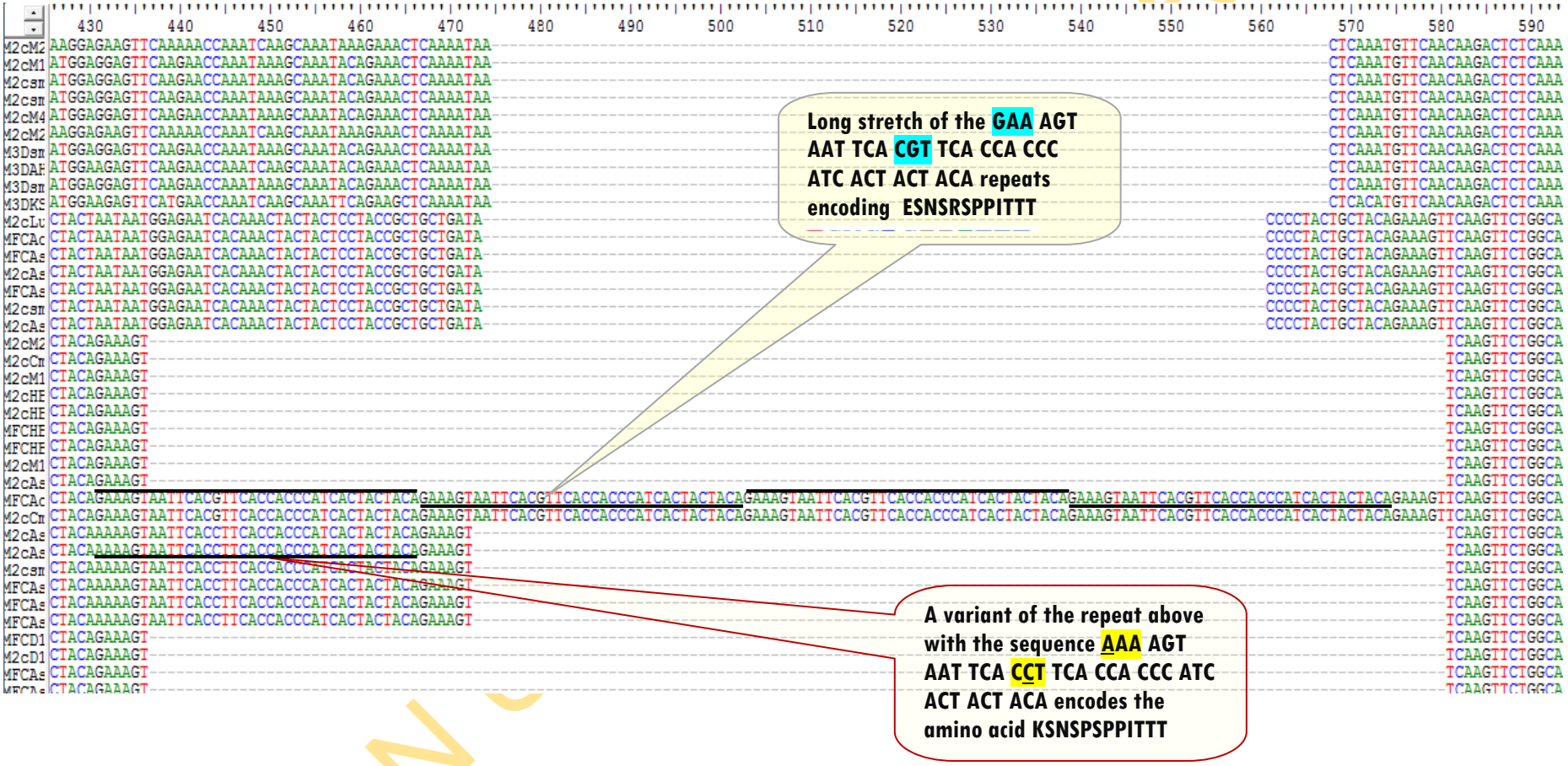


Figure 4.7: Portion of *MSP-2* gene sequence showing nucleotide sequence diversity, non-synonymous polymorphisms and variation in the number of repeat unit.

Lysine (K) instead of Glutamic acid (E). Another non-synonymous polymorphism (C→G) at the 5th amino acid position of the same 12-amino acid repeat unit leads to the synthesis of Arginine (R) instead of Proline (P). Likewise in subtype 2, a non-synonymous SNP (G→A) was observed at the last amino acid position of the 32-amino acid repeat motif resulting in the synthesis of Arginine (A) instead of Glycine (G) at that codon (Fig. 4.8). Furthermore, the 32-amino acid motif present in both subtype 1 and 2 are not identical. In subtype 1, the codon AGT encoded Serine (S) at the 3rd amino acid position of this motif while in subtype 2, a variant of the codon (AGG) encoded Arginine (R) at the same position of the motif. The C-terminal portion (block 4) as well as the N-terminal region of the MSP-2 FC27 allelic types in the isolates were however found to be relatively conserved (Fig. 4.9). The sequence variants of FC 27 were distributed across the three study variants and no particular sequence was found to be unique to a disease type.

4.3.2 *MSP-2 sequence diversity in the 3D7 allelic type*

Analysis of the 3D7-type allele showed extensive intra-allelic sequence diversity resulting from amino acid substitutions especially non-synonymous substitutions, deletions and repeat sequences .There were three subtypes of repetitive domains. These include the GSA-rich repeat unit, a TPA repeat motif and a poly-Threonine (poly-T) stretch (Fig. 4.10). At the GSA domain, the common haplotypes include **GGSA** which was present in 22% of the isolates that were sequenced, **GGAS** which was present in 11% of the isolates, and a **GASGSA** which was present in 61% of the isolates sequenced. The **GGSA** if present was found in 1 to 4 copies, while the **GGAS** was usually found in 4 copies in the isolates for which it was present. The **GASGSA** was found in only one copy (Fig. 4.10).

The 3-amino acid repeat motif having the sequence **TPA** was present in 1 to 7 copies in the parasite isolates having the 3D7 allelic type, except in one clone in which two non-synonymous polymorphisms led to the synthesis of PPV instead of TPA (Fig. 4.10). Four distinct haplotypes were found immediately preceding the **TPA** repeat domain. These haplotypes which consist of a 4-amino acid sequence include: GGSS (present in 33% of the sequenced isolates),

GGSR (present in 5% of the isolates sequenced), KSPS (present in 11% of the isolates) and RSPS (present in 50% of the isolates). Interestingly, the RSPS haplotypes was found to be significantly associated with multiple **TPA** repeat units (r = 0.89, P<0.001).

The poly-Threonine repeat unit consists of 8 to 14 copies of Threonine. There were however, single copies of Lysine (K) at the 6th amino acid position along this stretch in some isolates. It is not clear whether this is as a result of a non-synonymous mutation or an insertion. Furthermore, the amino acid domain immediately after the poly-Threonine was relatively conserved but this was soon punctuated by a polymorphic domain with lots of non-synonymous SNPs (Figure 4.10, 4.11). Meanwhile, no association was found between a particular sequence variant of 3D7 and a specific disease category as the sequence variants were distributed across the three study groups.

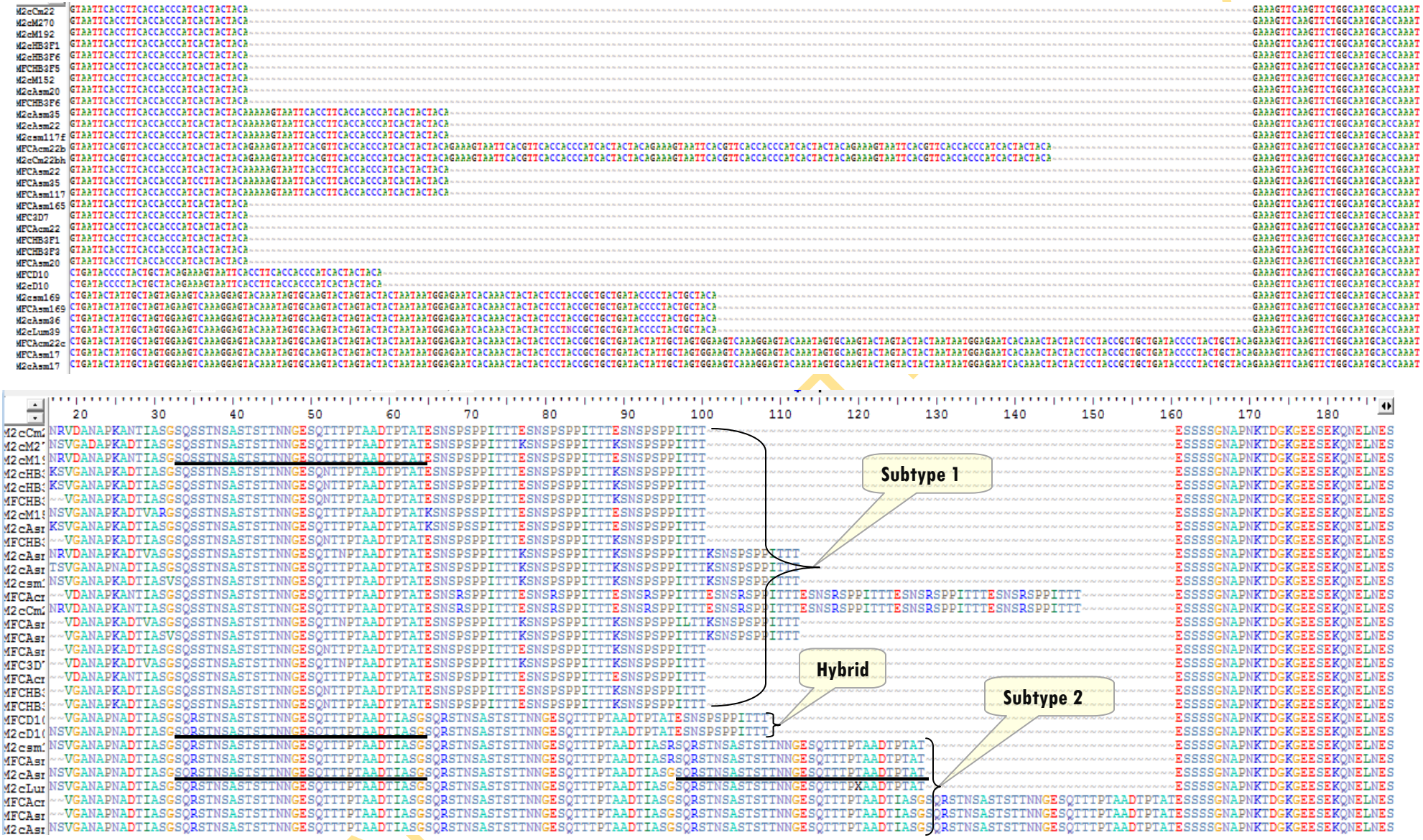


Figure 4.8: Multiple sequence alignment of the FC27 allelic type of the *MSP-2* gene showing intra-allelic variations along the central repeat region. Upper panel shows DNA sequence while the lower panel shows amino acid sequence.

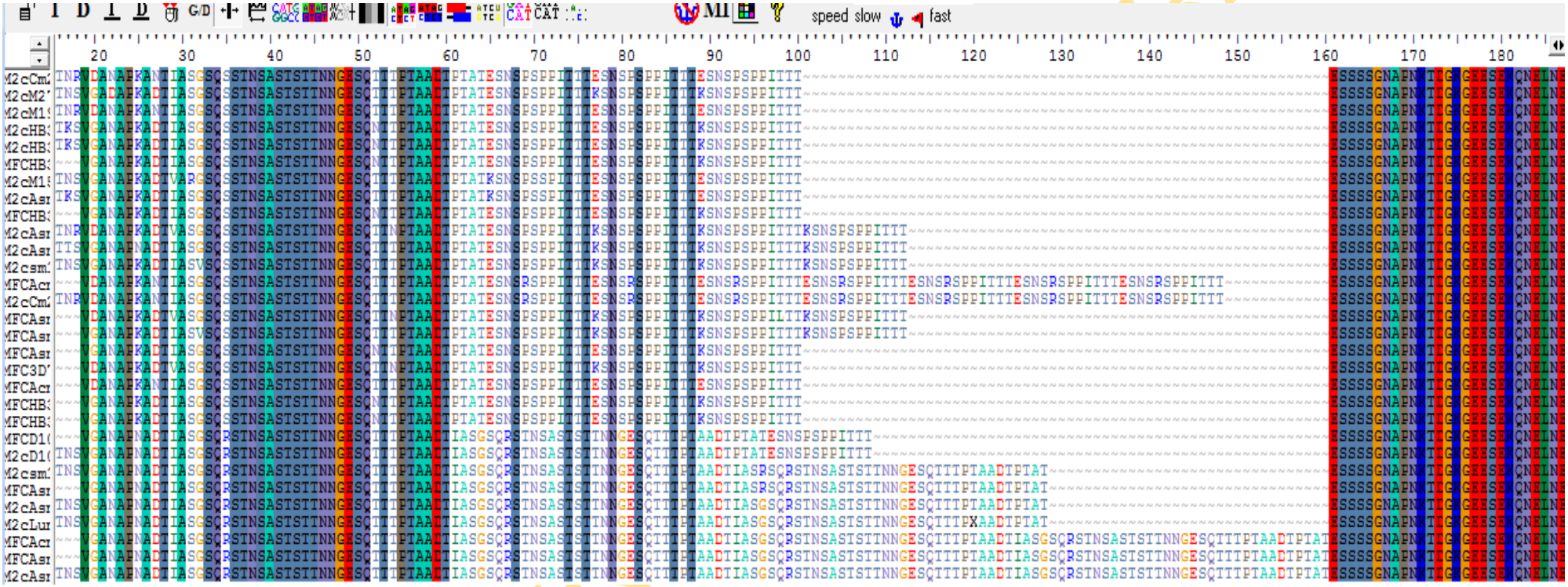


Figure 4.9: Central region of *MSP-2* gene showing conserved amino acid sequences at the C-terminal region of the FC27-type allele.

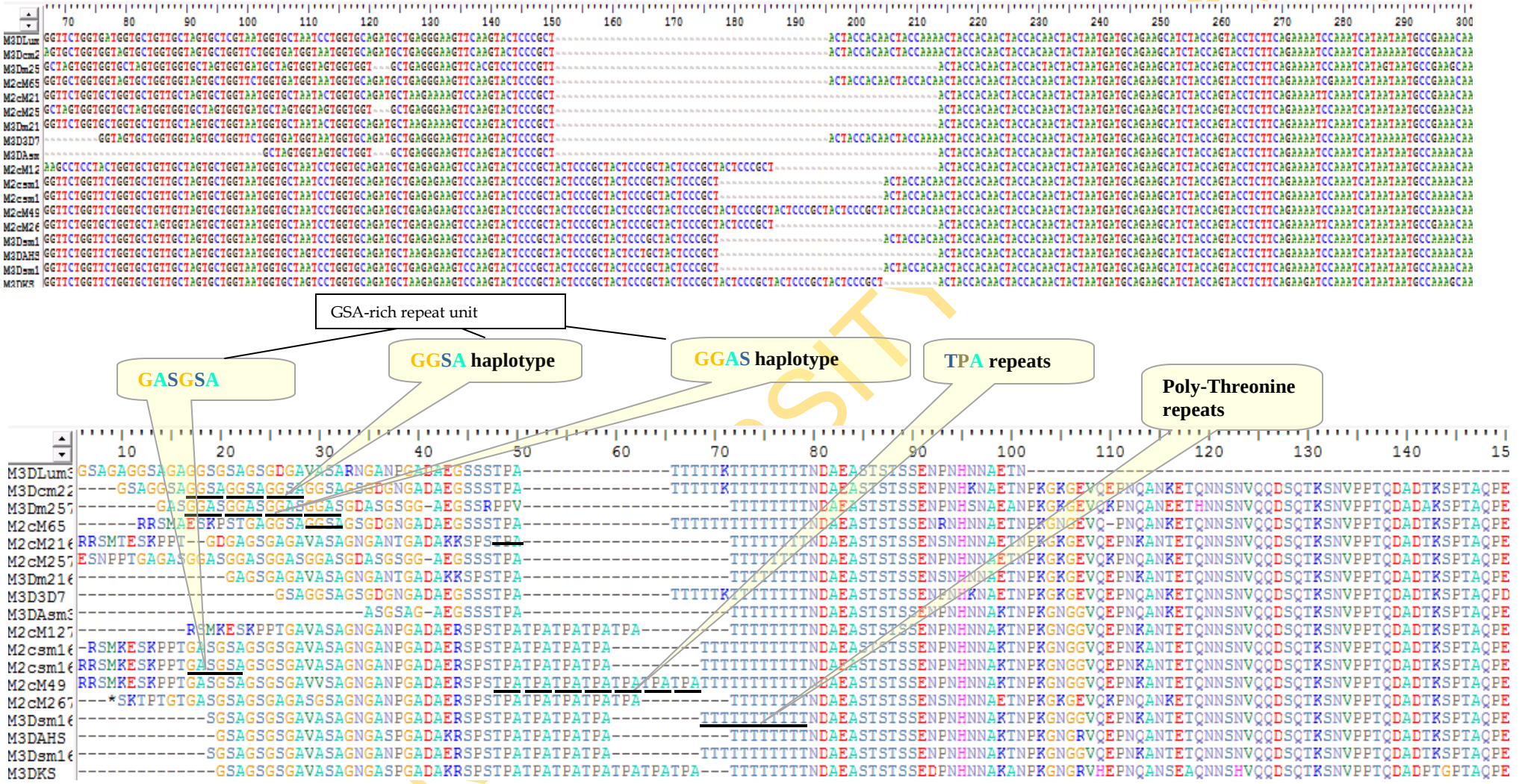


Figure 4.10: Multiple sequence alignment of the 3D7 allelic type of the *MSP-2* gene showing intra-allelic sequence diversity along the central repeat region. Upper panel shows DNA sequence while the lower panel shows amino acid sequence.

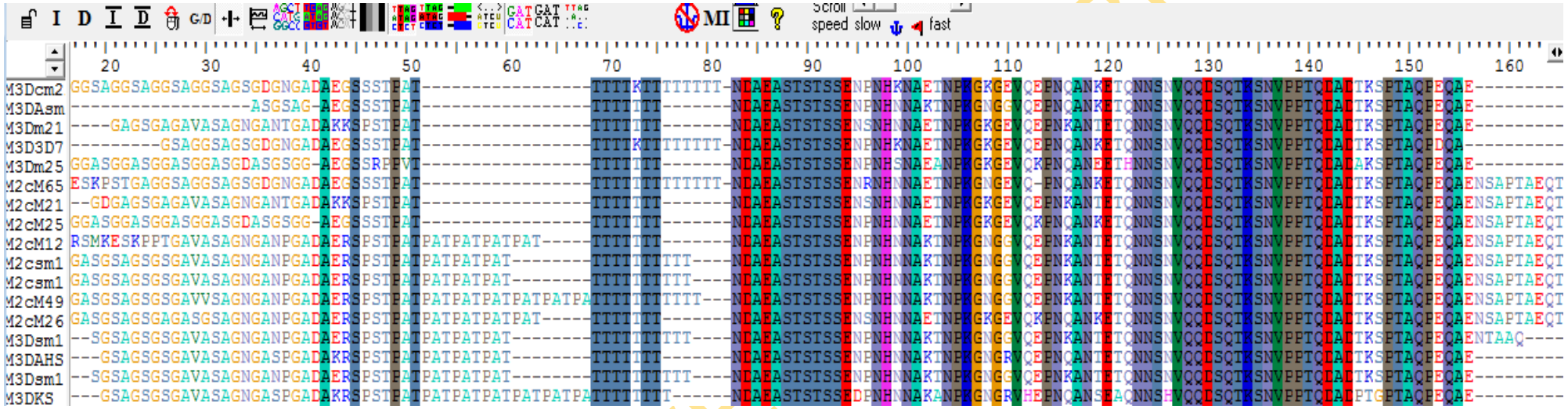


Figure 4.11: Central region of *MSP-2* gene showing partially conserved sequences at the C-terminal region of the 3D7-type allele.

4.4 IL-18 gene Promoter Polymorphisms

Genetic polymorphisms at the promoter region of IL-18 gene were determined for three previously described positions: -656 G/T, -607 C/A and -137 G/C, relative to the transcription start site as shown in Figure 3.2. All the three single nucleotide polymorphisms (SNPs) were present in the study population. The distribution of the allele and genotype frequencies for the three SNPs in the three study groups are shown in Table 4.5.

Chi-square test for compatibility with the Hardy-Weinberg equilibrium showed that the genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (Table 4.5). The -656 G/T and -607 C/A loci were found to be in complete linkage disequilibrium in the three study groups (r^2=1). The frequency of the -656 G/T and -607 C/A alleles were not significantly different between the asymptomatic control and the severe malaria group (P>0.05). However, the genotype frequency of -607AA was significantly higher in the AS group compared to SM cases (P<0.05; OR=1.68, 95% CI=0.95-3.04). Likewise, there was a statistically significant difference in the distribution of genotype frequencies at the -137 G/C loci between the asymptomatic control and severe malaria group (P=0.044), with a higher prevalence of the CC genotype in the severe malaria group (Table 4.5).

The electrophoretic pattern of the 1370bp PCR product for the IL-18 promoter is shown in Figure 4.12. The electropherogram showing the genotype variants of the three SNPs are presented in Figures 4.13 - 4.15.

4.5 IL-18Rα gene and Promoter Polymorphisms

Forty samples were selected at random from the asymptomatic group for DNA sequencing in order to determine polymorphic sites at the promoter region as well as the coding region of the IL-18Rα gene. The results showed the presence of conserved as well as polymorphic sequences across the length of the IL-18Rα gene, including sequences at the promoter region. Only variants in the promoter and coding regions of IL-18Rα gene, having

frequencies greater than 1 percent (0.01) were considered and genotyped in the three study groups.

Three single nucleotide polymorphisms were identified at the promoter region: -661 T/C, -175 G/A and -93 C/T relative to the transcription start site as shown in Figure 4.13. A synonymous polymorphism, which is a silent mutation that does not result in amino acid substitution in the transcribed cDNA, was found in Exon 1 of the IL-18Rα gene. This SNP consists of a C-to-G transition (CCC→CCG) that both results in the synthesis of Proline at the 7^{th} amino acid position [Pro^{7}→Pro^{7} (P7P)].

Exons 2 to 11 were also investigated for sequence variants. Except for Exon 7, all other Exons were highly conserved in the study population. A synonymous SNP or silent mutation was found in Exon 7, which consists of a C-to-T transition (TTC→TTT) that both results in the synthesis of Phenylalanine at the 21^{st} amino acid position [Phe^{21}→Phe^{21} (F21F)].

4.5.1 Distribution of genotype variants in IL-18Rα gene promoter

The allele and genotype frequencies for the -661 T/C, -175 G/A and -93 C/T SNPs are shown in Table 4.6. The distributions of the observed genotypes for the -175 G/A were in conformity with the expected distribution when tested for the Hardy-Weinberg equilibrium. Although the distributions of the -661 T/C and -93 C/T genotypes showed no deviation from the Hardy-Weinberg equilibrium in the asymptomatic and uncomplicated malaria group, the distributions in the severe malaria group displayed a significant departure from the Hardy-Weinberg equilibrium (χ^2= 8.21, P<0.01; χ^2= 5.94, P<0.05) for the -661 T/C and -93 C/T genotypes respectively. Furthermore, there were decreases in the observed genotypes for heterozygotes in the severe malaria group compared to the expected value for both the -661 T/C and -93 C/T polymorphic loci.

Further analysis to compare the distribution of genotype frequencies at the -661 position of the IL-18Rα gene in severe malaria group with the asymptomatic group showed that both the

CC and TT homozygotes were significantly higher in the severe malaria group compared to the asymptomatic group (P<0.05 and P<0.05 respectively). There was however, a significantly lower frequency of heterozygotes (CT) in the severe malaria group compared to the asymptomatic group which suggests a protective role for the IL-18Rα -661CT genotype (OR=2.06, 95% CI: 1.02-4.16, P<0.01). However, allele frequencies were not statistically different among the three study groups (P>0.05).

Similar trend was observed for the distribution of IL-18Rα -93C/T genotype where a significantly higher frequency of both homozygotes (CC and TT) were observed in the severe malaria group compared to the asymptomatic group (P<0.05 and P<0.05 respectively). The frequency of the heterozygotes (CT) was however, significantly lower in the severe malaria group compared to the asymptomatic control group (P=0.032). The electrophoretic separation of the 1409bp PCR products comprising the promoter region and Exon 1 of the IL-18Rα gene is shown in Figures 4.16. Furthermore, the electropherogram showing the genotype variants of the three SNPs are shown in Figures 4.17-4.19.

4.5.2 Distribution of promoter $(AC)_n$ repeats among the study groups

An adenine-cytosine $(AC)_n$ repeat polymorphism was found in the promoter region at the -430 position upstream of the transcription start site of the IL-18Rα gene. The frequency distribution of the $(AC)_n$ repeat among the three study groups are presented in Table 4.5. Chi-square test for conformity with the Hardy-Weinberg equilibrium showed that distributions of the observed genotypes were in conformity with the expected distribution across the three study groups.

The distribution of the number of AC repeats was trimodal, with one peak having seven $(AC)_7$ repeats, the second peak having eight $(AC)_8$ repeats and the third being heterozygous for $(AC)_7$ and $(AC)_8$. The common number of repeats was $(AC)_7$ in all the three groups. However, the distribution of $(AC)_8$ was higher in the asymptomatic group compared to the two clinical

cases though this did not reach a statistically significant level ($P > 0.05$). The electropherogram showing variants of the $(AC)_n$ repeats are shown in Figures 4.20.

Table 4.5 Genotype and allele frequencies of IL-18 -656G/T, -607 C/A, -137G/C

Locus	Asymptomatic Control n= 161(%)	Uncomplicated Malaria n= 160(%)	Severe Malaria n= 116(%)
IL18 -656G/T			
GG	75(46.58)	79(49.37)	63(54.31)
TG	67(41.62)	66(41.25)	44(37.93)
TT	19(11.80)	15(9.38)	9(7.76)
G allele	0.674	0.700	0.733
T allele	0.326	0.300	0.267
*HWE	(χ^2= 0.28, *P*=0.59)	(χ^2= 0.03, *P*=0.85)	(χ^2= 0.11, *P*=0.74)
IL18 -607C/A			
AA	19(11.80)	15(9.38)	9(7.76)
AC	67(41.62)	66(41.25)	44(37.93)
CC	75(46.58)	79(49.37)	63(54.31)
A allele	0.326	0.300	0.267
C allele	0.674	0.700	0.733
*HWE	(χ^2= 0.28, *P*=0.59)	(χ^2= 0.03, *P*=0.85)	(χ^2= 0.11, *P*=0.74)
IL18 -137G/C			
CC	5(3.11)	4(2.50)	6(5.17)
GC	40(24.85)	43(26.88)	31(26.72)
GG	116(72.05)	113(70.63)	79(68.10)
G allele	0.845	0.841	0.815
C allele	0.155	0.159	0.185
*HWE	(χ^2= 0.45, *P*=0.50)	(χ^2= 0.01, *P*=0.97)	(χ^2= 1.54, *P*=0.22)

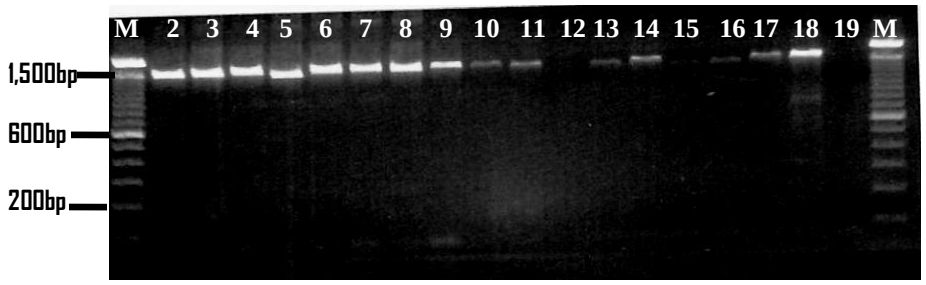
*HWE= Hardy-Weinberg equilibrium

Table 4.6 Genotype and allele frequencies of IL-18Rα -661T/C, -175G/A, -93C/T and $(AC)_n$ repeats.

Locus	Asymptomatic Control n= 161(%)	Uncomplicated Malaria n= 160(%)	Severe Malaria n= 116(%)
IL18Rα -661T/C			
CC	8(4.97)	10(6.25)	14(12.07)
CT	72(44.72)	63(39.38)	33(28.45)
TT	81(50.31)	87(54.38)	69(59.48)
C allele	0.273	0.259	0.263
T allele	0.727	0.741	0.737
*HWE	(χ^2= 2.55, *P*=0.11)	(χ^2= 0.10, *P*=0.75)	(χ^2= 8.21, *P*=0.004)
IL18Rα -175G/A			
AA	2(1.24)	0(0)	0(0)
AG	30(18.64)	20(12.50)	14(12.07)
GG	129(80.12)	140(87.50)	102(87.93)
A allele	0.106	0.063	0.060
G allele	0.894	0.937	0.940
*HWE	(χ^2= 0.03, *P*=0.86)	(χ^2= 0.71, *P*=0.40)	(χ^2= 0.48, *P*=0.49)
IL18Rα -93C/T			
CC	93(57.76)	103(64.38)	78(67.24)
CT	62(38.51)	51(31.88)	29(25.00)
TT	6(3.73)	6(3.75)	9(7.76)
C allele	0.770	0.803	0.797
T allele	0.230	0.197	0.203
*HWE	(χ^2= 1.24, *P*=0.27)	(χ^2= 0.01, *P*=0.92)	(χ^2= 5.94, *P*=0.015)
IL18Rα (AC)n			
$(AC)_7$	77(47.8)	95(59.4)	64(55.2)
$(AC)_{7/8}$	74(46.0)	55(34.4)	44(37.4)
$(AC)_8$	10(6.2)	10(6.3)	8(6.9)
*HWE	(χ^2= 2.012, *P*=0.156)	(χ^2= 0.285, *P*=0.594)	(χ^2= 0.014, *P*=0.907)

*HWE= Hardy-Weinberg equilibrium

(a)



(b)

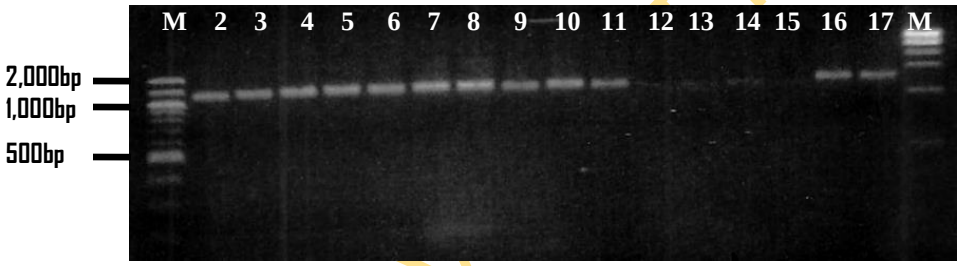


Figure 4.12: Agarose gel electrophoresis of IL18 gene promoter region showing ~1370bp of PCR product.

(a) PCR product before purification; Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants
(b) PCR product after purification for sequencing PCR; Lane M: 150-bp ladder. Lanes 2-17: DNA fragment of study participants.

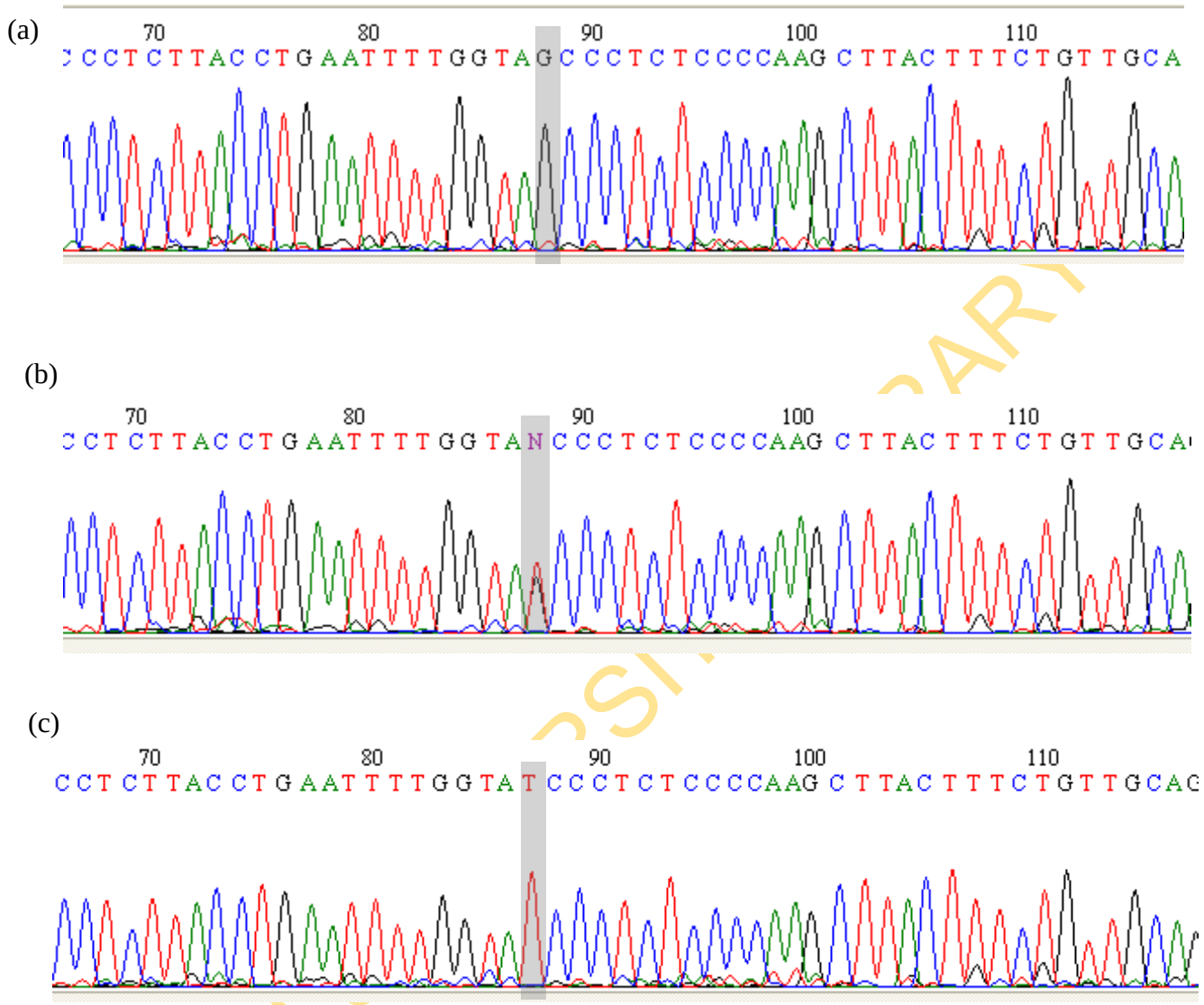


Figure 4.13: DNA sequence electropherogram showing the IL18 -656 G/T promoter polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18 -656 GG (b) Heterozygous IL18 -656 G/T (c) Homozygous IL18 -656 TT. *Sequences were done using the forward primer*.

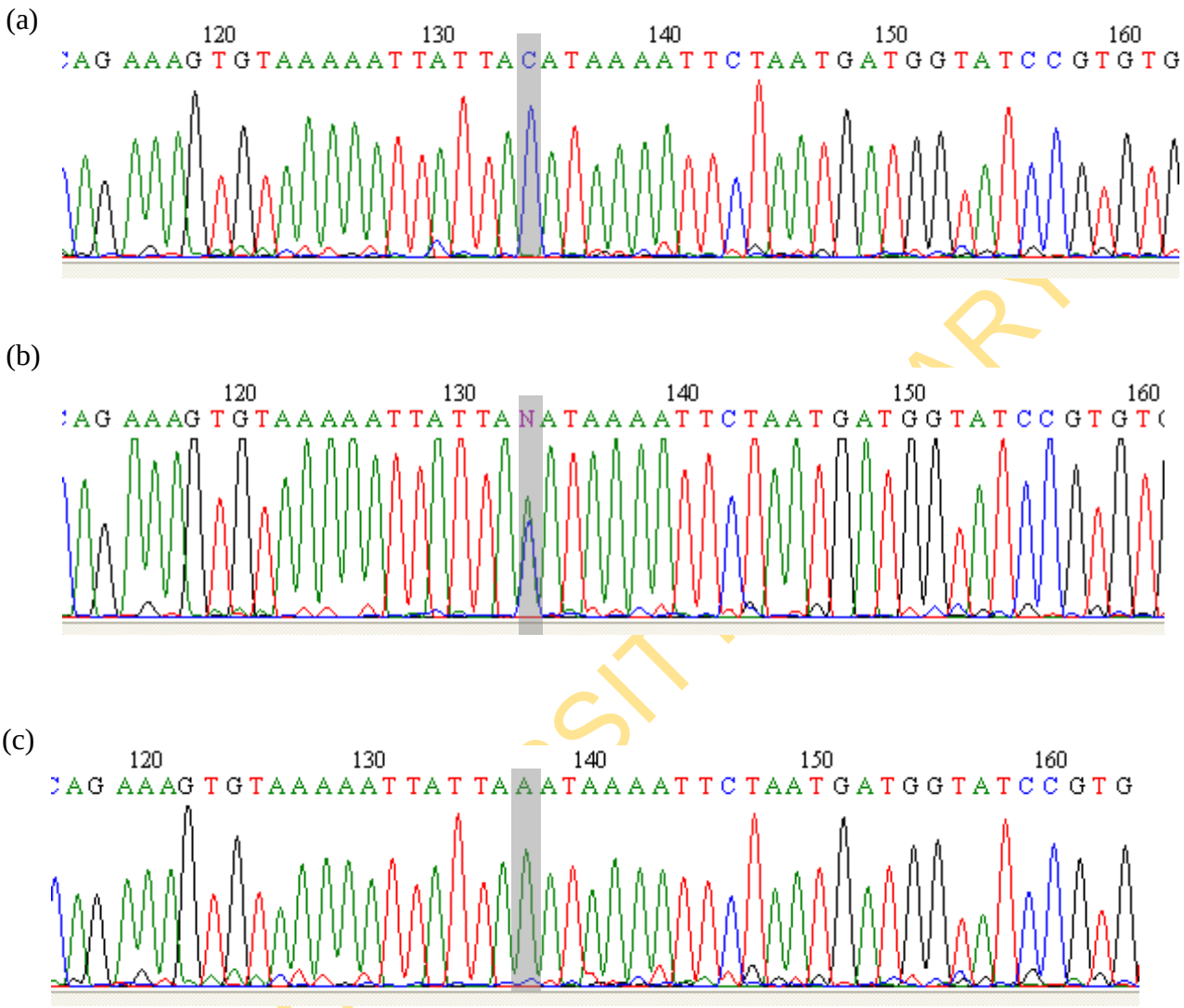
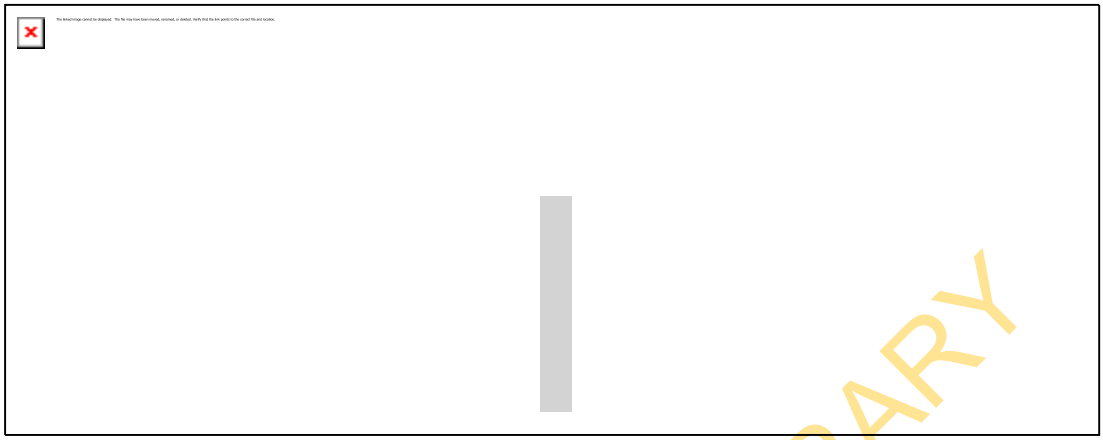


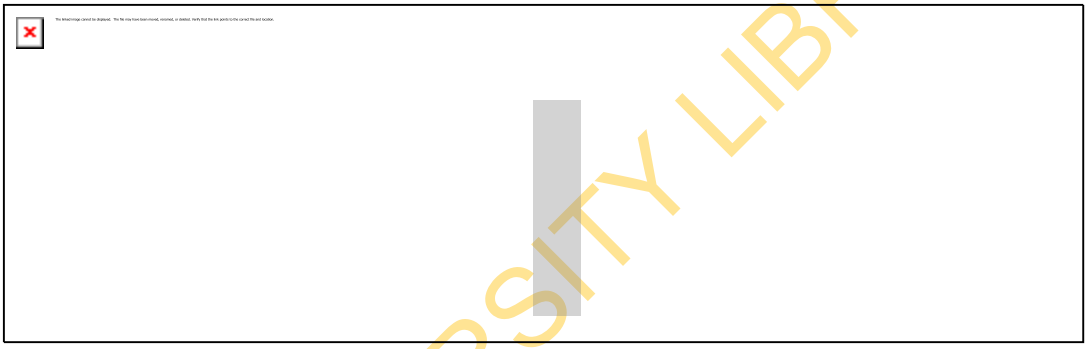
Figure 4.14: DNA sequence electropherogram showing the IL18 -607 C/A promoter polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18 -607 CC (b) Heterozygous IL18 -607 C/A (c) Homozygous IL18 -607 AA. *Sequences were done using the forward primer*.

(a)



(b)



(c)

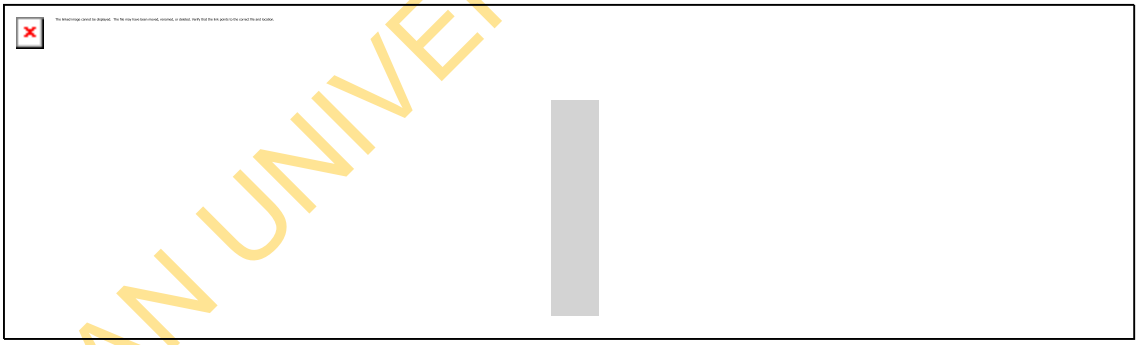
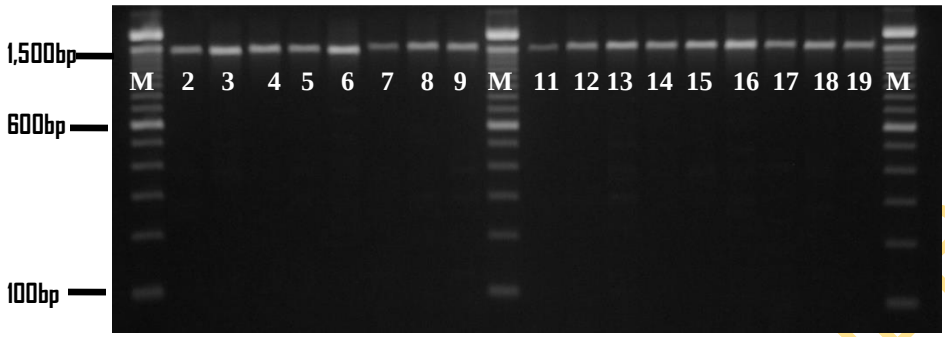


Figure 4.15: DNA sequence electropherogram showing the IL18 -137 G/C promoter polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18 -137 GG (b) Heterozygous IL18 -137 G/C (c) Homozygous IL18 -137 CC. *Sequences were done using the forward primer*.

(a)



(b)

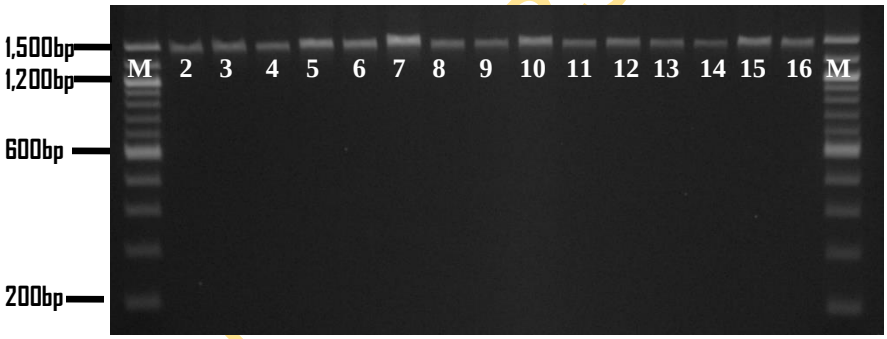
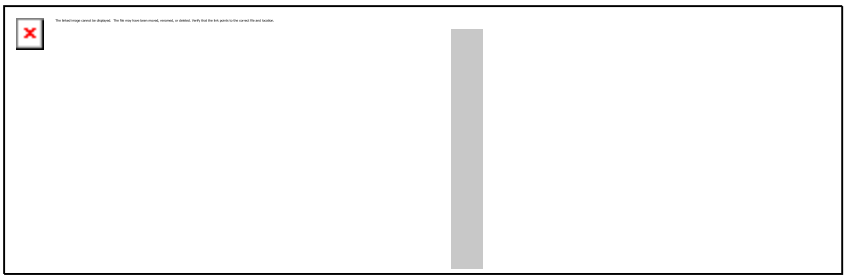


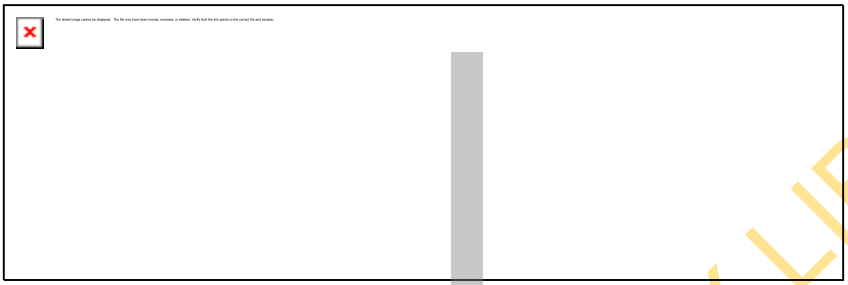
Figure 4.16: Agarose gel electrophoresis of IL18Rα Exon 1 showing ~1409bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

(a)



(b)



(c)

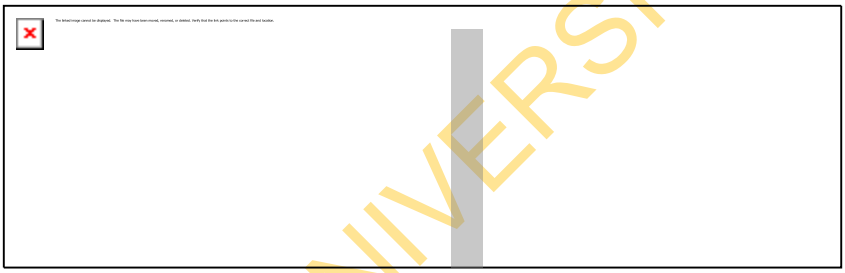
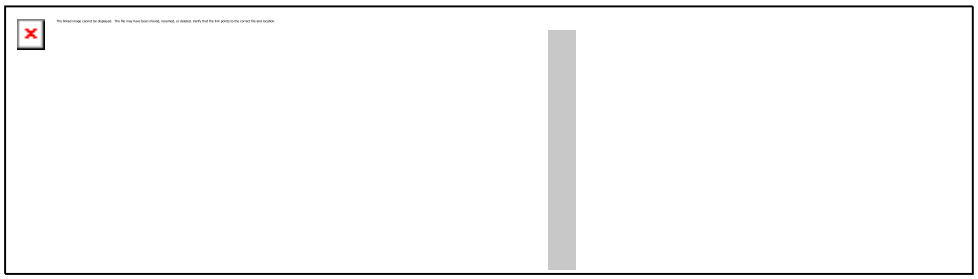


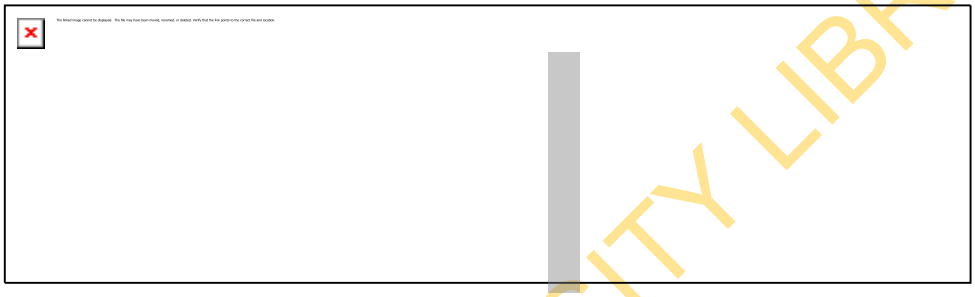
Figure 4.17: DNA sequence electropherogram showing the IL18Rα -661T/C polymorphism.

The locations of base substitution are highlighted in shaded boxes (a) Homozygous IL18Rα -661TT (b) Heterozygous IL18Rα -661T/C (c) Homozygous IL18Rα -661CC. *Sequences were done using the forward primer*.

(a)



(b)

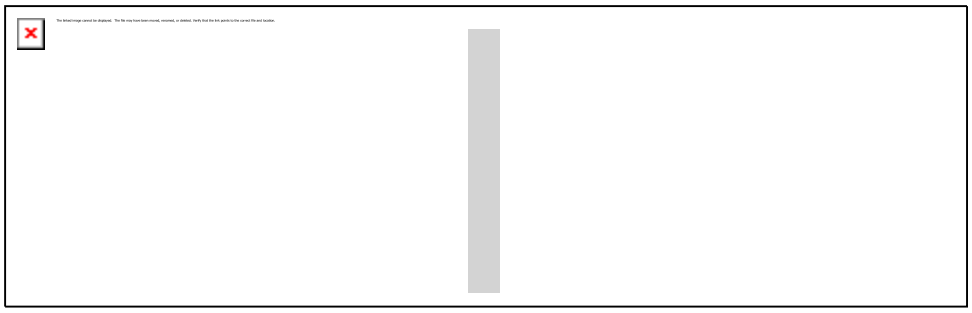


(c)

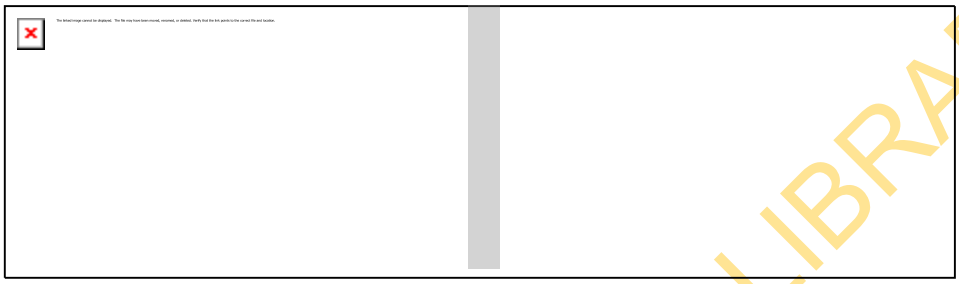
Figure 4.18: DNA sequence electropherogram showing the IL18Rα -175G/A polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18Rα -175GG (b) Heterozygous IL18Rα -175G/A (c) Homozygous IL18Rα -175AA. *Sequences were done using the reverse primer*.

(a)



(b)



(c)

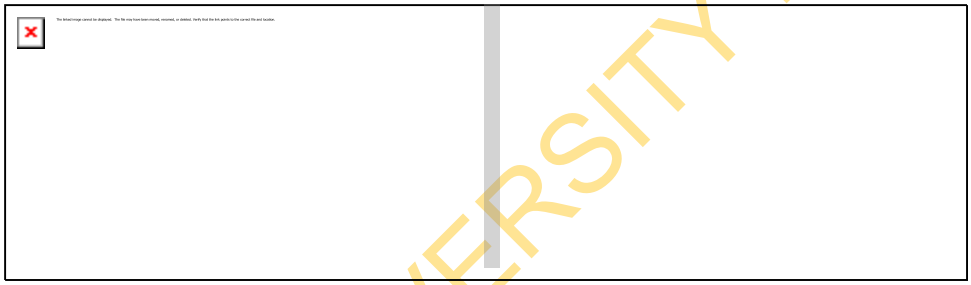
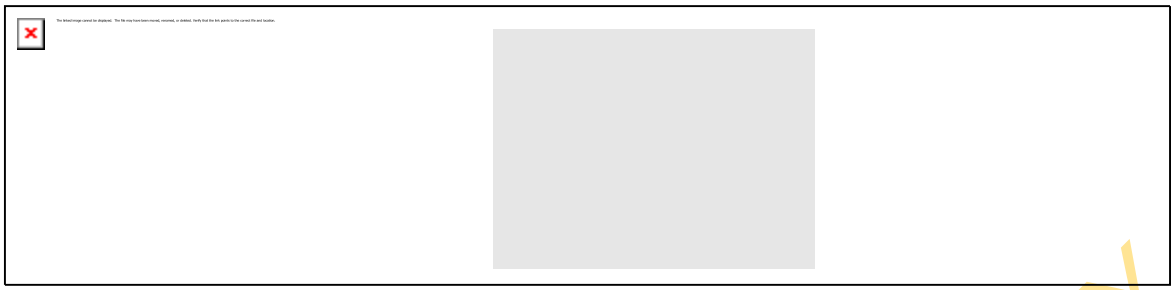


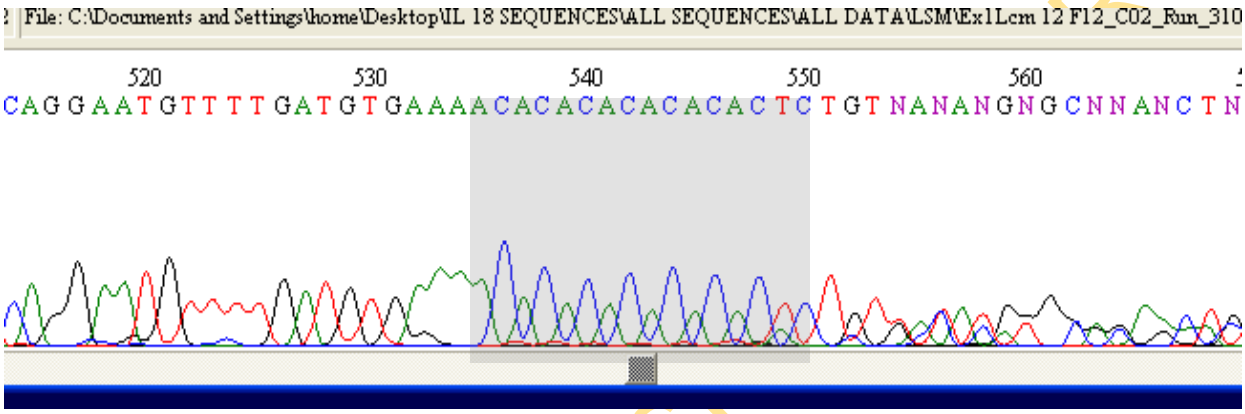
Figure 4.19: DNA sequence electropherogram showing the IL18Rα -93C/T polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18Rα -93CC (b) Heterozygous IL18Rα -93C/T (c) Homozygous IL18Rα -93TT. *Sequences were done using the reverse primer*.

(a)



(b)



(c)

Figure 4.20: DNA sequence electropherogram showing the IL18Rα -430 A/C microsatellite repeats.

The locations of repeat variants are highlighted in shaded boxes (a) Homozygous IL18Rα -430 $(AC)_7$ (b) Heterozygous IL18Rα -430 $(AC)_{7/8}$ (c) Homozygous IL18Rα -430 $(AC)_8$. *Sequences were done using the forward primer*.

4.5.3 Distribution of Exon 1 and Exon 7 genotypes

The allele and genotype frequencies for Ex1 +21 C/G are presented in Table 4.7. Chi-square test for conformity with the Hardy-Weinberg equilibrium showed that the genotype frequencies did not deviate from the Hardy-Weinberg equilibrium. Distribution of the heterozygous genotype CG was higher in the severe malaria group (12%). There was no statistically significant difference in the distribution of the Ex1 +21 C/G genotypes between the three study groups. The electrophoretic separation of the 1409bp PCR products of Exon 1 which also included the promoter region is shown in Figure 4.16. The electropherogram showing the genotype variants of the polymorphisms in Exon 1 are shown in Figure 4.21.

For the Exon 7 polymorphism, the allele and genotype frequencies of the Ex7 +63 C/T are presented in Table 4.7. The distributions of the observed genotypes for the Ex7 +63 C/T were in conformity with the expected distribution when tested for the Hardy-Weinberg equilibrium. The CC genotype was higher in the severe malaria group (87.1%) compared with the asymptomatic group (73.9%) or the uncomplicated malaria group (74.4%), though this was not statistically significant. There was however, a statistically significant difference in the distribution of the heterozygotes with significantly lower CT genotypes in the severe malaria group (*OR*=0.72, 95% CI: 0.51-1.02, χ^2= 4.795, *P*<0.05). The electrophoretic separation of the 410bp PCR products of Exon 7 is shown in Figure 4.22, while the electropherogram showing the genotype variants of polymorphisms in Exon 7 are shown in Figure 4.23.

4.5.4 Report of conserved Exons 2-6, 8-11

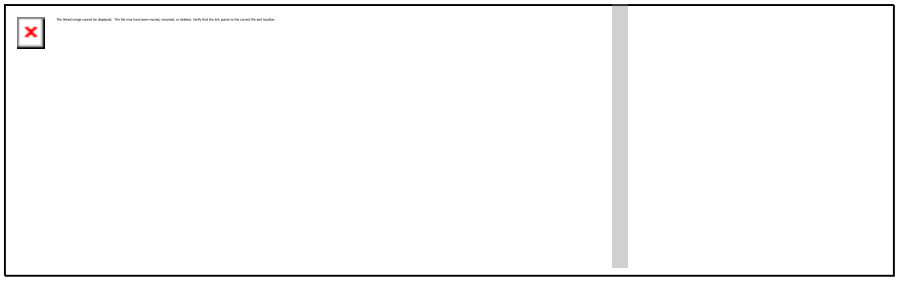
Exons 2-6, 8-11 were found to be conserved in the study population as no SNP was discovered in those coding sequences. The electrophoretic separation of PCR products and multiple alignments showing sequence conservation for Exons 2, 3, 4, 5, 6, 8, 9, 10 and 11 are shown in Figures 4.24-4.41.

Table 4.7: Genotype and allele frequencies of IL-18Rα Ex1 +21C/G and Ex7 +63C/T

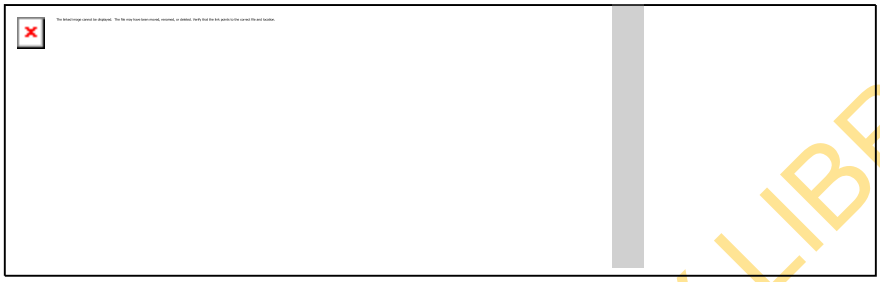
Locus	Non-Symptomatic Control n= 161(%)	Uncomplicated Malaria n= 160(%)	Severe Malaria n= 116(%)
IL18Rα Ex1 +21C/G			
CC	144(89.44)	138(86.25)	101(87.07)
CG	17(10.56)	19(11.88)	15(12.93)
GG	0(0)	3(1.88)	0(0)
G allele	0.053	0.078	0.065
C allele	0.947	0.922	0.935
*HWE	(χ^2= 0.50, *P*=0.48)	(χ^2= 4.93, *P*=0.03)	(χ^2= 0.55, *P*=0.46)
IL18Rα Ex7 +63C/T			
CC	119(73.9)	119(74.4)	92(87.07)
CT	40(24.9)	41(25.6)	24(12.93)
TT	2(1.2)	0(0)	0(0)
C allele	0.863	0.872	0.897
T allele	0.137	0.128	0.103
*HWE	(χ^2= 0.452, *P*=0.501)	(χ^2= 3.455, *P*=0.06)	(χ^2= 1.544, *P*=0.214)

*HWE= Hardy-Weinberg equilibrium

(a)



(b)

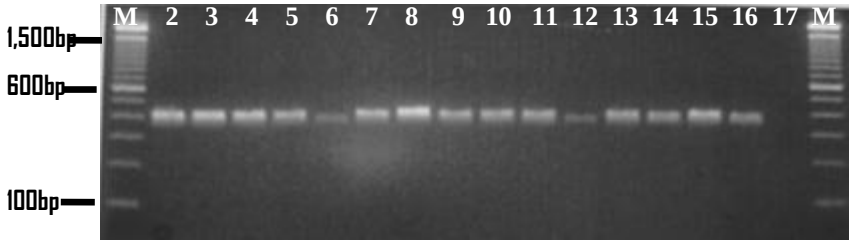


(c)

Figure 4.21: DNA sequence electropherogram showing the IL18Rα Ex1 +21C/G polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18Rα +21CC (b) Heterozygous IL18Rα+21G/C (c) Homozygous IL18Rα +21 GG.
This synonymous polymorphism is a silent mutation that does not result in amino acid substitution in the transcribed cDNA. *Sequences were done using the reverse primer*.

(a)



(b)

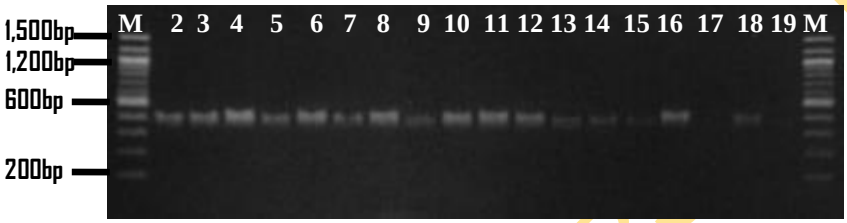
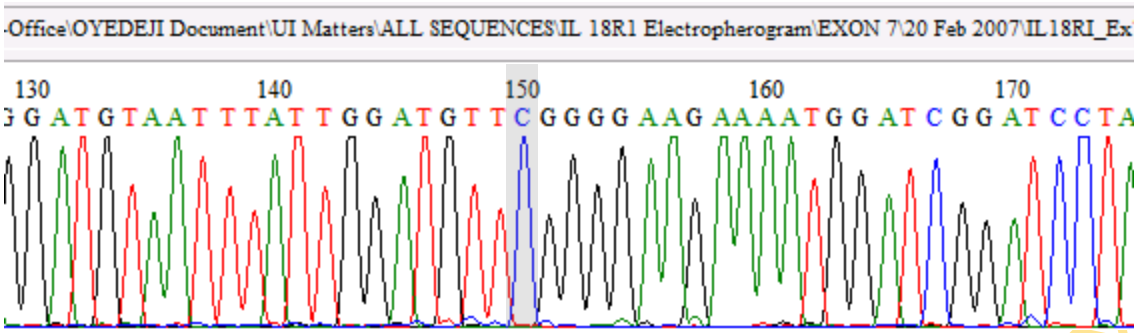


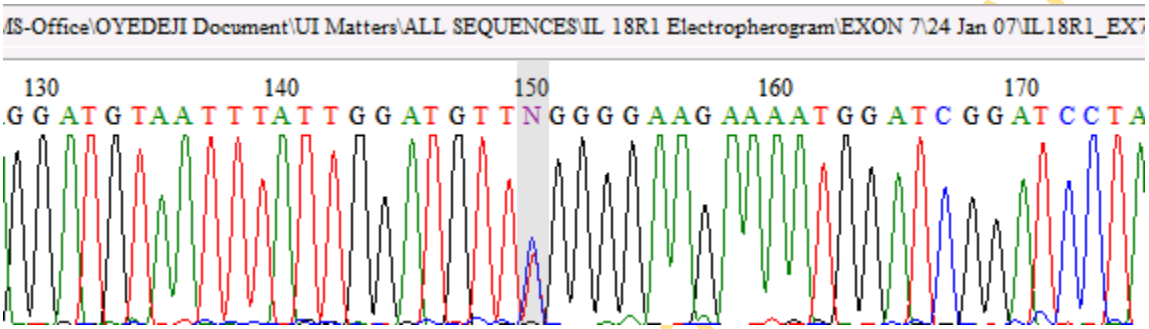
Figure 4.22: Agarose gel electrophoresis of IL18Rα Exon 7 showing ~410bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

(a)



(b)



(c)

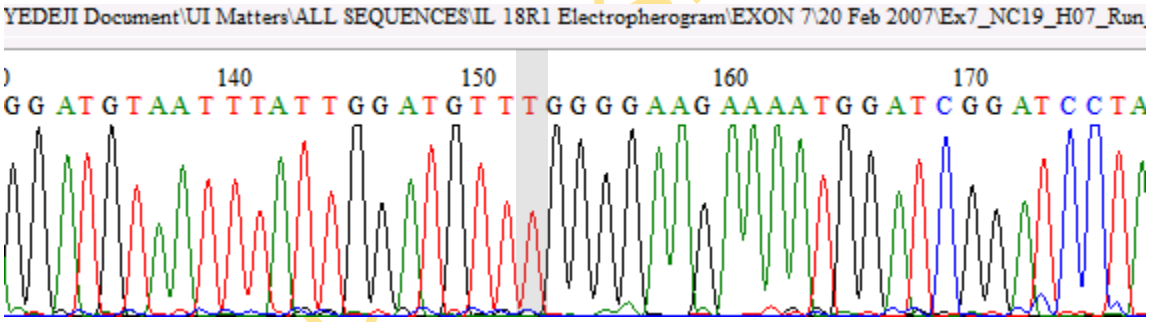
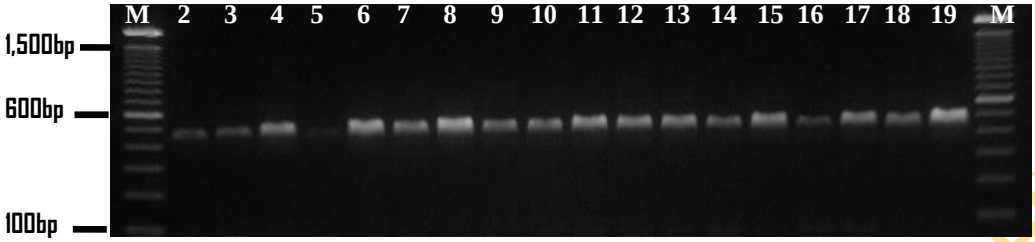


Figure 4.23: DNA sequence electropherogram showing the IL18Rα Ex7 +63C/T polymorphism.

The locations of base substitution are highlighted in shaded boxes. (a) Homozygous IL18Rα Ex7 +63CC (b) Heterozygous IL18Rα Ex7 +63CT (c) Homozygous IL18Rα Ex7 +63TT.
This synonymous polymorphism is a silent mutation that does not result in amino acid substitution in the transcribed cDNA. *Sequences were done using the forward primer*.

a)



b)

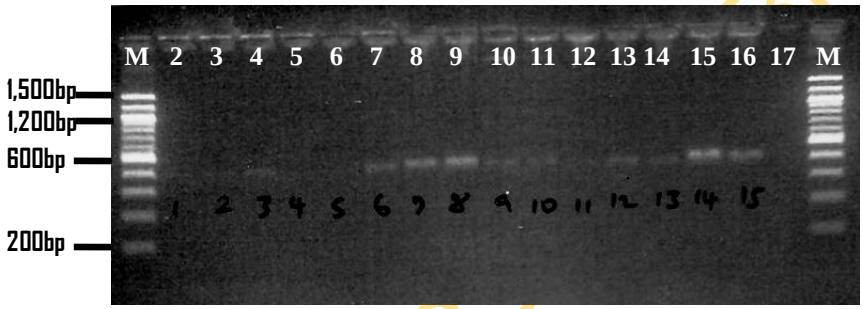


Figure 4.24: Agarose gel electrophoresis of IL18Rα Exon 2 showing ~525bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-18: DNA fragment of study participants

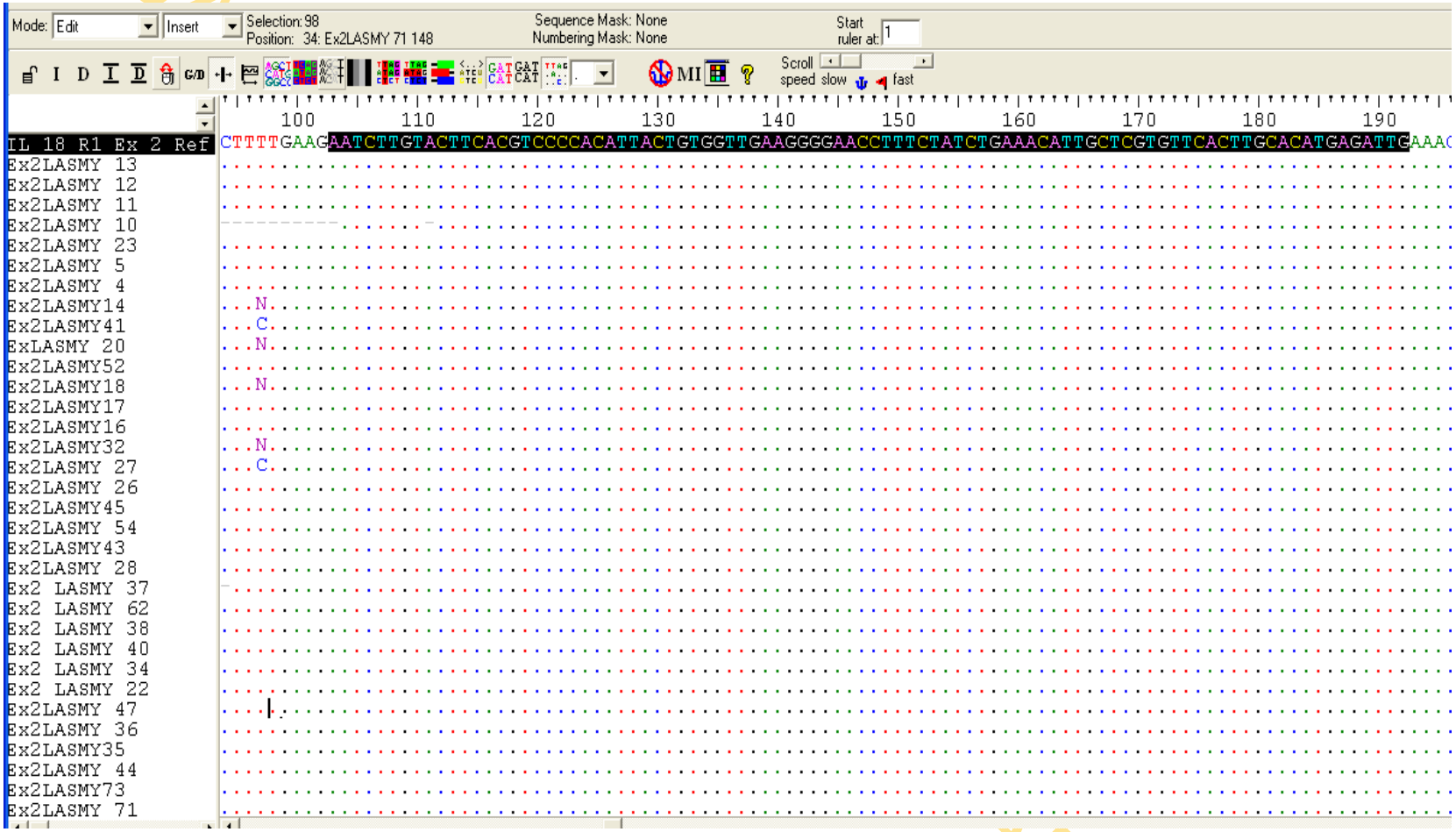
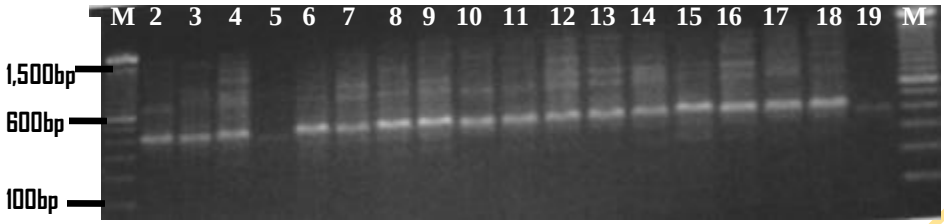


Figure 4. 25: Multiple alignment of Exon 2 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

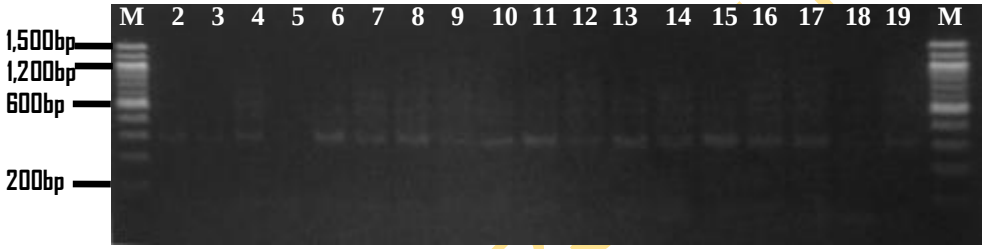


Figure 4.26: Agarose gel electrophoresis of IL18Rα Exon 3 showing ~415bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

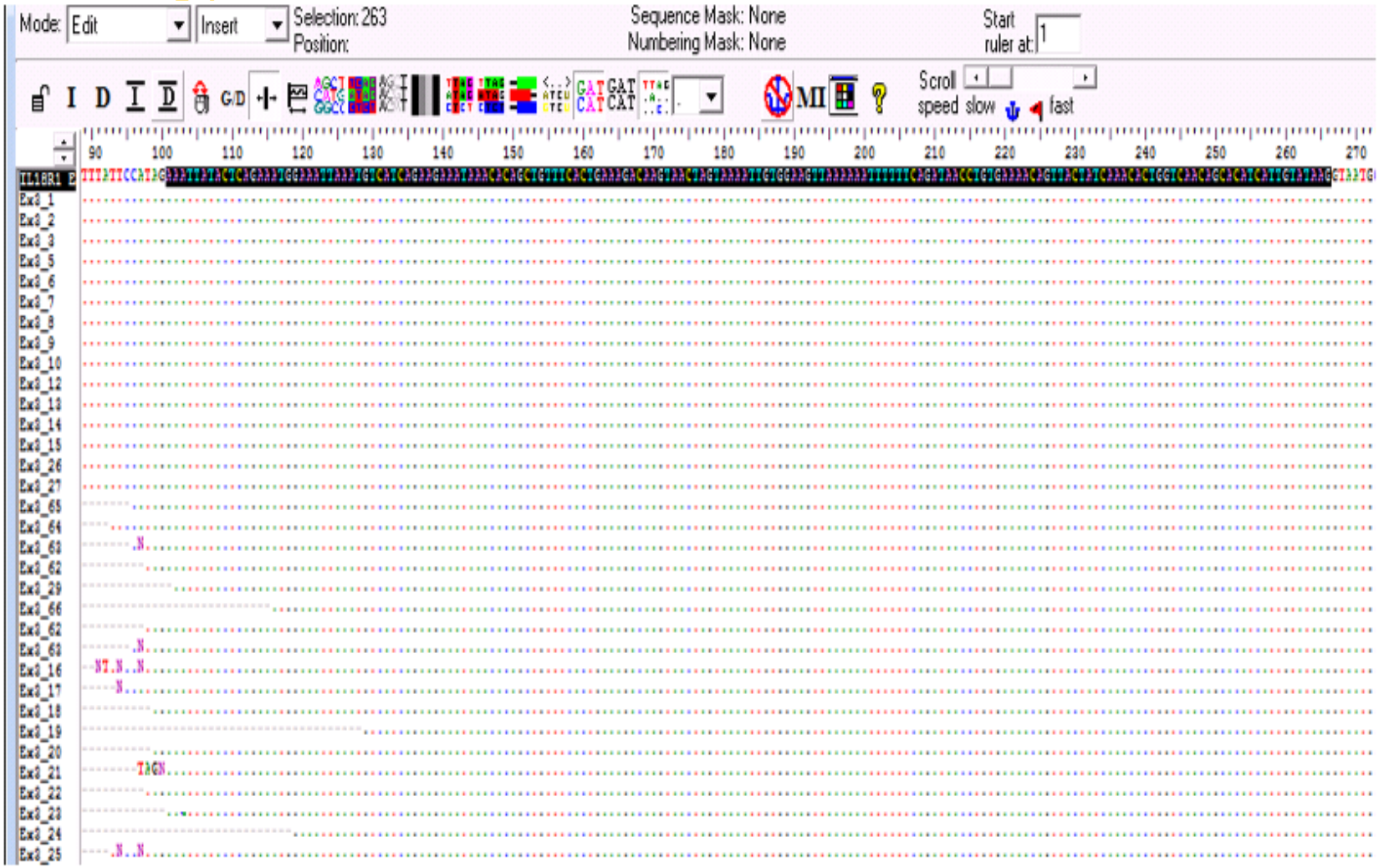
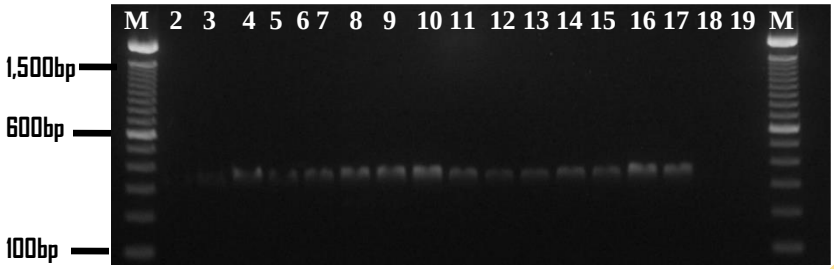


Figure 4. 27: Multiple alignment of Exon 3 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

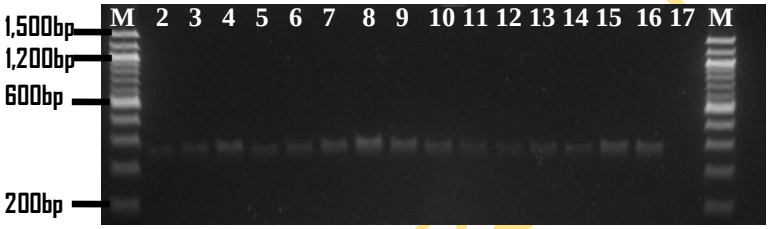


Figure 4.28: Agarose gel electrophoresis of IL18Rα Exon 4 showing ~285bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

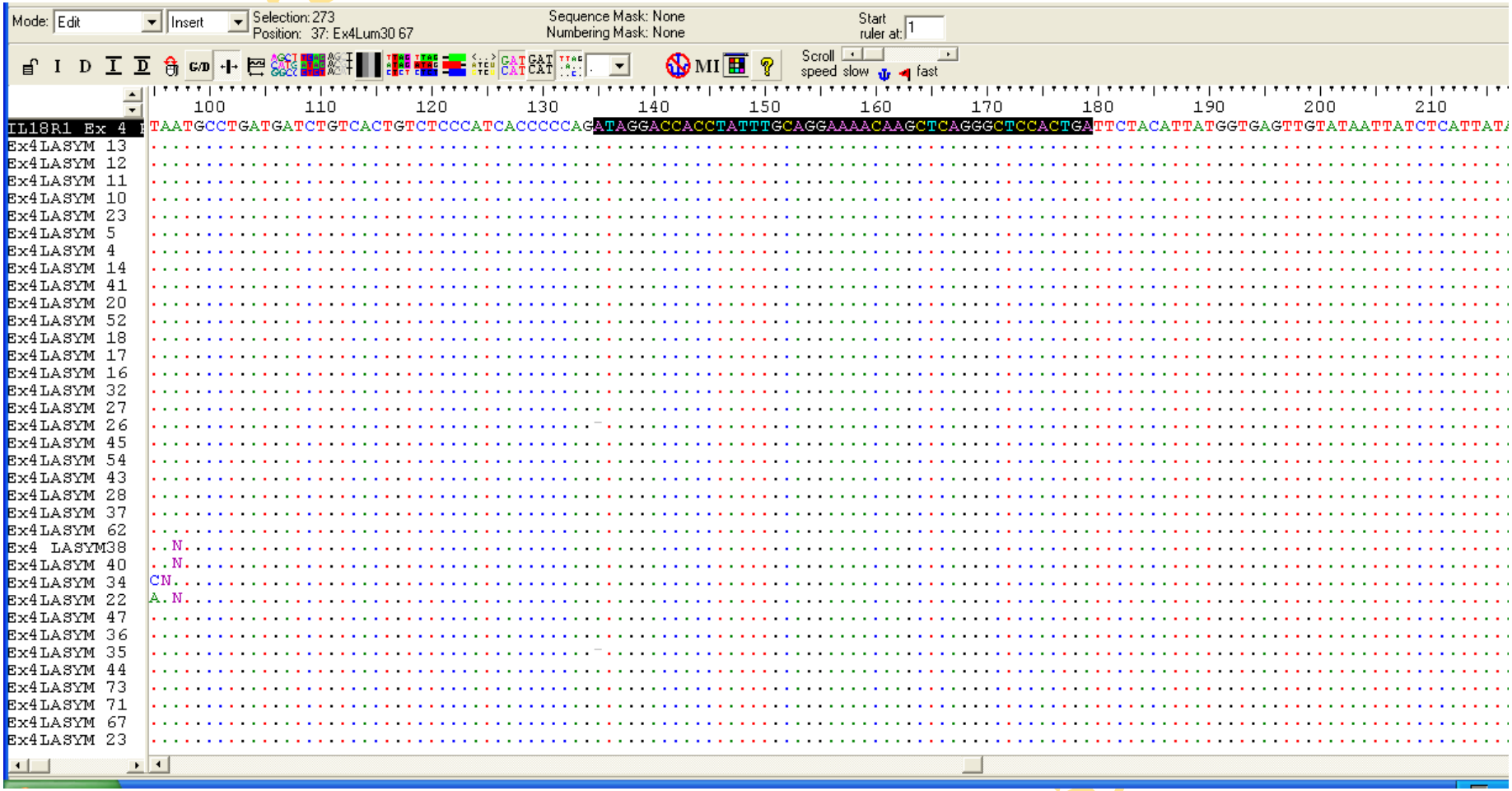
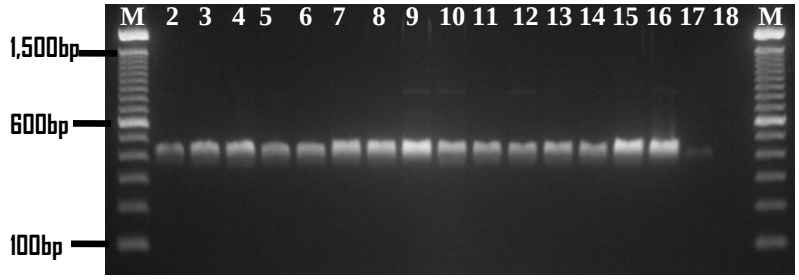


Figure 4. 29: Multiple alignment of Exon 4 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

Figure 4.30: Agarose gel electrophoresis of IL18Rα Exon 5 showing ~350bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-18: DNA fragment of study participants

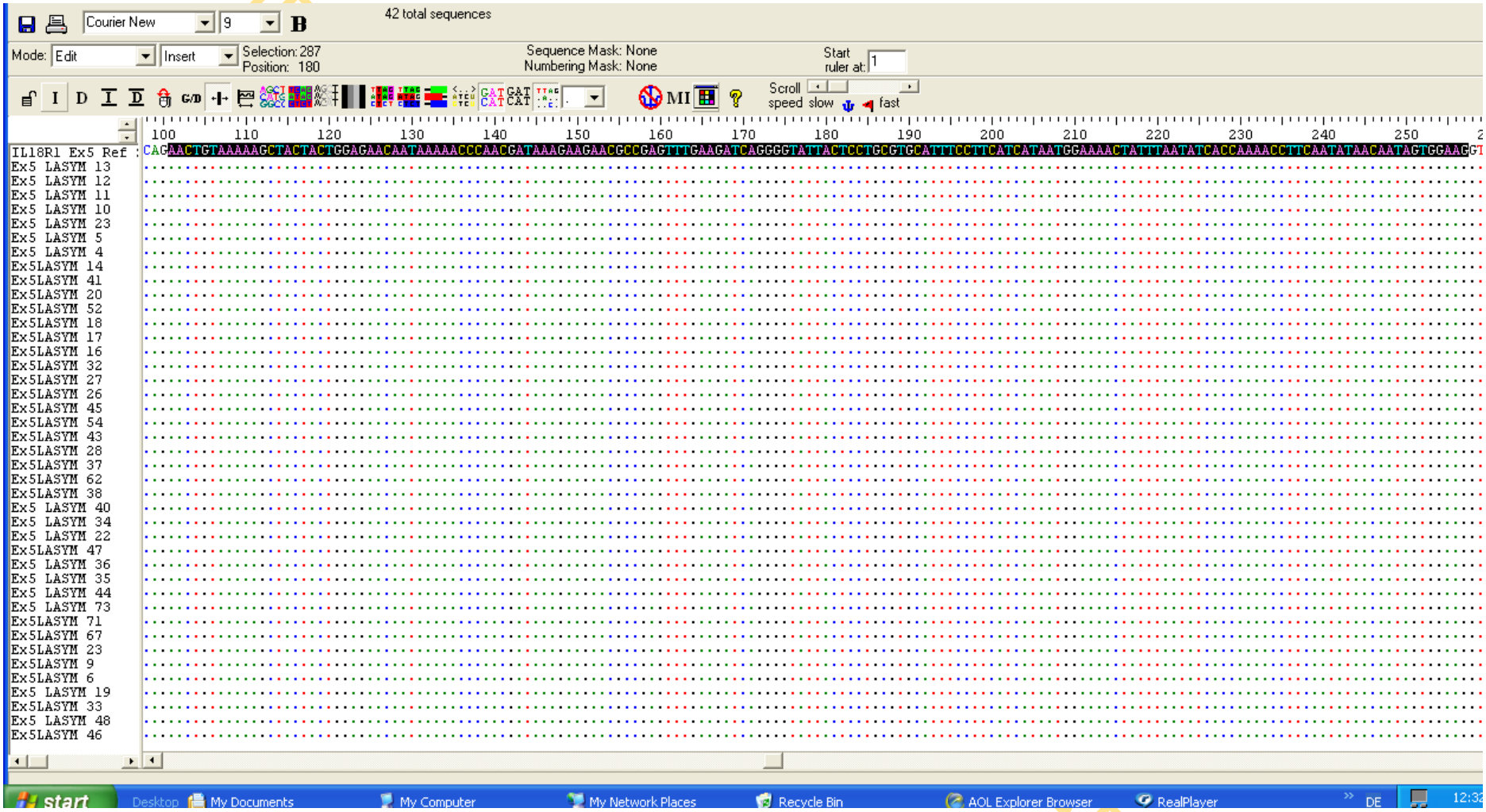


Figure 4. 31: Multiple alignment of Exon 5 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)

b)

Figure 4.32: Agarose gel electrophoresis of IL18Rα Exon 6 showing ~240bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-18: DNA fragment of study participants

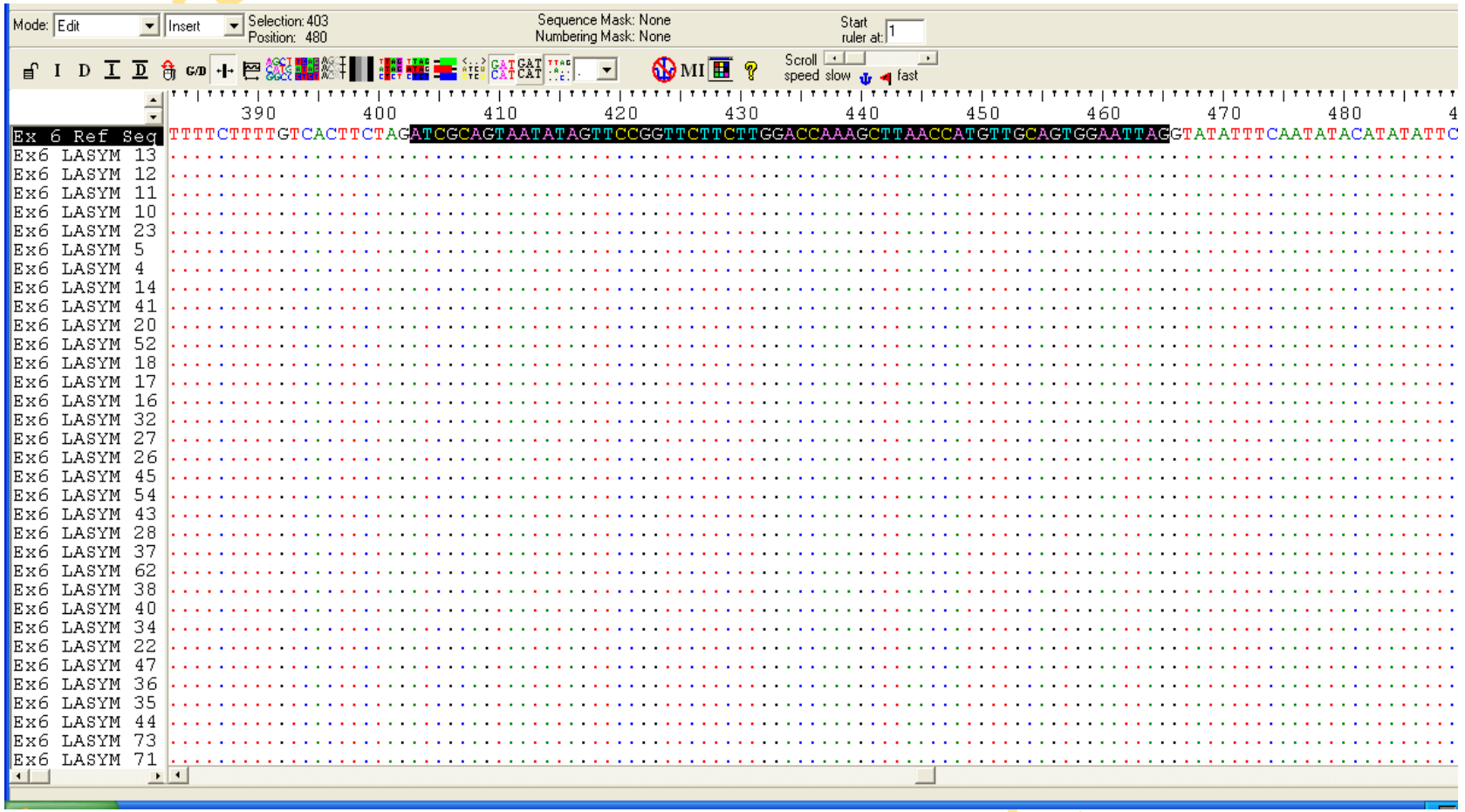
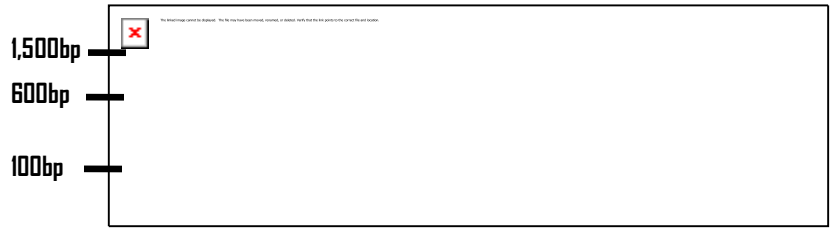


Figure 4. 33: Multiple alignment of Exon 6 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

Figure 4.34: Agarose gel electrophoresis of IL18Rα Exon 8 showing ~280bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-17: DNA fragment of study participants

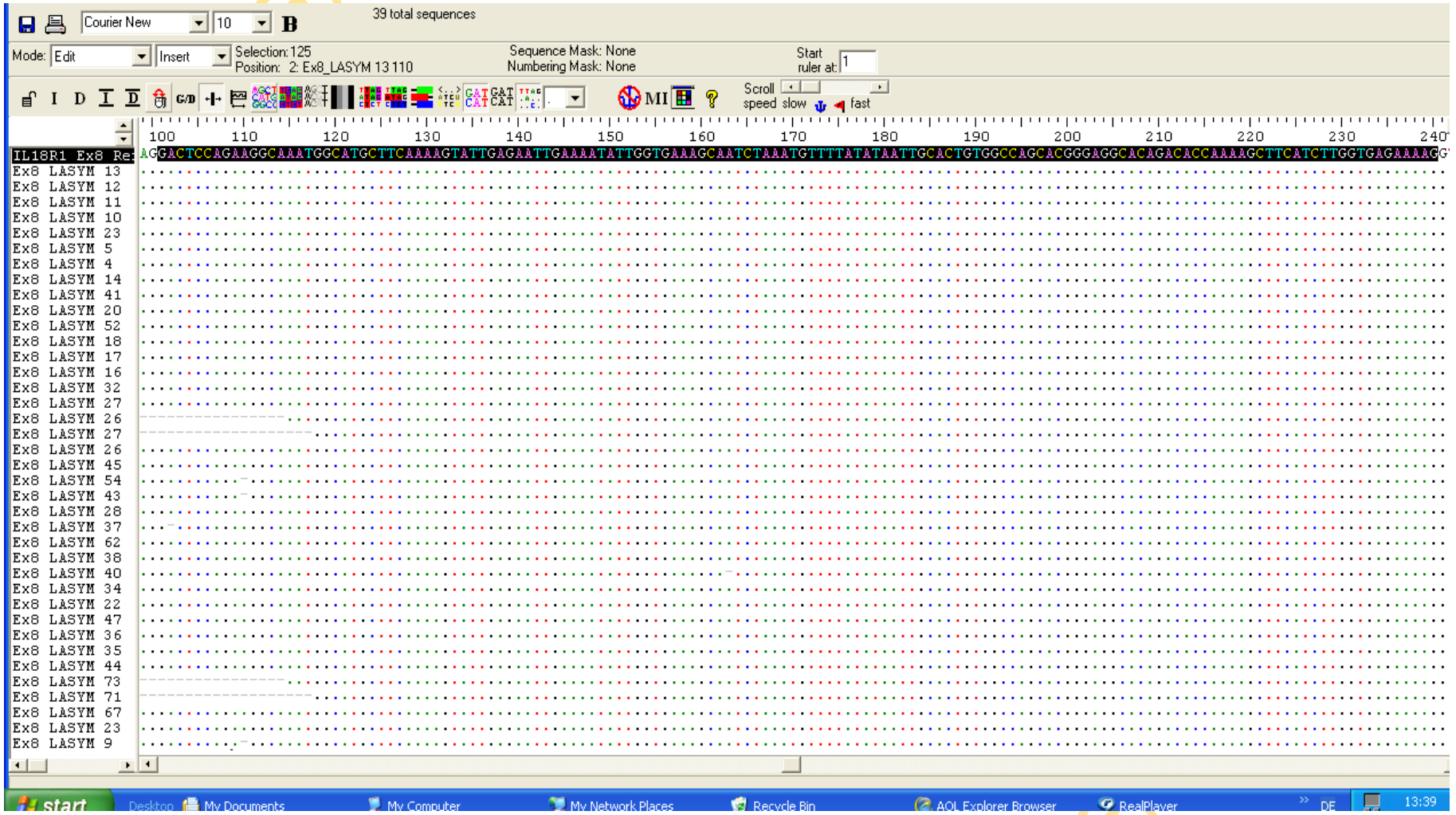
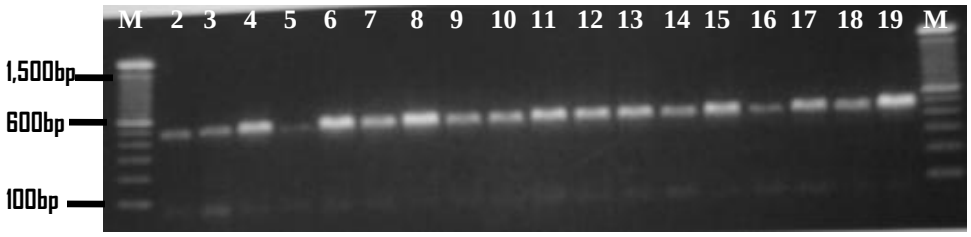


Figure 4. 35: Multiple alignment of Exon 8 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

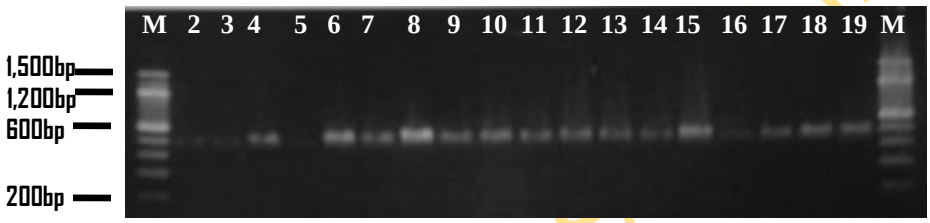


Figure 4.36: Agarose gel electrophoresis of IL18Rα Exon 9 showing ~370bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

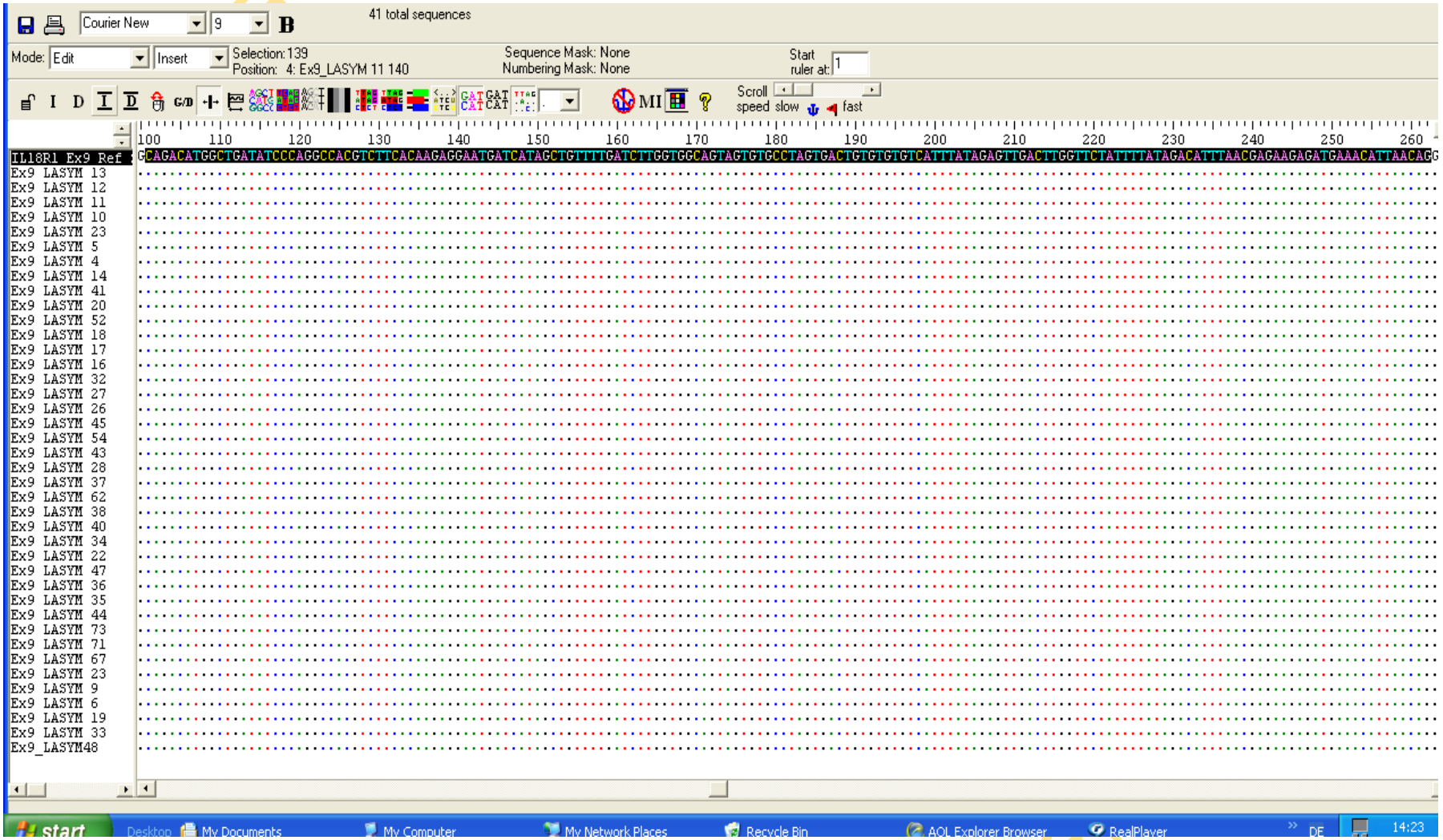
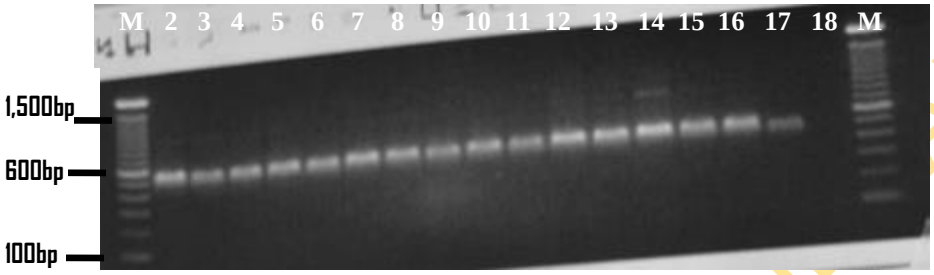


Figure 4. 37: Multiple alignment of Exon 9 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

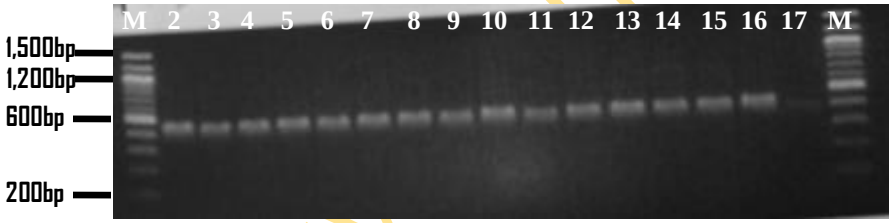


Figure 4.38: Agarose gel electrophoresis of IL18Rα Exon 10 showing ~402bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-18: DNA fragment of study participants

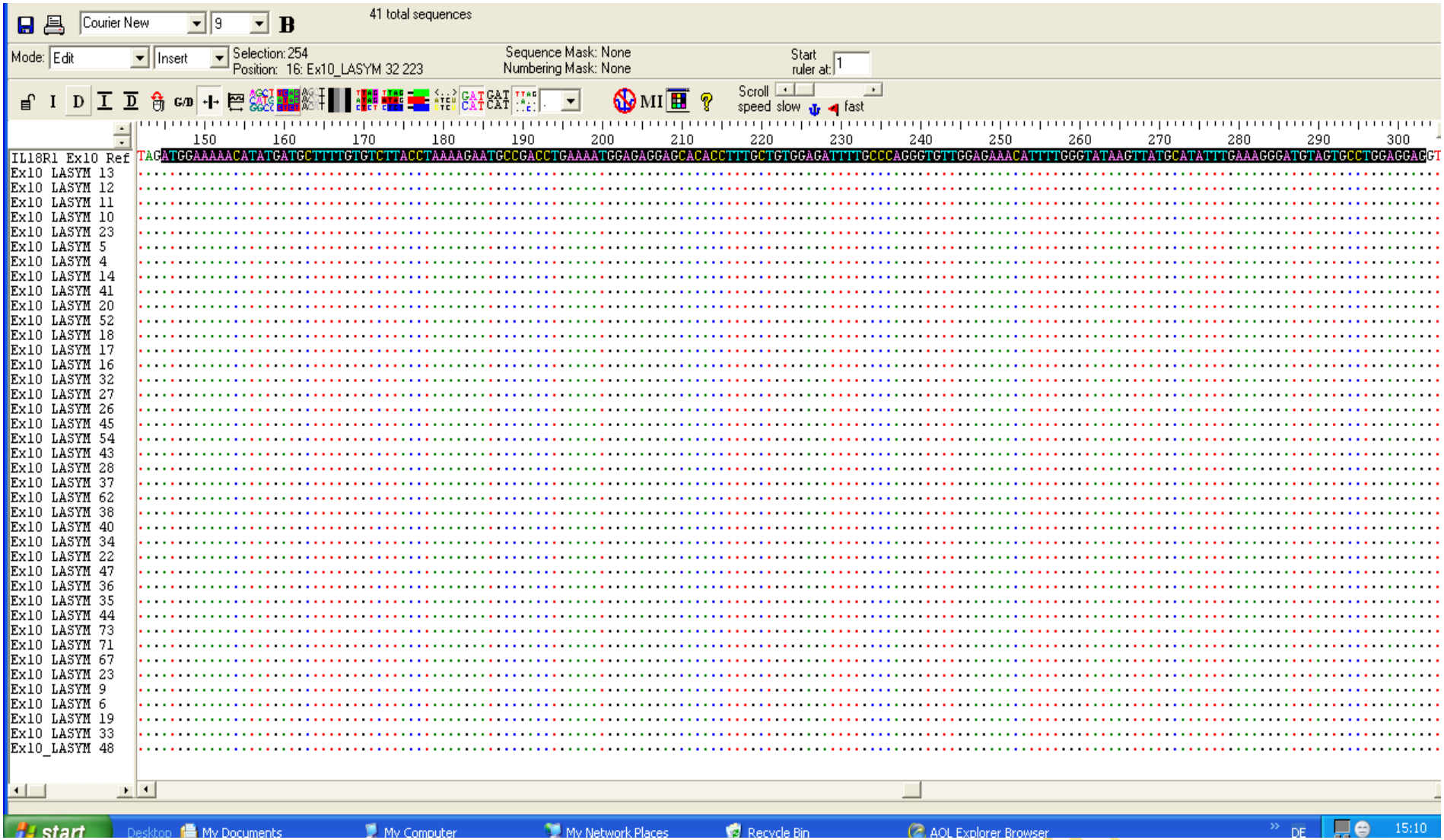
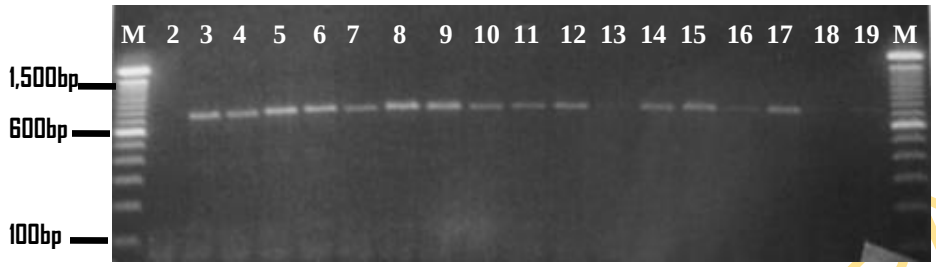


Figure 4. 39: Multiple alignment of Exon 10 sequences showing conservation to reference sequence (shaded in black) when identities were plotted as dot to the reference standard.

a)



b)

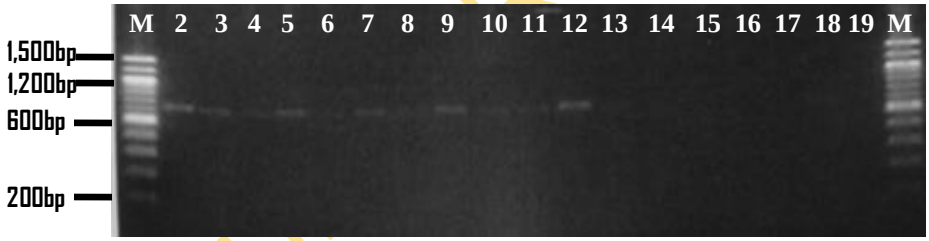


Figure 4.40: Agarose gel electrophoresis of IL18Rα Exon 11 showing ~750bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

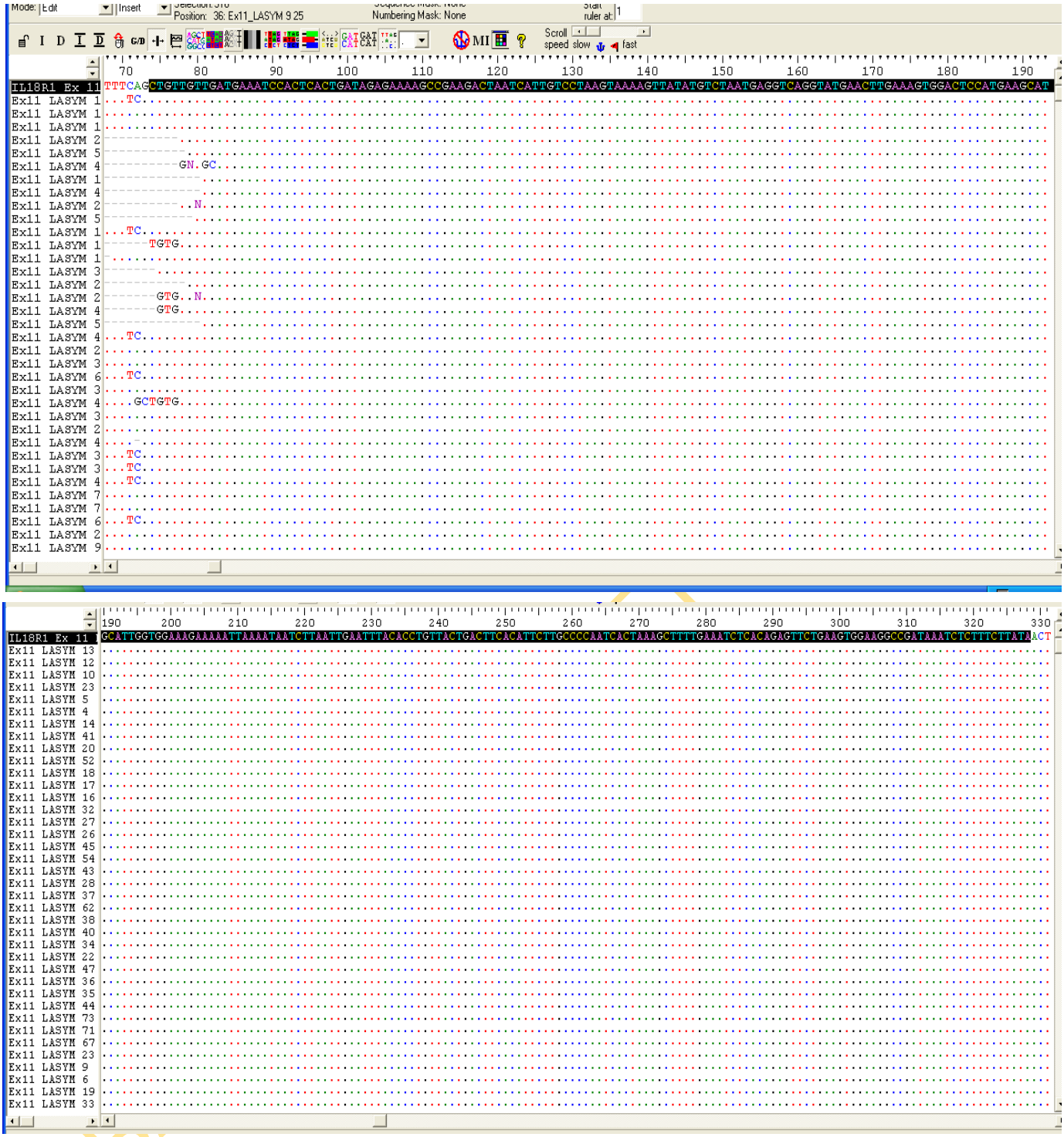


Figure 4.41: Multiple alignment of Exon 11 sequences showing conservation to reference standard (shaded in black) when identities were plotted as dot to the reference standard.

4.6 TNF-α gene Promoter Polymorphisms -308G/A and -238G/A

Genetic polymorphisms at the promoter region of TNF-α gene were determined for two previously reported variants: -308G/A and -238G/A relative to the transcription start site. Variants at these loci were found in the study population. The distribution of the allele and genotype frequencies for the -308G/A and -238G/A SNPs are shown in Table 4.8. The electrophoretic pattern of the PCR product spanning 832bp upstream of the transcription start site of TNF-α gene is shown in Figure 4.42.

The most common genotype in the study population for the TNFα -308 locus is homozygote GG. The AA homozygote genotype was not found in any of the study groups. Chi-square test for compatibility with the Hardy-Weinberg equilibrium showed that the genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (Table 4.8). No difference was also found in the distribution of the heterozygotes GA within the study groups (P>0.05). The electropherogram showing the genotype variants of the TNFα -308 are presented in Figures 4.43

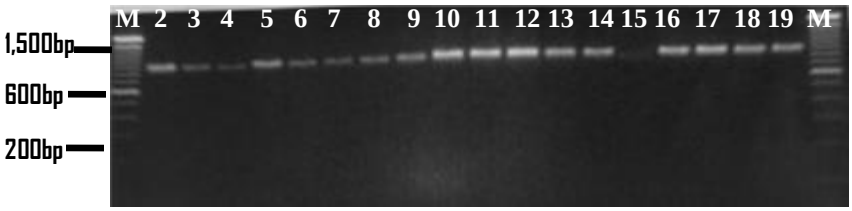
Similarly, the GG genotype was the most common amongst the three study groups for the TNFα -238 locus. When tested for conformity with the Hardy-Weinberg equilibrium, Chi-square analysis showed that the observed genotypes were not statistically different from the expected genotypes. The homozygote AA genotype was also not detected in the study population. No association was found between the allele and genotype distribution and disease outcome. The electropherogram showing the genotype variants of the TNFα -238 are presented in Figures 4.44

Table 4.8: Genotype and allele frequencies of TNFα -308G/A and -238G/A

Locus	Asymptomatic Control n= 161(%)	Uncomplicated Malaria n= 160(%)	Severe Malaria n= 116(%)
TNFα -238G/A			
GG	133(82.61)	128(80.0)	90(77.59)
GA	28(17.39)	32(20.0)	26(22.41)
AA	0(0)	0(0)	0(0)
G allele	0.896	0.824	0.786
A allele	0.104	0.176	0.214
*HWE	(χ^2= 1.46, *P*=0.23)	(χ^2= 1.98, *P*=0.16)	(χ^2= 1.85, *P*=0.17)
TNFα -308G/A			
GG	135(83.85)	130(81.25)	87(75.0)
GA	26(16.15)	30(18.75)	29(25.0)
AA	0(0.62)	0(1.25)	0(3.45)
G allele	0.904	0.894	0.836
A allele	0.096	0.106	0.164
*HWE	(χ^2= 1.24, *P*=0.27)	(χ^2= 1.71, *P*=0.19)	(χ^2= 2.37, *P*=0.12)

*HWE= Hardy-Weinberg equilibrium

(a)



(b)

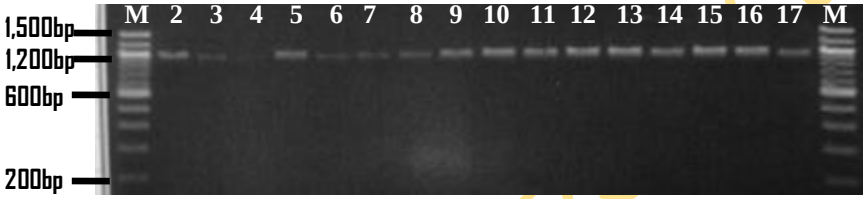
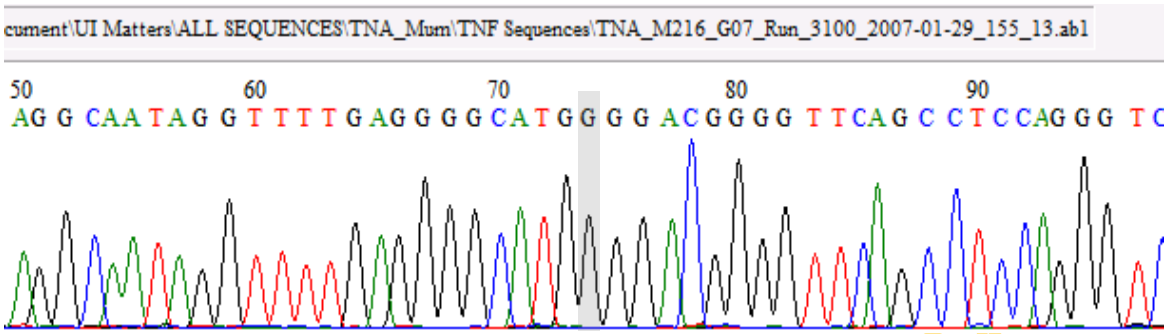


Figure 4.42: Agarose gel electrophoresis of TNF-α promoter region showing ~832bp of PCR product.

(a) PCR product before purification (b) PCR product after purification for sequencing PCR
Lane M: 100-bp ladder. Lanes 2-19: DNA fragment of study participants

(a)



(b)

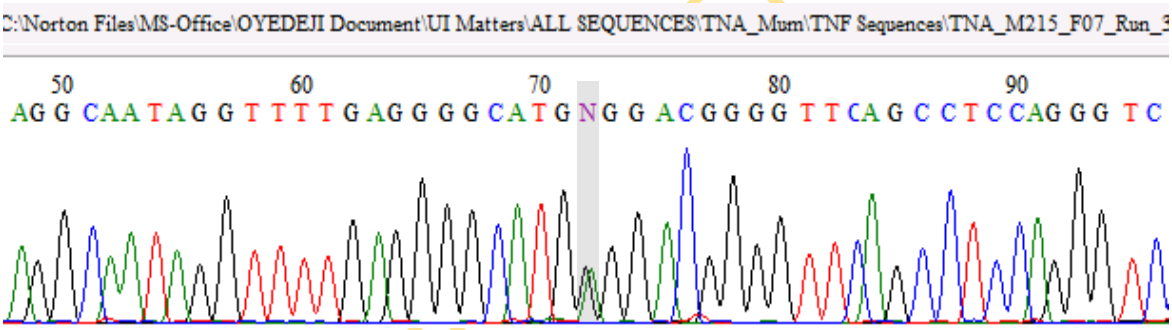
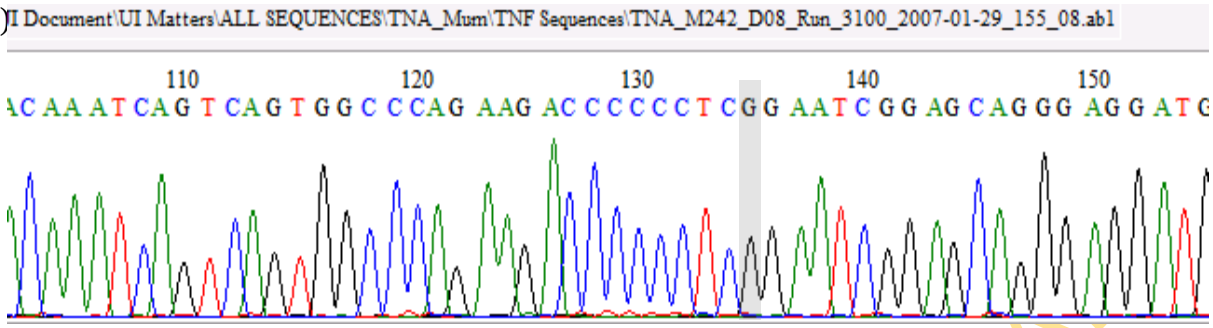


Figure 4.43: DNA sequence electropherogram showing the TNFα -308G/A polymorphism.

The locations of base substitution are highlighted in shaded boxes (a) Homozygous TNFα -308GG (b) Heterozygous TNFα -308GA. *Sequences were done using the forward primer*.

(a)



(b)

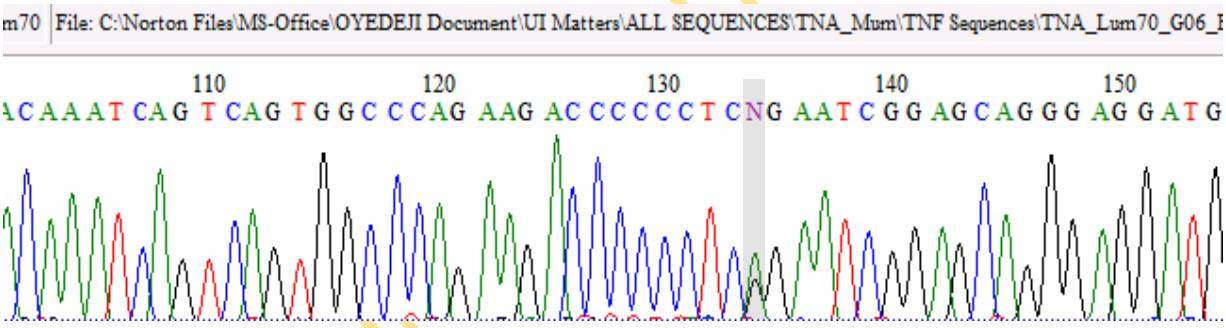


Figure 4.44: DNA sequence electropherogram showing the TNFα -238G/A polymorphism.

The locations of base substitution are highlighted in shaded boxes (a) Homozygous TNFα -238GG (b) Heterozygous TNFα -238GA. *Sequences were done using the forward primer*.

CHAPTER FIVE

DISCUSSION

The clinical outcome of an asymptomatic infection leading to severe disease and death has been shown to depend upon complexity of "many parasite, host, geographic and social factors" (Miller *et al.*, 2002; Rao *et al.*, 2012). Host-parasite interactions in malaria have led to a host's relative resistance to the parasite and parasite strain-specific susceptibility or virulence (Becker *et al.*, 2004; Grech *et al.*, 2006; Williams, 2009). Malarial parasites have co-evolved together with the human host for thousands of years (Kwiatkowski, 2005), which have led them to constitute an important driving evolutionary force behind common erythrocyte variants, such as sickle-cell disease, thalassaemia, and G6PD gluose-6-phosphate deficiency (Kwiatkowski, 2000; Koella and Boete, 2003; Kwiatkowski, 2005; Komba *et al.*, 2009; Driss *et al.*, 2011; Ferreira *et al.*, 2011). These genetic variants have been associated with resistance to malaria. Similarly, the level of antigenic diversity of *P. falciparum* populations in an area is likely to affect acquisition of immunity to malaria (Farnert *et al.*, 2009). Therefore, the understanding of the genetic structure of parasite population is necessary for planning of malaria control interventions.

5.1 Genetic Polymorphisms of *P. falciparum* MSP-2 alleles

In malaria endemic regions, it is now well established that infected individuals carry several complex mixture of parasite clones with different genetic and phenotypic characteristics (Babiker *et al.*, 1997; Missinou *et al.*, 2000; Muller *et al.*, 2001; Kang *et al.*, 2010). This is particularly true in Africa especially south of the Sahara, where studies have shown that considerable genetic diversity exists (Missinou *et al.*, 2000; Auburn *et al.*, 2012; Koukouikila-Koussounda *et al.*, 2012). This phenomenon appears to be important for the development of an efficient anti-malarial immunity which requires continuous exposure to a large number of parasite variants and malaria antigens. However, the parasites' genetic profile has not been systematically documented in several parts of Nigeria. This aspect of the study therefore,

investigated the genetic complexity and allelic diversity of *P. falciparum* parasites in children presenting with mild malaria, severe malaria and asymptomatic infection from north-central Nigeria.

In this study, a high genetic diversity of *P. falciparum* isolates was observed in the study population. This was reflected in the number of alleles found in each group studied (32, 35 and 28 for asymptomatic, mild and severe malaria respectively). This is consistent with data from other areas with high malaria transmission such as in north-eastern Tanzania (Magesa *et al.*, 2002), in Papua New Guinea (Fluck *et al.*, 2007), Ghana (Falk *et al.*, 2006b), Côte d'Ivoire (Silue *et al.*, 2006) and Congo Brazzaville (Mayengue *et al.*, 2011). Analysis of allele prevalence revealed interesting trends. A higher prevalence of 3D7 allelic type was found in the symptomatic groups, 51% for uncomplicated malaria and 54% for severe malaria compared to 38% found in the asymptomatic malaria group. Likewise, FC27 allelic types were more frequent in the asymptomatic group compared to the symptomatic groups. This may probably indicate a higher risk of developing symptomatic malaria with increasing carriage of isolates belonging to the 3D7 allelic family.

This observation is consistent with reports concerning clinical isolates from Senegal (Robert *et al.*, 1996) as well as south-western Nigeria (Amodu *et al.*, 2008). Similarly, in eastern Sudan, the FC27 genotype was noted to be over-represented in subjects with asymptomatic infections comparable to what was found in the present study (A-Elbasit *et al.*, 2007). Conversely, reports from other studies including north-eastern Tanzania (Magesa *et al.*, 2002), Benin (Issifou *et al.*, 2001) and Gabon (Aubouy *et al.*, 2003; Issifou *et al.*, 2003), showed no evidence for association between a particular genotype and clinical outcome. The present result also contrasts with the observations made in Papua New Guinea where MSP-2 FC27 alleles were found to be associated with clinical malaria (Engelbrecht *et al.*, 1995). However, the FC27 data from this study are compatible with the observation of a higher prevalence of FC27 allelic types in asymptomatic carriers in Senegal (Ntoumi *et al.*, 1995). In eastern Sudan, the genetic diversity of the parasite population was very high with 51 different genotypes (A-Elbasit *et al.*, 2007).

In the Sudan study, it was found that the ratio of the 3D7 to FC27 allele between SM and UM was comparable which is similar to what was found in the present study in Lafia. Furthermore, they noted that the FC27 genotype was overrepresented in subjects with asymptomatic infection (A-Elbasit *et al.*, 2007) comparable to what was found in the present study. Moreover, in an earlier study conducted in south-west Nigeria, the absence of FC27 alleles was found to be significantly associated with a 3.58-fold (95% CI = 2.0-7.3) increased risk of developing uncomplicated malaria and a 5.9-fold (95% CI = 2.2-9.6) increased risk of developing severe malaria (Amodu *et al.*, 2008). There is need therefore, for a larger, multi-regional study to be conducted in order to ascertain whether the discrepancies reflect geographical differences in parasite populations or a genuine tendency for 3D7 allelic types to be associated with clinical malaria.

Data on monoclonal infections in this study showed that multiplicity of infections and polyclonality was generally high in the asymptomatic as well as uncomplicated malaria group. Multiplicities of infections were 2.1 and 2.0 on the average per infected individual in the asymptomatic group and uncomplicated malaria group respectively. On the average, majority of participants in the asymptomatic as well as uncomplicated malaria group, were infected with more than one parasite genotype with polyclonality being 61% and 60% for asymptomatic and uncomplicated malaria respectively, while monoclonal infections were predominant in the severe malaria group as polyclonality was only 34%. A similarly high degree of multiple infections per infected individual with symptomatic malaria has been demonstrated in other areas of Africa with high malaria transmission such as in Senegal (Ntoumi *et al.*, 1995), Tanzania (Beck *et al.*, 1997), Cameroon (Basco and Ringwald, 2001) as well as the Republic of Congo (Mayengue *et al.*, 2011).

On the contrary, in regions of low intensity of malaria transmission such as Pahang, Malaysia a low multiplicity of infection (average of 1.2 per infected individual) has been reported in uncomplicated malaria, probably reflecting the transmission intensity of the area (Atroosh *et al.*, 2011). In the severe malaria group in the present study however, multiplicity of infection was significantly low (1.3). It is presumable that multiplicity of infections could be a

molecular marker for severe malaria since children in the severe malaria group had a very low, statistically significant multiplicity of infection (P<0.001) and polyclonality (P<0.001) compared to the asymptomatic or uncomplicated malaria groups. Moreover, limited parasite diversity in severe malaria subjects is consistent with data from other studies (Milner *et al.*, 2012).

Although the number of parasite types harboured was different between uncomplicated and severe malaria groups, there was a trend for reverse relationship between parasite density and complexity: severe malaria isolates had higher parasite density than mild malaria samples, yet their multiplicity of infection was lower. It is possible that severe malaria would be associated with high parasite density, resulting from overwhelming multiplication of a limited number of clones, while mild malaria would be caused by a large number of clones reaching a lower density, most of which were previously not exposed to. Indeed there are indications that the diversity of asymptomatic *P. falciparum* infections evident in the high multiplicity of infection, contribute to protective malaria immunity in children in an area of perennial transmission (Bereczky *et al.*, 2007).

It is obvious then that the genetic complexity of *P. falciparum* and in particular its ability to generate mutant variants, makes it a successful pathogen. Studies on the genetic complexity of *P. falciparum* infections therefore have wide variety of application including distinguishing between new infections and recrudescence in drug trials as well as the assessment of other control intervention studies such as vaccine trials and the use of insecticide treated bed nets. Genotyping of the *MSP-2* gene is a widely used protocol for epidemiological surveys investigating the genetic diversity of *P. falciparum* populations (Contamin *et al.*, 1995; Babiker *et al.*, 1998; Kang *et al.*, 2010; Mwingira *et al.*, 2011) or the effect of interventions on surviving parasites (Beck *et al.*, 1997; Snounou and Beck, 1998; Takala and Plowe, 2009; McCarthy *et al.*, 2011) because it has been demonstrated that *MSP-2* gene polymorphism is extensive within natural parasite populations (Felger *et al.*, 1994; Meyer *et al.*, 2002; Happi *et al.*, 2004; Ghanchi *et al.*, 2010; Hussain *et al.*, 2011).

One factor that might potentially compromise a comparison of genotypic characters of isolates collected from patients experiencing a clinical episode is uncontrolled drug intake before

presentation to the hospital. In Africa, majority of malaria cases occur in rural areas with considerable variation in the treatment seeking pattern and over 50% are self treatment at home (Hamel *et al.*, 2001; Muller *et al.*, 2003). Likewise, pre-hospital antimalarial treatment of febrile children has been reported to be a common practice among child care-givers in Nigeria (Mockenhaupt *et al.*, 2000; Ajayi and Falade, 2006; Orimadegun *et al.*, 2008). Since most malaria cases are first treated at home as fevers, a plausible reason for the observed reduced genotypes in the severe malaria group might be the clearance of susceptible clones as a result of self medication. So the resistant strains that have survived the scourge of treatment, with their high propagative ability probably became more virulent to cause severe disease (Olumese *et al.*, 2002).

Other probable explanations for the reduced multiplicity of infection in the severe malaria group include: (i) the likelihood of an artefact caused by single dominant clones diluting the PCR template of low-density co-infections (Smith *et al.*, 1999b) since at high ratios of template, rare clones are not amplified (Contamin *et al.*, 1995); (ii) likely consequence of the anti-parasite effect of the cytokines released during fever or of fever itself on the parasite populations (Smith *et al.*, 1999c).

5.2 Sequence diversity of *P. falciparum* MSP-2 gene

Although results from this study showed a high complexity of infection determined by gel electrophoresis of nested PCR product, the possibility that PCR product of a given size could compromise variants with subtle differences in DNA sequence or arrangement of their amino acids could not be disregarded. To assess the pattern of sequence diversity in the *P. falciparum MSP-2* gene, nucleotide sequences of 97 PCR positive samples that show single band by gel electrophoresis of PCR product were determined. Sequence analysis of the central region of *MSP-2* gene showed a high allelic diversity even at the level of intra-allele type. Therefore, genetic diversity of alleles in this region might be far higher than that reported from the results of gel electrophoresis.

Data from this study showed that there were mixed infections that were as a result of double infections with an FC27 family and a 3D7 family parasite as well as those containing more than one allele from the same family in contrast to findings from the Oksibil region of Irian Jaya (Eisen *et al.*, 1998). Two subtypes of FC27 alleles were found in this study based on the sequence of repeat units and a hybrid sharing sequences from the two subtypes. The 3D7 allele type on the other hand had three subtypes of repetitive domains including the GSA-rich, the TPA and the poly-threonine repeat units. There were no intermediate allele sharing sequences of both FC27 allele and 3D7 allele which is in conformity with a previous report (Tanabe *et al.*, 2004).

The sequences belonging to the FC27 family of *P. falciparum* isolates from the study region were relatively conserved with few synonymous and non-synonymous polymorphisms, but with varied number of repeats. The 3D7 allele however displayed more extensive sequence diversity with lots of synonymous and non-synonymous amino acid substitutions as well as variations in the type and number of repeat units. This is in conformity with previous reports from Africa and other parts of the world (Fenton *et al.*, 1991; Eisen *et al.*, 1998; Hoffmann *et al.*, 2006; Ferreira and Hartl, 2007). A frameshift mutation in the FC27-type sequences which was reported for isolates in Brazil (Tonon *et al.*, 2004) was not found in this study which is in agreement with findings from other parts of Africa (Ferreira and Hartl, 2007). This mutation was generated by two indels leading to a variant form AAG TTC TGG CAA TCG ACA (encoding KFWQCT), instead of the wild-type sequence AGT TCT GGC AAT GCA CCA (encoding SSGNAP) at positions 164-169 on the lower panel of Figure 4.9.

As evident in the sequencing data, that there were significant nucleotide substitutions in the 3D7 allelic family compared to the FC27 allelic family which may further explain why in the clinical groups, a predominance of 3D7-type were found compared to the non-symptomatic group where infections were largely of the FC27 allele type. In a recent study on the genetic analysis of sequence polymorphisms in MSP-2 gene in Senegal and other African Countries, a predominance of 3D7 allele type were also found in isolates from patients (Ahouidi *et al.*, 2010). This additionally strengthens the idea that carriage of parasites belonging to the 3D7 allelic family may lead to increased risk of developing clinical symptoms. However, the fact that the

different genetic variants observed were present in isolates from the three clinical groups as shown from the sequencing results also calls for caution in drawing at a conclusion.

Analyses of sequence variants especially those involving insertions and deletions of repeat units within dimorphic families are of practical significance in determining whether such variants affect the immune recognition of MSP-2 and thus, favour immune evasion. Previous studies have shown that antigenic proteins, especially the merozoite surface antigens including MSP-1, MSP-2 and MSP-3 though polymorphic, are recognized by the host's immune system and are targets for the development of vaccines against blood stage parasites (Genton *et al.*, 2002; Nwuba *et al.*, 2002; Mahajan *et al.*, 2005; Tanabe *et al.*, 2012). Naturally acquired antibodies to MSP-2 have been associated with clinical immunity to malaria in Africa (Metzger *et al.*, 2003; Polley *et al.*, 2006) but sequence diversity in the MSP-2 hampers its recognition by naturally acquired (Taylor *et al.*, 1995) and vaccine-induced (Fluck *et al.*, 2004) antibodies.

There are reports showing that antibody recognition of MSP-2 comprise of a type-specific component, which discriminates between dimorphic types, and a variant-specific component, which discriminates among variants within each dimorphic group (Franks *et al.*, 2003; Tonon *et al.*, 2004). Furthermore, studies have shown that: (i) deletions of 12-mer repeats in FC27 allelic type variants affect their recognition by naturally acquired antibodies (Ranford-Cartwright *et al.*, 1996; Franks *et al.*, 2003), (ii) a murine monoclonal antibody discriminates between 3D7 allelic type antigens differing in the number of copies of the tetrapeptide GGSA (Fenton *et al.*, 1991), and (iii) antibodies that discriminate between MSP-2 variants within the same allelic family are found during acute *P. falciparum* infections (Weisman *et al.*, 2001; Felger *et al.*, 2003; Kanunfre *et al.*, 2003; Tonon *et al.*, 2004), although examples of extensive cross-reactivity to structurally different MSP-2 variants have been described in African children (Franks *et al.*, 2003).

Studies of naturally acquired antibodies to MSP2 performed in Papua New Guinea demonstrated that significant numbers of people in the study area have antibodies only to the repeat regions of *MSP-2* and that antibodies to the conserved regions of this protein develop at later ages after more prolonged exposure to malaria (al-Yaman *et al.*, 1995). Similarly, anti-

MSP2 response occurring in Gambian adults was shown to be directed almost exclusively to polymorphic regions of the protein which consisted of the family-specific regions and the repeats (Taylor *et al.*, 1995). Thus, the repeat regions appear to be the immune-dominant regions of the protein. It is clear that anti-repeat regions antibodies may inhibit parasite growth (Epping *et al.*, 1988), but such antibodies may be much less effective against variant repeats. The conserved region has been shown to have a low frequency of non-synonymous nucleotide substitutions and are also poorly recognized by naturally acquired (Taylor *et al.*, 1995) and vaccine-elicited (Fluck *et al.*, 2004) antibodies. These data suggest a role for naturally acquired immunity in maintaining sequence diversity in the non-repetitive domains of MSP-2 dimorphic groups.

Since naturally acquired antibodies to MSP-2 which recognize predominantly polymorphic epitopes, have been shown to be associated with clinical immunity to malaria (Metzger *et al.*, 2003), sequence diversity in surface antigens of malaria parasites populations will have implications in determining susceptibility to the disease as well as for adequate design of MSP-2 based immunization strategy. For example, the GGSA motif present in 22% of the isolates sequenced in this study is a component of a 3D7-based malaria vaccine prototype used in clinical trials (Fluck *et al.*, 2007) and has been found to be relatively common in Africa (13.8% of 3D7-typed alleles), Asia (15.8%), and Oceania (11.5%), although it is substantially less prevalent in areas like Brazil (5.0%). Accordingly, recombinant antigens containing GGSA-type repeats are poorly recognized by antibodies of malaria-exposed subjects in areas like Brazil, while locally prevalent 3D7-type variants were readily recognized (Kanunfre *et al.*, 2003; Tonon *et al.*, 2004).

Therefore, it remains uncertain whether vaccines containing GGSA-type repeats may induce significant protection in areas where this MSP-2 variant is infrequent. Thus if immunity conferred by a monovalent vaccine is allele specific, a vaccine with high allele-specific efficacy would have low overall efficacy in populations where the target allele is in the minority. Without an understanding of the distribution of vaccine target haplotypes, this scenario could result in the premature abandonment of a promising vaccine that could be modified (perhaps by including additional target alleles) to be more universally protective. This possibility highlights the need to

include molecular endpoints in addition to conventional efficacy endpoints in clinical trials of malaria vaccines.

This is the first description of sequence diversity of field isolates of *Plasmodium falciparum* parasites in Lafia, north-central Nigeria and also to my knowledge, the first description in Nigeria. This information therefore, bridges an important gap in understanding the molecular characteristics of *P. falciparum* populations in Nigeria.

5.3 IL-18 promoter polymorphisms and disease outcome of malaria

Based on the historical presence of malaria, a large impact on the human genome has been exerted by the disease such that potentially harmful variants are preserved, largely because of the advantage offered in heterozygous individuals that are often protected from severe, complicated, and fatal malaria (Koch *et al.*, 2005; Kwiatkowski and Luoni, 2006; Hedrick, 2011). Polymorphic forms of a number of host genes involved in immunity have been associated with protection or susceptibility to malaria. It is thus expected that cytokine genes that regulate a Th1 dominant pathway may be associated with malaria. In recent years, increasing evidence has emerged from experimental and epidemiological data that IL-18, a pro-inflammatory cytokine involved in both innate and acquired immune responses, plays an important immune-regulatory role in malaria (Torre *et al.*, 2002a; Chaisavaneeyakorn *et al.*, 2003; Perkmann *et al.*, 2005; Torre, 2009). Information on the relationship between IL-18 polymorphisms and malaria is however limited. This study therefore investigated three known promoter polymorphisms in IL-18 at positions -656, -607 and -137 relative to the transcription start site.

All the three single nucleotide polymorphisms were detected among participants in the study region. The -656G/T and -607C/A loci were found to be in complete linkage disequilibrium. A higher frequency of the -607AA genotype was found among the asymptomatic group compared to participants in the severe malaria group. This is comparable to findings from south west England where AA genotypes were higher among controls compared to subjects with osteoarthritis (Hulin-Curtis *et al.*, 2011). It is also similar to a report from an earlier study that

showed a higher frequency of the AA genotype among controls in comparison to subjects with rheumatoid arthritis (Gracie *et al.*, 2005). In Kenya, the carriage of -607AA genotype was found to be associated with protection against severe malaria (Anyona *et al.*, 2011). Likewise, a higher prevalence of -137CC genotype was found among participants in the severe malaria group compared to the asymptomatic group. This is contrary to findings from Kenya where no difference was found in the distributions of the -137CC genotype between participants presenting with severe malarial anaemia and those in the non-severe malarial anaemia group, although carriers of the -137G/-607C (CG) haplotype were found to have increased susceptibility to severe malarial anaemia (Anyona *et al.*, 2011).

Functional assays have previously demonstrated that the production capacity of IL-18 by monocytes is significantly reduced in participants with -137C and -607A alleles (Giedraitis *et al.*, 2001; Arimitsu *et al.*, 2006). The -607C/A and -137G/C are believed to be located within a transcription initiation site and have consistently been associated with altered IL-18 transcriptional activity. These promoter regions are predicted to be the binding sites for cyclic AMP-responsive element-binding protein [cAMP] and the human histone H4 gene-specific transcription factor-1 [H4TF-1] respectively (Haus-Seuffert and Meisterernst, 2000; Giedraitis *et al.*, 2001). A change from G to C at position -137 changes the H4TF1 nuclear binding site into that for an unknown factor located within the granulocyte-macrophage colony-stimulating factor (GM-CSF) promoter, potentially reducing production of IL-18 (Giedraitis *et al.*, 2001). Furthermore, the C-to-A change at the -607 loci mediates transcriptional activity activity in response to cAMP by disrupting the binding site, thereby down-regulating transcription which may result in lower IL-18 production (Giedraitis *et al.*, 2001).

5.4 IL-18Rα polymorphisms and disease outcome of malaria

A total of three single nucleotide polymorphic loci were investigated at the promoter region of the IL-18Rα gene: -93C/T, -175G/A and -661C/T. No association was found between the 175G/A locus and disease outcome and the genotype distributions were in conformity with the Hardy-Weinberg equilibrium. Interestingly, the distributions of -93C/T and -661C/T in the severe malaria group significantly deviated from the Hardy-Weinberg equilibrium, even though

there was conformity with the Hardy-Weinberg equilibrium in the asymptomatic and uncomplicated malaria groups. The result showed that there was significant decrease in the frequency of heterozygotes for the two polymorphic loci (-93C/T and -661C/T), while the frequency of homozygotes increased in the severe malaria group. This may indicate a protective role for the heterozygotes (-93CT and -661CT genotypes) at the two loci.

To my knowledge, this is the first study to investigate the association of polymorphisms in the IL-18Rα gene and malaria and as such bridges an important gap in our knowledge of the contributions of host cytokine gene polymorphisms in malaria.

5.4 TNF-α polymorphisms and disease outcome of malaria

The TNF-308G/A and TNF-238G/A loci were investigated for associations with disease outcome in malaria in the study population. The genotype distributions were found to be in conformity with the Hardy-Weinberg equilibrium. The most common genotype at the two polymorphic sites was the GG genotypes. At both loci, the rare genotype AA was not found in the study population. No evidence was found for associations between the TNF-308G/A and TNF-238G/A polymorphisms in this study. This is in contrast to report from Gambia where the homozygotes for the TNF-308A allele were found to be at increased risk of cerebral malaria (McGuire *et al.*, 1994). In another study, Homozygousity for the TNF-308A allele was found to be a risk factor for pre-term birth and early childhood mortality and malaria morbidity in children in Western Kenya (Aidoo *et al.*, 2001). However, in another study in Mali, no association was found in the TNF-308G/A polymorhism with malaria (Cabantous *et al.*, 2006). Furthermore, associations were not found between the TNF-308G/A and TNF-238G/A loci and mild malaria or parasitaemia in Gabon (Migot-Nabias *et al.*, 2000) contrary to findings from Burkina Faso (Flori *et al.*, 2005).

CHAPTER SIX

CONCLUSION

The host-parasite interaction in malaria that leads to variability in individual susceptibility or resistance to the disease has been suggested to involve several host and parasite genetic factors. This study characterized and determined cytokine gene polymorphisms of IL-18, IL-18Rα and TNF-α as well as genetic polymorphisms in the *P. falciparum* MSP-2 gene in children in three categories: asymptomatic infection, uncomplicated malaria and severe malaria.

Data from this study showed that all cases of malaria among the study participants in Lafia were due to infection with *P. falciparum*. It was also found that multiplicity of infection was significantly lower in severe malaria group compared with uncomplicated malaria or asymptomatic infection; which probably suggests that in severe malaria, proliferation of certain dominant clones which could be more virulent, may be associated with disease pathology. In addition, the study showed a high allelic diversity of the MSP-2 gene in the study region, with a significantly higher distribution of 3D7 alleles in uncomplicated and severe malaria groups; which may indicate a higher risk of developing symptomatic malaria with increasing carriage of the 3D7 allele type isolates. Further analysis of the MSP-2 gene by sequencing showed several synonymous and non-synonymous amino acid substitutions in isolates from the study region, and with extensive sequence diversity in the 3D7 allele type.

Likewise, analysis of host cytokine gene polymorphisms showed a significantly higher distribution of the IL18 -607AA genotype in the asymptomatic group compared to the symptomatic groups; probably suggesting association with disease protection. This study finds no association of the promoter polymorphisms of TNF-α with disease outcome. However, promoter polymorphisms of the IL18Rα showed a significant deviation from the HWE in the distribution of genotypes at the -93C/T and -661C/T loci in the severe malaria group, with significantly higher frequency of homozygotes. This may indicate a protective role for the heterozygotes at the two loci. Thus, this study supports the idea that certain host and parasite genetic factors may indeed be associated with variable sucessibility or resistance to malaria.

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APPENDIX

Appendix 1: Nucleotide sequence of primers used in *Plasmodium spp* characterization

Primer Name	Nucleotide sequence
Fw PLU6	5'-TTA AAA TTG TTG CAG TTA AAA CG-3'
Rv PLU5	5'-CCT GTT GTT GCC TTA AAC TTC-3'
Fw FAL 1	5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3'
Rv FAL 2	5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3'
Fw MAL 1	5'-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3'
Rv MAL 2	5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3'
Fw OVA 1	5'-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3'
Rv OVA 2	5'-GGA AAA GGA CAC ATT AAT TGT ATC CTA ATG-3'

Appendix 2: Nucleotide sequence of primers used in MSA-2 genotyping study

Primer Name	Nucleotide sequence
MSA 2-1	5'-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3'
MSA 2-4	5'-TTA TAT GAA TAT GGC AAA AGA TAA AAC AAG-3'
MSA 2-2	5'-ACA TTC ATA AAC AAT GCT TAT AAT ATG AGT-3'
MSA 2-3	5'- GAT TAT TTC TAG AAC CAT GCA TAT GTC CAT -3'
FC 27-1	5'-GCA AAT GAA GGT TCT AAT ACT AAT AG-3'
FC 27-2	5'-GCT TTG GGT CCT TCT TCA GTT GAT TC-3'
3D7-1	5'-GCA GAA AGT AAG CCT TCT ACT GGT GCT-3'
3D7-2	5'-GAT TTG TTT CGG CAT TAT TAT GA -3'

Appendix 3: Nucleotide sequence of primers used for IL-18 gene promoter study

Primer Name	Nucleotide sequence
IL-18Pro -F	5'-GAC TTC CCG AAA TGA AAA CCC-3'
IL-18Pro -R	5'-ATG CAC TGG GAG ACA ATT CC-3'
IL-18Pro -Fb	5'-TCA AAT ATT TTA GGT CAG TCT TTG-3'

Appendix 4: Nucleotide sequence of primers used for IL-18Rα study

Primer Name	Nucleotide sequence
IL18R1 Ex1-F	5'-AGC CCA GGT TTG TGT GTT TC-3'
IL18R1 Ex1-Fb	5'-CCA CTG GGA CAC AGT CAA TG -3'
IL18R1 Ex1-R	5'- TCA GCA TCT TCA GTA GCC ACC-3'
IL18R1 Ex1-Rb	5'-ACA TTC TTC CTC AT TAC TCA TGA A -3'
IL18R1 Ex1-Rc	5'-GCC TGG TCT ACT AAA TCC TGC T -3'
IL18R1 Ex2-F	5'- TGC TAA CCT TGC TTC TTC ACC-3'
IL18R1 Ex2-R	5'- TTC AGA TTA CTG CAT ATT TGA GTT G-3'
IL18R1 Ex3-F	5'- AAG GGA AGA TGG GTG ATA TTT G-3'
IL18R1 Ex3-R	5'- ATG GTA GCT CTC AGC CCC TC-3'
IL18R1 Ex4-F	5'- GAT CCG CAG CTG CAT TAG AC-3'
IL18R1 Ex4-R	5'- TTT GGG GAT GAT TCA GGC-3'
IL18R1 Ex5-F	5'- GGA TCA CTG TAA TAT CAA TTT GGC-3'
IL18R1 Ex5-R	5'- GTG TGG TCA CAA CCC CAA C-3'

IL18R1 Ex6-F	5'- GGA ATC TTT GTT ACA TGA AAT GAG C-3'
IL18R1 Ex6-R	5'- TCA TAT TTA CGC TTG GAA GGC-3'
IL18R1 Ex7-F	5'-GCA CCA CGT TTT GCT TTA GG -3'
IL18R1 Ex7-R	5'-ATA CAC ATC AGC CAC CCA GTG -3'
IL18R1 Ex8-F	5'- TGT GAA TTC CCC TCT CAA GG-3'
IL18R1 Ex8-R	5'- TGG CCA TCT TTG AAA TGT CTC-3'
IL18R1 Ex9-F	5'- ACA AGC ACG TGA TGA TGG AC-3'
IL18R1 Ex9-R	5'- CCA TAG AAA ACC TCT CCC ACA G-3'
IL18R1 Ex10-F	5'- TTG CTT GGT TAG CAT GGG AG-3'
IL18R1 Ex10-R	5'- AAT GGG ATA GCT CTC TGG GG-3'
IL18R1 Ex11-F	5'- TGA CTT TTA TCT CAT GTT CCC C-3'
IL18R1 Ex11-R	5'- CAT CAC GTC CAG CTT CAC AC-3'

Appendix 5: Nucleotide sequence of primers used for TNF-α gene promoter study

Primer Name	Nucleotide sequence
TNFPro-F	5'-CCT GCA TCC TGT CTG GAA GT-3'
TNFPro-R	5'-CTC CCT ATC AGC GCA CAT CTT-3'
TNFPro-Fb	5'-ATC AGT CAG TGG CCC AGA AG-3'

Appendix 6: Ethical clearance granted by the Nasarawa State Ministry of Health, Lafia.

NASARAWA STATE OF NIGERIA
MINISTRY OF HEALTH

In replying, please quote reference and data all correspondence should be directed to the commissioner

Ministry of Health Headquarters
Private Mail Bag 032
Lafia, Nasarawa State.

Telephone:----------

S/MH/519/VOL.1/84

22nd December, 2005

Mr. Oyedeji Segun Isaac,
PhD Research Student,
Parasitoloty Unit,
Department of Zoology,
University of Ibadan,
Ibadan, Nigeria.

Re - Application for Ethical Clearance on the study titled:
"Determinants of Disease Outcome in Plasmodium Falciparum Infected Children in Lafia, North Central Nigeria"

Your letter dated 24th November, 2005 on the above subject matter refers.

2. Sequence to your interview with the Ethical Committee on the 21st December, 2005 and your submission of the modified version of the baseline study material to conform with the medical ethics.

3. You are by this letter granted the Ethical Clearance to go ahead and conduct your research in Lafia, Nasarawa State, with an instruction to maintain absolute confidentiality.

4. However, since you are not a licensed Medical Laboratory Scientist, you will be assisted by the Medical Laboratory Scientist/Medical Laboratory Technician in taking blood samples from infected children with a design consent form to each child's parent to give unqualified consent to have his/her blood taken for the research project.

5. At the end of the research, copies of the research document be given to the Ministry for her keep, information and utilization where necessary, please.

Dr. G. C. Monday,
Director, Clinical Services,
Chairman, Ethical Committee,
For: Hon. Commissioner

Appendix 7: Ethical clearance granted by the Dalhatu Araf Specialist Hospital, Lafia.

DALHATU ARAF SPECIALIST HOSPITAL, LAFIA.

RESEARCH PLANNING AND CONTINUING MEDICAL EDUCATION COMMITTEE.

Ref. No. DASH/ADM/MR/VOL.I/0001

TO: OYEDEJI SEGUN ISAAC
Pavasitology Unit
Department of Zoology,
University of Ibadan, Nigeria.

19th December, 2005

RE: APPLICATION FOR ETHICAL CLEARANCES

With reference to your application dated 1st December, 2005 on the research proposal titled: *Determinants of disease outcome in p. falcipavum infected children in Lafia; North-central Nigeria* and your appearance before the research committee on 8th November, 2005.

Management has given approval for you to proceed on your research topic as indicated following recommendation from the research committee. However, you are required to obtain separate approval for use of patients and facilities from the department(s) you intend to use for your research, please.

Signed
Committee's Chairman

CC CMD
DOA