DETECTION OF INTERLEUKIN-10 POLYMORPHISM AND *Plasmodium falciparum* INFECTION IN ARDAMATA IDP CAMP AL-GENEINA TOWN SUDAN

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2020

DETECTION OF INTERLEUKIN-10 POLYMORPHISM AND *Plasmodium falciparum* INFECTION IN ARDAMATA IDP CAMP AL-GENEINA TOWN SUDAN

by

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Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science (Biomedicine) Mixed Mode

AUGUST 2020

ACKNOWLEDGEMENT

I am grateful to Allah, the Almighty, for giving me the privilege and honor of doing this study. For the most part, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Rapeah Suppian for her continued support, patience, motivation, enthusiasm and immense knowledge. Her guidance has helped me to research and write this thesis all the time. I couldn't have imagined that my thesis would have been better supervised and mentored. Besides my co-supervisor, I would like to thank Dr. Khalid Mohamed Ali for his encouragement, insightful remarks and helpful guidance.

My sincere thanks also go to Dr. Wong Weng Kin and Assoc. Prof. Dr. See Too Wei Cun for his kind support, particularly during my graduation project. My special thanks also go to my beloved wife Fardowsa Abdi and my children, Safwan, Abdulkadir and Abdulsalam, for their emotional support and prayers before and during this struggle. Last but not least, I would like to thank my family: my parents Amina and Ali, and my elder brother Muktar Ali, for supporting me throughout my life and education.

TABLE OF CONTENT

ACKNOWLEDGEMENT ii

TABLE OF CONTENT iii

LIST OF TABLES v

LIST OF FIGURES vi

LIST OF ABBREVIATIONS AND ACRONYMNS vii

LIST OF SYMBOLS AND UNITS viii

ABSTRAK ix

ABSTRACT xi

CHAPTER 1 INTRODUCTION 1

1.1 Study background 1

1.2 Objective of the study 2

1.2.1 General objective 2

1.2.2 Specific objectives 3

2.1 Malaria 4

2.1.1 Global malaria 4

2.1.2 Malaria in Sudan 5

2.2 Malaria parasite 7

2.2.1 Plasmodium life cycle 7

2.3 Malaria prevention and control 9

2.3.1 Anti-malarial drugs 9

2.3.2 Vector control strategies 10

2.4 Pathophysiology of malaria 10

2.5 Immunity to malaria 12

2.5.1 Innate immunity to malaria 13

2.5.2 Adaptive immunity to malaria 14

2.6 The role of cytokines in immune pathogenesis of malaria 16

2.6.1 Interleukin 10 (IL-10) 18

2.6.2 Genetic polymorphism of Interleukin-10 20

CHAPTER 3 MATERIALS AND METHODS 22

3.1 Materials 22

3.1.1 Equipment and reagents 22
3.2.1(a) Ethanol 70% (v/v) 23
3.2.2(b) 10X Tris Borate EDTA (TBE) buffer 24
3.3 Methodology 24
3.3.1 Study Area 24
3.3.2 Study design 24
3.3.3 Sample size 24
3.3.4 Ethical Statements 24
3.3.5 DNA Extraction from archived blood samples 25
3.3.6 Primers preparation 26
3.3.7 Parasite identification using nested polymerase chain reaction (nPCR) 26
3.3.8 Agarose gel electrophoresis 27
3.3.9 Genotyping of 1L10-(1082A/G) SNP gene polymorphism 28
CHAPTER 4 RESULTS 29
4.1 Confirmation of malaria infection and *P. falciparum* identification by nested PCR 29
4.2 Comparison between nested PCR result with ICT and microscopic data 30
4.3 IL-10 gene (1082A/G) polymorphism in Sudanese malarial patients 30
CHAPTER 5 DISCUSSION 33
CHAPTER 6 CONCLUSION 37
REFERENCES 39

LIST OF TABLES

Table 3.1: List of chemicals, kits and consumables 22

Table 3.2: List of laboratory apparatus... 23

Table 3.3: List of primers used for parasite detection 26

Table 3.4: Primer for IL-10 1082A/G polymorphism 28

Table 4.1: Comparison between ICT, microscopic examination and PCR in malaria detection 30

Table 4.2: Summary of IL-10 genotyping 32

LIST OF FIGURES

Figure 1.1: Flow chart of the study 3

Figure 2.1: Global malaria distribution (Source: WHO, 2016). 6

Figure 2.2 Life cycle of a *Plasmodium* parasite (Source: CDC, 2016). 9

Figure 4.1 Agarose gel electrophoresis of blood samples for *P. falciparum* identification. 29

Figure 4.2: Agarose gel electrophoresis showing PCR products of IL-10 gene (1082A/G) polymorphism in *P. falciparum* positive samples and malaria negative samples 31

LIST OF ABBREVIATIONS AND ACRONYMNS

APC	Antigen Presenting Cell
°C	Degree Celsius
CSP	Circumsporozoite protein
CTL	Cytotoxic T-lymphocytes
ddH2O	double distilled water
e.g.	For example (Latin: exempli gratia)
IFN-g	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
PCR	Polymerase chain reaction
Pf	Plasmodium falciparum
RT	Room temperature
TNF- α	Tumour necrosis factor α
v/v	Volume to volume
WHO	World Health Organization

LIST OF SYMBOLS AND UNITS

bp	Base pairs
cm	Centimetre
et al	et alii— ‘and others’
g	Gram
kDa	Kilodalton
L	Litre
M	Molar
μ	Micro-
μg	Microgram
μL	Microlitre
min	Minutes
mL	Millilitre
mM	Millimolar
μg/mL	Microgram per millilitre
%	Percentage
Rpm	Revolutions per minute
Sec	Seconds
V	Voltage
X	Times
&	And
≈	Approximately

PENGESANAN POLIMORPHISME INTERLEUKIN-10 DEGAN JANKITAN *Plasmodium falciparum* DI ARDAMATA IDP CAMP DI SUDAN BANDAR AL-GENEINA

ABSTRAK

Malaria merupakan penyebab utama kematian dalam kalangan penduduk Sudan. Penyakit ini telah menyumbang kepada 7.5 juta kes malaria dan 35,000 kematian setiap tahun, terutama akibat jangkitan *Plasmodium falciparum* (*P. falciparum*). Genetik perumah dan patogen merupakan dua faktor penting yang menyumbang kepada keparahan sesuatu penyakit. Dalam promoter atau kawasan pengkodan gen sitokin, polimorfisme nuklear tunggal (SNP) mengubah pengaktifan transkripsi dan menghasilkan sitokin yang berbeza. Interleukin-10 (IL-10) merupakan sitokin anti-radang yang mungkin memainkan peranan penting dalam jangkitan *P. falciparum*. IL-10 mempunyai polimorfik yang tinggi pada peringkat promoter, antaranya pada gen 1082 G/A, 819 C/T dan 592 C/A. Walau bagaimanapun, polimorfisme yang paling kerap dikaji adalah pada gen 1082 G/A kerana kemampuan polimorfisme pada gen ini dalam merangsang penghasilan IL-10 dan berkait rapat dengan kerentanan atau perlindungan terhadap penyakit. Oleh itu, adalah penting untuk memahami kaitan antara jangkitan *P. falciparum* dan polimorfisme gen IL-10 bagi menentukan sama ada polimorfisme ini ada kaitan dengan jangkitan *P. falciparum*, supaya vaksin yang lebih berkesan dapat dibangunkan terhadap parasit ini. Ujian rantaian polimerase (PCR) merupakan salah satu kaedah yang paling biasa digunakan untuk membezakan parasit malaria daripada pelbagai spesies dan untuk pengesanan polimorfisme gen pada spesies tertentu. Kajian ini bertujuan untuk menyiasat kaitan antara jangkitan *P. falciparum* dengan polimorfisme gen IL-10-1082 G/A dalam kalangan penduduk Sudan. Tiga puluh empat (34) darah pesakit malaria dari Kem IDP Ardamata di

Bandar Al-Geneina, Sudan terlibat dalam kajian ini. Sampel darah pada awalnya ditentukan sama ada positif malaria atau tidak, sebelum disahkan berpunca daripada jangkitan

P. falciparum dengan menggunakan kaedah PCR bersarang (nPCR). PCR kemudian dilakukan untuk menilai polimorfisme gen IL-10 -1082 G/A pada sampel tersebut. nPCR menunjukkan daripada 34 sampel yang dikaji, 17 adalah positif malaria, dan 17 adalah negatif. Kesemua 17-sampel positif malaria telah disahkan dijangkiti *P. falciparum*. Walaupun nPCR dikatakan lebih sensitif dan spesifik, jumlah sampel positif yang dikesan menggunakan kaedah ini kurang berbanding kaedah mikroskopi dan analisis ICT. Analisis PCR juga mengesahkan tidak terdapat mutasi IL-10 -1082G / A pada sampel DNA yang positif malaria, yang menunjukkan tidak ada hubungan yang signifikan antara polimorfisme IL-10 -1082G / A dengan jangkitan *P. falciparum* dalam sampel yang dikaji.

DETECTION OF INTERLEUKIN-10 POLYMORPHISM AND *Plasmodium falciparum* INFECTION IN ARDAMATA IDP CAMP IN AL-GENEINA TOWN SUDAN

ABSTRACT

Malaria is the main cause of Sudanese morbidity and death. It contributes an estimated 7.5 million cases and 35,000 deaths annually, mainly because of *Plasmodium falciparum* (*P. falciparum*). Host and pathogen genetic factors are linked to the severity of the disease. In the promoter or coding region of cytokine genes, single nuclear polymorphisms (SNPs) alter their transcriptional activation and produce differential cytokine. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that may play an active role in *P. falciparum* infection. IL-10 polymorphisms are associated with several diseases, including malaria. IL-10 has a highly polymorphic promoter with variations gene transcription, including 1082 G/A, 819 C/T, and 592 C/A. However, polymorphism at 1082 G/A gene is the most extensively studied because of this polymorphism's ability to increase IL-10 production, which correlated with susceptibility or protection against infection. Therefore, it is important to understand the association of *P. falciparum* infection and IL-10 gene polymorphism to confirm if this polymorphism plays a role in the incidence of *P. falciparum* infection to develop a more efficient vaccine against the disease. Polymerase Chain Response (PCR) is one of the most common methods for distinguishing malaria parasites from species levels and gene polymorphism detection in specific species. This study aimed at investigating the association of *P. falciparum* infection with IL-10 gene promoter 1082 G/A polymorphism in the Sudanese population. Thirty-four (34) blood smears of malarial patients from the Ardamata IDP Camp in Al-Geneina Town, Sudan, were enrolled in

this study. The samples were first confirmed with nested PCR (nPCR) for *Plasmodium* infection before proceeding with *P. falciparum* identification. Further, PCR was then performed to evaluate IL-10 gene 1082 G/A polymorphism in the samples. nPCR showed that out of 34 samples, 17 were malaria positive, and 17 were negative. Interestingly, all the 17-malaria positive samples were confirmed as *P. falciparum*. Although nPCR has been claimed to be more sensitive and specific, the number of positive samples detected using this method was less than microscopy and ICT analysis. PCR analysis also confirmed that no gene mutation occurs in the malaria positive samples, indicating there was no significant association between IL-10 -1082G/A polymorphism and *P. falciparum* infection in these samples.

CHAPTER 1

INTRODUCTION

1.1 Study background

Malaria is a severe tropical parasitic disease. It is the main cause of morbidity and mortality in Sudan, contributing to 50% of all malaria cases in the country (Abdalla et al., 2007). malaria in Sudan was estimated to be about 9 million incidents in 2002, and the number of deaths due to malaria was about 44,000. Children under five years of age had the highest burden and males had the highest incidence and mortality (Abdalla et al., 2007). Symptomatic malaria accounts for 20-40% of outpatient clinic visits and around 30% of hospital admissions (Elfatih Mohd Malik et al., 2016).

The whole population of Sudan is at risk of malaria, although this occurs with different places. In the northern and western states, malaria is mainly low to moderate. In southern Sudan, malaria is moderate to high or highly intense, generally with the perennial transmission (Malik et al., 2006). In eastern Sudan, malaria transmission and intensity are perennial and moderate (Himeidan et al., 2005). In addition, there was a significant positive correlation between malaria cases with heavy rains (Himeidan et al., 2007). Malaria in Sudan mainly causes by *Plasmodium falciparum* (*P. falciparum*), whereas *P. ovale* is occasionally distributed. *P. malariae* is mainly correlated with Southern Sudan, while in Eastern Sudan, *P. vivax* is widely spread (Mohamed et al., 2016).

Human malarial parasites develop through two stages in humans: a liver stage and a blood stage. However, the asexual blood stage of the parasite is the cause of malarial

pathologies. Therefore, it is important to prevent the replication of the parasite at this stage. It is necessary to understand the mechanism of protective immunity against the blood stage of the parasite

during malaria infection. Over the centuries, the high prevalence of malaria infections in Africa has placed selective pressure on human genome. Several studies have shown that polymorphisms in cytokines lead to *Plasmodium* infections (Medina et al., 2011; de Paulo Ribeiro et al., 2016). Interleukin-10 (IL-10) is reported to be elevated in individuals with *Plasmodium* infection (Medina et al., 2011; Riccio et al., 2013). IL-10 is a dominant cytokine that was reported as being related to reduced risk of cerebral malaria and extreme anemia associated with malaria (Couper et al., 2008). The IL-10 gene is situated on chromosome 1q31-q32 of the promoter region with several variants associated with variable IL-10 manufacturing and infection phenotypes (Jin et al., 20120). Thus far, very limited studies investigating the combination of IL-10 genetic variants in Sudanese population with the incidence of *P. falciparum* clinical malaria. Therefore, in this study the association between IL-10 gene-polymorphisms in the Sudanese population will be determined to get more clear information on the SNP affect the disease susceptibility.

1.2 Objective of the study

1.2.1 General objective

To determine the association between IL-10 gene polymorphism and *P. falciparum* infection in malaria patients of Ardamata IDP Camp, Al-Geneina City, Sudan.

1.2.2 Specific objectives

1. To determine *Plasmodium falciparum* infection in blood samples of malaria patients from Ardamata IDP Camp, Al-Geneina City, Sudan using nested polymerase chain reaction (nPCR)
2. To determine the association between IL-10 gene (1082A/G) polymorphism with *Plasmodium falciparum* infection in malaria patients of Ardamata IDP Camp, Al-Geneina City, Sudan

1.3 Flowchart of the study

All the activities are summarized in Figure 1.1.

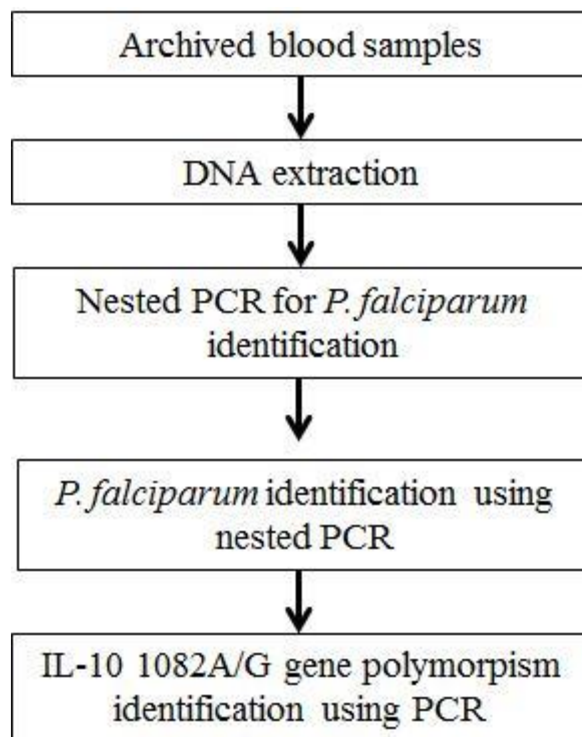

Figure 1.1: Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria

Malaria is a vector borne infectious disease caused by protozoan parasites of the genus *Plasmodium* and is transmitted by an infected female Anopheles mosquito. Until the late 19th Century, the contributory agent for malaria was largely unknown. The medieval Italian term, mala aria, meaning "bad air", or commonly known as march fever (Reiter & Compartments, 2001). was used to describe the flu-like symptoms, such as headaches, fever, shivering, joint pain, vomiting, haemolytic anaemia, jaundice, haemoglobin in the urine, retinal damage, and convulsions of patients infected with malaria (Price, 2008). It was not until 1880 that the true cause of malaria was discovered (Ghosh, 2014).

2.1.1 Global malaria

Malaria is a fatal disease. People who get malaria are typically, very sick with high fevers, shaking, chills, and flu-like illness. Because malaria causes so much illness and death, the disease is a great drain on many national economies (Suliman et al., 2016). At global scale, 3.4 billion people are at risk of contracting malaria (Figure 2.1). As reported by the WHO (World Health Organization, 2016), the majority of malaria cases and deaths are found in sub-Saharan Africa. In 2015, the region was home to 90% of malaria cases and 92% of malaria deaths. Children under five years of age are particularly vulnerable, accounting for an estimated 70% of all malaria deaths. Diagnostic testing enables health providers to rapidly detect malaria and prescribe life-saving treatment (316 ,هبر C.E.).. In 2015, 51% of children at a public health facility in 22 African countries received a diagnostic test for

malaria, compared to 29% in 2010 (Kastrati & Sahiti, 2019). Despite decades of control measures and intensive interventions, malaria continues to cause extensive morbidity and mortality throughout the widespread regions where it is endemic. The vast majority of research has been directed towards *P. falciparum*, which is the primary contributor to disease burden throughout sub- Saharan Africa (Sama et al., 2006).

Most countries of the Asia-Pacific region have *P. vivax* malaria infection and they account for over 80% of the global burden of *P. vivax* malaria (Mendis et al., 2001). Among these countries, India alone accounts for an estimated 24 million cases per year (WHO Factsheet, 2012). South America is also home to a significant number of malaria cases, where an estimated 427,000 cases, with 82 recorded deaths were reported in 2013 (WHO world malaria report, 2014). On the other hand, Europe has achieved a complete interruption of indigenous malaria transmission with the drastic drop of indigenous malaria cases from 90,712 in 1995 to zero cases in 2015 (WHO, 2015a). Despite these mixed pictures of success and failure with malaria painted above, one interesting fact is that the disease is no more confined to the so-called malaria-endemic regions; it has gone beyond its traditional enclaves. Increased globalization and population dynamics have increased the risk of malaria for many people from non-endemic areas of the world making it a global menace(Gowda, 2007). For example, an eight-year study found that most patients with severe malaria in Europe were tourists or migrants acquiring the infection in West Africa (Kurth et al., 2017).

2.1.2 Malaria in Sudan

Malaria mortality rates have fallen by 47% globally since 2000 and by 54% in the WHO African Region. Based on WHO estimation in 2010, the number of deaths due

to malaria in the Eastern Mediterranean Region were 15 000 (range 7000–24000 deaths), of which 70% were in children under 5 years of age. The reported deaths due to malaria were only 1148 with more than 53% from Sudan. The majority of malaria cases occur in Sudan are caused by *P. falciparum* ~95%, while cases caused by *P. vivax* is relatively rare, only ~5% (Suliman et al., 2016). However, current data showed that the incidence of *P. vivax* infection is rising in Sudan and has increased from about 5% in 2013 to 26% of the total malaria cases (Elgoraish et al., 2019).

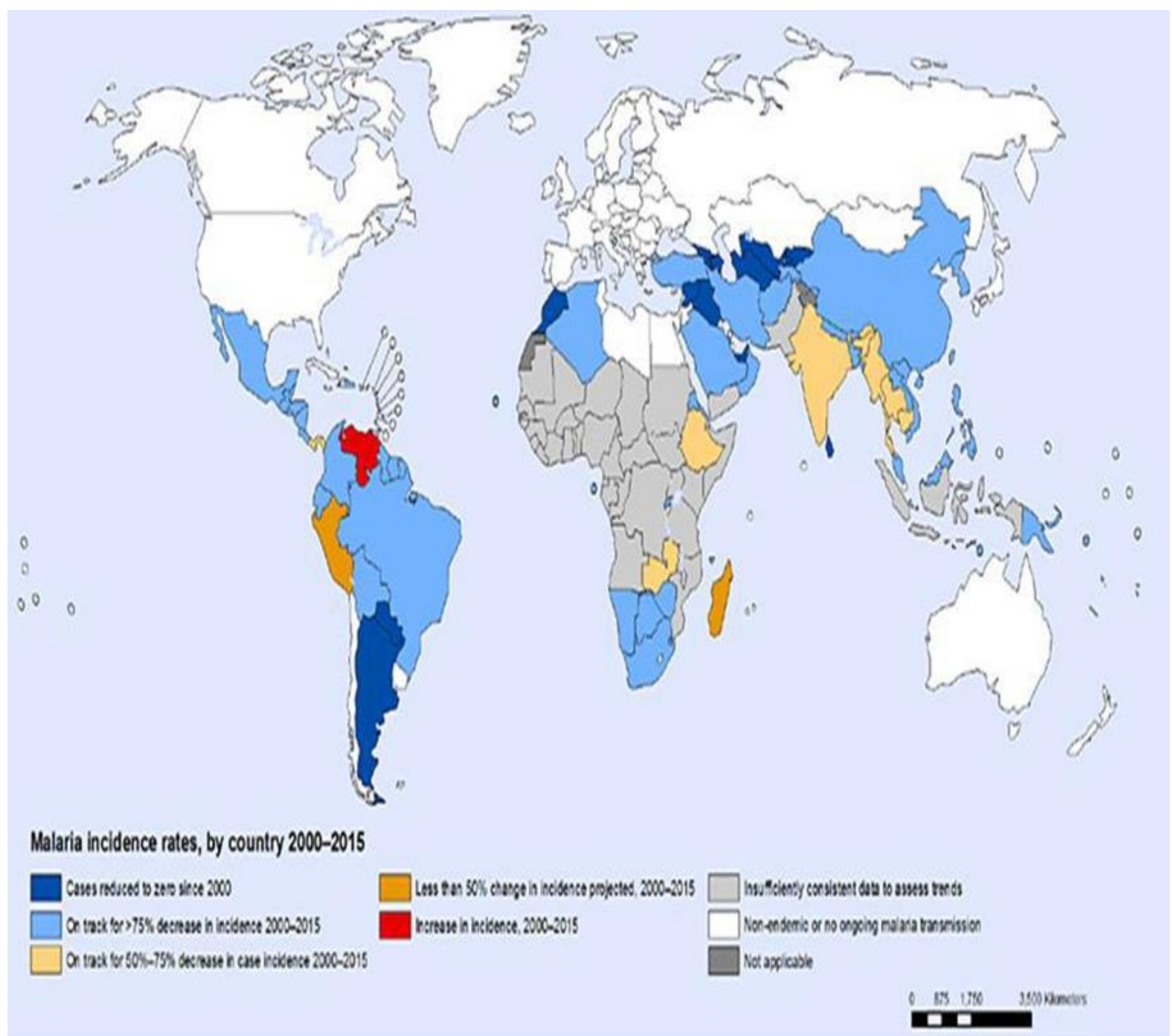

Figure 2.1: Global malaria distribution (Source: WHO, 2016).

2.2 Malaria parasite

An understanding of the *Plasmodium* life cycle is a major tool in malaria vaccine development. As pointed earlier, human malaria is caused by members of the Plasmodium species, a genus of unicellular parasites (CDC, 2015b). The malaria parasite, an obligate intracellular parasite has a complex life cycle which utilises two hosts in the cycle: a Dipteran insect; the Anopheline host and the human host. During the *Plasmodium* life cycle, sexual stage reproduction occurs in the Anopheles mosquito, making it the definitive host (CDC, 2015c). This host requirement is fundamental to all the five identified species of *Plasmodia* causing malaria in humans namely: *P. falciparum*, the causative agent of malignant tertian malaria and the one responsible for the highest global malaria rate, *P. vivax*, the major cause of benign tertian malaria, *P. ovale*, the minor cause of benign tertian malaria, *P. malariae*, the identified cause of benign quartan malaria and the relatively newer addition, *P. knowlesi*, the causative agent of severe quotidian malaria in South East Asia (Collins, 2012).

2.2.1 Plasmodium life cycle

The malaria parasite life cycle involves two human and one mosquito stages (Figure 2.2). The first human asymptomatic stage called pre-erythrocytic or exo-erythrocytic stage occurs in the liver, while the second; the symptomatic stage occurs in the red blood cells and thus is called the erythrocytic stage (Haque & Engwerda, 2014). The stage in the mosquitos, called the sporogony stage involves the gametes fusion and oocyst formation. During her egg nurturing blood meal, a malaria-infected female Anopheles mosquito inoculates the *Plasmodium* cells called sporozoites into the

human host. The Sporozoites travel towards the liver and infect the liver cells. The sporozoites now replicate and mature into schizonts, which rupture (Peter et al., 2010) and release merozoites in a process called exo-erythrocytic schizogony. The released merozoites invade the red blood cells and infect them. Within the red cells, the parasites undergo asexual multiplication, a process called erythrocytic schizogony, expanding the trophozoites population six to 20 times per cycle (Simpson et al., 2002). The ring stage trophozoites now mature into schizonts, which rupture releasing merozoites which infect more red blood cells. The manifestations of clinical symptoms of malaria, including fever occur at the point of rupture of the infected erythrocytes and the release of erythrocyte and parasite debris. Thus, the blood-stage parasites are responsible for the clinical manifestations of malaria, which can occur as early as three days from the beginning of the erythrocytic stage (Simpson et al., 2002).

At the third stage, the gametocyte, or sexual stage, in a small percentage of the merozoite-infected blood cells, the asexual reproduction would stop, and the *plasmodia* now mature into sexual forms of the parasites known as male and female gametocytes (Carter & Miller, 1979). The gametocytes which are divided into microgametocytes, the male gametes and macrogametocytes, the female, assume an Anopheles mosquito ingests various shapes as they mature during a blood meal. They travel to the mosquito's stomach. Within the mosquito's stomach, the microgametocytes fuse into the macrogametocytes forming a zygote which undergoes some morphologic changes and turn into an ookinete. These ookinetes invade the midgut wall of the mosquito, where they replicate and mature into oocysts. The matured oocysts then grow and rupture to release sporozoites. This cycle of multiplication in the mosquito is called the sporogonic cycle. All *Plasmodium* species

undergo a similar life cycle except *P. vivax* and *P. ovale* which have a quiescent liver-stage called the hypnozoites.

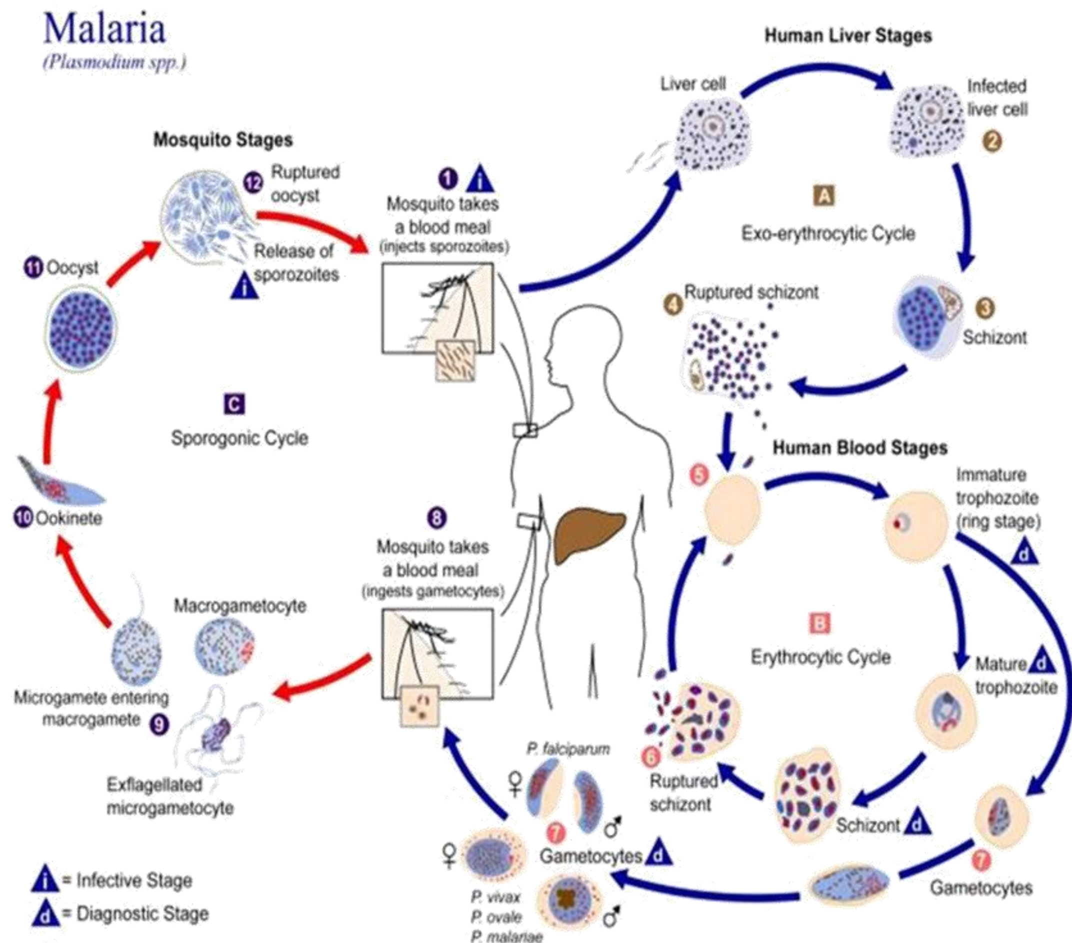

Figure 2.2 Life cycle of a *Plasmodium* parasite (Source: CDC, 2016).

2.3 Malaria prevention and control

2.3.1 Anti-malarial drugs

The only drug known to act effectively against pre-erythrocytic forms of *Plasmodium* is Primaquine which eliminates hypnozoites (Wells et al., 2010). Drugs that act against the blood-stage parasites are chloroquine, pyrimethamine, quinine, sulfadoxine, mefloquine,

or halofantrine. As a result of the appearance of parasites that were multi-drug resistant, various drug combinations were used, and the antimalarial properties of Artemisinin were discovered. Primaquine effectively eliminates the gametocytes of all *Plasmodium* (White, 2008). However, chloroquine and quinine are effective against *P. malariae* and *P. vivax* but not *P. falciparum* (Hunja, 2013).

2.3.2 Vector control strategies

Insecticide-treated nets (ITNs) kill or remove mosquitoes that come into contact with them as they are insecticide-treated nets. One type of net is a net treated conventionally, infused with an insecticide that has a temporary effect, and treatment needs to be repeated every three months or at least once a year to ensure a stable insecticide effect. Another type is a long-lasting insecticide-treated net (LLINs), impregnated with insecticide with the ability to maintain its efficacy for up to three to five years (WHO, 2007). Compared to untreated nets, ITNs have been shown to help reduce malaria cases by up to 50% (Greenwood et al., 2008). ITNs have helped to reduce child mortality by approximately 44% (Fegan et al., 2007). Residual indoor spraying (IRS) removes or kills mosquitoes that enter and rest in areas where the insecticide has been used. It is therefore considered to be most effective against endophilic vectors such as Anopheles gambiae (Pluess et al., 2010).

2.4 Pathophysiology of malaria

A single bite of infected mosquito inoculates about 100–200 sporozoites in human skin, most of which are destroyed by an innate immune system (Risco-Castillo et al., 2015). The blood-stage merozoite erythrocyte invasion process defines the onset of

malaria pathogenesis and all symptoms associated with the disease (White et al., 2014b). Successful invasion of red blood cells leads to the development of anaemia, which is the most frequent complication of malaria (Quintero et al., 2011), because of haemolysis and splenic sequestration of malaria-parasitized red blood cells. This destruction of red blood cells results in the release of toxins that cause endothelial damage and stimulate the production of pro-inflammatory cytokines by macrophages and other immunological cells, such as IL-1, IL-12, TNF-5-007 and the malaria-activating factor (Dondorp et al., 2004) responsible for malaria-associated pathophysiology (CDC, 2015a).

In uncomplicated malaria, red cell destruction is usually compensated for by erythropoiesis. Anaemia develops when this compensation is not sufficient (Chang et al., 2004). Increased sequestration of red blood cells compromises the blood supply, which in turn causes hypoxia and lactic acidosis and causes metabolic disturbances. These complications may affect multiple organs of the body, such as the brain, lungs, and placenta in the uterus. Hypoxia in the organs triggers vasodilation to improve the flow of blood through the affected organs. The immune response of the host and the parasite virulence mechanisms determine the progression of malaria. Thus, malaria may be asymptomatic or exhibit a prodrome of fever , chills, sweating, headache , and muscle aches that may degenerate into some serious life-threatening complications such as hyperparasitemia, hypoglycaemia, and hyperlactaemia that may lead to kidney failure, metabolic acidosis, cerebral malaria, severe malaria anemia, and respiratory distress in some cases (WHO, 2000).

2.5 Immunity to malaria

Humans with no previous experience of malaria almost invariably become ill on their first exposure to the parasite. They develop a febrile illness, which may become severe and, in a proportion of cases, may lead to death. In malaria-endemic areas, young children are particularly susceptible, and it has been estimated that a quarter of all childhood deaths are due to malaria (Duffy & Mutabingwa, 2005). However, with exposure, older children and adults develop essentially complete protection from severe illness and death, although sterile immunity is probably never achieved. Although vaccines may not be limited to mimicking natural immunity, as clear a picture as possible of the mechanisms of such immunity is an important starting point.

An important body of literature was generated using induced malaria in the treatment of neurosyphilis in the early twentieth century. Those studies have informed the paradigm of how humans respond to malaria, and re-analysis of the data has provided new insights into the immune response (Collins et al., 2004). At present, induced malaria in volunteers forms an important aspect of testing of some malaria vaccines and offers the opportunity of detailed studies of possible protective mechanisms (Krause et al., 2007). After an infected mosquito bite through the skin, sporozoites rapidly move from the dermis to the liver, where they go through an asymptomatic stage of rapid division before the parasite reenters the bloodstream. In the blood, exponential expansion of parasite populations leads to febrile illness. Typically, acute infection is controlled, and chronic infection is established at reasonably low parasite density, with intermittent episodes of fever associated with peaks of higher parasitemia (Miller et al., 2000). Such peaks are of progressively lower density until the infection is eliminated, usually after many months. There is the relatively rapid acquisition of immunity to the homologous

parasite, demonstrated as more rapid control of successive infections at lower parasite densities and less-severe or even absent clinical illness. Although less profound in terms of parasitological indices, there is also evidence of early acquisition of heterologous immunity, particularly in terms of clinical symptoms (Michon et al., 2007).

2.5.1 Innate immunity to malaria

Innate immune responses being the first line of defense against invading pathogens limit the initial phase of parasite replication, and lead to parasite elimination and the resolution of infection (Urban et al., 2005) as well as improve host survival (D'Ombrain et al., 2008) via IFN-γ derived from natural killer (NK) cells and/or γδ T cells (Fell and Smith, 1998). The wave of parasitaemia elicited following the inoculation of sporozoites is believed to be controlled by the onset of innate immune responses preceding the development of adaptive immune responses (Stevenson & Riley, 2004). Generally, malaria is associated with increased pro-inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α (Crutcher et al., 1995), as well as the anti-inflammatory cytokines; IL-4 and IL-10 (Rodrigues-da-Silva et al., 2014). During the early phase of the *P. falciparum* infection, Th1 subsets are activated in which IFN-γ, IL-12 and TNF-α are elevated, controlling primary parasitaemia, while the Th2 subset modulates cytokine IL-4 rise in the later stage of the infection (Mohapatra et al., 2014). In fact, malaria pathogenesis and the observed clinical manifestations are essentially due to excess inflammation (Stanisic et al., 2014) which can be elicited by *Plasmodium* parasite and vaccine administration through a poorly defined pattern (Kasturi, 2011). This immune reaction is initiated by the recognition of pathogen-associated molecular patterns, PAMPs by the specialised pattern recognition receptors, PRRs (Kumar et al., 2011) such as the two important sentinel cells; macrophages, which effect their functions via phagocytosis, cytokines production and

presentation of antigens (den Haan & Kraal, 2012) and dendritic cells (DCs) which are also primarily involved in recognizing pathogens, initiating cytokine responses, and processing and presenting antigens (Shortman & Heath, 2010). Once parasitaemia is under control the pro-inflammatory phase of the innate immune response is countered by a timed response by type 2 cytokines such as IL-10, transforming growth factor (TGF)-β, and IL-4, to avoid inflammatory host damage (Clark et al., 2006). Essentially, immunity to infection with blood-stage malaria is dependent on a well-coordinated, accurately timed release of IFN-γ, TNF-α, IL-12, in both innate, (Riley & Stewart, 2013) and adaptive immune responses (Langhorne et al., 2004) acting via dendritic, natural killer, CD4+ T helper, and B cells (McCall & Sauerwein, 2010). Thus, higher levels circulatory pro-inflammatory cytokines, such as TNF-α and IL-6 are observed in severe malaria alongside low levels of anti-inflammatory cytokines, such as IL-10 (Mbengue et al., 2016). While severe malarial anaemia induces higher production of IL-10 and TNF-α than cerebral malaria (Philippe et al., 2012). In essence, the principal component of innate immunity utilised in controlling malaria blood-stage infection and parasitic replication involves dendritic cells (Stevenson and Urban, 2006), monocytes/macrophages and neutrophils which act via phagocytic activity and antigen presentation (Serghides et al., 2003; Stevenson & Riley, 2004) as well as the release of cytokine and other inflammatory mediators (Chua et al., 2013).

2.5.2 Adaptive immunity to malaria

Naturally acquired immunity to malaria takes as long as 10-15 years of exposure to develop (Casals-Pascual et al., 2006). However, this acquired immunity is non-sterile and is species-, stage-, strain-, and variant-specific (Casals-Pascual et al., 2006). Residents in malaria-endemic areas frequently have premunition (parasitemia and

antibodies without symptoms (Casals-Pascual et al., 2006). Acquired immunity to malaria involves both antibody-mediated and cell-mediated immunity.

Antibodies play a crucial role in mediating acquired immunity to malaria. Blood stage merozoite antigens and variant surface antigens (VSA) expressed on infected erythrocytes (IE) are important targets of this protective immunity. Antibodies to merozoite antigens inhibit invasion of red blood cells (RBCs), prevent intra-erythrocytic growth (Richards & Beeson, 2009), and promote opsonization for phagocytic clearance and complement fixation (Osier et al., 2014). Antibodies to merozoite antigens of sufficient magnitude and function appear to contribute to immunity (Richards et al., 2013). In young children or those with limited malaria exposure, they may instead act as biomarkers of malaria exposure (Richards et al., 2013; Reynaldi et al., 2019). with the potential to inform surveillance and control activities (Stanisic et al., 2015). Among the tested antigens, merozoite surface protein 1 (MSP1) is the most copious protein found on the merozoite (Proellocks et al., 2007). Crucial for the primary interaction between merozoites and RBCs in parasite invasion (Jaskiewicz et al., 2019). MSP1 is a major target of opsonizing following natural exposure (Blank et al., 2020). MSP2 is another abundant, GPI-anchored surface protein necessary for merozoite invasion. Increased IgG level against MSP2 was associated with increasing age, higher hemoglobin level, and reduced parasitemia suggesting its protective effect (O'Donnell et al., 2001). Erythrocyte binding antigen 175 (EBA175) is released from micronemes (Reynaldi et al., 2019), and aggregates at the apical region of the merozoite surface. Antibodies to the RIII-V region of EBA175 have also been associated with protection from malaria (Touré, Bisseye, et al., 2006; Additional Details on Decoupling, 2012). Rhoptry-derived Rh2A9 helps binding to the RBC receptors after the primary interaction between the RBC and merozoite surface proteins is completed. The level of IgG against Rh2A9 in children (5–14 years) was

associated with a lower risk of malaria (Abagna et al., 2018). Antibodies to VSA diminish malaria risk by obstructing cytoadherence to different host receptors (Quintana et al., 2016), and initiating phagocytic clearance of IE. Several studies have reported associations between levels of anti-VSA antibodies and protection against symptomatic malaria (Bull et al., 2013), but few studies have examined the dynamics of naturally occurring anti-VSA IgG in infants in a malaria-endemic setting (Nhabomba et al., 2014; Dobbs & Dent, 2016). In one study, children up to 24 months of age did not acquire antibodies to VSA but in a high-transmission area of Tanzania (Vestergaard et al., 2008). children had dramatic increases in antibodies to VSA from 1 to 2 years of age. Recent studies in Papua New Guinea suggest that acquired antibodies to VSA play an earlier role in immunity to malaria than antibodies to merozoite antigens (Dent et al., 2016).

2.6 The role of cytokines in immune pathogenesis of malaria

Malaria disease is characterized by the production of a wide range of cytokines. Studies suggest that these come from both the innate arm and the adaptive arm of the immune system. Because parasites multiply very rapidly, the innate arm likely mediates early cytokines responses against malaria. An early interferon-gamma (IFN-γ) response is important in protecting against the development of severe disease symptoms (Robinson et al., 2008; Hill et al., 2013). Natural killer cells (NK) have been implicated as the source of early proinflammatory responses such as IFN-γ and TNF-α against malaria parasites (Artavanis-Tsakonas & Riley, 2002; Korbel et al., 2005). Activation of innate immunity depends on the broad recognition of pathogens. This recognition is driven by receptors that recognize pathogen-associated molecular patterns (PAMPs). Among the best characterized of these pattern recognition receptors

are Toll-like receptors (TLR). Upon recognition of PAMPs, TRLs induces a signaling cascade leading to secretion of proinflammatory cytokines, chemokines, and interferons. Malaria parasite glycophosphoinostol (GPI) has been shown to interact with TLR2 and, to some extent, TLR4 (Franklin et al., 2009; Krishnegowda et al., 2005). While some studies suggest that hemozoin, a product of hemoglobin digestion by malaria parasites, interacts with TLR9 ((Wagner, 2010). It is also possible that malaria parasites can induce the innate immune system through interaction between other non-TLR receptors and AT-rich parasite DNA fragments (Sharma et al., 2012).

γ After innate responses mediate early resistance to malaria infection, the adaptive immunity takes over with CD4+ T-cells becoming the main producers of cytokines. Traditionally mature CD4+ T-cells are placed in two groups that are associated with distinct cytokine profiles. Production of interferon-alpha/gamma (INF-α/γ), lymphotoxin-α (TNFβ), interleukin-12 (IL-12) defines type 1 helper cells (Th1). It is associated with a strong cell-mediated immunity while the production of IL-4, 5, 6, 9, 10, and 13 define type 2 (Th2) associated with antibody production. However, because some T-cells and non-T-cells can produce both Th1 and Th2 cytokines, it may be more appropriate to talk of type 1 (TR1) or a type 2 response (TR2) (Vasel et al., 2014). In malaria, the TR1/TR2 dichotomy is most evident in the mouse-P. chabaudi model ((De Weerdt et al., 2001; Wagner, 2010). In this model, TR1 dominates the early response of mice to acute P. chabaudi infection, and parasite killing is mediated by INF-γ, tumor necrosis factor (TNF-α) and nitric oxide (NO) secreted by activated Th1 CD4+, macrophages, and natural killer cells. In P. berghei and P. yoelii models, TR1 response induced through sporozoites vaccination has been shown to provide strong protection against challenge infections (Wagner, 2010). On the other hand, a shift towards TR2 leads to less symptomatic chronic infections (Wagner, 2010). Along with inhibiting both INF-γ

and TNF-α, type 2 cytokines also stimulate B-cells to secrete antibodies (Fell and Smith 1998, (Wagner, 2010)(Wagner, 2010). The dual anti-parasite/ pathogenetic nature of TR1 is also evident in P. berghei infections (Wagner, 2010). Other murine-malaria models display variable tendencies towards either type of response during acute or chronic infections (Wagner, 2010).

The distinction between type 1 and 2 responses is less clear in human malaria. Increased IFN-γ is associated with the resolution of parasitemia in acute malaria episodes (Wagner, 2010). and delay reinfection (Wagner, 2010). at the same time, reduced levels accompany hyperparasitaemia in children ((Wagner, 2010). Similarly, levels of type 1 response were lower among Malawian malaria patients than among patients of other diseases, with a reverse trend being observed for the type 2 responses (Wagner, 2010). IFN-γ levels were found to be higher in pregnant women who did not have placental malaria t (Wagner, 2010). These observations argue for a possible anti-parasite role of TR1 in humans. Furthermore, CD4+ secreted IL-2 and TNF−α are associated with the protection provided by the experimental vaccine RTS'S (Wagner, 2010). On the other hand, IL-10 and IL-4, both type 2 cytokines, have been associated with protection against malarial anemia (Wagner, 2010). However, reduced secretion of INF-γ by immune T-cells in response to malaria led to the conclusion that reduced pathology in immune individuals may be attributable to the downregulation of TR1 cytokines (Wagner, 2010). observed a striking increase in type 1 cytokines in immune adults (Wagner, 2010). Adequate immunity to malaria likely requires a balance between TR1 and TR2.

2.6.1 Interleukin 10 (IL-10)

Interleukin-10 (IL-10) was initially identified as a cytokine synthesis inhibitory factor (CSIF) produced by mouse Th2 cells and inhibited Th1 cells cytokine production. It is primarily produced by cells of the monocyte/macrophage lineage and to a lesser extent by activated T and B cells (Lucey et al., 1996). IL-10 has extensively diverse effects mediated through specific cell surface receptor complex (IL-10Rα and IL-10Rβ) expressed on most hemopoietic cell types. It acts as a suppressor of immune responses, principally through down-regulating the expression of the MHC class II, the costimulatory molecules, and cytokine genes in the antigen-presenting cells (Rojas et al., 2017). For example, IL-10 is known to inhibit the secretion of IFN-γ by Th1 cells (Takayama et al., 2001).

In various experimental models, induction or administration of IL-10 suppressed the antigen-specific T-cell proliferation. It contributed to the establishment 35 of persistent infection by several pathogens, including HIV, Mycobacterium tuberculosis (de Moreno de LeBlanc et al., 2011), Listeria monocytogenes Trypanosoma cruzi (Díaz et al., 2015). However, although generally perceived as an anti-inflammatory cytokine, IL-10 could have quite the opposite effects on some specific models [(Gabryšová et al., 2014). For instance, IL-10 was found to stimulate the expression of MHC-II molecules, the production of immunoglobulins in B cells (Rousset et al., 1992), and augment the proliferation cytotoxicity of NK cells when combined with IL-18 (Cai et al., 1999). Increasing evidence demonstrates the role of IL-10 in malaria protection and pathogenesis (Kossodo et al., 1997). Murine and in vitro studies have demonstrated IL-10’s ability to inhibit TNF-α production in response to malarial antigens and high levels early during malaria infection were found to inhibit Th1 type of immune responses in both mice and humans.

These raised levels were also associated with less effective clearance of Plasmodial parasites and subsequently, the development of severe malarial complications, particularly SMA (Hugosson et al., 2004). In contrast, IL-10 has been suggested to play a protective role against experimental cerebral malaria (Kossodo et al., 1997), and clinical studies associated insufficient IL-10 with acute and severe malaria (Nasr et al., 2014). Moreover, it was demonstrated that, in individuals with SMA, plasma levels of TNF-α tend to exceed those of IL-10, and low ratios of IL-10: TNF were found to be a risk factor for both CM and SMA, whereas higher ratios were more frequent among hyperparasitemic individuals (Nasr et al., 2014). Interestingly, low circulating IL-10 associated with high pro-inflammatory cytokine levels was noted in comatose patients with CM (Nasr et al., 2014), suggesting a protective effect on IL-10 against SM. Therefore, while it may be detrimental by decreasing cellular immune responses to the parasite, IL-10 may equally be 36 beneficial in preventing excessive inflammation that underlies the SM. It is currently assumed that the levels and the timely production of IL-10 are crucial for controlling the parasite growth and replication and preventing excessive inflammatory responses to *P. falciparum*.

2.6.2 Genetic polymorphism of Interleukin-10

The IL-10 gene promoter is highly polymorphic, it contains two informative microsatellites, IL10.G and IL10.R and eleven single nucleotide polymorphisms (SNPs), of which the three proximal ones located at positions (-1082 G/A [rs 1800896], -819 C/T 39 [rs 5289772] and -592 C/T [rs5289771]) are the most frequent points of mutations (Zienolddiny et al., 2004). Differences in the genotypes and allele frequencies in these SNPs were observed between different populations. These differences may influence the outcomes of certain infections in different populations

(Chong et al., 2004). Interestingly, the -1082G allele was associated with higher production of IL-10 compared to the A allele both in vitro and in vivo, whereas polymorphisms at positions -819 and -592 appear to have no influence on IL-10 production (Koss et al., 2000). Several studies have demonstrated the relevance of these SNPs to susceptibility and/or severity of a number of diseases including the inflammatory bowel disease, psoriasis, primary Sjogren's syndrome, rheumatoid arthritis, myocardial infarction, and systemic lupus erythematosus ((Myhr et al., 2003; Suárez et al., 2005). Similarly, several infectious diseases were associated with certain alleles in these SNPs. Genetic predisposition to high IL-10 expression has been reported to be associated with a higher rate of mortality in meningococcal disease (Westendorp et al., 1997). Chronically infected hepatitis C patients who are genetically predisposed to high IL-10 production were reportedly less likely to benefit from IFN-α therapy [(Wang et al., 2011). Moreover, associations have been reported between IL-10 polymorphisms and Epstein– Barr virus infection , leprosy severe malaria (Wilson et al., 2005), and recurrence of hepatitis C in liver transplant patients. Of particular significance, is the A/G transition at position -1082 which has been associated with rheumatoid arthritis, systemic lupus erythematosus [(Suárez et al., 2005), psoriasis , coeliac disease [369], graft-versus host disease and the severity of P. falciparum malaria in African children (Wilson et al., 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment and reagents

A variety of laboratory tools and apparatus was used throughout this study, including chemicals, kits, reagents, and consumables, , all of which are listed in Tables 3.1, and 3.2 respectively.

Table 3.1: List of chemicals, kits and consumables

Item	Manufacture/ supplier
100 bp DNA ladder	Thermo fisher, USA
Absolute ethanol	SDFCL, India
DNA loading dye	SMOBIO, India
Master Mix	Bioline,U.K
Pipette tips	Eppendorf, Germany
Automatic Micro Pipette	Eppendorf, Germany
PCR Tubes	Eppendorf, Germany
Latex Examination Gloves	Iron skin, Malaysia
Tris-base	Fisher, U.K

Table 3.2: List of laboratory apparatus

Item	Manufacturer/ supplier
Automatic Micro Pipette	Eppendorf, Germany
Centrifuge	Eppendorf, Germany
Centrifuge mini spin	Andreas Hettich, Germany
Gel Electrophoresis system	Bio-Rad, USA
Gel image analyzer	SYNGENE, U.S. A
Microwave	ELBA,Italy
PCR machine	MJ Research, USA
pH-meter	Hanna Instrument, USA
Refrigerator (-20ºC)	LG Electronics, South Korea.
Sensitive Balance	OHAUS,Germany
Thermo cycler PCR mechine	Eppendorf, Germany
Tomy autoclave	Nerima-ku, Japan
Vortex mixer	Elmi skyline, U.S.A

3.2 Preparation of buffer and stock solutions

3.2.1(a) Ethanol 70% (v/v)

A solution of 70% ethanol was prepared by mixing 700 ml of ethanol with 300 ml of ddH2O to make a solution of 1 L volume. The 70% ethanol solution was stored at room temperature.

3.2.2(b) 10X Tris Borate EDTA (TBE) buffer

The 10X TBE buffer was prepared by mixing 50 ml TBE buffer from the original stock to 950 ml of distilled water, then pH was adjusted to 8.0 before the final volume was made up to 1000 ml with distilled water (dH2O) for gel preparation.

3.3 Methodology

3.3.1 Study Area

This original study was conducted in Ardamata IDP camp established in Al-Geneina City, Sudan during July 2018 to December 2018.

3.3.2 Study design

This study was case control study design to study the Association between IL10- gene polymorphism and *Plasmodium falciparum* infection.

3.3.3 Sample size

A total of 34 samples, were used in the present study. Samples used this study were archived blood samples collected from Ardamata IDP Camp, Al-Geneina City, Sudan.

3.3.4 Ethical Statements

The study protocol was approved by ethical review committee of the research directorate, FEDERAL MINISTRY OF HEALTH SUDAN (fmoh/nhrc/rd/rec 2018).