Sudan University of Science and Technology

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Genetic Mutation of Malaria Drug Resistance Genes after Adoption of Artemisinin Combination Therapy in Sudan

الطفرات الجينية لجينات الملاريا المقاومة للعلاج بعد تنفيذ العلاج المتجمع للارتميسنين في السودان

A thesis submitted in fulfilment for the requirements of degree of Ph.D. in Medical Laboratory Science (Parasitology and Medical Entomology)

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الآية

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قال تعالى:

﴿إِنَّ اللَّهَ لَايَسْتَحْيِي أَنْ يَضْرِبَ مَثَلًا مَا بَعُوضَةً فَمَا فَوْقَهَا فَأَمَّا الَّذِين َآمَنُوا فَيَعْلَمُون َأَنَّهُ الْحَقّ ُمِن ْرَبِّهِم ْوَأَمَّا الَّذِين َكَفَرُوا فَيَقُولُون َمَاذَا أَرَاد َاللَّهُ بِهَٰذَا مَثَلًا يُضِل ُّبِهِ كَثِيرًا وَيَهْدِي بِهِ كَثِيرًا وَمَا يُضِلّ ُبِهِ إِلَّا الْفَاسِقِينَ﴾

صدق الله العظيم

(سورة البقرة، الاية 26)

Dedication

To my parents

To my wife and daughters

To my sister and brothers

To my colleagues

To my friends

I dedicate this work.

Acknowledgement

All great thanks are firstly to Allah. I would like to express my gratitude and thanks to my supervisors Prof. Mohammed Bhaa El-din and Dr. Tayseer Elamin for their guidance, helpful suggestions, solving problems and their precious advices as well as continuous assistance through the whole process of the research.

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Abstract

This study was carried out in three states in Sudan (Sennar, Khartoum and River Nile) to detect the prevalence of antimalrial genetic markers; *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt), P.f multidrug resistance gene-1 (Pfmdr-1), P.f dihydorofolate reductase (Pfdhfr) and P.f dihydropotrate synthesize (Pfdhps). A cross-sectional study was carried out during the period from July 2015 to December 2017.

300 patients were included in this study their age ranged between 1- 90 years old of mean age was 31 ± 23 years, from them 133 were males and 167 were females.

Blood samples were taken from all patients to detect malaria parasite by using Giemsa stained blood smears.

Out of 300 positive malaria samples, the prevalence rates of these genes were 32%, 36%, 36.7% and 33% respectively by using of restriction fragments length polymorphisms - polymerase chain reaction (RFLP-PCR) method.

The highest prevalence mutant allele rate (47%) was reported in Khartoum state with Pfmdr, while the lowest prevalence rate (28%) was reported in two genes (Pfcrt in Sennar and Pfdhfr in Khartoum). Among the gender, the rates were 22% and 10% in females and males respectively. The highest rate (15.7%) was reported in both Pfmdr-1 and Pfdhfr among age group 20-50 years old, while the lowest prevalence rate (3.7%) was reported in Pfdhps among age group 11-19 years old. Also, the highest prevalence rate (12.7%) was reported in Pfdhfr among parasite count ++, while the lowest prevalence rate 3% was reported among parasite count ++++.

The highest prevalence rate (37%) of single point mutation of Pfcrt K76T was reported in River Nile, while the lowest prevalence rate (28%) was reported in Sennar. In females reported 22% and males 10%. Among age groups the highest prevalence rate (10.7%) of Pfcrt K76T was reported in age group 20- 50 years, while the lowest prevalence rate (7.7%) was reported among both age groups (<10 and >50). The highest prevalence rate (12%) was reported among parasite count ++, while the lowest prevalence rate (3%) was reported among parasite count ++++.

The results revealed that, the highest prevalence rate (15.7%) of single point mutation of Pfmdr-1 N86Y was reported in Khartoum state, while the lowest prevalence rate (9.7%) was reported in Sennar state. The result showed equal rate of 18% among the gender with presence of mixed allele with a rate of 0.7%. Also, the results revealed that, the highest Pfmdr-1 N86Y rate of 15.7% was reported among age group 20-50 years, while the lowest prevalence rate of 4.7% was

reported among age group 11 -19 years . The prevalence rate among parasite count was 10.7% among the + and ++ followed by 10.3% and 4.3% among +++ and ++++ respectively.

From the results, the highest prevalence rate (14.3%) of single point mutation Pfdhfr C59Rwas reported in Khartoum, while the lowest prevalence rate (9.3%) was reported in River Nile with the presence of mixed allele (1.7% and 4%) in River Nile and Khartoum respectively. Among the gender, the prevalence rates of 20.7% and 16%were among females and males respectively with the presence of mixed allele 1.3% and 4.3% respectively. The highest rate (15.7%) was reported among age group 20 -50 years, while the lowest rate (5.7%) was reported among age group 11 -19 years with the presence of mixed allele among all age groups. The highest prevalence rate (12.7%) of Pfdhfr C59R was reported among parasite count ++, while the lowest prevalence rate (3%) was reported among parasite count ++++ with the presence of mixed allele among all parasite counts.

From the results, the prevalence rate of single point mutation of Pfdhps A436G (13%) was reported in Khartoum and (10%) in both Sennar and River Nile with the presence of mixed allele in Khartoum and River Nile with rates of 1.7% and 3% respectively. Among gender 19.3% rates was reported among females and 13.7% among males with mixed allele of 3% and 1.7% respectively. However, the highest prevalence rate (15.7%) was reported among age group 20-50 years, while the lowest rate (3.7%) was reported among age group 11-19 years with mixed allele among all age groups except the age group 11-19 years. The highest prevalence rate (11%) of Pfdhps A436G was reported among parasite count ++, while the lowest prevalence rate (3.7%) was reported among parasite count ++++ with the presence of mixed allele among all parasite counts.

The study indicated that, the genetic mutation of malaria drug resistance markers after adoption of artemisinin combination therapy were still found in the study areas.

المستخلص

أجريت هذه الدراسة في ثلاث ولايات سودانية (سنار والخرطوم ونهر النيل) للكشف عن انتشار الواسمات الجينية المضادة للأدوية، بلاسموديوم فالسيباروم مقاومة الكلوروكين (Pfcrt)، بف جينترال مقاومة الجينات (Pfmdr-1)، بف ديهيدوروفولات مختزلة (Pfdhfr) و بف ديهيدروبوترت توليف (Pfdhps) . الدراسة المستعرضة أجريت في الفترة بين يوليو 2015 إلى ديسمبر 2017.

شملت الدراسة علي 300 شخصا تراوحت أعمارهم بين 1- 90 سنة بمتوسط عمري 23±31 سنة, كان منهم 133 ذكورا" و167 إناثا".

أخذت عينات الدم من جميع الاشخاص موضوع الدراسة للتعرف على طفيل الملاريا بواسطة إستخدام مسحات الدم المصبوغة بصبغة جيمسا.

من بين 300 عينة إيجابية للملاريا كانت معدلات انتشار هذه الجينات32٪ و 36٪ و 36.7٪ و 33٪ على التوالي من خلال إستخدام شظايا التقييدات متعددة الأشكال- طريقة تفاعل البلمرة المتسلسلة.

أعلى نسبة انتشار أليل متحولة (47٪) سجلت في ولاية الخرطوم مع (Pfmdr-1)، في حين أن أدنى معدل انتشار (28٪) سجل في اثنين من الجينات (Pfcrt في سنار و Pfdhfr في الخرطوم). ومن بين الجنسين، كانت المعدلات 22 % و 10 % في الإناث والذكور على التوالي. أعلى معدل (15.7%) سجل في كل من (Pfmdr-1) و (Pfdhfr) في الفئة العمرية 20-50 سنة، في حين سجل أدنى معدل انتشار 3.7٪ في Pfdhps بين الفئة العمرية 11-19 سنة. كما تم تسجيل أعلى معدل انتشار 12.7٪ في Pfdhfr بين عدد الطفيليات ++، في حين بلغت أدنى معدل انتشار 3٪ بين عدد الطفيليات ++++.

أعلى معدل انتشار (37٪) من طفرة نقطة واحدة من Pfcrt K76T سجلت في نهر النيل، في حين أن أدنى معدل انتشار(28٪) سجل في سنار. سجلت في الإناث 22 % والذكور 10 % . بين الفئات العمرية أعلى معدل انتشار(10.7٪) من Pfcrt K76T سجل بين الفئة العمرية 20-50 سنة، في حين أن أدنى معدل انتشار (7.7٪) سجل بين كل من الفئات العمرية (10أقل وأكبر من 50 سنة) . اخيرا", أعلى معدل انتشار(12٪) سجل بين أعداد الطفيليات ++، في حين أن أدنى معدل انتشار (3٪) سجل بين أعداد الطفيليات++++ .

أظهرت النتائج أن أعلى معدل انتشار 15.7٪ من طفرات نقطة واحدة من Pfmdr-1 N86Y تم تسجيلها في ولاية الخرطوم، في حين أن أدنى معدل انتشار 9.7٪ سجل في ولاية سنار. أظهرت النتائج نسبة متساوية 18٪ وسط الجنسين مع وجود أليل مختلط بمعدل 0.7٪. أيضا" بينت النتائج أن أعلى معدل ل Pfmdr-1 N86Y 15.7 ٪ سجل بين الفئة العمرية 20 -50 سنة، في حين أن أدنى معدل انتشار 4.7٪ سجل في الفئة العمرية بين 11 -19سنة. أخيرا" معدل الانتشار وسط أعداد الطفيل كانت 10.7٪ بين (+ و ++) تليها 10.3٪ و 4.3٪ بين (+++ و ++++) على التوالي.

من النتائج، عن أعلى معدل انتشار 14.3٪ من طفرة نقطة واحدة Pfdhfr C59R سجلت في الخرطوم، في حين أن أدنى معدل انتشار 9.3٪ سجل في نهر النيل مع وجود أليل مختلط 1.7٪ و 4٪ في نهر النيل والخرطوم على التوالي. من بين الجنسين, معدلات الانتشار 20.7٪ و 16٪ بين الإناث والذكور على التوالي مع وجود أليل مختلط 1.3٪ و 4.3٪ على التوالي. أعلى معدل 15.7٪ سجل بين الفئة العمرية 20-50 سنة ، في حين أن أدنى معدل 5.7٪ سجل بين الفئة العمرية 11- 19 سنة مع وجود أليل مختلط بين جميع الفئات العمرية. أخيرا", أعلى معدل انتشار 12.7٪ من Pfdhfr C59R سجل بين عدد الطفيل (++)، في حين أن أدنى معدل انتشار 3٪ سجل بين عدد الطفيل (++++) مع وجود أليل مختلطة بين جميع اعداد الطفيل.

من النتائج معدل انتشار(13٪) من طفرة نقطة واحدة من Pfdhps A436G سجل في الخرطوم و 10٪ في كل من سنار ونهر النيل مع وجود أليل مختلط في الخرطوم ونهر النيل بمعدلات 1.7٪ و 3٪ على التوالي. بين الجنسين 19.3٪ سجل بين الإناث و 13.7٪ بين الذكور مع الأليل المختلط 3٪ و 1.7٪ علي التوالي. ومع ذلك، أعلى نسبة انتشار 15.7٪ سجل بين الفئة العمرية 20-50 سنة، في حين أن أدنى معدل 3.7٪ سجل بين الفئة العمرية 11-19 سنة مع أليل مختلط بين جميع الفئات العمرية باستثناء الفئة العمرية 11- 19سنة. أخيرا", أعلى معدل انتشار 11٪ من Pfdhps A436G سجل بين عدد الطفيل (++)، في حين أن أدنى معدل انتشار 3.7٪ سجل بين عدد الطفيل (++++) مع وجود أليل مختلط بين جميع اعداد الطفيل.

دلت الدراسة علي ان الطفرات الجينية لواسمات الملاريا المقاومة بعد تنفيذ العلاج المزدوج ما تزال موجودة في المناطق موضوع الدراسة.

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Abbreviations

A/L: Artemether /lumefantrine

Abs: Antibodies

ACT: Artemisinin-based combination therapy

ACTs: Artemisinin-based combination therapies

Ags: Antigens

AQ: Amodiaquine

ARDS: Acute respiratory destress syndrome

AS/ SP: Artesunate/Sulphadoxine-Pyrimethamine

ASP: Artesunate + sulphadoxine

AT: Artesunate

AT: Annealing temperature

C.S.F: Cerebral spinal fluid

CM: Cerebral Malaria

CNS: Central nervous system

CQ: Chloroquine

DCs: Dendritic cells

dH2O: Deionized or distilled water

dhfr: Dihydrofolate reductase gene

dhps: Dihydropetroate synthase gene

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide triphosphate

EDTA: Ethylene Diamine Tetra Acetate

EIR: Entomological Inoculation Rate

ELISA: Enzyme Linked Immunosorbent Assay

ETF: Early treatment failure

G6PD: Glucose-6 phosphate dehydrogenase deficiency

GPI: Glycosylphosphatidylinositol

HF: Halofantrine

HIV: Human immunodeficiency virus

ICT: Immunochromatographic tests

IFN-γ: Interferon-γ

Ig: Immunoglobulin

IL-1: Interleukin-1

IPTp: Intermittent treatment in pregnancy

MSP1: Merozoite Surface Protein1

msp1: Merozoite surface protein1 gene

MSP2: Merozoite Surface Protein2

msp2: Merozoite surface protein2 gene

NK: Natural Killer

NO: Nitric oxide

PBS: Phosphate Buffer Saline

PCR/RFLP: Polymerase chain reaction/restriction fragment length polymorphism

PCR: Polymerase Chain Reaction

pfcrt: Plasmodium falciparum chloroquine resistance transporter gene

PfEMP1*: Plasmodium falciparum* erythrocyte membrane protein1

pfemp1: Plasmodium falciparum erythrocyte membrane protein1 gene

PfHRP2: Histidine-rich protein 2 of *P. falciparum*

pfmdr: Plasmodium falciparum multidrug resistance gene

pLDH: Parasite lactate dehydrogenase

PMA: Pan malarial antigen

PRRs: Pattern-recognition receptors

QBC: Quantitative Buffy Coat

RBCs: Red blood cells

RDTs: Rabid diagnostic tests

SNP Single nucleotide polymorphism

SP: Sulphadoxine/Pyrimethamine

SP-IPTp: SP resistance and a strong predictor of intermittent preventive treatment in pregnancy

Taq: DNA polymerase enzyme

SPR: Sulphadoxine/Pyrimethamine resistance

TBE: Tris-Boric acid-EDTA Buffer

TCRint: Intermediate cells

TGF-β: Transforming growth factor-β

TH1: Helper 1

TLR-9: Toll-like receptor-9

TMAC: Tetramethyl ammonium chloride

UM: Uncomplicated Malaria

WHO: World Health Organization

Chapter 1

Introduction

1.1 Introduction

Malaria is one of the important diseases that affect people especially in tropical and subtropical regions, it is a great affliction that causes death in children and pregnant women and migrants (Chaianantakul *et al*., 2013). Malaria is in a same rank with Human Immunodeficiency Virus (HIV), influenza and tuberculosis (Petersen *et al*., 2011). Malaria is caused by intracellular protozoan parasite of the genus *Plasmodium,* there are four species of *Plasmodium* that infect human; *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale.* However, a primate species proposed to infect human was discovered which is known as *P. knowlesi* (Douradinha *et al*., 2008).

All species of malaria transmitted to human primary by bite of female mosquito of genus *Anopheles,* and occasionally through shared needles, blood transfusion and congenital from mother to her fetus (Abdullah and Karunamoorthi, 2016). Malaria now threatening about 40% of world population annually, most of them are children and pregnant women, however 90% of them in Africa (Modell and Darlison, 2008). Although *P. falciparum* is mostly responsible for death especially in children less than 5 years in sub-saharan Africa as a single infectious agent, also *P. vivax* becomes to spread (Hoffman, 2004).

Anti-malarial drug resistance is a major challenge to the control of *falciparum* malaria, the leading cause of morbidity and mortality especially in Africa and Southern Asia (Atroosh *et al*., 2012). The first *P. falciparum* chloroquine resistance (CQ) was reported in the late 1950 in Southeast Asia along the Thai-Cambodian border (Atroosh *et al*., 2012). Further spread of CQ resistance was shown later to include neighboring countries in Asia, Africa and South America. Moreover, *P. falciparum* has been also reported resistant to other antimalarial drugs including sulphadoxine/pyrimethamine drug combination, mefloquine, atovaquone and artemisinin (Atroosh *et al*., 2012). Historical evidence shows that the emergence of drug-resistant *P. falciparum* strains first originated in Southeast Asia and then, spread to Africa (Thomsen *et al*., 2011). East Africa has been a major focus of drug resistance spread, and development in sub Saharan Africa, probably originated at Southeast Asia (Lobo *et al*., 2014). The spread of CQ resistance has been attributed to the resurgence of malaria morbidity and mortality during the 1990s. In 2001, the World Health Organization (WHO) recommended the use of artemisinin-

based combination therapy (ACT) for treatment of uncomplicated *P. falciparum* malaria (Gresty *et al*., 2014). In general, resistance is most severe in East and Southern, compared with West Africa. Resistance to aminoquinolines, especially chloroquine (CQ), is widespread and CQ is no longer recommended to treat malaria in sub-Saharan Africa (Escobar *et al*., 2015). Resistance to amodiaquine (AQ), a related aminoquinoline, is less common, although its antimalarial efficacy is unsatisfactory in many areas (Gresty *et al*., 2014). Resistance to antifolates, notably sulfadoxine/pyrimethamine (SP), is increasing (Mobula *et al*., 2009). With increasing resistance to older drugs, highly efficacious artemisinin-based combination therapies (ACTs) are now the recommended first line antimalarials in nearly all countries in sub-Saharan Africa (Mobula *et al*., 2009).

The *P. falciparum* multi-drug resistance gene 1 (pfmdr1) and particularly, single nucleotide polymorphisms (SNPs) resulting in an amino acid change in codons 86 (N86Y), 184 (Y184F), and 1246 (D1246Y) have been associated with changes in parasite susceptibility to various drugs, including (ACT) (Uhlemann and Krishna, 2005). Initially, SNPs in pfmdr1 were associated with chloroquine (CQ) and amodiaquine (AQ) resistance (Lobo *et al*., 2014). For instance, the pfmdr1 86Y mutation has been associated with high CQ resistance, and the combination of pfmdr1 86Y, Y184, and 1246Y is likely selected by AQ monotherapy and associated with increased risk of treatment failure (Lobo *et al*., 2014), while 1034C, 1042D and 1246Y mutations have been reported to confer resistance against quinine (QN) and increased susceptibility to mefloquine (MQ), halofantrine (HF) and artemisinin (Kavishe *et al*., 2014). Although SNPs at positions 1034 and 1042 of *P. falciparum* multi drug resistance 1 (pfmdr1) were described as being related to phenotype modulation by altering a drug pocket in Pfmdr1, they are very infrequent in Africa (Ferreira *et al*., 2011). Sulphadoxine-pyrimethamne (SP) resistance is caused by mutation on two genes, the dihydrofolate reductase (Pfdhfr) and the dihydropteroate synthetase (Pfdhps) genes. Three Pfdhfr mutations: N51I, C59R and S108N, referred to as the triple mutation, and the Pfdhps mutations: A437G and G540E referred to as the double mutation, collectively form the quintuple mutations. An additional mutation on Pfdhps 581 has been associated with high level of SP resistance and a strong predictor of intermittent preventive treatment in pregnancy (SP-IPTp) failure and in addition to the quintuple forms the sextuple mutation. In East Africa SP resistance has reached over 90% and in some places the prevalence of the quintuple mutation is approaching fixation levels (Matondo *et al*., 2014).

Malaria is a major public health problem in the Sudan with an estimated 7.5 million cases and 35.00 death annually. Sudanese health authorities adopted a new antimalarial drug policy in June 2004, in which artemether based combination (ACT) are recommended and it identifies artesunate + sulphadoxine (ASP) as first line of treatment and artemether /lumefantrine (A/L) as second line of treatment (Elamin *et al*., 2006). In Sudan, *P. falciparum* causes over 95% of malaria infections and drug resistance to CQ and SP is well established (Gadalla *et al*., 2010). The wide-scale deployment of (ACT) across malaria-endemic zones is intended to counteract the emergence and spread of resistance (White, 2004). Artesunate/Sulphadoxine-Pyrimethamine (AS/ SP) and artemether-lumefantrine (AL) became the first and second-line of treatment, respectively for uncomplicated *P. falciparum* malaria in 2004. Recently, the efficacy of AS/SP has been questioned among practicing physicians with some patients returning with recurring infections. Furthermore the emergence of slow clearing parasites following artesunate treatment as in South East Asia has prompted closer monitoring of the efficacy of ACT and necessitated extensive research on molecular markers of resistance to artemisinins and partner drugs (Gadalla *et al*., 2013).

1.2 Rationale

Antimalarial resistance is a global threat and is extremely harmful to disease management particularly in low income countries. Baseline data are crucially needed to assess the situation and to develop successful management strategy. Therefore, tools for early detection and identification of such evolving resistant malaria parasites are of high importance to better monitoring and evaluation of resistance markers.

There is lack of updating for the current situation of the resistance levels in Sudan. This study will fill these gaps by using advanced molecular techniques to identify, characterize and determine the spread of hospital associated infections to allow infection control personnel to more rationally identify potential sources of infection, health worker and stakeholders to develop of treatment and control strategies against these rapidly evolving organisms.

Therefore, the use of molecular tools is very essential in identification and characterization of these organisms. In addition it will assist in the detection of the most encountered resistance genes and understanding disease epidemiology and may limit the spread of infections.

There is increasing anecdotal evidence from Sudan, reporting malaria treatment failures after artesunate and sulphdoxine – prymethamine (ASP) treatment (first line of malaria treatment in Sudan), indicating reduced sensitivity of the parasite towards the current first line treatment.

1.3 Objectives

1.3.1 General objective

To study genetic mutation of malaria drug resistance gene (Pfcrt, Pfmdr1, Pfdhfr and Pfdhps) in Sudan.

1.3.2 Specific objectives

To detect mutations of drug resistance genes in malaria parasite from different areas in Sudan.

To compare prevalence of drug resistance genes mutations in malaria parasite among age groups and gender.

To compare between drug resistance genes mutations in malaria parasite and level of parasitemia.

Chapter 2

Literature review

2.1 Background

Throughout human history, including prehistoric times, malaria has been one of the greatest afflictions, in the same ranks as bubonic plague, influenza, and tuberculosis. Even now, as the dominant protozoan disease, it threatens 40% of the world's population every year. The origin of the name is from the Italian words (mal, bad, and aria, air). The superstitions of the middle ages explained that evil spirits or mists and vapors arising from swamps caused malaria, because many victims came down with the disease after this sort of exposure (Taylor *et al*., 2012). Of course, we now know that a swamp was mainly involved as a habitat for the mosquito vector. The agent of malaria is an obligate intracellular sporozoan in the genus *Plasmodium*, which contains four species: *P.malariae, P.vivax, P.falciparum*, and *P.ovale*. The human and some primates are the primary vertebrate hosts for these species, which are geographically separate and show variations in the pattern and severity of disease. All forms are spread primarily by the female *Anopheles* mosquito and occasionally by shared needles, blood transfusions, and from mother to fetus (Trampuz *et al*., 2003).

2.2 Transmission of malaria

Human malaria is transmitted by the bite of female *Anopheles* mosquito. The male mosquito feeds exclusively on fruit juices, but the female needs at least two blood meals before laying the first batch of eggs. Also, malaria parasites of animals (apes, monkeys, rodents) are transmitted by *Anopheles*, but bird malaria parasites are carried by *Culex*, *Aedes* and other genera of mosquitoes (Paniker, 2007). Only about 50 species transmit malaria of more than 480 species of *Anopheles*, with every continent having its own species of these mosquitoes: *An. gambiae* complex in Africa, *An. freeborni* in North America, *An. culicifacies*, *An. fluviatilis*, *An. minimus*, *An. philippinensis*, *An. stephensi* and *An. sundaicus* in the Indian subcontinent (Paniker, 2007, Griffin *et al*., 2010). The adult female mosquito can survive under ideal conditions up to 50 to 60 days. Practically, however, female *Anophelines* survive on the average of 20 to 25 days, and consequently must be infected by malaria parasites in their early blood meals, taken every 3 to 4 days. Maturation of the parasites ingested with the blood meal requires 8 to 12 days before the mosquito has infective sporozoites in her salivary glands (Heggenhougen *et al*., 2003).

2.3 Epidemiology of malaria

Malaria has a worldwide distribution between 45°N and 40°S latitude. Transmission of malaria occur in many countries in Africa, South of Sahara desert, South East Asia and in Central and South America (Hoffman *et al.*, 2004, Guerra *et al.*, 2008). The prevalence of malaria infection has not changed in these areas in few past decades, from 2000 to 2015 world health organization (WHO) estimated 262 million cases of malaria globally, leading to 839, 000 death in 2000 against 214 million cases and 438, 000 death in 2015, sub Saharan Africa remain the region of highest disease burden and accounts for 88 and 90% of global clinical cases and death respectively (Hoffman *et al.*, 2004).Generally, at altitudes below 1800 m. *P. vivax* is the most widely distributed of the four species, and together with the uncommon *P. malariae* are found primarily in temperate and subtropical areas, *P. falciparum* is the dominant organism of the tropics, while *P. ovale* is rare and found principally in Africa (Reiter, 2001, Breman *et al.*, 2001). The major determinants of the epidemiology of malaria infection and disease relate to the *Plasmodium* spp. the transmission intensity, innate and acquired resistance of the human host and access to and efficacy of control measures. In addition, the immunocompromised states associated with pregnancy and infection with human immunodeficiency virus (HIV) affect the epidemiology of malaria (Farrar *et al*., 2013).

2.4 Life cycle

2.4.1 Human phase

The life cycle of malaria parasites comprises two stages an asexual phase occurring in humans and the sexual phase occurring in the mosquito. Usually, 20-30 sporozoites which are elongated spindle-shaped bodies become rounded inside the liver cells are transmitted to the host by a single mosquito bite and some of the sporozoites rapidly reach the liver of the host via the blood circulation and thereby invade hepatocytes (Heggenhougen *et al*., 2003, Abhay *et al*., 2009). Once inside the hepatocyte, the parasite undergoes a sexual division and develops into liver schizonts within the infected hepatocytes over a period of approximately 1-2 weeks, depending on the species of *Plasmodium*. Because the initial stage of development within the liver occurs outside the bloodstream, this hepatic stage is generally referred to as the exo-erythrocytic stage (Hafalla *et al*., 2011). Formerly, it was postulated that some merozoites released after the primary exoerythrocytic schizogony invaded other hepatocytes to initiate the secondary exoerythrocytic schizogony. Such exoerythrocytic schizogony was believed to be repeated for a few generations and was considered to explain the occurrence of relapses in *P. vivax* and *P.*

ovale infections (Reece *et al*., 2009). In *P. vivax* and *P. ovale*, two kinds of sporozoites are seen, some which multiply inside hepatocytes promptly to form schizonts and others which remain dormant. These latter forms are called hypnozoites (from hypnos-sleep). Hypnozoites remain inside the hepatocytes as uninucleate forms, 4 to 5 µm in diameter, for long periods. From time-to-time, some are activated to become schizonts and release merozoites, which go on to infect erythrocytes, producing clinical relapses. This is the present concept of relapses in *P. vivax* and *P. ovale*. Secondary exoerythrocytic schizogony is not believed to occur. In *P. falciparum* and *P. malariae* no hypnozoites are formed and the parasites do not persist in the exoerythrocytic phase. However, a small number of erythrocytic parasites persist in the blood stream, and in course of time, multiply to reach significant numbers, resulting in clinical disease (short-term relapse or recrudescence). In *falciparum* malaria, recrudescence are seen for one or two years, while in *P. malariae* infections, they may last for long periods, even up to 50 years (Paniker, 2007). *P. falciparum* can complete this liver stage within 7 days and each of its sporozoites produces about 40,000 daughter parasites, *P. vivax* in 6-8 days produces 10,000 merozoites; while *P. malariae* produces 2000 merozoites in 12-16 days and for *P. ovale* produces 15,000 merozoites in 9 days. The next stage of development, called the erythrocytic or blood stage, is initiated when exo-erythrocytic merozoites from the liver invade red blood cells (RBCs) (Führer, 2012).

At the end of the hepatic stage of development, a single sporozoite can develop into a schizont that contains thousands of daughter parasites that fill the hepatocyte. Infected hepatocytes burst and release numerous merozoites into the blood stream (Suarez *et al*., 2013). Merozoites are pear-shaped bodies about 1.5 µm in length, possessing an apical complex (rhoptry). They attach to erythrocytes by their apex, which has certain organelles that secrete a substance producing a pit on the erythrocyte membrane. The merozoite then enters the erythrocyte by endocytosis and the red cell membrane seals itself to form a vacuole (parasitophorous vacuole) enclosing the merozoite (Awuor, 2014). Merozoites of *P. falciparum* can infect RBCs of all ages, whereas those of *P. vivax* and *P. ovale* infect reticulocytes and those of *P. malariae* invade only older RBCs. Shortly after merozoites are released from hepatocytes, they invade RBCs and over a period of 2 or 3 days, develop asexually (Ménard *et al*., 2010, Roestenberg *et al*., 2011). The stages of asexual development include the ring (early trophozoite), trophozoite and schizont stages, the ring stage derives its name from its signet ring-like appearance, the trophozoite is the feeding stage of the parasite and contains a single nucleus with pigment granules, called hemozoin (a product of hemoglobin digestion), located within the cytoplasm of the parasite. The schizont stage is initiated

by the division of the trophozoite nucleus. Further nuclear division leads to enlargement of the parasite. Each individual nucleus then becomes surrounded by parasite cytoplasm to form a merozoite (Abhay *et al*., 2009). At maturation, the schizont bursts and releases merozoites into the blood circulation. Most of the released merozoites re-invade a new erythrocyte, thereby repeating their asexual life cycle (blood stage cycle) (Barry, 2005, Antinori *et al*., 2012). In some instances, however, invasion of an erythrocyte by a merozoite initiates sexual development instead of asexual development. Thus, merozoites may develop into male gametocytes (microgametocyte) or female gametocytes (macrogametocytes). These gametocytes can develop further only when they are taken up by an appropriate species of *Anopheles* mosquito during a blood meal. They subsequently mate within the gut of the mosquito the definitive host (Armson, 2011).

The mature gametocytes are round in shape, except in *P. falciparum*, in which they are crescent shaped. In all species, the female gametocyte is larger (macrogametocyte) and has cytoplasm staining dark blue with a small compact nucleus staining deep red. In the smaller male gametocyte (microgametocyte), the cytoplasm stains pale blue or pink and the nucleus is larger, pale stained and diffuse. Pigment granules are prominent. Female gametocytes are generally more numerous than the male (Despommier and Karapelou, 2012). Gametocytes appear in circulation 4 to 5 days after the first appearance of asexual form in the case of *P. vivax* and 10 to 12 days in *P. falciparum*. A person with gametocytes in circulation is a carrier or reservoir. Children are more effective carriers than adults. Gametocytes are more numerous in the early phase of infection. The gametocytes do not cause any clinical illness in the host, but are essential for transmission of the infection. The gametocytes do not develop further or divide in the vertebrate host and unless taken up by the vector mosquito, they die in a few days. A gametocyte concentration of 12 or more per mm^3 of blood in the human host is necessary for mosquitoes to become infected (Paniker, 2007).

2.4.2 The mosquito phase

When a female *Anopheles* mosquito ingests parasitised erythrocytes along with its blood meal, the sexual forms of malaria parasites are digested, but the gametocytes are set free in the stomach and undergo further development. Within 15 minutes of entry into the stomach of the mosquito, the male gametocyte divides into 8 nuclei, from each of which protrudes a long, actively motile whip-like filament. These flagella which are the male gametes (microgametes) lash about for some time and then break free. This process of formation of male gametes from the gametocyte

is called exflagellation (Gunn and Pitt, 2012). Detaching from the cell body, the flagella lash about vigorously in the plasma. At 25°C, the exflagellation is complete in 15 minutes for *P. vivax* and *P. ovale*, and 15 to 30 minutes for *P. falciparum*. The female gametocyte does not divide but undergoes a process of maturation to become the female gamete or macrogamete. It is fertilized by one of the microgametes to produce the zygote. Fertilization occurs in half to two hours after the blood meal. The zygote, which is initially a motionless round body elongates and within 18 to 24 hours, becomes a vermicular motile form with an apical complex anteriorly. This is called the ookinete ('travelling vermicule'). It penetrates the epithelial lining of the mosquito stomach wall and comes to lie just beneath its basement membrane. It becomes rounded into a sphere with an elastic membrane. This stage is called the oocyst. There may be up to several hundred pigmented oocysts in the stomach of a mosquito. It was the discovered by Ronald Ross of pigmented oocysts in the stomach walls of dissected mosquitoes that established the mosquito transmission of malaria (Paniker, 2007).

2.5 Pathology of malaria

Malaria is often classified as uncomplicated or complicated/severe. Uncomplicated malaria can be caused by all four species and is characterized by periodic fever and chills, mild anemia and splenomegaly. Uncomplicated malaria is rarely fatal unless it is left untreated and it progresses to severe disease (Carter *et al*., 2017). Severe or complicated malaria is almost exclusively caused by *P. falciparum* infections (although occasionally by *P. vivax* and other species) and is associated with higher parasite burdens and vital organ dysfunction including central nervous system (CNS) (coma, seizures.) and pulmonary compromise (pulmonary edema, acute respiratory distress syndrome (ARDS), respiratory distress.), acute renal failure, severe anemia and metabolic acidosis (Winstanley, *et al*., 2014). Anemia arises in part from the destruction of erythrocytes when merozoites burst out of the infected RBC and RBC production is further compromised by bone marrow suppression or dyserythropoiesis. In *falciparum* malaria, anemia can be dramatic and life threatening (Avery, 2010). The rise in temperature is also correlated with the rupture of schizonts with release of pyrogens together with merozoites from the bursting infected RBCs (Shio *et al*., 2010). The pathogenesis of general malaise, myalgia and headache is will-defined. The classic periodicity of the fever based on synchronous infections, is often not observed particularly early in the course of infection (Bing, 2015). In the early phase of infection, the growth of the parasites is not synchronous, RBC rupture is more random and consequently fever can be erratic. In addition, some infections may be due to two or more broods

of parasites, with the periodicity of one being independent of that of the others. This is more often seen in the case of severe *falciparum* malaria (Bogitsh *et al*., 2013). Most malaria deaths are associated with *P. falciparum* infections. RBCs infected with the maturing forms of this parasite express parasite proteins (PfEMP) associated with morphological structures ("knobs") that permit them to stick to endothelial cells lining the blood vessels and result in sequestration of these infected RBCs within the vascular bed of vital organs. When this occurs in the brain, the resulting cerebral malaria may lead to coma and death. Renal, pulmonary and gastrointestinal complications may also be seen. Congenital malaria and infection of the placenta may result in stillbirth, low birth weight infants, or perinatal mortality. After the initiation of blood stage infection by the parasite, the repeated infection of erythrocytes by merozoites results in exponential growth (Boddey and Cowman, 2013). As a result, the parasitized RBCs accumulate in the capillaries and sinusoids of blood vessels, causing general congestion in the peripheral blood circulation. The congestion causes organomegaly, notably splenomegaly and possibly hepatomegaly and contributes to anemia, leukopenia and thrombocytopenia (Ferrer *et al*., 2014). In *vivax* malaria, these processes occur rather acutely and the affected organs, particularly the spleen, become susceptible to rupture following trauma (Anstey *et al*., 2009). In severe *falciparum* malaria, the kidneys may show punctate hemorrhages and tubular necrosis. Severe hemolysis and damage in the renal tubules results in hemoglobinuria or in its most severe form "blackwater fever". In fact, the latter condition has been often associated with massive intravascular hemolysis in the context of prior treatment with quinine or treatment with primaquine in those with glucose-6 phosphate dehydrogenase deficiency (G6PD). Chronic *P. malariae* infection can be associated with nephrotic syndrome, a condition in which the kidney shows histological hypertrophy, caused by the deposition of immune complexes (Laveran, 2001, Abhay *et al*., 2009, Soderland *et al*., 2010). All the manifestations of malarial illness are caused by the infection of the red blood cells by the asexual forms of the malaria parasite and the involvement of the red cells makes malaria a potentially multisystem disease, as every organ of the body is reached by the blood (Fakhreldin *et al*., 2003). All types of malaria manifest with common symptoms such as fever, some patients may progress into severe malaria. Although severe malaria is more often seen in cases of *P. falciparum* infection, complications and even deaths have been reported in non-*falciparum* malaria as well. Induction of fever by malaria parasites (Ralf, 2007, Greenwood *et al*., 2008). The released products into the blood as red cell membrane products, hemozoin pigment, and other toxic factors such as

glycosylphosphatidylinositol (GPI) are activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor (TNF), interferon-γ (IFN-γ), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), macrophage colony-stimulating factor, and lymphotoxin (LT), as well as superoxide and nitric oxide (NO). Many studies have implicated the GPI tail, common to several merozoite surface proteins such as merozoite surface protein -1 (MSP-1), MSP-2, and MSP-4, as a key parasite toxin (Claire *et al*., 2004, Srabasti *et al*., 2008). The systemic manifestations of malaria such as headache, fever and rigors, nausea and vomiting, diarrhea, anorexia, tiredness, aching joints and muscles, thrombocytopenia, immunosuppression, coagulopathy, and central nervous system manifestations have been largely attributed to the various cytokines released in response to these parasite and red cell membrane products (Clark *et al*., 2006). In addition to these factors, the plasmodial DNA is also highly proinflammatory and can induce cytokinemia and fever. The plasmodial DNA is presented by hemozoin (produced during the parasite development within the red cell) to interact intracellularly with the Toll-like receptor-9 (TLR-9), leading to the release of proinflammatory cytokines that in turn induce COX-2-upregulating prostaglandins leading to the induction of fever (Ralf, 2007). Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Lamikanra *et al*., 2009).

2.5.1 Severe malaria

Severe *falciparum* malaria is defined by the demonstration of asexual forms of *P. falciparum* in a patient with a potentially fatal manifestation or complication of malaria in whom other diagnoses have been excluded. Even though the complications are almost unique to *P. falciparum* infection that does not mean that all cases of *P. falciparum* malaria invariably develop complications (Trampuz *et al*., 2003, Winstanley *et al*., 2004). The major complications of severe malaria include cerebral malaria, pulmonary edema, acute renal failure, severe anemia, and/or bleeding. Acidosis and hypoglycemia are the most common metabolic complications, any of these complications can develop rapidly and progress to death within hours or days (Trampuz *et al*., 2003, Idro *et al*., 2010, WHO, 2014b). The presentation of severe malaria varies with age and geographical distribution. In areas of high malaria transmission, severe malaria mainly affects children under five years of age. The mortality rate is higher in adults than in children but African children develop neuro-cognitive sequelae following severe malaria more frequently. In children, the complications include metabolic acidosis (often caused by hypovolemia), hypoglycemia, hyperlacticacidemia, severe anaemia, seizures and raised intracranial pressure and

concomitant bacterial infections occur more frequently. In adults, renal failure and pulmonary oedema are more common causes of death (Gething *et al*., 2011, Jain *et al*., 2013).

2.5.2 Cerebral malaria

Cerebral malaria is the most important complication of *falciparum* malaria. However, its pathophysiology is not completely understood. The basic underlying defect seems to be clogging of the cerebral microcirculation by the parasitized red cells. These cells develop knobs on their surface and develop increased cytoadherence properties, as a result of which they tend to adhere to the endothelium of capillaries and venules. This results in sequestration of the parasites in these deeper blood vessels. Also, rosetting of the parasitized and non-parasitized red cells and decreased deformability of the infected red cells further increases the clogging of the microcirculation. It has been observed that the adhesiveness is greater with the mature parasites (Medana *et al*., 2001, Clark and Cowden, 2003, Zaki and Shanbag, 2011). Obstruction to the cerebral microcirculation results in hypoxia and increased lactate production due to anaerobic glycolysis (van der Heyde *et al*., 2006). The parasitic glycolysis may also contribute to lactate production. In patients with cerebral malaria, cerebral spinal fluid (C.S.F). Lactate levels are high and significantly higher in fatal cases than in survivors (Patel *et al*., 2003). The adherent erythrocytes may also interfere with gas and substrate exchange throughout the brain. However, complete obstruction to blood flow is unlikely, since the survivors rarely have any permanent neurological deficit. Vascular permeability is found to be mildly increased, however, no definite evidence of cerebral edema has been found on imaging studies (Babikir, 2010, Zhao and Mackenzie, 2011). The mechanism of coma is not clearly known, increased cerebral anaerobic glycolysis, interference with neurotransmission by sequestered and highly metabolically active parasites have been blamed. Cytokines induce nitric oxide synthesis in leukocytes, smooth muscle cells, microglia and endothelium and NO is a potent inhibitor of neurotransmission (Babikir, 2010).

Cerebral malaria refers to unarousable coma (Glasgow Coma Scale <11), in malaria having excluded, as far as possible, other causes of coma. The proportion of patients with severe malaria who have cerebral malaria varies in time and place. In some parts of the tropics, cerebral malaria is the most common clinical presentation and the major cause of death in adults with severe malaria (Idro *et al*., 2005, Phu *et al*., 2010, Santos *et al*., 2012). While *P. vivax* infections may be associated with some central nervous system dysfunction particularly during paroxysms,

unarousable coma is extremely unusual. This section refers to coma in severe *falciparum* malaria (WHO, 2014a).

2.5.3 Respiratory distress

Acute pulmonary edema is a grave and usually fatal complication of severe *falciparum* malaria with more than 50% mortality (Taylor *et al*., 2012). Acute lung injury is defined as the acute onset of bilateral pulmonary infiltrates with an arterial oxygen tension/fractional inspired oxygen ratio of 300 mmHg or less, a pulmonary artery wedge pressure of 18 mmHg or less, and no evidence of left atrial hypertension (Yuan, 2014). Acute respiratory destress syndrome (ARDS) is defined as acute lung injury and an arterial oxygen tension/fractional inspired oxygen ratio of 200 mmHg or less. Volume overload and hypoalbuminemia may aggravate pulmonary capillary leakage. Chest radiograph abnormalities range from confluent nodules to basilar and/or diffuse bilateral pulmonary infiltrates. No cardiogenic pulmonary edema rarely occurs with *P.vivax* and *P. ovale* malaria (WHO, 2014a, Buregeya *et al*., 2014, Hamza, 2015).

2.5.4 Haemoglobinuria and blackwater fever

Classical descriptions of blackwater fever mention severe intravascular haemolysis with haemoglobinuria in patients with severe manifestations of *P. falciparum* infection, such as renal failure, hypotension and coma, but scanty or absent parasitaemia and mild or absent fever (Fernando *et al*., 2001, Cahill, 2011). Symptoms associated with a typical attack of malaria included loin pain, abdominal discomfort, restlessness, vomiting, diarrhoea, polyuria followed by oliguria and passage of dark red or black urine. Signs included tender hepatosplenomegaly, profound anaemia and jaundice. The exaggerated haemolytic response in the absence of hyperparasitaemia was attributed to immune lysis of quinine-sensitised erythrocytes (Beeson and Brown, 2002, Woodruff and Wright, 2013). However, direct evidence for autoimmune hemolysis in this condition was weak. Although the epidemiological evidence strongly suggests a close link with quinine use, the pathophysiological mechanism has not been identified. In more recent times, intravascular hemolysis with haemoglobinuria has also been observed in Africa among patients who had repeatedly used quinine or halofantrine to treat febrile episodes (Weatherall *et al*., 2009, Bodi *et al*., 2013). As G6PD deficiency is prevalent throughout the malaria affected world, with gene frequencies typically of 5-15% (but up to 35% in some areas), oxidant hemolysis is an important cause of (blackwater fever) independent of malaria, the patients, typically had a greyish pallor, vomiting, loin pain and passage of black/red (Coca-cola™ coloured) urine were common symptoms (Monteiro *et al*., 2014). In the context of severe malaria

treatment, blackwater has been slightly more common in artesunate or artemether recipients than quinine recipients (Dondorp *et al*., 2005a). It seems that some patients with severe malaria and no known enzyme deficiency in oxidant defense systems have severe hemolysis sufficient to cause haemoglobinuria which ever antimalarial drug they receive, severe hemolysis has recently been reported following recovery from severe malaria in artesunate-treated patients (Rolling *et al*., 2012, Jauréguiberry *et al*., 2015). Most of these patients were with hyper parasitaemia, and the hemolysis may be explained, at least in part, by the shortened survival of once infected 'pitted' erythrocytes (Newton *et al*., 2001a, Abdalla and Pasvol, 2004).

2.5.5 Pulmonary oedema

This is a grave and often fatal manifestation of severe *falciparum* malaria in adults which may develop suddenly after one or two days of treatment. Some cases show evidence of fluid overload, with raised central venous or pulmonary artery wedge pressures and grossly positive fluid balance (Estévanez and Hernández-Mora, 2016). Other patients develop pulmonary oedema with normal or negative fluid balance and with normal or reduced pulmonary capillary wedge pressure, this indicates increased pulmonary capillary permeability and so malaria pulmonary oedema resembles the acute respiratory distress syndrome (Taylor *et al*., 2012, Hanson *et al*., 2013). Patients with severe malaria are more vulnerable than those with sepsis to volume overload and readily develop ARDS, hyperparasitaemia, renal failure, and pregnancy are predisposing factors. Hypoglycemia and metabolic acidosis are commonly associated (Dellinger *et al*., 2013). Although pulmonary oedema may develop at any stage of the acute illness, it tends to occur later than the other acute manifestations of malaria, hypoxia may cause convulsions and deterioration in the level of consciousness, and the patient may die within a few hours (Brent *et al*., 2014, WHO, 2014a).

2.5.6 Hypoglycemia

Hypoglycemia is an important complication of *falciparum* malaria and its treatment, often hypoglycemia is not suspected clinically so blood glucose concentrations must always be checked in severely ill patients, the incidence of hypoglycemia at presentation has fallen as use of quinine has declined (Mehta and Das, 2006, Thien *et al*., 2006). The diagnosis of hypoglycemia may be overlooked because all these clinical features are also typical of severe malaria per se. In severe malaria, many of the usual diagnostic features of hypoglycemia may be absent and the diagnosis may be overlooked. Sweating is an inconstant sign, the pupils are frequently not dilated, and the breathing may be cyclical and deep, and there may be abnormal

posturing of the arms and legs. There is usually a deterioration in the level of consciousness (WHO, 2000b, Taylor *et al*., 2012). Following treatment with intravenous 50% glucose, clinical improvement is very variable from no apparent change to a change in the respiratory pattern and a lightening of coma. Hypoglycemia complicates malaria in three clinical settings which may overlap: in patients with severe disease especially young children, in pregnant women and in patients given quinine. Quinine-induced hyperinsulinemia is a common contributing cause of hypoglycemia particularly in pregnant women, and hypoglycemia in this context may be recurrent as glucose administration stimulates further hyperinsulinemia (Madrid *et al*., 2015). Hypoglycemia develops during treatment of malaria significantly more commonly in patients (adults and children) treated with quinine than those treated with artesunate or artemether (Dondorp *et al*., 2005b, Rosenthal, 2008). Hypoglycemia unrelated to quinine is associated with acidosis in severe malaria in adults and children and carries a poor prognosis, it is a direct result of the disease process (Haldar *et al*., 2007, WHO, 2014b). Hypoglycemia is one of the tricky complications of *falciparum* malaria and may often go unnoticed and may be asymptomatic, adding to the morbidity and mortality. Therefore, hypoglycemia, which is easily treatable, may be missed. Added to this, hypoglycemia can occur repeatedly and hence continuous monitoring of blood glucose levels is needed (Provias and Jeynes, 2014).

Causes are increased consumption of glucose by the host and the growing parasites, failure of hepatic gluconeogenesis and glycogenolysis as a result of impaired liver function and acidemia and hyperinsulinemia and stimulation of pancreatic insulin secretion by drugs like quinine. More than one of these factors may be at play in a given patient (Trampuz *et al*., 2003, WHO, 2014b).

2.5.7 Anemia

Anemia is a common manifestation of all types of malaria. It is more common and poses more problems in pregnancy and children. In developing countries of the tropics, pre-existing anemia, most commonly due to malnutrition and helminthiasis, compounds the problem. In *falciparum* malaria, anemia can develop rapidly due to profound hemolysis. The degree of anemia correlates with parasitemia and schizontemia. It is also associated with high serum bilirubin and creatinine levels. Pregnancy, secondary bacterial infections and bleeding disorders like disseminated intravascular coagulation can aggravate the anemia. Children may have severe anemia even with low parasitemia and in such cases the reticuloendothelial cells exhibit abundant malarial pigments. Anemia in malaria is multifactorial. The causes include obligatory destruction of red cells at merogony, accelerated destruction of non-parasitised red cells (major contributor in

anemia of severe malaria), bone marrow dysfunction that can persist for weeks, shortened red cell survival and increased splenic clearance. Massive gastrointestinal haemorrhage can also contribute to the anemia of malaria (Haldar *et al*., 2007, Dondorp *et al*., 2010).

2.5.8 Algid malaria

Algid malaria or hypotension due to peripheral circulatory failure may develop suddenly in severe malaria or it may be the presenting feature in some cases of malaria, with a systolic blood pressure less than 80 mmHg (10.7 kPa) in the supine position [less than 50 mmHg (6.67 kPa) in children], a cold, clammy, cyanotic skin, constricted peripheral veins and rapid feeble pulse. In some countries this clinical picture is often associated with a complicating Gram-negative septicemia and possible sites of associated infection should be sought in such patients, e.g. lung, urinary tract (especially if there is an indwelling catheter), meninges (meningitis), intravenous injection sites, intravenous lines (WHO, 2000b, Lalloo *et al*., 2007). Severe hypotension can also develop suddenly in patients with pulmonary edema, metabolic acidosis, sepsis, and/or massive hemorrhage due to splenic rupture or from the gastrointestinal tract (WHO, 2000a). Postural hypotension may be secondary to autonomic dysfunction (Patel *et al*., 2003). Most patients with shock exhibit a low peripheral vascular resistance and elevated cardiac output. Cardiac pump function appears remarkably well preserved despite intense sequestration of parasitized erythrocytes in the microvasculature of the myocardium (WHO, 2000a). The cardiac index may be elevated with low peripheral vascular resistance and low to normal ventricular filling pressures. Hypovolemia (due to reduced fluid intake, high grade fever, sweating, vomiting and diarrhoea) also may contribute to the reduced pressures. There may be reduction in visceral perfusion. Septicemia, metabolic acidosis and hypoxia may result in a drop in cardiac index. Gram negative septicemia has been blamed as an important cause of hypotension in some cases of *falciparum* infection. Gram negative organisms have been frequently cultured from the blood of patients with cerebral malaria. Septicemia is restricted to patients with severe *falciparum* infection and it may be due to reduced immunity, secondary infections from the gut, in dwelling catheters and intravenous lines and infections in the lung, urinary tract, meninges (Maitland and Newton, 2005, Hanson *et al*., 2009).

2.6 Immunity to malaria

During its complex, multi-stage life cycle, the malaria parasite not only expresses a great variety of proteins at different stages, but these proteins also keep changing often. As a result, a natural infection with malaria parasites leads to of only a partial and short lived immunity that is unable

to protect the individual against a new infection. The complex interplay of parasite proteins with the immune system of the host has also made it difficult or even impossible to develop an effective vaccine against the disease until now. Immunity against malaria can be classified into natural or innate immunity and acquired or adaptive immunity (Srinivas, 2016).

2.6.1 Natural or innate immunity

Innate immunity to malaria is an inherent refractoriness of the host that prevents the establishment of the infection or an immediate inhibitory response against the introduction of the parasite. The innate immunity is naturally present in the host and is not dependent on any previous infection. Alterations in the structure of hemoglobin or in certain enzymes have been found to confer protection against either the infection or its severe manifestations and these traits are often found in areas of high malaria transmission (McKenzie *et al*., 2008). Duffy negativity in red cells protects against *P.vivax* infection. It is found to be widely prevalent in Africa and this may be responsible for the virtual elimination of this parasite from the continent (Singer and Castro, 2011). Certain thalassemias (50% reduction in infection), homozygous hemoglobin C (90% reduction), hemoglobin E, and ovalocytosis carrier status have been reported to confer protection against *P. falciparum* or *P.vivax*. Glucose 6 phosphate dehydrogenase deficiency (50% protection) and sickle cell hemoglobin (90% protection) confer protection against severe malaria and related mortality (Doolan *et al*., 2009). Acute malarial infection also induces immediate, non-specific immune response that tends to limit the progression of disease. The humoral and cellular mechanisms of this 'nonspecific' defense are poorly defined. Primordial, extrathymic T Cells (Natural Killer (NK), intermediate toll cell receptor (TCRint) cells) and autoantibody producing B-1 cells have been considered as the prime movers of this response. Natural killer (NK) cells are found in blood, in secondary lymphoid organs as well as in peripheral non-lymphoid tissues. NK cells have been shown to increase in numbers and to be able to lyse *P.falciparum*-infected erythrocytes in vitro. NK cells in peripheral blood produce Interferon-gamma in response to *Plasmodium* infected erythrocytes, leading to parasiticidal macrophage activation, and this may be of greater importance for innate malaria immunity than their potential to lyse infected host erythrocytes (Sun and Bucala, 2014). These cells are also important in the initiation and development of adaptive immune responses. NK cells induce the production of the proinflammatory chemokine Interleukin-8, that in turn plays its role in the recruitment and the activation of other cells during malaria infection. Dendritic cells, macrophages, gamma delta T cells and NKT cells also sense the presence of the parasite and

participate in the immune response. NKT cells are potent inhibitors of liver-stage parasite replication in mouse malaria systems *in vitro*. Malaria infection gives rise to strongly elevated blood concentrations of non-malaria-specific immunoglobulin, but the importance of the underlying polyclonal B-cell activation for innate immunity is not known (Roetynck *et al*., 2006, Anoja *et al*., 2006).

2.6.2 Acquired or adaptive immunity

Adaptive immunity against malaria develops after infection and its protective efficacy varies depending on the characteristics of the host, place of stay, number of infections suffered. It has been graded as anti-disease immunity that protects against clinical disease, anti-parasite immunity protects against high parasitemia and sterilizing immunity (protects against new infections by maintaining a low-grade, asymptomatic parasitemia; also called premunition), with a considerable overlap between these (McNicholl *et al*., 2000). Following infection with malaria parasites, a nonimmune individual commonly develops an acute clinical illness with very low levels of parasitemia and the infection may progress to severe disease and death. After a couple of more infections, anti-disease immunity develops and causes suppression of clinical symptoms even in the presence of heavy parasitemia and also reduces the risk of severe disease (Bartoloni and Zammarchi, 2012). Frequent and multiple infection slowly lead to the development of anti-parasite immunity that results in very low or undetectable parasitemia. Sterilizing immunity, though never fully achieved, results in a high degree of immune responsiveness, low levels of parasitemia, and an asymptomatic carrier status. Premunition suggests an immunity mediated directly by the presence of the parasites themselves and not as much the result of previous infections (Doolan *et al*., 2009). The presence of genetically and antigenically distinct strains of the parasites in a given locality and the occurrence of clonal antigenic variation during the course of an infection force the host to mount immune response against these different strains and antigenic variants (Quaye, 2016). The acquisition of immunity against malaria is therefore, very slow and not very effective and remains species specific and strain specific. However, in areas with stable endemic malaria and intense malaria transmission, such as sub-Saharan Africa and forest areas in the most of Indian states, acquired immunity develops at a very early age. In these areas, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies. This passive immunity is followed by 1 or 2 years of increased susceptibility before acquisition of active immunity (Doolan *et al*., 2009). The risk of clinical disease increases from birth to about 6 months of age, depending on the transmission rate, and

beginning at around 3 to 4 months of age, infants become susceptible to severe disease and death. The risk of cerebral malaria increases with age in children 2 to 4 years old. At about 2 to 5 years of age, due to repeated and frequent infections, the frequency of clinical disease begins to diminish and the risk of mortality sharply decreases, and by adulthood, most inhabitants generally possess sterilizing immunity. On the other, people living in unstable endemic areas tend to acquire only partial immunity (Doolan *et al*., 2009, Ashwani *et al*., 2007). Thus, the level of antimalarial immunity influences the clinical outcome of the disease in different locations and age groups recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and cluster of differentiation 36 (CD36), or inflammatory cytokines, such as interferon- (IFN-γ), dendritic cells (DCs) mature and migrate to the spleen, the primary site of immune responses against blood-stage *Plasmodium* parasites (Doolan, 2009). Maturation of DCs is associated with the upregulation of expression of MHC class II molecules, CD40, CD80, CD86 and adhesion molecules and the production of cytokines including interleukin-12 (IL-12) (Stevenson and Riley, 2004). IL-12 activates natural killer (NK) cells to produce IFN-γ and induces the differentiation of T helper 1 (TH1) cells. The production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive CD4+ T cells. IL-2 produced by antigen-specific TH1 cells further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response (Basha *et al*., 2014). Cytokines such as IL-10 and transforming growth factor-β (TGF-β) negatively regulate both innate and adaptive responses (Good and Doolan, 2010).

The acquired anti-malaria immunity has been demonstrated to be strain specific and stage specific, with cross reactivity. Immune response have been documented against the various parasite antigens in pre-erythrocytic (sporozoite), asexual erythrocytic (merozoite) and sexual stages (gametocyte). Natural exposure to sporozoites does not induce complete (sterilizing) anti parasite and anti-disease immunity but only limit the density of parasitemia and thereby decrease the malaria-associated morbidity and mortality (Duffy *et al*., 2012). The acquired immunity is directed predominantly against the asexual erythrocytic stage, the primary targets being the extracellular merozoites in circulation. Although the pre-erythrocytic stage is also targeted by protective immune responses, it does not effectively block sporozoite invasion or intrahepatic development of the parasite (Doolan *et al*., 2009). Malaria infection induces both polyclonal and specific immunoglobulin production, predominantly IgM and IgG but also of other

immunoglobulin isotypes. Of these, 5% or more represent species- as well as stage-specific antibodies reacting with a wide variety of parasite antigens. Passive transfer of IgG from immune donors may be protective by reducing parasitemia and clinical disease (Rowe and Kyes, 2004). Antibodies may protect against malaria by a variety of mechanisms. They may inhibit merozoite invasion of erythrocytes and intra-erythrocytic growth or enhance clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration in small vessels and promoting elimination by the spleen. Opsonization of infected erythrocytes significantly increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells such as neutrophils and monocytes/macrophages. Interaction of opsonized erythrocytes with these effector cells induces release of factors such as TNF which may cause tissue lesions but which are also toxic for the parasites (Perlmann and Troye-Blomberg, 2002). Cell-mediated immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages. CD4 T cells are essential for immune protection against asexual blood stages in both murine and human malaria. However, the role of CD8 T cells, which have important effector functions in pre-erythrocytic immunity and which contribute to protection against severe malaria, is less clear. It has been proposed that CD8 T cells may regulate immunosuppression in acute malaria and down-modulate inflammatory responses (McCall and Sauerwein, 2010). As human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by CD8 cytotoxic T lymphocytes has no role in the defense against blood-stage parasites (Perlmann and Troye-Blomberg, 2002). Malaria parasites not only escape the host's immune response, owing to their antigenic diversity and clonal antigenic variation, but also modulate the immune response and cause significant immune suppression. The parasitized red cells, with the deposited hemozoin inside, have been found to inhibit the maturation of antigen presenting dendritic cells, thereby reducing their interaction with T cells, resulting in immunosuppression. Immune suppression in malaria increases the risk of secondary infections (such as nontyphoidal *Salmonella*, *Herpes zoster* virus, hepatitis B virus, Moloney leukemia virus and nematode infections and reactivation of Epstein-Barr virus) and may also reduce the immune response to certain vaccines (Owain *et al*., 2006). The acquired anti malaria immunity does not last long. In the absence of re-infection for about 6 months or 1 year, as may happen when the person leaves the malarious area, the acquired immunity turns ineffective and the individual becomes vulnerable to the full impact of a malarial infection once again. The immunity is also rendered less effective during pregnancy, particularly during the first and

second pregnancies, due to the physiological immunosuppression as well as the cytoadherence of erythrocytes to the newly available Chondroitin Sulfate A receptors on the placenta. Such loss of acquired immunity makes the pregnant woman more susceptible to malaria and its complications (Denise *et al*., 2009). Immunosuppression in HIV also increases the risks of clinical malaria, its complications and death (Laith *et al*., 2006)**.**

2.7 Diagnosis of malaria

Diagnosis of malaria involves identification of malaria parasite or its antigens products in the blood of the patient. Although this seems simple, the efficacy of the diagnosis is subject to many factors (Tangpukdee *et al*., 2009). The different forms of the four malaria species; the different stages of erythrocytic schizogony; the endemicity of different species; the population movements; the inter-relation between the levels of transmission, immunity, parasitemia, and the symptoms; the problems of recurrent malaria, drug resistance, persisting viable or nonviable parasitemia, and sequestration of the parasites in the deeper tissues; and the use of chemoprophylaxis or even presumptive treatment on the basis of clinical diagnosis can all have a bearing on the identification and interpretation of malaria parasitemia on a diagnostic test. The diagnosis of malaria is confirmed by blood tests and can be divided into microscopic and non-microscopic tests (Suwalka *et al*., 2012)

2.7.1 Microscopic tests

The microscopic tests involve staining and direct visualization of the parasite under the microscope. For more than hundred years, the direct microscopic visualization of the parasite on the thick and/or thin blood smears has been the accepted method for the diagnosis of malaria in most settings, from the clinical laboratory to the field surveys. The careful examination of a well-prepared and well-stained blood film currently remains the "gold standard" for malaria diagnosis. The most commonly used microscopic tests include the peripheral smear study and the Quantitative Buffy Coat (QBC) test. The simplest and surest test is the time-honoured peripheral smear study for malarial parasites. None of the other newer tests have surpassed the 'gold standard' peripheral smear study (Prescott *et al*., 2012)

2.7.1.1 Peripheral smear study for malarial parasites

The Light microscopy of thick and thin stained blood smears remains the standard method for diagnosing malaria. It involves collection of a blood smear, its staining with Romanowsky stains and examination of the Red Blood Cells for intracellular malarial parasites (Shapiro *et al*., 2013). Thick smears are 20-40 times more sensitive than thin smears for screening of *Plasmodium*

parasites, with a detection limit of 10–50 trophozoites/µl. Thin smears allow one to identify malaria species (including the diagnosis of mixed infections), quantify parasitemia, and assess for the presence of schizonts, gametocytes, and malarial pigment in neutrophils and monocytes (Wangai *et al*., 2011). The peripheral blood smear provides comprehensive information on the species, the stages, and the density of parasitemia. The efficiency of the test depends on the quality of the equipment and reagents, the type and quality of the smear, skill of the technician, the parasite density, and the time spent on reading the smear. The test takes about 20 to 60 minutes depending on the proximity of the laboratory and other factors mentioned above (Sreekanth *et al*., 2011). Before reporting a negative result, at least 200 oil immersion visual fields at a magnification of 1000× should be examined on both thick and thin smears, which has a sensitivity of 90%. The level of parasitemia may be expressed either as a percentage of parasitized erythrocytes or as the number of parasites per microliter of blood (Mangala, 2012). In non-*falciparum* malaria, parasitemia rarely exceeds 2%, whereas it can be considerably higher (>50%) in *falciparum* malaria. In nonimmune individuals, hyperparasitemia (>5% parasitemia or >250 000 parasites/µl) is generally associated with severe disease (White *et al*., 2003b). The smear can be prepared from blood collected by venipuncture, finger prick and ear lobe stab. In obstetric practice, cord blood and placental impression smears can be used. In fatal cases, post-mortem smears of cerebral grey matter obtained by needle necropsy through the foramen magnum, superior orbital fissure, and ethmoid sinus via the nose or through fontanelle in young children can be used (Srinivas, 2016 and Brown).

2.7.1.2 Thick smear

The thick smear of correct thickness is the one through which newsprint is barely visible. It is dried for 30 minutes and not fixed with methanol. This allows the red blood cells to be hemolyzed and leukocytes and any malaria parasites present will be the only detectable elements (Heilmeyer and Begemann, 2004). However, due to the hemolysis and slow drying, the plasmodia morphology can get distorted, making differentiation of species difficult. Thick smears are therefore used to detect infection, and to estimate parasite concentration (Mehta and Desai, 2013).

2.7.1.3 Thin smear

Air dry the thin smear for 10 minutes. After drying, the thin smear should be fixed in methanol. This can be done by either dipping the thin smear into methanol for 5 seconds or by dabbing the

thin smear with a methanol-soaked cotton ball. While fixing the thin smear, all care should be taken to avoid exposure of the thick smear to methanol (Mehta and Desai, 2013, Jan *et al*., 2017).

2.7.1.4 Staining

A number of Romanowsky stains like Field's, Giemsa's, Wright's and Leishman's are suitable for staining the smears. Thick films are ideally stained by the rapid Field's technique or Giemsa's stain for screening of parasites (Mehta and Desai, 2013). The sensitivity of a thick blood film is 5-10 parasites/µl. thin blood films stained by Giemsa's or Leishman's stain are useful for specification of parasites and for the stippling of infected red cells and have a sensitivity of 200 parasites/µl. The optimal pH of the stain is 7.2. Slides should be clean and dry (Mehta and Das, 2006). The thick film is first dehemoglobinized in water and then stained with Giemsa (Rapid Giemsa: Prepare a 10% Giemsa in buffered water at pH 7.1. Immerse the slide in the stain for 5 minutes. Rinse gently for 1 or 2 seconds in a jar of tap water. Drain, dry and examine. Standard Giemsa: Prepare a 4% Giemsa in buffered solution at pH 7.1. Immerse the slide (at least 12 hours old) in stain for 30 minutes. Rinse with fresh water, drain, dry and examine (Muhammad *et al*., 2009, Hayat, 2012).

2.7.2 Quantitative buffy coat (QBC)

The QBC test, developed by Becton and Dickenson Inc., is a new method for identifying the malarial parasite in the peripheral blood. It involves staining of the centrifuged and compressed red cell layer with acridine orange and its examination under UV light source. It is fast, easy and claimed to be more sensitive than the traditional thick smear examination (Tangpukdee *et al*., 2009). Precision glass hematocrit tube, pre-coated internally with acridine orange stain and potassium oxalate. It is filled with 55-65 microliters of blood from a finger, ear or heel puncture. A clear plastic closure is then attached. A precisely made cylindrical float, designed to be suspended in the packed red blood cells, is inserted. The tube is centrifuged at 12,000 rpm for 5 minutes. The components of the buffy coat separate according to their densities, forming discrete bands. Because the float occupies 90% of the internal lumen of the tube, the leukocyte and the thrombocyte cell band widths and the top-most area of red cells are enlarged to 10 times normal. The QBC tube is placed on the tube holder and examined using a standard white light microscope equipped with the UV microscope adapter, an epi-illuminated microscope objective. Fluorescing parasites are then observed at the red blood cell/white blood cell interface (Chotivanich *et al*., 2007). The key feature of the method is centrifugation and thereby concentration of the red blood cells in a predictable area of the QBC tube, making detection easy

and fast, red cells containing Plasmodia are less dense than normal ones and concentrate just below the leukocytes, at the top of the erythrocyte column. The float forces all the surrounding red cells into the 40 micron space between its outside circumference and the inside of the tube. Since the parasites contain DNA which takes up the acridine orange stain, they appear as bright specks of light among the non-fluorescing red cells. Virtually, all of the parasites found in the 60 microliter of blood can be visualized by rotating the tube under the microscope. A negative test can be reported within one minute and positive result within few minutes (Chigurupati and Srinivas, 2016).

2.7.3 Rapid diagnostic test (RDTs)

Immunochromatographic tests (ICT) for the detection of malaria antigens, developed in the past decade, have opened a new and exciting avenue in malaria diagnosis. However, their role in the management and control of malaria appears to be limited at present. The tests are based on the capture of the parasite antigens from the peripheral blood using either monoclonal or polyclonal antibodies against the parasite antigen targets. Currently, ICT tests can target the histidine-rich protein 2 (HRP2) of *P.falciparum*, a pan-malarial *Plasmodium* aldolase, and the parasite specific lactate dehydrogenase. These RDTs do not require a laboratory, electricity, or any special equipment (Tamboli *et al*., 2015). Histidine-rich protein 2 of *P. falciparum* (PfHRP2) is a water soluble protein that is produced by the asexual stages and gametocytes of *P.falciparum*, expressed on the red cell membrane surface, and shown to remain in the blood for at least 28 days after the initiation of antimalarial therapy. Several RDTs targeting PfHRP2 have been developed. *Plasmodium* aldolase is an enzyme of the parasite glycolytic pathway expressed by the blood stages of *P.falciparum* as well as the non-*falciparum* malaria parasites. Monoclonal antibodies against *Plasmodium* aldolase are pan-specific in their reaction and have been used in a combined 'P.f/P.v' (Leow *et al*., 2014). Immunochromatographic test that targets the pan malarial antigen (PMA) along with PfHRP2. Parasite lactate dehydrogenase (pLDH) is a soluble glycolytic enzyme produced by the asexual and sexual stages of the live parasites and its present in blood and released from the parasite infected erythrocytes. It has been found in all 4 human malaria species, and different isomers of pLDH for each of the 4 species exist. With pLDH as the target, a quantitative immunocapture assay, a qualitative immunochromatographic dipstick assay using monoclonal antibodies, an immunodot assay and a dipstick assay using polyclonal antibodies have been developed (Srinivas, 2016).

2.7.4 Polymerase chain reaction (PCR)

Using the non-isotopically labelled probe following PCR amplification, it is possible to detect malaria parasites. In travelers returning to developed countries, studies based on PCR have been found to be highly sensitive and specific for detecting all 4 species of malaria, particularly in cases of low level parasitemia and mixed infections (Image, 2013). The PCR test is reportedly 10-fold more sensitive than microscopy, with one study reporting a sensitivity to detect 1.35 to 0.38 parasites/µL for *P.falciparum* and 0.12 parasites/µL for *P.vivax*. The PCR test has also been found useful in unraveling the diagnosis of malaria in cases of undiagnosed fever (Mehta and Desai, 2013).

2.7.5 Detection of antimalarial antibodies

Antibodies to the asexual blood stages appear a few days after malarial infection, increase in titer over the next few weeks, and persist for months or years in semi-immune patients in endemic areas, where reinfection is frequent. In non-immune patients, antibodies fall more rapidly after treatment for a single infection and are undetectable in 3-6 months (Struik and Riley, 2004). Re-infection/relapse induces a secondary response with a rapidly increasing antibody titer (Mehta and Desai, 2013). Malarial antibodies can be detected by immunofluorescence or enzyme immune-assay. It is useful in epidemiological surveys, for screening potential blood donors and occasionally for providing evidence of recent infection in non-immunes. In future, detection of protective antibodies will be important in assessing the response to malaria vaccines (Drakeley and Cook, 2009).

2.7.6 Intraleukocytic malaria pigment

Intraleukocytic malaria pigment has been suggested as a measure of disease severity in malaria. The proportion of pigment-containing monocytes did not differ significantly between the mild malaria, asymptomatic malaria and no malaria groups but the cerebral malaria group had a higher median value than the other 3 groups. The ratio of pigment-containing neutrophils to pigment-containing monocytes showed the same trend across the groups of subjects as was observed with the number of pigment-containing neutrophils (Mohammed *et al*., 2003, De Langen *et al*., 2006, Hänscheid *et al*., 2007).

2.7.7 Flow cytometry

Flow cytometry and automated hematology analyzers have been found to be useful in indicating a diagnosis of malaria during routine blood counts. In cases of malaria, abnormal cell clusters and small particles with DNA fluorescence, probably free malarial parasites, have been seen on

automated hematology analyzers and it is suggested that malaria can be suspected based on the scatter plots produced on the analyzer. Automated detection of malaria pigment in white blood cells may also suggest a possibility of malaria with a sensitivity of 95% and specificity of 88%. On flow cytometric depolarized side scatter, the average relative frequency of pigment carrying monocytes was found to differ among semi-immune, non-immune and malaria negative patients (Hänscheid *et al*., 2001, Mehta and Desai, 2013).

2-7-8 Mass spectrometry

A novel method for the in vitro detection of the malarial parasite at a sensitivity of 10 parasites/µl of blood has been recently reported. It comprises a protocol for cleanup of whole blood samples, followed by direct ultraviolet laser desorption time-of-flight mass spectrometry. Intense ion signals are observed from intact ferriprotoporphyrin IX (heme), sequestered by malaria parasites during their growth in human red blood cells. The laser desorption mass spectrum of the heme is structure-specific, and the signal intensities are correlated with the sample parasitemia. Many samples could be prepared in parallel and measurement per sample may not take longer than a second or so. However, the remote rural areas without electricity are not hospitable for existing high-tech mass spectrometers. Future improvements in the equipment and technique can make this method deployable and useful (Demirev, 2004, Tangpukdee *et al*., 2009).

2-7-9 Other investigations

Total and differential count, hemoglobin, blood glucose, serum bilirubin, serum creatinine, blood urea nitrogen (BUN), AST, ALT, Prothrombin time, urine analysis may be done as needed. Widal test may be positive, even up to a dilution of 1:320 for 'O' and H' and at lower titres for 'AH' and 'BH'. Any or all the four may be positive, suggesting a nonspecific response. A positive Widal test in a patient with confirmed malaria should not therefore be considered as suggestive of typhoid fever (Srinivas, 2016).

2.8 Treatment of malaria

Mother Nature gave us the cinchona alkaloids and qinghaosu. World War II led to the introduction of chloroquine, chloroguanide (proguanil), and eventually amodiaquine and pyrimethamine (Gupta *et al*., 2010).The war in Vietnam brought mefloquine and halofantrine. These drugs are available now to treat malaria. It is difficult to see where the next generation of antimalarial drugs will come from there is little pharmaceutical industry interest in developing new antimalarial drugs (Miller *et al*., 2013). The risks are great, but the returns on investment are

low, if drug resistance in *P. falciparum* continues to increase at the current rate, malaria may become untreatable in parts of Southeast Asia by the beginning of the next millennium (Lindsay *et al*., 2004). The effectiveness of early diagnosis and prompt treatment as the principal technical components of the global strategy to control malaria is highly dependent on the efficacy, safety, availability, affordability and acceptability of antimalarial drugs. The effective antimalarial therapy not only reduces the mortality and morbidity of malaria, but also reduces the risk of resistance to antimalarial drugs (Tanner *et al*., 2015). Therefore, antimalaria chemotherapy is the keystone of malaria control efforts. On the other hand, not many new drugs have been developed to tackle malaria, of the 1223 new drugs registered between 1975 and 1996, only 3 were antimalarial. Hence the need for a rational antimalarial treatment policy (WHO, 2000a).

2.8.1 Classification

Anti-malarial drugs can be classified according to anti-malarial activity and according to structure (Wong *et al*., 2014).

2.8.1.1 According to anti-malarial activity

2.8.1.2 Tissue schizonticides for causal prophylaxis

These drugs act on the primary tissue forms of the plasmodia which after growth within the liver, initiate the erythrocytic stage. By blocking this stage, further development of the infection can be theoretically prevented. Pyrimethamine and Primaquine have this activity. However, since it is impossible to predict the infection before clinical symptoms begin, this mode of therapy is more theoretical than practical (Held *et al*., 2013).

2.8.1.3 Tissue schizonticides for preventing relapse

These drugs act on the hypnozoites of *P.vivax* and *P.ovale* in the liver that cause relapse of symptoms on reactivation. Primaquine is the prototype drug; pyrimethamine also has such activity (Baird *et al*., 2007).

2.8.1.4 Blood schizonticides

These drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in antimalarial chemotherapy. These include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines (Ibezim and Odo, 2008).

2.8.1.5 Gametocytocides

These drugs destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocytocidal

activity against *P.vivax* and *P.malariae*, but not against *P.falciparum*. Primaquine has gametocytocidal activity against all plasmodia, including *P.falciparum* (Bousema *et al*., 2010).

2.8.1.6 Sporontocides

These drugs prevent the development of oocysts in the mosquito and thus ablate the transmission. Primaquine and chloroguanide have this action (Kiszewski, 2010). Thus in effect, treatment of malaria would include a blood schizonticide, a gametocytocidal and a tissue schizonticide (in case of *P. vivax* and *P.ovale*). A combination of chloroquine and primaquine is thus needed in all cases of malaria (Carter *et al*., 2017).

2.8.2 According to the structure

2.8.2.1 Aryl amino alcohols

Quinine, quinidine (cinchona alkaloids), mefloquine, halofantrine (Griffin *et al*., 2012).

2.8.2.2 4-Aminoquinolines

Chloroquine, amodiaquine (Tekete *et al*., 2009).

2.8.2.3 Folate synthesis inhibitors

This two types: either competitive inhibitors of dihydropteroate synthase- sulphones, sulphonamides; or inhibit dihydrofolate reductase- biguanides like proguanil and chloroproguanil; diaminopyrimidine like pyrimethamine (Walawalkar, 2004).

2.8.2.4 8-Aminoquinolines

Primaquine (Tekwani, Walker, 2006).

2.8.2.5 Antimicrobials

Tetracycline, doxycycline, clindamycin, azithromycin, fluoroquinolones Peroxides: Artemisinin (Qinghaosu) derivatives and analogues- artemether, arteether, artesunate, artelinic acid (Burrows *et a*l., 2011).

2.8.2.6 Naphthoquinones

Atovaquone is a synthetic hydroxynaphthoquinone developed in the early 1980s, atovaquone has been found to be useful against the Plasmodia (as well as *Toxoplasma* and *Pneumocystis carinii*) (Rathore *et al*., 2005). It has a highly lipophilic molecule that supposedly interferes with the mitochondrial electron transport and thereby adenosine tri phosphate (ATP) and pyrimidine biosynthesis and in *Plasmodia*, it is found to target cytochrome bc1 complex and disrupt the membrane potential (Sharma *et al*., 2013, Mukherjee *et al*., 2016).

2.8.2.7 Iron chelating agents

Desferrioxamine (Srinivas, 2016).

2.8.2.8 The artemisinin derivatives

2.8.2.8.1 Artemisinin or qinghaosu

Artemisinin or Qinghaosu ("ching-how-soo") is the active principal of the Chinese medicinal herb Artemisia annua. It has been used as treatment of fevers in China for more than 1000 years. The antimalarial value of Artemisia annua was first documented in Zhou Hou Bei Ji Fang (Handbook of prescriptions for emergency treatments) written as early as 340 AD by Ge Hong of the Eastern Jin Dynasty. The active antimalarial constituent of this plant was isolated in 1971 and it was named artemisinin. The WHO accorded high priority to the development of fast acting artemisinin derivatives for the treatment of cerebral malaria as well as for the control of multi-drug resistant *P.falciparum* malaria. A water-soluble ester called artesunate and two oil soluble preparations called artemether and arteether (artemotil) have now been developed (Cui and Su, 2009).

2.8.2.8.2 Antimalarial activity

They act by inhibiting a *P. falciparum*-encoded sarcoplasmic-endoplasmic reticulum calcium ATPase, and not by inhibiting the haem metabolic pathway as previously supposed (Li and Zhou, 2010). Most clinically important artemisinins are metabolised to dihydroartemisinin (elimination half-life of about 45 min), in which form they have comparable antimalarial activity (Galusic, 2015). However, their use in monotherapy is associated with high incidences of recrudescent infection, suggesting that combination with other antimalarial might be necessary for maximum efficacy. It is the fastest acting antimalarial available. It inhibits the development of the trophozoites and thus prevents progression of the disease. Young circulating parasites are killed before they sequester in the deep microvasculature (Eastman and Fidock, 2009). These drugs starts acting within 12 hours. These properties of the drug are very useful in managing complicated *P.falciparum* malaria. These drugs are also effective against the chloroquine resistant strains of *P. falciparum*. Artesunate and artemether have been shown to clear parasitaemia more effectively than chloroquine and sulfadoxine/pyrimethamine. Meta-analysis of mortality in trials indicated that a patient treated with artemether had at least an equal chance of survival as a patient treated with quinine. It has also been reported that artemisinin drugs cleared parasites faster than quinine in patients with severe malaria but fever clearance was

similar. Also, parenteral artemether and artesunate are easier to use than quinine and do not induce hypoglycaemia (Cui and Su, 2009).

2.8.2.8.3 Gametocytocidal action

Artemisinin compounds have been reported to reduce gametocytogenesis, thus reducing transmission of malaria, this fact being especially significant in preventing the spread of resistant strains. These drugs prevent the gametocyte development by their action on the ring stages and on the early (stage I-III) gametocytes. In studies including over 5000 patients in Thailand, it was shown that gametocyte carriage was significantly less frequent after treatment with artemisinin derivatives than after treatment with mefloquine (Rajendran, 2013).

2.8.2.8.4 Absorption, fate and excretion

Artemisinin derivatives are absorbed well after intramuscular or oral administration. The drug is fully metabolized and the major metabolite is dihydroartemisinin, which also has anti-parasite effects. It is rapidly cleared, predominantly through the bile (Gautam *et al*., 2009)**.**

2.8.2.8.5 Toxicity

Toxic effects have been reported less frequently with the artemisinin than with other antimalarial agents. The most common toxic effects that have been identified are nausea, vomiting, anorexia, and dizziness; these are probably due, in many patients, to acute malaria rather than to the drugs (Rosenthal, 2008). More serious toxic effects, including neutropenia, anemia, hemolysis, and elevated levels of liver enzymes, have been noted rarely. Two cases of severe allergic reactions to oral artesunate have been reported, with an estimated risk of approximately 1 reaction per 3000 treatments (Walgren *et al*., 2005).

2.8.2.9 Artemether

Available as 80mg/ml Injection and 40 mg per capsule Injection: 3.2 mg/kg intramuscularly as a loading dose, followed by 1.6 mg/kg daily until oral therapy. Oral: 4 mg/kg on first day followed by 2mg/kg (Mathew, 2010).

2.8.2.10 Arteether

Also Known (Artemotil), available as 150 mg per 2 ml ampoule, dose: 3 mg/kg once a day for 3 days, as deep intramuscular injection (Patel *et al*., 2014).

2.8.2.11 Artesunate

Available as 50mg tablets and 60 mg/ml injection, also available as 100mg suppository and in Switzerland is available as 200mg recto cap, Oral: 4 mg/kg. Parenteral: Loading dose of 2.4 mg/kg followed by 2.4mg/kg after 12 hours, 24 hours and once daily thereafter (Srinivas, 2016).

Artesunate dosages need not be changed because of hepatic or renal failure or concomitant or previous therapy with other medications, including previous therapy with mefloquine, quinine, or quinidine. There are no known interactions between artesunate and other drugs (Rosenthal, 2008).

2.8.2.12 Chloroquine

Chloroquine is the prototype anti-malarial drug, most widely used to treat all types of malarial infections. It is also the cheapest, time tested and safe antimalarial agent (Gurib-Fakim, 2006).

2.8.2.12.1 Mechanism of action

The mechanism of action of chloroquine is unclear. Being alkaline, the drug reaches high concentration within the food vacuoles of the parasite and raises its pH. It is found to induce rapid clumping of the pigment (Awasthi and Das, 2013). Chloroquine inhibits the parasitic enzyme heme polymerase that converts the toxic heme into non-toxic hemozoin, thereby resulting in the accumulation of toxic heme within the parasite. It may also interfere with the biosynthesis of nucleic acids. Other mechanisms suggested including formation of drug-heme complex, intercalation of the drug with the parasitic DNA (Coronado *et al*., 2014).

2.8.2.12.2 Absorption, fate and excretion

About 90% of the drug is absorbed from gastro intestinal tract (G.I.T) and rapidly absorbed from intramuscular and subcutaneous sites. It has a large distribution volume due to extensive sequestration in tissues of liver, spleen, kidney, lung etc. Hence the need for a larger loading dose. Therapeutic blood levels persist for 6-10 days and elimination half-life is 1-2 months. Half of the drug is excreted unchanged by the kidneys, remaining is converted to active metabolites in the liver (Szakacs *et al*., 2008).

2.8.2.12.3 Antimalarial activity

It is highly effective against erythrocytic forms of *P. vivax*, *P. ovale* and *P. malariae*, sensitive strains of *P. falciparum* and gametocytes of *P. vivax* (Bloland and WHO, 2001). It rapidly controls acute attack of malaria with most patients became afebrile within 24-48 hours. It is more effective and safer than quinine for sensitive cases (Carter *et al*., 2017).

2.8.2.12.4 Adverse effects

Chloroquine is a relatively safer anti-malarial. At therapeutic doses, it can cause dizziness, headache, diplopia, disturbed visual accommodation, dysphagia, nausea, malaise, and pruritus of palms, soles and scalp. It can also cause visual hallucinations, confusion, and occasionally frank psychosis. These side effects do not warrant stoppage of treatment (Bloland and WHO, 2001). It

can exacerbate epilepsy. When used as prophylactic at 300 mg of the base/ week, it can cause retinal toxicity after 3-6 years (i.e. after 50-100 g of chloroquine). Intramuscular injections of chloroquine can cause hypotension and cardiac arrest, particularly in children (WHO, 2001b).

2.8.2.12.5 Contraindications

Chloroquine should be used with caution in patients with hepatic disease, (even though it is not hepatotoxic per se, it is distributed widely in the liver and is converted to active metabolites there; hence the caution), severe gastrointestinal, neurological or blood disorders. The drug should be discontinued in the event of such problems during therapy. It should not be co-administered with gold salts and phenylbutazone, because all the three can cause dermatitis. Chloroquine may interfere with the antibody response to human diploid cell rabies vaccine (Jain, 2011).

2.8.2.12.6 Availability

Chloroquine is available as Chloroquine phosphate tablets; each 250-mg tablet contains 150 mg of the base. Chloroquine hydrochloride injection contains 40 mg of the base per ml**.** and it's dose: Oral- 10 mg/kg stat., then three doses of 5 mg/kg, over 36-48 hours (WHO, 2010a).

2.8.2.13 Quinine

Quinine is the chief alkaloid of cinchona bark (known as 'Fever Bark'), a tree found in South America. It has a colourful history of more than 350 years. Calancha, an Augustinian monk of Lima, first wrote about the curative properties of cinchona powder in "fevers and tertians" as early as in 1633. By 1640, the bark had already found its way into Europe, thanks to the Jesuit fathers (hence the name 'Jesuit's bark'). Eminent philosopher Cardinal de Lugo popularised the bark in Rome (hence it is also called Cardinal's bark). In 1820, Pelletier and Caventou isolated quinine and cinchonine from cinchona. Even today, quinine is obtained entirely from the natural sources due the difficulties in synthesising the complex molecule (Maehara *et al*., 2011, Achan *et al*., 2011).

2.8.2.13.1 Mechanism of action

Quinine acts as a blood schizonticide although it also has gametocytocidal activity against *P. vivax* and *P. malariae*. Because it is a weak base, it is concentrated in the food vacuoles of *P. falciparum*. It is said to act by inhibiting heme polymerase, thereby allowing accumulation of its cytotoxic substrate, heme. As a schizonticidal drug, it is less effective and more toxic than chloroquine. However, it has a special place in the management of severe falciparum malaria in areas with known resistance to chloroquine (Achan *et al*., 2011, Delves *et al*., 2012).

2.8.2.13.2 Absorption, fate and excretion

Quinine is readily absorbed when given orally or intramuscularly. Peak plasma concentrations are achieved within 1-3 hours after oral dose and plasma half-life is about 11 hours. In acute malaria, the volume of distribution of quinine contracts and clearance is reduced, and the elimination half-life increases in proportion to the severity of the illness. Therefore, maintenance dose of the drug may have to be reduced if the treatment is continued for more than 48 hours. The drug is extensively metabolized in the liver and only 10% is excreted unchanged in the urine. There is no cumulative toxicity on continued administration (Achan *et al*., 2011, Carter *et al*., 2017).

2.8.2.13.3 Adverse effects

Functional impairment of the eighth nerve results in tinnitus, decreased auditory acuity and Quinine is a potentially toxic drug. The typical syndrome of quinine side effects is called as cinchonism and it can be mild in usual therapeutic dosage or could be severe in larger doses. Mild cinchonism consists of ringing in the ears, headache, nausea and disturbed vision. vertigo. Visual symptoms consist of blurred vision, disturbed colour perception, photophobia, diplopia, night blindness, and rarely, even blindness (Diener *et al*., 2002). These changes are due to direct neurotoxicity, although vascular changes may contribute to the problem. Gastrointestinal symptoms like nausea, vomiting, abdominal pain and diarrhoea may be seen. Rashes, sweating, angioedema can occur. Excitement, confusion, delirium are also seen in some patients. Coma, respiratory arrest, hypotension, and death can occur with overdosage (Dondorp *et al*., 2010). Quinine can also cause renal failure. Massive hemolysis and hemoglobinuria can occur, especially in pregnancy or on repeated use. Hypoprothrombinemia, agranulocytosis are also reported (Srinivas, 2016).

2.8.2.13.4 Contraindications

Hypersensitivity in the form of rashes, angioedema, visual and auditory symptoms are indications for stopping the treatment. It is contraindicated in patients with tinnitus and optic neuritis. It should be used with caution in patients with atrial fibrillation. Hemolysis is indication for immediately stopping the drug. It is also contraindicated in patients suffering from myasthenia gravis (Singhal, 2004).

2.8.2.13.5 Availability

It is available as tablets and capsules containing 300 or 600 mg of the base. It is also available as injections, containing 300mg /ml. and its dose**:** Oral: 10 mg/kg 8 hourly for 4 days and 5 mg/kg 8

hourly for 3 days. Intravenous: 20 mg of salt/kg in 10 ml/kg isotonic saline or 5% dextrose over 4 hours, then 10 mg of salt/kg in saline or dextrose over 4 hours, every 8 hours until patient is able to take orally or for 5-7 days. Intramuscular: 20 mg/kg stat, followed by 10 mg/kg 8 hourly by deep intramuscular injections for 5-7 days (WHO, 2010b).

2.8.2.14 Quinidine

The anti-arrhythmic drug related to quinine can also be used in the treatment of severe *P. falciparum* malaria. Dose is 10 mg of base / kg by infusion over 1-2 hours, followed by 0.02 mg/kg/min with ECG monitoring (White *et al*., 2003b, Parise and Lewis, 2005, Schwartz, 2009).

2.9.2.15 Chloroguanide (proguanil)

More popularly known as proguanil, this drug was developed by British antimalarial research in 1945. It is a biguanide derivative that is converted to an active metabolite called cycloguanil pamoate. It exerts its antimalarial action by inhibiting parasitic dihydrofolate reductase enzyme. It has causal prophylactic and suppressive activity against *P. falciparum* and cures the acute infection. It is also effective in suppressing the clinical attacks of *vivax* malaria. However, it is slower compared to 4-aminoquinolines. Chloroguanide is slowly but adequately absorbed from the gastrointestinal tract. Peak plasma levels are attained within 5 hours and elimination half-time is about 16-20 hours. Chloroguanide is available as tablets, each containing 100 mg of the drug. The dose for prophylaxis is 100-200 mg daily. Chloroguanide along with chloroquine is used as prophylaxis effective against *P.falciparum* malaria. At the prophylactic doses, it produces occasional nausea and diarrhoea. It is otherwise a safe drug and can be used in pregnancy (Riemsdijk *et al*., 2002, WHO, 2010b).

2.8.2.16 Sulfadoxine and pyrimethamine

Pyrimethamine and sulphadoxine are very useful adjuncts in the treatment of uncomplicated, chloroquine resistant, *P. falciparum* malaria. It is now used in combination with artesunate for the treatment of *P. falciparum* malaria. It is also used in intermittent treatment in pregnancy (IPTp) (Kremsner and Krishna, 2004, Barnes *et al*., 2006).

2.8.2.16.1 Antimalarial activity

Pyrimethamine inhibits the dihydrofolate reductase of plasmodia and thereby blocks the biosynthesis of purines and pyrimidines, which are so essential for DNA synthesis and cell multiplication. This leads to failure of nuclear division at the time of schizont formation in erythrocytes and liver. Sulfadoxine inhibits the utilisation of para-aminobenzoic acid in the

synthesis of dihydropteroic acid. The combination of pyrimethamine and sulfa thus offers two step synergistic blockade of plasmodial division (Ducati *et al*., 2013).

2.8.2.16.2 Absorption, fate and excretion

Pyrimethamine is slowly but completely absorbed after oral administration and is eliminated slowly with a plasma half-life of about 80-95 hours. Suppressive drug levels may be found in the plasma for up to 2 weeks. The drug is excreted in breast milk. Sulfonamides are rapidly absorbed from the gut and are bound to plasma proteins. They are metabolized in the liver and are excreted in the urine. They pass through the placenta freely. Sulfadoxine is a long acting sulfonamide with a half-life of 7-9 days (Deen *et al*., 2008, Tornio *et al*., 2012).

2.8.2.16.3 Toxicity and contraindications

Pyrimethamine can cause occasional skin rashes and depression of hematopoiesis. Excessive doses can produce megaloblastic anemia. Sulfonamides can cause numerous adverse effects. Agranulocytosis; aplastic anemia; hypersensitivity reactions like rashes, fixed drug eruptions, erythema multiform of the Steven Johnson type, exfoliative dermatitis, serum sickness; liver dysfunction; anorexia, vomiting and acute hemolytic anemia can also occur. The drug is contraindicated in patients with known hypersensitivity to sulfa, infants below 2 months of age, patients with advanced renal disease and first and last trimesters of pregnancy (Deen *et al*., 2008, Tornio *et al*., 2012).

2.8.2.16.4 Availability

Pyrimethamine and sulphadoxine is no longer used as a single drug, but only in combination with artesunate (Srinivas, 2016).

2.8.2.17 Halofantrine

Halofantrine was developed in the 1960s by the Walter Reed Army Institute of Research. It is a phenanthrene methanol structurally related to quinine. Its mechanism of action may be similar to that of chloroquine, quinine, and mefloquine; by forming toxic complexes with ferriprotoporphyrin IX that damage the membrane of the parasite. This synthetic anti-malarial is effective against multi drug resistant (including mefloquine resistant) *P. falciparum* malaria. Its bioavailability is low and variable (may be doubled if taken with a fatty meal). The peak plasma concentration is achieved in 4-8 hours after the oral dose. The elimination half-life is 1-3 days for the parent drug and 3-7 days for the active metabolite. Halofantrine is no more used in the treatment of chloroquine resistant and multidrug resistant, uncomplicated *P.falciparum* malaria (Kremsner and Krishna, 2004, Schlitzer, 2007). The dose for adults, three tablets of 500 mg

each, 6 hours apart. For children, three doses of 8 mg/kg of the salt 6 hours apart. Treatment should be repeated after 7 days. Side effects include abdominal pain, diarrhoea, and prolongation of QTC interval and arrhythmias that could be fatal. It is contraindicated in patients with prolonged QTC interval (congenital, electrolyte disorders, myocardial disease). However, it appears less toxic than quinine and mefloquine. It is also contraindicated in pregnancy and lactation, infants, and patients who have received mefloquine in the preceding 3 weeks (WHO, 2010b).

2.8.2.18 Mefloquine

Mefloquine was born during the Vietnam War, as a result of research into newer antimalarials, to protect the American soldiers from the multi drug resistant *falciparum* malaria. Nothing much has happened after that and hence this 'new' drug should be restricted for use against multi drug resistant *falciparum* only (Gelband *et al*., 2004).

2.8.2.18.1 Antimalarial activity

Mefloquine has been found to produce swelling of the *P.falciparum* food vacuoles. It may act by forming toxic complexes with free heme that damage membranes and interact with other plasmodial components. It is effective against the blood forms of falciparum malaria, including the chloroquine resistant types (Smithson *et al*., 2010).

2.8.2.18.2 Absorption, fate and excretion

Mefloquine is available for oral administration only because parenteral preparations cause severe local reactions. It is absorbed rapidly and is extensively bound to plasma proteins. Elimination half-life is about 2-3 weeks. It is mainly excreted in the faeces (Actor *et al*., 2005).

2.8.2.19 Primaquine

Primaquine is the essential co-drug with chloroquine in treating all cases of malaria. It is highly effective against the gametocytes of all plasmodia and thereby prevents spread of the disease to the mosquito from the patient. It is also effective against the dormant tissue forms of *P. vivax* and *P. ovale* malaria, and thereby offers radical cure and prevents relapses. It has insignificant activity against the asexual blood forms of the parasite and therefore it is always used in conjunction with a blood schizonticide and never as a single agent (Baird, 2012, Baird *et al*., 2012).

2.8.2.19.1 Mechanism of action

Is not well understood. It may be acting by generating reactive oxygen species or by interfering with the electron transport in the parasite (Vale *et al*., 2009).

2.8.2.19.2 Absorption, fate and excretion

It is well absorbed after oral administration and rapidly metabolised. Its elimination half-life is about 6 hours. The metabolites of primaquine have oxidative properties and can cause hemolysis in susceptible patients (Baird *et al*., 2012).

2.8.2.19.3 Adverse effects

In therapeutic doses, primaquine is well tolerated. At larger doses, it may cause occasional epigastric distress and abdominal cramps. This can be minimized by taking the drug with a meal. Mild anemia, cyanosis and methemoglobinemia may also occur. Severe methemoglobinemia can occur rarely in patients with deficiency of NADH methemoglobin reductase. Granulocytopenia and agranulocytosis are rare complications. Patients with deficiency of Glucose 6-phosphate dehydrogenase will develop hemolytic anemia on taking usual doses of primaquine. This problem is restricted to certain sections of the population. It may not be practical to test each and every patient for G 6 PD deficiency before administering primaquine. If a patient is known to be severely G6PD deficient, then primaquine should not be given. For the majority of patients with mild variants of the deficiency, primaquine should be given in a dose of 0.75 mg base/kg bw once a week for 8 weeks. If significant haemolysis occurs on treatment, then primaquine should be stopped (AlKadi, 2007).

2.9 Combinations of antimalarial drugs

Early and effective chemotherapy for malaria has a pivotal role in reducing morbidity and mortality especially since a vaccine is unlikely to emerge within the next decade. Multidrug resistance has been reported from most parts of the world and as a result, monotherapy or some of the available combination chemotherapies for malaria are either ineffective or less effective (Kremsner and Krishna, 2004). New antimalarial regimens are, therefore, urgently needed and antimalarial combination chemotherapy is widely advocated. Antimalarial combinations can increase efficacy, shorten duration of treatment (and hence increase compliance), and decrease the risk of resistant parasites arising through mutation during therapy (Li and Hickman, 2015). Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. The concept of combination therapy is based on the synergistic or additive potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination (Li and Hickman, 2015). Artemisinin based combinations are known to improve cure rates, reduce the development of resistance and they

might decrease transmission of drug-resistant parasites. The total effect of artemisinin combinations (which can be simultaneous or sequential) is to reduce the chance of parasite recrudescence, reduce the within-patient selection pressure, and prevent transmission (Kremsner, Krishna, 2004).

2.10 Drug resistance

Emergence of resistance to antimalarial drugs has become a major hurdle in the successful treatment of the infection, and has contributed significantly to global malaria-related mortality (WHO, 2010a). Till date, drug resistance has been documented in *P. falciparum*, *P. vivax*, and *P. malariae* (WHO, 2010a). *P. falciparum* has developed resistance to nearly all antimalarial drugs currently in use; *P.vivax* has been found to be resistant to chloroquine and primaquine; and *P.malariae* has been reported to be resistant to chloroquine and pyrimethamine in some areas (WHO, 2010a, Wells *et al*., 2009). In 1967, WHO defined drug resistance as the ability of the parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject. This definition was later modified to include the sentence: "The form of the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action". This addition took into account a new development in the understanding of human metabolism of sulfonamide (Talisuna *et al*., 2004). As the pharmacokinetics of antimalarial medicines varies widely among individuals, the definition of resistance is enhanced if the concentration profile of the active drug concerned is also taken into consideration (WHO, 2010b).

2.11 Treatment failure

Defined as an inability to clear malarial parasitaemia or resolve clinical symptoms despite administration of an antimalarial medicine. Treatment failure is not, however, always due to drug resistance, and many factors can contribute, mainly by reducing drug concentrations. These factors include incorrect dosage, poor patient compliance in respect of either dose or duration of treatment, poor drug quality and drug interactions (Cheng *et al*., 2012). Even after supervised administration of a full regimen of an antimalarial medicine, individual variations in pharmacokinetics might also lead to treatment failure because of poor absorption, rapid elimination (e.g. diarrhoea or vomiting) or poor biotransformation of prodrugs (WHO, 2010b).

2.12 Multidrug resistance

P. falciparum is seen when the parasite is resistant to more than two operational antimalarial compounds of different chemical classes and modes of action. Generally, the two classes first affected are the 4-aminoquinolines and the antifolates (diaminopyrimidine, sulfonamides). Drug resistance results in a delay in or failure to clear asexual parasites from the blood, which allows production of the gametocytes that are responsible for transmission of the resistant genotype (WHO, 2010b).

2.13 Development of resistance

The malaria parasite is well known for its frequent, de novo mutations, mostly single, and sometimes multiple. In the presence of heavy infection and inadequate drug levels, the resistant mutations survive and propagate (White and Pongtavornpinyo, 2003). Development of resistance requires a high grade of parasitemia, coupled with low or inadequate drug levels. Most cases of resistance have emerged out of SE Asia region. This region is known for low transmission and low immunity that lead to high parasitemia; it also has a long history of indiscriminate use of different antimalarial drugs (White, 2004). In such low-transmission areas, most malaria infections are symptomatic, and therefore proportionally more people receive treatment, providing more opportunities for selection of resistant strains (Sinha *et al*., 2014). One study also suggested that *P. falciparum* in Southeast Asia has an inherent propensity to develop drug resistance through genetic mutation. Areas with very high transmission, such as Africa, appear to be less susceptible to the emergence of drug resistance. In these areas, infections are acquired repeatedly throughout life, resulting in partial immunity (premunition), that in turn controls the infection, usually at levels below those that cause symptoms. Asymptomatic infection and often non-availability of drugs in these areas mean that these patients do not receive antimalarial drugs, and hence the chances of development of resistance are lower (WHO, 2010b). Immunity acts by non-selectively eliminating blood-stage parasites, including the rare de novo resistant mutants, and also improves cure rates, even with failing drugs, thereby reducing the relative transmission advantage of resistant parasites. Furthermore, complex polyclonal infections in semi-immune people allow possible outbreeding of multigenic resistance mechanisms or competition in the host or the mosquito between less-fit resistant strains and more-fit sensitive strains (Srinivas, 2016). Drug pressure leads to higher gametocyte release, and this facilitates the propagation of the resistant mutants that have escaped the drugs. Failure to use primaquine as a gametocytocidal agent for *P. falciparum* further aids such spread of resistance. Therefore,

gametocyte production from the recrudescent resistant infection must be prevented by administration of early, appropriate treatment (Flannery *et al*., 2013). Combined with primaquine. Administration of drugs with long elimination phases facilitates the spread of resistant mutant malaria parasites. The residual antimalarial activity that is present during the post-treatment period serves as a "selective filter", which prevents infection by sensitive parasites but allows infection by resistant parasites. Drugs such as chloroquine, mefloquine and piperaquine, which persist in the blood for months, provide a selective filter long after their administration has ceased (Burrows *et al*., 2017). Factors that promote the development of drug resistance are more intense with *P. falciparum* compared to *P. vivax* and this explains the higher incidence of resistance in *P. falciparum* (White, 2004).

2.14 Mechanisms of resistance

The biochemical mechanism of resistance has been well understood in cases of chloroquine, the antifolates, and atovaquone. The chloroquine-resistant strains of *P. falciparum* tend to accumulate the drug less efficiently than the sensitive ones (FGarcia-Bustos and Gamo, 2013). Polymorphism in the Pfcrt (for chloroquine resistance transporter) gene, particularly the one amino acid change, K76T, located in the first transmembrane domain, has been found consistently in chloroquine-resistant *P. falciparum* parasites. This critical K76T mutation could possibly alter the selectivity of CRT such that chloroquine more efficiently exits the food vacuole (Cooper *et al*., 2005). Another mutation could be at the pfmdr1 (multidrug resistance 1) gene encoding for the transporter for importing solutes into the food vacuole, including the drugs mefloquine, halofantrine, and artemisinin (and possibly chloroquine), but this may not confer resistance on its own (Sanchez *et al*., 2008). In the laboratory, *P. falciparum* resistance to chloroquine can be reversed by combining it with various drugs such as calcium inhibitors, phenothiazines, antidepressants, and antihistamine compounds, but clinical evidence is limited and the usefulness of this approach in humans has not been established (Bloland, 2001).

Cross-resistance between the 4-aminoquinolines, chloroquine and amodiaquine, is common and development of resistance to mefloquine may also lead to resistance to halofantrine and quinine (Wongsrichanalai *et al*., 2002). Resistance to SP results from the mutations in the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) domains, respectively, with the parasites highly resistant to SP having a combination of triply mutated DHFR and doubly mutated DHPS (Sridaran *et al*., 2010). Resistance to atovaquone is related to point mutations in the cytochrome b gene, and emerges rapidly when it is used in monotherapy. Some cases of

resistance to atovaquone and proguanil combination have also been reported, but all of these were not associated with mutation in the cytochrome b gene (Hyde *et al*., 2014). Resistance to artemisinin derivatives had also been reported recently; a study published in July 2014 had reported that slowly clearing infections (clearance half-life >5 hours) were strongly associated with single point mutations in the "propeller" region of the *P. falciparum* kelch protein gene on chromosome 13 (kelch13) (Ashley *et al.*, 2014).

2.15 Origin and distribution of antimalarial drug resistance

Drug-resistant strains have more often evolved out of areas of low malaria transmission, like Thailand, and then spread across to endemic areas like Africa, where they have contributed to worsening mortality (Wongsrichanalai *et al.*, 2001).

In 1955, WHO launched the most ambitious Global Malaria Eradication Programme; DDT was sprayed everywhere across Asia and South America and chloroquine was used extensively to treat malaria. In many areas, particularly in South East Asia and South America, mass administration of chloroquine was also started as a preventive measure and it was administered to even those who did not have malaria. As a result of this wide spread use, very soon chloroquine resistance in *P. falciparum* was observed; first in Thailand in 1957 and on the Colombia-Venezuela border in 1959. Pailin in Western Cambodia was a centre for gem mining in the 1950s having a lot of migrant labourers from Myanmar and Bangladesh. Chloroquine was used as mass prophylaxis to prevent malaria among these workers (Packard, 2014). Through the returning workers, choloroquine resistance spread to Myanmar and Bangladesh 1969-70 and reached the neighbouring Karbi-Anglong district of Assam, India by 1973 (Packard, 2014, Shah *et al*., 2011). By 1980s, chloroquine resistance had spread to sub-Saharan Africa and today chloroquine has lost its efficacy in all but a few areas such as Central America (north-west of the Panama Canal), the island of Hispaniola, and limited areas of the Middle East and Central Asia (Bloland, 2001). With chloroquine losing its efficacy, other drugs were introduced one after another in the South East Asia region, only to be lost to resistance in quick succession. Resistance to Sulfa-Pyrimethamine was reported in *P. falciparum* in the very year of its introduction in 1967, and it reached India by 1979 and now it is frequently seen in South America and Africa (WHO, 2010a, Farooq and Mahajan, 2004). During the Vietnam War, the Chinese developed artemisinin from the sweet wormwood; it turned out to be the fastest acting and safest antimalarial drug, and helped the North Vietnam army. During the same period, the American army had developed mefloquine for its own soldiers, but it came to use after the war

ended. Mefloquine was introduced in Thailand in 1977, but it became ineffective by 1982. Mefloquine resistance also meant cross-resistance to halofantrine and quinine (Bloland, 2001, WHO, 2014a). Mefloquine resistance is now common in Southeast Asia and has also been reported from Amazon region of South America and Africa (sporadically) (Lin *et al.*, 2010). Resistance to quinine has been reported from parts of Southeast Asia and South America (Bloland, 2001, Wongsrichanalai *et al.*, 2002). Vietnam, poor by war, could not afford this expensive mefloquine of United State and by 1991, developed its own production of artemisinin and started using it, largely as monotherapy, often in sub-therapeutic doses. Later, substandard artemesinin was smuggled to the entire region. The Roll Back Malaria initiative was launched by WHO in 1998. By then artemisinin was gaining ground as the fastest acting antimalarial, and some early cases of recrudescence were being reported (Gelband *et al.*, 2004). In view of this, WHO recommended a ban on artemisinin monotherapy and in 2001, recommended artemisinin combination therapy, combining a fast acting artemisinin compound with another slower acting antimalarial such as sulfa-pyrimethamine, mefloquine, amodiaquine and lumefantrine. The use of ACTs has since been going up all over the world, and as of now, this seems to be working, with 30% reduction in the incidence and about 47% reduction in mortality (WHO, 2014b).

2.16 Identifying drug resistance

Estimation of treatment failure rate and validation of drug resistance can only be done by objective study of therapeutic efficacy. Such therapeutic efficacy studies are conducted in a controlled environment, in which drug administration is supervised, the results of microscopic examinations of blood films are validated, and the origin and quality of the drugs are verified (WHO, 2010b). The outcome of such a study is influenced by a combination of a human factor (immunity), a parasite factor (drug resistance) and individual variation leading to differences in the availability of the drug (pharmacokinetics). For example, an adult living in an area of high transmission might be able to eliminate resistant parasites even if the medicine is not fully effective, because of acquired immunity. Conversely, a non-immune child infected with drug-sensitive parasites who has severe gastrointestinal problems may experience therapeutic failure because of poor absorption. While therapeutic efficacy studies can help to predict the likelihood of drug resistance, additional tools are needed to confirm antimalarial drug resistance. First, it must be proven that the parasites are recrudescent in a patient who recently received treatment. The parasites are genotyped to distinguish between those that are recrudescent and those that caused a new infection. Evidence must be obtained that the patient had an adequate blood

concentration of the drug or its metabolites, typically for at least four parasitic cycles. This can be confirmed by pharmacokinetic analyses of blood samples (WHO, 2010a). There are different methods to identify and assess drug resistance in malaria. An in vivo assessment of drug sensitivity involves monitoring of the parasitological and/or clinical response following treatment. Case reports and passive detection of treatment failure may also be used to indicate drug resistance, but all cases of treatment failure may not be due to drug resistance; many factors such as incorrect dosing, noncompliance with duration of regimen, poor drug quality, drug interactions, poor or erratic absorption, and misdiagnosis can all contribute to treatment failure (Boland, 2001b). A standardized in vivo test protocol for assessing the response of *P. falciparum* to chloroquine was first developed in 1965 and after many revisions, the latest protocol has been put forth by the WHO in 2003 (Bloland *et al.*, 2001). It involves a fairly simple, prospective evaluation of the clinical and parasitological response to treatment for uncomplicated malaria. It also provides for measuring blood levels of the drugs, extending the period of follow-up, and testing for molecular markers to help distinguish reinfection from recrudescence whenever technically and logistically feasible (Rosenthal, 2009). Worsening or less than expected resolution of clinical features and parasite density by third day after treatment is considered as early treatment failure. Worsening or reappearance of symptoms after day 4 and persistence of parasitemia at day 28 (day 14 in intense transmission areas) are considered as late treatment failure (Bloland *et al.*, 2001).

In areas with intense transmission, recurrence of symptoms after day 14 may be due to reinfection and molecular tools such as PCR may be needed to differentiate it from recrudescence due to treatment failure (Vestergaard, Ringwald, 2007). As younger children often have a less favorable therapeutic response to antimalarial drugs than do older children and adults, the evaluation of antimalarials for uncomplicated malaria should emphasize treatment efficacy in children <5 years with clinically apparent malaria (WHO, 2003). Wherever possible, blood or plasma levels of the antimalarial should also be measured in prospective assessments, so that drug resistance can be distinguished from treatment failures due to pharmacokinetic reasons (WHO, 2010a). Drug resistance can also be studied by in vitro methods, done by exposing finger-prick blood samples on microtitre plates to precisely known quantities of drug and observing for inhibition of maturation into schizonts. Animal model studies with in vivo tests conducted in nonhuman animal models and molecular tests using PCR to indicate the presence of mutations encoding drug resistance are the other methods used (WHO, 2010a). The results of

these additional tests must, however, be interpreted with caution, as they do not always correlate well with the results of therapeutic efficacy studies, and the predictive usefulness of some of these tests remains to be defined (Carpenter, 2010, WHO, 2010a).

2.17 Prevention and control measures for malaria

Prevention of malaria is currently based on two complementary methods: chemoprophylaxis and protection against mosquito bites. While several malaria vaccines are under development, none is available yet (Matthews, 2011).

2.17.1 Chemoprophylaxis

Malaria chemoprophylaxis is only for travelers to malaria endemic countries, which are classified in three (or four) groups, to determine which drug is recommended for chemoprophylaxis. The choice of drugs depends on the travel destination, the duration of potential exposure to vectors, parasite resistance pattern, level and seasonality of transmission, age and pregnancy. In endemic countries, chemoprophylaxis could also be recommended for autochthonous young children and pregnant women, depending on endemicity level and seasonality of transmission (Cullen and Arguin, 2014).

2.17.2 Personal protection measures against mosquito bites

Because of the nocturnal feeding habits of most of *Anopheles* mosquitoes, malaria transmission occurs primarily at night. Protection against mosquito bites include the use of mosquito bed nets (preferably insecticide-treated nets), the wearing of clothes that cover most of the body, and use of insect repellent on exposed skin. Type and concentration of repellents depend on age and status (Matthews, 2011).

2.17.3 Mosquito control

Vector control measures depend on vector species, mosquito biology, epidemiological context, cost and acceptability by populations. The main current measures are focused on reduction of the contact between mosquitoes and humans, the destruction of larvae by environmental management and the use of larvicides or mosquito larvae predators, and destruction of adult mosquitoes by indoor residual spraying and insecticide-treated bed nets (Campbell-Lendrum, *et al*., 2005).

Chapter 3

Materials and Methods

3.1 Study design

It's a descriptive cross sectional study.

3.2 Study area

The study was carried out in three areas in Sudan, Sennar state (holo-endemic malaria area), Khartoum state (meso-endemic area) and River Nile state (hypo-endemic area).

3.3 Study population

This study was conducted on patients with malaria in Sudan. Patients were recruited between July 2015 to December 2017 at hospitals and medical centers in different parts of the study area in Sudan. The areas were characterized by moderate perennial malaria transmission with a peak in December and January. Blood samples from febrile patients (auxiliary temperature of ≥37.5°C) from all age groups were microscopically confirmed as an uncomplicated *P. falciparum* mono infection and a parasite count of a minimum of 1,000 asexual parasites/µl. A structured questionnaire for socio demographic information and medical history was completed for each patient by a physician (appendix 1). The study was performed following the WHO guidelines for antimalarial drug efficacy surveillance methods (Gadalla *et al.*, 2013).

3.4 Sample size

The sample size was determined using the following equation:

$$N = (t^2 \times P(1-P))/M^2$$

N = Sample size

t = 1.96

P = Prevalence of disease (3.2).

M = 0.05

Based on the formula, individuals were enrolled in the study (N = 272). The sample size was completed to 300 samples and then the whole number of individuals was equally divided into the areas (100 sample for each area).

3.5 Methods

3.5.1 Collection and preparation of blood films

Blood samples were collected for malaria screening from both finger prick and venipuncture,this is to check the presence of healthy asexual parasites in the peripheral smear of patients. Safety procedures were adopted in the collection of finger-prick blood samples by swabbing the area to be sampled with 70% alcohol and allowing it to dry before collection. About 2-5 ml of blood was then drawn (venepuncture) with a sterile disposable syringe in EDTA container. The blood samples were transported to the laboratory. Drops of peripheral blood were placed on Whatman (Qiagen, Hilden, Germany) 3MM filter paper, air-dried and kept in plastic bags until use. Finger prick samples were taken from all participants. Thick smears were prepared and stained with 10X Giemsa stain and slides were read under a 100X oil immersion field. The number of parasites were counted and reported as described by Panikar (2007) using the following grading:

- 1- 10 parasites per 100 fields of thick film +.
- 11- 100 parasites per 100 fields of thick film ++.
- 1- 10 parasites per one field of thick film +++.
- 11- 100 parasites per one field of thick film ++++.

3.5.2 DNA extraction

Harris Uni-CoreTM puncher (Qiagen, Hilden, Germany) was used to punch out six of filter paper with dried blood sample 3 mm in diameter. The puncher was cleaned and blank filter paper pieces that were punched out in the last step of the washing process was subjected to DNA extraction, and then was followed by PCR between random samples to ensure no transfer of parasite DNA between samples using this cleaning method. DNA was extracted from the dried blood sample using a method with Chelex-100® Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA) and was soaked in 0.5% saponin in phosphate buffered saline (PBS) solution overnight, *Plasmodium* speciation was performed using species specific primers in a multiplex PCR to identify *P. falciparum*, *P. vivax*, *P. ovale* or *P. malariae* infections in blood samples.

3.5.3 Molecular methods

Polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) was used to determine the resistant genes and study the genetic diversity (genetic variation) of antimalarial resistant *P. falciparum.* DNA was extracted from patient's blood spotted on the filter paper as

mentioned above. The protocol for the extraction was carried out according to manufacturer's instruction.

3.5.4 Nested PCR and RFLP for Pfcrt mutation-specific detection

For amplification of the 1.6kb fragment of *Pfcrt,* a primary PCR was set up using the primers PfcrtF 5'-CCG TTA ATA ATA AAT ACA GGC-3' and PfcrtR 5'-CTT TTA AAA TGG AAG GGT GT-3'. Product from primary PCR (2µl of 10x dilution) was used in a follow-up, nested, allele-specific PCR amplifications to detect the codon for *pfcrt* 76K or 76T. These diagnostic PCR amplifications used a common inner primer pair Pfcrt1F 5'-GGC TCA CGT TTA GGT GGA-3' and Pfcrt1R 5'-TGA ATT TCC CTT TTT ATT TCC AAA-3' (detects the 76T codon) or Pfcrt2R 5'-GTT CTT TTA GCA AAA ATCT-3' (detects the 76K codon). The PCR stages for these diagnostic amplifications were at 94°C for 5 minutes, then were followed by 40 cycles at 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 45 seconds and a final extension of 72°C for 5 minutes. Purified genomic DNA from *P. falciparum* clones (chloroquine sensitive) and (chloroquine resistant) was used as positive controls, and water as negative control, extracted uninfected blood smears, and uninfected blood spots on filter paper was used as negative controls. The PCR products from the amplification reactions was evaluated by electrophoresis on 2% agarose gels containing ethidium bromide.

3.5.5 PCR and RFLP for detection of Pfmdr1 gene

Gene segments spanning codon 86 of the *Pfmdr*1 gene was amplified in 20µl of standard PCR mixture containing 3µl of extracted DNA and 1µl of the primers MDR1 5'-ATG GGT AAA GAG CAG AA A GA-3' and MDR2 5'-AAC GCA AGT AAT ACA TAA AGTCA-3'. The PCR amplification stages were at 94°C for 2 minutes, followed by 35 cycles at 94°C for 20 seconds, 52°C for 10 seconds, 48°C for 10 seconds, and 60°C for 1.5 minutes. A second, nested amplification from this segment was then performed under the same PCR conditions using 3µl of the product solution and primers MDR3 5'-TGG TAA CCT CAG TAT CAA AGA A-3' and MDR4 5'-ATA AAC CTA AAA AGG AAC TGG-3'. Presence of the mutant 86Y codon was detected by digestion of 10 µl of the second amplification product solution with 3U of the restriction enzyme *Apo*I-HF for 1 to 2 hours at 50°C as recommended by the manufacturer (New England Biolabs). The products of restriction digestion were separated by electrophoresis on a 2% agarose gel and detected by staining with ethidium bromide.

3.5.6 PCR assays for the detection of Pfdhps genes

PCR for *Pfdhps* performed as follow: two primers (primer *Pfdhps*-F 5-ATG ATT CTT TTT CAG ATG-3- and primer *Pfdhps*-R 5-CCA ATT GTG TGA TTT GTC CAC-3 was designed to amplify 747 bp of the region exhibiting mutations relevant to sulphadoxine resistance. PCR was performed with a volume of 20µl (each primer at 0.2M, dNTPs at 200M, and 1U of Hotstar-*Taq* with the appropriate buffer [Qiagen, Valencia, Calif.] and $MgCl_2$ at a final concentration of 2.0 mM) and approximately 80ng of template genomic human DNA, with parasite DNA concentrations corresponding to the parasite burden in the individual. After an initial denaturation (15 min at 95°C), 31 cycles of 30s at 94°C, 40s at 53°C, and 1min at 72°C was run. Elongation of the amplicons was completed by a final cycle of 10min at 72°C. Subsequently, a nested PCR was performed to increase the yields of the specific amplicons using primers primer *pfdhps*-F1 5-GTT GAA CCT AAA CGT GCTG-3 and *pfdhps*-R1 5-ATT ACA ACA TTT TGA TCA TTC-3. 3µl of the primary PCR product was used in a reaction volume of 25µl containing 0.2M of each primer, dNTPs at 200M, reaction buffer with $MgCl_2$ at a final concentration of 2.0 mM, and 1U of Hotstar-*Taq*). In the nested PCR, a high initial annealing temperature (AT), which ensures a high level of specificity of initial primer binding, was followed by a gradual decrease in the AT toward the pre-calculated optimal AT. The parameters consisted of an initial denaturation step (15 min at 95°C) and 43 cycles of 30s at 94°C, the AT for 40 s, and 72°C for 1 min, in which the ATs will be 65°C (5 cycles), 60°C (5 cycles), 56°C (7 cycles), 54°C (13 cycles), and 53°C (13 cycles). Fragment elongation was performed by use of a cycle of 10 min at 72°C. The amplicons were monitored for quality and the expected size on 1% ethidium bromide-stained agarose gels. Presence of the mutant R59 codon was detected by digestion of 10 µl of the second amplification product solution with 4U of the restriction enzyme *Xmn1* for 1 to 2 hours at 37°C as recommended by the manufacturer (New England Biolabs). The products of restriction digestion were separated by electrophoresis on a 2% agarose gel and detected by staining with ethidium bromide.

3.5.7 PCR assays for the detection of Pfdhfr genes

PCRs for *Pfdhfr* was performed as follow: two primers (primer *Pfdhfr*-F 5-CCA ATT GTG TGA TTT GTC CAC-3- and primer *Pfdhfr*-R 5-ATG ATT CTT TTT TCA GATG-3 was designed to amplify 521 bp of the region exhibiting mutations relevant to sulphadoxine resistance. PCR was performed with a volume of 20µl (each primer at 0.2M, dNTPs at 200M, and 1U of Hotstar-*Taq* with the appropriate buffer [Qiagen, Valencia, Calif.] and $MgCl_2$ at a final concentration of 2.0

mM) and approximately 80ng of template genomic human DNA, with parasite DNA concentrations corresponding to the parasite burden in the individual. After an initial denaturation (15 min at 95°C), 31 cycles of 30s at 94°C, 40s at 53°C, and 1min at 72°C was run. Elongation of the amplicons was completed by a final cycle of 10min at 72°C. Subsequently, a nested PCR was performed to increase the yields of the specific amplicons using primers primer *pfdhfr*-F1 5-ATT ACA ACA TTT TGA TCA TTC-3 and *pfdhfr*-R1 5-GTT GAA CCT AAA CGT GCTG-3. 3µl of the primary PCR product was used in a reaction volume of 25µl containing 0.2M of each primer, dNTPs at 200M, reaction buffer with MgCl2 at a final concentration of 2.0 mM, and 1U of Hotstar-*Taq*). In the nested PCR, a high initial annealing temperature (AT), which ensures a high level of specificity of initial primer binding, was followed by a gradual decrease in the AT toward the pre-calculated optimal AT. The parameters consisted of an initial denaturation step (15 min at 95°C) and 43 cycles of 30s at 94°C, the AT for 40 s, and 72°C for 1 min, in which the ATs will be 65°C (5 cycles), 60°C (5 cycles), 56°C (7 cycles), 54°C (13 cycles), and 53°C (13 cycles). Fragment elongation was performed by use of a cycle of 10 min at 72°C. The amplicons were monitored for quality and the expected size on 1% ethidium bromide-stained agarose gels. Presence of the mutant codon was detected by digestion of 10 µl of the second amplification product solution with 3U of the restriction enzyme *BtsC1* for 1 to 2 hours at 37°C as recommended by the manufacturer (New England Biolabs). The products of restriction digestion were separated by electrophoresis on a 2% agarose gel and detected by staining with ethidium bromide.

3.6 Statistical analysis

All information and data were analyzed by using Statistical Package of Social Science (SPSS) (version 16; Corp., College station, Tax), using Chi square test, *P*. value <0.05 considered as significant then data were presented in tables and graphs using excel.

3.7 Ethical consideration:

The approval was taken from Research Committee of College of Medical Laboratory Science, Sudan University of Science and Technology. Written informed consent was obtained from each study participant or from his/her guardians after explaining the study purpose (appendix 2) .

Chapter 4
Results

4.1 The overall results

300 patients were included in this study their age ranged between 1- 90 years old of mean age was 31 ± 23 years, from them 133 were males and 167 were females.

All the samples were examined for parasite count by cross method after stained the blood film by Geimsa stain, the results showed that, the highest rate (37%) was reported in Sennar state among (++), while the lowest rate (8%) was reported in Khartoum among (++++) (Table 4.1)

From the results, the highest rate (62%) was reported in female among (++), while the lowest rate (15%) was reported in male among (++++) (Table 4.2).

The results showed that, the highest rate (45%) was reported among age group (20-40) years old among (++), while the lowest rate (3%) was reported among age group 11-19 years old among (++++) (Table 4.3).

The samples were examined by RFLP- PCR to detect mutation in Pfcrt (figure 4.1), Pfmdr-1 (figure 4.2) RFLP for Pfmdr1 (figure 4.3), Pfdhfr (figure 4.4), RFLP for Pfdhfr (figure 4.5), Pfdhps (figure 4.6) and RFLP for Pfdhps (figure 4.7).

The overall prevalence rates of mutant alleles were reported in all area examined as follows:

Pfcrt (32%), Pfmdr-1 (36%), Pfdhfr (36.7%) and Pfdhdps (33%) (Table 4.4).

The results showed that, the highest prevalence of mutant alleles (47%) in four genes among each area was reported in Khartoum state from Pfmdr-1, while the lowest prevalence mutant alleles (28%) was reported in two areas with two genes (Pfcrt in Sennar and Pfdhfr in River Nile) (Table 4.5).

From the results, the overall prevalence rates of mutant alleles was high (22%) among females, while the lowest prevalence rates (10%) was reported among males (Table 4.6).

The overall prevalence rates among age groups revealed that the highest prevalence rates of mutant alleles in all genes (15.7%) was reported in both Prmdr-1 and Pfdhfr among the same age group (20- 50), while the lowest prevalence rates (3.7%) was reported in Pfdhps among age group 11- 19 years (Table 4.7).

The overall prevalence rates of mutant alleles when compared to parasitemia showed that the highest prevalence rate of mutant alleles (12.7%) was reported in Pfdhfr among parasite count (++), while the lowest prevalence rate (3%) was reported in the same gene (Pfdhfr) but among parasite count (++++) (Table 4.8).

4.2 The single point mutation Pfcrt K76T

The highest prevalence rate of Pfcrt single point mutation K76T (37%) was reported in the Sennar state, while the lowest prevalence rates (28%) was reported in River Nile state (Table 4.9). The difference in rates in the single point mutant allele of Pfcrt K76T among areas was found to be statistically insignificant at *P*.value= 0.381.

The highest mutant allele Pfcrt K76T (22%) was reported among females, while the lowest prevalence rate (10 %) was reported among males (Table 4.10). The difference in rates in single point mutant allele of Pfcrt K76T among gender was found to be statistically significant at *P*.value=0.002.

The highest prevalence rate of Pfcrt K76T mutant allele (10.7%) was reported among age group 20 -50, while the lowest prevalence rate (7.7%) was reported among two age groups less than 10 years and more than 50 years (Table 4.11). The difference in rates in single point mutant allele of Pfcrt K76T among age groups was found to be statistically insignificant at *P*.value=0.202.

The highest prevalence rate of Pfcrt K76T mutant allele (12%) was reported among parasite count (++), however, the lowest prevalence rate of Pfcrt K76T mutant allele (4.7%) was reported among parasite count (++++) (Table 4.12). The difference in rates in single point mutant allele of Pfcrt K76T among parasitemia was found to be statistically insignificant at *P*.value=0.445.

4.3 The single point mutation Pfmdr-1 N86Y

The highest prevalence rate (15.7%) of single point mutation in Pfmdr-1 N86Y was reported in Khartoum state, while the lowest prevalence rate (9.7%) was reported in Sennar (Table 4.13). The difference in rates in single point mutant allele of Pfmdr-1 N86Y among areas was found to be statistically significant at *P*.value=0.018.

The results showed that, the Pfmdr-1 N86Y mutant allele was reported among males and females equally (18%) with mixed wild and mutant allele (0.7%) which was only reported among females (Table 4.14). The difference in rates in single point mutant allele of Pfmdr-1 N86Y among gender was found to be statistically insignificant at *P*.value=0.167.

The highest prevalence rate of Pfmdr-1 N86Y mutant allele (15.7 %) was reported among age group 20- 50, while the lowest prevalence rate (4.7%) was reported among age group 11- 19 years (Table 4.15). The differences in rates in single point mutant allele of Pfmdr-1 N86Y among age groups were found to be statistically insignificant at *P*.value=0.399.

The results showed that, the prevalence rates of Pfmdr-1 N86Y mutant allele were closely equal (10.7%, 10.7% and 10.3%) among parasite count (+, ++, and +++) respectively, while the

prevalence rate among (++++) was 4.7% (Table 4.16). The difference in rates in single point mutant allele of Pfmdr-1 N86Y among parasite count was found to be statistically insignificant at *P*.value=0.627.

4.4 The single point mutation Pfdhfr C59R

The highest prevalence rate (14.3 %) of single point mutation in Pfdhfr C59R rates was reported in Khartoum state, while the lowest prevalence rate (9.3%) was reported in River Nile state (Table 4.17). The difference in rates in single point mutant allele of Pfdhfr C59R among area was found to be statistically significant at *P*.value=0.018.

The results showed that, the highest prevalence rate (20.7%) of Pfdhfr C59R mutant allele was reported among females, while it was 16% among males. The mixed wild and mutant alleles (4.3% and 1.3%) was reported among males and females respectively (Table 4.18). The difference in rates in single point mutant allele of Pfdhfr C59Ramong gender was found to be statistically significant at *P*.value=0.022.

The highest prevalence rate of Pfdhfr C59R mutant allele (15.7 %) was reported among age group 20- 50 years, while the lowest prevalence rate (5.7%) was reported among age group 11-19 years (Table 4.19). The difference in rates in single point mutant allele of Pfdhfr C59R among age groups was found to be statistically insignificant at *P*.value=0.395.

The results showed that, the highest prevalence rate of Pfdhfr C59R mutant allele (12.7%) was reported among parasite count (++), while the lowest prevalence rate (3%) was reported among (++++) (Table 4.20). The difference in rates in single point mutant allele of Pfdhfr C59R among parasite count were found to be statistically insignificant at *P*.value=0.307.

4.5 The single point mutation Pfdhps A436G

Of single point mutation in Pfdhps A436G our results revealed that, the highest prevalence rates (13 %) was reported in Khartoum state, while in Sennar and River Nile states the same prevalence rate (10%) was reported (Table 4.21). The difference in rates in single point mutant allele of Pfdhps A436G among area was found to be statistically significant at *P*.value=0.018.

The result showed that, the highest prevalence rate (19.3%) of Pfdhps A436G mutant allele was reported among females, while it was 13.7 % among males. The mixed wild and mutant alleles (1.7% and 3%) were reported among males and females respectively (Table 4.22). The difference in rates in single point mutant allele of Pfdhps A436G among gender was found to be statistically insignificant at *P*.value=0.569.

The highest prevalence rate of Pfdhps A436G mutant allele (15.7 %) was reported among age group 20- 50 years, while the lowest prevalence rate (3.7%) was reported among age group 11 – 19 years (Table 4.23). The difference in rates in single point mutant allele Pfdhps A436G among age groups was found to be statistically insignificant at *P*.value=0.147.

The results showed that, the highest prevalence rate of Pfdhps A436G mutant allele (11%) was reported among parasite count (++), while the lowest prevalence rate (3.7%) was reported among (++++) (Table 4.24). The difference in rates in single point mutant allele of Pfdhps A436G among parasite count were found to be statistically insignificant at *P*.value=0.901.

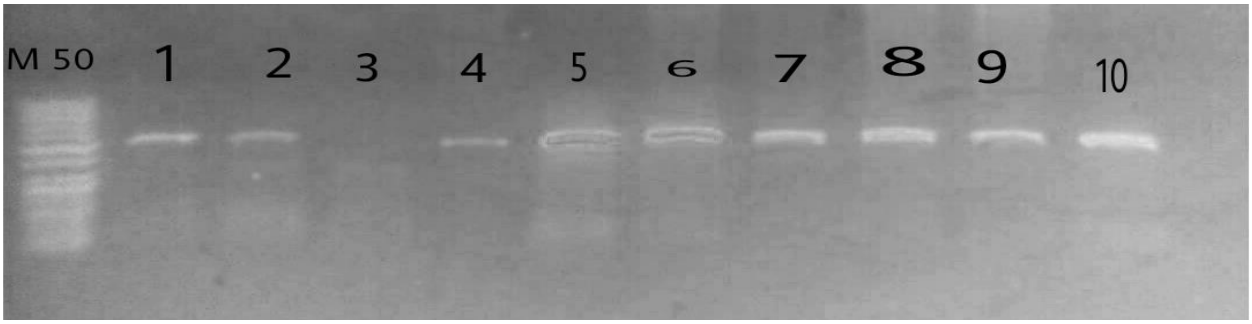

Figure 4.1: The allele specific PCR for Pfcrt K76T, Marker 50 pb, lane 1, 2,4,5,6,7,8,9,10 wild type K76, lane 3 mutant type 76T.

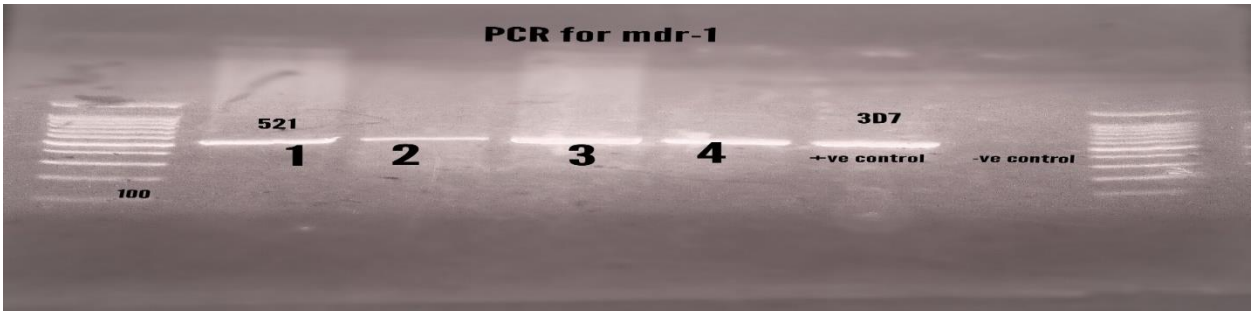

Figure 4.2: PCR for Pfmdr -1, Marker 100, lane 1,2,3,4 samples, lane 5 +ve control, lane 6 –ve control

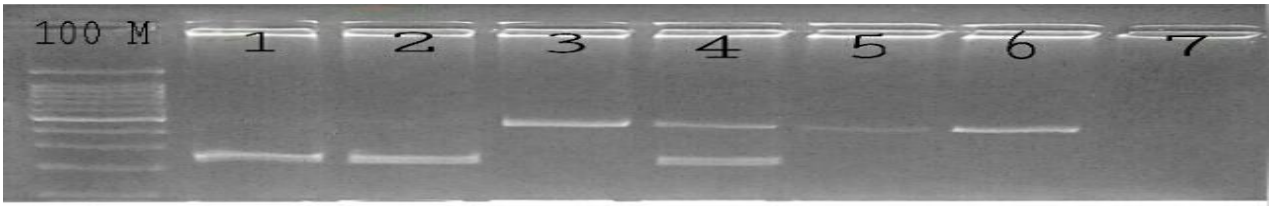

Figure 4.3: RFLP-PCR for Pfmdr-1 N86Y cutting with *Apo1*-HF enzyme, lane 1and2 mutant type Y86, lane 3,5,6 wild type N86, lane 4 mixed alelle N/Y86**.**

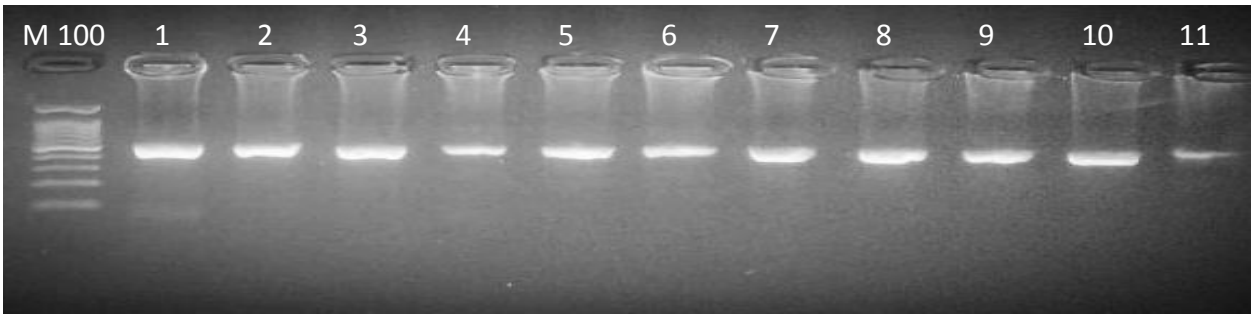

Figure 4.4: PCR for Pfdhfr, Marker 100, lane 15 +ve control, lane 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 samples.

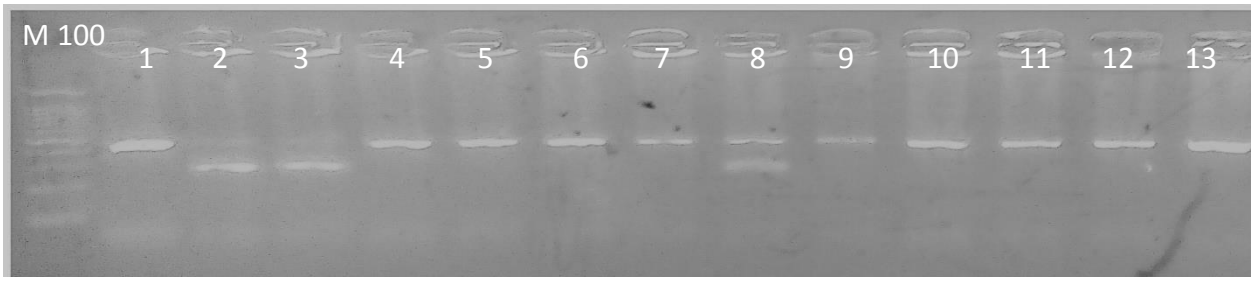

Figure 4.5: RFLP-PCR for Pfdhfr cutting with *BtsC*1 enzyme, Marker 100, lane 1,4,5,7, 9, 10, 11,12,13 wild type, lane 2 and 3 mutant type, lane 8 mixed type.

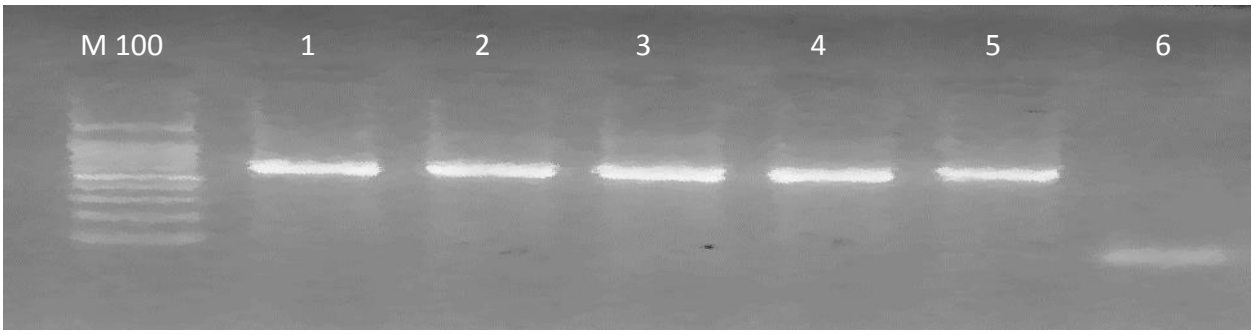

Figure 4.6: PCR for Pfdhps, M 100, lane 1,2,3,4, samples, lane 5 +ve control, lane 6 –ve control.

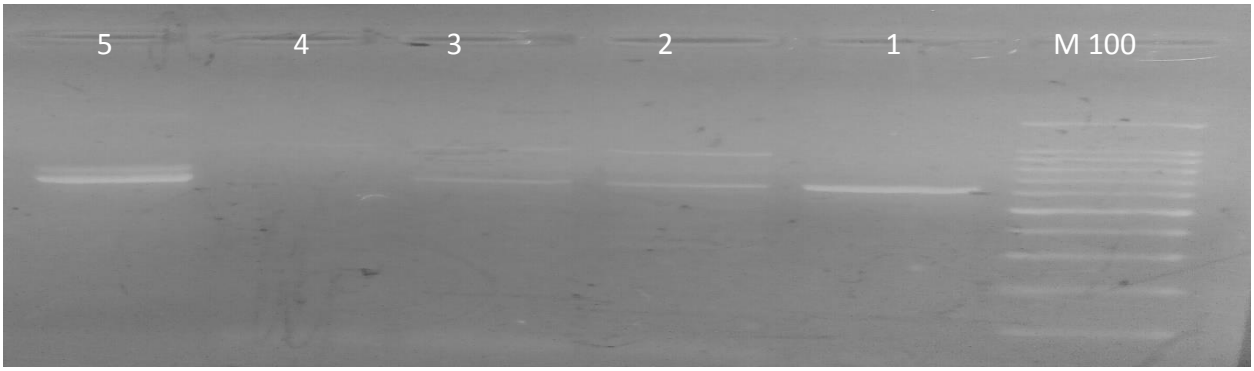

Figure 4.7: RFLP for Pfdhps cutting with *Xmn*1 enzyme, DNA marker (M 100), lane 1 and 5 mutant type, lane 4 wild type lane 2 and 3 mixed type**.**

4.1: Frequency of parasitemia in the study area

Parasitemia / Area	+	++	+++	++++
River Nile	25%	32%	27%	16%
Khartoum	32%	36%	24%	8%
Sennar	28%	37%	22%	13%

Table 4.2: Frequency of drug resistance gene according to gender in the study areas

Parasitemia / Sex	+	++	+++	++++
Male	42 %	43 %	33 %	15 %
Female	43%	62 %	40 %	22 %

Table 4.3: Frequency of drug resistance gene according to age groups in the study areas

Parasitemia / Age group (years)	+	++	+++	++++
>10	8 %	19 %	20 %	11 %
11 – 19	7 %	21 %	14 %	3 %
20 – 50	35 %	45 %	24 %	16 %
<50	35 %	20 %	7 %	8.7 %

Table 4.4: Frequency of mutation in drug resistance gene

	Pfcrt	Pfmdr1	Pfdhfr	Pfdhps
Frequency	32 %	36%	36.7%	33%

Table 4.2: Frequency of mutation of drug resistance gene in the study area

Mutation (%) / Area	Pfcrt (%)	Pfmdr1 (%)	Pfdhfr (%)	Pfdhdps (%)
River Nile	37%	32%	28%	30%
Khartoum	31%	47%	43%	39%
Sennar	28%	29%	39%	30%

Table 4.6: Frequency of drug resistance gene according to gender in the study areas

Gene / Sex	Pfcrt	Pfmdr1	Pfdhfr	Pfdhps
Male	10 %	18 %	16 %	13.7 %
Female	22 %	18 %	20.7 %	19.3 %

Table 4.7: Frequency of drug resistance gene according to age groups in the study areas

Gene / Age group (year)	Pfcrt (%)	Pfmdr1 (%)	Pfdhfr (%)	Pfdhdps (%)
<10	7.7 %	8 %	8.3 %	5 %
11 – 19	6 %	4.7 %	5.7 %	3.7 %
20 – 50	10.7 %	15.7 %	15.7 %	15.7 %
>50	7.7 %	7.7 %	7 %	8.7 %

Table 4.8: Frequency of drug resistance gene according to parasitemia in all areas

Gene / Parasitemia	Pfcrt (%)	Pfmdr1 (%)	Pfdhfr (%)	Pfdhdps (%)
+	9.3 %	10.7 %	12.3 %	10.3 %
++	12 %	10.7 %	12.7 %	11 %
+++	6 %	10.3 %	8.7 %	8 %
++++	4.7 %	4.3 %	3 %	3.7 %

Table 4.9: Association between wild and mutant allele of single point mutation Pfcrt K76T and area.

Area	**No. examined**	**Mutant allele (%)**	**Wild type (%)**
River Nile	100	28 (9.3%)	72 (24%)
Khartoum	100	33 (10.3%)	69 (23%)
Sennar	100	37 (12.3%)	63 (21%)

P.value=0.381.

Table 4.10: Association between single point mutation Pfcrt K76T and gender

Gender	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
Male	133	30 (10%)	103 (33.7%)	0 (0.0 %)
Female	167	66 (22%)	101 (34.3%)	0 (0.0 %)

P.value=0.002.

Table 4.11: Association between single point mutation Pfcrt K76T and age groups

Age groups(years)	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
<10	58	23 (7.7%)	35 (11.7%)	0.0 %
11 – 19	45	18 (6 %)	27 (9%)	0.0 %
20 – 50	120	32 (10.7 %)	88 (29.3 %)	0.0 %
>50	77	23 (7.7 %)	54 (18 %)	0.0 %

P.value=0.202.

Table 4.12: Association between single point mutation Pfcrt K76T and parasitemia

Parasitemia	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
+	85	28 (9.3%)	57 (19%)	0 (0.0%)
++	105	36 (12%)	69 (23%)	0 (0.0%)
+++	73	18 (6%)	55 (18.3%)	0 (0.0%)
++++	37	14 (4.7%)	23 (7.7%)	0 (0.0%)

P.value=0.445.

Table 4.13: Association between single point mutation Pfmdr-1 N86Y and area.

Area	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
Sennar	100	29 (9.7%)	71 (23.7%)	0 (0.0 %)
Khartoum	100	47 (15.7 %)	53 (17.7%)	0 (0.0 %)
River Nile	100	32 (10.7 %)	66 (22%)	2 (0.7 %)

P.value=0.018.

Table 4.14: Association between single point mutation Pfmdr-1 N86Y and gender.

Gender	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
Male	133	54 (18%)	79 (26.3%)	0 (0.0 %)
Female	167	54 (18%)	111 (37%)	2 (0.7 %)

P.value=0.167.

Table 4.15: Association between single point mutation Pfmdr-1 N86Y and age group.

Age groups (years)	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
<10	58	24 (8%)	34 (11.3%)	0 (0.0 %)
11 – 19	45	14 (4.7%)	31 (10.3%)	0 (0.0 %)
20 – 50	120	47 (15.7%)	71 (23.7%)	2 (0.7 %)
>50	77	23 (7.7%)	54 (18%)	0 (0.0 %)

P.value=0.399.

Table 4.16: Association between single point mutation Pfmdr-1 N86Y and area.

Parasitemia	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
+	85	32 (10.7%)	53 (17.7%)	0 (0.0%)
++	105	32 (10.7%)	72 (24.0%)	1 (0.3%)
+++	73	31 (10.3%)	41 (13.7%)	1 (0.3%)
++++	37	13 (4.3%)	24 (8.0%)	0 (0.0%)

P.value=0.627.

Table 4.17: Association between single point mutation Pfdhfr C59R and area.

Area	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
River Nile	100	28 (9.3%)	72 (24%)	0 (0.0 %)
Khartoum	100	43 (14.3%)	45 (15%)	12 (4.0 %)
Sennar	100	39(13 %)	56 (18.7%)	5 (1.7 %)

P.value=0.018.

Table 4.18: Association between single point mutation Pfdhfr C59R and gender.

Gender	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
Male	133	48 (16%)	72 (24%)	13 (4.3 %)
Female	167	62 (20.7%)	101 (33.7%)	4 (1.3 %)

P.value=0.022.

Table 4.19: Association between single point mutation Pfdhfr C59R and age group.

Age groups (year)	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
<10	58	25 (8.3%)	29 (9.7%)	4 (1.3 %)
11 – 19	45	17 (5.7%)	27 (9%)	1 (0.3 %)
20 – 50	120	47 (15.7%)	65 (21.7%)	8 (2.7 %)
>50	77	21 (7.0%)	52 (17.3%)	4 (1.3 %)

P.value=0.395.

Table 4.20: Association between single point mutation Pfdhfr C59R and parasitemia.

Parasitemia	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
+	85	37 (12.3%)	44 (14.7%)	4 (1.3%)
++	105	38 (12.7%)	63 (21%)	4 (1.3%)
+++	73	26 (8.7%)	40 (13.3%)	7 (2.3%)
++++	37	9 (3%)	26 (8.7%)	2 (0.7%)

P.value=0.307.

Table 4.21: Association between single point mutation Pfdhps A436G and area.

Area	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
Sennar	100	30 (10%)	70 (23%)	0 (0.0 %)
Khartoum	100	39 (13 %)	56 (18.7%)	5 (1.7 %)
River Nile	100	30 (10%)	61 (20.3%)	9 (3.0 %)

P.value=0.018.

Table 4.22: Association between single point mutation Pfdhps A436G and gender.

Gender	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
Male	133	41 (13.7%)	87 (29%)	5 (1.7 %)
Female	167	58 (19.3%)	100 (33.3%)	9 (3 %)

P.value=0.569.

Table 4.23: Association between single point mutation Pfdhps A436G and age group.

Age groups	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
<10	58	15 (5%)	41 (13.7%)	2 (0.7 %)
11 – 19	45	11 (3.7%)	34 (11.3%)	0 (0.0 %)
20 – 50	120	47 (15.7%)	66 (22%)	7 (2.3 %)
>50	77	26 (8.7%)	46 (15.3%)	5 (1.7 %)

P.value=0.147.

Table 4.25: Association between single point mutation Pfdhps A436G and parasitemia.

Parasitemia	No. examined	Mutant allele (%)	Wild type (%)	Mixed allele (%)
+	85	31 (10.3%)	49 (16.3%)	5 (1.7%)
++	105	33 (11%)	69 (23%)	3 (1.0%)
+++	73	24 (8%)	45 (15%)	4 (1.3%)
++++	37	11 (3.7%)	24 (8%)	2 (0.7%)

P.value=0.901.

Chapter 5

Discussion, Conclusion and Recommendations

5.1 Discussion

The study aimed to detect the *Pfcrt, Pfmdr-1, Pfdhfr, and Pfdhps* mutant alleles in three areas which comprise the holo, meso and hypo-endemic malarial transmission area in Sudan. The study focused on detection of different mutant allele among different age groups in the study areas.

This study revealed that, the polymorphism mutation rates of Pfcrt at codon 76, Pfmdr-1 at codon 86, Pfdhfr at codon 59 and Pfdhps at codon 436 were 32%, 36%, 36.7%, and 33% respectively among the 300 positive *P. falciparum* malaria samples isolated from three states in Sudan.

Plasmodium falciparum carrying mutations associated with drug resistance may be less fit to survive in the absence of drug pressure. As mentioned by Gadalla *et al.* (2013) that, the continuous use of chloroquine mono-therapy for decades has led to almost saturation of the *P. falciparum* population in Sudan carrying the mutant Pfcrt allele.

The results showed that, Sennar state (holo endemic) was the highest Pfcrt mutant allele K76T (12.3%) followed by Khartoum state (meso endemic) (10.3%) and River Nile state (hypo-endemic) (9.3%). In our opinion, this is not a major differences because the malaria endemic all over Sudan, so there are no obvious differences.

From the results, it was obvious that, the overall of PfcrtK76T mutant allele in the study areas was relatively high (32%). This rate was found to be closer to the rate reported by Schönfeld *et al.* (2007) in Tanzania (34.1 %), however, it was lower than the rate reported by Sousa-Figueiredo *et al.* (2010) in Angola (93.3 %).

The investigation revealed that, the highest Pfcrt mutant allele (10.7%) was found in age group 20-50 years old. This finding disagreed with the study done in Yemen by Al-Mekhlafi *et al.* (2011) who reported 71% in the same age group. Also, our finding to some extend with a study done in Nigeria by Muhammed *et al.* (2017) who reported a rate of 14.9%.

From the study, it is obvious that, the prevalence of Pfmdr-1 N86Y mutant allele was 36%, which means the chloroquine and other anti-malarial drugs resistance were found in Sudan, although the ministry of health in Sudan changed the first line of malaria treatment from chloroquine to artemisinin in 2004.

Our results showed that, Khartoum state was with the highest Pfmdr-1 N86Y (15.7%) followed by River Nile state (10.7%) and Sennar state (9.7 %).In our opinion, this is a normal distribution although Khartoum had the highest prevalence due to the high number of population, so there are no obvious differences.

From the results obtained in this study, it was obvious that, the overall of the Pfmdr-1 N86Y mutant allele in the study areas was relatively high (36%). It was higher than the 31.8% rate reported by Elhadi *et al.* (2015) in Eastern Sudan, and lower than the rate (55.5%) reported by Menegon *et al.* (2010) in Central Sudan. Also, higher than the rate (33.1%) Omer *et al.* (2010) in Central Sudan.

In this study, equal rate (18%) of Pfmdr-1 mutant allele was reported among gender, which was lower than the rate in males (58.6%) and in females (41.4%) in study done by Basheir *et al.* (2012) in Central Sudan. In our opinion, the equal prevalence of Pfmdr-1 N86Y mutant allele (18.0%) reported in this study among males and females proves that drug resistance is undoubtedly not affected by gender. .

The investigation revealed that, the highest Pfmdr-1 N86Y mutant allele (15.7%) was found in age group 20 – 50 years old. This finding was lower than the finding in Nigeria by Muhammad *et al.* (2017) who reported 27.6 % in the same age group.

Although sporadic cases of resistance to sulphadoxine (SP) was observed and reported many years back in the Sudan, no compiled study was carried out to quantify the resistance to SP in these regions before. The findings in this study suggested that, parasites resistant to SP was less likely to be sensitive to chloroquine (CQ). This statement is supported by the co-existence of SP and CQ resistance associated with single point mutation alleles in the Pfdhfr C59R and Pfdhps A436G. It is possible that, during the parasite evolution the occurrence of mutations in the genes associated with CQ resistance precedes but enhances the mutations associated with SP resistance. However, these findings were in contradiction with a recently published work from West Africa and Nigeria, where the combination of CQ with SP significantly improved the efficacy of the later in the treatment of uncomplicated malaria. While an earlier report from Gambia showed no additional privilege from the use of CQ in combination with SP except for symptomatic relief (Eltayeb *et al.*, 2015).

The results obtained from this study revealed that, it was obvious that the overall of Pfdhfr C59R mutant allele in the study areas was relatively high (36.7%), it was higher than the rate (1.4%)

reported by Marks *et al.* (2005) in Ghana, and lower than the rate (55.4%) reported by Tessema *et al.* (2015) in North Western Ethiopia.

The investigation revealed that, the highest Pfdhfr C59R mutant allele (15.7%) was found in age group 20-50 years old. This finding was disagreed with the finding in North Western Ethiopia by Tessema *et al.* (2015) who reported 43.1% in the same age group.

This study reported a high rate (20.7%) of Pfdhfr mutant allele C59R among females. This rate was lower than the rate reported by Isozumi *et al.* (2010) in Vietnam 42.4%.

From the results obtained from this study, it was obvious that, the overall of Pfdhps A436G mutant allele in the study areas was relatively high (33%). It was higher than the 1.3% rate reported by Marks (2005) in Ghana, and lower than the 67.7% rate reported by Tessema *et al.* (2015) in North Western Ethiopia.

The investigation revealed that, the highest Pfdhps mutant allele rate (15.7%) was found in age group 20-50 years old. This finding disagreed with the finding in North Western Ethiopia by Tessema *et al.* (2015) who reported 35.4% rate in age group 11-19 years old.

This study reported a high rate 19.3%of Pfdhps mutant allele A436G among females. This rate was lower than the rate (60%) reported by Isozumi *et al.* (2010) in Vietnam.

From the results, the prevalence rate of Pfcrt K76T mutant allele (12%) was reported among parasite count (++).This rate was lower than the rate reported by Berry *et al.* (2004) among the same parasite count in France (30%) and higher than the rate 6.5%. reported by Acharya *et al.* (2017) in Qatar. The prevalence Pfmdr-1 N86Y mutant allele was closely equal (10.7%) among parasite count (+ and ++) it was higher than the rate (6.4%) reported by Acharya *et al.* (2017) in Qatar among low parasitemia. The prevalence rate of Pfdhfr mutant allele (12.7%) was reported among parasite count (++), which was higher than the rate reported by Marks *et al.* (2005) among the same parasite count 6.0% in Ghana. Also from the results, the prevalence rate of Pfdhps mutant allele (11%) was reported among parasite count (++), which was higher than the rate reported by Marks *et al.* (2005) among the same parasite count 6.3% in Ghana. In our opinion, all the high rates were reported among (++) and this may be due to large number of participant in study were with (++) parasite count.

This was the first molecular study carried out in these regional areas including a considerable number of samples 300 and focused on the mutations of Pfcrt, Pfmdr1, Pfdhps and Pfdhfr genes, strongly associated with CQ and SP resistance. The results of the epidemiological study on the prevalence of genotypes associated with drug resistance, showed high presence of CQ resistance

and SP markers. This work might probably be of significance to evaluate the implementation of new therapeutic strategies based on combinations that include SP, like the protocol that is now implemented in Sudan (artesunate combined with SP) as first-line drug for uncomplicated malaria treatment. The main conclusions drawn from our observations, namely, that mutations that confer antimalarial drug resistance can be widely maintained in the absence of drug pressure, have the advantage of being only hypothetical. If, however, they should hold true and apply to other antimalarial drugs as well, the dramatic situation of malaria control in Sudan may be foreseen to deteriorate still more.

5.2 Conclusions

The study conclude that:

- Artemisinin is still used as the first line of treatment of malaria and remains a safe and effective first- drug for the treatment of uncomplicated *falciparum* malaria in Sudan. However, the presence of this mutation may lead to the failure of this effective drug.
- The presence of the mutation allele of the malaria drug resistance gene at these different areas indicates the spread of these gene all over Sudan and might be probably increase in the future.
- The presence of these drug resistant genes might in fact complicate the establishment and launching control programs for combating malaria in different parts of Sudan.
- There was association between the Pfmdr-1, Pfdhfr and Pfdhps gene polymorphism and area.
- There was association between the Pfmdr-1 and Pfdhfr gene polymorphism and gender.
- There was no association between these mutant allele and age groups.
- The level of parasitemia not affect in the mutant allele of all these genes.

5.3 Recommendations

- Monitoring the efficacy of artemisinin should continue as there is a high risk of drug failure due to sub-therapeutic levels resulting from inadequate use of anti-malarial drugs in the country.
- Surveillance for artemisinin resistance-associated molecular markers should also be monitored as a supportive tool to *in vivo* efficacy.
- Monitoring of the second line of treatment in Sudan which would be as appropriate option for treating uncomplicated *falciparum* malaria.
- Further studies in different areas of Sudan should be conducted to follow up all drug failures.
- The use of sequencing as the tool of detection for all mutation of drug resistance gene.
- Establishment of good strategies and planning through investigation to prevent the spread of the malaria parasite.

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Appendices

Appendix 1**:** Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Questionnaire for requirement of PhD degree

ID number …… **Date of collection** ……..……

Name of patient ……..…….……..…

Age : less than 5 years ☐ more than 5 and less than 10 years ☐ more than 10 ☐ and less than 20 years ☐ more than 20 years ☐

Sex: male ☐ female ☐

Specimen: finger prick ☐ venous blood ☐

Symptoms

……

……

Is this the first time suffering from malaria: Yes ☐ No ☐

If No, How many times you suffered from malaria?

Which drug you used: Chloroquine ☐ Quinine ☐ Fancidar ☐ Artusinate ☐ Artemether ☐ combination AS/SP ☐ Other ☐

Have you taken the dose and completed the course? Yes ☐ No ☐

Which one of them was effective?

When the drug is not effecive what would you do?

Go to the doctor ☐ Go to lab ☐ Directly change the drug by pharmacist

Change drug by your self ☐

Appendix 2: Informed consent

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

دراسة لنيل درجة الدكتوراة

اقرار بالموافقة والمشاركة

الاسم: أحمد بخيت عبد الله أحمد

لدي بحث لنيل درجة الدكتوراة في علم الطفيليات والحشرات الطبية بعنوان الطفرات الجينية لجينات الملاريا المقاومة للعلاج بعد تنفيذ العلاج المزدوج في السودان, ويتطلب البحث أخذ عينة من الدم الوريدي بواسطة حقنة وذلك بعد تعقيم منطقة أخذ العينة بواسطة مطهر, جميع المواد والادوات المستخدمة جديده وتستخدم مرة واحدة مع مراعاة كل وسائل السلامة, قد يحدث تورم بسيط ولمنه يزول بعد فترة, والغرض من اخذ العينة هو البحث العلمي وستحفظ النتائج بسرية تامة وسوف تبلغ بنتيجة الفحص.

الاسم: .....

اوافق انا المذكور أعلاه للدارس علي أخذ عينة مني عن طريق الحقن الوريدي وقد اطلعت علي الشروط أعلاه.

التوقيع: ..... البصمة:

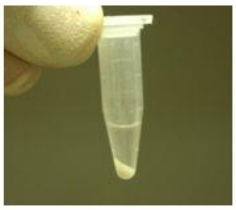
العنوان: .....

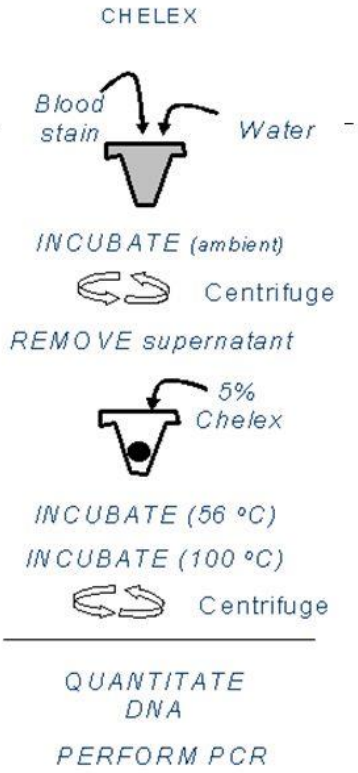
الجوال:.....

Appendix 3: DNA extraction protocol

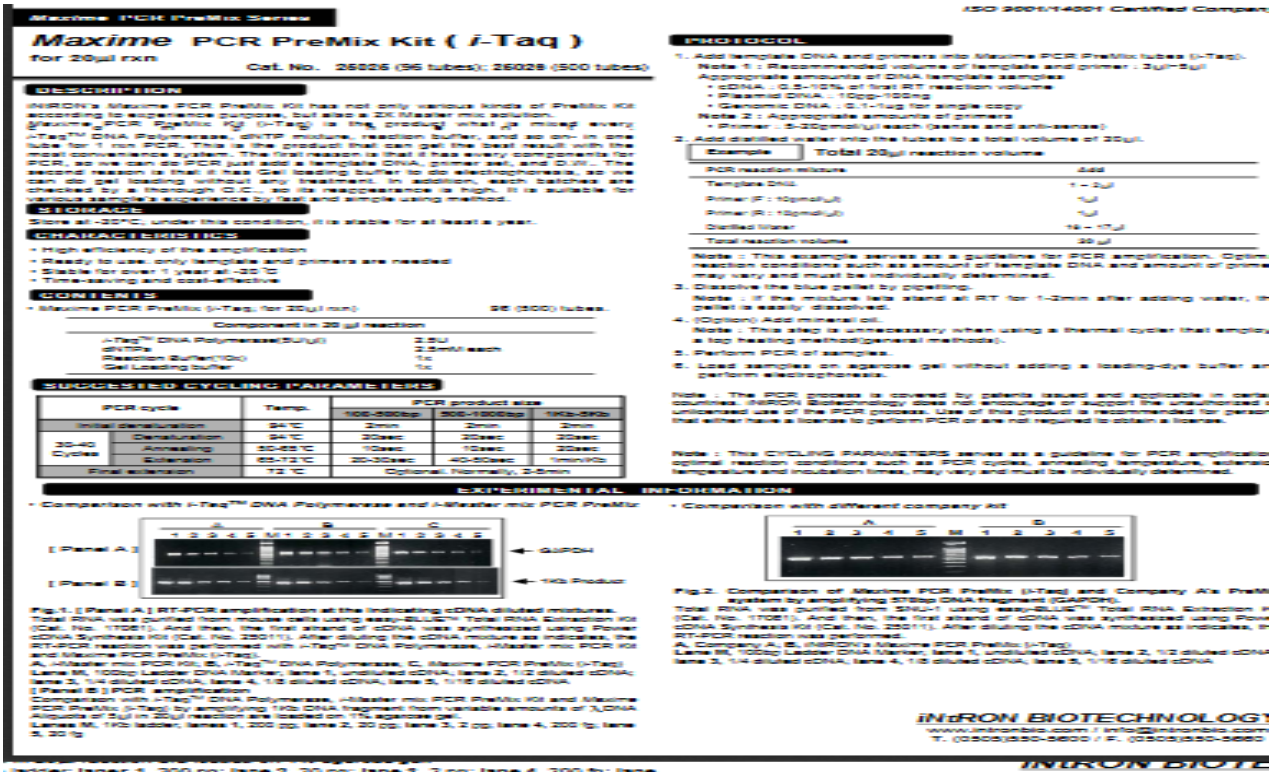
Chelex extraction

- Sample is suspended in 5% chelex and water and incubated at 56° C (½ h)
- Sample is **boiled** to lyse cells and destroy proteins
- Sample is centrifuged. The supernatant contains the DNA and can be directly used for PCR





Appendix 3: PCR Protocol



Maxime PCR PreMix Kit (i-Taq)

for 20μl rxn

Cat. No. 25025 (96 tubes); 25028 (500 tubes)

DESCRIPTION

STORAGE

CHARACTERISTICS

CONTENTS

SUGGESTED CYCLING PARAMETERS

PROTOCOL

EXPERIMENTAL INFORMATION

iNtRON BIOTECHNOLOGY

Appendix 4: Paper 1

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Detection of *Plasmodium falciparum* Chloroquine Resistance Transporter Mutant Allele K76T in Three Regional Areas in Sudan

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Abstract

Background

Chloroquine resistance in Sudan lead the ministry of health to change the first line of malaria treatment from chloroquine to artemisinin, this study was designed to detect *Plasmdium falciparum* Chloroquine Transporter (*Pfcrt*) mutant allele K76T in three regional areas in Sudan.

Materials and Methods

Three hundred (300) *P.falciparum* positive samples were collected from three regional areas in Sudan,positivesamples were confirmed by usingdirect microscopical stained blood films, DNA was extracted usingChelix method and then were amplified using Nested RFLP-PCR method to detect the mutant allele of *Pfcrt* K76T. Data were analyzed using SPSS 16.5 by Chi-squire test.

Results

Mutant allele of (*Pfcrt*K76T) was detected in the study areasincluding Al-Dinder (12.3%) , Khartoum (10.3%) and Al-Damer (9.3%) respectively, *Pfcrt*K76T among the female (22%) was detected higher than male (10%) which found to be statistically significant at *P*.value=0.002, finally the *Pfcrt*K76T in association with age groups, showed (7.7%, 6.0%, 10,7% and 7,7%) in age groups (<10, 10-19, 20-50 and >50) respectively which found to be statistically insignificant at *P*.value=0.202.

Key words:*Pfcrt*K76T, Antimalarial resistance, Al- Dinder, Al-Damer.

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Appendix 5: Paper 2

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Detection of Single Point Mutation of Plasmodium Falciparum Multi-Drug Resistance 1 Gene in Three Different Areas in Sudan

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Abstract

Background: *Chloroquine resistance in Sudan lead the ministry of health to change the first line of malaria treatment from chloroquine to artemisinin, this study was designed to detect single point mutation of Plasmodium falciparum multi-drug resistance-1 (Pfmdr-1) N86Y mutant allele in three different areas in Sudan.*

Materials and Methods: *Three hundred (300) P.falciparum positive samples were collected from three areas in Sudan, positive samples were confirmed by using direct microscopical stained blood films, DNA was extracted using Chelix method and then were amplified using Nested RFLP-PCR method to detect the Pfmdr-1 N86Y mutant allele. Data were analyzed using SPSS 16.5 by Chi-squire test.*

Results: *Single point mutation of Pfmdr-1 N86Y mutant allele was detected in the study areas including Khartoum (15.7%) , River Nile (10.7%) and Sennar (9.7%) respectively, Pfmdr-1 N86Y among the males and females were equal (18 %) which found to be statistically insignificant at P. value=0.167, finally the Pfmdr-1 N86Y in association with age groups, showed (8.0%, 4.7%, 15.7% and 7,7%) among age groups (<10, 10-19, 20-50 and >50) respectively which found to be statistically insignificant at P.value=0.399.*

Keywords: *Pfmdr-1 N86Y, Anti-malarial resistance, Sennar, River Nile.*

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I. Introduction

Malaria has been one of the greatest afflictions, in the same ranks as human immunodeficiency virus (HIV), influenza, and tuberculosis[1]. The agent of malaria is an obligate intracellular sporozoan in the genus Plasmodium, which contains four species; Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, and Plasmodium ovale[2]. Malaria is transmitted by the blood feeding of infectious female Anopheles mosquitoes, and understanding mosquito ecology and population dynamics can inform how best to defeat malaria[3]. Anti-malarial drug resistance is a major challenge to the control of falciparum malaria, the leading cause of morbidity and mortality especially in Africa and Southern Asia[4].

Chloroquine (CQ) and other quinoline-based drugs have been used for the prophylaxis and treatment of malaria for more than 50 years in all of the malarias countries because of its cost effectiveness, few side effects, and easy availability[5]. P. falciparum multi-drug resistance 1 (Pfmdr-1), a gene on chromosome 5 encoding a P-glycoprotein homolog 1 (Pgh1), also contributes to chloroquine resistance[6]. A few studies of resistant parasites from in vitro drug selection indicate that alteration of the Pfmdr-1 gene copy number contributes to changes in the level of chloroquine resistance[7,8]. Pfmdr-1 an Asn→Tyr mutation at amino acid 86 (N86Y) and other mutations in this gene correlated with chloroquine resistance[9]. Also, Pfmdr-1 has been associated with altered in vitro and in vivo parasite response to aryleaminoalcohols, including lumefantrine[9], molfequine and artemisinin[10]. Multidrug resistant P. falciparum malaria is common in Southeast Asia, but difficult to identify and treat. Genes that encode parasite transport proteins maybe involved in export of drugs and so cause resistance. Increase in Pfmdr-1 copy number predicts failure even after chemotherapy with the highly effective combination of mefloquine and 3 days' artesunate. Monitoring of Pfmdr-1 copy number will be useful in epidemiological