IN VITRO CULTIVATION OF *PLASMODIUM* SPECIES AND DETECTION OF ANTIPLASMODIAL ACTIVITY OF SOME DRUGS ON THE PARASITES

BY

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AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,

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ZARIA, NIGERIA

NOVEMBER, 2018

DECLARATION

I declare that this dissertation entitled "*In Vitro* Cultivation of *Plasmodium* species and Detection of Antiplasmodial Activity of some Drugs on the Parasites" was carried out by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

_____ _____ _____

ADAMU, IYANG COMFORT **SIGNATURE** **DATE**

CERTIFICATION

This dissertation entitled "*In Vitro* Cultivation of *Plasmodium* species and Detection of Antiplasmodial Activity of some Drugs on the Parasites obtained from Antenatal Patients in Zaria, Nigeria" by Adamu, Iyang Comfort meets the regulations governing the award of the master degree in Microbiology of Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to the ALMIGHTY GOD, The Father, The Son and The Holy Spirit without whom, this work would not have been possible and to my beloved parents, Rev. and Mrs. Sunday Adamu for their unending love, care and support.

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ABBREVIATIONS

ACTs	Artemisinin-Based Combination Therapies
AOFM	Acridine Orange Fluorescence Microscopy
BC	Before Christ
BP	Blood Pressure
CSA	Chondroitin sulphate A
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
EIR	Entomological Inoculation Rates
FIND	Foundation for Innovative New Diagnostics
FMOH	Federal Ministry of Health
FP	ferriprotoporphyrin IX
G-6PD	Glucose-6-Phosphate Dehydrogenase
GSM	Giemsa Stain Microscopy
HBV	Hapatitis B virus
HCMV	human cytomegalovirus
HCV	Hapatitis C virus
HIV/AIDS	Human Immunodeficiency virus/ Acquired Immune Deficiency Syndrome
IEs	Infected erythrocytes
IPT	Intermittent Preventative Therapy
IRS	Indoor Residual Spraying
LBW	Low birth weight
LLINs	Long-Lasting Impregnated Mosquito Nets
LSM	Larval Source Management
MAP	Mean Arterial Pressure
NMCP	National Malaria Control Programme
OD	Odd Ratio
PAM	Pregnancy Associated Malaria
PEP	phosphoenolpyruvate
PEPCK	PEP carboxykinase
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
PfEMP1	*Plasmodium falciparum*Erythrocyte Membrane Protein1
PfQRT	*Plasmodium falciparum* chloroquine resistance transporter
PM	Placental malaria
PPPK	2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase
ppy	Person Per Year
PTB	Preterm malaria
QBC	Quantitative Buffy Coat
RBC	Red Blood cell
RBM	Row Back Malaria
RDTs	Antigen-based Rapid Diagnostic Tests
RPMI	Rosewell Park Memorial Institute medium
TS	thymidylate synthase

WHO	World Health Organisation

ABSTRACT

Several reports have conducted resistance of *Plasmodium* to antimalarial in use. Successful cultivation and susceptibility will help to identify effectiveness of current antimalarial in use. The study was aimed at cultivating *Plasmodium falciparum in-vitro* and determination of the antiplasmodial activity of some drugs against malaria parasites. In this study, Blood samples were obtained from antenatal women attending some hospital in Zaria. Thin blood films were prepared stained using Giemsa stain and Acridine Orange stain. The films were observed microscopically using bright field microscope and fluorescence microscope (T6200 MIJEI). Antiplasmodial activity of some drugs to *Plasmodium falciparum* was carried out *in-vitro.* The prevalence of *Plasmodium* infection among antenatal patients was 27% and 53% by Giemsa and Acridine Orange Fluorescence microscopy. Factors found to be associated with the disease were lack of awareness of the aetiology of malaria (OR= 1.026; 95%; *p*-value= 1.000 CI= 0.585-1.799) and type of drainage (OR= 1.192; 95%; *p*- value= 0.129; CI= 0.456-3.113). The modified *Jatropha* medium supported the growth of the red blood cells with parasite density of 6500-12000as compared to 8000-14000 parasites/µl for those grown in Rosewell Park Memorial Institute medium. Results obtained from the *in-vitro* cultivation of *P. falciparum* with some drugs showed that chloroquine was able to reduce the parasite density from 7000 parasites/µl and 7500 parasites/µl with Rosewell Park Memorial Institute and *Jatropha* plant media at the 24^{th} and 12^{th} hour respectively compare to Artesunate, Quinine and Sulphadoxine + Pyrimethamine. There is need to further improve the antiplasmodial activity of some drugs to *Plasmodium* parasite in order to establish a malaria free nation.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Malaria

Malaria remains a major health challenge to mankind all over the world and over three billion people in the world stand the risk of having malaria (WHO, 2013). Despite local and international efforts towards the prevention of the disease, the morbidity and mortality rate as a result of malaria remains alarming (Adebayo *et al.*, 2015). In 2010 alone it was reported that about 216 million cases of malaria occurred worldwide, out of which 655,000 deaths were recorded. This report further revealed that 81% of the occurrence and 91% of those who died were recorded from sub-Saharan Africa (Okpere *et al.*,2010 and WHO, 2011).

Nigeria is one of the countries in Africa where the disease is endemic accounting for 25% of all reported cases of malaria in the 45 countries of Africa (WHO, 2008). The incidence of malaria and associated deaths in Nigeria is reportedly far more than those of any country in the world (United States Embassy in Nigeria, 2011; Omaka-Omari and Nwimo, 2015). This statement was underscored by another report that about 97% of people in Nigeria stand the risk of having malaria due to the geographical location of the country that makes the climate suitable for the transmission of the disease. The remaining 3% who live in the mountains in places like Jos are relatively at low risk of the disease (NMIS, 2010; Musbau *et al.*, 2014).

Some socio-demographic factors are known to influence the occourrance of malaria in pregnant women. These include education and place of residence (Sharma *et al.*,2007). High level of formal education is reported to increase the level of general knowledge and hence may positively influence healthy behaviour (Antwi, 2010). Similarly, some cultural beliefs and myths about illness perception often prevalent in many rural settings are likely to influence knowledge of malaria among rural dwellers (Sharma *et al.*,2007).

Pregnant women are three times more likely to develop severe form of the disease than non-pregnant women within the same area (Falade *et al.*,2008; CDC, 2013).

In order to relieve the economic burden caused by the disease in human, the Roll Back Malaria (RBM) campaign was initiated by the World Health Organization (WHO), United Nations Children's Fund (UNICEF), UNDP and the World Bank with different objectives, one of which was intended to halve the burden of malaria by 2010 (Agbaje and Aphia, 2007).

Natural transmission of *Plasmodium species* occurs throughmosquito vector of the genus*Anopheles*. The discovery that mosquitoes are involved in the transmission of *Plasmodium species* was first reported by Ronald Ross in 1897 (Gilles, 2002). There are about 430 species which transmit malaria and only 40 are of medical importance (Service and Townson, 2002). The four *Plasmodium species* that produce human disease are *Plasmodium vivax, P. falciparum, P. malariae* and *P. ovale*.

The signs and symptoms of malaria can be classified into two categories: uncomplicated and severe malaria. In uncomplicated malaria, symptoms are present, but there are no signs indicating severe infection or dysfunction of the vital organs. However, severe malaria could occur if not attended to quickly, and due to poor immunity status. Some strains of the parasite can have a longer cycle or cause mixed symptoms. Symptoms resemble those of flu; they could be undiagnosed or misdiagnosed in areas where malaria is less common. A sensation of cold with shivering, fever, headache, vomiting, seizures sometimes especially among the young diseased individuals, sweating followed by a return to normal temperature with tiredness (Luo, 2017) are the most common symptoms heralding the onset of a clinical case of uncomplicated malaria. Vital signs and symptoms of severe malaria include fever, chills, impaired consciousness, prostration, multiple convulsions, respiratory distress, abnormal bleeding and signs of anaemia, clinical jaundice, haematological complications (Akinosoglou

et al., 2012) haematological maglinancy (Rapoport and Uys, 2008) and evidence of vital organ dysfunction such as splenomegaly, hepatomegaly, hepatosplenomegaly and Central Nervous System (CNS) involvement (Luo, 2017).

Currently available anti-malarials fall into three broad categories according to their chemical structure and mode of action: Aryl aminoalcohol compounds or the quinolines: Quinolones target blood and hepatic stages of the parasite's lifecycle. Drugs in this class act by forming a complex with haem in the parasite food vacuole so, haem polymerization is inhibited. As a result, the haem is released during haemoglobin breakdown and builds up to poisonous levels, thereby killing the parasite with its own toxic waste. Examples of quinolines are chloroquine, mefloquinine and quinine (Tripathii, 2009; Saifi *et al.*, 2013).

Antifolate compounds (examples: Atovaquone, Trimethoprim, Dapsone, and pyrimethamine): These compounds inhibit the synthesis of folate cofactors that are required for nucleotide synthesis and amino acid metabolism. Antifolates prevent nuclear division of *Plasmodium* at the schizont stage within the hepatocytes and erythrocytes by acting on dihydrofolate reductase (DHFR) or dihydropteroate synthase (DHPS) (Cunha-Rodrigues *et al.*, 2006). Atovaquone inhibits electron transport in plasmodial mitochondria and depolarizes the membranes of plasmodial mitochondria (Nigussie,*et al.*, 2015).

Artemisinin compounds includes artemisinin, artesunate and hydroarteminsinin have a broad spectrum of activity against all parasite phases within erythrocytes, in particular younger ring forms and suppress gametocyte transmission. Artemisinin and its analogs are rapidly acting blood schizonticides against all human malaria parasites. However, it has no effect on hepatic stages (Wunderlich, *et al.*, 2012). The antimalarial activity of artemisinins results from the production of toxic free radicals, produced due to cleavage of the artemisinin endo-peroxide

Bridge in the parasite food vacuole or from inhibition of a parasite calcium ATPase (Mbengue, *et al.,* 2015; Nigussie,*et al.,* 2015).

In addition to first- and second-line antimalarial drug treatments, adjunctive and supportive care measures (e.g., intravenous fluids, blood transfusions, supplemental oxygen, and antiseizure medications) may be needed for severe manifestations. The aims of treatment are to prevent death or long-term deficits from malaria, to cut short the morbidity of an acute episode of illness, and to clear the infection entirely so that it does not recur.

1.2 Statement of Research Problem

Malaria is a major cause of obstetric complications (pre-term or complicated labour leading to caesarean section instead of spontaneous vaginal delivery), increased uterine activity, foetal death, low birth weight, severe maternal morbidity and mortality (Onah *et al.*,2006; Garner and Gülmezoglu, 2007; WHO, 2007a andFalade *et al.*,2008).It also causes maternal hypoglycaemia, acute pulmonary oedema, cerebral oedema and maternal death (FMH, 2000a; WHO, 2007b; Gajida *et al.,* 2010).

*Plasmodium falciparum*infection in pregnancy leads to parasite sequestration in the maternal placental vascular space, with consequent maternal anaemia and infant low birth weight (LBW) due to prematurity, anaemia, vitamin A and iron deficiencies (Richard *et al.*,2001). Malaria has been responsible directly or indirectly for 10% of maternal death in Calabar, 8% in Enugu, 7.8% in Lagos and 8.2% in Kano (Gajida *et al.,* 2010).

Despite the availability of numerous antimalarial drugs and control programs, malaria remains endemic and constitutes a major public health problem accounting 11% maternal mortality, 25% infant mortality, 30% under 5years old mortality with 60% cases among outpatients and 30% of admission (NMIS, 2010 and Musbau *et al.,* 2014). Thus this disease

exerts a huge social and economic burden on families, communities and the country at large and that an estimated 100 million cases and 300, 000 deaths of malaria occur every year in the country (United States Embassy in Nigeria, 2011 and Musbau *et al.*, 2014).

Pregnancy poses specific challenges for the diagnosis of malaria, because *P. falciparum* parasites may be present in the placenta but absent or undetectable in peripheral blood. This phenomenon is attributable to the accumulation of infected erythrocytes in the intervillous spaces of the placenta through specific adhesion to chondroitin sulphate A. Even infections with low parasite densities undetected by standard microscopy can have severe effects on the pregnant woman and her foetus. Thus, prompt and accurate detection of *P. falciparum* infection is fundamental for the control of malaria in pregnancy (Alfredo *et al.*,2012).

Giemsa stained microscopy is the recommended gold standard by World Health Organisation for the diagnosis of malaria.However, the detection of malaria parasites requires expertise because when it is performed by poorly trained personnel, very low sensitivity especially in healthcare facilities results leading to over diagnosis or under diagnosis with the consequent excessive use of anti-malarial drugs or negligence of treatment which invariably contributes to the increased development of resistance by the malaria parasites, as well as drug-induced pathological conditions (Coleman *et al.*,2002; Bates *et al.*,2004; Obare *et al.*,2013; Ugah, *et al.*,2017).

Anti-malarial drugs were majorly designed based on the major biochemical metabolism of malaria parasite such as nucleic acid metabolism, heme detoxification, oxidative stress and fatty acid biosynthesis. These drugs though effective yet are now limited due to emergence of resistance (Alam, *et al.*,2009). In addition, the mechanisms of emergence of resistance are poorly understood for some of these drugs. Genetic, molecular and pharmacological approaches have shown that different targets of older drugs are resistant due to mutations on

their key enzymes or transporters (Alam, 2014; Nigussie,*et al.*,2015) hence the need of a cheap and effective method of culturing *Plasmodium in-vitro* enabling more research on understanding the mode of action and potential targets of these drugs on *Plasmodium*, combat issue that arosed by emergence of resistance to existing drugs and explore new target proteins of malaria parasite for antimalarial drugs so as to completely eliminate *Plasmodium*.

1.3 Justification of the Study

A National Survey conducted in Nigeria in 2000 showed that out of the ailments of pregnant women, malaria account for 48.2%. Thus prevention of malaria in pregnancy has continued to be a major public health challenge, an initiative which is encapsulated in the Roll Mack Malaria (RBM) programme (Mathanga, 2005; Daboer *et al.,* 2010).

The development of a new technique in the rapid diagnosis of malaria such as the technique of acridine orange fluorescence microscopy has opened a new door for malaria diagnosis. It has been found that acridine orange fluorescence microscopy is more superior about eight (8) times better to Giemsa stained microscopy in the direct microscopic examination and a reliable method for detecting organisms in cultures early during incubation and can be substituted for blind subcultures because it gives striking differential straining between organisms and background cells and debris and the poor differential staining of organisms can be overcome with acridine orange stain (Siddique *et al.*,2003).

Therefore, acridine orange fluorescence microscopy staining technique is capable of detecting the presence of malaria parasites in direct clinical specimens and microscopically negative culture with a higher sensitivity and less time than the Giemsa stained microscopy (Mirrett, 1982; Mirrett *et al.,* 1982; Siddique *et al.*,2003).

In vitro culturing of malaria parasites was first reported in 1912 by Bass and Johns, and subsequent attempts were made to develop a better culture medium including such methods as short term cultures with Harvard growth medium (Ball *et al.*,1945; Anfinson *et al.*,1946), modified Harvard growth medium (Geiman *et al.*,1946)and Rosewell Park Memorial Institute (RPMI-1640) medium (Moore, 1967) developed originally for culture of human leukocytes. Rosewell Park Memorial Institute medium was proven to be significantly superior to Harvard medium, and allow for a successful continuous culture of the malaria parasite and is still being widely used throughout the world (Trager and Jensen, 1976).

Jatropha leaves extract medium is a cheap medium that is can easily be produced because the materials are readily available unlike other commercial medium probably because of its cost implication and/or storage problem as a result of instability in electricity for proper refrigeration. The mechanism responsible for the growth of malaria parasite is not known. Culture requirement for the culture of *Plasmodium*such as human serum, liver extract contains a growth-promoting factors treat that aid in the parasite proliferation for diagnosis and treatment of *Plasmodium* especially in a clinical situation to reducing malaria burden in Nigeria (Nassar *et al.*,2014).

1.4 Aim

To cultivate *Plasmodium falciparumin-vitro* and determine the antiplasmodial activity of some drugs against the parasite.

1.5 Objectives

The specific objectives of this research were to determine:

1. prevalences of *Plasmodium* infection among pregnant women using Giemsa staining and Acridine Orange Fluorescence Microscopy technique.
2. socio-demographic and risk factors that predispose pregnant women to malaria using structured questionnaires.
3. cultivate*Plasmodium falciparum in vitro* using Rosewell Park Memorial Institute (RPMI) and *Jatropha curcas*leaves extract media.
4. parasite density of *Plasmodium falciparum* in Rosewell Park Memorial Institute (RPMI) and *Jatropha curcas*leaves extract media.
5. antiplasmodial activity of some drugs *in-vitro* using Rosewell Park Memorial Institute (RPMI) and *Jatrophacurcas*leaves extract media.

CHAPTER TWO

2.0 History of Malaria

The history of malaria was first written in the Chinese document from about 2700 BC on clay tablets from Mesopotamia and Egyptian papyri from 2000 BC, and 1570 BC respectively and it was also recorded as far back in the 6^{th} century BC. Malaria awareness on its signs and symptoms were known by the early Greeks, Empedocles of Agrigentum and Hippocrates about 850BC, 550BC, and 400 BC respectively. Over 2500 years, malaria fevers were known to be caused by miasmas rising from persisted swamps.

The word malaria hail from Italian: mal'aria meaning 'spoiled air'. Over time, this has been disputed in 1676 by Antony Van Leeuwenhoek who discovered bacteria. Likewise the contributions of Pasteur and Robert Koch in 1878-1879 gave birth to another school of thought: the germ theory of infection. In 1880, Charles Louis Alphonse Laveran scientific studies was possible by discovering the parasites mosquitoes which were first incriminated as the vectors by Ronald Ross in 1897 and Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between 1898 and 1900 for avian malaria and then for human malaria respectively (Francis, 2010).

Charles Louis Alphonse Laveran, an unknown French army officer working in Algeria, challenged the perceived wisdom of Italian Corrado Tommasi-Crudeli and the German, Theodor Albrecht Edwin Klebs who claimed that they had isolated a bacterium *Bacillus malariae* from the waters of the Pontine Marshes, where malaria was prevalent, a bacterium, *Bacillus malariae,* which when isolated in culture and injected into rabbits caused febrile infections accompanied by enlarged spleens reminiscent of malaria (Klebs and Tommasi-Crudeli, 1888) and began in his own words 'to follow the pigment'. Beginning with the known fact that the spleens of malaria patients contained pigment he began to look for

pigment in the fresh unstained blood of patients and observed it first in leucocytes and then in or on red blood cells. He observed several forms of erythrocytic organism including crescents, spherical motionless bodies with pigment, spherical moving bodies with pigment and bodies that extruded flagella-like structures all of which he thought were on the outside of the red cells with a microscope. He also suggested a course of events that began with clear spots that grew, acquired pigment and filled the corpuscle which then burst coinciding with the fevers associated with malaria. After so much work, Laveran was awarded the Nobel Prize for Medicine in 1907 (Bruce-Chwatt, 1981; Francis, 2010).

A group of researchers discovered development of malaria parasites in human liver before entering the blood stream. The presence of dormant stages of this parasite in liver was only reported in 1982 (Cox, 2010).

2.1 Taxonomic Classification of *Plasmodium*species

*Plasmodium*belongs to the Phylum Apicomplexa, Order Haemosporidia, Family*Plasmodidae*. The genus *Plasmodium*is subdivided into the subgenus *Laverania*and the subgenus of the same name, i.e. *Plasmodium*. *P. falciparum*belongs to the subgenus *Laverania*and *P. vivax, P. ovale*and *P. malariae*belong to the subgenus *Plasmodium* (Cheesbrough, 2009). The most recent evidence indicates that *P. ovale* is composed of two subspecies, i.e., *P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type) (Sutherland, *et al.*, 2010). The unusual characteristics of malaria parasite in comparison to general eukaryotes include the presence of rhoptry, micronemes, and polar rings near the apical end (Sutherland, *et al.*, 2010).

2.2 Global Burden of Malaria

Globally, malaria ranks as the fifth cause of death from infectious diseases, after respiratory infections, diarrhoeal diseases, HIV/AIDS, and tuberculosis (WHO, 2012). Generally, the transmission of malaria is more intense in warmer regions closer to the equator, such as in areas of the Americas, many parts of Asia, and Africa. The prevalence of malaria in tropical regions is due to the significant amounts of rainfall with consistent high temperatures and humidity, along with stagnant waters, which allow the mosquito to thrive and breed. In contrast, malaria transmission is unstable with a low incidence (limited cases) at the high altitude areas over elevations (1,500 m,) desert areas (excluding oases), as well as during dry and colder seasons even in some parts of malaria-endemic countries (United States Embassy in Nigeria, 2011).

In Africa, malaria is the second leading cause of death after HIV/AIDS (WHO, 2013). Besides that, malaria is re-emerging as the first infectious killer and it is the number one priority tropical disease (WHO, 2012). Thirty countries in Sub-Saharan Africa account for 90% of global malaria deaths where Nigeria, Democratic Republic of Congo (DRC), Ethiopia, and Uganda account for nearly 50% of the global malaria deaths (United States Embassy in Nigeria, 2011).

United States Embassy in Nigeria (2011) estimated 216 million cases of malaria in 2010 with about 81% cases in the African region, resulting in 655,000 malaria deaths, 91% of which was in the African Region, and 86% were children under 5 years of age. Malaria is the 3rd leading cause of death for children under five years worldwide, after pneumonia and diarrheal disease (United States Embassy in Nigeria, 2011).

2.2.1 Burden of malaria in Nigeria

According to WHO, (2015), approximately 51 million cases and 207,000 deaths reported annually (approximately 30 % of the total malaria burden in Africa), while 97 % of the total population (approximately 173 million) is at risk of infection in Nigeria with 50.3% in SouthWest, 49.4% in North central, 48.2% in North West, 32.2% in South South, 30.9% in North East and 27.6% in South East. The remaining 3% of the population live in the malaria free highlands hence the world's greatest malaria burden (United States Embassy in Nigeria, 2011).

The differences in temperature and distance to water bodies where the risk factors associated with high malaria prevalence varying from 20% in certain areas to 70% in the Niger Delta states of Rivers and Bayelsa, areas surrounding the confluence of the rivers Niger and Benue, other regions such as isolated parts of the north-eastern and north-western parts of the country account for the high prevalence. Isolated patches of low malaria prevalence were found scattered around the country with northern Nigeria having more such areas than the rest of the country. The middle belt of Nigeria generally has malaria prevalence of 40% and above (Onyiri, 2015).

Malaria accounts for 60 % of outpatient visits to hospitals in Nigeria (FMOH, 2009; WHO, 2014) where about half of adults have at least one episode of malaria each year and seven (7) out of every 10 patients seen in Nigerian hospitals are due to malaria (FMOH, 2005). According to Holding and Kitsao-wekulo, (2004), "malaria is one of the principal reasons for poor school attendance in many settings in Nigeria because it accounts for 13 to 15 percent of medical reasons for absenteeism from school".

Malaria is responsible for approximately 11% maternal mortality and 30-42% child mortality, especially among children less than 5 years (FMOH, 2009; WHO, 2014). Likewise, Roll Back Malaria (RBM, 2005) reported that malaria was responsible for one death out of every

ten women during childbirth and three (3) out of every ten (10) deaths of under five years mortality (WHO, 2014).

The major malaria vectors are the *Anopheles gambiae* complex (*gambiae*s.s. and *arabiensis*) and the *funestus* group. These three species are widely distributed across the country, from the mangrove and coastal areas of the south to the Sahel savannah of the far north. Transmission studies show that entomological inoculation rates (EIR) range from 18-145 infective bites person per year (ppy) for *Anopheles gambiae* in the north central and 12-54 infective bites ppy for *Anopheles funestus* in the southwest and between 0-12 bites ppy for *Anopheles arabiensis* (FMOH, 2014).

Since 2008, the National Malaria Control Programme (NMCP) in Nigeria has adopted a specific plan, the goal of which is to reduce 50 % of the malaria burden by 2013. The country aimed at achieving at least 80 % coverage of long-lasting Impregnated mosquito nets (LLINs), together with other measures, such as 20 % of houses in targeted areas receiving indoor residual spraying (IRS), and treatment with two doses of intermittent preventative therapy (IPT) for 100 % of pregnant women who visit antenatal care clinics (Ye *et al.,* 2012; USAID, 2013; Adigun *et al.,*2015). Because of these measures, the percentage of households with at least one LLIN increased to over 70 % by 2010, compared to only 5 % in 2008 (WHO, 2010; Oyeyemi *et al.,*2010; Garley *et al.,*2013).

These reductions was complimented by a major scale-up of vector control interventions, treatment with artemisinin-based combination therapies (ACTs), public health education campaigns, and improvement on some socio-economic factors and more. While the risk of malaria as measured by prevalence in children is high in several region of the country, there are some differences between regions with high prevalence, over 50 % prevalence in Zamfara, Kebbi, Kwara and Osun while prevalence 40-50% recorded in 10 States: Sokoto,

Katsina, Niger, Oyo, Ekiti, Ondo, Kogi, Benue, Cross Rivers and Ebonyi. These differences can be attributed to variations in intervention coverage, socio-demographic factors, and capacity of the health systems, climate and ecology in the States (FMOH, 2014).

Presently, NMCP have strategized means of reducing malaria specific moratality by 2017 by implementing Seasonal malaria chemoprevention a new intervention for the first time in nine States in the Sahel region. This has the potential of reducing annual malaria burden to achieve a prevalence of below 5% and zero malaria mortality by 2020, building on the achievement from 2009-2013 (FMOH, 2014).

2.3 Malaria in Pregnancy

Malaria in pregnancy is one of the leading causes of maternal and child morbidity and mortality worldwide, mainly in high endemic areas (Dellicour *et al.*,2010; Rijken *et al.*,2012). About 125 million women are at risk of acquiring malaria infections during pregnancy (Dellicour *et al.*,2010), mainly in Africa where *Plasmodium falciparum*is the predominant malaria parasite. Outside Africa, *Plasmodium vivax*infections are predominant and every year about 90 million pregnant women are exposed to the risk of infection mostly in the Asia–Pacific region only and about three million in Latin America (Dellicour *et al.*,2010; Rijken *et al.*,2012).

About 50 million women who become pregnant in malaria-endemic regions each year (WHO, 2016) are infected with *Plasmodium*parasite (Desai *et al.*,2007). Pregnant women are three times more likely to suffer from severe disease from malarial infection than their non-pregnant counterparts, with close to 50% mortality rate from severe disease (Monif *et al.*,2004). Malaria, particularly *Plasmodium falciparum*infection increases the risk of maternal anaemia and other complications in pregnancy, often leading to abortions and stillbirths (WHO, 2004).

According to Ofori *et al.*(2002), immunity to *P. falciparum* develops slowly and naturally after repeated exposure (Mackintosh*et al.*,2008). The acquired anti-*Plasmodium*antibodies confer protective immunity (Marsh and Kinyanjui, 2006), which reduces morbidity (Kinyanjui, 2006; Stephens *et al.*,2017). However, clinical immunity to malaria is partial and shortlived, and requires constant 'longitudinal exposure' to parasites to be maintained (Stephens *et al.*,2017).

Malaria infection during pregnancy is associated with a broad spectrum of clinical manifestations ranging from uncomplicated to severe cases and death of both mothers and neonates (McGready *et al.*,2012; Lopez‑Perez *et al.*,2016).

2.4 Biology of *Plasmodium*species

Malaria is an infectious disease caused by protozoan which is a unicellular eukaryotic parasite from the genus *Plasmodium*. There are over 200 species of *Plasmodium*that produce disease in non-human primates, rodents, reptiles, and birds, in addition to humans. Thus far, about 30 species naturally infect non-human primates (monkeys, great apes, and others), whilst only five identified species, i.e.*P. vivax*, *P. falciparum*, *P. malariae*, *P. ovele*, and *P. knowlesi* cause malaria in humans (Singh *et al.* 2004; Antinori*et al.* 2013).

2.4.1 Plasmodium falciparum

*Plasmodium falciparum*is found in tropical areas i.e. the hotter and more humid regions which arein tropical and subtropical Africa and parts of Central America and South America, Bangladesh, Pakistan, Afghanistan, Nepal, Sri Lanka, South East Asia, Indonesia, Philippines, Haiti, Solomon Islands, Papua New Guinea and many islands in Melanesia. It also occurs in parts of India, the Middle East, and Eastern Mediterrane. It is the most disease causing human malaria parasite. *P. falciparum* is responsible for over 90% of the malaria morbidity worldwide (Hanscheid and Grobusch 2002; Cheesbrough, 2009).

The *P. falciparum* parasite belongs to the subgenus *Laverania* which has been suggested to be of more recent origin compared to other subgenus due to the low level of polymorphism within it's genome (Han *et al.*,2007; Njiru, 2012). Reports by Bossuyt *et al.*(2003) and Shakely *et al.*(2013) have shown that *P. falciparum* can infect both monkeys and gorillas, which may constitute a reservoir complicating efforts to eliminate the parasite.

*Plasmodium falciparum*species has the ability of causing the highest parasite load due to the fact that each schizont can harbour up to 32 merozoites that are able to infect RBCs of all ages. Hence, enabling the infection to become hyper parasitaemic with parasite densities of more than 5%, therefore causing massive lysis of RBCs and subsequent anaemia, a common cause of severe malaria especially children (Polley *et al.*,2010). Sequestrating characteristic to vital organs of falciparum infection is the pathogenesis of this deadly disease (Hay, *et al.*, 2009).

*Plasmodium falciparum*has an erythrocytic cycle of 48 hours in humans, produces malignant tertian malaria - 'tertian' because the fever was believed to recur every third day however, fever paroxysms generally might not show a distinct periodicity, 'malignant' because it is the most severe form of malaria and can be fatal hence, the name malignant and fatalistic malaria (WHO, 2012). *Plasmodium falciparum*does not have an exoerythrocytic stage, so if the erythrocytic stage is eradicated, relapse do not occur (Schmidt *et al.*,2014).

*Plasmodium falciparum*maturing stages expresses cyto-adherent proteins (such as *P. falciparum* Erythrocyte Membrane Protein1 (PfEMP1), encoded by the *var* gene family), forming knobs on the RBC surface (Shakely *et al.*,2013). These knobs make the infected cells "sticky" and responsible for binding uninfected RBCs to their surface forming rosettes (Shokoples *et al.*,2009; Steenkeste*et al.*,2009). Cyto-adherence proteins also mediate binding of the infected RBC to the endothelial of the deep vessels, known as sequestration. This

prevents the infected RBCs from being cleared from the circulation by the spleen (Dahlstrom *et al.,*2008; Schmidt *et al.,*2014). The sequestered parasites clog in the vessels hampering the blood circulation. When this occurs in the brain it can result in cerebral malaria (Schmidt *et al.,*2014).

2.4.2 Plasmodium vivax

Vivax malaria also known as benign tertian malaria is caused by *Plasmodium vivax.* It is biologically and morphologically very similar to *P. ovale*. *Plasmodium vivax*is the main *Plasmodium*species in South America (occurring as far south as northern Argentina), Mexico, the Middle East, northern Africa, India, Pakistan, Sri Lanka, Papua New Guinea and the Solomon Islands. It is also found in parts of South East Asia, Indonesia, Philippines, Madagascar, tropical and subtropical Africa, Korea, and China due to its ability to developing in mosquitoes at lower temperatures than *P. falciparum* and therefore has a widest global distribution (Cheesbrough, 2009). Even though *P. vivax* infection; therefore, known as benign malaria in many cases, however the surge of drug resistance strain, which is potentially fatal has weaken the malaria treatment and control (Mueller, *et al.,* 2009).

*Plasmodium vivax*normally requires the Duffy blood group antigen, expressed on the RBC surface to invade the cell and is therefore seldom seen in West Africa where Duffy negativity is common (Hsiang *et al.,*2010). However, a recent report has shown *P. vivax* infections among some Duffy negative individuals in Africa (Kamau *et al.,* 2013). *Plasmodium vivax*is generally seen as a benign form of malaria even though there are several reports on severe manifestations in the last years (FIND, 2012)

*Plasmodium vivax*has a dormant liver stage, hypnozoites that can activate and invade the blood (relapse) several months or years after the initial infection. Strains of *P. vivax* can develop in the mosquito in temperate climates where they often do not produce primary

attacks shortly after the infective bite; instead the clinical symptoms arise up to nine months later (Polley*et al.*,2013).

2.4.3 Plasmodium ovale

*Plasmodium ovale*has long been considered to have a dormant hypnozoite stage that can persist in the liver and cause relapses. *Plasmodium ovale*also has a tertian cycle andpreferably invades young RBC, i.e., reticulocytes, which make parasite densities therefore seldom exceed 1% just like *P. vivax.* Mixed infections with *P. falciparum* are common*Plasmodium ovale*has a low prevalence and found mostly in Africa (especially West Africa) where it accounts for up to 10% of malaria infections and has also been reported from other parts of Africa, and from the Philippines, Indonesia, China, and parts of the Far East, South East Asia, and South America (Cheesbrough, 2009).

2.4.4 Plasmodiummalariae

*Plasmodium malariae*has a much lower prevalence than *P. falciparum* and *P. vivax.* It is found in tropical and subtropicalregions. In tropical Africa it accounts for upto 25% of *Plasmodium*infections. It is also present inGuyana, India, Sri Lanka and Malaysia. In thesecountries it accounts for less than 10% of*Plasmodium*infections (Cheesbrough, 2009; Schmidt *et al.*,2014).

*Plasmodium malariae*is distributed over most of the malaria endemic area and is the only human malaria species that has a quartian (72 hours) cycle (Polley*et al.*,2013; Lucchi *et al.*,2010). Untreated, *P. malariae* often causes a long-lasting, chronic infection which often remains latent and may probably last a lifetime. Chronically infected patients with *P. malariae* could experience serious complications such as nephrotic syndrome (Petel *et al.*,2014). Mixed infections with *P. falciparum* and *P. ovale* are common in Africa and all

three species can even occur simultaneously.*Plasmodium malariae*preferably invades RBCs older than 100 days making parasite densities seldom exceed 1% (Schmidt *et al.*,2014).

2.4.5 Plasmodiumknowlesi

*Plasmodium knowlesi*as the most recent malaria-causing parasite discovered (WHO, 2012). *Plasmodium knowlesi*was recently recognized as the fifth human malaria that is widespread in the forest areas of Southeast Asia (as a natural pathogen of long and pig-tailed macaques i.e. old world monkeys of genus *macaca*) especially in Malaysian Borneo and extends to Malaysia and neighboring countries (Cox-Singh, *et al.,* 2008; White, 2008). Severe complications are common in knowlesi infection and potentially lethal associated with hyperparasitemia and development marked hepatorenal dysfunction. It has been shown to be a significant cause of zoonotic human malaria in that region, particularly in Malaysia where it accounts for up to 70% of human malaria cases (Cox-Singh, *et al.,* 2008; Daneshvar, *et al.,* 2009; Cox-Singh, *et al.,* 2010; William, *et al.,* 2011; Abdul-Ghani *et al.*,2012; Rajahram, *et al.,* 2012).

Morphologically *P.knowlesi* resembles *P. malariae* but unlike *P. malariae*, parasite numbers have been reported as high and clinical symptoms more severe (Cheesbrough, 2009). *Plasmodiumknowlesi* has a 24-hour replication cycle and can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported (Barber *et al.,* 2011; Schmidt *et al.*,2014).

2.5 Life Cycle of Plasmodium*species*

The life cycle of *Plasmodium*species is extraordinarily complex and specific for the particular parasite species (Florens, *et al.*, 2002). In general, the *Plasmodium*parasites live part of its life as an endogenous asexual phase (schizogony) with multiplication in the vertebrate host and as exogenous sexual phase (sporogony) with multiplication in certain female mosquitoes

(exclusively of the genus). Infected female anophelesmosquitoes play a role as vector in human-human transmission. Basically, it can be distinguished into three main stage-cycles, i.e., exo-erythrocytic and erythrocytic cycles in human host, and sporogonic cycle in mosquito (Ezeigbo, 2005).

2.5.1 Schizogony

Schizogony is an asexual endogenous (exo-erythrocytic and erythrocytic) phaseof Plasmodial'slife cycle.

2.5.1.1 Exo-erythrocytic phase

In human host, infection starts after the inoculation of few sporozoites into the blood stream during the blood meal of an infected mosquito. Within minutes of entering the blood circulation, sporozoites are able to travel quickly to the parenchyma cells of the liver and invade hepatocytes, where they replicate and develop into liver schizonts (Francis and Warrel, 1993; Ezeigbo, 2005).

The sporozoite will develop to form trophozoite and then mature into tissue schizont during the within 5-10 days. The sporozoites of *P. vivax* and *P. ovale* persist in hepatocytes as hypnozoites. The state of dormancy may last for months or years and are responsible for new for relapse is due to incomplete treatment. Basically, the rupture of tissue schizonts will subsequently release several thousands of infectious merozoites into blood stream. Schizonts of*P. falciparum, P. vivax, P. ovale* and *P. malariae* wouldliberatemerozoites into the host's blood stream thereby invading the red blood cells (RBCs) and thus initiating the erythrocytic cycle.

2.5.1.2 Erythrocytic cycle

Exo-erythrocytic merozoites invade the blood stream and penetrate the erythrocytes where they undergo schizogony. The trophozoite forms contain food vacuoles which displace the nucleus to one side like a signet ring. Hence this stage is referred to as the signet ring stage. Later the vacuole disappears and haemozoin pigment granules are deposited. Eventually the red blood cells rupture to release the merozoites, haemozoin and other metabolic wastes into the blood stream. The rupture of parasitized cells is synchronized such that they all rupture at definite intervals characteristic of each species. This periodically accounts for the bout fever at regular intervals caused by the metabolic wastes.The erythrocytic schizogony is repeated several times producing several generations of merozoites with the infection of new cells (Prescott *et al.,* 2005) and sexually differentiated into gametocytes (producing either macro- or micro-gametocytes)within the erythrocytes. No further development occurs until they are ingested by mosquito during a blood meal (Long, 1999).

2.5.2 Sporogony

In the sporogonic cycle, Ingested gametocytes develop into macrogamete and microgamete which are female and male gametes respectively after ex-flagellation. The gamete does not develop further in the venicle host but in the mosquito vector. The macrogamete is fertilized by one microgamete to form a zygote (an ookinette). This penetrates the cell wall of the mosquito and lies between the epithelium and the basement membrane (Philips, 2001).

Ookinette multiplies to form oocysts. The oocyst nucleus divides several times with the first division being a reduction division to produce a number of sporoblasts. These divide further to produce thousands of haploid thread-likes porozoites. Upon maturation the oocyst bursts releasing the sporozoites into the haemocoel of mosquitoes. Some of these sporozoites are released into mosquito salivary gland and injected with saliva into blood when the mosquito bites man for a blood meal (Greenwood *et al.,*2005).

At any time after the infection is established, the vast majority of plasmodia will be in some stage of asexual maturation leading to another round of multiplication within the patient's bloodstream. However, a few parasites will have transformed into sexual stages (gametocytes) that, once ingested by mosquitoes, can perpetuate the transmission cycle. Because each stage of the malarial life cycle exhibits distinct biochemical and other characteristics (i.e., it expresses different proteins or locates in different sites within the body), a drug may kill one stage but have little effect on another. In other words, in each life-cycle stage the parasite manifests unique biological properties that can offer a target for the action of one or more antimalarial drugs.

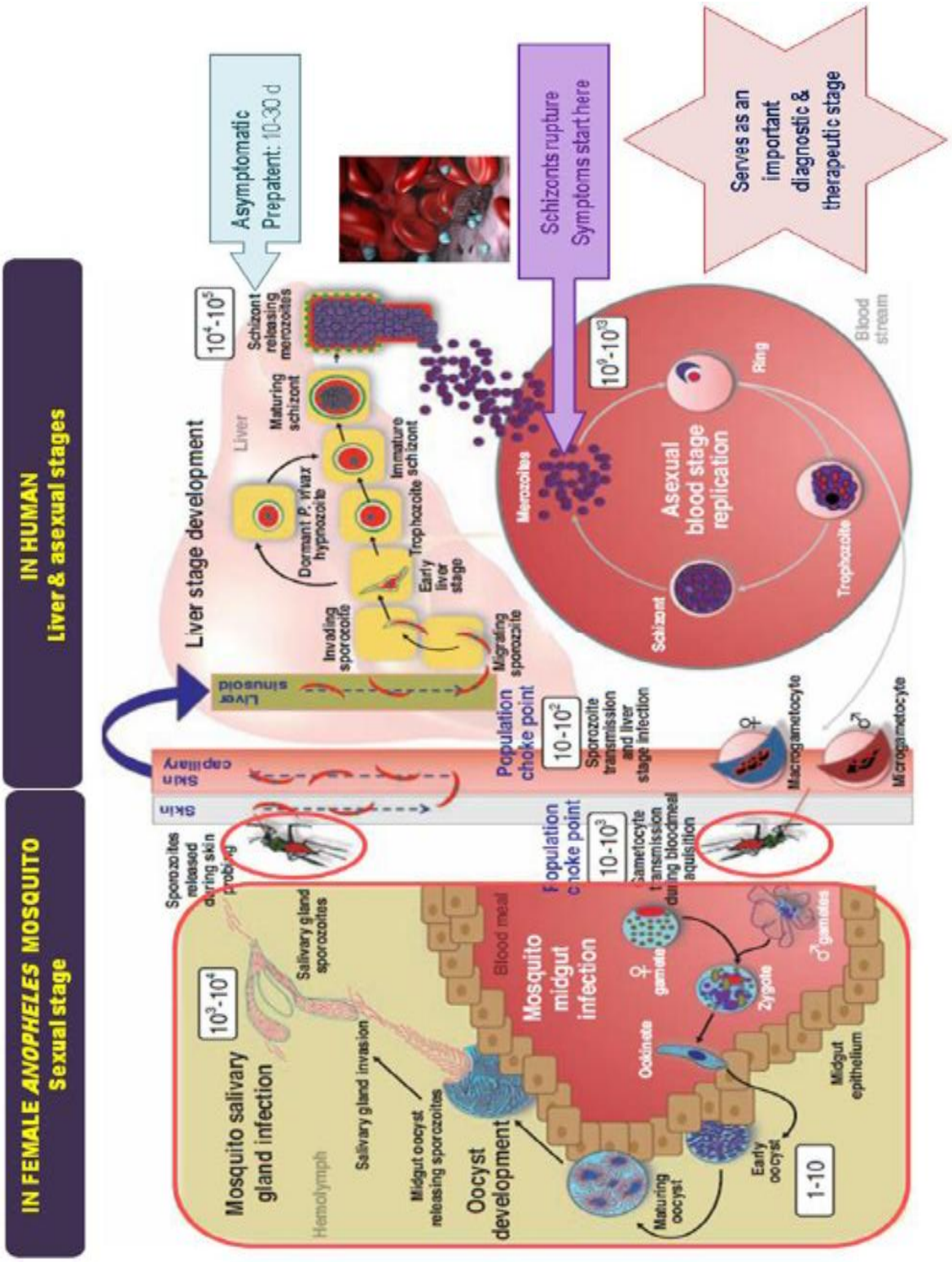


Fig 2.1: General life cycle of human *Plasmodium*species (adapted from Kappe, *et al.*, 2010).

2.6 Mode of Transmission of *Plasmodium*

The pattern of malaria transmission underpins several aspects of malaria epidemiology. It affects the prevalence, age profile of infection and types of syndromes as well as mortality. Knowledge of all these characteristics influences the choice of appropriate interventions to be used in malaria control and also affect the outcome of control efforts (Hay and Snow, 2006, Hay *et al.*, 2008).

Malaria is transmitted by *mosquitoes*, the number and type of which determine the extent of transmission of infection in a given area. It is transmitted to humans by the bite of female mosquitoes that are infected with protozoan parasite of the genus *Plasmodium.* The three most efficient species according to Azuike (1991) are *Anophelesgambiae, Anophelesarabienses* and *Anophelesfunestus.*

Transmission of malaria is affected by climate and geography and often coincides with the rainy season (WHO, 1998). It can be transmitted to man through three known ways which are (1) vector transmission (2) blood transmission (3) congenital transmission and (4) needlestick injury.

2.6.1 Vector transmission

Malaria parasites are transmitted from human to human through the bites of infected femalemosquito (a vector), typically at night time. Currently, there are approximately 530 *species* found around the world, only 70 to 80species transmit malaria in nature, with diverse breeding and feeding habits, and thus result in vast disease spectra in different population target groups and epidemiological settings. The intensity of malaria transmission depends on factors related to the vector, the parasite, the human host, and the environment (Manguin, *et al.*, 2008; Manguin, *et al.*, 2010; Alonso, *et al.*, 2011; Centers for Disease Control and Prevention (CDC), 2012; Choochote and Saeung, 2013).

A mosquito host is required by the parasite to complete their sexually development. When mosquito is infected, it is infected for life and continues to transmit infections, therefore the lifespan of mosquito also play a crucial role in disease transmission. Only female mosquitoes feed on blood as they require high protein diets in order to reproduce rafts of eggs. Even thoughmosquitoes breed in water, each of the *species* has its own breeding preference, for example some prefer shallow collections of fresh water, such as puddles, paddy fields, tire tracks, as well as irrigation water. The areas of distribution also differ, for instance, *gambiae* is the major vector in Africa that can breed in diverse habitats, *fluviatilis* and *minimus* are found in the foothill regions, whereas *philippinensis* and *culicifacies* are found in the plains, *stephensi* and *sundaicus* are found in the coastal regions. Species like *Anopheles stephensi* are highly adaptable and are found to be potent vectors of human malaria (CDC, 2012; WHO, 2013). Generally, mosquitoes are found worldwide except in the Antarctica. Environmental factors including climate conditions, such as temperature, humidity and rainfall pattern play a larger role in malaria transmission, since wet, tropical areas are conductive for mosquito breeding. In many places, transmission of malaria is seasonal, surging during and just after the rainy season (WHO, 2013). Besides that, the urbanization and land use might also bring changes to the ecosystem, which contribute to the changing pattern of malaria transmission (Gallup and Sachs, 2001; WHO, 2013).

Vector control measures, such as the use of insecticides and larva controls play a critical role in malarial elimination and eradication, however some of the mosquitoes may shift resistance to insecticide after prolong exposure over several generations, thus impairing the success in vector controls via insecticides (Alonso, *et al.*, 2011).

2.6.2 Blood transfusion

Induced malaria by blood transfusion was first reported in 1911 (Woolsey, 1991) The frequency of transfusion transmitted malaria showed values that vary from less than 0.2 in non-endemic countries to 50 or more cases per million in endemic countries and the species most frequently associated with transfusional transmission are *P. falciparum*, *P. malariae* and *P. vivax* (Wylie, 1993; Scuracchio *et al.*,2011). The transmission of malaria by blood transfusion is the fact that malaria parasites can survive in red blood cells at refrigerator temperatures (2-6^{o}C) until three weeks, with the estimated inoculum in transfusions from one to 10 parasites per donation (Sazama, 1991; Seed *et al.*,2005; Chattopadhyay *et al.*,2010). Whole blood and red blood cell units are the primary carriers of malaria transmitted by transfusion, although platelets and leukocytes may contain variable numbers of red cells and have, rarely, transmitted malaria (WHO, 2009).

2.6.3 Congenital transmission

According to Warrel, (1993), all four species of *Plasmodium*can produce congenital infection but *Plasmodium falciparum*and *Plasmodium vivax*are most common. Such infection results to low birth weight, abortion, still birth or death in new born babies. In endemic regions, the incidence of congenital malaria is usually extremely low or it may not result in any significant problem despite the high prevalence of placental infection. This is due to the fact that sufficient antibodies would have also crossed from maternal compartment to the foetal compartment to protect the new born babies in the early phase of life (Okonofua, 2001; Gülaş and Özdener, 2016). The incidence is much higher in infants born to non-immune mothers and increases during malaria epidemics. Outside the endemic areas, *Plasmodium*malaria causes a disproportionate number of cases because of its very long persistence.

Congenital malaria is defined as the presence of asexual malaria parasites in the erythrocytes of newborns aged less than seven days (Brabin, 1991; WHO, 2003; Gülaş and Özdener, 2016). It is also known as transplacental vertical transmission is the uterine transfer of malaria from mother to child in the womb. The transplacental transmission of *Plasmodium falciparum*from mother to fetus has long been well-described. Malaria density due to transplacental transmission is defined as umbilical cord blood parasitaemia (Ou´edraogo *et al.*,2012).The newborn child can manifest with fever, irritability, feeding problems, hepatosplenomegaly, anaemia, and jaundice. Although fever is a cardinal symptom of malaria, it may be absent in congenital malaria (Gülaş and Özdener, 2016).

The factors implicated in congenital malaria include: (i) pre-existing level of malaria immunity in thepregnant woman (Hviid, 2004; Bir *et al.*, 2006; Rogerson *et al.*, 2007); (ii) occurrence of severe malaria during pregnancy (Coll *et al.*, 2008; Lagerberg, 2008); (iii) absence of immunity especially pregnant women travelling to endemic areas (Coll *et al.*, 2008); (iv) placental malaria especially among primigravid and secundigravid women (Uneke, 2007; 2008); and (v) maternal human immunodeficiency virus (HIV) infection (Uneke, 2007; Perrault *et al.*,2009; Uneke, 2011).

2.6.4 Needle stick injury transmission

Needle stick injuries are most frequently caused while disposing or disassembling needles, which may contain blood and body fluids of another person. This is a rare means of contacting malaria because the occurance is very low compare to other means of transmission. In 2004, there was a report of a nurse from France that contacted malaria during her vacation in south of France from June 23 to June 26 and had been working as a substitute employee at various hospitals in the greater Paris area. In the course of the vacation, she sustained an accidental needlestick injury that caused a deep, blood-letting injury on the

anterior aspect of the left wrist while taking a blood sample with an 18-gauge, peripheral venous catheter that had no safety feature. She had no previous history of needlestick injury. She notified the hospital occupational medicine department of her injury on the day it occurred and was given a post exposure interview. In accordance with national postexposure management guidelines, she was tested for HIV and hepatitis C virus (HCV) antibody, and results were negative at baseline; her immunization against hepatitis B virus (HBV) was confirmed.

2.7 Clinical Manifestations

Clinical manifestations of malaria vary with geography, epidemiology, immunity, and age. Clinically malaria is classified into uncomplicated and complicated or severe malaria (AreÂvalo-Herrera *et al.*,2017).

Uncomplicated malaria is defined as symptomatic malaria without signs of vital organ dysfunction. Fever is the cardinal symptom of malaria. It can be intermittent with or without periodicity or continuous. Many cases have chills and rigours. The fever is often accompanied by headache, myalgia, arthralgia, anorexia, nausea and vomiting. The symptoms of malaria can be non-specific and mimic other diseases like viral infections and enteric fever (AreÂvalo-Herrera *et al.*,2017).

Complicated falciparum malaria or severe malaria is characterized organ involvemet and failure as evidenced by one or more of the following features: impaired consciousness /coma, repeated generalized convulsions, renal failure (Serum Creatinine >3 mg/dl; urine output <400ml/24hrs), jaundice (Serum Bilirubin >3 mg/dl), severe anaemia (Hb <5 g/dl), pulmonary oedema – cardiogenic and non-cardiogenic, hypoglycaemia (plasma glucose <40 mg/dl), metabolic acidosis (arterial pH <7.25 or plasma bicarbonate level of <12 mmol/l), circulatory collapse/shock (Systolic blood pressure (BP) <80 mm Hg, Mean Arterial Pressure

(MAP) <65 mmHg, core skin temperature difference of >10°C), abnormal bleeding and disseminated intravascular coagulation, haemoglobinuria, hyperthermia (Temperature >104º F) and hyperparasitaemia (>5% parasitized RBCs in low endemic and >10% in hyperendemic areas) (AreÂvalo-Herrera *et al.*,2017; Stephens *et al.*,2017).

2.8 Complications of malaria

Malaria present with several complications which vary from patients to another and the most common ones are discussed below:

2.8.1 Cerebral malaria

Cerebral malaria malaria is a medical emergency demanding urgent clinical assessment and treatmentwhere the cerebral capillaries and venules are engorge with parasitized and non-parasitized red blood (Oluwayemi, 2014).Altered consciousness, convulsions, ataxia, hemiparesis, other neurologic and psychiatric impairments should raise the possibility of cerebral malaria (Warrel, 1993; White, 2009; Bartoloni and Zammarchi, 2012; Yusuf *et al.*, 2017).

The diagnosis requires presence of neurologic symptoms and asexual forms the parasite on peripheral blood smears. It is important to exclude other causes of encephalopathy (e.g., hypoglycaemia, bacterial meningitis and viral encephalitis) and Glasgow coma scale score of 9 or less which depicts an already determined significant unconscious state due to any other cause (Idro *et al.*,2010). To distinguish cerebral malaria from transient postictal coma, unconsciousness should persist for at least 30 min after a convulsion (Bartoloni and Zammarchi, 2012).

All patients with *P. falciparum* malaria with neurologic basic underlying defect seems to clogg the cerebral microcirculation by the parasitized red cells as a result of increased cytoadherent properties due to which the parasites sequester in these deeper blood vessels.

Also, rosetting of both healthy and infected red cells further impairs healthy circulation. This results in hypoxia and brain ischemia as depicted by increased lactate and alanine concentrations along with decreased aspartate and adenosine triphosphate levels (Idro *et al.*,2010; Yusuf *et al.,* 2017).

2.8.2 Placental malaria

Some Pregnant women have evidence of peripheral or placental malaria at delivery (Steketee *et al.,* 1996; Uneke, 2011). It has been documented that malaria parasites may be detected in the placenta of women who are otherwise apparently uninfected; with a peripheral parasitaemia undetectable by microscopy (Beeson *et al.*,2002; De Beaudrap*et al.*,2013) and infection of the placenta by malaria parasites has been found to have no effect on the transmission of maternal antibodies to infants (Stephens *et al.*,2017).

Malaria during pregnancy is characterized by the sequestration of infected erythrocytes (IEs) in placental inter-villous spaces binding chondroitin sulphate A (CSA) resulting in placental malaria (Muthusamy *et al.*,2004). Placental malaria is associated with adverse pregnancy outcomes, such as miscarriage, stillbirth, preterm birth (PTB) (Tako *et al.*,2005; Stanisic *et al.*,2015), low birth weight (LBW) and congenital malaria (Omer *et al.*,2017).

Primigravidae are highly susceptible to malaria compared with multigravidae because multigravidae, who have developed anti-CSA from previous exposure, are less susceptible to PAM than the primigravidae who are experiencing the placenta for the first time (Steketee *et al.*,2000; Ricke *et al.*,2001; Omer *et al.*,2017).

Another reason for the high susceptibility of pregnant women is attributed to various hormonal and immunological changes during pregnancy, with a leaning from cell-mediated immunity to humoral immunity. Primigravidae have higher levels of cortisol than

multigravidae and therefore lose their acquired immunity more frequently and this increases their susceptibility to *P. falciparum* infection. These problems are more common in the first and second pregnancies since the parasitaemia level generally decreases with increasing numbers of pregnancies (Stephen *et al.*,2017).

2.8.4 Respiratory failure

Respiratory failure may rapidly develop at any stage of acute malaria but, in many cases, occurs when subjects are already recovering, parasitemia is cleared, and in comatose subjects, consciousness has been regained (WHO, 2010). Pregnant women are particularly prone to develop acute respiratory failure. The clinical picture is indistinguishable from the adult respiratory distress syndrome that develops as a result of a systemic or pulmonary infection, severe trauma orother systemic or pulmonary insults. Tachypnea, dispnea, and scattered rales and ronchi on auscultation are the earliest warnings. Patients with severe hypoxemia may require mechanical ventilation (Bartoloni and Zammarchi, 2012).

2.8.5 Acute renal failure

Renal dysfunction is frequently observed in otherwise uncomplicated malaria characterized by little change in renal parenchyma, there may be minimal tubular degeneration, mild renal parenchymal change and presence of vacuoles while acute renal failure is another complication of falciparum malaria where tubular degeneration with distal tubular necrosis, proximal tubules are often loaded with malaria pigments, and haemoglobin granules may be seen in the tubular cells mostly common in adults than among children which could be an isolated complication or as a component of maltiorgan failure Malaria (Bartoloni and Zammarchi, 2012).

Acute renal failure may be defined as a urine output of <400 mL in 24 hours in adults, or 12 mL/kg in 24 hours in children, in spite of rehydration, and a serum creatinine of more than

265 μmol/l (>3.0 mg/dL). In practice for initial assessment, the serum creatinine alone is used (Lee *et al.*,2009). Acute renal failure may be present at the subject admission in a context of a multiple organ involvement, or occurring in the recovery phase of severe malaria. In the former case prognosis is worse. Oliguria is the main feature of acute renal failure, although urine output may also be normal or increased. In oliguric patients, levels of blood urea nitrogen and serum creatinine are elevated and hyperkalaemia, hyperphosphatemia, hypocaelcemia, and metabolic acidosis usually develop. Dialysis may be necessary in some cases (Bartoloni and Zammarchi, 2012).

According to Ani (2015), malarial renal impairment/failure is mediated through several mechanisms such as:

- Effect of parasitized red blood cells on microcirculation. Knob-like processes are formed on surfaces of parasitized red blood cells which help them anchor on the endothelium.
- Cytoadherence occurs due to thrombospondin formation from vascular endothelium. This is specific of *Plasnodium falciparum*.
- Loss of deformability of parasitized red blood cells occurs according to need of microcirculation. This leads to sluggish circulation and resultant renal ischemia.
- Hypovolumia may occur due to fever, sweating, decreased intake of fluid and vomiting.
- There may be release of chemical mediators such as Tumor necrosis factors-α, cachectin, cytokines, interleukins etc. This causes vasoconstriction, increased vascular permeability, catecholamine release, shock and tubular necrosis.
- Plasma viscosity is increased due to infection.
- Hyperbilirubinaemia occurs due to hemolysis. Black water fever in G6PD deficiency patients is also associated with renal impairment in malaria.

2.8.6 Jaundice and hepatic dysfunction

Jaundice is common in adult with severe malaria, while relatively rare in children. It is generally mild or moderate, but in some cases may be marked. Hyperbilirubinemia may be predominantly unconjugated, in case of extensive haemolysis of both parasitized and unparasitized erythrocytes, or conjugated, indicating hepatocytic disfunction among the jaundiced subjects. Apart from jaundice, signs of severe liver cell damage are uncommon. Concentrations of transaminases may be increased up to ten fold, but never to the level observed in viral hepatitis. The liver, as well as the spleen, is enlarged and tender, especially in young children and non immune adults (Bartoloni and Zammarchi, 2012).

2.8.7 Severe anaemia

Severe malaria is associated with development of anaemia, usually normochromic and normocytic. However, in endemic areas the morphology may be influenced by the nutritional status of the subject and some helminthiasis, resulting in an associated microcytic (iron deficiency) and macrocytic (folic acid deficiency) component. The causative mechanisms are multifactorial, including haemolysis of infected and uninfected red blood cells, inappropriate bone marrow response, and numerous other individual factors (e.g. bacteraemia, HIV, hookworm, G6PD deficiency, vitamin A and vitamin B12 deficiency) (Calis *et al.*,2008). In areas of high endemicity, severe anaemia is a particular problem in children, where it can be fatal (Bartoloni and Zammarchi, 2012).

In epidemic rather than endemic areas, severe anaemia may also develop in adult population and pregnant women, especially primipagravidae, are those at major risk. In accordance with the WHO definition, severe malarial anaemia is defined in the presence of a haemoglobin level of<5g/dL, or haematocrit <15% (WHO, 2010). Pathological consequences of anaemia are particularly likely with such degree of anaemia; however they are also importantly related

to the rapidity at which it develops. In this perspective, decisions regarding blood transfusion should be taken at individual level (Bartoloni and Zammarchi, 2012).

2.8.8 Hypoglycaemia

Hypoglycaemia has been detected in approximately 8% of adults and up to 30% of children with cerebral malaria (White, 2009). Hypoglycaemia is a particular complication in pregnancy. It may be present before starting antimalarial treatment, representing a sign of poor prognosis. Hypoglycaemia can also result from rapid infusions or prolonged treatment with quinine, due to its capability to stimulate insulin secretion (Bartoloni and Zammarchi, 2012).

Early diagnosis is essential in order to assure a prompt treatment that may be lifesaving, but symptoms may be easily overlooked. In fact, classical symptoms of Hypoglycaemia, such as those induced by adrenaline secretion (sweating, tachycardia, breathlessness, tremor, anxiety and hunger) or those due to dysfunction of central nervous system (impairment of consciousness, seizures, extensor posturing), are difficult to distinguish from those caused by malaria. Any subject with malaria and altered behavior or consciousness, or seizure, should be evaluated immediately for blood glucose level and, if this cannot be possible, assumed as Hypoglycaemia and given glucose (Bartoloni and Zammarchi, 2012).

2.8.9 Blackwater fever.

Blackwater fever is a syndrome presenting with passage of mahogany-coloured (or 'Coca-Cola'-coloured) urine in association with severe intravascular haemolysis. The dark urine is due to haemoglobinuria. Blackwater fever occurs in subjects with Glucose-6-Phosphate Dehydrogenase (G-6PD) deficiency who takes antioxidant drugs or foodstuffs, in subjects with G-6PD deficiency who have acute malaria after the administration of quinine or artemisinin derivative, but also in subject with acute, often severe malaria, who have normal

red cell G-6PD concentrations. Blackwater fever has usually a good prognosis resolving without complications. Only a minority of subjects may develop acute renal failure as a consequence of acute tubular necrosis. (Severe falciparum Malaria, 2000)

2.8.10 Non-falciparum malaria complications

Although *P. falciparum* malaria is the major cause of severe malariaand death, increasing evidence has recently emergedthat *P. vivax* can also be severe and even fatal (Kochar *et al.*,2005; Arsteny *et al.*,2007; Genton *et al.*,2008) Besides the rare fatal haemorrhage due to the traumatic or spontaneous rupture of an enlarged spleen, other complications of *P. vivax* infection have been reported, including complications of seizure, shock, jaundice, renal failure, severe anaemia (Baird, 2007; Kochar *et al.*,2009), coma (Genton *et al.*,2008; Tjitra *et al.*,2008) and acute respiratory distress syndrome (Saleri, 2006). Studies conducted in Indonesia and Papua New Guinea showed rates of severe malaria in *P. vivax* infections as high or higher than those observed in *P. falciparum* malaria (Genton *et al.*,2008; Tjitra *et al.*,2008). *P. ovale* malaria is rarely severe.

However, respiratory distress has also been reported as a rare complication of ovale malaria (Rojo-Marcos *et al.*,2008). As far as *P.knowlesi* malaria is concerned, most cases are uncomplicated but complications are possible, and in a study conducted in Malaysia, approximately 1 in 10 patients developed potentially fatal complications, with a case fatality rate of 1.8%. The most frequent complication was respiratory distress, but also multiorgan failure, hypoglycaemia, and lactic acidosis were observed (Daneshvar *et al.*,2009; Bartoloni and Zammarchi, 2012).

2.9 Specific Population at Increased Risk of Developing Severe Malaria.

Non immune pregnant in second and third trimester are at increased risk of developing life threatening malaria and are particularly susceptible to develop pulmonary oedema and

hypoglycaemia. Foetal death and premature labour are common (Boel *et al.*,2012; Jimenez*et al.*,2012).

People with immunosuppression related to HIV show animpaired immune control of malaria. There is an increasing risk of illness, increased parasitemia and severe malaria. Therapeutic responses to antimalarial treatment are impaired so treatment failure rates are increased. Drug interactions between antiretrovirals and antimalarials have not yet been studied adequately. Trimethoprim-sulphamethoxazole prescribed as prophylaxis for opportunistic infection gives some protection also for malaria. Co-infected pregnant women are at risk for higher parasite density, anaemia and malarial infection of the placenta (Bartoloni and Zammarchi, 2012). Children born to women with HIV and malaria infection have low birth weight and are more likely to die during infancy (Abu-Raddad *et al.*,2006; Hewitt *et al.*,2006).

Malaria in transplant recipients can be caused by graft-born or blood born infection or reactivation of previous infection due to immunosuppression (Salutari *et al.*,1996; Menichetti *et al.*,2006; Shamsi *et al.*,2008). The clinical picture of malaria in transplant recipients is usually severe, owing to the impaired immune response (Barsoum, 2004; Bartoloni and Zammarchi, 2012). It is characterized by pyrexia, which may lack the typical periodicity or rigors. Anaemia is severe, being typically haemolytic and occasionally haemophagocytic Abdelkefi *et al.*,2004). Acute graft dysfunction may occur as a consequence of the haemodynamic consequences of falciparum infection (Barsoum, 2000; Bartoloni and Zammarchi, 2012).

Presence of the sickle cell trait confers some protection against malaria; however, for those with homozygous sickle-cell disease, malaria is regarded as a significant cause of morbidity and mortality, producing further haemolysis against the background of that due to sickle-cell disease itself (Serjeart, 2004)

Subjects who have no spleen or whose splenic function is severely impaired are at particular risk of severe malaria, and malarial parasitaemia in asplenic individuals may rise rapidly to very high levels (Chiodini *et al.*,2007). Post-splenectomy episode of *P. falciparum* malaria has been reported in immigrants (Bidegain *et al.*,2005). Other groups at increased risk for developing severe malaria are malnourished children, elderly and those with comorbidities (WHO, 2011; Muhlberger *et al.*,2013)

2.10 Laboratory Diagnosis of Malaria

Current diagnostic tools, parasite detection by microscopic examination of blood smears, antigen-based rapid diagnostic tests (RDTs), and sensitive DNA-based assays require blood sampling by finger-prick and their implementation has been limited by either their labour /time intensive nature and requirement for specialized training and skills (microscopic method), moderate sensitivity (RDTs, microscopy), or high cost of sample preparation and supporting infrastructure needed (DNA based methods).

2.10.1 Microscopy

The corner stone of the parasitological diagnosis of malaria is microscopy which has the advantage of assessing the various stages of the infection and quantifying parasitaemia. It is a cheap technique which requires high level of technical expertise.

2.10.1.1 Giemsa stain microscopy

Simple light microscopic examination of Giemsa stained blood films is the most widely practised and useful method for definitive malaria diagnosis. When used by skilled and careful technicians, microscopy can detect densities as low as 5–10 parasites per μl of blood.

Under general field conditions, the detection capabilities of a typical microscopist can be more realistically placed at 100 parasites per μl of blood (Jonkman *et al.,* 1995).

Observed parasites are characterized in terms of their species (*P. falciparum, P. vivax, P. ovale,* and/or *P. malariae*) and of the circulating stage (e.g. trophozoites, schizonts, gametocytes). Also, the parasite densities can be quantified (from ratio of parasites per number of leukocytes or erythrocytes). Such quantifications are needed to demonstrate hyperparasitaemia (which may be associated with severe malaria) or to assess parasitological response to chemotherapy and ability to distinguish clinically important asexual parasite stages from gametocytes which may persist without causing symptoms. These advantages can be critical for proper case-management and evaluating parasitological response to treatment. Specific disadvantages are that slide collection, staining, and reading can be time-consuming and Microscopists need to be trained and supervised to ensure consistent reliability (Jonkman *et al.,* 1995; Amalu *et al.*,2013).

2.10.1.2 Fluorescence microscopy

Kawamoto acridine orange interference filter technique:This system, designed by Dr. F Kawamoto, requires an interference filter specially designed for the fluorochrome acridine orange and located in the sub stage filter holder of an ordinary transmitted light microscope, a barrier filter (inserted in the eyepiece) and a 150 W or 200 W halogen lamps. The nuclei of malaria parasites and white cells fluorescent bright green and the cytoplasm of the parasites fluoresces red (particularly the cytoplasm of gametocytes). Compared with the Quantitative Buffy Coat (QBC) system, the Kawamoto fluorescence system is inexpensive. Most field evaluation of the system have found it to be as sensitive, but more rapid than Giemsa stained thick films in detecting *P. falciparum* parasites. Blood films must be prepared and stained correctly (Amalu *et al.*,2013).

Useful in survey work for screening blood donors and reduces wastage. The acridine orange stained films can be washed with methanol and restained with Giemsa (Amalu *et al.*,2013).

Although more rapid than examining thick stained blood films, Considerable skill and experience are required to process (Amalu *et al.*,2013).

2.11 *Iv-vitro* Culture of Malaria

Culture is not a routine identification technique for most of the parasitic infections. However, culture is useful for clinching the diagnosis in some protozoan parasitic infections, e.g. in case of Central Nervous System infections by free living amoebae; and also culture has immense role in research related to pathogenic parasites, differentiating clinical isolates and for demonstrating the characteristics of pathogenic parasites to medical students to facilitate understanding the morphology and physiology; while keeping a stock of cultured parasites is of enormous worth (Ahmed, 2014).

*In vitro*cultivation of *Plasmodium* is a conducted in outside the normal biological context (outside the human erythrocytes) i.e. it is a screening based on the ability of *Plasmodium falciparum*to survive in glasswares. *In vitro* cultivation of malaria parasites requires simulation of the sexual and asexual conditions with the specific characteristics and properties of each of them. Early studies developed culture conditions, which permitted the combination of *in vivo* and *in vitro* experiments with the same parasites, and methods for in vitro culture of gametocytes were rapidly described (Jambou *et al.*,2011).

Researches on *in vitro* cultivation of plasmodia pushed the door wide open for research into the malaria parasite such as the growth of plasmodia under a completely cell-free condition is more important for research(Schuster, 2002). Toward this end, successful development of *Plasmodium* species from merozoites to ring stage under cell-free conditions has been demonstrated (Trager and Lanners, 1984).The studies done have suggested that factors present in the erythrocyte but not necessarily the intracytoplasmic location of the parasite were needed for development (Trager and Lanners, 1984; Trager *et al.,* 1990). These

extracellular forms react to the same monoclonal antibodies that the intracellular forms respond to, suggesting a similar pattern of molecular differentiation (Zung *et al.,* 1991; Schuster, 2002). Further refinement of technique is the use of a system with merozoites embedded in a Matrigel substrate in a well containing a fluid medium overlay, (Trager and Williams, 1992; Trager *et al.,* 1992) which could be changed with minimal disturbance to the parasites developing within the Matrigel layer. Ring-stage parasites, gametocytes and shizonts; all have been demonstrated by different workers in this system (Trager and Williams, 1992; Williams *et al.,* 1995; Ahmed, 2014).

In vitro development of *Plasmodium* ookinetes was achieved later on, followed by a complete *in vitro* development of mosquito phases. Trager and Jensen (1976) first published the continuous culture of *Plasmodium falciparumin vitro*, another group, led by J. David Haynes, also demonstrated the same findings, which were published the same year in the journal *Nature* in October (Haynes 1976). Haynes demonstrated that in addition to human red cells, chimpanzee red cells also supported *P. falciparum* growth but red cells from rhesus monkeys and guinea pigs did not (Haynes 1976; Chauhan*et al.*,2017).

Significantly increases culture growth, prolongs culture synchrony, and reduces rate of multiple infections (Sherman, 2010). Despite the advances, the practice of culturing the parasite under static conditions inside a candle-jar remains widespread, as this can be used in laboratories almost anywhere in the world where there is an incubator, a candle and desiccator (Allen and Kirk, 2010). Differences exist between strains of *Plasmodium* (Schuster, 2002) and isolates also undergo change once in culture, perhaps due to selection. In *P. falciparum*, for example, gametocyte formation is typical of recently cultured strains but is lost with prolonged cultivation. In this regard, it is important to note that cryopreservation of isolates can maintain those characteristics in a strain that may be lost on prolonged cultivation (Ponnudurai, 1987; Ahmed, 2014)

According to Chauhan*et al.*(2017), the enormous impact of continuous *P. falciparum* culture cultivation on malaria research across the world cannot be gauged and probably there was no other single development has had such a profound and unparalleled influence on malaria research as observed with the laboratory culture of malaria parasites. It completely revolutionized malaria research and enabled studies on different aspects of malaria that are being pursued with great vigor even today in fields include biochemistry and molecular and cellular biology of the parasite; host–parasite interactions especially, with human red cells; immunology and vaccine development; drug development and resistance; pathogenesis; gametocytogenesis and mosquito transmission; and genetics of the parasite and red cell susceptibility. Basically, malaria parasite culture brought accessibility to scientists who otherwise did not have access to clinical malaria cohorts and thus allowed an astounding expansion in malaria research. Overall, the successful *in vitro* culture of *P. falciparum* remains the basis of present-day malaria research (Chauhan*et al.*,2017).

Many manuscripts cite this reference as the basis of their culturing protocol employ variations in media components, % haematocrit and parasitaemia, plus incubation gas mixtures. The consequences of variations in continuous culturing parameters of *Plasmodium falciparumin vitro* is an underrepresented area of malaria research, especially with respect to parasite tolerance and sensitivity to anti-malarial drugs and compounds (Jambou *et al.*,2011; Duffy and Avery, 2017).

In-vitro studies permit a simpler, precise and efficient ways of culturing *Plasmodium* without extrapolation from an experimental animal's cellular response culture (Vignais *et al.,* 2010), it is miniaturized and automated, yielding high-throughput screening methods for testing molecules in pharmacology or toxicology (*Quignot* et al., 2014) making it very rapid and convienent for resarch purposes. Large number of compounds can be evaluated at the same

time; Synergism or antagonism with drug combinations can be studied which also gives a better assessment of intrinsic activity of a drug (Kaira *et al.,* 2005).

Several strains with the same genetic background (Saul *et al.*,1997) have been derived from the original isolates with different sensitivity to drugs. They are used for drug testing in maturation assays, molecular studies or evaluation of vaccine efficacy. Selection of mouse strains with various degrees of sensitivity for the parasite paved the way for analysis of the pathophysiology of cerebral malaria (Grau *et al.*,1986) and of severe anaemia (Evans *et al.*,2006).

The major obstacle researchers faced when they were researching on malaria parasites i.e. focusing on describing the parasite, identifying it as the causative agent of malaria, understanding its mode of transmission, and uncovering the different stages of its complex life cycle after Ross's landmark discovery Century ago was the difficulty in culturing the parasite *in vitro* in the laboratory, due to which primary experiments were performed with primate models such as *Aotus* monkeys and even human volunteers. Initial attempts to culture *Plasmodium* parasites failed because the cultures were always short term, in which the parasite numbers were constantly decreasing till they completely died (Chauhan *et al.,* 2017).

In-vitro cultivation of parasite may also challenging in extrapolating from the results of *in vitro* work back to the biology of the intact organism hence drugs acting through active metabolite cannot be studied (Kaira *et al.,* 2005). Investigators doing *in vitro* work must be careful to avoid over-interpretation of their results, which can lead to erroneous conclusions about organismal and systems biology (Rothman, 2002);There is usually difficulty in reproducibility of pharmacokinetic effects; Toxic compounds also get selected; expertise and infrastructure needed and there is lack of clinical correlation (Kaira *et al.,* 2005).

2.12 Treatment of Malaria

The effectiveness of early diagnosis and prompt treatment to control malaria is highly dependent on the efficacy, safety, availability, affordability and acceptability of antimalarial drugs to prevent death or long-term deficits, recrudescence, transmission, emergence of resistance and disabilities (Saifi *et al.,* 2013).

The different stage of the malarial life cycle exhibits unique biological, biochemical and other characteristics (i.e., it expresses different proteins or progress in different sites within the body). These stages serve as target for the action of antimalarial drugs (Saifi *et al.,* 2013).

Currenly, available antimalarials fall into three categories according to their chemical structure and mode of action:

2.12.1 Aryl aminoalcohol compounds or quinoline based drugs

Quinoline based drugs includes quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine and tafenoquine. Quinoline and its derivative are blood schizontocides that contains a quinoline ring moiety with various aminoalkyl side chains. They possess activity against the erythrocytic stage of the malaria parasite. One of the members of this group is used to treat and prevent relapse of malaria due to its highly effective against the gametocytes of all the *Plasmodium*species as well as the hypnozoites of *P. vivax* and *P. ovale* (Saifi *et al.,* 2013).

Quinine and quinidine are stereoisomers and alkaloids derived from the bark of the cinchona tree. Both quinine and quinidine can be used to treat severe malaria *via* parenteral admission, but quinidine has cardio specific toxicity (Ashley *et al,* 2006). Chloroquine is a 4-aminoquinoline derivative of quinine obtained from cinchoma tree and first synthesized by two French pharmacists, P.J. Pelletier and J.B. Caventou (Kumar *et al.,* 2009). It has been the most widely used antimalarial drug because it is protonated (to CQ2+) making the digestive

vacuole is acidic (pH 4.7) and subsequently cannot leave the vacuole by diffusion. Chloroquine caps hemozoin molecules and prevents the further biocrystallization of heme, thus leading to heme buildup. Chloroquine binds to heme (or ferriprotoporphyrin IX (FP) a waste products of the parasite's haemoglobin diet) to form what is known as the FP-chloroquine complex; this complex is highly toxic to the cell and disrupts membrane function. The actions of the toxic FP-chloroquine complex and FP result in cell lysis and ultimately the auto-digestion of the parasite cell. In essence, the parasite cell drowns in its own metabolic products (kaur *et al.,* 2010; Saifi *et al.,* 2013).

Mefloquine (a quinolinemethanol derivative of quinine) is a drug with a long elimination half-life and is therefore an excellent prophylactic drug amongst non-immune travellers because of the once-a-week dosage (Shapiro & Goldberg, 2006; CDC, 2010). Due to neuropsychiatric side effects such as seizures, acute psychosis and nightmares, it is contraindicated in patients with epilepsy, a history of neuropsychiatric disease and patients recovering from cerebral malaria. A link between mefloquine use during pregnancy and stillbirths has been reported, but not yet confirmed (Winstanley *et al.*, 2004).

Primaquine, an 8-aminoquinoline, is a tissue schizontocide with intrahepatic activity and the ability to kill hypnozoites of *P. vivax* and *P. ovale* and thus prevent malaria relapse. In combination with a blood schizontocide, a radical malaria cure can be achieved. Primaquine is contra-indicated in patients with G6PD deficiency and may cause haemolytic anaemia (WHO, 2001; Vale *et al.,* 2009).

2.12.1.2 Mechanism of drug resistance against the quinoline derivatives

The ability of resistant strains of *P. falciparum* to prevent the accumulation of chloroquine in their digestive food vacuoles helped with the formation of hypotheses on how quinoline resistance is acquired.

The most supported hypothesis is the efflux of chloroquine out of the digestive food vacuole *via* a transporter protein. Mutations in the *pfcrt* gene produced strains of *P. falciparum* with a transporter protein in the membrane of their food vacuole called the *Plasmodium falciparum* chloroquine resistance transporter (PfQRT). It is believed to prevent quinoline accumulation and thus the formation of chloroquine-FP bindings (Carlton *et al.,* 2001).

2.12.2. Antifolate combination drugs

These drugs are various combinations of dihydrofolate- reductase inhibitors (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) a type 1 antifolates and sulfa drugs (dapsone, sulfalene, sulfamethoxazole, sulfadoxine,) a type 2 antifolates (Watkins, *et al.,* 1997; Saifi *et al.*, 2013). The type 1 inhibit dihydrofolate reductase (DHFR, a bifunctional enzyme in plasmodia coupled with thymidylate synthase (TS)), thus preventing the NADPH-dependent reduction of H2folate (DHF) to H4folate (THF) by DHFR.while type 2 prevent the formation of dihydropteroate from hydroxymethyldihydropterin catalyzed by dihydropteroate synthase (DHPS) by competing for the active site of DHPS (a bifunctional enzyme in plasmodia coupled with 2-amino-4-hydroxy-6- hydroxymethyl-dihydropteridine pyrophosphokinase (PPPK). The antifolate drugs inhibit either dihydrofolate reductase (DHFR) (pyrimethamine, cycloguanil) or dihydropteroate synthase (DHPS) (sulfadoxine). These are two key enzymes in *de novo* folate biosynthesis; inhibition of this metabolic pathway leads to the inhibition of the biosynthesis of pyrimidines, purines, and some amino acids. Antifolate antimalarial drugs interfere with folate metabolism, a pathway essential to malaria parasite survival (Saifi *et al.*, 2013).

Although these drugs have antimalarial activity when used alone, parasitological resistance can develop rapidly. When used in combination, they produce a synergistic effect on the parasite and can be effective even in the presence\ of resistance to the individual components.

Typical combinations include sulfadoxine/ pyrimethamine (SP or Fansidar1), sulfalenepyrimethamine (metakelfin), and sulfamethoxazole- trimethoprim (co-trimoxazole). However, resistance to antifolate drugs according to Saifi *et al.* (2013) is caused by point mutations in DHFR and DHPS, the two key enzymes in the folate biosynthetic pathway which are targeted by antifolates (Saifi *et al.,* 2013). Resistance to these drugs arises relatively rapidly in response to drug pressure and is now common worldwide (Saifi *et al.,* 2013).

2.12.3. Artemisinin compounds

Artemisinin is got from medicinal herb Artemisia annua (Whirl-Carrillo, 2012; Saifi *et al.,* 2013). Structurally, it is a sesquiterpene lactone containing an internal peroxide bridge which makes it different from other available drugs (Whirl-Carrillo et al., 2012). Semi synthetic derivatives of artemisinin include: artesunate, artemether, artenimol, artelinic acid and arteether (Whirl-Carrillo *et al.,* 2012). The parent drug of artemisinin is poorly soluble in water or oil, the carbonyl group of artemisinin was reduced to obtain DHA and its derivatives such as the water-soluble artesunate and oil- soluble artemether and arteether, which also show greater antimalarial activity.

Artemisinin also has been shown to bind to six (6) specific *P. falciparum* proteins, one of which is a member of the translationally controlled tumor protein family but the precise effect of this protein alkylation on the parasite is still to be determined (Saifi *et al.,* 2013).

2.12.3.1 Mechanism of action

The artemisinin free radical when endoperoxide bond in artemisinin structure reacts with Iron or heme or other parasite proteins to form heme-artemisinin covalently, is able to inhibit the production of hemozoin (Asawamahasakda *et al.,* 1994;Aderibigbe, 2017). Artemisinin half-live ranges between 2 to 5 hours, whereas the half-lives or artemether and artesunate range

between 2 to 4 hours and less than 1 hour, respectively (Whirl-Carrillo *et al.*,2012). The metabolism of artemisinin is primarily mediated by the genes CYP2B6 and CYP3A4. Artesunate is metabolised by CYP2A6 to dihydroartemisinin, an active metabolite which is responsible for its antimalarial activity. Artemether is also rapidly metabolised to dihydroartemisinin by CYP3A4 and CYP3A5 (Whirl-Carrillo *et al.*,2012). Arteether is metabolised by CYP3A4 to dihydroartemisinin. The active metabolite, dihydroartemisinin is converted to inactive metabolites by glucuronidation.

Dihydroartemisinin is eliminated in the bile as minor glucuronides (Whirl-Carrillo *et al.*,2012) The presence of the endoperoxide moiety is also responsible for the anticancer activity of artemisinin and its derivatives (Olliaro *et al.*,2001; Das, 2015). The absence of the endoperoxide moiety reduces significantly their cytotoxic effects. Iron and heme metabolism is important in the anticancer activity of artemisinin because itenhances its cytotoxicity (Lu *et al.*,2010; Lu *et al.*, 2011; Mercer *et al.*,2011; Das, 2015). It has been postulated that iron-activated artemisinin produces alkylating carbon-centered radicals and radical oxygen species that results in DNA damage, enhanced apoptosis, growth arrest and reduced angiogenesis (Das, 2015)

Artemisinin derivatives such as artesunate have been reported to exhibit antiviral activity. It hinders in vitro replication of human cytomegalovirus (HCMV) and β-herpes virus that are responsible for lifelong infections in humans. The mode of action was postulated that it inhibit central regulatory processes of HCMV-infected cells thereby interfering with host-cell type and metabolism requirements for HCMV replication (Qian *et al.*,1982;Kaptein*et al.*,2006; Blazquez *et al.*,2013).

2.12.3.2 Therapeutic applications of artemisinin and its derivatives

The therapeutic applications of artemisinin and its derivatives is wide and huge. These are treatment of severe malaria and have shown very rapid parasite clearance times, it is a faster fever resolution than occurs with quinine in various parts of the world. More importantly, they have proved effective against strains resistant to conventional antimalarials such as chloroquine and mefloquine (Winstanley, 2000).

Other applications of this drug and its derivatives used to reduce angiogenesis; they exhibit antitumor and anticancer activities (Blazquez *et al.*,2013); they are used for the treatment of joint pain; epilepsy; liver problems; menstrual pain and loss of appetite (Anonymous, 2016). They also exhibit anti-inflammatory activity, sedative activity, treat insomnia and gastrointestinal disease, exhibit antibacterial activity and are useful for the treatment of ulcers, skin diseases and wounds (Anonymous, 2016). They build-up the healthy cells, prevent infections, destroy existing infections and inhibit viral infections (Efferth *et al.*,2008; Anonymous, 2016). They are effective for the treatment of malaria and protozoan infections (Mutabingwa, 2005; Loo *et al.*,2016). Despite the effectiveness of artemisinin in the treatment of several diseases, it is limited in the treatment of malaria by its cost when compared to other antimalarials (Okell *et al.*,2014), poor bioavailability and solubility, short half-life, toxicity and drug resistance (Medhi*et al.*,2009; Aderibigbe, 2017). Due to the aforementioned pharmacological limitations, several researchers are designing derivatives with enhanced therapeutic effects and some researchers have developed delivery systems in which artemisinin and its derivatives are incorporated to produce improved therapeutic effects (Aderibigbe, 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Zaria is a major city in Kaduna State in Northern Nigeria, as well as being a Local Government Area. The 2006 population Census was estimated at 408,198 (2006). The town is home to Zazzau Emirate.

Zaria municipality comprises of four local government areas namely Kudan L.G.A., Giwa L.G.A., Sabon Gari L.G.A., and Zaria L.G.A. Two (2) Federal Government hospitals, the four State owned referral hospitals are the major healthin Zaria. The hospitals include: Hajiya Gambo Sawaba General Hospital, Zaria, Giwa General Hospital, Giwa, Kudan General Hospital, Kudan and Major Ibrahim B. Abdullahi (MIBA) General Hospital, Sabon Gari. Zaria has twenty community health facilities (Fig. 1).

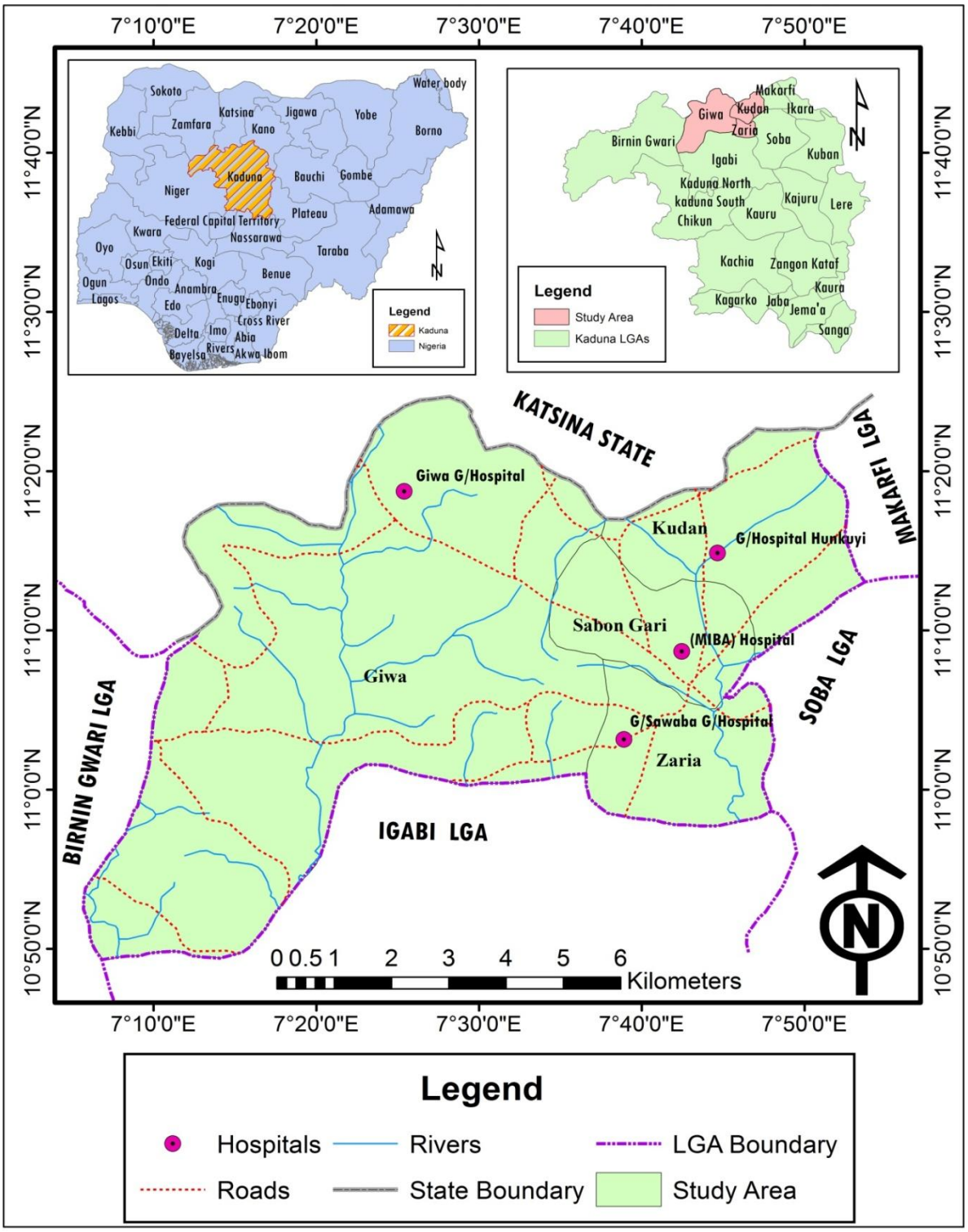


Figure: 3.1: Distribuion of Hospitals in the study Area.

Source: Modified From Administrative Map of Kaduna State.

3.2 Study Population

This study was conducted among antenatal patientsattending some hospitals in Zaria. These hospitals include: Hajiya Gambo Sawaba General Hospital, Zaria, Giwa General Hospital, Giwa, Kudan General Hospital, Kudan and Major Ibrahim Bello Abdullahi (MIBA) General Hospital, Sabon Gari.

3.3 Study Design

This study was cross-sectional and covered some selected hospitals in Zaria. Blood samples were collected intravenously from antenal patients that gave consent and transported to the Department of Microbiology, Ahmadu Bello University, Zaria where the samples were screened for Malaria.

3.4 Ethical Approval

Ethical clearance was obtained from Ministry of Health, Kaduna state and the ethicalcommittee of the hospitals. A copy is attached in Apendix XII page 113. The consent of the structured questionnaire was sorted.

3.6 Inclusion Criteria

The study population was antenatal patients that gave consent were recruited.

3.7 Exclusion Criteria

These were all women who didanttended antenatal clinics and those that give consent but later decline.

3.8 Sample Size

A prevalence of 19.2% as reported in a study conducted at Benin City, Southern Nigeria (Wogu and Obasohan,2014) was used to determine the sample size using the equation below (Niang, *et al*.2006).

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

Where Z= 1.96 at 95%
p= Prevalence rate
q= 1-p
d= Sampling error that can be tolerated (0.05)

Therefore:

$$n = \frac{(1.96)^2\ 0.192(1-0.192)}{0.05^2}$$

$$= \frac{3.841 \times 0.192\ (0.808)}{0.0025}$$

$$= \frac{0.5959}{0.0025}$$

$$= 238.36$$

$\approx$ 270 blood samples were used for the research.

3.9 Sample Collection

Four (4) milliliers of blood samples were collected from 270 women attending antenatal sessions in selected hospitals under study using convenient sampling techniques during clinics respectively with the help of the health facilities-based staff. The blood samples were collected in sterile Ethylene Diamine Tetraacetic Acid (EDTA) bottles and transported to the laboratory.

3.10 Preparation of Thin film

Thin blood film was prepare using about 1.5 µl of blood was transferred to the slide, The edge of a second slide (or a cover slip) was then placed on the drop of blood to spread the blood on the entire line of contact between the two slides and raised at an angle of 45° angle with the original slide and then moved to the opposite end of the slide to which the drop was originally located(CDC, 2013). Thin films were made in duplicate, air dried, lebelled and fixed using absolute methanol.

3.13 Giemsa staining of smears for malaria parasites

The prepared thin filmswere stained with 10% Giemsa for 30 minutes in the staining trough. The slides were removed,rinsedwith a running tap and allowed to dry on a wooden rack(Ugah *et al.*, 2017).

3.14 Microscopic Observation of Giemsa Stained Slides

Slides were observed under the oil immersion objective i.e. X100 magnification of a bright field microscope for *Plasmodium*species.

3.15 Detection of *Plasmodium* using Acridine Orange Fluorescence Microsopy

Prepared thin blood films were stained, observed and recorded as stated below:

3.15.1 Staining procedure

Thin films of the blood sample were prepared on greaseless slides and allowed to air dry. The slides were fixed with absolute methanol, and thenflooded with acridine orange stain for two minutes. The slides wererinsed and allowed to air dry (Singh *et al.*,2015). Stained slide was stored in the dark to prevent loss of fluorescence (Murray *et al.*, 2003).

3.15.2 Observation of *Plasmodium*specieson stained slide using fluorescence microscope

Prior to examination, the back of each slide was cleaned with cotton wool moistened with ethanol to remove excess acridine orange and to prevent interference with the dark background condenser. The stained slides were examined under a fluorescent microscope (T6200 MIJEI) at 40x objective (Murray *et al.*,2003; Singh *et al.*,2015).

3.16 Quantification of parasitaemia

Average count of Infected and non-infected erythrocytes under a microscopic field were recorded. Parasitaemia was determined by dividing parasitized erythrocytes present in 3 microscopic fields by the total number of erythrocytes present in those fields, and multiplied

by one hundred. The result indicates the percentage of erythrocytes that are infected by malaria parasites(Ugah *et al.,* 2017).

3.17 Preparation of *Jatropha curcas* leaves extract medium

*Jatropha curcas*was obtained from Hayin Danyaro, Samaru-Zaria. It was authenticated in Department of Botany, Ahmadu Bello University and given a voucher number022873.

The *Jatropha curcas* leaves extractmediumwas constituted, by adding 10ml of *Jatropha curcas*leaves extractextract, 1g of glucose, 1ml of PBS (pH 7.2), 12mls of liver extract and 20µl of(40mg/ml) gentamycin.

3.18 In-vitro cultivation of Plasmodium

A total of 200µl of Rosewell Park Memorial Institute (RPMI-1640) medium and *Jatropha*leaves extractconstituted medium respectively were dispensed aseptically into six macro-titre plates each. Gentamycin (20µl)was added to both RPMI-1640 and *Jatropha*leaves extract medium to mask the challenge in maintaining sterility during cultivation of the parasite.

Infected bloodwith *Plasmodium falciparum* (200µl) was inoculated directly into the wells containing RPMI-1640 and *Jatropha*leaves extract media respectively.The macro-titre plates were inocubated at 37^{o}C and samples (5µl) were taken at interval of six (6) hours for two days to prepare Giemsa-stain thin smear during culturing andreadings were taken at an interval of six hours for two days.

3.19 Determination of the antiplasmodia acivity of selected animalatial drugs

The drugs that were assessed for their *in vitro*antiplasmodialactivities against *Plasmodium falciparum* wereArtesunate, chlorquine, quinine and fansider (Sulphadoxine +

Pyrimethamine). These drugswere purchased from a pharmaceutical store. Stock solution of 30mg/ml, 40mg/ml, 5mg/ml and 52.5mg/mlwere prepared for each of the drug.

200µl of Rosewell Park Memorial Institute (RPMI-1640) medium wasdispensed into 10 wells of a tissue culture macro titre plate's wells. The first two wells do not containanydrug, thus served as the control while the remaining eight microtitre wells contained, in duplicate, 20µl of the prepared stock solution of quinine, chloroquine, artesunate, and fansider (Sulphadoxine + Pyrimethamine)respectively. A total of 20µl of unwashed whole blood was then seeded into all the wells of the macro titre plate. Similar procedure was carried out for the *Jatropha curcas* leaves extract medium.

The plates were incubated anaerobically in an anaerobic jar at 37^{o}C for 36 hours. After every 6 hours the media was removed using a sterile Pasteur pipette without disturbing the cells that settled down. The cells were mixed without frothing and a drop of blood was placed on the slide and thin films were prepared. Prepared films were air dried, stained with Giemsa staining technique and examined under a bright field microscope at X100 objectives.

CHAPTER FOUR

4.0 Results

A prevalence of27% and 53% of *Plasmodium falciparum* were obtained by Giemsa stained microscopy and acridine orange fluorescence microscopy respectively (Fig. 4.1).

Of the 270 blood samples examined by Giemsa stained microscopy and acridine orange fluorescence microscopy, 26.3% of the antenatal patients were true positive i.e. had malaria, 27.0% were false negative, 0.7% were false positive and 45.9% were true negative. Acridine orange fluorescence microscopy gave a better result with a sensitivity of 49% and specificity of 98% with a positive prediction value of 97% and negative prediction value of 63% compared to Giemsa stained microscopy (Table 4.1).

Plasmodium infection among antenatal patients in respect to the hospitals studied showed that pregnant women attending Ante-natal clinics at Hajiya Gambo Sawaba Hospital, Giwa General hospital, Hunkuyi General Hospital and Major Ibrahim Abdullahi Memorial Hospital had a prevalence of 4.4%, 6.7%, 7.0% and 8.9% by Giemsa stained microscopy and 12.6%, 11.9%, 12.2% and 16.3% by acridine orange fluorescence microscopy respectively. Antenatal patients attending Major Ibrahim Bello Abdullahi Memorial Hospital had the highest prevalence of 8.9% and 16.3% using Giemsa stained microscopyand acridine orange fluorescence microscopy. However, antenatal patients attending Giwa General Hospital had the lowest prevalence of *Plasmodium* infection 6.7% and 11.9% both in Giemsa stained microscopyand Acridine orange fluorescence microscopy as shown in Table 4.2.

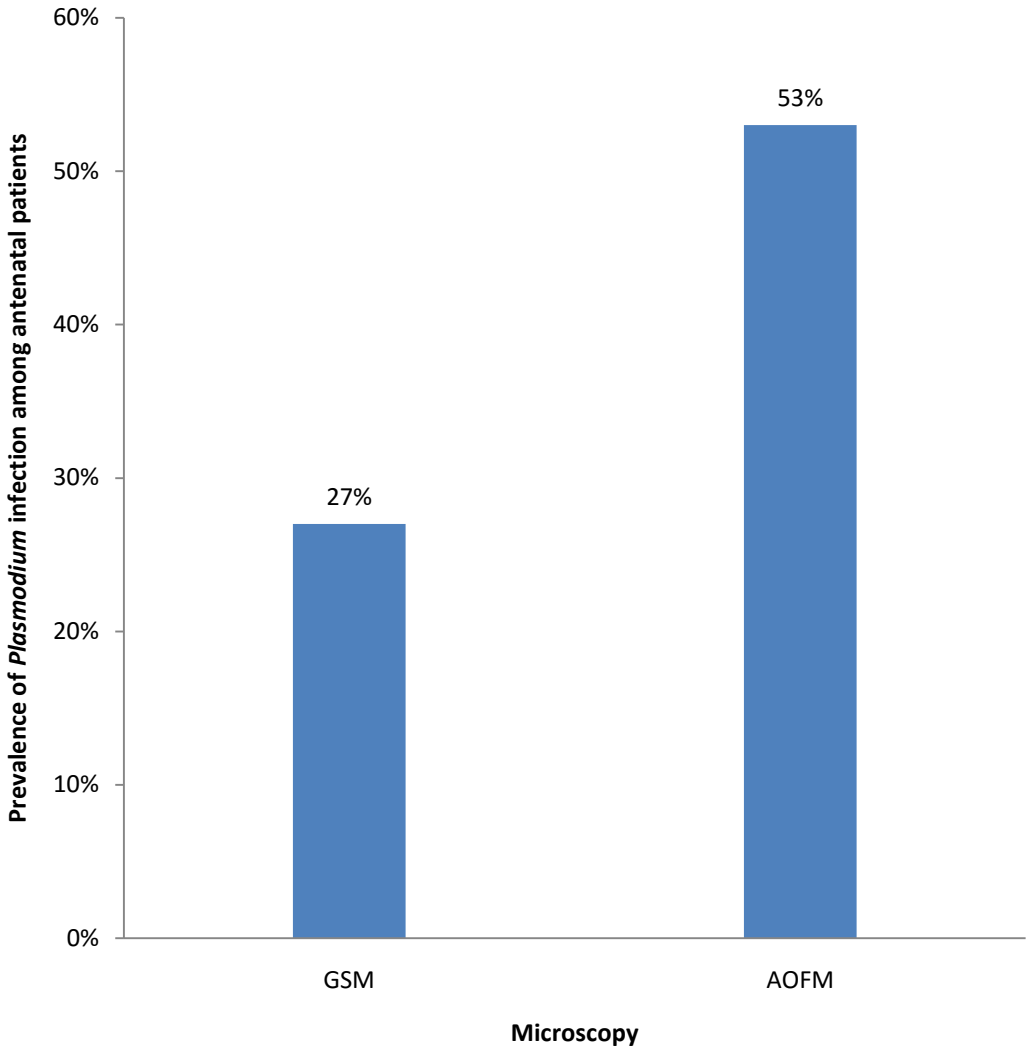


Fig. 4.1: Prevalence of *Plasmodium* infectionamong Antenatal patients using Giemsa StainMicroscopy (GSM) and Fluorescence Microscopy (AOFM).

Table 4.1: Comparative results of Giemsa stained microscopy (GSM) and Acridine orange fluorescence microscopyof *Plasmodium falciparum* obtained from the study population

		Giemsa stain microscopy (GSM)		
		Positive	Negative	Total
Acridine orange fluorescence microscopy	Positive	71(TP)	73(FN)	144
	Negative	2(FP)	124(TN)	126
	Total	73	197	270

Key:

TP: True positive

FN: False Negative

TN: True Negative

FP: False positive

Sensitivity= (TP/TP + FN) x 100 = (71/144) x 100 = 49%

Specificity= (TN/FP + TN) x 100 = (124/126) x 100 = 98%

Positive predictive value= (TP/TP + FP) x 100 = (71/73) x 100 = 97%

Negative predictive value= (TN/TN + FN) x 100 = 63%

Table 4.2: Distribution of *Plasmodium falciparum*infection among antenatal patients in some hospitals in Zaria

		Giemsa stain microscopy				Acridine Orange fluorescence microscopy			
Hospital	**Number Examined**	**Number positive (%)**	**N=270 (%)**	**Chi square**	***p*-value**	**Number positive (%)**	**N=270 (%)**	**Chi square**	***p*-value**
GSH	65	18(27.7)	4.4	3.532	0.317	32(49.2)	12.6	1.480	0.697
GGH	65	12(18.5)	6.7			34(52.3)	11.9		
HGH	65	19(29.2)	7.0			33(50.8)	12.2		
MIBA	75	24(32.0)	8.9			44(58.7)	16.3		
Total	270	73(27.0)	27			144(53.0)	53		

Key:

GSH: Hajiya Gambo Sawaba Hospital, Zaria City

GGH: Giwa General Hospital, Giwa

HGH: Hunkuyi General Hospital, Hunkuyi

MIBA: Major Ibrahim Bello Abdullahi Memorial Hospital, Sabon Gari

The analysis of socio-demographic factors in relation to *Plasmodium falciparum*infection in table 4.3 showed that Antenatal patients in age group ≤ 20 years had the highest prevalence of 11.5% by Giemsa stained microscopy and 23.3% by Acridine orange fluorescence microscopy; the least prevalence was observed in age group ≥31 years for Giemsa stained microscopy (4.4%) and Acridine orange fluorescence microscopy (8.9%) respectively. Multigravidae had the highest prevalence of 17.0% and 32.2% in both Giemsa stained microscopy and Acridine orange fluorescence microscopy while the least prevalence of 4.4% by Giemsa stained microscopy and 8.1% by Acridine orange fluorescence microscopy where observed (Table 4.3).

The prevalencesof *Plasmodium* infection by trimester were 2.6%, 10.4%, 14.1% and 59.1%, 53.7% and 50.4% in the first, second and third trimester by Giemsa stained microscopy and Acridine orange fluorescence microscopy respectively (Table 4.3). The prevalences of *Plasmodium* infection among antenatal patients with secondary school education were 10.0% (Giemsa stained microscopy) and 18.0% (Acridine orange fluorescence microscopy) respectively. While, antenatal patients with tertiary education had the least prevalence of 3.7% and 7.4% by Giemsa stained microscopy and Acridine orange fluorescence microscopy respectively (table 4.3).

The antenatal patients who were employed had a higher prevalence of 16.3% by Giemsa stained microscopy and 34.4% by Acridine orange fluorescence microscopy while among unemployed antenatal patients a prevalence of 10.7% and 18.5% by Giemsa stained microscopy and Acridine orange fluorescence microscopy respectively were observed. The employed antenatal patients were at risk of having malaria with an Odd Ratio (OD) value of 1.372 by Acridine orange fluorescence microscopy (Table 4.3).

In table 4.4, antenatal patients who were aware of *Plasmodium* infection had a prevalence of 26.7% and 50.0% while patients who have never heard of *Plasmodium* infection had

prevalences of 0.3% and 3.0% in Giemsa stained microscopy and Acridine orange fluorescence microscopy. Antenatal patients who were aware of malaria were at risk of having *Plasmodium* infection with an Odd Ratio (OD) of 4.670 although it's was not statistically significant. Antenatal patients who obtained information on malaria from home and dispensary had the highest prevalence of 11.1% by Giemsa stained microscopyand 21.9% by Acridine orange fluorescence microscopy while those who acquired knowledge from religious centers had least with 0.4% and 1.5% by Giemsa stained microscopy and Acridine orange fluorescence microscopy respectively.

Table 4.3: Distribution of *Plasmodium falciparum* infection in relation to socio-demographic factor in the study population

Factor	NumberExamined	Giemsa stain microscopy					Acridine orange fluorescence microscopy				
		Numberpositive (%)	N=270 (%)	Chi square	*p*-value/OD	CI	Numberpositive (%)	N=270 (%)	Chi square	*p*-value/ OD	CI
Age(years)											
≤ 20	126	31(24.6)	11.5	0.779	0.679		63(50.0)	23.3	1.601	0.457	
21-30	105	30(28.6)	11.1				56(53.3)	20.7			
≥31	39	12(30.8)	4.4				24(61.5)	8.9			
Parity											
Primigravidae	63	15(31.8)	5.6	0.474	0.802		34(54.0)	12.6	0.054	0.982	
Secundigravidae	41	12(29.3)	4.4				22(53.7)	8.1			
Multigravidae	166	46(27.7)	17.0				87(52.4)	32.2			
Trimester											
First	22	7(31.8)	2.6	2.110	0.376		13(59.1)	4.8	0.774	0.686	
Second	123	28(22.8)	10.4				67(53.7)	24.8			
Third	125	38(30.4)	14.1				63(50.4)	23.3			
Education											
Primary	64	19(29.7)	7.0	0.444	0.934		36(56.3)	13.3	1.300	0.734	
Secondary	101	27(26.7)	10.0				49(48.5)	18.1			
Tertiary	36	10(27.8)	3.7				20(55.6)	7.4			
Informal	69	17(24.6)	6.3				38(55.1)	14.1			
Occupation status											
Employed	166	44(26.5)	16.3	0.062	0.804/0.933	0.538-1.617	93(56.0)	34.4	1.621	0.203/ **1.376**	0.841-2.250
Unemployed	104	29(27.9)	10.7				50(48.1)	18.5			

Key: OR= Odds Ratio, CI = Confidence Interval.

Table 4.4: Prevalence of *Plasmodium falciparum*infection in relation toawareness of malaria among the antenatal patients

Factor	NumberExamined	Giemsa Stain Microscopy					Acridine Orange Fluorescence Microscopy				
		Numberpositive (%)	N=270 (%)	Chi-square	*p*-value/ OR	CI	Numberpositive (%)	N=270 (%)	Chi-square	*p*-value/ OR	CI
Awareness of Malaria											
Yes	257	72(28.0)	26.7	2.591	0.124/ **4.670**	0.596-36.573	135(52.5)	50.0	0.403	0.581/ 0.692	0.220-2.171
No	13	1(7.7)	0.3				8(61.5)	3.0			
Means of awareness of malaria											
Home	113	30(26.5)	11.1	1.964	0.602		59(52.2)	21.9	1.609	0.672	
Mass media	55	12(21.8)	4.4				26(47.3)	9.6			
Hospitals	96	30(31.3)	11.1				47(56.3)	20.0			
Religious center	6	1(16.7)	0.4				4(66.7)	1.5			
Vector of malaria											
Insects	95	26(27.4)	9.6	0.008	1.000/ **1.026**	0.585-1.799	48(50.5)	17.8	0.349	0.610/ 0.860	0.522-1.418
Mosquitoes	175	47(26.9)	17.4				95(54.3)	35.2			

Key: OR= Odds Ratio, CI= Class Interval

The risk factors associated with the prevalence of *Plasmodium* infection is presented in table 4.5. Antenatal patients with open drainages in their homes had a prevalence of 24.8% by Giemsa stained microscopy and 46.3% (*p-value* 0.045) by Acridine orange fluorescence microscopy while the least prevalence of 2.2% and 6.7% by Giemsa stained microscopy and Acridine orange fluorescence microscopy were observed for those who use closed drainages.

Those who clean their drainages daily, weekly, sometimes cleaning and those that never cleaned their drainage had prevalence of 17.8%, 4.8%, 1.1%, 3.3% in Giemsa stained microscopy and 53.3%, 54.9%, 66.7% and 43.3% for Acridine orange fluorescence microscopy respectively (Table 4.5).

Antenatal patients who used mosquito nets had a prevalence of *Plasmodium* infection of21.1% by Giemsa stained microscopy and 38.9% by Acridine orange fluorescence microscopy which is higher, follow by those who use insecticide(Giemsa stained microscopy: 41%; Acridine orange fluorescence microscopy: 7.8%) and the least prevalence was among those who use repellants with a prevalence of 1.9% by Giemsa stain microscopy and 6.3% by Acridine orange fluorescence microscopy respectively (Table 4.6).

The growth rates of *P. falciparum* in *Jatropha* leaves extract and Rosewell Park Memorial Institute media alone without any antiplasmodium agent is as shown in Fig. 4.2. There was an initial decrease in the growth rate which was followed by rapid growth after the 6^{th} hour of incubation in both *Jatropha* and Rosewell Park Memorial Institute media. There was gradual increase in the growth of *Plasmodium falciparum* without any antiplasmodium agent in both *Jatropha* leaves extract and Rosewell Park Memorial Institute media.

Table 4.5: Risk factors of *Plasmodium falciparum* infection among antenatal patients at some hospitals in Zaria

Factor	NumberExamined	Giemsa Stain Microscopy					Acridine Orange Fluorescence Microscopy				
		Number positive (%)	N=270 (%)	Chi-square	*p*-value/ OR	CI	Number positive (%)	N=270 (%)	Chi square	*p*-value/ OR	CI
Drainage											
Open drainage	245	67(27.3)	24.8	0.129	0.817/**1.192**	0.456-3.113	125(51.0)	46.3	4.008	**0.045**/ 0.405	0.163-1.005
Closed drainage	25	6(24.0)	2.2				18(72.0)	6.7			
Cleaning of drainage											
Daily	180	48(26.7)	17.8	0.389	0.948		96(53.3)	35.6	1.882	0.601	
Weekly	51	13(25.5)	4.8				28(54.9)	10.4			
Sometimes	9	3(33.3)	1.1				6(66.7)	2.2			
Never	30	9(30.0)	3.3				13(43.3)	4.8			

Key: OR= Odds Ratio, CI = Class Interval

Table 4.6: Prevalence of *Plasmodium falciparum*infectionin relation to vector control among antenatal patients

vectorscontrol	NumberExamined	Giemsa Stain Microscopy				Acridine Orange Fluorescence Microscopy			
		Numberpositive (%)	N=270 (%)	Chi-square	*p*-value	Numberpositive (%)	N=270 (%)	Chi square	*p*-value
Mosquito net	195	57(29.2)	21.1	3.657	0.164	105(53.8)	38.9	0.550	0.772
Repellant	36	5(13.9)	1.9			17(47.2)	6.3		
Spray	39	11(28.2)	4.1			21(53.8)	7.8		

Key: OR= Odds Ratio

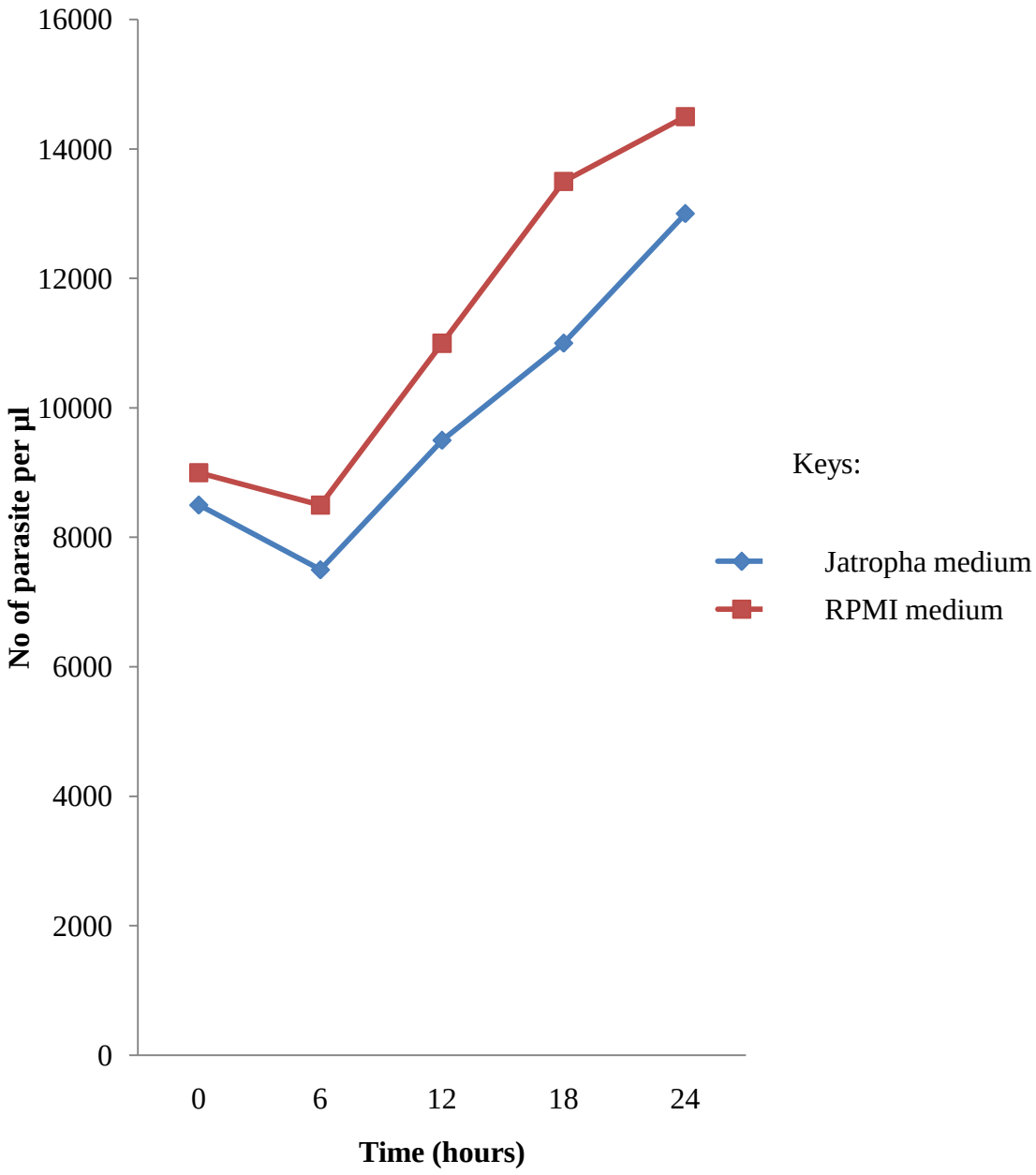


Fig 4.2:Growth rate of *P. falciparum* in *Jatropha*leaves extract and Rosewell Park Memorial Institute (RPMI) media at different time interval

There was successive decrease to an undetectable level in *Plasmodium falciparum* densityin RPMI medium after inoculation with 5mg/ml of Artesunate. Likewise, there was also a progressive decrease in *Plasmodium falciparum* density in *Jatropha* leaves extract medium within the first 18 hour of inoculation and then a gradual increase with increase in time when inoculated with artesunate (Fig. 4.5).

In Fig. 4.6, decreases in *Plasmodium falciparum*densities were observed in *Jatropha* leaves extract and RPMI media when inoculated with 40mg/ml of chloroquine. Microscopically, parasites were undetected observed after the 12^{th} and 24^{th} hour of incubation in RPMI and *Jatropha* leaves extract media respectively.

There were successive decreases between 0 - 24^{th} hour and undetectable levels of *Plasmodium falciparum* were observed from 24^{th}-36^{th} hourwheninoculated with 30mg/ml of quinine in both *Jatropha* leaves extract and RPMI media (Fig. 4.7).

The inoculation of *Plasmodium falciparum* in *Jatropha* leaves extract medium and RPMI media containing 52.5mg/ml of (sulphadoxine + Pyrimethamine) showed successive decrease in *Plasmodium falciparum* density with increase in incubation time. Undetectable levels of parasites were observed at the 30^{th} and 36^{th} hour of inoculation in RPMI and *Jatropha*leaves extractmedia respectively (Fig. 4.8).

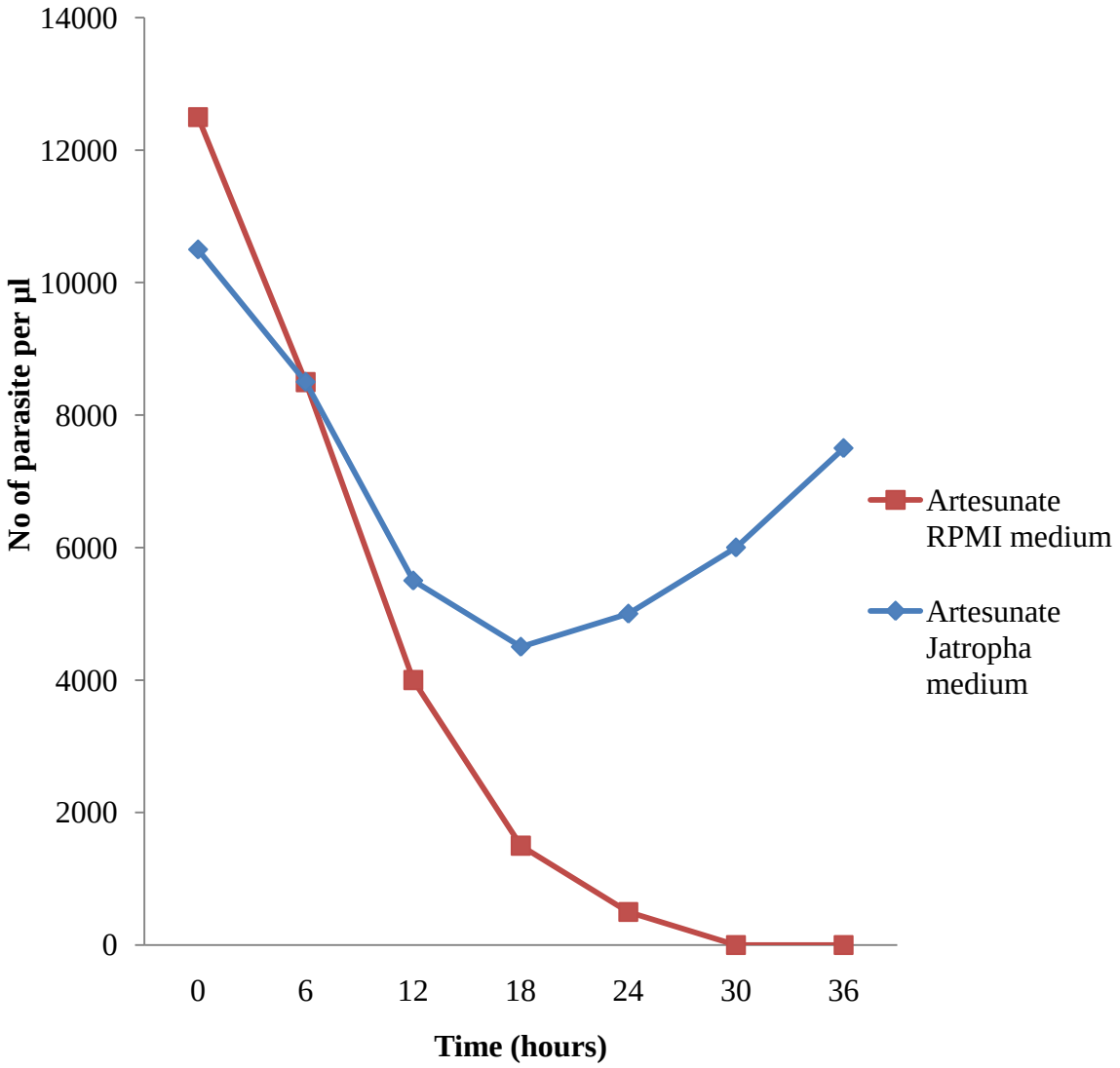


Fig 4.5:*Plasmodium falciparum* density during and after inoculation of *Jatropha* leaves extract and RPMI media with Artesunate

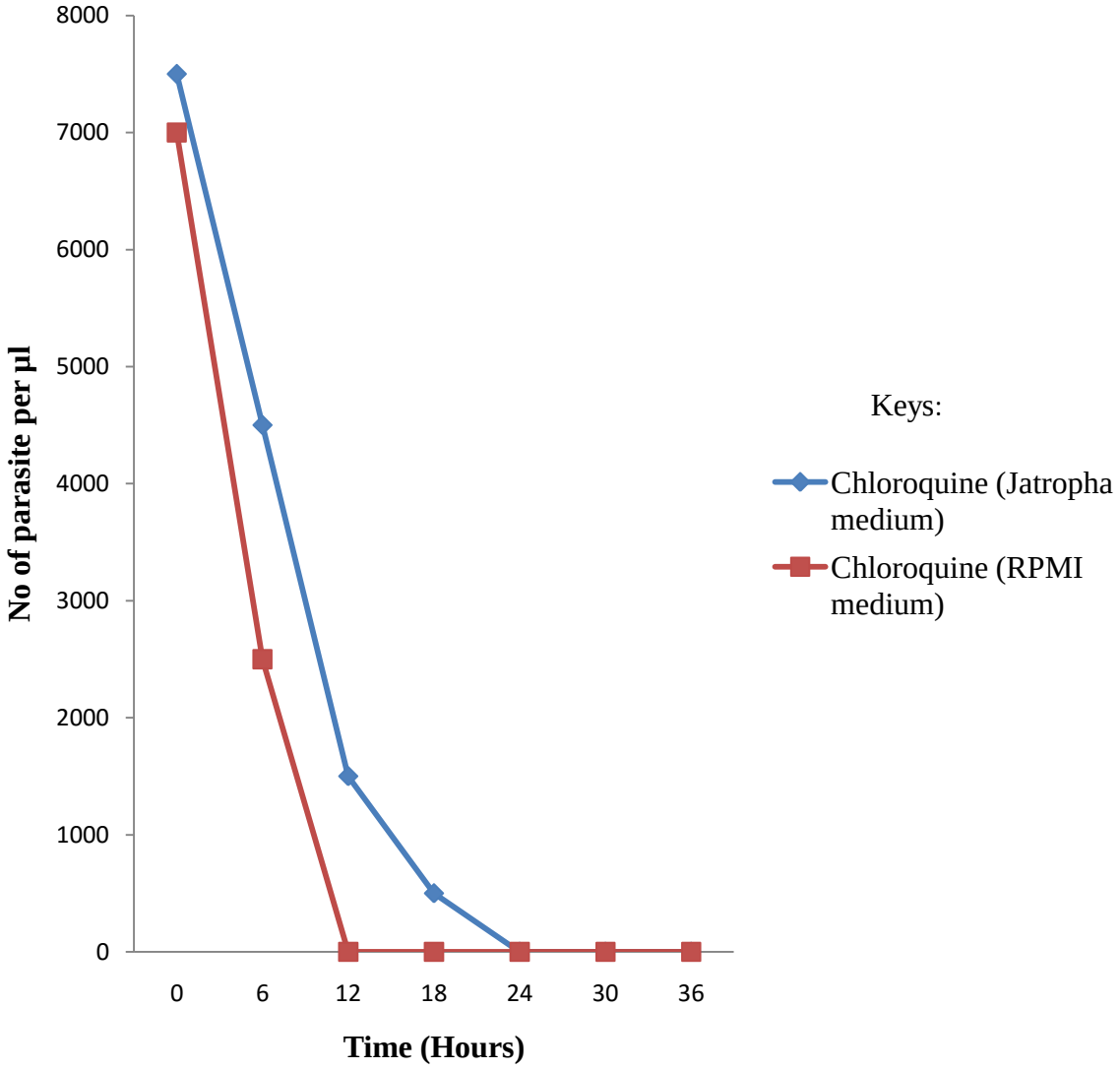


Fig 4.6:*Plasmodium falciparum* density during and after inoculationof *Jatropha* leaves extract and RPMI media with Chloroquine

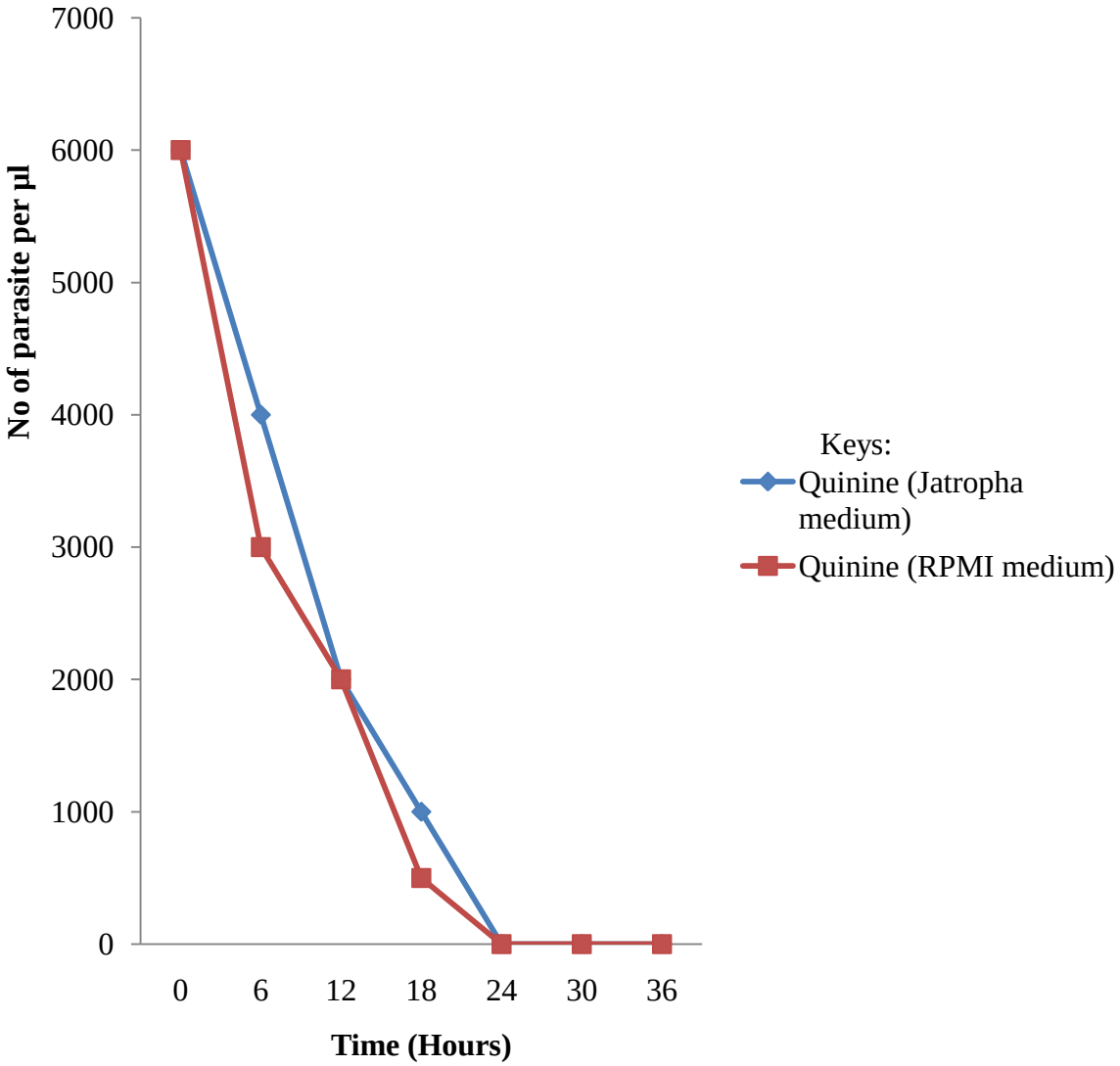


Fig 4.7:*Plasmodium falciparum* density during and after inoculationof *Jatropha* leaves extract and RPMI mediawith Quinine

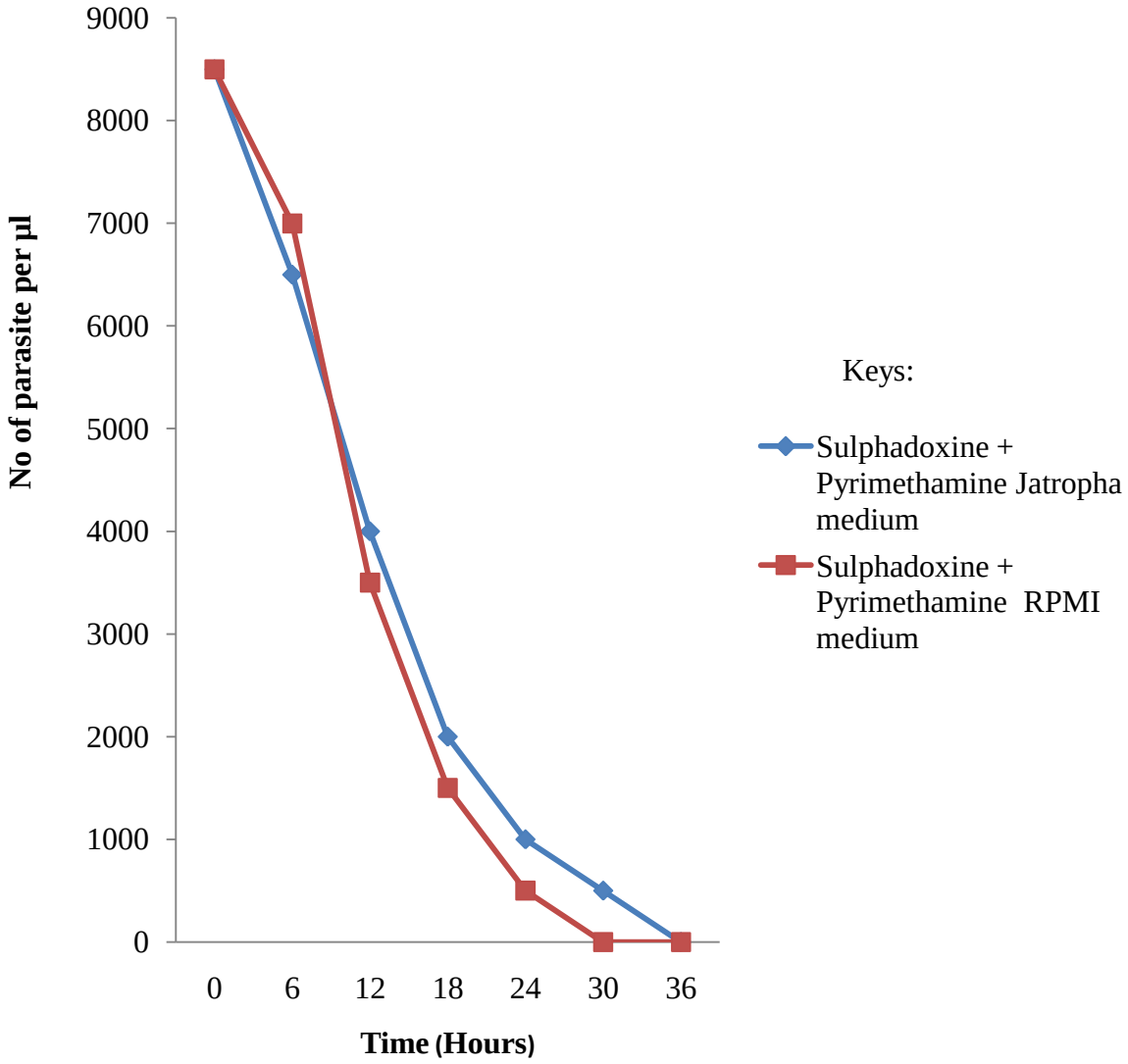


Fig 4.8:*Plasmodium falciparum* density during and after inoculationof *Jatropha* leaves extract and RPMI media with Sulphadoxine + Pyrimethamine

DISCUSSION

A total prevalence of 27% and 53% by Giemsa stained microscopy and Acridine orange fluorescence microscopy was obtained in the study. These prevalences may be attributed to the dry season and the onset of rainy season which is marked as a period of higher transmission when this study was conducted. Likewise, transient depression of cell mediated immunity that allows foetal allograft retention which interferes with resistance to various infections may be responsible for the high prevalence. This could lead to maternal anemia, delivering low birth weight babies and infant mortality generally could be attributed to these prevalences. These findings is in conformity with reports by Bawa *et al.* (2014) who reported a prevalence of 36.5% and stated that in all malaria endemic areas the frequency of malaria is higher in the pregnant women than the same women before pregnancy, non pregnant women.

High prevalence of *Plasmodium* infection observed by acridine orange fluorescence microscopy could be due its ability to stain the nucleic acids of the parasites which fluorescence brightly. Less sophisticated skill is required in identification of infecting parasite species and determination of parasite density in minimal timing compare to Giemsa stained microscopywhich is labourious and time consuming. This is similar with the reports of Keiser *et al.*, (2002) and Nag *et al.*, (2016).

Comparative results of Giemsa stained microscopy and Acridine orange fluorescence microscopy shows that Acridine orange fluorescence microscopy was better and this could be attributed to its ability to transmit longer wavelength of light (from 530-650nm) and they absorbs (maximally at 470) using appropriate filters that are responsible for its high sensitivity and specificity. This agrees with the research of Keiser *et al.*, (2002) and Jangale and Waghmare, (2016) who had high sensitivity using Acridine orange fluorescence microscopy compare to Giemsa stained microscopy.

The prevalences of *Plasmodium* infection in relation to hospitals were relatively low and not significant. This could be attributed to use of Insecticides Treated Nets (ITNs) and chemoprophylaxis by antenatal patients to boost their immunity.

The association of some socio-demographic factors (age, parity, trimester and occupation) with the prevalences of *Plasmodium* infection in both Giemsa stained microscopy and Acridine orange fluorescence microscopy was not significant. The degree of risk of contracting *Plasmodium* infection varies with the age of the pregnant woman, reflecting cumulative exposure to *Plasmodium* infection in women of ≤ 20 years age group had higher risk of *Plasmodium* infection in both diagnostic techniques. This prevalence could be that pregnancy is usually accompanied by general immune suppression leading to loss of acquired immunity. This agrees with the findings of Ohalete *et al.* (2011); Tay *et al.* (2013); Fana *et al.* (2015); Yaro *et al.* (2017) that stated that high prevalence of *Plasmodium* infection in the pregnant women of age group below 20 years showed a decrease in *Plasmodium* infection with increase in age.

There was no association between the educational level of the patients and *Plasmodium* infection even though higher prevalence was recorded among women with secondary education and least among women with tertiary education by Giemsa stained microscopy and Acridine orange fluorescence microscopy. There is need to stress the importance of education to improves citizen's education status in order to eradicate Malaria. Likewise, Government policies should be geared in improving our standard of living in our society to reduce poverty to minimal percentage.

In this study, it was observed that multigravidae had higher prevalence of *Plasmodium* infection by Giemsa stained microscopy and Acridine orange fluorescence microscopy. This contradic the findings ofAgomo and Oyibo, (2013); Patrick *et al.* (2013); Onuh *et al.*, (2015);

Aliyu *et al.,* (2017); Yaro *et al.,* (2017) who had rapid clearance of *Plasmodium* infection in multigravidae as a result of large inflammatory immune response thus, possessing a high titers antibody response that is specific and efficient in subsequent pregnancies.

The association between trimester and *Plasmodium* infection among the pregnant women examined shows that those in third trimester had higherprevalences of *Plasmodium* infection than those in first and second trimesters. This could be attributed to intrauterine growth restriction the foetus due to genetic disorders (aneuploidies, non-aneuploid syndrome), other infections, metabolic factors and placental disorders(Scifres and Nelson, 2009; Umbers *et al.,* 2011;Schmiegelow *et al.,* 2013).This result agrees with the report of other researchers that antenatal patients in the second and third trimester have higher prevalence of malaria (Akinboro *et al.,* 2010; Nasir *et al.,* 2016).

The prevalence of *Plasmodium* infection based on the means of awareness and vector of transmission by Giemsa stained microscopy and Acridine orange fluorescence microscopy require the responsibility of matron, nurse or health worker to deliver detailed education on malaria, its aetiology, transmission and control on eliminate the insect vector. This finding is similar with Ikeako *et al.* (2017) who had prevalences of 79.6% and 77.4 % among who were aware of *Plasmodium* infection in the dispensary/hospital and responded to mosquito as the vector in malaria transmission.

The prevalences of *Plasmodium* infection in relation to type of drainage by Giemsa stained microscopy and Acridine orange fluorescence microscopy respectively could be attributed to the fact that open stagnant drainages are suitable breeding environment for insect vector of *Plasmodium*. This is similar with the findings of De Silva and Marshall, (2012) and Mokuolu *et al.,* (2017) who reported that open water bodies serves as a breeding ground formosquitoes.

There was no association vector control and *Plasmodium* infection by Giemsa stained microscopy and Acridine orange fluorescence microscopy respectively.

The performance of *Jatropha* leaves extract mediumcompared suitably with RPMI-1640 in supporting human reds cells that are essential for adaptation and growth of parasite. This is particularly true in light of new findings on exosome-like particles, which can facilitate cellular communications (Mantel *et al.,* 2013; Regev-Rudzki *et al.,* 2013; Nassar *et al.,* 2014; White *et al.,* 2016).

The slow growth rate of *Plasmodium falciparum* observed in the first six hours could be due to the slow growth of the red cells and lag phase needed for adaptation of the cells in the new media. This is in agreement with White *et al.* (2016) who stated the time to adaptation is intrinsic properties of parasite during culture of *Plasmodium.*

Significant associations between the growth rates of *Plasmodium falciparum* in *Jatropha* leaves extract and Rosewell Park Memorial Institute media in the study justifies the uses of Rosewell Park Memorial Institute medium in the culture of *Plasmodium falciparum* (Mantel *et al.,* 2013; Regev-Rudzki *et al.,* 2013; White *et al.,* 2016).

The susceptibility of *Plasmodium falciparum* to artesunate in Rosewell Park Memorial Institute and *Jatropha* leaves extract media implies that artesunate exhibited a high degree of effectiveness in the inhibition of *Plasmodium falciparum* within the first 30 hours of inoculation. This is similar to the findings of Anaemene (2012) who reported decrease in antiplasmodial activity of artesunate.

Chloroquine, was also able to decrease the parasite densities to undetectable levels this proves that chloroquine remain effective in the control of *Plasmodium* infection. This result agrees with the findings ofAnaemene, (2012) who also observed decrease in parasite density.

However, the findings of this study contrast the findings of Yavo *et al.,* (2010) who observed resistanceto chloroquine by *Plasmodium.*

The antiplasmodial activity of quinine on*Plasmodium falciparum* in *Jatropha* leaves extract and Rosewell Park Memorial Institute media within the 24 hours suggest that quinine is still an effective drug for the control of the parasite. This result agrees with the report of Yavo *et al.,* (2010) who state that quinine remains the drug of choice for the treatment of uncomplicated and complicated malaria.

The susceptibility of *Plasmodium falciparum* to sulphadoxine + pyrimethamine was demonstratedby reduction of the parasite density to undetectable level*in vitro* in both media used. This justifies the recommendation of sulphadoxine + pyrimethamine in many malaria endemic communities. This research contradicts the report of Meberu *et al.,* (2000) who observed resistance of *Plasmodium falciparum* to sulphadoxine + pyrimethamine.

The study did not establish significant difference between the *Plasmodium* density after inoculation of the media (*in-vitro*) with artesunate, chloroquine, quinine and sulphadoxine + pyrimethamine suggesting similarity in the activity, effectiveness and antiplasmodial effect of the selected drugs in *Jatropha* leaves extract and Rosewell Park Memorial Institute media when cultured and incubated within thirty six (36) hours.

CONCLUSION

The overall prevalence of *Plasmodium* infection by Giemsa stained microscopy (GSM) was 27% while Acridine orange fluorescence microscopy gave an overall prevalence of 53% among antenatal patients which is an indication that malaria is endemic and of public health importance.

Comparatively, Acridine orange fluorescence microscopy gave a better result with a sensitivity of 49% and specificity of 98% with a positive prediction value of 97% and negative prediction value of 63% and it is simple, accurate and suitable for use than Giemsa stained microscopy the gold standard and suitable for use.

Multigravidae were found to be more susceptible to *Plasmodium* infection compared to secundigravidae and primigravidae. There was no relationship between other risk factors, living conditions, socio-demographic factors, vector control and knowledge or awareness and *Plasmodium* infection.

Result from this study shows that *Jatropha* leaves extract medium support the growth of malaria parasite. The antiplasmodial activity of chloroquine was better compare towith artesunate, chloroquine, quinine and sulphadoxine + pyrimethamine in Rosewell Park Memorial Institute media while Chloroquine and quinine did better than artesunate and sulphadoxine + pyrimethamine in *Jatropha* leaves extract medium.

RECOMMENDATIONS

Acridine Orange Fluorescence Microscopy which is less laborious and saves time should be used compared to Giemsa stained microscopy. Challenges in the managerial aspect of the technique could be address using battery-powered microscope fitted with interference filters could resolve the problems associated with halogen bulbs and unstable supply of electricity

There should be a better standard means of comparing results in Nigeria such as a particular level of parasitaemia when a certain quantity/standard of an inoculum in cultured and results should be expressed as a geometric mean which allows more precise comparisons of sites over time in a particular region.

Adequate utilization of insecticide treated net and intermittent preventive therapy is the corner stone in prevention of malaria in pregnancy. Hence, there is need to step up in the effort to prevent malaria in pregnancy through educational campaign with the use of audio visual aids and local languages at all levels to improve the knowledge and awareness on the efficacy of the preventive strategies.

*Jatropha*leaves extract medium which is cheaper and readily available was able to grow the parasite with a success rate of compare to the commercial sold Rosewell Park Memorial Medium (RPMI) hence could be used instead of the commercial medium where it is not readily available.

In vitro tests have offers a more objective approach to determining parasite susceptibility, as they are based on direct contact between parasites and incremental drug concentrations hence, standard protocol for conducting in vitro assays of drug sensitivity for field monitoring of drug-resistant malaria should be established.

A fundamental understanding of metabolism in *Plasmodium species* and the unique adaptations differentiating the parasite and its host is required to provide exciting new avenues for future therapeutic intervention strategies

CONTRIBUTION TO KNOWLEDGE

1. Giemsa stained microscopy and Acridine orange fluorescence microscopy gave an overall prevalence of 27% and 53% respectively.
2. In comparative study, Acridine orange fluorescence microscopy could be use as an alternative to Giemsa stained microscopy accounting for a sensitivity of 49%, specificity of 98%, a positive prediction value of 97% and negative prediction value of 63%.
3. Parity, Age, Education status and occupational status of pregnant women attending antenatal clinics are associated with *Plasmodium* infection in Acridine orange fluorescence microscopy while Trimester of the pregnancy was associated with the prevalences using Giemsa stained microscopy.However, there was no relationship between other risk factors, living conditions, socio-demographic factors and knowledge or awareness and *Plasmodium* infection.
4. *Jatropha*leaves extract medium support growth of *Plasmodium* just like Rosewell Park Memorial Institute medium
5. There was no difference in the growth rate of *Plasmodium falciparum* in *in-vitro* culture using *Jatropha*leaves extract medium and Rosewell Park Memorial Institute medium. Hence, *Jatropha*leaves extract medium which is cheaper can be use in the absent of Rosewell Park Memorial Institute medium.

6. The antiplasmodial activity of the antimalarial against *Plasmodium falciparum* shows that chloroquine performs better in Rosewell Park Memorial Institute medium while chloroquine and quinine performedbetter in *Jatropha* leaves extract medium.

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APPENDIX

Appendix I: Preparation of Giemsa Stock Culture.

About 3.8 g of Giemsa stain powder was transferred into a conical flask containing the 50 methanol-cleaned glass beads. Gently, 100mls of methanol was transferred into the same conical flask. The flask was then screw capped and shaken in a circular motion for 2–3 minutes to start dissolving the stain crystals. About 250mls glycerol and 150mls of methanol were transferred to the mixture and shaken for 3–5 minutes. The stock solution was shaken for 2-3minutes for 7 days. The stock solution was clearly labeled and stored in universal bottles before usage

Appendix II: Preparation of 10% Giemsa Working Culture.

About 90mls of prepared buffered water was transferred into beaker containing 10mls of Giemsa stock solution. The Giemsa working solution was used for staining the blood film(s) and discarded daily after used.

Appendix III: Procedure for the preparation of Acridine Orange stain

About 50mg of acridine orange (Sigma, USA) was dissolved in 4ml of glacial acetic acid and diluted with 71mls distilled water to make working stain. The solution was stored at $4^{o}C$ in an amber coloured bottle.

Appendix IV: Preparation of Phosphate Buffered Saline

Phosphate buffered saline was prepared by dissolving 0.16g of NaCl, 0.04g of KCl, 2.88g of Na_2HPO_4, 0.48g KH_2PO_4 into 150mls of distilled water, The pH was adjusted to 7.4 using

HCl. Distilled water was added to make up 200mls. It was autoclaved, labeled and stored at room temperature.

Appendix V: Preparation of Physiological Normal Saline

Using a weighing balance, about 0.9g of Sodium Chloride (NaCl) was weighed and transferred into a 90mls of distilled water then mixed. Distilled water was added to make up 100mls and stirred. It was transferred into a reagent bottle and labeled.

Appendix VI: Preparation of Chicken Liver extract

Using a weighing balance, 25g of the chicken liver purchased from Ahmadu Bello University community market was transferred into transparent polythene bag containing 100 mls of PBS; it was minced for 30 minutes (using a stomacher), filtered, centrifuged for 20 minutes at 2000 RPM, decanted and filtered with the aid of a filteration setup and suction machine. The extract was transferred into a sterile universal bottle, labeled and stored at 4^{o}C in a refrigerator.

Appendix VII: Preparation of *Jatropha curcas*leaf Extract

The leaves and stems of *Jatropha cucus* were collected within Samaru Zaria and was carried to the Department of Botany, Ahmadu Bello University, Zaria for authentication and its was given a authentication number.

About 20g of the fresh *Jatropha curcas*leaves were transferred into clean transparent polythene bag containing 100mls of the prepared phosphate buffer solution (PBS). It was placed in a stomacher and minced. After 40 minutes, the mixture was transferred into a beaker and subjected to heat at 100^{0}C for 20 minutes. It was allowed to cool down before

filtering. The prepared extract was transferred into universal bottles, labeled and stored at $4^{o}C$ in a refrigerator.

Appendix VIII: Preparation of *Plasmodium* RPMI culture medium

Preparation of complete medium RPMI 1640 1x (+) L-Glutamine (+) 25 mM HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) (Gibco, Life Technologies, UK) was prepared base on manufacturer's procedure.

Appendix XI:The chemical composition ofRPMI culture medium

Inorganic salts

$Ca(NO_3)_2$ _ $4H_2O$, KCl, $MgSO_4$ (anhydrous), NaCl, $NaHCO_3$and Na_2HPO_4 (anhydrous).

Amino acids

L-Arginine, L-Asparagine (free base), L-Aspartic acid, L-Cystine _ 2HCl, L-Glutamic acid, L-Glutamine, Glycine, L-Hisitidine (free base), L-Hydroxyproline, L-Isoleucine, L-Leucine, L-Lysine _ HCl, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine and L-Valine

Vitamins

D-Biotin, D-Ca pantothenate, Choline chloride, Folic acid, *I*-Inositol, Niacinamide, *para*-Aminobenzoic acid, Pyridoxine HCl, Riboflavin, Thiamine HCl, B12

Other components

D-Glucose, Glutathione (reduced), HEPES and Phenol red (Schuster, 2002)

Appendix XII: The chemical composition of *Jatropha curcas* leaves extract medium

This medium was composed of 0.2g/ml of glucose solution, 0.13g/ml of saline preparation, 0.126g/ml of Phosphate buffer (PBS), 0.25g/ml of liver extract, 0.05mg/ml of hypoxanthine solution and 0.2g/ml of *Jatropha curcas* leaves extract

Appendix XIII: The Phytochemical of *Jatropha curcas* leaves extract medium.

Chlorophyll a, Chlorophyll b, Catalase (CAT) activity, Peroxidases (POX) activity , Ascorbate peroxidase (APX) activity, Dehydroascorbate reductase (DHA-Rd) activity, Ascorbic acid (ASA), Dehydroascorbic acid, Reduced glutathione, Total phenols, Total carotenoids reduced glutathione, flavonoids, Anthocyanins, Tartaric acid esters derivatives and Tartaric acid esters derivatives (Papalia *et al.* 2017).

Appendix X: *P. falciparum* density during and after treatment with some antimalarial drugs in Jathropha medium.

	Parasite density per microlitre (µl) at various time interval (hours)						
Drugs	**0**	**6**	**12**	**18**	**24**	**30**	**36**
Artesunate	21	8500	5500	4500	5000	6000	7500
Chloroquine	7500	4500	1500	500	0	0	0
Quinine	6000	4000	2000	1000	0	0	0
Sulpha	8500	6500	4000	2000	1000	500	0

Appendix XI: *P. falciparum* density during and after treatment with some antimalarial drugs in RPMI medium.

	Parasite density per microlitre (µl) at various time interval (hours)						
Drugs	**0**	**6**	**12**	**18**	**24**	**30**	**36**
Artesunate	12500	8500	4000	1500	500	0	0
Chloroquine	7000	2500	0	0	0	0	0
Quinine	6000	3000	2000	500	0	0	0
Sulpha	8500	7000	3500	1500	500	0	0

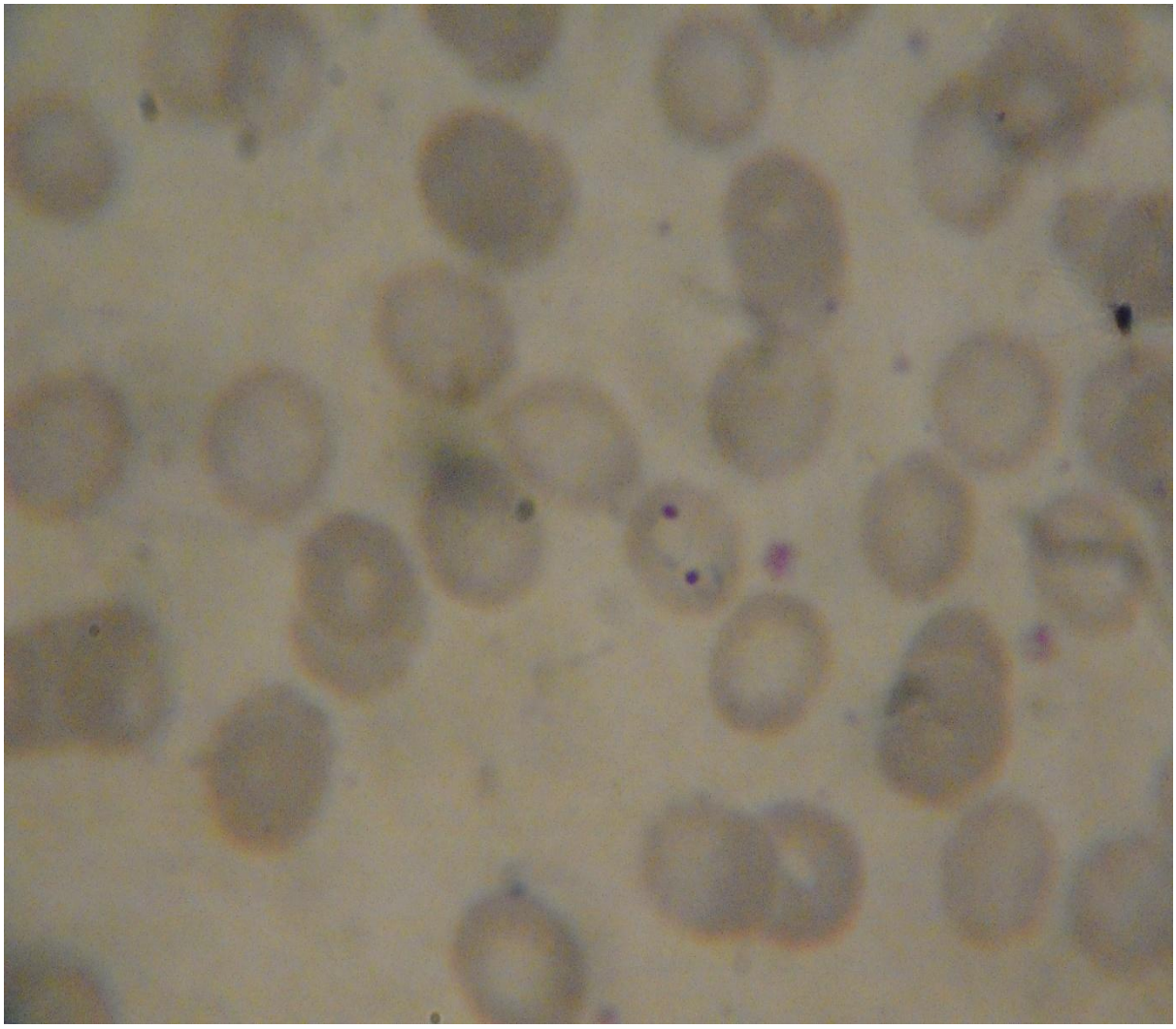


Plate I: *Plasmodium falciparum*stained with Giemsa under a bright field microscope

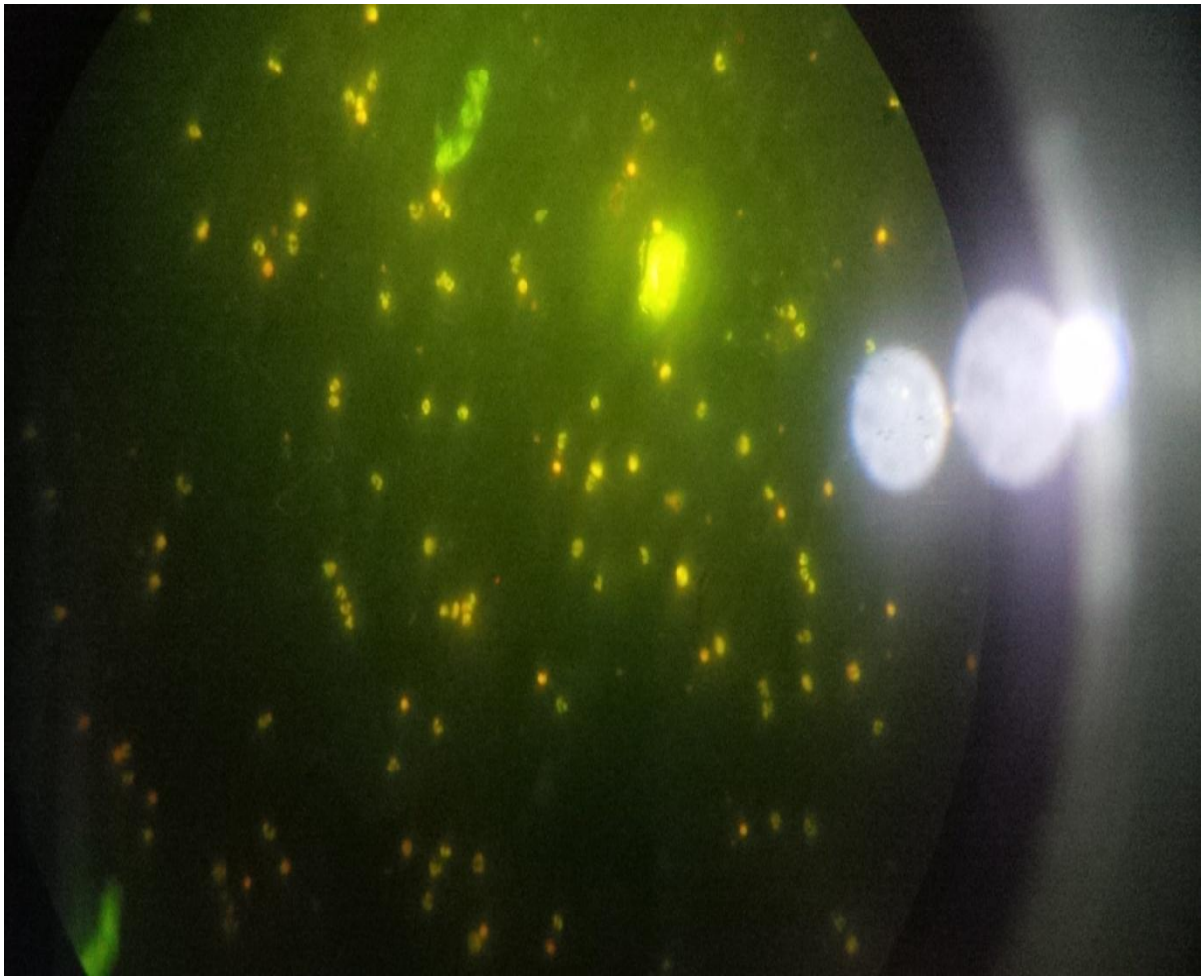


Plate II: *Plasmodium falciparum*stained acridine orange under a fluorescence microscope.

Appendix XII: Certificate of Consent

DEPARTMENT OF MICROBIOLOGY
SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

Serial No.: …….. Hospital No.: …… Age: …….. Phone No.: ……..……

This Informed Consent Form is basically for women attending some selected Hospitals in Zaria, Kaduna State. We are inviting you to participate in this research work titled "*In-Vitro* Cultivation of *Plasmodium* to determine the Antiplasmodia Activities of Some Drugs in Jatropha Plant Medium and RPMI Medium among Antenatal attendees *In Vitro* Cultivation of *Plasmodium* and Detection of Antiplasmodial Activity of some Drugs on *Plasmodium* obtained from Antenatal Patients in Zaria, Nigeria". Malaria is caused by *Plasmodium sp.* which is endemic in this part of the Country and result to complications, foetal/maternal morbidity or mortality. This research will involve the collection of blood samples of participants. The results obtained thereby may be used in any way to improve the understanding and management of the disease in our community. Individual data will be treated with strict confidentiality. Participation is strictly voluntary and refusal to participate in the research study will not in any way affect your right and benefit in this clinic/hospital.

CERTIFICATE OF CONSENT

I ……of …….. do hereby consent to participate in this study. The full procedures of the test/study have been explained to me by the investigator. I understand that blood sample will be taken for the test. I therefore give this consent voluntarily without being subjected to any pressure.

Name of Participant ……
Signature/right thumb print of participant …… Date …….

Statement by Witness:

I have witnessed the accurate reading of the consent form and detail explanation of the study to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness……
Signature of witness…… Date …….

Statement by the Researcher/Person Taking Consent

I confirm that sufficient information, including risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study, and all the questions asked by participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher…….Signature ……...Date ……

Appendix XIII: Informed Consent Form (ICF)

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

INFORMED CONSENT FORM (ICF)

This Informed Consent Form is for pregnant women attending antenatal clinics in some hospitals in Zaria, Kaduna State. We are inviting you to participate in this research work titled "*In Vitro* cultivation of *Plasmodium* and detection of antiplasmodial activity of some drugs on *Plasmodium* obtained from antenatal patients in Zaria, Nigeria".

Principal Investigator: Adamu, Iyang Comfort
Collaborating Investigators: Prof. H.I Inabo
Dr. E.E. Ella
Name of Organization: Department of Microbiology
Ahmadu Bello University, Zaria - Nigeria
Name of Sponsor: Parent
Name of Proposal: Postgraduate Research Proposal

This Informed Consent Form has two parts:

- Information Sheet (to share information about the research with you)
- Certificate of Consent (for signatures if you agree to take part)

You will be given a copy of the full Informed Consent Form

PART I: INFORMATION SHEET

Introduction

I am Adamu, Iyang Comfort,a postgraduate student of the Department of Microbiology, Ahmadu Bello University, Zaria, carrying out a research work under the supervision of Prof. H.I Inabo and Dr. E.E. Ella.

We are doing a research work on *In Vitro* cultivation of *Plasmodium* and detection of antiplasmodial activity of some drugs on *Plasmodium* obtained from antenatal patients in Zaria, Nigeria. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask them of me, the study doctor or the staff.

Purpose of the research

The purpose of this research is to determine the prevalence and risk factors of malaria, *In Vitro* cultivation of *Plasmodium* and detection of antiplasmodial activity of some drugs on *Plasmodium* obtained from antenatal patients in Zaria, Nigeria.

Participant selection

We are inviting all pregnant women who are attending antenatal clinics in some hospitals in Zaria, Kaduna State to participate in the research work on *In Vitro* cultivation of *Plasmodium* and detection of antiplasmodial activity of some drugs on *Plasmodium* obtained from antenatal patients in Zaria, Nigeria.

- *Do you know why we are asking you to take part in this study? YES..... NO……..*
- *Do you know what the study is about? YES..... NO. .....*

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this clinic will continue and nothing will change. If you choose not to participate in this research project, you will be offered the treatment that is routinely offered in this clinic/hospital for malaria patients and we will tell you more about it later. You may change your mind later and stop participating even if you agreed earlier.

- *If you decide not to take part in this research study, do you know what your options are? YES…… NO……*
- *Do you know that you do not have to take part in this research study, if you do not wish to? YES…… NO ……*
- *Do you have any questions? YES……. NO. ……*

Procedures and Protocol

When you have agreed to participate in this research, we will take blood from your arm using a syringe. Vial or other small container containing EDTA in it, will be used to show you the amount of blood that we will collect from you). At the end of the research, any leftover blood will be destroyed.

Description of the Process

During the research you will make just one visit to the clinic which is on your antenatal day.

In the visit, a small amount of blood will be taken from your arm with a syringe. This blood will be tested for the presence of Malaria and analysis will be carried out. We will also ask you a few questions about your general health and other measures so as to investigate the epidemiologic risk factors associated with the occurrence of Malaria.

Duration

The research will take place over two months. During that time, it will be necessary for you to come to the clinic just once. After that, the research will finish.

- *How many times are we asking you to come to the hospital to participate in the research?.....*
- *Can you tell me the number of time we will take your blood sample?.....*
- *Do you have any questions?.....*
- *Do you want me to go through the procedures again?.....*

Risks

There is a risk that you may share some personal or confidential information by chance, or that you may feel uncomfortable talking about some of the topics. However, we do not wish for this to happen. You do not have to answer any question or take part in the research if you feel the question(s) are too personal or if talking about them makes you uncomfortable.

Benefits

If you participate in this research, you will have the following benefits:

- ✓ You will have the opportunity of having tested for malaria.
- ✓ Your blood will be screened for Srcub Typhus.
- ✓ There may not be any benefit to the society at this stage of the research, but future generations are likely to benefit.
- ✓ Apart from these few benefits listed above, your participation is likely to help us find answer to the research questions.

Reimbursements

You will not be provided with any special incentive or travel allowance for you to take part in the research. But we advise that your visit to the clinic will have to be on your normal schedule clinic days. However, you will have the opportunity of being screened for malaria test.

Can you tell me if you have understood correctly the benefits that you will have if you take part in the study?.....

- *Do you know that the study will not pay for your travel costs? .....*

- *Do you know the kind of benefit you can get by participating in this research?.....*
- *Do you have any other questions?.....*

Confidentiality

With this research, it is possible that if others in the community are aware that you are participating, they may ask you questions. We will not be sharing the identity of those participating in the research.

The information that will be collected from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a number on it instead of your name. Only the principal investigator will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given toanyone except the principal investigator.

- *Did you understand the procedures that we will be using to make sure that any information that we as researchers collect about you will remain confidential?.....*
- *Do you have any questions about them?.....*

Sharing the Results

The knowledge that we get from doing this research will be shared with you through community meetings before it is made widely available to the public. Confidential information will not be shared. There will be small meetings in the community and these will be announced. After these meetings, we will publish the results in order that other interested people may learn from our research.

Right to Refuse or Withdraw

You do not have to take part in this research if you do not wish to do so, refusing to participate will not affect your treatment at this clinic in any way. You will still have all the benefits that you would otherwise have at this clinic. You may stop participating in the research at any time that you wish without losing any of your rights as a patient here. Your treatment at this clinic will not be affected in any way.

Who to Contact

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

Adamu, Iyang Comfort 08176132809

This proposal has been reviewed and approved by Scientific Ethical Committee (SEC) of the Ministry of Health and other hospitals, which is a committee whose task is to make sure that research participants are protected from harm. If you wish to find out more about the SEC, contact the office of the Assistant Dean, School of postgraduate studies, ABU, Zaria.

- *Do you know that you do not have to take part in this study if you do not wish to? ……*
- *Do you know that you can ask me questions later, if you wish to? ……..*
- *Do you know that I have given the contact details of the person who can give you more information about the study?.....*
- *You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?.....*

PART II: CERTIFICATE OF CONSENT

I have read the foregoing information, or it has been read and translated to me in language that I understand. I have also talked it over with the doctor to my satisfaction. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional sheet to keep for myself. I therefore consent voluntarily to participate as a participant in this research.

Name of Participant:.....Signature of Participant:.....
Date: .....
Day/month/year

Statement by Witness

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness: ..... AND Thumb print of participant

Signature of witness: _____

Date _____
Day/month/year

Statement by the Researcher/Person Taking Consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. *Blood sample will be taken*

I confirm that sufficient information, including about risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study, and all the questions asked by participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Name of Researcher/person taking the consent: .....

Signature of Researcher /person taking the consent: _____

Date _____
Day/month/year

Appendix XIV: Ethical Approval from Ministry of Health and Human Services

MINISTRY OF HEALTH AND HUMAN SERVICES
KADUNA STATE, NIGERIA

MOH/ADM/744/VOL.1/472 **14TH December, 2016**

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NOTICE OF APPROVAL AFTER FULL COMMITTEE REVIEW

PREVALENCE OF SCRUB TYPHUS AND MALARIA CO-INFECTION AMONG PREGNANT WOMEN ATTENDING ANTENATAL CLINICS IN SOME HOSPITALS IN ZARIA, NIGERIA

Name of Principal Investigator:	ADAMU, IYANG COMFORT
Address of principal Investigator:	Department of Microbiology, ABU Zaria
Date of receipt of application:	17th October, 2016
Date of Ethical Approval:	6th December, 2016

This is to inform you that the research described in the submitted protocol, the consent forms, advertisements and other participant information materials have been reviewed and given full approval by the Health research Ethics Committee (HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health, please.

DR. BUTAWA N.N
Secretary

Appendix XV: Ethical Approval from Giwa General Hospital, Giwa

GENERAL HOSPITAL GIWA
KADUNA, NIGERIA

MINISTRY OF HEALTH
AND HUMAN SERVICES
KADUNA STATE

7/9/2016

The H.O.D,
Microbiology Department,
Ahamadu Bello University,
Zaria.

ACCEPTANCE LETTER
(ADAMU IYANG COMFORT P14SCMC8013)

I am directed to write and acknowledge that the above student is accepted for collection of blood sample for her research, titled: Prevalence of Scrub Typhus and Malaria Co-infection among Pregnant Women attending Antenatal in some Hospital in Zaria Kaduna state.

We will appreciate any assistance rendered to her.

Thanks.

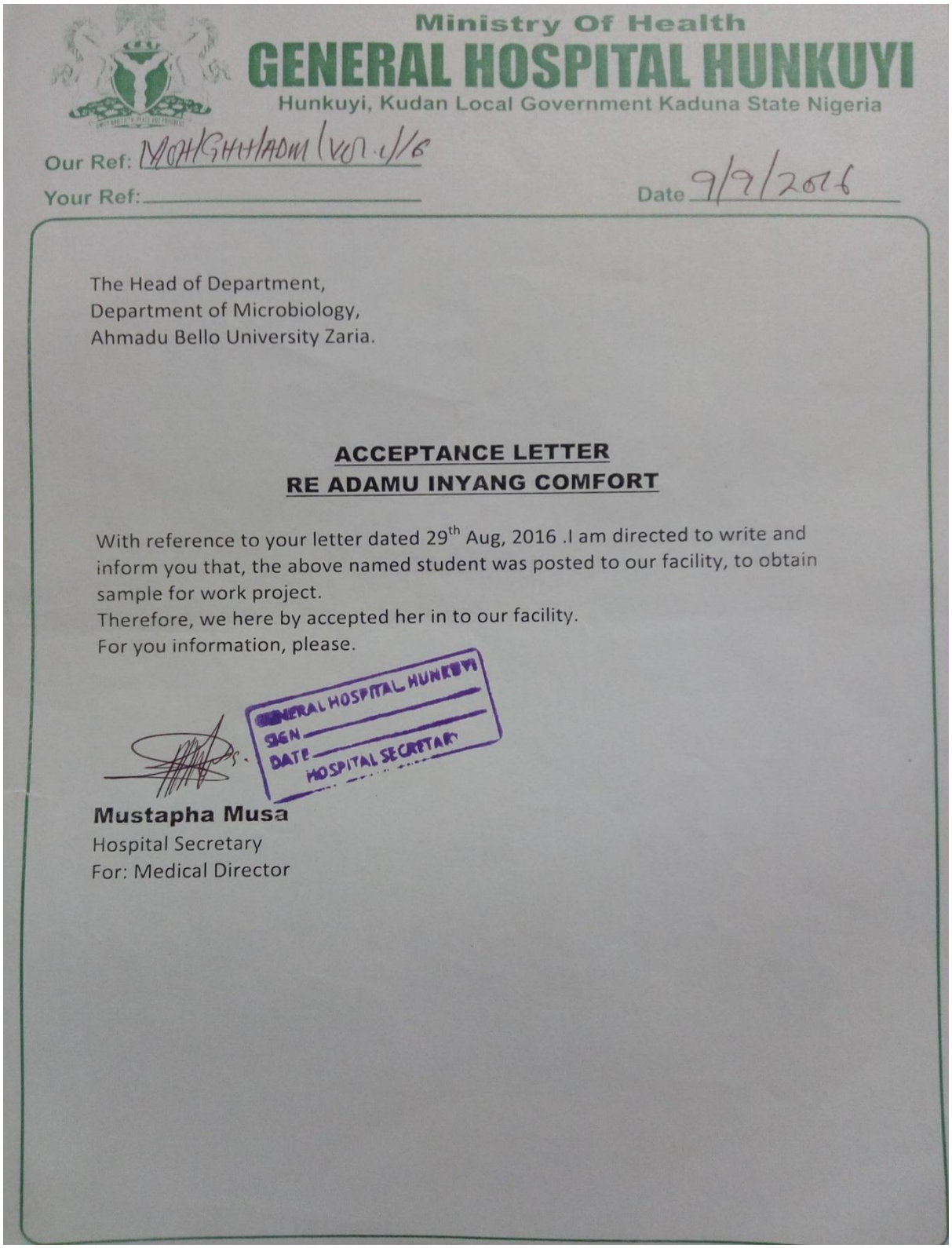
Yours faithfully,

Hospital Secretary
General Hospital Giwa
Kaduna State.

Ibrahim B. Shehu
For: Medical Director

OFFICE ADDRESS: Along Zaria Funtua, Express Way, P. M. B. 3 Giwa
Email: generalhospitalgiwa@gmail.com

Appendix XVI: Ethical Approval from Hunkuyi General Hospital, Hunkuyi



Ministry Of Health

GENERAL HOSPITAL HUNKUYI

Hunkuyi, Kudan Local Government Kaduna State Nigeria

Our Ref: MOH/GHH/ADM/VOL.I/6

Your Ref: _____ Date 9/9/2016

The Head of Department,
Department of Microbiology,
Ahmadu Bello University Zaria.

ACCEPTANCE LETTER
RE ADAMU INYANG COMFORT

With reference to your letter dated 29th Aug, 2016 .I am directed to write and inform you that, the above named student was posted to our facility, to obtain sample for work project.
Therefore, we here by accepted her in to our facility.
For you information, please.

GENERAL HOSPITAL HUNKUYI
SIGN
DATE
HOSPITAL SECRETARY

Mustapha Musa
Hospital Secretary
For: Medical Director