SEROPREVALENCE AND MOLECULAR IDENTIFICATION OF MALARIA PARASITES AMONG BLOOD DONORS IN KADUNA STATE, NIGERIA

BY

GARBA DOUGLAS DZINGINA

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCE,
ANMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

NOVEMBER, 2016

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GARBA, DOUGLAS DZINGINA
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DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA.

NOVEMBER, 2016

DECLARATION

I declare that the work in this thesis entitled '**Seroprevalence and Molecular Identification of Malaria Parasites among Blood Donors in Kaduna State, Nigeria'** was carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria under the supervision of Professor J.B. Ameh, Professor C.M.Z. Whong and Dr(Mrs.) M. Aminu-Mukhtar of the Department of Microbiology Ahmadu Bello University, Zaria. The information derived from literature had been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Garba, Douglas Dzingina	_____	_____
Name of Student	**Signature**	**Date**

CERTIFICATION

This thesis entitled **"Seroprevalence and Molecular Identification of Malaria Parasites among Blood Donors in Kaduna State, Nigeria**" by GARBA, Douglas Dzinginameets the regulations governing the award of the degree of Doctor of Philosophyin microbiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

_____ _____

Prof. J.B. Ameh Date
Chairman, Supervisory Committee
Department of Microbiology Ahmadu Bello University, Zaria

_____ _____

Prof. C.M.Z. Whong Date
Member, Supervisory Committee
Department of Microbiology Ahmadu Bello University, Zaria

_____ _____

Dr (Mrs) M. Aminu-Mukhtar Date
Member, Supervisory Committee
Department of Microbiology Ahmadu Bello University, Zaria

_____ _____

Prof. I.O. Abdullahi Date
Head,
Department of Microbiology Ahmadu Bello University, Zaria

_____ _____

Prof. K. Bala Date
Dean, School of Post Graduate Studies
Ahmadu Bello University, Zaria

DEDICATION

This thesis is dedicated to my beloved children, Wheynom, Ndwaghnom, Tsetnom and Yaghnom D. Garba.

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I give special thanks, all glory, honour and adoration to my heavenly Father for His guidance and protection throughout the period of the study.

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ABSTRACT

This research was undertaken to determine the seroprevalence and molecular identification of malaria parasites among blood donors in Kaduna State, Nigeria. Blood specimens were obtained from 360 blood donors for detection and identification using Giemsa stained thick and thin blood films, rapid diagnostic test kit and Polymerase chain reaction. A total of 27 (7.5%) of the subjects had malaria parasitaemia detected by microscopy with a mean parasite density of 126/µl of blood. The rapid diagnostic test (RDT) kit detected 31 (8.6%) positive cases. *Plasmodium falciparum* was determined to be responsible for all the cases. There were no mixed infections and no other blood parasites were observed. Malaria parasitaemia in blood donors was not significantly associated with the risk factors considered except occupation and blood group. Also, malaria parasitaemia had no significant effect on the haematological parameters considered. Compared with microscopy, the RDT kit was 70% sensitive and 97% specific. Out of the 23 cases tested by the polymerase chain reaction (PCR), six (26.1%) were positive. The species of the parasite was further confirmed through sequencing of the complete mitochondrial (mt) genome to be *Plasmodium falciparum* with 97% identity and accession number KT119883.1These findings indicate the need for blood banks to include malaria parasite species screening as a routine test of blood from donors to avert its deadly complications.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria remains an acute public health problem in many regions of the world despite tremendous progress in its control. According to the latest estimates released byWHO, (2015), malaria morbidity and mortality rates have fallen by 37% and 60% respectively globally since 2000.There were 214 million new cases of malaria and approximately 438,000 deaths in 2015 (WHO, 2015). More than two-thirds of malaria deaths globally were children under 5 years of age. The report also indicates that about 3.2 billion people (almost half of the world's population) are at risk of malaria (WHO, 2015).

Fifteen countries mainly in sub-Saharan Africa, accounted for 80 % of cases and 78% of deaths due to malaria globally in 2015 (WHO/UNICEF, 2015). Malaria has been the second leading cause of death from infectious diseases in Africa, after HIV/AIDS and is the 5th leading cause of death from infectious diseases in low-income countries, which are vastly concentrated in sub-Saharan Africa (Wong *et al*., 2009; WHO, 2011; CDC/USAID, 2012; CDC, 2015; UNICEF, 2015). The high volume of confirmed cases in sub-Saharan Africa in relation to the global statistic indicates that the disease is highly endemic in the region. It is also estimated that a child dies every one minute. There are multiple reasons why malaria is so prevalent in Africa. First, the predominant parasite, *Plasmodium falciparum* which is one of the four species of human malaria parasites, is the leading cause of severe malaria (Guerin *et al*., 2002; CDC, 2005). Second, the warm and wet weather conditions in equatorial regions provide the mosquito with ideal conditions in which to thrive

(Lomborg, 2009; CDC, 2015). Studies also show that global warming contributes to the crisis by increasing the areas in which the mosquito and the *Plasmodium* can survive (Brabin *et al*., 2001). Third, and perhaps most importantly, the lack of resources and the socioeconomic instability in sub-Saharan African countries increases the burden of malaria (FOA, 2000; Gallup and Sacks, 2001; UNECA 2005; CDC, 2015). Although it is a health crisis, malaria also has grave economic impact, burying communities in cycles of poverty (Ivy, 2012).

In Nigeria, malaria is characterized by a stable and perennial transmission in all parts of the country though transmission is higher in the wet than in the dry season(Ekanem, 1998). It is a major public health problem where it accounts for more cases and deaths than any other country in the world(CDC/USAID, 2012). Malaria is a risk for 97% of Nigeria's population, accounting for 60% of outpatient visits in all age groups in all parts of the country, 30% of hospitalizations, 11 % of maternal mortality, 10% of low birth weight and tops the mortality table, with pneumonia, measles and dysentery in that order. There are estimated 100million cases of malaria with over 300,000 deaths and 50% of the population has at least one episode of malaria every year, while children less than 5 years have on the average of 2-4 attacks in a year. Furthermore, the nation loses over 132 billion naira annually in form of treatment costs, prevention and loss of man-hours (FMOH, 2005; WHO, 2011; CDC/USAID, 2012).

Malaria is caused by blood protozoan parasites of the genus *Plasmodium*. The species of *Plasmodium* that infect humans under natural conditions are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. The predominant parasite species is *P. falciparum* which causes the most severe form of

malaria (CDC/USAID, 2012). It accounts for about 98% of malaria cases in Nigeria while *P. malariae* occurs as mixed infection with *P. falciparum*. *Plasmodium knowlesi* found in Southeast Asia as a natural malaria pathogen of Macaques Monkeys has recently been shown to be a significant cause of zoonotic malaria in that region (Patrick *et al*., 2005; CDC, 2012). Malaria diagnosis is largely based on microscopy, which after more than 100 years is still considered to be the gold standard of malaria diagnosis. However, with the widespread availability of Polymerase chain reaction (PCR), a new era in the detection of *Plasmodium* species has begun. Largely based on PCR results, the number of species of human malaria parasites has increased from four to six within only 10 years. The species now comprise of *Plasmodium falciparum, P. vivax, P. malariae, P. knowlesi,* the newly recognized species *Plasmodium ovale curtisi* (former classic type) and *Plasmodium ovale wallikeri* (former variant type) (Singh *et al*., 2004; Sutherland *et al*., 2010; Hans-Peter and Noed, 2014).

The infection is transmitted naturally via the bite of infected female *Anopheles* mosquitoes. It can also be transmitted through blood transfusion, sharing of needles and syringes and transplacentally from infected pregnant mother to the foetus (Haslett *et al*., 1999; Patrick *et al*., 2005). *Anopheles gambiae* is the main vector in Nigeria but *Anopheles arabiensis*, and *Anopheles funestus* are also commonly encountered while *Anopheles melas* is found in the coastal areas (Ekanem, 1998; FMOH, 2005; Oyewole *et al*., 2011).

Clinical manifestations of infection depend on a combination of several factors including the virulence of the isolate, host related factors such as the immune status and genetic makeup (El. Harith, 2004; Hisaeda *et al*., 2009). The symptoms of malaria are non - specific and include headache, pains in the back and limbs,

anorexia, nausea, chill, and continuous or remittent fever (Hauster and Sussman, 1989). Anaemia and frequent malaria relapses result in a marked reduction in macro-economic indices of malaria endemic nations (CDC, 2012).

Malaria maintains a vicious cycle of disease and poverty since many countries with malaria are already among the poorer nations. The disease imposes substantial costs to both individuals and government. Costs to individuals and their families include purchase of drugs for treating malaria at home, travel expenses and hospital based treatment costs. Inestimable losses are loss of work days, school attendance and expenses on preventive measures and burial in case of deaths. Costs to government include maintenance of health facilities, purchases and supplies of drugs, public health interventions against malaria, lost days of work with resulting loss of income and lost opportunities for joint economic ventures and tourism(CDC, 2012). Such costs add substantially to the economic burden of malaria on endemic countries and impede their economic growth. It has been estimated that economic growth per year of countries with intensive malaria was 1.3% lower than countries without malaria (CDC, 2012). While only 0.2% of global malaria deaths are found in the world's richest population quintile, 57.9% of global malaria deaths are concentrated among the world's poorest population quintile (Gwatkin and Guillot, 2000).

1.2 Statement of the Research Problem

Blood transfusion is necessary for the management of severe anaemia, deficiency of blood clotting factors, thrombocytopenia, immunodeficiency states, hypoalbunaemia and emergencies involving surgery (Talib, 1996; Ali *et al*., 2004). Although a life-saving venture, it also poses problems such as the risks of immunological adverse

reactions and an effective means of transmitting malaria parasites and other blood borne pathogens to other persons (Mungai *et al*., 2001; Cheesbrough, 2010).

Prevalence rates of malarial parasitaemia of between 4.1and 77.4% have been found in blood donors in Nigeria. (Akinboye and Ogunrinade, 1987; Okocha *et al*., 2005; Chigozie *et al*., 2006 and Ekwunife *et al*., 2011).These reports were based on blood microscopy and use of rapid diagnostic test (RDT). There is however no report indicating that these results were further confirmed by an advanced diagnostic technique such as the polymerase chain reaction (PCR). In Nigeria, as in other developing tropical countries, the occurrence of blood-demanding health conditions due to the increase in road accidents, pregnancy-related haemorrhage, armed robbery attacks, and violent events (such as insurgency and communal clashes) amplify the possibility of the transmission of blood-borne diseases (Chigozie *et al*., 2006). Most donors implicated in transfusion-transmitted malaria (TTM) are predominantly semi-immune with very low parasite loads. However, the infectious dose is estimated to be 1 - 10 parasites in a unit (450ml) of blood (Owusu-Ofori *et al*., 2010). Detection of such low parasitaemia is difficult with the peripheral smear examination (Seed *et al*., 2005; Kitchen and Chiodini, 2006; Jean-Pierre, 2010).

The long standing concern about transfusion-transmitted hepatitis and human immunodeficiency virus (HIV) infections has overshadowed the significance of the potential risk of other transfusion transmitted diseases such as malaria (Bove, 1990). For example, among donors, the prevalence of human immunodeficiency virus in the region ranges from 0.5 - 16%, the prevalence of hepatitis C virus infection ranges from 0.5 - 12.3%, and the prevalence of hepatitis B virus infection ranges from 2.5% to 20% (Allain *et al*., 2004). Compared with other transfusion-

transmitted infections, the very high prevalence of malaria among blood donors has been almost totally ignored.

The infectious dose of malaria parasites is estimated to be 1 - 10 parasites in a unit (450ml) of blood (Owusu-Ofori *et al*., 2010). Microscopy though the gold standard for malaria diagnosis in endemic areas, its best sensitivity of detection is approximately 10-30 parasites/µl of blood (Gilles, 1993). Furthermore, this level of detection is normally not attained in malaria endemic areas and especially during epidemiologic studies when many samples need to be screened in a relatively short time (Singh *et al*., 1999). Thus, incorrect speciation is common, mixed infections and low levels of parasitaemia may be missed. To overcome some of the limitations of microscopy for detection of malaria, PCR based assays have been developed to detect as low as 0.001 parasites / µl of blood (Jaureguiberry *et al*., 1990).

The National institute of Health (NIH) consensus conference in 1995 recommended that every donor blood should be screened for various infections including HIV, hepatitis Band C, malaria and syphilis (Agboola *et al*., 2010). However, the critical lack of convincing evidence about the clinical impact of transfusion-transmitted malaria and the absence of an approved effective and feasible screening method are preventing rational decision-making about whether or not to screen blood for malaria in Nigeria (Owusu-Ofori *et al*., 2010).

1.3 Justification of the Research

Post transfusion malaria may not only compound the already deplorable health condition of recipients but may also be fatal. Therefore the need for effective donor selection guidelines cannot be overstated. The outcome of this study will provide additional information on the prevalence of malaria parasites in apparently healthy

individuals in the whole state. This will certainly add to the available evidence of the potential risk associated with the transmission of malaria parasites through blood transfusion in the state. Though similar studies have been previously conducted in the state, they only covered part of the northern senatorial district of the state (Garba *et al*., 2009; Oche and Aminu, 2012).

This research will also provide documented molecular evidence of the species of malaria parasites present in Kaduna state as there seems to be no such evidence presently.

There is need for the determination of some of the haematological parameters which will provide information that could contribute immensely to the adoption of reference values of these parameters for use by clinicians in the study area as most clinical decisions are based on western values.Published literature has indicated that many of the reference values obtained from the developed countries differ significantly from those in most African localities (Lugada *et al*., 2004; Quinto *et al*., 2006; Koram *et al*., 2007; Adetifa *et al*., 2009; Karita *et al*., 2009). The Clinical and Laboratory Standards Institute (CLSI) and the International Federation for Clinical Chemistry recommend that each laboratory establishes its own reference values (Solberg, 1987; CLSI, 2008).

The findings of this research will provide more evidence about the potential risk associated with Transfusion related malaria (TRM)and some of the risk factors associated with malarial parasitaemia in the state which may help the concerned authorities in formulating a more useful policy on the control of malaria in the state.

There is need for continuous evaluation of the diagnostic performance of the RDT kits in a given areato determine the potential utility of RDTsfor the screening of donor-blood for malaria parasites prior to transfusion.

The mitochondrial DNA (mtDNA) is a useful and one of the most frequently used markers in molecular systematics (Sorension *et al*., 1999).It has been widely employed to address questions of genetic diversity, population structure, phylogeography and population evolution of animals (Anderson *et al*., 1981; Wallace, 1995; Ankel-Simons and Cummins, 1996). Molecular analysis mt-DNA of *P falciparum* isolates from the state is particularly useful in inferring phylogenetic relationship between closely related species from the state and global isolates(Hartl and Clark, 1997).

1.4 Aim of the Study

The study was aimed at detecting and molecularly identifying malaria parasites in donor blood with a view to providing molecular epidemiological bases for the prevention and control of transfusion related malaria (TRM) in the study area.

1.5 Specific Objectives

The specific objectives of the study were to

1. determine the prevalence rate and intensity of malaria parasites among blood donors in Kaduna state by microscopy and rapid diagnostic test (RDT) andevaluate the diagnostic performance of the RDT kit for screening of blood donors for malaria parasites.

2. determine the risk factors associated with malaria parasitaemia among blood donors and determine the effect of malaria parasitaemia on some haematological parameters
3. confirm the species of malaria parasites detected by microscopy and RDT using PCR.
4. determine variations in the sequences and the phylogenetic relatedness of some of the malaria parasites isolates by senatorial districts.

CHAPTER TWO

2.0 LITERATURE REVIEW

Malaria has a long history and it is most likely that humans and malaria have probably evolved together (Sheman, 1998). Although people were likely to be exposed to malaria since ancient times, virulent malaria probably originated in Africa, was common in Europe by the 15th century and became worldwide by the early 18th century (Sheman, 1998; Davis and Icke, 2004). Despite its long history, it was not until 1880 that Charles Louis Alphonse Laveran, a French army surgeon discovered parasites in the blood of a patient suffering from malaria (CDC, 2006). Very early works on malaria parasites included their taxonomy and systematic. The Italian investigators Giovanni Batista Grassi and Raimondo Filetti first introduced the names *P. vivax* and *P. malariae* in 1890 while *P. falciparum* and *P. ovale* were named by William H. Welch and John William Watson in 1897 and 1922 respectively. In 1897, Ronal D. Ross demonstrated that malaria parasites could be transmitted from infected patients to mosquitoes. Giovanni Batista Grassi led a team of Italian investigators that discovered the transmission *Plasmodium* parasites by infected mosquitoes to humans (CDC, 2015).

The earliest studies on malarial parasitaemia in blood donors in Nigeria were in relation to ABO blood group. Akinboye and Ogunrinade (1987), Ibhnesebhor *et al*. (1996), Mbanugo *et al*. (2004), Nrnor and Egwunyenga (2004), Okocha *et al*. (2005), Umeanaeto *et al.* (2006) and Chigozie *et. al*. (2006) provided valuable information on the prevalence of malarial parasitaemia among blood donors in the southern part of Nigeria.

Transmission of malaria parasites through blood transfusion is a well known serious risk. Screening of blood donors for malaria as recommended by WHO is currently not included in the protocols of many Nigerian blood banks. Presence of asymptomatic *Plasmodium* species carriers (APCs) in some parts of the state has already been demonstrated using rapid diagnostic tests (RDTs) and microscopic examination of Giemsa stained blood films(Garba *et al*., 2009; Ocheh and Aminu, 2012).

The works carried out in the northern part of the country include the prevalence of transmissible blood infections among blood donors at the University Teaching Hospital, Maiduguri by Chikwem *et al*. (1997). He found that 4.1% of the donors had *P. falciparum* infection. lkeh and Okeke (2005) studied the prevalence of malaria parasitaemia in screened blood donors at Jos University Teaching Hospital, Nigeria. The parameters considered included age, gender, donation frequency and month of the year. He also determined the parasite density of the blood donors. The results showed that malaria parasitaemia was significantly associated with, age group and gender.

2.1 Morphology and Life Cycle of Malaria Parasites

The malaria parasite has a complex life cycle involving asexual stages within human hepatocytes and erythrocytes and a sexual stage within the mosquito gut. Malaria is typically transmitted by the bite of an infected female anopheles mosquito, however, it can also be transmitted transplacently during pregnancy, by blood transfusion or needle sharing (Haslett *et al*., 1999). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. Sporozoites may invade hepatocytes using region Ii of the circumsporozoite protein (CSP-II) on their

surface as the ligand for binding to heparin sulfate proteoglycans (HSPGs) and the low-density lipoprotein receptor on the hepatocyte surface (Krogstad, 2000). Sporozoites in the liver cells mature into schizonts, which rupture and release 20,000-40,000 merozoites into circulation in about 10 to 15 days depending upon the species (Fujioka and Aikawa, 1999). Of note, in *P. vivax* and *P. ovale* a dormant stage (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later). After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony) releasing more Merozoites which infect red blood cells. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. The merozoites are small, ovoid cells 1.5-2.5 um long and 1.0-2.0 um wide, having slightly narrower apical pole. Three types of membrane bound organelles namely rhoptries, micronemes and dense granules are located at the apical poel and appear to play a role in binding and entry of the merozoite into RBCs (Fujioka and Aikawa, 1999). Invasion of erythrocyte by the merozoites is a complicated process involving four steps:

a. Initial recognition and loose attachment of merozoite to erythrocyte membrane.
b. Juxtrapositioning of apical prominence of merozoite to the RBC membrane and formation of tight junction.
c. Movement of junction and invagination of RBC membrane.
d. Resealing of parasitophorous vacuole membrane and RBC membrane after completion of invasion (Fujioka and Aikawa, 1999).

Duffy Binding Protein (DBP) of *P. vivax* merozoites binds with the Duffy factors on the erythrocytes and forms a tight junction and initiates invasion. Similarly, the

normocyte binding Proteins (NBPs) of *P. falciparum* merozoites help their merozoites specifically to target normocytes and initiate loose attachment between the merozoites and erythrocytes. This initial interaction between merozoites and RBCs enables the merozoite to reorient its apical pole towards the red cell surface and may trigger the release of Erythrocyte binding antigen-175 (EBA-175) from the micronemes of P. falciparum merozoites. The EBA-175 specifically binds with the sialic acid residue of Glycophorins A on the erythrocyte and initiates invasion of merozoite. Additionally, merozoite surface proteins (MSP) and Apical merozoite antigen (AMP) particularly MSP-1 and apial AMA-1 also play a role in erythrocyte invasion (Fujioka and Aikawa, 1999; Chitins, 1999). Blood stage parasites are responsible for the clinical manifestations of the disease (CDC, 2015). Some of these merozoites then differentiate into sexual erythrocytic stages (gametocytes). Factors such as immunological stress, host response to the parasites and cell debris and chemotherapy may play important roles. It has been shown also that chloroquine may induce gametocytogenesis whereas sulfadiazine and sulfamethazine may induce extended period of high gametocytemia (Sinden, 1998).

When mature gametocytes are taken up by a female anopheline mosquito with a blood meal, microgametocytes and macrogametocytes mature inside the mosquito gut into male and female gametes respectively. The microgamete then fertilizes macrogamete and produces a diploid zygote. The zygote matures to an ookinete and undergoes a meiotic reduction division to produce the haploid sporozoites than might migrate to the salivary gland and are prepared to infect the human host during the next blood meal (Fujioka and Aikawa, 1999; CDC, 2015). A diagrammatic representation of the generalized life cycle of *Plasmodium* species is shown below (Figure2.1).

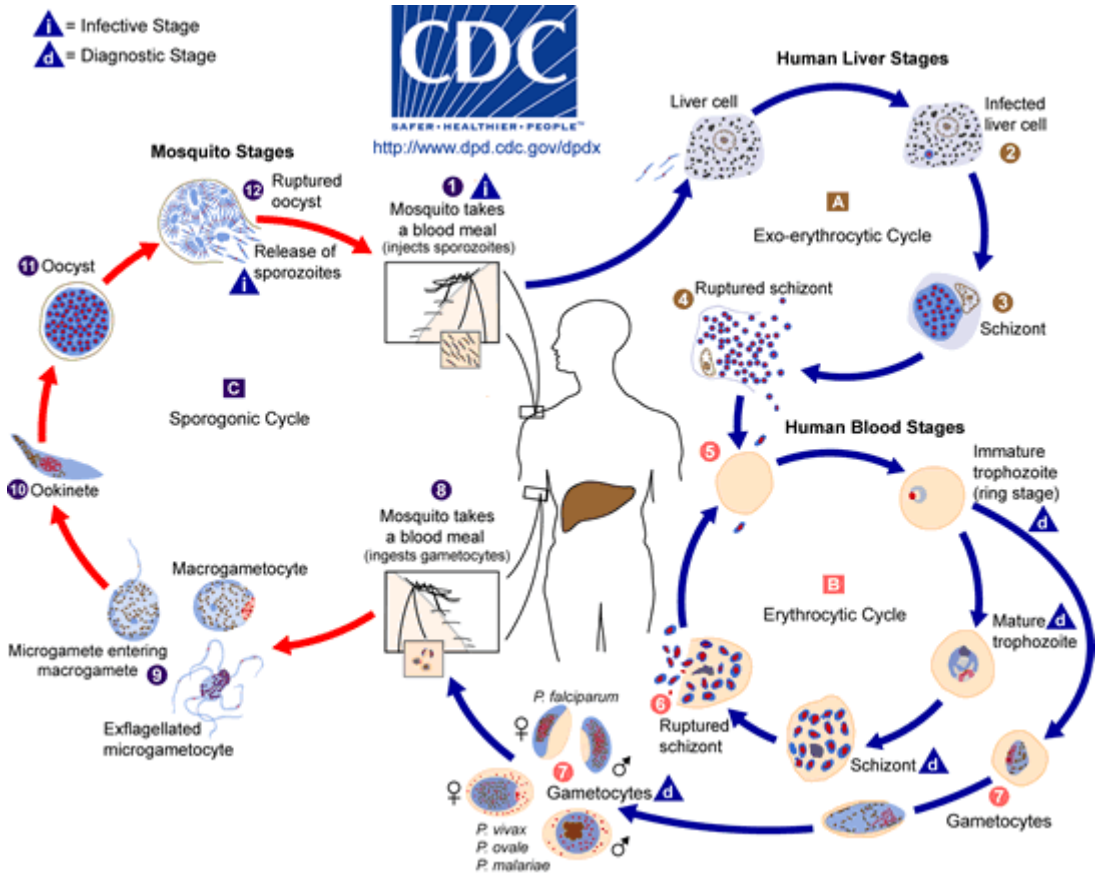


Figure 2.1: A Diagrammatic Representation of the Life Cycle of Malaria parasites (CDC, 2012).

2.2 Epidemiology

Malaria is widely distributed in tropical and subtropical countries between latitude 49^0 north and 30^0South. The risk of malaria is highly dependent on interactions between the host, parasite, mosquito vector, and environment, a relationship known as the epidemiologic triad of disease (CDC, 2015). The major factors that contribute to the epidemiology of malaria include: Ideal climatic conditions of temperature, humidity and rainfall, presence of human carriers, presence of appropriate and susceptible mosquito hosts which are in contact with humans and presence of malaria parasites.

2.2.1 Climatic factors

The most important climatic factors that directly affect malaria transmission are temperature, rainfall and humidity. Rainfall and relative humidity have been found to show positive correlations with major peaks of malaria prevalence observed following the peaks of rainfall (Sena *et al*., 2015). Rainfall provides "breeding sites" for the *Anopheles* mosquito such breeding sites may dry up in the dry season, or are flushed by flood. Temperature influences anopheline mosquito feeding intervals, population density, and longevity, as well as the reproductive potential of the *Plasmodium* parasite (Small *et al*., 2003; EFMOH, 2016).

Relative humidity affects malaria transmission through its effect on the activity and survival of mosquitoes. Mosquitoes survive better under conditions of high humidity. They also become more active when humidity rises. This is why they are more active and prefer feeding during the night – the relative humidity of the environment is higher at night (CDC, 2004; EFMOH, 2016).

The ranges of minimum and maximum temperature greatly affect the development of the malaria parasite and its mosquito vector, which determines malaria transmission. A rise in ambient temperature shortens the duration of the extrinsic cycle, thereby increasing the chance of transmission. Conversely, a decrease in ambient temperature prevents the completion of the extrinsic cycle and malaria transmission. Then optimal temperatures for *P. falciparum* and *P. vivax* are 20°C (68^0F) and 15^0C (59^0F). Therefore even within tropical and subtropical areas transmission will not occur at high altitudes due to lower temperature. However, malaria transmission may be higher in the oasis. In warmer regions where *P. falciparum* predominates, transmission will be more intense and malaria transmitted year round while in cooler regions, transmission will be less intense, more seasonal and *P. vivax* more prevalent because it is more tolerant of lower ambient temperatures (CDC, 2004; EFMOH, 2016).

Development of the mosquito larva also depends on temperature – it develops more quickly at higher temperatures. Higher temperatures also increase the number of blood meals taken and the number of eggs laid by the mosquitoes, which increases the number of mosquitoes in a given area. The minimum temperature for mosquito development is between 8–10°C; the optimum temperature is 25–27°C, and the maximum temperature for is 40°C (CDC, 2004; EFMOH, 2016).

Altitude (elevation above sea level) is one of the most important factors that determine the pattern of malaria transmission in Ethiopia. As altitude increases, temperature decreases, highlands are therefore colder and lowlands are warmer (EFMOH, 2016). Newer hypothesis suggest that current trends of global warming enhances the spread of malaria (CDC, 2004).

2.2.2 Vector factors

Malaria is transmitted exclusively by different species of female *Anopheles* mosquitoes, depending on the region and the environment. Across Nigeria from 1900 to 2010, the overall highest number of vector species reported included *An. gambiae* complex (65.2%), *An. funestus* complex (17.3%), *An. gambiae* s.s. (6.5%). *An. arabiensis* (5.0%) and *An. funestus* s.s. (2.5%), with the molecular forms *An. gambiae* M and S identified at 120 locations (Okorie *et al*., 2011).In Ethiopia there are about 40 different species of *Anopheles* mosquitoes, but only four of them are known to transmit malaria parasites, and just one of them, *Anopheles arabiensis*, is responsible for more than 95% of malaria transmissions (EFMOH, 2016).

Different species of *Anopheles* mosquitoes differ in their capacity to transmit malaria. This depends on the biology and behaviour of the mosquitoes. Mosquitoes in the *Anopheles gambiae* group (which includes *A. arabiensis),* are the most efficient malaria vectors in the world. These mosquitoes are found only in Africa. In fact, the higher incidence of malaria in Africa compared to other parts of the world is mainly due the efficiency of these mosquitoes in transmitting the parasites(CDC, 2015). Factors that affect a mosquito's ability to transmit malaria include its innate susceptibility to *Plasmodium,* its host choice and longevity. The primary malaria vectors in Africa *A. gambiae* and *A. funestus* are strongly anthropophilic and consequently are two of the most efficient malaria vectors in the world (CDC, 2015; EFMOH, 2016). *A. gambiae* mosquitoes breed in a wide range of habitats, including small water collections such as hoof-prints, water-filled holes in rocks and trees, as well as dams, river beds and lake shores. Because *A. gambiae* vectors can breed in so many different habitats, they are responsible for much of the malaria transmission in Africa. It is not possible to measure directly the lifespan of mosquitoes in nature.

However, estimates of daily survival of *A. gambiae* in Tanzania ranged from 0.77 to 0.84 meaning that at the end of one day between 77% and 84% will have survived; this also means that more of mosquitoes will survive longer than 14 days intrinsic incubation period (Charlwood *et al.*, 1997). Since mosquitoes can have many generations per year high levels of resistance can rise very quickly. There are over 125 mosquito species with documented resistance to one or more insecticides (CDC, 2015; EFMOH, 2016). This enhances the chance of transmitting malaria. Some *Anopheles* species are poor vectors of malaria. They have an immune response that encapsulates and kills the parasites after they have invaded the mosquito's stomach wall. Female mosquitoes require blood meals for ovulation. Only thirty to fifty of the approximately four hundred and fifty known species of *Anopheles* transmit malaria and the mosquito host does not noticeably suffer from the presence of the parasites in them (CDC, 2015; EFMOH, 2016).

After repeated application of insecticides, the mosquitoes develop insecticideresistance, which means that they are no longer killed by the insecticides. This means a large number of mosquitoes will survive in the community, and the risk of malaria infections rises and many people can be affected (EFMOH, 2016).

2.2.3 Human factors

Human biologic characteristics and behavioural traits can influence an individual's risk of developing malaria and on a larger scale, the intensity of transmission in a population. The biologic characteristics are genetic factors and acquired immunity.

Genetic factors: People with the sickle cell traits (HbAS) are relatively protected against *P. falciparum* malaria and thus enjoy a biologic advantage termed balanced polymorphism (Graben and Beryl, 2001). It has also been found that persons who

are negative for the Duffy blood group have red blood cells that are resistant to *P. vivax* infection. Since majority of Africans are Duffy negative, *P. vivax* infections is rare especially in West Africa. In this area, the niche of *P.vivax* has been occupied by *P. ovale,* a very similar parasite that infect Duffy- negative persons. Persons with β- thalassaemia, class I (HLA-BW 53) and class II (HLA-DR B1 1302) human leucocytes antigen (HLA) and newly horn infants with HbF in their red blood cells have protection against severe malaria. Ovalocytosis also protects against *P. falciparum* and *P. vivax* infections while IL. 4-524T allele increases resistance to malaria (Cheesbrough, 2010).

Acquired immunity: After repeated attacks of malaria a person develops a partially protective immunity. Such persons though still susceptible to malaria parasites but do not develop severe diseases and frequently lack typical malaria symptoms. This is termed Immunological premunition (Doolan *et al*., 2009). In areas with high *P. falciparum* transmission (most of Africa south of the Sahara), newborns are protected during the first few months of life presumably by maternal antibodies transferred to them through the placenta. As these antibodies decreases with time, these young children become vulnerable to diseases and death by malaria. If they survive to an older age (2-5 years) they will have reached a protective semi-immune status. Thus in high transmission areas, young children are a major risk group and targeted preferentially by malaria control interventions (Doolan *et al*., 2009; CDC 2015; EFMOH, 2016).

In areas with lower transmission (such as Asia and Latin America), infections are less frequent and a larger proportion of the older children and adults have no protective immunity. In such areas, malaria syndromes can be found in all age groups, and epidemics can occur. Pregnancy decreases immunity against many

infectious diseases. Hence, women who have developed protective immunity against *P.falciparum* tend to lose this protection when they become pregnant (especially during the first and second pregnancies) (Doolan *et al*., 2009; CDC, 2015; EFMOH, 2016).

Behavioural factors: Human behavior, often dictated by social and economic reasons can influence the risk of malaria for individuals and communities. Poor rural populations often cannot afford the housing and nets that would protect them from mosquitoes. These persons often lack the knowledge to recognize malaria and to treat it promptly and correctly. Often, cultural belief result in use of traditional methods of treatment with doubtful effects. Poverty and inaccessible health care facilities limit the compliance of the populace to malaria control programmes (Heggenhougen *et al*., 2003; EFMOH, 2016).

Agricultural mechanization, land reclamation irrigation programmes and mining markedly increase the number of mosquito bites. Agricultural work such as harvesting may force increased nighttime exposure to mosquito bites. On the other hand, animal husbandry near the household may provide alternative source of blood for zoophilic *Anopheles* mosquitoes and thus decreases human exposure. War, migrations (voluntary or forced) and terrorism may expose non-immune individuals to high malaria transmission environment (CDC, 2015; EFMOH, 2016).

2.2.4 Parasitic factors

Largely based on PCR results, the number of species of human malaria parasites has increased from four to six. The species now comprise of *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, the newly recognized species *P. ovale*

curtisi(former classic type) and *P. ovale wallikeri* (former variant type) (Singh *et al*., 2004; Sutherland *et al*., 2010; Hans-Peter and Noed, 2014).

P. falciparum the agent of severe and potentially fatal malaria is found mainly in the hotter and more humid regions of the world. It is the main species found in tropical countries. *P vivax* is capable of developing in mosquitoes at lower temperature and therefore has a wide distribution in temperate and subtropical areas. It is the main species in South America and is also found in East Asia; tropical and subtropical Africa. *P. malariae* has a much lower prevalence than *P. falciparum* and *P. vivax*. It is found in tropical and subtropical regions. It accounts for up to 25% of *Plasmodium* infections in Asia. *P ovale* has a low prevalence, it accounts for up to 10% of malaria infections in West Africa and has also been reported in other parts of Africa, Philippines, Indonesia, China, Far East, South East Asia and South America. *P. vivax* and *P. ovale* have hypnozoites which can cause relapse months or years after apparently successfully control efforts or can introduce malaria in an area that was malaria free *P. malariae* causes long-lasting infections that can persist asymptotically in the human host even a life time if left untreated. *P. falciparum* (and to a lesser extent *P.vivax*) have developed strains that are resistant to antimalaria drugs and these are considered one cause for repeated episodes of malaria because immunity built up against one strain is not necessarily protective against another. Such strains are not uniformly distributed (CDC, 2015; EFMOH, 2016).

Malaria transmission has been eliminated in many countries of the world since 1946. These include the United States and countries of Western Europe. However, Cases

of malaria still occur in these countries mostly in returning travelers or immigrants (imported malaria) (CDC, 2004).

2.3 Pathophysiology and Clinical Course

The characteristic feature of malaria is fever, caused by the release of toxins (when erythrocytic schizonts and the infected erythrocytes rupture) which stimulate the secretion of cytokines from the leucocytes and other cells. Clinical illness associated with malaria is caused by the erythrocytic stage of the parasite. When infected erythrocytes and mature erythrocytic stage schizonts rupture, merozoites along with haemozoin pigment and other toxic factors such as glucose phosphate isomerase (GPI) are dumped in the blood stream. These stimulate macrophages and other cells to produce cytokines such as interleukin 1 (ILI) and Tumor necrosis factor alfa (α-TNF). This causes the release of prostaglandin which interferes with the temperature regulatory function of the hypothalamus causing vasoconstriction of the cutaneous blood vessels and preventing heat loss through the skin. This leads to fever, chills, sweat weakness and other systemic symptoms associated with malaria. *P. falciparum*-infected erythrocytes (IES) adhere to the endothelium of capillaries and post capillary venules on the hyaluronic acid (HA) and chondroitin sulfate A (CSA) receptors on the epithelial cells of the endothelium of capillaries and post capillary venules and appear to be involved in mediating parasite accumulation in the placenta. This is the cause of cerebral malaria(Kwiatkwoski *et al*., 1989; CDC, 2015). In the intestines it can cause ischemia and ulceration leading to gastrointestinal bleeding and to bacteremia secondary to the entry of intestinal bacteria into the systemic circulation. The severity of malaria associated anaemia tends to be related to the degree of parasitaemia, haemolysis or phagocytosis of

parasitized erythrocytes, phagocytosis of uninfected erythrocytes and an auto immune haemolytic anaemia. Active malaria infections also through unknown mechanisms induce bone marrow dyscrasias and suppress normal development. Haemolysis may also occur after the use of certain antimalarials (especially primaquine) in patients with glucose-6-phosphate dehydrogenase deficiency (Kwiatkwoski *et al*., 1989; Schofield and Hackett, 1993; Oyerinde, 1999).

The incubation period following the infective bite by the female *Anopheles* mosquito varies according to the species of the parasite, the size of the dose of the sporozoites received and the resistance of the host. The average figures are 12 days for *P. falciparum* and 30 days for *P. malariae.* During this period, the pre-erythrocytic cycle as well as few erythrocytic cycles occurs. Infection with malaria parasite may result in a variety of symptoms ranging from absent or very mild symptoms to severe disease and even death (CDC, 2015). The severity of the symptoms depends on factors such as the species of the infection parasite, the human's acquired immunity and genetic background. *P. malariae* causes a long-lasting, chronic infection (can last a life time in some cases) and serious complication such s the nephritic syndrome in some patients. *P. vivax* and *P. ovale* have "hypnozoites" that can activate and cause "relapse" several months or years after the infecting mosquito bite (Kwiatkwoski *et al*., 1989; Schofield and Hackett, 1993; Oyerinde, 1999; CDC, 2015).Malaria disease has been categorized as uncomplicated or complicated (severe) as discussed below.

2.3.1 Uncomplicated Malaria

The classical (but rarely observed) malaria attack lasts six to ten hours. It consists of a cold stage characterized by sensation of cold and shivering (this phase that lasts for ten to thirty minutes depending on the species of the malaria parasite), a hot stage characterized by a rise in body temperature to a peak of about 40^0C (105^0 F), fever, headaches, vomiting, seizures in young children. This lasts for between three and eight hours and finally a sweating stage when there is sweat, return to normal temperature and tiredness. These attacks occur at regular interval governed by the length of the erythrocytic cycle of the *Plasmodium* species involved. Thus, *P. vivax* and *P. ovale*, which completes their erythrocytic cycle in 48 hours, produce fever third day ("tertian"malaria). *P. malariae* completes its erythrocytic cycle in 72 hours and so produce its attack every fourth day ("quartan" malaria). *P. falciparum* during the irregular cycle produces its attack irregularly between 36 and 48 hours. Double or mixed infection running concurrently with overlapping schizogonic cycle produces its attack irregularly between 36 and 48 hours. Patients more commonly present with a combination of fever, Chills, sweats, headaches, nausea and vomiting, body aches and general malaise at about 11 days after the sporozoites inoculation when there are an estimated 20-50 parasites/µl of blood that are detectable by microscopy (White, 1998; Oyerinde, 1999). The fever usually begins two days later, when an adult harbors about 10^8 malaria parasites in the body (20-20,000 parasites/µl). However, in endemic areas people with partial immunity usually tolerate parasitaemia up to 10,000/µl or 10^7 per ml of blood without feeling ill (Armstrong-Schellenberg *et al*., 1994; Smith *et al*., 1994).

Physical findings may include elevated temperature, perspiration, weakness and enlarged spleen. In *P. falciparum* malaria, additional findings may include mild jaundice, enlargement of the liver and increased respiratory rate (CDC, 2015).

2.3.2 Complicated or Severe Malaria

Severe malaria occurs most often in persons who have no immunity to malaria or whose immunity has decreased. These include all residents of areas with low or no malaria transmission, young children and pregnant women in endemic areas (in all cases, severe malaria is a medical emergency and should be treated urgently and aggressively (CDC, 2006).

Most complications attributed to malaria infection are mainly due to *P. falciparum* but not exclusively. Cytoadherence and sequestration are central to the pathophysiology of *P. falciparum* infection. This occurs mainly in the venules of vital organs including the brain, heart, kidney and intestine. Also, *P. falciparum* has the ability of invading red cells of all ages (Miller *et al*., 1994; White, 1998; WHO, 2015). The manifestations of severe malaria include:

Cerebral malaria: Malaria with coma persisting for > 30 minutes after a seizure is considered to be cerebral malaria (WHO, 2010).It is Characterized by abnormal behaviour, impairment of consciousness, seizures, coma or other neurological abnormalities and death. This is a very serious complication of *P. falciparum* infection but can be induced by *P. vivax* and *P. malariae*. Children usually become restless but mainly there is loss of appetite, convulsion and haemolysis(CDC, 2015).The basic underlying defect seems to be clogging of the cerebral micocirculation by the parasitized red cells. These cells develop knobs on their

surface and develop increased cytoadherent properties, as a result of which they tend to adhere to the endothelium of capillaries and venules. This results in sequestration of the parasites in these deeper blood vessels. Also, rosetting of the parasitized and non-parasitized red cells and decreased deformability of the infected red cells further increases the clogging of the microcirculation. Obstruction to the cerebral microcirculation results in hypoxia and increased lactate production due to anaerobic glycolysis. The parasitic glycolysis may also contribute to lactate production. The adherent erythrocytes may also interfere with gas and substrate exchange throughout the brain. Increased cerebral anaerobic glycolysis, intereference with neurotransmission by sequestered and highly metabolically active parasites have been blamed for the mechanism of coma (Andrej *et al*., 2003).

Severe anaemia: In accordance with the WHO definition, severe malarial anaemia is defined in the presence of a haemoglobin level of < 5 g/dL, or haematocrit < 15% (WHO, 2010). The severity of malaria associated anaemia tends to be related to the degree of parasitaemia, haemolysis or phagocytosis of parasitized erythrocytes, phagocytosis of uninfected erythrocytes and an auto immune haemolytic anaemia. Active malaria infections also through unknown mechanisms induce bone marrow dyscrasias and suppress normal development. Haemolysis may also occur after the use of certain antimalarials (especially primaquine) in patients with glucose-6-phosphate dehydrogenase deficiency (CDC, 2015).

Haemoglobinuria (Black water 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 (Bartoloni and Zammarchi, 2012).It results from acute haemolysis of erythrocytes

with the liberation of haemoglobin into the plasma. It is one of the complications of *P. falciparum* infection. Other suspected contributing factors include administration of irregular or overdose of quinine, Glucose-6-phosphate dehydrogenase deficiency and autohaemolysis. It is characterized by sudden attack of chills fever and passage (after a few hours) of brown or almost black urine. The disease usually terminates fatally in severe cases (CDC, 2015).

Bilious remittent fever: It is characterized by Jaundice. The liver usually enlarged and tender bile is passed in the urine and vomitus. The characteristic symptoms include abdominal pain, nausea and vomiting. There may be diarrhea and or dysentery (Bartoloni and Zammarchi, 2012).

Algid malaria: This results when the adrenals are involved. There is sudden fall in temperature, may be associated with abdominal pain, vomiting, diarrhea, generalized vascular collapse, shock and terminating fatally (CDC, 2015).

Malaria hyperpyrexia: Occurs as a result of damage to the heat controlling mechanism of the hypothalamus by endogenous pyrogens (cytokines) and prostaglandins released at the end of the erythrocytic cycle. The temperature rises quickly to 108°F or higher. There is coma and death unless prompt treatment is instituted (CDC, 2015).

Malaria in pregnancy: Malaria during pregnancy (especially *P. falciparum*) may cause premature delivery or low birth weight (LRW) babies.

Other manifestations include pulmonary edema or acute respiratory distress syndrome (ADRS), which may occur even after the parasite count has decreased in response to treatment. On rare occasions, *P. vivax* infection can cause rupture of the

spleen or ARDS. There may be abnormalities in the blood coagulation thrombocytopenia, cardiovascular collapse and shock acute renal failure, metabolic acidosis, often in association with hypoglycemia (may also occur in pregnant woman with uncomplicated malaria, or after treatment with quinine).Other manifestations Include Nephrotic syndrome resulting from or repeated infections with *P. malariae*, hyper reactive malarial spleenomegaly known as "tropical spleenomegaly syndrome" occurs infrequently and is attributed to an abnormal Immune response to repeated malaria infections. Tropical spleenomegaly syndrome is marked with much enlarged spleen and liver, abnormal immunologic findings anaemia and a susceptibility to other infections such as skin or respiratory infection (Oyerinde, 1999; Krogstad, 2000; Muhilberger *et al*., 2004; CDC, 2015).

2.4 Haematological Effects of Malaria

Malarial parasitaemia has been found to cause significantly lower white blood cells, red blood cells, platelets and haemoglobin levels while a parallel trend in thrombocytopenia with parasitaemia was found to be associated with both P. *falciparum* and *P vivax* infection. Persons with platelets of <150, 000/µL were 12-15 times more likely to have malaria than persons with platelet counts ≥150,000µl (Erhart *et al*., 2004). However, asymptomatic malarial infection does not much influence haemoglobin concentration (Stoltzfus *et al.,* 1997) thrombocytopenia is almost invariable in malaria. However, severe thrombocytopenia (<50 x 10^9) is rare. Though activation of the coagulation cascades has been suggested in acute infection particularly in adults, factor XIII is inactivated during malaria infection. Therefore patients may present with bleeding at infection sites or mucous membranes (Weatherall *et al*., 2002). Parasitized cells also lose their deformability and are

rapidly phagocytosed and destroyed in the spleen. The production of red cells in the bone marrow is also reduced and there is slow reticulocyte response. An immune destruction of non-parasitized red cells also occurs. A combination of these factors leads to anaemia. Consequently, there may be difficulty in determining the blood group because of auto agglutination of the person's red cells (Cheesbrough, 2010).

2.5 Diagnosis

Prompt and accurate diagnosis of malaria has been identified as the key to the effective management and timely treatment of the patient and prevention of further spread of infection in the community (CDC, 2015). Diagnosis of malaria can be very difficult especially in non-endemic settings where health care providers are not familiar with (or often forget) the clinical manifestations of the disease and do not order the needed diagnostic test. In some areas, malarial transmission is so intense that a large proportion of the population is infected but not made ill by the parasites. In such instances finding malaria parasites in an ill person does not necessarily mean that the illness is caused by the parasites (CDC, 2015). In many malaria-endemic countries, lack of resources is a major barrier to reliable and timely diagnosis. Health personnel are under trained; under equipped and underpaid they often face excessive patient's loads, and must divide their attention between malaria and other equally severe infectious disease such as pneumonia diarrhoea, tuberculosis and HIV/AIDS (CDC, 2015). However, malaria presents a diagnostic challenge to laboratories of both developed and developing countries. Endemic malaria, population movements, and travelers coupled with the changing patterns of morphological appearances of malaria species due to drug pressure or strain variation, also contribute to this challenge (Moody, 2002). There has been a rapid

development in the field of malaria diagnosis in the last couple of decades and currently, several malaria diagnostic options are available although, most of these options are feasible only in the research laboratories in the developed world. Current methods of malaria diagnosis include light microscopy, fluorescent microscopy and Quantitative Buffy Coat (QBC), flow cytometry, automated blood cell analyzers, detection of antigens and antibodies, molecular methods, laser adsorption mass spectrometry and Microarrays (Murray *et al*., 2003; Tangpukdee, 2009; Lie *et al*., 2016; Nam *et al*., 2016)).Diagnosis of malaria is both clinical (presumptive) and laboratory (definitive)

2.5.1 Clinical diagnosis

Clinical diagnosis is based on the patients' symptoms and on physical findings at examinations. Signs attributed to malaria are non-specified and are also found in other diseases such as the "flu" and common viral infections. Likewise, the physical findings are often not specific. The symptoms of malaria include fever, chills, sweat, headache cough catarrh, muscle pains, diarrhoea, yellowness of eyes, nausea and vomiting. The signs (physical findings) include pyrexia, Palor, jaundice, spleenomegaly, Hepatomegaly, and bone/muscle tenderness. In severe malaria there may be confusion coma, neurologic focal signs, severe anemia and respiratory difficulties. These clinical findings in malaria are usually not typical and need to be confirmed by a laboratory test (CDC, 2015).

2.5.2 Laboratory diagnosis

The laboratory diagnosis of malaria could be achieved through the following techniques:

Conventional microscopy: Malaria parasites could be demonstrated in the patient's blood either by the time honored conventional microscopy or fluorescent microscopy. Microscopic examination of blood smears stained with Giemsa, Wright's, or Field's stain has been the method of malaria diagnosis ever since the discovery of malaria parasites by Laveran in 1880 (Sheman, 1998) and introduction of the thick smear by Ronald Ross in 1903 (Fabre *et al*., 2004).

The method is simple, does not require highly equipped facilities and often enables differentiation among four human *Plasmodium* species while providing parasites density and stage (Murray *et al*., 2003). Although, blood obtained by pricking of finger or earlobe is the ideal because of the higher density of developed trophozoites or schizonts in blood from these capillary rich areas (Giles, 1993). Blood obtained from venipuncture and stored with EDTA may conveniently be used for malaria diagnosis (Moody, 2002).

Thick blood film concentrates the layers of RBCs by 20-30 times and provides enhanced sensitivity detecting up to five parasites/µl (Lanar *et al*., 1989). Thus, it is better suited to detect low level of parasitaemia, recrudescence or relapse. However, lysis of RBCs during the staining may make species identification more difficult. Thin blood film provides the fixed monolayer of RBCs with preserved morphology and makes this procedure more specific than thick film in identifying the *Plasmodium* species. However, thin blood film is much less sensitive than the thick blood smear (Moody, 2002).

Although several methods of estimation of parasitaemia have been described using either the thin or thick blood films (WHO, 1984), there is no single, universally

accepted standard method for the quantification of parasites and parasitaemia is often estimated using different methods, making it difficult to compare the performance of the different diagnosis tools (Hanscheid, 1999). A well stained and not too thick area of film is examined using the oil immersion (x100) objective of the microscope. 10-50 high power (x100 objective) microscope fields are examined for parasites and the approximate number of parasites (trophozoites, Schizonts and gametocytes) are reported. Malaria pigments if seen in white blood cells are also reported. The parasites number are normally reported as

1. + for 1-10 parasites per 100 high power fields.
2. ++ for 11-100 parasites per 100 high power fields.
3. +++ for 1-10 in every high field.
4. ++++ for more than 10 in every high power field (Cheesbrough, 2010).

The parasites number/µl of blood can be estimated in a thick film by multiplying the average number of parasites per high power field by 500 after examining 10-50 fields. This method was found to be more accurate and quicker than counting the parasites against white cells (Cheesbrough, 2010). In high parasitaemia however, the percentage of red blood cells are estimated by counting the number of parasitized red blood cells in eight fields (approximately 200 cells) and divining by 20 in an area where the total number of red cells is approximately 250 per field (Cheeshrough, 2010).

Routine microscopy, when examined carefully by an expert microscopist is a sensitive (can detect as low as 10-30 parasites per µl of blood) (Giles, 1993),

relatively inexpensive (US $0.12 - 0.40 per slide examined), can differentiate among various species and also provide a permanent record useful for quality control (Wongsrichanalai *et al*., 2007). This conventional light microscopy of a well-prepared and well-stained thick and thin blood films has thus stood the test of time and is still considered the 'gold standard' for malaria diagnosis (Warhurst, *et al*., 1996) as no other reliable and relatively rapid method for the detection and quantification of the parasite burden has been available (Farcas *et al*., 2004).

However, there is no international agreement on the precise number of microscopic fields to be examined (Chiodini *et al*., 1999) and the microscopic 'gold standards' varied widely (Hanscheid andValadas, 1999) making it difficult to make valid comparisons. It is a labour intensive, time-consuming procedure requiring well-trained and well-supervised technicians, a condition often difficult to meet in many developing countries particularly in rural settings (Wongsrichanalai *et al*., 2007). There are often long delays in providing the diagnostic results to the clinicians and clinical decisions are often taken without the benefit of the results. Moreover, substantial number of patients with low levels of parasitaemia may be missed by this method (Makler *et al*., 1998). Screening the blood donor by this method cannot reliably prevent transfusion transmitted malaria as many asymptomatic blood donors in endemic harbor the parasites at low levels which may not be detectable by routine microscopy (Schindler *et al*., 2001) but still be high enough to cause disease in the blood recipients. Moreover, the sensitivity actually achieved in the busy routine examination clinics is 10 fold or more lower (-100 parasites/µl) than that achieved in the research laboratories by expert microscopists (Wongsrichanalai *et al*., 2007).

This technique remains the gold standard for laboratory confirmation of malaria. However, it depends on the quality of the reagents, microscope and on the experience of the laboratorian to process, examine and report malaria blood films with minimum delay. Standardized and adequately controlled methods for preparing, staining and reporting blood films should be used. Also, a "safe code of practice" should be followed when collecting and handling blood specimens and disposing of contaminated lancets, syringes and needles (CDC, 2015).

Malaria blood films are best prepared directly from capillary blood. However, EDTA (sequestrene) anticoagulated venous blood can also be used provided the blood films are made soon after collection. Blood should be collected as soon as malaria is suspected and before the patients receives anti-malarial drugs (Cheesbrough. 2010). Both thick and thin blood films are essential for the diagnosis of malaria parasites. The thin film provides a monolayer of red blood cells which remain intact after staining and is used for identification of the parasites, in the thick films, the red blood cells are lysed before staining and only the blood cells, platelets and parasites are visible. Thick films allow a larger amount of blood to be examined thereby concentrating the parasites and increasing the possibility of detecting light infections. Unfortunately, increased distortion of the parasites makes species identification difficult using the thick film. Proper use of this technique usually requires a great of expertise and experience. Giemsa stain gives the best staining of malaria parasites in thin films and differentiation of the different species in thin film is excellent (Patrick *et al*., 2005: Cheesbrough. 2010).

Quantitative Buffy Coat (QBC) and fluorescent microscopy: Alternative microscopic methods for malaria diagnosis have been introduced with the hope of

overcoming the limitations of conventional microscopy. QBC® (Quantitative Buffy Coat) (Beckton Dickinson, Franklin Lakes, NJ) is one such system (Rickman *et al*., 1989). In QBC, 55-65ul of whole blood is centrifuged at 12,000 rpm x g for 5 minutes in a commercially supplied capillary tube coated internally with acridine Orange (AO) (Wardlaw and Kumuda, 1987; Tangpukdee, 2009; Mannali *et al*., 2012). The tube is then placed on the paraviewer ® microscopic tube holder and scanned for parasites in the interface between RBCs and granulocytes using a standard white light microscope equipped with the ParaLense ® U-V Microscopic adaptor. Since Acridine orange (AO) has an affinity for the nucleric acids, it differentially stains the malaria parasites (Kawamato and Kumuda, 1987; Tangpukdee, 2009; Mannali *et al*., 2012) and since malaria infected RBCs, being lighter than the normal RBCs but heavier than the granulocytes, concentrate in the upper most layer of the erythrocytes during centrifugation (Woo and Hauck, 1987; Tangpukdee, 2009; Mannali *et al*., 2012). Moreover, a cylindrical float that has the same density as that of WBCs is also inserted into the capillary tube and thus compresses the Buffy coat and the lighter protion of the RBCs against the wall of the capillary tube during centrifugation (Spielman *et al*., 1988; Mannali *et al*., 2012). Thus, a large volume of potentially infected blood could be observed through the capillary tube using a special, long-focal-length objective (paralens) with a fluorescence microscope (Spielman *et al*., 1988; Clendeman *et al*., 1995; Tangpukdee, 2009; Mannali *et al*., 2012).

Several trials using QBC techniques have been performed under laboratory and field conditions (Spielman *et al*., 1988; Rickman *et al*., 1989; Clendeman *et al*., 1995; Lema *et al.*, 1999; Pinto *et al*., 2001; Mannali *et al*., 2012). It has been claimed that the malaria diagnosis by QBC was unambiguous (Spielman *et al*., 1988), easier to

use and faster than the conventional microscopy. The QBC often detected more malaria cases than the microscopy and thus may have higher sensitivity than conventional microscopy Spielman *et al*., 1988; Clendeman *et al*., 1995; Lema *et al.,* 1999; Pinto *et al*., 2001; Manali *et al*., 2012).

The malaria detection limit of the QBC was substantially lower than that of the microscopy and QBC detected infection when concentration of samples was halved a mean of 3.3 times beyond the dilution detectable by conventional microscopy (Spielman *et al*., 1988). However, negative QBC results in samples having high parasitaemia have also been reported (Lowe *et al*., 1996). QBC is more technically demanding and requires specialized equipment to separate the cell layers by centrifugation and a good fluorescence microscope and is costly (-2.25$ per test vs. $0.02 for field stain) (Lema *et al.,* 1999). There is no standard method of parasite quantification in QBC although attempts have been made to express relative quantity of parasites in the samples. Lema *et al.* (1999) graded the parasitemia as 'few' (≤2 parasites per field) ≥, 'Moderate' (3-5 parasites per field) or "many" (> 5 parasites per field). Alternatively, Pinto *et al.* (2001) used the plus (+) system as follows.

+ (1+) <1 parasite per QBC field

++ (2+) 1-10 parasites per QBC field

+++ (3+) 11-100 parasites per QBC field

++++ (4+)>100 parasites per QBC field.

However, the practical significance of these classifications in terms of their correlation with in vivo parasitaemia is unknown.

In spite of some reservations about the use of QBC for malaria diagnosis, it remains a viable and rapid alternative to Giemsa staining as a screening test because of its higher sensitivity in order to eliminate negative slides that generally predominate in surveys. The conventional method used for the diagnosis is the thick-and-thin peripheral blood smears (PBS). Modified quantitative buffy coat (QBC) method is less expensive than routine QBC and the sample is easier to prepare than microscopy. The availability of competent technician to make appropriate smears for correct interpretation of results is a disadvantage of PBS microscopy which is also a very tedious and time-consuming method.The time required for the routine QBC^{TM} technique in detecting malarial parasites is 8–15 minutes which is a definite advantage over PBS microscopy which requires more than 60 minutes of sample examination.The QBC detection limit is approximately five parasites/μl of blood and establishes diagnosis earlier than the PBS microscopy which limits the detection in low parasitaemia cases to 50–100 parasites/μl by an average microscopist (Tangpukdee *et al*., 2009).QBC technician can be trained in only 3–5 days as compared to a microscopist who requires years of experience before doing the detection optimally. In the QBC method, the staining is easily done by just rolling the QBC tubes unlike in PBS microscopy which involves a tedious method of staining.However, the main disadvantage of routine QBC is that it is very expensive and modifying the QBC renders it much less expensive (Tangpukdee *et al*., 2009; Manali *et al*., 2012).

Immunodiagnosis: The majority of immunodiagnostic methods have been used as aids in the diagnosis of malaria. These methods include serologic tests that are based on the detection of specific antibody response to the presence of the parasites. The serologic procedures commonly used include immunoflourescence antibody test

(IFA) and enzyme linked immunosorbent assay (ELISA). Serology does not detect only current infection but also past experience (CDC, 2015). Serologic findings usually remain positive for about one year even after complete treatment and there could be cross-reaction with other plasmodia (Patrick *et al*., 2005).

In contrast to antibody, antigen detection is a more appropriate marker for the presence of active infection. Several commercial test kits are now available for detection of antigens derived from malaria parasites. "Immunochromatographic" tests most often use a dipstick or cassette format, and provide results in 2-15 minutes. These are therefore referred to as “Rapid Diagnostic Tests (RDTs)”. They offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available (CDC, 2015).

The use of RDT minimizes delay in diagnosis. It is recommended that all RDTs are followed-up with microscopy to confirm the results and to quantify the proportion of infected red blood cells. This is because RDT may not be able to detect some infection with lower parasites density. Also there is insufficient data to determine the ability of this test to detect the two other species of malaria, *P. ovale and P. malariae.* However, before malaria RDTs can be widely adopted, several issues must be addressed i.e. improving their accuracy, lowering their cost and ensuring their adequate performance under adverse field conditions (CDC, 2015). Antigens detected by malaria RTDs include HRP2 (Histidine-rich protein), species-specific lactate dehydrogenase enzyme (pLDH) and aldolase enzyme.

HRP-2 is a water-soluble protein produced by asexual stages and young trophozoites of *P. falciparum* only. It is expressed in abundance on the surface membrane of

infested erythrocytes. However, it has been shown to persist for 7-28 days after successful treatment.

The enzymes pLDH and aldolase are metabolic enzymes in the glycolysis of malaria parasites produced by asexual stages and gametocytes of all four human malaria parasites species. The RDTs have been developed with monoclonal antibodies specific for *P. falcioparum* pLDH and monoclonal antibodies that are pan-specific (i.e recognize all four malaria species). This enables non *P. falciparum* infections to be distinguished from *P. faciparum* or mixed species infection. With the isolation of specific isomers of pLDH, species specific enzyme-based RDTs are becoming available. Monoclonal antibodies are produced against aldolase from *Plasmodium* species as well as *P. falciparum*. Aldolase pan-specific anti bodies do not have the same binding sensitivities for *P. falciparum* as HRP-2 and may be negative when an HRP-2 capture line is positive in low parasitaemia. The activity of the enzymes pLDH and aldolase correlates with active viable parasites and rapidly disappear with successive treatment. Hence, pLDHdetecting RDTs can be used for monitoring cure as well as initial detection (Cheesbrought, 2010).

HRP-2 is localized in the RBC cytoplasm and released into plasma in high amounts from the RBCs infected with *P. falciparum*, and may persist in blood up to 28 days after cure of infection and may not be a suitable target for devices that intend to monitor therapeutic response (Humar *et al*., 1997). Moreover, devices that target this antigen, could detect only *Plasmodium falciparum*. People in endemic area with low persistence detect only *P. falciparum*. People in endemic area with low persistence parasitemia may be positive but the clinical value of such result is doubtful whereas some individual may have a gene deletion for the production of HRP-2 and will never give HRP-2 positive results (Moody, 2002).

Parasite lactate dehydrogenase (pLDH), is produced by the viable blood stage parasites of all four human *Plasmodium* species and each species produces a distinct isomer (Moody, 2002; Saad *et al*., 2014). Thus, MRDDs that target this enzyme could detect all four species, differentiate falciparum from non-falciparum and may be suitable for monitoring therapeutic response (Smith *et al*., 1994). OptiMAL is a prototype of MRDD that targets this enzyme (Wongsrichanalai *et al*., 2007; Maha *et al*., 2012).

Plasmodium aldolase is also produced by all four species of *Plasmodium* and is an enzyme in glycolytic pathway (Moody, 2002). Monoclonal antibodies produced against *Plasmodium* aldolase are pan-specific and have been used in combination with HRP-2 to detect *P. falciparum* as well as other plasmodium species (Moody, 2002). Previous studies with MRDDs based on this enzyme demonstrated the low sensitivity of detecting *Plasmodium vivax*, particularly at low parasitaemia (Tjitra, 1999; Moody, 2002; Olusola *et al*., 2013; Dzaka *et al*., 2014; Pembele *et al*., 2015; Oyetunde *et al*., 2015).

Performances of the several MRDDs have been reviewed (Hanscheld, 1999; Moody, 2002; Murray *et al*., 2003; Wongsrichanalai *et al*., 2007; Olusola *et al*., 2013; Dzaka *et al*., 2014; Pembele *et al*., 2015; Oyetunde *et al*., 2015). These reviews of the earlier studies generally proved that the MRDDs have good sensitivity (>90%) and specificity (100%) for *Plasmodium falciparum* at parasite densities >100-500 parasites/µl of blood but sensitivity decline rapidly at lower parasite densities and the performance (sensitivity) for *Plasmodium vivax* is generally lower. A review (Murray *et al*., 2003) has shown that the overall sensitivity and specificity of Optimal varies from 62 - 99% and 84 - 94%, respectively. Similarly, the overall

sensitivity and specificity of ICT Pf/Pv varies from 63 - 84% and from 89 - 97%, respectively.

The Now® ICT P.f/P.v. (Binax, Inc, Portland, ME) that detects *P. falciparum* HRP-2 and pan-Plasmodium aldolase was evaluated in symptomatic patients attending the malaria clinics in endemic areas in Thalland (Wongsrichanalai *et al*., 2007). A similar product ParaSight f+v (Beckinson Dickinson) was also evaluated (Forney *et al*., 2003). The overall sensitivity and specificity of the Now® ICT P.f/P.v and ParaSight f+v were excellent (>90%). However, again as before the detection of plasmodium vivax was somewhat lower (sensitivity 87.3%). Similarly,studies in malaria endemic areas in India (Singh *et al*., 2003) and multiple hospitals in US (Palmer *et al*., 2003) were also very encouraging, the sensitivity approaching almost 100%. Although, the specificity was somewhat lower in field study in India continuous improvement in the latest versions of the MRDDs is encouraging and MRDDs may become a good screening tool for malaria diagnosis (Singh *et al*., 2003; Olusola *et al*., 2013; Dzaka *et al*., 2014; Pembele *et al*., 2015; Oyetunde *et al*., 2015).

Although some versions of the MRDDs have good sensitivity with high parasitaemia, their sensitivity is still low when parasitaemia is low (Ricci *et al*., 2000, Iqbal *et al*., 1999), they failed to detect gametocytes (Wongsrichanalai *et al*., 2007) and could not differentiate among various non-falciparum parasites. These antigen detection methods are fast and simple to perform and are good for use in clinical diagnosis. They are not yet reliable for conducting epidemiological studies, screening blood donors and monitoring drug effectiveness or development of resistance. Moreover, their high cost is the main limiting factor for their wider use

(Makler *et al*., 1998; Wongsrichanalai *et al*., 2007). Some rapid diagnostic tests (RDTs) especially those that detect both aldolase and pLDHwith potential for malaria parasites detection have been developed (Olusola *et al*., 2013; Dzaka *et al*., 2014; Saad *et al*., 2014; Oyetunde *et al*., 2015 Pembele *et al*., 2015).

Molecular diagnosis: Molecular diagnosis based on nucleic acid hybridization takes advantage of the fact that all organisms contain nucleic acid sequences that may be used in a hybridization assay to distinguish among general species and strains. This depends on the specificity of the nucleic acid probe used these nucleic acid-based detection systems are independent of the patients immunologic state or previous infection history, thereby identifying active infection. The development of target amplification techniques, such as the polymerase chain reaction (PCR), provides exquisite sensitivity, allowing the detection of as one organism in a biologic sample. This technique is more accurate than microscopy (Haditsch, 2004; CDC, 2006). However, it is expensive and requires a specialized laboratorian. A target sensitivity of 91-96% on circumsporozoites gene of *P. vivax* has been obtained through PCR using dried blood-spotted filter paper samples (Patrick *et. al., 2005).* However, PCR-based assays are poor at identifying mixed infection and need to be improved particularly for the correct identification of non-falciparum and mixed infections (Gatti *et al.*, 2007).

Molecular methods have gathered pace over the years and all major scientific disciplines have found molecular techniques increasingly useful in research, diagnosis, prognosis, and clinical management. The molecular revolution began in 1953 when Watson and Crick proposed the structure of double-stranded deoxyribonucleic acid (DNA) (Watson and Crick, 1953). Subsequently, purified

restriction enzymes were developed, which allows further manipulation of DNA (Nathans and Smith 1975). Development of southern blotting by EM Southern allows detection of sequences of DNA fragments separated by an agarose gel using radioactive probes (Southern, 1975). In 1987 a unique DNA amplification method called the polymerase chain reaction was developed, which was followed by the development of reverse transcriptase PCR (RT-PCR) for the amplification of RNA has been further developed in recent years to provide real-time fluorescent methods using taqman and molecular Beacon, chemistries and quantification of parasites by this approach has become possible (Lee *et al*., 2002; Fabre *et al*., 2004; Perandin *et al*., 2004).

Molecular techniques have been used in diagnosing *Plasmodium* for several years. Initial nucleic acid based probes were focused on *P. falciparum* targeting the 21-bp-repetitive sequences found throughout the *P. falciparum* genome (Franzen *et al*., 1984). Although, these probes were very specific (Barker, 1990), their sensitivity was lower than that of microscopy particularly with the patients having low parasitemia (Lanar *et al*., 1989). Subsequently, nucleic acid probes targeting species-specific rRNA molecules and labeled with radioisotope were used to diagnose the malaria and a sensitivity of nucleic acid based hybridization techniques (Chen e*t al*., 1991), a number of candidate DNA probes were found to be less useful than previously thought (Lanar *et al*., 1989) and efforts were switched to PCR once it became available (Weiss, 1995). Snounou *et al.* in 1993 used species-specific oligonucleotide primers targeted to 18S Small subunit of ribosomal RNA (SSU RRNA) gene of all human *Plasmodium* species for PCR and detected a large number of mixed infections (Snounou *et al*., 1993). The multiply 18S small subunit rRNA genes of *Plasmodium* species that infect human have been found to be highly

stable and conserved do not cross react to human or other human pathogens' DNA or RNA (Snounou *et al*., 1993; Waters, 1994).

Several polymerase chain reaction (PCR) assays have been developed and evaluated for diagnosing malaria most often based on genus or species specific sequences of the parasites' 18S small subunit rRNA gene (Snounou *et al*., 1993; Rubio *et al*., 1999; Singh *et al*., 1999) and generally PCR particularly the nested PCR was found much more sensitive and specific than those of the other diagnostic methods when compared either with microscopy or rapid diagnostic tests (Weiss, 1995; Rubio *et al*., 1999; Singh *et al*., 1999; Tham *et al*., 1999). Moreover, multiplex PCR, where more than one target sequences can be amplified by including more than one primer sets in a single reaction and thus potentially could save time and effort has been shown to be a valuable tool in infectious disease diagnosis could also theoretically be used to diagnose malaria (Elnifro *et al*., 2000).

However, loss of sensitivity due to differences in sample collection and storage (Farnert *et al*., 1999) and geographical differences in the sensitivity of PCR has been reported (Jelinek *et al*., 1996). Furthermore, multiplex PCR has often failed (Snounou *et al*., 1993, Singh *et al*., 1999) or found to be less sensitive for malaria diagnosis (Jelinek *et al*., 1996). Similarly, although nested PCR increases the sensitivity (Singh *et al*., 1999), it also requires multiple cycles and thus could be inefficient and increases the risk of contamination (Morassin *et al*., 2002).

Real time PCR in malaria diagnosis was first reported in 2001 for quantifying the parasitaemia during the *Plasmodium falciparum* infection (Hermsen *et al*., 2001) and was then thought to be only a potentially interesting method (Hanscheid, 2003).

However, it has been used in recent years for the routine diagnosis of malaria (Fabre *et al*., 2004). Real time PCR has several advantages over the conventional PCR. Real time PCR uses capillary tubes that are better for heat spreading than conventional tubes and thus the amplification step is shortened. Final visualization by electrophoresis or hybridization is not required since amplicon production is measured by fluorescence. Moreover, operating the PCR reaction in sealed vials dramatically limits the risk of contamination (Fabre *et al*., 2004). Recent studies indicated that the real time PCR is more sensitive than microscopy and QBC, more specific than the currently available RMDDs, faster and easier to use than conventional PCR and certainly quantifiable (Fabre *et al*., 2004).Consequently, various modifications of the molecular tests are continually being developed and proposed. Recently, a two-stage sample-to-answer system based on nucleic acid amplification approach for timely (results within 1 hour) and highly sensitive (<1parasite/µl of blood) detection of malaria parasites have been developed (Liu *et al*., 2016). Also, a high-throughput malaria parasite separation using a viscoelastic fluid for an ultrasensitive PCR has been proposed (Nam *et al*., 2016). However, the affordability of these techniques in low resource settings like ours is doubtful for reasons ranging from the poverty level of these malaria endemic countries to political issues.

2.6 Treatment

Malaria is an entirely preventable and treatable disease. The primary objective of treatment is to ensure a rapid and complete elimination of the *Plasmodium* parasite from the patient's blood in order to prevent progression of uncomplicated malaria to severe disease or death, and to chronic infection that leads to malaria-related

anaemia (WHO, 2015).Treatment of malaria is based on the history regarding travel to endemic areas, prompt clinical review and differential diagnosis, accurate and rapid laboratory work and correct use of anti malaria drugs (Patrick *et al.*, 2005). Presumptive treatment based on clinical suspicions without laboratory confirmation is commonly practiced in highly endemic areas through it allows the treatment of potential fatal disease but often leads to misdiagnosis and mistreatment due to overlap between symptoms of malaria and other frequent disease especially acute lower respiratory tract infection (Red, 1992). Effective treatment of malaria consists of supportive treatment and rational malaria chemotherapy (Ogala, 1998).

There are guidelines for treatment of malaria in different parts of the world. In the United States, Chroquine phosphate is the drug of choice for treatment of uncomplicated malaria and Quinine phosphate for Chloroquine resistant or unknown resistance malaria while quinine Gluconate plus Doxycyline or tetracycline or Clindamycin is recommended for severe malaria (CDC, 2015). In Nigeria, Artemether-Lumefantrine (AL) is the drug of choice for treatment of uncomplicated malaria while quinine is recommended for severe malaria (FMOH, 2005; CDC, 2015). Supportive measures include hydration, administration of antipyretics and or analgesics, anti emesis and blood transfusion in cases of severe anaemia. In all cases, bed rest and adequate feeding must be ensured to maintain caloric requirement of the patient (Ogala, 1998; CDC, 2015).

2.6.1 Antimalarial drugs resistance

Antimalaria drugs resistance has been defined as the ability of a parasite strain to survive and multiply despite the administration and absorption of a drug given in does equal to or higher than those usually recommended but within tolerance of the

subject. This definition was later modified to specify that the drugs in question must gain access to the parasite or the infected red blood cell for the duration of time necessary for its action (Bruce-Chwatt *etal*., 1986).In order for a case to be defined as resistant, the patient under question must have received a known and observed anti-malarial therapy whilst the blood drug and metabolite concentrations are monitored concurrently.

Chloroquine-resistant forms of *Plasmodium falciparum* malaria first appeared in Thailand in 1957 (Packard, 2007).Resistance to Chloroquine then spread rapidly through South America, Southeast-Asia to East Africa and eventually to all endemic countries of the continent. A similar process hashappened to Sulfadoxine–Pyrimethamine (SP) except that resistance to the drug in Africa is low in WestAfrica, and is beginning to increase in East African countries where Sulfadoxine–Pyrimethamine (SP) hasreplaced Chloroquine as the first line antimalarial drug (FMOH, 2005).

To date, parasite resistance to antimalarials has been documented in three of the five malaria species known to affect humans: *P. falciparum*, *P. vivax* and *P. malariae*(WHO, 2014).

There is no single factor that confers the greatest degree of influence on the spread of drug resistance, but a number of plausible causes associated with an increase have been acknowledged. These include aspects of economics, human behaviour, pharmokinetics, and the biology of vectors and parasites.

The most influential causes are examined below:

1. The biological influences are based on the parasites ability to survive the presence of an anti-malarial thus enabling the persistence of resistance and the potential for further transmission despite treatment. In normal circumstances any parasites that persist after treatment are destroyed by the host's immune system, therefore any factors that act to reduce the elimination of parasites could facilitate the development of resistance. This attempts to explain the poorer response associated with immunocompromised individuals, pregnant women and young children.
2. There has been evidence to suggest that certain parasite-vector combinations can alternatively enhance or inhibit the transmission of resistant parasites, causing 'pocket-like' areas of resistance.
3. The use of anti-malarials developed from similar basic chemical compounds can increase the rate of resistance development, for example cross-resistance to chloroquine and amiodiaquine, two 4-aminoquinolones and mefloquine conferring resistance to quinine and halofantrine. This phenomenon may reduce the usefulness of newly developed therapies prior to large-scale usage.
4. The resistance to anti-malarials may be increased by a process found in some species of *Plasmodium*, where a degree of phenotypic plasticity was exhibited, allowing the rapid development of resistance to a new drug, even if the drug has not been previously experienced.
5. The pharmokinetics of the chosen anti-malarial are key; the decision of choosing a long half-life over a drug that is metabolised quickly is complex and still remains unclear. Drugs with shorter half-life's require more frequent administration to maintain the correct plasma concentrations, therefore

potentially presenting more problems if levels of adherence and compliance are unreliable, but longer-lasting drugs can increase the development of resistance due to prolonged periods of low drug concentration.

6. The pharmokinetics of anti-malarials is important when using combination therapy. Mismatched drug combinations, for example having an 'unprotected' period where one drug dominates can seriously increase the likelihood of selection for resistant parasites.
7. Ecologically there is a linkage between the level of transmission and the development of resistance, however at present this still remains unclear.
8. The treatment regime prescribed can have a substantial influence on the development of resistance. This can involve the drug intake, combination and interactions as well as the drug's pharmokinetic and dynamic properties.In general, resistance appears to occur through spontaneous mutation.

Only a single point or multiple mutations may be required depending on the drug. Provided the mutation is not deleterious to the survival or reproduction of the parasite, susceptible parasites will be removed while resistant strains survive. The resistance of *P. falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization (Foley and Tilley, 1997). Specific gene mutations encoding for resistance to both dihydropteroate synthase (DHPS) and dihydrofolate reductase enzymes have been associated with varying degrees of resistance to antifolate combination drugs such as sulfadoxine plus pymethamine (Plowe *et al.,* 1981). Resistance to Atovaquone is conferred by single-point mutations in the cytochrome-b gene (WHO, 2005).

In Africa, the first confirmation of chloroquine resistance to *P. falciparum* malaria was in the south-east and North-east of the continent in the late 1980's.There were reports of chloroquine resistance to *P. falciparum* malaria in Zaria in the late 1980's (Lege-Ogunteye, 1989).However, resistance to pyrimethamine has long existed in Nigeria since the late 1950s.Resistance to anti malarial drugs has been described for *P. falciparum* and *P. vivax* has been shown to be resistant to chloroquine and or primaquine (Murphy,1993*;* Looaresuwann *et. al.,* 1997*).*However, *P. falciparum* resistance is now emerging to artemisinin having detected in five countries of the Greater Mekong Subregion: Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam (WHO, 2014).

Key drivers of antimalarial drug resistance include; Unusual genetic structure of malaria parasites in regions known for antimalarial drug resistance, Counterfeit or substandard treatments, Unregulated or poorly administered antimalarial drug use, Artemisinin drug use without a complementary combination treatment, such as lumefantrine (James, 2016). The problem of antimalarial drug resistance is compounded by cross resistance, in which resistance to one drug confers resistance to other drugs that belong to the same chemical family or which have similar modes of action. During the past decades, several antimalarials had to be removed from markets after spread of parasite resistance (WHO, 2014).

There are concerns that *P. falciparum* malaria in Cambodia and Thailand is becoming increasingly difficult to treat, and that multi-drug resistance could spread to other regions with dire public health consequences. Consequently, WHO's Malaria Policy Advisory Committee in September 2014 recommended adopting the goal of eliminating *P. falciparum* malaria in this subregion by 2030. WHO

launched the Strategy for Malaria Elimination in the Greater Mekong Subregion (2015–2030) at the World Health Assembly in May 2015 (WHO, 2016).

There are currently no licensed vaccines against malaria or any other human parasite. One research vaccine against *P. falciparum*, known as RTS, S/AS01, received a positive opinion by the European Medicines Agency in July 2015 and pilot implementationsin a limited number of African countries has been adopted by WHO. These pilot projects could pave the way for wider deployment of the vaccine in 3 to 5 years, if safety and effectiveness are considered acceptable (WHO, 2016).

2.7 Prevention and Control

The principle of malaria prevention and control include; Individual Protection, Vector Control, Anti-Plasmodia Measures and Social Participation(CDC, 2015).

2.7.1 Individual protection

This is aimed at reduction of human mosquitoes contact and involves the use of mosquitoes repellants, insecticides treated bed-nets and other materials. Others include wearing of protective clothing, house screening, house spraying and use of any mosquito's' fumigants.Four of the key prevention and control interventions recommended by WHO and used by programs worldwide to fight malaria are: use insecticide-treated bed nets (ITNs) to protect people from mosquitoes; Rapid diagnostic tests and treatment with effective high-quality drugs such as artemisinin-containing combination therapies (ACTs); Treatment to protect pregnant women and their newborn children; intermittent preventive treatment (IPTp) for pregnant women; Indoor spraying of homes to protect people from mosquitoes (IRS).

2.7.2 Vector control

The aim is to reduce vector-breeding habit and reduction of mosquitoes-human contact. This includes environmental modification and manipulations, chemical and biological larviciding, reduction of vector densities and insecticides space spraying. Vector control is an essential component of malaria prevention. Such control has been proven to successfully reduce or interrupt malaria transmission when coverage is sufficiently high.

In 2004, the WHO adopted integrated vector management (IVM) globally for the control of all vector-borne diseases.Integrated vector management (IVM) is defined as "a rational decision-making process for the optimal use of resources for vector control" and includes five key elements: 1) evidence-based decision-making, 2) integrated approaches 3), collaboration within the health sector and with other sectors, 4) advocacy, social mobilization, and legislation, and 5) capacity-building.While interventions using only ITNs and/or IRS successfully reduce transmission intensity and the burden of malaria in many situations, it is not clear if these interventions alone will achieve those critical low levels that result in malaria elimination.Integrated vector management (IVM) is not a new concept and the basic principles of IVM have been used over the past century in the U.S.A. and elsewhere for mosquito control (Hatch *et al*., 1973; Challet, 1991; Challet, 1994; WHO, 1993). These operations are designed to protect people from nuisance-biting and vector species of mosquitoes and are guided by the following basic principles: 1) to effectively reduce adult vector populations and pathogen transmission; 2) that interventions should be ecologically, environmentally, socially, economically and politically acceptable; 3) that management strategies should not create adverse side

effects such as environmental contamination or the development of resistance, nor should they have a negative impact on non-target organisms, including beneficial insects, humans, domestic animals and wildlife; 4) to understand the transmission cycle, the life history of the vector species, and the natural factors regulating vector survivorship are critical, 5) that the most effective programmes develop descriptive and predictive models for population dynamics and transmission potential; 6) to have flexibility in terms of changing strategies and tools in response to surveillance and biological data; and 7) that management strategies should be dynamic and able to respond to the results of an active and sensitive mosquito/pathogen surveillance programme.As part of the global plan to combat neglected tropical diseases for 2008–2015, the WHO has called for the strengthening of IVM and capacity building as one of the strategic areas for action (WHO, 2001).Historically, the implementation of integrated approaches to vector control has been a slow and complicated process. In the 1970's, WHO listed five reasons for the slow uptake of integrated vector control: 1) over-estimation of the requirements of integrated control: 2) over-sophistication of integrated control: 3) insufficient conviction of the advantages of integrated control; 4) misconceptions about the use of pesticides in integrated control: and 5) insufficient consciousness of comparative costs, effectiveness, benefits and risks (Rafatja, 1982). Additional reasons for the slow uptake of integrated vector control, as of late, include a lack of capacity building, poorly defined roles for advocacy and legislative activities, and a general lack of intersectoral linkage within the health sector (John *et al*., 2008).Like traditional approaches to vector control, IVM relies on an understanding of how environmental factors affect the distribution and densities of different species of vectors, and how

effectively control measures reduce vector-human contact, vector survival and the overall intensity of pathogen transmission.

Long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are the two most important vector-control measures that protect humans from the bites of mosquitoes carrying the malaria parasite (WHO, 2016).When optimally employed, these vector control measures can reduce malaria parasite transmission by 90% or more and can correspondingly reduce malaria incidence, malaria prevalence, high parasite density, and clinical malaria (Nyarango *et al*., 2006; Protopopoff *et al*., 2007; Protopopoff *et al*., 2008).Other aspects of IVM that have impacted positively in malaria vector control include; a variety of environmental management techniques, including source reduction and drainageGilroy and colleagues (1945), a combination of drainage, filling larval habitats, and bed netsUtzinger and colleagues (2002), a combination of water drainage techniques and larviciding, Schliessmann and colleagues (1973), a combination of water-source reduction activities and biological control in larval habitatsSharma and colleagues (Sharma and Sharma, 1986; Sharma *et al*., 1986).The absence of IVM evaluations, as well as evaluations of packages of interventions, has been noted recently by several researchers (Goodman and Mills, 1999; Goodman *et al*., 2000; Breman *et al*., 2006).

However, there are many reasons why vector-borne diseases remain a major public health problem: 1) entomologists and vector-control specialists often fail to convince decision-makers of the importance of sound vector control; 2) intersectoral collaboration has been recommended as a method to improve mosquito control, but in practice, this type of collaboration has proved difficult; 3) modeling and adaptive management have not been used effectively to quantify the effect of disease and

vector control interventions on the economic and social development of communities and nations; 4) failed management of insecticides has created a conflict of "to spray or not to spray"; 5) there has been a failure to connect the true disease burden with other societal burdens to establish the public health importance of vector-borne diseases in low-transmission settings; 6) the interaction between proper utilization of law enforcement and health education has not been explored in sufficient detail; 7) economics plays a central role in mosquito-borne disease control but has not been fully integrated in the evaluation of disease control programmes; 8) entomologists and public health specialists have failed to convince decision-makers that they have an ethical duty to control mosquitoes and the pathogens they transmit; and 9) biomedical strategies, such as active and passive case-detection and treatment, are often used instead of preventative vector control measures, as a result of international donor requirements (Impoinuil *et al*., 2007).In an Institute of Medicine review of Vector Borne Diseases, David Morens cites five fundamental deficiencies that inhibit our ability to find realistic solutions for vector-borne disease problems:deterioration of public health infrastructure, lack of adequate funding, lack of adequate training and training models, over-specialization in the biomedical sciences, driven by emerging technology and emphasis on the basic sciences, and bureaucratization (Morens, 2008).

In recent years, mosquito resistance to pyrethroids has emerged in many countries. In some areas, resistance to all 4 classes of insecticides used for public health has been detected (WHO, 2016).

2.7.3 Anti-Plasmodia measures

These are aimed at elimination of parasites and prevention of their transmission. Such measures include early diagnosis and prompt and effective treatment of acute cases of malaria and mass treatment in case of epidemics. Malaria prophylaxis is the preventive treatment of malaria. Chloroquine may be used where the parasite is still sensitive. (Jacquerioz and Croft 2009)Jacquerioz FA, Croft AM (2009). However, due to resistance one of three medications: mefloquine(*Lariam*), doxycycline (available generically), and the combination of atovaquone and proguanil hydrochloride (*Malarone*) is frequently needed (Jacquerioz and Croft 2009).In choosing the agent, it is important to weigh the risk of infection against the risks and side effects associated with the medications (Julia *et al.,* 2010) Prophylaxis against *Plasmodium vivax* requires a different approach given the long liver stage of this parasite (Schwartz *et al.*, 2003).

This is a highly specialist area.Some of the factors in deciding whether to use chemotherapy as malaria pre-exposure prophylaxis include the specific itinerary, length of trip, cost of drug, previous adverse reactions to antimalarials, drug allergies, and current medical history (Kathrine *et al.*,2010). Pregnant women receive ‘’intermittent preventive treatment’’ (IPT) with anti-malaria drugs during the second and third trimesters of pregnancy. Intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) is an integral part of WHO’sthree-pronged approach to the prevention and treatment of malaria in pregnancy,which also includes the use of insecticide-treated nets and prompt and effective casemanagement (WHO, 2013).

The first treatment identified is thought to be Quinine, one of four alkaloids from the bark of the Cinchona tree.Quinine remained the only available treatment for malaria until the early 1920s. During the First World War German scientists developed the first synthetic antimalarial compound—Atabrin and this was followed by Resochin and Sontochin derived from 4-aminoquinoline compounds. American troops, on capturing Tunisia during the Second World War, acquired, and then altered the drugs to produce Chloroquine.The development of new antimalarial drugs spurred the World Health Organization in 1955 to attempt a global malaria eradication program. This was successful in much of Brazil, the US and Egypt but ultimately failed elsewhere. Efforts to control malaria are still continuing, with the development of drug-resistant parasites presenting increasingly difficult problems.

The CDC publishes recommendations for travels advising about the risk of contracting malaria in various countries.Some of the factors in deciding whether to use chemotherapy as malaria pre-exposure prophylaxis include the specific itinerary, length of trip, cost of drug, previous adverse reactions to antimalarials, drug allergies, and current medical history (Kathrine *et al*., 2010).

2.7.4 Social participation

This is aimed at motivation for personal and family protection. This involves (a) health education (also called information-Education-Communication (IEC), where the communities are informed of what they can do to prevent and treat malaria. (b) Training and supervision of health workers, to ensure that they carry out their tasks correctly, (c) provision of equipment and supplies (e.g. microscopes, drugs bed nets) to allow the health workers and the communities to carry out their interventions. In

the North central Nigeria, educational level and marriage have been found to have a positive impact on knowledge about the disease. Also, accessibility to information on malaria and general knowledge of the modes of transmission and control was generally low, and to a large extent influenced by cultural beliefs, values and low economic strengths (Godwin *et al*., 2010).

Successful malaria control activities require coordinated action by National authority (especially the ministry of health), international organizations (such as the world health organization and UNICEF), Government and non-Governmental Agencies, the private sectors and the communities. To enhance co-ordination, various partnerships have been formed the most prominent being the roll back malaria global partnership (CDC, 2015).

In Nigeria, CDC has also provided direct staff support to WHO; UNICEF; the Global Fund to Fight AIDS, Tuberculosis, and Malaria; and the World Bank – all stakeholders in the Roll Back Malaria (RBM) Partnership (CDC, 2015). Roll Back Malaria (RBM) in Nigeriais an initiative to improve malaria control in the context of health sector reform. It was initiated in1998 through a joint partnership of WHO, UNICEF, UNDP and the World Bank. RBM consists of twophases - the inception phase and the implementation phase (FMOH, 2005).

The WHO Pesticide Evaluation Scheme (WHOPES) coordinates the testing and evaluation of pesticides for public health, and makes recommendations on vector-control tools such as LLINs, insecticides for IRS, and other vector-control interventions as appropriate (WHO, 2016).The WHO Vector Control Advisory Group (VCAG) on new tools and the Technical Expert Group on Malaria Vector

Control (VCTEG) are the two main advisory bodies addressing policy topics related to malaria vector control (WHO, 2016).

2.7.5 Barriers to malaria control and prevention

Malaria control is hampered by several technical and administrative problems. These include:

1. Drug-resistance by malaria parasites which hinders case management by decreasing the efficiency of anti-malaria drugs and by requiring the use of alternative drugs that are often more costly, less safe and less easy to administer
2. Insecticide resistance by mosquitoes which reduces the efficiency of intervention that relies on insecticides such as insecticides treated bed-nets and indoor spraying(WHO, 2016).
3. Inadequate health infrastructures in poor countries making them enable to conduct the recommendable interventions
4. The people most expose to malaria are often poor and lack education. They do not know how to prevent or treat malaria. They often do not have the financial means to purchase the necessary products such as drugs or bed-nets (Amajoh, 1998 and CDC, 2006; Godwin *et al*., 2010).

The bane of malaria control in endemic region in addition to above factors, include inadequate budgetary allocation, a grossly under-remunerated and under-supervised work force, the absence of appropriate policies and laws (Arrese, 2001; CDC, 2015). Others include, emerging resistance to artemisinin drugs, widespread availability of substandard and counterfeit malaria treatments, inadequate disease

surveillance systems, waning country and donor attention as malaria burden drops, and unexpected crises such as the Ebola and Zika outbreaks (CDC/USAID, 2016).

In recent years, 5 countries have been certified by the WHO Director-General as having eliminated malaria: United Arab Emirates (2007), Morocco (2010), Turkmenistan (2010), Armenia (2011) and Maldives (2015). Recently 3 other countries started the certification process: Argentina, Kyrgyzstan and Sri Lanka (WHO, 2016).

The WHO Global Technical Strategy for Malaria 2016-2030 – adopted by the World Health Assembly in May 2015 – provides a technical framework for all malaria-endemic countries. It is intended to guide and support regional and country programmes as they work towards malaria control and elimination by the year 2030 (WHO, 2016).

Malaria elimination is defined as the interruption of local transmission of a specified malaria parasite in a defined geographical area as a result of deliberate efforts. Malaria eradication is defined as the permanent reduction to 0 of the worldwide incidence of malaria infection caused by human malaria parasites as a result of deliberate efforts (WHO, 2016).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The Study Design

The study was a cross-sectional, facility based study. Samples were collected from General Hospital Kafanchan, Mamman Tsoho Memorial Hospital, Tudun Wada, Kaduna and Hajiya Gambo Sawaba Memorial Hospital, Zaria representing the southern, central and northern senatorial districts of the state respectively. These hospitals were considered because they have facilities for and provide blood transfusion services in their respective senatorial districts.

3.2 The Study Area

Kaduna State is a state in North-western geo-political zone of Nigeria with a population of 6,066.562. It is made up of 23 local government areas which are further grouped into 3 senatorial districts (north, central and south). It is a metropolitan as well as a cosmopolitan industrialized state with over 80 commercial and manufacturing industries. It is one of the education centers in Nigeria with many colleges and one of the most premier universities in Nigeria. Agriculture is the mainstay of the state with about 80% of the people actively engaged in farming. It is defined by longitude 10°20' N and latitude 9° 03' E. The vegetation characteristic is that of the guinea savanna with scattered trees and shrubs. There are two distinct seasons, the wet (rainy) which lasts from April to October and dry season that occurs from November to March. It experiences a rainfall of 1530mm in Kafanchan-Kagoro in the southeast and 1015mm in Ikara/Makarfi districts in the northeastern

part of the state (Encyclopedia Britanica, 2012). The map of Kaduna state showing the location of the sampling sites is shown in Figure 3.1.

3.3 Ethical Approval

Approval for the research was obtained from the Ethical Committee of the Kaduna State Ministry of Health (Appendices I-III). Patients' anonymity was maintained and the findings treated with utmost confidentiality. Patients who were positive either by microcopy, RDT and PCR were advised to go for malaria treatment.

3.4 Sample Size

The minimum Sample size was determined using the formula described by Naing *et al*. (2006).

$$n = \frac{zp\,(1-p)}{d}$$

Where:

n = Sample size

p = Expected prevalence (which is 11% by Ike and Okeke, 2009).

z = the standard normal distribution at 95% confidence interval (Which is equal to 1.96)

d = Precision/allowable error (which is 5% = 0.05)

$$\text{Thus } n = \frac{(1.96)^2 \times 0.11 \times (1-0.11)}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.11 \times 0.89}{0.0025}$$

$$= 150$$

However, a total of 360 samples were collected in order to have a more representation of the study population.

3.5 Inclusion Criteria and Exclusion Criteria

Inclusion criteria include individuals who,

i. Consented
ii. were negative for HIV, HBV and HCV.
iii. were between 18 and 65 years.
iv. Weighed 50kg and above.
v. had a Haematocrit ≥ 36%.

Exclusion criteria include individuals who,

i. did not consent.
ii. had evidence of HI V, HBV and HCV infections and history of medical illness.
iii. were < 18 years and >65 years.
iv. had Haematocrit < 36%.

3.6 Administration of Questionnaire

Prior to the sample collection, a well-structured questionnaire was used to obtain clinical information, socio-demographic data and information on risk factors likely to be associated with TRM from consented donors (Appendices IV and V).

3.7 Collection of Samples

Two milliliters (2ml) of whole venous blood was collected from each of the donors by the Phlebotomist for routine preliminary screening and haematological

investigations to determine the fitness of the donor) into ethylene diamine tetra acetic acid (EDTA) vacutainers. Haematological tests were performed on the same sample according to normal hospital protocol. The samples were stored in the refrigerator until the thick and thin blood films were prepared, RDT carried out and microscopy tests were performed almost immediately and the remaining whole blood stored at -20^{0}C for PCR (Singh *et al*., 1999).

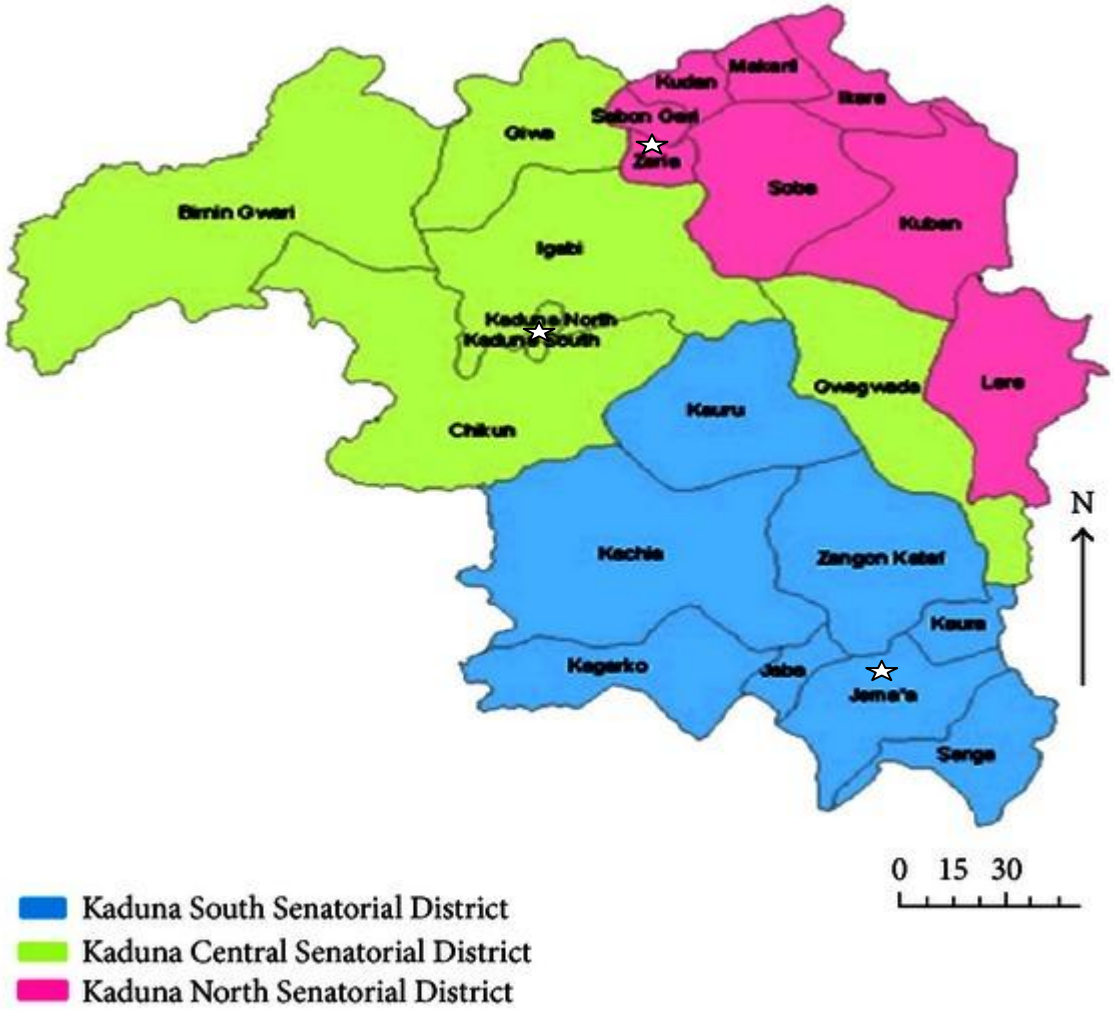


Figure 3.1: Map of Kaduna state showing the sampling sites

3.8 Laboratory Analysis

Malarial parasitaemia was determined through detection of malaria parasite antigen using a rapid diagnostic test (RDT) kit, microscopic examination of Giemsa stained thin and thick blood films and PCR.

3.8.1 Rapid diagnostic test

The detection of the malaria parasite antigen was carried out with the aid of SD BIOLINE ONE STEP Malaria HRP-II(P.f) and pLDH(Pan) Antigen Rapid Test kit manufactured by STANDARD DIAGNOSTICS, INC. Kyonggi-do, Korea.

The test principle: The SD BIOLINE Malaria Ag P.f/Pan test cassette is an in-vitro immunochromatographic assay designed for the rapid qualitative and differential diagnosis between HRP-II(Histidine-rich protein II) specific to *P. falciparum* and pLDH(Plasmodium lactate dehydrogenase) specific to *Plasmodium* species(Pan) in human blood samples. The test contains a membrane strip which is pre-coated with mouse monoclonal antibodies specific to HRP-II of *P. falciparum* on test line P.f region and with mouse clonal antibodies specific to lactate dehydrogenase of *Plasmodium* species (*P.falciparum, P. vivax, P. malariae* and *P. ovale*) on test line Pan region respectively. This mixture of these monoclonal antibodies colloid gold conjugate reacts with the malaria antigen in the sample. They move along the membrane chromatographically to the test region and form a visible line as the antibody-antigen-antibody gold particle complex with high degree of sensitivity and specificity. All the test lines and control line in the result window are not visible before applying any sample. The control line is used for procedural control and should always appear if the test procedure is performed correctly.

Test procedure: The test was carried out according to manufacturer's instructions. Prior to use, all kit components and sample were allowed warm to room temperature. The test device was then removed and placed on a flat dry surface. The whole blood specimen was drawn with a 5µl capillary pipette provided. The blood sample was transferred from the capillary tube to the sample well by holding the capillary tube vertically and gently touching the sample containing end against the pad within the sample addition sport until all the blood was transferred. Four drops of the assay diluents were added into the assay diluents well. The reaction was allowed to proceed for a minimum of 15 minutes. The results were then read not later than 30 minutes.

Interpretation of results: The presence of three colour bands ("P.f", "Pan" Test lines and "C" control line) within the result window indicates a positive result for *P. falciparum* or mixed infection of *P. falciparum* and *P. vivax* or *P. ovale* or *P. malariae*. The presence of two colour bands ("P.f" Test line and "C" control line) indicates a positive result for *P. falciparum*. While two colour bands ("Pan" Test lines and "C" control line) indicates positive for *P. vivax*, *P. ovale* or *P. malariae*. The presence of only one colour band ("C" control line) indicates a negative result. The result was invalid if the control band ("C" control line) fails to appear and was repeated using a new cassette.

3.8.2 Microscopy

Thin and thick blood films were made, stained with Giemsa stain and examined using the x100 oil immersion objective of the microscope (Cheesbrough, 2010).

Preparation of thin blood film: A drop of blood was placed at approximately 1 cm from one end of a clean, grease-free microscope slide. Immediately, a smooth edged

cover slip was placed in front of the drop of blood at an angle of 45° to the slide. The cover slip was pulled backward to touch and allow the drop of blood to spread to the edges of the cover slip. The cover slip was then gently pushed forward until the whole blood was smeared. The film was then air dried, away from dust and flies.

Preparation of thick blood film: A large drop of blood was placed approximately 2cm from one of a clean grease-free Microscope slide. The edge of another slide was used to spread the drop of blood in circular motion to a diameter of 0.5cm. The thick smear was air-dried away from dust and flies.

Staining of thin blood films: The dried thin film was fixed in absolute methanol for 2 minutes, allowed to dry and placed in a Coplin Jar. A 1 in 10 dilution of Giemsa stain was prepared, poured into the Coplin jar and allowed to stain for 30 minutes. The slides were removed from the jar and rinsed in buffered distilled water pH 7.2. They were placed in a draining rack and allowed to air-dry.

Staining of thick blood films: The dried, unfixed thick films were placed in a coplin jar. A 10% dilution of Giemsa stain was prepared, poured into the Coplin jar and allowed to stain for 30 minutes. The slides were removed from the Coplin jar, rinsed in buffered distilled water pH7.2. They were placed in a draining rack and allowed to air-dry.

Examination of the stained blood films: When the stained blood films were completely dried, a drop of immersion oil was applied around the edges of the films and spread to cover an area of about 10mrn in diameter. A well-stained area was selected and examined under the 100 x or oil immersion objective of the microscope. The malaria parasites seen were identified and the parasite density determined.

3.8.3 Estimation of parasites density

The malaria parasite numbers/µl of blood was estimated by counting the number of malaria parasites seen against 200 white blood cells (WBC) in a Giemsa stained thick film and result expressed using this formula described by Cheesbrough (2010)

Number of parasites/µl of blood

$$= \frac{8000 \times \text{Number of parasites counted against 200 WBC}}{200}$$

3.8.4 Molecular analysis

Twenty three samples were tested for presence of malaria DNA using the polymerase chain reaction (PCR) assay at DNA labs Kaduna.

3.8.4.1 Extraction of the deoxyribonucleic acid (DNA): The genomic (Mitochondrial) DNA was extracted from the whole blood according to the standard Phenol-Chloroform extraction procedure (Singh *et al*., 1996). Two hundred micro liters of whole blood was mixed with 400µl of the lysing fluid, vortexed and incubated at 65^0C for 1h. Exactly 400µl of phenol-chloroform was then added, vortexed and centrifuged at 14000 revolutions per minute (rpm) for 10 minutes. The supernatant was then harvested into fresh empty microtubes by pipetting out. To the supernatant, 400 µl of chloroform was added, vortexed and spun at 13000rpm for 5 minutes. The supernatant fluid was again harvested into fresh micro tubes. Furthermore, 1000 µl of absolute ethanol and 40 µl of sodium acetate were added to the supernatant fluid and mixed by inversion. The eluted DNA was stored at -20^0C overnight.

3.8.4.2: Polymerase chain reaction assay (DNA Amplification): The Cox 1 mitochondrial gene of *Plasmodium falciparum* was used as a target for

amplifications. Amplification was carried out in a DNA thermal cycler type 480™ (Perkin-Elmer, Courtaboeuf, France). Conventional PCR was performed using the primers designed by Tham *et al*. (1999) and Morassin *et al*. (2002)shownin Table 3.1.

The PCR master mix was prepared by adding 2µl of genomic DNA, 1 µl of forward primer (Pf1), 1 µl of reverse primer and 16 µl of deionized water to PCR premix kits containing 1.5 Mm Mg^{2+} and 250µM of each dNDP lyophilized unto the surfaces of Hot-start appendoff tubes. The PCR was then carried out as follows:incubation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute and extension at 72^0c for 1 minute. Denaturation, annealing and extension were repeated 35 times. In the final cycle, an additional four-minute extension step at 72^0C was included.

The PCR products were analyzed by gel electrophoresis using 1.5% agarose stained with ethidium bromide. The bands were then read using the 100bp plus ladder as a marker and visualized using ultraviolet (UV) light and photographed.

Table 3.1: The Sequences of the Primers used for the PCR

Species	Primer	Sequence (5'-3')	Size of amplicon (bp)
P. falciparum	PF1(forward)	5'-GGA ATG TTA TTG CTA ACA-3'	422
	PF2(Reversed)	5'-AAT GAA GAG CTG TGT ATC-3'	

Detection of PCR products:Amplified PCR products were detected by electrophoresis. A standard 1.5% agarose gel stained with ethidium bromide was loaded with 2µl samples of the PCR products. Samples were subjected to electrophoresis at 100V for 1h. The gel was then visualized with ultraviolet light at a wavelength of 625nm and photographed. A 422 base pair fragment was detected for *P. falciparum.*

Sequencing of DNA**:**Direct sequencing of PCR products were carried outat DNA Laabs, Kaduna. This was to determine possible mutations and to determine the phylogenetic relatedness of the isolates. Sequencing reactions were performed using an applied biosystems (ABI) PrismSequencer™ (Perkin-Elmer).

A. Elution of DNA from the gel band: The band was cut from the gel and weighted. An aliquot of 300 µg of gel binding (GB) buffer was added to each 100mg of the band. The above mixture was heated at 50^0C for 10 minutes or until the gel is dissolved with inversion of the tube after every 2-3 minutes. Approximately equal volume of Isopropanol was added to the above suspended mixture (for Base pairs less than 500). They were mixed and transferred to binding columns. It was spun at 10,000 rpm for 1 minute and the content of the flow tube discarded (containing the gel) (while DNA would be bound to the binding column). To the binding column, 750µl of protein extraction (PE) Buffer was added and allowed to stand for 5 minutes. It was spun at 13,000 rpm for 1 minute and the flow tube discarded. The empty tube was spun again rpm for 2 minutes. The content of the column was transferred to a new collecting tube and the column tube discarded. To this new collecting tube, 25µg ultrapure water was added and allowed to stand for 5 minutes. It was then spun at 13,000rpm for 1 minute.

B. Sequencing PCR reaction: Approximately10µl of the above DNA was mixed with 2µl of reverse/or forward primer and 8µl of Dye terminator cycle (DTC) mixture added. The above mixture was put in a PCR machine and conditions set at96^{0}C for 20 seconds, 50^{0}C for 20 seconds and 60^{0}C for 4 minutesFor 30 cycles.

C. Sequencing clean-up: Exactly100µl of prewarmed buffer (Pb) was added to the product of the above sequencing reactionand mixed by pipetting in and out. The entire reactionwas transferred into the binding column and spun at 10,000rpm for 1 minute. The flow-trough was discarded and the collection tube re-used. Approximately 650µl of protein extraction (PE) Buffer was added; the mixture allowed to stand for 3 minutes and spun at 13,000rpm for 1 minute. The empty column was spun at 13,000 rpm for 2 minutes. New collection tube was used. To the above reaction, 13µl of Sample Loading Solution (SLS) was added, allowed to stand for 5 minutes and spun at 13,000rpm for 1 minute. The elution step was repeated and the product was spun immediately.The above product was then loaded in the applied biosystems (ABI) Prism Sequencer™ (Perkin-Elmer). The DNA sequences were compared with data in GenBank.

3.9 Determination of Haematological Parameters

Full blood/differential count was carried out using the fully automated Sysmex Haematology analyzer manufactured by Sysmex Corporation Kobe, Japan, at the haematology departments of the study facilities (General Hospital Kafanchan, Mamman Tsoho Memorial Hospital, Tudun Wada, Kaduna and Hajiya Gambo Sawaba Memorial Hospital, Zaria representing the southern, central and northern senatorial districts of the state respectively).

3.9.1 Procedure Determination of haematological parameters

The instrument was switched-on and allowed to carry out a default check. The sample was then sufficiently mixed and the plug of the vacutainer removed. The tube was set to the sample probe and the start button pressed. The tube was then removed when the LCD screen displayed "Analysing". The instrument was then allowed to execute automatic analysis and the result was displayed on the LCD screen.

3.10 Data Analysis

All statistical analyses were carried out using Statistical Analysis System (SAS) and Statistical Package for the Social Sciences (SPSS) version 20. The Pearson Chi-square test was used to determine the associations between the variables at 95% confidence interval while differences in haematological parameters was subjected to analysis of variance (ANOVA). Values of $p \leq 0.05$ were considered significant.The diagnostic performance of the RDT kit was evaluated using2 x 2 contingency tables and McNemar test (Pembele *et al*., 2015). The kappa coefficient (Cohen's kappa coefficient as a measure of agreement for qualitative items) was determined to confirm the consistency of the results among the diagnostic tools. The kappa values were used to categorize the strength of agreement between the microscopic examination and RDT. Values were interpreted with the Landis and Koch classification (Pembele *et al*., 2015): poorly correlated (<0), slightly correlated (0-0.20), fairly correlated (0.21-0.40), moderately correlated (0.41-0.60), substantially correlated (0.61-0.80), and perfectly correlated (0.81-1.0) (WHO, 1993; Mbarakurwa, 1996; Agomo *et al*., Tjitra*et al*., 1999; 1998; Garba *et al*., 2011 and Pembele *et al*., 2015). The key variables considered were;

$$\text{Sensitivity (\%)} = \frac{\text{number of true positives (TP)}}{\text{number of true positives (TP)} + \text{number of false negatives (FN)}} \text{ X } 100$$

$$\text{Specificity (\%)} = \frac{\text{number of true negatives (TN)}}{\text{number of true negatives (TN)} + \text{number of false positives (FP)}} \text{ X } 100$$

$$\text{Positive Predicted Value (PPV, \%)} = \frac{\text{TP}}{\text{TP+FP}} \text{ X } 100$$

$$\text{Negative Predicted Value (NPV, \%)} = \frac{\text{TN}}{\text{TN+FN}} \text{ X } 100$$

$$\text{False Positive Rate (FPR, \%)} = \frac{\text{FP}}{\text{FP+TN}} \text{ X } 100$$

$$\text{False Negative Rate (FNR, \%)} = \frac{\text{FN}}{\text{FN+TN}} \text{ X } 100$$

$$\text{Efficacy/Accuracy} = \frac{\text{TP+TN}}{\text{TP+TN+FP+FN}} \text{ X } 100$$

$$\text{Test Reliability} = \frac{(TP \times TN - FP \times FN}{(TP+FN)(TN+FP)}$$

CHAPTER FOUR

4.0 RESULTS

A total of 360 donors were enrolled for this study consisting of 314(87%) males and 46(12%) females. Out of the 360 blood donors studied, the overall prevalence rate of malaria parasite was 27(7.5%) by microscopy and 31(8.6%) by RDT. Only the asexual stages (ring forms) of malaria parasites were seen microscopically. *Plasmodium falciparum* was the only malaria parasite species encountered by both microscopy and RDT. The 27 donors that positive by microscopy had parasite densities of between 80-240 parasites/µl of blood with an average of 126 parasites/µl of blood.

The highest prevalence rate of 11(9.2) was recorded by both microscopy and RDT in Kafanchan, while the least prevalence rate of 6(5.0%) and 9(7.5%) were detected by microscopy and RDT respectively in Zaria. The difference was not statistically significant (χ^2 = 1.6817, df =1, p= 0.4313 for microscopy and χ^2 = 0.2824, df =1, p=0.8683 for RDT) (Table 4.1).

With respect to gender, the male donors had a higher prevalence rate of 25(8.0%) and 23(7.3%) by RDT and microscopy respectively compared tothe female donors with prevalence rates of 6(13%) and 4(8.7%) by RDT and microscopy respectively. However, this difference was statistically insignificant (χ^2 = 1.1087, df =1, p= 0.2512 for microscopy and χ^2 = 1.3166, df =1, p=0.7417 for RDT)(Table 4.2).

Table 4.1: Prevalence of Malarial Parasites among Blood Donors in Kaduna State by Senatorial District.

Senatorialdistrict	Number examined	Microscopy Positive (%)	P value	RDT Positive (%)	P value
Northern	120	6 (5.0)	0.4313	9 (7.5)	0.8683
Central	120	10 (8.3)		11 (9.2)	
Southern	120	11 (9.2)		11 (9.2)	
Total	360	27(7.5)		31(8.6)	

Donors in the age group 18<20 years had the highest prevalence rate of 3(15.8%) by RDT and 2(10.5%) by microscopy, while those of the age group20-29years had the least prevalence of 9(6.9%) by RDT and 8(6.1%) by microscopy. However, this difference was statistically insignificant (χ^2 = 0.8616, df =4, p= 0.9300 for microscopy and χ^2 = 2.1524, df =4, p=0.7078 for RDT) (Table 4.2).

In relation to marital status, the prevalence was highest among the married donors who had 23(11.1%) by RDT and 21(10.1%) by microscopy while no infection was detected by either techniques among the divorced. The difference was not statistically significant (χ^2 = 4.9563, df =2, p= 0.0839 for microscopy and χ^2 = 3.9386, df =2, p=0.1396 for RDT). Based on type of settlement, result of microscopy showed that donors whole live in the urban areas had the highest prevalence of 26(9.6%) while those who live in the rural areas had the least of 3(7.5%). However, result of the RDT showed that donors who live in the semi urban areas had the highest prevalence rate of 3(9.7%) while the east of 2(5%) was found among the donors who live in the rural areas. The difference was not statistically significant (χ^2 = 0.5775, df =2, p= 0.7492 for microscopy and χ^2 = 0.3010, df =2, p=0.8603 for RDT). In relation to the educational status, those who had informal education had the highest prevalence rate of 8(15.7%) by both techniques while no infection was recorded among the donors who were not educated donors. The difference was not statistically significant (χ^2 = 0.5775, df =4, p= 0.7492 for microscopy and χ^2 = 0.3010, df =4, p=0.8603 for RDT) (Table 4.2).

The result of the analysis of malaria parasites among blood donors in relation to occupational status in the study area showed that donors who belong to undisclosed occupational groups had the highest prevalence rate of 2(33.3%) by both techniques

while those who belong to the business class had the least prevalence rate of 7(6%) and 5(3.9 %) by RDT and microscopy respectively. The difference was statistically significant (χ^2 = 24.0845, df =6, p= 0.0.0005 for microscopy and χ^2 = 14.5993, df =6, p=0.0236 for RDT) (Table 4.2).

Also, in relation to the Body mass index (BMI), the under weighed (<18.5 kg/m^2) donors had the highest prevalence rate of 1(11.1%) by both techniques while the obese donors had the least prevalence rate of 1(5.9%) by RDT and 0% by microscopy. This difference was not statistically significant (χ^2 = 1.8031, df =3, p= 0.6143 for microscopy and χ^2 = 0.5062, df =3, p=0.9175 for RDT). This is shown in Table 4.3.

The result of this study also shows that a higher prevalence rate of 23(10.6%) and 20(9.2%) of the malaria parasite infections were recorded in wet season compared to prevalence rates of 8(5.6%) and 7(4.9%) recorded in the dry season by RDT and microscopy respectively. However, this difference was not statistically significant (χ^2 = 2.2333, df =, p=0.1351 for microscopy and χ^2 = 2.6414, df =1, p=0.1041 for RDT) (Table 4.4).

Table 4.2: Prevalence of Malarial Parasites in the Blood of Blood Donors in Kaduna State in Relation to Demographic and Socio-Economic Factors

Factor	Number examined	Microcopy positive (%)	P value	RDT Positive (%)	P value
Gender					
Male	314	23(7.3)	0.2512	25(8.0)	0.7417
Female	46	4(8.7)		6(13.0)	
Age group					
<20	19	2(10.5)	0.9300	3(15.8)	0.7078
20-29	131	8.(6.1)		9(6.9)	
30-39	144	12(8.3)		14(9.7)	
40-49	54	4(7.1)		4(7.1)	
50-59	1	1(10)		1(10)	
Marital status					
Single	151	6(4.0)	0.0839	8(5.3)	0.1396
Married	204	21(10.1)		23(11.1)	
Divorced	2	0(0)		0(0)	
Settlement type					
Urban	289	22(7.6)	0.7492	26(9.6)	0.8603
Semi-urban	31	3(9.7)		2(6.5)	
Rural	40	2(5.0)		3(7.5)	
Educational status					
None	9	0(0%)	0.7492	0(0%)	0.8603
Informal	51	8(15.7)		8(15.7)	
Primary	34	2(5.9)		3(8.8)	
Secondary	118	7(5.9)		11(9.3)	
Tertiary	148	10(6.8)		9(6.1)	
Occupation					
Unemployed	129	5(3.9)	0.0005	9(7.0)	0.0236
Civil servants	54	8(14.8)		6(11.1)	
Business	116	5(4.3)		7(6.0)	
Artisans	6	1(16.7)		1(16.7)	
Farmers	49	6(12.2)		6(12.2)	
Others	6	2(33.3)		2(23.3)	

Table 4.3: Prevalence of Malarial Parasites in the Blood of Blood Donors in Kaduna State in Relation to Body Mass Index (BMI).

Parameter	Number examined	Microcopy positive (%)	P value	RDT Positive (%)	P value
BMI					
<18.5	9	1(11.1)	0.6143	1(11.1)	0.9175
18.5-249	244	18(7.4)		20(82	
25-29.9	90	8(8.9)		9(10)	
30 ≤	17	0(0)		1(5.9)	
Total	360	27(7.5)		31(8.6)	

In relation to the use of insecticide treated nets (ITNs), those who slept under insecticide treated bed nets had the highest prevalence of 16(10.3%) by microscopy. However, RDT detected more infections among donors who did not sleep under insecticide treated bed nets with a prevalence rate of 18(8.9%). Statistical analysis reveals that these differences were not significant (χ^2 = 0.0571, df =1, p=0.2169 for microscopy and χ^2 = 0.1264, df =1, p=0.9388for RDT). This is shown in Table 4.4.

When considering the relationship between prevalence of the infection and some social habits, donors who did not smoke had a higher prevalence of 29(8.8%) by RDT and 25(7.6%) by microscopy while those who smoked had 2(6.9%) for both techniques. However, statistical analysis shows that this difference was not significant (χ^2 = 0.01166, df =1, p= O.8976 for microscopy and χ^2 = 0.1178, df =1, p=0.7314 for RDT)(Table 4.5).

Those who eat Kola nut had 3(8.3%) by RDT and 3(8.6%) prevalence rate by microscopy while those who do not had prevalence rate of 28(8.6%) by RDT and 24(7.5%) by microscopy. Statistical analysis showed that this difference was not significant (χ^2 = 0.0166, df =1, p= 0.8000 for microscopy and χ^2 = 0.0.0001, df =1, p= O.9930 for RDT). Those who take alcoholic drinks had prevalence rates of 1(12.5%) by both techniques while those who do not had prevalence rates of 30(8.5%) by RDT and 26(7.4%) by microscopy However, statistical analysis showed that this difference was not significant (χ^2 = 0.1264, df =1, p= O.9388 for microscopy and χ^2 = 0.2948, df =1, p=0.5871 for RDT)(Table 4.5).

Table 4.4: Prevalence of Malarial Parasites in the Blood of Blood Donors in Kaduna State in Relation to Season and use of ITN

Factor	Number Examined	Microcopy Positive (%)	P value	RDT Positive (%)	P value
Season					
Wet	218	20(9.2)	0.1351	23(10.6)	0.1041
Dry	142	7(4.9)		8(5.6)	
ITN use					
Yes	157	16(10.03)	0.2169	13(8.3)	0.9388
No	203	11(5.4)		18(8.9)	

Key: ITN= Insecticide treated net

Table 4.5: Prevalence of Malarial Parasites in the Blood of Blood Donors in Kaduna State in Relation to Some Social Habits

Social habit	Number Examined	Microscopy Positive (%)	P value	RDT Positive (%)	P value
Smoking					
Yes	29	2(6.9)	0.8976	2(6.9)	0.7314
No	331	25(7.6)		29(8.8)	
Kolanut consumption					
Yes	35	3(8.6)	0.8000	3(8.3)	0.9930
No	325	24(7.5)		28(8.6)	
Alcohol Consumption					
Yes	8	1 (12.5)	0.9388	1 (12.5)	0.5871
No	352	26(7.4)		30 (8.5)	

With respect to ABO blood group and donation history, blood group B donors had the highest prevalence rates of 11(14.1%) by RDT and 10(12.8%) by microscopy while no infection (0%) was detected among blood group AB donors by both techniques However, statistical analysis shows that this difference was significant for microscopy (χ^2 = 10.589, df =4, p= O.032) but was not significant for RDT (χ^2 = 9.7612, df =4, p=0.055) (Table 4.6).

Relation or replacement donors had prevalence rates of 24(8.8%) and 27(9.9%) by microscopy and RDT respectively, Commercial donors had 0% by microscopy and 1(4.8%) by RDT while voluntary donors 3(4.6%) by both techniques. Furthermore, first time donors had prevalence rates of 16(6.5%) and 19(7.7%) by microscopy and RDT respectively while regular donors had 11(9.7%) and 12(10.5%) prevalence rates by microscopy and RDT respectively. Statistical analysis showed no significant difference in the prevalence rate of both donor type (χ^2 =3.1892, df =2, p=0.2030 for microscopy and χ^2 = 2.3491, df =2, p=3090for RDT) and donation frequency (χ^2 = 1.1107, df =1, p=0.2030 for microscopy and χ^2 = 0.7776, df =1, p=0.3779for RDT). This is shown in Table 4.6.

In relation to previoushistory of malaria and treatment, donors who have previously had malaria fever had prevalence rates of 27(9.4%) and 24(8.4%) by RDT and microscopy respectively while those who never had malaria fever prior to this research had prevalence rates of 4(5.5%) and 3(4.1%) by RDT and microscopy respectively.

Table 4.6: Prevalence of Malarial Parasites in the Blood of Blood Donors in Kaduna State in Relation to Blood Group and Donation History

Factor	Number examined	Microscopy positive (%)	P value	RDT Positive (%)	P value
Blood group					
A	57	2(3.5)	0.032	5(8.8)	0.055
B	78	10(12.8)		11(14.1)	
AB	7	0(0)		0(0)	
O	218	15(6.9)		15(6.9)	
Donor Type					
Commercial	21	0(0)	0.2030	1(4.8)	0.3090
Replacement	273	24(8.8)		27(9.9)	
Voluntary	66	03(4.6)		03(4.6)	
Donation frequency					
First time	246	16(6.5)	0.2030	19(7.7)	0.3779
Repeat	114	11(9.7)		12(10.5)	

Those who previously treated malaria by traditional means had 2(9.1%) prevalence rates by both RDT and microscopy and those who previously treated malaria by orthodox means had prevalence ratesof 25(9.6%) by RDT and 23(8.9%) by microscopy. No infection was detected in those who previously treated malaria using a combination of both traditional and orthodox means. The results also reveal that those who had malaria within 2-4 weeks prior to the research had the highest prevalence rates of 2(16.7%) by both diagnostic methods while the least prevalence rates of 3(4.2%) and 4(5.6%) by RDT and microscopy respectively was recorded among those who have never had malaria treatment. However, these differences were not statistically significant in both history of previous malaria (χ^2 = 1.5172, df =1, p=0.2180 for microscopy and χ^2 = 1.1411, df =1, p=0.2854for RDT), type of treatment (χ^2 = 3.5602, df =3, p=0.6143 for microscopy and χ^2 = 1.7592, df =3, p=0.8814for RDT) and date of last treatment (χ^2 = 0.5377, df =4, p=0.5377 for microscopy and χ^2 = 2.6554, df =4, p=0.6170for RDT) as shown in Table 4.7.

Out of the 360 blood donors examined, 21 (5.8%) were positive (True positives) and 323 (89.7%) were negative (True negatives) by both techniques. The result also showed that 10 were negative by microscopy but positive by RDT (false positives) whereas 6 were negative by RDT but positive by microscopy (False negatives) Table 4.8.

Table 4.7: Prevalence of Malarial Parasites in the Blood of Blood Donors in Kaduna State in Relation to History of Malaria Fever and Treatment

Factor	Number Examined	Microscopy Positive (%)	P value	RDT Positive (%)	P value
History of Malaria					
Yes	287	24(8.4)	0.2180	27(9.4)	0.2854
No	73	3(4.1)		4(5.5)	
Type of Treatment					
Traditional	22	2(9.1)	0.6143	2(9.1)	0.8814
Orthodox	260	23(8.9)		25(9.6)	
Both	6	0(0.0)		0(0.0)	
Nil	72	2(2.8)		4(5.6)	
Date of Treatment					
≤1week	10	1(10)	0.5377	1(10)	0.6170
2-4weeks	12	2(16.7)		2(16.7)	
≥month-1year	145	10(6.9)		11(7.6)	
>1year	122	11(9.0)		13(10.7)	
Nil	71	3(4.2)		4(5.6)	

Table 4.8: Comparison of RDT with Microscopy as Gold Standard

	MICROSCOPY		**Total**
RDT	Positive	Negative	
Positive	21	10	31
Negative	06	323	329
Total	27	333	360

Analysis of the results using the standard format showed that the RDT kit had an overall efficacy of 95.5 %, sensitivity of 77.8, specificity of 97%, positive predictive value (PPV) of 67.7%, a negative predictive value (NPV) of 98.2% a false positive rate (FPR) of 3%, a false negative rate (FNR) of 1.8% and a substantially correlated κ value of 0.700 (Table 4.9).

The mean values of RBC of the donors were slightly below the lower limit of the normal values (Lewis, 2006)while the mean value of LYM of the male donors was slightly higher than the male corresponding normal value. The mean values of otherhaematological parameters of the blood donors were within the normal ranges(Table 4.10).

The Tukey test result showed no significant difference between the meanvalues of all the haematologicalparameters of the positive and negative cases(Table 4.11).

Parasite density was found to be significantly but negatively correlated with RBC (CC= -0.16979, P.value= 0.0015) and PLT (CC= -0.12903, P. value= 0.0159) and significantly but positively correlated with HB (CC= 0.14870, p= 0.0054) as shown in Table 4.12.

Table 4.9: Diagnostic Performance of RDT with Microscopy as Gold Standard

Feature	Sensitivity	Specificity	PPV	NPV	FPR	FNR	Efficacy	Reliability	κ value
Value (%)	77	97	67.7	98.2	3	1.8	95.6	74.7	0.700

Key:

PPV = Positive predictive value

NPV = Negative predictive value

FPR = False positive rate

FNR = False negative rate

κ =Kappa

Table 4.10: Over all mean values of Haematological parameters Among Blood Donors in Kaduna state

Parameter	Sex	Normal range	Mean	Standard deviation	Standard error
HB (g/dl)	Male	13-18	13.3	2.20	0.12
	Female	12-16	12.8	1.90	0.28
PCV (%)	Male	40-52	41.5	5.96	0.34
	Female	37-47	38.1	6.36	0.94
WBC($\times 10^9$/µl)	Male	4.5-11	5.9	2.48	0.14
	Female	4.5-11	6.1	1.38	0.20
RBC($\times 10^{12}$/µl)	Male	4.9-5.9	4.6	0.94	0.05
	Female	4.1-5.1	4.0	0.97	0.14
PLT ($\times 10^9$/µl)	Male	150-400	206.6	6756	3.81
	Female	150-400	213.0	76.6	11.29
LYM (%)	Male	20-40	41.9	131	0.74
	Female	20-40	39.2	8.9	1.30
NEUT (%)	Male	50-70	49.6	32.0	1.81
	Female	50-70	51.1	51.12	1.82

KEY: HB= Haemoglobin concentration

PCV= Packed cell volume

WBC= White blood cells count

RBC= Red blood cells count

PLT= Platelets count

NEUT= Neutrphils count

NR= Normal range

Table 4.11: Tukey test for significant difference in the means of the haematological parameters between the malaria parasites negative and positive donors.

Parameter	Group	F value	Pr>F
HB (g/dl)	NF Vs PF	0.27	0.6080
	NM Vs PM	2.80	0.0955
PCV (%)	NF Vs PF	0.12	0.7256
	NM Vs PM	2.57	0.1101
WBC ($^{x}10^{9}$/µl)	NF Vs PF	0.20	0.6590
	NM Vs PM	0.04	0.8441
RBC (x10^{12}/µl)	NF Vs PF	0.60	0.4415
	NM Vs PM	0.12	0.7324
PLT (x10^{9}/µl)	NF Vs PF	0.65	0.4245
	NM Vs PM	0.32	0.5846
LYM (%)	NF Vs PF	0.25	0.6185
	NM Vs PM	0.30	0.5846
NEUT (%)	NF Vs PF	0.06	0.8080
	NM Vs PM	0.07	0.7872

Difference is significant when Pr≥0.0

Key: NF = Negative female PCV = Packed cell volume

PF = Positive female WBC = White blood cells

NM = Negative male RBC = Red blood cells

PM = Positive male PLT = Platlets

HB = Haemoglobin LYM = Lymphocytes

NEUT= Neutrophils

Table 4.12: Correlation between Parasite Density and some Haematological Parameters

Parameter	HB	PCV	WBC	RBC	PLT	LYM	NEUT
CC	0.1487	0.0600	0.0902	- 0.1697	- 0.1290	0.0910	0.0652
P. value	0.0054	0.2634	0.0925	0.0015	0.0159	0.0895	0.2239

Key:CC = Correlation coefficience

HB = Haemoglobin

PCV = Packed cell volume

WBC = White blood cells

RBC = Red blood cells

PLT = Platlets

LYM = Lymphocytes

NEUT = Neutrophils

Twenty three samples were used for PCR comprising of 13 that were positive by both RDT and microscopy, 6 positive by RDT but negative by microscopy, 3 positive by microscopy but negative by RDT and 1 that was negative by both RDT and microscopy was also included for the PCR. Amplification with the *P. falciparum* species-specific primers gave rise in 6 samples to a 422-bp PCR product which correlates to the *P. falciparum* primer set Pf1-Pf2 (Plate 1).

Out of the 13 that were positive by both RDT and microscopy, 5 were positive by PCR. Out of the 6 that were positive by RDT but negative by microscopy, none was positive by PCR. Out of the 3 that were positive by microscopy but negative by RDT, 1 was positive by PCR. While the 1 that was negative by both RDT and microscopy was confirmed negative by PCR (Plate 1).

Three*P. falciparum* isolates (one from each senatorial district) were sequenced and their sequences compared with GenBank sequences using the BLAST system. The result showed that they were all *P falciparum* with the closest having E values of 2e (for KAF 54) and 0.0 (for TW 032 and GS 110), percentage identifications of 97, 99 and 100 (for KAF 54, TW 032 and GS 110) respectively and Accession numbers of KT119883.1(for KAF 54) and KT191163.1 (for TW 032 and GS 110) respectively (Table 4.13).

The sequences of these three *P. falciparum* were aligned and their nucleotide sequences compared for possible mutations. Sequence changes observed were: sequence rearrangements, additions, deletions, and nucleotide substitutions (Figure 4.1).

Isolate	Description of closest GenBank isolate	Max score	Total score	Query cover	E value	Ident	Accession
GS 110	*Plasmodium falciparum* voucher SR_Ban_COXI_IV cytochrome c oxidase subunit I (COX I) gene, partial cds; mitochondrial	689	689	100%	0.0	100%	KT191163.1
TW 032	*Plasmodium falciparum* voucher SR_Ban_COXI_IV cytochrome c oxidase subunit I (COX I) gene, partial cds; mitochondrial	689	689	100%	0.0	99%	KT191163.1
KAF 54	*Plasmodium falciparum* isolate MAN14 mitochondrion, complete genome	375	375	71%	2	97%	KT119883.1

Table 4.13: Result of Blast Showing Sequences Producing Significant Alignment with the *P. falciparum* Isolates



Plate I: Agarose gel image of the PCR for Plasmodium falciparum (M=100bp marker, N=Negative control, P=Positive control, Lanes 1-6= samples).

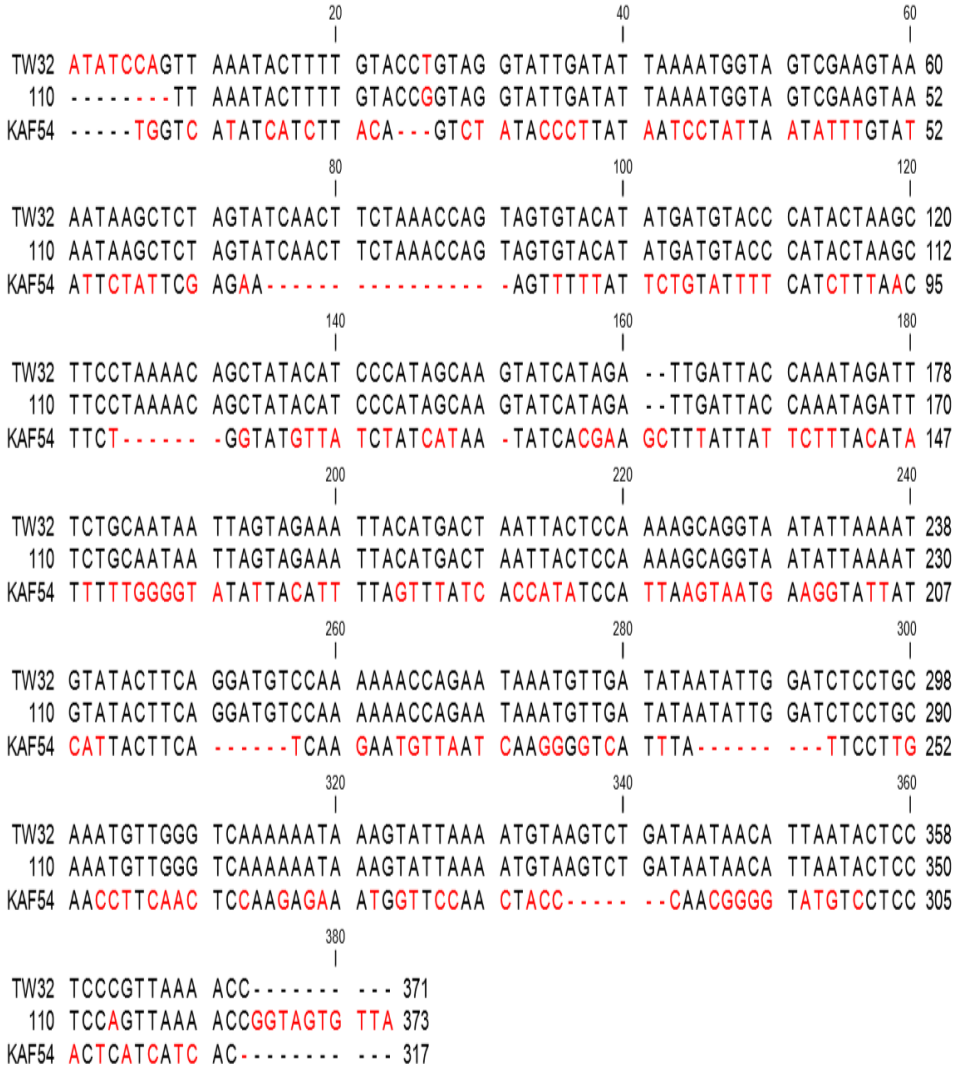


Figure 4.1: Nucleotide sequence alignment of the whole mitochondrial genomes of *P. falciparum* isolates from different senatorial districts of Kaduna state.

Apopulation phylogenetic tree was constructed using the neighbour-joining (NJ) method implemented in MEGA v.7 computer program of these three *P. falciparum*isolates was carried out along with three other *P. falciparum*isolatesfrom the Genbank and the result indicated that the isolates have a common ancestral origin. However, isolates from the northern and central senatorial districts were more closely related than the isolate from the southern senatorial district and were placed in the same clade. The population phylogenetic tree placed the isolates from the northern and central senatorial districts with the global isolates, while the isolate from the southern senatorial district was an outlier. The *mt* genome sequences of *P. falciparum* utilized here for comparative and evolutionary studies bear the GenBank accessions KT119856.1, KT191163.1 and KT119873.1(Figure 4.2).

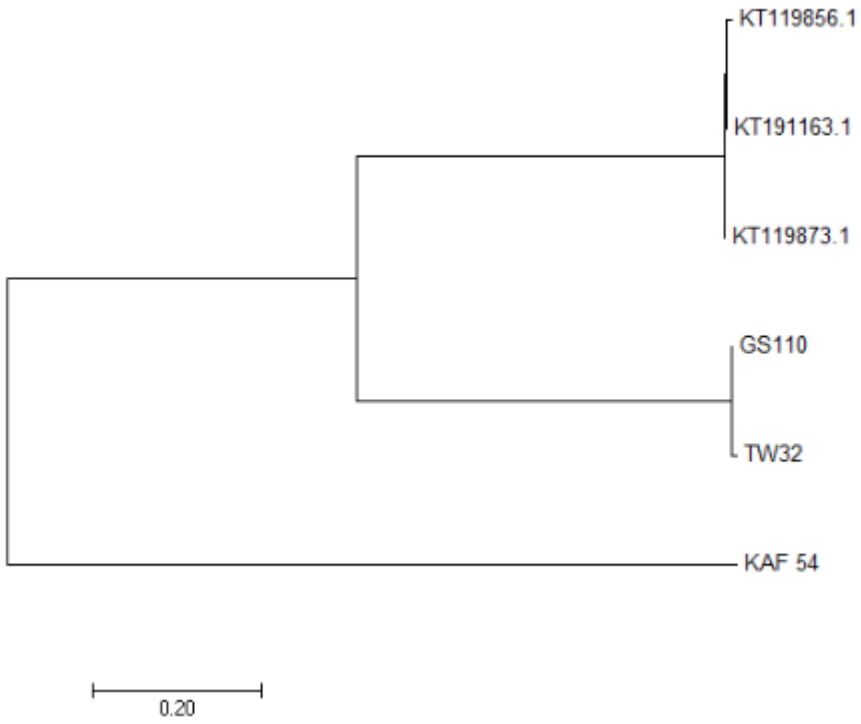


Figure 4.2: Neighbour-joining Phylogenetic tree of *P. falciparum* isolates from different senatorial districts of Kaduna state and Genbank.

CHAPTER FIVE

5.0 DISCUSSION

The malaria parasites prevalence rate of 27(7.5%) by microscopy and 31(8.6%) by RDT obtained in the present study (Table 4.1), though not very high, is a reflection of the potential role that blood transfusion could play in the transmission of malaria in the state. This result is similar to the 6.8%reported by Garba *et al.* (2009) among blood donors in Ahmadu Bello University Teaching Hospital, Zaria and and 7.8%in the southwest regions of the country by Akinboye and Ogunrinade (1987) respectively. This is slightly higher than 4.1% by Chikwem *et al.* (1997) among blood donors at the University of Maiduguri Teaching Hospital, Maiduguri. These values are however by far lower than 29.4% reported by Oche and Aminu (2012) among blood donors in Zaria, 32.5% by Zama *et al*. (2013) in Sokoto, North western Nigeria, 11% and 50% by Ikeh and Okeke (2005) at the Jos University Teaching Hospital, and Udeze *et al*.(2013) in Ilorin respectively in the North Central Nigeria.

Prevalence rates of 17.5 % (Okonko *et al*., 2012), 28% (Agboola *et al*., 2010) and 46.5% (Alli *et al*., 2010) have been reported in the South western Nigeria. Also, 30.0% (Okocha *et al.*, 2005), 40.9% (Chigozie *et al.*, 2006), 46% (Umeanaeto *et al.*, 2006) and 77% (Mbanugo and Emenalo, 2004) prevalence rates have been reported in the South eastern Nigeria. In the South-South region, prevalence rates of 12.56% (Pondei *et al*., 2012), 30.4% (Nmor and Egwungenga, 2004), 40% (Ibhanesebhor *et al*., 1996), 45.5% (Momoh *et al*., 2013), 67.5% (Wariso and Oboro, 2015) and 76.8% (Otajevwo, 2013) have been reported.This variation in the prevalence rate of malaria parasites in blood donors from the different regions of the country is a reflection of the level of malaria endemicity in these parts of the country. These

regional differences in the country could be attributed to variation in the pre-disposing factors such as *Anopheles* species present, environmental conditions, climatic conditions, period of study, the study populations and the diagnostic test methods used in the study. Similar conclusions have been made by previous researchers (Nega, 1993; Ali *et al.*, 2004; Okonko *et al*., 2012 and Otajevwo, 2013).

The detection of *Plasmodium falciparum* as the sole *Plasmodium spp*. identified in this study is neither surprising nor solitary. This finding is consistent with other reported studies where *P. falciparum* was either the predominant or only *Plasmodium* species detected (Akinboye and Ogunrinade, 1987; Ibhanesebhor *et al*., 1996; Chikwem *et al*., 1997); Chigozie *et al*., 2006; Wariso and Oboro, 2015; Umaru and Uyaiabasi, 2015). *P. falciparum* has been the most endemic species of malaria parasite in sub-Saharan Africa and has been associated with the high morbidity and mortality rate and the greatest propensity for developing resistance in the sub region (Happi *et al*., 2009; WHO, 2010; CDC, 2015). It is notable that *P. falciparum* malaria is not only highly endemic in Africa, but based on the high genetic diversity of isolates from this continent, Africa is considered to be the homeland of this species (Conway *et al*., 2000; Joy *et al*., 2003).

All the infected donors had low levels of parasitaemia with an average density of 126 parasites/µl of blood. This is similar to between 10-400 parasites/µl and 285 parasites/µl of blood average reported by Chigozie *et al*. (2006) and Aina *et al*. (2013) respectively. Also,mild to moderate levels of parasitaemia were recorded by Wariso and Oboro (2015) among donors in Port Harcourt. This is higher than the density of 3.43 parasites/ µl reported by Ojo, (2006) among apparently healthy individuals in ABU, Zaria, and 20parasites/ µl among blood donors in ABUTH,

Zaria by Garba *et al*. (2009). However, other studies have reported higher densities of 10,000 - 250,000 parasites/µl of blood (Ali *et al*., 2010; Pondei *et al*., 2012). The low parasitaemia levels observed in our study is not unusual in view of the fact that the participants were apparently healthy free from HIV, hepatitis B and C, weighed ≥50kg and between 18 and 65 years old.It is also in concurrence with the assertion that older children and adult sometimes develop protective immunity to the disease and can be asymptomatic carriers for a long time without clinically manifesting symptoms/signs of malaria (Boland, 2001; Aweeka *et al*., 2008; Umaru and Uyaiabasi, 2015).

The higher prevalence of the parasites seen in the southern senatorial district represented by the General Hospital Kafanchan (Table 4.1) could be due to the higher rainfall of 1530mm usually experienced in Kafanchan-Kagoro in the southeastern part compared to 1015mm in Ikara/Makarfi districts in the northeastern part of the state (Encyclopedia Britanica, 2012). Incidence of malaria has been found to be significantly and positively correlated with rainfall (Kayode *et al*., 2009). Rainfall provides sufficient breeding sites for the mosquitoes and relative humidity that enhances the survival of the adult mosquitoes.

The higher prevalence rate observed for the females in this study (Table 4.2) is in concordance with the findings of several other researchers (Vlassoff and Bonilla, 1994; Ibekwe *et al*., 2009; Okonko *et al*., 2009; Alli *et al*., 2010; Okonko *et al*., 2012; Oche and Aminu, 2012; Udeze *et al*., 2013; Inabo and Umaru, 2011; Otajevwo, 2013 Wariso and Oboro, 2015). This is also consistent with the assertion that in Africa female are considered more vulnerable to this disease (WHO, 2004). This may also be a reflection of differences in disease susceptibility. However, other

investigators have found higher prevalence among the males than the females (Eke *et al*., 2006; Epidi *et al*., 2008; Ilozumba and Uzozie, 2009; Muntaka and Opoku-Okrah, 2013). There appears to be no scientific evidence linking malaria prevalence to gender (Udeze *et al*., 2013).

Available evidence suggests that given equal exposure, adult men and women are equally vulnerable to malaria infection, except for pregnant women who are at greater risk of severe malaria in most endemic areas. Evidence from some countries indicates that restricted mobility of women may also impede their attendance at primary health care clinics for malaria testing thereby increasing their risk of infection (Muller *et al*., 1998; Lampieti *et al*., 1999).The higher prevalence rate reported among females in this study could just be by chance (Alli *et al*., 2010).

In relation to the age group, the highest prevalence rate was recorded among the donors that were<20years (Table 4.2). This finding is in agreement with the reports of other researchers (Inabo and Umaru, 2011; Umaru and Uyaiabasi, 2015). This buttresses the early assertions that younger ones are more disposed to the infection than older individuals in malaria endemic areas of sub-Saharan Africa (WHO, 2003). This may be due to the fact that individuals build up an acquired immunity and become relatively protected against disease as they grow older, after continued exposure from multiple malaria infections over time (Inabo and Umaru, 2011; Umaru and Uyaiabasi, 2015). This further demonstrates that adults living in endemic regions have a form of protective immunity against the disease than the younger ones, even during peak transmission periods. However, this result is at variance with the report of several other researchers who detection higher rates in the older individuals (Umeanaeto *et al*., 2006; Ikeh and Okeke, 2006; Osaro *et al*., 2007;

Ajayi *et al*., 2010; Alli *et al*., 2010; Oche and Aminu, 2012; Okonko *et al*., 2012; Muntaka and Opoku-Okrah, 2013; Wariso and Oboro, 2015).

The findings from this study indicated a higher prevalence among married compared to the unmarried blood donors though the difference was not statistically significant (Table 4.2). This is contrary to the report of Chigoze *et al.*(2006) where the single donors had a higher prevalence rate attributing it to their greater involvement in outdoor and nocturnal activities that enhance contact with mosquitoes, which may be social or occupational in nature. Alli *et al*. (2010) reported a higher PR among the married though those who did not disclosed their marital status had the highest. The reason for this present outcome could be their greater number in the study population or by chance.

There was no significant variation of the infection with respect to type of settlement (Table 4.2). While microscopy indicated a highest prevalence among semi-urban settlers, RDT results showed that the prevalence was highest among urban. This finding is similar to the report of Okonko *et al*. (2012) who reported higher prevalence in blood donors from the urban areas of Ibadan. This is contrary to the findings of Atif *et al*. (2009) who reported malaria infections to be prevalent among majority of patients belonging to the rural areas of Hyderabad, in Pakistan. It has been shown that high population densities and possible lower immunity may result in more disease impact in urban setting (Udeze *et al*., 2013).

Those with informal education had the highest prevalence in relation to educational status, the difference was however not statistically significant (Table 4.2). This finding is in consonance with the findings of Alli *et al*. (2010). This observation is in line with the fact that education is one of the potentially protective socio-economic

factors against malaria risk. Similar observations were made by Ndawala *et al.* (2000) who reported that women with lower levels of education were likely to have fever than those with higher levels of education.

In this study, those with undisclosed occupation had significant higher ($p=0.0005$ for microscopy and $p=0.0236$ for RDT) prevalence (Table 4.2). It may be that these individuals are engaged in activities that predispose them to contact with mosquitoes especially if such are nocturnal in nature. It could also be by chance.There is need more studies on larger populations in other parts of Nigeria to unravel the possible reasons for this observation.

In relation to the Body Mass Index (BMI), the underweight donors ($BMI>18.5Kg^2/_m$) higher prevalence (Table 4.3). This is in agreement with the findings of Shulman *et al.* (2001) in Kenya who found increased presence of placental malaria among women of low body mass index. Also, Ivy (2012) reported that the severely thin were at a higher risk (12.8%) of having malaria, whereas the pre-obese and obese had no (0%) malaria parasites. This observation is in contrast to the report of Garba *et al*. (2010) who reported the highest prevalence among the obese. Nevertheless, undernourishment continues to be the main issue in many populations of sub-Saharan Africa. Due to the fact that nutrient deficiencies impair resistance to infection, low BMI has been associated with the pathogenesis of severe malaria (Nacher *et al*., 2001). In other words, there are several mechanisms through which malaria could impact nutritional status. Malaria-related symptoms, such as diarrhea and abdominal pain, may lead to mal-absorption of nutrients and decreased intake, respectively. Parasitaemia has also been found to have a detrimental effect on nutritional status through host elaboration of pro-inflammatory cytokines. TNF-α is

a known mediator of anorexia and cachexia seen in many human disease states (David *et al*., 1983; Davis *et al*., 1998; Friedman *et al*., 2003; Cross, 2015).

The preponderance of infection in the rainy season compared to dry season in this study (Table 4.4) is consistent with reports by other previous researchers attributing it to the peak of mosquito biting density and is probably due to favourable conditions for mosquito vector breeding during the warm humid summer associated with the high rainfall (Rowland *et al*., 1997; Ali *et al*., 2004; Okocha *et al*., 2005).

The result of this study also shows that the infection was higher among donors who used insecticide treated bed nets than those who did not (Table 4.4). This result is in contrast to the reports of most researchers having reported higher prevalence among those who do not use ITNs. In fact, the sharp drop in malaria morbidity and mortality globally has been attributed to the use of ITNs (WHO/UNICEF, 2015). However, The higher prevalence of infection among donors who use insecticide treated bed nets could be attributable to factors such as presence of pyrethroid resistant *Anopheles* in the area, inconsistent use of ITNs, incorrect hanging of ITNs, use of expired ITNs, proportion of those using ITNs in the study area and the biting behaviour of some *Anopheles* mosquitoes (Renne *et al*., 2008; CDC, 2015). These are factors that could undermine effectiveness of ITNs in the study area. More than half of the people in a community must use ITNs for it to be effective (CDC, 2015). Promoting the culture of appropriate net use based on effective education, promotion and marketing is essential to the success of the use of ITNs as far as public health is concerned (Guillet *et al*., 2001). There is need for a deeper investigation on the use of ITNs in the study area in order to unravel the possible cause of this finding.However, the result of RDT showed that the prevalence was higher among

donors that used ITNs. This agrees with the fact that the use of ITNs confers protection against the bites of mosquitoes. WHO (2015) considers the use of ITNs as one the factors responsible for the reduction in malaria morbidity and mortality in the past 15 years (2000-2015).

The relationship between parasitaemia and some social habits was considered (Table 4.5). Those who drink alcohol had higher prevalence than those that did not. This is not surprising because alcohol ingestion has been shown to stimulate mosquito's attraction thereby increasing contact with malaria vectors (Shirai e*t al*., 2002). Also, most of the drinking is done outdoor and could expose them to mosquito bites. Non-smokers were found to be more infected than smokers. Smoking has been found to be a risk factor for some other diseases and malaria could be one of such diseases. On kola nut consumption, a higher prevalence was found among Kola nut eaters than those who do not. Though the differences were not statistically significant, there is need for more work on this as it will contribute to the fight against the scourge of the disease.

When malarial parasitaemia was associated with ABO (Table 4.6), higher prevalence was found in blood group B donors. This difference was statistically significant. This finding is similar to the findings ofSule *et al*.(2014)and Chigoze *et al*. (2006) who reported a higher prevalence among blood group B and AB donors in Zaria and south-eastern Nigeria respectively. Similar studies have been yielding conflicting results (Facer and Brown, 1979; Martin *et al.,* 1979; Montoya *et al.,* 1994; Alli *et al*., 2010). The findings in this study are almost similar with the observation that individuals with A and B group cells are at greater risk of developing severe cerebral malaria (Carlson, 1992; Udonsangpetch *et al.,* 1993).

Since the differences obtained were not significant in both methods, the result suggests that no particular blood group precludes the susceptibility to malaria. Similar conclusions were drawn by Fischer and Boone (1998) in Zimbabwe, Cavasini *et al.* (2006) in Brazil, Ibheanesebhor *et al*. (1996) and Chigozie *et al.* (2006)in south-eastern Nigeria. However, blood group B has been found to confer weaker protection against plague (Eupedia, 2016).Interestingly, a recent case-control study carried out in a tertiary care centre in Odisha (India) revealed a significant association of blood group B, but not A and AB, with severe malaria (Panda *et al*., 2011).

Replacement or family-related donors were most infected though the difference was not statistically significant (Table 4.6). This is similar to the report of Ikeh and Okeke (2005) and Alli *et al*. (2010). This is in contrast with the findings of Mbanugo and Emenalo (2004) and Osaro *et al.* (2007) who reported higher prevalence among commercial donors. Replacement donors are usually family members and friends who are determined to save lives by all means and may not want to be disqualified even when they have slight fever or headaches. However, they may consist of poor members since poverty has been identified as the principal cause of malaria in donors.

Repeat or regular donors were most infected (Table 4.6). This is in concurrence to the report of Alli *et al*. (2010). The reason for the observation in this study is not clear even though the difference was not statistically significant.However it may not be unconnected with the fact that first time donors are usually apprehensive and those who are positive could be discouraged with the slightest feel of unwell.

When considering malarial parasitaemia prevalence with regards to history of past malaria infection (Table 4.7), it was higher among those who have previously had the infection. This may suggest the possibility of the presence of some antimalaria resistant strains of malaria parasites in the area though the difference was not significant. It may also be dependent on the type of antimalaria used as is indicated by the preponderance of the infection among those who took antimalarials not long ago (between two to four weeks ago). Similar observations were made by Alli *et al*. (2010). This finding agrees with the observation that inability to prevent or properly treat malaria (which could be due to poverty) is one the major causes of malaria (Okrah *et al.,* 2002). Previous studies have reported Chloroquine resistant *Plasmodium falciparum* (CRPf) rates of 15.3% (Midala *et al.,* 1988) and 15% (Lege-Oguntoye *et al.,* 1989) in some parts of the state.

The detection of more cases by RDT compared to microscopy (Table 4.8) is attributable to crossreactivity with rheumatoid factors in the blood, but replacement of IgG with IgM in rescent products reduces this problem (Laferl *et al*., 1997; Grobusch *et al*., 1999;Mishra *et al*., 1999), cross reactivity with the heterophile antibodies (Moody and Chiodini, 2002) and persistence of residual antigens for weeks after treatment (Heutmekers *et al*., 2012).A similar observation was reported by Pembele *et al*. (2015).

Compared with microscopy, the RDT test kit was 77.8% sensitive and 98% specific (Table 4.9). This finding is similar with The findings of Azikiwe *et al*. (2012) observed that RDT was as specific as microscopy and even more sensitive in their study of 200 febrile patients in Ogun state. RDT detected 128(64%) malaria parasite positive cases while 118(59%) cases were detected by microscopy. Also, Pembele *et al*. (2015) reported that SD BIOLINE Ag. RDT kit was significantly more

sensitive than microscopy in their study in Luanda, Angola where the RDT kit detected 87.65% malaria parasite cases and microscopy detected 71.60%. This observation is in agreement with several reports that have demonstrated an overall high sensitivity of HRP-2 based diagnostics kits in patients (Beadle *et al.,* 1994*;* Dietz *et al*., 1995; Humar *et al.,* 1997; Newton, 2006).

The sensitivity of 77.8% reported in this study is lower than the recommended value of >95% (WHO, 2000 and Whonsrichanalai *et al*., 2015). It is also lower than the 80-100% previously reported in Nigeria (Ajumobi *et al*., 2015;) and other parts of the world (Buchachat *et al*., 2004; Hopkins *et al*., 2008; Msellem *et al*., 2009; Nicastri *et al*., 2009; Pemble *et al*., 2015). However, this sensitivity is higher than 42.5% reported elsewhere in Nigeria (Oyeyemi *et al*., 2015). The high specificity of 97%, efficiency 95.6% and substantial correlation ($\kappa = 0.700$) with the reference method makes it a potential tool for the detection of asymptomatic malarial parasitaemia since the RDT is unlikely to miss-out the non-infected individuals. The type of samples evaluated and the regions from which they were collected have also been found to influence the performance of RDTs (Dzakah *et al*., 2014).

The accuracy (sensitivity and specificity) of RDT is mostly dependent on the parasite species, transmission intensity, parasite density, and amount of circulating antigens, local polymorphisms of target antigen and persistence of antigens after treatment (Murray *et al*., 2008; Ishengoma *et al*., 2011). RDT sensitivity has been found to decline at parasite densities <500/µl of blood for *P. falciparum* (Whonsrichanalai *et al.,* 2015). Extremely low sensitivity had been reported earlier for both HRP-2 and Pldh tests and batch specific problems were suspected (Rubio *et al*., 2001; Mason *et al*., 2002;Coleman *et al*., 2002; Huong *et al*., 2002; Iqbal *et al*.,

2002). The storage temperature the RDT product was subjected to by the marketers could also cause a low sensitivity. Exposure of RDT kit to high temperatures has been implicated as a possible cause of poor performance in the tropics. Denaturation of antibodies in the test membrane can impair binding to the target antigen at high temperature. Heat can also cause damage to the nitrocellulose membrane forming the strip thus changing its flow characteristics or causing the antibody to detach from the membrane (Chiodini *et al*., 2007).

The diagnostic performance of the RDT kit indicates that it is a potential alternative to microscopy in areas with poor facilities for microscopy. The kit is reliable, easy to use, and simple to interpret. Hence the RDT can be used for the rapid screening of blood donors for malaria parasites and epidemiological studies.

Out of the23 microscopy and RDT positive samples analyzed by PCR, only 6 were confirmed positive (Table 4.8). This low detection rate by PCR was surprising because generally PCR particularly the nested PCR has been found to be much more sensitive and specific than those of the other diagnostic methods when compared either with microscopy or rapid diagnostic tests (Weiss, 1995; Rubio *et al*., 1999; Singh *et al*., 1999; Tham *et al*., 1999). The low detection by PCR in this study could be as a result of the low parasite density, residual phenol used for the DNA extraction, storage and geographic differences in parasite populations. It could also result from poor performance of microscopy at low parasite densities rather than poor performance of PCR. These data are however not unusual (Coleman *et al*., 2006). A similar observation was reported by Davis *et al.* (2009)where microscopy identified parasites in 1 sample that had tested negative with the standard PCR assaydespiteexclusion of the possibility of the presence of PCR inhibitors by

successful quantification of parasite DNA that was added to the PCR-negative sample.

Although the majority of studies have shown that PCR is both sensitive and specific for the diagnosis of malaria, there are limitations that can affect the accuracy of the method. Selection of appropriate primers, methods used for collection and storage of blood samples, parasite density, geographic differences in parasite populations and extraction methods can all affect PCR performance (Kain *et al*., 1993; Barker *et al*., 1994; Singh *et al*., 1996; Jelinek *et al*.,1996;Farnert *et al*., 1999). Other studies have reported that PCR may occasionally yield false negative results (Kain *et al*., 1993;Singh *et al*., 1996; Barker *et al*., 1994). Barker *et al*. (1994) carefully analyzed discrepancies between microscopy and PCR, and although true false negative PCR results did occur, the majority of discrepancies resulted from problems with microscopy.Furthermore, multiplex PCR has often failed (Snounou *et al*., 1993, Singh *et al*., 1999) or found to be less sensitive for malaria diagnosis (Jelinek *et al*., 1996). Similarly, although nested PCR increases the sensitivity (Singh *et al*., 1999), it also requires multiple cycles and thus could be inefficient and increases the risk of contamination (Morassin *et al*., 2002).It had also been observed that PCR-positive results were obtained only when DNA was extracted from samples containing live parasites (Tham *et al*., 1999). Dead parasites or parasites cleared by drug treatment or immune system pressure do not give positive results by PCR amplification. Similar results have been reported by several other researchers (Aurelius *et al*., 1991; Kain *et al*., 1993; Nocton *et al*., 1994). Residual phenol can also cause inhibition of *Taq* polymerase (Shariff *et al*., 2016).Performance of PCR has also been found to decrease markedly at parasite densities <500/µl, with sensitivity of

only 20% for *P. falciparum* and 24% for *P. vivax* at densities <100 parasites/µl (Coleman *et al*., 2006).

There was a highrate of nucleotide mutations in the isolate from the Southern senatorial district (KAF 54)compared to the few in the isolates from the North and Central senatorial districts (Figure 4.1).In the derivation of phylogeneticrelationships, most emphasis has been placed on nucleotidesubstitutions (Wilson *et al*., 1985).The changes observed in the sequences of the isolate from the Southern senatorial district could be due to the high rate of nucleotide substitutions in the isolate from the Southern senatorial district. These results corroborate earlier observations on high genetic diversity in different genes in African *P. falciparum* (Conway *et al*., 2000, Joy *et al*., 2003). This observation is also in consonance with the report that the overall substitution rates inmt-DNA have been estimated to be about 5-10 times greater than in single-copy nuclear DNA (Brown *et al*., 1979; Fliss *et al*., 2000). The higher rate of nucleotide substitution observed in the isolate from the southern senatorial district could be responsible for its being placed in a different clade. The observed genetic similarities between the sequences of the isolates from the North (GS110) and Central (TW 32) senatorial districts may be due to their more geographical closenessand can be explained by the fact that *P. falciparum* populations often follow the isolation-by-distance (IBD) model of population structure (Tanabe *et al*., 2010) and are also maintained as genetically sub-structured populations (Anderson *et al*., 2000).

The fact that the mean values of the haematological parameters considered were all within normal values suggest that asymptomatic malarial parasitaemia has slight, but not significant effects on haematological indices(Table 4.10). This could be

attributed to the low parasite density recorded in the study. This is similar to conclusion of Soltyzfus *et al.* (1997) that asymptomatic malarial parasites infection does not produce much influence on haemoglobin concentration. A significant effect on the haematological parameters could lead to manifestation of symptoms that could disqualify the donor through pre-transfusion screening process.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Out of the 360 blood donors studied, the overall prevalence rate was 27(7.5%) by microscopy and 31(8.6%) by RDT. The 27 microscopy positive donors had parasite densities of between 80-240 parasites/µl of blood with an average of 126 parasites/µl of blood. There were no mixed infections and no other blood parasites were observed. Malarial parasitaemia in blood donors was significantly associated with occupation and blood group. The study also demonstrated a high sensitivity (70%), accuracy/efficacy (95.6%) and specificity (97%) of HRP-2 and aldolase based diagnostic assays and their potential utility for the screening of malarial parasitaemia in blood donors. Also, malarial parasitaemia had no significant effect on the Haematological parameters considered.

Out of the 23 cases tested by PCR, 6 (26.1%) were positive. Sequencing of the complete mitochondrial (mt) genome of the *Plasmodium falciparum* confirmed the previous species identification by the PCR with the closest having E values of 2e (for KAF 54) and 0.0 (for TW 032 and GS 110), percentage identifications of 97, 99 and 100 (for KAF 54, TW 032 and GS 110) respectively and Accension numbers of KT119883.1(for KAF 54) and KT191163.1 (for TW 032 and GS 110) respectively. The sequences of these three *P. falciparum* were aligned and their nucleotide sequences compared for possible mutations. Sequence changes observed were: sequence rearrangements, additions, deletions, and nucleotide substitutions. A

phylogenetic study of these three *P. falciparum* species was carried out and the result indicated that the isolates have a common ancestral origin.

6.2 RECOMMENDATIONS

Based on the findings in this study I wish to recommend that:

1. Blood donors should be routinely screened for malarial parasites and the blood marked negative or positive as the case may be.
2. Transfusion recipients of malaria positive blood, especially when they fall into the malaria vulnerable group should be routinely treated with anti-malarial as a prophylactic measure.
3. The accuracy and reliability of available and affordable RDTs be determined with a view to adopting RDTs for screening of blood donors as is obtainable in some countries.
4. Other aspects of the Epidemiology of malaria especially factors responsible for low or moderate prevalence in the area be extensively studied as the findings may be of great help towards the reduction or control of the infection in areas with high prevalence rates.
5. Commercial blood donors should be included in the targeted groups for malaria control and be given free LLITNs as an incentive for blood donation.

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APPENDICES

APPENDIX I: LETTER OF INTRODUCTION FROM THE DEPARTMENT

DEPARTMENT OF MICROBIOLOGY
AHMADU BELLO UNIVERSITY
ZARIA

***Vice Chancellor: Professor Abdullahi Mustapha, B.Sc (Hons) Pharm* (ABU), Ph.D. (London), FPSN**

Head of Department: DR. S.A. ADO; BSc, MSc, PhD (ABU)
E-mail: Microbiology@abu.edu.ng

Your Ref:

17/07/12

Dear Sir,

LETTER OF INTRODUCTION – GARBA, DOUGLAS DZINGINA (Ph.D/SCIE/02801/10-11)

The above mentioned is a Postgraduate student of this Department. He is carrying out his Ph.D. project research work with topic: "Molecular Epidemiology of Malaria Parasites among blood donors in Kaduna State, Nigeria".

He wishes to collect some samples from some selected hospitals in your State for his laboratory analysis.

We will appreciate any assistance rendered to him.

Yours faithfully,

DR. S.A. ADO
Head of Department

HEAD
Department of Microbiology
Ahmadu Bello University
Zaria

APPENDIX II: LETTER OF REQUEST FOR ETHICAL APPROVAL

Department of Microbiology,

Ahmadu Bello University,

Zaria.

30th July, 2012.

The Honourable commissioner,

Ministry of Health,

Kaduna State.

Dear Sir,

REQUEST FOR ETHICAL APPROVAL

I hereby request for ethical approval to conduct a Ph.D research titled "Molecular epidemiology of malaria parasites among blood donors in Kaduna state".

I am a Medical Laboratory Scientist and a post graduate student of the above stated institution and wish to use some of your hospitals to collect blood samples from fit blood donors, screen for malaria parasites and use the haematology auto analysers(free of charge) in the hospitals to determine the haematological parameters of the blood donors.

This will help in establishing the prevalence rate of asymptomatic malaria in apparently healthy individuals and the associated risk factors using molecular techniques, base line values of haematological parameters and assess the progress of the current Government efforts in the control of malaria in the state.

Patients' anonymity would be maintained, good laboratory practice ensured and every finding treated with utmost confidentiality.

Attached is a copy of the proposal and letter of introduction from my head of department.

Thank you in anticipation of your kind and favourable consideration.

Yours faithfully,



Garba Douglas Dzingina

APPENDIX III: ETHICAL APPROVAL

MINISTRY OF HEALTH, KADUNA STATE

All Communications to be Addressed to:
THE HON. COMMISSIONER
Quoting Reference and Data
Tel: (062) 248084
(062) 248252

Independence Way,
P.M.B. 2014,
Kaduna,
Kaduna State, Nigeria.

MOH/ADM/744/Vol. 1/188

5th September, 2012.

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ETHICAL APPROVAL

I am directed to convey the Honorable Commissioners' wish to grant approval to Mr. Garba Douglas Dzingina, a Ph. D student of the department of Microbiology, ABU – Zaria submitted a proposal to carry out a research on MOLECULAR EPIDEMIOLOGY OF MALARIA PARASITES AMONG BLOOD DONORS Kaduna State. It is expected that necessary assistance be accorded to him in the process of his research, please.

However, it is mandatory for the to submit a copy of his research finding(s) to the office of the Honorable Commissioner, Health as soon as it is concluded as you have the assurances of my highest regards.

5/9/12.

JACOB KAYIT MUSA
SECRETARY, ETHICAL COMMITTEE

APPENDIX IV: CONSENT FORM

A research on molecular epidemiology of malarial parasitaemia in blood donors is hereby being conducted.

The purpose of this research is to determine the frequency of asymptomatic malarial infection in persons who come to donate blood. The results will help in the formulation of a treatment policy for patients who receive blood transfusion. Your kind participation in this research will also enable us accurately describe the changes that occur due to malaria in the blood of persons who appear healthy inspite of the infection. This will be related to social and other factors in all participants.

You are expected to give 5ml (1 ½ teaspoonful) of your blood. In the course of giving your sample, you will experience a sharp pain due to needle prick which usually wanes within six (6) hours and in most cases no further treatment is required. If your blood sample is positive for malaria you will be given a prescription of antimalarial treatment.

If you agree to participate in this research please sign below.

Name and address of participant
_____
_____

Signature/Thumb print_____
Tel: _____
Date: _____

Name and address of witness
_____
_____

Signature/Thumb print_____
Tel: _____
Date: _____

Name of researcher
_____
_____

Signature/Thumb print_____
Tel: _____
Date: _____

APPENDIX V: CASE RECORD FORM

Donor's name (optional) ----------

Study no. ----------

A. QUESTIONARE

1. Age (a) <20 (b) 20-29 (c) 30-39 (d) 40-49 (e) 50-59 (f) 60 and above
2. Sex (a) Male (b) Female
3. Marital status (a) Single (b) Married (c) Divorced (d) Widow
4. Educational status (a) None (b) Primary (c) Secondary (d) Tertiary (e) Islamic (f) Others (specify) ----------
5. Occupation (a) Unemployed (b) Student (c) Civil servant (d) Business (e) Artisan U) Farmer (g) Others (specify) ----------
6. Where do you reside? ----------
7. What is the aim of donation?

   (a) To be given money, job or favour (b) For an ill-relative/friend (c) Voluntary
8. (a) Is this your first time of donation? Yes ( ) No ( )

   (b) I f no, (i) how many times have you donated before? ----------

   (ii) When last did you donate? ----------
9. (i) Have you ever had malaria? Yes ( ) No ( )

   (ii) If yes, when last did you have malaria?

   (a) <1 week ago (b) 2-4weeks ago (c) > 1 month- 1 year ago (d) > 1 year ago

   (iii) How did you treat it?

   (a) Using traditional medicine

   (b) Using orthodox medicine (indicate type) ----------
10. (i) Do you use mosquito/bed net? Yes ( ) No ( )

    (ii) If yes, is it insecticide treated? Yes ( )
11. Do you eat kola nut? Yes ( ) No ( )
12. Do you smoke? Yes ( ) No ( )
13. Do you take alcoholic drinks Yes ( ) No ( )

B. LABORATORY FINDINGS

1. Blood group ----------
2. Weight ----------
3. Weight ----------
4. Hb (g/dl) ----------
5. PC V ----------
6. WBC x 10^9 /L ----------
7. RBC ----------
8. PLTS x 10^9 /L ----------
9. Lymphocytes (%) ----------
10. Neutrophils (%) ----------
11. Eosinophils (%) ----------
12. Basophils (%) ----------
13. Monocytes (%) ----------
14. Malarial parasitaemia ----------

(a) Screening (RDT) ----------

(b) Microscopy ----------

(c) Parasite density/count ----------

(d) PCR ----------

15. Other blood parasites seen ----------