New Standards in Laboratory Diagnosis of Malaria

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By

Caroline Amuche Okoli

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I dedicate this book to my children Otitodirichukwu, Ofufedirichukwu and Ekenedirichukwu and all the children I attended to while working as a Medical Laboratory Scientist in the Pediatrics Research Laboratories of the University of Jos.

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PREFACE

There have been several reports on the increasing incidences of drug resistant plasmodium species, insecticide-resistant mosquitoes, morbidity and mortality due to malaria infection, and re-emergence of malaria in once malaria free areas. To reduce some of these problems, the World Health Organization (WHO) introduced and recommended the use of ACT based drugs as second-line drugs to deal with chloroquine-resistant malaria. These drugs, because of their high cost, the looming threat of plasmodial resistance to artemisinin and greater potential for adverse effects are advisably administered only when plasmodium species are identified in a patient's blood. Consequently, this has led to the WHO's shift from presumptive clinical diagnosis of malaria to confirmatory laboratory diagnosis. As a result, accurate, precise and timely laboratory diagnosis of malaria has become a matter of great concern to all involved in research and diagnosis of the disease. Laboratory diagnosis of malaria is however, not without its challenges. Errors in technique application, poor reagent quality, deficiency in personnel expertise, poor equipment, and unavailability of malaria reference laboratories, especially in malaria endemic areas, for proper quality assessment of malaria test reports are some of the challenges facing laboratory diagnosis of malaria, especially in developing countries. This book proffers some solutions to these challenges and is the result of over ten years of experience working in research and routine laboratories where malaria diagnosis is one of the major investigations. It is also a product of personal research, seminars and workshops on malaria.

This book is divided into four sections. Section one deals with the fundamentals of malaria and the use of microscopes in the diagnosis of malaria. It is essentially designed to acquaint the student or microscopist with the essentialities in proper diagnosis of malaria by the use of a microscope.

Section two deals with the use of antigen-antibody reactions in diagnosis of malaria. Immunochromatic and serological tests are described.

In section three, particular attention is paid to molecular diagnosis of malaria by polymerase chain reactions and the use of microsatellite markers. The use of metric equipment is also described.

Section four deals with some other aspects of proper diagnosis of malaria including the procedures in evaluation of a new test method, quality control and assurance as well as laboratory bio-safety.

The passion to write this book stems mostly from my experience working as a medical laboratory scientist and researcher in the Research Laboratory of the Special Care Baby Unit of Jos University Teaching Hospital in Jos, Plateau State, Nigeria. Malaria poses a great menace especially to children and pregnant women. Accurate and proper laboratory diagnosis of malaria in neonates, in the author's opinion, is the greatest challenge in laboratory diagnosis of malaria as far as age is concerned. This stems mostly from the low parasitaemia rate common in this age group. Having worked with this age group for several years, I have the passion to share the knowledge I have acquired. "We are open to sound scientific ideas and knowledge that could help take the laboratory diagnosis of malaria to higher heights, so as to help save lives which could be yours' or mine or our neighbors' far or near". This book is the author's second book. The first book was on 'Congenital Malaria and Birth weight in North Central Nigeria'.

Caroline A. Okoli

FOREWORD

New Standards in Laboratory Diagnosis of malaria is a well-researched text covering laboratory and diagnostic procedures in malariology. It was born out of several years of laboratory practice in some of the best laboratories in Nigeria. The detailed manner in which the author handled each aspect of the book depics a meticulous attention to detail hardly seen in most texts on the subject. There cannot be too many texts of equal measure of attention on malariology. It is highly recommended to both laboratory science practitioners and medical students alike.

ACKNOWLEDGEMENTS

I am passionately grateful to the Almighty God, who is the Eternal Light. He has given birth to me and by reason of this; I am an eternal light under the Almighty Eternal Light.

I am indebted to my children Otitodirichukwu, Ofufedirichukwu and Ekenedirichukwu for all their encouragement and understanding in the course of reviewing and updating this book.

I am sincerely thankful to my lecturers who taught me malariology and its laboratory investigations at different spheres at the Federal School of Medical Laboratory Sciences, Jos, Nigeria. Notable among them are, Dr Darlington Elekwe, Mr. Olotu, Mr. Olowu, Prof. G. Imade, Prof. I. Ike, just to mention a few. I remain indebted to my professional mentor, Mr. Ben Amamasi, a focused, meticulous and seasoned medical laboratory scientist, of the MD Ben Medical Laboratories Ltd, Maiduguri, Nigeria, for all the professional training I received from him.

Thanks to the University of Jos management for giving me the opportunity to serve as a medical laboratory scientist and researcher with the Research Laboratory of the Special Care Baby Unit (S.C.B.U) from where the passion to write this book sprang up.

I am indebted to one of the fathers of Biochemistry in Nigeria. A scientist of renown, a man who made indelible marks on the soil of sciences in Nigeria, Africa and the world at large, distinguished (late) Professor G.E Anekwe (O.O.N) for editing and reviewing this piece of work. I am also grateful to Mercy N. Sylva-Ugoh (Mrs) a paragon of the English Language for also reviewing this work. Your constructive criticisms are the pivot on which the success of this work rests. Thanks.

Caroline A. Okoli

ABOUT THE AUTHOR

Caroline A. Okoli hails from Eluama in the Orlu Local Government Area of Imo State, Nigeria. She studied Medical Laboratory Science from the prestigous Federal School of Medical Laboratory Sciences in Jos, Nigeria, where she obtained the Associate and Fellows Certificates of the Medical Laboratory Science Council of Nigeria with options in Chemical Pathology and Medical Microbiology respectively. She has M.Sc and PhD degrees in Nutritional and Clinical Biochemistry from the Universities of Jos and Ilorin, Nigeria, respectively. She is currently a lecturer with the Department of Medical Laboratory Science at the University of Jos, Nigeria. She has research interests in; Malariology, Clinical Paediatrics and Clinical Biochemistry. She has several research publications in several reputable journals both nationally and internationally. Her first book was titled 'Congenital Malaria and Birth weight in North Central Nigeria', published by Lambert Academic Publishers, Germany. She is married with children Otitodirichukwu, Ofufedirichukwu and Ekenedirichukwu (UpJesus).

SECTION ONE:

FUNDAMENTALS OF MALARIA AND MICROSCOPY DIAGNOSIS OF MALARIA

This section deals with the fundamentals of malaria infection and its diagnosis by the use of a microscope. We start with an introduction of malaria infection, its cause and control and we end with the Quantitative Buffy Coat test. Principles, methods and standard patterns for examination of both thin and thick blood films are fully described. Basic knowledge and use of a microscope are important. The microscopist needs to be very familiar with the morphological appearances of the different species and stages of plasmodium in both thin and thick blood films plus the appearances of the host red blood cells in the thin film in accordance to the infecting species of plasmodium. Constant and frequent practice, research and up-to-date knowledge are very necessary for proper use and mastering of these methods.

CHAPTER 1

INTRODUCTION

1.1 History of Malaria

The history of malaria stretches from its prehistoric origin as a zoonotic disease in the primates of Africa through the 21st century. Malaria infested every continent, except Antarctica (Carter and Mendis, 2002); references to its unique, periodic fevers are found throughout recorded history beginning in 2700 BC in China (Nghina *et al.,* 2010).

Malaria was the most important health hazard encountered by U.S. troops in the south pacific during World War II, where about 500.000 men were infected. According to Joseph Patrick Byrne, "Sixty thousand American soldiers died of malaria during the African and south pacific campaigns" (Byrne, 2008).

1.1.1 Origin and prehistoric period

Human malaria likely originated in Africa and co-evolved with its host, mosquitoes and non-human primates. Malaria protozoa are diversified into primate, rodent, birds and reptile host lineages (Joy *et al.,* 2003; Hayakawa, *et al.,* 2008). Humans may have originally caught *plasmodium falciparum* from gorillas (Liu, *et al.,* 2010). *P. vivax*, another malaria *plasmodium species* among six that infect humans, also likely originated in African gorillas and chimpanzees (Lui, *et al.,* 2014). Another malaria species recently discovered to be transmissible to humans, P. *knowlesi,* originated in Asian macaque monkeys (Lee, *et al.,* 2011). Meanwhile, *p. malarae* is highly host-specific to humans.

Molecular methods have confirmed the high prevalence of *P. falciparum* malaria in ancient Egypt (Brier, 2004). The ancient Greek historian Herodotus wrote that the builders of Egyptian pyramids (circa 2700-1700BCE) were given large amounts of garlic (Macaulay, 1890), probably to protect them against malaria. The presence of malaria in Egypt from circa

800 BCE onwards has been confirmed using DNA-based methods (Lalremruata, *et al.,* 2013).

1.1.2 Classical period

The term μίασμα (Greek for miasma), "stain, pollution", was coined by Hipocrates of Kos who used it to describe dangerous fumes from the ground that are transported by winds and can cause serious illness. Hippocrates (460-370 BCE), the "father of medicine", related the presence of intermittent fevers with climatic and environmental conditions and classified the fever according to periodicity: fever every third day and fever every fourth day (Pappas, *et al.,* 2008).

1.1.3 European Renaissance

The name malaria derived from mal aria ('bad air' in Medieval Italian). This idea came from the ancient Romans who thought that this disease came from the horrible fumes in the swamps. The word malaria has its roots in the miasma theory, as described by historian and chancellor of Florence Leonardo Bruni (Hempelmann and Krafts, 2013). The coastal plains of southern Italy fell from international prominence when malaria expanded in the sixteenth century. William Shakespeare was born at the start of the cold period that climatologists call the "little ice age", yet he was aware enough of the ravages of the disease to mention it in eight of his plays (Reiter, 2000).

1.1.4 Spread to the American Regions

European settlers and their West African slaves likely brought malaria to the Americas in the 16th century (De Castro and Singer, 2005; Yalcindag *et al.,* 2011). As malaria spread, places such as the tidewater of Virginia and South Carolina which had previously been habitable by white people became endemic with malaria. Malaria caused huge losses to British forces in the South during the revolutionary war as well as to Union forces during the civil war. Malaria also weakened the Native American population and made them more susceptible to other diseases.

1.2 Geographical Distribution of Malaria

Worldwide, an estimated 300 milion malaria infection occurs each year with 2 to 3 million deaths, making it one of the most common infectious diseases worldwide. Most deaths are from infections with *P.falciparum.* Malaria

occurs in over 90 countries worldwide. According to the figure provided by the World Health Organization (WHO): 36% of the global population live in areas where the risk of malaria transmission is high; 7% reside in areas where malaria has never been under meaningful control and 27% live in areas where malaria was once transmitted at low levels or not at all, but where significant transmission has been re-established. Young children and pregnant women have the highest risk of malaria infection. WHO estimates that more than 90% of 1.5-2.0 deaths due to malaria each year occur in African children.

Although Africa is the most affected, an estimated 10% of malaria cases occur in Southeast Asia and 45% occur in the Eastern Mediterranean. Transmission occurs primarily in tropical and subtropical regions, in Sub-Saharan Africa, Central and South America, the Caribbean of Hispaniola, the Middle East, the Indian sub-continent, and Southeast Asia.

In areas where malaria occurs, however, there is considerate variation in the intensity of transmission and risk of malaria infection. Highland (>1500m) and arid areas (<1000mm rainfall /year) typically have less malaria, although they are also prone to epidemics when parasitaemic individuals provide a source of infection and the environmental condition is favorable to mosquito development.

Table 1: Geographical Distribution of Malaria

Region	*P. falciparum*	*P. vivax*	*P. ovale*	*P.malariae*
North Africa	Frequent	Predominant	Absent	Frequent
West Africa	Predominant	Very rare	Frequent	Rare
Central Africa	Predominant	Very rare	Rare	Frequent
East Africa	Predominant	Rare	Rare	Frequent
Indian Ocean	Predominant	Rare	Rare	Frequent
Central America	Frequent	Frequent	Absent	Rare
South America	Frequent	Predominant	Absent	Rare
Indian subcontinent	Frequent	Predominant	Very rare	Rare
Southeast Asia	Predominant	Frequent	Rare	Rare
Pacific Islands	Frequent	Frequent	Rare	Rare

From Basic Laboratory Techniques, WHO 1982, modified.

1.3 Epidemiology of Malaria

Malaria is a serious health burden of developing nations, including Nigeria. It claims more lives than any other infectious disease in the world (Narasinhan and Attarann, 2003). Malaria deaths in African children younger than 5 years increased by about three times from 377,000 (95% uncertainty of 182,00-602,000) in 1980 to a peak of 1,047,000 (716,000-1,479,000) in 2004. Accelerated decreases in the past years translate to 699,000 (415,000-1,112,000) deaths in 2010 (Murray *et al.,*2012). Malaria is highly endemic in Nigeria where it accounts for 60% of outpatient visits to health facilities, 30% of childhood deaths, and 11% of maternal deaths (4,500 die yearly). The financial loss of man-hours etc. (Nigerian Demographic and Health Survey, 2011).

Plasmodium falciparum causes most severity and deaths attributed to the disease, which is most prevalent in Africa south of the Sahara, where Nigeria has the largest population. Country-specific evidence shows that Nigeria has the largest population at risk of malaria in Africa. The disease malaria is a major health problem in the country, with stable transmission throughout the country. It accounts for about 50% of outpatient consultations, 15% of hospital admissions, and is also prime among the top three causes of death in the country (National Malaria Control Plan of Action 1996 to 2001). More importantly, it is an economic problem, as it consumed about US$3.5 million in government funding and US$2.3 million from other stakeholders in various control attempts in 2003 (World Health Organization, 2005).

Approximately 50% of the Nigerian population experience at least one episode per year. However, official estimates suggest as much as four bouts per person per year on average (WHO, 1995 and 2002). The trend is rapidly increasing due to the current malaria resistance to first line anti-malarial drugs (WHO, 2000). The magnitude of incidences and deaths due to it is a multiple of all other tropical diseases put together. It is responsible for over 90% of reported cases of tropical disease in Nigeria (Alaba and Alaba, 2003).

The worst malaria situations occur in remote, rural areas and among the marginalized poor populations, ethnic minorities and forest dwellers. Travelers from malaria-free regions to endemic areas are highly vulnerable and may suffer from sever malaria due to lack of immunity (Were, 2004). Most malaria deaths occur at home, without confirmation of diagnosis. The reality is that in the poorest rural areas, where malaria takes its highest toll,

it is difficult to obtain accurate data and to derive meaningful malaria statistics. During their illness, many patients struggle, often unsuccessfully to access basic health care. For those that succeed, the care may be of dubious quality and ineffective (Hemingway and Bates, 2003).

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CHAPTER 2

FUNDAMENTALS OF MALARIA

2.1 Introduction

Malaria must be recognized promptly in order to treat the patient on time and prevent the complications associated with the disease and further spread of the infection in the community via local mosquitos. Malaria can be suspected based on the signs and symptoms manifested by the patient, the physical findings at examination as well as the patient's travel history. However, for a definite diagnosis to be made, laboratory tests must demonstrate the malaria parasites or their components. Malaria in humans is mainly caused by infection by four plasmodium species (*P. falciparum, P.vivax, P.ovale* and *P.malariae*).

2.2 Cause of Malaria

2.2.1 The Vector

Female anopheles mosquitoes transmit human malaria parasites. Of the 460 species of anopheles described, only 68 are known vectors of malaria. The best vectors are abundant and long-lived, prefer to feed on humans and usually prefer to live in close proximity to human habitation. *Anopheles gambiae* has all these characteristics and is, consequently, the best vector. This explains why 90% of malaria is found in Africa, where this particular mosquito is present (Roger and Hommel 2008).

2.2.2 Plasmodium

Blood parasites of the genus plasmodium are approximately 156 named species, which infect various species of vertebrate. Malaria in humans is caused by four species of plasmodium (*P.falciparum*, *P. vivax*, *P.ovale* and *P. malariae*). These plasmodia utilize humans almost exclusively as a natural intermediate host. However, there have been periodic reports of simian malaria parasites infecting humans.

P. falciparum

P. falciparum is the most important malaria parasite. It is life-threatening, especially when it is not properly diagnosed and treated. It is responsible for about 50% of all the malarial cases throughout the world. Its distribution is restricted to warm and tropical countries.

P. vivax

It causes the benign tertian form of malaria. It is generally non-life threatening, though it can cause severe, acute malaria. Globally, it is responsible for about 43% of malaria cases and has the widest geographical distribution.

P.malariae

It produces a quartan malaria, which is responsible for about 7% of malaria cases.

P. ovale

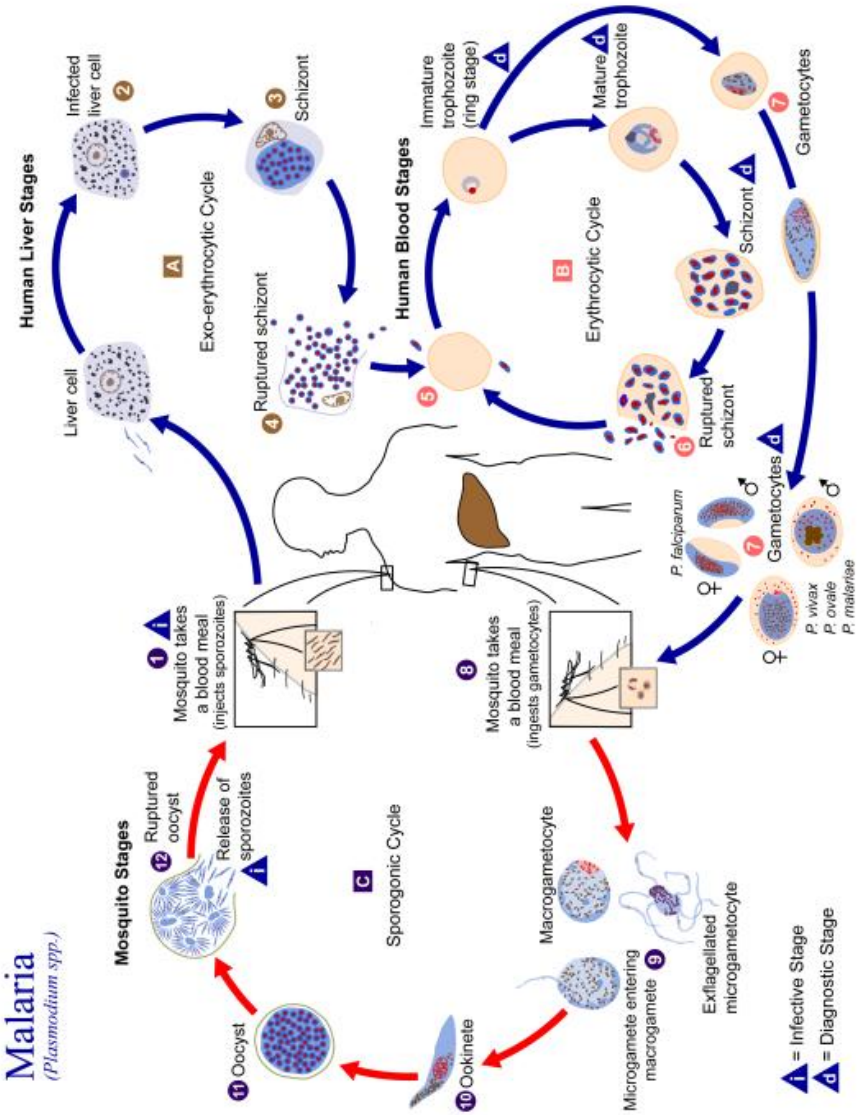
It produces ovale tertian fever, which is milder than the benign tertian fever of *p. vivax.* It is rare and has been reported in the tropics and subtropics.

Simian Malaria

Molecular methods have revealed the possible existence of some other species or morphological variants apart from the four distinct plasmodium species infecting humans. P.simian has been identified in Malaysia as infective to humans. Simian plasmodium species are of zoonotic origin. They are originally from monkies and apes. They have a single S-type like gene and several A-type-like genes. The Asian simian plasmodia species are *Plasmodium coatneyi, Plasmodium cynomolgi, Plasmodium fragile, Plasmodium inui, Plasmodium fieldi, Plasmodium hylobati, Plasmodium simiovale* and *Plasmodium knowlesi*.

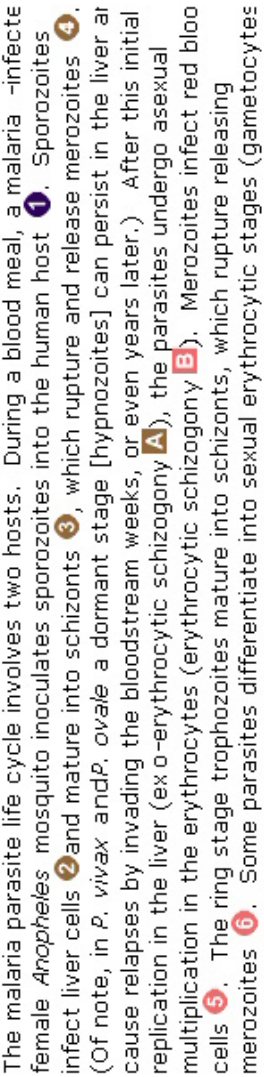
P. knowlesi is a *P. simian* parasite that is most implicated in human infection.

2.3 Life Cycle of Plasmodium



Adapted from: http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm

Explanation of the illustration by CDC:



The malaria parasite life cycle involves two hosts. During a blood meal, a malaria -infecte female *Anopheles* mosquito inoculates sporozoites into the human host (1). Sporozoites infect liver cells (2) and mature into schizonts (3), which rupture and release merozoites (4). (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver ar cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony (A)), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony (B)). Merozoites infect red bloo cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). Some parasites differentiate into sexual erythrocytic stages (gametocytes . Blood stage parasites are responsible for the clinical manifestations of the disease.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingeste by an *Anopheles* mosquito during a blood meal (8). The parasites' multiplication in the mosquito is known as the sporogonic cycle (C). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10) which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites which make their way to the mosquito's salivary glands. Inoculation of the sporozoites in new human host perpetuates the malaria life cycle (1).

2.4 Clinical Features of Malaria

The symptoms of uncomplicated malaria can be rather non-specific. Untreated malaria can progress to severe forms that may be rapidly (<24 hours) fatal. The most frequent symptoms include: fever and chills which can be accompanied by headache, myalgias, arthralgias, weakness, vomiting and diarrhoea. Other clinical features include splenomegaly, aneamia, thrombocytopenia, hypoglyceamia, pulmonary or renal dysfunction and neurologic changes.

The clinical presentation can vary substantially depending on the species, the level of parasitaemia and the immune status of the patient. Infections caused by *P.falciparum* can progress to severe, potentially fatal forms with central nervous system involvement (cerebral malaria), acute renal failure, severe anaemia or adult respiratory distress syndrome. Complications of *P.vivax* malaria include splenomegaly (with rare splenic rupture) and those of *P.malariae* include nephritic syndrome (Greenwood et al, 2005; Greenwood,2005).

2.5 Factors Influencing Vectorial Capacity of Mosquitoes

The potential of the mosquito to serve as a vector depends on the ability to support sporogony, mosquito abundance, and contact with humans, which are all influenced by climatic and ecological factors (Martin & Lefesvre, 1995). The ability to support sporogony is largely dependent upon species in that not all species of Anopheles are susceptible to Plasmodium infection. Temperature and mosquito longevity are other key factors affecting the parasite's interaction with the vector (Coetzee et al, 2000; Protopoff et al, 2009; Ceccato et al, 2012; Matsuoka & Kai 1995; Martin & Lefesvre, 1995). Development of *P. falciparum* requires a minimum temperature of 20^{o}C, whereas the minimum temperature for the other species is 16^{o}C. Temperature also affects the time of development in that the duration of sporogony is substantially shorter at higher temperatures (Coetzee *et al*, 2000; Protopoff et al, 2009; Ceccato *et al*, 2012). A shorter duration of sporogony increases the chances that the mosquito will transmit the infection within its lifespan. Mosquito density and feeding habits also influence the transmission of malaria. Mosquito density is affected by temperature, altitude, rainfall and the availability of breeding places, whereas human-mosquito contact will be influenced by the mosquito behavior. The preferred feeding time and whether the mosquito feeds predominantly indoors or outdoors will influence the transmission

dynamics (Martin & Lefesvre, 1995; Ceccato *et al*, 2012). For example, outdoor feeding mosquitoes are more likely to find a human blood meal in the early evening than those feeding late at night when most people are inside. The behavior of the mosquito also needs to be considered in control activities.

2.6 Control of Malaria

The control of malaria involves three living organisms: man (the host), plasmodium (the parasite) and anopheles mosquito (the vector). The WHO Ministerial conference in October, 1992 in Amsterdam evolved a Global strategy for malaria control. The strategy broadly suggests less emphasis on vector control and renewed emphasis on treatment. Early diagnosis and treatment, prevention of deaths; promotion of personal protection measures like use of insecticide treated nets (ITNs); epidemic forecasting, early detection and control; monitoring, evaluation and operative research and integration of activity in Primary Health Care Centers are salient aspects of this strategy (WHO, 1992).

For effective malaria control, man is the first target followed by mosquito control and development of effective drugs and vaccines against the parasite.

2.6.1 Man as the First Target in Malaria Control

Prompt and accurate diagnosis plus early and proper treatment are very important factors in malaria control (WHO, 1992). The increasing prevalence of malaria, its associated morbidity and mortality, especially in children and pregnant women, coupled with the progressive increase in the resistance of anopheles mosquitos and malaria parasites to insecticides and anti-malarial drugs respectively have called for an up-to-date research report on malaria. Concerning prevention and control, in a recent study by Okoli and Enna (2014), it was discovered that at community levels in Jos, Nigeria, there was an increased ownership and usage of insecticide treated bed nets. However, the level of environmental/personal hygiene was low and availability of mosquito breeding sites was high. Thus, in malaria control strategies, efforts need to be intensified to make adequate information relating to the importance of environmental/personal hygiene, elimination of mosquito breeding sources and safety of insecticide treated bed nets more available and accessible in the community.