VALIDATION AND COMPARISM OF CYSCOPE MICROSCOPE, QUANTITATIVE BUFFY COAT MICROSCOPE AND RAPID DIAGNOSTIC KIT FOR MALARIA DIAGNOSIS AMONG CLINIC ATTENDEES IN IBADAN, NIGERIA

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A DISSERTATION IN THE DEPARTMENT OF EPIDEMIOLOGY AND MEDICAL STATISTICS SUBMITTED TO THE FACULTY OF PUBLIC HEALTH, COLLEGE OF MEDICINE IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF PUBLIC HEALTH (LABORATORY EPIDEMIOLOGY PRACTICE) OF THE UNIVERSITY OF IBADAN

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ABSTRACT

The unavailability of accurate, rapid, reliable and cost effective malaria diagnostic instruments constitute a major challenge to malaria elimination campaign. This compels many laboratories to depend on the conventional method of detecting malaria parasitaemia using light microscopy. This method has challenges such as labour-intensiveness, poor expertise, resulting in delayed turnaround time for diagnosis and thereby promoting high morbidity and mortality. Alternative diagnostic instruments like cyscope fluorescent microscope (Cyscope), quantitative buffy coat fluorescent microscope (QBC) and CareStart™ rapid diagnostic kit (CareStart™) with the potential to address these challenges have been developed but their validity and cost effectiveness have not been determined in Nigeria. This study was, therefore, designed to validate these instruments and assess their comparative cost effectiveness.

Using evaluative study design, five hundred and two (502) out of one thousand eight hundred (1,800) patients with signs and symptoms suggestive of malaria at the University College Hospital, Adeoyo State Hospital, and Kola Daisi Foundation Health center in Ibadan between January and April, 2014, were selected by systematic random sampling. Blood samples were collected and evaluated for malaria parasites; using Cyscope, QBC and CareStart™. The blood samples were then evaluated for malaria parasites using light microscopy as gold standard. For each instrument, validity indices assessed were sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV). Cost per hour of use of each instrument was also determined. Data were analyzed with McNemar Chi-square and Kappa statistics at level of significance set at $p = 0.05$.

Malaria prevalence in the samples was 19.5%, 21.7%, 30.7% and 32.7% for CareStart™, light microscopy, Cyscope, and QBC respectively. The sensitivity of the instruments compared with light microscopy was 76.0% for CareStart™, 95.0% for Cyscope and 98.1% for QBC; while specificity for QBC was 85.5%, Cyscope had 87.3% and 96.0% for CareStart™. Positive Predictive Value for the instruments was 65.2% for QBC, 67.5% for Cyscope, and 84.7% for CareStart™; with Negative Predictive Value of 93.6%, 98.6% and 99.4% for CareStart™, Cyscope, and QBC respectively. Inter instrument agreement index, Kappa values (Ka) was 0.71

(CI= 0.64 - 0.77) for QBC, 0.72 (CI= 0.65 - 0.78) for Cyscope and 0.75 (CI= 0.68 - 0.82) for CareStart™. Average cost per hour of use for Cyscope was $2.04, CareStart™: $5.61, QBC: $5.89 and $10.77 for light microscopy. The turnaround time per result output was Cyscope: 5minutes, QBC: 10minutes, CareStart™: 20minutes and 45 minutes for light microscopy.

Cyscope fluorescent microscope had the least turnaround diagnostic time and it is the most cost effective of all the laboratory diagnostic instruments evaluated. Cyscope fluorescent microscope is therefore strongly recommended for malaria parasite detection.

Keywords: Malaria diagnosis, Rapid diagnostic test, Malaria parasites, Cyscope microscope.
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I pray God will keep us all to achieve greater heights in all our endeavors in Jesus name. Amen.

DEDICATION

This work is dedicated to God Almighty for providing me life, good health and strength to get to this stage of the programme. May His name be glorified.

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CERTIFICATION

We certify that Abiodun Olakunle Ogunniyi carried out this work in the Department of Epidemiology and Medical Statistics, College of Medicine University of Ibadan under our supervision.

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CHAPTER ONE

1.0 INTRODUCTION

Background of the study

Approximately half of the world's population is at risk of malaria, and an estimated 243 million infected cases resulted in nearly 863,000 deaths in 2008 (WHO, 2009). In sub-Saharan Africa (SSA), where 91% of all malaria-related deaths take place, malaria is estimated to result in an annual loss of 35.4 million Disability Adjusted Life Years with 85% of the deaths amongst children below five years of age. In the same region, around 40% of all public health spending is related to malaria (WHO, 2010).

Malaria is responsible for about a 1.3 per cent reduction in the average annual rate of economic growth for those countries with the highest burden (Oyindamola *et al.*, 2010). In Nigeria, malaria is the major cause of morbidity and mortality, especially among children below five years of age (Alaba, 2007). Malaria is a social and economic problem, which consumed about US$ 3.5 million in government funding and US$ 2.3 million from other stakeholders in the form of various control attempts in 2003 (WHO, 2005).

Human infection begins when the malaria vector, a female anopheline mosquito, inoculates plasmodial sporozoites from its salivary gland into humans during a blood meal. The sporozoites mature in the liver and are released into the bloodstream as merozoites. These invade red blood cells, causing malaria fevers. Some forms of the parasites (gametocytes) are ingested by anopheline mosquitoes during feeding and develop into sporozoites, restarting the cycle (Mendis *et al.*, 2001).

Patients with malaria typically become symptomatic a few weeks after infection, although the host's previous exposure or immunity to malaria affects the symptomatology and incubation period. In addition, each *Plasmodium* species has a typical incubation period. Importantly, virtually all patients with malaria present with fever. Clinical symptoms include headache, cough, fatigue, malaise, shaking chills, arthralgia, myalgia. In severe cases, patient could present with convulsions, loss of consciousness, etc. Paroxysm of fever, shaking chills, and sweats (every 48hr or 72 hr, depending on species). The classic paroxysm begins with a period of shivering and chills, which lasts for approximately 1-2 hours and is followed by a high fever. Finally, the patient experiences excessive diaphoresis, and the body temperature of the patient drops to normal or below normal (Emilio, 2012).

Malaria is the most common single diagnosis made in most countries in Africa (WHO, 2003). In many endemic countries, clinical diagnosis is the only method used to decide on treatment even though its accuracy is limited by the low specificity of signs and symptoms of malaria (Chandramohan *et al.*, 2002, Källander *et al.*, 2004 and Mwangi *et al.*, 2005). Presumptive antimalaria treatment for any fever with no obvious alternative cause is widely practiced, and studies suggest that this leads to significant overuse of antimalaria drugs throughout Africa (Amexo *et al.*, 2004, Barat *et al.*, 1999, Mwangi *et al.*, 2005).

Reliable diagnosis of malaria requires laboratory confirmation of the presence of malaria parasites in the blood of a febrile patient (Nevill, 1990), hence a prompt and accurate diagnosis of malaria is the key to effective disease management. Laboratory confirmation of malaria infection requires the availability of a rapid, sensitive, and specific test at an affordable cost. However, in many endemic countries, conventional laboratory technique through the use of Light microscopy using Giemsa stain, to confirm the clinical diagnosis of malaria are considered to be too labour-intensive (Bojang *et al.*, 2000) and unreliable due to lack of skilled and certified microscopists, limited supplies, inadequate maintenance of microscopes and reagents, and inadequate or absence of quality control systems (Coleman et al., 2002).

In general, the screening of blood slides by Light microscopy using Giemsa stain for detecting malaria parasite is still considered as the gold standard (Jonkman *et al.*, 1995). This method is cheap and simple but labour-intensive, time-consuming and requires well-trained and certified utilization of microscopy and the development of alternative diagnostic techniques could substantially improve malaria control (Cook, 1992). Such objectives prove particularly relevant to the Roll Back Malaria initiative, a global movement that emphasizes better application of existing tools and the development of new ones (WHO, 2000).

There exists challenge of mis-diagnosis of malaria, high turnaround time, inadequate number of skilled malaria microscopists. These have effects on timely and appropriate case management of malaria and the implementation of WHO's guideline of parasite based diagnosis before malaria treatment. Cyscope fluorescent microscope (Cyscope), Quantitative Buffy Coat fluorescent microscope (QBC), CareStart rapid diagnostic kit (CareStart) are some of the new malaria diagnostic tools with the potential to provide a better alternative to Light microscopy using Giemsa stain , the routinely available and conventional diagnostic tool. This will significantly resolve the challenges earlier identified with the use of the conventional tool. However, the validation/performance assessment of these tools and their cost effectiveness has not been done in Nigeria, an endemic and resource limited setting. Hence no in country/ local data to support their effective use and justify possible consideration as tools for inclusion in the malaria elimination program of the country.

1.3 Rationale and Justification for the study

In public health especially in low resource settings, it is preferable to have cost-effective tools that can be deployed at the peripheral centres that are the only health facilities accessible to people in hard-to-reach areas. Performance assessment of Cyscope, QBC, and CareStart using light microscopy as gold standard needs to be established; knowing well that it is feasible to deploy these instruments at primary/secondary/tertiary health facilities as their individual usage may be required or implied. Also there is visual fatigue associated with having to read hundred of slides in high malaria burden environment using light microscopy, which suggests the need to consider alternative tool which is relatively user friendly with less rigorous training on how to operate as opposed to what is obtained using conventional light microscopy.

The burden of definitive diagnosis of malaria relies majorly on the laboratory and the outcome is dependent on the expertise of the laboratory personnel, accuracy of the diagnostic equipment, Turnaround time for result of diagnosis, availability and affordability of relevant diagnostic tools.

Malaria is greatly over diagnosed by clinicians owing to perceived equivalence of malaria with febrile illness due to the endemic nature of the disease. This disease is also mis-diagnosed due to lack of expert microscopists especially at the rural areas where majority of the population resides. Inability of clinicians to wait for the result of Light microscopy using Giemsa stain due to long turnaround time have resulted on reliance on clinical judgment, thus presumptive treatment with its attendance consequences.

Failure of the laboratory to provide timely diagnostic response with equipments that will ensure high predictive values, the subjective nature of malaria light microscopy and non availability of rapid diagnostic equipments has contributed to the perceived high burden of the disease in Nigeria. There is paucity of information on comparability of Cyscope microscopy with Giemsa-stained light microscopy and other routinely available diagnostic tools. There still exist issues with long turnaround time of conventional microscopy in facilities with high patient load; complicated by inadequate number of skilled malaria microscopists. These affects rational use of antimalarials for cases that do not need it and the resultant effects of these on management of malaria and the implementation of WHO's guideline of parasite based testing before treatment.

Until recently, presumptive diagnosis of malaria by healthcare professionals was the routine method of diagnosis of malaria. Similarly, self-medication is a common practice among the general populace. These underscore the need for accurate and prompt diagnosis of malaria before treatment to achieve better disease control. These challenges necessitated the development of easier and faster diagnostic methods including rapid diagnostic tests (RDTs). Generally, RDTs are immunochromatographic tests targeting specific antigens of one or more *Plasmodium* species. They produce easily interpretable results within a short time; require minimal training and less expertise. A WHO product testing round 4 done in 2012, showed that CareStart has a parasite detection score of 100% using cultured or clinical sample, however performance of CareStart™, an HRP-II-based RDT has not been evaluated in south-west Nigeria. Also, there are only few reports evaluating Cyscope® - a fluorescent microscopic device for the rapid diagnosis of malaria. Unlike most malaria RDTs which consist of lateral-flow immunochromatographic devices that detect parasite-specific antigens in the blood, Cyscope 'RDT" is a portable, battery-operated fluorescent microscopy manufactured by Partec, Germany.

The principle of Cyscope is based on the detection of intraerythrocytic *Plasmodium* DNA, which results in a bright intracellular dot-shaped fluorescence if the red blood cells are infected with *Plasmodium* spp. A affordable pricing, portability and compact design of the CyScope®, and the fact that reagents do not require cold storage, battery operated , make the method a potentially attractive alternative for malaria diagnosis in the field and rural setting. These tools have not been validated alongside their cost-effectiveness in Nigeria, a resource limited setting, hence the need to assess their performance and diagnostic accuracy, turnaround time and cost effectiveness for possible consideration in malaria diagnosis protocol in National Malaria Elimination Program

1.4 Research Questions

1. What is the sensitivity of Cyscope microscope, QBC and CARESTART for the diagnosis of malaria in this setting, using light microscopy as gold standard?
2. What is the specificity of Cyscope microscope, QBC and CARESTART for the diagnosis of malaria in this setting, using light microscopy as gold standard?
3. What is the predictive value of Cyscope microscope, QBC and CARESTART for the diagnosis of malaria in this setting, using light microscopy as gold standard?
4. What is the likelihood ratio of Cyscope microscope for the diagnosis of malaria using light microscopy as gold standard?
5. How does light microscopy compare with Cyscope microscope, Carestart and QBC for the diagnosis of malaria in terms of cost effectiveness and Turnaround time?

1.5 Hypothesis

Using light microscopy as gold standard, there is no difference in the diagnostic performance of Cyscope fluorescent microscope, Quantitative Buffy Coat fluorescent microscope and CareStart™ rapid diagnostic kit, in malaria diagnosis.

1.6 Aim of the study

The aim of this study is to test and compare the diagnostic performances characteristics of Cyscope (Partec, Germany), Quantitative Buffy Coat (QBC) and CareStart™ (AccessBio, Inc. New Jersey) in malaria parasite detection among clinical suspected malaria cases in a malaria-endemic environment using light microscopy as the gold standard.

1.7 Specific Objectives of the study

The objectives of this study were to:

1 Determine the diagnostic performance characteristics of Cyscope microscope, QBC microscope and Care Start™ rapid diagnostic kit, in malaria diagnosis using conventional light microscopy as gold standard.

2 Compare the diagnostic performance characteristics of Cyscope microscope, QBC microscope and Care Start™ rapid diagnostic kit, in malaria diagnosis using conventional light microscopy as gold standard.

3 Assess the turnaround time of Cyscope microscope, QBC microscope and Care Start™ rapid diagnostic kit, under routine laboratory working condition

4 Determine the average cost of malaria diagnosis using Cyscope microscope, QBC microscope and Care Start™.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of malaria

Malaria was once thought to be caused by breathing in foul swamp vapour: the name is from the Italian word "mal" - bad - and "aria", air (Suh et al., 2004). Swamps are indeed a cause, because they are breeding grounds for mosquitoes, which spread the malaria parasite from person to person through their bite (Suh et al., 2004). Towards the end of the 19th century, Charles Louis Alphonse Laveran, a French army surgeon, noticed parasites in the blood of a patient suffering from malaria and Dr Ronald Ross (Tuteja, 2007), a British medical officer in Hyderabad, India, discovered that mosquitoes transmitted malaria. The Italian professor Giovanni Battista Grassi subsequently showed that human malaria could only be tr\ansmitted by Anopheles mosquitoes (Tuteja, 2007).

2.1.1 The parasite

Malaria is transmitted through the bite of an infected female Anopheles mosquito. During the life cycle in peripheral blood, the different species may be observable in the four different life-cycle-stages which are generally morphologically distinguishable: ring, trophozoite, schizont, and gametocyte. The species differ in the changes of the shape of the infected (occupied) cell, presence of some characteristic dots (Schüffner's dots, Maurer's clefts, Ziemann's Stippling) and the morphology of the parasite in some of the life-cycle-stages (WHO, 1991). The life-cycle-stage of the parasite is defined by its morphology, size (i.e. maturity), and the presence or absence of malarial pigment (i.e. Haemozoin). Illustrations can be found in various sources, as reported by WHO in 1991 and Coatney *et al*, (1971).

Of the approximately 400 species of Anopheles throughout the world, about 60 are malaria vectors under natural conditions, 30 of which are of major importance (Tuteja, 2007). Malaria parasites are eukaryotic single-celled microorganisms that belong to the genus *Plasmodium*

(Tuteja, 2007). Only 4 of the species of plasmodia are infectious to humans (Tuteja, 2007). The majority of cases and almost all deaths due to malaria are caused by *Plasmodium falciparum* (Snow et al., 2004). *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* cause less severe disease (Suh et al., 2004). These four species differ morphologically and immunologically, in their geographical distribution, in their relapse patterns and in their drug responses (Tuteja, 2007). *Plasmodium falciparum* is the agent of severe, potentially fatal malaria and is the principal cause of malaria deaths in young children in Africa (Snow et al., 2004) and generally 90% of all cases in Africa (Suh et al., 2004).

2.1.2 Parasite Distribution

Malaria is endemic in 109 countries and is found throughout the tropics (WHO, 2008b) (Figure 1). In Africa, *P. falciparum* predominates, as it does in Papua New Guinea and Haiti, whereas *P. vivax* is more common in Central and parts of South America, North Africa, the Middle East and the Indian subcontinent (Cook et al., 2008). The prevalence of both species is approximately equal in other parts of South America, South-east Asia and Oceania. *P. vivax* is rare in sub-Saharan Africa (except for the horn of Africa), whereas *P. ovale* is common only in West Africa (Cook et al., 2008). *P. malariae* is found in most areas, but it is relatively uncommon outside Africa. Malaria was once endemic in Europe and northern Asia and was introduced to North America but it has been eradicated from these areas. In northern China and North Korea, *P. vivax* strains (*P. vivax hibernans*) with long incubation periods and long intervals (10–12 months) between relapses may still be found (Cook et al., 2008).

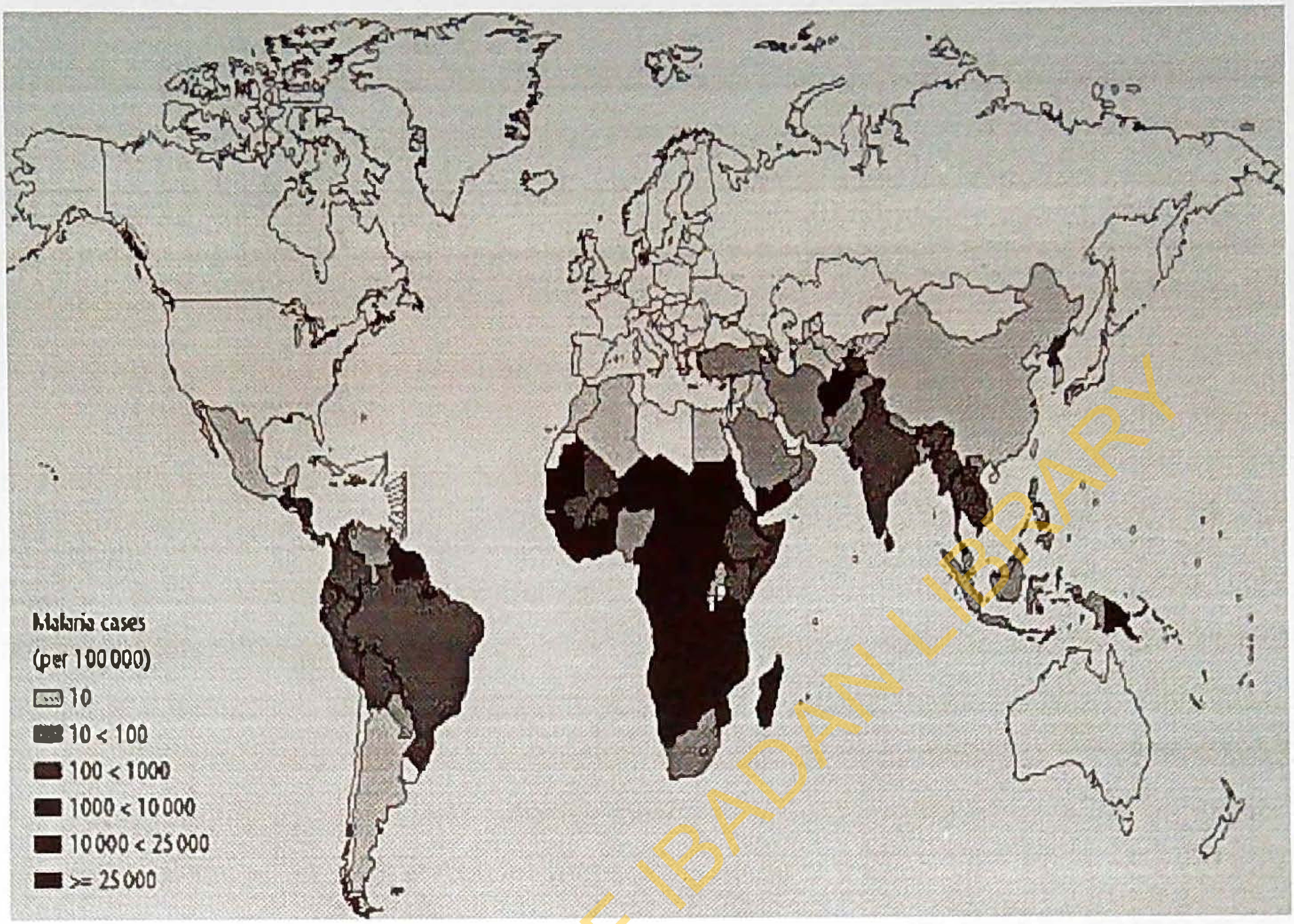

Figure 1: Global distribution of malaria by WHO map

(Source: http://gamapserver.who.int/mapLibrary/Files/Maps/global_cases.jpg, 2008)

2.1.3 Life cycle of the malaria parasite

The life cycle of malaria parasites is extremely complex and requires specialized protein expression for survival in both the invertebrate and vertebrate hosts (Tuteja, 2007). These proteins are required for both intracellular and extracellular survival, for the invasion of a variety of cell types and for the evasion of host immune responses (Tuteja, 2007). Once injected into the human host, *P. falciparum* and *P. malariae* sporozoites trigger immediate schizogony, whereas *P. ovale* and *P. vivax* sporozoites may either trigger immediate schizogony or lead to delayed schizogony as they pass through the hypnozoite stage. The life cycle of the malaria parasite is shown in Figure 2 and can be divided into several stages, starting with sporozoite entry into the bloodstream.

2.1.3a Tissue schizogony (pre-erythrocytic schizogony)

Infective sporozoites from the salivary gland of the Anopheles mosquito are injected into the human host along with anticoagulant-containing saliva to ensure an even-flowing blood meal (Tuteja, 2007). It was thought that sporozoites move rapidly away from the site of injection, but a recent study using a rodent parasite species (*Plasmodium yoelii*) as a model system indicates that the majority of infective sporozoites remain at the injection site for hours, with slow release into the circulation (Yamauchi et al., 2007). Once in the human bloodstream, *P. falciparum* sporozoites reach the liver and penetrate the liver cells (hepatocytes) where they remain for 9-16 days and undergo asexual replication known as exo-erythrocytic schizogony. The mechanism of targeting and invading the hepatocytes is not yet well understood, but studies have shown that sporozoite migration through several hepatocytes in the mammalian host is essential for completion of the life cycle (Mota et al., 2001).

The receptors on sporozoites responsible for hepatocyte invasion are mainly the thrombospondin domains on the circumsporozoite protein and on thrombospondin-related adhesive protein (Miller et al., 2002). These domains specifically bind to heparin sulfate proteoglycans on the hepatocytes (Frevert et al., 1993). Each sporozoite gives rise to tens of thousands of merozoites inside the hepatocyte and each merozoite can invade a red blood cell (RBC) on release from the liver. In an interesting study, also using rodent malaria parasites

(Plasmodium berghei), it has been shown that liver-stage parasites manipulate their host cells to guarantee the safe delivery of merozoites into the bloodstream (Sturm et al., 2006). Hepatocyte-derived merosomes appear to act as shuttles that ensure the protection of parasites from the host immune system and the release of viable merozoites directly into the circulation (Sturm et al., 2006). The time taken to complete the tissue phase varies, depending on the infecting species; (8-25 days for *P. falciparum*, 8-27 days for *P. vivax*, 9-17 days for *P. ovale* and 15-30 days for *P. malariae*) and this interval is called the **prepatent period**.

2.1.3b Erythrocytic schizogony

Merozoites enter erythrocytes by a complex invasion process, which can be divided into four phases: (a) initial recognition and reversible attachment of the merozoite to the erythrocyte membrane; (b) reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of substances from the rhoptry and microneme organelles, leading to formation of the parasitophorous vacuole; (c) movement of the junction and invagination of the erythrocyte membrane around the merozoite accompanied by removal of the merozoite's surface coat; and (d) resealing of the parasitophorous vacuole and erythrocyte membranes after completion of merozoite invasion (Miller et al., 2002). This is because the invasion of erythrocytes by *P. falciparum* requires a series of highly specific molecular interactions; it is regarded as an attractive target for the development of interventions to combat malaria (Frevert et al., 1993).

Asexual division starts inside the erythrocyte and the parasites develop through different stages therein (Tuteja, 2007). The early trophozoite is often referred to as the ring form, because of its characteristic morphology (Tuteja, 2007) (Figure 3). Trophozoite enlargement is accompanied by highly active metabolism, which includes glycolysis of large amounts of imported glucose, the ingestion of host cytoplasm and the proteolysis of hemoglobin into constituent amino acids (Tuteja, 2007). Malaria parasites cannot degrade the heme by-product and free heme is potentially toxic to the parasite (Tuteja, 2007). Therefore during hemoglobin degradation, most of the liberated heme is polymerized into hemozoin (malaria pigment), a crystalline substance that is stored within the food vacuoles (Miller et al., 2002).

The end of this trophic stage is marked by multiple rounds of nuclear division without cytokinesis resulting in the formation of schizonts (Miller et al., 2002) (see figure 2). Each mature schizont contains around 20 merozoites and these are released after lyses of the red blood cells (RBC) to invade further uninfected RBCs. This release coincides with the sharp increases in body temperature during the progression of the disease (Tuteja, 2007). This repetitive intraerythrocytic cycle of invasion multiplication release invasion continues, taking about 48 h in *P. falciparum*, *P. ovale* and *P. vivax* infections and 72 h in *P. malariae* infection (Miller et al., 2002; Tuteja, 2007). It occurs quite synchronously and the merozoites are released at approximately the same time of the day (Tuteja, 2007). The contents of the infected RBC that are released upon its lyses stimulate the production of tumor necrosis factor and other cytokines, which are responsible for the characteristic clinical manifestations of the disease.

A small proportion of the merozoites in the red blood cells eventually differentiate to produce micro and macrogametocytes (male and female, respectively), which have no further activity within the human host (Carter et al., 1980). These gametocytes are essential for transmitting the infection to new hosts through female Anopheles mosquitoes (Carter et al., 1980). Normally, a variable number of cycles of asexual erythrocytic schizogony occur before any gametocytes are produced (Tuteja, 2007). In *P. falciparum*, erythrocytic schizogony takes 48hours and gametocytogenesis takes 10-12 days (Tuteja, 2007).

Gametocytes appear on the fifth day of primary attack in *P. vivax* and *P. ovale* infections, and thereafter become numerous; they appear at anytime from 5-23 days after a primary attack by *P. malariae* (Tuteja, 2007).

2.1.3c Sexual phase in the mosquito (sporogony)

A mosquito taking a blood meal on an infected individual may ingest these gametocytes into its midgut, where macrogametocytes form macrogametes and exflagellation of microgametocytes produce microgametes (Tuteja, 2007). These gametes fuse, undergo fertilization and form a zygote. This transforms into an ookinete, which penetrates the wall of a cell in the midgut and develops into an oocyst (Tuteja, 2007).

In a recent study, it has been shown that gamete surface antigen Pfs230 mediates human RBC binding to exflagellating male parasites to form clusters termed exflagellation centers, from which individual motile microgametes are released. This protein thus plays an important role in subsequent oocyst development, which is a critical step in malaria transmission (Eksi et al., 2006). Sporogony within the oocyst produces many sporozoites and when the oocyst ruptures, they migrate to the salivary glands for onward transmission into another host. This form of the parasite is found in the salivary glands after 10-18 days and thereafter the mosquito remains infective for 1-2 months. When an infected mosquito bites a susceptible host, the Plasmodium life cycle begins again (Tuteja, 2007).

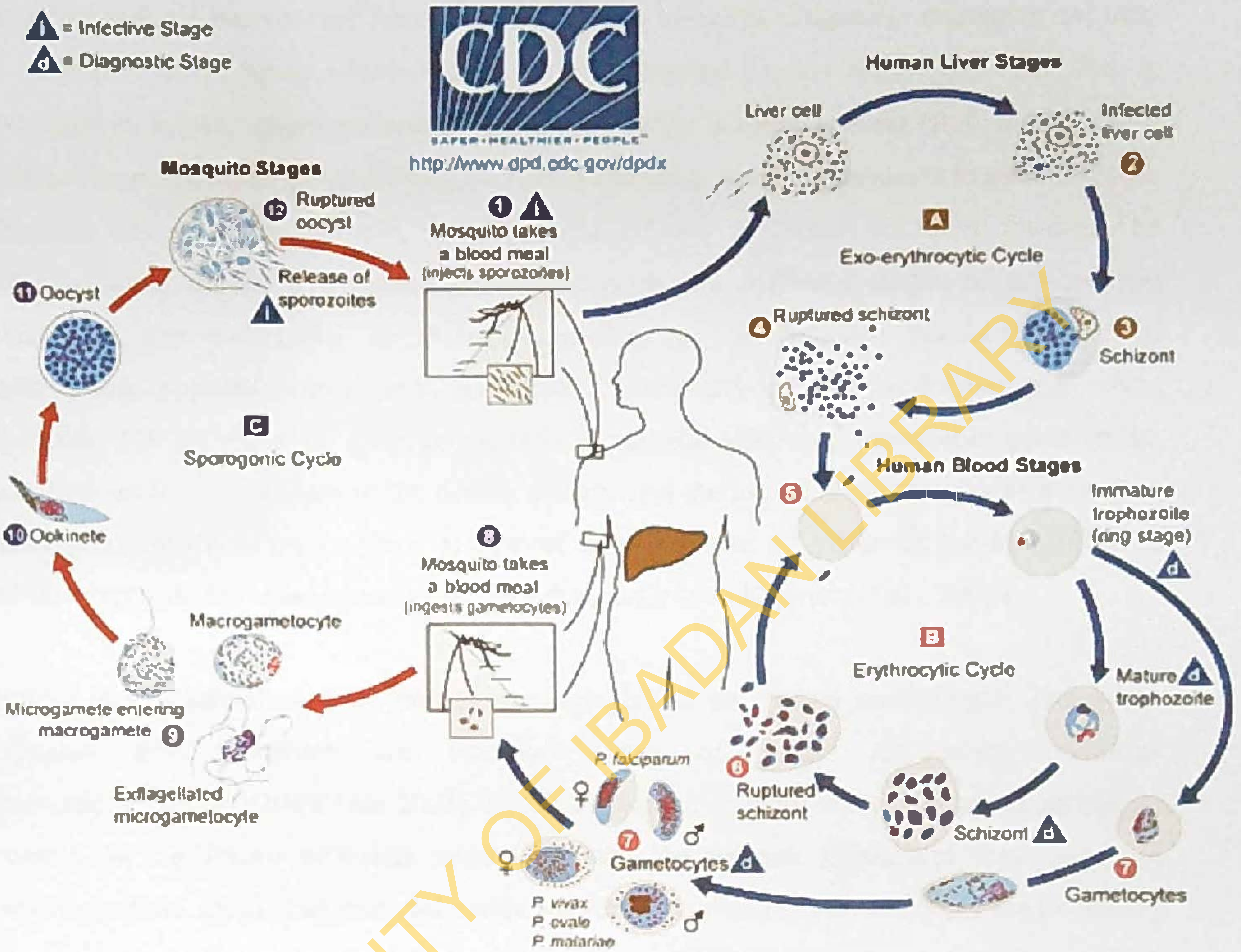

Figure 2: **Schema of the Life Cycle of Malaria.**

(Source: http://www.cdc.gov/malaria/biology/life_cycle.htm)

2.2. Diagnosis of malaria

Prompt and accurate diagnosis is critical to the effective management of malaria. The global impact of malaria has spurred interest in developing effective diagnostic strategies not only for resource-limited areas where malaria is a substantial burden on society, but also in developed countries, where malaria diagnostic expertise is often lacking (Bell et al., 2005). Malaria diagnosis involves identifying malaria parasites or antigens/products in patient blood. Although this may seem simple, the diagnostic efficacy is subject to many factors: The different forms of the five malaria parasite species, the different stages of erythrocytic schizogony, the endemicity of different species, the interrelation between levels of transmission, population movement, parasitemia, immunity and signs and symptoms, drug resistance, the problems of recurrent malaria, persisting viable or non-viable parasitemia, sequestration of the parasites in the deeper tissues, and the use of chemoprophylaxis or even presumptive treatment on the basis of clinical diagnosis, can all influence the identification and interpretation of malaria parasitemia in a diagnostic test (Reyburn et al., 2007).

Malaria is a potential medical emergency and should be treated accordingly. Delays in diagnosis and treatment are leading causes of death in many countries (www.cdc.gov/malaria/facts.htm 2008). Diagnosis can be difficult where malaria is no longer endemic for healthcare providers unfamiliar with the disease. Clinicians may forget to consider malaria among the potential diagnoses for some patients and not order the necessary diagnostic tests. Some Medical Laboratory Scientists may be unfamiliar with, or lack experience with malaria, and fail to detect parasites when examining blood smears under a microscope. In some areas, malaria transmission is so intense that a large proportion of the population is infected but remains asymptomatic, e.g., in Africa.

Such carriers have developed sufficient immunity to protect them from malarial illness, but not infection. In such situations, finding malaria parasites in an ill person does not necessarily mean that the illness is caused by the parasites. In many malaria-endemic countries, the lack of resources is a major barrier to reliable and timely diagnosis. Health personnel are undertrained, under- equipped, and underpaid. They often face excessive patient loads, and must divide their attention between malaria and other equally severe infectious diseases, such as tuberculosis or HIV/AIDS.

2.2.1 Clinical Diagnosis of Malaria

A clinical diagnosis of malaria is traditional among medical doctors. This method is least expensive and most widely practiced. Clinical diagnosis is based on the patients' signs and symptoms, and on physical findings at examination. The earliest symptoms of malaria are very nonspecific and variable, and include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus (Looareesuwan, 1999). A clinical diagnosis of malaria is still challenging because of the non-specific nature of the signs and symptoms, which overlap considerably with other common, as well as potentially life-threatening diseases, e.g. common viral or bacterial infections, and other febrile illnesses.

The overlapping of malaria symptoms with other tropical diseases impairs diagnostic specificity, which can promote the indiscriminate use of antimalarials and compromise the quality of care for patients with non-malarial fevers in endemic areas (Mwangi et al., 2005; Reyburn et al., 2004; McMorrow et al., 2008). The Integrated Management of Children Illness (IMCI) has provided clinical algorithms for managing and diagnosing common childhood illnesses by minimally trained health-care providers in the developing world having inappropriate equipment for laboratory diagnosis.

A widely utilized clinical algorithm for malaria diagnosis, compared with a fully trained pediatrician with access to laboratory support, showed very low specificity (0-9%) but 100% sensitivity in African settings (Perkins et al., 1997; Weber et al., 1997). This lack of specificity reveals the perils of distinguishing malaria from other causes of fever in children on clinical grounds alone. Recently, another study showed that use of the IMCI clinical algorithm resulted in 30% over-diagnosis of malaria (Tagbo et al., 2005). Therefore, the accuracy of malaria diagnosis can be greatly enhanced by combining clinical-and parasite-based findings (Kyabayinze et al., 2008).

2.2.2 Laboratory Diagnosis of Malaria

Rapid and effective malaria diagnosis not only alleviates suffering, but also decreases community transmission. The non-specific nature of the clinical signs and symptoms of malaria may result in over-treatment of malaria or non-treatment of other diseases in malaria-endemic areas, and misdiagnosis in non-endemic areas (Bhandari et al., 2008). In the laboratory, malaria is diagnosed using different techniques, e.g. conventional microscopic diagnosis by staining thin and thick peripheral blood smears (Ngasala et al.,2008), other concentration techniques, e.g. Quantitative Buffy Coat (QBC) method (Bhandari et al., 2008), rapid diagnostic tests e.g., OptiMAL (Tagbor et al.,2008, Zerpa et al., 2008), ICT (Ratsimbasoa et al.,2008), Para- HIT-f (McMorrow et al., 2008), ParaScreen (Endeshaw et al., 2008), SD Bioline (Lee et al., 2008), Paracheck (Harvey et al., 2008), and molecular diagnostic methods, such as polymerase chain reaction (PCR) (Holland et al., 2008)

Some advantages and shortcomings of these methods have also been described, related to sensitivity, specificity, accuracy, precision, time consumed, cost-effectiveness, labor intensiveness, the need for skilled microscopists, and the problem of inexperienced Medical Laboratory Scientists. (Holland et al., 2008)

2.2.2a Microscopic diagnosis using Giemsa-stained thin and thick peripheral blood smears (PBS)

Malaria is conventionally diagnosed by microscopic examination of stained blood films using Giemsa, Wright's, or Field's stains. This method has changed very little since Laverran's original discovery of the malaria parasite, and improvements in staining techniques by Romanowsky in the late 1,800s. More than a century later, microscopic detection and identification of Plasmodium species in Giemsa-stained thick blood films (for screening the presenting malaria parasite), and thin blood films (for species' confirmation) remains the gold standard for laboratory diagnosis (Bharti et al., 2007). Malaria is diagnosed microscopically by staining thick and thin blood films on a glass slide, to visualize malaria parasites.

Briefly, the patient's finger is cleaned with 70% ethyl alcohol, allowed to dry and then the side of fingertip is picked with a sharp sterile lancet and two drops of blood are placed on a glass slide. To prepare a thick blood film, a blood spot is stirred in a circular motion with the corner of the slide, taking care not make the preparation too thick, and allowed to dry without fixative. After drying, the spot is stained with diluted Giemsa (1: 20, vol. /vol.) for 20 min, and washed by placing the film in buffered water for 3 min. The slide is allowed to air- dry in a vertical position and examination using a light microscope as they are unfixed, the red cells lyse when in a water-based stain is applied.

A thin blood film is prepared by immediately placing the smooth edge of a spreader slide in a drop of blood adjusting the angle between slide and spreader to 45 degrees and then smearing the blood with a swift and steady sweep along the surface. The film is then allowed to air dried and fixed with absolute methanol. After drying, the sample is stained with diluted Giemsa (1:20, vol. /vol.) for 20 minutes and washed by briefly dipping the slide in and out of a jar of buffered water (excessive washing will decolorize the film). The slide is then allowed to air dry in a vertical position and examined under a light microscope (Chotivanich et al., 2006). The wide acceptance of this technique by laboratories all around the world can be attributed to its simplicity, low cost, its ability to identify the presence of parasites, the infecting species and assess parasite density/ all parameters useful for the management of malaria.

Recently, a study showed that conventional malaria microscopy diagnosis at primary health care facility in Tanzania could reduce the prescription of antimalarial drugs, and also appeared to improve the appropriate management of non-malarial fevers (Ngasala et al., 2008). However, the staining and interpretation processes are labor intensive, time consuming, and require considerable expertise and trained healthcare workers, particularly for identi- fying species accurately at low parasitemia or in mixed malarial infections. The most important shortcoming of microscopic examination is its relatively low sensitivity, particularly at low parasite levels. Although the expert microscopist can detect up to 5 parasites/μl, the average microscopist detects only 50-100 parasites/ μl (Payne, 1988).

This has probably resulted in underestimating malaria infection rates, especially cases with low parasitemia and asymptomatic malaria. The ability to maintain required levels of malaria diagnostics expertise is problematic, especially in remote medical centers in countries where the disease is rarely seen (Ohrt et al., 2002). Microscopy is laborious and ill-suited for high-through-put use, and species determination at low parasite density is still challenging. Therefore, in remote rural settings, e.g. peripheral medical clinics with no electricity and no health-facility resources, microscopy is often unavailable (Erdman et al., 2008).

The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wrights, or Fields stain (Warhurst et al., 1996). However in resource-poor areas, microscopic diagnosis has been shown to be insensitive and non-specific, especially when parasitaemia is low or mixed infections are present (Amexo et al., 2004). In field conditions, sensitivities and specificities as low as 71–72% have been reported (Snow et.al. 2005). Other limitations include false negativity due to relatively small amount of blood examined or low parasitaemia, and false positivity due to debris (Salako et al., 1999). In an attempt to enhance the detection of malaria parasites, alternative methods have been introduced.

2.2.2b Rapid Diagnostic Tests (RDTs)

Malaria rapid diagnostic tests, sometimes called "dipsticks" or malaria rapid diagnostic devices (MRDDs) are simple immunochromatographic tests that identify specific antigens of malaria parasites in whole or peripheral blood (Hopkins et al., 2008). RDTs are available as a simple dipstick, a cassette (dipstick in a plastic holder), or in a card format.
Simplicity of format (e.g. cassettes) may be important to overall sensitivity (Tagbo et al., 2005). Pictorial representation of the RDT formats is contained on the appendices page (ref. appendix).

2.2.2bi Target Antigens

Malaria antigens currently targeted by RDT are Histidine-rich protein II of *P. falciparum* (PfHRPII), Plasmodium aldolase and Parasite lactate dehydrogenase (pLDH). Histidine-rich protein II of *P. falciparum* (PfHRPII) is a water soluble protein that is produced by the asexual stages and gametocytes of *P. falciparum*, expressed on the red cell membrane surface, and shown to remain in the blood for at least 28 days after the initiation of antimalaria therapy. Several RDTs targeting PfHRP II have been developed (McCutchan et al., 2008) Plasmodium aldolase is an enzyme of the parasite glycolytic pathway expressed by the blood stages of *P. falciparum* as well as the non-falciparum malaria parasites. Monoclonal antibodies against Plasmodium aldolase are pan-specific in their reaction and have been used in a combined 'P.f/P.v' immunochromatographic test that targets the pan malarial antigen (PMA) along with Pf HRPII (McCutchan et al., 2008).

Parasite lactate dehydrogenase (pLDH) is a soluble glycolytic enzyme produced by the asexual and sexual stages of the live parasites and it is present in and released from the parasite infected erythrocytes. It has been found in all 4 human malaria species, and different isomers of pLDH for each of the 4 species exist (Piper et al., 1999). With pLDH as the target, a quantitative immunocapture assay, a qualitative immunochromatographic dipstick assay using monoclonal antibodies, an immunodot assay, and a dipstick assay using polyclonal antibodies have been developed.

2.2.2bii Test performance of RDTs

Dipstick RDTs

Dipsticks are the commonly used forms of RDTs. This may be due to the fact that they are readily available in the market at a relatively cheap price US$ 0.5/test (Guthmann et al., 2008). They are easy and quick to use and thus require very little training and a shorter turn-around time. Dipsticks do not offer enough protection against blood contamination (Kakkilaya, 2003), but if protective gloves are used during testing procedures, this problem can be solved or minimized. Most formats detect only the HRPII antigen which is specific for *P. falciparum* and therefore areas where non falciparum malaria is predominant may not find this type of RDT formats very useful (Moody, 2002).

Cassette and Cards RDTs

These RDT formats are much safer to use. This is because they prevent blood contamination. They are also readily available but at a 40% price higher than the dipsticks (WHO, 2004).

These tests are not as simple as the dipsticks and thus require proper training before use and they also require much time for results to be ready. Unlike the dipsticks, most of these RDT formats target two antigens (HRPII/Pan pLDH, HRPII/Pan aldolase or HRPII/pLDH) (Moody, 2002) making it possible to detect all the plasmodium species.

2.2.3 Fluorescence Microscopy

The fluorescence microscope is based on the phenomenon that certain materials emit energy detectable as visible light when irradiated with light of a specific wavelength. The sample can either be fluorescing in its natural form like chlorophyll and some minerals, or treated with fluorescing chemicals (Moody, 2002). In malaria diagnosis, many methods have been developed based on this technique. Some of these methods are the Quantitative Buffy-Coat (QBC) method which is available as a commercial kit (QBC®; Becton Dickinson, Franklin Lakes, NJ); the Kawamoto Acridine-Orange (KAO) process (Kawamoto, 1991; Kong et al., 1995; Bosch et al., 1996), the Benzothiocarboxypurine (BCP) procedure (Makler et al., 1998) and recently the Cyscope rapid malaria test® (Partec GmbH, Münster, Germany).

2.2.3a Test Performance of the fluorescent microscopy

Quantitative Buffy Coat (QBC) method

The QBC technique was designed to enhance microscopic detection of parasites and simplify malaria diagnosis (Clendennen et al., 1995). This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, e.g. acridine orange, and its subsequent detection by epi-fluorescent microscopy. Briefly, finger-prick blood is collected in a hematocrit tube containing acridine orange and anticoagulant. The tube is centrifuged at 12,000 revolutions /minute for 5 minute and immediately examined using an epi-fluorescent microscope (Chotivanich et al., 2006). Parasite nuclei fluoresces bright green, while cytoplasm appears yellow-orange.

The QBC technique has been shown to be a rapid and sensitive test for diagnosing malaria in numerous laboratories settings (Bhandari et al., 2008, Pornsilapatip et al., 1990; Salako et al., 1999; Barman et al., 2003; Adeoye et al., 2007). While it enhances sensitivity for *P.*

falciparum, it reduces sensitivity for non-falciparum species and decreases specificity due to staining of leukocyte DNA (Moody, 2002). Recently, it has been shown that acridine orange is the preferred diagnostic method (over light microscopy and immunochromatographic tests) in the context of epidemiologic studies in asymptomatic populations in endemic areas, probably because of increased sensitivity at low parasitemia (Ochola et al., 2006).

The QBC method uses Acridine Orange (AO) as the fluorochrome to stain the nucleic acids of any malarial parasites in the sample (Figure 3). Although AO is a very intense fluorescent stain, it is non-specific and stains nucleic acids from all cell types (Moody, 2002). AO is considered hazardous and needs special disposal requirements, making it inappropriate for use in the field. Comparing methodologies, the QBC is more demanding technically (Agabani et al., 1994) and require special equipment and supplies making it more expensive (Craig et al., 1997). However, QBC is rapid and has a high *P. vivax* detection rate with sensitivity and specificity: 87.2% and 95% respectively as found by Wang et al., in 1996; but lower *P. falciparum* detection rate with sensitivity and specificity of 55.9 % and 88.8% respectively in the findings of Adeoye et al., 2007.

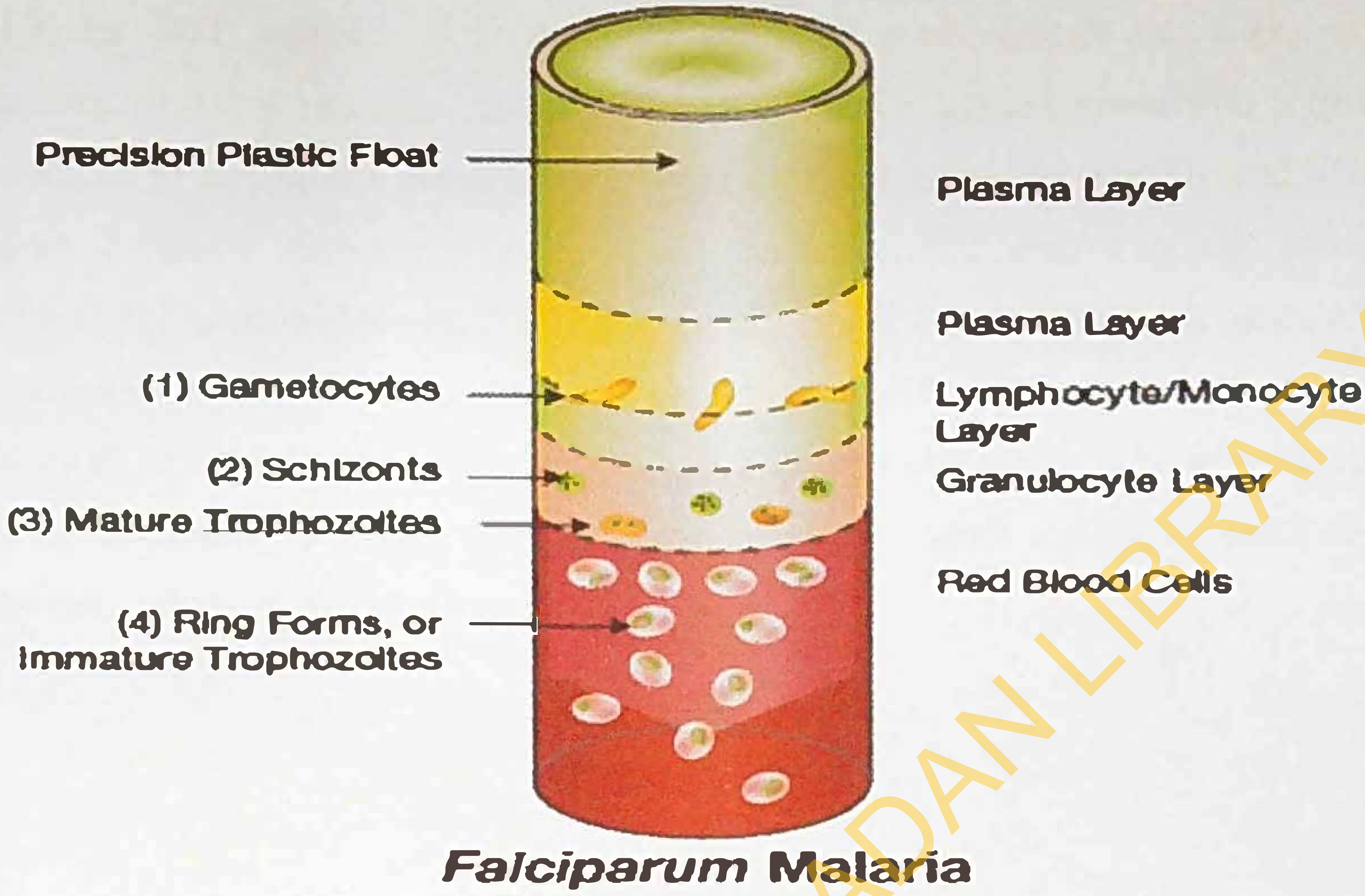

Figure 3: QBC Test showing discrete bands and a parasite

(Source: QBC Diagnostics manufacturer's manual)

2.2.4 KAO and BCP method

Whilst the Kawamoto Acridine-Orange (KAO) method uses AO as the fluorochrome to stain the nucleic acids of any malaria parasite in the sample, the Benzothiocarboxypurine (BCP) is used for the BCP method. Both methods are rapid even though the KAO is simpler (Kawamoto, 1991a; Kawamoto, 1991b). The BCP can be applied directly to a lysed blood suspension or to an unfixed but dry thick blood film and stains the nucleic acid of viable *P. falciparum* parasites intensely (Figure 4) and has a reported sensitivity and specificity of >95% for *P. falciparum* (Moody, 2002). The sensitivity of AO staining with parasite levels of <100 parasites/ ml has been reported to range from 41.7%- 93% (Lowe et al., 1996) and specificity of AO staining for *P. vivax* infections appears to be about 52%, whereas that for *P. falciparum* infections is around 93% (Clendennen, 1995). Both methods cannot distinguish between the various plasmodium species.

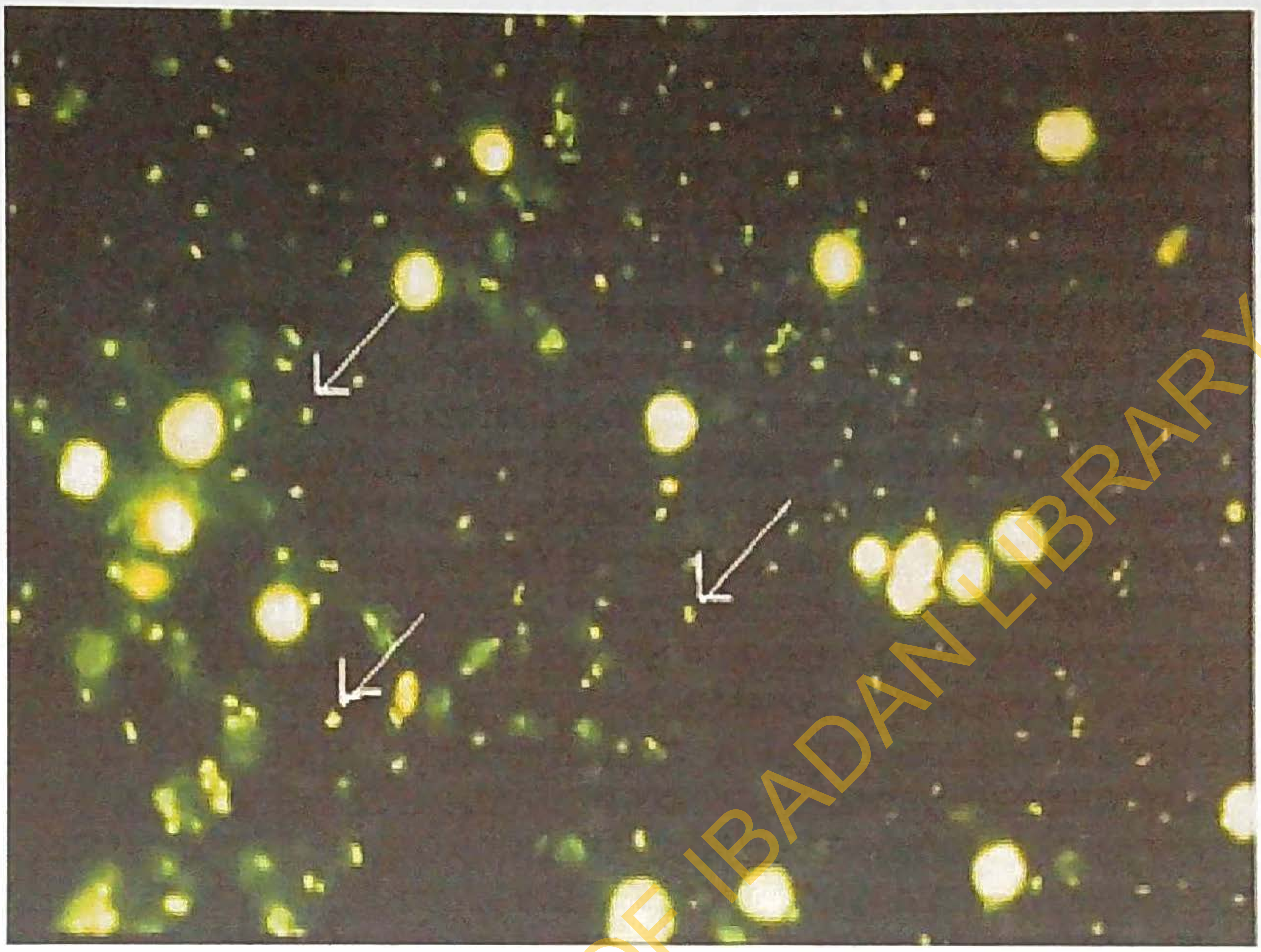

Figure 4: Trophozoites of P. falciparum (arrowed) stained with BCP in the fluorescence method. Source: Moody, 2002

2.2.5 Cyscope Rapid Malaria Test®

The test is done using the Partec CyScope®. It is a new innovative microscope that uses both UV fluorescence light and transmitted light simultaneously or in separate and integrates the most recently available generation of powerful light emitting diode (LED) light sources (Figure 5). It is battery-operated and mobile, designed for several hours of use completely independent from any regular power supply. The Partec CyScope® is perfectly suited for all applications in light and fluorescence microscopy (Nkrumah et al., 2010) and has ready-prepared and ready-to-use test slides which carry the dried-in reagents (DAPI), (emission 443 nm, wavelength 365 nm, safely on the slide surface). Therefore long-term storage and shipment are significantly supported, making malaria testing easier, faster and more affordable than ever before (Nkrumah et al., 2010). Like the other fluorescent methods, this method may not be ideal for species identification.

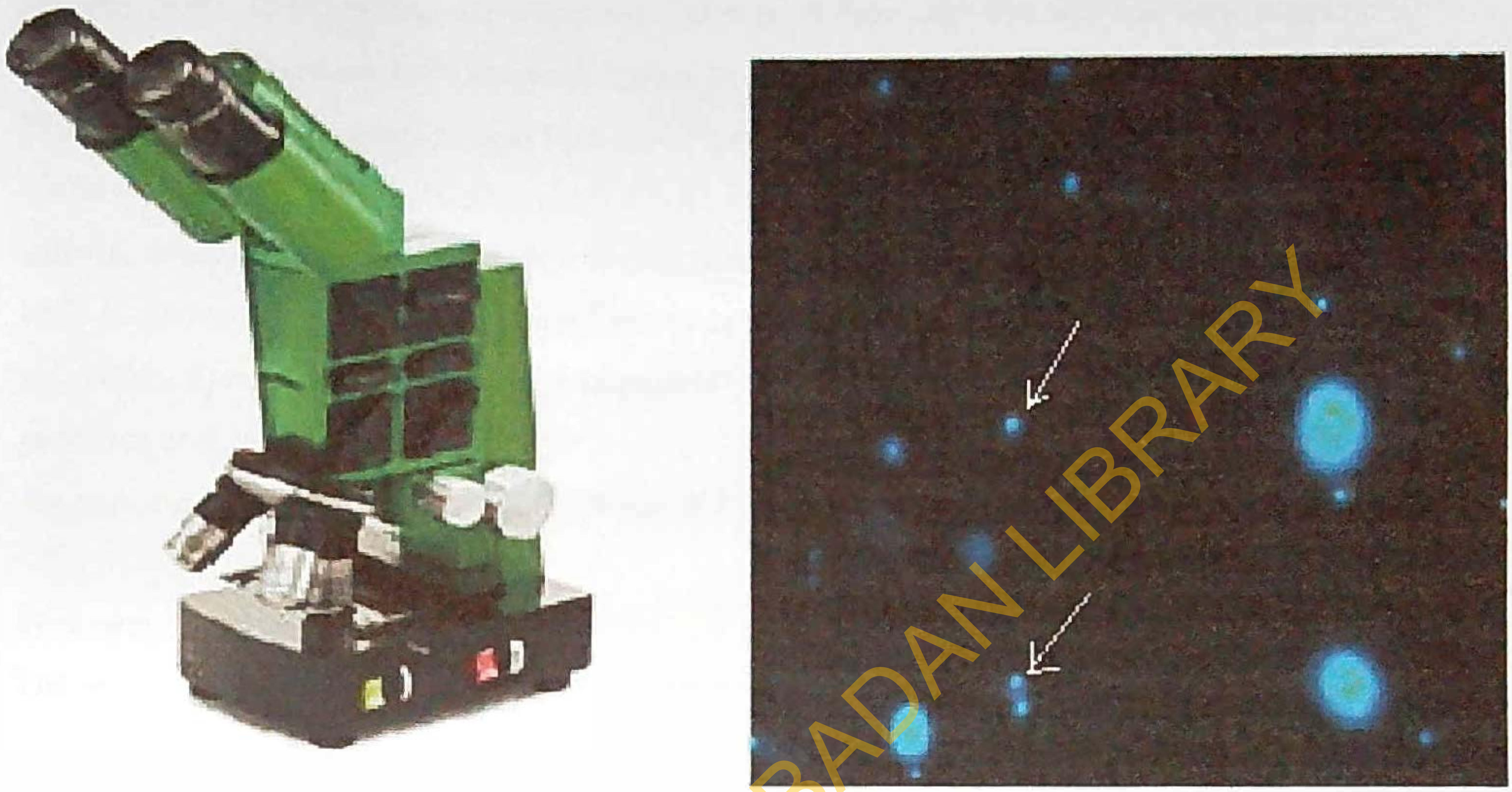

Figure 5: Fluorescent nuclei of Plasmodium parasites (arrowed) within unstained peripheral erythrocytes beside the CyScope microscope. The large fluorescent round areas represent the nuclei of leukocytes. LED fluorescence light (365 nm), 1000-fold magnification. (Source: www.partec.com)

2.2.5 Molecular Techniques

Molecular techniques such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence-Based Amplification (NASBA) have been recently developed in the molecular diagnosis of malaria. Since 1990, several experimental assays have been reported that use various primers, extraction and detection techniques (Snounou et al., 1993b). Several reports have shown that PCR has a higher sensitivity (infection with five parasites or less per µl can be detected with 100% sensitivity and specificity) (Kawamoto et al., 1996) than examination of thin blood smears, especially in cases with low parasitaemia or mixed infection (Makler et al., 1998). PCR is said to have a lower detection limit of between 0.7 and 0.02 parasites/µl (Schneider et al., 2005). Quantitative-nucleic acid sequence-based amplification (QT-NASBA) can detect parasites at a level as low as 0.02 parasites/µl blood and allows for precise quantification of the parasite load over a range of 20–108 parasites/ml blood (Schoone et al., 2000).

However, most published PCR assays are gel based with (Brown et al., 1992)or without (Alves et al., 2002) subsequent probe hybridizations, resulting in a lengthy procedure not optimal for clinical use. The need for a more sensitive and time-efficient assay has led to the development of molecular assays involving Real time PCR (Lee et al., 2002). Real time PCR assays have the potential to detect low levels of parasitaemia, identify mixed infections, and allow for precise differentiation of species via melting curve analysis (Mangold et al., 2005). In a study conducted by Mangold et al., (2005), patient specimens infected at 0.01 to 0.02% parasitaemia densities were detected by Real time PCR, and analytical sensitivity was estimated to be 0.2 genome equivalent per reaction (Mangold et al., 2005).

2.2.5a Test Performance of Real time PCR

Real time PCR is much easier to perform because it offers the option of using single probes instead of multiple probes with complex procedures. It is less time consuming since results interpretation is not gel based but rather on melting curve analysis which takes less time with 100% sensitivity and specificity (Boonma et al., 2007). Real time PCR also prevents carryover contamination as products are not reopened for gel based electrophoresis. On the other hand, the thermal cyclers, primers, probes etc used for the amplification processes are very expensive and therefore cannot be used for district hospitals and malaria endemic areas where they are needed most. It also requires much expertise and experience which are not available in these endemic areas (Brown et al., 1992 and Mangold et al., 2005).

2.2.5b Loop-mediated isothermal DNA amplification (LAMP) technique: The LAMP technique, that detects 1-6 parasites /μl with minimal sample processing and requires no sophisticated equipment which can be read with the naked eye have been launched. It is claimed to be a simple and inexpensive molecular malaria-diagnostic test that detects the conserved 18S ribosome RNA gene of *P. falciparum* (Poon et al., 2006). Other studies have shown high sensitivity and specificity, not only for *P. falciparum*, but also *P. vivax*, *P. ovale* and *P. malariae* (Han et al., 2007 and Aonuma et al., 2008). These observations suggest that LAMP is more reliable and useful for routine screening for malaria parasites in regions where vector-borne diseases, such as malaria, are endemic. LAMP appears to be easy, sensitive, quick and lower in cost than PCR. However, reagents require cold storage, and further clinical trials are needed to validate the feasibility and clinical utility of LAMP (Erdman et al., 2008).

2.2.6 Flow Cytometry

Flow cytometry carries some potential as an alternative tool for malaria diagnosis. Whereas this method appears to be too expensive for malaria-endemic countries, it might be of great value in affluent countries where flow cytometric blood cell differentiation is used routinely in hematology laboratories. An advantage of the method is its potential to detect cases in the absence of clinical suspicion (Hänscheid et al., 1999; Hänscheid et al., 2000). Recent studies using automated hematology analyzers have demonstrated unexpected abnormalities in differential white blood cell plots and reticulocyte histograms from patients with malaria.

Normal monocytes can be discriminated from monocytes that have ingested the malarial breakdown product hemozoin because of the ability of hemozoin to depolarize laser light used for routine differentiation of eosinophils. Nuclear material of intraerythrocytic malaria parasites could be discriminated by fluorescent nucleic acid dye used in routine quantification of reticulocytes. The presence of infected erythrocytes leads to a distinct fluorescent spike in reticulocyte histograms, referred to as pseudoreticulocytosis. It has been suggested that this novel method is a useful addition to conventional microscopy (Hanscheid et al., 2001, Hoffmann et al., 1999 and Mendelow et al., 1999).

Recently, depolarized light scatter of white blood cells has been applied to automated malaria diagnosis using commercial hematology analyzers such as the Cell-Dyn® 3500 (CD3500) (Abbott, Santa Clara, CA) (Mendelow et al., 1999). This allows malaria diagnosis by detecting malaria pigments in white blood cells during routine full blood counts. Compared to microscopy its sensitivity and specificity is 95% and 88% respectively (Hanscheid et al., 2001).

2.2.7 Mass Spectrometry

A novel method for the in vitro detection of the malaria parasite at a sensitivity of 10 parasites/µl of blood has been recently reported. It comprises a protocol for clean-up of whole blood samples, followed by direct ultraviolet laser desorption time-of-flight mass spectrometry. Intense ion signals are observed from intact ferriprotoporphyrin IX (heme), sequestered by malaria parasites during their growth in human red blood cells. The heme group is photoactive and turns out to be easily detectable by direct laser-desorption mass spectrometry. The laser-desorption mass spectrum of the heme is structure-specific, and the signal intensities are correlated with the sample parasitaemia. Many samples could be prepared in parallel and measurement per sample may not take longer than a second. However, even though this technique may be fast, it is expensive thus cannot be used in developing countries let alone rural areas (Demirev et al., 2002 and Mann, 2002).

2.2.8 Malaria Magnetic Deposition Microscopy (MDM)

In an attempt to overcome some problems inherent to blood smear microscopy, a magnet-based approach to concentrate malaria parasites and augment detection of malaria-infected erythrocytes by microscopy has been developed. This system, malaria magnetic deposition microscopy (MDM), exploits the fact that Plasmodium parasites produce a crystalline by-product, hemozoin, from heme which is liberated during hemoglobin digestion (Nalbandian et al., 1995 and Paul et al., 1981). MDM captures parasitized erythrocytes in a narrow magnetic field and deposits them directly onto a small region of a polyester slide, which is immediately ready for fixation and ~~staining~~. By concentrating parasites, MDM increases the sensitivity of diagnosis and decreases the time it takes to read the slide and it the ability to concentrate parasites of all four human malaria parasite species, including efficient capture of *P. falciparum* gametocytes.

P. falciparum–infected blood samples were enriched 40-fold from a parasitaemia of 2.7% to nearly 100% whilst *P. vivax*–infected blood samples were enriched up to 250-fold, from an initial parasitaemia of 0.1% to clusters with 25% infected erythrocytes (Zimmerman et al., 2006).

2.2.9 Light microscopy

The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wrights, or Fields stain (Warhurst et al., 1996).

2.2.9a Test Performance

Giemsa stain

Giemsa microscopy is still regarded as the gold standard and the most suitable diagnostic instrument for malaria control because it is believed to be inexpensive to perform, able to differentiate malaria species and quantify parasites (Jonkman et al., 1995). However, microscopy is labour-intensive, time-consuming, requires well-trained (Reyburn et al., 2004), expert microscopists and rigorous maintenance of functional infrastructures plus effective quality control (QC) and quality assurance (QA) (Wongsrichanalai et al., 2007). Giemsa stained light microscopic diagnosis has been shown to be insensitive and nonspecific, especially when parasitaemia are low or mixed infections are present (Amexo et al., 2004) . Sensitivities and specificities as low as 71–72% have been reported (Arai et al., 1996 and Snounou et al., 1993a).

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Fields stain

Field's stain is widely used as a rapid staining technique for thick and thin blood films for the diagnosis of malaria. This is mostly due to the fact that this technique is easy, quick and the stains are commercially prepared, ready for use and malaria films stained by this method show adequate staining of all stages of Plasmodium including the Schueffner's and James's dots of *P. vivax* and *P. ovale* respectively. However, just as Giemsa stain, a considerable amount of expertise is needed to identify malaria parasites and the stain also fades with time. Compared to Giemsa, the sensitivity of Field's stain is low; 34.57% even though it has an excellent specificity, 100% (Mendiratta et al., 2006 and Moody et al., 1985) but other studies have reported a higher sensitivity of 96.3% and a specificity of 96.3% (Ibrahim, 2002).

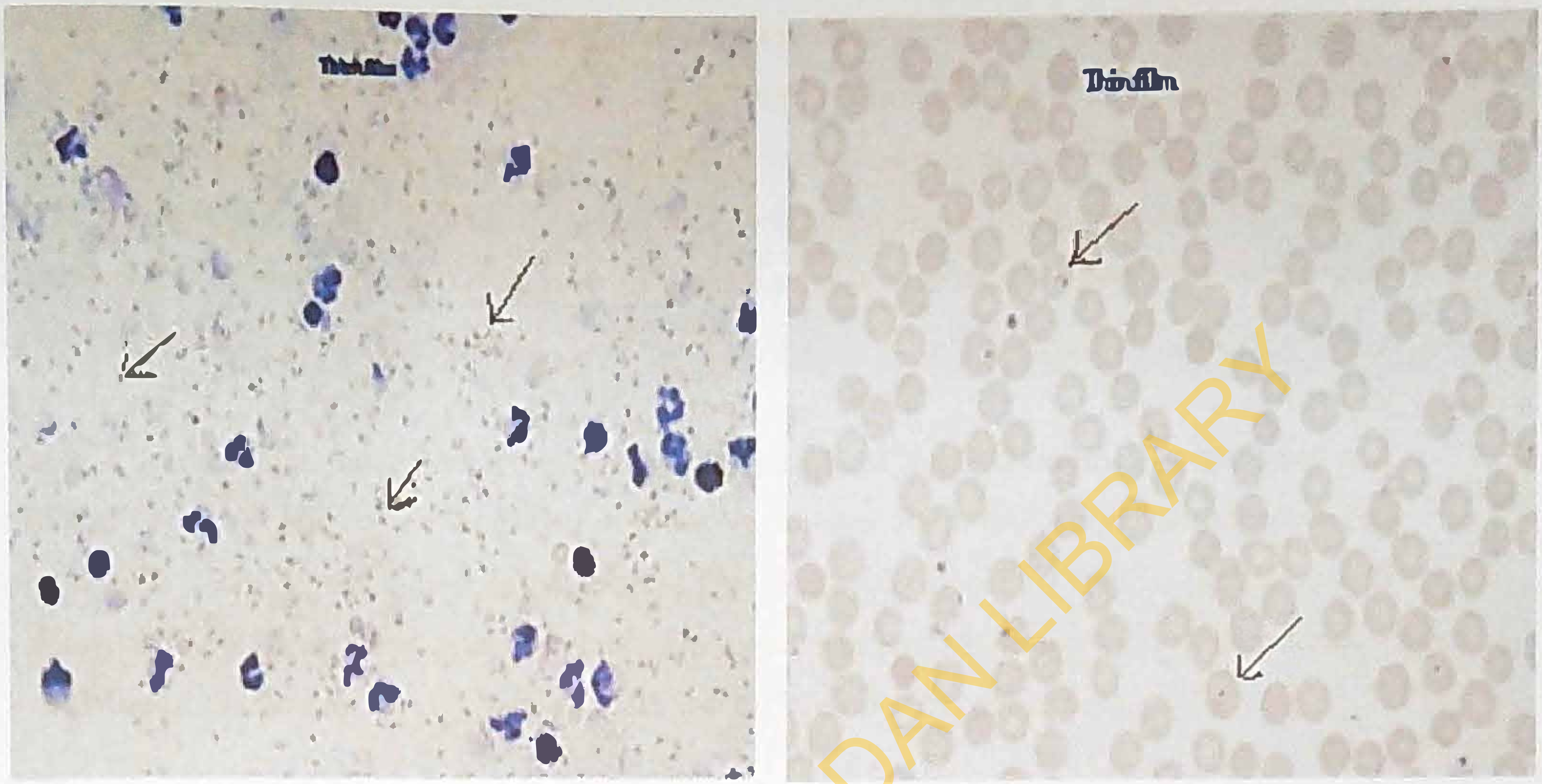

Figure 6: Giemsa stained malaria parasites (arrowed) as they appear in the thick and thin films under the light microscope (Source: MDCoE, Kisumu, Kenya).

2.3 Laboratory diagnosis in summary

The WHO practical microscopy guide for malaria provides detailed procedures for laboratory practitioners (WHO, 1991). Diagnosis initially requires determining the presence (or absence) of malarial parasites in the examined specimen. Then, if parasites are present two more tasks must be performed: 1) identification of the species and life-cycle stages causing the infection and 2) calculation of the degree of infection, by counting the ratio of parasites vs. healthy components (i.e. parasitaemia). However, these tasks are not necessarily performed separately or hierarchically.

Using a microscope, visual detection and identification of the *Plasmodium* is possible and efficient via a chemical process called staining. A popular stain, Giemsa, slightly colors red blood cells (RBCs) but highlights the parasites, white blood cells (WBC), platelets, and various artifacts. In order to detect the infection it could be sufficient to divide stained objects into two groups such as parasite/non-parasite and differentiate between them.

However, to specify the infection and to perform a detailed quantification, all four species of *Plasmodium* at four life-cycle-stages must be differentiated. Despite that the term 'artifact" is not very definitive, any stained object that is not a regular blood component or a parasite is referred here using this term: these include bacteria, spores, crystallized stain chemicals, and particles due to dirt (WHO, 1991). It must be noted that other peripheral blood parasites and RBC anomalies (e.g. Howell-Jolly bodies, iron deficiency, reticulocytes) are included in this artifact class definition. They could be examined in individual dedicated classes if their identification is also required.

A specimen for manual microscopy diagnosis can be prepared (on a glass slide) in two different forms: 1) a *thick blood film* enables examination of a larger volume of blood; hence it is more sensitive to detect parasites (as low as 50 parasites/μl (Moody A., 2002). However, the thick film preparation process destroys RBCs and thus makes identification of species difficult. 2) On the other hand, a *thin blood film* preserves RBC shapes and parasites and is thus more suitable for species identification. A common practice in manual diagnosis is to perform positive/negative type decisions in thick blood films and identify species and life-stages in the thin films. Parasitaemia can be calculated in both types of smears (WHO, 1991). Polymerase chain reaction (PCR) methods are known to be more sensitive and more specific than (manual) microscopy (Berry *et al.*, 2008, Coleman *et al.*, 2006 and Moody, 2002). Recent advances in the technique allow high-throughput applications and promote its use in routine diagnosis (Safeukui *et al.*, 2008; Muldrew *et al.*, 2009). Mueller *et al.* (2009), showed that Post-PCR ligase detection reaction fluorescent microsphere assay is more accurate than light microscopy in resolving species in the presence of mixed infections, which are common in the areas where malaria is endemic. PCR-based methods may replace microscopy examination as the gold-standard (Berry *et al.*, 2008); however, costs are significantly higher and more expensive instruments are required (Erdman *et al.*, 2008). On the other hand, emerging new technologies such as Rapid Diagnostic Tests Kits do not require any special equipment and training. The detection sensitivity is lower but comparable to manual microscopy. However, they provide poor species discrimination and do not provide quantification of the results (Chilton *et al.*, 2006).

In the tropics, practitioners are preoccupied by malaria diagnosis, not only in feverish patients but also for many undiagnosed systemic disorders (WHO, 2003; UNICEF, 2003). Such malpractice is not limited to treatment of false positive malaria, but presumptive treatment is also frequently practiced (Amexo *et al.*, 2004 and Zurovac *et al.*, 2006). The low accuracy of malaria diagnosis is widely recognized in malaria endemic countries (Zurovac *et al.*, 2006). Misdiagnosis of malaria is costly and results in considerable morbidity and mortality, because it contributes to both delay in treatment of the correct diagnosis and to increasing antimalarial drug pressure and thus resistance, thereby speeding up the obsolescence of affordable drugs (Bell *et al.*, 2006).

The WHO recommends malaria case management, where possible, to be based on parasitological diagnosis, except when considering young children in areas of high transmission where lack of resources or urgency of response temporarily limits its application (World Malaria Report WHO, 2008). However, most malaria cases in resource constrained areas tend to go undiagnosed and, more often than not, untreated, as clinical diagnosis has limited specificity (Amexo *et al.*, 2004) and quality malaria microscopy is difficult to implement at rural clinic levels. The expansion of parasitological diagnosis leading to better case management even in the most remote areas endemic for malaria will soon rely predominantly on rapid diagnostic tests (RDTs) (Bell *et al.*, 2008), and until recently they all consisted of lateral-flow immunochromatographic devices that detect parasite-specific antigens in the blood (WHO/TDR/FIND/CDC, 2010).

Prompt parasitological confirmation by microscopy or alternatively by rapid diagnostic tests (RDTs) is recommended for all patients with suspected malaria before treatment is started (Abba *et al.*, 2011). Microscopy is the corner stone in malaria diagnosis; it is a valuable technique when performed correctly, but it is unreliable and wasteful when poorly executed. In addition, the technique has its own inherent limitations.

Traditional microscopy using giemsa-stained blood smears and high-power light microscopes has a number of problems. Giemsa stain is unstable at high temperatures and so has to be freshly prepared; the technique is labour intensive and time-consuming; and low parasite numbers, below 20 parasites/µl of blood, may be missed. The number of slides that can be examined without undue strain is limited, and tired microscopists may be even more likely to miss occasional parasites in a Giemsa-stained smear (Guy *et al.*, 2007). Alternative staining techniques using fluorescent stains have been described, and have the advantage of allowing rapid scanning of slides at lower magnification that both reduces microscopist fatigue and increases rates of detection where the parasitaemia is low (Mendiratta *et al.*, 2006). The fluorescent dye SYBR-Green 1 has been shown to be the most useful in the detection of malaria parasites (Guy *et al.*, 2007). However, the need for a special microscope with UV light limited the value of such techniques.

Many diagnostic procedures have been developed to reduce the time, preparation, and training needed to diagnose malaria. The use of Plasmodium nucleic acid fluorescent dyes was found to facilitate detection of the parasites even in low parasitaemia conditions due to the contrast with the background (Guy *et al.*, 2007).

More recently, however, a new type of 'RDT' has been developed using fluorescent microscopy: a portable fluorescence microscope was developed in Germany called "Cyscope", by Partec GmbH, Munster, and Germany). The portable, battery-operated CyScope® (Partec, Germany) aims at reducing time and training needed for diagnosis (for information on technology see Guy *et al.*, 2008).

Furthermore, the microscope is capable of both fluorescent and transmitted light operation, and incorporates powerful high-efficiency light-emitting diodes (LED) as light sources. It is battery-powered and portable and can be used independently of mains power for about 12 hours. A built-in camera interface enables images of the slides to be taken for further investigation by image analysis software if desired. Slides that are pre-coated with fluorescent stains can be used in combination with the Cyscope to provide a rapid, affordable and practical alternative to traditional microscopy methods of parasite detection.

Albeit classifiable as a 'RDT' (average time per diagnosis under 10 min), the CyScope® is also thought able to quantify infection parasitaemia, by counting the number of malaria parasites per white blood cells, a feature missed by all lateral-flow tests, as well as, being used for direct morphological inspection of red blood cells. Understanding parasitaemia levels pre- and post- treatment is crucial particularly for in-patient case management in health centres, clinics or hospital wards.

Cyscope® microscope is a mobile, battery-operated microscope with ready slides with malaria parasite DNA specific staining reagents in the dried form. All that is needed is the addition of a drop of blood and viewing the slide under the microscope, saving time and preparation and relatively cheap (£818 for the microscope and £0.40 per test). An optional add-on enables viewing the slides on a computer to facilitate the diagnostic procedure and storage and retrieval of results. However, few published data on Cyscope® microscope in malaria diagnosis are available (Guy *et al.*, 2007, Hassan *et al.*, 2010, Sousa-Figueiredo *et al.*, 2010 and Nkrumah *et al.*, 2011). Given the fact that the test is relatively cheap, this technique offers the possibility of a useful test especially for malaria endemic and resource limited regions.

In 2006, Mendiratta et al., from India suggested that the use of acridine orange in fluorescent microscopy as obtainable in Cyscope microscope can be used for malaria parasite screening. Recently, a pioneering cross-sectional facility-based study of the diagnostic performance of the CyScope® was conducted in Sudan with very promising results: sensitivity of 98.2% and specificity of 98.3% (gold standard: light microscopy) was obtained (Hassan *et al.*, 2010). The affordable pricing, use of solar powered battery, portability, compact design of the CyScope microscope, and the fact that reagents do not require cold storage, make the method a potentially attractive alternative for malaria diagnosis in the rural setting.

A similar work done in neighbouring country of Ghana by Bernard Nkrumah et.al, published in 2010, found that the results of malaria diagnosis for the Cyscope microscope were obtained more quickly and at less cost than those for the light microscope (using PCR as gold standard) and that while the performance characteristics of the cyscope microscope were almost equal to those of the light microscope, the operational characteristics were better, and cyscope can therefore be considered as an alternative method for light microscope.

However, in 2013, Rabiu et. al., in Ibadan, Nigeria reported that Paracheck-Pf®, a HRP-2 RDT demonstrated a better diagnostic performance than Cyscope®mini (a miniature size of Cyscope microscope) for diagnosis of *falciparum* malaria and will be a good diagnostic tool for field studies. This finding was inconsistent with other reports about Cyscope microscope and raised another reason to investigate without using the miniature size microscope, while employing the service of an experienced microscopist and under a similar environment to ascertain the true picture of this tool in the Nigeria population. When this is done alongside the cost effective analysis of some of the routinely used diagnostic instruments, it will give a better guidance in the choice of malaria diagnostic tool towards ensuring malaria elimination in Nigeria.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study Area

The study area was the municipal area of Ibadan, which is made up of five local government areas. Ibadan is the capital city of Oyo State located in the forest zone of southwestern Nigeria. Ibadan city lies on the longitude 3°5' East of Greenwich meridian and latitude 7°23' North of the Equator. Besides being the largest indigenous city in Africa south of Sahara, the city is an important trade and educational centre. It also houses one of the largest and foremost teaching hospitals in Africa. However, the city is characterized by low level of environmental sanitation, poor housing, overcrowding, lack of potable water and improper management of wastes especially in the indigenous core areas characterized by high density and low income populations; these predisposes to malaria infection (Okonkwo et. al., 2012).

3.2 Study Site

The University College Hospital (UCH) Ibadan is a tertiary healthcare facility that has been over saddled with providing secondary and primary healthcare services due to a near collapse of the last two. This health facility has about 1,000 bed spaces with almost 500 outpatients flow daily, wherein more than 50% of these patients present with fever and are queried for malaria diagnosis. The UCH recently acquired Cyscope fluorescent microscope and QBC fluorescent microscope to support the existing light microscope for malaria diagnosis in her Medical Microbiology and Parasitology department. With access to an array of WHO trained malaria diagnostic personnel who participate in external quality assessment programs, this facility was chosen for conducting the laboratory analysis of the study.

3.3 Study Design

This was an evaluative cross-sectional study

3.4 Study Period

The study was conducted within four months (January 2014 to April 2014).

3.5 Inclusion / Exclusion Criteria

Patients of any age with fever, having axillary body temperature above 37.5 °C, and/or wherein the clinician suspects malarial, presenting in the selected clinics in Ibadan, Nigeria, and consented were included in the study. However, patients with symptoms suggestive of malaria but who had taken any antimalaria drugs within fourteen days of reporting to the hospital were not included in this study.

3.6 Sample Size Calculation

For the calculation of the sample size, the following equation was used:

$$n_x = \frac{(Z\alpha + Z\beta)^2 \quad x \quad P(1-P)}{(P - P_0)^2}$$

Sensitivity and specificity is given with the 95% Confidence Interval (CI)

P: estimated sensitivity (85%)

P_0: recommended minimum sensitivity (95%)

n_x: estimated number of infected with disease

$$n_x = \frac{(1.96+1.28)^2 \quad x \quad 0.85(1-0.85)}{(0.85 - 0.95)^2}$$

$$n_x = 133.8$$

Where Px is the prevalence obtained from similar study, n (total positive samples expected) for this type of study is calculated

$n = n_x/P_x$

Using P_x = *76.4% malaria in children < 5 yrs (*Tiddi & Akogun, 2005)

$$n = \frac{133.8}{0.764}$$

n = 175

Accounting for 10% non response

NR = n/1-f

= 175 X 1/0.9

=194

Using a design effect of 3, the total sample size was calculated to be 582 (194x3).

However of the 582 samples only 502 were valid for processing due to transportation and other logistics challenges, giving 13.75% invalid samples.

3.8 Sampling Technique

A multi-stage sampling technique was used

- Stage 1: One tertiary health facility (HF), one secondary HF and two primary HFs were selected by simple random sampling
- Stage 2: At each HF, phlebotomy units were selected by simple random sampling
- Stage 3: Patients who presented with laboratory forms requesting for malaria parasite investigation at the selected phlebotomy units and consented to participate were selected by systematic random sampling until the sample size was reached.

About 95 % of patients enlisted in this study were attending University College Hospital, Adeoyo State Hospital and Kola Daisi comprehensive health center while the rest participants were from other HF in Ibadan (Remi Babalola health center).

3.8.1 Sample and data collection

Patients with laboratory request form sent in by the requesting physician for malaria parasite test, who consented or assented to participate in the study were enrolled. Certified phlebotomists collected one millimeter of blood sample by venepuncture from each participant into an EDTA anticoagulant specimen bottle, which was used to test for malaria parasite using all the diagnostic instruments.

A structured data collection register was used to record the bio data and the results of the tests.

3.8.2 Sample Transportation

Samples collected daily were stored/ preserved at 4^0C in a refrigerator when daily processing was not feasible. Samples from HF not situated very close to the laboratory where analysis was done were transported within 24 hours of collection in a geostat ice pack container to the processing laboratory site at the University College Hospital, Ibadan ensuring cold chain and sample integrity was maintained.

3.8.3 Sample Processing

Light microscopy: The Giemsa stained thick and thin films were examined with a Zeiss light microscope (Aziostar plus, Carl Zeiss Microimaging, Germany) using the high power (40x) and the oil immersion (100x) objectives by a WHO certified microscopist and double checked by a WHO trained microscopy expert of about 10 years experience. The microscopists were blinded to the outcome of other diagnostic instruments. Details of the procedure are contained in the annex.

Cyscope fluorescent microscopy: A Cyscope® malaria fluorescent microscope from Partec Company, Germany, was used to diagnose malaria by other trained and experienced laboratory personnel, blinded to the other diagnostic instruments' result. According to the manufacturer's instruction, ten (10) microlitre of blood sample was applied to the pre-prepared slides of the fluorescence microscope, wet mount, and viewed with the microscope. Presence (or absence) of malaria parasites was confirmed by viewing the fluorescent DNA of plasmodia under the microscope (objective × 40). Details of the procedure are contained in the annex.

Quantitative Buffy Coat fluorescent microscopy: The QBC malaria test was done by a trained expert with 7 years experience, using QBC Paralens Advance from QBC Diagnostics, Port Matilda, U.S.A. Details of this procedure is contained in section 2.2.3a.

CareStart™ rapid diagnostic kit: CareStart™ targeting histidine rich protein 2 specific for plasmodium falciparum specie, manufactured by Acess Bio, Inc. New Jersey, USA, with Lot number MO3B10 and expiry date slated for July 2015 was used for this study. Trained and experienced personnel followed manufacturer's instruction on standard operating procedures. Detail procedures are contained in the annex.

3.9 Turn-Around Time (TAT)

This was the time taken starting from when sample is received by the laboratory personnel who processed it, and the time taken for completing all stages of the laboratory procedure and results was generated following the standard operating procedure for each diagnostic instrument. This was monitored by using a stop watch and separately recorded in another structured data collection register/ 'time sheet'. Time spent on each stage of processing according to the SOP until result was ready for each diagnostic instrument was recorded and compared.

3.10 Cost-Effectiveness Analysis

Cost effectiveness analysis, is an economic study in which the costs are expressed in monetary units, here in U.S Dollars (Naira equivalent slated at 162 naira for 1 U.S dollar at the time of this study), and the results/outcome in non-monetary units, here in number of tests done by each of the instruments. It is also the ratio between the resources used and the related effects which is determined by comparison of the costs/input and consequences/outcome. The 'Standard Guidelines on Health Economic Evaluation' as put together in 2006 by Evelyn Walter and group of health economics experts from the Institute for Pharmaeconomic Research, Vienna, was used for this analysis.

3.10.1 Cost/ Input

The costs were divided into machine or equipment cost, reagents /consumables cost, manpower/personnel, electricity or other miscellaneous.

Assumptions: The following assumptions were made;

- That machine cost is per unit time of use, assuming uniform depreciation over time / lifespan of the equipment (fixed lifespan is 3 years for the entire machine).
- That period of use was fixed at 8 hours per day.

Acquisition cost was divided by total life span of equipment (in real use days i.e. excluding weekends) to get machine cost per unit time of use. Cost of reagents/consumables was calculated per session of use (8hours/day) (table 5).

- Manpower/Personnel cost was calculated using standard monthly wage of basically qualified staff to operate each diagnostic tool; expressed in wage/hour. Basic staff qualification for CARESTART (RDT) usage is post primary education while the staff qualification for other diagnostic tools is post-secondary education. Using the United States Office of Personnel Management , General Schedule Qualification Standards, 2014; The General Schedule 2 (GS 2) is the standard for secondary education equivalent in Nigeria, which is acceptable staff qualification for operating the CARESTART, while the Cyscope fluorescent microscope can be operated by a staff on GS 5 (i.e, a degree holder). Both QBC fluorescent microscope and light Microscope will require a staff on GS 7 (i.e, a professional degree).

At GS 2, the standard salary is $25,114 per annum, which is equivalent to $12.50 per hour (given 21 working days/ month and 8 hours/ day). For GS 5, the equivalent salary per hour is $17.10 (given $34, 415 per annum). The standard schedule for GS 7 will provide a salary per hour of $21.14 (at $42,631 per annum). Electricity was estimated at $0.08/ hour (equivalent of N12.99k).

3.10.2 Consequences/Output

It is expected that each diagnostic tool produces result (yield) for malaria parasite test as the output or consequence of the use. The yield per procedure over the work/ allotted time was calculated. Hence, cost per hour of use was determined as the ratio between the resources used and the related effects, classified by comparison of the costs /input and consequences/outcome per hour.

3.11 Quality Control

Standard operating procedures (SOPs) was developed and validated for every clinical and laboratory procedure to ensure compliance with international practicing standard. Laboratory procedures were repeated by another experienced professional for each of the tool and a tie breaker observer was engaged where there are conflicting results between observers using same diagnostic instruments, to ensure agreement before results were entered. Double data entry and confirmation was done to ensure data integrity.

3.12 Data Management and Analysis

Microsoft Excel (2008) was used for data entry, data cleaning, and analysis. Quantitative data were summarized using proportions and means. With the results from the light microscope as the standard/reference, sensitivity, defined as the probability that a test correctly classifies people with disease as positive, for each of the malaria diagnostic tools were calculated as true positives/ (true positive + false negatives). Specificity, defined as probability that a test correctly classifies people without disease as negative, was calculated as true negatives/ (true negatives + false positives); while positive predictive value (PPV), defined as the proportion of people with a positive test who have the disease, by each instrument was calculated as true positives/(true positives + false positives); and negative predictive value (NPV), defined as the proportion of people with a negative test who do not have the disease as determined by the instruments was also calculated as true negatives/ (true negatives + false negatives).

Statistical analyses of the validity indices were done using Mcnemar chi-square and inter instruments agreement was analyzed using Kappa statistics. This was done at 95% confidence interval with level of significance set at < 5%.

3.12 Ethical Approval

Ethical approval for the study was obtained from the University of Ibadan/University College Hospital Ethics Committee. Informed consent was obtained from participants before enrolling them into the study while their confidentiality was maintained by ensuring their names cannot be linked to the samples and information they gave. The participant's benefit of participation was presentation of their results to the requesting clinicians and advised on treatment. To ensure minimal discomfort, experienced phlebotomists were employed to perform venepuncture. All eligible and consented/assented participants were enlisted without prejudice. Copy of ethical approval as obtained is attached under appendix 4.

3.13 Conflict of Interest: I declare that there is no conflict of interest in this study.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of the Study Participants

A total of five hundred and two (502) participants were involved in this study. Out of this, 378 (75.2%) were males. Participants' age ranged from 3 to 47 years with the median age being 18years.

4.2 Prevalence of Malaria across the Tested Diagnostic Instruments

Table 1: Malaria parasite detection by the diagnostic instruments

(n =502)

Diagnostic Instrument	Malaria parasite detection rate (%)
Light microscope	109 (21.7)
Cyscope	154 (30.7)
Quantitative Buffy Coat (QBC)	164 (32.7)
CareStart	98 (19.5)

4.3 CareStart™ (HRP2) and Light Microscopy Results for Malaria Parasite Diagnosis

A total of 502 blood samples were tested for malaria parasite using the conventional light microscopy and CareStart™ Rapid Diagnostic Test (CARESTART) test kit (HRP2). Of the 502 blood samples tested for malaria parasites, 109 (21.7%) and 98 (19.5%) were positive for light microscopy and CareStart respectively. Also by specificity and sensitivity of CARESTART in comparison with light microscopy, CareStart had 96% and 76% respectively. Furthermore, both Positive Predictive Value (PVP) and Negative Predictive Value (NVP) were respectively 84.7% and 93.6% for CareStart when compared with light microscopy (table 2).

Table 2: CareStart (HRP2) and Light Microscopy Results for Malaria Parasite Diagnosis

		Light Microscopy		
		Positive	Negative	**Total (%)**
CareStart	**Positive**	83 (TP)*1	15 (FP)*2	**98 (19.5%)**
	Negative	26 (FN)*3	378 (TN)*	404
Total		**109 (27.1%)**	393	502

*TN: True Negative *1TP: True Positive *2FP: False Positive *3FN: False Negative

Sensitivity (Se) = TP / (TP + FN)*100

Sensitivity of CareStart = 83/109 * 100

Sensitivity of CareStart = 76%

Specificity (Sp) = TN / (TN + FP)*100

Specificity of CareStart = 378/404 * 100

Specificity of CareStart = 96%

Positive Predictive Value (PPV) = TP/ TP+FP * 100

PPV of CareStart = 83/98 * 100

PPV of CareStart = 84.7%

Negative Predictive Value (NPV) = TN/ TN+FN

NPV of CareStart = 378/404

NPV of CareStart = 93.6%

4.4 Cyscope Fluorescent Microscopy and Light Microscopy Results for Malaria Parasite Diagnosis

Table 3 shows the comparison of Cyscope florescent microscopy with the conventional light microscopy in the laboratory diagnosis of malaria parasites. A total of 502 blood samples were tested for malaria parasite using the conventional light microscopy and Cyscope florescent microscopy. Of the 502 blood samples tested for malaria parasites, 109 (21.7%) and 154 (30.7%) were positive for light microscopy and Cyscope florescent microscopy respectively. Also by specificity and sensitivity of Cyscope florescent microscopy in comparison with light microscopy, Cyscope florescent microscopy had 95% and 87.3% respectively. Furthermore, both Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were respectively 67.5% and 98.6% for Cyscope florescent microscopy when compared with light microscopy (table 3).

Table 3: Cyscope Fluorescent Microscopy and Light Microscopy Results for Malaria Parasite Diagnosis

		Light Microscopy		
		Positive	Negative	Total
Cyscope	Positive	104 (TP)	50 (FP)	**154 (30.7%)**
	Negative	5 (FN)	343 (TN)	348
TOTAL		**109 (27.1%)**	393	502

Sensitivity of Cyscope = 104/109 * 100

Sensitivity of Cyscope = 95%

Specificity of Cyscope = 343/393 * 100

Specificity of Cyscope = 87.3%

PVP of Cyscope = 104/154 * 100

PVP of Cyscope = 67.5%

NPV of Cyscope = 343/348

NPV of Cyscope = 98.6%

4.5 Quantitative Buffy Coat (QBC) Fluorescent Microscopy and Light Microscopy Results For Malaria Parasite Diagnosis

Table 4 shows the comparison of Quantitative Buffy Coat (QBC) microscopy with the conventional light microscopy in the laboratory diagnosis of malaria parasites. A total of 502 blood samples were tested for malaria parasite using the conventional light microscopy and QBC microscopy. Of the 502 blood samples tested for malaria parasites, 109 (21.7%) and 164 (32.7%) were positive for light microscopy and QBC microscopy respectively (table 3). Also by specificity and sensitivity of QBC in comparison with light microscopy, QBC microscopy had 98.1% and 85.5% respectively. Furthermore, both Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were respectively 65.2% and 99.4% for QBC microscopy in comparison with light microscopy.

Table 4: Quantitative Buffy Coat (QBC) Fluorescent Microscopy and Light Microscopy Results for Malaria Parasite Diagnosis

		Light Microscopy		
		Positive	Negative	Total
QBC	Positive	107	57	**164 (32.7%)**
	Negative	02	336	338
Total		**109 (27.1%)**	**393**	**502**

Sensitivity of QBC= 107/109 * 100

Sensitivity of QBC = 98.1%

Specificity of QBC = 336/393 * 100

Specificity of QBC = 85.5%

PVP of QBC= 107/164 * 100

PVP of QBC = 65.2%

NPV of QBC = 336/338

NPV of QBC = 99.4%

4.6 Comparison of the Diagnostic Accuracy of CareStart, Cyscope Fluorescent Microscopy and QBC Fluorescent Microscopy

Comparison of the diagnostic accuracy of the three methods for the laboratory diagnosis of malaria parasites was carried out using Rapid Diagnostic Test (CareStart), Cyscope fluorescent microscopy, Quantitative Buffy Coat (QBC) fluorescent microscopy against the conventional light microscopy for the 502 blood samples for malaria parasite analysis (table 5). For the sensitivity of the three methods for the laboratory analysis of malaria parasites in this study, QBC had the highest rate of 98.1% while the lowest rate of 76% was obtained for CareStart. However for specificity of the test, highest rate of 96% was obtained for CareStart while lowest rate (87.3%) was obtained for Cyscope. Furthermore, in terms of Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for the three laboratory test methods, highest rate (84.7%) and lowest rate (65.2%) were obtained for CareStart and QBC respectively. On the other hand, highest rate (99.4%) and lowest rate (93.6%) were obtained for QBC and CareStart respectively (table 5).

Table 5: Diagnostic Accuracy of CareStart, Cyscope Fluorescent Microscopy and QBC Fluorescent Microscopy Using Light Microscopy as Gold Standard

Diagnostic Instrument	Sensitivity (95% CI)	Specificity (95% CI)	Positive Predictive Value (95% CI)	Negative Predictive Value (95% CI)
CareStart	76 (72.26 - 79.74)	96 (94.29 - 97.71)	84.7 (81.44 - 87.76)	93.6 (91.46 - 95.74)
Cyscope	95 (93.09 - 96.91)	87.3 (84.39 - 90.21)	67.5 (63.4 - 71.6)	98.6 (97.57 - 99.63)
QBC	98.1 (96.91- 99.29)	85.5 (82.42 - 88.58)	65.2 (61.03 - 69.37)	99.4 (98.72 - 100.08)

4.7 Comparison of Agreement Index amongst CareStart, Cyscope Fluorescent Microscopy and QBC Fluorescent Microscopy Using Light Microscopy as Gold Standard

The inter-instrument agreement, using Kappa statistical analysis to generate the Kappa values amongst the diagnostic instruments tested in this study showed that CareStart, Cyscope fluorescent microscopy and QBC fluorescent microscopy all have good agreement with the Light microscopy has shown by Kappa values 0.71 (CI = 0.64 - 0.77), 0.72 (CI=0.65 - 0.78), 0.75 (CI=0.68 - 0.82) respectively. (Table 6)

Table 6: Agreement Index amongst CareStart, Cyscope Fluorescent Microscopy and QBC Fluorescent Microscopy Using Light Microscopy as Gold Standard (n= 502)

Diagnostic Instrument	Expected Agreement (%)	Observed Agreement (%)	Standard Error	95% Confidence Interval	Kappa Value
CareStart	(59.80)	(88.25)	0.034	0.64 - 0.77	0.71
Cyscope	(60.93)	(89.1)	0.035	0.65 - 0.78	0.72
QBC	(67.24)	(91.83)	0.037	0.68 - 0.82	0.75

4.8 Comparison of the Operational Characteristics of CareStart, Cyscope Fluorescent Microscopy and QBC Fluorescent Microscopy Using Light Microscopy as Gold Standard

With reference to available findings from this study and information from the manufacturers website· the operational characteristics assessed in this study included parameters like Turnaround Time, Cost of equipment

Table 7: Operational characteristics of all the diagnostic instruments**

Parameters	Light Microscope	Cyscope	QBC	CareStart
Average time/test	45 minutes	5 minutes	8 minutes	20 minutes
Blood qty. needed/test	10 ul	8 ul	50 ul	3 ul
Electric current by standby battery	No	Yes	No	NA
Average cost of equipment	$1,197	$1,155	$14,970	NA
Number of Test/hour	1	12	7	3

** Findings as at March, 2014 obtained from this study procedures and manufacturers website.

4.9 Cost Effectiveness Analysis of the Diagnostic Accuracy of CareStart, Cyscope Fluorescent, QBC Fluorescent and Light Microscopy

(a) Input:

The costs are divided into machine or equipment cost, reagents /consumables cost, manpower/personnel, Electricity or other miscellaneous. The following assumptions were made; that machine cost is per unit time of use, assuming uniform depreciation over time / lifespan of the equipment (fixed lifespan is 3 years for the entire machine). Acquisition cost was divided by total life span of equipment (in real use days i.e. excluding weekends) to get machine cost per unit time of use. Period of use assumed to be fixed at 8 hours per day. Cost of reagents/consumables was calculated per session of use (8hours/day) (table 8).

Manpower/Personnel cost was calculated using standard monthly wage of basically qualified staff to operate each diagnostic tool; expressed in wage/hour. Basic staff qualification for CareStart usage is post primary education while the staff qualification for other diagnostic tools is post-secondary education. Using the United States Office of Personnel Management , General Schedule Qualification Standards, 2014; The General Schedule 2 (GS 2) is the standard for secondary education equivalent in Nigeria, which is acceptable staff qualification for operating the CareStart, while the Cyscope fluorescent Microscope can be operated by a staff on GS 5 (i.e, a degree holder). Both QBC fluorescent microscope and Light Microscope will require a staff on GS 7 (i.e, a professional degree). At GS 2, the standard salary is $25,114 per annum, which is equivalent to $12.50 per hour (Given 21 working days/ month and 8 hours/ day). For GS 5, the equivalent salary per hour is $17.10 (Given $34, 415 per annum). The standard schedule for GS 7 will provide a salary per hour of $21.14 (At $42,631 per annum). Electricity is estimated at $0.08/ hour (equivalent of N12.99k).

(b) Output/Consequences:

It is expected that each diagnostic tool produces result (yield) for malaria parasite test as the output or consequence of the use. The yield per procedure over the work/ allotted time is thus calculated as shown in (table 7). On the estimate, based on the turnaround time for each diagnostic tool; for instance, Light microscopy takes about 45 minutes to yield a test result. The CareStart takes about 20 minutes, while the QBC fluorescent microscopy takes about 10 minutes to produce result respectively. Cyscope on the other hand takes less than 5 minutes to yield result. As such, per hour, the Light microscope can yield about 1 test results; CareStart can yield approximately 3 test results, while QBC and Cyscope can yield approximately 7 and 12 test results respectively (table 7).

Cost Effectiveness Analysis

Table 8 describes the Cost Effectiveness Analysis of the three diagnostic tools in comparison with light microscopy as gold standard. Using Light Microscopy, with $21.54 spent per hour, the yield will be 2 test results. This is equivalent to $10.77 per test. On the other hand, CareStart cost per hour of use is $16.82 with a yield of 3 test results. This implies $5.61 per test. With Cyscope fluorescent microscopy, $24.53 per hour of use generated 12 test results. This is equivalent to $2.04 per test. The QBC fluorescent microscopy, $35.27 per hour of use produced approximately 6 test results, which brings the cost to $5.89. In summary, light microscopy, QBC, CareStart and Cyscope tools of malaria laboratory diagnosis costs $10.77, $5.89, $5.61 and $2.04 respectively per hour of use and per yield/test result. This also could be interpreted when compared with the cost of other tools that; x 5.28, x 2.89 and x 2.75 teams of light microscopy, QBC fluorescence microscopy and CareStart can be established for Cyscope fluorescent microscopy and also increase the yield/test result by multiples of 5.28, 2.89 and 2.75 respectively with improved turnaround time (table 8) if Cyscope microscope were used.

Table 8: Cost Effectiveness Analysis for the Diagnostic Performance of CareStart, Cyscope Fluorescent, QBC Fluorescent and Light Microscopy

INPUT	LIGHT	CARESTART	CYSCOPE	QBC
Machine/ Equipment cost ($)	1,197	NA	1,155	14,970
Cost per unit time of use ($/hr)	0.2	NA	0.2	2.5
Reagent/consumables ($/test/hr)	0.12	4.32	7.2	11.4
Manpower/Personnel ($/hr)	21.14	12.5	17.10	21.14
Electricity ($/hr)	0.08	NA	0.03	0.23
Total Cost per test ($)	**10.77**	**5.61**	**2.04**	**5.89**

CHAPTER FIVE

5.1 DISCUSSION

Malaria as a disease condition has continued to cause human and economic loss to developing countries of the world due to lack of facilities and appropriate diagnostic tool for precise laboratory diagnosis of malaria parasites (Badaru, 2010). It is also known that African countries including Nigeria are worst hit with attendant public health problems on malaria as health condition (Tagbo and Henrieta, 2007; Uzochukwu, 2009). The wide range of 200 million in the frequently quoted "300–500 million cases per year" in itself reflects the lack of precision of current malaria statistics. Any attempt to estimate the number of malaria cases globally is likely to become subject to argument most especially in developing countries of Africa (Snow *et al.*, 2005 and Bill *et al.*, 2005).

Malaria is a major public health problem in Nigeria where it accounts for more cases and deaths than any other country in the world. The disease is a risk for 97% of Nigeria's population while the remaining 3% of the population live in the malaria free highlands. The country also has an estimated 100 million malaria cases with over 300,000 deaths per year. This compares with 215,000 deaths per year in Nigeria from HIV/AIDS. Malaria also contributes to an estimated 11% of maternal mortality, accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age in Nigeria with greatest prevalence, close to 50%, in children age 6-59 months in the southwest. The south west region of the country also has the least (20.3%) Insecticide Treated Nets ownership.

In the present study, while comparing the available different methods which included Cyscope florescent microscopy, Quantitative Buffy Coat (QBC) florescent microscopy and CareStart Rapid Diagnostic Test (CareStart) for the detection of Malaria parasites with gold standard light microscopy, the prevalence rates of Malaria parasites detection were 32.7% (QBC), 30.7% (Cyscope) and 19.5% (CareStart) when compared with 21.7% detection rate with light microscopy. These rates are lower compared to findings by Badaru et.al, of 76.3%, 76.4%, and of 84.7% at Maiduguri, Yola, and Ota respectively in Nigeria (Bell and Peeling, 2006; WHO, 2000; Badaru, 2010). This could be due to the fact that the present study was largely carried out in the low transmission season. Thus, indiscriminate use of antimalarials should be avoided based on the level of malaria prevalence recorded in this study and there seems to be no place for presumptive treatment of febrile illnesses as malaria given the revealed specificity of the Cyscope, QBC and CareStart. A similar conclusion was reached on validity of Malaria parasites test diagnostic tools in a study in rural and urban Zambia (Salako, 1999).

Moreover, had accurate malaria diagnosis been achieved together with an improved public health data reporting system and healthcare access, inaccurate malaria parasites detection resulting in serious health issues would be lessened. Clinical diagnosis is imprecise but remains the basis of therapeutic care for the majority of febrile patients in malaria endemic areas, where laboratory support is often out of reach. Scientific quantification or interpretation of the effects of malaria misdiagnosis on the treatment decision, epidemiologic records, or clinical studies has not been adequately investigated.

Despite an obvious need for improvement, malaria diagnosis is the most neglected area of malaria research, accounting for less than 0.25% ($700,000) of the U.S.$323 million investment in research and development in 2004 (Malaria and RD Alliance, 2005 and Mepham *et al*., 2009)

Furthermore, this study also validated the malaria parasites diagnostic test tools in comparison with light microscopy of which the specificity and sensitivity rates were CARESTART (96%, 76%), Cyscope (95%, 87%) and QBC (98.1%, 85.5%). This finding is in concordance with earlier studies on sensitivity and specificity of diagnostic tools for malaria parasite detection. Previous studies conducted using *P. falciparum* only, rapid diagnostic kits in north-eastern Tanzania and in Uganda, showed sensitivities of 95.4%, 97.2% and 97.6% for Parachek, Parachek Pf and and ParaHIT *f*, respectively (Pekins *et al*., 1999 and Jeremiah *et al*., 2007).

However, the result differs from other studies which showed lower sensitivities. Studies conducted in Yola, Enugu, Port-Harcourt, Nigeria and in Ethiopia which found a sensitivity

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However, the result differs from other studies which showed lower sensitivities. Studies conducted in Yola, Enugu, Port-Harcourt, Nigeria and in Ethiopia which found a sensitivity of 69.7% for Global device rapid diagnostic kit, 42.3% for a *P.f* rapid diagnostic kit, 47% for SD Bioline rapid diagnostic kit *pf/pv* and 47.5% for Parascreen, an HRP -2 and pLDH based rapid diagnostic kit respectively (Kadeshaw *et al*., 2008, WHO 2010a).

The specificity of CareStart obtained in this study (98.5%) is consistent with a study conducted in Ethiopia (98.5%), but lower than 100% for global device rapid diagnostic kit assessed in Yola-Nigeria (Kadeshaw *et al.*, 2008, WHO 2010a). It is slightly higher than results of similar studies conducted in north-eastern Tanzania and in Uganda which showed 95.9%, 88.8%, and 87.7% for Parachek, Parachek Pf and ParaHIT *f* (Pekins *et al.*, 1997, Jeremiah *et al.*, 2007).

However,it is at variance with findings of 42.9% for SD Bioline rapid diagnostic kit in Obafemi-Owode area of Ogun state, Nigeria.

The absolute necessity for rational therapy in the face of rampant drug resistance places increasing importance on the accuracy of malaria diagnosis (Jager *et al.*, 2011). Giemsa microscopy and rapid diagnostic tests (RDTs) represent the two diagnostics most likely to have the largest impact on malaria control today. These two methods, each with characteristic strengths and limitations, together represent the best hope for accurate diagnosis as a key component of successful malaria control (Agomo *et al.*, 2003, and Murray *et al.*, 2008).

This is the main reason why malaria parasite diagnosis should not depend only on the conventional light microscopy when other tools with high specificity and sensitivity are now available. In spite of a variation in the basic targets of malaria control from elimination of mortality and minimizing morbidity to reducing prevalence or eradication, all malarious countries share a common need for reliable laboratory-diagnostic services to ensure early and rational treatment, reliable epidemiologic information, and epidemic preparedness (Maguire *et al.*, 2006).

The positive predictive value (PPV) and negative predictive value (NPV) detected in this study showed value rates of 84.7%, 93.6% (CareStart), 67.5%, 98.6% (Cyscope) and 62.5%, of 69.7% for Global device rapid diagnostic kit, 42.3% for a *P.f* rapid diagnostic kit, 47% for SD Bioline rapid diagnostic kit *pf/pv* and 47.5% for Parascreen, an HRP -2 and pLDH based rapid diagnostic kit respectively (Kadeshaw *et al.*, 2008, WHO 2010a).

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The positive predictive value (PPV) and negative predictive value (NPV) detected in this study showed value rates of 84.7%, 93.6% (CareStart), 67.5%, 98.6% (Cyscope) and 62.5%, 99.4% (QBC) respectively. The Negative predictive values obtained by the three malaria parasites diagnostic test tool validated and compared with light microscopy in this study are consistent with study in Ebute-Metta Lagos-Nigeria, and higher than that (99.3%) reported in north-eastern Tanzanian (Pekins *et al.*, 1997; Okolie, 2006). Lower NPVs of 62.9% and 68% respectively have been reported in other studies conducted in Nigeria (Hamer *et al.*, 2007 and Endeshaw *et al.*, 2008). Positive predictive values of 84.7%, 67.5% and 62.5% obtained in this study respectively for CareStart, Cyscope and QBC microscopy are also at variance with findings of 68% and 77% in other studies (Pekin *et al.*, 1997and Hamer *et al.*, 2007). Although it is pertinent to note that the lower sensitivity of the light microscopy which is the reference has an effect on the predictive values of the other diagnostic instruments in this study.

The turnaround time (TAT) for the malaria parasites diagnostic tools employed in this study when compared with the conventional light microscopy plays a vital role considering the input and output yields or results generated with the tools involved in this study. According to previous findings on turnaround time (TAT) to produce trained personnel in malaria diagnosis, it takes twelve days intensive training for Giemsa microscopy (Ohrt *et al.*, 2007) while Partec rapid malaria test takes about three days. In terms of turnaround time to yield result output, it takes only about 5 minutes to obtain results from the Partec Rapid Malaria Test® (PM) and 15 minutes from the Binax NOW® rapid diagnostic test (BN RDT) compared to 25 minutes for the Giemsa Stain (GS). However in the present study on the estimate, based on the turnaround time for each diagnostic tool. Light microscopy takes about 45 minutes to yield a test result. The CareStart took about 20 minutes, while the QBC fluorescent microscopy took about 10 minutes to produce result respectively. Cyscope on the other hand took less than 5 minutes to yield result. As such, per hour, the light microscope can yield about 2 test results; CareStart can yield approximately 3 test results, while QBC and Cyscope can yield approximately 6 and 12 test results respectively.

Also in this study, the cost effective analysis of the malaria parasites diagnostic tools employed when compared with the conventional light microscopy plays a significant role regarding the input and output yields or results of the test. With $21.54 spent per hour using Light Microscopy, the yield will be 2 test results which is equivalent to $10.77 per test. On the other hand, CareStart cost per hour of use is $16.82 with a yield of 3 test results. This implies $5.61 per test. With Cyscope fluorescent microscopy, $24.53 per hour of use generated 12 test results. This is equivalent to $2.04 per test. The QBC fluorescent microscopy, $35.27 per hour of use produced approximately 6 test results, which brings the cost to $5.89. This implies that there is a great advantage of these three diagnostic test tools for malaria parasite diagnosis over Giemsa stain microscopy (Ohrt *et al.*, 2007) as reported in our findings.

In terms of cost, light microscopy, QBC, CareStart and Cyscope instruments for malaria laboratory diagnosis costs $10.77, $5.89, $5.61 and $2.04 respectively per hour of use and per yield/test result. This also could be interpreted when compared with the cost of other tools; x 5.28, x 2.89 and x 2.75 teams of Light microscopy, QBC fluorescence microscopy and CareStart can be established for Cyscope and also increase the yield/test result by multiples of 5.28, 2.89 and 2.75 respectively with improved turnaround time.

This study has reaffirmed that there is the need to expand malaria diagnostic services as part of a greater framework of health system strengthening within resource-limited settings. Increasingly, countries and implementing partners have identified that limited diagnostic capacity represents a major barrier to implementation and sustainability of prevention, treatment and care programs for malaria (Maputo Declaration, 2008).

It was found that Cyscope fluorescent microscope is a reliable diagnostic tool that is very sensitive and specific in diagnosing falciparum malaria. Since this is the predominant species in Nigeria, causing most mortality and complications, this is very relevant and useful. It is expected that the CyScope will show similar results for other malaria species especially *P. vivax*, but this could not be ascertained by this study. Further studies are needed to determine its effectiveness in diagnosing other Plasmodium species.

Based on the ASSURED criteria and the need to expand malaria diagnostic services as part of a greater framework of health system strengthening within resource-limited settings (Maputo.Declaration, 2008), Cyscope should be considered as a point-of-care diagnostic device for resource limited and endemic areas.

5.2 Limitations to Study

Based on our findings from this work, the following limitations were observed when validating and comparing the malaria parasites diagnostic test tools with light microscopy as gold standard:

1. The Cyscope malaria diagnostic equipment is specific for *Plasmodium falciparum* and does not detect other malaria parasite species; hence there is limitation in detecting other circulating species in this population with this tool. However, it is on record that 95% of malaria cases in Nigeria children for instance are caused by *Plasmodium falciparum* (NIMS, 2010). However, an effort to hybridize the DNA of other plasmodium species into the present composition used by cyscope malaria diagnostic equipment by the manufacturer in Germany is in progress.
2. Also, parasite quantification was not comprehensively done for all positive samples by the microscopists due to time constraints and hence evaluation of these tools as regards malaria parasitaemia quantification was not done. However, the parasite quantification done with the few positive samples gave a good comparison but too few to be generalized in reporting.
3. The sensitivity of light microscopy used as reference in this study is low due to different objectivity of the microscopists. This might have negatively affected the performance indices of other instruments which have higher sensitivity. However, the malaria parasite detection rate of the diagnostic instruments revealed that the strength of individual instrument detection under routine laboratory working conditions.
4. Expected sample size for positive samples not met due to the level of malaria prevalence during the study period. This could have affected the strength of the argument, but the number gotten was still sufficient to make argument as suggested by subject matter experts.

5. In Africa over 70% of malaria cases do not present initially to health facilities but diagnosed and managed at home with traditional remedies or drugs bought from local shops (Amexo *et al.*, 2004). Patients only attend health centers after self-treatment fails (Chandramohan *et al.*, 2002). This might have affected the performance of some of the test methods especially CareStart RDT.

5.3 CONCLUSIONS AND RECOMMENDATIONS

All the three diagnostic instruments namely Cyscope florescent microscopy (Cyscope), Quantitative Buffy Coat (QBC) florescent microscopy and CareStart Rapid Diagnostic Test (Carestart) were very sensitive, specific, and had high predictive values for the laboratory diagnosis of malaria parasites when compared with light microscopy used as gold standard in this study. QBC had the highest sensitivity rate of 98.1% while the lowest rate of 76% was obtained for CareStart. However for specificity of the test, highest rate of 96% was obtained for CareStart while lowest rate (87.3%) was obtained for Cyscope. The sensitivity and specificity were stable irrespective of levels of parasitaemia and parasite rate. Furthermore, in terms of Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for the three laboratory test methods, highest and lowest rates were obtained for Carestart and QBC respectively. On the other hand, highest rate and lowest rate were obtained for QBC and Carestart respectively

This study has shown that Carestart, QBC fluorescent microscopy and Cyscope fluorescent microscopy are valuable complement to light microscopy because they help expand the coverage of parasite-based diagnosis to the periphery and minimize exclusively clinical diagnosis. The cost of improved malaria diagnosis will inevitably increase, whether by investment in light microscopy or Carestart or both. However, such investment offers a more promising strategy to deal with increasing costs of therapy driven by drug resistance. Today's multi-million dollar investment in anti-malarial drug development should be accompanied by a parallel commitment to improve diagnostic tools and their availability to those living in malaria endemic areas such as Nigeria.

Cyscope fluorescent microscope had the shortest turnaround diagnostic time and it is the most cost effective of all the laboratory diagnostic instruments evaluated.

5.4 Recommendations

This study has highlighted the substantial burden attributable to inadequate malaria parasites diagnostic tools in our laboratories in southwestern Nigeria as a public health problem especially in a resource limited and endemic setting. I therefore recommend as follows:

1. Cyscope fluorescent microscope is strongly recommended for malaria parasite detection and the primary health care board in Oyo state should consider its deployment to her health facilities to complement light microscopy and in areas without access to light microscopy.
2. There should be provision for adequate capacity for malaria diagnosis using various tools adopted in this study for accurate diagnosis of malaria parasites rather than adopting the conventional light microscopy alone.
3. The governments and healthcare stakeholders should support the need for the training and retraining of laboratory staff in our General Hospitals for those who lack the requisite experience and skills for malaria diagnosis using these three tools for laboratory staff that have not undergone formal training on Cyscope, QBC malaria microscopy and Carestart to help enhance usage as there exist paucity of experienced laboratory professionals who can use this tools.
4. There is a need to create awareness and educate both the public and caregivers in the communities in Ibadan Nigeria on the need to use well equipped malaria laboratory services for the diagnosis of malaria parasites rather than treating presumptively at home or at patent medicine stores.
5. The already existing community-based interventions to roll out malaria from our communities in Nigeria and the delivery of malaria diagnostic and treatment services should be strengthened.
6. The local government areas in Ibadan, Nigeria, as a matter of urgency should strengthen malaria laboratory services in health facilities through provision of basic laboratory reagents, equipment and materials, supervisory and quality assurance mechanism while ensuring adequate and proper training of laboratory staff on malaria diagnostic services.

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APPENDICES

Appendix 1

Test Principle for Partec CyScope®

The Partec CyScope® uses DAPI (4', 6-diamidino-2-phenylindole) as the dried-in reagent on the test slide. DAPI is a fluorescent stain that binds strongly to DNA (intraerythrocytic DNA). It is used extensively in fluorescence microscopy. Since DAPI can pass through an intact cell membrane, it may be used to stain both live and fixed cells. For fluorescence microscopy, DAPI is excited with ultraviolet light. When bound to double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm (Du *et al.*, 1998). DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA (Hard *et al.*, 1990).

Partec CyScope® Test Procedure

- ☐ Remove the test slide(s) from the slide box.
- ☐ Label them with the corresponding pathology number(s).
- ☐ Take a drop of blood from a finger prick directly or from a well mixed blood in an EDTA tube.
- ☐ Put the drop of blood onto the Test Slide (delivered ready-prepared, already containing the necessary reagents dried-in for long and safe storage) making sure not to mismatch the samples. The drop of blood must be placed at the portion containing the reagent.
- ☐ Cover the slide with a cover glass, wait for a minute and analyze the slide with the Partec CyScope® in a dark room.
- ☐ The presence of bright shinny dots (Fig. 11) indicates a positive slide for malaria parasites.
- ☐ To prevent the slides from drying out, they must be kept in a wet chamber.

Cell Count

- ☐ Parasites were counted against 200 or 500 WBC's.
- ☐ For very heavy parasitaemia (>100 parasites/field), an approximate count was done using a quarter of the field.
- ☐ The approximate count was then multiplied by four to get the overall total count.

Appendix 2

Light Microscopy

Thick film Preparation

- Using a micro-pipette place 12 μL drop of blood in the larger circle of the slide template on a pre-labeled slide.
- Place the micro-pipette tip in the 12 μL drop of blood and, using a circular motion, spread the blood so that it fills the larger circle or use a second microscope slide or an applicator to spread the blood.
- Air dry the slide on a flat surface. This slow drying avoids cracking.
- If the thick film dries too quickly it may "crack". A dry smear can be easily recognized by holding it to light and noting any wet areas. (Dry slides can then be stored vertically in slide boxes up to 72 hours.)
- The blood must be well mixed before the films are prepared.

Diameter of thick smear 15mm

Amount of blood for thick smear 12μl

Amount of blood for thin smear 2μl

Area covered by thick blood film 176.78sq.mm

Slide ID

Date

Study Name

Template for thick and thin blood film preparation

Thin film Preparation

- Using a micro-pipette, place a 2μL drop of blood in the smaller circle of the slide template. Do not delay between applying the drop and spreading the drop.
- Obtain a second slide and place it in front of the 2μL drop of blood at a 30° - 45° angle. Pull back the slide and hold until the suspension is evenly spread along the width of the spreader slide. Use a spreader slide with a clean and polished end.

□ Push the slide forward in a smooth, continuous motion. Avoid hesitation or jerky motions when spreading the blood. (The feathered end of the film should have RBCs that are in one single, distinctive layer).

□ To avoid cross contamination, do not re-use the same slide for another subject's blood sample.

Fixing

□ Fix the thin film by gently immersing it into absolute methanol (never ethanol) in a Coplin jar. Allow the film to dry naturally in a vertical position. Care must be taken not to accidentally fix any portion of the thick film. The thin film is dipped into methanol and immediately removed.

□ Heat fix by blowing hot air (about 45□ C) over the slides for 20 – 30 minutes or placing it in a dry box for 40 minutes. This gentle "heat fixation" allows thick films to adhere to the microscope slide much better.

□ At all times during preparation and storage, slides should be protected from exposure to insects and dust.

Giemsa Staining

Preparation of Giemsa buffer

a. Preparation of buffered water using buffer pellets

□ Measure 1000 mL distilled or de-ionized water into a graduated cylinder

□ Transfer the 1000 mL distilled or de-ionized water into a buffer bottle.

□ Using a forceps or spatula, pick one pellet and put in the buffer bottle.

□ Put a magnetic stirring bar into the bottle and close it tightly.

□ Place the cylinder on a stirring plate. Allow the contents to stir until the reagents are dissolved completely.

□ Check the pH as outlined in (d) below

b. Checking the pH of the buffered water

☐ Prepare the pH meter in accordance with manufacturer's instruction

☐ Remove the probe from the store solution, rinse with distilled water and wipe excess water with paper towel.

☐ Put the probe into the buffer solution and read the pH as displayed on the meter.

☐ The pH of the buffered water should be between 7.0 and 7.2.

☐ If the pH of the buffered water is too acidic, add small quantities of the 2% Na_2HPO_4 and recheck with the pH meter. Repeat this process until the desired pH is obtained.

☐ If the pH of the buffered water is too alkaline, add small quantities of the 2% KH_2PO_4 and recheck with the pH meter. Repeat this process until the desired pH is obtained.

☐ Store the buffer in a plastic container. The container should be labeled with contents, date prepared, expiration date, and technician initials.

☐ The buffer is considered expired 7 days after preparation.

Preparation of Giemsa working solution and staining

a. For routine malaria blood film (10% solution) (Working Solution)

☐ Pour 90 mL of buffered water (pH 7.0- 7.2) into a 100 mL graduated cylinder.

☐ Using a serological pipette, draw up 10 mL of Giemsa stain. Add the stain to the buffered water in the graduated cylinder.

☐ Cover the top of the graduated cylinder with Para film or protected hands. Gently invert the cylinder several times (or use a magnetic stirrer) until completely mixed.

☐ Label the cylinder with contents, date prepared, time prepared, expiration time, and laboratory personnel's initials.

☐ Buffered Giemsa stain (working solution) must be discarded and prepared afresh after 6 hours.

b. Technique of staining with Giemsa stain solutions

☐ Each malaria blood film is stained singly on a staining rack, rather than together in batches, to avoid cross-contamination.

- Routine and QC malaria blood films (both thick and thin films) will be stained in 3% Giemsa by flooding the slide (diluted in buffered water of pH 7.0-7.2) for 45-60 minutes.
- Acute malaria and quality control blood films (both thick and thin films) will be stained in 10% Giemsa by flooding the slide for 10-15 minutes.
- Rinse the slide briefly and gently by gentle running tap water or by a gentle flow of clean water from a beaker.
- Let the slides dry in a vertical position. (Drying may be hastened by use of a blow drier or slide warmer).
- Keep the slides in the slide box/folder in sequential order according to subject identification numbers.
- At all times during preparation and storage, slides should be protected from exposure to insects and dust.

Reading and quantification of parasites

a. Quantification of parasites in thick films

- The following method was used for quantifying asexual *Plasmodium* ~~forms~~ (in either single or mixed species infections) as well as sexual (gametocyte) forms. (If different species are observed, this fact will also be recorded).
- Piano-type tally counters will be used for counting asexual parasite forms and for counting WBCs.
- If parasites are observed, count them while simultaneously counting WBCs, up to a total of 200 WBCs. (But ensure that all parasites in the final HPF are counted even if a count of 200 WBCs has been exceeded.)
- A malaria blood film was considered negative if 100 HPFs have been scanned and no parasite observed.
- A recent laboratory WBC count is used to convert a parasite count to a parasite density (per µl of blood) by the following formula:# Parasites x (WBCs per µl blood) / # WBCs = Parasites/µl
- If the parasite/field exceeds 100 in a thick film, discontinue the thick film count and switch to the thin film instead.

b. Quantification of infected RBCs in Thin films

- Identify an area in the thin film where RBCs do not overlap, preferably in the tail (feathered edge) of the thin film.
- Upon observation of malaria parasites, begin to count parasitized RBCs per 1,000 total RBCs.
- Perform the count across the width of the thin film using the "battlement method" and stop the count on the 1000th RBC.
- After the first reading, slides should be kept in the same order in the slide box/folder for the second reader (who will follow the same procedure, but will record the results on a different Microscopist work sheet).
- Acute slides are read in an expedited fashion. This system is used ONLY to guide clinical management of subjects, whereas parasite densities are used to determine final results.
- Record all the HPFs scanned, parasites counted, WBCs counted, and parasitized RBCs counted into the "Microscopist Worksheet" or the Malaria Microscopy Logbook provided.

Appendix 3

Immunochromatographic Test Principle

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Immunochromatographic tests are based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line. Incorporation of a labeled goat antimouse antibody capture ensures that the system is controlled for migration (Piper *et al.*, 1996). Migration depends on several physical characteristics of the component reagents, primarily the porosity of the membrane controlling the flow rate and the components of the buffer solution used to transport the labeled antigen-antibody complex in the lysed blood sample (Moody, 2002).

Procedure for CareStart RDT

Check the expiry date on the test packet. Put on the gloves. Use new gloves for each patient. Open the alcohol swab. Grasp the 4th finger on the patient's left hand. Clean the finger with the alcohol swab. Allow the finger to dry before pricking. Open the lancet. Do not allow the tip of the lancet to touch anything before pricking the patient's finger. Prick patient's finger to get a drop of blood. Discard the lancet in the Sharps Box. Gently release the pipette bulb to draw blood after pricking to the graduated 3 ul line of the pipette. Touch the tip of the pipette to the sample hole marked "S". Squeeze gently to transfer the blood. Discard the pipette in the Sharps Box.

Do not set the lancet down before discarding it. Put two (2) drops of buffer into the assay hole marked "A". Wait 20 minutes after adding buffer. Read test results.

(NOTE: Do not read the test sooner than 20 minutes after adding the buffer. You may get FALSE results.)

How to read the test results:

A line in "C" AND a line in "T" means the patient does have *Plasmodium falciparum*

A line in "C" and NO LINE in "T" means does not have *Plasmodium falciparum*

The test is POSITIVE even if the line in "T" is faint.

NO LINE in "C" and a line or no line in "T" means the test is INVALID.

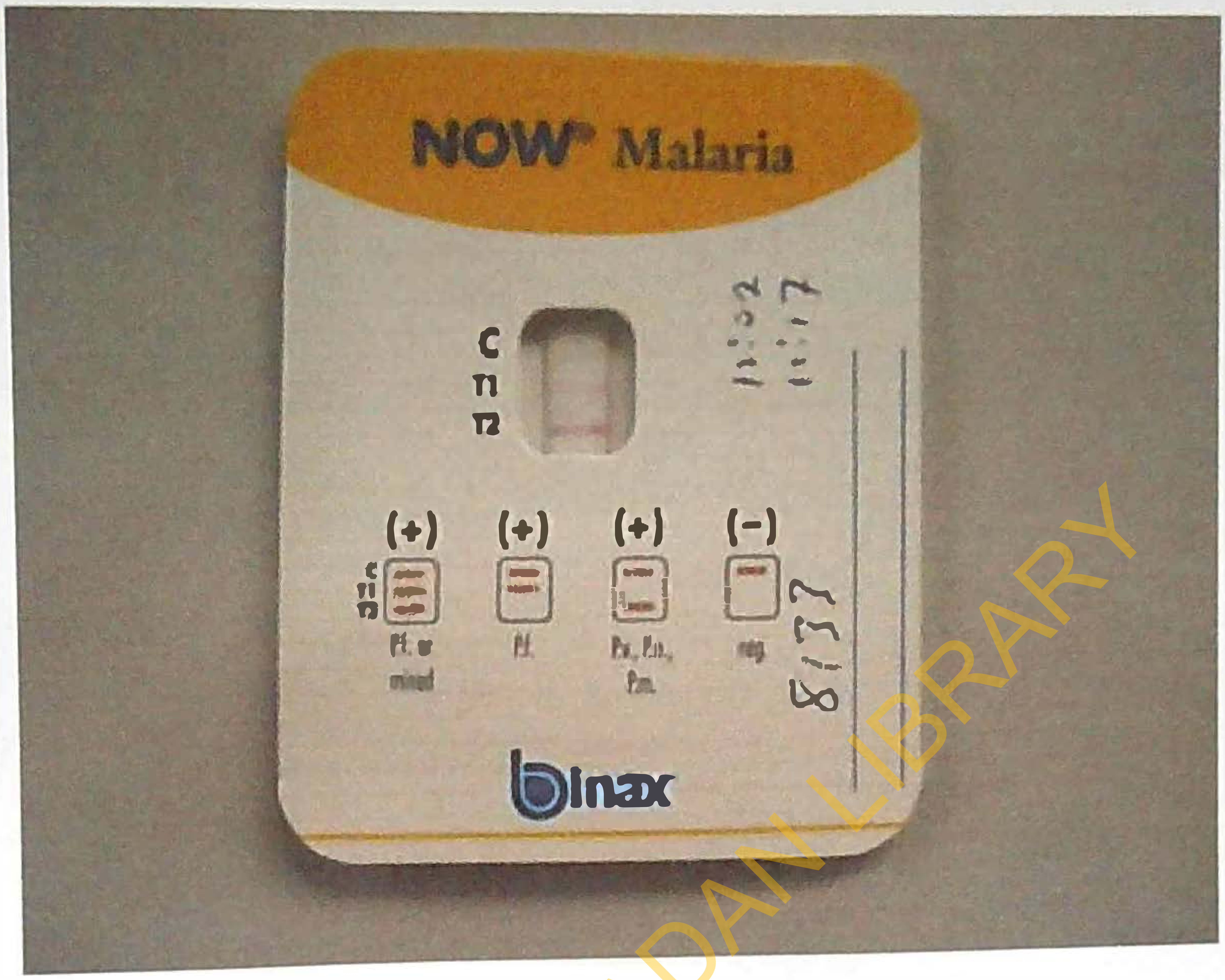
Repeat the test using a new RDT if no control line appears.

Appendix 4

RDT Format (Pictorial Representation)

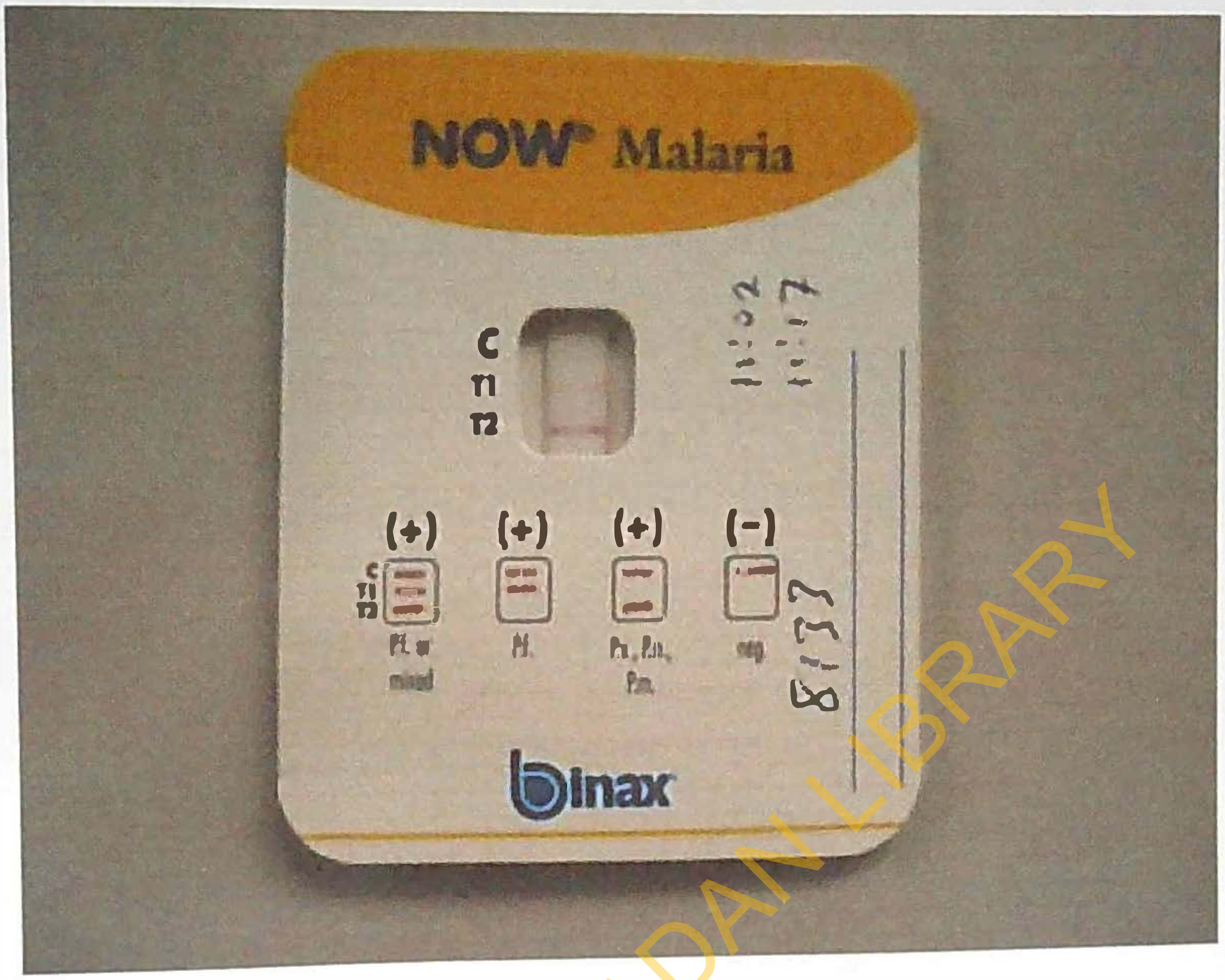
Dipstick format

Cassette format



Card format

(Source: Dipstick and Cassette (MDCoE, Kisumu, Kenya); Card (Original))



Card format

(Source: Dipstick and Cassette (MDCoE, Kisumu, Kenya); Card (Original))

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UI/UCH EC Registration Number: **NHREC/05/01/2008a**

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Validation of Cyscope Microscope, Quantitative Buffy Coat and Rapid Diagnostic Kit for Malaria Diagnosis among Clinic attendees in South West Nigeria

UI/UCH Ethics Committee assigned number: UI/EC/13/0357

Name of Principal Investigators: Abiodun O. Ogunniyi

Address of Principal Investigators: Department of Medical Microbiology, University College Hospital, Ibadan

Date of receipt of valid application: 25/10/2013

Date of meeting when final determination on ethical approval was made: N/A

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and *given full approval by the UI/UCH Ethics Committee.*

This approval dates from 27/12/2013 to 26/12/2014. If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the UI/UCH EC assigned number and duration of UI/UCH EC approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. No changes are permitted in the research without prior approval by the UI/UCH EC except in circumstances outlined in the Code. The UI/UCH EC reserves the right to conduct compliance visit to your research site without previous notification.

27 DEC 2013
APPROVED

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