Chapter one

Introduction and literature review

1.1 Introduction:

Malaria is a disease of global importance that results in 300-600 million cases annually and an estimated 2.2 billion people are at risk of infection (Singh *et al.*, 2010). More than a century after identification of the causative parasites and more than half a century after finding effective drugs and insecticides, the disease as old as humanity itself (Mehta and Desai, 2013). Numerically the most important of the life threatening protozoan disease is malaria, which is responsible for at least 750,000 death a year, mostly in young children in Africa (Greenwood *et al.*, 2012 ; WHO-a, 2015). Over half of the world's population is at risk from catching malaria. Malaria is currently endemic in 109 countries in four continents and of the 500 million cases of malaria estimated to occur annually, approximately one million result in death. Most of the fatalities are in children under the age of five years old and pregnant women (Lamb, 2012). Malaria accounts for at least $12 billion in economic losses each year in Africa and a reduction in annual economic growth estimated at 1.3 percent (NIH-a, 2010).

1.2 Literature review:

1.2.1 Definition:

Earlier theories were that malaria was caused by bad air ("mala aria" in Italian) (CDC-a, 2012). The parasites in the blood were first seen in 1880 by French army surgeon Alphonse Laveran, who was looking for a bacterial cause of malaria. He is immediately realized that parasites rather than bacteria were responsible for the disease (Ridley, 2012) (figure 1.1). Malaria parasites are micro-organisms that belong to the genus *Plasmodium*. There are more than 100 species of *Plasmodium*, which can infect many animal species such as reptiles, birds and various mammals.

Four species of *Plasmodium* have long been recognized to infect humans in nature. In addition, there is one species that naturally infects macaques which has recently been recognized to be a cause of zoonotic malaria in humans (CDC-b, 2012). The species infecting humans are: *P.falciparum*, which is found worldwide in tropical and subtropical areas. It is estimated that every year approximately 1 million people are killed by *P.falciparum*, especially in Africa where this species predominates. *P.vivax*, which is found mostly in Asia, Latin America and in some parts of Africa. Because of the population densities especially in Asia it is probably the most prevalent human malaria parasite. *P.ovale* is found mostly in Africa (especially West Africa) and the islands of the western Pacific. It is biologically and morphologically very similar to *P.vivax*. However, differently from *P.vivax*, it can infect individuals who are negative for the duffy blood group, which is the case for many residents of sub-Saharan Africa. This explains the greater prevalence of *P.ovale* (rather than *P.vivax*) in most of Africa. *P.malariae*, found worldwide, is the only human malaria parasite species that has a quartan cycle (three-day cycle). *P.knowlesi* is found throughout Southeast Asia as a natural pathogen of long-tailed and pig-tailed macaques. It has recently been shown to be a significant cause of zoonotic malaria in that region, particularly in Malaysia. *P.knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported (CDC-b, 2012).

1.2.2 Malaria Transmission:

The malaria parasite typically is transmitted to people by mosquitoes belonging to the genus Anopheles (NIH-b, 2009). The female anopheles mosquito is the chief vector and the most common means for transmitting malaria to humans. Some 60 species of this mosquito have been identified as vector for malaria. The infection is transmitted by the bite of an

infected female anopheles mosquito (Ridley, 2012). The mosquito most frequently bites at dawn and at dusk, as this is the most active feeding times for mosquitoes. The mosquito is infected by biting a patient infected with malaria, where it aspirates the sexual forms of the parasite, the gametocyte continue the sexual phase of the cycle and the sporozoites fill the salivary glands of the infested mosquito (Ridley, 2012). In rare cases, a person may contract malaria through contaminated blood. Malaria also may be transmitted from a mother to her fetus before or during delivery ("congenital" malaria). Because the malaria parasite is found in red blood cells, malaria can also be transmitted through blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood (NIH-b, 2009).
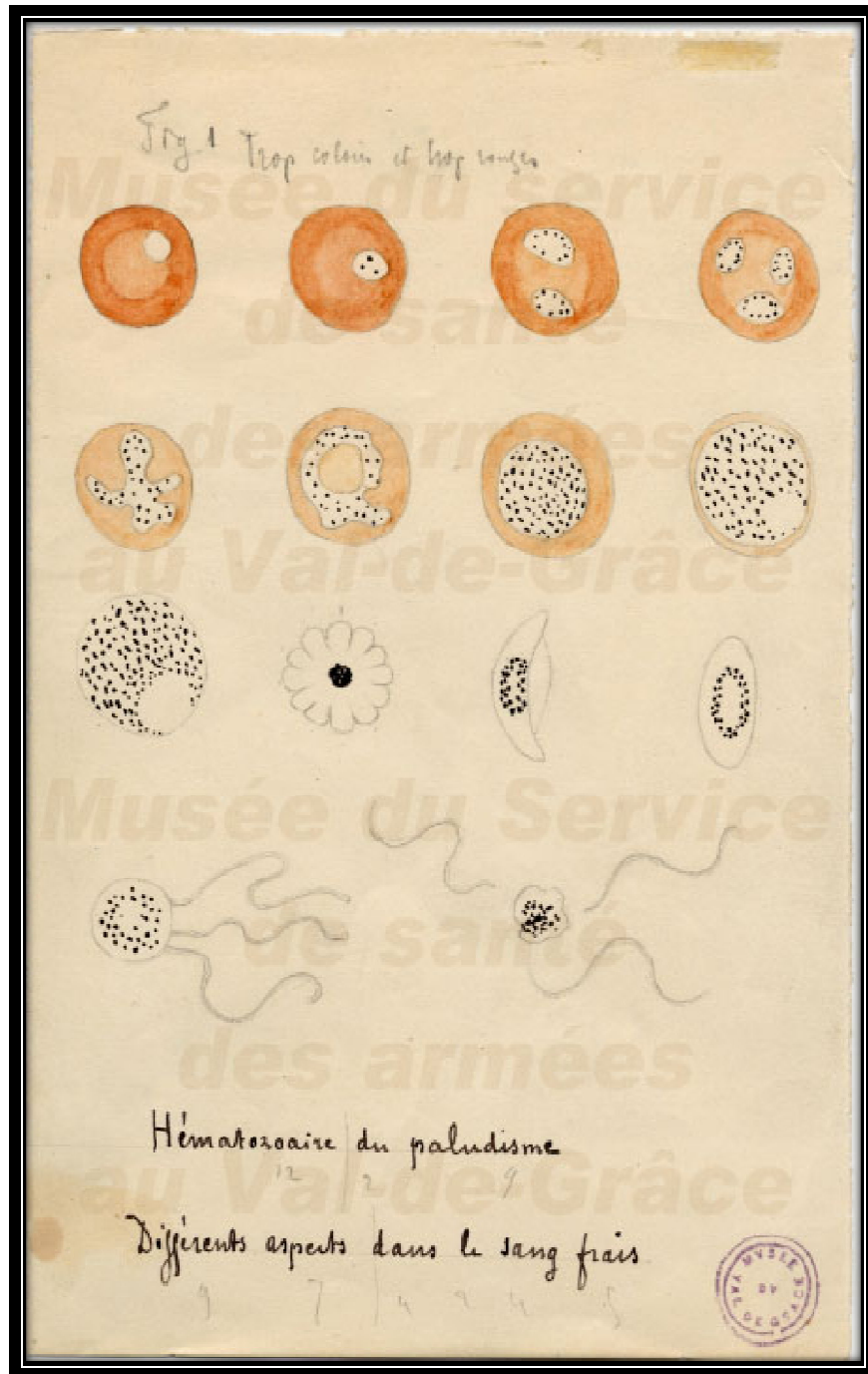
Figure (1.1): Illustration drawn by Laveran of various stages of malaria parasites as seen on fresh blood. Dark pigment granules are present in most stages (CDC-a, 2012).

1.2.3 Malaria life cycle:

The life cycle is a very complex one that begins when an infected anopheles mosquito injects sporozoites, the infectious stages, into the blood of it is host (Ridley, 2012).

1.2.3.1 Sporozoites in the skin:

Malaria infection is initiated upon deposition of sporozoites into the a vascular tissue of the skin from the salivary glands of a female mosquito as it probes a blood meal. Within one minute, the sporozoites become highly motile, traverse the capillary wall and enter the blood stream (Lamb, 2012). Sporozoite injected into the blood stream leave the blood vascular system within 40 minutes and subsequently invade the parenchymal cells of the liver (John and Petri, 2006).

1.2.3.2 Liver- stage malaria:

Once in the liver, sporozoite glide along the sinusoidal epithelium traversing several kupffer cells (resident liver macrophage) before invading a final hepatocyte, in which a parasitophorus vacuole (pv) forms (Lamb, 2012). They undergo a process of multiple nuclear division, followed by cytoplasmic division (schizogony) (Greenwood *et al.*, 2012). The infected hepatocyte then grows into a large exo-erythrocytic form, which eventually gives rise to between 10,000 and 20,000 merozoites over a 7-10 day time period. The liver stage of the life cycle is not associated with notable disease in malaria infection, but allows the parasite to multiply. Relapsing malaria infections caused by *P.vivax* and *P.ovale* arise from arrested liver-stage parasites known as hypnozoites, which are generally resistant to anti-malarial drugs (Lamb, 2012).

1.2.3.3 Asexual erythrocytic cycle:

Once merozoites burst from hepatocyte they invade red blood cells (RBCs) and enter into the asexual erythrocytic cycle. Invasion of new RBCs by merozoites involves secretion of proteases from structures

found at the apical end of the merozoites called micronemes, rhoptries and dense granules. One of the major surface proteins of merozoites is a 200 KDa protein called merozoite surface protein (MSP)-1. This protein is essential for asexual cycling in RBC stages; it is proteolytically processed in a number of stages after it reaches the merozoite surface, a process necessary for invasion. *P.vivax* requires the presence of a glycoprotein on the RBC surface (known as Duffy antigen) to attach. This species is not a major contributor to the malaria burden across sub-Saharan Africa, where most of the population are of the Duffy-negative blood type, making their RBCs refractory to invasion by *P.vivax.* RBCs do not have nuclei and are essentially metabolically inactive cells. Replicating parasites obtain the amino acids they require by digesting haemoglobin. At schizogony, once infected RBCs burst, between 10-32 merozoites are released, and these invade fresh RBCs to begin a new erythrocytic cycle (Lamb, 2012).

1.2.3.4 Transmission back to mosquitoes:

A small proportion of infected RBC differentiate in to transmissible male and female gametocytes, but the exact molecular cues leading to the development of male and female gametocyte are unknown. Once inside the mosquito midgut, the temperature shift and pH change induces gametogenesis and fertilization leading to the formation of motile diploid ookinetes that leave the blood meal bolus and traverse the midgut epithelium to become sessile oocysts. Over 10-14 days sporozoite develop within the oocyst via mitosis, and these escape via an enzymatic process into the mosquito body cavity. The sporozoites circulate via the haemolymph and attach onto the basal lamina of the mosquito salivary glands, ready for introduction into the next host (Lamb, 2012) (figure 1.2).
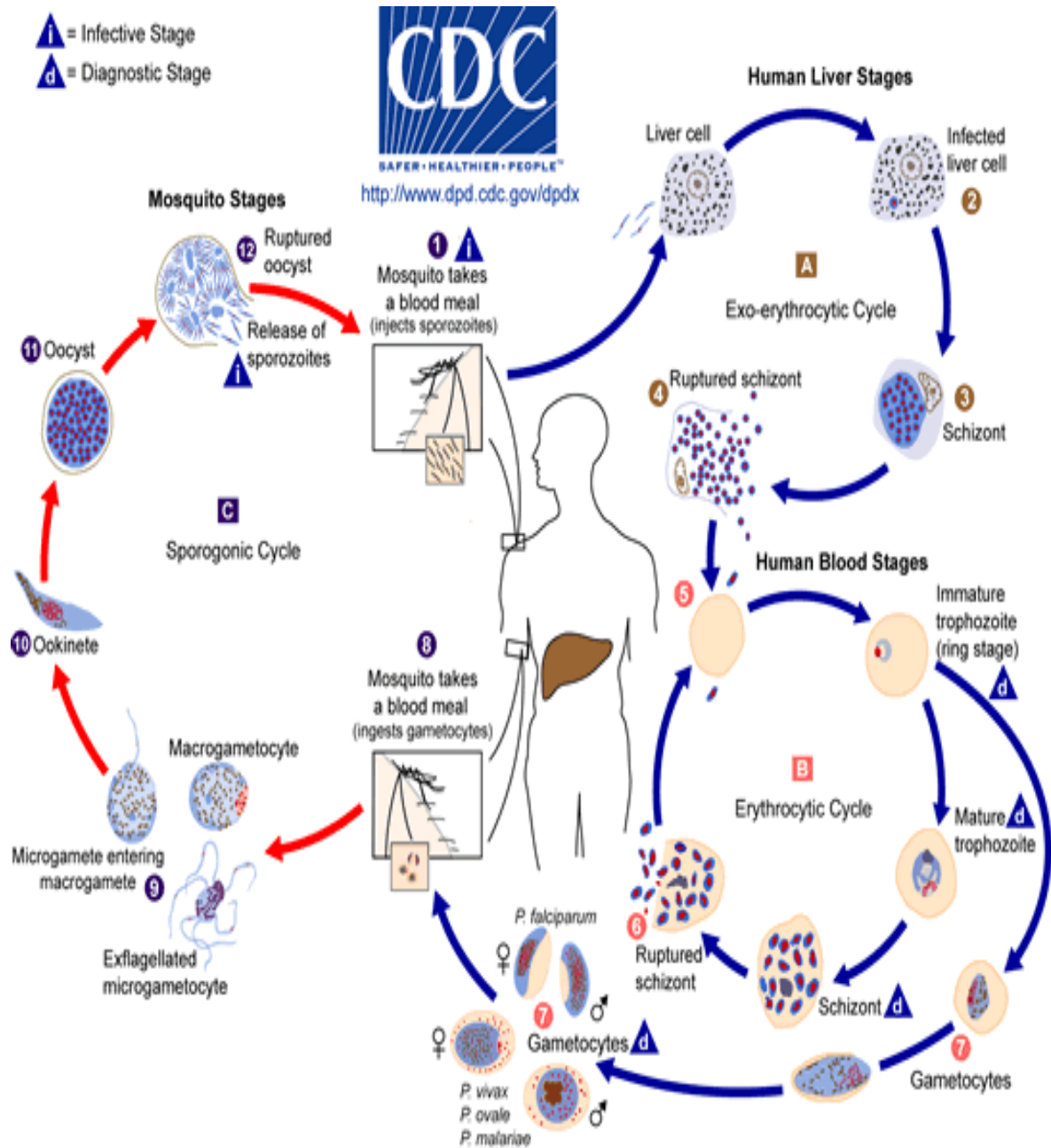
Figure (1.2): Life cycle of malaria (CDC-c, 2012).

1.2.4 Pathogenesis, pathology and symptomatology:

The pathogenic effect of a malarial infection have been considered to be directly related to hemolysis of infected red cell and uninfected cell, liberation of the metabolites of the parasite and the immunologic response of the host to this antigenic material and the formation of malarial pigment, additionally, in *falciparum* malaria the phenomenon of cytoadherence is basic to the locally diminished tissue perfusion seen in it is more severe complications, cytoadherence is the result of the expression on the surface of the parasitized red cell of strain and stage-specific parasite-derived ligands, which adhere to a specific receptor complex on the endothelial cells. In persons subjected to repeat attack of malaria anaemia is disproportional to the number of red blood cells infected, and indicates that non infected red blood cells may become sensitized and be destroyed (John and Petri, 2006).

1.2.4.1 The classical symptoms of malaria:

The clinical symptoms of malaria are primarily due to schizont rupture and destruction of erythrocytes (Trampuz *et al.*, 2003). Malaria typically produces a string of recurrent attacks, or paroxysms, each of which has three stages-chills, followed by fever and then sweating. Along with chills, the person is likely to have headache, malaise, fatigue, muscular pains, occasional nausea, vomiting and diarrhea. Within an hour or two, the body temperature rises, and the skin feels hot and dry. Then, as the body temperature falls, a drenching sweat begins. The person, feeling tired and weak, is likely to fall asleep. The symptoms first appear some 10 to 16 days after the infectious mosquito bite and coincide with the bursting of infected red blood cells (RBCs). When many RBCs are infected and break at the same time, malaria attacks can recur at regular time periods-every two days for *P.vivax* malaria and *P.ovale* and every three days for *P.malariae* (NIH-c, 2009).

1.2.4.2 Severe malaria:

Almost all severe forms and deaths from malaria are caused by *P.falciparum*. Rarely, *P.vivax* or *P.ovale* produce serious complications, debilitating relapses and even death (Trampuz *et al.*, 2003).

1.2.4.2.1 Complications in patients with severe malaria:

1.2.4.2.1.1 Cerebral malaria:

This has a high case fatality and is a pathological condition resulting from infection with *P.falciparum*. A number of hypotheses have been proposed to explain the phenomenon of cerebral malaria, but in general it is thought to stem from immune responses against sequestered infected RBCs (Lamb, 2012). The onset may be dramatic with a generalized convulsion, or gradual with initial drowsiness and confusion, followed by coma lasting from several hours to several days (Trampuz *et al.*, 2003). Sections of brain tissue from fatal *P.falciparum* infections reveal microvascular obstruction in the brain due to the accumulation of sequestered infected RBCs, autoagglutinates (where by infected RBC adhere to each other) and rosettes of infected RBCs, as well as infiltrates of lymphocytes. Brain-resident macrophages, or macrophage/monocyte population that migrate to the brain tissue as a result of inflammatory immune responses against sequestered infected RBCs, directly contribute to the pathogenesis of cerebral malaria (Lamb, 2012).

1.2.4.2.1.2 Severe anaemia:

Severe malarial anaemia (SMA) is often associated with chronic and repeated infections of malaria, and it can lead to a drop in haemoglobin in the blood to <5 g/dl (normal value are between 10-15 g/dl for humans). Anaemia in malaria infection can be due to loss of RBCs during parasite replication, as well as removal of infected RBCs as part of immune - mediated clearance mechanism. In addition, increased phagocytic mechanisms in the spleen lead to premature clearance of un infected

RBCs; around ten times more uninfected RBCs are removed from the circulation that infected RBCs. RBCs loss is normally compensated for by the development and release of new RBCs from progenitor cells in a process known as erythropoiesis. Parasite products such as haemozoin, and anti-malarial immune responses to these products, can depress normal haematopoietic mechanisms in the bone marrow and spleen (Lamb, 2012).

1.2.4.2.1.3 Renal complications:

Acute renal failure is usually oliguric (<400 ml/day) or anuric (<50 ml/day), rarely nonoliguric, and may require temporary dialysis. Urine sediment is usually unremarkable. In severe cases, acute tubular necrosis may develop secondary to renal ischemia. The term 'blackwater fever' refers to passage of dark red, brown, or black urine secondary to massive intravascular hemolysis and resulting hemoglobinuria. Usually, this condition is transient and not accompanied by renal failure (Trampuz *et al.*, 2003).

1.2.4.2.1.4 Pulmonary complications:

Acute lung injury usually occurs a few days into the disease course. It may develop rapidly, even after initial response to antimalarial treatment and clearance of parasitemia. The first indications of impending pulmonary edema include tachypnea and dyspnea, followed by hypoxemia and respiratory failure requiring intubation. Pulmonary edema is usually noncardiogenic and may progress to acute respiratory distress syndrome (ARDS) with an increased pulmonary capillary permeability. Acute lung injury is defined as the acute onset of bilateral pulmonary infiltrates with an arterial oxygen tension/fractional inspired oxygen ratio of 300 mmHg or less, a pulmonary artery wedge pressure of 18 mmHg or less, and no evidence of left atrial hypertension. ARDS is defined as acute lung injury and an arterial oxygen tension/fractional inspired oxygen ratio

of 200 mmHg or less. Volume overload and hypoalbuminemia may aggravate pulmonary capillary leakage. Chest radiograph abnormalities range from confluent nodules to basilar and/or diffuse bilateral pulmonary infiltrates. Noncardiogenic pulmonary edema rarely occurs with *P.vivax* and *P.ovale* malaria (Trampuz *et al.*, 2003).

1.2.4.2.1.5 Metabolic acidosis:

The development of metabolic acidosis, whereby the pH of the blood lowers due to increased production of hydrogen in the body or defective removal of bicarbonate from the body by the kidneys, is often accompanied by respiratory distress and is strongly correlated with fatal malaria infection. Metabolic acidosis is exacerbated by the lack of circulating RBCs in patients with SMA, due to a reduction in the amount of oxygen delivered to the tissues and anaerobic metabolism. Hypovolaemia, whereby the volume of circulating blood decreases (presumably volume loss is partially due to lost RBC mass), is associated with severe anaemia, and this also exacerbates metabolic acidosis (Lamb, 2012).

1.2.4.2.1.6 Hypoglycemia:

Hypoglycemia is a common feature in patients with severe malaria. It may be overlooked because all clinical features of hypoglycemia (anxiety, dyspnea, tachycardia, sweating, coma, abnormal posturing, generalized convulsions) are also typical of severe malaria itself. Hypoglycemia may be caused by quinine- or quinidine-induced hyperinsulinemia, but it may be found also in patients with normal insulin levels (Trampuz *et al.*, 2003).

1.2.5 Immunity to malaria:

Immunity produced following infection with malaria parasites is species-specific, stage-specific and strain-specific. Immunity in malaria is of two types:

1.2.5.1 Innate immunity:

This refers to inherent, non-immune mechanisms of host defense against malaria. This is due to: age of red blood cells, *P.falciparum* infects both young and old red blood cells while *P.vivax* and *P.ovale* infect only young erythrocyte and *P.malariae* only old erythrocyte. Nature of haemoglobin: presence of abnormal haemoglobin like thalassemia haemoglobin and foetal haemoglobin confers resistance against all plasmodium species, while sickle cell anaemia trait and haemoglobin E protect against *P.falciparum* and *P.vivax* respectively. Enzyme content of red blood cells: a genetic deficiency known as glucose-6-phosphate dehydrogenase (G6PD) trait confers some protection against *P.falciparum* infection. This enzyme is essential for respiratory process of the parasite. Presence or absence of certain factors: the presence of the Duffy factor increases the susceptibility to malaria. It is believed that Duffy factor present on the surface of erythrocytes acts as receptor for attachment of malaria parasite (Arora and Arora, 2010).

1.2.5.2 Acquired immunity:

Acquired immunity in malaria involves both humoral and cellular immunity. Antibodies against sporozoites and asexual and sexual blood stages develop in malaria patients. Antibodies (Immunoglobulin M (IgM), Immunoglobulin G (IgG) and Immunoglobulin A(IgA)) against asexual blood stages may protect by inhibiting red cell invasion and antibodies against sexual stages are believed to reduce malaria transmission. A variety of cellular mechanisms may play a role in conferring protection against malaria. These include natural killer activity and activated macrophages. The latter phagocytose and induce extracellular killing of target cells. T.cells are crucial for malaria immunity. Their major function seems to provide help for the production of antibodies and to activate macrophages. Malaria parasites like many

other microorganisms, are capable of periodically changing the expression of their antigens. This provides the parasite with a powerful means for evading host immunity. The ability of *P.falciparum* to remain sequestered by cytoadherence to the capillary lining of certain tissues is regarded as a selective advantage as such parasites can avoid frequent passage through spleen and thus exposure to immune effector mechanisms. Sequestration does not exist in other human malaria parasites and this is considered the main reason for the difference in disease severity (Arora and Arora, 2010).

1.2.6 Malaria diagnosis and treatment:

Malaria presents a diagnostic challenge to laboratories in most countries (Singh *et al.*, 2010). Malaria should be considered a potential medical emergency and should be treated accordingly (CDC-d, 2012).

1.2.6.1 Clinical diagnosis:

Clinical diagnosis is based on the patient's symptoms and on physical findings at examination. The first symptoms of malaria (most often fever, chills, sweats, headaches, muscle pains, nausea and vomiting) are often not specific and are also found in other diseases (such as the "flu" and common viral infections). Likewise, the physical findings are often not specific (elevated temperature, perspiration, tiredness). In severe malaria (caused by *P.falciparum*), clinical findings (confusion, coma, neurologic focal signs, severe anemia, respiratory difficulties) are more striking and may increase the index of suspicion for malaria. If possible, clinical findings should always be confirmed by a laboratory test for malaria (CDC-d, 2012).

1.2.6.2 Microscopic diagnosis:

The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wright's, or Field's stain. Blood obtained by pricking a finger or

earlobe is the ideal sample because the density of developed trophozoites or schizonts is greater in blood from this capillary-rich area. Blood obtained by venipuncture collected in heparin or sequestrine (EDTA) anticoagulant-coated tubes is acceptable if used shortly after being drawn to prevent alteration in the morphology of white blood cells (WBC) and malaria parasites. Both thick and thin blood films should be prepared. The thick blood film concentrates the layers of red blood cells (RBC) on a small surface by a factor of 20 to 30 and is stained as an unfixed preparation using Field's stain or diluted Wright's or Giemsa stain. The thick blood film provides enhanced sensitivity of the blood film technique and is much better than the thin film for detection of low levels of parasitemia and reappearance of circulating parasites during infection recrudescence or relapse. The lyses of the RBC during the staining process can make the process of scanning for parasites more difficult until experience is gained in finding the parasites among the WBC and platelets. The thin blood film is methanol fixed and stained with diluted Giemsa or Wright's stain using buffered water at pH 7.2 to emphasize the parasite inclusions in the RBC. Because of the fixed monolayer of RBC available in this procedure, the morphological identification of the parasite to the species level is much easier and provides greater specificity than the thick-film examination. The thin blood film is often preferred for routine estimation of the parasitemia because the organisms are easier to see and count. The ability to count parasites in sequential blood films enables the response to therapy to be monitored, particularly for *P.falciparum* infections (Moody, 2002).

Microscopy remains the gold standard for diagnosing malaria infections in clinical practice and research. However, microscopy is labour intensive, requires significant skills and time, which causes therapeutic delays. The objective of obtaining result quickly from the examination of

blood samples from patients with suspected malaria is now made possible with the introduction of rapid malaria diagnostic tests (RDTs) (Singh *et al.*, 2010).

1.2.6.3 Rapid diagnostic tests (RDTs):

Several RDTs are available, which are fast, reliable and simple to use and can detect *P.falciparum* and non-*falciparum* infections or both (Singh *et al.*, 2010). RDTs are based on the detection of antigens derived from malaria patients in lysed blood, using immunochromatographic methods. Most frequently they employ a dipstick or test strip bearing monoclonal antibodies directed against the target parasite antigens (Arora and Arora, 2010). 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. 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). Malaria antigens currently targeted by RDT are HRP-2, pLDH and *Plasmodium* aldolase. HRP-2 is a water-soluble protein produced by asexual stages and young gametocytes of *P.falciparum*. It is expressed on the RBC membrane surface, and because of its abundance in *P.falciparum*, it was the first antigen to be used to develop an RDT for its detection. The

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pLDH, an enzyme found in the glycolytic pathway of the malaria parasite, is produced by sexual and asexual stages of the parasite. Different isomers of pLDH for each of the four *Plasmodium* spp. Infecting humans exist, and their detection constitutes a second approach to RDT development. Several other enzymes of the malaria parasite glycolytic pathway, notably aldolase, have been suggested as target antigens for RDT for species other than *P.falciparum* (Moody, 2002).

1.2.6.4 Buffy coat concentration technique :

Centrifuging of EDTA anticoagulated venous blood gives excellent concentration of prasitized red cells just below the white cells and platelets (buffy coat layer). Detection of the parasites in stained buffy coat preparations, especially late stage trophozoites and gametocytes, is often possible when no parasites are found in thick and thin blood films. In buffy coat preparations, phagocytosed malaria pigment is more easily detected because the white cells are also concentrated. While preparations can be made from centrifuging blood in capillary tubes and then breaking the tubes to obtain the buffy coat layer and red cells bellow it, this technique is dangerous and should be avoided. A safer technique by using glass test tubes with EDTA anticoagulated venous blood (Cheesbrough, 1987).

1.2.6.5 Quantitative buffy coat technigue (QBC):

The principle of QBC technique is based on the fact that on centrifugation at a high speed, the whole blood separates into plasma, buffy coat and packed red cell layer. Blood cells in the buffy coat layer separated according to their densities. As the parasites within erythrocytes mature, they reduce the buoyant density of infected erythrocyte. These two properties are exploited in QBC technique for malaria diagnosis (Suthar *et al.*, 2013). The use of the dye is based on the premise that infected red cells appear to be less dense than uninfected and is concentrated primarily

within the zone at the interface-a small one to two mm region near the top of the RBC column. These parasites fluoresce as green and orange objects because of the uptake of dye (Kocharekar *et al.*, 2014). Certain fluorescent dyes have an affinity for the nucleic acid in the parasite nucleus and will attach to the nuclei. When excited by UV light at an appropriate wavelength, the nucleus will fluoresce strongly. Two fluorochromes have frequently been used for this purpose, acridine orange (AO) and benzothiocarboxypurine (BCP), which are both excited at 490 nm and exhibit apple green or yellow fluorescence. Rhodamine-123 is also useful for assessing the viable state of parasites, since its uptake relies on an intact, working parasitic membrane. Several methods have been published in which AO is used either as a direct-staining technique or combined with a concentration method such as a thick blood film. The centrifugal quantitative buffy coat combines an AO-coated capillary tube and an internal float to separate layers of WBC and platelets using centrifugation. Parasites concentrate below this layer of cells, appearing in the upper layer of RBC but also sometimes appearing within the layers of platelets and WBC. Parasites can be viewed through the capillary tube using a special long-focal-length objective (paralens) with a fluorescence microscope (Moody, 2002).

1.2.6.6 Polymerase chain reaction (PCR):

PCR cannot strictly be considered a rapid technique for the initial diagnosis of malaria. Its value lies in its sensitivity, with the ability to detect five parasites or less/µl of blood. Nested and multiplex PCR methods can give valuable information when difficult morphological problems arise during attempts to identify parasites to the species level. A number of PCR assays have been developed for the detection of malaria DNA from whole blood as either single or multiplex methods. These assays have been used for the initial diagnosis, following the response to

treatment and as sensitive standards against which other nonmolecular methods have been evaluated. The major advantages of using a PCR-based technique are the ability to detect malaria parasites in patients with low levels of parasitemia and identify them to the species level. Infection with five parasites or less per μl can be detected with 100% sensitivity and equal specificity. The additional sensitivity obtained using PCR may provide positive results from subpatent infections. Although many organisms may remain sequestered in the capillary beds, these parasites may be released into circulation but in insufficient numbers to be detected by peripheral-blood microscopy alone (Moody, 2002). Although this technique may be slightly more sensitive than smear microscopy, it is of limited utility for the diagnosis of acutely ill patients in the standard healthcare setting. PCR results are often not available quickly enough to be of value in establishing the diagnosis of malaria infection. PCR is most useful for confirming the species of malaria parasite after the diagnosis has been established by either smear microscopy or RDT (CDC-d, 2012).

1.2.6.7 Treatment:

Malaria is an entirely preventable and treatable disease. The primary objective of treatment is to ensure a rapid and complete elimination of the *Plasmodium* parasite from the patient's blood in order to prevent progression of uncomplicated malaria to severe disease or death, and to chronic infection that leads to malaria-related anaemia. From a public health perspective, treatment is meant to reduce transmission of the infection to others, by reducing the infectious reservoir and to prevent the emergence and spread of resistance to antimalarial medicines (WHO-b, 2015). Patients who have severe *P.falciparum* malaria or who cannot take oral medications should be given the treatment by continuous intravenous infusion. Most drugs used in treatment are active against the parasite forms in the blood (the form that causes disease) and include:

chloroquine, atovaquone-proguanil, artemether-lumefantrine, mefloquine, quinine, quinidine, doxycycline (used in combination with quinine), clindamycin (used in combination with quinine), artesunate. In addition, primaquine is active against the dormant parasite liver forms (hypnozoites) and prevents relapses. Primaquine should not be taken by pregnant women or by people who are deficient in G6PD (glucose-6-phosphate dehydrogenase). Patients should not take primaquine until a screening test has excluded G6PD deficiency (CDC-e, 2012).

1.2.7 Prevention and control:

Vector control is the main way to reduce malaria transmission at the community level. It is the only intervention that can reduce malaria transmission from very high levels to close to zero. For individuals, personal protection against mosquito bites represents the first line of defence for malaria prevention. Two forms of vector control are effective in a wide range of circumstances: insecticide-treated mosquito nets (ITNs): long-lasting insecticidal nets (LLINs) are the preferred form of ITNs for public health distribution programmes. WHO recommends coverage for all at-risk persons; and in most settings. The most cost effective way to achieve this is through provision of free LLINs, so that everyone sleeps under a LLIN every night. Indoor spraying with residual insecticides: indoor residual spraying (IRS) with insecticides is a powerful way to rapidly reduce malaria transmission. Its full potential is realized when at least 80% of houses in targeted areas are sprayed. Indoor spraying is effective for 3-6 months, depending on the insecticide used and the type of surface on which it is sprayed. Dicholoro Diphenyl Trichloroethane (DDT) can be effective for 9-12 months in some cases. Longer-lasting forms of existing IRS insecticides, as well as new classes of insecticides for use in IRS programmes, are under development.

Antimalarial medicines can also be used to prevent malaria (WHO-a, 2015).

1.2.8 Malaria in Sudan:

There is a high burden of malaria-related morbidity and mortality in Sudan. However, the national malaria control programme, with WHO's support, has reduced the number of malaria cases from more than four million in 2000 to less than one million in 2010. Between 2001 and 2010, the number of deaths due to malaria reduced by 75%. WHO works in close collaboration with the national malaria control programme to implement appropriate and cost-effective malaria control interventions. These include the distribution of artemisinin-based combination therapy treatments, rapid diagnostic tests and long-lasting insecticidal nets, and the introduction of the home-based management of malaria strategy. Artemisinin-based combination therapy treatments: in 2011, around 4666 health facilities provided free artemisinin-based combination treatments. This was 89% of the total number of health facilities targeted. First introduced in Sudan in 2005, artemisinin-based combination treatments are recommended as the first-line treatment for malaria caused by *P.falciparum*, the most deadly of parasites that infect humans. Rapid diagnostic tests: to help detect malaria parasites in human blood promptly, rapid diagnostic tests were distributed to health facilities in villages. The number of health facilities with rapid diagnostic tests has reached 3363 or 73% of the total targeted facilities. Long-lasting insecticidal nets: considered as the most effective intervention, WHO has been supporting the free distribution of long-lasting insecticidal nets to families in risk areas. Home-based management of malaria: In Sudan's far-flung villages, access to curative and diagnostic services is limited. The home-based management of malaria has been identified as one of the strategies to reduce the burden of malaria, especially in malaria-endemic

areas. So far the strategy has been introduced in 988 villages across the country. With home-based management of malaria, diagnosis and treatment has been brought nearer the home and the community, so that treatment can be given within 24-hours of the onset of symptoms (WHO-c, 2015).

Rationale:

Malaria present a diagnostic challenge for laboratories in most countries. Microscopy remains the gold standard for diagnosing malaria infection in clinical practice and research. However, microscopy is labour intensive requires significant skills and time, which causes therapeutic delays. The objective of obtaining quickly result is now made possible with the introduction of rapid malaria diagnostic tests (RDTs). Initially the use of (RDTs) met stiff resistant by the malaria community because of it's cost. However, a number of reports from policy makers have acknowledged that (RDTs) may have their place because expert microscopy in malaria-endemic area is hard to establish and the cost has been gently reduced. Buffy coat concentration technique was intended mainly for the diagnosis of *Plasmodium* species, it concentrates the malaria parasite so easy to detect. The speed of buffy coat concentration technique in detecting malarial parasites is a definite advantage in laboratories which screen large number of samples. In addition, low levels of parasitaemia can easily be detected. Evaluation and comparison of these techniques will assist the policy makers and program managers in taking decisions and improve the quality of malaria diagnosis techniques.

Objectives:

General objective:

To evaluate malaria diagnostic methods in Medical Military Hospital-Khartoum state.

Specific objectives:

1. To detect the prevalence of malaria using blood films, ICT and buffy coat techniques in the study area.

2. To evaluate the efficiency of blood films, rapid diagnostic tests and buffy coat techniques in the detection of malaria parasite.

3. To study prevalence of malaria in relation to gender, age and symptoms.

Chapter two

Materials and Methods

2.1 Study design

Cross-sectional study design.

2.2 Study area and study duration

The study was conducted at Medical Military Hospital-Khartoum State, in the period between March - December 2015. Khartoum State lies between longitude 31.5-34 east and latitude 15-16 north in an area about 28.165 square kilometers. It is bordered on the north and the east side's by the River Nile State, on the north-western side by the Northern State and on the eastern and southern sides by Kassala, Gedaref and Gezira States (Ministry of Human Development and Labour, 2015).

2.3 Study population

The study was carried out on patients that were clinically suspected to have malaria.

2.4 Sample size

The sample size was obtained according to the following equation:

$N = (t^2 * P (1-p)/M^2)$

N = Sample size

t = the normal standard deviate (t = 1.96)

P = the frequency of occurrence of malaria (1.6%)

M = degree of precision (0.05%)

N = 1.96x1.96x.16x(1-.16)/.05x.05 =206

According to the above finding, the study was conducted on 200 clinically suspected patients.

2.5 Sampling

Two hundred blood samples were collected from all participants. The blood specimens obtained by venipuncture and collected in EDTA anticoagulant-coated tubes. Two hundred questionnaires were filled by

participants. Each specimen was tested by the three methods (blood films, ICT and buffy coat concentration technique).

2.6 Data collection

Designed questionnaire (appendix) contained the following variables: Gender, age, symptoms, previous infection, duration of previous infection, previous treatment, the type of previous treatment and parasitological results.

2.7 Methods

2.7.1 Stained blood films

2.7.1.1 Thick blood films

Three drops of blood were added to clean and dry slide, mixed and allowed to dry. Then the slides were stained by 10% Giemsa stain, washed and air dried. Then a drop of oil was added and examined under microscope. The number of parasites was counted and reported by using the following grading as described by Cheesbrough (1987)

1-10 per 100 high power fields ……. +

11-100 per 100 high power fields ……. ++

1-10 in every high power field ………..+++

More than 10 in every high power field …++++

2.7.1.2 Thin blood films

A drop of blood was added below the level of slide (2/3) and by spreader the blood was pushed forward with suitable speed, allowed to air dry, then fixed with absolute methanol, allowed to air dry and stained with 10% Giemsa stain. Then washed and allowed to dry, drop of oil was added and examined under microscope (100x oil immersion).

2.7.2 Immunochromatography test (ICT)

Five µl of whole blood was added into sample well in the pf/pv antigen test kits, two drops (80µls) of assay buffer were added into the developer well. Then the results were read in 20 minutes as follow: the presence of

two color bands ″c″ and ″pf″, indicates a positive result for *P.falciparum*, two color bands ″c″ and ″pv″ indicates a positive result of *P.vivax*, three color bands indicates a positive result for *P.falciparum* and *P.vivax*. The presence of only one band, ″c″ within the result window indicates a negative result, as manufacturer's instructions (Rapid Malaria pf/pv Antigen Test).

2.7.3 Buffy coat concentration technique

Glass test tubes with EDTA anticoagulated venous blood were centrifuged at 2000 RPM for about 5 minutes, by small plastic pipette the supernatant plasma above the buffy coat layer was removed and discarded. The buffy coat layer and red cells immediately below it was transferred to one end of slide and mixed (thick film), by spreader made an evenly spread thin preparation. The preparations were allowed to air dry, fixed with absolute methanol (just for thin films), stained with 10% Giemsa stain, allowed to air dry and examined under microscope first with 40x objective and then with 100x objective (Cheesbrough, 1987).

2.8 Data analysis

Result obtained were analyzed by the computerized program of statistical package of social science (SPSS) version 11.5. Frequencies, mean and Chi-squire test were used. Data were presented in figures and tables.

2.9 Sensitivity and specificity of techniques

Sensitivity and specificity was calculated as described by Kocharekar *et al.* (2014)

Sensitivity = TP/(TP+FN) x 100%

Specificity = TN/(TN+FP) x 100%

TP= True positive

FN= False negative

2.10 Ethical consideration:

Approval was taken from the College of Medical Laboratory Science-Sudan university for Science and Technology. A consent was taken from all participants or their guardians before being enrolled in the study. All participants were informed on the nature of the study.

Chapter three

Results

3.1 General characteristics of study population:

The study was conducted on 200 subject, 92 (46%) were males and 108 (54%) were females (table 3.1). The age was between 1-65 years old, the mean age was 27±18 years old. Study subjects were divided into 5 age groups as follows: 1-15, 16-25, 26-35, 36-45 and 46-65 years old, the frequency of each age group was 77 (38.5%), 25 (12.5%), 32 (16%), 30 (15%) and 36 (18%) respectively (table 3.2).

Table (3.1): Frequency of study subjects according to gender

Gender	Frequency	Percentage (%)
Male	92	46%
Female	108	54%
Total	200	100%

Table (3.2): Frequency of study subjects according to age groups

Age groups (years)	Frequency	Percentage (%)
1-15	77	38.5%
16-25	25	12.5%
26-35	32	16%
36-45	30	15%
46-65	36	18%
Total	200	100%

3.2 Parasitological results:

3.2.1 Prevalence of malaria by using stained blood films, ICT and buffy coat concentration technique:

Out of 200 blood samples, 20 (10%), 25 (12.5%) and 20 (10%) were positive for *P.falciparum* by using stained blood film, ICT and buffy coat concentration technique respectively. Out of 200 blood samples, 4 (2%) were positive for *P.vivax* by using the three methods. The difference between rates was found to be highly significant at p=0.000 (table 3.3).

Table (3.3): Prevalence of malaria by using blood films, ICT and buffy coat concentration technique

Techniques	*P.falciparum* (+ve)	*P.vivax* (+ve)
Blood films	20	4
ICT	25	4
Buffy coat technique	20	4

p=0.000

3.2.2 The prevalence of malaria by using blood films according to gender:

Out of 24 positive blood samples,13 (6.5%) were positive for males and 11 (5.5%) were positive for females. The difference between rates was found to be insignificant at p=0.392 (figure 3.1).
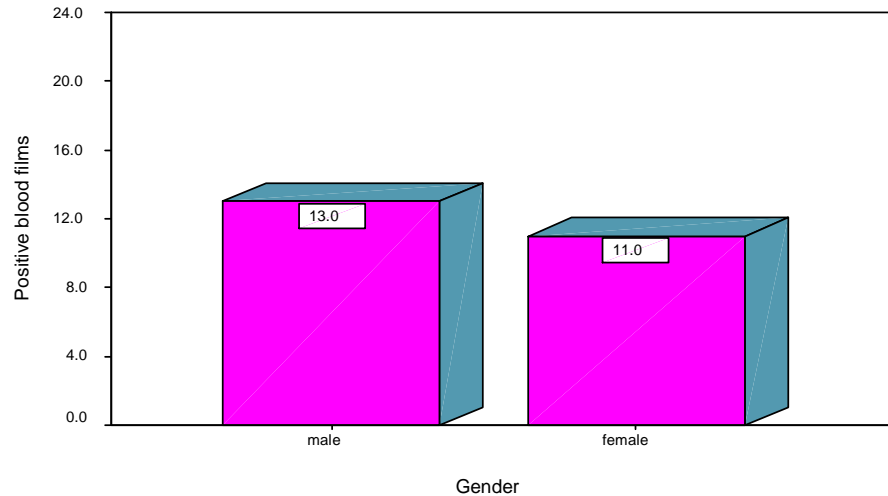
Figure (3.1): The prevalence of malaria by using blood films according to gender

3.2.3 The prevalence of malaria by using blood films according to age groups:

The prevalences of malaria were, 12 (6%), 2 (1%), 2 (1%), 4 (2%) and 4 (2%) in age groups between 1-15 years, 16-25, 26-35, 36-45 and 46-65 respectively. The difference between rates was found to be insignificant at p=0.664 (table 3.4).

Table (3.4): The prevalence of malaria by using blood films according to age groups

Age groups (years)	Blood Films		Total
	Positive	Negative	
1-15	12	65	77
16-25	2	23	25
26-35	2	30	32
36-45	4	26	30
46-65	4	32	36
Total	24	176	200

p=0.664

3.2.4 Relationship between the three different methods in detection of malaria parasites:

When blood films compared with ICT, 23(11.5%) were positive by two methods, while 1(0.5%) blood sample was positive by blood film and negative by ICT, 6(3%) were positive by ICT and negative by blood film. While blood film and buffy coat concentration techniques showed similar results (table 3.5).

Table (3.5): Relationship between the three different methods in detection of malaria parasites

		Blood films		Total	P value
		Positive	Negative		
ICT	Positive	23	6	29	p=0.000
	Negative	1	170	171	
Total		24	176	200	
Buffy coat	Positive	24	0	24	p=0.000
Technique	Negative	0	176	176	
Total		24	176	200	

3.2.5 Sensitivity and specificity of techniques:

Sensitivity and specificity of ICTs according to the formula mentioned in materials and methods was 96% and 96.6% respectively (table 3.6). Buffy coat concentration technique sensitivity and specificity was 100% (table 3.7).

Table (3.6): Sensitivity and specificity of ICTs

		Blood films	
		Positive	Negative
ICTs	Positive	23	6
	Negative	1	170

Table (3.7): Sensitivity and specificity of buffy coat concentration techniques

		Blood films	
		Positive	Negative
Buffy coat	Positive	24	0
Technique	Negative	0	176

Table (3.8): Relationship between malaria and fever

		Fever		Total
		Yes	No	
B.F for	Positive	24	0	24
Malaria	Negative	158	18	176
Total		182	18	200

p=0.101

Table (3.9): Relationship between malaria and headache

		Headache		Total
		Yes	No	
B.F for	Positive	18	6	24
Malaria	Negative	131	45	176
Total		149	51	200

p=0.952

Table (3.10): Relationship between malaria and vomiting

		Vomiting		Total
		Yes	No	
B.F for	Positive	21	3	24
Malaria	Negative	66	110	176
Total		87	113	200

p=0.000

Table (3.11): Relationship between malaria and diarrhea

		Diarrhea		Total
		Yes	No	
B.F for	Positive	2	22	24
Malaria	Negative	25	151	176
Total		27	173	200

p=0.430

3.2.6 Intensity of malaria parasites:

Out of 24 positive cases, 6(3%) presented as (+) for *P.falciparum*, 4(2%) and 1(0.5%) presented as (++) for *P.falciparum* and *P.vivax* respectively, 7(3.5%) and 2(1%) presented as (+++) for *P.falciparum* and *P.vivax* respectively, 3(1.5%) and 1(0.5%) presented as (++++) for *P.falciparum* and *P.vivax* respectively (figure3.2).
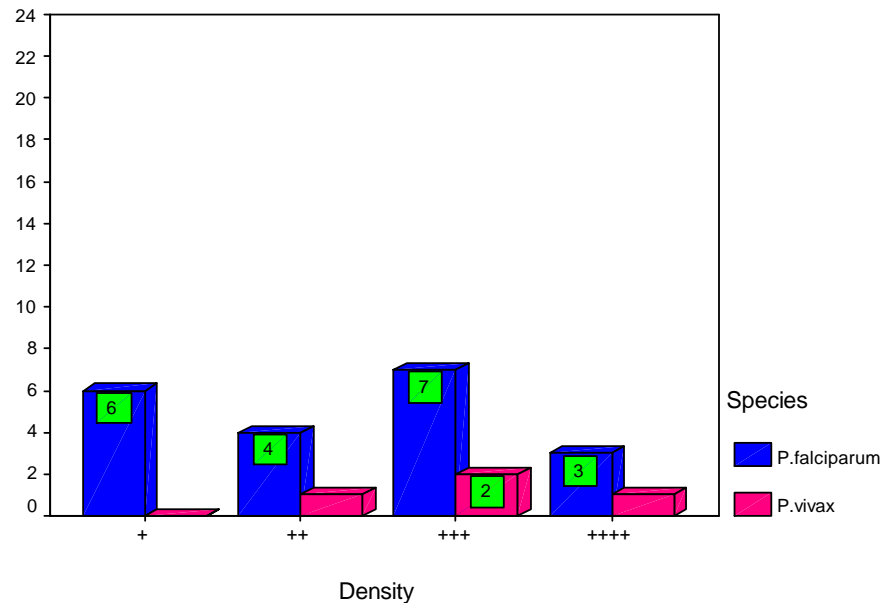

Figure (3.2): Intensity of malaria parasites

3.2.7 The prevalence of malaria according to stages:

Out of 24 positive cases, *P.falciparum* stages were 16(8%) trophozoite, 3(1.5%) gametocyte and 1(0.5%) were trophozoite and gametocyte. *P.vivax* stages were 3(1.5%) trophozoite and gametocyte and 1 (0.5%) were trophozoite, schizont and gametocyte. The difference between rates was highly significant at p=0.000 (figure 3.3).
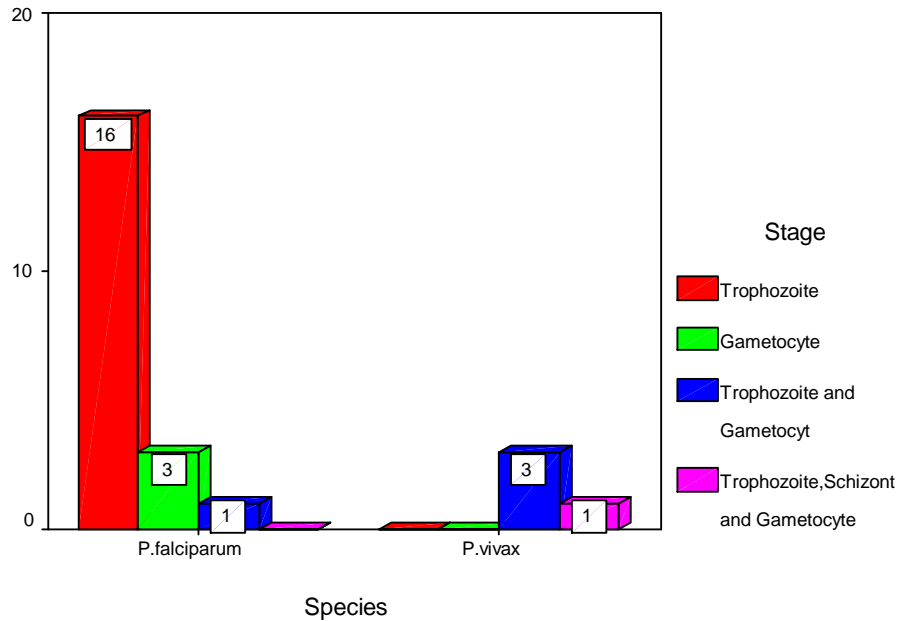
Figure (3.3): The prevalence of malaria according to stages

3.2.8 Prevalence of malaria according to duration of previous infection:

Out of 29 positive cases by using ICT, 7 (3.5%), 1(.5%) and18 (9%) were previously infected in the time period of about 1-30 day, 31-60 day and more than yaer respectively, while 3(1.5%) were not infected previously. The difference between rates was highly significant at p=0.000 (figure 3.4).
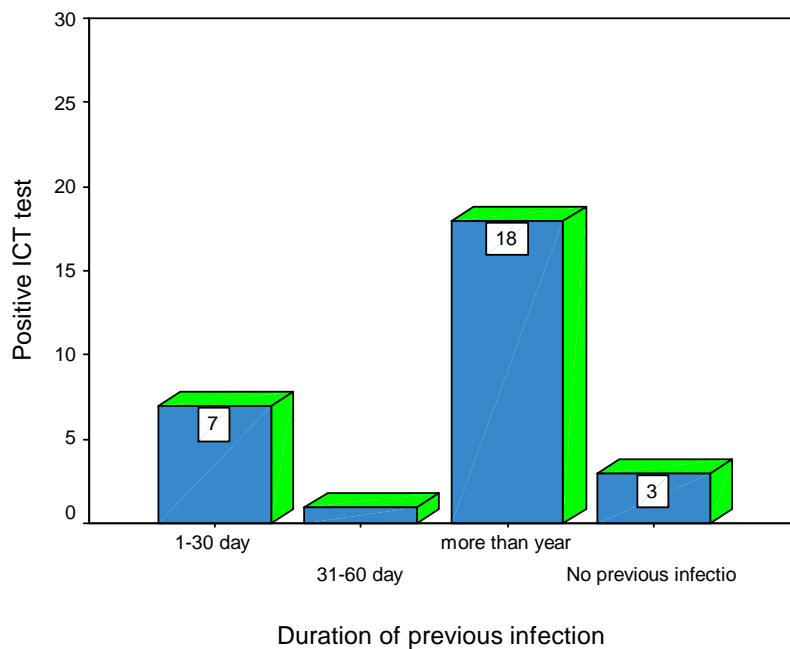
Figure (3.4): Prevalence of malaria according to duration of previous infection

3.2.9 Prevalence of malaria according to types of previous treatment:
Out of 24(12%) positive cases by blood films, 20 (10%) were treated previously while, 4(2%) were not treated previously. From 20 (10%) treated cases, 12 (6%), 7(3.5%), 1(0.5%) were previously treated by artemether, artesunate, artemether+primaquine respectively. The relationship between malaria and previous treatment was significance (p=0.030) (figure 3.5).
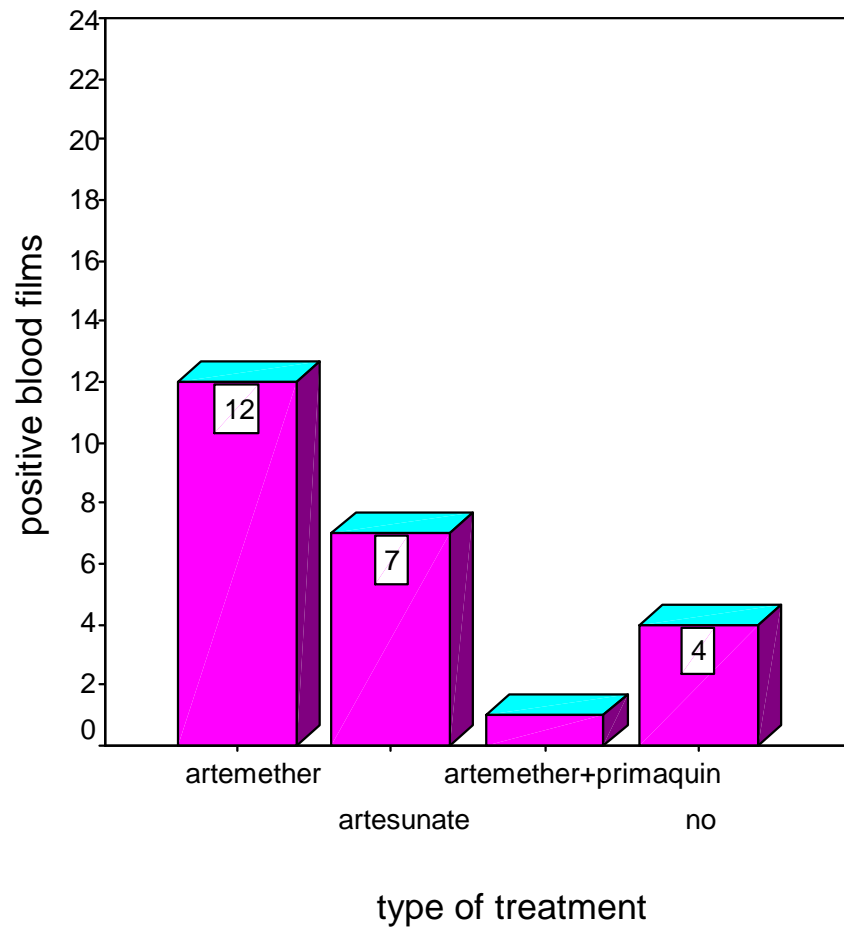
Figure (3.5): Prevalence of malaria according to types of previous treatment

Chapter four

Discussion

The present study was carried out on 200 blood samples collected from patients with clinical symptoms of malaria at Medical Military Hospital-Khartoum State. Out of 200 blood samples, 108(54%) were females, while 92(46%) were males with ratio of 1.2:1, this finding was in contrast to the Rashmi *et al.* (2015) findings. The present study showed that the prevalence of malaria in the study area was 24(12%), which was more in males (6.5%) than in females (5.5%), these findings were in agreement with Rashmi *et al.* (2015) findings, while the relationship between malaria and gender was insignificant (p=0.392). The prevalence was high (6%) in the age group 1-15 years old, due to their lack of efficient immunological response against the infection. The relationship between age and malaria was found to be statistically insignificant (p=0.664), which means that the infection is not affected by the age.

Out of 24 positive cases, 20(10%) were positive for *P.falciparum*, 4(2%) were positive for *P.vivax* which indicates that *P.falciparum* is the predominant species than *P.vivax* in the study area. These results were similar to results obtained by Medhi *et al.* (2015).

The prevalence of malaria according to fever was 24(12%) (p=0.101), headache was 18(9%) (p=0.952), vomiting was 21(10.5%) (p=0.000), while diarrhea was 2(1%) (p=0.430). From these findings, there is no relationship between malaria infection and clinical symptoms except with vomiting.

Out of 24 positive cases 6(3%) presented as (+) for *P.falciparum*, 4(2%) and 1(0.5%) presented as (++) for *P.falciparum* and *P.vivax* respectively, 7(3.5%) and 2(1%) presented as (+++) for *P.falciparum* and *P.vivax* respectively , 3(1.5%) and 1(0.5%) presented as (++++) for *P.falciparum* and *P.vivax* respectively.

Out of 24 positive cases, *P.falciparum* stages were 16(8%) trophozoite, 3(1.5%) gametocyte and 1(0.5%) were trophozoite and gametocyte. *P.vivax* stages were 3(1.5%) trophozoite and gametocyte and 1 (0.5%) were trophozoite, schizont and gametocyte. The difference between rates was found to be highly significance at p=0.000.

Out of 200 blood samples, 29(14.5%) were found to be positive by immunochromatographic test (ICT), while 1 case which was positive for *P.falciparum* by blood film was not detected by ICT. This false negative result is due to low parasitaemia. Out of 29 positive cases, 6(3%) were negative by blood film and positive by ICT for *P.falciparum*. This false positive result due to antigen (HRP-II) persistent in circulation after parasite clearance, while 23(11.5%) cases were detected by the above two methods. The result showed that, out of 200 blood samples, 24(12%) cases were positive by buffy coat concentration technique which is similar to results obtained by blood films.

The results showed that the sensitivity of blood films and buffy coat concentration technique was (100%), while ICT sensitivity was (96%). A similar study done by Binesh *et al.* (2011), showed sensitivity of (97.77%), (80.76) and (97.10%) for blood films, buffy coat concentration technique and ICT respectively. Sensitivity of blood films and buffy coat concentration technique was slightly higher in the present study, while ICT sensitivity was slightly lower in the present study.

The present study indicated that buffy coat concentration technique provides a reliable, quick, easily mastered method for diagnosis of malaria. The method is useful in laboratories in endemic areas where parasite level is low, so it concentrated the low number of parasites. The present study showed that the antigen detection test had lower accuracy than blood films and buffy coat concentration technique. These findings were similar to the findings obtained by Salmani and Peerapur (2011),

and the gold standard for laboratory diagnosis is microscopic examination of thick and thin blood films. This result was agreement with Binesh *et al.* (2011) study.

Chapter five
Conclusions and recommendations

5.1 Conclusions:

The present study concluded that**,** the prevalence rate of malaria was high in the study area. Blood films examination is the gold standard for malaria diagnosis. Buffy coat concentration technique method provided a reliable, quick, accurate method for diagnosis of malaria while ICT test showed less accuracy in malaria diagnosis. *P. falciparum* is the predominant species in the study area. Most affected age group was the 1-15 years age group.

5.2 Recommendations:

1- The gold standard for malaria diagnosis is Giemsa stained microscopy while ICT should be used as confirmatory test.

2- Buffy coat concentration technique could be the method of choice for laboratory diagnosis for malaria as an alternative to conventional microscopy.

3- Further studies should be done to evaluate the modified QBC technique by using fluorescence dye and fluorescence microscope for malaria parasite detection.

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Appendix

Sudan University of Science and Technology

College Of Graduate Studies

M.Sc. in Parasitology and Medical Entomology

Questionnaire form

Date: ………………..   ID: ………………

General information:

Name:………………………………………………………………………..

Gender:…………………………………………………………………

Age:……………………………………………………………………

Information Related To Malaria Infection:

Are You Suffering From:

Fever	Yes	☐	No	☐
Headache	Yes	☐	No	☐
Vomiting	Yes	☐	No	☐
Diarrhea	Yes	☐	No	☐
Are you infected with malaria before?	Yes	☐	No	☐

If Yes when you infected?

Before 1-30 day ☐   Before 31-60 day ☐   More than year ☐

Information Related to Treatment:

Are you taken any treatment before?   Yes ☐   No ☐

Which Type Of Treatment?

Artemether ☐   Artesunate ☐   Quinine ☐   Primaquine ☐

Laboratory Investigations:

Blood Films:

a-**Thick**   +Ve ☐   -Ve ☐

Density: + ☐   ++ ☐   +++ ☐   ++++ ☐

b-**Thin:**   Species………………………..   Stage……………………..

Buffy Coat Film: +Ve ☐   -Ve ☐

Species …………………………   Stage……………………**.**

ICT: +Ve ☐   **-**Ve ☐