

Chapter one

Introduction and literature review

Chapter one

Introduction and literature review

1.1 Overview

Malaria is caused by the protozoan parasite *Plasmodium* that is transmitted to humans via the bite of an infected female *Anopheles* mosquito. Four *Plasmodium* species can infect humans and cause disease; i.e. *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is the most pathogenic species and this parasite is highly prevalent on the African continent (WHO/UNICEF, 2005).

Malaria is one of the leading parasitic diseases in the world causing 200-300 million clinical cases and over 1 million deaths each year, especially in children under five years of age (Amexo, *et al.*, 2004).

In general, the disease burden is almost 50 million disability adjusted life years (WHO, 2004). Furthermore, malaria endemic countries are not only poorer than non-malarial countries, but they also have a lower economic growth rate; i.e. 1.3% lower in countries with intensive malaria transmission compared to countries without malaria (Sachs and Malaney, 2012).

Malaria has a large variety in clinical presentation and severity of the disease. The symptoms of a mild infection are general, with fever as the main characteristic and can be combined with symptoms like headache, myalgias, arthralgias, weakness, vomiting and diarrhea. Other clinical features include splenomegaly, anemia, thrombocytopenia, hypoglycemia and pulmonary or renal dysfunction. In severe infections, neurological damage, coma and even death can be caused by rosetting of the red blood cells, leading to impaired microvascular blood flow in the brain, and the

release of cytokines such as TNF - α which can in turn trigger the release of harmful substances such as nitric oxide that damage the brain (Reyburn, *et al.*, 2004; Guerra, *et al.*, 2006).

1.2 Malaria: life cycle of *Plasmodium falciparum*

P. falciparum is the most prevalent species and causes the highest number of casualties, therefore this chapter is focused on the description of the life cycle of this species of the *Plasmodium* genus (Fig1.1). The parasite is able to successfully infect humans as well as its vector, the female *Anopheles* mosquito. When a person is bitten by a an infected mosquito, sporozoites are injected into the blood stream. The sporozoites are transported to the liver where they invade the liver cells (exo-erythrocytic cycle). In the liver cells, the parasite multiplies and develops into a schizont. When the schizonts are mature, they rupture and the parasites are released into the blood stream again where they are able to invade red blood cells (erythrocytic cycle). In the red blood cells, maturation occurs and again, by forming schizonts and releasing the merozoites, the red blood cells are ruptured which gives rise to the clinical symptoms in the patient. This cycle is the so called asexual cycle of the parasite. The released merozoites can continue the asexual cycle by invading other red blood cells (Tuteja, 2007; CDC, 2015).

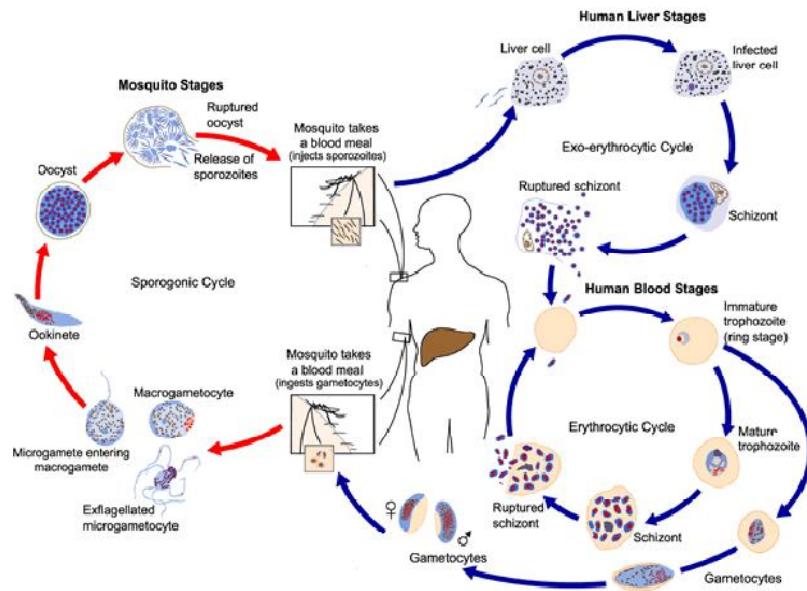


Figure (1.1): Lifecycle of Malaria Parasite (Source: CDC, 2015)

1.3 *Plasmodium* species:

1.3.1 *Plasmodium falciparum*:

It causes *falciparum malaria* (tropical malaria, malaria tropica, sub-tertian malaria, malignant tertian malaria), which is the most severe form of *malaria* (Loban and Pozolok; 1983). It is characterized by cycles of fever occurring every 36-48 hours. *Plasmodium falciparum* is the dominant species throughout most of the tropics and is responsible for 80-90% of *malaria* infections in these areas. The liver stage of this species in man lasts for 8-10 days, and there is thought to be only one population of parasites developing at any one time. The fully developed liver schizont contains about 40,000 merozoites, considerably more than in the other species. The cycle of infection in the erythrocytes is more rapid than in other species. The increase in numbers of parasites in this species is therefore, much more rapid than in the others, and this together with the ability of the parasite to attack red blood cells (RBCs) of all ages, gives rise to a rapid deterioration in the clinical condition in the patient,

hence the term "malignant tertian" got formed. Parasitaemia is often heavy, 20-40% of erythrocytes may be infected, and the sudden destruction of this proportion of RBCs has serious consequences for the patient. Although erythrocyte schizonts occur in *Plasmodium falciparum*, the great majority of these forms, particularly when parasitaemia is relatively low, are in the blood vessels of the deeper tissues and are not seen in peripheral blood. Schizonts are normally seen only in blood films from moribund patients (Abushama, 2004).

1.3.2 *Plasmodium vivax*:

Causes benign tertian malaria; in which the cycle of fever recurs every 48 hours. It is the second most important species and occurs over a much wider geographical area than *Plasmodium falciparum*, from the tropics and subtropics to some temperate areas of the world. The erythrocytic stages are morphologically distinct from those of *Plasmodium falciparum*, and the liver and erythrocyte schizonts contain fewer merozoites, (Abushama, 2004). Older erythrocytes are preferentially infected, in contrast to *Plasmodium falciparum* infection, and the percentage of cells infected may be much lower than in that condition. *Vivax* malaria is less severe than *falciparum* infection in the non-immune patient, hence the term "benign tertian malaria". Relapses of parasitaemia and fever are frequently seen in *vivax* infection. Infection is maintained for months and years because there are two or more populations of parasites in the liver cells. One population developing as the typical liver schizonts and the other (the hypnozoites) persisting for some time as small non-developing uninucleate parasites, giving rise to relapses, (Abushama, 2004). *Vivax* can induce clinical infection after 10-21 Days or a prolonged 6-14 Months incubation period, (Loban and Polozok; 1983). *Vivax* invades selectively young RBCs and the number of infected

erythrocytes does not exceed 2% usually being under 1% (Loban and Polozok; 1983).

1.4 Diagnoses of malaria:

Malaria is a global health problem and although it mainly affects the poorer regions of the world, the disease can be encountered in the developed world as well, mainly as so called imported or travel malaria (Gjorup, *et al.*, 2007). This fact makes the subject of good diagnosis a worldwide issue. The capabilities of proper diagnosing are however not universally distributed. When discussing diagnostic methods and its advantages and drawbacks, it is important to keep this in mind since the applicability of diagnostic methods largely depends on the resources available in a certain place (Hawkes and Kain, 2007).

Malaria in the developed world is not endemic anymore and therefore diagnosis possesses its own problems. Laboratories are well equipped but do not encounter the disease regularly which could lead to not recognizing the *Plasmodium* parasite in a clinical sample (Warre and Gilles, 2012). In the developing world, the disease is encountered very frequently but resources are often lacking (Rafael, *et al.*, 2006).

1.4.1 Clinical diagnosis:

In many parts of the world, the diagnosis malaria and the subsequent treatment will be made without a laboratory test and the physician will often rely on the clinical symptoms of the patient (Othnique, *et al.*, 2006). Although malaria has several characteristic features such as intermittent fevers and typical symptoms, presumptive diagnosis is very unspecific and often confused with other diseases like respiratory tract infections or

typhoid fever. In areas of high endemicity, fever in children is often regarded and treated as malaria (Schellenberg, *et al.*, 2006).

Although in some areas and during high transmission seasons this may be the case for a large proportion of children, this also means that in a large proportion of patients other diseases causing a fever are not treated as such and unnecessary anti-malarial drugs are given instead (Reyburn, *et al.*, 2004). In the light of wide spread drug resistance against affordable drugs and changing of drug policies towards expensive ACTs the importance of laboratory confirmed diagnosis is evident.

1.4.2 Microscopy:

Microscopy has been used since the time of Laveran to diagnose malaria in the blood of an infected patient. The method relies on the microscopic identification and morphological determination of the parasite, usually Giemsa or Leishman stained, thin and thick films (Makler,*et al.* ,2008).

The method has several advantages above other methods such as the ability to differentiate between the different species, that is important in determining the treatment of a patient, and differentiation between asexual and gametocyte stages which also has consequences for the treatment. In many areas, patients are gametocyte carriers but do not harbour asexual parasites and are therefore often not treated (Alves, *et al.*,2005). Another advantage of microscopy is the ability to quantify the parasitaemia, which is an important indicator for the clinical outcome (Gjorup, *et al.*,2007). In addition, microscopy allows for an easy identification of blood abnormalities (Hawkes and Kain, 2007). However, this method has its disadvantages as well. Microscopy can be very sensitive but under normal field conditions an expert microscopist can only reach a sensitivity of 100 parasites/ μ l blood in a thin smear and 40-50 parasites/ μ l blood in a thick film (WarrelandGilles, 2012).

Other obstacles are maintenance of microscopes, electricity and the relatively long processing, staining and reading time that is required. To overcome some of these obstacles, methods such as Field's stain, that give very good quality slides and are much faster to stain, have been developed (Hawkes and Kain, 2007).

Adjustments of microscopes towards battery powered systems have enabled microscopy diagnosis to be performed where no electricity is available (Jones, *et al.*, 2007). To circumvent the need of highly trained and experienced laboratory personnel staining with acridine orange has been introduced which stains the parasites that are easily recognized under a fluorescent microscope (Adeoye and Nga, 2007).

Quantitative Buffy Coat (QBC) is also based on acridine orange staining in a microcentrifuge tube. The parasites can be easily seen under an ultraviolet light. Albeit easy to perform, these methods are very expensive and require additional equipment to the traditional microscope (Guy, *et al.*, 2007). Furthermore, QBC is, under field conditions, just as sensitive as conventional microscopy but species identification and quantification is not possible with this technique making Giemsa stain the optimal method for microscopy diagnosis (Adeoye and Nga, 2007).

1.4.3 Rapid diagnostic tests:

In recent years, a variety of rapid diagnostic tests (RDTs) has been developed to overcome the limitations of microscopy. These tests are fast, easy to perform and do not require electricity or specific equipment and cost currently, depending on the manufacturer and/or supplier, around Euro 2.0/test (Hawkes and Kain, 2007). RDT's are immunochromatographic lateral flow assays and are based on the recognition of *Plasmodium* antigens circulating in the blood of the patient (Cruciani, *et al.*, 2004).

Few targets have been used in commercialized RDT's, i.e. parasite specific aldolase, parasite lactate dehydrogenase (pLDH) and histidine rich protein-2 (HRP-2) with the latter two most frequently used (Craig, *et al.*, 2002). HRP-2 is a water soluble antigen that is present during the whole erythrocytic cycle of the parasite (Dondorp, *et al.*, 2005). It is a very specific antigen but has a drawback that the antigen persists for at least a week after treatment making follow-up monitoring and recognition of resistant parasites difficult (Cruciani, *et al.*, 2004). In contrast, pLDH, a metabolic enzyme that is actively produced during the growth of the parasite in RBCs, is cleared rapidly after the patient is successfully treated and is used in several RDT's (Craig, *et al.*, 2002). The pLDH tests are less sensitive than the HRP-2 based tests (Iqbal, *et al.* 2003).

In a study performed in Uganda, the sensitivity of HRP-2 based tests was 92% whereas LDH based tests had a sensitivity of 85%. These differences were mainly due to the ability of HRP-2 tests to detect lower parasite densities. On the other hand HRP-2 based tests are prone to give false positive signals in patients with rheumatoid factors, and patients that recently have cleared a *Plasmodium* infection (Hopkins, *et al.*, 2007). Several studies including the study in Uganda (Hopkins, *et al.*, 2007) found 98-100% specificity for LDH-based tests and 90-93% specificity for HRP-2 based tests. This lower specificity is primarily due to the persistence of antigens after parasite clearance. In general, RDTs can detect around 100 parasites/ μ l but at lower parasitaemia their sensitivity decreases, making these tests unsuitable for patients with low numbers of parasites (Rubio, *et al.*, 2001). Another drawback is the lack of stability of the tests under tropical conditions and their inability to discriminate between the different species of *Plasmodium* although some recently

developed tests are able to distinguish between *P. falciparum* and non-*falciparum* infections (vandenBroek, *et al.*, 2006).

Despite their shortcomings, in areas where microscopy is unavailable or in situations where it is difficult to perform microscopic slide examinations, such as emergencies or during the night, RDTs can be very useful (Hashizume, *et al.*, 2006).

1.4.4 Molecular methods:

The application of molecular techniques circumvents the limitations of conventional malaria diagnosis. Polymerase chain reaction (PCR) based assays are sensitive and can be converted to a quantitative format if SYBR green or molecular probes (e.g. a Taqman probe or a molecular beacon) are used in real time assays (Perandin, *et al.*, 2004). Other applications, such as the identification of drug resistant strains (Wernsdorfer and Meshnick, 2002), make these techniques very suitable for epidemiological and vaccine studies as well (Felger, *et al.* 2003; Andrews, *et al.* 2005). However, molecular techniques are not routinely implemented in developing countries because of the complexity of these tests and the lack of resources to perform these tests adequately and on a routine basis (Whitcombe, *et al.*, 2008).

Major obstacles are the need for continuous supply of electricity and complex apparatus like PCR machines. Furthermore, the analysis of the end product (amplicons) involves the handling of labour intensive read-out systems such as electrophoresis systems that use very toxic ethidium bromide stained gels and hazardous UV light transilluminators. These read-out systems are expensive and require well organised laboratories. Nevertheless, many see the potential of these highly sensitive techniques and therefore possibilities to overcome the above mentioned limitations are explored (VanNess, *et al.*, 2003).

Isothermal reactions such as Loop-mediated isothermal amplification (LAMP), exponential amplification reaction (EXPAR), or Nucleic Acid Sequence Based Amplification (NASBA) circumvent the use of expensive and maintenance dependent thermocyclers and might be an alternative for PCR methods(Mekata , *et al.*, 2006).

1.4.5 Nucleic acid sequence based amplification:

Nucleic acid sequence based amplification (NASBA) is a technology which uses the activity of three enzymes (AMV-RT, RNase H and T7 RNA polymerase) for the isothermal amplification of RNA molecules (Whitcombe, *et al.*, 2008). The low reaction temperature (41 0C) and the addition of a T7 polymerase sequence on one of the added primers ensures the amplification of only single stranded RNA (Whitcombe, *et al.*, 2008).

The reaction does not require a DNA denaturing step hereby preventing amplification of genomic DNA in case of contamination. Therefore, NASBA can be performed in a background of DNA in a sample and, in addition, allows easy detection of stage specific expressed genes (e.g. Pfs25 for the specific detection of the gametocyte stage of *P. falciparum*) (Schneider, *et al.* 2004). Moreover, with some technical adaptations and appropriate controls, NASBA can be used in a quantitative format to determine the number of infectious agents in a clinical sample (Schneider, *et al.*,2005).

The technique has been successfully applied for the detection and quantification of several infectious agents such as HIV-1 (Ayele, *et al.*,2005), Hepatitis viruses, respiratory syncytial virus, *Leishmania* spp, and dengue (Hutamai, *et al.*, 2007). NASBA has also proven its value in several areas of malaria research, because this highly specific and

sensitive technique also allows for quantification of very low parasite densities (Schneider, *et al.*, 2004).

These properties make NASBA a very effective tool for epidemiological studies, monitoring of drug resistance and the analysis of parasite dynamics even at sub-microscopical level (Schneider, *et al.*, 2006).

Furthermore, quantitative NASBA has proven to be very well suited to monitor treatment efficacy in malaria as well as other parasitic diseases, such as leishmaniasis (deVries, *et al.*, 2006). These assays have shown to be even more sensitive than other molecular techniques such as PCR or RT-PCR (Schneider, *et al.*, 2005). Quantification of *P.falciparum* in a sample using 18S ribosomal RNA (18S rRNA) as a target can still be achieved with as little as 10 parasites/ ml of blood (Schoone, *et al.*, 2010).

1.4.6 Nucleic acid lateral flow immuno assay:

Nucleic acid lateral flow immuno assay (NALFIA) is a simple test format that can be used for the visualization of nucleic acids after amplification. This simple read out system has been successfully applied for the detection of food borne pathogens such as *Bacillus cereus* and *Salmonella* (Amerongen and Koets, 2005).

This assay combines the lateral flow assay, that is widely known for its serological applications like the above mentioned RDT's, with the detection of labeled nucleic acid-amplification-products on a nitrocellulose stick. The nitrocellulose is coated with specific antibodies that capture the amplicons which are labeled with specific primers that contain a biotin molecule and a hapten. This complex is detected by direct hybridization with a colloidal, avidine labeled carbon particle and shows a product line if the sample is containing the product. The combination

of the proven successful methods of lateral flows assays and molecular tools can overcome the need for expensive or laborious read out systems when performing assays such as PCR (Bogdanovic, *et al.*, 2006).

1.5 Malaria in Sudan:

According to National Malaria Control Programme, (2010) and based on climate models, it is estimated that 75% of the population (37 millions) are at risk of endemic malaria, while 25% are at risk of epidemic malaria. Most of the country below north latitude 15° is endemic zone with relatively high transmission in southern states, while parts of the north are exposed to epidemics following the heavy rains or floods from River Nile. Transmission of malaria in north Sudan south to Khartoum is seasonal and depends on rainfall except in urban cities and irrigated schemes. Sudan's rainy season lasts for about three months (July to September) in the north, and up to six months (June to November) in the south. Hence, the duration of transmission varies from 3-6 months with an average of 4 months, while a longer season is noticed in the southern areas. The transmission season may last from July/August to November/December, with an earlier beginning in June in the southern areas (e.g., Kadugli, El Damazin) and later start in August in northern areas (Wad Medai, Kosti, Kassala, El Obeid). Longer transmission up to 9 months takes place in certain agriculture schemes areas, while the urban cities may have another transmission during winter (December- February) due to broken water pipes; a clear cut example of manmade malaria *Plasmodium falciparum* is responsible for more than 95% of malaria cases in Sudan. However, an increase in *P. Vivax* cases has been noticed in the last years.

In 2011, in comparison with 2009, incidence of reported confirmed cases has decreased by 36%, and reported number of deaths by 46%. Reported

total (probable and confirmed) malaria incidence has decreased by 71% in 2011 in comparison to 2000. The incidence of inpatient cases decreased by 25% in 2011 in comparison with 2000 and the proportion of inpatient malaria cases to all cause cases decreased from 26% to 9%. Parasite prevalence at national level was 1.8% in 2009 and 3.3% in 2012. Gedaref and south and west Darfur states were the main contributors to the rise in the parasite prevalence. The objective of the national strategy in areas targeting for malaria free status is to have reported malaria incidence, with 100% laboratory confirmation and reduction of confirmed incidence by at least 80% as compared to 2009 and will reach the level of 10 cases per 1000 in Northern, Red Sea, River Nile, Gezira and White Nile states. *Plasmodium falciparum* is responsible for more than 95% of malaria cases in Sudan. However, an increase in *P. vivax* cases has been noticed in the last years. The primary vector is *Anopheles arabiensis* and is widely distributed in Sudan although has been reported in southern parts of Sudan (White Nile state) (National Malaria Control Programme, 2013).

Rationale:

Plasmodium vivax accounts for the majority of malaria with approximately 80-100million cases annual and more people area risk from infection with *P.vivax* than with *P.falciparum*.

Malaria in Sudan is a major health problem. It leads to 7.5 million cases and 35,000 deaths every year.

Malaria has serious consequences in pregnancy and childhood. Besides that, there are other causes of fever such as tonsillitis, chest infection, measles, abscess, typhoid, urinary tract infection, etc...

Hence, this study aims to study prevalence of *plasmodium vivax* and *P.falciparum* in Ed babeker Shergalnile locality.

Objectives:**General objective:**

To study the prevalence of *Plasmodium vivax* and *Plasmodiumfalciparum* in Ed babeker in Shergalnile locality in Khartoum Stateusing blood film and PCR technique.

Specific objectives:

- To estimate the prevalence rate of *Plasmodium vivax* and *Plasmodium falciparum* in Ed babeker in Shergalnile locality according to gender and age.
- To compare the efficiency of blood film (Bf) and polymerase chain reaction (PCR) as methods for diagnosing *Plasmodium* species in blood.

Chapter two

Material and methods

Chapter two

Material and methods

2.1 Study design:

It is a descriptive cross-sectional study

2.2 Study area :

The study was conducted in Ed babeker health center which is located in shergnile locality in Khartoum stateof Sudan country which is stretching from east bank of the Blue Nile to the east bank of the Nile River ordered to the west of the blue Nile and Nile river, north of the river Nile state East of the Gadaref, kassala states and south Aljazeera state.

2.3 study population :

This study was conducted on all outpatient attending the target primary health center in Ed babeker . The population was categorized according to gender and age groups (10-20,21-30,31-40,41-50, and more than 50)

2.4 Study duration :

The study commenced on March 2016 and ended on September 2016.

2.5 Sample size :

The study was carried out on 100 suspected individuals .

2.6Data collection:

A questionnaire was designed for data collection (appendix).

2.7 Ethical considerations:

The patients were informed and consent was obtained before sample collection.

2.8 methodology :

2.8.1Collection of blood :

Peripheral blood was collected for applying BF (blood film) and 1-3 drop of it had been obtained for PCR (polymerase chain reaction) on the Whitman type of filter paper .

2.8.2 Thick and thin blood films :

2.8.2.1 Thick blood film :

The slide was labeled, small drop of blood was placed on one end of pre cleaned new slide. By using the corner of another slide, the drop was spread in a circular pattern to reach the size of 1.5 cm diameter and the smear was allowed to air dry.

2.8.2.2 Thin blood film:

small drop of blood was placed in the same slide of thick blood film and smear was made, with cover glass or another slide with angle 35_40 ,and the thin blood film was fixed by using absolute ethanol .

2.8.2.3Staining procedure and quality control:

2.8.2.4 Working Giemsa stain (10%)

Each slide was covered with working giemsa stain in staining rack for 10 minutes. Distilled water was dropped on the slide to avoid staining deposits and the slide was left to air dry.

2.8.2.5Examination of blood film:

Thick blood film was examined to detect the parasite.

Thin blood film was examined to identify species of malaria by using 100x objective .

2.8.3 PCR procedure and technique:

2.8.3.1 DNA extraction

Solutions required	Equipment required
5M NaOH	Sterile scalpel and blades
5M HCl	Small piece of glass plate
1 x PBS (autoclaved)	Beakers, tissues, forceps
1 x PBS/0.5% saponin (autoclaved)	1.5ml and 0.5ml microfuge tubes (sterile)
20% Chelex-100 solution in 1 x PBS (autoclaved)	Waterbath at 100°C (or PCR machine or heated block)
Water (autoclaved)	Microfuge, vortex mixer

- The scalpel, glass and forceps were cleaned before beginning and between each sample by dipping or wiping with 5M HCl, followed by neutralisation with 5M NaOH, and a final wash with distilled water, wiping between each treatment with tissue
- The area of filter paper with the blood spot (**figure 2**) was excised using a scalpel on a piece of glass. The piece of filter paper was transfer to a sterile 1.5ml microfuge tube using the forceps.
- 1ml 0.5% saponin was added in 1 x PBS (sterile: autoclaved solution) to each tube. Invert each tube several times to mix and place at 4°C overnight.
- The brown solution (**figure 3**) was removed and replace with 1ml of PBS (sterile: autoclaved). Invert the tube again, and then place at 4°C for 15-30 minutes.
- The PBS buffer was removed. 200µl of stock 5% chelex-100 solution (sterile: autoclaved) was added to the filter paper.
- Heated to 100°C in a PCR machine (**figure 4**) or a waterbath or heated block.

- Vortex vigorously for 30 seconds, and then replace in the PCR machine (etc) at 100°C for a further 10 minutes, it was vortex again once during the incubation and once afterwards.
- The tube was spun at 10000g for 2 minutes and the supernatant was removed to a fresh tube. This tube was spun for a further 2 minutes at 10 000g, and then transfer the supernatant to another fresh tube. Stored at -20°C (short term) or at -70°C (long term).

Figure 2: Spot of blood on whiteman filter paper type 3



Figure 3: Brown solution after incubation over night



2.8.3.2 Outer PCR for amplification of 18ssrRNA:

Table (1) Master mix preparation for one sample of 25 μ l

Reagents	1	6	10	20	25	30	40	45	60	65
H ₂ O	15.2	91.2	152	304	380	456	608	684	912	988
buffer	2.5	15	25	50	62.5	75	100	112.5	150	162.5
dNTPs	0.5	3	5	10	12.5	15	20	22.5	30	32.5
Plus5	0.75	4.5	7.5	15	18.75	22.5	30	33.75	45	48.75
Plus6	0.75	4.5	7.5	15	18.75	22.5	30	33.75	45	48.75
Taq	0.3	1.8	3.0	6	7.5	9	12	13.5	18	19.5

Method:

1. 5 μ l of DNA was added to 20 μ l of master mix in to labeled PCR tubes (working was on ice).
2. The tubes were loaded in to thermocycler of the following temperature profile :

Table (2) Thermocycler temperature profile

Step	Denaturation	Annealing	Extention	Cycle
Outer step <i>(p.falciparum ,p.vivax)</i>	94°C for 1 min	60°C for 2 min	72°C for 1 min 30s	30

2.8.3.3 Multiplex nested PCR for amplification of *P.falciparum* and *P.vivax*:

Table (3) Master mix preparation for one sample of 25 μ l

Reagents	1	6	10	20	25	30	40	45	60	65
H ₂ O	19.2	115.2	192	384	480	576	768	864	1152	1248
Buffer	2.5	15	25	50	62.5	75	100	112.5	150	162.5
dNTPs	0.5	3	5	10	12.5	15	20	22.5	30	32.5
FAL-1	0.75	4.5	7.5	15	18.75	22.5	30	33.75	45	48.75
Fal-2	0.75	4.5	7.5	15	18.75	22.5	30	33.75	45	48.75
Taq	0.3	1.8	3	6	7.5	9	12	13.5	18	19.5

Methods

- 1 μ l outer PCR products was added to 24 μ l master mix in to the labeled PCR tube .
2. The tubes were loaded into the thermocycler of the following temperature profile :

Table (4) Thermocycler temperature profile for nested PCR:

Step	Denaturation	Annealing	Extension	Cycle
Nested step (<i>p.falciparum</i> ,<i>p.vivax</i>)	94°C for 1 min	55°C for 2 min	72°C for 1 min 30s	30

Table (5): The sequence of *P.falciparum* and *P.vivax* primer using in PCR:

Species	Primer	Sequence (5-3)	Size (bp) of PCR product
<i>Plasmodium</i> sp.	rPLU5 rPLU6	CCTGTTGTTGCCTTAAACTTC TTAAAATTGTTGCAGTTAAAACG	1,100
<i>P. falciparum</i>	rFAL1	TTAAACTGGTTGGGAAAACC AAATATATT	205
	rFAL2	ACACAATGAACTCAATCATGA CTACCCGTC	
<i>P. vivax</i>	rVIV1 rVIV2	CGCTTCTAGCTTAATCCACAT AACTGATAC ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA	120

Figure(4): The PCR machine



2.8.3.4 Electrophoresis and result interpretation :

DNA already has uniform negative charge, two nucleotides are connected together by a phosphodiester bond which gives one negative charge . When placed in an electric field in a processes called electrophoresis , negatively charged DNA molecules were migrate toward the positive electrode in a semisolid matrix like an agarose gell . The migration of the molecules in gel electrophoresis is directly proportional to the size of the molecule. The gel sieves the movement of the molecules based on their size. Small molecules migrate faster and then bigger ones as small molecules can move more easily through gel pore.

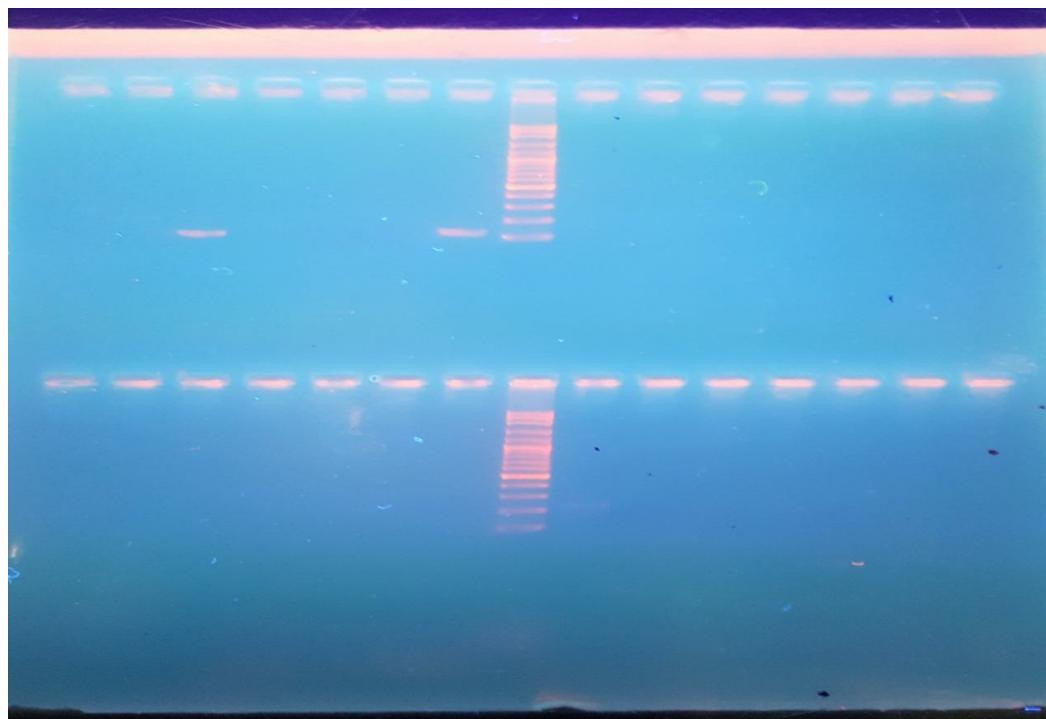
Method :

- Agarose gels are prepared as percentage weight/volume solutions. prepare standard 1.5% in TBE buffer, boiled in microwave oven and 3 μ l ethidium bromide (10mg /ml)was added to this solution at 60°C .
- The casting tray was filled by agarose solution after inserting of the combs .
- Combs was removed from the casting tray after the cooled of gel.
- Agarose was transferred to electrophoresis apparatus and cover with TBE buffer .
- Molecular weight marker called (DNA ladder) was added in to the meddle well.
- μ lloading dye was added to 10 μ l of DNA product , well mix and loaded to the well.
- After the well were filled with sample ,the power supply was connected .
- The power supply was disconnected after one hour.
- Agarose was transferred to visualize under UV to read the positive result in band forms (**figure 5**).

2.9Data analysis :

Data were analyzed by using Statistical Package for the Social Sciences(SPSS) program . Then data were presented in tables.

Figure 5: positive result in band forms.



CHAPTER THREE

RESULTS

Chapter three

Results

The results showed that out of the 100 patients in Ed babekir in Sharg Elneel locality, 27 were found infected by *Plasmodium spp*, this constituted an overall infection rate of 27%. The prevalence of *P.falciparum* was found to be 17%, *P.vivax*, 7% and mixed infection, 3% (table 6).

The results also showed that out of 51 males, 10 (19.6%) were infected with *P.falciparum*, 5 (9.8%) were infected with *P.vivax* and 1 (2%) was infected with the two species, while out of 49 females, 7 (14.3%) were infected with *P.falciparum*, 2 (4.1%) were infected with *P.vivax* and 2 (4.1%) were infected with the two species, this difference in rates was found to be statistically insignificant at p.value= 0.526 (table 7).

The highest prevalence rate of *P.falciparum* was found among 21-30 age group (25.9%), while the lowest prevalence rate was found among 41-50 (12.5%). The highest prevalence rate of *P.vivax* was found among the more than 50 age group (20%), while the lowest prevalence rate was found among 21-30 age group (0%) and highest prevalence rate of mixed species was found among 41-50 (6.3%), while the lowest prevalence rate was found among the 31-40 and more than 50 age group (0%), this difference in rates was found to be statistically insignificant at p.value= 0.537 (table 8).

When using blood films and polymerase chain reaction for all samples, polymerase chain reaction proved to detect higher rate of *Plasmodium spp* encountered. The highest rate found for *P.falciparum* (17%), followed by *P.vivax* (7%) and 3% for mixed species, while when using blood films. The highest rate found for *P.falciparum* 12%, followed by *P.vivax* 3% and 2% for mixed species, This difference in rates was found to be statistically highly significant at p.value= 0.000 (table 9).

Table 6: The overall prevalence of *Plasmodium spp* among the study population:

<i>Plasmodium spp</i>	Number examined	Number positive	Prevalence
<i>P.falciparum</i>	100	17	17%
<i>P.vivax</i>	100	7	7%
Mixed	100	3	3%
Total	100	27	27%

Table 7: Prevalence of *Plasmodium spp* according to gender:

Gender	Number examined	<i>P.falciparum</i> (Prevalence)	<i>P.vivax</i> (Prevalence)	Mixed (Prevalence)
Male	51	10 (19.6%)	5 (9.8%)	1 (2%)
Female	49	7 (14.3%)	2 (4.1%)	2 (4.1%)
Total	100	17 (17%)	7 (7%)	3 (3%)

p.value= 0.526

Table 8: Prevalence of *Plasmodium spp* according to age groups:

Age groups (Years)	Number examined	<i>P.falciparum</i> (Prevalence)	<i>P.vivax</i> (Prevalence)	Mixed (Prevalence)
10 - 20	30	4 (13.3%)	1 (3.3%)	1 (3.3%)
21 – 30	27	7 (25.9%)	0 (0%)	1 (3.7%)
31 – 40	22	3 (13.6%)	2 (7.4%)	0 (0%)
41 – 50	16	2 (12.5%)	3 (18.6%)	1 (6.3%)
More than 50	5	1 (20%)	1 (20%)	0 (0%)
Total	100	17 (17%)	7 (7%)	3 (3%)

p.value= 0.537

Table 9: Comparison between blood films and polymerase chain reactions among all populations:

<i>Plasmodium spp</i>	Number examined	BF (+ve/ rate)	PCR (+ve/ rate)
<i>P.falciparum</i>	100	12 (12%)	17 (17%)
<i>P.vivax</i>	100	3 (3%)	7 (7%)
Mixed	100	2 (2%)	3 (3%)
Total	100	17 (17%)	27 (27%)

p.value= 0.000

Chapter four

Discussion

Chapter four

Discussion

Plasmodium vivax has the widest geographic distribution among the human malaria parasites in Sudan, *P.falciparum* has a dominant distribution specially in Khartoum state. Recently *P. vivax* appears with wide distribution and its control in individuals and populations is complicated due to its ability to relapse weeks to months after initial infection (Barcus. *et al.*2007).

In this study, the prevalence rate reported was 27%. This result was less than the result of a survey, which was carried out during the transmission season in north Sudan by Omer (1978). The study revealed that, there was no statistically significant difference between gender and infection with malaria, as both genders are almost equally exposed to infection.

Also in this study, there was no statistically significant difference between age groups and infection with malaria. This finding was different from other findings obtained by Munyekeny *et al.* (2005) who found that the age group 1-9 years was the most infected with malaria in western Kenya.

P.falciparum was the more common species in Shergalnile locality followed by *P. vivax* and there was statistically significant difference between them, This results is in agreement with the result obtained by Zeinab (2013) in Dardoug area. She found that the prevalence of *P.falciparum* was more than *P. vivax* and also there was a mixed infection.

Also in another study done by Ahmad (2010), *P. falciparum* was more common species in al Dewaim area.

The present study is also in agreement with those of Omer (1978) who showed that *P.falciparum* is the predominant species in north and south sudan.

Microscopic results were initially considered as the reference standards for true positive and true negative results. Many studies have demonstrated the greater sensitivity and specificity of PCR compared to thick blood films (Zakeriet *al.* 2002).

In this study there was statistically significant difference between PCR and microscopy, 27 samples out of 100 sample were positive by PCR, (*P.falciparum* 17(17%), *P. vivax* 7(7%) and 3(3%) mixed species, while 17 samples out of 100 were positive by BF 12(12%) *P.falciparum*, 3 (3% *P. vivax*), 2 (2%) mixed species. From these results, can concluded that PCR is moer accurate than BF. Finding from their result is in agreement with those of Ahmad (2010), who showed that the PCR is more sensitive than BF in the diagnosis of malaria parasites.

Chapter five

Conclusion and Recommendation

Chapter five

Conclusion and Recommendation

5.1 Conclusion:

*The prevalence of *P.vivax* in Khartoum state especially in Shergalnile is increasing. However *P.falciparum* is still considered the more common malaria parasite in the area .*

Although the PCR was the more accurate method for malaria diagnosis, the BF is still the gold standard test because it is cheaper, easy, not time consuming and available than PCR .

5.2 Recommendation:

- Despite the fact that *P. falciparum* was the more common species in Shergalnile locality, *P.vivax* should be put into consideration as its spread is increasing now a days .
- *P. vivax* should be diagnosed and treated correctly.
- More epidemiological studies should be done to precisely estimate the prevalence of *P.falciparum* and *P. vivax* in al-Khartoum state.

Reference

References

Abushama, F., Tag El-Din, (2004). Flies, Mosquitoes and Disease in the Sudan. Published by Khartoum University .(2004).

Adeoye, G.O. and Nga, I.C, (2007). Comparison of Quantitative Buffy Coat technique (QBC) with Giemsa -stained thick film (GTF) for diagnosis of malaria. *Parasitol Int.* **56**:308-12.

Ahmad, A. (2010). *El Dueim, prevalence of malaria in Ed Dueim*, M.Sc. thesis, Alazhari University.

Alves, F.P., Gil, L.H., Marrelli, M.T., Ribolla, P.E., Camargo, E.P., D.a and Silva, L. (2005). Asymptomatic carriers of *Plasmodium spp.* as infection source for malaria vector mosquitoes in the Brazilian Amazon. *J Med Entomol.* **42**:777-9.

Amerongen van A, Koets M. (2005). Simple and rapid bacterial protein and DNA diagnostic methods based on signal generation with colloidal carbon nanoparticles. In: Rapid methods for biological and chemical contaminants in food and feed. Eds. A. van Amerongen, D. Barug and M. Lauwaars, Wageningen Academic Publishers, ISBN. **90**:105-126.

Amexo, M. Tolhurst, R. Barnish, and G. Bates I. (2004). Malaria misdiagnosis: effects on the poor and vulnerable. *Lancet.* **364**:1896-8.

AndrewsL.,AndersenR.F.,WebsterD.,DunachieS.,Walther,R.M.,Bejon,P.,Hunt-Cooke,A.,BergsonG.,SandersonF.,HillA.V. , and GilbertS.C. (2005). Quantitative real-time polymerase chain reaction for malaria diagnosis and its use in malaria vaccine clinical trials. *Am J Trop Med Hyg.* **73**:191-8.

Ayele,W.,Baar,M.P.,Goudsmit,J.,Kliphuis,A.,Tilahun,T.,Dorigo-Zetsma,W.,Wolday,D.,Abebe,A.,Mengistu,Y.,and Pollakis,G.(2005). Surveillance technology for HIV-1 subtype C in Ethiopia: an env-based NASBA molecular beacon assay to discriminate between subcluster C and C'. *J Virol Methods.* **130**:22-9.

Bogdanovic,J.,Koets,M.,Sander,I.,Wouters,I.,Meijster,T.,Heederik,D.,van Amerongen,A.,and Doekes,G.(2006). Rapid detection of fungal alpha-amylase in the work environment with a lateral flow immunoassay. *J Allergy Clin Immunol.* **118**:1157-63.

Barcus, M.J., Demographic risk factors for severe and fatal vivax and falciparum malaria among hospital admissions in northeastern Indonesian Papua. *The American journal of tropical medicine and hygiene*, (2007). **77(5)**: p. 984-991.

CDC (2015). Lifecycle of Malaria Parasite, available in <http://www.dpd.cdc.gov/>, accessed May 1st 2016.

Craig,M.H.,Bredenkamp,B.L.,Williams,C.H.,Rossouw,E.J.,Kelly,V.J ..Kleinschmidt,I.,Martineau,A.,and Henry,G.F.(2002). Field and

laboratory comparative evaluation of ten rapid malaria diagnostic tests. *Trans R Soc Trop Med Hyg.* **96**:258-65.

Cruciani,M.,Nardi,S.,Malena,M.,Bosco,O.,Serpelloni,G.,and Mengoli,C. (2004). Systematic review of the accuracy of the ParaSight-F test in the diagnosis of Plasmodium falciparum malaria. *Med Sci Monit.* **10**:MT81-8.

DeVries,P.J.,vanderMeide,W.F.,Godfried,M.H.,Schallig,H.D.,Dinant,H.J.,and Faber,W.R.(2006).

Quantification of the response to miltefosine treatment for visceral leishmaniasis by QT-NASBA. *Trans R Soc Trop Med Hyg.* **100**:1183-6.

Dondorp,A.M.,Desakorn,V.,Pongtavornpinyo,W.,Sahassananda,D.,Silamut,K.,Chotivanich,K.,Newton,P.N.,Pitisuttithum, P.,Smithyman,A. M.,White,N.J.,and Day,N.P.(2005).

Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med.* **2**:e204.

Felger,I.,Genton,B.,Smith,T.,Tanner,M.,and Beck,H.P.(2003).

Molecular monitoring in malaria vaccine trials. *Trends Parasitol.* **19**:60-3.

Gjorup,I.E.,Vestergaard,L.S.,Moller,K.,Ronn,A.M.,and Bygbjerg,I.C . (2007).

Laboratory indicators of the diagnosis and course of imported malaria. *Scand J Infect Dis.* **39**:707-13.

Guerra, C.A.,Snow,R.W.,and Hay, S.I.(2006).

Mapping the global extent of malaria in 2005. *Trends Parasitol.* **22**:353-8.

Guy, R., Liu, P., Pennefather, P., and Crandall, I. (2007). The use of fluorescence enhancement to improve the microscopic diagnosis of falciparum malaria. *Malar J.* **6**:89.

Hashizume, M., Kondo, H., Murakami, T., Kodama, M., Nakahara, S., Lucas, M.E., and Wakai, S. (2006).

Use of rapid diagnostic tests for malaria in an emergency situation after the flood disaster in Mozambique. *Public Health.* **120**:444-7.

Hawkes, M., Kain, K.C. (2007).

Advances in malaria diagnosis. *Expert Rev Anti Infect Ther.* **5**:485-95.

Hopkins, H., Kambale, W., Kamya, M.R., Staedke, S.G., Dorsey, G., and Rosenthal, P.J. (2007). Comparison of HRP2- and pLDH-based rapid diagnostic tests for malaria with longitudinal follow-up in Kampala, Uganda. *Am J Trop Med Hyg.* **76**:1092-7.

Hutamai, S., Suwonkerd, W., Suwannchote, N., Somboon, P., and Prapanthadara, L.A. (2007). A survey of dengue viral infection in *Aedes aegypti* and *Aedes albopictus* from re-epidemic areas in the north of Thailand using nucleic acid sequence based amplification assay. *Southeast Asian J Trop Med Public Health.* **38**:448-54.

Iqbal, J., Khalid, N., and Hira, P.R. (2003).

Performance of rapid malaria Pf antigen test for the diagnosis of malaria and false-reactivity with autoantibodies. *Adv Exp Med Biol.* **531**:135-48.

JonesD,NyalwidheJ,TetleyL,BarrettMP(2007).
McArthurrevisited:fluorescencemicroscopesforfielddiagnostics.*Trend sinParasitol***23**:468-469.

Loban, K. M.and Polozok, E. S.,(1983). Mir publishers; Malaria.
Makler M.T., Palmer C.J.,and Ager A.L. (2008). A review of practical techniques for the diagnosis of malaria. *Ann Trop Med Parasitol.* **92**:419-33.
Mekata T., Kono T., Savan R., Sakai ,M., Kasornchandra J., Yoshida T.,and Itami T. (2006). Detection of yellow head virus in shrimp by loop-mediatedisothermal amplification (LAMP). *J Virol Methods.* **135**:151-6.
Munyekeny, O. G., Githcko, A. K. Zuh, G., Mushiuzimana, E., Minakawa, N . and Yan, G. (2005). Plasmodium falciparum spatial analysis, Western Kenya . *CDC.* 11(10).

National Malaria Control Programme (2013). Sudan Malaria Program Performance Review. The Federal Ministry of Health, Sudan .

National Malaria Control Programme (2010). Five years Strategic Plan for the National Malaria Control Programme Sudan 2011-2015, The Federal Ministry of Health, Sudan .

Omer, E. S. (1978). Species prevelance of malaria in northern and southern Sudan , and control by mass chemoprophylaxis.*Am. J. Trop. Med. Hyg.* **27(5)**: 858-863.

Othnigue,N.,Wyss,K.,Tanner,M., andGenton,B. (2006). Urbanmalaria in the Sahel:prevalence and seasonality of presumptive

malaria and parasitaemia at primary care level
in Chad. *Trop Med Int Health*. **11**:204-10.

Perandin, F., Manca, N., Calderaro, A., Piccolo, G., Galati, L., Ricci, L., Medici, M.C., Arcangeletti, M.C., Snounou, G., Dettori, G., and Chezzi, C. (2004). Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol*. **42**:1214-9.

Rafael, M.E., Taylor, T., Magill, A., Lim, Y.W., Girosi, F. and Allan, R. (2006).

Reducing the burden of childhood malaria in Africa: the role of improved diagnostics. *Nature diagnostics*. **26**:39-47.

Reyburn, H., Mbatia, R., Drakeley, C., Carneiro, I., Mwakasungula, E., Mwerinde, O., Saganda, K., Shao, J., Kitua, A., Olomi, R., Greenwood, B.M. and Whitty, C.J. (2004).

Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study. *BMJ*. **329**:1212.

Rubio, J.M., Buhigas, I., Subirats, M., Baquero, M., Puente, S., and Benito, A. (2001). Limited level of accuracy provided by available rapid diagnosis tests for malaria enhances the need for PCR-based reference laboratories. *J Clin Microbiol*. **39**:2736-7.

Sachs, J., Malaney, P. (2012). The economic and social burden of malaria. *Nature* **415**:680-685.

Schellenberg,D.,Abdulla,S., a n d Roper,C.(2006).
Current issues for anti-malaria drugs to control *P.falciparum* malaria. *Curr Mol Med* **6**:253-260.

Schneider,P.,Bousema,T.,Omar,S.,Gouagna,L.,Sawa,P.,Schallig,H., and Sauerwein,R. (2006).
(Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenya
children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate. *Int J Parasitol*. **36**:403-8.

Schneider,P.,Schoone,G.,Schallig,H.,Verhage,D.,Telgt,D.,Eling,W.,and Sauerwein,R.(2004).
Quantification of *Plasmodium falciparum* gametocytes in different stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol*. **137**:35-41.

Schneider,P.,Wolters,L.,Schoone,G.,Schallig,H.,Sillekens,P.,Hermans,R.,Sauerwein,R.(2005). Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. *J Clin Microbiol*. **43**:402-5.

Schoone,G.J.,Oskam,L.,Kroon,N.C.,Schallig,H.D.,Omar,S.A.(2010).
Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. *J Clin Microbiol*. **38**:4072-5.

Tuteja,R.(2007). Malaria-an overview FEBSJ.274:4670-9.

VandenBroek,I.,Hill,O.,Gordillo,F.,Angarita,B.,Hamade,P.,Counihan, H.,Guthmann,J.P. (2006). Evaluation of three rapid tests for diagnosis of *P.falciparum* and *P.vivax* malaria in Colombia. *AmJTropMedHyg.* **75**:1209-15.

VanNess,J.,VanNess,L.K.,Galas,D.J.(2003). Isothermal reactions for the amplification of oligonucleotides. *Proc Natl Acad Sci USA.* **100**:4504-9.

Warrel,D.A.andGilles,H.M.(2012). Essentialmalariaiology Eighth Edition Arnoldp.:37-46.

Wernsdorfer,W.H.,Meshnick,S.R.(2002). Epidemiology of drug-resistant malaria. *Lancet Infect Dis.* **2**:209-18.

Whitcombe,D.,Newton,C.R.,and Little,S. (2008). Advances in approaches to DNA-based diagnostics. *Current Opinions in Biotechnology* **9**:602-608.

WHO, (2004). The World Health Report 2004. World Health Organization. Geneva Switzerland, p.126.

WHO/UNICEF (2005). World Malaria report, World Health Organization and UNICEF, WHO/HTM/MAL/2005.1102 WHO, Geneva, Switzerland.

Zakeri, S., et al., Detection of malaria parasites by nested PCR in south-eastern, Iran: evidence of highly mixed infections in Chahbahar district. *Malaria journal*, (2002). **1**: p. 2.

Zeinab.A.Ahmed,Daardogvillage,Association between fever and malaria among patients in Dardog village.(2013).

Appendex
The Questionnaire
Sudan University of Science and Technology
Collage of graduate studies
Department of parasitology and medical entomology
Detection of P.vivax and P.falciparum among population in Ed babeker

1- Number.....

2- Name

3- Sex.....

4- Age

5- Occupation

Symptom

If patient under malaria treatment or not:

Yes()

No ()

Notes

.....
.....
.....