Diagnosis of Malaria Using Real-time (PCR) in Comparison with Microscopy and Immuno Chromatographic Test in A Malaria Endemic Area, Gezira State, Sudan (2011 – 2014)

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A thesis

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Diagnosis of Malaria using Real-time (PCR) in Comparison with Microscopy and Immuno Chromatographic Test in A Malaria Endemic Area, Gezira State, Sudan (2011 – 2014)

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Date: March, 2014
Diagnosis of Malaria using Real-time (PCR) in Comparison with Microscopy and Immuno Chromatographic Test in A Malaria Endemic Area, Gezira State, Sudan (2011 – 2014)

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Date of Examination: 25 /3/ 2014
Declaration

This is original study was carried out by me at the Faculty of medical Laboratory Sciences, University of Gezira, Sudan under the supervision of Dr. Bakri Yousif Mohamed Nour. The data collection work has been carried out in Al karabia clinic, west Wad medani city Gezira locality, Sudan. The DNA Molecular studies were carried out in the Diagnostic and Research Unit of Parasitic Diseases (DRUP) and Laboratory Molecular Medical Parasitology (LMMP), Department of Medical Parasitology, Kasr Al-Ainy School of Medicine, Cairo University, Cairo – Egypt, under the supervision of Prof. Dr. Ayman Al-Badry.
Dedication

This work is dedicated

To

The soul of my father

The soul of my mother,

my family,

my wife and my daughter
ACKNOWLEDGEMENT

In the name of Allah the Beneficent the Merciful

I am very much grateful for my research supervisor, Dr. Bakri Yousif Mohamed Nour, for his supervision, valuable advices and assistance, and my Co-Supervisor, Prof. Osman Khalafalla Saeed, for his supervision, suggestions and direction, those who have assisted me throughout the research with full support, proper guidance and well planning in determination to have this work come out true.

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Diagnosis of Malaria using Real-time (PCR) in Comparison with Microscopy and Immuno Chromatographic Test in A Malaria Endemic Area, Gezira State, Sudan (2011 – 2014)

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Abstract

Malaria is one of the top five causes of death in low income countries. More than 95% of malaria cases in Sudan are due to *P. falciparum*. In malaria endemic areas, *P. falciparum* and *P. vivax* are the predominant species. Early and accurate diagnosis of malaria is essential for case management and malaria control programs. 160 samples positive for *P. falciparum* and 40 negative samples were collected for the study. All 200 samples were re-tested by the RDTs, and species-specific Real-Time PCR. Statistical analysis was performed by the Chi-square test using SPSS programme. Sensitivity was calculated as the number of true positive results divided by the sum of true positives and false negatives multiplied by 100. Specificity was calculated as the number of true negatives results divided by the sum of true negatives and false positives multiplied by 100. *P. falciparum* species-specific real-time PCR methods showed 98.6% sensitivity, 86.8% specificity and high significant difference (P-value <0.05), *P. vivax* species-specific real-time PCR methods showed with 100% sensitivity, 88.4% specificity and a significant difference (P-value <0.05), when it was used as gold standard. ICT for *P. falciparum* methods showed 90% sensitivity, 92.5 specificity and (P-value <0.05), when compared to microscopy method. Compared to species-specific real-time PCR, both microscopy and RDTs had lower sensitivities. The species-specific real-time PCR used in this study provided a more accurate, sensitive and specific in the differentiation of the *P. falciparum* and *P. vivax* than light microscopy. Its use in Gezira area, Central Sudan may be limited as a confirmatory tool in reference laboratories due to cost. The use the ICT Pf/Pv for diagnosis of malaria and species differentiation in the study could be recommended as alternative diagnostic tool to microscopy in suspected malaria cases.
تشخيص الملاريا باستخدام تفاعل البلمرة المتسلسل في الوقت الحقيقي مقارنة مع المجهر الروتيني وإختبار المناعة اللونى في منطقة موبوءة بالملاريا - الجزيرة - وسط السودان (2011-2014)

محمد يوسف أحمد باكر

ملخص الدراسة

تعتبر الملاريا أحد أعلى الأسباب للوفاة في الأطفال ذوي الدخل المنخفض، أكثر من 95% من حالات الملاريا في السودان بسبب الإصابة بالملاريا المنتشرة والمتصورة المحلية، وتشخيص المبكر والدقيق لملاريا مهم لتناسب الحالة وبرامج مكافحة الملاريا. تم جمع 160 عينتين لموجبة للفحص المتسرع المتسلسل والقطة عينة سلبية لدراسة كل المنتوان عينة تم إعادة فحصها باستخدام تقنية اختبار التشخيص السريع (RDT) وتقنية تشخيص البلازموديوم تقنية تحديد كل من نوعي المتسلسل (Real-time PCr) وتقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي (PCR). تم استخدام برنامج (Chi-square) باستخدام برنامج SPSS الأساسي من نتائج العينات الحقيقية الموجبة مقومة على حاصل جميع الحقيقية الموجبة مع الغير حقيقية سلبية والنتائج مضروب في النتائج. تم حساب التخليص من نتائج العينات الحقيقية السلبية مقومة على حاصل جميع الحقيقية سلبية مع الغير حقيقية موجبة والنتائج مضروب في النتائج. أظهرت تقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي النوع المحدد للمتسلسل بنسبة 98.6% و86.8% تخصص وفرق عالي مهم (P < 0.05). كما أظهرت تقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي النوع المحدد للتحفيز النشط في الموجبة بنسبة 100% و88.4% تخصص وفرق عالي مهم (P < 0.05). عندما استخدمت كمقرر ذهبي تقنية واختبار المناعة اللونى (ICT) للمتصورة المحلية أظهرت درجة حساسية 90% و92.5% تخصص عندما استخدم المجهر كمقرر ذهبي. مقارنة بتقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي- المجهر أظهر كل من المجهر واختبار التشخيص السريع ذو حساسية منخفضة. تقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي المستخدمة في هذه الدراسة أعطت أكثر صحة، حساسية وتحديد في تصور المتسلسل المحلية والمتصورة النشطة من التشخيص بالمجهر الضوئي. إن استخدامها في منطقة الجزيرة - وسط السودان كتقنية تأكد من المحتوى أن يكون محدود على المختبرات المرجعية ذلك بسبب التكلفة. استخدم تقنية واختبار المناعة اللونى للمتصورة المحلية والمتصورة النشطة (ICT Pf/Pv) يمكن أن يوصى بها كتقنية تشخيصية بديلة للمجهر في حالات الملاريا المشتبه بها.
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<td>AQ</td>
<td>Amodiaquine</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>EIR</td>
<td>Entomological Inoculation Rate</td>
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<td>ELISA</td>
<td>Enzyme Linked Immuno- Sorbent Assay</td>
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<td>HRP II</td>
<td>Histidine-Rich Protein II</td>
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<tr>
<td>ICT</td>
<td>Immuno Chromatographic Test</td>
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<tr>
<td>IPT</td>
<td>Intermittent Preventive Treatment</td>
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<td>IRS</td>
<td>Indoor Residual insecticide Spraying</td>
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<td>MAbs</td>
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<td>MoH</td>
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<td>OPD</td>
<td>Ortho Phenylene-Diamine</td>
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<td>RBM</td>
<td>Roll Back Malaria</td>
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<td>Polymerase Chain Reaction</td>
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<td>Rapid Diagnostic Test</td>
</tr>
<tr>
<td>SIR</td>
<td>Sporozoite Infection Rate</td>
</tr>
<tr>
<td>SMC</td>
<td>Seasonal Malaria Chemoprevention</td>
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<tr>
<td>SP</td>
<td>Sulphadoxine Pyrimethamine</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for Social Science</td>
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<tr>
<td>SR</td>
<td>Sporozoite Rate</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction and literature review

1.1 Introduction

Malaria remains a huge burden for human populations living in tropical areas and a major public health threat in many African countries, with the heaviest mortality rates in children living in sub-Saharan Africa (WHO 2012). It is one of the top five causes of death in low income countries (Fig 1.1). The disease is endemic in 106 countries and accounted for 655,000 deaths in 2010, 86% of which occurred among children. *Plasmodium falciparum*, the most lethal species, is the predominant species in tropical regions. *Plasmodium vivax* is mainly found in South-America and Asia, where it co-occurs with *P. falciparum*, as well as in the horn of Africa (WHO 2011). *P. vivax* is the most widely distributed human-infecting species, with up to 2.6 billion people at risk for infection (Mueller I. *et al*. 2009), in Iran it represents the most reported cases of the malaria parasites each year (Edrissian G; 2006), 71-81% in Central and South America and 5% in Eastern and Southern Africa of total malaria cases (Khalil, Alifrangis *et al.*, 2011). In Southeast Asia, *P. vivax* co-occur with *P. falciparum*, hence accurate diagnosis is required to differentiate these two species in areas of co-existence (The Global Health Group 2011).

*Plasmodium ovale* is mainly restricted to sub-Saharan Africa while *Plasmodium malariae* is present in most malaria endemic regions. *Plasmodium knowlesi* infections are frequently encountered in Malaysian Borneo (Singh B. *et al*. 2004) and have also been observed in other south-east Asian countries (Antinori S. *et al*. 2012).
In the case of *P. falciparum* infection, patients can quickly develop symptoms of severe malaria. Severe malaria cases have also been described for *P. vivax* infections (Kochar DK. *et al.* 2009).
Half the world's population is at risk for malaria. Africa account for 78% of the 225 million malaria estimated episodes and 91% of all malaria-related deaths worldwide in 2009 (WHO 2010).

The congenital malaria in sub-Saharan Africa is variable from (0 - 46%). Anti-malarial drugs are prescribed for sick newborns by health care workers. Data on prevalence of congenital malaria in high-risk newborns will inform appropriate drug use and timely referral of sick newborns (Enweronu-Laryea, Adjei et al. 2013).

Each year more than 500,000 women die during pregnancy or childbirth (WHO; 2004) and more than four million babies die in the first 28 days of life, accounting for 38% of mortality in children five years of age or under worldwide (Lawn JE et al., 2005, Adam T et al., 2005).

Pregnant women have a higher risk of malaria compared to non-pregnant women. The main effects of maternal risk factors for malaria in pregnancy includes maternal anaemia, low birth weight (LBW), preterm delivery and increased infant and maternal mortality. *P. falciparum* infected erythrocytes sequester in the placenta by expressing surface antigens, that bind to specific receptors, mainly chondroitin sulphate A. Recently, placental sequestration has been described also for *P. vivax* infections (Takem and D’Alessandro 2013). A recent review carried out in sub-Saharan Africa between 2000 and 2011, reports that malaria prevalence in pregnant women attending antenatal clinics was 29.5% in East and Southern Africa, and 35.1% in West and Central Africa, while the prevalence of placenta malaria was 26.5% in East and Southern Africa, and 38% in West and Central Africa (Chico RM, et al., 2012).

### 1.2 An overview of malaria epidemiology

The epidemiological pattern of the disease varies from place to place and even from time to time and occurs predominantly in rural areas (Deressa W. et al. 2006). In urban areas malaria transmission has increased due to the rapid growth of cities with lack of proper sanitation, increased migration of people from malaria rural areas to urban areas, poor
drainage of surface water, weak health services, limited tradition of indoor residual insecticide spraying (IRS) and bed net use, increased number of man-made mosquito breeding sites, and unplanned irrigation schemes and water collection reservoirs (Alemu A et al. 2011). The epidemiology of malaria in islands often varies from what occurs in the mainland. A general baseline health survey report carried out in 1991 concluded that six of the nine villages surveyed were hyperendemic, and the other three villages were mesoendemic (Mitja, Paru et al., 2013). Equatorial Guinea is an area of stable malaria transmission, where more than 85% of reported cases are due to *P. falciparum* infection (WHO 2008).

Iran is situated in the Eastern Mediterranean region, where about 45% of the population live with the risk of both *falciparum* and *vivax* malaria. Countries situated in Afrotropical region like Somalia and Sudan, in other regions like Pakistan, south-eastern Iran, part of Afghanistan, Turkmenistan, Uzbekistan and Tajikistan are eco-epidemiological zones regarding malaria disease (Sadrizadeh B; 2001).

Malaria is one of the public health problems in Ethiopia, there are more than 5 million clinical cases and thousands of deaths due to malaria each year (Ministry of Health of Ethiopia; 2012). Situation and knowledge about malaria is necessary to establish appropriate preventive and control measures (Abate, Degarege et al. 2013).

Kakuma refugee camps in sub-Saharan Africa, where malaria is a major health concern for persons occupying them. The Camp is located in a dry area of north-western Kenya, has hosted around 60,000 to 90,000 refugees since 1992. Refugees were from Sudan and Somalia. Malaria in the camp was due mainly to infection with *P. falciparum* and transmission was sustained by *Anopheles arabiensis* (Bayoh, Akhwale et al. 2011).

Data on the severity of malaria from the Republic of Congo is limited. The disease remains the main cause for 49 to 51.5% of hospital admissions and 35.4% of children deaths at health facilities (Ministère de la Santé Publique 2010). Diagnosis of malaria is through microscopy despite availability of rabid diagnostic tests (RDTs). At country level, performances of laboratory technicians using microscopy or RDTs are not yet assessed (WHO 2009).

Malaria is hyper-endemic in Ghana and accounts for 18% of deaths reported (Ghana Health Service Annual Report 2007). From outpatient attending the health facilities, malaria accounts
for 38.6% and 20% of mortality are among children under 5 years of age. Among pregnant women, malaria accounts for about 8% of hospital admissions (Ghana Statistical Service 2009).

In Nigeria, malaria is reported to be responsible for 25% of infant mortality and 30% of childhood mortality (FMOH Nigeria, 2005) and self-medication is a common practice among the general populace (Klemenc-Ketis Z et al., 2010). The overlap of malaria symptoms with other febrile illnesses resulted in overdiagnosis and development of drug-resistant stages of the parasite (WHO 2000).

Malaria in Brazil affects mainly the Northern region with approximately 450,000 cases per year (Brazilian Ministry of Health 2009). Malaria in the Atlantic Forest in the southern and southeastern regions of Brazil is characterized by atypical cases involving asymptomatic or oligosymptomatic individuals (Cerutti C Jr et al. 2007).

It is estimated that 99% of malaria cases occur in Amazon areas. The number of cases per year decreased from 500,000 in the year 2006 to 300,000 in the year 2011. The Artemisin-based Combination Therapy, used by Brazilian Health Ministry decreased the falciparum malaria incidence from 26.5 in 2006 to 12.4 in 2011 (Covell G; 1960).

In the Republic of Korea, P. vivax malaria has been endemic for centuries. In the 1960s and 1970s, active and passive vivax malaria case detection and vector control were combined in an eradication project by the government (Paik YK. et al. 1988). In the mid-1970s, transmission cases of malaria were greatly reduced and in 1979, the World Health Organization (WHO) declared the country as a malaria free zone (Ree HI, 2000). P. vivax re-emergence in the early 1990s, comprise of (36.8%) from total malaria cases in the country and P. falciparum account for (41.5%) cases (KCDC 2012).

In Bangladesh, malaria is endemic in and P. falciparum and P. vivax are the two main parasites (Alam MS. et al. 2011). Malaria incidence is seasonal in the country, where the warm and wet months from May to October define as the peak season. The dry and cooler months from November to April define as the off season (Rahman A. et al. 2006). Epidemics of malaria in Sri Lanka occurred every three to five years, in 1934 and 1935 a major epidemic led to an estimated 5.5 million cases (Karunaratne WA, 1959). Malaria transmission in the country is seasonal, typically peaking at the end of the northeast from December to March, with a smaller peak from June to October (van der Hoek et al., 1997).
It has been estimates that about 11,000 Indonesia’s die annually due to infections with *P. falciparum* (Murray CJ *et al.* 2012), the country has two dozen known malaria vectors and a high prevalence of both *P. falciparum* and *P. vivax* (Elyazar IRF *et al.* 2011). Indonesia has set the year 2030 as its deadline for elimination of malaria in the region according to present levels of malaria endemicity and strength of health infrastructure (Herdiana, Fuad *et al.*, 2013).

Within Southeast China, malaria occurs mainly at the border areas, in the Yuanjiang River (WHO 2010). While parasite-based diagnosis is increasing, most suspected cases of malaria are not properly identified (McMorrow ML *et al.*, 2008).

In Senegal, seasonal malaria chemoprevention (SMC) is a new strategy for malaria control (Lo, Faye *et al.*, 2013) The WHO now recommends that children living in areas of high seasonal transmission should receive treatment with sulphadoxinepyrimethamine (SP) plus amodiaquine (AQ) each month for up to four months during the peak transmission period (WHO; 2012).

During 2008 *P. falciparum* malaria accounts for 43.9% reported cases and 25.5% of malaria deaths in Odisha State, India (National Vector Borne Disease Control Programme, India; 2009). The disease is geographically distributed in poor population groups, in hilly and forested areas (Sharma VP, 2003).

There was a major reduction in malaria intensity in all age groups in Papua New Guinea in the year 2011 (Hetzel MW *et al.*, 2011) where 6.8% individuals and 7% children under the age of five years were infected with malaria parasites (Roll Back Malaria 2010).

Approximately 125 million non-immune travellers visit malaria-endemic countries yearly and over 10,000 cases were reported after returning home (WHO 2011). Among United Kingdom travellers, 52 to 196 cases out of 1,000 travelers between 2003 and 2006 (Behrens RH *et al.*, 2008). In France the incidence of imported malaria from endemic areas was 178 cases per 1,000 travelers (Malvy D *et al.*, 2006).

Soon after the Second World War, malaria was eliminated from Mediterranean countries, the rise in the average temperature of the earth, environmental modifications and the increase of international travel have raised the concern about the possible re-emergence of malaria in some of these countries, such as Italy, where malaria had been endemic before,
since the five-year eradication in 1947-1951, one single confirmed case of *P. vivax* malaria has been recorded (Gould EA. *et al.* 2009).

In Greece, malaria epidemics have been recorded since as early as the fifth century BC. A local outbreak of malaria was reported in the summer to autumn of 2011 (Kousoulis, Chatzigeorgiou *et al.* 2013). The presence of competent vectors and of humans carrying gametocytes in their blood may play a major role in malaria re-emergence. There were 36 reported cases of *P. vivax* malaria infection in 2011. *Anopheles sacharovi* has been recognized as the dominant vector in this region. Half of the cases were Greek citizens, who had no history of travel to an endemic country, and the rest were migrant workers mainly from Pakistan (Danis K. *et al.* 2011).

### 1.3 Malaria epidemiology in Sudan

Sudan is the largest country in Africa, malaria is dominates in most parts of the country, including the eastern and central areas with unstable transmission (Al Gadal AA, 1986). The total population of Sudan is estimated to be 39.2 million inhabitants, of whom 75% live in rural areas. The entire country is at risk of malaria, the endemicity is mainly low to moderate with predominantly seasonal transmission and epidemic outbreaks in the northern, eastern, and western states (Sudan, Country Profile, 2005). Malaria is the leading cause of morbidity and mortality in Sudan, with an annual estimated 7.5 million clinical cases and 35,000 deaths, mainly because of *P. falciparum* parasite infection and the principal mosquito vectors are *A. arabiensis, Anopheles gambiae* and *Anopheles fenstus* (Malik EM, Khalafalla OM, 2004).

The widely extended irrigated schemes floods, insecticide resistance and the spread of *P. falciparum* resistant strains are factors leading to spread of malaria disease in Sudan (Himeidan Yel-S 2004). The disease constitutes 30% of all attendance to health facilities, and it is the main cause of hospital death, that is because of the increasing parasite
resistance to chloroquine and vector resistance to insecticides used (Roll Back Malaria 2002).

Malaria risk map for malaria control in Sudan was done up to the year 2010. *P. falciparum* parasite rate (*PfPR*) data were assembled. Surfaces of aridity, urbanization, irrigation schemes, and refugee camps were included. It was stated that areas of meso- and hyperendemic risk were located in the south. About 80% of Sudan's population in 2011 was in low risk areas, in the desert, urban centers, or where risk was < 1% *PfPR*, (Noor, ElMardi *et al.* 2012).

Malaria transmission has shown difference between non-irrigated and irrigated semi-arid areas of Eastern Sudan. Very little information is available regarding malaria transmission along the seasonal river's basin. Understanding malaria vector mosquitoes and their infectivity is of importance in malaria control programmes. (Himeidan, Elzaki *et al.*, 2011).

In eastern and central Sudan, insecticide treated nets (ITNs) has been dramatically scaled up. (Himeidan, Muzamil *et al.* 2011). IRS and ITNs strategies in reducing and elimination malaria disease as a public health problem are a feasible objective (Tatem AJ *et al.*, 2010).

International efforts have been made to access approximately 289 million ITNs in sub-Saharan Africa, to cover 76% of the 765 million people at risk of malaria (WHO 2010).

Maternal and perinatal mortality have been observed in the different regions of Sudan; the major cause of high mortality level is due to malaria and anaemia (Elhassan EM *et al.* 2009). Data from hospital-based study from Kassala maternity hospital, which is located in eastern Sudan and Medani maternity hospital that located in central Sudan, the areas of unstable malaria transmission, showed that pregnant women are more susceptible to severe *P. falciparum* malaria, which can lead to poor maternal and fetal outcomes. Early detection and prompt treatment of severe malaria, insecticide-treated bed nets and chemoprophylaxis may be beneficial in areas of unstable malaria transmission (Ali, Elhassan *et al.* 2011). At Wad Medani Teaching Hospital in Medani City, Sudan, around 37% of all maternal deaths between 1985 and 1999 were due to malaria that has severe effects on the foetus causing both foetal loss and low birth weight (Dafallah SE *et al.*, 2003). Both malaria and pre-
eclampsia were among the common causes of high maternal mortality in Sudan (Elhassan EM. et al. 2009). With the exception of a small area at the Sudan–Egypt border, the whole population is considered to be at risk of malaria infection. More than 95% of malaria cases in Sudan are due to *P. falciparum* (Elhassan IM. et al., 1995).

1.4 Mixed Plasmodium infection

Plasmodium species traditionally associated with human infections are *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* (Lee KS et al., 2011). *P. falciparum* is the most prevalent malaria infection in Africa and accounts for the largest proportion of clinical cases. *P. malariae* infections are frequently found in sympatry with *P. falciparum* infections (Bruce MC et al., 2011). *P. ovale* is thought to be highest in sub-Saharan Africa (Mueller I et al., 2007). Much of the research concentrates on *P. falciparum* and *P. vivax*. There is nomuch data about the prevalence of *P. falciparum* co-exists with *P. malariae* and *P. ovale* across the human population (Gneme, Guelbeogo et al., 2013). In malaria endemic areas, the four human malaria species often co-exist, but with *P. falciparum* and *P. vivax* being the predominant species (Jiang N et al., 2010). *P. vivax* can co-occur with other *Plasmodium* species causing further complications for accurate diagnosis and treatment (Mayxay M et al., 2004).

Using sensitive polymerase chain reaction (PCR) methods in endemic areas, the prevalence of malaria is much higher than the microscopy, a high rates, around 20%, of mixed blood stage infection have been reported (Gupta B et al., 2010).

Lower rates of mixed species infection occur in symptomatic non-immunes presenting with acute *falciparum* malaria in low transmission settings. Studies showed that mixed infection could occur from simultaneous inoculation, and that in the blood stage infection *P. falciparum* tended to suppress *P. vivax*, so low level *P. vivax* parasitaemia might still be missed by current diagnostic methods (Steenkeste N et al., 2010). In South-East Asia where transmission of malaria is low, seasonal and unstable, about 30 to 50% of acute *P.
*falciparum* malaria infections are followed soon afterwards by an infection with *P. vivax* (Douglas NM et al., 2011).

*P. vivax* may also suppress *P. falciparum*; in Thailand acute vivax malaria may followed shortly by *P. falciparum* infections without re-infection (Mason DP et al., 2001). In Asia and Oceania studies suggested that simultaneous inoculation of the two malaria species does occur, and the majority of mixed species infections do not derive from a single mosquito bite. Asymptomatic individuals are able to control the infections and so are much more likely to have a multiplicity of different genotypes present and also mixed species infections. It seems likely that the majority of mixed infections in humans are acquired from separate mosquito inoculations (Mallika Imwong et al., 2011).

*P. falciparum* is the most pathogenic of the five *Plasmodium* species. In malaria endemic regions co-infection are mostly found within individual human hosts or the mosquito vector population (Collins WE et al. 2007). *Plasmodium* inter-species interactions have been the focus of interest of number of studies (Bousema JT et al., 2008).

### 1.5 Malaria transmission

Malaria transmission in sub-Saharan Africa may vary from stable to unstable, depending on the climatic conditions. Malaria can be a chronic disease. The acute infection can often switch into asymptomatic parasitaemia that may last for months (Babiker et al., 1998).

In areas with stable malaria transmission, repeated exposure to *P. falciparum* develop clinical immunity against the disease. In intense malaria transmission areas, protection against severe malaria is acquired early in life (Gupta S et al., 1999) while protection against mild malaria or asymptomatic infection develops later in life (Cham GK et al., 2010), in children multiple malaria infections result in increased exposure that leads to semi-immunity limiting the severity of the disease (Rogerson SJ; 2010).

90% of the global malaria cases were recorded in Africa (fig: 1.2), which is due to the spread of drug resistant parasites strains, the vector resistance to insecticide, and poor economic status of populations.

For an effective control, an integrated approach against both the parasites and vectors is necessary (WHO; 2011). ITN and intermittent preventive treatment (IPT) in pregnancy are recommended for the prevention of malaria in pregnancy in stable transmission settings. ITNs have been shown to reduce malaria infection in pregnancy by 28–47%. Treatment of
malaria in pregnancy should be started immediately with the most readily available drug (Takem and D’Alessandro 2013).

Malaria transmission are thought to be driven by many factors; including host genetics, socio-behavioural characteristics, housing construction, and how far these houses from mosquito breeding sites (Coulibaly, Rebaudet et al., 2013).

The environment is a key determinant of the spread of disease. Temperature, moisture and water quality determine the presence of Anopheles breeding sites, vector densities, adult mosquito survival rate and vector capacity that lead to malaria incidence in populations (Texier, Machault et al., 2013).

Breaking the vector-human link, either by reducing the mosquito’s population to avoiding infective bites or reducing house entry rates of the vectors is a best ways to control malaria transmission (Kampango, Braganca et al. 2013).

Malaria parasite and vector are sensitive to mean temperatures and daily temperature variation that control malaria transmission (Cator, Thomas et al., 2013). Water temperature influences larval development rates whereas air temperature determines adult longevity and the rate of parasite development within the adult mosquito (Garske, Ferguson et al., 2013). The role of anophelines in transmitting malaria depends on their distribution, preference to feed on humans and their susceptibility to Plasmodium gametocytes, all of which are affected by local environmental conditions (Animut, Balkew et al., 2013).

A strong association exists between human uses of water and the production of adult malaria vectors from aquatic environments containing the larval stages, and subsequent malaria transmission (Killeen GF et al., 2006). Reservoirs created by damming rivers are often believed to increase malaria incidence risk and stretch the period of malaria transmission (Yewhalaw, Getachew et al. 2013). The requirements for irrigation, water for animals and for other domestic uses can create intensive pathogen transmission through formation of adult stages of vectors (Keiser J et al., 2005).

The spread of chloroquine resistance among malaria parasites, the emergence of insecticide-resistant mosquitoes and population movements within or through endemic countries were factors contributing to the spread of malaria disease (Phillips RS, 2001).
Fig: 1.2 Parts of the world showing malaria transmission

Reviewed by April Cashin-Garbutt, BA Hons (Cantab)

Sources

Human mobility plays an important role in transmission of malaria parasites between regions of variable intensity. Asymptomatic individuals can carry parasites to regions where mosquito vectors are available when they travel importing parasites between regions, therefore are a principal sources of malaria that can be an important target in elimination and control of malaria transmission (Buckee, Wesolowski et al., 2013). Asymptomatic malaria is a case where an individual been infected with malaria parasite, without showing symptoms, often occurs at low parasite level, between 50 and 200 parasites per µL, and is capable of transmitting the disease (McMorrow et al., 2011). Asymptomatic carriers, whom had continuous exposure to Plasmodium parasites in malaria endemic areas, provide a fundamental reservoir of malaria parasites (Zoghi, Mehrizi et al., 2012), and they might become gametocyte carriers, contributing in the persistence of malaria transmission (Bousema JT et al., 2004).

Malaria can be transmitted for over a week after the clearance of the asexual parasites following standard therapy, as current antimalarial drug treatment does not effectively kill mature P. falciparum gametocytes. In combinational with currently available anti-malarials therapy, a new generation of malaria gametocytocidal drug is needed to control the spread of the disease and facilitate eradication efforts (Tanaka, Dehdashti et al. 2013).

The sporozoite infection rate (SIR) of the mosquito population is a powerful and sensitive measure for describing the epidemiology of malaria in a particular area. There are substantial gaps in the annual Entomological Inoculation Rate (EIR) across Africa, the past estimates of EIR were found to be available in only 23 of the 54 African countries, with 56% of the measures coming from only four countries; Kenya, Burkina Faso, Tanzania, and The Gambia (Kelly-Hope LA, McKenzie FE 2009).

Studies have reported significant variations in the transmission intensity of malaria between rural and urban settings within the same locality and even between villages separated by short distances (Robert V. et al. 2003). Local rural health centres can reflect the dynamic nature of malaria transmission, as these local rural health centres has a sufficiently developed infrastructure and use rapid diagnosis that can deliver critical information to assist in planning in elimination of malaria. Data collected over a five-year period in Zambia showed the importance of ecological zones and the seasonal fluctuation of malaria cases (Shiff, Stoyanov et al., 2013).
The near absence of Duffy positive phenotype in the local populations seems to exclude transmission of *P. vivax* in sub-Saharan Africa (Culleton RL *et al.*, 2008) except in countries such as Djibouti, Eritrea, Ethiopia, Somalia, and Sudan (Snow and Omumbo 2006). Controlling the spreading of *P. vivax* has proved more difficult than *P. falciparum*, with successful malaria control programmes in some countries leading to an increase in the incidence of *P. vivax* as overall malaria rates drop (Oliveira-Ferreira J *et al.*, 2010).

1.5.1 Malaria transmission in Sudan

In eastern Sudan, the EIR estimates are only available in non-irrigated, rain-dependant agricultural areas. This region as reported where two to three infective bites per person per year occurring entirely at the end of the rainy season (Hamad AA *et al.* 2002). A different pattern of malaria transmission, from perennial to moderate transmission has been observed in irrigated semi-arid areas of eastern Sudan (Himeidan YE *et al.* 2007). However, very little information is available about the seasonal variation and intensity of malaria transmission along the river basin. Such information is required for the design of effective vector control strategies. The present longitudinal entomological surveys examined seasonal variations of the EIR at two villages along the Rahad River basin in eastern Sudan.

Understanding malaria vector mosquitoes and their infectivity dynamics is of importance in setting up intervention and control programmes. Patterns of malaria transmission have been shown to differ between non-irrigated and irrigated semi-arid areas of eastern Sudan. However, very little information is available regarding malaria transmission dynamics along the seasonal river's basin. Such information is required for the design of effective vector control strategies (Himeidan, Elzaki *et al.*, 2011).

1.6 Malaria Control

The WHO has put many efforts to control malaria leading to the significant reduction of malaria cases; still transmission of about 30,000 malaria cases is reported annually from non-endemic area, including industrialized countries (Schwartz E, 2012). Malaria control has resulted in considerable reductions in morbidity and mortality associated with malaria in parts of Africa (O’Meara WP *et al.* 2010).
The strategies of the National Malaria Elimination programme 2010–2020 include early diagnosis; prompt treatment, distribution of long-lasting, insecticide-treated bed nets and basic and applied research on *P. vivax* and *P. falciparum* (Ministry of Health, China 2010). The use of antigen-detecting RDTs forms a vital part of this strategy, in areas where good quality microscopy cannot be maintained (WHO 2009). Two types of RDTs have been extensively used based on co-existence of *P. falciparum* and *P. vivax* in this area (Yan, Li *et al.* 2013).

In children, the IPT was considered effective on malaria control due to the reduction of its incidence in some areas with seasonal malaria in Africa (Katsuragawa, T *et al.*, 2013). Global control efforts have resulted in a reduction in the incidence of malaria and malarias specific mortality rates (WHO 2011). In areas of a low transmission, where most of the asymptomatic carriers are not treated, threat of malaria epidemics is potential (Lee PW *et al.*, 2010).

Tools in malaria control in sub-Saharan Africa include a wide use of artemisinin-combination therapies, ITNs and in some places targeted IRS of insecticides. The intensity of malaria has declined in some countries while other countries were unable to provide accurate data on their malaria situation (WHO; 2011).

The goals set by the World Health Assembly and the Roll Back Malaria (RBM) Partnership to reduce the number of malaria cases and deaths recorded in 2000 by 50% or more by the end of 2010, and by 75% or more by 2015 have not yet been achieved (Kant R, 2011).

The estimation of malaria risk factors is important for planning and evaluating the effectiveness of vector control programs. Climate, physical and chemical conditions in mosquito habitats, vector abundance, human biting rate, parity, sporozoite rate (SR) and EIR are all influenced by local and seasonal environmental factors (WHO 1975).

Mosquitoes, the malaria vector, are very sensitive to changes in environmental conditions suitable for larval habitats might affect their species distribution, survival and density (Molyneaux DH, 1998); Deforestation in western Kenya resulted in an increase in temperature that led to a decrease in the duration of sporogony of *P. falciparum* (Afrane YA *et al.*, 2008).
Studies conducted in the northern Peruvian Amazon and western Brazilian Amazon have shown that the degree of deforestation and ecological alterations influence the risk of malaria (Olson SH et al., 2010). Human biting rate was higher in areas with more deforestation and development associated with road construction in Peru (Vittor AY et al., 2006), while a 4.3% increase in deforestation resulted in a 48% increase in malaria incidence in Brazil (Olson SH et al., 2010).

To design an efficient anti-malarial vaccine, worldwide information on circulating antigenic variants is necessary for the formulation of a polyvalent vaccine, which would be effective in different regions (Cui L et al., 2003; Moon et al., 2010). Considering the emergence of chloroquine resistance and evidence of P. vivax strains with lower sensitivity to primaquine (Arias and Corredor, 1989), the development of an effective and a safe vaccine would be an important concern in control and elimination strategies. Despite decades of research, effective malaria vaccines are still not available and one of the major hurdles in designing a successful vaccine could be the presence of antigenic diversity that has led to the failure of several licensed and test malaria vaccines. Studies performed in animals and human subjects (Anders R.F. et al., 1998; Stowers A.W et al., 2002) have facilitated the development of several P. vivax vaccine candidates and in particular asexual antigens, such as Duffy-binding protein, merozoite surface protein-1 and apical membrane antigen-1. These antigens have received specific attention for their role in the invasion process and the generation of antibody response (Remarque E.J et al., 2008; Arévalo-Herrera M et al., 2010).

Genetic diversity of P. falciparum is an important parameter for malaria surveillance that plays a major role in the natural acquisition of malaria immunity. Exposure to several strains positively influences the development of partial immunity to malaria in people living in malaria endemic areas (Conway DJ et al., 2000).

Malaria control measures led to a decline in clinical malaria morbidity and mortality in a number of African countries. This can be attributed to the use of ITNs, IRS and artemisinin-based combination therapies (WHO 2009). In 2002, the Ministry of Health (MoH), Kenya developed an intensive ITN delivery programme targeting pregnant women and children under the age of 5 years, to reach a target of 60% coverage of populations at risk by 2005 (The World Bank website 2010).
Eextensive efforts are being made to reduce the impact of malaria disease in Zambia by developing strategies that can focus on identifying reservoirs and on targeting periods of the seasonal cycle of malaria transmission (Chizema-Kawesha E et al., 2010). It was estimates that “you cannot control what you cannot measure”, this applies to malaria, and what is not measured accurately could be misleading (Steketee RW et al., 2008). Republic of Congo from the year 2009 established malaria control program by improving malaria diagnostic skills of laboratory technicians and providing appropriate stocks of RDTs and reagents (Koukouikila-Koussounda F et al., 2012).

Reduction in the annually reported number of malaria cases in Sri Lanka from 264,549 in the year 1999 to 196 in the year 2007 was achieved by adopting the Global Malaria Control Strategy recommended by WHO in 1994. There is a plan for the elimination of malaria from the country by the end of 2014 (Anti-Malaria-Campaign 2008, 2011). There is a possible increasing risk of re-introduction of malaria to the country from imported cases. Increase in international travel has contributed to an increase in number of malaria cases (Galappaththy, Fernando et al. 2013). A scheme for IRS using DDT for malaria control was developed in 1945 by Sri Lanka and was quickly expanded to all malarious areas (Karunaratne WA, 1959).

Vaccines that interrupt malaria transmission are of increasing interest and would promote their development by providing a biologically relevant means of evaluating potential vaccine candidates (Miura, Deng et al., 2013)

Vaccination is considered to be one of the most cost-effective control methods for a range of infectious diseases, to date only one malaria vaccine candidate against the pre-erythrocytic stages, the RTS, S vaccine, has shown encouraging clinical protection and a large phase 3 trial is underway in Africa (Agnandji ST et al., 2011). There is increasing interest in a transmission-blocking vaccine (TBV) which is designed to induce antibodies in human hosts against sexual stage malaria antigens or to antigens found in the mosquito vector. The TBV-induced antibodies are ingested by Anopheline mosquitoes along with parasites in the blood and subsequently inhibit parasite development in the mosquito host (Wu Y et al., 2008).
1.7 Malaria pathogenicity

Malaria remains the most significant parasitic infection in humans, claiming over a million lives annually (WHO; 2008). The understanding of malaria causative agents begins in 1880, when Charles Louis Alphonse Laveran was the first to discover protozoan parasites in the blood of affected patients (Cox FE, 2010). The genus Plasmodium comprises about 172 species, infecting a wide range of mammals, birds, reptiles, and amphibians. Plasmodium species; *P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi* are the causative agent for malaria in human (Rogers WO, 2007).

The life cycle of the plasmodium is completed in two hosts, a vector, a female *Anopheles* mosquito and a vertebrate host (fig: 1.3). Female *anopheline* mosquitoes introduce infectious sporozoites into the host dermis when taking a blood meal. Sporozoites migrate to enter a blood vessel, and are carried to the liver (Yamauchi LM et al., 2007) where the parasite undergoes liver-stage development, and release of tens of thousands of merozoites (Vaughan AM et al., 2008).

Invasion of host erythrocytes by the parasite initiates the blood stage of infection responsible for the symptoms of malaria. Invasion involves extracellular protein interactions between host erythrocyte receptors and ligands on the merozoite, (Bartholdson, Crosnier et al., 2013). After entering the erythrocyte, the merozoite grows to a ring-shaped, a trophozoite, a schizont, the parasite divides several times to produce new merozoites that travel within the bloodstream to invade new red blood cells. The parasite feeds by ingesting haemoglobin and other materials from red blood cells and serum (Leitgeb AM et al., 2011).

All of the symptoms and pathology of the disease are caused by the asexual blood stage of the infection when the infected red blood cell burst to release numerous invasive merozoites (Cowman and Crabb 2006).

Erythrocytes infected by *P. falciparum* tend to form clumps - rosettes - and these have been linked to pathology caused by vascular occlusion (Leitgeb AM et al., 2011). Rosette formation has also been reported in *P. vivax* (Marín-Menéndez A et al., 2013).

A switch from asexual to sexual development resulting in the production of gametocytes is essential for transmission of the malaria parasite to the mosquito vector (Talman AM et al., 2004). There is strong evidence that commitment to gametocytogenesis is sensitive to
environmental conditions suggesting that a signalling pathway between the environment and the parasite must be involved (Neves SR et al., 2002).

Some merozoites differentiate into male or female sexual forms, the gametocytes. Gametocytes appear in the blood after 7 to 15 days post infection in case of *P. falciparum* while in others they appear after 1 to 3 days. Gametocytes are taken up by the female mosquito during its blood meal (Robert, Boudin 2003).
In the mosquito's midgut, fertilization of gametes resulting in formation of a diploid zygote, to ookinetes, that penetrate the midgut, attach onto the gut membrane where they differentiate into oocysts that divide many times to produce large numbers of tiny elongated sporozoites. The sporozoites migrate to the salivary glands of the mosquito to be transmitted into the blood of the next host (Raibaud A, et al., 2001). When Plasmodium sporozoites invade the salivary glands, the mosquito becomes infectious (Sidjanski et al., 1997).
1.8 Diagnosis of malaria parasite

Early and accurate diagnosis of malaria is essential for case management and for malaria control programs (WHO; 2010) and is needed to prevent progress to severe malaria (Leslie T et al., 2012). Current diagnostic methods include microscopy, RDTs that target parasite proteins, and PCR-based molecular tests. Each method has advantages and limitations (Mens P et al., 2007).

Microscopy is considered the gold standard for malaria diagnosis but has limitations. Enzyme Linked Immunosorbent Assay (ELISA), PCR, and Real-Time PCR are also used to diagnose malaria in reference laboratories, although their application at the field level is currently not feasible (Mohon, Elahi et al., 2012) and RDTs have become an essential tool in malaria control and management programmes in the world (Yan, Li et al., 2013).

Measurement of malaria burden is critical, due to the natural history of the disease, delays in seeking treatment, patterns of infection, and asymptomatic infection (Crowell, Yukich et al., 2013). Identification of the four Plasmodium species is important for successful treatment. In cases of co-infection, diagnosis and subsequent treatment is even more complicated (Snounou G et al., 1993). In regions such as Papua and Indonesia, misdiagnosis of P. vivax potentially leading to increased morbidity and transmission of the disease (WHO 2011). Elimination of the malaria disease require that malaria diagnosis must actively detect infections in asymptomatic and low-parasite-load patients as well as diagnosing ill patients (Cheng, Sun et al., 2013).

There is a clear need for diagnostic tools that are accurately enough to detect the different Plasmodium species, to identify the transmission foci of malaria reservoirs and to monitor the success of malaria control and elimination programs (WHO; 2010).

1.8.1 Microscopy in malaria diagnosis

Light microscopy, established over 100 years ago and considered the reference standard for clinical diagnosis, remains the most accessible method for parasite quantitation, drug efficacy monitoring, and as a reference of assessing other diagnostic tools (Ashraf, Kao et al., 2012).

Microscopy technique can be used to differentiate parasite species as well as provide quantitative data on the level of parasitemia (Kahama-Maro J et al., 2011). Species differentiation is possible by microscopic blood smear examination when conducted by
trained and experienced staff. Low levels of parasitaemia, mixed *Plasmodium* infections, altered morphology of parasites following self-treatment and poor smear quality challenge the abilities of even the most skilled microscopists (Gillet *et al*., 2009), as identification of the infecting *Plasmodium* species using conventional microscopy in malaria diagnosis relies on parasite morphological characteristics, such as size, shape, pigment granules, in addition to size and shape of the parasitized red blood cells and presence of cell inclusions (Obare, Ogutu *et al*., 2013). Correct diagnosis is crucial for appropriate treatment. Species misidentification by microscopy has been reported in areas co-endemic for *vivax* and *falciparum* malaria (Barber, William *et al*., 2013).

Giemsa-stained thick blood film analysis by microscopy is cheap and enables identify the sexual stage, the gametocytes, from asexual stages. Its quality in endemic areas is often inadequate due to problems with electricity, microscopes, stains and shortage of competent staff (Alonso PL *et al*., 2011). It is difficult to use microscopes in the field. In a rural centre health clinic, there is frequently a life-threatening delay in obtaining a diagnostic, hence the clinical diagnosis becoming the best option, but it is non-specific, and this can lead to wastage of anti-malarial drugs (Mc Morrow ML *et al*., 2008).

For a large numbers of samples, microscopy technique is impractical as it cannot differentiate between *Plasmodium* species with similar morphology, required time to examine a slide and sensitivity is low (Di Santi *et al*., 2004). Inaccuracy in confirmation of malaria status using microscopy can lead to patient mismanagement (Rolland E *et al*., 2006).

During *P. vivax* infections the parasite density is often low and cannot be detected by microscopic examination. The use of molecular methods for the diagnosis of malaria has proved to be highly sensitive and specific (Kho WG *et al*., 2003).

### 1.8.2 RDT in malaria diagnosis

RDTs have become very popular in various malaria endemic areas (Lubell Y *et al*., 2007), especially in areas where microscopic expertise is lacking. They are now an essential tool during the malaria elimination and eradication (Bisoffi Z *et al*., 2009).

RDTs are immunochromatographic tests (ICT) targeting specific antigens of one or more *Plasmodium* species (Abeku TA *et al*., 2008). RDTs are designed using antibodies against parasite species-specific or genus-specific antigens, such as *P. falciparum*-specific
histidine-rich protein-2 (PfHRP2) and P. falciparum-lactate dehydrogenase (Pf-LDH) (Wongsrichanalai C et al., 2007).

RDTs detect Plasmodium antigens by an antigen-antibody reaction on a nitrocellulose strip (fig: 1.4) When blood and buffer are applied to the sample and buffer pad, respectively, they will be attracted by the capillary action of the absorption pad and start to migrate, passing the conjugate pad, which contains a detection antibody targeting a Plasmodium antigen, this detection antibody is a is conjugated to a mostly colloidal gold signal. The Plasmodium antigen, if present in the sample, will bound to this detection antibody-conjugate. The antigen-antibody-conjugate complex then migrates further until it is bound to the capture antibody, which binds to another site of the Plasmodium target antigen. As the capture antibody is applied on a narrow section of the strip, the complex with the conjugated signal will be concentrated and by virtue of the colloidal gold will become visible as a coloured line. The excess of detection antibody-conjugate that was not bound by the antigen and the capture antibody moves further until it is bound to a goat-raised antimouse antibody, thereby generating a control line.
RDTs require no specific training and equipment and provide results rapidly. Sensitivity for *P. falciparum*, with detecting the PfHRP2 gives better result than Pf-pLDH. Sensitivity for *P. vivax* is about 66 to 88%, *P. ovale* about 5.5 to 86.7%, and *P. malariae*, about 21.4 to 45.2% (Maltha, Gillet *et al.*, 2013).

Higher sensitivities for *P. falciparum*, 99.4%, was reported in Ethiopia with parasite densities above 100/μl (Mekonnen Z *et al.*, 2010) at parasite densities of less than100/μl, sensitivity was low, 60.0%, and showed 100% sensitivity at parasite densities of more than 500/μl in Madagascar (Ratsimbason A *et al.*, 2007). Sensitivity for *P. vivax* with pLDH detection was significantly higher than that found in Ethiopia, 91.0% (Ashley EA *et al.*, 2009).
RDTs are easy to use, provide quick results, and are useful alternatives when there is no access to microscopic diagnosis (Moody A et al., 2002). It has some limitations; poor sensitivity at low parasite densities, susceptibility to the prozone effect, cross-reactions between Plasmodium antigens and detection antibodies, false-positive results by other infections and susceptibility to heat and humidity, delayed reading, incorrect sample and buffer volumes and interpretation results (Maltha, Gillet et al., 2013). False positive results may because of the antigens that may remain in the circulation of a patient after treatment (Wongsrichanalai C et al., 2007). Most of RDTs had poor performance for low levels of parasitaemia, less than 500 parasites/μL, which is especially true for vivax malaria (Coleman RE et al., 2002) so it cannot distinguish P. vivax from other species, and they cannot be used to determine parasite densities with respect to antimalarial drug resistance and malaria control programs (Erdman, Kain, 2008).

Malaria RDTs overcome many of the limitations of microscopy. They are designed as instrument-free tests that can be used by community health workers (Counihan H et al., 2012). In endemic areas, the performance of RDT for the detection of P. falciparum has been shown to be equal to routine microscopy, indeed they are not as accurate as expert microscopy and do not generate all information provided by microscopy (Batwala V et al., 2010).

RDT diagnostic accuracy depend on the RDT product used, local epidemiology of malaria, availability of infrastructure, the expert laboratory technicians and the reference method used; microscopy or PCR, (WHO 2011). In the case of negative RDT results repeating the test may be useful, in a developing infection, when antigen concentrations are still too low to be detected. RDTs cannot be used for treatment follow-up (Senn N et al., 2012).

The Cyscope-mini RDT is a portable, battery-operated fluorescent microscopy. Its principle is based on the detection of intra-erythrocytic Plasmodium DNA, which results in a bright intracellular dot-shaped fluorescence if the red blood cells are infected with Plasmodium species (Nkrumah B et al., 2010).

With the limitations of both microscopy and RDTs, there is a need for more sensitive diagnostic tools for the detection of infections, to guide and monitor malaria elimination programs (mal ERACGoD 2011).
1.8.3 PCR in malaria diagnosis

Malaria Molecular methods based on DNA amplification have been applied to malaria diagnosis since the late 1980s (Snounou G et al., 1993). PCR-based techniques have a higher sensitivity by detecting infections at an earlier time, when parasite density is still under the limit of detection of microscopy (Carneiro I, et al. 2010). High sensitivity has been achieved by using a common target, the conserved small subunit ribosomal RNA 18S locus which in the *P. falciparum* chromosomal genome exists in five to eight copies depending on the strain (Snounou G et al., 1993) with a sensitivity of one to ten parasites per microlitre (p/μl) of blood using nested PCR with 18S as the target gene (Polley SD et al. 2010).

Different PCR-based methods for malaria diagnosis have been developed, mainly in reference centers (Rantala AM et al. 2010). Most of them have shown to be more sensitive than microscopy, and some are able to detect mixed infections (Coleman RE et al. 2006). There are many types of molecular techniques for malaria diagnosis, conventional PCR-based assays, real-time PCR assays and isothermal amplification assays (Erdman, Kain, 2008). The nested PCR described by Snounou et al. in 1993, remains commonly used and often regarded as a gold standard and a reference method. Nested PCR is a highly sensitive and specific, has low cost-efficiency but it is time-consuming, contamination is possible (Polley SD et al., 2012). Conventional PCR assays have higher sensitivity and specificity than microscopic examination and have been used as alternative diagnostic methods (Igbinosa et al., 2010). The methods although provide accurate results but are long running time, expensive and require technical expertise, a well-equipped laboratory, that most malaria-endemic countries cannot offer (Patel, Oberstaller et al., 2013).

Real-time PCR assays depend on the use of fluorophores or DNA intercalating fluorescent dyes. These molecular methods are and simpler than conventional PCR methods, but the reagents are expensive and require special handling, which became obstacle for field use in malaria endemic countries. A less complicated real-time PCR technique is the use of SYBR Green, SYTO-9, and calcein, which emit fluorescence signals when bound to double stranded DNA. As in the cases of TaqMan probes, molecular beacons and scorpion, sequence-specific oligonucleotide probes are dual labeled with a fluorescent dye and
quencher. Other alternatives for the detection of real-time PCR assays, is the direct labeling of one of the primers forward or reverse, with a single fluorophore that facilitates self-quenching without the need of a quencher. These self-quenching primers facilitate the use of real-time PCR without the need for internal dual-labeled sequence specific probes (Nazarenko I, 2006).

The real-time PCR methods are particularly capable for large scale screening and they can be used in malaria control and elimination programs (Lucchi, Narayanan et al., 2013). It is a fast, automated, accurate and efficient technique for applications with large numbers of samples, with a high degree of sensitivity and specificity when compared to microscopy (Swan et al., 2005), and has advantages over the nested PCR, as it involves a single step, been performed in a closed system, which reduces the contamination, reduce handling of toxic reagents and agarose gel electrophoresis, the results are obtained more faster than those from a nested PCR and this technique allows the detection and quantification of parasites (Perandin et al., 2004).

1.9 Rationale and Justification

In the recent years, there was a great potential for traveling across the country due to the feasibility of the transportation, seeking jobs or coming to the irrigated areas for work during the rainy seasons. *P. falciparum* is endemic species in the area. Recently patients attending Wad medani hospitals and its peripheries showed the presence of *P. vivax* species, which was not reported in Al Gezira area.

The difficulty in maintaining competent microscopists for malaria diagnosis prompted the development of non-microscopic alternatives, to be clinically useful and to overcome diagnostic microscopy limitations, such a test should be rapid, easy to handle, highly sensitive and detecting low levels of parasitemia (Playford and Walker 2002).

1.10 The objectives of the study

1.10.1 The general objectives
The aims of the present study was to evaluate the most efficient and sensitive diagnostic methods for rapid diagnosis of *P. falciparum* and *P. vivax* in malaria endemic areas, Gezira State, Central Sudan.

The goal of the study was to use a qualitative monoplex real-time PCR for the simultaneous detection of *P. falciparum, P. vivax*, and its evaluation on blood samples, with microscopy used as the gold standard comparative method and RDTs used.

### 1.10.2 The specific objectives

- To assess the efficacy of the molecular biology method, real-time PCR, in detection of *P. falciparum* species from samples diagnosed by routine microscopic examination using Giemsa stain and ICT technique.

- To assess the efficacy of the molecular biology method, real-time PCR, in detection of *P. vivax* based on routine microscopic examination using Giemsa stain and ICT technique.

- To detect the sensitivity of species-specific Real-Time PCR in patients showed negative results by routine microscopic examination and ICT; and who were clinically showed malaria symptoms.

- To compare between the species-specific Real-Time PCR, ICT and the routine microscopic techniques in diagnosing malaria species.
Materials and Methods

2.1 Study area and population

The study was conducted between July 2011 to December 2012 at Al Karaiba area Western Wad Medani City, Gezira State, Central Sudan. Malaria in this region, caused mainly by *P. falciparum*, recently few cases of *P. vivax* infections were reported, The area shows seasonal malaria transmission, which occurs mostly in the rainy season from June to November. A total of 200 patients attending Al Karaiba local clinic were recruited in the study based on the following criteria: suspected of having malaria infection, having fever with temperature above 37.5°C at the time of examination and willing to participate in the study.

2.2 Collection of the specimens

Three ml fresh blood sample was collected from each patient, out of it, 2 ml was transferred to EDTA blood containers and mixed gently. Few drops were spotted on Whatman filter paper grade 3, air-dried at room temperature and kept in a zipper plastic bag. The collected blood samples were then stored at −20°C for further ICT analysis and DNA extraction. 160 samples were collected from patients diagnosed as having *P. falciparum* malaria and/or *P. vivax* based on routine microscopic examination on thin smears using Giemsa stain. 40 samples were collected from patients suspected as having malaria and showed negative result for the presence of malaria parasite based on routine microscopic examination on thick and thin blood smears using Giemsa stain.

2.3 Clinical evaluation

A medical history, epidemiological data; name, sex, age, number of malaria attacks per year and physical examination for each study subject was recorded. The study protocol was reviewed and approved by the Department of Medical Parasitology, Faculty of Medical Laboratory Sciences, University of Gezira - Sudan. Informed consent for participation in the study was obtained from each patient or legal guardians in case of children.

2.4 Microscopic examination

Microscopy of thick and thin Giemsa-stained smears was considered the reference method. Blood films were prepared from peripheral blood. The slides were stained with Giemsa and...
screened for the presence of parasites and identification of parasite species. Stained blood films were examined with (a 100×) oil immersion lens.

Parasite density was determined by counting the parasites and leucocytes, assuming 8,000 leucocytes/μL of blood (WHO 2010). Smears were considered negative if no parasite was seen in 100 oil immersion fields on a thick blood film. All the slides were double checked blindly by a second, independent microscopist and the results were combined.

2.5 RDTs for malaria diagnosis

All 200 samples in this study were examined by the RDTs, SD Bioline Malaria Ag Pf/Pv (Cat. No. 05FK80, Standard Diagnostics, Inc., Korea). The Pv/Pf test device is based on PfHRP2 antigen and Pv-pLDH antigen, which are specific for *P. falciparum* and *P. vivax*, respectively. Five microlitres of fresh whole blood was added to the card pad, and three drops of specific lying agent was added. The RDT result was read in 15–20 minutes according to the manufacturer’s instructions and immediately recorded. The test was considered valid when the control line on the immune-chromatographic test strip was shown. It was counted as *P. falciparum*-positive if the line detecting *P. falciparum*-specific PfHRP2 was positive or *P. vivax*-positive if the line detecting *P. vivax* specific Pv-pLDH was positive and count positive for the two species if both specific test lines were positive, along with the control line. The test device was counted invalid if the line detecting the control did not show positive. The readers of RDTs were blinded to the results of the microscopy.

2.6 Diagnosis by PCR

2.6.1 DNA extraction for blood parasites from filter paper

Parasite's genomic DNA was extracted from dried blood spots using QIAamp DNA Mini Kit (Qiagen, Germany), catalog No. 51304.

The lyses buffer (ATL), binding buffer (AL), elution buffer (AE) and proteinase K solution was supplied ready-to-use.

Washing buffer 1 (AW1) and washing buffer 2 (AW2) was made ready for use by adding the specific volume of ethanol as recommended by the manufacturer and labeled as ethanol added.

2.6.1.1 Lyses of blood spots
Three blood spots, about 3mm in diameter each, was cut from dried blood spot on filter paper and was put in eppendorf tube (1.8 ml). 180 μl of ATL buffer and 20 μl protinase k was added, vortexes, incubated at 95°C for 1 hour and vortexes 3-4 times during incubation. After incubation the content was briefly centrifuged. 200 μl AL buffer was added, vortexes and incubated at 95°C for 10 minute. Samples after lyses and incubation were briefly centrifuged.

200 μl Ethanol (96-100%) was added, vortexes and brief centrifuged

2.6.1.2 DNA Binding
The mixture was applied to spin column and centrifuged at 8000 runs per minutes (rpm) for 1 minute. The filtrate was discarded and the column was place in a clean collection tube.

2.6.1.3 Washing
500 μl of AW1 buffer was added and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and the column was place in a clean collection tube. 500 μl of AW2 buffer was added and centrifuged at 14000 rpm for 3 minutes. The filtrate was discarded; the column was placed in a clean collection tube and centrifuged at 14000 rpm for 1 minute. The filtrate was discarded and the column was place in a clean collection tube.

2.6.1.4 DNA Elution
50 μl of AE buffer was added, incubated at room temperature for 5 minutes and centrifuged for 8000 rpm 1 minute. The above step was repeated and the DNA was recovered.

2.6.2 Conventional PCR screening for the 18S rRNA gene in malaria parasite for the four plasmodial species
For screening purposes, Primer sequence was selected from the small subunit of rRNA (18S). A forward primer, rPLU5 (5’- CCT GTT GTT GCC TTA AAC TTC -3’), and a reverse primer, rPLU6 (5’- TTA AAA TTG TTG CAG TTA AAA CG -3’), was designed to amplify 1100 bp segment of the four plasmodial 18S genes.

The reaction was performed in a final volume of 25 μl containing 5 μl of DNA 1 μl of 200 nM concentration of each forward primer (rPLU5) and 1μl reverse primer (rPLU6), 12.5 μl master mix (Emerald Amp GT PCR Master Mix, Japan) 0.1μl Taq DNA polymerase and 5.4 μl double distilled water. The reaction was performed under the following conditions: initial step (95°C for 3 min) and 35 cycles (Denaturation at 94°C for 60 seconds, annealing
at 50°C for 30 seconds and extension at 72°C for 2 min) and a final extension at 72°C for 7 minutes.

The PCR products were separated in a 2% agarose gels after electrophoresis at 200 V for 40 minutes. DNA bands were stained with Ethidium bromide and visualized under a UV light. Known positive samples from previous malaria diagnosed individuals and negative samples from uninfected individuals were used as controls. The primers and expected sizes of the PCR fragments of the SSU rRNA genes are shown in (Table 2.1).

Table 2.1: Primers based on the 18S rRNA gene in malaria parasite for the screening of the four plasmodial species

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer</th>
<th>Sequence (5' – 3')</th>
<th>Size (bp) of PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium sp.</td>
<td>rPLU5</td>
<td>5’-CCT GTT GTT GCC TTA AAC TTC-3’</td>
<td>1,100</td>
</tr>
<tr>
<td></td>
<td>rPLU6</td>
<td>5’-TTA AAA TTG TTG CAG TTA AAA CG-3’</td>
<td></td>
</tr>
</tbody>
</table>


2.6.3 Plasmodium species-specific real-time PCR

The Plasmodium species-specific real-time PCR is a monoplex PCR for the determination of the two Plasmodium species. Primers sequence was shown on (Table 2.2). Amplification and detection of the amplified product were performed on a LightCycler®2.0 (Serial Number 1415000) Roche Applied Science (Germany). The reaction was performed in a final volume of 20 μl containing 5 μl of DNA, 1 μl of 200 nM concentration of each parasite species-specific forward and reverse primer set, 1 μl of 200 nM concentration of each corresponding probe, 7.4 μl enzyme, 1.3 μl magnesium chloride and 3.3 double distilled water. The reaction was performed under the following conditions: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each reaction of the monoplex PCRs included one reaction mixture without DNA as a negative control and DNA positive samples; that diagnosed and the positivity was confirmed to the species level; was added for each reaction as positive controls in order to facilitate sensitivity assessment.

Each reaction of the monoplex PCRs was performed in triplicate in order to assess the reproducibility, and the same conditions were used for the amplification steps as well as for the interpretation of a positive versus negative result. The sample was considered positive by identifying the threshold cycle number (Ct) at which normalized reporter dye emission raised above background noise. If the fluorescent signal did not increase within 40 cycles (Ct 40), the sample was considered negative.
Table 2.2 Primers sequence for Plasmodium species-specific real-time PCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer or probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. falciparum</td>
<td>FAL-Forward</td>
<td>(5′-CTTTTGGAGAGTTTTTTTACTTTTGAGTAA-3′)</td>
</tr>
<tr>
<td></td>
<td>FAL-Reverse</td>
<td>(5′-TATTTCCATGCTGATGTATTCAAACACAA-3′)</td>
</tr>
<tr>
<td></td>
<td>probe (FAM)</td>
<td>(5′-TGTTCAACAGACGGGTAGATGATTGAGTTCA-3′)</td>
</tr>
<tr>
<td>P. vivax</td>
<td>VIV-Forward</td>
<td>(5′-ACGCTTCTAGCTTAATCCACATAACT-3′)</td>
</tr>
<tr>
<td></td>
<td>VIV-Reverse</td>
<td>(5′-ATTATCTCAANAGTAACAGGACTTCAAGC-3′)</td>
</tr>
<tr>
<td></td>
<td>probe(TET)</td>
<td>(5′-TTCGTATCGACTTTTGCGCATTTTC-3′)</td>
</tr>
</tbody>
</table>

2.7 Sensitivity of the real-time PCR

The sensitivity of primer and probe sets was assessed on 10-fold serial dilutions of positive control DNAs to test the efficiency of the amplification when the relative amount of human DNA increased. Different concentrations of the control DNA from each species were mixed and tested by the Plasmodium species-specific real-time PCR to ascertain the ability of the assay to co-amplify different DNA targets in order to detect mixed infections.

2.8 Specificity of the real-time PCR

The specificity of the genus-conserved Plasprobe was evaluated by testing a DNA of other 18S-possessing eukaryotes, a human genomic was performed in duplicate and a negative control that contains no template was also tested.

2.9 Statistical analysis

Statistical analysis was performed by the Chi-square test using the Statistical Package for Social Science (SPSS) software version 16.0 and the significance level was set as < 0.05. Sensitivity was calculated as the number of true positive results divided by the sum of true positives and false negatives multiplied by 100.
Specificity was calculated as the number of true negatives results divided by the sum of true negatives and false positives multiplied by 100.

κ values expressed the agreement beyond chance (Altman 1991) and were calculated with a 95% confidence interval (CI). A κ value of 0.21 to 0.60 is moderate, a κ value of 0.61 to 0.80 is good, and a κ value over 0.80 is an almost perfect agreement beyond chance.

2.10 Ethical approval

Ethical approval for the study was obtained from the Research Board at the Faculty of Medical Laboratory Sciences, University of Gezira and from the State Ministry of Health, Wad Medani, Sudan. The study received ethical clearance.
Results

3.1 Study subjects

Hundred and sixty samples positive for *P. falciparum* malaria, and 40 samples negative for *p. falciparum* malaria, using the microscopy, were included in the study.

The mean age of the study subjects was (25.3 ± 13.8) years; the minimum age was 1 year old and the maximum age was 79 years old.

The pattern of the study subjects according to sex was shown in (Fig 3.1), 145 (72.5%) were males and 55 (27.5 %) were females. The highest incidence of the disease was among the age group (22 – 28) years (Fig 3.2).
Fig 3.1 The distribution of the study subjects according to sex
3.3 The degree of parasitemia of the study subjects

The degree of parasitemia in the study subjects was shown in (Fig 3.4). 68 (34.0 %) of them with moderate parasitemia (500 - <5000 parasites/µl) which showed the higher frequency in the study subjects, 44 (22.0 %) of them were the high parasitemia (5000 - <50000 parasites/µl), 39 (19.5 %) of them were the very high parasitemia (≥250000 parasites/µl), 9 (4.5 %) of them were the lower parasitemia (50 - <500 parasites/µl), and the negative samples represented 40 (20.0 %) of the study subjects.
3.4 The frequency of positive and negative results in the different diagnostic methods used in the study

The frequency of positive cases by microscopy was 80% (160/200), ICT falciparum was 73.5% (147/200) and ICT vivax was 5.5% (11/200).

The frequency of negative cases by microscopy was 20% (40/200), ICT falciparum was 26.5% (53/200) and ICT vivax was 94.5% (189/200) (Fig 3.5).
Fig 3.5 The frequency of positive results of microscopy, ICT *falciparum* and ICT *vivax*
3.5 The frequency of positive results by conventional PCR and Real-Time PCR

The frequency of positive results by conventional PCR was 185 (92.5%), real-time PCR for *P. falciparum* was 152 (76%) and real-time PCR for *P. vivax* was 33 (16.5%) samples (Fig 3.6).
Fig 3.6 The frequency of positive results of the conventional PCR and Real-Time PCR.
3.6 Microscopy as a diagnostic method

The total microscopically positive samples included in the study were 160 samples (80%), positive for *P. falciparum* malaria, and 40 samples (20%) were negative by microscopy. There was no mixed infection.

3.6.1 The microscopy and the conventional PCR results

Hundred and eighty five out of 200 samples (92.5%) were found positive for malaria parasite and 15 samples (7.5%) were found negative by conventional PCR.

Hundred and sixty out of 200 samples (80%) were positive by both microscopy and the conventional PCR, whereas 15 samples (7.5%) were negative by both methods, giving a concordance rate of 87.5% (175 of 200) between microscopy and the conventional PCR. 25 samples (12.5%) out of the 40 negative microscopy samples were found positive by conventional PCR method.

The result showed significant difference between the microscopy method and the conventional PCR method (*P*-value <0.05), (κ-value: 0.49) with 86.5% sensitivity, 100 % specificity and 87.5% agreement (Table 3.1).
Table 3.1 Microscopy and the conventional PCR results

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>Conventional PCR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>160</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
<td>15</td>
</tr>
</tbody>
</table>

\( \chi^2 = 64.86, \quad df = 1, \quad P\text{-value} = 0.000 \)

Sensitivity 86.5\%, specificity 100\%, agreement 87.5\%  \( \kappa: 0.49 \)

3.6.2 The microscopy and the *P. falciparum* species-specific real-time PCR results

The total positive samples by *P. falciparum* species-specific real-time PCR were 152 samples (76\%) and the negative samples were 48 (24\%).

Hundred and forty two samples out of 200 (71\%) were positive for *P. falciparum* by both microscopy and the *P. falciparum* species-specific real-time PCR and 30 samples (15\%) were negative by both methods, giving a concordance rate of 86\% (172 of 200) between microscopy and the *P. falciparum* species-specific real-time PCR. 18 samples (9\%) which were positive for *P. falciparum* by microscopy, were found negative by real-time PCR for *P. falciparum*. 10 samples (5\%) out of the 40 (20\%) negative samples for *P. falciparum* by microscopy, were found positive by *P. falciparum* species-specific real-time PCR.

The result showed a significant difference between the microscopy and *P. falciparum* species-specific real-time PCR methods (\( P\text{-value} <0.05 \)), (\( \kappa\text{-value}: 0.59 \)) with 88.8\% sensitivity, 75\% specificity and 86\% agreement (Table 3.2).
Table 3.2 Microscopy and *P. falciparum* species-specific real-time PCR results

<table>
<thead>
<tr>
<th><em>P. falciparum</em> species-specific RTPCR</th>
<th>Microscopy</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>142</td>
<td>10</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>30</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>40</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

\( (\chi^2 = 71.299, \text{ df } = 1, \text{ P-value } = 0.000) \)

Sensitivity 88.8%, specificity 75%, agreement 86% \( \kappa: 0.59 \)

3.6.3 The microscopy and the *P. vivax* species-specific real-time PCR results

The total positive samples for *P. vivax*, by *P. vivax* species-specific real-time PCR, were 33 samples (16.5%) and the negative samples were 167 samples (83.5%).

Eighteen samples (9%) were found positive by both microscopy and *P. vivax* species-specific real-time PCR, whereas 25 samples (12.5%) were negative by both methods.

Fifteen samples (7.5%) out of the 40 negative samples by microscopy, were found positive for *P. vivax*, by *P. vivax* species-specific real-time PCR. 142 samples (71%) which were positive for *P. falciparum* by microscopy, were found negative for *P. vivax* by *P. vivax* species-specific real-time PCR.

The result showed a significant difference between the microscopy and the *P. vivax* species-specific real-time PCR methods (P-value = 0.006), with 11.3% sensitivity, 62.5% specificity (Table 3.3).
Table 3.3 Microscopy and the *P. vivax* species-specific real-time PCR results

| *P. vivax* species- | Microscopy |
|---|---|---|
| specific RTPCR | Positive | Negative | Total |
| Positive | 18 | 15 | 33 |
| Negative | 142 | 25 | 167 |
| Total | 160 | 40 | 200 |

\( \chi^2 = 16.004, \quad df = 1, \quad P\text{-value} = 0.006 \)

Sensitivity 11.3%  
Specificity 62.5%

### 3.7 RDT as a diagnostic method

The 160 positive samples (80%) by microscopy, 158 samples (79%) were positive by RDT, out of it, 147 samples (73.5 %) were *P. falciparum* positive, 11 samples (5.5 %) were *P. vivax* positive and there was no mixed infection of *P. falciparum* and *P. vivax* (Table 3.5, Table 3.8).

#### 3.7.1 The microscopy and the ICT for *P. falciparum* results

Hundred and forty four samples (72%) were positive for *P. falciparum*, by both microscopy and the ICT for *P. falciparum*, whereas 37 samples (18.5%) were negative by both methods, giving a concordance rate of 90.5% (181 of 200) between the ICT for *P. falciparum* and microscopy. 16 samples (8%) which were positive for *P. falciparum* by microscopy, were found negative for *P. falciparum* by ICT for *P. falciparum*. 3 samples (1.5%) which were negative for *P. falciparum* by microscopy were found positive by ICT for *P. falciparum*. The result showed a significant difference between the microscopy and the ICT for *P. falciparum* methods (P-value <0.05), (κ-value: 0.74) with 90% sensitivity, 92.5 specificity and 90.5% agreement (Table 3.4).
Table 3.4 The ICT for *P. falciparum* and the microscopy results

<table>
<thead>
<tr>
<th>ICT for <em>P. falciparum</em></th>
<th>Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>144</td>
</tr>
<tr>
<td>Negative</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
</tr>
</tbody>
</table>

$(\chi^2 = 1.118, \quad df = 1, \quad P\text{-value} = 0.000)$

Sensitivity 90%, specificity 92.5%, agreement 90.5% $\kappa$: 0.74

### 3.7.2 The conventional PCR and the ICT for *P. falciparum* results

Hundred and forty seven samples (73.5%) were found positive for *P. falciparum* by both ICT for *P. falciparum* and the conventional PCR, 15 samples (7.5%) were negative by both methods, giving a concordance rate of 81% (162 of 200) between the ICT for *P. falciparum* and conventional PCR. 38 samples (19%) which were negative for *P. falciparum* by ICT for *P. falciparum* method, were found to be conventional PCR positive. The result showed a significant difference between the ICT for *P. falciparum* and conventional PCR methods ($P\text{-value} <0.05$), ($\kappa$-value: 0.37) with 79.5% sensitivity, 100% specificity and 81% agreement (Table 3.5).
Table 3.5 The pattern of ICT for *P. falciparum* the conventional PCR results

<table>
<thead>
<tr>
<th>ICT for</th>
<th>Conventional PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>147</td>
</tr>
<tr>
<td>Negative</td>
<td>38</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
</tr>
</tbody>
</table>

\( \chi^2 = 44.98, \quad df = 1, \quad P\text{-value} = 0.009 \)

Sensitivity 79.5%, specificity 100%, agreement 81% κ: 0.37

3.8 Species specific real-time PCR as a gold standard method
The total positive samples detected by the real-time PCR were 185 samples (92.5%), positive for *P. falciparum* and *P. vivax*. 15 samples (7.5%) were negative for *P. falciparum* and *P. vivax* when examined by the real-time PCR (Table 3.6, Table 3.8). The pattern of positive and negative results by the real-time PCR was shown in figure 4.1 and 4.2.

3.8.1 The *P. falciparum* species-specific real-time PCR and conventional PCR results
Hundred and fifty two samples (76%) were positive by both *P. falciparum* species-specific real-time PCR and the conventional PCR, 15 samples (7.5%) were negative by both methods, giving a concordance rate of 83.5% (167 of 200) between *P. falciparum* species-specific real-time PCR and the conventional PCR. 33 samples (16.5%) which were positive by the conventional PCR method were found to be negative by *P. falciparum* species-specific real-time PCR method.

The result showed a significant difference between the *P. falciparum* species-specific real-time PCR and the conventional PCR methods (\(P\text{-value} < 0.05\)), (κ-value: 0.41) with 82.2% sensitivity, 100 % specificity and 83.5% agreement (Table 3.6).
Table: 3.6 The *P. falciparum* species-specific real-time PCR and conventional PCR results

<table>
<thead>
<tr>
<th><em>P. falciparum</em> species-specific RTPCR</th>
<th>Conventional PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>152</td>
</tr>
<tr>
<td>Negative</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
</tr>
</tbody>
</table>

\[(\chi^2 = 51.4, \ df = 1, \ P-value = 0.000)\]

Sensitivity 82.2\%, specificity 100\%, agreement 83.5\%  \(\kappa: 0.41\)

3.8.2 The *P. falciparum* species-specific real-time PCR and ICT for *P. falciparum* results

Hundred and forty five samples (72.5\%) were found positive by both *P. falciparum* species-specific real-time PCR and ICT for *P. falciparum*, 46 samples (23\%) were negative by both methods, giving a concordance rate of 95.5\% (191 of 200) between *P. falciparum* species-specific real-time PCR and ICT for *P. falciparum*.

Seven samples (3.5\%) which were negative for *P. falciparum* by ICT for *P. falciparum* were found positive by *P. falciparum* species-specific real-time PCR method. Two samples (1\%) which were positive for *P. falciparum* by ICT for *P. falciparum* were found negative by *P. falciparum* species-specific real-time PCR method.

The result showed a significant difference between the *P. falciparum* species-specific real-time PCR method and the ICT for *P. falciparum* method (\(P\)-value < 0.05), (\(\kappa\)-value: 0.88) with 98.6\% sensitivity, 86.8\% specificity and 95.5\% agreement (Table 3.7).
3.7 The *P. falciparum* species-specific real-time PCR and ICT for *P. falciparum* results

<table>
<thead>
<tr>
<th><em>P. falciparum</em> species-specific RTPCR</th>
<th>ICT for <em>P. falciparum</em></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>145</td>
<td>7</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>46</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>147</td>
<td>53</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

\( \chi^2 = 1.559, \quad df = 1, \quad P\text{-value} = 0.007 \)

Sensitivity 98.6\%, specificity 86.8\%, agreement 95.5\% \( \kappa: 0.88 \)

3.8.3 The *P. vivax* species-specific real-time PCR and ICT for *P. vivax* the results

Eleven samples (5.5\%) were found to be positive for *P. vivax* by both *P. vivax* species-specific real-time PCR and ICT for *P. vivax*, 167 samples (83.5\%) were negative by both methods, giving a concordance rate of 89\% (178 of 200) between *P. vivax* species-specific real-time PCR and ICT for *P. vivax*.

Twenty two samples (11\%) which were negative for *P. vivax* by ICT for *P. vivax* method, were found to be positive by *P. vivax* species-specific real-time PCR.

The result showed a significant difference between the *P. vivax* species-specific real-time PCR and ICT for *P. vivax* methods (\( P\text{-value} <0.05 \)), (\( \kappa\text{-value}: 0.46 \)) with 100 \% sensitivity, 88.4\% specificity and 89\% agreement (Table 3.8).
Table: 3.8 The *P. vivax* species-specific real-time PCR and ICT for *P. vivax* the results

<table>
<thead>
<tr>
<th><em>P. vivax</em> species-specific RTPCR</th>
<th>ICT for <em>P. falciparum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
</tr>
</tbody>
</table>

\( \chi^2 = 58.9, \quad df = 1, \quad P-value = 0.000 \)

Sensitivity 100\%, specificity 88.4\%, agreement 89\%  \( \kappa: 0.46 \)
Fig: 3.7 The positive results and a positive control by the real-time PCR
Fig: 3.8 The positive results and a negative control by the real-time PCR
DISCUSSION

4.1 Study area and population
People living at Al Karaiba area Western Wad Medani City in irrigated areas Gezira scheme regions that shows seasonal malaria transmission, the rainfall during autumn season make the area rich with water resources that lead to the multiplication of the malaria vector that transmits the malaria disease, mainly P. falciparum species.

At Al Karaiba area there is a central animal market, the area is inhabited with animal breeders coming from Eastern and Central Sudan.

Hundred and sixty samples positive for P. falciparum malaria, confirmed using the microscopy and 40 patients whom show clinically malaria symptoms but their blood samples showed negative microscopy results for P. falciparum malaria were included in the study.

The minimum age was 1 year old and the maximum age was 79 years old.

The pattern of the study subjects according to sex was shown in (Fig 3.1), 145 (72.5%) were males and 55 (27.5 %) were females. The highest incidence of the disease was among the age group (22 – 28) years that might be due to the fact that this age group is the most frequent in the area where dealing with animal breeding and working in farms in the area (Fig 3.2).

4.2 The distribution of the different tribal stocks in the study
There were nine different tribal stocks included in the study subjects.

Juhaina Arab group had the higher frequency among the study subjects (26.5 %), the tribes might be genetically susceptible to malaria infection and seeking the local clinic for treatment not having the habit of self medication as well as they were more represented in the population in the study area. The Moraccoan and Egyptian group, and Nilotic group were the least represented in the study it might be they were less presented in the study area (Fig 3.3) (Appendix 2).

4.3 Comparison of microscopy to RDT method
Many of the hospitalized pregnant women with acute illness in Medani Hospital - Sudan, whom were considered positive for malaria by light microscopy showed no evidence of malaria when re-examined with experienced micoscopist (Elhassan ME et al., 2010).

As it is difficult to maintain a competent microscopists for malaria diagnosis, non-microscopic alternatives is needed to overcome diagnostic microscopy limitations. It should be rapid, sensitive, easy to perform and able to detect low levels of parasitemia (Playford, E. G., and J. Walker. 2002).
WHO recommends that patients of all ages with suspected malaria should receive a parasitological confirmation of diagnosis by either microscopy or RDT (WHO 2011). The Sudanese National Malaria Control Programme recommends the use of RDT in areas where no expert microscopy is available (Elmardi KA et al., 2009) putting in mind that the diagnostic accuracy of RDTs can be affected by quality of the products, storage temperature, humidity, and users' performance (WHO 2008), moreover, the sensitivity of malaria RDT decreases with the decrease level of the parasitaemia (Ratsimbasoa A et al., 2008).

The frequency of positive cases for *P. falciparum* by microscopy was 80%, and the negative was 20%. The same samples when screened by ICT methods showed a different result in species differentiation, only 73.5% were positive for *P. falciparum* by ICT for *P. falciparum* and 5.5% were positive for *P. vivax* by ICT for *P. vivax* methods (Fig 3.5), which gives a clue that the microscopist has missed some *P. vivax* species and reported all the samples as positive for *P. falciparum*. ICT for *P. falciparum* and ICT for *P. vivax* methods were able to differentiate between the two species.

The current study showed acceptable sensitivity (90%) and specificity (92.5%) for diagnosing *P. falciparum* malaria when compared to microscopy method. The performance of the RDT kit for malaria diagnosis used in the current study is in agreement with the findings of Yan, Li et al., who showed that RDT kits had a sensitivity of 88.6% for detecting *P. falciparum* infections (Yan, Li et al., 2013).

A higher sensitivity (98.8%) of RDTs compared to microscopy for detection of *P. falciparum* has been obtained by ICT Paracheck in a study conducted by Alam, Mohon et al., 2011, and in a study during a nationwide school survey in Kenya, when three RDT brands were compared with expert microscopy, RDT showed overall sensitivity of 96.1% and specificity of 70.8% (Gitonga, Kihara et al., 2012), also the RDTs showed 96.8% sensitivity in a study among febrile pregnant women in a hyper-endemic region in Uganda for the diagnosis of *P. falciparum* malaria, when compared with microscopy as the gold standard (Kyabayinze DJ et al., 2011).

The 100% sensitivity and 94.3% specificity of the ICT versus direct microscopy was shown in a study conducted in the Ahmed Gasim Hospital Khartoum North, Sudan among blood donors screened for malaria parasites (Ali, Yousif et al., 2005).

Also 100% sensitivity of the ICT versus direct microscopy corresponds to the results in India, France, and in Senegal (Singh B. et al., 1996, Cavallo JD et al., 1997, Caye O et al., 1998).
4.4 Comparison of molecular methods to RDTs

Chou, Monidarin et al., in their study using a real-time PCR as reference method, showed that the best performances of VIKIA Malaria Ag Pf/Pan™ test for both *P. falciparum* and non-*P. falciparum* detection were observed at 20–30 minutes with 93.4% sensitivity and 98.6% specificity for *P. falciparum* (Chou, Monidarin et al., 2013) and this is in agreement with similar studies by Playford EG et al., 2002, Grobusch MP. et al., 2003 in travellers returning to Berlin, Germany, and Hopkins H et al., 2008 in Uganda and Ratsimbasoa A et al., 2008.

In a study done at China-Myanmar endemic borders, the CareStart™ ICT kit’s for the diagnosis of malaria showed 88.52% sensitivity, 98.26% specificity for *falciparum* malaria and 90.77% sensitivity, 100% specificity for *vivax* malaria (Xiaodong et al., 2013).

4.4.1 ICT for *P. falciparum* as screening method

When the samples were screened with conventional PCR for the presence of plasmodial species, ICT for *P. falciparum* showed modest sensitivity (79.5%) and full specificity (100%) when conventional PCR was used as the reference standard method (Table 3.5), which agreed in full specificity (100%) with a study reported for RDT kits used during pregnancy in Uganda, and disagreed with the same study in low sensitivity (31.8%) reported, when PCR was used as the gold standard (Dhorda M. et al., 2012).

The performance of the RDT kit versus conventional PCR in the current study was found slightly more sensitive and full specific (79.5% sensitivity, 100% specificity) when compared with a study done in Kassala, Eastern Sudan among febrile, suspected of uncomplicated malaria patients, found to have a sensitivity of 69% and specificity of 84% when PCR was compared to RDT (Osman MM et al., 2010).

4.4.2 ICT for *P. vivax* as species differentiation method

When the *P. vivax* species-specific real-time PCR method was used as gold standard, the ICT for *P. vivax* failed to detect 22 samples out of the 33 positive samples detected by the *P. vivax* species-specific real-time PCR method (Table 3.8), and that could be technical error in reading the test card at the specific time mentioned by the manufacturer, or the faint unclear line in the card test line was been considered as negative due to the low parasitemia. All the 167 samples (83.5%) that was detected negative by the ICT for *P. vivax* method was confirmed negative by the *P. vivax*
species-specific real-time PCR method, there was no samples which was negative by ICT for \(P. \textit{vivax}\) method found positive by the \(P. \textit{vivax}\) species-specific real-time PCR method (a full specificity, 100%).

A better sensitivity (66.7%) of ICT for screening malaria parasites and 94.9% specificity was found in a study conducted among blood donors, in Khartoum state, Sudan that applied PCR as a standard technique (Ali, Yousif \textit{et al.} 2005), and in a study conducted by Yan, Li \textit{et al.}, the sensitivity of Pf/Pan test for \(P. \textit{falciparum}\) was 81.7% and for \(P. \textit{vivax}\) was 64.6% (Yan, Li \textit{et al.}, 2013).

\textbf{4.4.3 Molecular methods in species differentiation}

A study comparing the \(P. \textit{falciparum}\) species-specific real-time PCR to ICT for \(P. \textit{falciparum}\), \(P. \textit{falciparum}\) species-specific real-time PCR showed 98.6% sensitivity, 86.8% specificity and 95.5% agreement when it was used as gold standard.

This result disagreed with the studies that were reported by Alam, Mohon \textit{et al.}, 2011, comparing RDTs to real-time PCR assay for detection of \(P. \textit{falciparum}\), RDT gave a higher sensitivity of (97.6%) than the real-time PCR assay and also in another study reported 83.3% sensitivity and 92.0%, specificity of RDT evaluation as compared with PCR as the gold standard (Kashif, Adam \textit{et al.}, 2013). A low sensitivity (76.9%) with very high specificity (100%) was obtained of RDTs compared to real-time PCR assay (Alam, Mohon \textit{et al.}, 2011).

\textbf{4.5 Comparison of microscopy to molecular methods}

When the samples were screened for the four Plasmodial species by conventional PCR, the frequency of positive results was 185 (92.5%), which indicates that some of the negative microscopy samples were positive by conventional PCR (Table 3.1).

For species differentiation by \(P. \textit{falciparum}\) real-time PCR, 152 (76%) were positive for \(P. \textit{falciparum}\) (Table 3.6). 33 (16.5%) were positive for \(P. \textit{vivax}\) by \(P. \textit{vivax}\) real-time PCR (Table 3.6), which confirmed that the microscopist has missed the diagnosis of some \(P. \textit{vivax}\) and some \(P. \textit{falciparum}\). Missing the diagnosis of some negative samples may be due to low parasitemia, that was clear during amplification carves, some samples show weak positive in the amplification (Fig 3.6).
out of the 200 samples 185 were found positive and 15 samples were found negative by conventional PCR. 160 samples were found positive by both microscopy and the conventional PCR, whereas 15 samples were negative by both methods, giving a concordance rate of 87.5%. 25 samples out of the 40 negative microscopy samples were found positive by conventional PCR method.

The result showed significant difference between the microscopy method and the conventional PCR method (\(P\)-value <0.05), (\(\kappa\)-value: 0.49) with 100% sensitivity, 92.5% specificity and 87.5% agreement (Table 3.1).

In a study among 606 samples the sensitivity of microscopy for \(P. falciparum\) was 71.0% and for \(P. vivax\) was 73.3% if the PCR was used as the gold standard (Yan, Li et al., 2013).

### 4.5.1 The microscopy and the \(P. falciparum\) species-specific real-time PCR results

There is a need for reliable malaria diagnostic tests, sensitive and specific to replace the microscopy the current gold standard test. The use of a molecular-based assay for detecting \(Plasmodium\) parasites would be an excellent choice to avoid false-negative microscopy results (Berry A. et al., 2008). Out of the 80% positive microscopy samples for \(P. falciparum\), 71% of the samples were confirmed positive for \(P. falciparum\) by the \(P. falciparum\) species-specific real-time PCR, with 88.8% sensitivity, 75% specificity and 86% agreement and a significant difference between the two methods (\(P\)-value <0.05) (Table 3.2), and the same 71% samples that were confirmed positive for \(P. falciparum\) samples were found negative for \(P. vivax\) by \(P. vivax\) species-specific real-time PCR, which confirmed that there was no mixed infection in the study samples (Table 3.3).

A high sensitivity (94.1%) and 100% specificity of real-time PCR ssrRNA method for \(P. falciparum\) and a full sensitivity for \(P. vivax\) (100%) with 99.1% specificity was reported by Mangold et al., when microscopy was used as gold standard (Mangold et al., 2005). A similar finding of a high sensitivities was detected in a study compared the microscopy to real-time PCR methods, showed a sensitivity of 97% and 97% specificity for the detection of \(P. falciparum\) (Alam, Mohon et al., 2011) and sensitivity of 96.6% and specificity of 89.4% was reported by Boonma et al. 2007. A slightly higher sensitivity of 98.5% and specificity of 94.3% was reported by Han et al. 2007 and 99.41% sensitivity and specificity 90.88% was reported by Khairnar et al. 2009.

### 4.5.2 The microscopy and the \(P. vivax\) species-specific real-time PCR results
Fifteen (9%) of the 40 negative samples (20%) for *P. falciparum* by microscopy were confirmed positive for *P. vivax* by the *P. vivax* species-specific real-time PCR methods (Table 3.3).

Out of the 40 negative samples (20%) by microscopy for *P. falciparum*, 5% of the samples were confirmed positive for *P. falciparum* by the *P. falciparum* species-specific real-time PCR (Table 3.2), where as 7.5% of the samples were confirmed positive for *P. vivax* by the *P. falciparum vivax* species-specific real-time PCR methods (Table 3.3), which conclude that the microscopist wasn’t able to distinguish; at the species level; the *P. vivax* from the positive samples, reporting all the samples positive for *P. falciparum*.

Also the microscopist had missed the diagnosis of *P. falciparum* and *P. vivax* from the 20% negative samples (Fig 3.2), that might be due to the very low parasitemia in these samples, as what was shown on the amplification curves by species-specific real-time PCR methods, or when the parasites have lost their shape after the beginning of treatment or in case of hyper-parasitemia, he did not follow the WHO criteria in screening 100 field in the microscopy slides before reporting the result as positive or negative, immediately discarding the microscopy slide, reporting the sample as positive without carefully observing to differentiate species or to determine mixed infection if any. A better sensitivity 77.8% and 84.9% specificity of the RTD compared to microscopy were detected in a study conducted in Kassala area, Eastern Sudan (Osman MM et al., 2010).

4.6 CONCLUSION

Compared to species-specific real-time PCR, both microscopy and RDTs had lower sensitivities. The species-specific real-time PCR used in this study provided a more accurate, sensitive and specific in the differentiation of the *P. falciparum* and *P. vivax* than light microscopy. It is useful in asymptomatic infections, in suspected malaria cases, in detection of malaria parasite with low parasitemia that are missed on light microscopy. Its use in Gezira area, Central Sudan may be limited as a confirmatory tool in reference laboratories due to cost. The use the rapid SD Bioline Malaria Pf/Pv test for diagnosis of malaria and species differentiation in the study could be recommended as a complementary method but not as a primary method and it can be considered as alternative diagnostic tools to microscopy in suspected malaria cases.

Microscopy should be the standard diagnostic tool following the WHO criteria.

4.7 Recommendations
1. There is a need for efficient diagnostic method for malaria parasite; this can be achieved at present by improved quality-control measures of microscopy for accurate diagnosis and species differentiation at Wad Medani hospitals and their periphery clinics, where many patients are coming for medication.

2. State ministry of health laboratories administrative should train microscopists for the four plasmodium malaria species.

3. Microscopists should following the WHO recommendations in reading each microscopic slide, especially slides with low parasitemia.

4. State health authority should screen the area for the presence of \( P. \text{vivax} \), \( P. \text{malarae} \) and \( P. \text{ovale} \) species.

5. Supplying RDT kits with higher sensitivity and good specificity for the diagnosis of different plasmodial species.

6. State health authorities should supply the reference laboratories, in hospitals and medical education centers in the Gezira area, with Real-time PCR machines for screening purpose and for further species screening and differentiation in the area.
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5.2 Appendixes

6.2.1 Appendix 1  Clinical Evaluation Form

Hospital: .................................................................

Individul No: .........................................................

Name: ........................................................................

Referred by DR.: ....................................................

Age: ......................... Yrs ...........................................

Sex: Male ( )  Female ( )  مذكر ( )  مؤنث ( )

Tribe: .................................................................

Marital status: married ( ) single ( )  متزوج ( )  أعزب ( )

Address: ..................................................................

B.F for malaria: Positive ( ) Negative ( )  موجب ( )  سالب ( )

Parasitemia: + ( ) ++ ( ) +++ ( )  كثافة الطفيل: + ( ) ++ ( ) +++ ( )

P. falciparum ( )  P. vivax ( )  بلازموديوم فالسبارم ( )  بلازموديوم فيفاكس ( )

Malaria in last 4 weeks: Yes ( ) No ( )  الملاريا خلال 4 أسابيع مضت: نعم ( )  لا ( )

Fever:  Yes ( ) No ( )  الحمى: نعم ( )  لا ( )

Headache:  Yes ( ) No ( )  الصداع: نعم ( )  لا ( )

Vomiting:  Yes ( ) No ( )  الاستفراغ: نعم ( )  لا ( )

Diarrhea:  Yes ( ) No ( )  الإسهال: نعم ( )  لا ( )

Anti-malaria used (1 week) ........................................

TWBCs count ....................... cells/mm$^3$  عدد كريات الدم البيضاء

Haemoglobin concentration .................... g/dl ................... نسبة الهموغلوبين
5.2.2 Appendix 2 Some of the Sudanese tribal stocks

<table>
<thead>
<tr>
<th>CHAD ORIGIN</th>
<th>NIGERIAN</th>
<th>BEJA</th>
<th>NILOTIC</th>
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<tbody>
<tr>
<td>Bargo</td>
<td>Fellata</td>
<td>Hadandawa</td>
<td>Nuir</td>
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<td>Burno</td>
<td>Fulani</td>
<td>Bani Amir</td>
<td>Shulok</td>
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<td>Hawsa</td>
<td>Bushari</td>
<td>Dainka</td>
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<td>Silihab</td>
<td>Naira</td>
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<tr>
<th>NILE NUBIAN</th>
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<th>NUBA</th>
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<td>Tagalia</td>
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<thead>
<tr>
<th>BAGGARA ARABS</th>
<th>MOROCCAN &amp; EGYPT GROUP</th>
<th>GAALI GROUP</th>
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