

**ASYMPTOMATIC MALARIA IN PREGNANCY: PREVALENCE OF  
PERIPHERAL PARASITEMIA AND ANEMIA, IN KINSHASA,  
DEMOCRATIC REPUBLIC OF THE CONGO**

**Submitted by**

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**As part fulfilment of the requirements for award of the degree of Master of  
Science in One Health Molecular Biology, Sokoine University of Agriculture,**

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## ABSTRACT

**Background:** Malaria infection in pregnancy is an enormous public health problem in Sub-Saharan Africa. In areas of high malaria transmission, *P. falciparum* infection during pregnancy is usually characterized by malaria related anemia in the mother and placental malaria. This situation has been associated with poor pregnancy outcomes across many populations. The aim of this study was to determine the extent of asymptomatic *P. falciparum* infection and its relation with anemia in healthy pregnant women living in Kinshasa, Democratic Republic of the Congo, an endemic area for malaria transmission.

**Methods:** A cross-sectional study was conducted in healthy pregnant women attending prenatal care consultations. Information on socio-demographic characteristics was collected using a questionnaire. *P. falciparum* infection was diagnosed using Rapid diagnostic test (RDT), microscopy and Polymerase Chain Reaction. Hemoglobin concentration was determined at the same time. Bivariate and multivariate logistic regression models were used to determine the association between variables.

**Results:** From 332 pregnant women enrolled, complete RDT and microscopy data was available for 332 blood samples and of these 166 samples were analyzed by PCR. The prevalence of asymptomatic *P. falciparum* infection using microscopy TBS, RDTs and PCR, were respectively 21.6% (95% CI 17.4-26.6%), 27.4% (95% CI 22.7-32.6%) and 29.5% (95% CI 22.7-37.1%). The medium parasite density was 126 (IQR =105-162). Women who spent last night under a mosquito net were less

likely to have asymptomatic *P. falciparum* infection compared to women who did not use a mosquito net (OR 0.1; 95%CI:0.03-0.5,  $p<0.01$ ); being less than 20 years was strongly associated with asymptomatic *P. falciparum* infection (AOR 2.6; 95% CI 1.1-6.1,  $P=0.04$ ) and married women were less likely to have asymptomatic *P. falciparum* infection than single women (AOR 0.3; 95% CI 0.1-0.9,  $p = 0.02$ ). Prevalence of anemia was 61.1%. The likelihood of having anaemia for pregnant women with asymptomatic malaria was about 3.8 times more AOR 3.8 (95% CI 1.0-14.8;  $p=0.04$ ).

**Conclusion:** These alarming results emphasize the need to actively diagnose and treat asymptomatic malaria infection during prenatal care visits, regardless of Intermittent Preventive Treatment with Suldoxine-Pyrimethamine, and to increase efforts in promoting the use of Insecticide Treated Nets in DRC.

### DECLARATION

I, Junior Matangila Rika, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been nor is it concurrently being submitted for a higher degree award in any other university.



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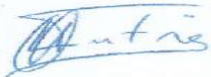
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## **DEDICATION**

To my wife Ornella Matangila Kipasa and our children Ryan and Isaac Matangila who patiently accepted and afforded to have me away, for my studies, even at times when they needed me the most.

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**LIST OF ABBREVIATIONS**

AIDS	Acquired Immunodeficiency Syndrome
ANC	Antenatal Clinic
CHK	Centre Hospitalier de Kingasani
CSA	Chondroitin Sulfate A
DBL	Duffy-Binding Proteins Like
DDT	Dichloro-diphenyl-trichloroethane
DRC	Democratic Republic of the Congo
EDTA	Ethlene Diamine Tetra Acetic Acid
HIV	Human Immunodeficiency Virus
HRP-II	Histidine Rich Protein II
ITN	Insecticide Treated Net
ITNs	Insecticide Treated Nets
IPT	Intermittent Preventive Treatment
IPTp	Intermittent Preventive Treatment in pregnancy
IPTs	Intermittent Preventive Treatments
MoH	Ministry of Health
NaOH	Sodium Hydroxide
NMCP	National Malaria Control Program
NPV	Negative Predictive Value
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

<i>P.f.</i>	<i>Plasmodium falciparum</i>
PNC	Prenatal Clinic
PPV	Positive Predictive Value
RBC	Red Blood Cell
RBL	Reticulocytes-Binding Proteins Like
RDT	Rapid Diagnostic Test
RDTs	Rapid diagnostic Tests
SACIDS	Southern African Center for Infectious Diseases Surveillance
SP	Sulphadoxine Pyrimethamine
Taq	<i>Thermus aquaticus</i>
TBS	Thick Blood Smear
TDRC	Tropical Diseases Research Centre
WHO	World Health Organization



## CHAPTER I

### 1.0 INTRODUCTION

#### 1.1. Background

Malaria infection during pregnancy is an enormous public health problem, with substantial risks for the mother, her foetus and the neonate. In areas of low transmission of *Plasmodium falciparum*, where levels of acquired immunity are low, women are susceptible to episodes of severe malaria, which can result in stillbirths or spontaneous abortion or in the death of the mother (Steketee et al, 1996).

In areas of high transmission of *P. falciparum*, where levels of acquired immunity tend to be high, women are susceptible to asymptomatic infection, which can result in maternal anaemia and placental parasitaemia, both of which can subsequently lead to low birth weight (Nosten et al, 2004). Low birth weight in turn is an important contributor to infant mortality (Sullivan et al, 1999). It has been estimated that malaria during pregnancy is responsible for 5–12% of all low birth weight and 35% of preventable low birth weight and contributes to 75 000 to 200 000 infant deaths each year (Verhoeff F.B et al, 1999).

In malaria-endemic areas, it is estimated that about 25% of pregnant women are infected with malaria, with the greatest risk of infection and morbidity in primiparous adolescents, and those co-infected with HIV (Schantz-Dunn et al, 2009). A recent review of studies, carried out in sub-Saharan Africa between 2000 and 2011, reports that malaria prevalence in pregnant women attending antenatal clinics was 29.5% (95%CI: 22.4–36.5) in East and Southern Africa, and 35.1% (95%CI: 28.2–41.9) in

West and Central Africa, while the prevalence of placental malaria was 26.5% (95%CI: 16.7–36.4) in East and Southern Africa, and 38% (95%CI: 28.4–47.6) in West and Central Africa (Chico RM et al, 2012).

The Democratic Republic of Congo (DRC) is currently the second most affected by malaria in the world (WHO, 2013). Nearly 95% of the population is exposed to *P. falciparum* and 60% of pregnant women have anemia (Unpublished data). Maternal mortality is the highest in Africa (NMCP, 2009). However, there is very little data on asymptomatic *P. falciparum* infection among pregnant women. The latest studies were conducted in 1988 in Kinshasa (Mbanzulu et al, 1988) and in 2003 in Lubumbashi ( Kalenga et al, 2003) and reported a prevalence of asymptomatic malaria infection to be respectively 22% and 49%. These prevalences were determined using microscopy.

Diagnosis of asymptomatic *P. falciparum* infection during pregnancy is essential to prevent poor pregnancy outcomes but presents a great challenge (Greenwood et al, 2007). The absence of clinical signs decreases the chances of a clinical diagnosis and a management guided by a decisional algorithm. If untreated it develops into chronic infection, sometimes with fluctuating and very low parasite densities. Placental parasite sequestration, the most common form of asymptomatic malaria, usually leads to a lack of peripheral parasitaemia (Brabin et al, 2004). Optical microscopic examination of peripheral-blood smears for parasitemia during pregnancy is known to miss a significant portion of asymptomatic placental infections (Leke et al., 1999). Cross-sectional studies conducted at delivery in endemic settings have shown that rapid diagnostic tests (RDTs) detecting *P. falciparum* histidine-rich protein-2

(HRP2) are more sensitive than blood smears and appear to be reliable predictors of adverse outcomes of malaria in pregnancy (Mockenhaupt *et al.*, 2006). Polymerase Chain Reaction (PCR), a research “gold standard,” has been estimated to detect as little as 5 parasites or less per microliter. (Mayor *et al.*, 2012). This underlines the importance of using appropriate techniques, to determine a true prevalence of asymptomatic malaria in pregnant women in endemic areas.

## **1.2. Justification**

In malaria-endemic areas, most of the prevalence estimates of malaria in pregnancy were done using microscopy, unless specified otherwise, (Chico *et al.*, 2012 and Teklehaimanot *et al.*, 2009) and they would probably be higher if more sensitive methods like RDT and PCR (Polymerase Chain Reaction) were incorporated (Rantala *et al.*, 2010).

To determine the extent of asymptomatic *P. falciparum* infection in pregnant women, using microscopy, rapid diagnostic test and polymerase chain reaction, was the aim of this study. Kinshasa is an area of stable transmission for malaria and therefore an ideal ground to perform this study.

### **1.3 Objectives**

#### **1.3.1 Main objective**

To determine the prevalence of *P.falciparum* infection and related anaemia among healthy pregnant women attending antenatal clinics in Kinshasa, DRC.

#### **1.3.2 Specific objectives**

1.3.2.1 To determine the prevalence of parasitaemia asymptomatic malaria and its relationship with various sociodemographic characteristics of pregnant women in Kinshasa.

1.3.2.2 To determine the association between asymptomatic *P.falciparum* infection and anemia in pregnancy in Kinshasa.

1.3.2.3 To determine the internal validity of microscopy and RDT for the diagnosis of asymptomatic *P. falciparum* infection in pregnancy in Kinshasa, using PCR as gold standard.

## CHAPTER II

### 2.0 LITERATURE REVIEW

#### 2.1 Historical aspect

##### 2.1.1 History

The history of malaria predates humanity, as this ancient disease evolved before humans did (Reiter., 2000). As malaria remains a major public health problem, causing more than 225 million clinical cases each year (Phillips et *al.*, 2010), killing about 781,000 people each year according to the World Health Organisation's 2010 World Malaria Report, 2.23% of deaths worldwide (WHO, 2010b).

Human malaria likely originated in Africa and has coevolved along with its definitive hosts, mosquitoes and intermediate hosts, non-human primates (Poinar, 2005). The first evidence of malaria parasites was found in mosquitoes preserved in amber from the Paleogene period that were approximately 30 million years old (Poinar, 2005). Malaria may have been a human pathogen for the entire history of the species (Hayakawa et *al.*, 2008). Close relatives of the human malaria parasites remain common in chimpanzees, the closest evolutionary relative of modern humans (Roy and Irimia, 2008). Malaria started having a major impact on human survival about 10,000 years ago which coincides with the introduction of agriculture (Neolithic revolution) (Hempelmann et *al.*, 2009). The consequence was natural selection for sickle-cell disease, thalassaemias, glucose-6-phosphate dehydrogenase deficiency, ovalocytosis, elliptocytosis and loss of the Gerbich antigen (glycophorin C) and the Duffy antigen on the erythrocytes because such blood disorders confer a

selective advantage against malaria infection (Canali, 2008). The three major types of inherited genetic resistance (sickle-cell disease, thalassaemias, and glucose-6-phosphate dehydrogenase deficiency) were present in the Mediterranean world by the time of the Roman Empire, about 2000 years ago (Sallares *et al.*, 2004).

The name malaria, derived from “mal”aria” (bad air in Medieval Italian) was probably first used by Leonardo Bruni in a publication (Bruni, 2004). Malaria was once common in most of Europe and North America, where it is now for all purposes non-existent (Knottnerus, 2002). The coastal plains of southern Italy, for example, fell from international prominence (the Crusaders going by sea to the Holy Land took ship at Bari) when malaria expanded its reach in the sixteenth century (Knottnerus, 2002).

At roughly the same time, in the coastal marshes of England, mortality from "marsh fever" or "the ague" (from Latin “febris acuta”) was comparable to that in sub-Saharan Africa today (Knottnerus, 2002). William Shakespeare was born at the start of the especially cold period that climatologists called the "Little Ice Age", yet he was aware enough of the ravages of the disease to mention it in eight of his plays (Reiter, 2000).

Throughout history the most critical factors in the spread or eradication of the disease has been human behaviour (shifting population centers, changing farming methods etc.) and living standards (Breman, 2001). Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care (Breman, 2001). As a consequence, the majority of cases are undocumented (Breman, 2001). Poverty has been and remains a reason for the

disease to remain today while it has undergone a decline in other locations (Worrall *et al.*, 2005).

### **2.1.2 Discovery of malaria parasite (1880)**

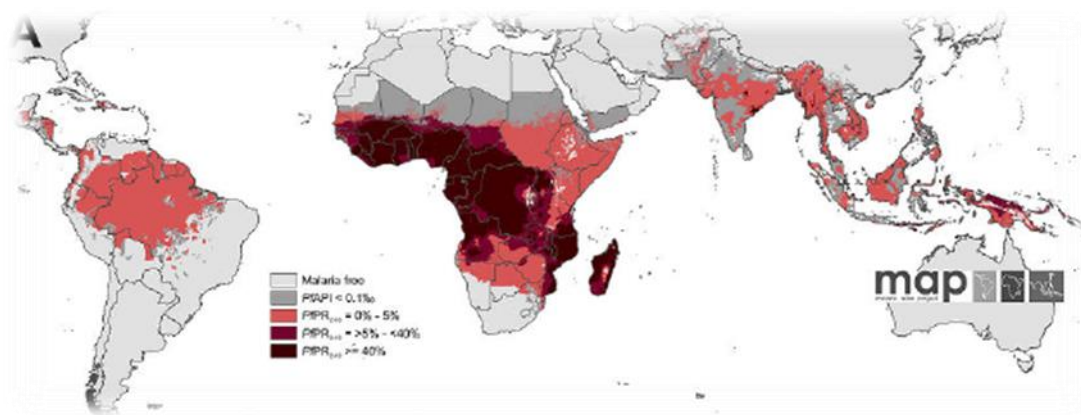
The causal relationship of pigment to the parasite was established in 1880, when the French physician Charles Louis Alphonse Laveran, working in the military hospital of Constantine, Algeria, observed pigmented parasites inside the red blood cells of people suffering from malaria (Cox , 2010). He also witnessed the events of exflagellation and became convinced that the moving flagella were parasitic microorganisms (Cox , 2010). He noted that quinine removed the parasites from the blood. Laveran called this microscopic organism *Oscillaria malariae* and proposed that malaria was caused by this protozoan (Cox , 2010).

It took over eighteen years from the time the malaria parasite was discovered to understand the complete picture of the parasite transmission cycle to human. In 1886, the Differentiation of Species of Malaria Parasite was made by Camillo Golgi. Giovanni Batista Grassi, Raimondo Filetti, Laveran and John William Watson named the four human malaria parasites (1890 -1897). The role of Mosquitoes as the vector of malaria was described by Ronald Ross (1897-1898). Giovanni Batista Grassi, Amico Bignami and Giuseppe Bastianelli discovered of the Transmission of the Human Malaria Parasites *Plasmodium* (1898-1899).

(<http://www.cdc.gov/malaria/history/index.htm>).

## 2.2. Malaria situation in the world

Malaria occurs in over 100 tropical and subtropical countries including Sub-Saharan Africa, Asia, the Pacific and Latin America (Figure 1). The estimates indicate that 216 million episodes of malaria in 2010, with a wide uncertainty interval (5th to 95th percentile) ranging from 149 to 274 million cases. Almost 81%, or 174 million cases (between 113 and 239 million), took place in the Africa region and 13% in the South-East Asia (WHO, 2011) region. In 2010, deaths due to malaria were estimated to be 655,000 (between 537,000 and 907,000), 91% (or 596,000 in a range between 468,000 and 837,000) in the Africa region. Globally, 86% of malaria deaths have involved children under 5 years.



**Figure 1: Malaria situation in the world in 2010** Source: Gething *et al.*, 2011



### 2.3. The vector agent and ecology

The Anopheles mosquito is an insect of the *Pterygota* subclass and *Culicidae* family. The Anopheles genus includes about 484 species, of which sixty are vectors and out of these thirty are good vectors, whose distribution and efficiency vary by geographic region. In sub-Saharan Africa, it is considered that there are some 150 species of Anopheles, a dozen are excellent vectors such as *A. gambiae*, *A. arabiensis*, *A. funestus*, *A. nili*, *A. moucheti*. In stable malaria-endemic regions such as savannah areas of Burkina Faso and Nigeria, parasite transmission is mostly due to *A. gambiae* and *A. arabiensis* during rainy seasons and *A. funestus* at the beginning of the dry season (Carnevale and Robert, 2009). The breeding sites are varied; Anopheles can develop in:

- fresh or brackish water in sub-Saharan Africa, on the western and eastern side, South America, Southeast Asia in the Indochina peninsula and in the waters over-salted.
- sunny sites in tropical Africa, America, Southeast Asia or in shady forests in Southeast Asia, Central America.

### 2.4. The Parasite

*Plasmodium falciparum* parasite is a member of *Haemosporidea* class and *Plasmodidae* family (Bannister and Sherman, 2009). The four species of *Plasmodium* that can infect the human host are: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. In the forest areas of Southeast Asia, infections also include *P. knowlesi*, a parasite of monkeys (Singh et al., 2004). *P. falciparum*, the most

dangerous and most widespread species in the warm regions (sub-Saharan Africa, Asia, Oceania, Central and South America) is responsible for the majority of fatal cases of malaria. *P. vivax* responsible for benign tertian fever in Asia, South and Central America, is poorly represented in Africa. Long considered responsible for mild infection, *P. vivax* is now recognized as a cause of severe malaria (Karyama et al. 2008). Present in Africa, *P. malariae* is not fatal but may be responsible for relapse cases of the disease twenty years after the first infection. *P. ovale* is present mainly in Africa and sporadically in Amazonia, Oceania and Asia. It is not fatal but can recur 4-5 years after the initial infection. A fifth species, *P. knowlesi*, head of the monkey malaria parasites has been found in humans and is responsible for quartan Borneo (Southeast Asia). This infection attributed to *P. malariae*, is in fact due to *P. knowlesi*, its evolution is potentially serious: it must be treated as *P. falciparum* (Figtree et al., 2010).

## **2.5. Life cycle of the parasite**

### **2.5.1. Cycle in human host**

#### **2.5.1.1. Exo-erythrocytic cycle**

During the bite, the infected female Anopheles injects sporozoites into a blood capillary. These pass into the general circulation and in a few minutes, they invade hepatocytes through a specific interaction between the major surface protein of the sporozoite and a specific receptor on the plasma membrane of the hepatocyte-side in direct contact with the circulating blood (Roestenberg et al., 2011). The sporozoite then enters a phase of intracellular replication and proliferation that pushes the nucleus at the periphery of the cell and eventually forms a mass called a multi-

nucleate schizont which leads to the release of tens of thousands of merozoites into the circulation. This multiplication phase is asymptomatic and lasts 8 to 15 days, depending on the species. Unlike *P. vivax*, *P. falciparum* does not have forms of liver or hypnozoites persistence (Chamchod F and Beier JC. 2012).

#### **2.5.1.2. Intra-erythrocytic cycle**

Only the blood phase is responsible for the symptoms that may be of varying intensity. Merozoites released upon rupture of the hepatocyte will begin the asexual blood cycle proliferation of *P. falciparum* and infect erythrocytes. The merozoite enters the erythrocyte through active parasitic processes and differentiates into trophozoite, at which point an intense replicative phase begins. It then gives rise to the schizont, which shows a characteristic segmentation rosette shape and then releases 8-32 merozoites rapidly, which reinfect healthy erythrocytes. This entire cycle lasts 48 hours in *P. falciparum*. The appearance of gametocytes usually takes place the second week after infection and these forms can persist for several weeks after recovery. Following another injection by *Anopheles*, male and female gametocytes are ingested with the blood meal.

#### **2.5.2. Cycle in the Anopheles**

During a blood meal on an infected individual, the female mosquito ingests gametocytes in male or female sexual potential. They reach the stomach of the mosquito and become gametes. The male gamete undergoes a process exflagellation after which the female gametes are fertilized. The result is called an ookinete, a motile zygote. It is implanted in the stomach wall to form the oocyst. This brief diploid stage ends with a meiotic division and is followed by thousands of mitosis

leading to the development of sporozoites. Sporozoites enter preferentially salivary glands where they can be injected with the saliva during a bite. In the mosquito, this whole cycle takes place in 10 to 40 days, depending on the outside temperature and the species involved (Bannister Sherman, 2009).

## **2.6. Pathogenesis of malaria**

In humans, the parasite needs to proliferate and survive without being destroyed. To achieve this, *P. falciparum* uses several techniques to avoid the host immune system and cause serious illness. The best-known mechanisms: the invasion of erythrocytes, cytoadherence and sequestration, rosetting and antigenic variation (Howard and Barnwell, 1984).

### **2.6.1. Invasion of erythrocytes**

*P. falciparum* can invade red blood cells at different stages of their development, from reticulocytes to mature stages. Proteins such as Duffy-Binding Proteins Like (DBL) and Reticulocytes-Binding Proteins Like (RBL) of *P. falciparum* recognize receptors on the surface of red blood cells, allowing their invasion (Rayner et al., 2005).

### **2.6.2. Cytoadherence**

*P. falciparum* is responsible for the sequestration of parasitized erythrocytes. Surface of infected red cells is covered with protrusions that are knobs called the point of contact with the cells of the host. Membership protects infected (ERi) erythrocyte destruction because circulating Eris are eliminated in the spleen. Several parasite proteins are localized in these projections and participate directly or indirectly in the

cytoadherence. The studies focus specifically on family PfEMP1 (*P. falciparum* erythrocyte membrane protein-1) involved in the adhesion of mature forms of the parasite (trophozoites and schizonts). One of sixty variable proteins is expressed. The parasitized erythrocytes adhere to the endothelium of the capillaries of deep organs (brain, lung, kidney, placenta). This sequestration plays a major role in the pathophysiology of severe malaria cases and as such the subject of numerous studies (Ndam and Deloron, 2007; Crompton et al., 2010). The formation of rosettes or rosetting is the ability of parasitized erythrocytes to bind uninfected erythrocytes and autoagglutination which corresponds to the adhesion between them. Parasitized erythrocytes are also involved in erythrocyte sequestration.

## **2.7. Antigenic variability of *Plasmodium***

The PfEMP1 protein is involved in antigenic variation of malaria due to *P. falciparum*. The var genes of the human malaria parasite *Plasmodium falciparum* are highly polymorphic loci coding for the erythrocyte membrane proteins 1 (PfEMP1), which are responsible for the cytoadherence of *P. falciparum* infected red blood cells to the human vasculature. Cytoadhesion, coupled with differential expression of var genes, contributes to virulence and allows the parasite to establish chronic infections by evading detection from the host's immune system. In addition, frequent recombination and genetic alterations in the process of merging and splitting in the mosquito and human erythrocytes can cause great genetic and antigenic diversity of the parasite (Flick and Chen, 2004).

## **2.8. Clinical manifestations**

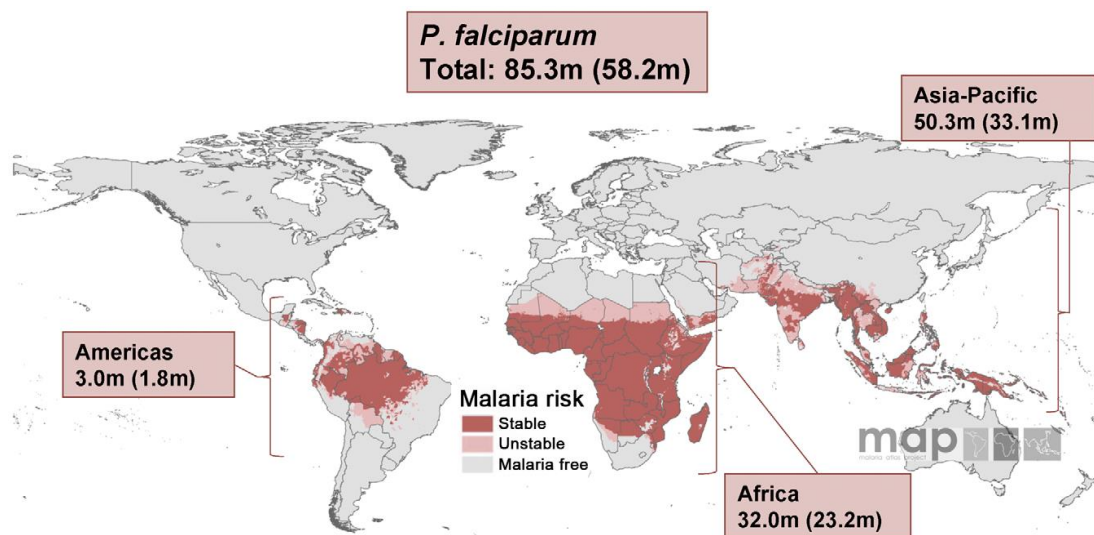
The most common clinical manifestation is uncomplicated malaria which comprises usually a febrile syndrome with fatigue, headache and upset stomach. This condition is related to the breakdown of parasitized erythrocytes and release of pyrogens. This erythrocyte lysis and, to a lesser extent, dyserythropoiesis can cause life-threatening anemia, particularly in young children. Severe malaria is complex and affects several organs. Severe malaria usually occurs by one or more of the following: coma (cerebral malaria), metabolic acidosis, severe anemia, hypoglycemia, acute renal failure or acute pulmonary oedema. At this stage of the disease, if left untreated, severe malaria is always fatal.

- Severe anemia is the low concentration of erythrocytes or hemoglobin less than (5 g /dL) with high parasitaemia. The mortality rate is about 1%.
- Cerebral malaria indicates a neurological disease. The events range from a single prostration to impaired consciousness and coma. The mortality rate is around 7%.
- Respiratory distress is the most obvious clinical manifestation of metabolic acidosis syndrome and also with the highest mortality rate of about 24% (Mangano , 2008).

## **2.9. Malaria and pregnancy**

The malaria infection in pregnant women is a very serious public health problem because it poses risks to the mother, foetus and newborn. Pregnant women are three times more likely to suffer from severe malaria infection compared to non-pregnant

women. In malaria-endemic areas, it is estimated that at least 25% of pregnant women are infected with malaria, with the greatest risk of infection and morbidity in primiparous adolescents, and those co-infected with HIV (Schantz-Dunn et al, 2009). In areas of low transmission of *P. falciparum*, where rates of acquired immunity are low, women are exposed to bouts of severe malaria. In areas of high transmission of *P. falciparum*, where rates of acquired immunity are generally high, women are exposed to asymptomatic infection, which can lead to maternal anemia and placental parasitaemia. Figure 2 shows the number of pregnant women living in areas at risk of infection with *P. falciparum*. The total presents the number of pregnant women in the area, whereas in bracket is presenting the number of pregnant women at risk of *P. falciparum* infection.



**Figure 2: Mapping the number of pregnant women living in areas at risk of malaria due to *P. falciparum* in 2007** Source: Dellicour et al., 2010

### **2.9.1. Placental cytoadherence**

Gestational malaria is associated with sequestration of parasitized erythrocytes by *P. falciparum* in the intervillous spaces of the placenta (syncytiotrophoblast). Indeed, a protein family PfEMP1 called var2CSA expressed on the surface of parasitized erythrocytes is responsible for the adhesion of the RBCs. These bind to a sugar, chondroitin sulfate A (CSA) and hyaluronic acid (HA) present in the placenta. (Rogerson *et al*, 2007b). Parasites chondroitin sulfate A variants setting are only found in pregnant women. Vaccination strategies considered in the fight against gestational malaria recreate protective immunity by blocking the adhesion of parasitized erythrocytes to the placenta (Duffy and Fried, 2011).

### **2.9.2. Maternal effects**

For various reasons, anemia is more common in pregnant women than non-pregnant women. This could be, least partly, explained by hemodilution due to increased intravascular volume during the second trimester of pregnancy, the increased used by the foetus of iron and folic acid (Brabin B *et al.*, 1990) and direct destruction of a large number of infected erythrocytes.

When severe, it increases the risk of death for the mother and it is estimated that anemia due to malaria may be responsible for 10,000 maternal deaths per year in Africa (WHO, 2005).



### **2.9.3. Effects on child health**

Malaria infection in the mother can cause abortion, stillbirth or congenital infection. During the second half of pregnancy, it may, by association with maternal anemia, interfere with foetal weight gain and contribute to intrauterine growth retardation or prematurity with a low score birth weight. The underweight at birth is one of the major causes of reduced survival and very little development in infants. According to WHO estimates, malaria in pregnancy is responsible for 8-14% of all cases of underweight at birth and is the source of 75, 000 to 200, 000 infant deaths each year (Rogerson et al., 2007a). There is not enough data on the long-term consequences of malaria during pregnancy for the child. Essentially nutritional studies indicate that exposure to an abnormal intrauterine environment affects the mental, metabolic and anthropometric development and can lead to an increased risk of disease later in life (Desai et al., 2007).

### **2.9.4. Asymptomatic malaria in pregnant women**

Symptoms and complications of malaria during pregnancy differ depending on the intensity of transmission and the level of immunity acquired by pregnant women. In areas of low transmission or epidemic (unstable) malaria, pregnant women have not acquired a high level of immunity and generally fall sick when infected with *P. falciparum*. In areas of stable malaria transmission, most adult women have developed sufficient immunity, even during pregnancy; infection with *P. falciparum* usually does not causes fever or other clinical symptoms. In these areas, malaria infection is mainly characterized by the onset of a secondary anaemia and the

presence of parasites in the placenta. Nutrient deficiencies resulting in the foetus contribute to low birth weight (WHO, 2005).

#### **2.9.5. Recommendation in the prevention and the fight against malaria in pregnant women in areas of stable transmission interventions (WHO Africa, 2005)**

The guidelines for the fight against malaria during pregnancy in areas of stable transmission should emphasize the association of intermittent preventive treatment (IPT) and insecticide-treated nets (ITNs) and ensure effective management of malaria cases and anaemia (WHO, 2005).

##### **2.9.5.1. Intermittent Preventive Treatment**

Intermittent Preventive Treatment should be given to all pregnant women living in areas of stable transmission at least two doses of IPT from the moment they begin to perceive the movements of the foetus. The World Health Organization recommends a schedule of four antenatal visits, with three after the onset of foetal movements. Providing administration of IPT at three consultations will ensure that a large proportion of pregnant women receive at least two doses. Because of its safety during pregnancy, its effectiveness in women of childbearing age and ease of use in the programs, sulfadoxine-pyrimethamine (SP) is currently the most effective drug for IPT. This medicine is given as a single dose in the presence of the health worker and should not be taken more than once a month.

### **2.9.5.2. Insecticide Treated Nets ( ITNs)**

Insecticide Treated Nets should be provided to women as soon as possible after the beginning of their pregnancy. It is encouraged to use ITNs throughout the period of pregnancy and the postpartum period. ITNs are made available to women in either antenatal care or by other actors in the public or private sector (WHO, 2005).

### **2.9.5.3. Effective management of malaria and anaemia cases**

Effective case management of malaria illness for all pregnant women in malarious areas must be ensured. Iron supplementation for the prevention and treatment of anaemia should be given to pregnant women as part of routine antenatal care. Pregnant women should also be screened for anaemia, and those with anaemia should be managed according to national reproductive health guidelines.

## **2.10. Diagnosis of malaria**

An effective management of the disease requires diagnosis without delay. Diagnosis is based on clinical suspicion of malaria, and the search for parasites by microscopic examination certifies the diagnosis by demonstrating the parasite in the circulating blood.

### **2.10.1. Microscopic diagnosis by thick blood smears (TBS) and thin film (TF)**

Microscopic examination of thin blood film and thick blood film is the reference technique advocated by WHO. It allows rapid diagnosis and monitoring of treatment efficacy of malaria by monitoring parasitaemia. This is inexpensive and remains the most widely used technique. However, its performance in terms of sensitivity and

reliability are directly dependent on the experience of the microscopist and the level of parasitemia of the infected. The blood smear allows better examination of the morphology of parasites and red blood cells and therefore makes diagnosis of Plasmodium species easier. It also allows calculating the parasitaemia. The detection threshold of BS is 40 to 50 parasites / $\mu$ L. Its sensitivity is much lower than the PCR that can detect low parasitaemia (10 parasites /  $\mu$ L). The microscopic diagnosis may also encounter difficulties in identifying species particularly in the presence of parasites altered by presumptive treatment or in case of very low parasitemia (Moody, 2002; Rogier et al, 2009; Siala et al., 2010).

#### **2.10.2. Detection of malaria antigens by rapid diagnostic tests (RDTs)**

The RDT is based on the principle of immunochromatography using strips sensitized by specific monoclonal antibodies detecting malarial antigens. They are made with a drop of blood placed on a strip and require no equipment.

- Antigen detection Histidin Rich Protein 2 (HRP2): This specific glycoprotein of *P. falciparum* is generated by all asexual erythrocytic stages of the parasite and can persist in the peripheral blood more than 15 days after the disappearance of parasites. These tests are credited with a greater than 96% sensitivity compared to conventional microscopic techniques, when evaluated on the BS parasitemia is greater than 100 parasites / $\mu$ L. Their detection threshold varies from 100 to 300 parasites / $\mu$ L. Persistent antigenemia after healing and monospecificity vis-à-vis *P. falciparum* are the major drawbacks of these tests. False positives were also associated with cross-reactions with rheumatoid factor. False negatives are possible

and are due to mutations in the gene encoding the HRP2 or presence of antibodies HRP2 (Rogier *et al.*, 2009; Siala *et al.*, 2010.).

- Detection of Parasite Lactate Dehydrogenase (LDH) they are glycolytic enzymes which have the advantage of being common to the four *Plasmodium* species, detected at all asexual and sexual stages of the parasite. LDH have a detection threshold identical to that of HRP2, their clearance is faster, and they do not persist in the blood after death of *Plasmodium*, hence their interest in the monitoring of patients (Siala *et al.*, 2010).
- Aldolase: antibodies capable of recognizing the aldolase of all human plasmodia can be used. The sensitivity of detection of these antigens is, however, still lower than that of tests detecting HRP2 and LDH (Rogier *et al.*, 2009).

RDTs are quick and easy to read and can be performed by moderately trained personnel. They are suitable especially in non-specialized structures where microscopy is not available. Their performance depends essentially on parasitemia. They are also less effective with species other than *P. falciparum*, particularly *P. ovale*. The RDTs should be considered as a complement to other diagnostic methods. Their results should be checked and possibly supplemented by microscopic examination. Their positivity provides patients adequate and timely support. However, their negativity does not rule out malaria positivity (Rogier *et al.*, 2009).

### **2.10.3. QBC Malaria Test and Quantitative Buffy Coat**

The principle of this microscopic fluorescence technique based on the use fluorochrome (acridine orange) capable of binding to the nucleus of the parasite. The

research of *Plasmodium* is done in 50 $\mu$ l of blood collected in a hematocrit tube after centrifugation and concentration reading with a fluorescence microscope.

The sensitivity of this technique is comparable to that of microscopy BS for more than 100 parasites /  $\mu$ L infections. It varies from 41% to 93% with less than 100 parasites / $\mu$ L parasitemia. Specificity for *P. falciparum* is high (93-98%) but drops to about 50% for infections caused by other species. The QBC Malaria Test is easy to use and fast realization. It is currently the best screening test for non-biologists and structures for treating a large number of tests of *Plasmodium*. Unfortunately, its use requires expensive equipment and reagents, which limits its use. It does not allow the diagnosis of species and calculation of parasitemia.

#### **2.10.4. Nucleic acid detection by PCR techniques**

Gene amplification by PCR is the most widely used technique. This is the most sensitive technique to detect very low parasitaemia of approximately 0.3 parasite / $\mu$ L of blood with a possibility of plasmodial DNA quantification using quantitative PCR. The amplification of the gene encoding the small subunit 18S ribosomal RNA of *Plasmodium* also allows the identification of the species involved using a nested PCR. Despite its advantages, molecular biology cannot replace the current practice . It is not compatible with the urgency of malaria diagnosis, due to relatively long time to complete. PCR is primarily indicated for the detection of low parasitemia in case of strong suspicion and difficulty of microscopic confirmation particularly among travellers under chemoprophylaxis. It is also a valuable contribution to the identification of *Plasmodium* species, post-treatment monitoring and study of genes

involved in resistance to antimalarial drugs. It's hardware requirements and cost make it to be restricted to specialized laboratories (Siala et al., 2010).

#### **2.10.5. Antiplasmodial antibody detection**

Serology has no role in the diagnosis of acute malaria attacks due to the late appearance of antibodies (Ab) against the malarial emergence of parasites in the blood. Serological diagnosis is also facing difficulties in interpretation. Indeed, the presence of specific Ab may reflect either a current malaria infection or a previous malaria since the antibodies may persist 2-3 years after infection. The immunological diagnosis is indicated in certain chronic clinical forms such as evolutionary visceral malaria and hyper-immune malarial splenomegaly in which antibodies are high rates while parasitological research is most often negative. Serology is also useful in case of retrospective presumptive treatment or self-medication. It is also widely used in the screening of blood donors in the prevention of post-transfusion malaria and epidemiological investigations (Siala et al., 2010).

## CHAPTER III

### 3.0 MATERIALS AND METHODS

#### 3.1 Study site

Kinshasa, the third largest city in Africa, with a population of 8 million, is located in the western part of DRC. Kinshasa has a largely tropical wet climate. It features a lengthy rainy season which spans from October through May and a relatively short dry season which runs between June and September.

Malaria in Kinshasa is endemic with stable transmission year round. Seasonal fluctuations in malaria transmission occur during the rainy season from September/October to May. The entomological inoculation rate in some peripheral areas of Kinshasa reaches 1200 infective bites/person/year (Coene J *et al.*, 1993).. According to the 2007 Demographic and Health Surveys, 95.7% of pregnant women in Kinshasa received some antenatal care (ANC) provided by qualified staff (DHS, 2007). Kinshasa features a low coverage of IPT despite the high rate of use of ANC and the high rate of assisted births (7% in 2007) (NMCP, 2009).

This study was conducted at the Centre Hospitalier de Kingasani II (CHK), commonly known as *Maternité des Soeurs*. It is by its attendance, the largest maternity hospital in Kinshasa and the country. Located in the heart of the peri-rural areas of the south-eastern suburbs of Kinshasa, the maternity hospital provides invaluable health care at a rate appropriate to the level of local life with twenty to thirty (20-30) daily deliveries (DHS, 2007).





**Figure 3: Map of Kinshasa province of the DRC and Kingasani**

### **3.2. Study design**

This study was designed as a cross sectional survey, targeting healthy pregnant women living in Kinshasa, presenting at CHK maternity hospital for the first antenatal visit. It was conducted from from 27 July 2012 to 27 August 2012.

#### **3.2.1. Inclusion criteria**

- Pregnant women living in Kinshasa for 10 consecutive years
- Pregnant women aged 17 years or older
- Age of pregnancy:  $\geq 7$  weeks
- Signing of informed consent form

#### **3.2.2. Exclusion criteria**

- Pregnant women coming from non-endemic areas for malaria

- Age of pregnancy less than 7 weeks
- Women who presented fever, muscle aches or other symptoms that suggested malaria
- Women who received anti-malarial treatment within the past 4 weeks
- Women who did not provide their written informed consent

### **3.2.3. Sample size**

The population of pregnant women in the health zone of Kingasani was estimated at 7187 (NMCP, 2009) with an expected malaria prevalence of 30%, a desired accuracy set at 5% and a confidence interval of 95%. Using Epi-info<sup>TM</sup>7 (CDC, Atlanta, USA), the minimum sample size with these parameters was calculated at 309 pregnant women.

## **3.3. Study procedure**

### **3.3.1. Patient recruitment**

For this study all pregnant women attending the hospital for a prenatal visit, were screened for eligibility, after a trained physician or nurse had given a clear explanation about the study and had answered all questions. Eligible women were enrolled after providing a written informed consent.

### **3.3.2. Epidemiological data collection**

Socio-demographics data and a complete medical history were collected using a questionnaire.

### **3.3.3. Blood sample collection**

#### **3.3.3.1. Venipuncture**

The site for venipuncture was selected at the antecubital. Five milliliters of blood were taken into a labeled EDTA tube for all analyses. For PCR analysis, blood was drawn from the EDTA tube, collected on a filter paper (Wattman® 3 mm), dried at room temperature and stored in a zip log with silica gel.

### **3.4. Laboratory analysis**

#### **3.4.1 Microscopy for *P.falciparum* detection in peripheral blood**

Both thick and thin smears were made on the same slide. Each slide carried the following information: patient initials, individual code and date of collection.

For each patient, two slides were prepared: one was read by two readers and kept in the archives there and a second was retained for external quality control.

The slides were stained with 10% Giemsa for 10 minutes. The thin films were fixed with pure methanol for 3 seconds before staining. Blood slides were examined using light microscopy (Olympus, USA), using immersion oil at 1000 × magnification. Hundred microscopic fields were examined in the thick smear before concluding that a blood slide was negative. All slides were read twice by experienced microscopists. If the discrepancy was greater than 15%, a third reader was used to confirm diagnosis. The parasite density per microliter of blood was computed using the

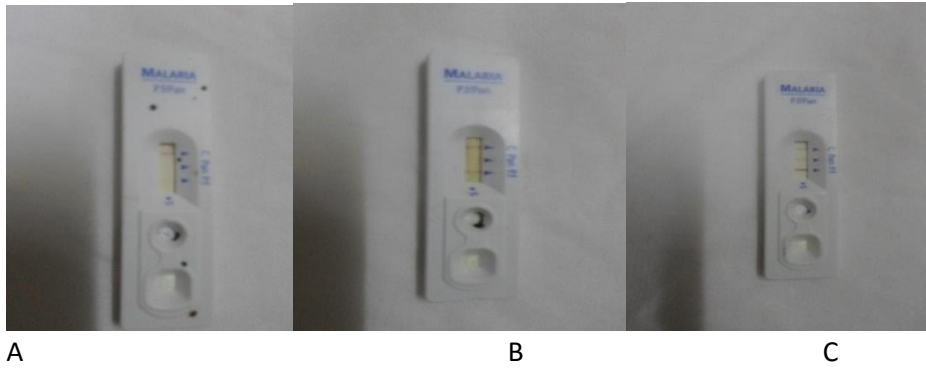
following formula: (Number of trophozoites x 8000)/Number of leucocytes.

Microscopy was done at the Parasitology Department of University of Kinshasa.

#### **3.4.2. Rapid Diagnostic Test for HRP-II detection (SD Bioline Malaria Ag Pf/Pan®).**

Test principle: The SD Bioline Malaria Ag Pf/Pan® test cassette contains a membrane strip, which is precoated with mouse monoclonal antibodies specific to HRP-II of *P. falciparum* on test line P.f region and with mouse monoclonal antibodies specific to lactate dehydrogenase of Plasmodium species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) on test line Pan region, respectively. The mixture of mouse monoclonal antibodies specific to HRP-II of P.f and mouse monoclonal antibodies specific to pLDH of pan - colloid gold conjugate reacts with the malaria antigen in the sample. They move along the membrane chromatographically to the test region (*P.f* and Pan) and form a visible line as the antibody-antigen-antibody gold particle complex with high degree of sensitivity and specificity. All the test lines and control line in the result window are not visible before applying any sample. The control line is used for procedural control and should always appear if the test procedure is performed correctly.

With a 5µl capillary pipette provided, whole blood sample was drawn from the EDTA tube and transferred into the round sample well. Then 4 drops of assay diluent were added into the square assay diluent well. The result was read after a minimum of 15 minutes (up to 30 minutes). Reading too late could give false results.



**Figure 4: RDT results. (A) Negative RDT;(B)RDT positive for *P.f*; (C) RDT positive for *Pf* and Pan**

### **3.4.3. Molecular analyses**

In order to circumvent the problem of false negatives due to the inability of microscopy in detecting submicroscopic infections or false positives due to the detection of circulating HRP-2 antigen by RDT even without infection or after parasite clearance, all the RDT positive but microscopy negative samples as well as a convenient 166 of the total samples were analyzed using nested *Plasmodium* species diagnostic PCR assay.

#### 3.4.3.1. *P.falciparum* DNA extraction from filter paper

The blood spot samples of 3mm diameter size were first soaked in 0.5% saponin in 1x phosphate-buffered saline (PBS), incubated for 10 minutes at room temperature in a 1.5-ml tube, and centrifuged at 14000 rpm. After the supernatant had been discarded, blood spots were washed in 1ml of 1x phosphate-buffered saline. One hundred fifty microlitres of a work solution of 2% Chelex-100 resin (Bio-Rad Richmond, CA) and 50 µl of water (pH 9.5) were added in a 1.5-ml tube and then boiled at 100°C for 10 minutes. After centrifugation at 10000-x g for 1 min, the supernatant was then collected into a new tube and stored at -20°C, for polymerase chain reaction (PCR) investigation.

#### 3.4.3.2. Polymerase Chain Reaction (PCR)

Nested PCR assay was performed as a two-step procedure. Firstly, amplification of *Plasmodium* genus specific fragment was carried out as follows; PCR mixture containing buffer, dNTPs, MgCl<sub>2</sub>, primers, Taq polymerases and sterile water. All PCR reactions were carried out in a total volume of 20 µl. One µl of the purified template DNA was used for the first reaction, in which the fragment spanned by rPLU5 (5' CCTGTT GTTGCCTTAAACTTC 3') and rPLU6 (5' TTAAAATTGTT GCAGTTAAAACG 3') was amplified. Secondly, a 1 µl aliquot from the product of the first PCR reaction was then used as a template for amplification of the *Plasmodium falciparum*-specific fragment using FAL1 (5' TTAAACTGGTTTGGGAAAACCAAATATATT 3') and FAL2 (5' ACACAATGAACTCAA TCATGACTACCCGTC 3') specific primers (Applied

Biosystems) (Snounou *et al.*, 1993). A negative control without DNA template and a positive control with appropriate template (3D7) were always included. The template was added to the mix in a PCR tube. Each reaction contained the components listed in table 1.

**Table 1: Reaction Mixture for PCR**

	μl
Go Taq flexi buffer 5X	2
dNTP mix (5mM each)	0.5
MgCl <sub>2</sub> (25 mM)	1.6
Primer rPLU5 (10μM)	0.5
Primer rPLU6 (10μM)	0.5
GoTaq flexi DNA polymerase	0.08
H <sub>2</sub> O Molecular Biology grade	13.82
DNA	1

The cycling parameters for the primary and nested amplification reaction were the same except that 30 PCR cycles were performed for secondary amplification (Snounou *et al.*, 1993).

**Table 2: Thermal cycling parameters for PCR**

Step 1: Primary denaturation	95°C, 5min
Step 2: Denaturation	94°C, 1min
Step 3: Annealing	58°C, 2min
Step 4: Extension	72°C, 2min
Step 5: Go to step 2 for	24 cycles (total 25)
Step 6: Final extension	72°C 5min
Step 7:	Hold at 20°C

#### **3.4.3.3. Detection of PCR product on Agarose gel**

PCR products were detected by running 20 µl of DNA product on 3% agarose gel, (Eurogentec®), which was subsequently stained with a 0.5 µg/ml ethidium bromide solution and visualized under ultraviolet transillumination. The specific size of the PCR product (second amplification) was 205 bp for *Plasmodium falciparum*.

Molecular analyses were performed at the Tropical Diseases Research Centre (TDRC) Ndola, Zambia.

#### **3.5. Data analysis**

Data were entered and stored in Epi info version 3.5.1. Frequencies were used to assess prevalence of asymptomatic malaria and anemia in pregnant women. The  $\chi^2$  test was used to investigate associations between 2 categorical variables and to compare proportions. For significant associations, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.  $P < 0.05$  was considered statistically significant. To identify factors we hypothesized to be associated with asymptomatic malaria and anemia in pregnancy, we constructed separate multivariate logistic regression models using a backward selection process. Based on prior knowledge, we included sociodemographic variables in the model and then used backward elimination to identify other covariates. An alpha level of 0.05 was used to determine which variables remained in the model. Statistical analyses were performed using SPSS statistical program, version 17 (SPSS, Chicago, IL, USA).



The Sensitivity, specificity, PPV and NPV of microscopy and RDT (SD bioline malaria Ag Pf/pan) were determined with PCR as gold standard, in only selected 50% of all samples.

### **3.6. Ethical consideration**

Ethical clearance for the study was obtained from the Ethical Review Committee of the School of Public Health of University of Kinshasa. Permission to undertake the study at the CHK II maternity was sought and granted by the hospital management. The subjects under study were provided with informed consent forms to sign before they were recruited into the study.

## CHAPTER IV

### 4.0 RESULTS

#### 4.1 Socio-demographic characteristics of the pregnant women

Table 3 shows the socio-demographic characteristics of the pregnant women.

In total, three hundred and thirty two (332) pregnant women agreed to participate in the study. Pregnant women were 18 to 39 years old with a median age of 27 years old (interquartile range (IQR) = 22-33). Of the surveyed women, 86 (25.9%; 95% CI 20.7-31.6) were primigravida, and the median gravidity was 3 (IQR = 1-5). The vast majority of the subjects (80.9%; 95% CI 76.1-85.0) were married and 64.9% had not completed secondary school. Fifty-nine percent of the pregnant women (59.2%) had started their antenatal care in the second trimester against 37.6% in the third trimester and only 3.2% in the first trimester. The mean gestational age in primigravidae at the time of the first prenatal visit was  $21.5 \pm 4.2$  weeks, vs.  $23.6 \pm 5.8$  in multigravidae ( $t = 3.11, p < 0.01$ ) (Table 3).



**Table 3: Socio demographic characteristics of pregnant women.**

		N	Total	Percentage
<i>Sociodemographic Characteristics</i>				
Age	<20	47	332	14.2
	20-49	285		85.8
Gravidity	Primigravidy	86	332	25.9
	multigravidy	246		74.1
Gestational age*	1 <sup>st</sup> and 2 <sup>nd</sup> trimester	191	306	62.4
	3 <sup>rd</sup> trimester	115		37.6
Marital Status	Married	259	320	80.9
	Single	61		19.1
Ownership and use of bed net	No	188		56.8
	Yes but not used	20	315	15.8
	Yes and used	107		84.3
Presence of lattice on windows	Yes	50	324	15.4
	No	274		84.6
Education	Illiterate	4		1.25
	Primary school	19		5.9
	Secondary school: uncompleted	184	319	57.7
	Secondary school	80		27.9
	University: incomplete	10		3.1
	University degree	13		4.1

#### **4.2. Prevalence of asymptomatic *P. falciparum* infection in pregnant women at the first prenatal care (PNC) visit**

From 332 pregnant women enrolled, complete RDT and microscopy data was available for 332 blood samples and of these 166 samples were analyzed by PCR. The prevalence of asymptomatic *P. falciparum* infection using microscopy TBS, RDTs and PCR, were respectively 21.6% (95% CI 17.4-26.6%), 27.4% (95% CI 22.7-32.6%) and 29.5% (95% CI 22.7-37.1%). Non-*falciparum* species were rare and were diagnosed by microscopy but not confirmed by PCR (five *Plasmodium malariae* cases). The median parasite density was 126/ $\mu$ l (IQR= 105-162). In bivariate analyses, age, gravidity, marital status and use of bed net have demonstrated a significant association with asymptomatic malaria. The regression model shows that being less than 20 years was strongly associated with asymptomatic *P. falciparum* infection (AOR 2.6; 95% CI 1.1-6.1, P=0.04), married women were less likely to have asymptomatic *P. falciparum* infection than a single women (AOR 0.3; 95% CI 0.1-0.9, p = 0.02), and infection with *P. falciparum* was less common among women who slept under a bed net compared to women who did not (AOR 0.3; 95% CI 0.2-0.8, p <0.01)(Table 4).

**Table 4: Parameter estimates from univariate and multivariate logistic regression model predicting the likelihood that a pregnant woman has asymptomatic *P.falciparum* infection**

		N	Positive TBS	OR	P value	AOR CI	95% CI	P value
<i>Sociodemographic Characteristics</i>								
Age	<20	47(14.2)	24(51.1)	5.2	<0.001	<b>2.6</b>	<b>(1.1-</b>	0.04*
	20-49	285(85.8)	48(16.9)	1		<b>6.4)</b>		
Gravidity	Primigravida	86(25.9)	27(31.4)	2.0	0.01	0.8	(0.3-	0.5
	multigravida	246(74.1)	45(18.3)	1		1.7)		
	1 <sup>st</sup> et 2 <sup>nd</sup> trimester	191(62.4)	40(20.9)	1				
Gestational age	3 <sup>rd</sup> trimester	115(37.6)	28(24.4)	1.2	0.49	0.7	(0.4-	0.3
						1.3)		
Marital Status	Married	259(80.9)	41(15.8)	0.22	<0.001	<b>0.3</b>	<b>(0.1-</b>	0.02*
	Single	61(19.1)	28(45.9)	1		<b>0.9)</b>		
Ownership and use of bed net	No	188(56.8)	48(25.5)	1				
	Yes but not used	20(15.8)	8(40)	1.9	0.17	1.9	(0.8-	0.2
	Yes and used	107(84.3)	12(11.2)	0.37	0.004	<b>0.3</b>	<b>(0.2-</b>	0.009*
Presence of lattice on windows	Yes	50(15.4)	11(22.0)	1.1	0.90	1.1	(0.5-	0.7
	No	274(84.6)	58(21.2)	1		2.5)		

\*significant at  $p < 0.05$ , OR- odds ratio, AOR- adjusted odds ratio, C.I- confidence interval.

### 4.3. Prevalence of anemia

The mean hemoglobin concentration in pregnant women was  $10.5 \pm 1.4$  g / dL and the prevalence of anemia was 61.1% (95% CI 55.7-66.4). Of these anemic pregnant women, 70 (21.3%), 132 (40.2%), 1 (0.3%), had respectively, mild, moderate and severe anemia (Table 5). In multivariate analyses only asymptomatic *P. falciparum* infection showed an association with anemia. The likelihood of having anaemia for pregnant women with asymptomatic malaria was about 3.8 times more (AOR 3.8; 95% CI 1.0-14,  $p = 0.04$ ). (Table 5, 6). No significant difference in the density of parasitemia was found in those with mild and moderate anemia ( $P=0.09$ ).

**Table 5: Prevalence of anemia in pregnant women**

	N	Percentage
Hemoglobin concentration	Severe anemia <7 g/dl	1 0.3
	Moderate anaemia 7 - 10 g/dl	132 40.
	Slight anaemia 10 - 11 g/dl	70 21.3
	Absence of anaemia $\geq 11$	125 38.1

**Table 6: Parameter estimates from univariate and multivariate logistic regression model predicting the likelihood that a pregnant woman is anemic**

		N	Anemia	OR	P value	AOR 95% CI	P value
<i>Sociodemographic Characteristics</i>							
Age	<20	47	34(72.3)	1.8	0.09	1.02 (0.2-6.1)	0.9
	20-49	285	169(59.3)	1			
Gravidity	Primigravidy	86	59(68.6)	1.5	0.1	0.8 (0.3-2.7)	0.8
	multigravidy	246	144(58.5)	1			
Gestational age*	1 <sup>st</sup> and 2 <sup>nd</sup> trimester	217	134(61.8)	1			
	3 <sup>rd</sup> trimester	115	69(60.0)	1.1	0.7	0.4(0.2-1.1)	0.09
Marital Status	Married	259	153(59.1)	0.7	0.2	0.6(0.1-2.4)	0.5
	Single	61	41(67.2)	1			
Ownership and use of bed net	No	190	115(60.5)	1			
	Yes but not used	20	14(70.0)	1.5	0.1	0.8(0.3-2.9)	0.7
	Yes and used	105	62(59.0)	0.6	0.2	0.5(0.2-1.3)	0.6
Malaria	Yes	72	60(83.3)	4.1	<0.001*	3.8(1.0-14)	0.04*
	No	260	143(55.0)	1			
Geophagia	Yes	179	102(57.0)	0.6	0.03*	0.9(0.2-2.9)	0.8
	No	152	100(65.8)	1			
Presence of lattice on windows	Yes	50	31(60.2)	1.1	0.8	1.6(0.5-4.4)	0.4
	No	274	164(59.9)	1			

\*significant at  $p < 0.05$ , OR- odds ratio, AOR- adjusted odds ratio, C.I- confidence interval.



#### **4.4. Possession and use of bed nets**

Of all participants, 42% possessed a mosquito net. Of these, 69.8% stated that their nets were treated with insecticide and 85.7% reported having slept under bed net the previous night. Married women were more likely to own a mosquito net compared to single women (OR 1.4 95% CI (1.0-3.5); P=0.03).

#### **4.5. Internal validity of microscopy and RDT using species specific PCR as goldstandard.**

PCR was performed on 166 randomly chosen samples. Compared to PCR, microscopy and RDTs had a sensitivity of respectively 67.3% (95%CI: 52.5-80.1) and 85.7% (95% CI: 72.8-94.1) to diagnose asymptomatic *P. falciparum* infection. Specificity was 96.6% (95% CI: 91.5-99.1) and 97.4% (95%CI: 92.7-99.5) for microscopy and RDTs, respectively. RDT demonstrated a positive predictive value (PPV) of 91.3% (95%CI 79.2-97.6%) and a negative predictive value (NPV) of 94.2% [95%CI 88.4-97.9%). Microscopy had similar PPV 91.7% (95% CI 77.5-98.2%) and the NPV 87.7% (95% CI 80.8-92.8%) was not significantly different compared to RDT (Table 7). thirteen out of 20 RDT positive but microscopy negative samples were positive by PCR (65%; 95%CI: 15.4- 59.2) (Table 8).

**Table 7: Comparison of RDT and microscopy performance using PCR as gold standard on the 166 samples where PCR results were available**

	PCR results			Performances				
	Positive	Negative	Total	Sensitivity	Specificity	PPV	NPV	
Microscopy	+	33	3	36	67.3%	97.4%	91.7%	87.7%
					(52.5-80.1)	(92.7-99.5)	(77.5-98.2)	(80.8-92.8)
	-	16	114	130				
RDT	+	42	4	46	85.7%	96.6%	91.3%	94.2%
					(72.8-94.1)	(91.5-99.1)	(79.2-97.6)	(88.4-97.9)
	-	7	113	120				

**Table 8: PCR analysis results of samples that were RDT positive but microscopy negative**

No. microscopy negative samples	PCR results	
	Positive	Negative
20	13(65%)	7(35%)

## CHAPTER V

### 5.0 DISCUSSION

The purpose of this study was to determine the prevalence of asymptomatic malaria and to assess the extent of anemia among pregnant women living in the south eastern suburbs of Kinshasa, attending the CHK II for their first PNC visit. The study revealed that the prevalence of asymptomatic malaria was 27% and 21% respectively, using RDT and microscopy TBS detection methods. Polymerase Chain Reaction performed on 166 samples showed a prevalence of 29%. The microscopy results showing prevalence of 21% are similar to previous results of 22% prevalence reported in 1988 by Mbanzulu *et al.*(1988). However, these values are paradoxically lower than the 42% prevalence found in Lubumbashi, an area of low malaria transmission (Kalenda *et al.* 2003). Asymptomatic infection is characteristic in areas of high malaria transmission; one would expect a higher prevalence in Kinshasa. The increased use of ITNs in Kinshasa could explain this discrepancy. In addition, the conduct of this study during the dry season, period subjected to a mild/low malaria transmission, suggests that the prevalence reported here could be largely underestimated. Parasite density was also found to be very low in all infected pregnant women. These results could be explained by malaria control programs efforts (changes in first-line treatment of malaria and the introduction of long-lasting insecticide-treated nets) in endemic areas (Ceesay, *et al.*, 2008, Roucher *et al.*, 2012).

In this study women who slept under mosquito nets were less likely to have asymptomatic *P. falciparum* infection compared to women who did not use a mosquito net. Our results are in agreement with other studies that found a protective effect of bed nets against malaria in pregnancy (Feng et al., 2010).

Asymptomatic *P.falciparum* infection was more frequent among young women compared to older women. Studies have shown that young women of child-bearing age may be more susceptible than older women to malaria because they are still in the process of acquiring natural immunity (Shi et al. 1995, Oeuvray et al. 2000).

Single women were more likely to have asymptomatic *P. falciparum* infection compared to married women. In addition, owning a mosquito net was associated with being married, as previously reported by Audrey Pettifor in 2008 ( Pettifor et al., 2008). This suggests that marriage or living with a partner, offers women an ideal setting to promote maternal health.

Gravidity did not show association with asymptomatic *P. falciparum* infection. This contrasts with the theory of immunity against malaria in pregnancy, which increases with successive pregnancies. Similar observations, however, were made in other malaria-endemic areas (Agan et al., 2010, Zoenabo . et al., 2012).

Almost half of the surveyed women, (42%), declared to own a bed net. These results are higher than the 26%, 33% and 7% previously reported by UNDP (UNDP, 2009), Pettifor ( Pettifor et al., 2008) and the National Malaria Control Program (NMCP, 2007). This positive evolution could contribute to the low prevalence of asymptomatic infections found in this study.

On the other hand, high prevalence (61.1%) of anemia was observed and it was strongly correlated with asymptomatic *P. falciparum* infection (OR 3.8,  $p < 0.04$ ). This prevalence is similar to 65% prevalence reported in Kisangani in 2000 (Kanku *et al.*, 2000) and Lubumbashi (average prevalence of 65%) in 2003 (Kalenda *et al.*, 2003). However, Kinshasa, Kisangani and Lubumbashi belong to three different epidemiological malaria patterns, hence to three different levels of transmission. This underlines the involvement of other risk factors for anemia in pregnant women, although the contribution of malaria seems to be predominant. The aim of the study was not to assess the risk factors for anemia in pregnant women; a dedicated study would be more appropriate to disclose this useful information. The severity of anemia could not be associated to gestational age and use of bed nets. This is not in agreement with results reported by Nwagha *et al.*, 2009. In addition, there was no significant difference in the density of parasitemia in those with mild and moderate anaemia. This observation was also reported in Nigeria (Nwagha *et al.*, 2009). The present study corroborated previous studies which showed that bed nets were not protective against anaemia (Gamble *et al.*, 2006; Lengeler *et al.*, 2004).

In the present study, no significant difference was found between microscopy and RDT when compared to PCR as gold standard based on 166 samples. This observation may be due to the small sample used to assess the performances of the three tests. In addition all the RDT positive but microscopy negative samples were checked by PCR and the analyses showed that 65% of the RDT positive but microscopy negative samples were in fact submicroscopic infections. However, 35% were negative by PCR. This could be explained by the persistence of HRP-2

circulation in blood for more than two weeks even after successful clearance of IEs in the bloodstream (Humar et al. (1997). The message portrayed here is that the RDT used in this study was able to capture over 65% of sub-microscopic infections missed by microscopy after PCR correction. A well-designed study may be useful to bring more insight regarding the performances of RDT and microscopy in diagnosing asymptomatic malaria in endemic areas of DRC.

## CHAPTER VI

### 6.0 CONCLUSION AND RECOMMENDATIONS

#### 6.1. CONCLUSION

This study reports:

1. A high prevalence of asymptomatic *P.falciparum* infection, mostly in younger women, single women and women who do not use bed nets.
2. A high prevalence of anemia which was strongly correlated with asymptomatic *P. falciparum* infection.

#### 6.2. RECOMMENDATIONS

These results are quite alarming and emphasize the need to actively diagnose and treat asymptomatic malaria infection using appropriate techniques during PNC visits in areas of high malaria transmission, regardless of IPTp. On the other hand, the use of ITNs in DRC is slightly increasing; however, the promotion of bed net use should be further encouraged. In addition, well-designed studies carried out to assess other risk factors of anemia in pregnancy in Kinshasa and the performances of RDT and microscopy in diagnosing asymptomatic malaria in endemic area of DRC.

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## APPENDICES

### Appendix i: English questionnaire

#### Asymptomatic *P.falciparum* infection in pregnancy in Kinshasa

Study ID : \_ \_ \_    Initials: \_ \_ \_

#### A. Sociodemographic data

1. Age : |\_\_|\_\_|    years

2. Gestity: |\_\_|\_\_|

3. Gestational age: |\_\_|\_\_| weeks

4. Possession of Bed net:  Yes  No

Is your bed net insecticide treated?  Yes     No     I do not know

Last night did you sleep under your bed net? :  Yes     No

If no, why?     I have forgotten to set it up     it is hot under a bed net

I do not like it     others (to be precise) .....

5. Geophagia :  Yes  No

Quantity:  slight     moderate     too much

6. Marital status :  married     single

7. Education:  Illiterate  Primary school degree  Secondary schooluncompleted  Secondary school degree  University: uncompleted  University degree

**B. Questions**

1. presence of lattice on windows ?  Yes  No
2. If yes, in what conditions ?  Good  bad

## Appendix ii: Lingala questionnaire

### Bokono ya Malaria ya kobombama epayi ya mwisi ya zemi

Motango ya moyekoli : \_ \_ \_ Kombo: \_ \_ \_

#### A. Makambo oyo etali yo

1. Mbula : |\_\_|\_\_|

2. Ba zemi: |\_\_|\_\_|

3. Mbula ya zemi: |\_\_|\_\_| weeks

4. Kozala na moustiquaire:  ya solo  te

Moustiquaire na yo ezali na kisi ya ngungi?  ya solo  te  na yebi te

Lobi na butu o lalelaki yango? :  ya solo  te

Soki te, pona nini?  na bosanaki ko tia yango  e pesaka moto

na lingaka yango te  mosusu .....

5. kolia mabele :  ya solo  te

Ndenge nini:  muke  mwa mingi  mingi penza

6. libala:  obala  likombe

7. kelasi:  atanga te  ecole primaire  asilisa ecole primaire te

asilisa co  asilisa kelasi ya monene te  asilisa kelasi ya monene

**B. mituna**

1. miyungulu na fenetre ? ya solo  te
2. soki ya solo ezali ndenge nini ? malamu yako beba



### Appendix iii: French questionnaire

#### Fiche de renseignement

Numéro d'identification : .....

#### A. Renseignements sociodémographiques

1. Age : |\_|\_| | années

2. gestité : |\_|\_|

3. Age gestationnel : |\_|\_| semaines

4. Utilisation de la moustiquaire imprégnée d'insecticide :  oui  non

Votre moustiquaire est-elle imprégnée d'insecticide ?  Oui  Non  je ne sais pas

La nuit dernière, avez-vous dormi sous la moustiquaire en question :  Oui  Non

Si non, pourquoi ?  j'ai oublié de la monter  à cause de la chaleur

je ne supporte pas ça  autre (à préciser) .....

5. Prise d'un vermifuge :  oui  non

Si oui ; - lequel ? \_\_\_\_\_

- et quand ?  moins d'un mois  un à trois mois  3 à 6 mois

6 mois à 1 année  plus d'une année

6. géophagie :  oui  non

Quantité :  faible  modérée  importante

7. Etat civil actuel :  mariée  union libre  célibataire  veuve   
séparée  divorcée

8. Niveau d'étude :  analphabète  primaire non achevé  primaire  
achevé  secondaire non achevé  secondaire achevé  universitaire  
non achevé

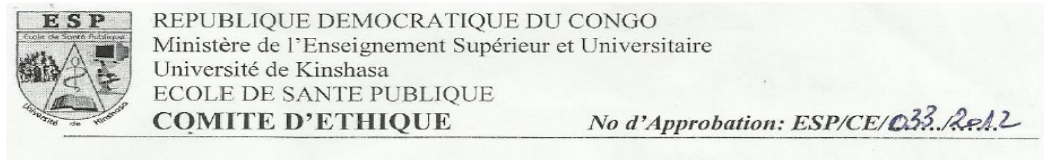
universitaire achevé  post universitaire  non applicable

## **B. Questions**

1. Votre habitation dispose-t-elle des treillis aux fenêtres ?  Oui  Non

2. Si oui, en quel état ?  Bon  Délabré

## Appendix iv: Ethical approval



Kinshasa le 11 mai 2012

**A Monsieur l'investigateur Principal  
Faculté de Médecine  
Université de Kinshasa**

Concerne : Décision du Comité d'Ethique sur l'étude : « *infection à P. falciparum asymptomatique chez la femme enceinte dans la Zone de Santé de Kingasani à Kinshasa RDC* »

Monsieur l'Investigateur Principal,

Le Comité d'Ethique de l'Ecole de santé Publique de l'Université de Kinshasa a bien reçu le protocole dont le titre est repris en marge et nous vous remercions.

Le Comité d'Ethique a examiné le protocole susmentionné selon les normes d'éthique nationales sur les études impliquant les êtres humains. Le Comité a approuvé et l'autorise à être exécutée à travers les sites ciblés. Cette autorisation est valable pour une année, allant du 11 Mai 2012 au 10 Mai 2013.

Veuillez agréer, monsieur l'investigateur Principal, l'expression de notre considération distinguée.



Prof. BONGOPASI MOKE SANGOL  
 Vice Président du Comité Ethique

Université de Kinshasa Faculté de Médecine : B.P 11850 Kin I.

## **Appendix v: English informed consent**

Study title: Asymptomatic *P.falciparum* infection in pregnancy: Prevalence of peripheral parasitaemia and anemia in Kingasani health Zone, Kinshasa DRC

We ask your authorization to participate in this research study. The aim of this formulary is to give you all the explanation regarding this study and to get your consent to participate in this study. You are not obliged to agree and if you do not agree or withdraw yourself from the study at any moment there will be no inconvenience for you.

### ***Information about the study***

In most endemic areas of Africa, pregnant women are the main adult risk group for malaria. The symptoms and complications of malaria during pregnancy differ with the intensity of malaria transmission and thus with the level of immunity acquired by the pregnant woman. In areas of high and moderate (stable) malaria transmission, most adult women have developed sufficient immunity that, even during pregnancy, *P. falciparum* infection does not usually result in fever or other clinical symptoms. In these areas, the principal impact of malaria infection is malaria related anaemia in the mother and the presence of parasites in the placenta, named placental malaria (PM).

The aim of this study is to determine the extent of asymptomatic *P.falciparum* infection in pregnant women in Kingasani health zone, in Kinshasa DRC.

We therefore invite you to participate to this study by signing the informed consent paper and by letting us collect 5ml of blood and do and a fingerpick. And by agreeing the data we will collect in exception of your personal data to be used in the study. Laboratories analysis will be made in a laboratory outside the country. But all positive blood smears will be supplied by anti malarial drug.

***Study procedure***

Nurses and medical doctor will collect the blood samples and will ask you some questions related to your health status and your pregnancy.

***Benefices and risks***

The information generated from the present study will help to improve pregnant women's health care. The bit of the needle during blood collection could lead to an infection in the site of collection but this will be done by professional staff using sterilized single use material. This precautions will reduce the post infection risk.

***Willing participation***

The decision to participate in the study is totally willing to you and if you do not agree, there will be none consequences.

***Cost and compensation***

You will not pay anything and no incentive will be provided by the team research if you accept to be included in the study.

***Confidentiality***

The data collected will stay strictly confidential. Your name or any other personal information will not be published in written or oral reports. You will be given a study ID. Collected samples will only be used for laboratory analysis according to this study.

***Questions***

For any further question and information, please contact the principal investigator, Dr Junior MATANGILA from Tropical medicine Department, Kinshasa University. Phone number: +243 97 07 56 890 e-mail :matangilaj@yahoo.fr or the local supervisor of the study : Pr Pascal Lutumba : +243 81 81 58 961 e-mail : pascal\_lutumba@yahoo.fr .

***Written consent***

I have been explained the aim of this study and I understand the objectives and the conditions. I have been answered to each of my questions. I understand that the participation to this research study is willingness and that I am free to withdraw myself at any moment without any inconvenient in the future. Personal information obtained from me will be kept confidential and will not be published in any publication. I understand that if I have questions to ask or I want to withdraw myself I can contact the principal investigator or the local supervisor on the address given.

I agree to participate to this study:

Name

Signature

.....

.....

Name and signature of participant witness ( in case the participant cannot write):

Name

Signature

.....

.....

Name and signature of the person who explained the informed consent

Name

Signature

.....

.....

Place : .....

Date : .....

Time : .....

**Appendix vi: French informed consent**

Titre de l'étude : « Infection asymptomatique à P.falciparum chez la femme enceinte ; prevalence de l'infection et de l'anémie dans la Zone de santé de Kingasani à Kinshasa RDC »

Nous demandons votre autorisation pour que vous participiez à cette étude de recherche. Le but de ce formulaire est de vous donner toutes les explications concernant cette étude et d'avoir votre consentement pour votre participation. Vous n'êtes pas obligé d'accepter et si vous vous retirez de cette étude, il n'en résultera aucun inconvénient pour vous.

**INFORMATIONS SUR L'ETUDE**

En zone d'endémie palustre, particulièrement en Afrique, les femmes enceintes représentent le principal groupe d'adultes à risque pour l'infection à Plasmodium falciparum. En zone de transmission stable du paludisme, tel qu'à Kinshasa, les principales manifestations du paludisme chez la femme enceinte sont l'anémie et l'accumulation du parasite dans le placenta appelée Malaria Placentaire. Cette dernière peut entraîner l'avortement, la mort in utero, le faible poids de naissance et la mort infantile.

Cette étude a pour but de déterminer la prévalence de l'infection palustre asymptomatique et de l'anémie chez la femme enceinte dans la Zone de Santé de Kingasani à Kinshasa RDC.

Nous vous invitons donc à participer à cette étude en signant ce formulaire de consentement éclairé et en nous permettant de prélever 5 ml de sang pour les différentes analyses de laboratoire. Les données collectées à l'exception de vos données personnelles seront utilisées dans notre étude. Certaines analyses de laboratoires seront faites dans des laboratoires en dehors du pays. Toutes les femmes enceintes ayant des signes de malaria avec ou sans présence de parasites dans le sang recevront des médicaments contre la malaria.



**Procédure de l'étude**

Des infirmiers et des médecins prélèveront les échantillons de sang et de placenta, feront un examen général et poseront des questions sur les épisodes de fièvres des mois précédent.

**Bénéfices and risques**

La piqure de l'aiguille durant le prélèvement sanguine pourrait entrainer une infection au site de prélèvement mais ceci sera fait par un personnel qualifié utilisant du matériel à usage unique. Ces précautions réduisent donc ce risque d'infection.

**Participation volontaire**

La décision de participer à cette étude est totalement volontaire de votre part et si vous n'acceptez pas, il n'y aura aucune conséquence.

**Cout et compensation**

Vous ne payerez rien et aucune motivation ne sera vous donnée par l'équipe de recherche si vous acceptez que votre enfant participe a l'étude.

**Confidentialité**

Les données collectées resteront strictement confidentielles. Votre nom ou des autres informations personnelles ne seront pas publiées dans les rapports écrits ou oraux et vous aurez un numéro d'identification dans l'étude. Les échantillons collectés ne seront utilisées que pour des analyses de laboratoires conformément au protocole de cette étude.

### Questions

Pour plus d'informations, veuillez contacter le principal investigateur, Dr Junior Matangila du Département de Médecine Tropicale de la Faculté de Médecine, Université de Kinshasa. Numéro de telephone : +243 97 07 56 890 e-mail : matangilaj@yahoo.fr ou le superviseur local de l'étude : Pr Pascal Lutumba : +243 81 81 58 961 e-mail :pascal\_lutumba@yahoo.fr .

### Consentement écrit

L'on m'a expliqué le but de cette étude et je comprends les objectifs ainsi que les conditions. L'on a répondu à chacune de mes questions. Je comprends que ma participation a cette étude est volontaire et que je suis libre de me retirer à tout instant sans aucun inconvénient dans le future. Les données personnelles sur moi demeureront confidentielles et ne seront publiées dans aucune publication. Je comprends que si j'ai d'autres questions, a poser ou que si je veux me retirer de l'étude , je dois contacter l'investigateur principal ou le superviseur local.

Moi ..... J'accepte  
de participer à cette étude:

Nom et signature de la participante ou de son tuteur légal

Nom

Signature

.....

.....

Nom et signature du Témoin de la participante (si cette dernière ne sait pas écrire) :

Nom

Signature

.....

.....

Nom et signature de la personne qui a expliqué le consentement éclairé

Nom

Signature

.....

.....

Place: .....

Date: .....

Heure: .....

### **Appendix vii: Lingala informed consent**

**Kombo ya moyekoli :BOkono ya malaria epayi ya mwasi ya zemi ya zone de sant » ya Kingasani na Kinshasa RDC.**

To sengi ndingisa na yo po été okota na moyekoli oyo. Tina ya mokanda oyo ezali ko yebisa yo nyonso oyo etali moyekoli oyo. Okoki ko ndima to ko boya kokota na moyekoli oyo. Soki ondimi te, mabe moko te eko yela yo.

### **OYO ETALI MOYEKOLI**

Bokono ebengami malaria e tungisaka mingi mingi mwasi ya zemi na mwana na ye. Epayi ya basi ya zemi oyo ba fandi na esika ebengami milieu de transmission stable du paludisme, bokona ya malaria ekendeke ko fanda na Placenta nde yango ebengami “Malaria Placentaire” ba consequences na yango ezalaka mingi mingi, avortement, kobotama ya mwana na kilo ya moke to pe kokufa ya mwana mikolo moke sima ya kobotama na aye. Mwasi ya zemi oyo azali na bokono ya Malaria Placentaire ayebanaka te ti mokolo oyo ako bota wana nde placenta na ye eko talisa été a zalaki na bokono yango

Tina ya moyakoli oyoy ezali po na ko yeba basi ya zemi boni bazali na bokono oyo na Kinshasa.

To zo senga bino bo ndima kokota na moyekoli oyo na ko koma mokoloto na yo na mokanda mua bolimboli oyo pe na ko ndima to benda 5 cc ya makila epayi na yo, pe to zwa eteni ya placenta soki o uti ko bota. No kondima na yo, to ko salela ba donnee nyonso longola oyo etali yo moko na moyekoli na biso. Ba examens ya labo misusu

ekosalama libanda ya mboka. Mutu oyo akomonana na bilembo ya malaria na nzoto na yo ba ko pesama kisi, examen emonisa microbe ya malaria to emonisa yango te.

### **Lolenge moyekoli eko tambola**

Ba munganga pe ba doctotolo ba ko benda basi ya zemi makila, bako sala bango examen ya nzoto pe ba ko tuna mituna na oyo etali ba fievres eyaki na ba sanza eleki.

### **Matabisi na ba risques**

Esika ba ko tuba tonga ekoki ki koma pota kasi lokola yango ekosalema na batu ba yebi musala na bango pe ba ko salela materiel ya usage unique, ezali pasi po pota eya.

### **Kokota na nguyana na yo**

Yo nde okondima okota na moyekoli oyo. Soki oboyi mabe moko te ekoyela yo.

### **Kofuta**

Oko futa eloko te pe ba ko futa yo eloko te soki ondimi kokota na moyekoli oyo.

### **Ko bomba ba sango**

Ba sango nyonso to ko zua to kobomba yango. Kombo to pe ba sango misusu ya yo ekokomama te, pe ekolobama te. To ko pesa yo numero na moyekoli oyo. Ba examens to ko sala yo ezali kaka oyo to tangi na moyekoli na biso.

**Mituna**

Soki ozali na mituna misusu, yeba ko benga principal investigateur, Dr Junior Matangila ya Département de Médecine Tropicale, Faculté de Médecine, Université de Kinshasa. Numéro de telephone : +243 97 07 56 890 e-mail : matangilaj@yahoo.fr te pe superviseur local ya moyekoli : Pr Pascal Lutumba : +243 81 81 58 961 e-mail :pascal\_lutumba@yahoo.fr .

**Bolimboli**

Ba limboleli nga tina ya moyekoli oyo pe na sosoli tina na yango. Ba pesi nga biyano na mituna nyonso. Na sosoli été ko kota na nga na moyekoli oyo ezali na nguya na nga pe soki na boyi, na koki ko bima na ngai tango nyonso, pe mabe miko eko yela nga te, na sima. Ba sango oyo etali ngai eko lobama pe ko komama na publication moko te. Na sosoli été soki na zali na mituna to pe na lingi ko longwa na ngai na moyekoli oyo na koki ko benga investigateur principal to pe superviseur local.

Ngai .....

Na ndimi kokota na moyekoli oyo:

Kombo pe mokoloto ya mwasi ya zemi to tuteur na ye

Kombo

Mokoloto

.....

.....

Kombo pe mokoloto ya Témoin ( soki mwasi ya zemi a yebi kokoma te) :

Kombo

Mokoloto

.....

.....

Kombo pe mokoloto ya mutu oyo a tangisi mokanda mwa bolimboli

Kombo

Mokoloto

.....

.....

Esika: .....

Mokolo: .....

Tango: .....