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AN INQUIRY INTO SULFADOXINE-PYRIMETHAMINE RESISTANCE AMONG THE PREGNANT WOMEN WHO RECEIVE INTERMITTENT PREVENTIVE TREATMENT

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ABSTRACT

Bateko, J.P.K. An Inquiry Into Sulfadoxine-Pyrimethamine Resistance Among The Pregnant Women Who Receive Intermittent Preventive Treatment. Near East University Institute of Health Sciences, M.Sc. Thesis in Medical Microbiology and Clinical Microbiology Programme, Nicosia, 2015.

Malaria which is caused by *Plasmodium* parasites continues to be a major health problem. The majority of malaria cases and deaths from malaria occur in Sub-Saharan Africa region. Malaria threatens several risk groups including pregnant women. The infection in this group has adverse outcomes for the mother, the fetus as well as the newborn. In order to control malaria infection in pregnant women, World Health Organization (WHO) recommends the administration of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP). However, the increasing resistance against SP can be a limiting factor for its prophylactic use during pregnancy. This research was conducted to evaluate the efficiency of SP against malaria in a group of pregnant women from the Democratic Republic of Congo (DRC). Two hundred and fifty women who received SP prophylaxis during the pregnancy were included in this study. Following the delivery, blood samples were collected from the women. *Plasmodium* parasites in the blood samples were investigated by microscopy, rapid diagnostic test (RDT) and polymerase chain reaction (PCR). Nested PCR was conducted for detection of *Plasmodium* parasites at the species level. The prevalence of malaria infection determined by microscopy, RDT and PCR were 32.4%, 37.2% and 36.8%, respectively. RDT presented a sensitivity of 59.3% and specificity of 73.4% when microscopy was taken as the gold standard. Sensitivity and specificity of RDT were 55.4% and 73.4%, respectively, when PCR was taken as the gold standard. *P. falciparum* was isolated as the only agent in 94.5% of the positive samples detected by nested PCR. This species was isolated concurrently with *P. vivax* and *P. malariae* in two (2.2%) and one (1.1%) samples, respectively. *P. vivax* was also detected as the only agent in two (2.2%) samples. In this study, *P. ovale* and *P. knowlesi* were not isolated. Mutations in *Pfdhfr* and *Pfdhps* genes that confer resistance to SP were examined by DNA sequencing. Among 20 samples sequenced for *Pfdhfr* gene, C59R mutation was detected in 10 of 20 samples, while no mutation was observed in the remaining samples. No mutation was detected in the other 10 samples sequenced for *Pfdhps* gene. Results of this study indicated that, malaria infection was detected in a considerable amount of women who participated in the research. This suggested that SP prophylaxis was not effective in terms of protection of these women against malaria during pregnancy.

Key words: Malaria, *Plasmodium*, pregnancy, sulfadoxine-pyrimethamine

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Bateko, J.P.K. Aralıklı Koruyucu Tedavi Uygulanan Hamile Kadınlarda Sulfadoksin-Primetamin Direncinin Araştırılması. Yakın Doğu Üniversitesi Sağlık Bilimleri Enstitüsü, Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji Programı, Yüksek Lisans Tezi, Lefkoşa, 2015.

Plasmodium türü parazitler tarafından gelişen sıtma hastalığı önemli bir sağlık sorunu olmaya devam etmektedir. Sıtma olguları ve buna bağlı gelişen ölümlerin çoğu Sahraaltı Afrika bölgesinde görülmektedir. Sıtma hastalığı hamile kadınlar dahil bazı risk grupları için tehdit oluşturmaktadır. Hamilelik sırasında oluşan sıtma enfeksiyonu anne, fetus ve yenidoğan açısından riskler taşımaktadır. Hamile kadınlardaki sıtma enfeksiyonunun önlenmesi için, Dünya Sağlık Örgütü (WHO) sulfadoksin-primetamin (SP) ile aralıklı koruyucu tedavinin (IPTp-SP) verilmesini önermektedir. Ancak, SP'ye karşı görülen dirençteki artış bu ilacın hamilelik dönemindeki profilaktik tedavide kullanılması konusunda sınırlayıcı bir etken olabilmektedir. Bu araştırma Kongo Demokratik Cumhuriyeti'ndeki bir grup hamile kadında SP'nin sıtma hastalığına karşı olan etkinliğinin değerlendirilmesi için yapılmıştır. Hamilelik döneminde SP profilaksisi verilen 250 kadın bu çalışmaya dahil edilmiştir. Doğumun ardından sözkonusu kadınlardan kan örnekleri toplanmıştır. Kan örneklerindeki *Plasmodium* parazitleri mikroskopi, hızlı tanı testi (RDT) ve polimeraz zincir reaksiyonu (PCR) ile araştırılmıştır. *Plasmodium* parazitlerinin tür sevitesinde saptanması için nested PCR uygulanmıştır. Sıtma enfeksiyonunun prevelansı mikroskopi, RDT ve PCR ile sırasıyla %32.4, %37.2 ve %36.8 olarak bulunmuştur. Mikroskopi altın standart olarak kabul edildiği zaman, RDT'nin duyarlılığı %59.3, özgüllüğü ise %73.4 olarak bulunmuştur. PCR altın standart olarak kabul edildiği zaman ise RDT'nin duyarlılığı ve özgüllüğü sırasıyla %55.4 ve %73.4 olarak bulunmuştur. Nested PCR ile pozitif saptanan örneklerin %94.5'inde *P. falciparum* tek etken olarak izole edilmiştir. Bu tür, *P. vivax* ve *P. malariae* ile, sırasıyla iki (%2.2) ve bir (%1.1) adet örnekte birlikte izole edilmiştir. *P. vivax* aynı zamanda iki (%2.2) adet örnekte tek etken olarak saptanmıştır. Bu çalışmada, *P. ovale* ve *P. knowlesi* izole edilmemiştir. SP direncine neden olan *Pfdhfr* ve *Pfdhps* genlerindeki mutasyonlar DNA dizi analizi ile incelenmiştir. *Pfdhfr* geninin araştırıldığı 20 örnekten 10 tanesinde C59R mutasyonu saptanırken, geri kalan örneklerde herhangi bir mutasyon gözlenmemiştir. *Pfdhps* geninin araştırıldığı 10 örnekte de herhangi bir mutasyon saptanmamıştır. Bu çalışmanın sonuçları, araştırmaya katılan kadınların önemli bir kısmında sıtma enfeksiyonunun saptandığını ortaya koymuştur. Bu durum, sözkonusu kadınların hamilelik döneminde sıtma hastalığına karşı korunması için SP profilaksisinin etkili olmadığına işaret etmiştir.

Anahtar kelimeler: Sıtma, *Plasmodium*, hamilelik, sulfadoksin-primetamin

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SYMBOLS AND ABBREVIATIONS

ANC	Antenatal care
ACT	Artemisinin-based combination therapy
CSA	Chondroitin sulfate A
DRC	Democratic Republic of Congo
EIR	Entomological inoculation rate
EPV	Expanded Programme on Vaccination
EDTA	Ethylenediaminetetraacetic acid
EBA	Erythrocyte-binding antigen
G6PD	Glucose-6-phosphate dehydrogenase
HRP-2	Histidine-rich protein 2
IPT	Intermittent preventive treatment
IPTi	Intermittent preventive treatment in infants
IPTi-SP	Intermittent preventive treatment in infants with sulfadoxine-pyrimethamine
IPTp	Intermittent preventive treatment in pregnancy
IPTp-SP	Intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine
IRS	Indoor residual spraying
ITN	Insecticide-treated net
LDH	Lactate dehydrogenase
PCR	Polymerase chain reaction
pLDH	*Plasmodium* lactate dehydrogenase
P. falciparum	*Plasmodium falciparum*
P. vivax	*Plasmodium vivax*
P. ovale	*Plasmodium ovale*
P. malariae	*Plasmodium malariae*
P. knowlesi	*Plasmodium knowlesi*
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
Pfcrt	Gene encoding *P. falciparum* chloroquine resistance transporter
Pfmdr1	Gene encoding *P. falciparum* multidrug resistance 1 protein

Pfdhfr	Gene encoding *P. falciparum* dihydrofolate reductase
Pfdhps	Gene encoding *P. falciparum* dihydroptereoate synthase
PNLP	National Malaria Control Programme (*Programme National de la Lutte contre le Paludisme*)
QBC	Quantitative Buffy Coat
RBC	Red blood cell
RDT	Rapid Diagnosis Test
RES	Reticuloendothelial system
rpm	Revolutions per minute
SMC	Seasonal malaria chemoprevention
SP	Sulfadoxine-pyrimethamine
WHO	World Health Organization

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1. INTRODUCTION

Malaria continues to be the major cause of morbidity and mortality in sub-Saharan Africa (World Health Organization, 2011). Each year, 216 million people are affected worldwide. Nearly 655,000 children and adults die from the disease each year worldwide despite the availability of effective preventive and curative measures (WHO, 2014). Approximately 94% of these deaths occur in sub-Saharan Africa (Bryce et. al., 2005). This high prevalence of malaria is due to a situation of poverty and inadequate health services (Koudou et. al., 2009). Moreover, malaria itself tends to keep people in a state of poverty, because it causes the loss of more than $12 billion annually to all sub-Saharan countries, foreign investment and tourism (Goesch et. al., 2008). *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi* are the species that cause malaria. Among these, *P. falciparum* causes the highest mortality rate and the most severe clinical manifestations. These parasites are transmitted to humans by the vectors belonging to *Anopheles* species (Ramasamy, 2014).

In the Democratic Republic of Congo (DRC), 97% of the populations live in areas of high level of malaria transmission. Three commonly encountered parasite species are *P. falciparum*, *P. ovale* and *P. malariae*. Among these, *P. falciparum* is the most common species (95%), and the most commonly encountered vector is *Anopheles gambiae* (92%). In the DRC, malaria is responsible for 30% of hospitalizations and 47.1% of deaths among children under 5 years (Menendez and Ordi, 2000). According to WHO, the vast majority of victims are children aged 0-5 years and vulnerable pregnant women. Malaria infection in pregnant women is a very serious public health issue because it poses significant risks for the mother, the fetus and the newborn. Several studies have demonstrated that placental and fetal distress resulting from the infection had a correlation with low birth weight and neonatal mortality (Mulumba, 2006).

In low transmission areas of *P. falciparum*, where the acquired immunity rates are low, women are exposed to severe malaria attacks, which can cause the birth of a stillborn baby, a miscarriage or maternal death (Stephens et. al., 2014). In areas of high transmission of *P. falciparum*, where the acquired immunity rates are

generally high, women are exposed to asymptomatic infection, which can lead to maternal anemia, placental parasitemia and therefore insufficient birth weight (Steketee et. al., 2001). Low birth weight is an important factor that contributes to infant mortality (Padonou et. al., 2013).

Due to the presence of chondroitin sulfate A (CSA), a molecule found in the intervillous space, the parasites can accumulate in placenta. The presence of parasites in the placenta has a risk ratio of 1 to 26 times greater than in the peripheral blood (Mulumba, 2006). According to estimation, malaria is responsible for 5 to 12% of all cases of low birth weight during pregnancy and it is also responsible for 75.000-200.000 children deaths each year (Steketee et. al., 2001).

To avoid this situation, WHO with National Malaria Control Programme (*Programme National de la Lutte contre le Paludisme: PNLP*) in the DRC currently recommends a series of interventions against malaria in pregnant women and in the areas of *P. falciparum* stable transmission. These include the use of insecticide-treated nets (ITNs), the administration of intermittent preventive treatment in pregnancy (IPTp), and proper management of malaria in pregnant women. According to WHO, sulfadoxine-pyrimethamine (SP) is recommended for the intermittent preventive treatment (IPT) of malaria in pregnancy (WHO, 2013a).

In this study, the efficiency of intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP) against placental malaria infection was inquired. For this purpose, a total of 250 women from Bandundu city, the DRC, who received SP prophylaxis during pregnancy were included in this study. Firstly, capillary blood samples from these women soon after the birth were collected. Blood smears and rapid diagnostic test (RDT) were performed on the samples in order to determine the presence of malaria infection. For the identification of malaria parasites in the pregnant women at the species level, polymerase chain reaction (PCR) was performed. Finally, DNA sequencing was conducted in terms of detection of the resistance genes that confer resistance against SP in *Plasmodium* species.

This study has two major advantages. First of all, this research could help vulnerable people keep their lives stable in the presence of malaria infection that

remains a major public health problem. Besides, this study could assist national health authorities to establish better strategies for eradication of malaria in the DRC.

2. GENERAL INFORMATION

2.1. Epidemiology of Malaria

2.1.1. Malaria in the World

With 198 million episodes and 584.000 deaths reported in recent years, malaria remains one of the most common and most deadly parasitic diseases worldwide. Fifty-eight percent of deaths were documented among children under 5 years (WHO, 2014).

The African region alone accounts for 81% of malaria cases and 91% of deaths due to malaria (WHO, 2014). Six countries in this region, Nigeria, the DRC, Burkina Faso, Mozambique, Cote d'Ivoire and Mali account for 390.000 (60%) of deaths caused by malaria (De Beaudrap et. al., 2013). After Nigeria, the DRC has the second highest numbers of morbidity and mortality caused by malaria (WHO, 2013b).

2.1.2. Malaria in the DRC

Profile of the DRC

The DRC is the largest and most populated country in Central Africa, with an area of 2.345.409 km^2, also an estimated population of 86.453.301 (PNLP, the DRC, 2013). It is a highly decentralized unitary state which includes 11 provinces, 25 administrative districts and 21 cities (National Statistics Institute, the DRC, 2014).

The DRC is located on the Equator. The country has a hot and humid climate in the central region and a tropical equatorial climate in the south and north parts. Both climates are favorable for mosquitoes and malaria transmission. Due to the mountains found in the region, the DRC has a climate with average temperatures ranging from 16-18°C in the East which does not allow the spread of mosquitoes. The water surface represents 52% of the total reserves of the continent, covering about 86.080 km^2 (3.5%) of the land area. Distribution of the population is disproportionate in the country, since 69.6% of the people live in the rural areas, while 30.4% resides in the urban areas (National Statistics Institute, the DRC, 2014).

According to the latest data, more than half (56.3%) of the population is under the age of 15, indicating that the population of the DRC is predominantly young. However, children under 5 years (18.5% of the population) and women of childbearing age (42.4%) are the main target of the first intervention against malaria (PNLP, the DRC, 2013).

Situation of Malaria in the DRC

P. falciparum, which causes severe forms of malaria, remains the most common species with at least 98% of infections in the DRC. *P. malariae* and *P. ovale* are present at very low rates and generally found in mixed infections with *P. falciparum*. The presence of *P. vivax* has been documented but it is extremely rare in the DRC (Er-Rami et. al., 2011; Taylor et. al., 2011a; WHO, 2013b).

A number of *Anopheles* species that have role in the transmission of malaria in the DRC include *A. gambiae* (92%), *A. funestus*, the main vector for the highlands of the east, and to a lesser extent *A. nili*, *A. moucheti*, *A brunnipes* and *A. paludis* (Menendez et. al., 2006).

As the epidemiological data on malaria was established more than twenty years ago, it requires an update to enable a better understanding of the factors and the risk areas. In the DRC, malaria transmission is evaluated according to three epidemiological patterns: (i) the equatorial area consists of forests and savanna post where transmission is intense and the entomological inoculation rate (EIR) is up to 1.000 infective bites per person per year (bites/person/year); (ii) the tropical area (wet savanna) where transmission is seasonal but long (EIR: 100-400 bites/person/year); (iii) and the mountainous region (areas between 1.000 and 1.500 meters, EIR <2 bites/person/year) with short periods of transmission (WHO, 2012).

The Annual Report of the PNLP 2013 shows a progressive increase in the number of cases and deaths from malaria. This increase is explained by the insufficient intervention practice against malaria. In the DRC, malaria remains a major public health problem with 8 million cases and more than 23.000 deaths. It should be noted that until 2013, the use of microbiological diagnosis was limited and

malaria treatment was based on presumptive clinical diagnosis (PNLP, the DRC, 2013).

Dynamics of Malaria Transmission and Level of Endemicity in the DRC

In the DRC, 97% of the population lives in equatorial and tropical regions with stable malaria transmission (Mukadi et. al., 2011). The transmission is perennial in the central basin that has the characteristics of hyper-endemic areas (50 to 75% of the infections) and holoendemic (more than 75% of the infections). The rest of the populations (3%) live in mountainous areas of the eastern DRC where malaria transmission is unstable. In these regions, there is a risk of occurrence of epidemics which are sporadic and seasonal in the highlands of the east. The sporozoite rate can reach 7% in urban areas (National Statistics Institute, the DRC, 2014). Malaria increases poverty because it reduces productivity and deteriorates social stability significantly in the populations (Mulumba, 2006).

The DRC through its PNLP has adopted the global strategy "Roll Back Malaria" by 2015. The purpose of the global strategy is to improve the health status of the population in the DRC. The main objective was to reduce the morbidity caused by malaria up to 50% by 2015 (PNLP, the DRC, 2014).

In addition, the DRC became a partner of the African Charter for Health Development with the strategy of Primary Health Care. This programme aims to ensure the whole community to access to the health care services without any commitment for free medication. Based on this plan, several intervention strategies have started in the DRC, including the fight against vectors, pharmacovigilance of antimalarial drugs, and prevention and treatment of malaria (PNLP, the DRC, 2013).

Fighting Against Vectors:

The fight against malaria vectors in the DRC focuses on three interventions: promoting the usage of ITNs, anti-larval control and indoor residual spraying (IRS) focused at certain areas (PNLP, the DRC, 2013). Efforts are currently being made in the implementation of ITN distribution campaigns since 2006, so the proportion of households owning at least one net in the DRC increased from 9.2% in 2010 to

50.9% in 2012 (PNLP, the DRC, 2013). The map that indicates the distribution of malaria vectors in the DRC was designed in 1960s and has not yet been updated. In addition, national strategies in entomology and vector control techniques are insufficient. It is therefore necessary to develop a management strategy for insecticide resistance in order to maintain and enhance the efficiency of vector control (PNLP, the DRC, 2013).

Pharmacovigilance of Antimalarial Medicines:

Based on the data that demonstrated the resistance of *P. falciparum* to chloroquine, the DRC has changed the policy to fight against malaria. The new policy recommends the use of therapeutic combinations such as artesunate plus amodiaquine and artemether plus lumefantrine for the treatment of uncomplicated malaria since 2012; the use of quinine tablet in case of treatment failure of the first-line drugs; and the use of injectable quinine or artesunate for treating severe malaria. SP is reserved for IPTp and intermittent preventive treatment in infants (IPTi) (PNLP, the DRC, 2013).

Prevention and Treatment of Malaria in the Vulnerable Groups:

Pregnant women are the most vulnerable targets of malaria and received specific measures for prevention and care since the last National Strategic Plan 2007-2011. However, several surveys revealed that despite high coverage of prenatal consultation (87%), the rate of IPT was low with only 21% of women who received at least two doses of SP during pregnancy. Unavailability of SP; insufficient training of health care workers on IPT and use of monotherapies are the concerns of SP prophylaxis during pregnancy (PNLP, the DRC, 2013).

2.2. Biology of *Plasmodium*

2.2.1. Taxonomy

Plasmodium is an intracellular parasite that has asexual reproduction cycle (schizogony) in the vertebrate host and sexual reproduction cycle in the invertebrate host (Feagin and Drew, 1995).

Plasmodium parasites belong to the kingdom *Protista*, the phylum *Apicomplexa*, the class *Hematozoa*, and the family *Plasmodidae*. The genus *Plasmodium* includes over 172 species of intraerythrocytic parasites that infect a wide range of mammals, birds, reptiles and amphibians (Mulumba, 2006). Four species have been documented to infect specifically humans for a long time: *P. falciparum, P. ovale, P. vivax* and *P. malariae* (Ramasamy, 2014).

Recently, it became clear that a fifth species, *P. knowlesi*, which normally infects long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*), is a major cause of malaria in humans in South Asia. Four cases of *P. knowlesi* malaria were documented in Malaysian hospitals (Cox-Singh et. al., 2008), and other human cases were reported from Thailand (Putaporntip et. al. 2009), Singapore (Ng et.al., 2008), Philippines (Luchavez et.al., 2008) and Western travelers returning from South Asia (Bronner et. al., 2009).

It is noted that among the five species, *P. falciparum* is the most dangerous because it causes the highest mortality rates. In the DRC, it causes 80% of all human malaria infections and 90% of deaths (Messina et. al., 2011).

All *Plasmodium* species share the following features: naked bodies, intracellular seat, mode of nutrition by osmosis and a life cycle consisting of schizogony in humans and sporogony in mosquitoes. The differential features are based on morphological, biological (life cycle, pathogenicity) and epidemiological criteria (Vaughan, 2007).

2.2.2. Transmission

While the male mosquitoes feed on flower nectar, the blood-sucking females bite. Blood meal is necessary for egg maturation (Hall et. al., 2005). Malaria parasite is transmitted by the infected female mosquito of the genus *Anopheles*, (CDC, 2013). Transmission depends on the presence and abundance of the vector *Anopheles* and the infected human reservoirs; or in case of *P. knowlesi,* infected macaques are the reservoirs (WHO, 2013b).

Transmission cannot occur at the temperature ranges out of 16-33°C or higher altitude (1.500-4.000 m) because development in the mosquito cannot occur. *P. knowlesi* infections can only occur in areas where there is the presence of long-tailed and pigtail macaques (Kar et. al., 2014).

In sub-Saharan Africa, the most serious morbidity and mortality occur in early childhood where the majority of deaths are due to severe anemia. Children of 0 to 5 years and pregnant women who have low immune response are classified in the category of those most exposed to the transmission (WHO, 2014).

More than 80% of malaria cases generally depend on the wide variety of factors; altitude, temperature, demographics, housing, agricultural conditions, nutritional factors and presence of the local *Anopheles* vector, and existence of the reservoirs (Kar et. al., 2014).

On the other hand, abnormal hemoglobins encountered more frequently in certain ethnic groups appear to have a protective effect against malaria infection. This would be the case of sickle cell anemia, thalassemia and hemoglobin E; although it is not an absolute protection (Mangano et. al., 2015).

According to a previous study, malaria transmission is lower in urban environment than in rural areas (Mungai et. al., 2001). Malaria transmission occurs primarily through the bite of an infected female *Anopheles*, penetration of the placenta, transplantation, and blood transfusion (Nansseu et. al., 2013)

2.2.3. Life Cycle

The life cycle development of *Plasmodium* is same for all human *Plasmodium* species. It includes a schizogonic (asexual) cycle in the vertebrate host, and a sporogonic (sexual) cycle in the invertebrates (Feagin and Drew, 1995; Price, 2007).

The Schizogony (Asexual Cycle)

It consists of two phases in which hepatocytes and erythrocytes are infected by *Plasmodium* species.

Invasion of Hepatocytes:

Sporozoites in the salivary glands of female *Anopheles* are inoculated into humans during the infective bite, remains 30-60 minutes into general circulation. Much of the sporozoites (over 90%) are captured and destroyed by the reticuloendothelial system (RES) and the remaining join the hepatocytes where they will initiate the pre-erythrocytic cycle (Figure 2.1). The penetration of the parasite to the hepatocyte needs specific membrane receptors on the hepatocyte (Price et. al., 2007). After four days, the parasite (hepatozoon) multiplies by simple binary division of the nucleus and develops into a schizont (Feagin and Drew, 1995). At the end of the nucleus division, the division of the cytoplasm occurs. Eventually, each cell forms many merozoites; 8 to 20 nuclei for *P. malariae*, 24 nuclei for *P. falciparum* and more than 24 nuclei for *P. vivax* and *P. ovale*. In the mature schizont, membrane of the host cell bursts and merozoites are released. The merozoites enter into the general circulation and finally infect the red blood cells (RBCs) (Feagin and Drew, 1995). Duration of the exo-erythrocytic stage depends on *Plasmodium* species; such as 7-11 days in *P. vivax* and *P. malariae* (Ashley and White, 2014).

An individual may have different generations of parasites that were injected at different times in the infected liver. Each schizogonic cycle begins its evolution in a random schedule. These parasitic forms (hypnozoites) are responsible for relapses that develop 18-36 months or more after inoculation by the mosquito. This type of

Plasmodium increases the chance of encounter with *Anopheles* in different climates, including winter (Ashley and White, 2014).

Invasion of Erythrocytes or Blood Stage:

Merozoites which enter the RBC grow into trophozoites where they use the host hemoglobin. Development results in the formation of endo-erythrocytic schizont, including pigment clusters. Then, the schizonts burst, releasing merozoites to infect other erythrocytes, while the pigment mass is engulfed by leukocytes (Figure 2.1). The bursting schizont in the host erythrocyte causes an abrupt febrile period. The febrile illness may be mild or absent because of the number of parasitized erythrocytes at the beginning. The concentration of merozoites in mm^3 is about 300 for *P. vivax*, 2.000 for *P. falciparum* and 150 for *P. malariae*. The duration of this cycle is 48 hours for *P. falciparum* and 72 hours for *P. malariae* (Feagin and Drew, 1995).

The process of erythrocyte invasion has two phases which are cyto-adhesion of merozoites to erythrocyte and penetration phase. The cyto-adhesion requires the intervention of specific membrane receptors (Duffy) for *P. vivax* and glycophorine receptor for *P. falciparum* (Ashley and White, 2014). During the penetration phase, merozoites invaginate the erythrocyte membrane and form the parasitophorous vacuole. Inside the erythrocyte, as the parasite grows, the amount of nuclear chromatin and the volume of the cytoplasm increase. Incomplete digestion of the erythrocyte's hemoglobin results in accumulation of the pigment hemozoin. The parasite digests more than 50% of the RBC mass and the hemoglobin (Mulumba, 2006). The infected erythrocytes undergo ultrastructural alterations such as Schuffner and Maurer dots, which are the small cavities on the erythrocyte membrane (Feagin and Drew, 1995).

The schizogony cycle is completed in the RBC, within 36-48 hours for *P. falciparum*, 48 hours for *P. vivax* and *P. ovale*, and 72 hours for *P. malariae*. *P. vivax* shows a preference for young RBCs and reticulocytes, *P. malariae* selects old erythrocytes, while *P. falciparum* can infect erythrocytes of any age. This explains the reason of low-density parasitemia observed in *P. vivax, P. ovale* and *P. malariae*, unlike *P. falciparum* where the density is very high (Ashley and White, 2014; Price,

2007). For *P. vivax* and *P. ovale*, blood passages may be repeated at intervals of several months due to prolonged time of parasitic forms in liver (hypnozoites or cryptozoites) (Ashley and White, 2014).

After one or more generations of schizonts, some merozoites specialize in male sexual elements called microgametocytes and some other merozoites form female sexual elements called macrogametocytes. These are the sexual forms that continue the life cycle in the mosquito. They can persist in the circulating blood for nearly two weeks, because they use protective mechanisms that enable escaping into the reticuloendothelial system (Figure 2.1) (Strickland, 2000).

The Sporogony (Sexual) Cycle

The female mosquito feeds on blood to complete the maturation of eggs. During the blood sucking, the mosquito receives different forms of parasites, however, only mature gametocytes continue their development, and other schizonts and trophozoites are digested. In the stomach of the mosquito, the gametocytes are resistant to digestion (Figure 2.1) (Feagin and Drew, 1995).

The female and male gametocytes grow in one macrogamete and eight microgametes, respectively. One of the mobile microgametes fertilizes the single female gamete and the egg which is called ookinete is formed. It moves through the stomach of mosquitoes and encysts, then grows into an oocyst. After 48 hours, the nucleus of the oocyst divides and this is immediately followed by the division of the cytoplasm called sporogenesis. At the end of their maturity, thousands of sporozoites migrate to the salivary glands of the mosquito where they will be injected during the next blood sucking. Sporozoites remain infective in the salivary glands of the mosquito for 12 weeks (Figure 2.1). From one blood sucking to another, the mosquito may become secondarily infected, increasing its load of sporozoites (Vaughan, 2007).

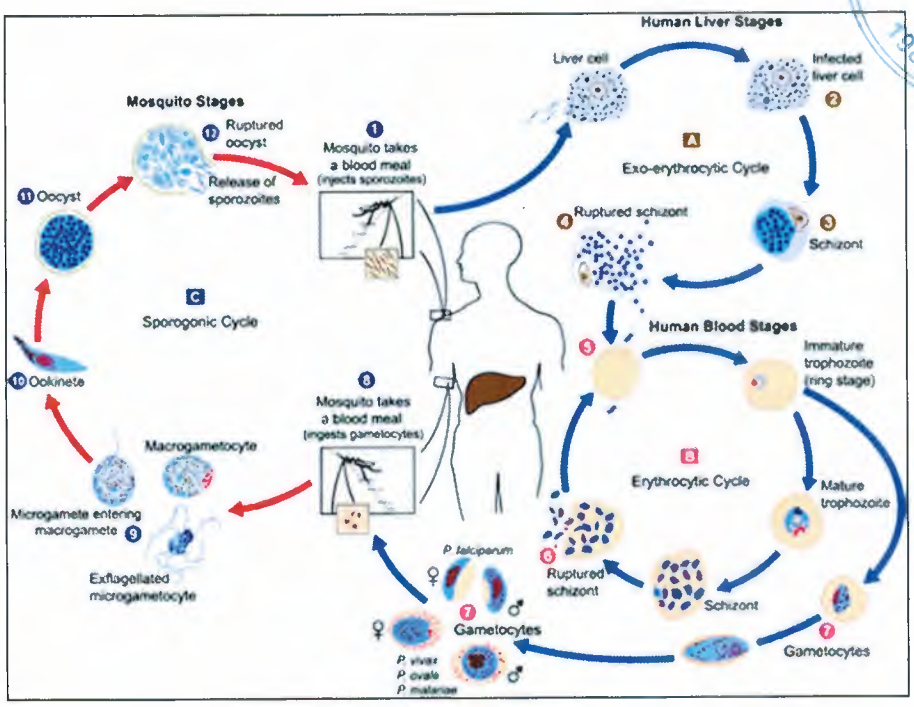

Figure 2.1. The life cycle of *Plasmodium* species (CDC, 2013)

Mechanisms of Parasite Survival

During their life cycle, *Plasmodium* species present different surface antigenic patterns which belong to sporozoites, hepatic schizonts, erythrocytic shizonts and gametocytes (Ashley and White, 2014). Recombination of genes during the sexual reproduction in the mosquito's stomach contributes to the antigenic variation (Borst et. al., 1995). *P. falciparum* was shown to change about 20% of the primary structure of its surface proteins within 48 hours (Fievet et. al., 1997).

The phenomenon of cyto-adhesion of infected RBCs to the vascular endothelium is due to the presence of complex adhesins such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and erythrocyte-binding antigen (EBA) which are secreted by surface of the parasitized RBC. These molecules allow the parasite to be present in the deep capillary microcirculation to accomplish the schizogony. This explains why older trophozoites (having spent more than 20 hours in the RBCs) and schizonts of *P. falciparum* are not usually noticed in peripheral circulation (Mulumba, 2006).

2.3. Clinical Forms

Malaria consists of two clinical forms which are uncomplicated and severe.

2.3.1. Uncomplicated Malaria

Febrile Paroxysm

The milder form of malaria is generally characterized by a febrile paroxysm, arthralgia, myalgia and headache. This is the most common clinical form of malaria, which may progress to the severe form. The febrile illness is characterized by series of repeating febrile every 24, 48 and 72 hours (Snow et. al., 2005). Hyperthermia is particularly common in children. Above 38.5°C (threshold temperature) attacks are triggered, between 38.5 and 42°C there is delirium; and beyond 42°C is coma (Barry et. al., 2007).

RBC destruction according to its importance (duration, intensity of infection) can cause anemia. Two main mechanisms were proposed to explain anemia. These are intravascular and extravascular hemolysis. In the intravascular hemolysis, direct destruction of the RBCs by *Plasmodium* occurs. In extravascular hemolysis, the infected RBCs are destructed by erythrophagocytosis or reticuloendothelial system. In severe malaria, there is a transient myelosuppression due to hyper secretion of erythropoietin as a result of cytokine production. Hemolysis is generally due to the action of antimalarial drugs or by antibody on infected RBC (Murphy and Breman, 2001).

Regarding the intravascular hemolysis, *P. falciparum* is the most aggressive species affecting the humans because it can achieve a high parasitemia of 30% in extreme cases. In severe forms of malaria, normocytic anemia with 15% hematocrit and hemoglobin with a rate of 5g/100ml was associated with a parasitemia greater than 100.000 trophozoites per microliter of blood. In case of dehydration, the rate of hematocrit increased to 20 or even 25% (Barry et. al., 2007). Destruction of RBCs due to the immune system was attributed to the lytic actions of complement on erythrocytes bearing the antigen-antibody complex, and also, the cytotoxic activity of reticuloendotelial system cells on the erythocytes. The destruction (erythrophagocytosis) takes place mainly in the spleen and liver. The degree of

hypertrophy of these organs reflects the intensity of destructive action (Ashley and White, 2014). The syndrome called tropical hypersplenism is associated with hypergammaglobulinemia that is often observed in hyperendemic areas. The anemic syndrome is often accompanied by disruption of hemostasis due to thrombocytopenia (gingival bleeding, epistaxis, petechiae, and subconjunctival hemorrhage). Disseminated intravascular coagulation is associated with substantial gastrointestinal bleeding. It is often observed in non-immune patients (Price et. al., 2007).

2.3.2. Severe Malaria

Blackwater Fever

Blackwater fever is a febrile syndrome that is associated with intravascular hemolysis and hemoglobinuria in malaria. This syndrome follows sensitization to quinine, however other factors are still not identified. This syndrome occurs due to the glucose-6-phosphate dehydrogenase (G6PD) deficiency (Bodi et. al., 2014).

Cerebral Malaria

Cerebral malaria is a neurological syndrome that occurs in nearly 10% of all hospitalized malaria cases and represents more than 80% of fatal forms (Soumaré et. al., 2008). This syndrome is associated with symptoms including blurred consciousness or behavior, focusing signs, convulsions or coma (Li and Weina, 2010).

Nephritis and Renal Failure

The syndrome of acute renal failure is due to tubular necrosis and the chronic insufficiency of the glomerular lesions. It can occur in malaria as a complication of *P. falciparum* or *P. malariae* infections. In case of *P. falciparum* infection, kidney damage is usually reversible. However, in *P. malariae* infection, the lesions gradually lead to chronic renal failure (Strickland, 2000).

Hypoglycemia

Hypoglycemia is a common manifestation of severe malaria that is often unrecognized. Patients at the age of five years, pregnant women and non-immune

people are at risk. The symptoms include profuse sweating, dyspnea, tachycardia, altered consciousness, generalized convulsions and coma. In pregnant women, administration of quinine could lead to physiological hyperinsulinemia (Murphy and Breman, 2001).

Cardiovascular Collapse

The algid malaria is characterized by a systolic blood pressure lower than 80 mm Hg (50 mm Hg in children) in supine position. This condition occurs with severe dehydration, hypoglycemia, digestive tract hemorrhage and pulmonary edema (WHO, 2010).

2.3.3. Malaria in Pregnancy and Childhood

Malaria and pregnancy are two situations that deteriorate each other. Malaria is more serious and more common during pregnancy, causing significant morbidity, maternal mortality, fetal and perinatal mortality. In hyperendemic areas, infection rate in pregnant women is usually higher than that of non-pregnant women. The effect of malaria depends on the level of immunity of the person. Likewise, the consequences will differ depending on whether a woman is immunized or not (Lagerberg, 2008).

The pregnancy makes women susceptible to malaria during the first pregnancy. Susceptibility of malaria infection in placenta of primigravida is unlike multigravida; this is due to the inexperience of their immune system's response to *P. falciparum* (Duffy and Fried, 2003).

In women infected with *P. falciparum*, the placenta contains RBCs parasitized in large quantities. The presence of parasites in the placenta is 26 times greater than in the peripheral blood, particularly in the gravid (Tako et. al., 2005). The massive presence of infected RBCs with *Plasmodium* causes an inflammatory reaction in the placenta which leads to destruction and necrosis of placenta, maternal morbidity or mortality, fetal and perinatal mortality. The infection disrupts the flow of nutrients between the mother and fetus, thus reducing the weight of the child at birth. This is an indirect source of neonatal morbidity and mortality in developing countries. The parasites can also be sequestered in the placental tissue as a result of

cytoadherence of the infected RBCs (Bourée, 2013). Cytoadhesion molecules which serve as ligands on the surface of the parasitized RBCs bind to the receptor present on the syncytiotrophoblasts of the placenta. The CSA molecule acts as the receptor for the parasitized erythrocytes (Boudová et. al., 2014; Duffy and Fried, 2003).

Other chronic placental infection caused by *P. falciparum* results in the development of an inflammatory chorionitis. This leads to the thickening of basement membrane of chorionic villi that affects the quality and quantity of fetal-maternal exchanges. The pregnant women's sensitivity to malaria is explained by the presence of parasite strains that would bind specifically to CSA in the placenta (Matangila et. al., 2014; Murphy and Breman, 2001).

Pregnancy is accompanied by a reduction of acquired immunity, especially in primigravida. This leads to an increase in the frequency and intensity of parasitemia. Besides, an alteration in the antibody levels occurs. The high protein requirements associated with nutritional deficiency can account for the lack of production of gamma globulin. In the first pregnancy, malaria is more common and severe, and the newborns are more seriously affected than those of subsequent pregnancies. The uterus and the placenta form a new location for the parasites. It is very likely that it induces a local response, providing protection against future infections. Parasitemia decreases with the parity and maternal age. The ratio of the malaria infection in primigravida to the sixth pregnancy was found to be 9:2 (Guyatt and Snow, 2004).

During pregnancy, the prevalence and intensity of malaria increased in the first weeks and returned to normal levels in the following weeks. In the non-immunized women (tourists) in the tropics, all forms of malaria can occur, ranging from mild to cerebral malaria. Fever causes abortion or preterm delivery in late pregnancy. This is mainly for malaria contracted at the end of pregnancy that can produce congenital fetal manifestations in nearly 10% of cases. Without diagnosis and treatment, the prognosis of the mother and fetus can be rapidly fatal (WHO, 2011).

Pregnancy which is an immunological stress causes a decrease in anti-malarial immunity, and therefore, can unmask latent malaria or increase the risk of severe disease especially cerebral malaria (Briand et. al., 2008). *Plasmodium* can be

found in the placenta, while the blood tests are negative. In Panama, out of 400 placentas examined, 11 samples were found to be infected, while peripheral blood smears of the same patients were negative. In Dakar, 130 placentas were examined, and 15% of the samples were determined to be positive while 1.6% of the fetal blood samples were positive (Van Eijk et. al., 2011).

Different consequences depend on the diversity of the rates in malaria-endemic areas. In hyperendemic areas, immunity is robust and pathological manifestations are rare. In hypoendemic areas, immunity is unstable and the risk of contracting malaria is quite high in pregnant women. Regardless of the stage of pregnancy, *P. falciparum* can develop into cerebral malaria when there is no treatment. In the subsequent pregnancy, there is often a risk of aggravation which can result in premature delivery or sudden death of the mother (Steketee et. al., 2001).

Identification of the parasites is unreliable in endemic areas where self-care obscures the diagnostic limits. Although the existence of a possible correlation between the levels of anti-malarial drug and age of pregnancy is controversial, malaria is considered the leading cause of anemia in the pregnancy. Anemia appears in the 20^{th} week and can be hemolytic, normocytic and normochromic. Anemia is important especially in the first pregnancy. Severe anemia increases the risk of maternal and fetal mortality. On the other hand, disappearance of the hemolytic anemia was demonstrated to follow the administration of proper chemoprophylaxis (Hatabu et. al., 2003).

Repeated bouts of malaria may disrupt the pituitary function and cause infertility (Menendez et. al., 2000). In late pregnancy, there is a correlation between the level of parasitemia, duration of fever and risk of abortion, particularly in the endemic areas. The stillbirth and premature delivery are more frequent. The placenta is an important reservoir for parasites, even without detectable parasitemia. The lesions formed due to the inflammatory and hormonal response in the placenta are more common in primigravida. Infected mothers usually have placenta with a lower weight than that of healthy ones. Malaria is a major factor in prematurity, especially in the primigravida. Dynamic dystocia is common and probably related to uterine hypoxia (Newman et. al., 2003; Tako et. al., 2005).

The risk of congenital malaria is more common in hypoendemic areas due to the low maternal immunization. The mechanism of placental transfer of parasitized erythrocytes is poorly understood. It is estimated that the parasite density remains 300-1000 times lower in the fetus than in the mother. Furthermore, there is no exoerythrocytic stage in the absence of transcutaneous inoculation by the mosquito (Hatabu et. al., 2003).

Congenital malaria occurs due to maternal-fetal transfer of *Plasmodium* at the time of delivery. The absence of CSA in the newborn limits the parasite's ability to replicate after the delivery (d'Acremont et. al., 2010). From birth to about 6 months of life, infants are relatively resistant to malaria because of the natural immunity and the presence of maternal anti-*Plasmodium* IgG antibodies transmitted. In the first three months of life, maternal IgG transmitted to the newborn are protective. The presence of fetal hemoglobin is an additional factor explaining the absence of malaria in infants born to mothers living in endemic areas (Mockenhaupt et. al., 2005). Children aged six months to five years are the most vulnerable group of the population and they are the worst affected by malaria with 1 to 3 million deaths annually (Greenwood, 2002). The major complications in children are cerebral malaria, anemia and hypoglycemia. Malnourished children present relatively less possibility of cerebral malaria compared with other clinical forms (Mwangi et. al., 2006).

2.4. Diagnosis

Several approaches can be adapted for malaria diagnosis. Apart from the clinical diagnosis, the diagnosis of malaria infection is made in the laboratory by examination of the parasites in the patient's blood through the thick film (microscopic diagnosis). There are several other diagnostic methods which are more sensitive and expensive such as Quantitative Buffy Coat (QBC). This method is generally difficult to use in the routine diagnosis. In the recent years, immunochromatographic methods that detect *Plasmodium* antigens and PCR have been used for rapid detection of malaria. Each diagnostic method has several advantages and drawbacks including cost, ease of application and accuracy which determine their feasibility in different settings (Barker et. al., 1992).

2.4.1. Clinical Diagnosis

In several regions of Africa, malaria diagnostic tests, even thick film, are not available. In areas of stable malaria transmission, a positive thick film does not prove the existence of malaria disease (Chippaux et. al., 1989). Malaria diagnosis in those regions depends largely on clinical examination and does not rely on the laboratory results. The thick film results may remain negative for several days due to the recent acquisition of malaria (Mwangi et. al., 2006).

2.4.2. Microscopic Diagnosis

One of the key elements in the containment and elimination strategy of malaria is to perform accurate diagnosis. The diagnostic process is initiated by suspected malaria based on the clinical criteria, since 2010. WHO recommends prompt microscopic confirmation of the parasites in malaria suspected patients, prior to treatment. Microscopic examination confirms malaria diagnosis by demonstrating the parasites in the blood. It must be completed immediately before the antimalarial treatment without waiting for a thermal peak. Microscopic examination allows identification of malaria parasites at the species level, the developmental stage of the parasites including gametocytes, and quantification of the parasite density for monitoring response to the treatment (Chippaux et. al., 1989).

Microscopic examination is the method of choice to investigate the reason for treatment failure. Giemsa is the conventional method of staining malaria parasites for the microscopic diagnosis. Microscopic examination requires the analysis of both thick and thin smears of the same patient and it is the gold standard when compared to other diagnostic methods (PNLP, the DRC, 2013). Smears can be made by using capillary blood sample collected from the fingertip. Blood can also be collected by venipuncture and kept in a tube containing anticoagulant (ethylenediaminetetraacetic acid: EDTA). This allows increased possibility for detection of the parasites in the blood sample (WHO, 2012).

Microscopic examination of thick and thin smears is the reference method because it has good sensitivity and specificity for monitoring the efficiency of malaria treatment by determination of parasitemia. The thick smear is an inexpensive

method and remains the most widely used technique (Murphy et. al., 2013). However, its reliability depends on the experience of the microscopist and the level of parasitemia of the infected patient. The thin smear can identify the parasites at the species level according to the morphological criteria in the parasitized erythrocytes. This is essential to assess the severity of disease and establish the appropriate treatment. Infection with *P. falciparum* is of particular concern since it can result in serious complications and lead to drug resistance (Greenwood, 2002). The thin smear also allows calculation of the parasitemia as the percentage of the infected erythrocytes, which is a useful diagnostic approach in case of infection with *P. falciparum* (Chippaux et. al., 1989).

Microscopic examination is cost-effective and gives accurate results that have an impact on treatment. Hyperparasitemia, when greater than or equal to 4% in a non-immune person is an indicator of the severity of malaria. The detection threshold of the blood smear is 100 parasites/µl. The thick smear detects lower parasitemia of about 10-20 parasites/µl. The thick film does not allow definitive diagnosis of malaria species due to the erythrocyte lysis that reduces the possibility of morphological identification (Chippaux et. al., 1989).

Although microscopy is a sensitive method, it can sometimes give false negative results. Such problems can be encountered particularly in the samples collected from the patients receiving chemoprophylaxis and in patients infected with *P. falciparum*, where parasites are sequestered in the capillaries of deep organs and are not sufficiently present in the circulating blood. In patients with high clinical suspicion and negative microscopy result, repeat of blood sampling 6-12 hours later is recommended. This should not delay the initiation of a specific treatment in a severe clinical condition (Hatabu et. al., 2003).

Identification of species by microscopic diagnosis may be difficult when the parasites are altered by presumptive treatment or the parasitemia is low. The automated hematology instruments cannot reliably detect malaria parasites. Negative results obtained from alternative diagnostic methods should be confirmed by examination of the repeated thick films (Mwangi et. al., 2006).

2.4.3. Rapid Diagnostic Test (RDT)

RDTs used in malaria diagnosis are immunochromatographic assays that can promptly detect *Plasmodium* antigens. RDTs are very useful in both endemic and non-endemic areas. Besides, they can be used by laboratory workers who are inexperienced in malaria diagnosis (Murray et. al. 2008). RDTs use monoclonal antibodies that can detect the antigens produced by *Plasmodium* species. These antigens include histidine-rich protein 2 (HRP-2), aldolase enzyme, and lactate dehydrogenase enzyme (LDH) (Grigg et. al., 2014).

HRP-2 is a protein of *P. falciparum* that has a role in the polymerization of actin and heme and also localized in the cytoplasm of the infected erythrocyte (Beadle et. al., 1994). Immunochromatographic assays which are based on detection of HPR-2 antigen for the diagnosis of *P. falciparum* have been extensively tested. RDTs for the diagnosis of *P. falciparum* malaria generally achieve a sensitivity of >90% at densities above 100 parasites per microliter of blood and the sensitivity decreases markedly below that level of parasite density (Kakkilaya, 2003). The tests based on HRP-2 are not convenient for monitoring the treatment, as HRP-2 antigens can remain positive for more than 28 days after successful treatment (Grigg et. al., 2014).

HPR-2 is specific for *P. falciparum*, however aldolase enzyme in the glycolytic pathway is found in all species of *Plasmodium*. Monoclonal antibodies against the aldolase enzyme recognize all *Plasmodium* species that infect humans. Recently, immunochromatographic assays using antibodies against both HRP-2 and aldolase have been designed for detection of *P. falciparum* (Grigg et. al., 2014).

An alternative test uses monoclonal antibodies that capture *Plasmodium* lactate dehydrogenase (pLDH) enzyme (Grigg et. al., 2014). Preliminary studies suggested that the LDH based assay had a high sensitivity and specificity (Baker et. al., 2005). Both HRP-2 and pLDH based tests lose their sensitivity when parasitemia is low (<50 to 100/mm3). Although these tests will not replace the microscopy in the near future, they can be important adjuncts to the microscopic diagnosis of malaria (Abba et. al., 2011).

2.4.4. Molecular Diagnosis

PCR is the process used to amplify DNA samples. The products can be used for sequencing or analysis, and this process is a key element in many research laboratories in genetics. PCR requires primers complementary to the target DNA (Foster et. al., 2014; Singh et. al., 2004).

A number of PCR variations have been developed over the past 20 years for specific research applications. Some of these changes include inverse PCR, in situ PCR, long PCR, real-time PCR and nested PCR. When there is a possibility of the primer binding to the template DNA sequence with the exception of the target area (e.g., when using degenerate primer), nested PCR can increase the yield and specificity of amplification of the target DNA (Brown et. al., 1992).

Nested PCR is a modification of PCR to reduce non-specific binding in the product due to the amplification of unexpected binding sites of the primers. Nested PCR is used in situations where it is necessary to increase the sensitivity and/or specificity of PCR. Applications of nested PCR include the amplification of a particular member of a polymorphic gene family, or the amplification of a DNA copy of an mRNA which is present at very low quantity in a clinical sample containing several different cell types (a heterogeneous population of cells) (Duraisingh et. al., 2000).

Nested PCR uses two sets of primers. The first set of primers binds to sequences outside of the target DNA, as provided in the standard PCR, but can also bind to other regions of the matrix. The second set of primers binds to sequences in the target DNA that are within the region previously amplified by the first set of primers. Thus, the second set of primers binds and amplifies the target DNA in the products of the first reaction. If the first set of primers binds and amplifies an unwanted DNA sequence, the second set of primers will less likely bind the unwanted region. This is the main advantage of nested PCR (Duraisingh et. al., 2000).

It is currently recommended that prior to the nested PCR, primers that have not been incorporated into the PCR products in the first reaction must be removed so as not to amplify the target DNA in the second PCR cycle. To remove the primers,

exonuclease enzyme is added to the PCR products of the first reaction. After exonuclease enzyme digests the primers, the enzyme must be inactivated to prevent digesting the second set of primers that will be added to the next PCR reaction. Following the treatment with exonuclease enzyme and inactivation of the enzyme, the PCR products generated in the first PCR cycle must be diluted (Brown et. al., 1992). Because of dilution, the PCR products will be less likely contaminated by the unwanted sequences in the next reaction and nested PCR will be very effective (Duraisingh et. al., 2000).

2.5. Treatment

Treatment of malaria is done primarily through the administration of antimalarial drugs which are proven to be effective for all existing malaria strains (Pasvol, 2006). National malaria treatment policy should aim to offer highly effective antimalarials. For this purpose, each country or region should establish standards and implement an appropriate and effective treatment regimen, depending on the rate of occurrence of antimalarial resistance rates. The therapeutic efficacy of antimalarial drugs also determines the policy on the treatment of malaria (WHO, 2010).

Monitoring the therapeutic efficacy includes an analysis of clinical and parasitological evaluation at least during the first 28 days of appropriate treatment. In this period, reappearance of parasites in the blood is investigated. Antimalarial treatment should be evaluated on the basis of parasitological cure rates (Batty et. al., 2002).

National policy on drug treatment is recommended to be changed when the overall rate of treatment failure revealed through monitoring of therapeutic efficacy in vivo is equal to or greater than 10% (Pasvol, 2006). Since the finding of several cases of treatment failures in 2006, most countries where *P. falciparum* is endemic have updated their treatment policies from chloroquine and SP to artemisinin-based combination therapies (ACTs) (WHO, 2001). The latter is currently regarded as the most promising treatment against uncomplicated falciparum malaria. The implementation of this strategy has been delayed for a number of reasons, including the high cost (WHO, 2010).

A combination therapy is a treatment which requires a drug with rapid action and a short half-life plus a partner drug with slow action and a long half-life (WHO, 2010). Both partner drugs in a combination should be independently effective. The therapy includes simultaneous administration of two or more blood schizontocidal drugs having different biochemical targets in the parasite. The combination therapy is based on two principles: (i) the combination therapy is often more effective, (ii) in case a mutant parasite resistant to one drug appears spontaneously, it is killed by the other drug (WHO, 2011). This mutual protection prevents the emergence of resistance (WHO, 2013b). There are two strategies that include combination therapies. These are combination therapies which are not based on artemisinin and combination therapies which are based on artemisinin.

Combination Therapies Which Are Not Based On Artemisinin:

The therapy includes SP combined with chloroquine or amodiaquine. The existence of a high level of resistance to these drugs when used alone reduces efficiency, even in combination. Since combining chloroquine with SP has not been shown to be advantageous over SP alone, this combination is not recommended. The combination of amodiaquine with SP may be more effective than drug being used alone, but it is generally less effective than the artemisinin-based combination therapies (ACTs) and no longer recommended for the treatment of malaria (WHO, 2011).

Combination Therapies Which Are Based On Artemisinin:

In this strategy, either artemisinin itself or its various derivatives (artesunate, artemether, dihydroartemisinin) are combined with the associated drug. Artemisinin causes rapid clearance of the parasite and the rapid resolution of symptoms by reducing the number of parasites in 48 hours (Duraisingh et. al., 2000)

In terms of public health, artemisinins have an additional advantage of reducing gametocyte carriage and thus the transmissibility of malaria. This is important for disease prevention, especially in areas of low to moderate transmission (WHO, 2011).

Different drug combinations that are currently recommended for the treatment of uncomplicated falciparum malaria are artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, and artesunate plus SP (WHO, 2011).

2.5.1. Treatment of Uncomplicated *P. falciparum* Malaria

Use of combination therapy with artemisinin for treating uncomplicated *P. falciparum* malaria is important to eliminate the drug resistance and improve the treatment outcomes. Each country systematically monitors the outcome of ACT for the treatment of malaria. Efficiency of different regimens using ACTs differs from one geographic region to another (Pasvol, 2006).

The First-Line Treatment

The combination of artemether plus lumefantrine should be used for this treatment. Each tablet contains 20 mg artemether and 120 mg lumefantrine. This treatment is administered for three days (Batty et. al., 2002). A single dose of primaquine at a dose of 0.75 mg/kg should be given to the patient on the first day to remove mature gametocytes in the blood (Li and Weina, 2010).

The Second-Line Treatment

This treatment consists of atovaquine plus proguanil. Each tablet contains 250 mg atavaquine and 100 mg proguanil. This treatment is administered for three days with four tablets daily with meals (WHO, 2013b).

The Third-Line Treatment

For this treatment, quinine tablets of 10 mg/kg are given three times a day at every eight hours during seven days. Quinine will be necessarily associated with doxycycline or clindamycin (Batty et. al., 2002).

2.5.2. Treatment of Severe *P. falciparum* Malaria

Delay in diagnosis or lack of treatment of uncomplicated malaria leads to increase of the parasite load that eventually contributes to development of severe malaria within a few hours (Davis et. al., 2001). Severe malaria is a medical emergency. Parenteral therapy should be initiated in the patients in serious condition. Although some laboratory tests are not available, they should not delay critical care (Dondorp et. al., 2005; WHO, 2010).

First-Line Treatment

The injectable artesunate is recommended as the first-line treatment of severe malaria and cases with multivisceral involvement. Compared to quinine, artesunate is rapidly absorbed and kills the young parasites rapidly. Thus, artesunate is a more effective treatment which is tolerated better and easier to use (Li and Weina, 2010).

Second-Line Treatment

In case artesunate is unavailable, the injectable quinine is the therapeutic alternative. A loading dose of 20 mg quinine/kg as a continuous infusion for 4 hours is followed by a dose of 10 mg quinine/kg every 8 hours in 10% serum glucose to avoid the risk of hypoglycemia. This treatment must be administered at hospital (Li and Weina, 2010).

2.5.3. Treatment of Other *Plasmodium* Species

Malaria cases caused by *P. vivax* and *P. ovale* are treated with the same protocol using chloroquine, which is still effective. Administration of the treatment is carried out in the presence of health care workers (Baird et. al, 2011). Radical treatment involves the administration of a combination of chloroquine and primaquine (Pasvol, 2006).

In case travelers stay for 3 months or more in an endemic area of *P. vivax*, primaquine should be administered as a prophylactic treatment for 14 days (WHO, 2012). Mainly in Southeast Asia, particularly in Indonesia and Oceania, the treatment is done by artemether associated with primaquine, which is same as the treatment of

uncomplicated falciparum malaria (Baird et. al. 2011). Although amodiaquine, mefloquine and quinine were previously considered to treat *P. vivax* resistant to chloroquine, ACTs containing amodiaquine, mefloquine or piperaquine were preferred over monotherapy and became the treatment of choice (WHO, 2010).

In case of infection with *P. malariae*, chloroquine is given as the usual treatment. A radical therapy with primaquine is not necessary because there is not any hypnozoite phase for *P. malariae* (Baird et. al., 2011). For infection with *P. knowlesi*, treatment with chloroquine or amodiaquine remains very effective (Mendez et. al, 2002).

2.6. Preventive Treatments

Preventive chemotherapy is a key element in the prevention of malaria. The overall objective of these interventions is to prevent malaria infection by maintaining therapeutic blood levels during the period when the risk of transmission is the highest (Boudová et. al, 2014). The preventive treatments recommended by WHO include IPTp, IPTi and seasonal malaria chemoprevention (SMC) (WHO, 2014).

2.6.1. Sulfadoxine-Pyrimethamine

SP acts through synergistic inhibition of two key enzymes involved in the folic acid synthesis. Sulfadoxine inhibits dihydroptereoate synthase enzyme that enables the incorporation of *p*-aminobenzoic acid in the folic acid synthesis. Pyrimethamine inhibits the step mediated by dihydrofolate reductase enzyme in the folic acid synthesis and indirectly prevents the nucleic acid synthesis in the parasites. Pyrimethamine which is active against the schizont forms also prevents the sporozoite development in *Anopheles* vector (WHO, 2010).

SP is indicated for the treatment of uncomplicated *P. falciparum* malaria in the areas where the parasites are resistant to amino-4-quinolines. SP is generally used for chloroquine-sensitive *P. falciparum* and chloroquine-resistant *P. malariae, P. ovale* and *P. vivax* (WHO, 2013b).

2.6.2. Intermittent Preventive Treatment in Pregnancy (IPTp)

Malaria infection during pregnancy is a major public health issue, with significant risks to the mother, the fetus and the newborn. Each year, more than 30 million African women living in malaria endemic areas become pregnant and they are at risk of infections with *P. falciparum*. Malaria is dangerous for both these women and their babies. Up to 437.000 infant deaths occur each year due to the presence of malaria in pregnancy (Hutton et. al, 2009; WHO, 2014).

IPT has been established for pregnant women in regions with a high prevalence of malaria. IPT for pregnant women is a full antimalarial treatment regimen administered during routine prenatal visits, regardless of the presence of such infection in the recipient. This treatment reduces episodes of malaria in the mother, the maternal and fetal anemia, placental parasitemia, the risk of low birth weight and neonatal mortality (Hommerich et. al, 2007; Huynh et. al, 2012).

WHO recommends at least four antenatal care (ANC) visits during pregnancy. According to the guidelines, IPTp-SP is recommended to be administered at each scheduled ANC visit starting from the second trimester with doses given at least one month apart. SP is not recommended to be given in the first trimester. According to WHO, IPTp-SP could be administered until the time of delivery with doses given at least one month apart. By this programme, women are considered to receive at least three doses of SP during pregnancy (WHO, 2013a).

IPTp-SP is recommended to be given as directly observed therapy consisting of three tablets. Since each tablet contains 500 mg/25 mg SP, the required dosage of 1500 mg/75 mg SP is administered. Due to a high risk of side-effects, SP is not recommended to be given to women taking co-trimoxazole. Besides, a dose equal to or greater than 5 mg of folic acid neutralizes the antimalarial efficacy of SP, therefore folic acid at this dose is not recommended to be administered with SP (WHO, 2013a).

The previous WHO recommendation proposed at least two doses of SP. Of approximately 55 million people at risk of malaria in malaria-endemic countries in sub-Saharan Africa, it has been estimated that 32 million pregnant women could benefit from IPT each year. However, in recent years, there is a decrease in efforts to

scale up IPT for pregnant women in a number of African countries (Kayentao et. al, 2005; WHO, 2014). In heavily affected countries, IPT for pregnant women lags behind the other malaria control measures. This appears to be due to the low levels of attendance at antenatal clinics. The uncertainty of health workers about the administration of SP within the IPT for pregnant women may also have played a role in this (Mendez et. al, 2002).

2.6.3. Other Preventive Strategies

Intermittent Preventive Treatment in Infants (IPTi)

In sub-Saharan Africa, young children are more vulnerable to malaria in terms of severity and mortality, followed by pregnant women (Hutton et. al, 2009). Recently, WHO has recommended a new intervention against *P. falciparum* that targets another high-risk group: intermittent preventive treatment in infants with sulfadoxine-pyrimethamine (IPTi-SP) (WHO, 2014). This treatment consists of delivering a therapeutic dose of an antimalarial drug to infants during the first year of life (Hutton et. al, 2009). IPTi-SP includes administration of a full regimen of SP along with the Expanded Programme on Vaccination (EPV) (Kayentao et. al, 2005; WHO, 2012). In areas where this treatment is implemented, SP is recommended to be given to each child for three times during the first year of life, at the time of routine vaccination treatment (WHO, 2011).

Seasonal Malaria Chemoprevention (SMC)

Formerly known as IPT of children under five years, SMC is defined as intermittent administration of antimalarial treatment during the malaria transmission season to prevent resulting morbidity and mortality (Nansseu et. al, 2013). SMC includes a complete processing cycle of a therapeutic combination of amodiaquine plus SP administered to children aged 3 to 59 months with intervals of one month starting from the beginning of the transmission season (WHO, 2012). Throughout sub-Saharan Africa, the highest mortality and morbidity attributed to malaria are observed during the rainy season. It was shown that intermittent administration of a

full course of an antimalarial drug during this period was effective in preventing the disease and it reduced the number of malaria deaths in children (WHO, 2014).

The aim of this strategy is to prevent malaria infection by maintaining therapeutic blood levels during the period when the risk of transmission is highest and reduce the incidence of malaria. It was shown that SMC was an effective, safe, inexpensive and feasible strategy to prevent malaria in children in areas with seasonal malaria transmission which did not last for more than four months (Kayentao et. al, 2005). SMC can be administered by community health workers. In areas where it is implemented, IPTi should not be applied (Nansseu et. al, 2013).

2.7. Antimalarial Drug Resistance

Antimicrobial drug resistance is the ability of a strain to survive or multiply despite the administration and absorption of a drug used at recommended doses (Basco and Ringwald, 2000). Drug resistance does not always mean treatment failure, but it is one of its causes. With the development of medicine in the last fifty years, the drug pressure caused by the massive use of synthetic antimalarials has selected parasites resistant to most molecules circulating (Hastings et. al., 2002).

Drug resistance is also defined by WHO as the ability of a *Plasmodium* strain to survive or multiply despite administering a drug at or above the usually recommended doses but within the tolerance limits, and the normal metabolism of the drug is maintained ensuring its bioavailability (Bloland, 2001).

The first massively used antimalarial was pyrimethamine in Indochina but the rapid selection of resistant parasites led to the use of chloroquine. Excessive use of antimalarial drugs for decades has provoked the acquisition of resistant parasites on the border of Thailand-Cambodia, Colombia, Venezuela, Papua New Guinea and the Philippines. Resistance to chloroquine has gradually reached to East Africa in the late 1970s from Asia (Hastings et. al., 2002).

Some cases of mefloquine resistance have been described in Brazil. In these areas, quinine resistance occurs due to the existence of cross resistance with

mefloquine. In Africa, mefloquine and quinine demonstrate high efficiency (Hastings et. al., 2002).

Detection of resistance mechanisms allows the development of new drugs that will reduce the resistance, identification of targets for new antimalarials and finally determination of molecular markers for monitoring antimalarial drug resistance (Hastings et. al., 2002)

2.7.1. Genes Associated with Antimalarial Drug Resistance

Several genes involved in antimalarial drug resistance were demonstrated. These included the genes encoding *P. falciparum* chloroquine resistance transporter (*Pfcrt*) and *P. falciparum* multidrug resistance 1 protein (*Pfmdr1*) (Fidock et. al., 2000; Sanchez et. al., 2008). The *Pfmdr1* gene modulates the level of resistance to chloroquine, quinine, mefloquine, halofantrine and artemisinin (Sanchez et. al., 2008).

Resistance to SP occurs due to the mutations in the genes encoding *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and *P. falciparum* dihydroptereoate synthase (*Pfdhps*) (Rouhani et. al., 2015; Sutherland et. al., 2009). Different mutations were reported at several codons of *Pfdhfr* and *Pfdhps* genes. The mutations in *Pfdhfr* gene include substitution of serine by asparagine at codon 108 (S108N), asparagine by isoleucine at codon 51 (N51I), cysteine by arginine at codon 59 (C59R), and isoleucine by leucine at codon 164 (I164L). The mutations in *Pfdhps* gene include substitution of alanine by glycine at codon 437 (A437G) and lysine by glutamic acid at codon 540 (K540E) (Pearce et. al., 2003).

2.7.2. Mechanisms of Drug Resistance

The acquisition of resistance by a *Plasmodium* strain against an antimalarial drug is a spontaneous process related to the mutations. The large-scale appearance of drug resistance in *Plasmodium* population depends on the selective pressure applied by the drug. This enables the parasites to survive in the presence of the drug. These mutants escape the destructive action of immunity and spread by *Anopheles* mosquitos to other hosts (Hastings et. al., 2002).

The spread of drug resistance depends on a combination of several factors. The most important reason is the widespread use of antimalarial drugs. Secondarily, the possibility of hybridization between susceptible and resistant strains in the stomach of the mosquito could induce the resistance (Hastings et. al., 2002).

Other factors that contribute to the emergence of resistance are (i) misuse of antimalarial drugs by infected individuals (self-medication) resulting in incomplete treatment; (ii) unavailability of effective drugs or application of inadequate medication as monotherapy; (iii) and consumption of low dose compounds that enable viable parasites to survive at suboptimal concentrations of antimalarial drugs (Hastings et. al., 2002).

The resistance is often associated with (i) alteration of key enzymes that are targets for the drug; (ii) and alteration of the factors that affect accumulation of the drug inside of the parasite such as decrease in entry and/or increase in output (efflux). Epidemiological data, modes of action, mechanisms of resistance and molecular markers of resistance are presented for each antimalarial drug currently used (Nzila et. al., 2000).

2.7.3. Dynamics of Resistance

With the identification of genotypic resistance determinants, many retrospective studies on the evolution of resistance to chloroquine and SP were performed. Microsatellite markers and neutral point mutations found in areas adjacent to these resistance determinants were analyzed. According to the studies, neutral point mutations were not polymorphic and had a low mutation rate (Hastings et. al., 2002). However, microsatellites were highly polymorphic and evolved faster (Ferdig and Su, 2000). The study of microsatellites around *Pfcrt* on chromosome 7 indicated that among all haplotypes of chloroquine-resistant strains that emerged over time, only four strains survived and these were still identified 50 years later. These four haplotypes were from New Guinea, South America and Asia (Hastings et. al., 2002). Some data suggested the presence of two additional origins of chloroquine resistance in Cambodia (Lim et. al., 2003) and the Philippines (Chen et. al., 2003). Therefore, resistance to chloroquine in Africa resulted from the spread of a mutant that emerged in Indochina in the 1950s (Wootton et. al., 2002).

The mechanism of resistance to antimetabolites appears with the mutations in *Pfdhfr* gene in vivo then turns into multiple resistances against SP (Hailemeskel et. al., 2013). Studies confirmed this hypothesis for simple *Pfdhfr* mutants. However, the presences of double or triple mutations in the *Pfdhfr* gene (codon positions 51, 59, 108) are at the origin of the high level of resistance (Cortese et. al., 2002; Roper et. al., 2003). Resistance against SP in Africa occurs due to the intercontinental migration of parasites from Asia (Naidoo and Roper, 2010).

The emergence of mutations that were scattered in endemic areas was shown to originate usually in Asia. These data suggest that the emerging resistance occurs more easily in areas of low malaria transmission due to the polyclonality and reduced immunity. The polyclonality in these areas is limited, and the competition among resistant and susceptible clones is reduced as the recombination initiates the transmission of the alleles. In addition, the lack of immunity results in more frequent contact with a large number of parasites since almost all infections are symptomatic (Hastings et. al., 2002).

In high transmission areas, competition and recombination between parasites are numerous and resistance disappears more easily. It is therefore important to examine the resistant parasites (Walliker et. al., 2005). Fitness is defined as the probability that the offspring of a strain with a special character has to survive and multiply. In the various models used to predict the distribution of the resistance, a loss of 10% of threshold of resistant parasites is generally accepted (Kublin et. al., 2003).

The dynamics of resistance is also strongly influenced by the half-life of the drug and its speed of action. Drugs with a long half-life such as atovaquone and mefloquine have the disadvantage of long persistence in the blood. Persistence increases drug pressure on the parasite population (Plowe et. al., 1995). Due to the rapid spread of global resistance in recent years and its impact on humans who are at risk of malaria, the efficiency of different antimalarial drugs should be monitored carefully (Djimde et. al., 2003).

3. MATERIALS AND METHODS

3.1. Study Area

This study was conducted in Bandundu, capital of the province of Bandundu, the DRC, specifically in its three municipalities: Disasi, Mayoyo and Basoko. The choice brought to this city was due to the presence of many breeding sites. This area has been a provincial health observatory and provincial office of the PNLP for decades. Three major species of *Plasmodium; P. falciparum, P. malariae* and *P. ovale* were documented from this region (PNLP, the DRC, 2013).

Bandundu is located at the junction of two rivers and on a swampy environment that promotes the breeding of mosquitoes. The region is surrounded by the cities where the rate of *Anopheles* index is too high. In those cities, malaria rate among children aged 0-5 years was reported to be 80%. Besides, malaria infection was documented in 65% of pregnant women, with a mortality rate of 38% (PNLP, the DRC, 2014).

In recent decades, an increase in out-migration from rural to urban areas has been observed since the installation of provincial institutions. Population activities revolve around its port, which has a fairly heavy traffic when crossing boats that go to others city. Moreover, its agro-pastoral activities, use of source water and well water all increase the risk of contact between humans with mosquitoes belonging to *Anopheles* species. The mosquito breeding sites are numerous and difficult to spot in the different areas (PNUD/UNOPS, the DRC, 1998).

3.2. Sampling

Blood sampling was carried out from March 2014 to May 2014 (short rainy season), and in September 2014 (end of the dry season). High transmission of malaria infection and many episodes of clinical disease are documented in this period (Er-Rami et. al., 2011).

The sampled population consisted of 250 delivered women from St. Joseph Hospital, Musaba Health Center, and Marie Kwango Health Center in Bandundu

from March 2014 to May 2014 and September 2014. The women were selected on the basis of being pregnant and having received two doses of SP during pregnancy.

Ethical approval was obtained from the DRC Ministry of Health and "Ankara Numune Eğitim ve Araştırma Hastanesi Etik Kurulu" from Turkey.

In this study, the transverse prospective methods were used to collect current data, and the experimental method was accomplished to perform the laboratory experiments.

A sample of capillary blood was obtained from the fingertip of each woman. The first drop of blood was taken to achieve RDT which detects possible *Plasmodium* antigens in the blood. Another drop of capillary blood sample was applied on an object holder plate. With this drop, thick blood smears were performed on the slides for the microscopic examination. In the last step, few drops of the capillary blood samples were applied on four different places of the Whatman filter paper for molecular techniques. Labeled filter papers were dried in the air and stored at 25°C, before being transported to the laboratory. Along with the blood smears, the filter papers were sent from the DRC to North Cyprus for analysis. The filter papers were permanently stored in foil bags containing silica gel at 4°C, then were transported from North Cyprus to Turkey for further molecular analyzes.

3.3. Microscopy

Capillary puncture and blood smears were performed according to the following procedures:

1. After wearing gloves, pre-cleaned slides were labeled with patient's information.
2. The middle finger was selected for puncture. Then, the area to be punctured was cleaned with 70% alcohol.
3. After the puncture of the fingertip, the first drop of blood was wiped with clean gauze.
4. The next drop of blood was applied on the clean slide.

5. A circular thick smear with about 1 cm of diameter was prepared by using the corner of another clean slide.
6. The preparations were dried in the air and protected against dust, flies, sunlight and extreme heat. After the preparations were completely dried, they were immediately stained and stored in slide boxes.

Staining of the Thick Blood Smears by Giemsa Method

After the preparations were dried in the air, the thick blood smears were stained by Giemsa according to the following procedure:

1. The smears were stained with 10% Giemsa solution for 15 minutes.
2. The slides were rinsed with buffer solution for 10 seconds.
3. The slides were dried in the air properly.
4. Immersion oil was applied onto the slides and the smears were examined under 100X objective of the microscope.
5. The results were recorded and interpreted.

3.4. Rapid Diagnostic Test (RDT)

In this study, RDT which is an immunochromatographic technique (SD BIOLINE Malaria Ag P.f/Pan) was performed. RDT uses monoclonal antibodies that can detect the antigens produced by the parasites. One of the antigens was HRP-2 which is specific for *P. falciparum*. The other antigen was pLDH which is produced by all of the *Plasmodium* species (pan-specific or species-specific). The reaction of blood antigens with antibodies present in the dipstick in the presence of buffer solution was marked by acridine orange. This specific stain is used for visualization of antigen-antibody reaction.

RDT was performed according to the manufacturer's protocol:

1. After capillary blood was collected, one drop of blood was applied into the well on the test strip by means of a glass stick.

2. One drop of buffer was added into the well. The test strip was left at room temperature for 15 minutes. The blood moved gradually to the level of different reaction sites.
3. A positive test result was obtained when the presence of red bands on both of the control and the test lines were visualized.
4. The presence of a red band on the control line and a lack of reaction on the test lines were interpreted as a negative test result.
5. In case a red line on the control band was not detected, the test was regarded as invalid.

3.5. Molecular Methods

In this study, presence of *Plasmodium* species in the delivered women was also examined by PCR. In the next step of molecular tests, a total of 30 samples determined to be positive by PCR were randomly selected for DNA sequencing. The mutations in the *Pfdhfr* and *Pfdhps* genes which are associated with SP resistance were analyzed by the sequencing method.

3.5.1. DNA Extraction

In this study, dried blood samples on Whatman filter papers were used for molecular methods. The fragments of filter papers having the dried blood samples were cut about 1 cm^2 and the pieces were placed into the 2 ml Eppendorf tubes.

DNA extraction was conducted according to the manufacturer's protocol (EURx GeneMATRIX Bio-Trace DNA Purification Kit):

1. 350 µl of Lyse BT buffer and 10 µl of Proteinase K were added and incubated for 60 min at 56°C.
2. 350 µl of Sol BT buffer were added and incubated for 10 min at 70°C.
3. 180 µl of 96-100 % ethanol were added and centrifuged for 2 min at 14000 rpm.

4. 600 µl of supernatant were transferred into the DNA binding spin-column in the collection tube.
5. The tube was centrifuged for 1 min at 12000 rpm and the spin-column was removed.
6. The spin-column was placed back in the collection tube.
7. DNA binding spin-column was removed and 500 µl of Wash BTX1 buffer were added to the spin-column. The tube was centrifuged for 1 min at 12000 rpm.
8. DNA binding spin-column was removed and 500 µl of Wash BTX2 buffer added to the spin-column. The tube was centrifuged for 2 min at 12000 rpm.
9. The spin-column was placed in a new collection tube (2 ml) and 50 µl of Elution buffer was added. The tube was incubated for 5 min at room temperature.
10. The spin-column was centrifuged for 1 min at 12000 rpm
11. DNA was collected and stored either at 2-8°C for short period or at -20°C for longer period.

3.5.2. Polymerase Chain Reaction (PCR)

The amplification method used for the detection and identification of malaria parasites in this study was nested PCR (Jelinek et. al., 1997). In the first PCR reaction (Nest 1), the *Plasmodium* genus–specific primers rPLU1 and rPLU5 were used for screening malaria parasites at the genus level in the samples. For detection at the species level; oligonucleotide primers specific to five *Plasmodium* species were used in the second PCR (Nest 2) reactions. These primers were rFAL 1 and rFAL 2 for *P. falciparum* (product of 205 bp), rMAL 1 and rMAL 2 for *P. malariae* (product of 144 bp), rVIV 1 and rVIV 2 for *P. vivax* (product of 120 bp), rOVA 1 and rOVA 2 for *P. ovale* (product of 387 bp) and Pmk 8 and Pmkr 9 for *P. knowlesi.*

Primers used for nested PCR are described in the following:

Genus-specific primers (Nest 1):

(Foster et. al., 2014; Singh et. al., 1999; Singh et. al., 2004)

rPLU 1 : 5'-TCAAAGATTAAGCCATGCAAGTGA-3'

rPLU 5 : 5'-CCTGTTGCCTTAAACTCC-3'

Species-specific primers (Nest 2):

***P. falciparum*:** (Dinko et. al., 2013; Singh et. al, 1999; Snounou et. al., 1993)

rFAL 1: 5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'

rFAL2: 5'- ACACAATGAACTCAATCATGACTACCCGTC-3'

***P. vivax*:** (Dinko et. al., 2013; Singh et. al, 1999; Snounou et. al., 1993)

rVIV 1: 5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3'

rVIV 2: 5'-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3'

***P. malariae*:** (Dinko et. al., 2013; Singh et al., 1999; Snounou et. al., 1993)

rMAL1: 5'-ATAACATAGTTGTACGTTAAGAATAACCGC-3'

rMAL 2: 5'-AAAATTCCCATGCATAAAAAATTATACAAA-3'

***P. ovale*:** (Dinko et. al., 2013)

PovaFWD: 5'-CTGTTCTTTGCATTCCTTATGC-3'

RVS common: 5'-GTATCTGATCGTCTTCACTCCC-3'

***P. knowlesi*:** (Singh et. al., 2004)

Pmk8: 5'-GTTAGCGAGAGCCACAAAAAAGCGAAT-3'

Pmkr9: 5'-ACTCAAAGTAACAAAATCTTCCGTA-3'

Nested PCR Assay Protocol:

Nested PCR was conducted according to the manufacturer's protocol (Solis BioDyne, FIREPol® DNA Polymerase). Each of the 30 µl reaction mixture for Nest 1 PCR contained 1 µl of DNA template, 0.4 µl of each primer (rPLU 1 and rPLU 5), 1.8 µl $MgCl_2$, 3.1 µl PCR buffer (50 mM KCl, 10 mM Tris-HCl), 0.3 µl of each

deoxynucleoside triphosphate, 0.4 µl of DNA polymerase and 23.8 µl PCR grade water.

The following cycling conditions were applied for Nest 1 PCR reaction: 94°C for 4 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute; and a final extension at 72°C for 4 minutes.

Two microliters of the Nest 1 PCR product served as the DNA template for each of the 20 µl Nest 2 reaction mixture. The concentration of the Nest 2 primers and other constituents were identical to those of the Nest 1 reaction except that 0.5 units of Taq DNA polymerase were used. Nest 2 PCR conditions were identical to those of Nest 1 reaction except that the cycle numbers were 20 for the species-specific primers. The PCR products of the Nest 2 reaction were analyzed by agarose gel electrophoresis. The bands were visualized by staining with ethidium bromide and observed under UV light.

3.5.3. Identification of Drug Resistance Genes

Amplification of Resistance Genes by PCR

A total of 30 samples that were previously determined as positive for *P. falciparum* by PCR were randomly selected and analyzed for the genes associated with SP resistance. Twenty samples were searched for *Pfdhfr* gene, and 10 samples were tested for *Pfdhps* gene. Prior to DNA sequencing, the resistance genes *Pfdhfr* and *Pfdhps* were amplified by PCR.

The following primers were used for the amplification of *Pfdhfr* and *Pfdhps* (Pearce et. al., 2003):

***Pfdhfr* gene:**

Fr-F: TTTATGATGGAACAAGTCTGC

Fr-R: CTAGTATATACATCGCTAACA

***Pfdhps* gene:**

Ps-F: GATTCTTTTTCAGATGGAGG

Ps-R: TTCCTCATGTAATTCATCTGA

PCR Assay Protocol:

Each of the 33 µl reaction mixture contained 5 µl of DNA template, 0.5 µl of each primer (forward and reverse primers), 1.5 µl of $MgCl_2$, 2.5 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl), 0.2 µl of each deoxynucleoside triphosphate, 0.3 µl of FIREPol® DNA polymerase and 15.9 µl of PCR grade water.

The following conditions for the first and second PCR cycles for *Pfdhfr* gene were applied: 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. For *Pfdhps* gene, the following cycling conditions were applied: 94°C for 3 minutes; 34 cycles of 94°C for 45 seconds, 51°C for 45 seconds, and 60°C for 45 seconds; and a final extension at 60°C for 5 minutes. The PCR products were analyzed by agarose gel electrophoresis. The bands were visualized by staining with ethidium bromide and observed under UV light.

Detection of Resistance Genes by DNA Sequencing

Following the amplification of resistance genes, the PCR products were sent to Macrogen Laboratory Company in Amsterdam/Netherlands for sequencing. Mutations in the *Pfdhfr* and *Pfdhps* genes were analyzed by Sanger's method.

DNA polymerases copy single-stranded DNA templates by adding nucleotides to a growth chain (extension product). Chain extension occurs at the 3' end of a primer. Deoxynucleotides added to the extension product are selected as pairs corresponding to the model. The extension product is developed by the formation of a phosphodiester bond between the 3'-hydroxyl of the primer and the 5'-phosphate group of the incoming deoxynucleotide, and growth occurs in the 5'-3' direction. In the DNA sequencing method developed by Sanger, dideoxynucleotides are used as substrates. When dideoxynucleotides are incorporated to the 3' end of the growing chain, further extension is prevented and the elongation is completed with adenine, guanine, cytosine, or thymine base (Sanger et. al., 1977).

3.6. Statistical Analysis

The Finch TV and NCBI Blast genetic software were used for analyzing the sequencing data. Different nucleotides on DNA were opened and decoded by the Finch TV software. Mutations associated with SP resistance in *P. falciparum* were identified by NCBI Blast programme. The statistical analysis (ki square) of the data obtained during the investigations was done according to the SPSS Version 22 software.

4. RESULTS

The 250 delivered women from Bandundu/the DRC who participated in this study had received SP prophylaxis for two times during their pregnancy. The participants aged between 16 and 39 years. There were 93 (37.2%) women in the age group of 15-24. Age groups of 25-34 and 35+ included 112 (44.8%) and 45 (18.0%) women, respectively.

The samples were collected from three hospitals, among them, 146 (58.4%) specimens were from St. Joseph Hospital, 67 (26.8%) specimens were from Musaba Health Center, and 37 (14.8%) samples from Marie Kwango Health Center.

In the examination of thick blood smears, out of 250 capillary blood samples of delivered women, 81 (32.4%) samples were found to be positive, while 169 (67.6%) samples were negative for *Plasmodium* species (Table 4.1).

Table 4.1. Malaria infection rates detected by examination of thick smear in 250 delivered women in Bandundu/the DRC, 2014.

Thick Smear		
Results	Samples	
	Number (n)	Percentage (%)
Positive	81	(32.4)
Negative	169	(67.6)
Total	250	(100.0)

The rate of malaria infection detected by microscopy was evaluated according to the age groups. In the age group of 15-24, 28 (30.1%) women were positive for malaria, while 65 (69.9%) participants were found to be negative. In the age group of 25-34, 36 (32.1%) positive and 76 (67.9%) negative samples were detected. In the age group of 35+, the number of positive and negative samples were 17 (37.8%) and 28 (62.2%), respectively. The difference in the distribution of infection among the age groups was not statistically significant (p= 0.663) (Table 4.2).

Table 4.2. Distribution of microscopy results according to the age groups of 250 delivered women in Bandundu/the DRC, 2014.

Age Group	Microscopy		Total n (%)
	Positive n (%)	Negative n (%)	
15-24	28 (30.1)	65 (69.9)	93 (100.0)
25-34	36 (32.1)	76 (67.9)	112 (100.0)
35+	17 (37.8)	28 (62.2)	45 (100.0)
Total	81 (32.4)	169 (67.6)	250 (100.0)

The microscopy results according to the number of birth were evaluated. Thirty-one (31.0%) cases of primigravida were identified as positive and 69 (69.0%) cases were determined to be negative. For multigravida, 50 (33.3%) cases were positive and 100 (66.7%) cases were negative. The difference of infection rates between two groups was not statistically significant (p= 0.669) (Table 4.3).

Table 4.3. Distribution of microscopy results according to the number of deliveries among 250 delivered women in Bandundu/the DRC, 2014.

Childbirth	Microscopy		Total n (%)
	Positive n (%)	Negative n (%)	
Primigravida	31 (31.0)	69 (69.0)	100 (100.0)
Multigravida	50 (33.3)	100 (66.7)	150 (100.0)
Total	81 (32.4)	169 (67.6)	250 (100.0)

Distribution of positive microscopy results among three hospitals was evaluated. In St. Joseph Hospital 39 (48.2%), in Musaba Health Center 27 (33.3 %), and in Marie Kwango Health Center 15 (18.5%) samples were found as positive by microscopic examination. The difference was not statistically significant (p= 0.075).

Of the 250 capillary blood samples analyzed for *Plasmodium* spp. antigens by RDT, 93 (37.2%) cases were detected as positive and 157 (62.8%) cases were determined to be negative (Table 4.4).

Table 4.4. Malaria infection rates detected by RDT in 250 delivered women in Bandundu/the DRC, 2014.

RDT		
Results	Samples	
	n	(%)
Positive	93	(37.2)
Negative	157	(62.8)
Total	250	(100.0)

RDT results were evaluated according to *Plasmodium* antigens detected. HRP-2 which is specific for *P. falciparum* was detected in 40 (43.0%) cases. *P. falciparum* (HRP-2) and pan-*Plasmodium* (pLDH) antigens were concurrently detected in 50 (53.8%) tests, while three (3.2%) samples gave positive reaction for pan-*Plasmodium* (pLDH) antigens only (Table 4.5).

Table 4.5. Distribution of *Plasmodium* species among 93 samples detected positive by RDT in Bandundu/the DRC, 2014.

Positive RDT Results		
Species	n	(%)
P. falciparum (HRP-2)	40	(43.0)
P. falciparum (HRP-2) + pan-*Plasmodium* (pLDH)	50	(53.8)
pan-*Plasmodium* (pLDH)	3	(3.2)
Total	93	(100.0)

Distribution of RDT results according to the number of deliveries was determined. Thirty-nine (39.0%) cases of primigravida were found to be positive and 61 (61.0%) cases were determined to be negative. Fifty-four (36.0%) cases of multigravida were observed to be positive and 96 (64.0%) cases were found to be negative. The difference of infection rates between two groups was not statistically significant (p= 0.631) (Table 4.6).

Table 4.6. Distribution of RDT results according to the number of deliveries among 250 delivered women in Bandundu/the DRC, 2014.

Childbirth	RDT Results		Total n (%)
	Positive n (%)	Negative n (%)	
Primigravida	39 (39.0)	61 (61.0)	100 (100.0)
Multigravida	54 (36.0)	96 (64.0)	150 (100.0)
Total	93 (37.2)	157 (62.8)	250 (100.0)

The rate of malaria infection detected by RDT was evaluated according to the age groups. In the age group of 15-24, 37 (39.8%) women were positive for malaria, while 56 (60.2%) samples were tested negative for *Plasmodium* spp. In the age group of 25-34, 38 (33.9%) positive and 74 (66.1%) negative samples were detected. In the age group of 35+, the number of positive and negative samples were 18 (40.0%) and 27 (60.0%), respectively. The difference in the distribution of infection among the age groups was not statistically significant (p= 0.628) (Table 4.7).

Distribution of positive RDT results among three hospitals was evaluated. In St. Joseph Hospital, Musaba Health Center and Marie Kwango Health Center, the number of positive results detected by RDT were 59 (63.4%), 17 (18.3%), and 17 (18.3%), respectively. The difference of infection rates between three hospitals was not statistically significant (p= 0.053).

Table 4.7. Distribution of RDT results between the age groups of 250 delivered women in Bandundu/the DRC, 2014.

Age group	RDT Result		Total n (%)
	Positive n (%)	Negative n (%)	
15-24	37 (39.8)	56 (60.2)	93 (100.0)
25-34	38 (33.9)	74 (66.1)	112 (100.0)
35+	18 (40.0)	27 (60.0)	45 (100.0)
Total	93 (37.2)	157 (62.8)	250 (100.0)

In this study, number of positive and negative samples obtained from microscopy and RDT were compared. When microscopy was taken as the gold standard, sensitivity and specificity of RDT were determined to be 59.3% and 73.4%, respectively (Table 4.8).

Table 4.8. Number of positive and negative samples determined by microscopy and RDT in 250 delivered women in Bandundu/the DRC, 2014.

		Microscopy		
		Positive	Negative	Total
RDT	Positive	48	45	93
	Negative	33	124	157
	Total	81	169	250

In this study, blood samples dried on Whatman filter papers were tested by molecular methods. Nested PCR was conducted in order to detect malaria parasites at genus and species levels in the capillary blood of 250 delivered women. Ninety-two (36.8%) samples were found as positive for *Plasmodium* species, while 158 (63.2%) samples gave negative result for *Plasmodium* parasites (Table 4.9).

Table 4.9. Prevalence of malaria infection detected by nested PCR among 250 delivered women in Bandundu/the DRC, 2014.

Nested PCR		
Results	Samples	
	n	(%)
Positive	92	(36.8)
Negative	158	(63.2)
Total	250	(100.0)

The rate of malaria infection detected by nested PCR was evaluated according to the number of childbirth. In primigravida, 36 (36.0%) women were positive, while 64 (64.0%) women were tested negative for malaria. In multigravida, 56 (37.3%) women were detected as positive; however 94 (62.7%) women were negative for malaria. The difference of infection rates between two groups was not statistically significant (p= 0.830) (Table 4.10).

Table 4.10. Distribution of nested PCR results according to the number of deliveries among 250 delivered women in Bandundu/the DRC, 2014.

Childbirth	Nested PCR Results		Total n (%)
	Positive n (%)	Negative n (%)	
Primigravida	36 (36.0)	64 (64.0)	100 (100.0)
Multigravida	56 (37.3)	94 (62.7)	150 (100.0)
Total	92 (36.8)	158 (63.2)	250 (100.0)

The presence of malaria infection detected by nested PCR was assessed according to the age groups. In the age group of 15-24, 29 (31.2%) women were determined to be positive for malaria parasites, while 64 (68.8%) samples were found to be negative. In the age group of 25-34, 46 (41.1%) cases of malaria

infections were detected, while 66 (58.9) samples were negative for *Plasmodium* spp. In the age group of 35+, the number of positive and negative samples were 17 (37.8%) and 28 (62.2%), respectively. The difference in the distribution of infection among the age groups was not statistically significant (p= 0.340) (Table 4.11).

Table 4.11. Distribution of nested PCR results between the age groups of 250 delivered women in Bandundu/the DRC, 2014.

Age groups	Nested PCR		Total n (%)
	Positive n (%)	Negative n (%)	
15-24	29 (31.2)	64 (68.8)	93 (100.0)
25-34	46 (41.1)	66 (58.9)	112 (100.0)
35+	17 (37.8)	28 (62.2)	45 (100.0)
Total	92 (36.8)	158 (63.2)	250 (100.0)

Distribution of positive PCR results among three hospitals was evaluated. In St. Joseph Hospital, Musaba Health Center and Marie Kwango Health Center, the number of positive results detected by nested PCR were 46 (50.0%), 33 (35.9%) and 13 (14.1%), respectively. The difference of infection rates between three hospitals was statistically significant (p= 0.045).

Positive PCR results were evaluated according to the *Plasmodium* species isolated. *P. falciparum* was detected as the only agent in 87 (94.5%) of 92 samples, while it was isolated concurrently with *P. vivax* and *P. malariae* in two (2.2%) and one (1.1%) samples, respectively. Apart from mixed infection with *P. falciparum*, *P. vivax* was also detected as the only agent in two (2.2%) samples. In this study, *P. ovale* and *P. knowlesi* were not isolated (Table 4.12).

Table 4.12. Distribution of *Plasmodium* species among 92 positive PCR samples of 250 delivered women in Bandundu/the DRC, 2014.

Plasmodium species	n	(%)
P. falciparum	87	94.5
P. falciparum + *P. vivax*	2	2.2
P. falciparum + *P. malariae*	1	1.1
P. vivax	2	2.2
P. ovale	0	0.0
P. knowlesi	0	0.0
Total	92	100.0

In this study, number of positive and negative samples obtained from PCR and RDT were compared. When PCR was taken as the gold standard, sensitivity and specificity of RDT were found to be 55.4% and 73.4%, respectively (Table 4.13).

Table 4.13. Number of positive and negative samples determined by PCR and RDT in 250 delivered women in Bandundu/the DRC, 2014.

		PCR		
		Positive	Negative	Total
RDT	Positive	51	42	93
	Negative	41	116	157
	Total	92	158	250

In this study, 30 samples that were tested positive for *P. falciparum* by PCR were randomly selected and analyzed for *Pfdhfr* and *Pfdhps* genes. Twenty samples were searched for *Pfdhfr* gene, while 10 samples were tested for *Pfdhps* gene. In 10 samples, replacement of cysteine by arginine (mutant type) was detected at the codon position 59 (C59R) of *Pfdhfr* gene (Figure 4.1). No mutation was detected in *Pfdhfr*

gene of the remaining 10 samples. Likewise, no mutation was observed in *Pfdhps* gene of the 10 samples sequenced (Table 4.14).

Table 4.14. DNA sequencing results of *Pfdhfr* and *Pfdhps* genes among 30 samples of 250 delivered women in Bandundu/the DRC, 2014.

Gene	Number of samples sequenced	Number of mutations detected	Type of mutation detected	Codon position
Pfdhfr	20	10	TGT → CGT (Cys → Arg)	59
Pfdhps	10	-	-	-

Figure 4.1. DNA sequencing results of *Pfdhfr* gene.

(Cysteine was replaced by arginine at the codon position 59 (C59R) of *Pfdhfr* gene.)

5. DISCUSSION

Malaria which is an important health issue threatens several risk groups including pregnant women. The infection in this group has adverse outcome for the mother, the fetus, and the newborn. For this reason, WHO recommends the administration of SP prophylaxis against malaria in pregnancy (WHO, 2013a). In this study, the presence of malaria infection was evaluated in a group of 250 women from the DRC who have recently given birth.

Blood samples were collected from 250 delivered women from three hospitals in Bandundu city (Bandundu province/the DRC). The presence of *Plasmodium* species in the samples was determined by microscopic examination, RDT and PCR.

In this study, three different hospitals were selected for the collecting of blood samples. From St. Joseph Hospital, Musaba Health Center and Marie Kwango Health Center, 146 (58.4%), 67 (26.8%) and 37 (14.8%) blood samples were collected, respectively.

The prevalence of malaria infection detected by microscopy among delivered women was 32.4% in this study (Table 4.1) and this finding can be attributed to increased transmission of malaria with high rate of reinfection between the last doses of SP (at the 28^{th} week) until delivery. The presence of infection can also be explained by incorrect use of SP because of the lack of trained health care worker staff who administered the drug as monotherapy; lack of *Plasmodium* screening in pregnancy, and use of self-medication by the pregnant women.

In this study, the prevalence of malaria infection detected by microscopy was found to be higher than those reported by Minang et. al. (21.6%) and Tonga et. al. (22.9%) in Cameroon (Minang et. al., 2004; Tonga et. al., 2013). Our result was also higher than those of recent studies conducted in Uganda (17.5%) (Arinaitwe et. al., 2013), Angola (10.9%) (Campos et. al., 2012), and Tanzania (29.7%) (Mpogoro et. al., 2014). The prevalence of malaria infection in the women who participated in this study was lower than those of Amoran et. al. (47.8%) and Aribodor et al. (64.4%), which were conducted in Nigeria that has a high rate of malaria (Amoran et. al., 2012; Aribodor et. al., 2009). The microscopy result in this study was also lower than

the recent study conducted in the DRC (74.1%) (Wumba et. al., 2015). The different rates of malaria infection among delivered women in the three border countries can be explained by the geographical variation of malaria transmission, lack of proper management of malaria in the region and the immune status of participants in this study.

Malaria rates detected by microscopy among the age groups of 15-24, 25-34 and 35+ were 30.1%, 32.1% and 37.8%, respectively. The difference in the distribution of infection among the age groups was not statistically significant (p= 0,663) (Table 4.2). This finding indicated that the age of the women probably did not influence the malaria infection in pregnancy. This finding was incompatible with other studies where the younger women were more susceptible to malaria than older women. This can be explained by the younger women's being in the process of acquiring natural immunity to pregnancy related malaria (Matangila et. al., 2014).

According to the microscopy results, the rate of malaria infection between primigravida and multigravida were 31.0%, 33.3%, respectively. The difference of infection rates between two groups was not statistically significant (p= 0.669) (Table 4.3). Our result was similar with the recent report in Nigeria, where no statistically difference was found between primigravida and multigravida in terms of prevalence of malaria infection, although prevalence was higher in multigravida (Efunshile et. al., 2011). Our results are not compatible with a previous report where the prevalence determined by microscopy was higher among primigravida (26.5%) than multigravida (18.8%) in Kinshasa capital of the DRC (Lukuka et. al., 2006).

The presence of malaria infection detected by microscopy was found to be 48.2%, 33.3% and 18.5% in St. Joseph Hospital, Musaba Health Center and Marie Kwango Health Center, respectively. St. Joseph Hospital where the highest amount (58.4%) of blood samples were collected presented the highest rate of malaria infection. This was followed by Musaba Health Center and Marie Kwango Health Center. The difference of malaria rates among three hospitals was not statistically significant (p= 0,075).

In this study, the rate of malaria infection determined by RDT was 37.2% (Table 4.4). This finding was higher than the RDT results of two previous studies

which were conducted on pregnant women receiving SP prophylaxis. In these studies which were reported by Lukuka et. al. and Matangila et. al., the rates of malaria infection were found to be 19.7% and 27.4%, respectively (Lukuka et. al., 2006; Matangila et. al., 2014). These observations indicated that malaria infection increased gradually in pregnant women in the DRC. The result of our study was also higher than the prevalence of asymptomatic malaria infection detected among delivered women in Burkina Faso which were 25.8% (Tiono et. al., 2009) and 30.0% (Douamba et. al., 2012).

RDT results were evaluated according to *Plasmodium* antigens detected. HRP-2 which is specific for *P. falciparum* was detected in 40 (43.0%) cases. *P. falciparum* (HRP-2) and pan-*Plasmodium* (pLDH) antigens were concurrently detected in 50 (53.8%) tests, while three (3.2%) samples gave positive reaction for pan-*Plasmodium* (pLDH) antigens only (Table 4.5). In a recent study conducted by Gatton et al., 57.1% of the samples were determined to be positive for HRP-2 and pLDH antigens, while 42.9% of the samples were positive for HRP-2 antigen only (Gatton et. al., 2015). Our results were inconsistent with the recent study conducted in Burkina Faso, where among the *Plasmodium* positive samples, HRP-2 antigen was positive in all samples, while pLDH antigen missed one (Maltha et. al., 2014).

RDT results revealed that the rate of malaria infection was higher in primigravida (39.0%) when compared to multigravida (36.0%), however the difference of malaria prevalence between two groups was not statistically significant (p= 0.631) (Table 4.6). Similar finding was obtained from another study in the DRC, where primigravida presented a higher rate (26.5%) than multigravida (18.8%) (Lukuka et. al., 2006).

The rate of malaria infection detected by RDT was evaluated according to the age groups. Malaria prevalence was found to be 39.8%, 33.9% and 40.0% in the age groups of 15-24, 25-34 and 35+, respectively. The difference in the distribution of infection among the age groups was not statistically significant (p= 0.628) (Table 4.7). This suggested that the age did not influence the development of malaria infection during pregnancy. Similar finding was shown in a previous study where no

significant association between the prevalence of malaria infection and age groups was documented (Efunshile et. al., 2011).

The rates of malaria infection in three hospitals were evaluated according to RDT results. St. Joseph Hospital where the highest amount (58.4%) of blood samples were collected presented the highest prevalence (63.4%) of malaria infection. This was followed by Musaba Health Center (18.3%) and Marie Kwango Health Center (18.3%). The difference of infection rates between three hospitals was not statistically significant (p= 0.053).

In this study, presence of malaria infection was also evaluated by nested PCR. Ninety-two (36.8%) samples were determined to be positive for *Plasmodium* species (Table 4.9). This result was higher than those of previous studies conducted in the DRC. In these studies which were reported by Taylor et. al. and Matangila et. al., the rates of malaria infection were found to be 31.2% and 29.5%, respectively (Taylor et. al., 2011a; Matangila et. al., 2014). Malaria prevalence in this study was also found to be higher than a previous research where 33.5% of pregnant women were reported to be positive for malaria infection (Taylor et al., 2011b). The PCR result obtained from our study was lower than that of a previous study from Ethiopia where malaria infection rate was documented to be 73.1% (Alemu et. al., 2014).

Malaria prevalence determined by nested PCR was evaluated according to the number of childbirth. The difference between malaria infection rates detected by nested PCR in primigravida (36.0%) and multigravida (37.3%) was not statistically significant (p= 0.830) (Table 4.10).

Malaria prevalence detected by nested PCR was recorded as 31.2%, 41.1% and 37.8% in the age groups of 15-24, 25-34 and 35+, respectively. The difference of infection rates between the age groups was not statistically significant (p= 0.340) (Table 4.11). This finding indicated that malaria infection occurred during pregnancy was not influenced by the age groups.

The rates of malaria infection in three hospitals were compared according to the nested PCR results. St. Joseph Hospital where the highest amount (58.4%) of blood samples were collected presented the highest prevalence (50.0%) of malaria infection. This was followed by Musaba Health Center (35.9%) and Marie Kwango

Health Center (14.1%). The difference of infection rates between three hospitals was statistically significant (p= 0.045).

In our study, three *Plasmodium* species were detected; *P. falciparum, P. vivax* and *P. malariae* (Table 4.12). Previously, only three *Plasmodium* species; *P. falciparum, P. malariae* and *P. ovale* were documented in the DRC (WHO, 2013b). Absence of *P. vivax* is attributed to the lack of Duffy receptor in the population of sub-Saharan Africa. This receptor is found on the RBCs which is essential for invasion of *P. vivax* parasite (Dhorda et. al., 2011).

In our study, *P. falciparum* was present in a high percentage (94.5%) of positive samples analyzed by PCR (Table 4.12). This finding was similar to the result of a previous report where *P. falciparum* was the most abundant species (96.3%) detected among pregnant women in Kisangani/the DRC (Bassandja et. al., 2014). This confirms that malaria infection caused by *P. falciparum* remains a public health problem in the DRC particularly in Bandundu and extends throughout the DRC. This species is more dangerous than other *Plasmodium* species because it has the ability to infect the RBCs of any stage (young or mature erythrocytes). This leads to obstruction of the blood microcirculation and results in dysfunction of multiple organs such as brain in cerebral malaria (Dondorp et. al., 2004). *P. falciparum* has adhesins that interact with CSA molecules found in the placenta. This interaction provokes the obstruction of blood microcirculation, leading to localized, limited or widespread tissue lesions in the placenta with the consequences of low birth-weight, spontaneous abortions and maternal death or stillbirth (Mulumba, 2006).

Among the positive samples, *P. malariae* was concomitantly detected with *P. falciparum* in one (1.1%) sample (Table 4.12). *P. malariae* is rarely documented in Bandundu. Similar findings were obtained from previous studies where the presence of *P. malariae* was reported in the eastern region of the DRC (PNLP, the DRC, 2013; Taylor et. al., 2011b). The reasons for this situation can be explained by the increased immigration from rural areas to urban areas due to the business activity and the installation of provincial institutions in Bandundu (PNUD/UNOPS, the DRC, 1998).

In this study, *P. vivax* was detected in four (4.4%) samples. *P. vivax* was isolated as the only agent in two (2.2%) samples, while it was concomitantly detected with *P. falciparum* in the other two (2.2%) samples (Table 4.12). *P. vivax* is generally rare or absent in the DRC (WHO, 2013b) and especially in Western Central Africa Region (Liu et. al., 2014). Similar findings were documented in a previous study from Uganda where three cases of *P. vivax* were detected. Three women infected with *P. vivax* were found to be Duffy-positive (Dhorda et. al., 2011).

Absence of Duffy antigen confers resistance against *P. vivax* infection in the sub-Saharan population; since *P. vivax* has not ability to infect the RBCs lacking Duffy receptor (Culleton et. al., 2009; Liu et. al., 2014). Although Duffy-negative individuals are generally protected from the blood stage infections, the recent studies in Madagascar and Ethiopia have shown that *P. vivax* infection is not dependent on the Duffy receptor (Liu et. al., 2014; Ménard et. al., 2010; Woldearegai, 2013). Other studies also found the presence of malaria infection caused by *P. vivax* in Duffy-negative individuals from Equatorial Guinea and Angola (Mendes et. al., 2011). This finding suggests that *P. vivax* is able to use receptors other than Duffy to invade erythrocytes, which may have an impact on the current distribution of this species.

P. vivax is one of the *Plasmodium* species that often causes epidemics in non-endemic areas and is also responsible for relapses of malaria infections in endemic areas (White, 2011). Infection caused by *P. vivax* in apes is highly prevalent especially in west central Africa. Thus wild-living chimpanzees and gorillas could serve as an infection reservoir, particularly in areas where an influx of Duffy positive humans through commerce and travel coincides with increasing forest encroachment by ape habitat destruction (Liu et. al, 2014). The presence of *P. vivax* in the DRC can be explained by the presence of a large number of tourists, salesmen or businessmen coming from South Africa, Asia and South America (Ramasamy, 2014).

In this study, mixed infections of *P. falciparum* with *P. vivax* (2.2%), and *P. falciparum* with *P. malariae* (1.1%) were documented. The prevalence of mixed infection of *P. falciparum* with *P. malaria* (1.1%) was lower than the results of previous studies. One of these studies conducted in the DRC (Taylor et. al., 2011b) reported the rate of mixed infection to be 4.9%, while another study from Nigeria

(Agomo and Oyibo, 2013) documented the mixed infection of *P. falciparum* with *P. malariae* to be 3.6%.

In the mixed infections consisting of *P. falciparum* and *P. vivax*, *P. falciparum* can lead to a transient increase in RBC production which promotes *P. vivax* to infect young RBCs (*P. vivax* hyper parasitemia). This also reduces the susceptibility of RBCs against *P. falciparum*. The mixed infection of *P. falciparum* with *P. vivax* also confers protection against severe malaria caused by *P. falciparum* (Mayxay et. al., 2004; McQueen and McKenzie, 2006).

The prevalence of malaria infection determined by microscopy, RDT and PCR in the blood samples of delivered women were 32.4% (Table 4.1), 37.2% (Table 4.4) and 36.8% (Table 4.9), respectively. The difference between microscopy and RDT results was statistically significant (p= 0.001). Likewise, the difference between microscopy and PCR results was also statistically significant (p= 0.001). The results of RDT revealed a higher rate of infection than those of PCR and microscopy. Among positive RDT results (n= 93), number of samples that were found to be negative by microscopy and PCR were 45 (48.4%) and 42 (45.2%), respectively (Table 4.8 and Table 4.13). The false positive results of RDT could be either explained by the persistence of HRP-2 circulation in the blood more than two weeks even after successful clearance of infected erythrocytes in the bloodstream or by the sequestration of the parasites in the placenta while HRP-2 circulate (Matangila et. al., 2014).

In our study, the prevalence of malaria infection found by three methods was higher than the results of a recent study that searched for asymptomatic *Plasmodium* infection among delivered women in the DRC. In that study the results of microscopy, RDT and PCR were reported as 21.6%, 27.4% and 29.5%, respectively (Matangila et. al., 2014). The rate of malaria infection detected by microscopy and RDT in our research was also higher than that of a previous study conducted in Burkina Faso. In that study, the prevalence of asymptomatic malaria infection among delivered women was found to be 24.0% and 30.0% by using microscopy and RDT, respectively (Douamba et. al., 2012).

In this study, when microscopy was taken as the gold standard, the sensitivity and specificity of RDT were found to be 59.3% and 73.4%, respectively (Table 4.8). The finding in our study was lower when compared to another report from Burkina Faso where RDT had a sensitivity of 89.0% (Singer et. al., 2004). Likewise, the result of our study was lower than that of a research conducted in Burkina Faso where RDT had a sensitivity and specificity of 100.0% and 92.0%, respectively, when compared to conventional microscopy (Douamba et. al., 2012). When PCR was taken as the gold standart, RDT had a sensitivity of 55.4% and a specificity of 73.4% in our study. This finding was lower than that of a recent report, where RDT had a sensitivity of 81.6% and a specificity of 94.9% to diagnose asymptomatic *P. falciparum* infection, when PCR was taken as the gold standard (Matangila et. al., 2014).

In this study, mutations in *Pfdhfr* and *Pfdhps* genes which are associated with SP resistance were investigated. Twenty samples were sequenced for *Pfdhfr* gene, while 10 samples were tested for *Pfdhps* gene. The data obtained from DNA sequencing were analyzed and compared with the sequences of the wild-type reference. In 10 samples, a single mutation (C59R) was detected in *Pfdhfr* gene, where cysteine (TGT) was replaced by arginine (CGT) at the codon position 59. No mutations were observed in the other 10 samples sequenced for *Pfdhfr* gene. Likewise, no mutations were detected in 10 samples searched for *Pfdhps* gene.

A previous study from the DRC reported the presence of quintuple mutations in *Pfdhfr* gene (N51I, S108N, and C59R) that were associated with SP resistance and treatment failure (Swarthout et. al., 2006). A recent study conducted in Burkina Faso during rainy season documented triple mutations (N51I, C59R, S108N) in *Pfdhfr* gene as 1.3%, 35.3% and 54.3% in 2000, 2009 and 2011, respectively (Geiger et. al, 2014). In a previous study from Congo Brazzaville, a neighbouring country of the DRC, approximately 97.5% of the samples were reported to have C59R mutation in *Pfdhfr* gene (Ndounga et. al., 2007). In a research from Angola, another neighbouring country of the DRC, C59R single mutation in *Pfdhfr* gene was detected in 90.3% of the samples (Fortes et. al., 2011). A study conducted in West Africa reported the mutations (S108N, N51I and C59R) in *Pfdhfr* gene as 100%, 93% and 57%, respectively (Fortes et. al., 2011).

The presence of mutations in both *Pfdhfr* and *Pfdhps* genes predicts SP treatment failure. C59R mutation is regarded as a useful indicator for the failure of treatment with pyrimethamine which can subsequently affect the SP combination therapy (Mayxay et. al., 2007). In this study, C59R polymorphism was detected in *Pfdhfr* gene of 10 samples, indicating that the mutation led to resistance against pyrimethamine. Eventually, SP prophylaxis was ineffective in the women these samples obtained from. In the remaining 10 samples, any mutation associated with pyrimethamine resistance was not detected in *Pfdhfr* gene. Likewise, mutations indicating sulfadoxine resistance in *Pfdhps* gene were not detected in the other 10 samples. According to nested PCR results, these samples were reported to be positive for *P. falciparum*. This suggests that, in those samples where no mutation was detected in *Pfdhfr* gene, polymorphisms in *Pfdhps* gene should be investigated. The samples without any mutation in *Pfdhps* gene should also be sequenced for the possible polymorphisms in *Pfdhfr* gene. Another reason for detection of *P. falciparum* in those samples without any mutation in *Pfdhfr* or *Pfdhps* genes could be explained by the dose of SP given to the women. In this study, the participants received two doses of SP in the 16^{th} and 28^{th} weeks of pregnancy. According to the new guidelines of WHO, at least four ANC visits are recommended during pregnancy where SP prophylaxis is given at least one month apart starting from the second trimester. By this programme, at least three doses of SP could be ensured during pregnancy (WHO, 2013a).

Several studies have shown that although the implementation of the SP does not prevent malaria infection during pregnancy, particularly in the presence of a high prevalence of SP-resistance markers, there is a significant protection against severe complications of malaria in pregnancy, such as low birth weight, maternal and neonatal mortality, especially when more than two doses of IPT is administered (Matondo et. al., 2014).

In our study, malaria infection was detected in a considerable amount of pregnant women by microscopy, RDT or PCR. This suggests that, two doses of SP remain ineffective in these women and SP prophylaxis can be more effective by increasing the number of doses. Efficiency of SP in the pregnant women should be monitored continuously in the regions where malaria remains an important health

issue. More comprehensive data on SP resistance will enable administration of the correct treatment scheme. Further studies should also examine the activity of alternative drugs in terms of prevention of malaria in pregnancy. This will enable protection of the mother, the fetus and the newborn against the adverse outcomes of malaria.

6. CONCLUSION

In this study, the presence of malaria infection was investigated in a group of 250 women from the DRC who received SP prophylaxis during pregnancy. The presence of *Plasmodium* species in the blood samples collected from 250 women was examined by microscopy, RDT and PCR.

Of the 250 blood samples tested, 81 (32.4%), 93 (37.2%) and 92 (36.8%) samples were determined to be positive by microscopy, RDT and PCR, respectively. RDT had a sensitivity of 59.3% and specificity of 73.4% when microscopy was taken as the gold standard. Sensitivity and specificity of RDT were 55.4% and 73.4%, respectively, when PCR was taken as the gold standard.

P. falciparum was isolated as the only agent in 94.5% of the positive samples detected by nested PCR. It was isolated concurrently with *P. vivax* and *P. malariae* in two (2.2%) and one (1.1%) samples, respectively. Apart from mixed infection with *P. falciparum*, *P. vivax* was also detected as the only agent in two (2.2%) samples. In this study, *P. ovale* and *P. knowlesi* were not isolated.

In this study, DNA sequencing was performed in order to examine the mutations in *Pfdhfr* and *Pfdhps* genes that confer resistance to SP. In 10 of 20 samples sequenced for *Pfdhfr* gene, C59R mutation was detected. In the remaining 10 samples, no mutation was detected in *Pfdhfr* gene. Likewise, no mutation was detected in the other 10 samples sequenced for *Pfdhps* gene.

The results of our study indicated that, malaria infection was detected in a considerable amount of pregnant women. The presence of infection suggested that the SP prophylaxis was not effective against malaria in these women. Since the participants received two doses of SP in the 16^{th} and 28^{th} weeks of pregnancy, the findings obtained from our study suggested that the number of doses could be increased according to new guidelines documented by WHO.

Further studies on the efficiency of IPTp-SP should be conducted in the regions where malaria is an important health problem. Investigation of SP resistance in these regions will enable the implementation of accurate treatment and encourage the studies where alternative drugs are tested. This will contribute to the prevention

of malaria in pregnancy, and enable protection of the mother, the fetus and the newborn.

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