SOKOINE UNIVERSITY OF AGRICULTURE

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DISSERTATION SUBMITTED IN PARTIAL FULLFILLEMENT OF THE REQUIERMENTS FOR THE AWARDS OF THE DEGREE OF MASTER IN SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE MOROGORO, TANZANIA

PREVALENCE OF *PLASMODIUM* INFECTION AND ACCURACY OF DIAGNOSTIC TESTS FOR MALARIA INFECTION IN CHILDREN UNDER FIVE IN THE HEALTH ZONE OF MONT NGAFULA1, AN ENDEMIC AREA FOR MALARIA IN KINSHASA, DRC

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ABSTRACT

Democratic Republic of Congo (DRC) is one of the five countries that carry half of the global disease burden. Yet, malaria is an entirely preventable and treatable disease, when currently recommended interventions are properly implemented. Such interventions include confirmation of malaria diagnosis through microscopy or malaria rapid diagnostic tests (MRDTs) for every suspected case, even in children under five years of age.

This study aimed to assess the prevalence of malaria infection and the performances of MRDT, the SD-Bioline a HRP2/PanLDH test using microscopy and PCR as the gold standard in a population based survey in children under five years of age living in endemic transmission settings.

This is a cross sectional based survey conducted in the health areas in the health zone of Mont Ngafula1 during the dry season from April to August 2012. A total of 812 children of 3 to 59 months of age were included from the 2 selected HA.

The sensitivity, specificity, positive and negative predictive values with their CI 95% were 93.5% (90.0-97.1), 81.1% (77.9-84.2), 60.6% (55.0-66.3) and 97.5% (96.2-98.9), respectively, in the overall study population when using microscopy as the gold standard and 88%.2 (79.2-97.3), 92.0% (84.3-99.7), 88.4% (79.6-97.3) and 91.8% (84.0-99.7) respectively, when PCR was used as the reference test . The prevalence of malaria with microscopy was 24.9% (CI 95%: 21.0-26.7).

The differences between PCR and microscopy with the specificity or and the PPV in the overall population might be due to the threshold detection of microscopy that does not detect very low parasite density. The results of this study show the limitation of the MRDT SD-Bioline, a HRP2/PanLDH test, on population based survey because of the risk of an overestimation of the infection prevalence in children aged less than five years.

DECLARATION

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

16th October 2014 .

Vivi MAKETA Tevuzula Date

(Msc **OHMB candidate**)

The above declaration is confirmed by following supervisors;

…… …. .....

Prof Paul Gwakisa PhD. Date
(Sokoine University of Agriculture)

16th October 2014

Pr Dr Pascal Lutumba PhD. Date
(University of Kinshasa)

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DEDICATION

To all mines: my friends, my family, my beloved husband (for his unconditional love and support) and especially to Eustache and Elliot (for the happiness they brought to my life)

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LIST OF ABBREVIATIONS

ACT	Artemisinin based combination therapy
CI95%	Confidence Interval at 95%
DNA	Deoxyribo Nucleic Acid
DRC	Democratic Republic of Congo
HPF	High Power Field
HRP2	Histidine Rich Protein 2
Ig	Immunoglobulin
IQI	Inter quartile Interval
ITN	Insecticide Treated Net
MRDT	Malaria Rapid Diagnostic Test
NPV	Negative Predictive Value
OR	Odd Ratio
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
Pk	*Plasmodium knowlesi*

PLDH	Plasmodium Lactate Dehydrogenase
Pm	*Plasmodium malariae*
PNLP	“Programme National de Lutte contre le Paludisme »
Po	*Plasmodium ovale*
PPV	Positive Predictive Value
Pv	*Plasmodium vivax*
WHO	World Health Organization

Chapter I

1. Introduction

1.1. Background

Malaria is a major public health problem in the world. According to World Health Organization (WHO) the disease affects half of the world's population and there were an estimated 216 million episodes of malaria in 2010, of which approximately 81%, or 174 million cases were in the African region. The estimated number of deaths due to malaria was 655 000 in 2010, of which 91% were still in Africa (WHO, 2010).

Democratic Republic of Congo (DRC) is one of the five countries that carry half of the global disease burden. According to the National Malaria control program, the "Programme National de Lutte contre la Malaria" (PNLP) 100% of the DRC population is exposed to the disease and 97% among them live in stable area. Malaria is the main cause of morbidity and is responsible for 83% of the direct mortality rate in the country (Rapport direction de lute contre la maladie 2009). The real contribution may even be higher due to the indirect mortality rate through malnutrition, anemia and other infections. In addition to the losses of human lives, a severe episode of malaria costs around 35 US$ and hence, malaria is an aggravation factor for poverty in DRC (Greenwood *et al*. 2005). Yet, malaria is an entirely preventable and treatable disease, when currently recommended interventions are properly implemented. Such interventions include confirmation of malaria diagnosis through microscopy or malaria rapid diagnostic tests (MRDT) for every suspected case, even in children under five years of age (WHO 2010, DÁcremont *et al.* 2009, Bisoffi *et al* 2008, English *et al*. 2009, Gerstl *et al*. 2010, Bisoffi *et al*. 2009).

Unfortunately, in an endemic country malaria diagnosis is often based on clinical symptoms that may yield high sensitivity but extremely low specificity (WHO 2009). Although microscopy remains the gold standard, optimal conditions for microscopy are lacking in remote rural areas. MRDTs are a good alternative as they allow rapid diagnosis and therefore a fast case management of malaria (Ashley *et al.* 2009, Abeku *et al*. 2008, Counihan *et al*. 2007, Muhindo *et al*. 2012). With MRDT, malaria infection is revealed, mainly through the detection of either Histidine-Rich Protein II (HRP2) or Plasmodium Lactate Dehydrogenase (PLDH). HRP2 is a protein specific to *Plasmodium falciparum* (*Pf*) expressed in the membrane of infected erythrocytes (Dondorp *et al*. 2005, Rock *et al*. 1987) while PLDH is an intracellular metabolic enzyme produced by all the *Plasmodium* species that infect humans (Mackler et al 1998, Fogg *et al*. 2008, Iqbal *et al*. 2002, Iqbal *et al*. 2004).

Yet, limited information is available about accuracy and predictive value of MRDT in population based surveys of malaria prevalence, where people are more likely to have lower parasitemia than in clinical settings (Endeshaw *et al*. 2008, Coleman *et al*. 2002).

1.2. Justification

Several studies, mostly in endemic areas, have assessed the accuracy of MRDT in clinical settings and the disease was assessed as the presence of *Plasmodium* infection using microscopy as the gold standard (Abeku *et al*. 2008, Muhindo *et al*. 2012, Endeshaw *et al*. 2008, Swarthout *et al*. 2007).

This study aimed to assess the prevalence of malaria infection and the performances of MRDT using microscopy and PCR as the gold standard in a population based survey in children under five years of age living in endemic transmission settings. This could result in a better estimation of the concordance of malaria diagnosis when MRDT are used in endemic settings, especially in a vulnerable stratum of the population. The MRDT used in the study was the one selected by the PNLP, the test based on *Pf*HRP2/PanPLDH from SD-bioline.

1.3. Objectives

1.3.1. *Overall objective*

- To comparatively assess the prevalence of malaria infection in a population-based survey in children under five years of age living in endemic transmission settings using microscopy, MRDT and PCR.
- To assess the performance of MRDT using microscopy and PCR as the gold standard

1.3.2. *Specifics Objectives*

- To determine the number of positive results for malaria infection based on microscopy in samples collected from children under five years of age living in endemic transmission settings.
- To compare the MRDT results with microscopy results in samples collected from children under five years of age living in endemic transmission settings.
- To compare the concordance of the positive MRDT with microscopy results using PCR as an internal control.

1.4. Research question

What is the prevalence of Plasmodium infection and the performance of MRDT in samples collected from children under five years of age living in endemic transmission settings when microscopy and PCR are used as the gold standard?

Chapter II

2. Literature review

2.1. History of malaria

Malaria or a disease resembling malaria has been present for more than 4,000 years. From the Italian expression for "bad air," mal'aria has influenced to a great extent human populations and human history.

Symptoms of malaria were already described in ancient Chinese medical writings. Several characteristic symptoms of what would later be named malaria were described in the Nei Ching, The Canon of Medicine) in 2700 BC, edited by the Emperor Huang Ti. Malaria became widely recognized in Greece by the 4th century BCE, and it was responsible for the decline of many of the city-state populations. Hippocrates described the principal symptoms. By the age of Pericles, there were extensive references to malaria in the literature and depopulation of rural areas was recorded. In the Susruta, a Sanskrit medical treatise, the symptoms of malarial fever were described and attributed to the bites of certain insects while a number of Roman writers attributed malarial diseases to the swamps. In China, during the second century BCE, the Qinghao plant (Artemisia annua) was described in the medical treatise (Fong *et al*. 1971). Nowadays, this plant is known as the annual or sweet wormwood. The Ge Hong of the East Yin Dynasty was the first to describe its anti-fever properties. The active ingredient of Qinghao, known as artemisinin was not isolated until 1971 by Chinese scientists. Derivatives of this extract, known collectively as artemisinins, are very potent today and effective antimalarial drugs, especially in combination with other medicines.

The protozoan parasite was only discovered in 1880 by Charles Louis Alphonse Laveran. While he was working in the military hospital in Constantine, Algeria, he observed the parasites in a blood smear taken from a patient who had just died of malaria (Bruce-Chwatt *et al.* 1981).

2.2. Malaria etiology

Malaria is a mosquito-borne infectious disease caused by eukaryotic protists of the genus *Plasmodium*. The disease results from the multiplication of malaria parasites within red blood cells, causing symptoms that typically include fever and headache, in severe cases progressing to coma, and death. Five species of *Plasmodium* can infect and be transmitted to humans. Severe disease is largely caused by *Plasmodium falciparum*. Malaria caused by *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* is generally a milder disease that is rarely fatal. A fifth species, *Plasmodium knowlesi*, is a zoonosis that causes malaria in macaques but can also infect humans (Fong *et al*. 1971, Bruce-Chwatt *et al.* 1981).

2.3. Epidemiology of malaria

Malaria causes an estimated more than 200 million cases of fever and approximately one million deaths annually (WHO 2010). The most vulnerable populations are pregnant women and children under five years of age in which the vast majority of cases occur. Despite tremendous efforts to reduce transmission and increase treatment, little has changed in areas at risk of this disease since 992 (WHO 2010, Greenwood *et al.* 2005). Indeed, if the prevalence of malaria stays on its present upwards course, death rates could double in the next twenty years (Hay *et al*. 2004). Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care (Breman *et al.* 2001). As a consequence, the majority of cases are not documented. Malaria is presently endemic in a broad

band around the equator, in areas of the Americas, many parts of Asia, and much of Africa; however, it is in sub-Saharan Africa where 85– 90% of malaria fatalities occur (Layne 2006).

2.4. Malaria diagnostic tests

Malaria treatment needs a fast and accurate diagnostic prior to treatment (WHO 2010, WHO 2006). This is essentially due to the widespread resistance against antimalarial drugs and the high cost of artemisinin based combination treatment (ACT). In the field two diagnostic methods namely microscopy and MRDT are used. Although PCR is not carried out in routine it is also a reference test for the diagnosis of the disease.

2.4.1. Microscopy (thin and thick blood smears)

The detection of parasite is done through a thick blood smear and malaria parasite species differentiation is done by a thin blood smear. The thick smear is micro-concentration method that aims to destroy the erythrocytes in order to increase the chance to detect the presence of the parasite. This method is considered as the gold standard for biologic diagnosis of malaria. However, its realization requires a microscope, good staining of the slide and a reading expertise.

2.4.2. Malaria rapid diagnostic tests

MRDTs are designed to detect malaria infection. They are immuno-chromatographic test based on the parasite antigen detection in the blood. They are constituted of a nitrocellulose membrane that holds monoclonal antibodies directed against parasite antigen. Ig (Immunoglobuline) M antibodies are the most used to avoid cross reaction.

To ease the erythrocyte membrane destruction as well as the sample migration trough the nitrocellulose membrane, few drops of a buffer/lyse solution are added while using the test. In case of a positive result, the antigen/antibody complex will migrate through the membrane and a violet line will appear.

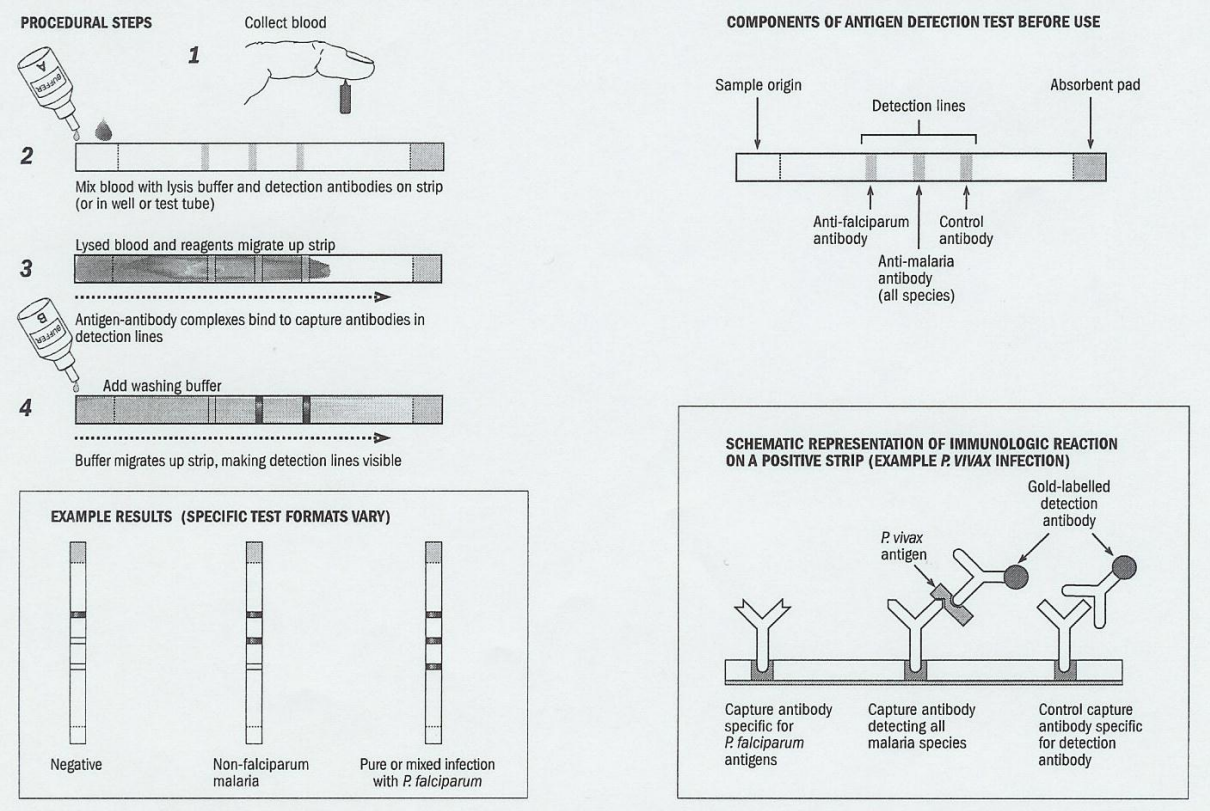

Figure 1: Procedural steps of MRDT:

Main groups of MRDT can detect three principal targets:

- *Plasmodium* Lactate Dehydrogenase (PLDH): an intracellular metabolic enzyme produced by all the *Plasmodium* species that infect humans. It is found in the glycolytic

pathway of the malaria parasite, and produced by sexual and asexual stages. PLDH is only produced by viable parasites and hence is cleared more quickly from the bloodstream than HRP2 (Makler *et al.* 1998, Fogg *et al.* 2008, Iqbal *et al.* 2002, Iqbal *et al*. 2004).

- Histidin Rich Protein 2 (HRP2): is a water-soluble protein produced by asexual stages and young gametocytes of *P.f.* It is expressed in the erythrocyte membrane and is slowly eliminated from the blood stream (Dondorp *et al.* 2005, Rock *et al.* 1987). HRP2 is the antigen used by the MRDT SD-Bioline.

In general, MRDT detecting HRP2 are most commonly used, because they are less expensive, more stable across a wider temperature range and have a lower detection threshold than pLDH-based tests (Chiodini *et al*. 2007, WHO 2008, WHO 2011). HRP2-based tests, however, detect only *P. falciparum*, and antigenic variation of this antigen may cause false negative results (Lee *et al.* 2006).

- Aldolases (« panmalarial antigens »): common antigens to all *Plasmodium* species. In the field, they are often combined with HRP2 based test to differentiate species.

2.4.3. Polymerase Chain reaction

PCR is considered to have the most sensitive detection level of parasites but requires highly trained staff and specialized equipment, which are not always available in resource-poor settings (Uneke *et al.* 2007, Uneke *et al.* 2008, Omo-Aghoja *et al.* 2008).

In limited resource countries, where malaria has the highest burden, most conditions for microscopy are not always found, leading authors to agree that MRDT may be a good alternative in those areas (Rafael *et al.* 2006, Murray *et al.* 2008, Shillcutt *et al.* 2008, Anash *et al.* 2010, WHO 2011). In the field, the choice of test should take into account malaria transmission dynamics (Rosenthal 2012). However, (i) performance, (ii) operational characteristics and (iii) the cost have to be considered (Mayxay *et al.* 2001, Moody *et al.* 2000).

Chapter III

3. *Material and Methods*

3.1. Study area and duration

Malaria is endemic for more than 90% in DR Congo, the level of transmission is high and perennial in the country. Kinshasa, DR Congo's capital has a surface of 13 195 Km² and more than 8 million of inhabitants (Rapport de l'Inspection Provinciale de Kinshasa 2009). The climate is tropical with two seasons, a rainy season for seven months extended from the middle of September till the middle of May and a dry season from June to August.

Entomological Inoculation rate reaches 1200 infectious bites/person/year in some peripheral areas in Kinshasa (Coene 1993). *Plasmodium falciparum* is responsible for 97% malarial cases and the plasmodic indice mean is 17% (Kazadi *et al.* 2004). Lethality due to malaria is estimated at 18% in children below 5 years in Kinshasa (Rapport de l'Inspection Provinciale de Kinshasa 2009).

According to the health system, Kinshasa is divided in 6 health districts (Nsele, Ndjili, Kalamu, Funa, Lukunga, Gombe). Each district is further divided into health zones which are the

operational level of the health system in DR Congo but they are further divided in health areas. The total number of health zones in Kinshasa is 35 and the number of health areas is 384 (Rapport de l'Inspection Provinciale de Kinshasa 2009). For pragmatic reasons (accessibility, time, money) the study was conducted in Lukunga district in Mont Ngafula1. Details about Lukunga are given in the Table 1.

3.2. Study Design and Sampling Procedure

This is a cross sectional study conducted in the health zone of Mont Ngafula 1, which is divided into 16 health areas. Samples were drawn from children under 5 years of age in 2 randomly selected health areas: Kindele and Cite Pumbu. A population based survey was performed in the household, a blood smear and MRDT test were performed and a blood sample was collected on a filter paper for molecular analysis. Prior to the collection, the children's parents or legal tutors had to give their informed consent and filled in an epidemiological form.

Table 1 : Details on Lukunga district

Lukunga district			
Health zones	**Surface (km^2)**	**Number of Health areas**	**Population**
BANDALUNGUA	7	7	147,288
BINZA METEO	36	11	325,445
BINZA OZONE	46	10	303,325
KINTAMBO	3,9	8	84,832
KOKOLO	0	15	217,269
MT NGAFULA I	202	16	200,030
MT NGAFULA II	153	14	117,183
SELEMBAO	23	18	282,164
S/Total district	**378**	**99**	**1, 677,539**

3.3. Study population

The study population was children under five years of age living in the health zone of Mont Ngafula1, an endemic area for malaria.

3.3.1. *Inclusion criteria*

In order to be eligible, patients had to satisfy the following inclusion criteria:

1. Males and females aged between three months and 6 sixty months inclusive.
2. Signed (or thumb-printed and witnessed by an impartial witness, whenever parents/guardians are illiterate) informed consent by the parents or guardians.

3.3.2. *Exclusion criteria*

Patients with at least one of the following criteria were excluded:

3. Presence of undercurrent illness or any condition (cardiac, renal, hepatic diseases) which in the judgement of the investigator would place the subject at undue risk or interfere with the results of the study.

3.4. Sample size

Assuming a prevalence of 50% and 80% of power, with a precision of 5%, the minimal required sample size was 384. Considering a possible cluster effect due to the heterogeneity of malaria endemicity around the city of Kinshasa, the sample size has been doubled (minimal sample size = 768).

3.5. Concept definitions of the study

Prevalence rate: is the proportion of the individuals who have a *Plasmodium* infection (*Pf, Pm, P.o, P.v, P.k*) in the study population.

Sensitivity: is the proportion of individuals having positive MRDT results among those having positive microscopy or PCR results.

Specificity: is the proportion of individuals having negative MRDT results among those having negative microscopy or PCR results.

Positive predictive value (PPV): is estimated as the probability to have the infection (microscopy or PCR positive) when the MRDT is positive.

Negative predictive value (NPP): is estimated as the probability to not have the infection (microscopy or PCR negative) when the MRDT is negative.

3.6. Sample collection and laboratories analysis

3.6.1. *Detection of Plasmodium falciparum*

Blood for thick/ thin smears and MRDT were collected from the same finger prick. Blood smears were prepared on the same slide bearing the patient's identification code. Slides were horizontally air-dried in a slide tray and stored in boxes. At the end of the day, blood slides were stained with 10% Giemsa but the thin smears part was previously fixed with methanol. Blood slides were read by senior laboratory technicians with over fifteen years of work/research experience at the Kinshasa University Parasitology laboratory. The slides were classified as either negative, *Pf*-positive, *P malariae*-positive, or mixed infection. The parasitic density was also calculated. One hundred high power fields of the thick film were examined at a 1000x magnification before identifying a slide as negative or positive. When positive, the thin film was read to determine the species.

Parasite density was calculated by counting the number of asexual parasites per 200 leukocytes in the thick blood film, based on an assumed WBC of 8,000 /µl by light microscopy at 1000 X magnification (100X objective and 10X eyepiece). One hundred high-powered fields (HPF) were examined (independent of presence or absence of asexual parasite stages). The parasite density (PD) per microlitre was calculated using the following formula:

$$\text{Parasite density / µl} = \frac{\text{Number of parasites counted x 8,000}}{\text{Number of leukocytes counted}}$$

3.6.2. *MRDT*

Blood samples collected from the children were tested for malaria parasites using the MRDT SD-Bioline. The test uses approximately 5 µl of blood and is readable after 15 minutes according to the manufacturer's instructions. The tests were classified negative, positive for *Pf* , positive for other species than *Pf* or positive with a mixed infection (*Pf* + other species).

3.6.3. *Molecular analysis*

-

A. Method

    a. DNA extraction

The first step was to make a fresh 2% Chelex solution by mixing 2.5 ml of distilled water with 0.05 g of Chelex (20 %) and a 1X PBS with 0.1% Saponin weight/volume by taking 50 ml of 1X PBS and added 0.05 g Saponin. Then, the puncher was cleaned by dipping it in a 70% Ethanol and passed it through a flame.

The 1.5 ml microcentrifuge tubes were labeled with the appropriate number according to the worksheet number and sample ID. For each filter paper sample a three mm disk (holds approx. 3-5 µl of dried blood) was cut and put into the corresponding 1.5 microcentrifuge tube. Then 1µl of the Saponine buffer solution was added in the tube ensuring that filter papers were totally soaked. The 1.5 microcentrifuge tubes were then left for 10 minutes incubation at room temperature.

After the incubation, the 1.5 microcentrifuge tubes were centrifuged at 14,000 rpm for 2 min and the supernatant was discarded using a clean pipette tip for each sample.

Then, in each microcentrifuge tubes were added 150 µl of 2% Chelex solution and 50 µl of distilled water. After 10 minutes incubation at 99 ºC at Waterbath, tubes were centrifuged at 14,000 rpm for 1 min and store at 4 ºC for use in the PCR

N.B: The PCR was always performed the following day.

b. Polymerase Chain Reaction mix

Each 20µl of PCR mix per sample contained 2 µl of Go Taq flexi buffer 10X, 0,5 µl of dNTP mix (5mM each), 1,6 µl of MgCl2 (25 mM), 0,5 µl of Primer rPLU5 (10µM), 0,5 µl of Primer rPLU6 (10µM), 0,08 µl of GoTaq flexi DNA polymerase, 13,82 µl of H 20 Molecular Biology grade and 1 µl of DNA.

Primers sequences were as follows:

rPLU5 : CTTGTTGTTGCCTTAAACTTC

rPLU6 : TTAAAATTGTTGCAGTTAAAACG

c. PCR program

The PCR program was set as follows:

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

d. Gel electrophoresis

The PCR product that was considered as positive was a 1200bp.

3.7. Data analysis

Data were double-entered and validated in Epi info version 3.5.1 software and analysed using Stata version 11 (Stata Corp, Lakeway, College Station, Texas, USA). For the different statistic tests (Chi2, linear regression) the level of significance was set at 5%.

3.8. Ethical consideration

3.8.1. Informed consent

All interviews were conducted in the native language of the patients by the study personnel. Consent forms in the local language were provided to the parents or legal tutors for their review. The parents or legal tutors were asked to sign (or thumb-print whenever the parents/guardians are illiterate) consent to participate in a research study. The informed consent described the purpose

of the study, the procedures to be followed, and the risks and benefits of participation. If a parent or guardian was unable to read or write an impartial witness took part in the informed consent discussion and signed the consent form. Parents or legal tutors were informed that participation in the study is completely voluntary and that they may withdraw their child from the study at any time without any negative consequences.

The Investigator agrees to conduct the present study in full agreement with the principles of the "Declaration of Helsinki".

All research activities were run in accordance with the standards and codes of conduct accepted by the International Conference on Harmonisation guidelines.

Prior to the beginning of the study we had the ethical clearance of the Public Health school of Kinshasa. All interviews were conducted in the native language of the patients by the study staff. Consent forms written in the local language (Lingala) were provided to the legal tutor of the children. The legal tutors were asked to sign (or thumb-print in front of a witness whenever he/she was illiterate) the consent form to let his/her child to participate in the study. All the children found with clinical sign of malaria and/or with a positive MRDT were supplied with antimalarial drugs according to the DRC national guidelines.

Chapter IV

4. *Results*

From April to August 2012, a total of 812 children were included in the study among whom, 376 (46.3%) were females. The median age was 42 months with an inter quartile interval (IQI) of 26. The minimum and the maximum age of the children were respectively 2 and 60 months.

According to the 812 slides read by microscopy in this study, the prevalence of *Pf* and of *Pm* was 21.7% (176) and 5.6% (45), respectively. The prevalence of the *Pf-Pm co-infection* was 3.6% (29) as shown in Table 3. Solely, *Pf* gametocytes were detected in 11 (1.4%) slides by microscopy but this was considered as a negative outcome of microscopy. However, within the *Pf* gametocyte slides MRDT results showed 6 (54.5%) mixed infections, 4 (36.4%) *Pf* infections and only 1(9.1%) carried negative result.

During the visit, 58 (7.1%) children had fever (36 boys and 22 girls) ranging from 37.6°C to 39.3°C. Among them 19 (32.8 %) had a *Pf* infection and 1 (1.7%) had a *Pm* infection. None of the children took anti-malarial drugs two2 weeks prior to the study and even the one with *Pm* infection only took antipyretics. Furthermore, among children having fever, only 14 (24.1%) slept under a bed net the day previous to our visit and 44 (74.9) did not. Still there was a borderline statistically significant difference with a P value of exactly 0.50.

Only 313 (38.6%) children were living in a household owning a bed net. Among the 309 parents or legal tutors who were able to tell the provenance of the nets, 230 (74.4%) said the nets came from mass distribution of ITNs and only 79 (25.6) bought them.

The bivariate analysis showed that categories of children who were protected from the presence of *Pf* infection detected by microscopy were: the children under one year compared to the older one, children who were living in Kindele compared to those living in Cite Pumbu, children who slept under ITN the night previous data collection compared to those who did not own or sleep under a ITN and children who had no fever compared to those who had it. Still after modeling considering only variables that were significantly different, the absence of fever was removed as a protector factor (Table 2). When taking the age in consideration, we spared children under one year in two subclasses. There were 12 children under 6 months and 38 being more than 6 months. Analysis showed that children under 6 months were the ones really protected from the presence of *Pf* with no children of that subclass carrying *Pf* in the blood an OR of 3.0 significantly different between the two subclasses. Within children under 6 months, only 4 (33.3%) slept under a bed net the day prior to our visit.

Table2. Basic information of the study population with their odd ratio (OR) based on the presence of *Pf* detected by microscopy

Variable		**Presence of Pf**	**OR**	**P value**	**Adjusted OR***	**Adjusted P value***
	n (%)	n (%)				
Sex						
Female	*376 (46.3)*	77 (20.5)	1			
Male	*436 (53.7)*	99 (22.7)	1.1	0.44		
Age						
3 -11.9 Months	*50 (6.2)*	4 (8.0)	1		1	
12-23.9 Months	*94 (11.6)*	20 (21.3)	3.1	**0.05**	3.6	**0.03**
24-35.9 Months	*175 (21.5)*	42 (24.0)	3.6	**0.02**	5.1	**0.004**
36-47.9 Months	*166 (20.4)*	33 (19.9)	2.9	**0.06**	3.7	**0.02**
48-60 Months	*327 (40.3)*	77 (23.6)	3.5	**0.02**	4.8	**0.004**
Health areas						
Cite Pumbu	*408 (50.2)*	121 (29.7)	2.7	**<0.001**	3.2	**<0.001**
Kindele	*404 (49.8)*	55 (13.7)	1			
Individuals per household						
<=5	*222 (27.4)*	44 (19.8)	1.5	0.10		
6-10	*451 (55.5)*	94 (20.8)	1.1	0.76		
>11	*139 (17.1)*	38 (27.3)	1			
Owned a Net						
No	*499 (61.5)*	118 (23.7)	2.0	**0.003**	2.1	**0.002**
Yes and slept under	*206 (25.4)*	28 (13.6)	1		1	
Yes but did not slept under	*107 (13.1)*	30 (28.0)	2.5	**0.002**	3.2	**<0.001**
Presence of fever						
Yes	*58 (7.1)*	19 (32.7)	1.9	**0.04**	1.4	0.25
No	*754 (92.9)*	157 (20.8)	1		1	

*: adjusted OR and P-value were only calculated in a final model that took variable that significant OR in the bivariate analysis

Table 3: Prevalence of *Plasmodium* infection detected by microscopy categorized by age and health areas.

Prevalence of malaria	**3mois-1an**	**1-2ans**	**2-3ans**	**3-4ans**	**4-5ans**	**Total**
Overall n=812	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**% (CI95%)**
Pf n=176	4 (8.0)	20 (21.3)	42 (24.0)	33 (19.9)	77 (23.6)	21.7 (18.8-24.5)
Pm n=45	1 (2.0)	7 (7.5)	9 (5.1)	12 (7.2)	16 (4.9)	5.6 (3.9-7.1)
Pf+*Pm* n=29	0 (0.0)	4 (4.3)	6(3.4)	6 (3.6)	13 (4.0)	3.6 (2.2-4.9)
Cite Pumbu n=408	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**% (CI95%)**
Pf n=121	4 (9.8)	13 (22.4)	22 (27.9)	23 (30.3)	59 (38.3)	29.7 (25.2-34.1)
Pm n=38	1 (2.4)	6 (10.3)	8 (10.1)	9 (11.8)	14 (9.1)	9.3 (8.7-9.4)
Pf+*Pm* n=26	0 (0.0)	3 (5.2)	5 (6.3)	6 (7.9)	12 (7.8)	6.4 (4.0-8.6)
Kindele n=404	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**% (CI95%)**
Pf n=55	0 (0.0)	7 (19.4)	20 (30.8)	10 (11.1)	18 (10.4)	13.6 (10.3-17.0)
Pm n=7	0 (0.0)	1 (2.8)	1 (1.0)	3 (3.3)	2 (1.1)	1.7 (0.5-3.0)
Pf+*Pm* n=3	0 (0.0)	1 (2.8)	1 (1.0)	0 (0.0)	1 (0.6)	0.7 (0.0-1.5)

Table4. Performance of MRDT using microscopy or PCR as gold standard in the 2 HA

	Sensitivity		**Specificity**		**PPV**		**NPV**	
	%	95%CI	%	95%CI	%	95%CI	%	95%CI
Overall population								
Microscopy	93.5	90.0-97.1	81.1	77.9-84.2	60.6	55.0-66.3	97.5	96.2-98.9
PCR*	88.2	79.2-97.3	92.0	84.3-99.7	88.4	79.6-97.3	91.8	84.0-99.7
Cite Pumbu								
Microscopy	94.0	89.9-98.0	80.1	75.3-85.0	70.2	63.5-77.0	96.4	93.9-98.9
PCR*	87.5	73.7-101.3	85.7	72.2-99.2	84.0	69.0-99.0	88.9	76.5-101.3
Kindele								
Microscopy	92.5	85.3-99.7	81.8	77.6-86.0	45	35.5-54.4	98.5	97.1-100
PCR*	88.9	79.5-101.2	100	---	100	---	88.0	74.7-101.3

*: Only 101 randomly selected samples has been performed for the PCR analysis.

Table 5: Sensitivity of MRDT over the different levels of parasitemia

Microscopy ranges		**Sensitivity**	
	N	%	CI 95%
<1000 (n=34)	30	88.2	77.1-99.3
1000-2000 (n=44)	44	100	--
>2000 (n=91)	86	94.5	89.8-99.2

Chapter V

5. *Discussions*

The overall prevalence of *Pf* infection found in the health zone of Mont Ngafula I was 21.7% with specific prevalences of 29.7 (CI95%: 25.2-34.1) and 13.6 (CI95%:10.3-17.0), respectively in Cite Pumbu and Kindele and an odd ratio of 2.7 between the two health areas. This might lead to reconsider the malaria classification of the health zone Mont Ngafula I that has always been considered as a hyper-endemic zone for malaria by the PNLP. In addition, the prevalence of *Pm* infection reached 9% in the health area of Cite Pumbu in a zone that has been established to be a zone restricted to *Pf* infections. This shows that extended prevalence studies to update the classification of the health zones/health areas and to update the epidemiology of *Plasmodium* species will be useful to map the health zone of Mont Ngafula I. In extension the update might also be needful to all health zones of Kinshasa because those "low" prevalence found may be a consequence of some health interventions like mass distribution of ITNs to the households that occurred in 2008 in Kinshasa. Therefore more extended studies are needed to be conducted in Kinshasa and in the other provinces of DRC. Moreover, they should be repeated over time to monitor the impact of the interventions that have been/are currently performed in the field such as mass distribution of ITNs or systematic intermittent preventive treatment at specific interval for the pregnant woman during antenatal care. Still, we take into account that the classification done by the PNLP was an overview of all the overall population while our study was only conducted within the stratum of children under the age of 5.

There was a statistical difference between children under one year old age class and the other age groups with OR ranking from 2.9 to 3.6. These results show that children under the age of one are less susceptible to malaria than the older children. One explanation can be due to the fact that

they may be overprotected (possibly with ITNs or other locally improvised methods) and therefore less exposed to mosquito bites. But a further look shows that in reality the subclass of children under 6 months was the "subclass" that was more protected than the others. But the affirmation of an overprotection by ITN was extravagant as only 33% of them were living in a household owing a net and slept under it the day previous to our visit. Thus, the argument given by the protection of children under the age of 6 months by IgG maternal antibodies and the presence of Fetal hemoglobin which confer them a protection against *Pf* parasite might explain that protection (Amaratunga *et al.* 2011, Billig *et al*. 2012).

Table 2 shows that living in a household that owned a bed net was not enough to be protected against *Pf* infection. The real protection from *Pf* infection was encountered in children who slept under a bed net the previous day. Furthermore, the use of bed net was also associated with the protection against fever. Therefore, the authorities should emphasize the mass distribution of ITNs but also encourage the population / society, especially in the strata of children under the age of 5, that use of bed nets alleviates risk of symptomatic and asymptomatic *Pf* infection.

In the overall population and in both health areas of the study, there was no statistically significant difference of sensitivity and NPV of MRDTs taking either PCR or microscopy as the reference test despite studies with PCR having shown that HPR-based MRDT sensitivity for malaria infection may be higher than that of standard microscopy (Nicastri *et al.* 2009, Stauffer *et al.* 2009). However, the change of reference technique lead to a statistical difference in either specificity or PPV in the overall population and in the Kindele but not in Cite Pumbu. This can be explained by the difference of prevalence in the two health areas but this latter explanation would have affected specificity, sensitivity and both predictive values. Therefore, the absence of false positive in Cite Pumbu when PCR was used as the gold standard is the most appealing

reason. The differences between PCR and microscopy with the specificity or/and the PPV in the overall population might be due to the threshold detection of microscopy that does not detect very low parasite density at the difference of PCR. However, PCR analysis was only performed on 101 samples limiting the study power.

When using solely microscopy as the gold standard, the SD-Bioline a HRP2/PanLDH test, has a good sensitivity (93.5%, CI95%:90.0-97.1) and NPV (97.5%, CI95%:96.2-98.9) although sensitivity was below the threshold of 95% required by the WHO (9). On the other hand, the specificity (81.1%, CI95%:77.9-84.2) and PPV (60.6%, CI95%:55.0-66.3) were low. Also, the results show that sensitivity ranges widely, varying according to the parasite density found in children and reach an optimal point between 1000 and 2000 parasite/µl. Under 1000 parasites/µl and over 2000P/µl the sensitivity was less than the 95%.

At low parasite density, false negative results might be relatively harmless as shown in a study in Uganda which showed that missed treatment for patients with a false negative malaria microscopy did not result in severe disease (Njama-Meya *et al.* 2007). However, at high parasite density (more than 2000 parasites/µl) the MRDT sensitivity was only 94.5%. This worrisome result has been described by others for HRP-based tests (Drakeley *et al.* 2009), and might be explained by the pro-zone effect but also batch-to-batch variation or failure to maintain the cold chain. (WHO 2011, Risch *et al.* 1999, Gillet *et al.* 2009).

The results of this study show the limitation of the MRDT SD-Bioline, a HRP2/PanLDH test, on population based survey because of the risk of an overestimation of the infection prevalence in children having aged less than five years. Indeed, a specificity of 81% means that almost one out of five people (19%) are falsely positive. The probability to have a real *Plasmodium* infection when the test is positive is only 60% (PPV). This leads the SD-Bioline a HRP2/PanLDH test

being limited as a tool for endemicity assessment in children aged less than five years in population based survey. Maybe these results are related to the choice of the test as HRP2 antigens can circulate longer than a month in the bloodstream even after a successful treatment and the presence of gametocytes could render the test positive (Swarthout *et al.* 2007, Mueller *et al.* 2007). However data collected from the parents/legal tutors of the children did not mention an antimalarial treatment two weeks prior to the visit (although the question was explicitly asked). Likewise, the 11 slides detected by microscopy as carrying solely gametocytes that represent only 1.4% of the slides are too few for a significant implication.

Only 14% of children having parasitemia above 2000 parasite/µl had fever. This means that 86% of children having more than 2000 parasite/µl had no fever despite the high parasitemia found. Therefore, the defective performance in specificity of microscopy using clinical malaria (the presence of fever) as gold standard is high. This is a reality in endemic countries where up to 50% of children are carriers of malaria parasites (Ogutu *et al.* 2010, Baliraine *et al.* 2009). These results tend to show that asymptomatic carriers can bear high parasitemia without symptom. Still, asymptomatic *Plasmodium* infection (*Pf* in particular) is not a harmless condition. As epidemiological evidence shows that asymptomatic malaria infections have an indirect impact on the children health, we cannot consider asymptomatic carriers as healthy individuals (Greenwood *et al.* 2005, Stauffer *et al.* 2009, Bisoffi *et al.* 2012). But on the other hand, we have also to pay attention to the consequence of over-diagnosis that is also harmful for children (Akpede *et al.* 1993, English *et al.* 1996, Molyneux *et al*. 1998, Berkley *et al.* 1999, English *et al*. 2003, Evans *et al.* 2004, Kallander *et al.* 2004, Berkley *et al.* 2005a, Berkley *et al.* 2005b, Jakka *et al.* 2006) as it leads to death of children from other pathologies such as invasive bacterial infection or other etiologies (Barat *et al.* 1999, Berkley *et al.* 2005a, Reyburn *et al.* 2004, Reyburn *et al* 2006,

Makani *et al*. 2003, Zurovac *et al.* 2006). Furthermore, this low specificity makes clear-cut policies for patient management really problematic as some studies also showed a disappointing specificity (Bisoffi *et al.* 2010). Up to 70% of the test can remain positive for weeks after disappearance of trophozoites (Swarthout *et al.* 2007).

Although the current study produced interesting results, we were however constrained with resources. For example, due to financial constrains, only 101 samples could be analyzed by PCR targeting *Pf*. This lead to a loss of power according to PCR results and it did not allow us to search for the occurrence of other *Plasmodium* species in the samples.

Conclusion and recommendation

Conclusions

The prevalence of *Pf* infection was statistically different between the Cite Pumbu and Kindele and the 2 health areas should not be classified as the same. And, the sensitivity, specificity, positive and negative predictive values of SD-Bioline assessed with microscopy or PCR was not optimal in children of less than five years in Mont Ngafula 1.

Difference found between specificity and NPV when the performance of the SD-Bioline was assessed with microscopy or PCR might be due to the threshold detection of PCR that is lower than microscopy

SD-Bioline is limited as a tool for endemicity assessment in children of less than five years in Mont Ngafula 1 due to an overestimation of the infection prevalence by the test.

Recommendations

Further studies should be done to assess prevalence *Pf* infection in the different health areas in Mont Ngafula 1 for an accurate mapping of the health zone in children having aged less than five years in population based surveys. Such studies must be repeated in the dry and the rainy season to assess the prevalence over the year.

Further MRDT should be tested in the health zone of Mont Ngafula I to find a test that will allow good performance for endemicity assessment in children of less than five years in population based surveys.

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Appendices

Appendix 1: Consentement éclairé en français

Titre de l'étude : **Prévalence et morbidité de la malaria chez les enfants de moins de cinq dans la zone de santé de Mont Ngafula1 in Kinshasa, RD Congo.**

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

INFORMATIONS SUR L'ETUDE

La malaria cause environ 200,000 morts par an en RD Congo et le les enfants de moins de 5 ans ainsi que les femmes enceintes sont les plus touches. Un individu sain tombe malade en se faisant piquer par un moustique anophèle infecté. Plusieurs interventions ont ete menées dans les communautés particulièrement les campagnes de distribution des Moustiquaires impregnées d'insecticide. Cependant il n'ya aucun moyen de savoir si ces interventions ont atteints leurs objectifs ou pas étant donné qu'il n'y a pas de donné sur le niveau précédant et actuel de la prévalence et de la morbidité causée par la malaria chez nos enfants de moins de 5 ans.

Le but de cette étude est d'établir la prévalence et la morbidité dus a la malaria chez nos enfants de moins de 5 ans dans la zone de santé de Mont Ngafula1 a Kinshasa, RD Congo. Ceci va nous aider parce que ca va produire des données sur la prévalence actuelle et la morbidité due a la malaria chez nos enfants et cela va aider à évaluer de prochaines interventions dans le but de les améliorer, les adapter et les remplacer si elles sont trouvé non efficaces.

Nous vous invitons donc a faire participer votre enfant a cette étude en signant ce formulaire de consentement éclair et en nous permettant de prélever 3 ml de sang et de faire une goutte épaisse. Et, en acceptant que les données collectées a l'exception de vos données personnelles soient utilisées dans notre étude. Certaines analyses de laboratoires seront faites dans des laboratoires en dehors du pays. Toutes les enfants ayant un test de diagnostic rapide positif des médicaments contre la malaria.

Procédure de l'étude

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

Bénéfices and risques

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

Participation volontaire

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

Cout et compensation

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

Confidentialité

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

Questions

Pour de plus amples informations, veuillez contacter le principal investigateur, Dr Vivi Maketa du Département de Médecine Tropicale de la Faculté de Médecine, Université de Kinshasa. Numéro de telephone : +243 99 83 67 773 e-mail : vmaketa@yahoo.fr ou le superviseur local de l'étude : Pr Pascal Lutumba : +243 81 81 58 961 e-mail : pascal_lutumba@yahoo.fr **.**

Consentement écrit

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

J'accepte la participation de mon enfant nommé

…….a cette étude:
Nom et signature du parent ou du tuteur légal de l'enfant

Nom Signature

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

Appendix 2: Informed Consent in English

Study title: **Prevalence and morbidity of malaria in the children stratified by age in the health zone of Mont Ngafula1 in Kinshasa, DR Congo**

We ask your authorization to let your children participate in this research study. The aim of this formulary is to give you all the explanation laying to this study and to get your consent for your

children to participate in this study. You are not obliged to agree and if you don't agree or if you want to withdraw your child from the study at any moment there will be no problem for you.

INFORMATIONS ON THE STUDY

Malaria causes approximately 200,000 deaths per year in DR Congo and the highest burden is shared between pregnant women and children below five years old. A healthy individual get the disease trough the bit of infected anopheles mosquitoes. Several interventions have been held in the community to wane the number of deaths cause by malaria in children especially the campaign of mass distribution of Insecticide treated nets. But there is no way to know if those interventions reached their target or not as long as there is no data on the previous and the actual level of prevalence and morbidity in our children.

The aim of this study is to assess the prevalence and the morbidity of malaria in children below five years old in the health zone of Mont Ngafula1 in Kinshasa, DR Congo. It will be very helpful cause it will yield data on the actual prevalence and morbidity of malaria in our children and it will help to evaluate further interventions in order to improve, adapt or to replace them if they are found non efficient.

We therefore invite you to let your child below 5 years old to participate to this study by signing the informed consent paper and by letting us collect 3ml of blood and do and a fingerpick. And by agreeing the data we will collect in exception of your personal data to be used in the study. Laboratories analysis will be made in a laboratory outside the country. But all positive blood smears will be supplied by anti malarial drug.

Study procedure

Nurses and medical doctor will collect the blood samples and will ask you question about the fever episode the previous month.

Benefices and risks

The survey on malaria will help us to assess the number of malaria episode and to correlate it with the parasitemia and the level of antibodies. The bit of the needle during blood collection could lead to an infection in the site of collection but this will be done by professional staff using sterilized single use material. This precautious will reduce the post infection risk.

Voluntary participation

The decision to let your child participate in the study is totally voluntary and if you don't agree, there will be no consequences.

Cost and compensation

You won't pay anything and no incentive will be provided by the research team if you accept your child to be included in the study.

Confidentiality

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

Questions

For any further question please contact the principal investigator, Dr Vivi Maketa from Tropical medicine Departemnt, Kinshasa University. Phone number: +243 99 83 67 773 e-mail : vmaketa@yahoo.fr or the local supervisor of the study : Pr Pascal Lutumba : +243 81 81 58 961 e-mail : pascal_lutumba@yahoo.fr **.**

Written consent

I've been informed about the aim of this study and I understand the objectives and the conditions. All my questions have been answered. I understand that the participation to this research study is voluntary and that I am free to withdraw my child at any moment without any inconvenience in the future. Personal information obtained from my child will be kept confidential and won't be published in any publication. I understand that if I have questions to ask or I want to withdraw my child I can contact the principal investigator or the local supervisor at the address given.

I agree for the participation of my child named

……to this study:

Name and signature of the parent/guardian of the child

Name Signature

Name and signature of the person who explained the informed consent

Name Signature

Appendix 3: Mokanda mua bolimboli na lingala

Kombo ya moyekoli : **Prévalence et morbidité de la malaria chez les enfants de moins de cinq dans la zone de santé de Mont Ngafula1 in Kinshasa, RD Congo.**

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

OYO ETALI MOYEKOLI

Bokono ebengami malaria esilaka ko boma batu 200,000 na mbula na mboka na biso RD Congo pe bana oyo nanu ba kokisi mbila 5 te pe basi ya zemi nde mingi mingi bakufaka na bokono yango. Bokono ezuamaka soki moustique anophele oyo azali na microbe ya malaria a sui moto. Ba interventions mingi esalamaki na bisika na biso, lolenge ya campagnes ya ba moustiquaires impregnées d'insecticide. Kasi lolenge ya ko yeba soki ba interventions wana esali mosala na yango ezali te po to yebi te bana boni bazalaki to bazali na bokono pe pasi nini bokono yango ekopesaka te epayi ya bana na biso oyo nanu ba kokisi mbula 5 te.

Tina ya moyekoli eye ezali ko lakisa bana boni ba zali na bokono ebengami malaria pe ba pasi nini bokono yango epesaka epayi ya bana na biso nanu bakokisi mbula 5 te na zone ya santé de Mont Ngafula1 a Kinshasa, RD Congo. Yango nde ekosalisa biso po to yeba ko tala na mikolo mi ko ya ndenge nini tokoki ko bongisa, ko kokanisa, to pe kolonga ba intervention yango soki to moni esimbi te.

To zo senga bino bon dima bana na bino ba kota na moyekoli oyo na ko koma mokolot na yo na mokanda mua bolimboli oyo pe na ko ndima to benda 3cc ya makila epayi ya muana pe to sala goutte epaisse. No kondima na yo, to ko salela ba donnee nyonso longola oyo etali yo moko na moyekoli na biso. Ba examens ya labo misusu ekosalama libanda ya mboka. Bana nyoso ba ko zuama na malaria ya makasi, to ko pesa bango kisi.

Lolenge moyekoli eko tambola

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

Matabisi na ba risques

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

Kokota na nguya na yo

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

Kofuta

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

Ko bomba ba sango

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

Mituna

Soki ozali na mituna misusu, yeba ko benga principal investigateur, Dr Vivi Maketa ya Département de Médecine Tropicale, Faculté de Médecine, Université de Kinshasa. Numéro de telephone : +243 99 83 67 773 e-mail : vmaketa@yahoo.fr te pe superviseur local ya moyekoli : Pr Pascal Lutumba : +243 81 81 58 961 e-mail : pascal_lutumba@yahoo.fr **.**

Bolimboli

Ba limobleli nga tina ya moyekoli yo pe na sosoli tina na yango. Ba pesi nga biyani na mituna nyonso. Na sosoli été ko kota na nga na moyekoli oyo exali na nguya na nga pe soki na boyi, na koki ko bimisa muana na ngai tango nyonso, pe mabe miko eko yela nga te, na sima. Ba sango oyo etali muanan na ngai eko lobama pe ko komama na publication moko te. Na sosoli été soki na zali na mituna to pe na lingi ko longola muana na ngai na moyekoli oyo na koki ko benga investigateur principal to pe superviseur local.

Na ndimi été muana na ngai, oyo kombo ya ye

…… A kota na moyekoli oyo:

Kombo pe mokoloto ya moboti to mobaletli ya muana

Kombo Mokoloto

…….. ……

Kombo pe mokoloto ya mutu oyo a tangisi mokanda mwa bolimboli

Kombo Mokoloto

Appendix 4 PROCEDURE OPERATOIRE STANDARD LA COLLECTE DES

PROCEDURE OPERATOIRE STANDARD :

LA COLLECTE DES ECHANTILLONS POUR LA PCR SUR PAPIER FILTRE

ECHANTILLONS POUR LA PCR SUR PAPIER FILTRE:

LA COLLECTE DES ECHANTILLONS POUR LA PCR SUR PAPIER FILTRE

1. Objectif / utilisation prévue
Collecter le sang total sur du papier filtre pour l'analyse de la PCR.

2. Principes de base
Les acides nucléiques peuvent être extraites du sang total qui est transféré sur le papier filtre et sécher. ce genre d'extraction peut être utilisé pour les analyses de la PCR sur le sujet et ou sur un agent infectieux qui peut se trouver dans le sang. Le sang est appliqué sur un papier épais avec une grande et forte absorption. le sang séché sur papier filtre est stocké avec du dessicant (silicagel) pour prévenir la dégradation des acides nucléiques immobilisées dans le papier filtre. Le sang couvre la totalité du bord du papier filtre pour permettre des coupes proches du sang au moment de l'extraction

3. Echantillon nécessaire
Le sang total obtenu par une ponction veineuse

4. Matériels
papier filtre Whatman 3mm marqueur indélébile

Sachet en plastique scellé (zip lock) avec étiquette

silicagel

seringue avec aiguille

Gants jetable

Poubelle a aiguille

Poubelle ordinaire

Ouate

Alcool dénaturé

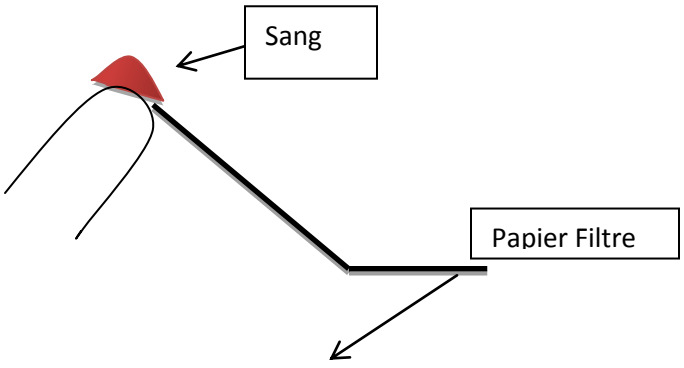
Garrot

5. Réactifs

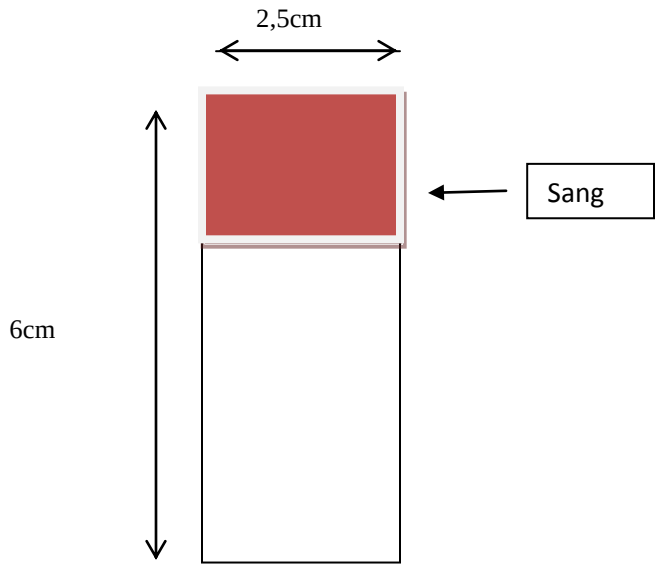
Non applicable

6. Mode opératoire

1. Préparer le matériel
2. Noter les cordonnées du patient (les initiales, le numéro de l'étude du patient, le jour de la visite et la date de la visite) sur l'étiquette colée sur le sachet en plastique ainsi que sur la bande de papier filtre.
3. Placer la bande de papier filtre sur le sachet en plastique.



4. Faire un prélèvement veineux
5. Couvrir complètement le bord du papier filtre avec du sang, pour le permettre d'être complètement imprégné. Le sang doit être totalement absorber a travers le papier (le 1/4 du papier filtre doit être couvert)
6. schéma représentatif de la bande de papier filtre après le prélèvement :



7. laisser le sang sécher une nuit à température ambiante dans un endroit propre et sec. classer chaque papier filtre sur son propre sachet tout en évitant la contamination de la surface ou les échantillons sont gardés.
8. Quand les gouttes de sang sont complètement sèches, le jour suivant, mettre les bandes dans les sachets en plastiques distincts, ajouter un paquet de silicagel dans chaque sachet et sceller.
9. Garder les sachets dans un endroit frais et sec à l'abri de la lumière
10. Enregistrer ces échantillons dans le registre de la collecte des échantillons PCR '

7. Résultats & Interprétation

NA

8. contrôle de qualité

- toujours vérifier la face avant et arrière du papier filtre, le sang doit être visible sur les deux faces.

- s'assurer que le papier filtre est placé loin des rayons solaires ou de tout autre source de chaleur pour éviter que le sang soit 'cuit' sur le papier filtre car ceci peut le rendre impropre à d'autres analyses

- étiquetage*:* s'assurer que les étiquettes sur les sachets en plastiques sont correctement remplies et que les détails repris ci dessus correspondent à ceux se trouvant sur la bande de papier filtre.

- la *contamination croisée:* chaque bande de papier filtre doit être placée sur son propre sachet en plastique pour prévenir la contamination de la surface sur laquelle elle est placée. Ne pas le faire peut entrainer la contamination des échantillons subséquents.

9. Sécurité

Tous les échantillons biologiques devraient être considérées comme potentiellement infectieux et doivent être traité selon les précautions générales. Ceci est basé sur les prémices que les travailleurs de soins de santé doivent considérer le sang et tous les humeurs du corps comme potentiellement infectieux.

les précautions générales qui doivent être respectées:

- *Porter des vêtements de protection personnels comme les gants et blouses de laboratoire.*
- *Si vous avez une plaie ou une écorchure* à *la main, le recouvrir avec du pansement adhésif.*
- *Les objets tranchants utilisés pendant la collecte des échantillons doivent être jeté dans une poubelle appropriée.*
- *Enlever les gants et laver les mains après avoir terminer une tache impliquant la manipulation des matériaux biologiques.*

10. Documents relatifs

- SOP N° L010: ponction veineuse pour la collecte de sang veineux
- Registre de la collecte des échantillons PCR

11. Références

- Manuel de procédures du laboratoire central

Appendix 5 Standard Operating Procedure: HOW TO DO RDT Test

Standard Operating Procedure

SOP title	**HOW TO DO RDT Test**

I. INTRODUCTION

The SD malaria Antigen P.f/Pan intended for the detection of Malaria infection in human blood sample, indicating qualitative and differential diagnosis between HRP-II specific to Plasmodium *falciparum* and pLDH specific to Plasmodium species in human blood sample.

II. PROCEDURE

- Explain the procedure you are going to do
- Take the RDT kit
- Put a new pair of glove
- Open the alcohol swab and clean the 4th finger (allowed it to dry)
- Once the patient's finger is dry, open the lancet and prick the patient finger
- Discard the lancet in an appropriate sharp container immediately after use

  NB : Never use a lancet on more than one person
- Ensure a good sized drop is on the finger before collecting. Collect just to the mark by placing the tip of the capillary tube on the blood drop of the finger
- Once you are collected a sufficient amount of blood, you may hand the alcohol swab back to the patient finger
- Use the loop to add blood to the small round hole
- Push the loop vertically into the hole until its touches the pad at the base

- Discard the blood collection loop after use
- Add 4 drops of diluent to the cassette
- Wait 15mn after adding assay diluent before reading test results
- Remove and discard your glove at this time
- Interpretation of the test
    a) Only line « C » = Negative
    b) Line « P.f » and a line « C » = *P.f* positive
    c) Line « Pan » and line « C » = *P.v* or *P.m* or *P.o* positive
    d) Line « P.f », « Pan », and line « C » = *P.f* positive or mixed infection of *P.f*, and *P.v* or *P.m* or *P.o*
    e) No line « C » and others line = invalid

NB: In case of invalid results repeat the test by using a new cassette

III. QUALITY CONTROL

Test lines and control line in result window are not visible before applying any sample. Control line should always appear if the test procedure is performed properly and the test result

IV. REFERENCES

- sd_bioline_malaria_ag_pf_manual_020510

Title	DNA Extraction

Appendix 6 Standard Operating Procedure: DNA Extraction

Samples

This method is designed to be performed on dried blood spot (DBS) samples.

Required Materials and Equipment

1.5 ml microcentrifuge tubes

1-20 μl single channel automatic pipettes

100-200 μl single channel automatic pipette

1000 μl single channel automatic pipette

Filter pipette tips for the above pipettes

Fine tip marker pens

Ball point pen

Paper towels or wipes

Distilled water

Phosphate Buffered Saline (PBS)

Bleach (5 %) in a beaker or wash bottle

Distilled water in a beaker or wash bottle

Ethanol (70 %) in a beaker or wash bottle

Chelex®-100 Resin

Scissors or 1/8 inch hole punch (plus spare filter paper if using a punch)

Timer

Microcentrifuge

Heating block or waterbath at 56 ºC

Waterbath at boiling temperature (96 ºC or above)

Vortex

Procedural steps

Important points to remember:

- Ensure the punchers are thoroughly cleaned before beginning the procedure, in between cutting filter papers and at the end of the procedure. Unclean puncherss can lead to cross contamination of samples and poor quality results.
- Ensure pipette tips are of a high quality, sterile and endonuclease free.
- Do not touch pipette tips.
- Make sure pipettes are calibrated and cleaned regularly.

1. Print out a PCR worksheet and record the sample ID of each DBS to be tested on a separate numbered line.
2. Gather all required supplies. *NB. If samples have been stored at +4 ºC or -20ºC they must be brought to room temperature in the sample bag prior to opening.*
3. Gather all required supplies.
Make 2% Chelex : Take 2.5 ml distilled water, add 0.05 g chelex (20 %). *NB. Chelex reagent should be made fresh each day it is required.*

4. Clean the scissors or punch by dipping in ethanol (70%) and passing through a flame.
5. Label an appropriate number of 1.5 ml microcentrifuge tubes (label both the lid and the side of the tube) with the worksheet number and sample ID.
6. Punch a 3 mm disk (holds approx. 3-5 µl of dried blood) from the filter paper and put it into the corresponding 1.5 microcentrifuge tube. NB. *Clean the scissors between each sample as detailed in step 5. Clean the punch by punching clean filter paper 3 times.*
7. Make 1X PBS with 0.1% Saponin weight/volume: Take 50 ml of 1X PBS, add 0.05 g Saponin *NB. Ensure filter papers are soaked inSaponin- buffer solution.*
8. Incubate at room temperature for 10 min.
9. Centrifuge at 14,000 rpm for 2 min and discard supernatant using a clean pipette tip for each sample.
10. Add 1 ml PBS
11. Centrifuge at 14,000 rpm for 2 min and discard supernatant using a clean pipette tip for each sample.
12. Add 150 µl of 2% chelex solution
13. Add 50 µl of distilled water
14. Incubate at 99 ºC for 10 min.
15. Centrifuge at 14,000 rpm for 1 min.
16. Store supernatant at +4ºC for use in PCR. *NB. If storing samples for longer than a day, transfer supernatant into a fresh microcentrifuge tube and stored at -20* ºC.

Appendix 7: Polymerase chain reaction

PCR conditions (PCR mix 20µl):

No. of reactions

	Lot number	µl
Go Taq flexi buffer 10X	2	
dNTP mix (5mM each)	0,5	
MgCl2 (25 mM)	1,6	
Primer rPLU5 (10µM)	0,5	
Primer rPLU6 (10µM)	0,5	
GoTaq flexi DNA polymerase		0,08
H2O Molucular Biology grade		13,82
DNA	1	

Program: PLU

PCR cycler n°:

PCR block n°:

Checked:

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

Step 7: Hold at 20°C

PCR product :1200bp

Primers

rPLU5 : CTTGTTGTTGCCTTAAACTTC

rPLU6 : TTAAAATTGTTGCAGTTAAAACG

Study identification: SD-optimal

Executor: Junior

Code Nested FAL: 2

PCR conditions (PCR mix 20µl):

No. of reactions

	Lot number	µl
Go Taq flexi buffer 10X		2
dNTP mix (5mM each)		0,5
MgCl2 (25 mM)		1,6
Primer rPLU5 (10µM)		0,5
Primer rPLU6 (10µM)		0,5
GoTaq flexi DNA polymerase		0,08
H2O Molucular Biology grade		13,82
DNA		1

Program: FAL

PCR cycler n°:

PCR block n°:

Checked:

Step 1: Primary denaturation 95°C, 5min

Step 2: Denaturation 94°C, 1min

Step 3: Annealing 58°C, 2min

Step 4: Extension 72°C, 2min

Step 5: Go to step 2 for 29 cycles (total 30)

Step 6: Final extension 72°C 5min

Step 7: Hold at 20°C

PCR product: 205bp

Primers

rFAL1 TTAAACTGGTTTGGGAAAACCAAATATATT

rFAL2 ACACAATGAACTCAATCATGACTACCCGTC (Check accuracy of primer sequences)