SAPIENZA
UNIVERSITÀ DI ROMA

Ph.D. in Infectious Diseases, Microbiology and Public Health

XXXIst Batch

Department of Public Health and Infectious Diseases

Plasmodium vivax, susceptibility factors and pharmacogenetics of treatment among febrile patients in the West and South Regions of Cameroon

Tutors:

Dr Gianluca Russo

Dr Sanou Sobze Martin

PhD Student:

Ghyslaine Bruna Djeunang Dongho

Matricule: 1693700

ACADEMIC YEAR 2017-2018

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ACKNOWLEDGEMENTS

My sincere gratitude goes to all those who contributed in one way or the other to the success of this work. More specifically, they are;

My thesis tutors: Dr **Gianluca Russo** and Dr **Martin Sanou Sobze**;

Doctors, find in these few lines the expression of my gratitude for believing in me and giving me the chance to add my contribution to this hot topic of *Plasmodium vivax* malaria; for your advices, your support and most especially the dedication of your time and efforts to supervise and direct this work right from the elaboration of the protocol to this final phase;

Botswana UPenn Partnership team especially Dr **Giacomo Maria Paganotti**, Mrs. **Lesego Sethole**, Mr. **Tawe Leabaneng**, Mr. **Charles Muthoga,** Mr. **Moses Vurayai,** Dr **Thato Motshoge,**

Your availability, devotion and support made my journey towards Ph.D. research an amazing trip full of beautiful discoveries and learning, Thank you very much;

Istituto Superiore di Sanità, malaria research team: Dr **Carlo Severini**, Dr **Michela Menegon** and Miss **Mariangela L'Episcopia**,

I am grateful for your great contribution consisting in training me and supervising parasite detection (both through PCR and microscopy);

The Department of Infectious Diseases and Public Health staff especially Pr **Stefano D'Amelio**,

Your openness, availability and collaboration made this outcome possible, I will forever be grateful;

PIPAD research team: Miss **Tsi Kien-Atsu**, Mr. **Armand Tiotsia Tsapi**, Mr. **Isidore Bell Pallawo**, and Miss **Rose Sangong Efeutmecheh**

Your great working spirit, encouragement, collection of samples and data made the field work more convenient, Thank you;

My colleagues: Dr **Christian Doll** and Mr. **Alaric Talom Tamuedjoun**

Your encouragements, support and advices were highly welcome, Thank you;

My lovely family especially my uncle and brothers: Mr. **Roger Nguefack**, Mr. **Serge Tonfack** and Mr. **Franck Tonfack**,

Your moral, material and financial support during hard times will never be forgotten.

And finally, my friends: Dr **Stephane Tekam**, Miss **Marinolle Zamke**, Mr. **Ingrid Biabaka**, Mrs. **Mehta Claudia**, Mrs. **Marina Mensi**, Mr. **Raoul Ngako** and Mr. **Amalesh Ngambo,**

Your help, attention and constant support brought me towards the end of this journey.

A Special thanks goes to the Italian organization **Mingha Africa Onlus**, which granted me a scholarship to cacrry on this Ph.D course.

It is almost impossible to mention everyone, I am forever grateful for every single input.

ABBREVIATIONS AND ACRONYMS

A	Adamaoua region
AFR	WHO African Region
Ag	Antigen
AHD	Ambam Health District
AIDS	Acquired Immuno-deficiency Syndrome
AMR	WHO Region of the Americas
AS	Activity Score
C	Centre region
CAMBIN	Cameroon Bioethics Initiative
CI	Confidence Interval
CIOMS	Council for International Organizations of Medical Sciences
CSP	Circumsporozoite Protein
CSP-LSP-N	CSP-long synthetic peptides N-terminal
CSP-LSP-R	CSP-long synthetic peptides R-terminal
CYP2D6	Cytochrome Superfamily450 family 2 subfamily D member 6
CYP450	Cytochrome superfamily 450
D	Coefficient of linkage disequilibrium
D'	Normalized coefficient of linkage disequilibrium
DARC	Duffy Antigen Receptors for Chemokines
DBS	Dried Blood Spots
DCs	Dendritic Cells
DHD	Dschang Health District
DNA	Deoxyribonucleic Acid
E	East region
EM	Expectation-Maximization
EMR	WHO Eastern Mediterranean Region
ERCC	Ethics Review and Consultancy Committee
FDA	Food and Drug Administration
FN	Far-North region
FY	Duffy Antigen allele
G6PD	Glucose-6-Phosphate Dehydrogenase

G6PDd	Glucose-6-Phosphate Dehydrogenase deficiency
GLA	Glucopyranosyl Lipid A
GPI	Glycosylphosphatidylinositol
HCF	Healthcare Facility
HD	Heterozygous defect
HIV	Human Immuno-deficiency Virus
HWE	Hardy-Weinberg Equilibrium
ICH-GCP	International Council for Harmonization in Good Clinical Practices
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IM	Intermediate metabolizer
L	Littoral region
LD	Linkage Disequilibrium
LLIN	Long Lasting Impregnated Net
MAOA	Monoamino-oxidase A
ML	Maximum-likelihood
MΦ	Macrophages
N	North region
NHP	Non-Human Primate
NM-F	Normal Metabolizer-Fast
NM-S	Normal Metabolizer-Slow
NT	N-Terminal
NW	North-West region
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
***Pf*-RDT**	*Plasmodium falciparum* Rapid Diagnostic test
PIPAD	*Projet Intégré pour la Promotion de l'Auto-Développement*
Pk	*Plasmodium knowlesi*
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
PQ	Primaquine
Pv	*Plasmodium vivax*

***Pv*CSP**	*Pv* Circumsporozoite Protein
***Pv*DBP**	*Pv* Duffy Binding Protein
***Pv*MSP1**	*Pv* erythrocytic antigens Merozoite Surface Protein 1
***Pv*TRAP**	Thrombospondin-Related Adhesive Protein
RBC	Red blood cell
RDT	Rapid Diagnostic tes
RT-PCR	Real-Time Polymerase Chain Reaction
S	South region
SEAR	WHO South-East Asia Region
SHD	Santchou Health District
SNP	Single Nucleotide Polymorphism
SW	South-West region
Th	T-helper
TNF-α	Tumor Necrosis Factor-alpha
VNTR	Variable Number of Tandem Repeats
W	West region
WHO	World Health Organization
WPR	WHO Western Pacific Region

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SUMMARY

Thanks to the implementation of molecular detection techniques, *Plasmodium vivax (Pv)* infections have been reported in several sub-Saharan African countries recently, including Duffy-negative populations of West and Central Africa. This work aimed to assess the possible circulation of *Pv* and factors linked to the susceptibility to *Pv* and the pharmacogenetics of its treatment (Duffy antigen, G6PD deficiency, CYP450 genetic variability) among outpatients of Santchou, Dschang and Ambam Health Districts.

A cross-sectional descriptive and analytical study was performed in 4 healthcare facilities (HCFs). Data was collected in two different periods, the rainy season in Santchou (August 2016-December 2016) and rainy season in Dschang and Kyé-ossi/Ambam (May 2017-September 2017). Interviews were conducted using a structured questionnaireand consenting febrile patients were consecutively recruited and drops of blood were collected on dried blood spots and smears. All samples were analysed by molecular and microscopic methods. All data was analysed using Microsoft Excel 2013 and Epi info version 7.2.

In total, 1001 samples were collected and *Plasmodium spp* parasite DNA was detected in 486 (48.6%) samples. In particular, 441 cases of *Plasmodium* mono-infections (287 *P. falciparum*, 146 *P. vivax*, 2 *P. ovale*, 6 *P. malariae*) and 45 cases of mixed-infections (37 *falciparum/vivax,* 2 *falciparum/ovale*, 4 *falciparum/malariae,* 2 *vivax/malariae)* were detected. Globally, *Pv* has been detected in 185 cases (38.1% of positive samples), mainly from Dschang (n=181) than Santchou (n=2) and Ambam (n=2). *Pv* occurrence appeared to be linked with environmental factors more than biological factors. Locally used malaria diagnosis methods had less specificity and sensibility to respect to PCR. Duffy blood group genotyping showed alleles frequencies of 0.30% (2/666) positive (-33TC) and 99.70% (664/666) negative (-33CC). All the *Pv* positive cases have shown a Duffy-negative genotype (-33CC). G6PD 968 SNP did not show any variability (968 A+, TT). G6PD deficiency prevalence was 2.78% (5/180) evaluated according to 202G>A SNP. The deficient allele frequency (G6PD 202A) among females was 2.72%. Two (2) *Pv* infected people were G6PD heterozygous (202GA; A+/-). Looking at *CYP2D6* gene variability, the mutant alleles found were *2 (56.71%), *17 (13.63%) and *4 (4.24%). Most of them were normal metabolizers (98.82%; 504/510). Twelve (12) *Pv* infected people were intermediate and normal-slow metabolizers.

These data show a relatively high circulation of *Pv* in the West Region of Cameroon among Duffy-negative autochthonous individuals, with a prevalence possibly depending from altitude and seasonality. Further study are necessary, in order to assess the real *Pv* local circulation and transmission, as well as to identify Duffy-independent *Pv* erythrocyte invasion pathway. It is important to improve the local malaria control program in Dschang taking in account the challenges of *Pv* malaria management (therapeutic toxicity, attacks and relapses).

Key words: Plasmodium vivax, malaria, pharmacogenetics, susceptibility factors, Cameroon.

INTRODUCTION

1. Definition of key concepts

Malaria: life-threatening disease caused by parasites (P*lasmodium*) transmitted to people through the bites of infected mosquitoes (*Anopheles*) (WHO, 2018).

Susceptibility factors: susceptibility is the degree to which a person or a population is sensitive to either adverse or protective exposures; "susceptibility factors" include populations at risk (e.g., the very young, sex, or genetically at risk), known risk factors, and known protective factors (Yeatts *et al.*, 2006).

Pharmacogenetics: single genes that dominate the effects of a drug response (Altman, Flokart and Goldstein 2012).

2. Malaria burden and distribution

Malaria currently occurs mostly in tropical and subtropical areas of the world, with 2.5 billion people living in countries affected. In malaria endemic countries, this infection is the leading cause of illness and death, and young children (< 5 years-old) and pregnant women are the most vulnerable groups. Moreover, other at risk population are patients affected by HIV/AIDS, as well as non-immune migrants, mobile populations and travellers (WHO, 2018).

According to the latest estimates, there were 216 million cases (95% confidence interval [CI]: 196–263 million) of malaria in 2016 distributed in 91 countries, thus showing an increase of 5 million of cases over 2015. Malaria deaths reached 445 000 in 2016, a similar number (446 000) to 2015 (WHO, 2018).

WHO Member States are grouped into six regions, each region having a regional office (figure 1).

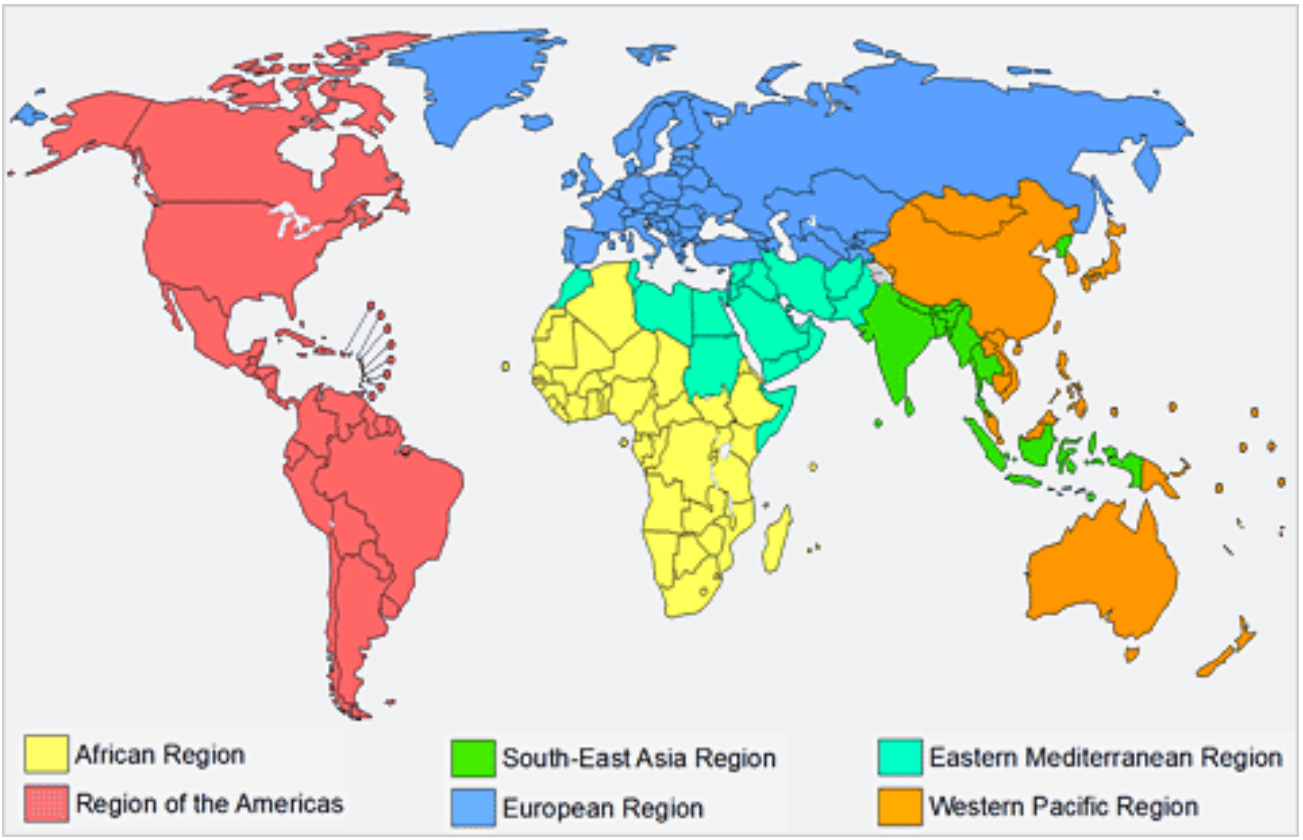
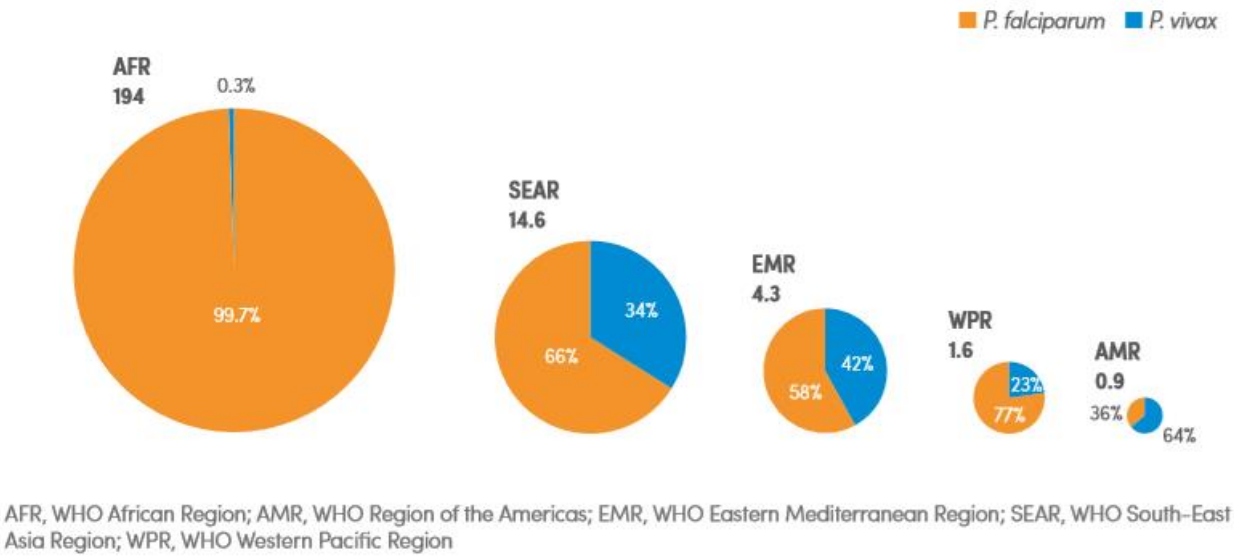


Figure 1: WHO regions ***(source: https://www.who.int/about/regions/en/)***

The WHO African Region continues to carry a disproportionately high share of the global malaria burden (90%) followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%). Concerning the malaria related mortality, 91% of the cases are in WHO African region. Globally, 15 countries – all in sub-Saharan Africa, except India – accounted for 80% of the global malaria burden in terms of morbidity and mortality (WHO Global Malaria Programme, 2017).

Between 2010 and 2016, the incidence rate of malaria is estimated to have decreased by 18% globally, from 76 to 63 cases per 1000 population at risk. The WHO South-East Asia Region recorded the largest decline (48%) followed by the WHO Region of the Americas (22%) and the WHO African Region (20%) (WHO, 2018).

Plasmodium falciparum (Pf) is the most prevalent malaria parasite in sub-Saharan Africa, accounting for 99% of estimated malaria cases in 2016 (figure 2).



AFR, WHO African Region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean Region; SEAR, WHO South-East Asia Region; WPR, WHO Western Pacific Region

Figure 2 : Estimated malaria cases (millions) by WHO region, 2016.

The area of the circles is proportional to the estimated number of cases in each region (WHO Global Malaria Programme, 2017).

Outside of AFR, *Plasmodium vivax (Pv)*, the most prevalent specie responsible of malaria, has the greatest geographic range and burden in the AMR, representing 64% of malaria cases, and is above 30% in the SEAR and 40% in the EMR. Worldwide, estimates of *Pv* infections range between 130 and 390 million, with 2.6 billion individuals living at risk of infection (Guerra *et al.*, 2006; Hay *et al.*, 2004; WHO Global Malaria Programme, 2017).

Scientists have believed for a long time that *Pv* originated in Asia, whereas recent studies on great apes of Central Africa show its African origin. It is clearly demonstrated that human *Pv* is phylogenetically linked to *Pv*-like parasite which endemically infects different non-human primates (NHPs), in particular chimpanzees, gorillas and bonobos (Culleton and Carter, 2012; Liu *et al*., 2014).

Actually, *Pv* epidemiology is a complex set of characteristics and patterns. It includes settings and populations, influenced by many features including local mosquito vector species, transmission intensity, relapse behaviors, host risk factors, availability and efficacy of treatment, malnutrition and prevalence of comorbidities (Howes *et al*., 2015).

The burden of malaria due to *Pv* in sub-Saharan Africa varies according to local climatic conditions, as well as to the population susceptibility factors (i.e. Duffy antigen, age) (Lo *et al*., 2015; Poirier *et al*., 2016; World Health Organization-WHO, 2015). In countries where there is co-existence of Duffy negative and positive population, the circulation of *Pv*

is higher (Golassa and White, 2017). For many years, people from sub-Saharan African countries were considered as resistant to *Pv* infection because of the absence of the Duffy antigen, on the surface of their erythrocytes, that mediate the entry of the parasite (Miller, *et al.*, 1976). Some surveys in Africa showed a prevalence of malaria cases due to *Pv* among the population study as follows: Republic of Congo (13%) (Culleton *et al.*, 2009), Equatorial Guinea (9.5%) and Angola (2.8%) (Mendes *et al.*, 2011), Ethiopia (59.5% of malaria cases) (Lo *et al.*, 2015), Mauritania (34.5%) (Ba *et al.*, 2016), Botswana (93.37% of malaria cases) (Motshoge *et al.*, 2016), Benin (1.05%) (Poirier *et al.*, 2016), Senegal (53% of malaria cases) (Niang *et al.*, 2017), Mali (2-2.5%) (Niangaly *et al.*, 2017).

In the last decade, several studies have reported the circulation of *Pv* in sub-Saharan Africa (figure 3), including Duffy-negative populations.

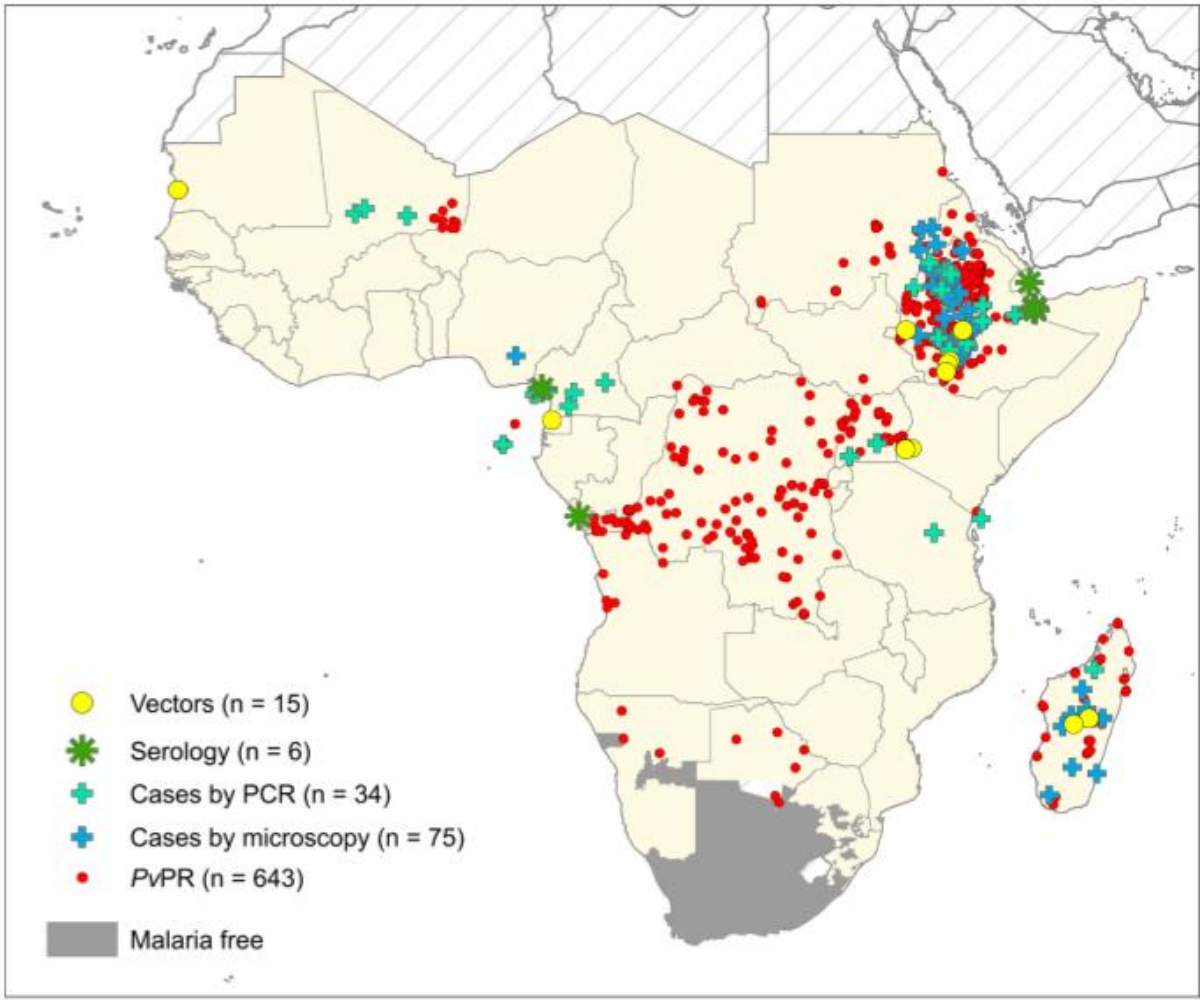


Figure 3: Evidence based of *Pv* occurrence in Sub-Saharan Africa (Howes *et al.*, 2015).

A review summarises all the studies published in sub-Saharan African until early 2017 (Zimmerman, 2017). *Pv* in Africa was known to be circulating in Madagascar, Ethiopia, Sudan and Mauritania due to their genetic susceptibility (Gunalan *et al*., 2018). These studies explored both symptomatic and asymptomatic subjects.

Concerning the malaria epidemiology in Cameroon, during 2016, 1,675,264 cases were reported as microscopically confirmed in healthcare facilities, with 2,639 related deaths (WHO, 2016). These reported figures were three times lower than the estimations (WHO, 2016). More than 90% Cameroonians are living in either high or low transmission area (Mbenda *et al.*, 2014). According to official data, all malaria cases in 2016 are related to *Pf* (WHO, 2016). In the last five years, four published studies showed evidence of *Pv* circulation among Cameroonian autochthonous from six regions of the country (figure 4) (Fru-Cho *et al*., 2014; Mbenda *et al.*, 2016; Mbenda and Das, 2014; Russo *et al*., 2017). Dschang in the west (W) region has shown the highest prevalence of *Pv* (38.6%) among the malaria infected patients (Russo *et al*., 2017), followed by Douala (23.2%) in the littoral (L) region (Mbenda *et al.*, 2016). Not all the regions of Cameroon are explored to assess *Pv* circulation and may be other non-*falciparum* malaria parasites. More widespread assessment is required to have the real picture of *Pv* prevalence and distribution among symptomatic and asymptomatic subjects.

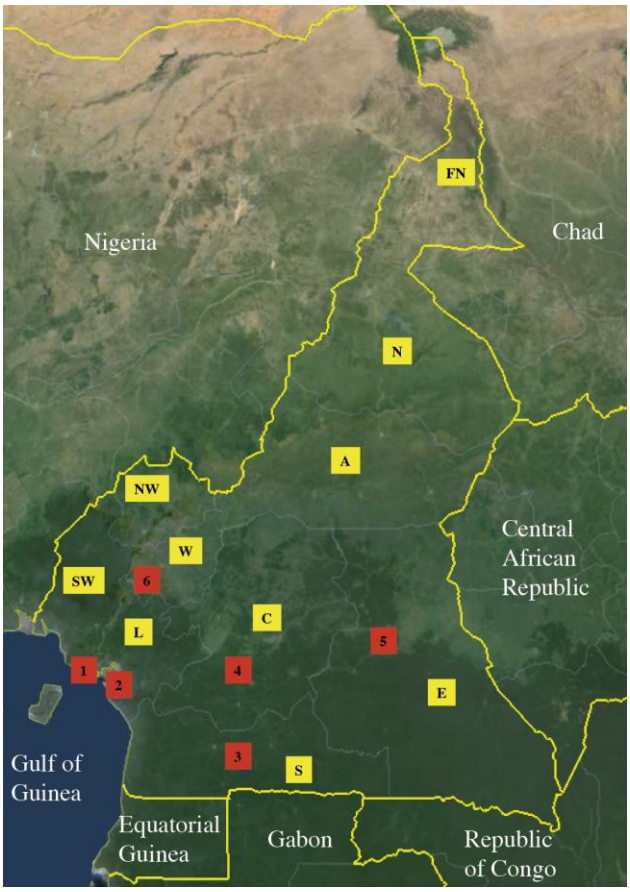


Figure 4: *Pv* circulation in Cameroon (Russo *et al.*, 2017)

FN: Far North, N: North, A: Adamaoua, NW: North-West, SW: South-West, W: West, L: Littoral, C: Centre, S: South, E: East.

3. Causes and determinants of malaria occurrence

There are four *Plasmodium* species that cause malaria in humans: *Plasmodium falciparum (Pf), Plasmodium vivax (Pv), Plasmodium malariae (Pm), Plasmodium ovale (Po).* Some human cases of malaria have also occurred with *Plasmodium knowlesi (Pk),* a specie that causes malaria among monkeys and occurs in certain forested areas of South-East Asia. *Pf* and *Pv* are the most common. *Pf* is the most deadly and *Pv* the most geographically widespread (WHO, 2018).

A case of severe *Po* infection in a patient presenting jaundice, respiratory distress, severe thrombocytopenia, petechiae, and hypotension has been reported (Strydom *et al.*, 2014). Among the non-falciparum malarias, severe and threatening illness also frequently occurs with both *Pv* and *Pk* (Cox Singh *et al*., 2008; Kochar *et al*., 2005).

Environment is the key determinant of malaria transmission and endemicity. It refers to the climate (temperature and precipitations), the landscape (hills, mountains, valleys and

more), the vegetation, the urbanisation, the population density and distribution and more (Bigoga *et al.*, 2007; Mbenda *et al.*, 2014). These factors influence the presence of malaria vectors which helps defining areas as high or low risks for malaria transmission (WHO, 2013a; WHO Global Malaria Programme, 2017). Climate change is therefore a big dilemma since the direct effects on the environment appear strongly and play a major role in infectious diseases transmission (Patz *et al*., 2008). There is relationship between house characteristics and malaria vector biting risk, an evidence shown in Tanzania (Kaindoa *et al.*, 2018).

Moreover, Malaria occurrence is linked to economy, social organization, belief system and behaviours (Brown, 2017; Heggenhougen *et al*., 2003). A really surprising determinant of malaria occurrence was found in Indonesia. In this study, the presence of livestock in households is shown to play a more efficient role in preventing malaria, than prophylaxis (Hasyim *et al.*, 2018).

Biological factors also determine malaria occurrence in various groups of population. Duffy antigen receptor for chemokines (DARC)-coding gene is polymorphic with multiple alleles which encode for the two antigens – Fya and Fyb; the codominant alleles being FY*A and FY*B. Four genotypes are possible, Fy(a+b+), Fy(a+b-), Fy(a-b+) and Fy(a-b-). The last genotype results from a point mutation, -33T>C, in the promoter region of allele FY*B (Mendes *et al*., 2011). People with the three other genotypes have an increased risks of being infected by *Pv* malaria. The human Duffy antigen act as receptors for *Pv* Duffy binding protein-1 (*Pv*DBP-1) to invade red blood cells (RBCs) (Zimmerman *et al*., 2013). Africans are generally Duffy null and non-endemicity of *Pv* in Africa has been attributed to this fact. However, recent reports describing *Pv* infections in Duffy-negative Africans from West and Central parts of Africa have surfaced including Cameroon. This suggests that there could be a different pathway through which *Pv* invades RBCs (Gunalan *et al*., 2016; Mendes *et al*., 2011). The first molecular evidence of *Pv* in Cameroon was done in both Duffy positive and negative individuals. This suggest that there might be a significant prevalence in this country (Fru-Cho *et al*., 2014).

Looking at the available data, the picture of Duffy negative- Fy (-a-b)- frequency is surprisingly overlapping with *Pv* circulation in sub-Saharan Africa (figures 5 and 6).

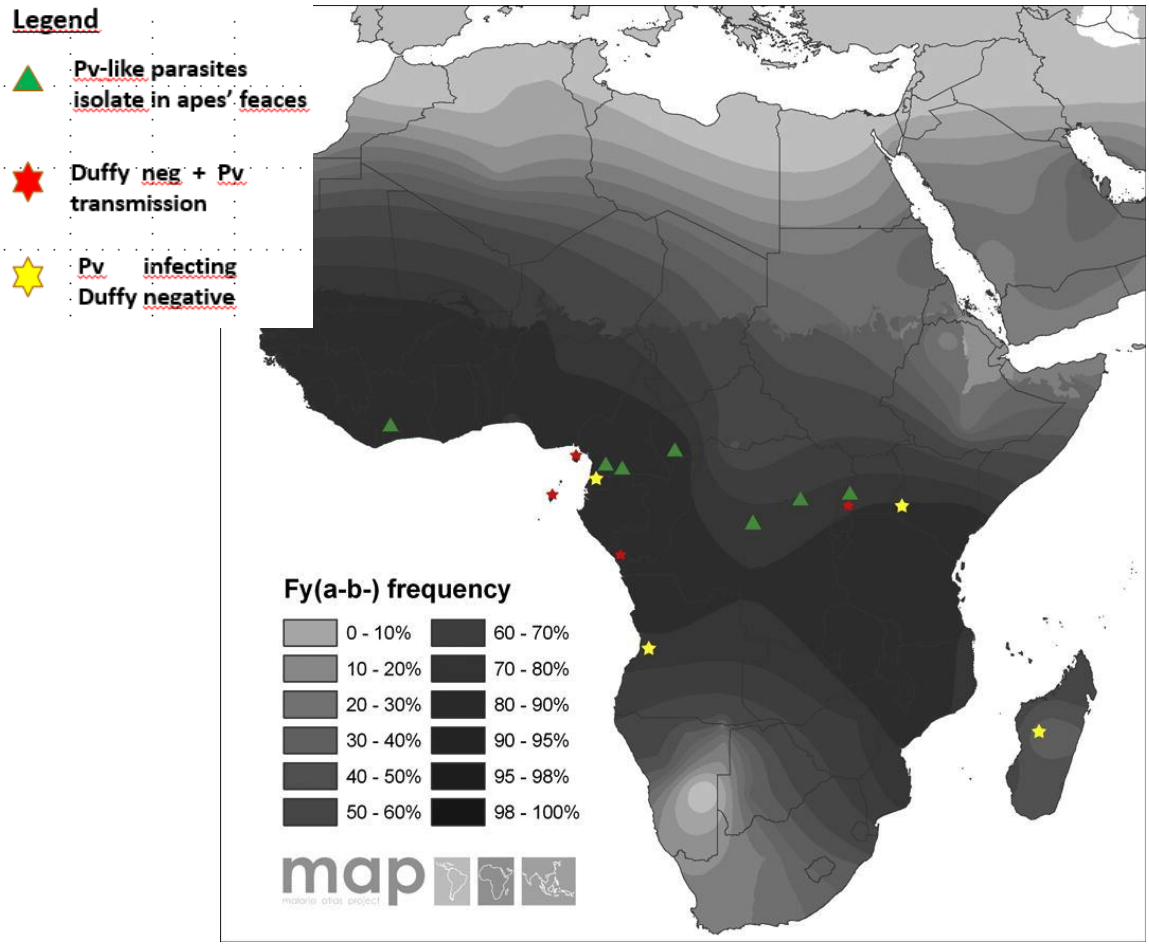


Figure 5:.Frequency of Duffy negativity in Africa, and the locations of surveys reporting evidence for the transmission of *Pv* in local human populations (Culleton and Carter, 2012).

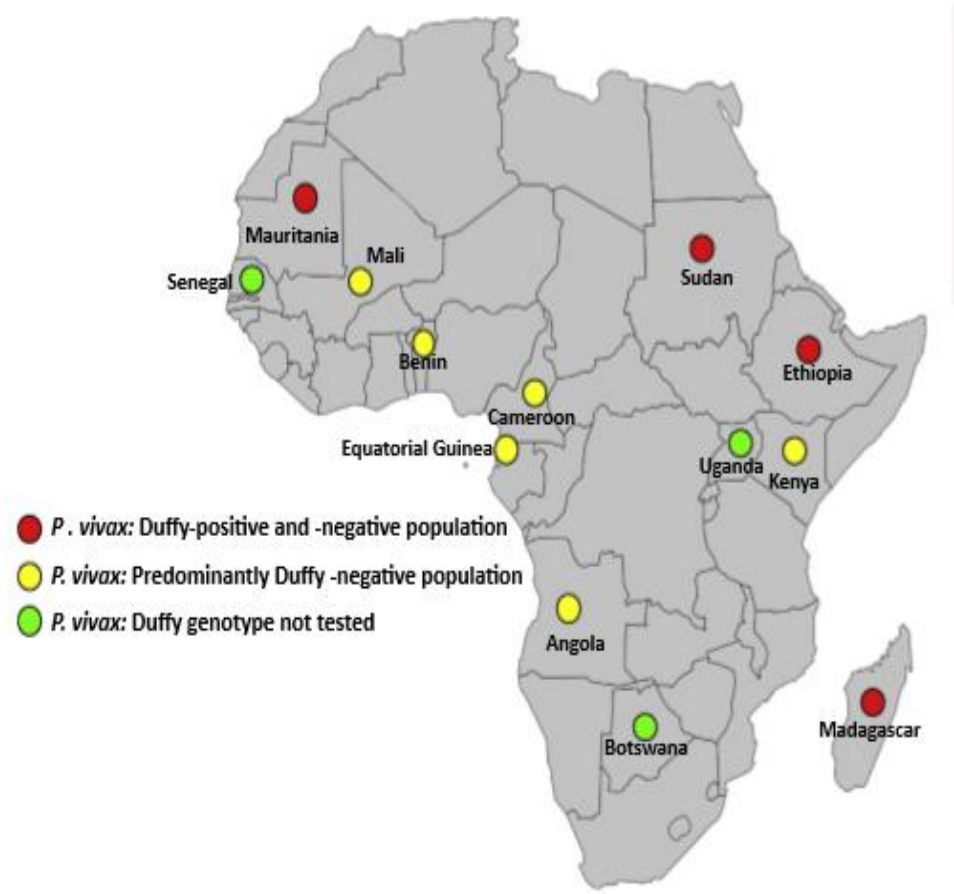


Figure 6: .*Plasmodium vivax* Infection in Duffy-Negative Populations in Africa (Gunalan *et al*., 2018).

More than 400 million people worldwide are thought to be G6PD deficient. RBCs with low G6PD activity offer a hostile environment to malaria parasite growth, since the deficiency reduces the oxidative stress. (Wajcman and Galactéros, 2004). Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency, referred to as G6PD A-, is commonly found in people living in malaria-endemic countries, since it is known to be a protective factor against malaria (Motshoge *et al*., 2018). G6PD A- has an estimated median allele frequency in these endemic areas of 8.0% (50% CI: 7.4-8.8) (Baird *et al*., 2018; Howes *et al*., 2013). A recent meta-analysis shows that G6PD A- can potentially protect against uncomplicated malaria in African countries, but not severe malaria and this protection is mainly in heterozygous (females), the gene being x-linked (Mbanefo *et al*., 2017). The deficient variants of the G6PD gene are distributed according to countries and malaria endemicity in Africa (figure 7).

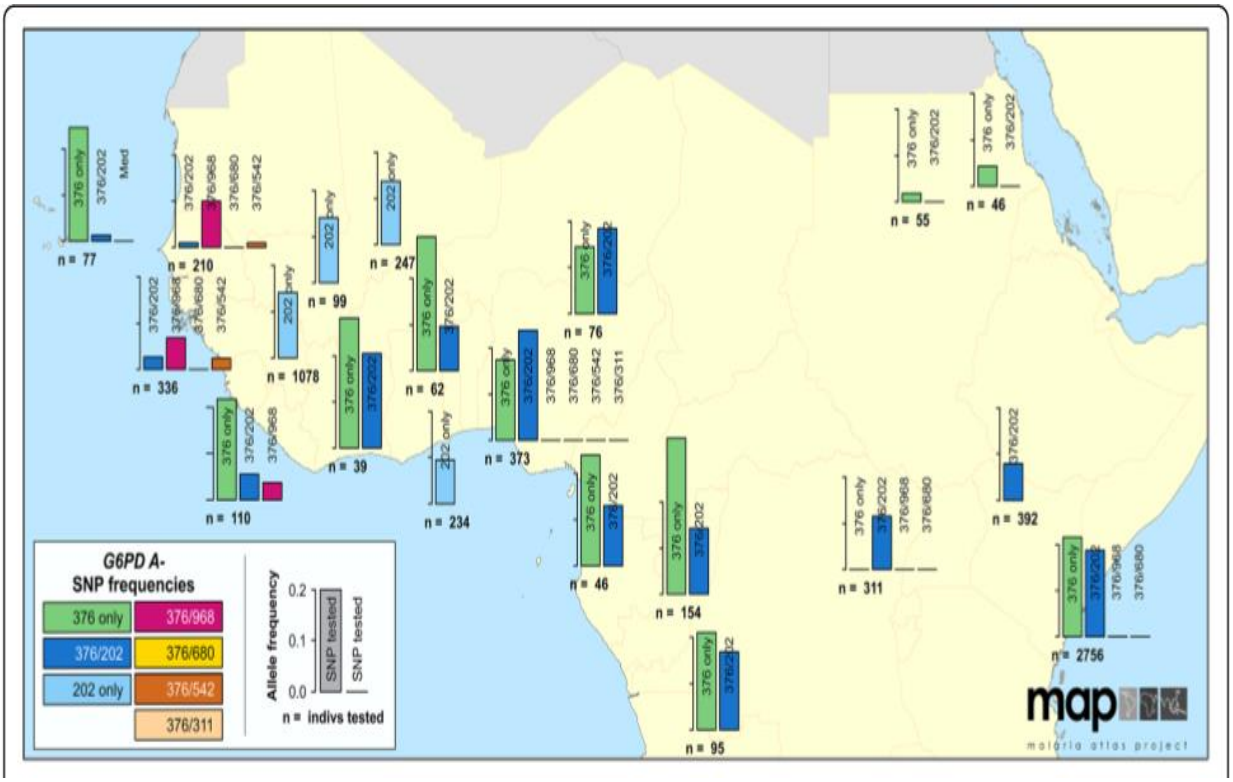


Figure 7: G6PD A- variant SNP frequency map for Africa (map series 2). (Howes *et al.*, 2013)

Bar charts representing the frequencies of G6PD variants. The variants that were tested for in each location are listed above the x-axis. Empty spaces along the x-axis indicate that the named variant was tested for but not identified in the population sample. Sample size is listed under each plot.

This distribution map shows available data in countries sharing borders with Cameroon, on SNPs 202 and 376. More studies are required on other SNPs frequencies to have a complete picture of the situation on G6PD A- in these countries and also in Cameroon, all having a long story with malaria, thus permitting to have a proper idea of the range of population "protected" against malaria.

4. *Pv* malaria diagnosis

Early diagnosis and treatment of malaria reduces disease and prevents deaths. It also contributes to reducing malaria transmission. WHO recommends all cases of suspected malaria to be confirmed, using parasite-based diagnostic testing (either microscopy or rapid diagnostic test-RDT), before administering the treatment. Results of parasitological confirmation can be available in 30 minutes or less (WHO, 2018).

According to Cameroon available guidelines, malaria diagnosis is performed either by malaria rapid diagnosis test (RDT), which detects specific antigens (proteins) produced by

malaria parasites, or by microscopy, the diagnostic test of reference for malaria, when a well-trained laboratory technician and well equipped laboratory are available (MINSANTE, 2017). RDTs are a relatively new innovation, an antigen based-stick, cassette or card test for malaria in which a coloured line indicates the presence of plasmodial antigens (Guerra *et al*., 2006). These tests are easy to use and do not require laboratory equipment or specialist skills (Wongsrichanalai *et al*., 2007). Recommendation of use of RDTs has raised the challenge of deciding on the proper treatment of febrile illness when the RDT result is negative (figure 8), especially for healthcare facilities not having microscopes (Bisoffi *et al*., 2009; Mma *et al*., 2017; WHO, 2013b).

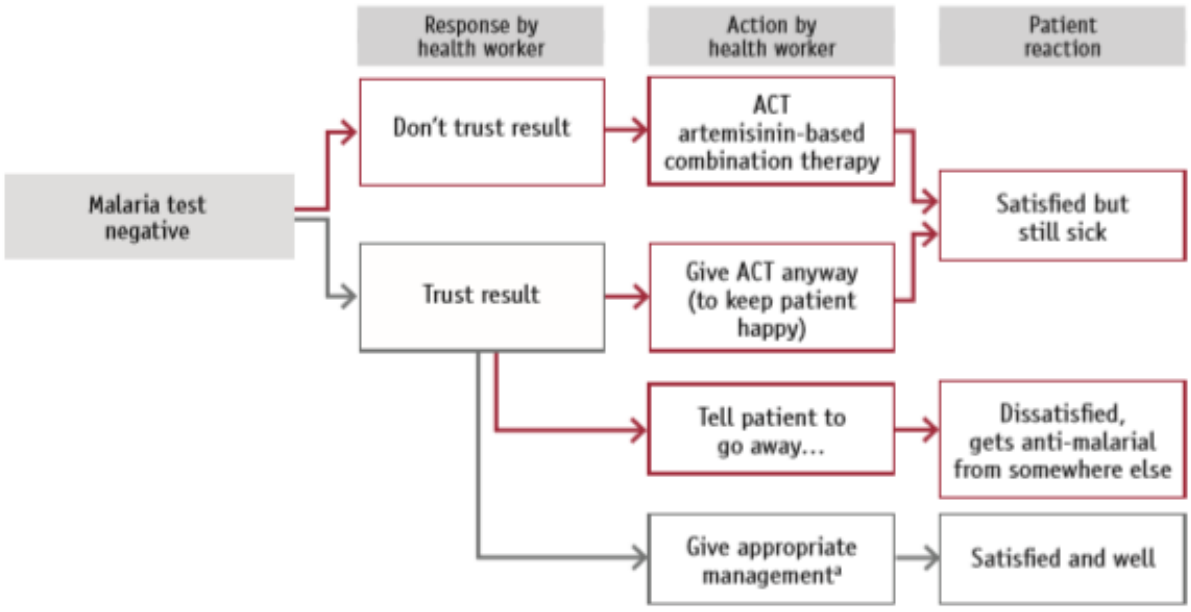


Figure 8: **Choices faced by health care workers when the results of a malaria RDT is negative (WHO, 2013b)**

Nevertheless, RDTs are available in remote areas where there is no power supply. In sub-Saharan African countries, where WHO assumes the significant circulation of *Pf*, only RDTs sensitive to the Histidine rich protein 2 and 3 (HRP-2 and HRP-3) are available. For this reason, it becomes more difficult to assess the circulation of all malaria parasites in these countries if not through research. HRP-2 and HRP-3 are genes which can easily be subject to deletion or mutation, making malaria diagnosis in the healthcare facilities more challenging (Tahar *et al*., 2013; Verma *et al*., 2018).

Microscopy is still used frequently as confirmation test even if it highly depends on the availability of well-trained lab technicians (WHO, 2013b). Both thick and thin blood films should be prepared for a more accurate diagnosis. The thick blood film provides enhanced sensitivity of the blood film technique and is much better for detection of low levels of parasitemia and reappearance of circulating parasites during infection recrudescence or relapse. The thin blood film allows the morphological identification of the parasite and is often preferred for routine estimation of the parasitemia because the organisms are easier to see and count (figure 9). Moreover, a study from Kenya confirms the high sensitivity of thin film for parasite identification (Moody, 2002, Ohrt *et al*., 2008). Still, microscopy can be less efficient with low parasitemia making it an imperfect gold standard. In fact, *Pv* malaria symptoms appear with a very low parasitemia to respect to *Pf* malaria (Ohrt *et al*., 2008).

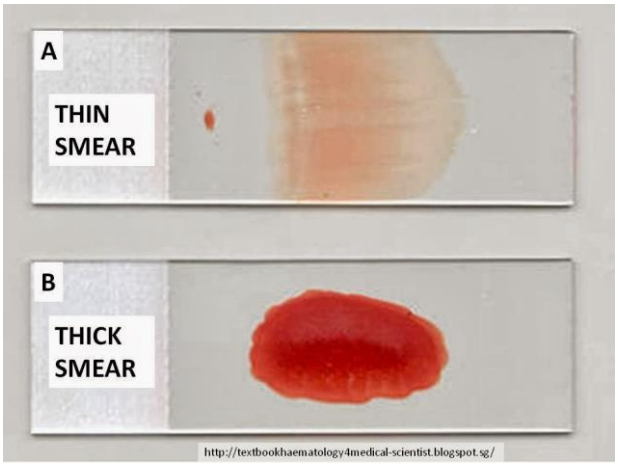


Figure 9: A-thin film; B-thick film

The need of obtaining results quickly from the examination of blood samples from patients with suspected acute malaria makes more sensitive methods for malaria diagnosis impractical for routine laboratory use. Nevertheless, due to challenges of proper diagnosis of low parasitamia cases of malaria routine technics and need for in-depth research concerning various species of malaria parasites, more sensitive methods such as enzyme linked immuno-sorbent assay (ELISA), polymerase chain reaction (PCR) and real-time PCR (RT-PCR) are used.

ELISA is a useful diagnostic tool which, however, has only limited application in malaria diagnostics and research since it focuses in detecting malaria parasites antigens and measuring malarial antibody titres. It allows the testing of large numbers of samples within

a short time frame (Sirichaisinthop *et al*., 2006). The DiaMed ELISA malaria antibody test, an ELISA kit for malaria screening developed more than 10 years ago, is based on binding of anti-*Plasmodium* antibodies present in a serum sample to antigens immobilized on 96-well plates (Doderer *et al*., 2007). ELISA method is also used by entomologists for malaria parasite detection (circumsporozoite proteins-CSP) because of the huge amount of mosquitoes they have to screen, its low cost, and its easy processing (Bigoga *et al.*, 2007; Kerah and Gru, 2006).

Amplification of DNA by the PCR has provided the opportunity to devise highly sensitive methods of malaria parasite detection. A single parasite can be reproducibly detected in the DNA template directly purified from 10 μL of blood (0.000002% parasitemia). The genes of the small subunit ribosomal RNA (ssrRNA) have been characterized from a wide range of *Plasmodium* species. This method is made of two consecutive amplification reactions. In the first amplification reaction (Nest 1), a pair of oligonucleotide primers (genus specific rPLU1 and rPLU5), which will hybridize to sequences in the ssrRNA genes of any *Plasmodium* parasite, are used to amplify a 1.6–1.7 kb fragment of these genes (the size varies with the species). The product of this first reaction is then used as the DNA template for a second amplification reaction (Nest 2), in which the oligonucleotide primers used recognize sequences contained within the DNA fragment amplified in the first reaction (Snounou and Singh, 2002). Conventional PCR is mainly qualitative.

The quantitative PCR is the RT-PCR, which can be very useful in epidemiological studies, drug trials or human diagnosis. The great disadvantage of this method in routine use or high-throughput analysis is the cost of the consumables. A multiplex RT-PCR was developed to detect *Pf, Pv* and *Pm* together. It uses primers and a probe specific for 18S rRNA of each *Plasmodium* specie (Veron *et al.*, 2009).

The specificity and the sensitivity of these various diagnosis technics are continuously assessed and more innovative technics are been developed such as the Malaria System MicroApp or the xRapid-Malaria: the automated diagnostic test for smartphones (Muzamil Abdel Hamid, Mona Awad-Elgeid, Nasr, 2017; Ohrt *et al*., 2008; Singh *et al*., 2014; Sirichaisinthop *et al*., 2006; Veron *et al*., 2009; Walk *et al*., 2016). Such assessment is useful to continuously improve the routine diagnosis.

5. Physiopathology and immune response of *Pv* malaria

5.1. Physiopathology of Pv malaria

Pv parasite life cycle occurs in three phases, the sporogonic cycle (mosquito), the exo-erythrocitic cycle (liver) and the erythrocytic cycle (RBC). It includes a special pathway producing hypnozoites (dormant liver stage) which is absent in *Pf* malaria (figure 10).

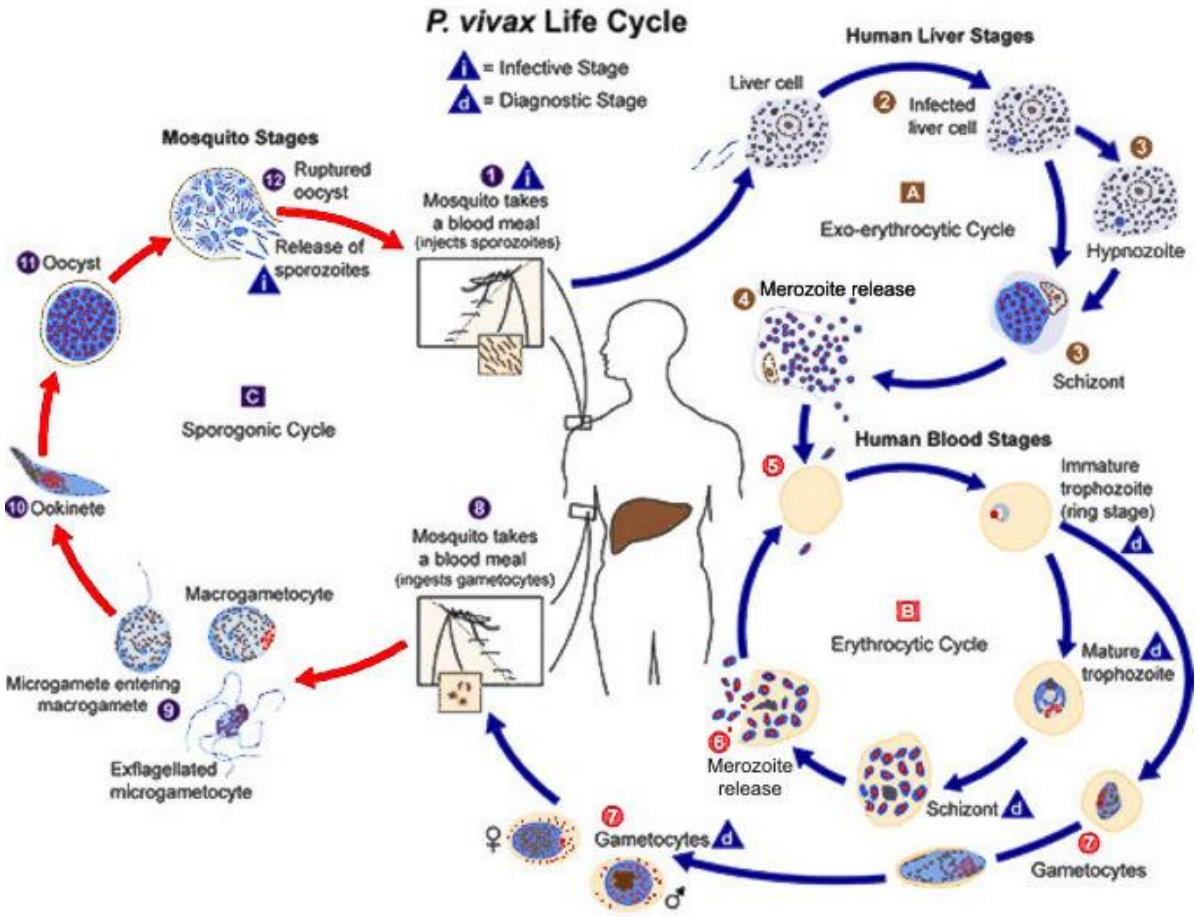


Figure 10 : *Pv* life cycle (source : https://www.cdc.gov/malaria/about/biology/index.html)

Pv has a preference for infecting immature RBCs, a property that seems to limit its reproductive capacity. Parasitemia in *vivax* malaria rarely exceeds 2% of circulating RBCs. Cytokine production during *Pv* infections is higher than that in *Pf* infections of similar parasite biomass. *Pf* 'toxins' thought to cause fever include glycosylphosphatidylinositol (GPI). It is not known whether there are structural differences in the *Pv* GPI that make it

more pyrogenic. These "toxins" are released together with RBCs merozoites (Price *et al*., 2009).

Fever is one of the most common symptoms of infectious diseases which is also present in case of malaria (Mma *et al*., 2017). It is also referred to as common neurologic event among children worldwide which is more common in tropical regions (Birbeck, 2010) and is the leading reason for health-care-seeking in resource-limited countries (Baltzell *et al*., 2013; Birhanu *et al*., 2016; Higgins-Steele *et al*., 2015; Kassile *et al*., 2014; Tinuade *et al*., 2010). *Pv* is known to be less symptomatic than *Pf*, still uncomplicated and severe cases of symptomatic *Pv* infected patients have been documented (Elizalde-Torrent *et al*., 2018; Price *et al*., 2009).

Pf infection of the liver leads to a single blood-stage infection. "Infection" in *Pf* context is an event which is singular and clear, whereas the same in *Pv* becomes a complex plurality and ambiguity in an epidemiological sense (Figure 11).

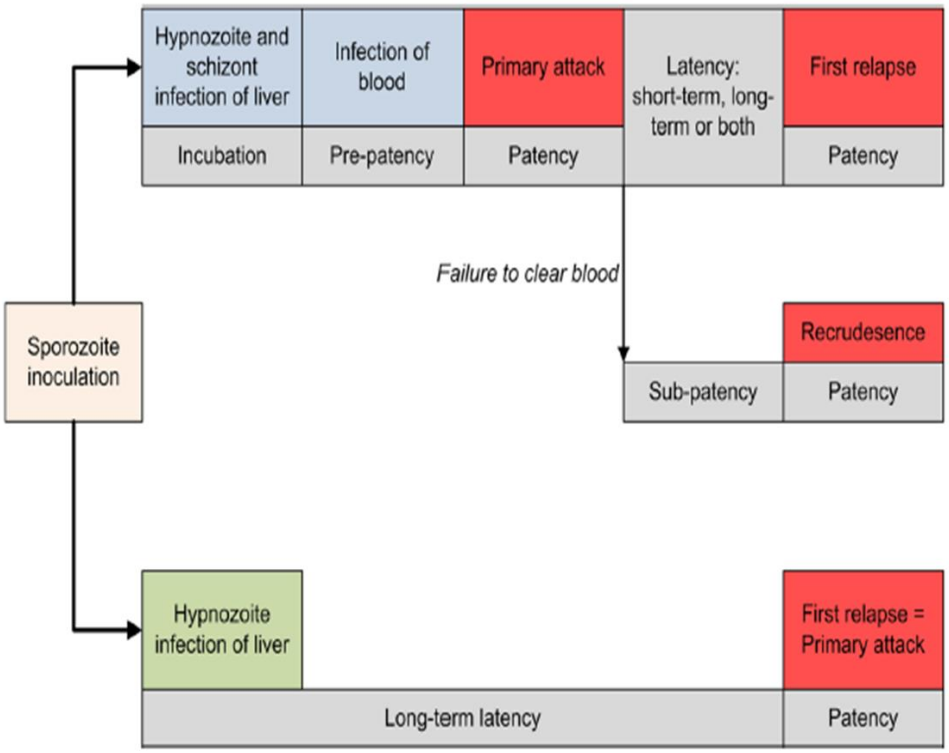


Figure 11: Pathways to infection of blood and clinical attacks in *Pv* malaria (Howes *et al*., 2016)

Potential mechanisms underlying the syndromes of uncomplicated and severe vivax malaria were assessed through a review and summarised in the table I.

Table I : Pathobiology of *Pv* and potential mechanisms of severe disease (Price *et al.*, 2009)

Pathobiological process in *P. vivax*	Potential contribution to severe malaria
Destruction of non-infected RBCs	Severe anemia despite small parasite biomass
Invasion and destruction of reticulocytes	Very short RBC lifespan and failure to replace destroyed RBCs: contribution to severe anemia
Increased fragility of infected and non-infected RBCs	Severe anemia
Probable faster parasite growth rates in chloroquine-resistant *P. vivax*	Severe anemia
Possible pooling of RBCs in the spleen	Potential contribution to anemia
Increased deformability of infected RBCs	Unlikely to contribute to microvascular obstruction or severity
Relapse	Recurrent hemolysis and severe anemia
Recrudescence of chloroquine-resistant *P. vivax*	Recurrent hemolysis and severe anemia
Greater cytokine production relative to *P. falciparum*	Organ-specific inflammation, including increased alveolar-capillary membrane permeability and acute lung injury Hypothesized contribution to impaired utero-placental circulation and low birth weight Dyserythropoiesis and anemia Endothelial activation Greater hemodynamic compromise in setting of acute or chronic comorbidity
Rosetting	Potential contribution to hypothesized but as-yet uncharacterized microvascular obstruction and end-organ pathology
Leukocyte aggregation by paroxysm plasma	Possible organ-specific inflammation, including acute lung injury Possible contribution to hypothesized but as-yet uncharacterized microvascular obstruction and end-organ pathology
Maternal anemia	Low birth weight
Splenic hematoma (±trauma) and thrombocytopenia	Splenic rupture
Less well characterized processes	
Putative cytoadherence of *P. vivax*-infected RBCs	Possible targeting of inflammatory responses to the lung: acute lung injury Speculative contribution to hypothesized, but as-yet uncharacterized, microvascular obstruction and end-organ pathology
Endothelial activation, dysfunction and/or injury	Increased alveolar-capillary permeability and acute lung injury Impaired utero-placental circulation and low birth weight Possible contribution to hypothesized, but as-yet uncharacterized, microvascular obstruction and end-organ pathology
Altered thrombostasis, thrombocytopenia and microvascular thrombosis	Possible contribution to hypothesized but as-yet uncharacterized microvascular obstruction and end-organ pathology
Comorbidities	Potential for a non-fatal comorbidity to become fatal with the fever and anemia of acute vivax malaria exacerbating hypoxia and/or hemodynamic compromise.

5.2. *Anti-vivax Immunity*

It is obvious that an infectious disease with a huge impact like malaria should be fought with multiple strategies. Few studies have explore the immune response of the human body when exposed to *Pv*. Vaccine development is considered one of the most promising strategies for controlling malaria, despite the great genetic variability of malaria parasites with multiple strains (some already identified and others not). The *Pv*12 protein, expressed on merozoite surface, is involved in RBCs invasion and has been recognised by antibodies from individuals exposed to the *Pv* infection. Two regions of this protein (containing peptides 39038, 39040, 39113 and 39117) have been characterised as promising vaccine candidates against *Pv* malaria (Yepes-Perez et al., 2018).

Trials on *Pv* vaccines candidate are being carried on here is a summary of one of them. After sporozoites have been inoculated into the skin by *Anopheles* mosquitoes, they travel to the liver via the bloodstream and enter hepatocytes thereby initiating the preerythrocyte stage. *Pv* circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (PvTRAP) are involved in hepatocyte recognition and binding in a mammalian host. In CSP, the N-terminal and repeat region facilitate parasite binding to hepatocytes. Adaptive immune responses against PvCSP and PvTRAP control invasion of hepatocytes by cytokines [CD4+ T-helper 1 (Th1) and CD4+ T-helper 2 (Th2) cells], cytophilic antibodies, and CD8+ T-cells. Interferon gamma (IFN-γ) increases and interleukin (IL)-4 decreases after vaccination with CSP-long synthetic peptides [CSP-LSP-N terminal; CSP-LSP-R (repeat region), and CSP-LSP-N terminal]. Cytophilic antibodies (IgG1 and IgG3) are produced after vaccination with CSP-LSP-N; CSP-LSP-R. Immunization with PvCSP recombinant vaccine (VMP 001) combined with CpG10104 has induced protection and activation of B-cells, macrophages (MΦ), and dendritic cells (DCs). When this recombinant vaccine is formulated with glucopyranosyl lipid A (GLA), there is activation of CD4+ T-cells, production of tumor necrosis factor-alpha (TNF-α), and reduction of IL-2. Immunization with PvTRAP, expressed in viral vectors, induces activation of CD8 T-cells and production of IFN-γ, TNF-α, and IL-2 (figure 12) (López *et al*., 2017).

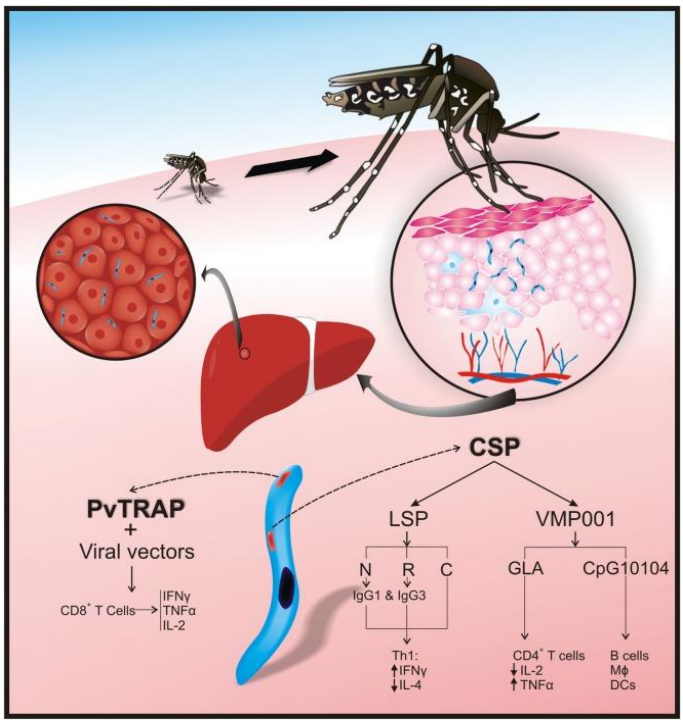


Figure 12: *Pv* preerythrocyte stage protein immunogenicity (López *et al*., 2017)

Polymorphism of the DARC is associated with susceptibility to and the severity of *Pv* malaria in humans. The frequency and level of naturally-acquired antibodies against the PvMSP1 (Pv erythrocytic antigens merozoite surface protein 1) and PvDBP varies with the host DARC genotypes. The lower IgG3 and IgG1 components of the total IgG suggested a down-regulating humoral responses against erythrocytic invasion and development with FY*A/FY*B and FY*B/FY*B genotypes (Maestre *et al.*, 2010). These studies ameliorate the understanding of the immune response towards *Pv* when it comes to RBCs invasion and vaccine candidates operative mode. Still few is known concerning the real mechanism towards infected RBCs and need to be deeply explored (Chuangchaiya *et al*., 2010).

There is huge gap concerning the understanding of the markers of *Pv* parasites which helps in differentiating the parasites responsible for a primary infection to respect to the parasites inducing relapses. Moreover, very few things are known concerning the relapses and their induction mechanisms (immunity might be playing a role). Which factors are implicated in the hypnozoites re-activation?

6. Treatment of *Pv* malaria

6.1. Recommended therapeutic protocols for Pv malaria

Chloroquine is the first-line treatment for *Pv* malaria in most endemic countries, but resistance is increasing. Monitoring of antimalarial efficacy is essential, but in *Pv* infections the assessment of treatment efficacy is confounded by relapses from the dormant liver stages (Bright *et al*., 2013). This currently exacerbates the wastage of antimalarial drugs and leads to over-treatment and consequent risk of possible toxicity and parasitic resistance insurgence (Anderson *et al*., 2016; Takala-harrison and Laufer, 2015; WHO, 2013b).

The most striking and potentially dangerous of the above-mentioned consequences may be appreciated by considering the chemotherapy against dormant forms of *Pv* in the liver (called hypnozoites). Primaquine (PQ), an 8-aminoquinoline antimalarial-drug, is the only drug currently indicated to treat relapsing strains of *Pv*. These strains cause multiple clinical attacks over months (up to approximately 2 years) following a single infectious bite by the anopheline mosquito vector (Ashley *et al*., 2014; Bright *et al*., 2013; Chu and White, 2016).

Tafenoquine, another 8-aminoquinoline antimalarial-drug, is a newly US food and drug administration (FDA) approved drug for prevention of relapse of vivax malaria. It is a synthetic analog of primaquine which has a long elimination half-life (average terminal half-life, 15 days). The drug is active against all stages of *Pv*, including the dormant liver hypnozoite and has slow clearance of blood stage parasites, so it is taken in combination with a faster-acting blood schizonticide, chloroquine (Watson *et al*., 2018).

6.2. Pharmacogenetics factors linked to Pv malaria treatment

PQ has been in continuous use for over 60 years, but this extraordinary longevity should not be misconstrued as lasting suitability: it is a seriously flawed drug, causing a mild to severe acute hemolytic anemia in patients with an inborn deficiency of glucose-6-phosphate dehydrogenase (G6PDd). This highly diverse and complex X-linked disorder affects approximately 500 million people, most of them living in regions where malaria is endemic and where the prevalence of G6PDd averages 8% (Baird, 2018; Baird *et al*., 2018). Nevertheless, a study highlighted the fact that primaquine could lead to acute hemolysis even when the patient is not G6PDd (Shekalaghe *et al*., 2010).

Human cytochrome P-450 isoenzyme 2D6 (CYP2D6) is an enzyme responsible for the metabolism and elimination of approximately 25% of clinically used drugs. It is clearly established to be the key enzyme involved in metabolizing primaquine/tafenoquine into redoxactive metabolites against hypnozoites in the liver (Marcsisin *et al*., 2016; Potter *et al*., 2015; Pybus *et al*., 2013; Watson *et al*., 2018). However, the genomic variation, including the Cytochrome P450 (CYP450) super-family, affects the safety and efficacy of therapeutic drugs. *CYP2D6* gene accounts for 120 variants already identified and categorized. Most of these variants studied among Caucasians have very few data available for black Africans (Gaedigk *et al*., 2018, 2017). A translation of the genotypes into the estimation of the phenotype through the activity score (AS) was developed and adopted but the scientific community. Till date five main metabolizers groups are known, poor, intermediate, normal, extensive and ultra-rapid with the activity score ranging from 0 to 4 , 0.5 being the interval between each score (Baird, 2018; Gaedigk *et al*., 2017).

Mono-amino-oxidase-A (*MAOA*) gene could be associated with behavioral or physiological variability in humans. Several different polymorphisms have previously been identified (Balciuniene *et al*., 2001; Lung *et al*., 2011; Sabol *et al*., 1998). MAOA and CYP2D6 enzymes are known to the involved in the metabolism of primaquine, producing respectively carboxy/aldehyde and phenolic metabolites (Marcsisin *et al*., 2016) (figure 13).

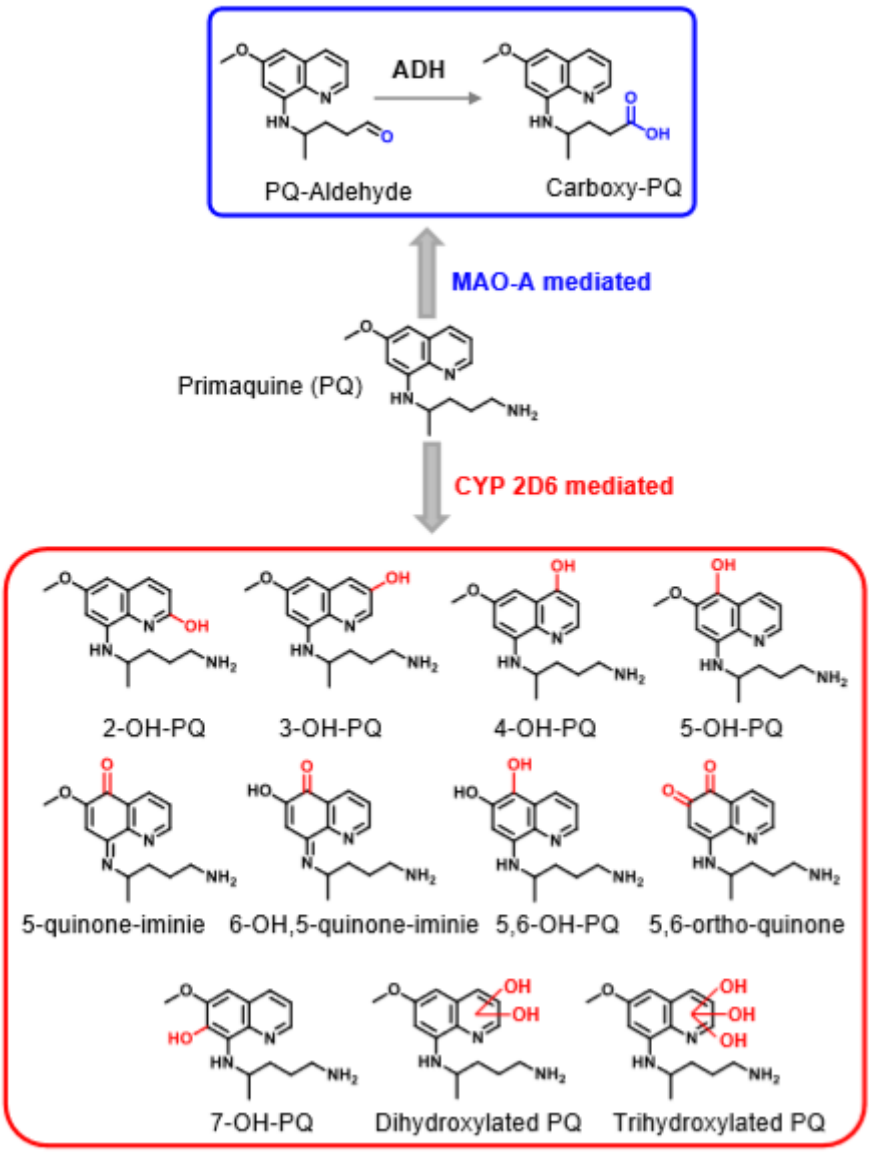


Figure 13 : Primaquine CYP2D6 and MAOA mediated metabolic pathways (Marcsisin *et al.*, 2016)

The primaquine aldehyde and subsequent carboxy metabolite formed through MAO-A metabolism is highlighted by the blue box. The primaquine phenolic metabolites produced through CYP 2D6 metabolism are highlighted by the red box.

The important variability of *CYP2D6* gene makes some groups more reactive to primaquine treatment than others and even more susceptible to toxicity. As part of the genetic background, it is compulsory to consider G6PDd (common in malaria endemic areas) as an exacerbating situation which could lead to death. This raises the question of the eligibility and ineligibility to primaquine treatment against liver stage *Pv* parasites (Baird *et al.*, 2018) (figure 14).

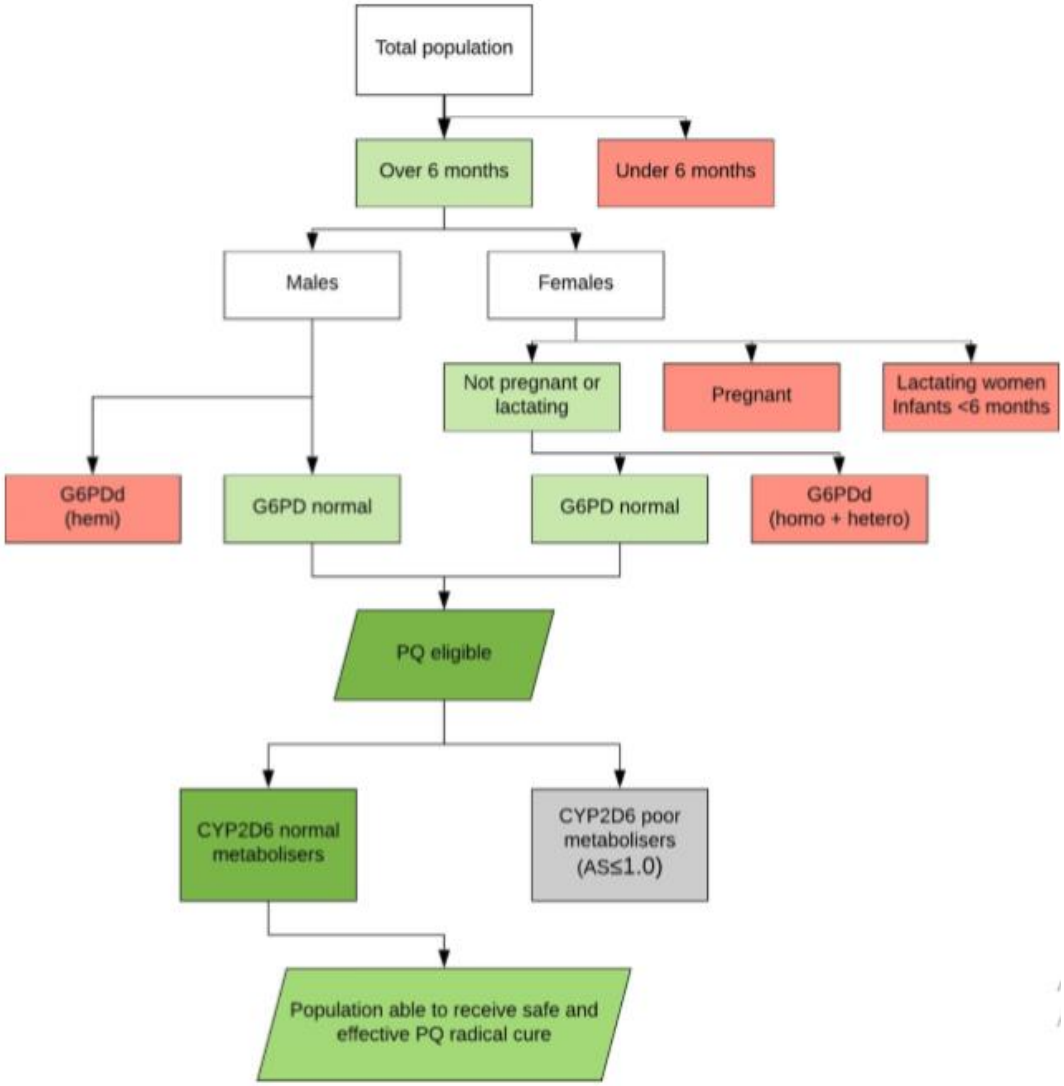


Figure 14: Stepwise exclusions of population subsets ineligible for Plasmodium vivax radical cure by primaquine (Baird *et al*., 2018).

Red boxes indicate excluded subsets, while green are eligible. AS: activity score, G6PDd: G6PD deficient, hemi: hemizygote, hetero: heterozygote, homo: homozygote, PQ: primaquine

7. Transmission of *Pv* malaria

Malaria is transmitted exclusively through the bites of *Anopheles* mosquitoes. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment (Atangana *et al*., 2009). About 20 different *Anopheles* species are locally important around the world. All the important vector species bite at night (Erlank *et al*., 2018). *Anopheles* mosquitoes breed in water and each species has its own breeding preference; for example some prefer shallow collections of fresh water, such as puddles, rice fields, and hoof prints. Transmission is more intense in places where the mosquito lifespan is longer (so that the parasite has time to complete its development inside the

mosquito) and where it prefers to bite humans rather than other animals. For example, the long lifespan and strong human-biting habit of the African vector species is the main reason why about 90% of the world's malaria deaths are in Africa (WHO Global Malaria Programme, 2017).

Cameroon has three malaria transmission seasons that vary by geographic region (see Figure 15). The forest zone (southern part) has a permanent transmission season of seven to twelve months. The N and A regions have a tropical climate but a shorter transmission season of four to six months. The FN region has a tropical and Sahelian climate with a short seasonal transmission period of one to three months (USAID, 2017).

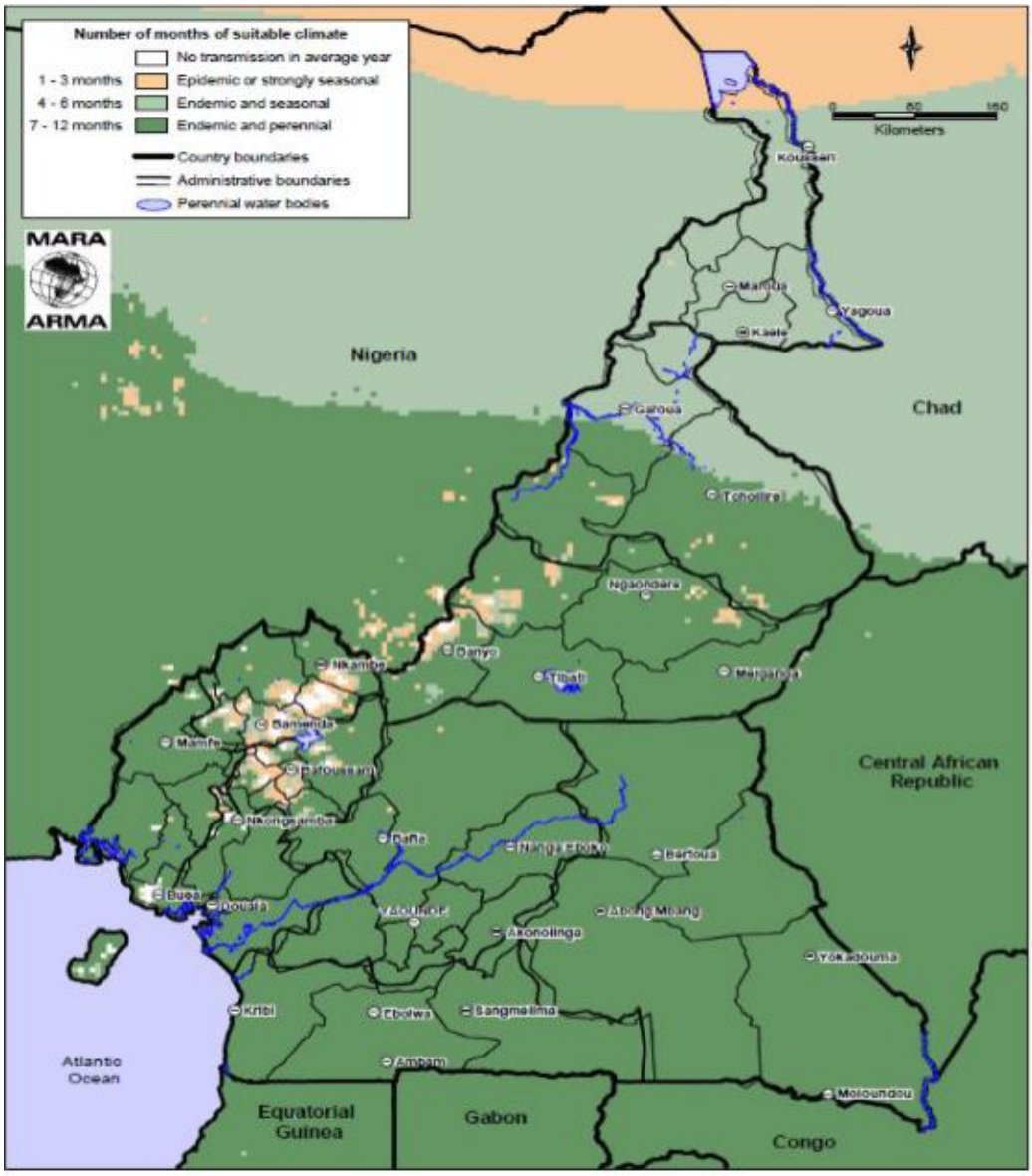


Figure 15: Malaria transmission pattern across Cameroon (USAID, 2017)

8. Prevention and control of *Pv* malaria

Still self-medication (Ocan *et al*., 2014) and non-adherence to WHO guidelines (Akinyode, *et al*., 2018) remain the most challenging aspects of malaria control and prevention. In malaria endemic countries such as Cameroon, fever is perceived as malaria both by caretakers and care-seekers (Birhanu *et al*., 2016; Hertz *et al*., 2013; Kassile *et al*., 2014) and occurs to be treated with antimalarial drugs even if the rapid diagnosis test (RDT) result is negative (Akinyode, *et al*., 2018; WHO, 2013).

New data from improved surveillance systems in several countries in the WHO African Region indicate that the number of malaria cases presented in this year's report are conservative estimates. WHO decided to review its malaria burden estimation methods for sub-Saharan Africa in 2018 (WHO Global Malaria Programme, 2017).

PART1: OBJECTIVES, MATERIALS AND METHOD

1. Problem statement

Malaria has a serious burden in sub-Saharan African countries as Cameroon. It induces unproductive periods for symptomatic infected people and even death in all age groups. This is unacceptable in developing countries needing healthy people to work, on a daily basis in order to optimize available financial resources, for a better quality of life. There are malaria management guidelines available, but, these guidelines are shaped only for *Pf*. This is understandable since sub-Saharan Africa was assumed to be an area free from *Pv*, the local population being in majority Duffy null. With the surfacing reports of *Pv* transmission among Duffy null symptomatic and asymptomatic Africans in many sub-Saharan countries, it becomes a necessity to think of adjusting the guidelines. Especially because a single infection of *Pv* leads to several malaria episodes (relapses) and because of the hypnozoïtes (*Pv* liver stage) which are hard to clean with the classical antimalarial drugs. The challenge of "Rolling back malaria" becomes impossible if the population is full of reservoir hosts (*Pv* malaria being mainly "benign" or asymptomatic). It appears clearly that to help updating the malaria management guidelines in Cameroon, it is important to have a proper idea of *Pv* circulation in different areas and assess the feasibility of primaquine safety use to clear *Pv* liver stages. To find a solution to the above mentioned problem, the study stands on the following objectives.

2. Objectives and hypothesis

2.1. General objective

To contribute to the improvement of fever and malaria management in African resource-limited settings.

2.2. Specific objectives

a. To assess the possible circulation of *Pv* among febrile outpatients attending the selected healthcare facilities (HCFs);
b. To identify biological factors that can permit the assessment of the susceptibility to the *P. vivax* infection and the feasibility of its treatment (Duffy antigen, G6PDd, CYP2D6 variants) among those suffering for malaria in the study population;
c. Assess the specificity and sensitivity of rapid diagnostic test used routinely in Cameroon healthcare facilities to respect to polymerase chain reaction technique in malaria diagnosis;

d. Determine the factors of malaria occurrence as well as the fever management challenges at the community level among the study population

2.3. Hypothesis

From the specific objective 4, we could draw the following hypothesis.

Socio-economic, clinical, environmental and behavioral factors such as age, sex, type of malaria test in the healthcare facilities, self-medication household features, and mosquito's net usage are associated with the occurrence of malaria.

3. Materials and methods

3.1. Study design and period

It is a cross sectional descriptive and analytic study which was carried out using a structured questionnaire and blood sample collection on febrile outpatients coming for consultation in the selected healthcare facilities in the three health districts. Data was collected in two different periods, rainy season in Santchou (August 2016-December 2016) and rainy season in Dschang and Ambam (May 2017-September 2017).

3.2. Study area

The study was carried out in Santchou Health District (SHD), Dschang Health District (DHD) and Ambam Health district (AHD).

SHD is a health district made of five (05) health areas with ten (10) health centers. SHD is a rural area with an estimated population of 25,500 people in 2016. The most important activities are farming and trade. Given the geographical accessibility of some healthcare facilities and the attendance rates in various healthcare facilities, 02 healthcare facilities have been chosen (Santchou District Hospital, Santchou Catholic Health center) to carry out this study.

DHD as SHD is situated in the West Region of Cameroon. It is bounded to the South by the Santchou and Bandja Health District, Penka-Michel to the East, Batcham to the North-East and the South-West Region to the West and North-West regions. Santchou (5°15'N; 9°50'E) is located at an altitude of 750m at 22km from Dschang (5°27'N; 10°04'E) which altitude is 1400m. DHD population was estimated to be 226 563 inhabitants in 2016. It is made up of 22 Health areas with 56 Health Facilities: it has 01 District Hospital, 03 private hospitals, 01 socio medical center (the University of Dschang), 03 medicalized health

centers, 02 medical cabinets, 28 integrated health centers, 18 private health centers and 03 Day Care Hospitals. We have chosen the reference healthcare facility to collect samples (Dschang District Hospital) since the population comes from all areas in case of serious health issues.

AHD includes Kyé-ossi which is the area of interest. Kyé-ossi is a joint and exchange point between Cameroon, Equatorial Guinea and Gabon. Its population was estimated to be 26 102 inhabitants in 2016, who seek for care in 4 healthcare facilities (2 public and 2 private). Kyé-ossi (2°10'N; 11°20'E) is located at an altitude of 556m. The population is mostly made of traders and very few civil servants and farmers are mostly found in remote areas. We selected one principal healthcare facility to collect samples, the medicalized health center of Kyé-ossi. The geographical localization of the study sites is shown in figure 16.

Figure 16: Localisation of the study sites-red circles (*source: Corpenicus landsat image*)

3.3. Study population

Using the appropriate formula (Daniel, 1999) for cross-sectional studies in investigating qualitative phenomenon, which is fever, $$\text{Sample size} = \frac{Z_{1-a/2}^2 p(1-p)}{d^2}$$

Here

$Z_{1-a/2}$ = is standard normal variate (at 5% type 1 error ($P<0.05$) it is 1.96 and at 1% type 1 error ($P<0.01$) it is 2.58). As in majority of studies P-values are considered significant below 0.05 hence 1.96 is the value of $Z_{1-a/2}$.

p = Expected proportion in population based on previous studies or pilot studies. In this case p=42% which is the proportion of children having fever in health facilities according to the Ministry of Health Cameroon.

d = Absolute error or precision – 0.04 for this study.

After all calculation, the minimum sample size is 585. Considering that 10% of the participants could deny to participate, we have as minimum sample size 644 samples for the study to be statistically significant.

Since the target population lives in three different areas, the proportion of participants is distributed according to the population proportion in each area (**table II**).

Table II: Distribution of sample population by area

Health District	Total population in 2016	Percentage (%)	Sample size
Santchou	25 500	9.17	59
Dschang	226 563	81.45	525
Kyé-ossi	26 102	9.38	60
Total	278 165	100	644

An exhaustive sampling permitted to include every febrile outpatients in the study during the sample collection period.

Inclusion criteria: febrile outpatients of all age range coming for consultation in the selected healthcare facilities of SHD, DHD and AHD.

Exclusion criteria: febrile outpatients who withdrawn during the data/sample collection, patients which questionnaire was not having at least 70% of answers.

Non-inclusion criteria: febrile outpatients transferred from another healthcare facility, patient coming for the second time during the data/sample collection period and those who did not give their consent to participate to the study.

3.4. Recruitment process

Participants were recruited at the reception of the selected healthcare facilities after detecting a temperature above 37.5°C (fever) or reporting fever history the day before seeking for care. These patients were informed about the study, its objectives and all the requirements (blood collection and questionnaire answering) in order to obtain their written consent by signing the informed consent form. The languages used were principally French and English but in case the participant was not able to express himself in any of the above mentioned languages, a translator was available for *Bamiléké* language especially *Yemba* and for Southern language especially *Ntumu*. Those are the main local languages of the study areas. The informed consent form was signed in two copies and each participant kept his own copy. They were also aware of their possible withdrawal from the study while going through the process without any issue or consequences. The patients less than 21 years (Cameroonian legal age) old were enrolled after the authorization of their parents or guardians. All the consenting patients fitting with the criteria were enrolled in the study.

3.5. Data and samples collection

Authorizations were taken from the different health administrative and healthcare facilities administratives before any data or sample was collected. After obtaining the consent of the participants, field surveyors administered a two pages questionnaire by on the spot question and answer. The questionnaire administration lasted between 5-10 minutes. Data was collected on weekly bases, by trained field surveyors. For those not able to express themselves, the guardians or parents answered the questionnaire. The questions to answer encountered demographic data (age, sex, profession, matrimonial status), medical data (symptoms, previous or ongoing treatment), and living conditions including a description of the environment. Further information, documentary-based, were collected in the laboratory registers (malaria diagnosis test performed and results)

The blood samples were collected using filter papers (Whatman) and slides in the normal check-up scheme of the patients in the healthcare facility. For all patients needing to do complementary tests before final diagnosis, their blood was collected from the sample they

donated to the hospital laboratory. For healthcare facilities not having laboratories, the blood was collected through a finger prick. The blood samples collected was put on filter papers (in duplicates), dried (dried blood spots –DBSs) on a rack, kept in individual plastic bags and stored in boxes containing silica gel bags in a cool dry place at room temperature (figures 17 and 18).

A drop of blood was used to prepare a thin film and stained following the malaria microscopy standard operating procedure for the rapid (10% stain working solution) method (Gonzales, 2016). The prepared thin films were stored in slides boxes and kept at room temperature.

All the collected material (DBs and thin films) was transferred to the University of Botswana-University of Pennsylvania Joint molecular laboratory for analysis (DBSs only for human genetic) and the Istituto Superiore di Sanità (DBSs and thin films for parasite detection).

Figure 17: Field surveyor collecting blood sample

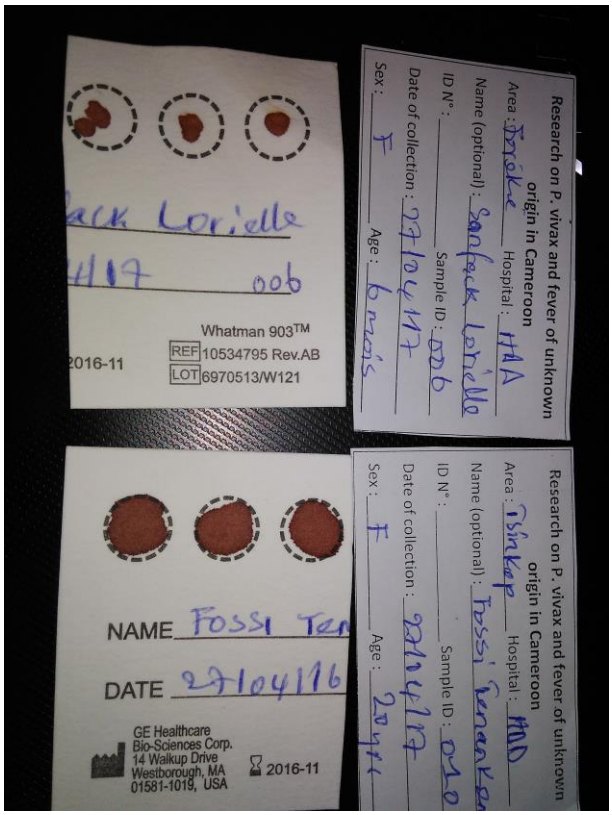


Figure 18: Samples collected

3.6. Sample analyses

3.6.1. *Deoxyribonucleic acid (DNA) extraction*

Human and parasite DNA was extracted from DBSs using an Invitrogen kit (PureLink™ Genomic DNA Mini kit) (Life tecnologies, 2013).

a. **Constitution of the Invitrogen PureLink™ Genomic DNA Mini kit**

- 10mL PureLink™ Genomic Lysis/Binding Buffer
- 9mL PureLink™ Genomic Digestion Buffer
- 10mL PureLink™ Genomic Wash Buffer 1
- 7.5mL PureLink™ Genomic Wash Buffer 2
- 10mL PureLink™ Genomic Elution Buffer
- 1mL Proteinase K (20 mg/mL)
- 50 each PureLink™ Spin Columns with Collection Tubes
- 100 PureLink™ Collection Tubes (2.0mL)

This kit contains enough reagents for 50 DNA preps.

The extraction protocol used was as follows:

b. **Preparing the Lysate**

Follow the procedure below to prepare a lysate from the 50-100 µl blood sample.

- Set a water bath at 55°C
- Place 5 punches of DBS (2-3 mm in size) in a sterile microcentrifuge tube
- Add 180µl of PureLink Genomic Digestion Buffer and 20µl Proteinase K (supplied with the kit) to the tube. Mix well by vortexing and ensure the pieces of paper are completely immersed into the buffer.
- Incubate at 55°C with occasional vortexing for 30 minutes and short spin.
- Prepare a master buffer/ethanol mix by mixing 200µl Lysis/Binding Buffer and 200µl of 99% ethanol for each samples
- Add 400µl of master buffer/ethanol mix to the lysate. Mix well by vortexing for 5 seconds and short spin.
- Proceed to Binding DNA.

c. **Binding the Lysate**

- Remove a PureLink™ Spin Column in a Collection Tube from the package.
- Load the lysate (~640 µL) with Lysis/Binding Buffer and ethanol prepared as described on pages 16–21 to the PureLink® Spin Column.
- Centrifuge the column at 10,000 rpm for 1 minute at room temperature.
- Discard the collection tube and place the spin column into a new collection tube.
- Proceed to Washing DNA.

d. **Washing DNA**

- Add 96–100% ethanol to PureLink™ Genomic Wash Buffer 1 and PureLink™ Genomic Wash Buffer 2 according to instructions on each label. Mix well. Mark on the labels that ethanol is added. Store both wash buffers with ethanol at room temperature.
- Wash the column with 500 µL Wash Buffer 1 prepared with ethanol.
- Centrifuge the column at 10,000 rpm for 1 minute at room temperature. Discard the collection tube and place column into a new collection tube.
- Wash the column with 500 µL Wash Buffer 2 prepared with ethanol.
- Centrifuge the column at 13700 rpm for 3 minutes at room temperature. Discard the collection tube.
- Proceed to Eluting DNA.

e. **Eluting DNA**

- Place the spin column in a sterile 1.5-mL microcentrifuge tube.
- Elute the DNA with 100 µL of PureLink™ Genomic Elution Buffer.
- Incubate the column at room temperature for 5 minute.
- Centrifuge the column at 13700 rpm for 2 minutes at room temperature. The tube contains purified DNA.
- Remove and discard the column.
- Store the purified DNA at 4ºC for immediate parasite detection and human genotyping and –20°C for long-term storage at the end of the analyses.

3.6.2. *Parasite detection*

Parasite detection was performed at the Italian Higher institute of Health, malaria research unit laboratory. The extracted DNA was used for nested conventional polymerase chain reaction (N-PCR), confirmation by real-time PCR (RT-PCR) and parasite DNA sequencing.

a. **Nested conventional PCR**

The PCR analysis of the four main *Plasmodium* species (*Pf*, *Pv*, *Po*, *Pm*) was performed by a nested PCR of a specific 18S ribosomal ribonucleic acid (rRNA) gene fragments as previously described (Snounou and Singh, 2002). Four positive control DNA for all Plasmodium species (laboratory strains for *Pf* 3D7, and isolates from sick patients for other species) and two negative (for the outer and nested PCR, respectively) were used to ensure the efficiency of the amplification. Specific primers were used for outer (Nest1) and nested (Nest2) PCRs (Figure 19).

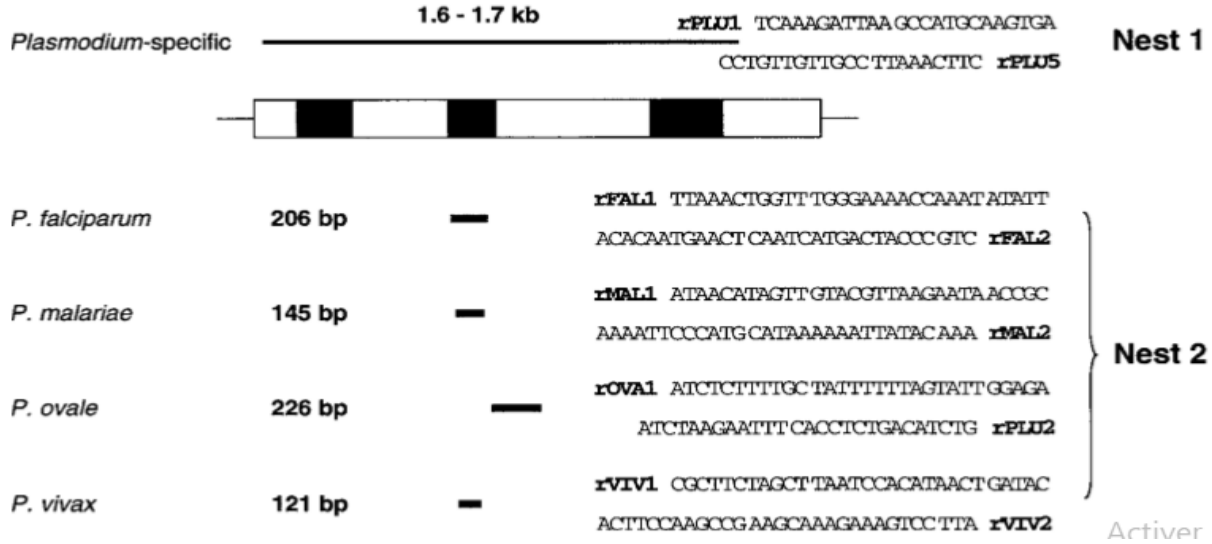


Figure 19 : Nest PCR analysis scheme and primers sequences (Snounou and Singh, 2002)

b. **Real-time PCR**

All the positive samples for *Pv* were selected for real-time PCR analysis using a protocol previously described (Veron *et al*., 2009). Briefly, RT-PCR was performed with a Light Cycler® 480 System (Roche) according to the following steps: pre-incubation 95°C for 10 min; amplification 95°C for 10 sec, 50°C for 20 sec and 72°C for 5 sec for 45 cycles.

PCRs was optimized according to the LightCycler® 480 Probes Master Kit, and 0.5 μM of each primer and 0.2μM of each probe, used in combination, was employed in this study (Table III).

Table III: **Real time PCR primers and probes**

Species	**Primer or probe**	**Sequence (5'–3')**
P. vivax	Pv-1	CGCTTCTAGCTTAATCCACATAACTG
	Pv-2	AATTTACTCAAAGTAACAAGGACTTCCAAG
	Pv-probe (VIC-MGB)	CGCATTTTGCTATTATGT

c. ***Pv*** **DNA Sequencing**

Samples, showing the best results with RT-PCR, were amplified using a protocol previously described (Koepfli *et al*., 2009). The target was specifically *Pv*MSP-1-*F3*. Positive results were obtained by amplification through outer and nested PCR with primers *Pv*MSP1-F3. The amplified samples were sent to EUROFINS genomics (Ebersberg, Germany) for sequencing.

d. **Microscopy**

Thin films, selected among samples showing *Pv* mono-infection results by PCR, stained with 10% Giemsa solution, were examined at x100 under oil immersion by two expert microscopists from the Istituto Superiore di Sanità. The examination aim was to identify *Pv* parasites in the slides.

3.6.3. Human genotyping

Human genotyping was performed at the University of Botswana-University of Pennsylvania Joint molecular laboratory. The extracted DNA was used for polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) and a single PCR. The targeted genes were *DARC* (-33T>C), G6PD (202G>A and 968 T>C), *CYP2D6**2 (2938 C>T), *CYP2D6**4 (1934 G>A) and *CYP2D6**17 (1023 C>T) (table IV).

Table IV: Human DNA polymorphisms subjected to genotyping analysis

Gene	Position SNP rs Number	Primers sequences (5'–3')	Restriction enzyme	Protocol applied
DARC	-33T>C rs2814778	AGGCTTGTGCAGGCAGTG GGCATAGGGATAAGGGACT	*StyI*- High fidelity	(Mendes *et al.*, 2011)
CYP2D6	*17/1023C>T rs28371706	TTTTGCACTGTGGGTCCTCGG CCCGGGTCCCACGGAAATCT	*FokI*	(Naveen *et al.*, 2006)
	*4/1934G>A rs3892097	CAAGAAGTCGCTGGAGCAGT GAGGGTCGTCGTACTCGAAG	*BstNI*	
	*2/2938C>T rs16947	GCTGGGGCCTGAGACTT GGCTATCACCAGGTGCTGGTGCT	*HhaI*	
G6PD	202 G>A rs1050828	TTACAGCTGTGCCCTGCCCT AGGGCAACGGCAAGCCTTAC	*NlaIII*	(Mombo *et al.*, 2003)
	968 T>C rs76723693	TCCCTGCACCCCAACTCAAC CCAGTTCTGCCTTGCTGGGC	*NciI*	(Hamel *et al.*, 2002)

After genotyping CYP2D6 alleles, the estimation of the phenotypic activity score was performed according to the following (Gaedigk *et al*., 2017) (Table V).

Table V: Allele calls according to, Activity Score (AS), phenotype prediction and genotyping combination in the study population

Genotyping combination	Allele calls	Activity score (AS)	Phenotype prediction
2938CT-1934GA-1023CT; 2938CC-1934GA-1023CT; 2938TT-1934GA-1023CT	*4/*17	0.5	Intermediate Metabolizer (gIM)
2938CC-1934GA-1023CC	*1/*4	1	Normal metabolizer- slow (gNM-S)
2938CC-1934GG-1023TT	*17/*17	1	
2938CT-1934GA-1023CC; 2938TT-1934GA-1023CC	*2/*4	1	
2938CC-1934GG-1023CT	*1/*17	1.5	Normal metabolizer- fast (gNM-F)
2938CT-1934GG-1023CT; 2938TT-1934GG-1023CT; 2938TT-1934GG-1023TT; 2938CT-1934GG-1023TT	*2/*17	1.5	
2938CC-1934GG-1023CC	*1/*1	2	Normal metabolizer- fast (gNM-F)
2938CT-1934GG-1023CC	*1/*2	2	
2938TT-1934GG-1023CC	*2/*2	2	

The AS was evaluated according to the AS-model developed by Gaedigk and colleagues (Gaedigk, *et al.*, 2008). The phenotype prediction was done according to a scheme developed recently (Baird *et al.*, 2018) with PM referring to Poor metaboliser and IM referring to Intermediate Metaboliser.

3.7. Data Management

Before entering the data for analysis, the different files were cross-checked by the PI and the field surveyor to ensure consistency. Each questionnaire was coded in order not to disclose information on any participant.

3.8. Data analysis plan

For the analysis of the results, the variables used in the questionnaires were defined according to Tables VI and VII.

Table VI: The quantitative and qualitative variables of the febrile outpatients

Quantitative	Qualitative
- **Activity score (AS)** - **Age** - **Duration of LLIN availability in the household** - **Number of time antimalarial drugs were taken in the last 6 months** - **Number of years of education**	- *CYP2D6**17 variants - *CYP2D6**2 variants - *CYP2D6**4 variants - Duffy (-33CC) variants - G6PD 202 variants - G6PD 968 variants - Marital status - Occupation - *Pf* - *Pf*-RDT results - *Pm* - *Po* - *Pv* - Self-medication - Sex - Thick films results

Table VII: **The independent and dependent variables of the febrile outpatients**

Independent	Dependent
- **Age groups (>5years old; <5years old)** - **All family members sleeping in the same room** - **Availability of curtains at the door** - **Availability of LLIN** - **Availability of mosquito net** - ***Pf*-RDT results** - **Plastering of the house** - **Power supply in the house** - **Presence of countertop in the house** - **Presence of livestock in the household** - **Regular use of mosquito net** - **Self-medication** - **Sex** - **Thick films results**	- *Pf* occurrence - *Pv* occurrence

With the aid of Microsoft Excel 2013, Epi info version 7.2 and Arlequin 3.5 software the data was analysed emphasizing on the mean age of the participants, the sex ratio M/F, personal and environmental hygiene, prevention measures taken to avoid malaria, the prevalence of *Pv*, among West and South regions' Cameroonians proportion. Duffy Antigen, G6PDd, CYP2D6 variants distribution and the association between socio-economic, clinical and environmental factors, *Pv* and *Pf* occurrence in participants was assessed.

The sensitivity and the specificity of the thick film and *Pf*-RDT to respect to the standard test which is nested-PCR in this case will be calculated thanks to the following formula:

$$\text{sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}}$$

$$= \frac{\text{number of true positives}}{\text{total number of sick individuals in population}}$$

$$= \text{probability of a positive test given that the patient has the disease}$$

$$\text{specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}}$$

$$= \frac{\text{number of true negatives}}{\text{total number of well individuals in population}}$$

$$= \text{probability of a negative test given that the patient is well}$$

Proportions and means were estimated using 95% confidence interval (CI). Histograms, pie charts, tables were used to present the results. Chi-square test was used to compare the observed proportions among sexes, age groups and districts. Linear and logistic regressions were used to assess the association between the independent and the dependent variables. Hardy-Weinberg Equilibrium (HWE) test was used to assess the normal distribution of genes variants in the study population. Linkage disequilibrium analysis was performed to assess the loci linkage. The significance level was set at alpha= 0.05.

3.9. Ethics and human subject issues

Malaria is a public health concern that causes death of many people without distinction of age or sex within the country. It will be of great advantage to investigate and get informed about the prevention measures people take to avoid malaria and also to know which genetic characteristics can ensure a good treatment or not (social value of the study). Every client suffering from fever, who came to the various healthcare facilities chosen, was proposed to participate in the study freely and was informed about the different steps (Questionnaire administration and sample collection).

Signed informed consent for multiple genetic and epidemiological surveys was obtained from all adults and from all children's parents/guardians.The protection of the participant was assured by the Helsinki declaration of 2000, the Council for International Organizations of Medical Sciences (CIOMS), International ethical guidelines for biomedical research involving human subjects, and the International Council for Harmonization in Good Clinical Practices (ICH-GCP).

The Ethics Review and Consultancy Committee (ERCC) of Cameroon Bioethics Initiative (CAMBIN) gave a favourable ethical opinion to this study, by delivering the ethical clearance CBI/427/ERCC/CAMBIN.

3.9.1. Withdrawal

To withdraw from the study, the participant just needed to stop everything by refusing to go till the end and it was mentioned by the field surveyor on the partially filled questionnaire or on the field register. Moreover, the two copies of signed informed consent were destroyed. If some blood has being already collected from that participant, it was discarded.

3.9.2. Confidentiality

Before entering the data for analysis, the different questionnaires were cross-checked by the PI and the field surveyor to ensure consistency. Each questionnaire was then coded in order not to disclose information on any participant. The names was not recorded in the data base. And all the data were be stored on an online dropbox embedded in the laptop of the PI. Only few people had access to that database (PI, Supervisor, Co-Supervisor and Collaborators).

3.10. Dissemination of results

Results dissemination was done by writing and publishing articles (ongoing), participating to conferences with abstracts of the work (6th International Conference for *Plasmodium vivax* Research 2017, Multilateral Initiatives on Malaria Conference 2018), organizing a thesis defense where a jury specified by my University will participate (upcoming). Concerning the participants, the results of the study were not disclosed to them because the samples were not going to analyzed as soon as they were collected. There was a delay between the sample collection and availability of laboratory results. We made it clear to them that we were planning to assess their samples with more specific tests but they just needed to refer to the results of the hospital laboratory and the follow up was in accordance with what they found.

PART 2: RESULTS

1. Description of the study population

One thousand and four (1004) patients were recruited for this study, distributed as follows, 400 from SHD, 500 in DHD and 104 in AHD. The mean age and distribution by sex is presented in **table VIII**.

Table VIII: **Distribution of the study population by sex and mean age**.

Sex	Freq	%	Mean	Std Dev	Min	25%	Median	75%	Max	Mode
Female	568	56.57	24.62	19.72	0.08	7	22	35	84	1
Male	436	43.43	18.43	16.76	0.17	5	17	25	84	1

The overall mean age was 21.95 years old with a standard deviation of 18.75 years old. The sex ratio M/F was 0.76 approximatively 3/4.

The distribution of the study population by sex and age groups is presented in the **table IX**.

Table IX: **Distribution of the study population by sex and age groups**

	age groups (years old)									
	[0-5]		**[6-17]**		**[18-59]**		**[60-84]**		**Total**	
Sex	**N**	**%**	**n**	**%**	**N**	**%**	**n**	**%**	**n**	**%**
Female	150	14.94	95	9.46	287	28.59	36	3.59	568	56.57
Male	149	14.84	88	8.76	182	18.13	17	1.69	436	43.43
Total	**299**	**29.78**	**183**	**18.23**	**469**	**46.71**	**53**	**5.28**	**1004**	**100.00**

Almost one third (29.78%; 299/1004) of the study population was children under five years old. The occupational activities and the marital status are shown in **table X**.

Table X: **Distribution of the study population by profession and marital status**

Profession	**Absolute Frequency**	**Percent**
Housewife	113	15.5
Farmer	69	9.5
Hairdresser	8	1.1
Tailor	8	1.10
Driver	5	0.7
Trader	42	5.8
Teacher	23	3.1
Civil servant	13	1.8
Unemployed	7	0.9
Hustler	7	0.9
Others (student; manson; welder; traditional healer; retired)	434	59.5
Subtotal 1	**729**	**100.00**
Marital status	**Absolute Frequency**	**Percent**
married (monogamy)	165	30.3
married (polygamy)	67	12.3
Widow	36	6.6
Divorced	4	0.7
Single	273	50.1
Subtotal 2	**545**	**100.00**

The most common occupations were students (59.5%; 434/729) and housewives (15.5%; 113/729). The marital status was led by singles (50.1%; 273/545) and monogamy married (30.3%; 165/545).

2. Assessment of *Pv* circulation

1001 samples fulfilled the conditions to proceed for analyses through molecular biology technics and microscopy. The first molecular biology technic applied was conventional nested-PCR (Snounou and Singh, 2002). The proportion of positive cases was shown in the figure 20.

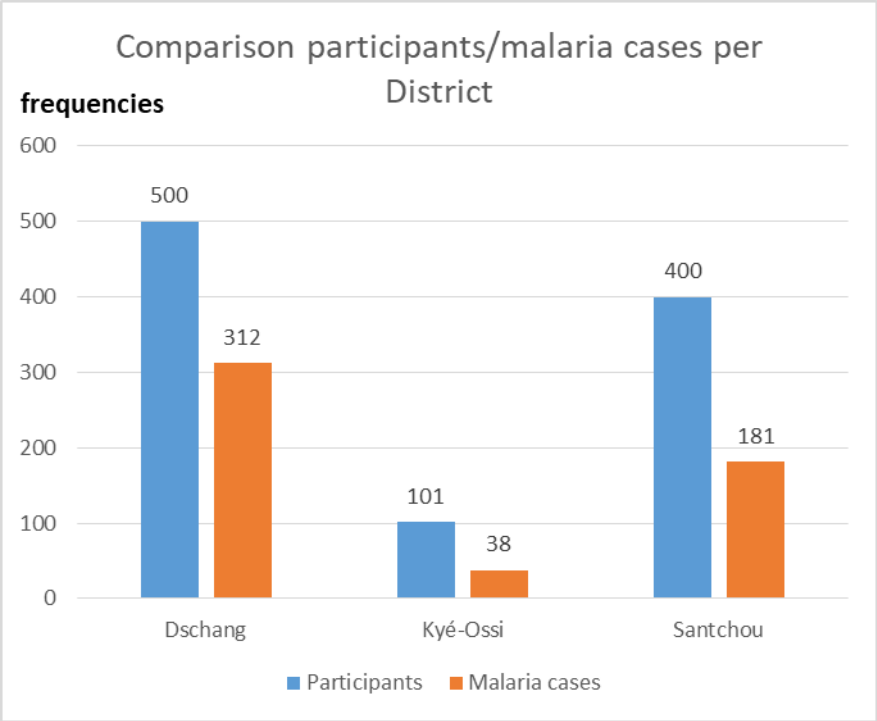


Figure 20: Comparison of participants with malaria cases per district

Four hundred and eighty-six (486) out of 1001 patients were infected by at least one *Plasmodium spp.*, almost half of them (48.55%). Fourty-five (45) patients had co-infections (4.50%). Figure 21 shows the general distribution of the circulating *Plasmodium spp*.

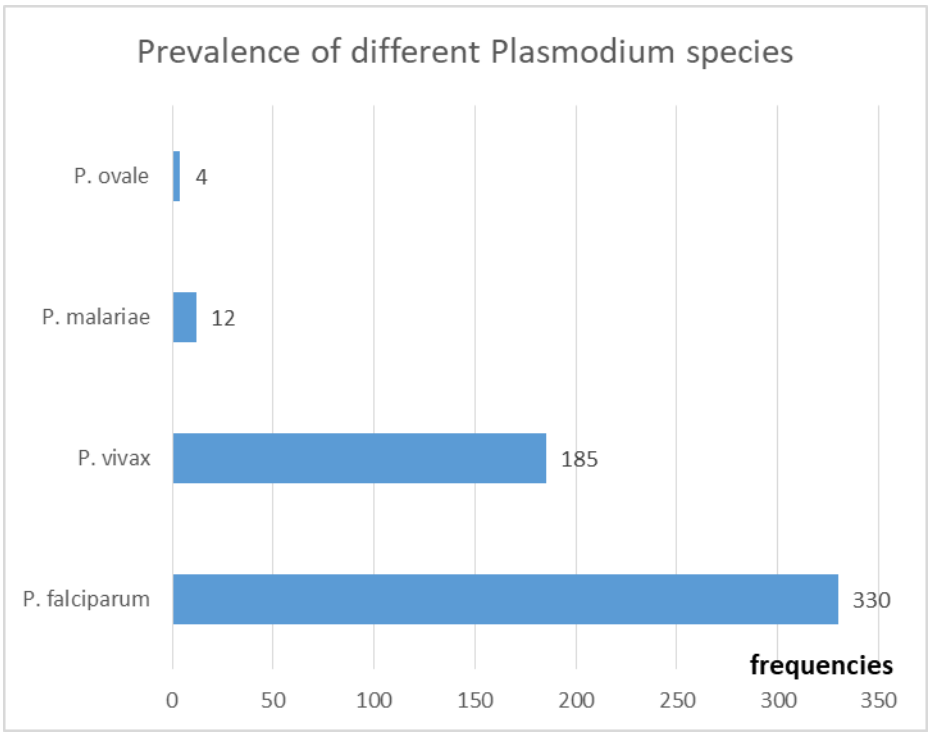


Figure 21 : Prevalence of different *Plasmodium spp*.

Pf was the main specie with 62.15% (330/531), followed by *Pv* with 34.84% (185/531). A very small proportion was attributable to *Pm* or *Po*, 3.01% (16/531). The mixed-infection map is shown below (figure 22).

MIXED-INFECTIONS

	P. ovale	*P. vivax*	*P. malariae*
P. vivax	**0**		
P. malariae	**0**	**3**	
P. falciparum	**2**	**37**	**3**

Figure 22: Mixed-infection map

Thirty-seven (82.22%) of the mixed-infections were cases of *Pf-Pv*, almost all of which came from Dschang District (36/37). The distribution of *Pv* cases according to age groups and sex are in the table XI.

Table XI: Distribution of *Pv* cases by age groups and sex

	***P. vivax* and Age Group**					
Age Group (years old)	**Female**		**Male**		**Total**	
	n	**%**	**n**	**%**	**n**	**%**
[0-5]	34	18.38	33	17.84	67	36.22
[6-17]	11	5.95	15	8.11	26	14.05
[18-59]	51	27.57	33	17.84	84	45.41
[60-84]	4	2.16	4	2.16	8	4.32
Total	**100**	**54.05**	**85**	**45.95**	**185**	**100.00**

Almost all cases of *Pv* (97.84%; 181/185) were detected in Dschang district. The proportion of women infected with *Pv* (54.05%) was slightly higher than that of men (45.95%) with no significant difference (X^2: 0.4146; P-value: 0.5197). No age group was spared (Table 4).

The malaria burden attributable to *Pv* and Duffy blood group specification is shown in the table XII.

Table XII: *Pv* infections among malaria cases

Study site	**N**	**Malaria cases n(%)**	***P. vivax* malaria cases**	
			N	**[1]%**
Santchou	400	177 (44.3)	2	1.1
Dschang	500	273 (54.6)	181	66.3
Kyé-ossi	101	36 (35.6)	2	5.5
Total	1001	486 (48.5)	185	38.1

[1] relative proportion of *P. vivax* infection among total malaria cases

In Dschang district, the burden attributable to *Pv* is the highest (66.3%) whereas, it is lowest in Santchou district (1.1%). All *Pv* cases belonged to Duffy negative blood group.

The summary of *Plasmodium spp*. distribution by districts is shown in the table XIII.

Table XIII: **Distribution of *Plasmodium spp* detected by health districts**

Study site	***Pf***	***Pv***	***Po***	***Pm***	**Co-inf *Pf-Pv***	**Co-inf *Pf-Po***	**Co-inf *Pf-Pm***	**Co-inf *Pv-Pm***	**n**	**N**
Santchou	170	1	2	0	1	2	1	0	177	400
Dschang	88	143	0	3	36	0	1	2	273	500
Kyé-ossi	29	2	0	3	0	0	2	0	36	101
Total	**287**	**146**	**2**	**6**	**37**	**2**	**4**	**2**	**486**	**1001**

All *Plasmodium spp*. were found as single infections and *Pf* (28.7%) was leading followed by *Pv* (14.7%). *Pf* had co-infections with *Pv* (3.7%), Po (0.2%) and *Pm* (0.4%). *Pv* had co-infections also with *Pm* (0.2%).

Pv mono-infected samples detected through nested-PCR were processed through RT-PCR. Only samples from the second sample collection period were cross-checked (figure 23).

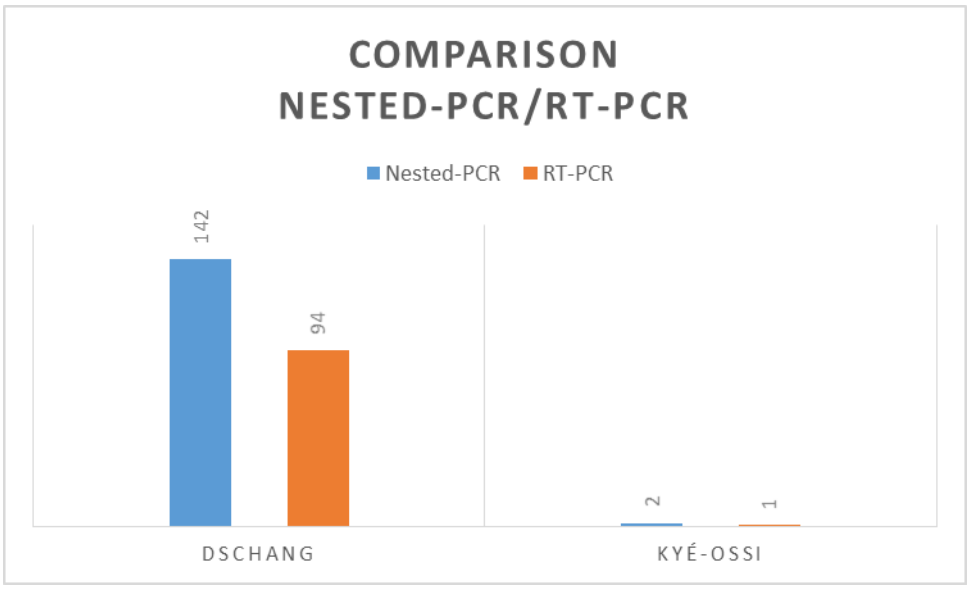


Figure 23 : Comparison between Nested-PCR and RT-PCR results for *Pv* positive samples.

Out of the positive samples detected through nested-PCR, 66% (95/144) showed positive results through RT-PCR.

Ten (10) *Pv* positive samples through real-time PCR were selected according to the quality of their results, amplified using MSP-1 primers (outer and nested) and sent to EUROFINS, Gemarny branch, for sequencing. Six (6) out of ten (60%) isolates sequences could be obtained, below are the sequences (figure 24).

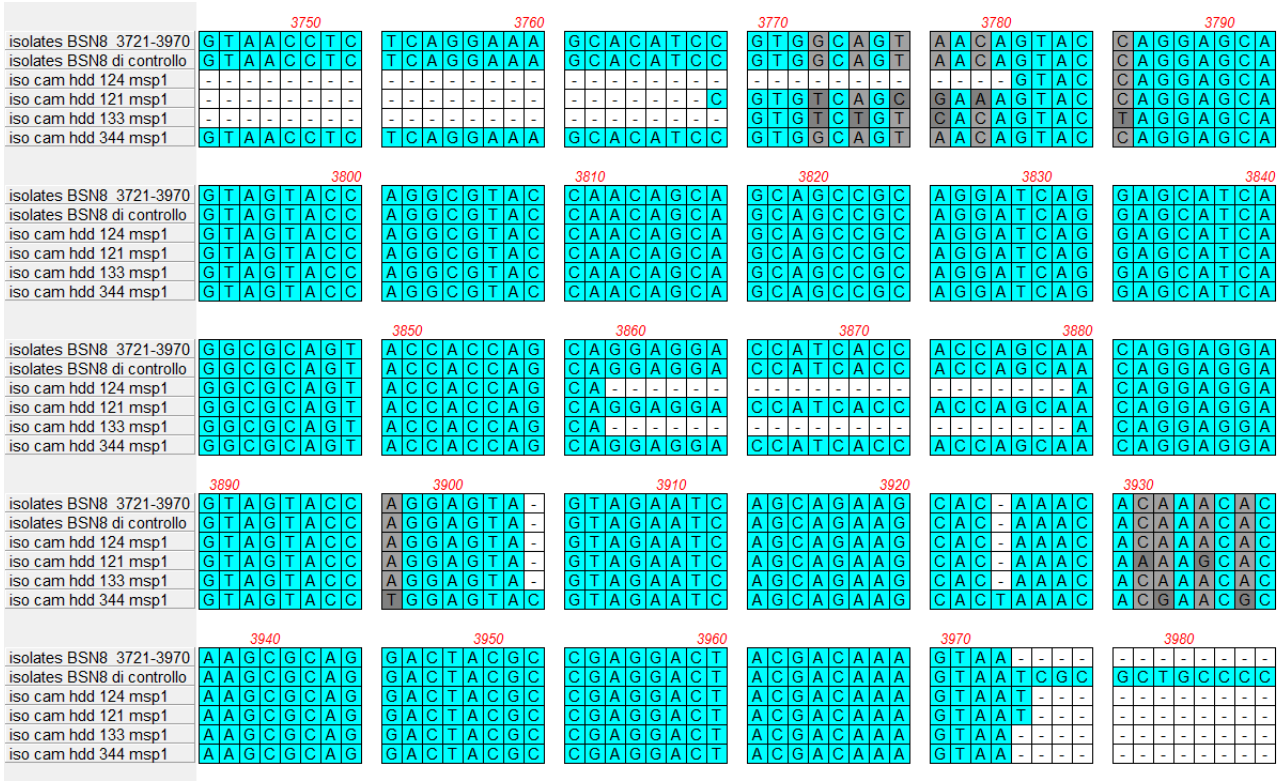


Figure 24 : Sequences of four samples from Dschang Health District

Sequence *Pv* MSP1 isolate Cameroon hdd 121

CGTGTCAGCGAAAGTACCAGGAGCAGTAGTACCAGGCGTACCAACAGCAGCA
GCCGCAGGATCAGGAGCATCAGGCGCAGTACCACCAGCAGGAGGACCATCAC
CACCAGCAACAGGAGGAGTAGTACCAGGAGTAGTAGAATCAGCAGAAGCACA
AACAAAAGCACAAGCGCAGGACTACGCCGAGGACTACGACAAAGTAAT (rev compl)

Sequence *Pv* MSP1 isolate Cameroon hdd 124

GTACCAGGAGCAGTAGTACCAGGCGTACCAACAGCAGCAGCCGCAGGATCAG
GAGCATCAGGCGCAGTACCACCAGCAACAGGAGGAGTAGTACCAGGAGTAGT
AGAATCAGCAGAAGCACAAACACAAACACAAGCGCAGGACTACGCCGAGGAC
TACGACAAAGTAAT (rev compl)

Sequence *Pv* MSP1 isolate Cameroon hdd 133

GTGTCTGTCACAGTACTAGGAGCAGTAGTACCAGGCGTACCAACAGCAGCAGC
CGCAGGATCAGGAGCATCAGGCGCAGTACCACCAGCAACAGGAGGAGTAGTA

CCAGGAGTAGTAGAATCAGCAGAAGCACAAACACAAACACAAGCGCAGGACT
ACGCCGAGGACTACGACAAAGTAA (rev complem)

Sequence *Pv* MSP1 isolate Cameroon hdd 331

GTTGATCCTGCTGCTGCTCCTGGTCCTGTTGCCGGTACTGAGCCTGCTGGTGGT
GGTGGCGTTAGCCCCGCCGCCGCCCCTGGTACTCCTGCTCCAGGTAATCCTCCT
GGTACCGCCGACGGTACCGTAAC

Sequence *Pv* MSP1 isolate Cameroon hdd 344

GCCTACCAAGAATTGATCCCCAAAGTAACCTCTCAGGAAAGCACATCCGTGGC
AGTAACAGTACCAGGAGCAGTAGTACCAGGCGTACCAACAGCAGCAGCCGCA
GGATCAGGAGCATCAGGCGCAGTACCACCAGCAGGAGGACCATCACCACCAG
CAACAGGAGGAGTAGTACCTGGAGTACGTAGAATCAGCAGAAGCACTAAACA
CGAACGCAAGCGCAGGACTACGCCGAGGACTACGACAAAGTAA

Sequence *Pv* MSP1 isolate Cameroon hdd 398

GGAGAACATAAGCAACCTGTCCAGTGGACTGCACCACGTCTTGACAGAGCTGA
AGGAAATTATCAAAAACAAGAAGTAATCCGGTAACGCCCACACGAAGAACAT
TGCAGCTGTTAAGGAAGTCTTCAGGCCTACCAAGAATTGATCCCCAAGGTGAC
CACTCAGGAAGGCGCATCCACAACAGCGCCAACATTACCAGTAACAGTACCAT
CAGCAGTACCAGGAGGATTACCTGGAGCAGGAGTACCAGGAGCAGCAGCAGG
ACTAACACCACCACCACCAGCAGGATCAGTACCAGCAACAGGACCAGGAGCA
GCAGCAGGATCAACAGAAGAAAACGTAGCAGCAAAAGCGCAGGACTACGCCG
AGGACTACGACAAAGTAATTGACCCTCCCTCTGCTCGGCCCAACGATGACGAC
TGGGAGGAAGACCAAGTAACAAC

Five strains were identified among the six samples successfully sequenced. All the succesfully sequenced samples came from Dschang district. The samples hdd124 and hdd344 appeared to be infected by the same strain. The length of the sequences for *Pv*msp-1 is different because of the different efficiency in sequencing reaction.

Thin films from few samples with very good results through PCR were assessed using a microscope. *Pv* was observed in some of them. Below are pictures from the slide hdd221 (figures 25 and 26).

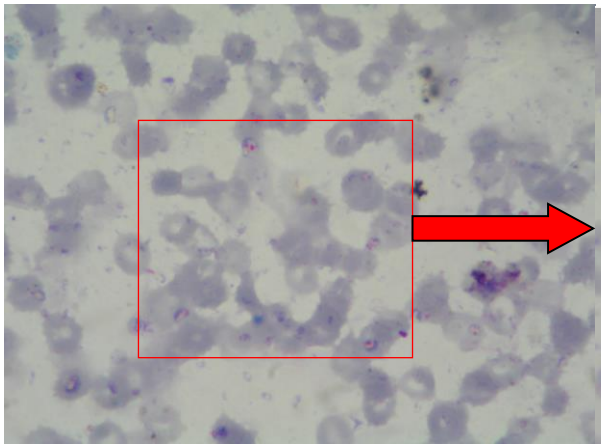


Figure 25: *Pv* trophozoites observed at 63X focus

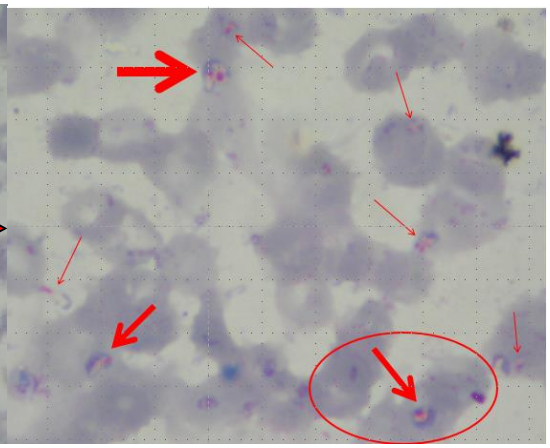


Figure 26: *Pv* trophozoites observed at 100X focus

NB: Red arrows in the figure 26 are pointing Pv parasites in thin film. The Red circle is showing a typical Pv young throphozoite.

3. Assessment of biological factors of the susceptibility to the *Pv* infection and the feasibility of treatment

The human genotyping and Hardy-Weinberg Equilibrium (HWE) results are recapitulated in tables XIV and XV below.

Table XIV: Genotype and allele frequencies for *CYP2D6* *17-*4-*2 SNPs and comparisons among the groups.

District	CYP2D6*17 1023 C>T						CYP2D6*4 1934 G>A						CYP2D6*2 2938 C>T					
	CC n (%)	CT n (%)	TT n (%)	Total n (%)	f(T) %	HWE P	GG n (%)	GA n (%)	AA n (%)	Total n (%)	f(A) %	HWE P	CC n (%)	CT n (%)	TT n (%)	Total n (%)	f(T) %	HWE P
Dschang	338 (77.35)	82 (18.76)	17 (3.89)	437 (100.00)	13.27	HD 0.0001	457 (91.77)	41 (8.23)	0 (0.00)	498 (100.00)	4.11	OK	109 (23.04)	200 (42.28)	164 (34.67)	473 (100.00)	55.77	HD 0.0019
Kyé-Ossi	72 (75.79)	17 (17.89)	6 (6.32%)	95 (100.00)	15.26	HD 0.0026	93 (90.29)	10 (9.71)	0 (0.00)	103 (100.00)	4.85	OK	21 (20.79)	37 (36.63)	43 (42.57)	101 (100.00)	60.89	HD 0.0203
Total n (%)	**410 (77.07)**	**99 (18.61)**	**23 (4.32)**	**532 (100.00)**	**13.63**	**HD 0.0000**	**550 (91.51)**	**51 (8.49)**	**0 (0.00)**	**601 (100.00)**	**4.24**	**OK**	**130 (22.65)**	**237 (41.29)**	**207 (36.06)**	**574 (100.00)**	**56.71**	**HD 0.0001**

The SNP 1934G>A is normally distributed in the sample population. The allele*4 has the frequency 4.24%. The SNPs 1023C>T and 2938C>T are not in Hardy-Weinberg Equilibrium (HWE), showing systematically a significant heterozygous defect (HD).

Table XV: **Genotype and allele frequencies for G6PD 202-968 SNPs and Duffy blood groups**

District	Duffy -33T>C						G6PD 202 G>A females						G6PD 968 T>C					
	TT	TC	CC	Total	f(C)	HWE	GG	GA	AA	Total	f(A)	HWE	TT	TC	CC	Total	f(C)	HWE
	n (%)	n (%)	n (%)	n (%)	%	P	n (%)	n (%)	n (%)	n (%)	%	P	n (%)	n (%)	n (%)	n (%)	%	P
Santchou	0 (0.00)	0 (0.00)	65 (100.00)	65 (100.00)	100.00	N/A	52 (98.11)	1 (1.89)	0 (0.00)	53 (100.00)	0.94	OK	/ /	/ /	/ /			N/A
Dschang	0 (0.00)	1 (0.20)	497 (99.80)	498 (100.00)	99.90	OK	130 (95.59)	5 (3.68)	1 (0.74)	136 (100.00)	2.57	HD 0.0018	498 (100.00)	0 (0.00)	0 (0.00)	498 (100.00)	0	N/A
Kyé-Ossi	0 (0.00)	1 (0.97)	102 (99.03)	103 (100.00)	99.51	OK	46 (92.00)	3 (6.00)	1 (2.00)	50 (100.00)	5.00	HD 0.0091	103 (100.00)	0 (0.00)	0 (0.00)	103 (100.00)	0	N/A
Total	**0**	**2**	**664**	**666**	**99.85**	**OK**	**228**	**9**	**2**	**239**	**2.72**	**HD**	**601**	**0**	**0**	**601**	**0**	**N/A**
%	**(0.00)**	**(0.30)**	**(99.70)**	**(100.00)**			**(95.40)**	**(3.77)**	**(0.84)**	**(100.00)**		**0.0000**	**(100.00)**	**(0.00)**	**(0.00)**	**(100.00)**		

Duffy Antigen (SNP -33T>C) is showing a normal distribution with the frequency of the mutant allele (-33CC) being around 99.85%. G6PD variants of the SNP 202 G>A are normally distributed among the study population in Santchou. But show a significant heterozygous defect in Dschang and Kyé-ossi districts. The frequency of the deficient allele is 2.72% among females. G6PD variants of the SNP 968 T>C did not show any variation (all samples being wild type) among the study population. In case of absence of allele variation in the gene, HWE was non-applicable (N/A).

Given the specificity of G6PD 202 G>A SNP which is linked to X chromosome, a specific data analysis was made for male sex (table XVI). An overview of the various allele's frequencies and Pv associated cases was done (table XVII).

Table XVI: Genotype and allele frequencies for G6PD 202 males

G6PD 202 G>A males			
District	**G**	**A**	**Total**
	n(%)	**n(%)**	**n(%)**
Santchou	45 (100.00)	0 (0.00)	45 (100.00)
Dschang	106 (97.25)	3 (2.75)	109 (100.00)
Kyé-Ossi	24 (92.31)	2 (7.69)	26 (100.00)
Total	**175 (97.22)**	**5 (2.78)**	**180 (100.00)**

Table XVII: G6PD deficiency distribution

G6PD Phenotype	**G6PD 202 alleles**	**Frequency**	**Percent**	***Pv* cases**
Deficient (A-)	A	5	1.2	0/5
	AA	2	0.5	0/2
Def +/- (A)	GA	9	2.1	2/9
Normal (B)	G	175	41.8	30/175
	GG	228	54.4	31/228
Total		419	100	63/419

The study population shows 2.78% (A-) G6PD deficient people globally, with two *Pv* infected people being G6PD A+ (202GA).

Knowing the metabolic role of *CYP2D6* allele in primaquine metabolism, the genotypes were translated into the activity score for a better phenotype estimation (table XVIII).

Table XVIII: **Distribution of the Phenotype Estimation (AS) by study sites**

	Phenotype Estimation - Activity scores									
	IM (AS=0.5)		**NM-S (AS=1)**		**NM-F (AS=1.5)**		**NM-F (AS=2)**		**Total**	
Sites	**N**	**%**	**N**	**%**	**n**	**%**	**n**	**%**	**N**	**%**
Dschang	6	1.18	30	5.88	90	17.64	291	51.06	417	81.76
Kyé-Ossi	0	0.00	10	1.96	23	4.50	60	11.76	93	18.24
Total	**6**	**1.18**	**40**	**7.84**	**113**	**22.16**	**351**	**68.82**	**510**	**100.00**

IM: intermediate metabolizer; NM-S: normal metaboliser slow; NM-F normal metaboliser fast

The majority (98.82%, 504/510) of the study population was normal metabolizers (NM) population. Among the intermediate/normal-slow metabolizers, 12 (26.10%) were *Pv* infected.

The HWE results for CYP2D6 SNPs suggested to perform linkage disequilibrium (LD) analysis and haplotypes reconstruction using Arlequin software version 3.5 (tables XIX and XX).

Table XIX: **Pairwise LD analysis for the three polymorphic loci. Chi-square values and *P*-value for LD analysis were obtained using Arlequin.**

District	Comparison	D	D'	Chi-square	*P*-value (1 df)
Dschang	2938 vs 1934	0.0124	0.55	6.52487	0.01064 *
Dschang	1934 vs 1023	0.0041	0.76	1.67584	0.19548
Dschang	2938 vs 1023	0.032	0.55	17.25230	0.00003 *
Kyé-ossi	2938 vs 1934	0.026	0.81	9.68206	0.00186 *
Kyé-ossi	1934 vs 1023	0.0084	1	3.49339	0.06161
Kyé-ossi	2938 vs 1023	0.0604	1	17.85110	0.00002*

**significant P- value (P<0.05)*

A significant linkage was observed between the loci 2938 and 1934 and 2938 and 1023 in Dschang with a similar D' (0.55). A significant linkage was found only between 2938 and 1934 in Kyé-ossi with a higher D' (0.81). The linkage of the loci 1934 and 1023 was significantly complete in Kyé-ossi.

Maximum-likelihood (ML) haplotype frequencies were calculated using the EM (expectation-maximization) algorithm in Arlequin software.

Table XX: **Haplotype frequencies by district and for all the samples combined.**

Haplotypes	***CYP2D6* alleles**	**Absolute and (ML) frequencies**	
		Dschang	**Kyé-ossi**
2938C-1934A-1023C	*4	24 (0.029)	9 (0.047)
2938C-1934A-1023T[§]	Not categorized	1 (0.0001)	0 (0.000)
2938C-1934G-1023C	*1	318 (0.382)	63 (0.340)
2938C-1934G-1023T[§]	Not categorized	21 (0.026)	0 (0.000)
2938T-1934A-1023C[§]	Not categorized	9 (0.011)	1 (0.006)
2938T-1934A-1023T[§]	Not categorized	1 (0.001)	0 (0.000)
2938T-1934G-1023C	*2	371 (0.445)	84 (0.451)
2938T-1934G-1023T	*17	89 (0.106)	29 (0.156)
Total		834 (1.000)	186 (1.000)

[§] *Haplotypes not yet categorized into alleles*

Four haplotypes not yet categorized, were found in the study population in relatively low frequencies. *2 (44.5%) and *1 (38.2%) were the main haplotypes found.

4. Specificity and sensitivity of routinely used diagnosis test in Cameroon healthcare facilities

Malaria diagnosis was performed by *Pf*-RDT only in 42.7% (308/721) of cases, thick-film only in 35.0% (252/721), both tests in 22.3% (161/721). A discordance in results was observed in 6.2% (10/161) of cases. Almost a third (27.5%; 274/996) of the study population did not benefit for any laboratory diagnosis test (**table XXI**).

Table XXI*:* Distribution of *Pf*-RDT and Thick films in the study population

		***Pf*-RDT**			
		Negative (%)	**Positive (%)**	**Not performed (%)**	**TOTAL (%)**
Thick film	**Negative**	37 (3.7)	3 (0.3)	78 (7.8)	**118 (11.8)**
	Positive	7 (0.7)	114 (11.4)	174 (17.5)	**295 (29.6)**
	not performed	125 (12.6)	184 (18.5)	274 (27.5)	**583 (58.6)**
	Total	**169(17.0)**	**301 (30.2)**	**526 (52.8)**	**996 (100)**

Chi-square P-value< 0.0001

Plasmodium falciparum PCR was positive in 330/1001 (32.9%) individuals. The discordance between PCR and *Pf*-RDT results was evident in 38.6% (181/469) of the cases, whereas it was 55.7% (230/413) by comparing PCR and thick-film methods (**table XXII**).

Table XXII : **Comparison of PCR results with thick films and *Pf*-RDT results**

		Nested PCR		**Discordance (%)**
HCF Diag		**Positive (%)**	**Negative (%)**	
Thick film	Positive	93 (22.51)	202 (48.91)	230 (55.69)
	Negative	28 (6.78)	90 (21.79)	
Total 1		121 (29.29)	292 (70.70)	413(100.00)
***Pf*-RDT**	Positive	154 (32.84)	147(31.34)	181 (38.59)
	Negative	34 (7.25)	134 (28.57)	
Total 2		188 (40.09)	281 (59.91)	**469 (100.00)**

Based on the comparison between the Nested PCR results with thick film and *Pf*-RDT respectively, the specificity and the sensitivity of these tests were calculated. *Pf*-RDT is relatively more specific and sensitive than thick film (**table XXIII**).

Table XXIII: Specificity and sensitivity of *Pf*-RDT and Thick film to respect to PCR

	***Pf*-RDT**		**Thick film**	
Nested PCR	**negative**	**positive**	**negative**	**Positive**
Negative (n)	134	147	90	202
Positive (n)	34	154	28	93
Specificity (%)	**65.6**		**59.1**	
Sensitivity (%)	**84.7**		**81.2**	

Thick film results were more likely to be positive for female sex. This relation was not significant among both age groups (**table XXIV**).

Table XXIV: Comparison of thick film results among sex and age groups

		Thick film				
Age groups	**Sex**	**no**	**yes**	**not performed**	**Total**	**P-value**
<5 years old	**Female n (%)**	57 (5.70)	123 (12.30)	237 (23.60)	**417 (41.60)**	0.25
	Male n (%)	28 (2.80)	94 (9.40)	166 (16.60)	**288 (28.80)**	
	Sub-total 1 n (%)	**85 (8.50)**	**217 (21.70)**	**403 (40.20)**	**705 (70.40)**	
>5 years old	**Female n (%)**	19 (1.90)	31 (3.10)	98 (9.80)	**148 (14.80)**	0.095
	male n (%)	14 (1.40)	47 (4.70)	87 (8.70)	**148 (14.80)**	
	Sub-total 2 n (%)	**33 (3.30)**	**78 (7.80)**	**185 (18.50)**	**296 (29.60)**	
	Total n (%)	**118 (11.80)**	**295 (29.50)**	**588 (58.70)**	**1001 (100.00)**	

Pf-RDT results were more likely to be positive for female sex. This relation was significant (P-value=0.005) in the group of those aged more than 5 years old (**table XXV**).

Table XXV: Comparison of *Pf*-RDT results among sex and age groups

		Pf-RDT				
Age group	**Sex**	**No**	**yes**	**Not performed**	**Total**	**P-value**
<5 years old	**Female n (%)**	75 (7.50)	116 (11.60)	226 (22.60)	**417 (41.70)**	0.005*
	Male n (%)	28 (2.80)	100 (10.00)	159 (15.90)	**287 (28.70)**	
	sub-total 1 n (%)	**103 (10.30)**	**216 (21.60)**	**385 (38.50)**	**704 (70.40)**	
>5 years old	**Female n (%)**	29 (2.90)	47 (4.70)	71 (7.10)	**147 (14.70)**	0.424
	Male n (%)	37 (3.70)	39 (3.90)	71 (7.10)	**147 (14.70)**	
	sub-total 2 n (%)	**66 (6.60)**	**86 (8.60)**	**142 (14.20)**	**294 (29.40)**	
	Total n (%)	169 (16.90)	302 (30.20)	527 (52.70)	998 (100.00)	

5. Factors of malaria occurrence at the community level

More than two thirds of participants (735/1001) reported to have started a treatment at home (26.3% antipyretics only, 13.6% ACT, 7.6% Quinine, 5.3% traditional drugs, 6.6% drug not specified and 14.1% other drugs) (**figure 27**).

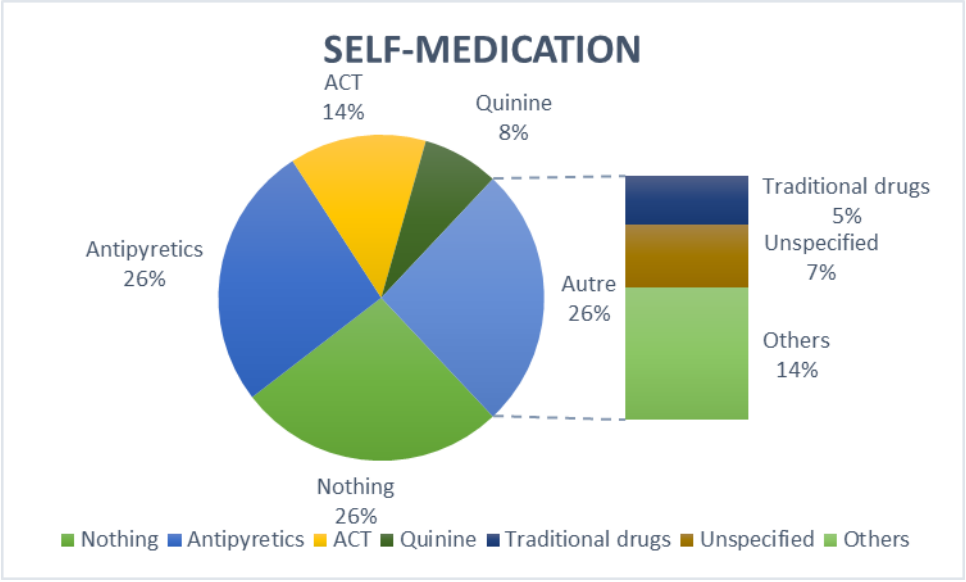


Figure 27: **Self-medication pattern in the study population**

Self-medication was more likely to happen among female. This relation was significant within both age groups (**table XXVI**).

Table XXVI: **Comparison of self-medication proportions in sex and age groups**

		Self-medication			
Age group	**Sex**	**no**	**yes**	**Total**	**P-value**
<5 years old	**Female n (%)**	132 (13.20)	285 (28.50)	417 (41.70)	0.034*
	Male n (%)	69 (6.90)	218 (21.80)	287 (28.70)	
	sub-total 1 n (%)	201 (20.10)	503 (50.30)	704 (70.40)	
>5 years old	**Female n (%)**	24 (2.40)	124 (12.40)	148 (14.80)	0.024*
	Male n (%)	41 (4.10)	107 (10.70)	148 (14.80)	
	sub-total 2 n (%)	65 (6.50)	231 (23.10)	296 (29.60)	
	Total n (%)	266 (26.60)	734 (73.40)	1000 (100.00)	

**P-value <0.05 showing significant dependence in variables*

Three quarter (74.97%) of self-medicated people decided themselves. While almost all the remaining (23.27%) received advices from health personnel (figure 28).

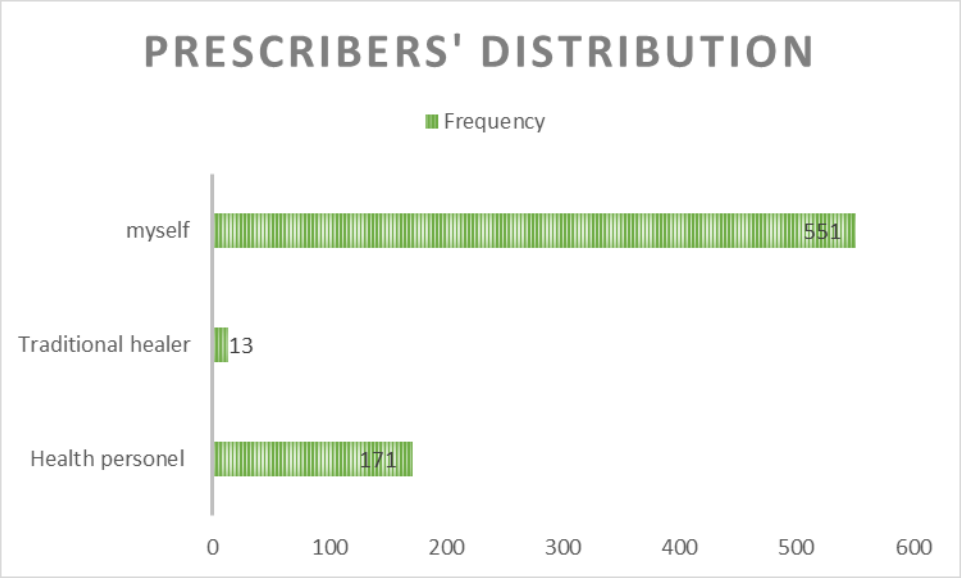


Figure 28: Distribution of drug prescribers

Outpatients who started a treatment before arriving to the health facilities were classified according to the person who oriented them to the treatment to respect to their parasite detection results through PCR (**Table XXVII**).

Table XXVII: Comparison between parasite detection results among the groups with different prescribers

		P. falciparum		*P. vivax*	
Prescriber	**Total**	**Negative**	**positive**	**Negative**	**positive**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Health Personel	**170 (23.22)**	120 (16.39)	50 (6.83)	130 (17.76)	40 (5.46)
Traditional Healer	**13 (1.78)**	9 (1.23)	4 (0.54)	12 (1.64)	1 (0.14)
Myself	**549 (75.00)**	357 (48.77)	192 (26.23)	449 (61.34)	100 (13.66)
TOTAL	**732 (100.00)**	**486 (66.39)**	**246 (33.61)**	**591 (80.74)**	**141 (19.26)**
Chi-square (P-value)	**2df**	1.8468 (0.3972)		3.4966 (0.1741)	

Forty outpatients (5.46%) infected with *Pv* and 50 (6.83%) infected with *Pf* were advised by a health personnel to start medication before going to the healthcare facility. Hundred outpatients (13.66%) infected with *Pv* and 192 (26.23%) infected with *Pf* decided to start treatment on their own. There was no significant difference in comparing respectively *Pf* and *Pv* detection results among the three groups.

Anterior history of antimalarial drugs use was assessed. The frequencies of antimalarial drugs use within the six month preceding the survey in reported in table XXVIII.

Table XXVIII: Distribution of antimalarial drug use in the last 6 months

Number of Antimalarial treatment (n)	Absolute frequency	Percent %	Wilson 95% LCL	Wilson 95% UCL
0	454	46.00	42.91	49.12
1	409	41.44	38.40	44.54
2	97	9.83	8.12	11.84
3	19	1.93	1.24	2.99
4	4	0.41	0.16	1.04
5	1	0.10	0.02	0.57
6	2	0.20	0.06	0.74
7	1	0.10	0.02	0.57
Total	**987**	**100.00**		

The largest proportion (46%) of the study population did not use antimalarial drugs in the last months. Four hundred and nine (41.44%) took antimalarial drugs once within 6 months.

Pv occurrence associated factors were assessed first through linear regression (table XXIX) then through logistic regression (table XXX).

Table XXIX: Correlation between *Pv* occurrence and social, clinical, behavioural and environmental factors

Variable	Coefficient	95% Confidence	Limits	Std Error	F-test	P-value
≤ 5-ys old(0=N; 1=Y)	-0,049	-0,110	0,012	0,031	2,5023	0,114113
all household sleep in same local (1=Y/0=N)	0,079	0,004	0,154	0,038	4,2768	0,038988
Countertop_(1=Y/0=N)	0,011	-0,055	0,077	0,034	0,1042	0,746994
Curtains door(1=Y/0=N)	-0,025	-0,089	0,040	0,033	0,5697	0,450640
electricity_(1=Y/0=N)	0,071	-0,026	0,167	0,049	2,0496	0,152674
Livestock(1=Y/0=N)	0,056	0,006	0,107	0,026	4,7409	0,029774
MILDA _(1=Y/0=N)	0,022	-0,119	0,162	0,072	0,0915	0,762325
ongoing therapy _(0=N; 1=Y)	0,029	-0,036	0,093	0,033	0,7504	0,386646
Mosquitoes net 1=Y/0=N	0,046	-0,175	0,267	0,113	0,1677	0,682252
Plastering(1=Y/0=N)	0,048	-0,010	0,106	0,030	2,6473	0,104158
Sex (0=F;1=M)	0,029	-0,028	0,086	0,029	0,9931	0,319307
RDT results	0,029	-0,009	0,067	0,019	2,2683	0,132475
regular use of mosquito net(1=Y/0=N)	-0,086	-0,155	-0,016	0,036	5,7919	0,016348
Thick_ film results	0,009	-0,031	0,049	0,020	0,2108	0,646275
CONSTANT	-0,029	-0,231	0,173	0,103	0,0805	0,776681

Correlation Coefficient: r^2 = 0,04

Source	df	Sum of Squares	Mean Square	F-statistic	p-value
Regression	14	4,6010	0,3286	2,2440	0,0055
Residuals	727	106,4717	0,1465		
Total	741	111,0728			

Significant P- value (P<0.05)

Regular use of mosquitoes net (r=-0.086; P=0.016), the presence of livestock in or around the household (r=0.056; P=0.030) and the fact that all family members were sleeping in the same room (r=0.079; P=0.039) were significantly correlated with the occurrence of *Pv*. Since the correlation coefficients of almost all the factors were suggesting interactions, factors were all analysed with the logistic model.

Table XXX: Association between *Pv* occurrence and social, clinical, behavioural and environmental factors

Term	Odds Ratio	95%	C.I.	Coefficient	S.E.	Z-Statistic	P-Value
≤ 5-ys old(0=N; 1=Y) (Yes/No)	0,6971	0,4501	1,0796	-0,3608	0,2232	-1,6167	0,1059
all household sleep in same local (1=Y/0=N) (Yes/No)	1,6199	1,0119	2,5932	0,4824	0,2401	2,0093	0,0445
Countertop_(1=Y/0=N)	1,0988	0,6964	1,7338	0,0942	0,2327	0,4049	0,6855
Curtains door(1=Y/0=N)	0,8356	0,5356	1,3036	-0,1797	0,2269	-0,7917	0,4286
Livestock(1=Y/0=N)	1,4340	1,0326	1,9914	0,3605	0,1675	2,1517	0,0314
MILDA _(1=Y/0=N)	1,2156	0,4211	3,5091	0,1953	0,5409	0,3610	0,7181
Mosquitoes net 1=Y/0=N (Yes/No)	1,1480	0,2374	5,5518	0,1380	0,8042	0,1716	0,8638
ongoing therapy _(0=N; 1=Y) (Yes/No)	1,2341	0,7764	1,9618	0,2104	0,2365	0,8897	0,3736
regular use of mosquito net(1=Y/0=N) (Yes/No)	0,5937	0,3829	0,9205	-0,5214	0,2237	-2,3301	0,0198
Sex (0=F;1=M) (Yes/No)	1,1998	0,8138	1,7689	0,1822	0,1981	0,9197	0,3577
RDT results	1,2400	0,9435	1,6296	0,2151	0,1394	1,5431	0,1228
Thick_ film results	1,0597	0,8025	1,3992	0,0580	0,1418	0,4086	0,6828
electricity_(1=Y/0=N)	1,8939	0,8553	4,1936	0,6386	0,4056	1,5747	0,1153
Plastering(1=Y/0=N)	1,3633	0,9504	1,9555	0,3099	0,1841	1,6837	0,0922
CONSTANT	*	*	*	-3,0688	0,7507	-4,0880	0,0000

Convergence: **Converged**
Iterations: **4**
Final -2*Log-Likelihood: **676,3107**
Cases Included: **742**

Test	Statistic	D.F.	P-Value
Score	30,8018	14	0,0059

Significant P- value (P<0.05)

Regular use of mosquitoes net (OR= 0.59; P=0.019), the presence of livestock in/or around the household (OR= 1.43; P=0.031) and the fact that all family members were sleeping in the same room (OR= 1.62; P=0.044) were significantly associated with the occurrence of

Pv. To be aged more than 5 years old (OR= 0.70; P=0.11) and putting curtains at the door (OR= 0.84; P=0.42) were non-significant protective factors to *Pv* malaria occurrence. Whereas presence of power supply (OR= 1.89; P=0.11) and plastering (OR= 1.36; P=0.09) of the house were non-significant risk factors.

Pf occurrence associated factors were assessed first through linear regression (table XXXI) then through logistic regression (table XXXII).

Table XXXI: Correlation between *Pf* occurrence and social, clinical, behavioural and environmental factors

Variable	Coefficient	95% Confidence	Limits	Std Error	F-test	P-value
≤ 5-ys old(0=N; 1=Y)	-0,093	-0,166	-0,020	0,037	6,1964	0,013023
all household sleep in same local (1=Y/0=N)	-0,065	-0,155	0,024	0,046	2,0406	0,153578
Countertop_(1=Y/0=N)	-0,089	-0,168	-0,009	0,040	4,8292	0,028295
Curtains door(1=Y/0=N)	0,033	-0,044	0,110	0,039	0,7087	0,400152
electricity_(1=Y/0=N)	-0,103	-0,219	0,013	0,059	3,0208	0,082628
Livestock(1=Y/0=N)	0,024	-0,037	0,085	0,031	0,6198	0,431383
MILDA _(1=Y/0=N)	0,104	-0,065	0,273	0,086	1,4480	0,229246
ongoing therapy _(0=N; 1=Y)	0,022	-0,056	0,099	0,040	0,2964	0,586331
Mosquitoes net 1=Y/0=N	-0,255	-0,521	0,010	0,135	3,5579	0,059661
Plastering(1=Y/0=N)	0,004	-0,065	0,074	0,036	0,0149	0,902817
Sex (0=F;1=M)	0,027	-0,041	0,095	0,035	0,5888	0,443148
RDT results	-0,009	-0,055	0,036	0,023	0,1576	0,691535
regular use of mosquito net(1=Y/0=N)	0,046	-0,038	0,129	0,043	1,1436	0,285247
Thick_ film results	0,035	-0,013	0,083	0,024	2,0225	0,155414
CONSTANT	0,520	0,277	0,763	0,124	17,6541	0,000030

Correlation Coefficient: r^2 = 0,04

Source	df	Sum of Squares	Mean Square	F-statistic	p-value
Regression	14	6,5084	0,4649	2,1969	0,0068
Residuals	729	154,2644	0,2116		
Total	743	160,7728			

Significant P- value (P<0.05)

Having a mosquitoes net (r=-0.255; P=0.059); being aged more than 5 years old (r=-0.093; P= 0.013) and having a house with a countertop (r=-0.089; P=0.028) were significant negatively correlated factors with *Pf* occurrence. Since the correlation coefficients of almost all the factors were suggesting interactions, factors were all analysed with the logistic model.

Table XXXII: Association between *Pf* occurrence and social, clinical, behavioural and environmental factors

Term	Odds Ratio	95%	C.I.	Coefficient	S.E.	Z-Statistic	P-Value
≤ 5-ys old(0=N; 1=Y) (Yes/No)	0,6371	0,4464	0,9092	-0,4509	0,1815	-2,4842	0,0130
all household sleep in same local (1=Y/0=N) (Yes/No)	0,7160	0,4571	1,1214	-0,3341	0,2289	-1,4595	0,1444
Countertop_(1=Y/0=N)	0,6629	0,4602	0,9548	-0,4111	0,1862	-2,2085	0,0272
Curtains door(1=Y/0=N)	1,1657	0,8098	1,6780	0,1533	0,1859	0,8247	0,4095
Livestock(1=Y/0=N)	1,1272	0,8473	1,4994	0,1197	0,1456	0,8221	0,4110
MILDA _(1=Y/0=N)	1,7477	0,7173	4,2582	0,5583	0,4544	1,2288	0,2191
Mosquitoes net 1=Y/0=N (Yes/No)	0,2861	0,0786	1,0412	-1,2515	0,6591	-1,8987	0,0576
ongoing therapy _(0=N; 1=Y) (Yes/No)	1,1055	0,7613	1,6052	0,1003	0,1903	0,5269	0,5982
regular use of mosquito net(1=Y/0=N) (Yes/No)	1,2505	0,8306	1,8829	0,2236	0,2088	1,0708	0,2843
Sex (0=F;1=M) (Yes/No)	1,1399	0,8248	1,5754	0,1309	0,1651	0,7930	0,4278
RDT results	0,9577	0,7707	1,1902	-0,0432	0,1109	-0,3898	0,6967
Thick_ film results	1,1910	0,9405	1,5082	0,1748	0,1205	1,4506	0,1469
electricity_(1=Y/0=N)	0,6401	0,3808	1,0760	-0,4461	0,2650	-1,6836	0,0923
Plastering(1=Y/0=N)	1,0224	0,7457	1,4019	0,0222	0,1610	0,1376	0,8906
CONSTANT	*	*	*	0,0805	0,5655	0,1423	0,8868

Convergence: **Converged**
Iterations: **4**
Final -2*Log-Likelihood: **897,5969**
Cases Included: **744**

Test	Statistic	D.F.	P-Value
Score	30,1187	14	0,0073

Significant P- value (P<0.05)

To be aged more than 5 years old (OR= 0.63; P=0.013), to have a house with countertop (OR= 0.662; P=0.027) and to have a mosquitoes net (OR= 0.286; P=0.057) were significant protective factors against malaria. To have a long lasting impregnated net

(LLIN) (OR= 1.74; P=0.22) and the regular use of mosquitoes net (OR= 1.25; P=0.28) appeared to be non-significant risk factors of *Pf* occurrence. Whereas the fact that all family members were sleeping in the same room (OR= 0.72; P=0.144) and the presence of power supply in the household (OR= 0.64; P=0.09) were non-significant protective factors.

PART3: DISCUSSION

The present study included only symptomatic patients seeking for care in three different health districts and selected the leading HCF of the districts, thus suggesting a selection bias. Moreover, no enquiry about fever management from the perspective of the health personnel was done, thus appearing as a limitation.

This cross-sectional study provides us with a picture of *Plasmodium spp* circulation in Cameroon; with *Pf* (62.15%) as the main circulating malaria parasite, followed by *Pv* (34.84%), *Pm* and *Po* (3.01%) being present in a very low proportion. These results are in line with those already available from Cameroon, reporting *Pf* as the leading cause of malaria (Fru-Cho *et al*., 2014; WHO, 2016). It highlights the presence in Cameroon of all the main *Plasmodium spp* available in Africa (WHO Global Malaria Programme, 2017). Excepted the newly found *Po* (SHD), this is in line with the findings of other studies on *Plasmodium spp* circulating in Cameroon, which reported the presence of *Pf, Pv, Pm* (Fru-Cho *et al*., 2014; Russo *et al*., 2017). The previous mentioned studies reported *Pf-Pv*/*Pf-Pv-Pm/ Pf-Pm* mixed-infections, which is also the case in this study were 45 mixed-infections were found with 37 being *Pf-Pv.* These mixed-infections representing 8.5% of *Pf* and *Pv* mono-infections suggest that there could be a lower probability of developing severe malaria among this group of outpatients (McKenzie *et al*., 2006; P. Zimmerman *et al*., 2013).

Pv circulation in Cameroon was reported in a relatively high rate (38.1%; 185/486). This is the highest *Pv* prevalence ever displayed in Cameroon (Fru-Cho *et al*., 2014; Mbenda *et al*., 2016; Mbenda and Das, 2014). Most of the cases arose from DHD (181/185; 97.84%), representing 66.3% (181/273) of malaria cases (more than *Pf*). Thus, showing results similar to those of Russo and colleagues which represented 38.6% (27/70) of malaria cases on a total population of 484 (Russo *et al*., 2017). *Pv* prevalence in the present study could be this high because Russo and colleagues assessed febrile outpatients in a low transmission period contrary to the high transmission period targeted. *Pv* prevalence was very low in SHD (1.1%) and AHD (5.5%). This could be due to the difference in altitude between the two other health districts (Santchou: 750 m; Kyé-ossi: 556m) and Dschang (1400m), correlating the hypothesis of *Pv* transmission to be linked to seasonality and altitude (Russo *et al*., 2017). Still, the question of the specific vector responsible of *Pv* transmission in Africa in general and in Cameroon in particular have never been addressed with accuracy, despite few studies assessing the presence of *Plasmodium spp*. in anopheles

mosquitoes (Atangana *et al*., 2009; Bigoga *et al*., 2007; Howes *et al*., 2015; Mendes *et al*., 2011).

Women (54.05%) were more likely to be infected by *Pv* than men (45.95%) even if there was no significant difference between under five and more than five years old age groups. The most infected group was [18-59] years old group (45.41%), probably due to their behaviours, being the most active age group, spending more time outside the house. This is in line with the findings of Zhu and colleagues concerning the importance of combining indoor and outdoor vectors control methods to reduce malaria transmission in rural area (Zhu *et al*., 2017). Considering the age range, [0-5] years old age group was the most affected group (36.22%), suggesting that Dschang could be an endemic area for *Pv* malaria, with an infective path similar to those of endemic regions (Battle *et al*., 2012; Iwagami *et al*., 2017).

Out of *Pv* infected outpatients, 66% (144/185) were mono-infections. These samples were confirmed using RT-PCR and 66% (95/144) were positive. This relatively low positivity rate could be explained by the fact that, nested-PCR performed immediately after extraction, was more sensitive because of the double PCR, whereas the RT-PCR was performed on already stored samples with a high probability of *Pv* DNA degradation (Barker *et al*., 1994). Ten of the RT-PCR confirmed samples with good results, were amplified for *Pv*MSP1 and sequenced. Sequences were obtained for 6 of them (60%) showing 5 different *Pv* strains. Further phylogenetic analysis are required to understand the origin of the strains found in Dschang. Some of the *Pv* mono-infected samples were microscopically assessed and despite the bad quality of the thin film (related to the bad quality of the Giemsa used and the working conditions), it was possible to find *Pv* trophozoites fulfilling approximately 70% of the CDC criteria. This slide was quite surprising because of its high parasitamia.

Duffy-blood group assessment showed most of the study population to be Duffy negative. All the *Pv* cases belonged to this group. A similar situation was reported in recent years in many African countries including Cameroon, where all the *Pv* cases were Duffy negative (Howes *et al*., 2015; Mbenda *et al*., 2016; Mbenda and Das, 2014; Mendes *et al*., 2011; Niangaly *et al*., 2017; Zimmerman, 2017). Some studies reported cases of Pv in Duffy positive and negative Africans (Fru-Cho *et al*., 2014; Gunalan *et al*., 2016; Menard *et al*., 2010), which could be due to a recent adaptation of *Pv* over the time, raising the point of

the new pathway used by *Pv* parasites to invade Duffy negative RBCs (Gunalan *et al*., 2018). Some studies explored the role of the *Pv*-DBP in Duffy null individual RBCs invasion, still no definitive conclusion is drawn (Gunalan *et al*., 2016; Hoque *et al*., 2018). Duffy variants were in HWE among the study population.

G6PD deficiency gene assessment showed a general G6PD deficiency prevalence of 2.78% among the overall study population. Looking into the different health districts, we realised there was a gradient in G6PDd prevalence (Santchou: 0%; Dschang: 2.75%; Kyé-ossi: 7.69%) suggesting more people at risk of haemolysis after primaquine administration as we move from Santchou to Kyé-ossi (Ashley et al., 2014). This tendency remain the same as we consider G6PDd as a protective factor against malaria, despite, it does not provide protection against severe malaria (Mbanefo, *et al*., 2017). This gradient could be explained by the past history of malaria in these areas, presenting Kyé-ossi as a previous malaria endemic area and Dschang and Santchou as less endemic area (Baird, 2018). Moreover, only two outpatients infected with *Pv* showed and heterozygous genotype, from which the phenotype was not easy to estimate, since we did not perform any phenotypic evaluation of the G6PD deficiency among the patients. G6PD 968 T>C SNP showed no variability whereas G6PD 202 G>A SNP (among women) showed a variability, which was in HWE in Santchou but not in the other health districts. Systematically, there was significant heterozygous defect, which could be due to a reduce accuracy of the restriction enzyme.

CYP2D6 alleles assessed were *2, *4 and *17. *CYP2D6 *2* allele was the main mutation found, followed by *CYP2D6*17* and *CYP2D6*4.* These results were consistent with the findings of Gaedigk and collaborators among Africans (Gaedigk *et al*., 2017). Only *CYP2D6* 1934G>A SNP presented variants in HWE. The other two SNPs were consistently not in HWE showing a significant heterozygous defect. The linkage disequilibrium analysis among the three loci of the CYP2D6 gene, performed separately in the two involved districts, provided information about the incomplete linkage among 2938 vs 1934 and 2938 vs 1023 in Dschang. Moreover, it showed that the linkage was incomplete between 2938 vs 1934 but complete between 2938 vs 1023 in Kyé-ossi, thus giving rise to four new haplotypes in Dschang and 1 in Kyé-ossi. The HWE failure for 2938 C>T and 1023 C>T SNPs variants distribution could also be explained by the gene duplication or deletion which is known to be common among the study population (Beoris *et al*., 2016; Gaedigk *et al*., 2017; Steijns and Van Der Weide, 1998). Furthermore, the

activity of the gene was predicted based on the assessed allele mutation. The population was mainly normal metabolisers fast or slow. Very few of them were intermediate metabolizers as found previously among African population (Gaedigk *et al*., 2017).

Malaria diagnosis in Cameroon, as is many other endemic countries, relies on microscopy and *Plasmodium falciparum* rapid diagnosis test. This study shows that these diagnosis methods were used in HCFs, but not systematically as required in the national guidelines for malaria management (MINSANTE, 2017). Almost a third of the study population did not benefit for malaria diagnosis at the HCF level. This could be due to the fact that some health personnel are prone to prescribe a malaria treatment only looking at the symptoms and especially in case there is fever (WHO, 2013b). Another reason could be the inadequation between the guidelines and the income of care-seekers together with issues related the diagnosis facilities (Crump *et al.*, 2011; Mma *et al.*, 2017). Only 161 people benefited for both diagnosis methods among which 10 (6.2%) were showing discordance in results. This relatively low discordance in results could be explained by the fact that in all the HCFs, all the sections, serology and parasitology, are found in the same room and health personels are more likely to ask for the results of the RDT to direct their miscrocope observation in order to make sure their results are online. It could also be due to the poor quality of the available microscopes not allowing to have a proper look at the thick film.

Assessing the specificity and the sensitivity of the locally available diagnosis methods we were able to have an idea on the accuracy of the latest. The standard method used was nested PCR developped by Snounou and Singh (Snounou and Singh, 2002). We found out that generally, the ability to detect the true negative patients with these methods was relatively poor to respect to the ability to detect true positive. In the same scope, we observed huge discrepencies while comparing thick films and *Pf*-RDT results respectively with nested PCR results. The discrepency between thick films and nested PCR results could be due to the poor facilities available as well as poor capacities of the health personels (Crump *et al*., 2011; WHO, 2013b). Whereas the discrepency between *Pf*-RDT and nested PCR results could be principally due to the deletion of histidine rich protein 2 (HRP-2) and the low parasitaemia as mentioned by studies carried on in resource-limited settings including Cameroon (Netongo, *et al.*, 2016; Tahar *et al.*, 2013; Verma *et al*., 2018). It is also known that, *Pf*-RDT can remain positive for a while after an anti-malarial

treatment (Dalrymple *et al.*, 2018). This could be the reason why an important amount of positive cases through *Pf*-RDT were negative through PCR.

Women tended to be more infected than men especially comparing the results of *Pf*-RDTs among people aged more than 5 years old. This finding goes in line with that of Jenkins *et al*. in Kenya showing women as 50% more likely than men to have malaria (Jenkins *et al.*, 2015). This was paradoxical, since there was no dependence between the under five years old age group, the sex and the results of locally available diagnosis methods, which age group is always referred to as part of the most susceptible individuals (Hertz *et al.*, 2013; World Health Organization, 2007).

Care-seeking behaviours were also assessed among the study population and revealed that 21.2% (212/1001) of the study population have started antimalarial therapy at home. ACT and Quinine reported as medicines used. This is a common behaviour in malaria endemic countries where population always assimilate fever to malaria (Birhanu *et al*., 2016; Hertz *et al*., 2013; Ocan *et al*., 2014; WHO, 2013b). Moreover, this practice can lead to a huge bias in diagnosing the disease (Netongo, *et al.*, 2016) as well as wastage of antimalarial drugs and over-treatment with consequent risk of possible toxicity and parasitic resistance insurgence (Anderson *et al*., 2016; Takala-harrison and Laufer, 2015; WHO, 2013b). This is more alarming when we observed that women were more likely to undergo self-medication and this, independently of the age group concerned. Especially because self-medication is also known as a reason for delay in care-seeking (Kassile *et al.*, 2014).

Potential protective factors related to *Pv* occurrence were mainly the regular use of mosquitoes net and the installation of curtains at the door. Whereas the potential risk factors were mainly the presence of livestock in/or around the household and the fact that all family members were sleeping in the same room. Potential protective factors related to *Pf* occurrence were to be under five years old, to have a house with countertop and to have mosquitoes net. Meanwhile having a LLIN and using regularly the mosquitoes net appeared to be potential risk factors. These findings suggest a potential diversity of the vectors which could be indoor and outdoor mosquitoes according to the area (Zhu *et al*., 2017). Moreover, contrary to this study presence of livestock was found to be a protective factor against malaria incidence (Hasyim *et al*., 2018). In line with this study, house gaps were found to be risk factors for malaria occurrence (Kaindoa *et al*., 2018; Peterson *et al*., 2009). Also, behavioural and environmental factors were found to be a key aspect for

malaria occurence as described previously (Heggenhougen *et al*., 2003; Niringiye and Douglason, 2010). The potential risks and protective factors described in this study, cannot be considered as real determinants of *Pv* and *Pf* occurrence, because it requires a longitudinal case-control study to point them out as proceeded by the previous-mentioned studies.

CONCLUSION

This study reports the high circulation of *Plasmodium vivax* in Cameroon especially in DHD with *Pv* representing 66.5% of malaria cases. It shows that G6PD 202 G>A SNP and CY2PD6 genes variability might not be a barrier to adopting new guidelines for the malaria control program in Cameroon, since relatively low proportions of the study population are G6PDd and CYP2D6 intermediate metabolizers. Moreover, it reveals an approximative diagnosis of malaria with laboratory tests available in the HCFs as well as relatively limited anti-malarial self-medication in Santchou, Dschang and Kyé-ossi health districts. Furthemore, it shows the tight interaction between the socio-economic, behavioural and environmental factors giving the appropriate conditions for *Pv* or *Pf* occurrence. This study highlights the need to improve the equipment and the skills of health personnel working in HCFs of the study site, but underlines also the need to improve the quality control of health services delivered in remote areas of resource-limited countries in order to strengthen the fight against malaria. Moreover, this study shed a light on self-medication pattern in the West and South regions of Cameroon where the misuse of antimalarial drugs appears to be a real challenge at the community level to roll back malaria.

RECOMMENDATIONS AND PERSPECTIVES

The results and conclusion of this work suggest us to draw the following.

Recommendations

- Raising the awareness of the population about self-medication dangers and behaviours which can help them avoid malaria infection and improve community management of fever,
- need to improve the local microscopic diagnosis capacity in order to ensure a more effective and safer therapeutic management of *vivax* malaria attacks and relapses by the practitioners,
- improvement of the quality control of health services delivered in remote areas of resource-limited countries in order to strengthen the fight against malaria by the policy makers,
- update and implementation of fever and malaria management guidelines in Cameroon including *Pv* especially in the West Region by introducing RDT targeting the four *Plasmodium spp* and the appropriate package for *Pv* malaria management after diagnosis by the policy makers.

Perspectives

- Assessment of the real *Pv* local circulation among asymptomatic population,
- identification of the susbtypes of *Pv* circulating in Cameroon,
- assessment of Duffy-independent *Pv* erythrocyte invasion pathway,
- carry out an in-depth pharmacological assessment including more CYP2D6 mutations and MAOA,
- carry out an entomological survey to find out real *Pv* vectors in Cameroon.

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APPENDIX

ETHICAL CLEARANCE

Cameroon Bioethics Initiative

Secretariat of the CAMBIN Ethics Review and Consultancy Committee
The ARK/L'ARCHE, Montée Jouvence, B.P. 31489, Biyem-Assi, Yaoundé, Cameroon.
OHRP IORG/IRB Registration IRB00008269; FWA N° 17337
Tel: +237-22-31-03-28; www.cambin.org Email: ercc@cambin.org cambin.ercc@gmail.com
Banker: Union Bank of Cameroon PLC; Yaoundé Branch; Acc. No: 10023 00040 004130000 35; Swift: UCMACMCX

Our Ref **CBI/ 427/** ERCC/CAMBIN

Ethics Review and Consultancy Committee (ERCC),

Cameroon Bioethics Initiative (CAMBIN),
P. O. Box 31489, Biyem-Assi, Yaoundé, Cameroon.

26th March 2018

Ghyslaine Bruna Djeunang Dongho, Université de Rome la Sapienza

Dear Ghyslaine Bruna,

RESEARCH ETHICS CLEARANCE

Re: **« Plasmodium vivax Malaria and Fever of Unknown Origin in the West and South Regions of Cameroon »**

Reference number: CBI/427/ERCC/CAMBIN **Protocol number:** 1134

The CAMBIN Ethics Review and Consultancy Committee (ERCC) gave a favourable ethical opinion to this study on 26th March 2018. You may now proceed with your research. Please note the following standard requirements of approval:

1. This approval is valid for a period of twelve months (26 March 2018-25 March 2019). At the end of this period, if the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a full report on the project. If you complete the work earlier, you must submit a final report as soon as the work is completed. At the end of the 12 months period, if the project is still ongoing, you should submit an application for renewal of the approval.
2. Please note that after this approval, the ERCC must be notified of any alteration to the project.

3. You must notify the ERCC immediately, in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability of the project.

4. Kindly note that, as the Principal Investigator (P.I) of this research protocol, you remain the person in charge of ensuring that it is carried out in an ethical manner and that this ethics clearance in no way dispenses you nor takes over any of your duties as principal investigator of the research.

5. As P.I, it is your responsibility to facilitate follow-up review or monitoring of this study by the ERCC.

6. At all times, you are responsible for the ethical conduct of this study in accordance with national and international guidelines. Please note that this is a research ethics clearance and not an administrative authorization.

Yours Sincerely,

Chi Primus Che (PhD, MPH,
M.Sc)

Chair of the CAMBIN
ERCC

cc: CAMBIN Secretariat

QUESTIONNAIRE

Code_____ Date _____

Health facility _____ surveyor _____

Demographic Information : Age _____ ; Sex : M ☐ F ☐

Home _____ Education (nombre of years of studies)

_____

Civil status : married ☐ (monogamy☐, polygamy☐) single ☐ widow ☐ divorced☐

Occupation : housewife ☐ farmer ☐ haidresser ☐ tailor ☐ driver ☐ trader ☐ teacher ☐ civil servant ☐ unemployed ☐ resourceful ☐

other_____

Reasons for consultation : onset of symptoms (date) _____

Fever	Yes ☐ No ☐	Rashes	Yes ☐ No ☐
headache	Yes ☐ No ☐	Muscle ache	Yes ☐ No ☐
cough	Yes ☐ No ☐	Joint pain	Yes ☐ No ☐
Diarrheoa	Yes ☐ No ☐	Abdominal pain	Yes ☐ No ☐
Vomiting	Yes ☐ No ☐		

Other (precise)

_____

Prior to this consultation, did you start treatment at home? Yes ☐ No ☐

If yes, which medication did you take ?

_____

Since when did you start taking this medicine ? (date) _____

Who prescribed the medicine ? : health staff ☐ ; traditional healer ☐ ; yourself ☐

Medical history:

Diabetes : Yes ☐ No ☐ ; Hypertension : Yes ☐ No ☐ ; Known HIV infection : Yes ☐ No ☐

Other (precise)

_____

For women : DLM_____ Currently pregnant : Yes ☐ No ☐

Number of : Born children: _____ ; Children alive _____ ; abortion _____ ;

Underfive years old deceased children _____

During the last <u>three months</u> (excluding the current consultation) have you suffered :

Fever	Yes ☐ No ☐	Rashes	Yes ☐ No ☐
headache	Yes ☐ No ☐	Muscle ache	Yes ☐ No ☐
cough	Yes ☐ No ☐	Joint pain	Yes ☐ No ☐
Diarrheoa	Yes ☐ No ☐	Abdominal pain	Yes ☐ No ☐
Vomiting	Yes ☐ No ☐	Other (precise) _____	

Over the last <u>six months</u> how many times did you take antimalarial treatment? _____

Do mosquitoes bite you ? : Often ☐ rarely ☐ sometimes ☐ never ☐

They bite during : the day ☐ the night ☐ sunset ☐ sunrise ☐

The site of the sting is often *(can be more than one answer)*

inflated ☐ (much☐, a bit☐) reddened ☐ (much ☐ a bit ☐)

we see nothing ☐ itching ☐ (much☐, a bit☐)

Do you treat the sting with *(if possible more than one answer)* :

Herbs ☐ Saliva ☐ Pharmaceutical products ☐ Nothing ☐ Other_____

Information on the surrounding of the house

Is it possible to find the following around the house *(if possible more than one answer)*:

Lake ☐ creek ☐ swamp ☐ river ☐ torrent ☐ None of those ☐

Do you (or your neighbours) have pets ? Yes ☐ No ☐

If Yes, which ones ? _____

Wher do you fetch water for the house ?

Tap at home ☐

Source or drilling or well (private or public) far from the house: <200 m ☐ >200 m ☐

Are there mosquito nets at home ? Yes ☐ No ☐ (treated Yes ☐ No ☐)

If Yes, how long have you been having it (year) ? _____

Do you often sleep under the mosquito net ? Yes ☐ No ☐

Si non, pourquoi ?

_____

Information about the house

Building material : bricks ☐ modern bricks☐ Other_____

Are the walls inside plastered ? Yes ☐ No ☐

Roofing material : metal sheets ☐ straws ☐ Other _____

Is there ceiling ? Yes ☐ No ☐ ; *If yes*, specify the material _____

Number of windows _____ Number of rooms _____

Restroom : internal ☐ external ☐ kitchen : internal ☐ external ☐

Is there electricity at home ? Yes ☐ No ☐

Do all inhabitants of the house all sleep in the same room ? Yes ☐ No ☐

Is there a curtain at the door : Yes ☐ No ☐

Lab

Thick film : pos ☐ neg ☐ (*if pos*, parasitemia _____) ; RTD malaria: pos ☐ neg ☐ Not done☐

Stools :

_____

Other tests

_____

_____

_____

Notes

_____

_

_____

_____

_____

_____

INFORMATION SHEET

STUDY TITLE: *PLASMODIUM VIVAX* MALARIA AND FEVER OF UNKNOWN ORIGIN IN THE WEST AND SOUTH REGIONS OF CAMEROON.

Principal Investigator: Ghyslaine Bruna DJEUNANG DONGHO Tél: 00237 694543116, E-mail: djeunabru@gmail.com, Sapienza University of Rome

We invite you to participate in this research, which focuses on the causes of fever, in order to help us draft our Doctoral Thesis in Public Health, Microbiology and Infectious Diseases at Sapienza University in Rome.

Objective: Contributing to improved management of fever and malaria in resource-limited settings

Period and study population: This study will be carried out from December 15, 2017 to April 15, 2018; in all the feverish patients who were consulted in the health facilities selected for our study.

Project development: Your participation in this study will consist of answering questions about your health and your environment and living conditions and allowing us to take your blood (three drops). It is also about giving access to your laboratory results so that you can compare them with those we will get with advanced techniques.

The become of collected data: All information gathered will be kept in a locked database hosted in an online box with restricted access to anyone other than the investigators and will be used later for further research.

The become of remaining samples: for all the planned analyzes we will extract a quantity of DNA (100µl) which will be sufficient only for the analyzes considering a margin of error for need of repetition. For that reason no sample will remain and all the samples tubes will be discarded at the end of the study.

Voluntary participation and possibility of withdrawal: Your participation in this study is voluntary, so you have the free choice to give us access to your laboratory results. You could also give up at any time while making your decision known to the investigator.

Ethical considerations:

Risks to the research project: The risks to which participants in this study are exposed are: confidentiality rupture and violation of your autonomy.

Control measure of risk: Access to information is restricted to the research team only

Benefits: Strengthen community and hospital management of fever and malaria in resource-limited settings

Compensation: There is no compensation for your participation.

Our contacts:

Ghyslaine Bruna DJEUNANG DONGHO Tel: 00237 694543116, E-mail: djeunabru@gmail.com, Université de Rome la Sapienza (Principal Investigator)

Dr. SANOU SOBZE Martin Tél: 0023797719601 E-mail: martinsobze@hotmail.com, Université de Dschang, BP 67 Dschang, Cameroun. (Co-Supervisor)

The Ethics Review and Consultancy committee of the Cameroon Bioethics Initiative (ERCC-CAMBIN), Tel : 00237 222310328 Email : ercc@cambin.org, cambin.ercc@gmail.com

INFORMED CONSENT FORM

I Undersigned……declare have been invited to participate in the study entitled ***PLASMODIUM VIVAX*** **MALARIA AND FEVER OF UNKNOWN ORIGIN IN THE WEST AND SOUTH REGIONS OF CAMEROON.**

By Miss Ghyslaine Bruna Djeunang Dongho

Principal investigator

I have carefully read the information sheet (or it was read and explained to me)

I understand the objectives of the study

The research process was well described and I understand what is expected of me

The risks and benefits of the study were presented to me

I had enough time to ask questions, which have received satisfying answers

I also understand that my participation in this research is voluntary; I am free to participate or not in this study and to withdraw my consent at any time without reprisals.

Given all the above, I voluntarily agree to participate in the study.

Date and signature of participant

Date and signature of the Principal Investigator

Parent Consent Form

STUDY TITLE: *PLASMODIUM VIVAX* MALARIA AND FEVER OF UNKNOWN ORIGIN IN THE WEST AND SOUTH REGIONS OF CAMEROON.

Principal Investigator: Ghyslaine Bruna DJEUNANG DONGHO Tel: 00237 694543116, E-mail: djeunabru@gmail.com, Sapienza University of Rome

Name of Child …… Date of Birth ……

Parent/ Guardian ……

Address: ……

Postcode ……

Tel: ……

Mobile: ……

E-mail: ……

Relationship to child: ……

CONSENT (please read carefully)

a) I confirm I read carefully and understood the information sheet.
b) I agree to my son/ daughter taking part in the activities of the study.
c) I consent to my son/ daughter being pricked for blood sample collection and I will be the one to explain to him/her what is going to be done.
d) I consent to participate in this study by answering questions about the health and the environment and living conditions on behalf of my son/ daughter.
e) I understand that the activities include accessing laboratory results of my son/ daughter so that it can be compared with those you will get with advanced techniques.

Signature …… (Parent/ Guardian) Date: ……

X