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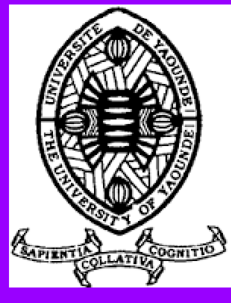

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DEPARTEMENT DE BIOCHIMIE

BIOTECHNOLOGY CENTRE

MOLECULAR PARASITOLOGY AND DISEASE VECTOR RESEARCH LABORATORY

CO-INFECTION OF MALARIA AND HELMINTHIASIS: EFFECT ON THE GENETIC DIVERSITY OF TARGETED VACCINE CANDIDATES AND ANTI-MALARIAL RESISTANCE MARKERS IN MFOU HEALTH DISCTRICT

A master dissertation submitted in partial fulfilment of the requirements for the award of a Master of science degree in Biochemistry

Option: **Biotechnology and Development**

by

KAMGMO TAPPE Ange Patricia

Bachelor's Degree

Registration Number: 17N2755

Under the supervision of:

Jude DAIGA BIGOGA

Professor

University of Yaounde I

ACADEMIC YEAR 2024-2025

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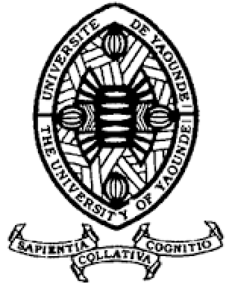

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DEDICATION

To my mother MEKAMTA Marie Mirabelle

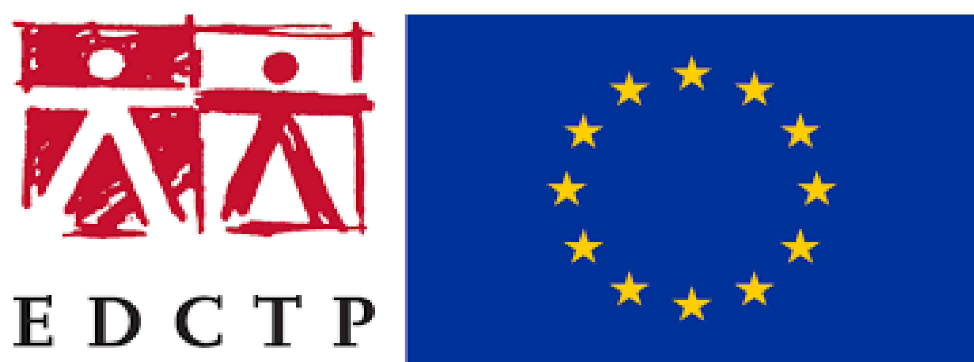
ACKNOWLEDGMENTS

At the end of this Master's dissertation, I would like to sincerely thank:

- The Almighty God for the gift of life, for the courage, strength and favor given me to carry out this work and also for His continuous blessings on me;
- **Professor Jude DAIGA BIGOGA,** Director of this work and Head of the Molecular Parasitology and Disease Vector Research Laboratory at the University of Yaounde I Biotechnology centre for having offered me an appropriate framework during this work, for his teachings, his criticism, invaluable advices and patience. Please accept the expression of my deepest gratitude;
- **Doctor Francis ZEUKENG,** for introducing me to the realities of research and the different molecular biology techniques and above all for his advice, his support, his understanding and patience towards me;
- **Professor Paul Fewou MOUNDIPA**, Head of the Biochemistry Department of the Faculty of Science of the University of Yaoundé I for the academic training and numerous advice;
- **Professor Pierre François DJOCGOUE**, Director of the Biotechnology Centre of the University of Yaoundé I for having made available to me the infrastructures for which he is responsible;
- **Mrs. NYONGLEMA Philomina,** our Microscopy Slides Reader expert for her intensive training and her patience towards me;
- To my laboratory seniors, Honoré AWANAKAM, Loic KEUMO, Hillary MATABOU, DJOUNDA Romeo, Loic NCHANKOU, Annie AGNENGA and the whole great team at the Malaria research unit for their physical and moral support during the manipulations;
- To my classmates and friends, especially those in the option Biotechnology and Development for their support and friendliness;
- To **Mr. Clinton NOMBO**, for his support, encouragement and reassurance;
- To my mother **MEKAMTA MIRABELLE** for the different forms of support provided throughout the production of this work and especially for her indefectible love;
- To my family and all those who directly or indirectly helped in any way to achieve this work.

FINANCIAL SUPPORT

This Master's dissertation was conducted within the framework of the CANTAM-3 Project at the Biotechnology Centre of the University of Yaoundé 1, under the supervision of Professor Jude DAIGA BIGOGA. The CANTAM-3 Project is funded by the EDCTP3 program, which is supported by the European Union. The views and opinions of authors expressed herein do not necessarily state or reflect those of the funders.



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LIST OF ABBREVIATIONS

A. lumbricoides	:	*Ascaris lumbricoides*
ABC	:	ATP-binding Casette
ABZ	:	Albendazole
ACT	:	Artemesinin Combination Therapy
AIDS	:	Acquired Immunodeficiency Syndrome
AMA-1	:	Apical Membrane Antigen 1
AL	:	Artemether-lumefantrine
AQ		Amodiaquine
µl	:	Microliter
CDC	:	Centers for Disease Control and Prevention
CSP		Circum Sporozoite Ptrotein
CQ	:	Chloroquine
DEC	:	Diethylcarbamazine
DNA	:	Deoxyribonucleic acid
DV	:	Digestive Vacuole
EDTA	:	Ethylene Diamine Tetra Acetic-Acid
ELISA	:	Enzyme-Linked Immunosorbent Assay
FCM	:	Flow Cytometry
GPI	:	Glycosylphosphatidylinositol
GSK	:	GlaxoSmithKline
He	:	Heterozygosity
HF	:`	Halofantrine
HRP2	:	Histidine Rich Protein 2
IFN	:	Interferon
Ig	:	Immunoglobulin
IL	:	Interleukin
IPTi	:	Intermittent Preventive Treatment for infants
ITNs	:	Insecticide Treated Mosquitos Nets
LAMP		Loop Mediated Isothermal amplification
LDH	:	Lactate Deshydrogenase
LMIC	:	Low Middle and Income Countries

LLINs	:	Long Lasting Insecticide-treated Nets
LSA-1	:	Liver Stage Antigen 1
Mg	:	Milligram
MOI	:	Multiplicity of Infection
MHC	:	Major Histocompatibilty Class
Ml	:	Milliliter
MIFs	:	Migrating Inhibiting Factors
MIPs	:	Macrophages Inhibiting Proteins
MS	:	Mass Spectrometry
MSP-1	:	Merozoite Surface Protein-1
MSP-2	:	Merozoite Surface Protein-2
MQ	:	Mefloquine
NBDs	:	Nucleotide Binding Domains
NMCP	:	National Malaria Control Strategy
NKs	:	Natural Killers
P.	:	*Plasmodium*
PCR	:	Polymerase Chain Reaction
PCT	`	Preventive Chemotherapy
Pfcsp	:	Plasmodium falciparum circum sporozoite protein
Pfmdr	:	*Plasmodium falciparum* multidrug resistance
Pfmsp1	:	*Plasmodium falciparum* merozoite surface protein 1gene
Pfmsp2	:	*Plasmodium falciparum* merozoite surface protein 2 gene
PRR	:	Pattern Recognition Receptors
PNLSHI	:	National Programme for the Control of Shistosomiasis and Soil transmitted Helminthiasis
PZQ	:	Praziquantel
RBCs	:	Red blood cells
RDT	:	Rapid Diagnostic Test
RNA	:	Ribonucleic acid
RTS,S	:	Repeat T cell epitope Surface, Surface
SMC	:	Seasonal Malaria Chemoprevention
STHs	:	Soil transmitted helminthiasis
SP	:	Sulfadoxine-pyrimethamine

SPC	:	Seasonal Preventive Chemother
SPZ	:	Sporozoites
T. *trichiura*	:	*Trichuris trichiura*
TGF-β	:	Transforming Growth Factor beta
Th	:	T-helper cell type
TMD	:	Transmembrane Domain
TNF-α	:	Tumor Necrotic Factor Alpha
TRAP	:	Thrombospondin Related Anonymous Protein
Ug	:	Microgram
WBC	:	White Blood Cells
WHO	:	World Health Organisation

ABSTRACT

Malaria and helminthiasis are major parasitic diseases that impose a significant global burden. Due to their overlapping geographical distributions, co-infection with these parasites is common. Helminth infections have been shown to modulate the immune response in individuals infected with malaria, thereby altering the selection pressure on the genetic structure of *Plasmodium falciparum* populations. However, the impact of helminth parasites on the genetic diversity of *Plasmodium* drug resistance markers and vaccine candidates remains poorly understood in regions where both infections are endemic. This study investigated the effect of malaria and helminth co-infection on the genetic diversity of targeted *Plasmodium falciparum* vaccine candidates (*Pfmsp-1* and *Pfmsp-2* genes) and the antimalarial multi-drug resistance marker (*Pfmdr-1* gene) in the Mfou Health District, Centre Region of Cameroon. A total of 521 participants were enrolled during various cross-sectional studies in Nkassomo, Vian, Lobe and Ndangueng. Malaria diagnostics were confirmed using Rapid Diagnostic Tests (RDT), microscopy, and PCR in blood samples, while the Kato-Katz method was used to confirm helminth infections in stool samples. The *Pfmsp-1, Pfmsp-2* and *Pfmdr-1* genes were genotyped by nested-PCR in mono- and co-infected individuals to assess the impact of soil-transmitted helminthiasis (STHs). The prevalence of malaria in the study population was 46.64% (243/521) by RDT, 35.70% (186/521) by microscopy, and 17.66% (92/521) by PCR. Helminth infections and co-infections with malaria were found in 10.75% (56/521) and 3.65% (19/521) of the participants respectively. *Ascaris lumbricoides* and *Trichuris trichiura* constituted the main helminthiasis in the study communities. Overall, all *Pfmsp-1* and *Pfmsp-2* allelic families were found in mono- and co-infected individuals. A total of 09 and 09 different alleles were respectively found in mono- and co-infected individuals for the *Pfmsp-1* gene, and 12 and 13 alleles for the *Pfmsp-2* gene. The overall Multiplicity of infection (MOI) was higher in the co-infected population (MOI = 4.06) compared to the mono-infected population (MOI = 2.83). *P. falciparum* mono-infected individuals exhibited an overall expected heterozygosity (He) of 1.47, while the coinfected ones with STHs showed an He of 1.09. The *Pfmdr-1* gene was highly diverged in coinfected individuals (03 different alleles, MOI = 1.38) compared to mono-infected individuals (02 different allele, MOI = 1.11). These preliminary data highlighted that coinfection by soil transmitted helminthiasis can increase the genetic diversity of *P. falciparum* vaccine genes and drug resistance makers, affecting the potential efficacy of antimalarial drugs and vaccines. Continuous monitoring of the effects of STHs on the multiplicity of *Plasmodium* infection could guide towards decision-making in the selection of efficient drugs and vaccines for coinfected individuals with malaria and STHs in co-endemic areas.

Key words: malaria, helminthiasis, co-infection, genetic diversity, vaccines candidates

RESUME

Le paludisme et l'helminthiase sont des maladies parasitaires majeures qui représentent un fardeau mondial considérable. En raison du chevauchement de leurs distributions géographiques, la co-infection par ces parasites est fréquente. Il a été démontré que les infections helminthiques modulent la réponse immunitaire chez les personnes infectées par le paludisme, modifiant ainsi la pression de sélection sur la structure génétique des populations de *Plasmodium falciparum.* Cependant, l'impact des helminthes sur la diversité génétique des marqueurs de résistance aux médicaments et des candidats vaccins de *Plasmodium* reste mal compris dans les régions où les deux infections sont endémiques. Cette étude a examiné l'effet de la co-infection par le paludisme et les helminthes sur la diversité génétique des candidats vaccins ciblés contre *Plasmodium falciparum* (gènes *Pfmsp-1* et *Pfmsp-2*) et du marqueur de multirésistance aux médicaments antipaludiques (gène *Pfmdr-1*) dans le district sanitaire de Mfou, région du Centre, au Cameroun. Au total, 521 participants ont été recrutés au cours de diverses études transversales à Nkassomo, Vian, Lobe et Ndangueng. Les diagnostics de paludisme ont été confirmés à l'aide de tests de diagnostic rapide (TDR), de la microscopie et de la PCR dans les échantillons de sang, tandis que la méthode Kato-Katz a été utilisée pour confirmer les infections par helminthes dans les échantillons de selles. Les gènes *Pfmsp-1, Pfmsp-2* et *Pfmdr-1*) ont été génotypés par PCR nichée chez les personnes mono- et co-infectées afin d'évaluer l'impact des helminthes transmis par le sol. La prévalence du paludisme dans la population étudiée était de 46,64% (243/521) par TDR, 35,70% (186/521) par microscopie et 17,66% (92/521) par PCR. Les infections à helminthes et les co-infections du paludisme ont été trouvées chez 10,75% (56/521) et 3,65% (19/521) des participants respectivement. *Ascaris lumbricoides* et *Trichuris trichiura* constituaient les principales helminthiases dans les communautés étudiées. Dans l'ensemble, toutes les familles alléliques *Pfmsp-1* et *Pfmsp-2* ont été trouvées chez les individus mono- et co-infectés. Au total, 09 et 09 allèles différents ont été trouvés respectivement chez les individus mono- et co-infectés pour le gène *Pfmsp-1*, et 12 et 13 allèles pour le gène *Pfmsp-2*. La multiplicité de l'infection (MOI) était plus élevée dans la population co-infectée (MOI = 4,06) par rapport à la population mono-infectée (MOI = 2,83). Les individus mono-infectés par *P. falciparum* ont présenté une hétérozygotie globale attendue (He) de 1,47, tandis que les individus co-infectés par des helminthiases présentaient une He de 1,09. Le gène *Pfmdr-1* était très divergent chez les individus coinfectés (03 allèles différente, MOI =1.38) par rapport aux individus mono-infectés (02 allèles différente, MOI = 1.11). Ces données préliminaires ont mis en évidence que la co-infection par des helminthiases peut augmenter la diversité génétique des gènes vaccinaux de *P. falciparum* et des facteurs de résistance aux médicaments, ce qui affecte l'efficacité potentielle des médicaments et des vaccins antipaludiques. La surveillance continue des effets des helminthiases sur la multiplicité de l'infection à *Plasmodium* pourrait guider la prise de décision dans la sélection de médicaments et de vaccins efficaces pour les personnes co-infectées par le paludisme et les helminthiases dans les zones co-endémiques.

Mots clés : paludisme, helminthiase, coïnfection, diversité génétique, candidat vaccin

INTRODUCTION

INTRODUCTION

Malaria is an endemic disease that causes widespread economic devastation and a major public health problem in sub-Saharan Africa. Pregnant women and children under the age of five years are the two most vulnerable groups. Malaria is a febrile illness caused by an infectious parasitic protozoan of the genus *Plasmodium* through the bite of an infected female *Anopheles* mosquito, of which there are more than 140 species and only six are pathogenic to humans. These are *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and *P. cynomolgi,* the most recent species (Ta *et al*., 2014). An estimated 263 million malaria cases were reported in 2023 that led to 597,000 deaths in total in 83 malaria endemic countries, with most of this increase coming from countries in the WHO African Region (WHO, 2024). Malaria is the most widespread endemic disease in Cameroon, responsible for about 7,343, 000 reported cases, and absenteeism from school and work annually (WHO, 2024). Adding to this very high burden of malaria is the frequent co-existence of parasitic helminths among children living in Low and middle-income countries (LMIC).

Helminthiasis, also known as worm infection, is any macroparasitic disease of humans and other animals in which a part of the body is infected with parasitic worms, known as helminths. It is estimated that 1.5 billion individuals are infected with helminths (WHO, 2023), with more than 800 million children in LMIC affected by Soil Transmitted Helminths (STH) primarily hookworm (*Ancylostoma duodenale* and *Necator americanus*), roundworm (*Ascaris lumbricoides*), and whipworm (*Trichuris trichiura*) (Degarege & Erko, 2016).

In Cameroon, both malaria and helminth infections coexist and are ranked among the major causes of parasitic mortality and morbidity with *Plasmodium falciparum* being the most prevalent and virulent species of malaria parasite. Although the nature of interaction remains uncertain, studies showed that an apparently true biological association exists between *Plasmodium* and helminths when they coexist in a host. Hence, the presence of helminth can affect the risk of malaria and severity of the disease; or the occurrence of *Plasmodium* infection may in turn impact the upcoming helminth infections and related morbidities (Mwangi *et al*., 2006); (Nacher, 2011). While some studies have reported that helminth infection promotes protection against clinical malaria(Degarege *et al*., 2012), others showed an increased susceptibility to *Plasmodium* infection (Nacher *et al*., 2002).

Over the past 10 years, there has been substantial progress in reducing the enormous burden of malaria globally through the use of interventions such as; the use of long-lasting insecticide-

treated nets (LLINs), artemisinin combination therapy (ACTs), intermittent preventive treatment for infants (IPTi), seasonal malaria chemoprevention (SMC), and vaccination programs are concentrated on this under five years-of-age group to significantly reduce the risk of death (WHO,2023). Also, Preventive chemotherapy (PCT) through school-based periodic administration of praziquantel (PZQ) and albendazole (ABZ) to school-age children remains the cornerstone of schistosomiasis and STH control respectively in endemic foci in Cameroon (*PNLSHI – National Programme for the Control of Schistosomiasis and STH*, n.d.). Despite the current efforts to control malaria in Cameroon, the situation remains alarming, mainly due to the increasing vector resistance to insecticides (Boussougou-Sambe *et al*., 2018) and parasite resistance to certain anti-malarial drugs (Metoh *et al*., 2020). This parasite resistance is mainly due to constant evolution in the parasite population due to its great genetic diversity resulting to high polymorphism of certain proteins among which Merozoite surface proteins 1and 2, with the emergence of resistance gene such as *Plasmodium falciparum* multi-drug resistance 1 (*Pfmdr-1*) gene. The *Pfmdr-1* gene has been associated with parasite susceptibility to a variety of currently available antimalarial drugs including chloroquine, lumefantrine, amodiaquine, mefloquine and quinine (Adamu *et al*., 2020) .

Merozoite Surface Protein-1 (MSP-1) and Merozoite Surface Protein-2 (MSP-2) are two antigens challenging the human immune system and are important candidates for development of blood stage malaria vaccines (Patel *et al*., 2017). The genetic diversity of the *msp-1* and *msp-2* genes in *Plasmodium falciparum* is a key factor in the parasite's ability to evade the host's immune system. Coinfection with malaria and helminths can influence this diversity. For instance, studies have shown that helminth infections may affect the immune response to malaria, potentially altering the selection pressure on the *msp-1* and *msp-2* genes. However, the exact mechanisms and outcomes of such interactions are complex and still under investigation. There is a lack of information about *Pfmdr*1 genetic diversity in a context of coinfection between malaria and helminthiasis. Continuous monitoring of the genetic diversity of malaria parasites and antimalarial resistance markers is an important action that could guide decision-making in the selection of efficient drugs and vaccines for a specific intervention within an endemic locality. Thus, the study aimed to determine the impact of malaria and helminth coinfection on the genetic diversity of targeted vaccines candidates.

RESEARCH QUESTION

Does malaria and helminth co-infection influence the genetic diversity of *Pfmsp-1* and *Pfmsp-2* genes and antimalarial resistance marker *Pfmdr-1* gene in Mfou health district?

RESEARCH HYPOTHESIS

Concomitant infection of malaria and helminth co-infection has no effect on the genetic diversity of *Pfmsp-1* and *Pfmsp-2* genes and the antimalarial resistance marker *Pfmdr-1* gene in Mfou health district in the Centre region of Cameroon.

STUDY OBJECTIVES

1. General objective

To investigate the effect of malaria and helminth co-infection on the genetic diversity of *P. falciparum* targets (*Pfmsp-1*, *Pfmsp-2)* and the multidrug resistance marker *Pfmdr-1* in Mfou health district, Centre region of Cameroon.

2. Specific objectives

1- To determine the prevalence of malaria, helminthiasis and malaria and their coinfection in the study population by RDT, PCR and Microscopy**.**

2- To assess the genetic diversity of *Pfmsp-1* and *Pfmsp-2* gene and *Pfmdr-1* resistance gene by nested PCR.

3- To compare the *P. falciparum* multiplicity of infection (MOI) between mono- and co-infected individuals with soil transmitted helminthiasis (STHs) targeting *Pfmsp-1*, *Pfmsp-2* and *Pfmdr-1 genes*.

CHAPTER I: LITERATURE REVIEW

CHAPTER I: LITERATURE REVIEW

I.1 MALARIA

I.1.1 DEFINITION AND HISTORY

Malaria is a severe disease caused by parasites of the genus *plasmodium*, which is transmitted to humans by a bite of an infected female mosquito of the species *Anopheles*. The history of malaria extends from its prehistoric origin as a zoonotic disease in the primates of Africa through the 21st century (Ernst, 2010). The first description of malaria was attributed to Hippocrates in 400 BC. The work carried out by Hippocrates established a relationship between the presence of marshes and seasonal fevers. The association between marshes and intermittent fevers yielded the name 'mal-aria' (bad air), thus the idea that malaria comes from bad air has been validated for nearly 2500 years (Hippocrates, 1994).

The search for the exact cause of malaria grew exponentially with the discovery of microbes by Leeuwenhoek in 1676 and also due to the theory of infections set out by Pasteur and Koch between 1878 and 1879, which stated that « all infections are caused by microorganisms ». They, therefore incriminated various algae and bacteria such as Bacillus malariae. Alphonse Laveran, while studying the lesions in blood, noticed the presence of black granular pigments. He therefore, put forward the hypothesis that these granules were the cause of malaria. Laveran later discovered spherical bodies that were free or adherent to erythrocytes. He finally observed the exflagellation of a male gametocyte: a life stage of the *Anopheles* mosquito. The mobility of these elements convinced Laveran that he discovered the cause of malaria and that it was a protozoan (Cox, 2010).

Various natural compositions from plants have been used for years to fight against malaria. The first effective treatment came from « Quinquina », which contains quinine. Once the link between mosquito and the parasite was established in the 20th century, elimination measures such as the use of DDT for spraying breeding sites and swamps have been put in place. Indoor spraying and the use of impregnated mosquito nets had also been initiate. Nowadays, artemisinin is the molecule common to all types of anti-malarial drugs; it's discovery last for several decades, but in 2015 Pr Tu Youyou demonstrated that it is the essential molecule in the treatment of malaria (Su & Miller, 2015). In October 2021, the World Health Organization (WHO) recommended RTS, S/AS01 (RTS, S), the world's first malaria vaccine, for children at risk in sub-Saharan Africa and in other regions with moderate to high transmission of malaria caused by *Plasmodium falciparum*.

I.1.2. MALARIA EPIDEMIOLOGY

Malaria is one of the deadliest infectious diseases of humanity, which causes significant mortality and morbidity in the tropics, particularly in Africa (Skeet, 2005). Malaria is endemic throughout most of the tropics. Globally, in 2023, there were 263million estimated malaria cases that led to 597,000 deaths in 83 malaria endemic countries, an increase of 11 million cases compared with 2022 reported by the World Health Organization (WHO, 2024).

Between 2000 and 2019, the number of annual estimated malaria cases remained stable, varying between 227 million and 248 million across the 108 countries that were malaria endemic in 2000. Since 2020, the number of estimated malaria cases has steadily increased, and most of this increase occurred in countries in the WHO African Region.

Malaria in human is known to be caused by five species of *Plasmodium* (*P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi*) ((Jongwutiwes *et al*., 2004), (Singh & Daneshvar, 2013)). The majority of infections in Africa are caused by *Plasmodium falciparum*, the most dangerous of the five human malaria parasites. Malaria affects the lives of almost all people living in the area of Africa defined by the southern fringes of the Sahara Desert in the north, and a latitude of about 28° in the south. Most people at risk of the disease live in areas of relatively stable malaria transmission infection and occurs with sufficient frequency that some level of immunity develops.

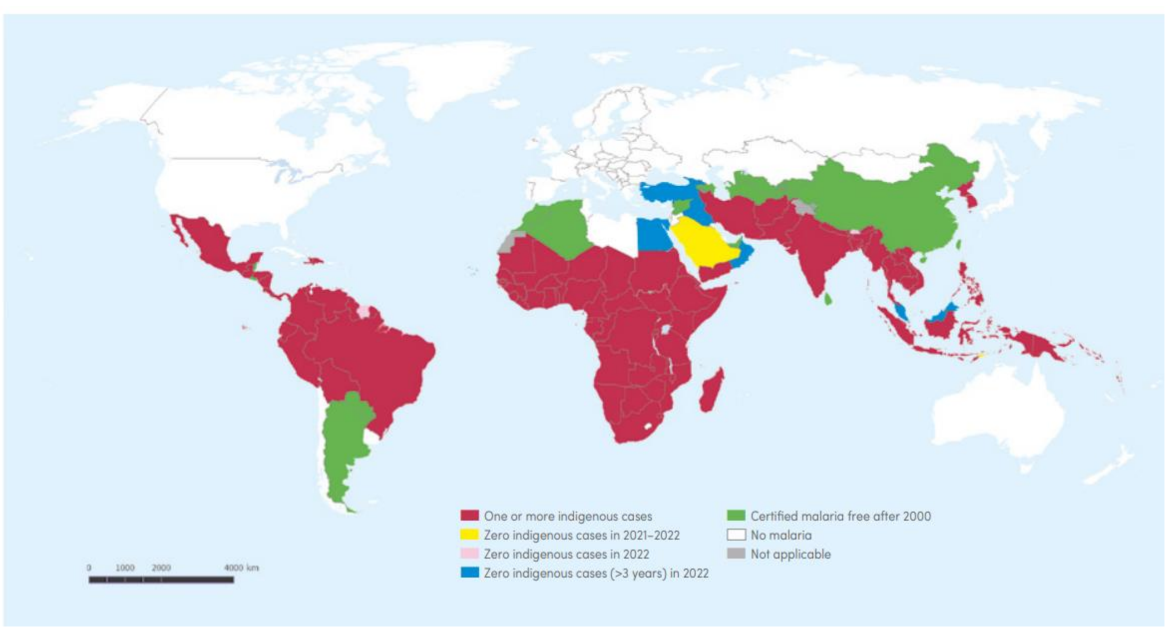


Figure 1: Countries with indigenous cases in 2000 and their status by 2022 (WHO, 2024).

I.1.3 MALARIA IN CAMEROON

Malaria is the most widespread endemic disease in Cameroon, annually responsible for greater than 2 million reported cases as well as absenteeism from school and work (World Malaria Report, 2023). The number of reported deaths in 2022 was 12,600, and the estimated number of infections was about 7 million (World Malaria Report, 2024). The country has three epidemiological zones: the Sahelian, sudano-sahelian and the equatorial. The forest zone in the south has a permanent transmission season of seven to twelve months (Global Fund, 2023.). The North and Adamawa regions have a tropical climate but a shorter transmission season of four to six months. The Far North region has a tropical and Sahelian climate with a short seasonal transmission period of one to three months. Transmission of malaria is highest from July to October**.** The incidence of malaria is highest in the East region, while malaria mortality remains highest in the North and Far North. Overall, Cameroon is among the 15 highest burden malaria countries, with 2,7% of all global malaria cases and deaths, and 2.3% of malaria deaths in 2021 (*U.S. President's Malaria Initiative: Cameroon Malaria Operational Plan FY 2023*, n.d.); this represents the 3rd highest number of malaria cases in Central Africa.

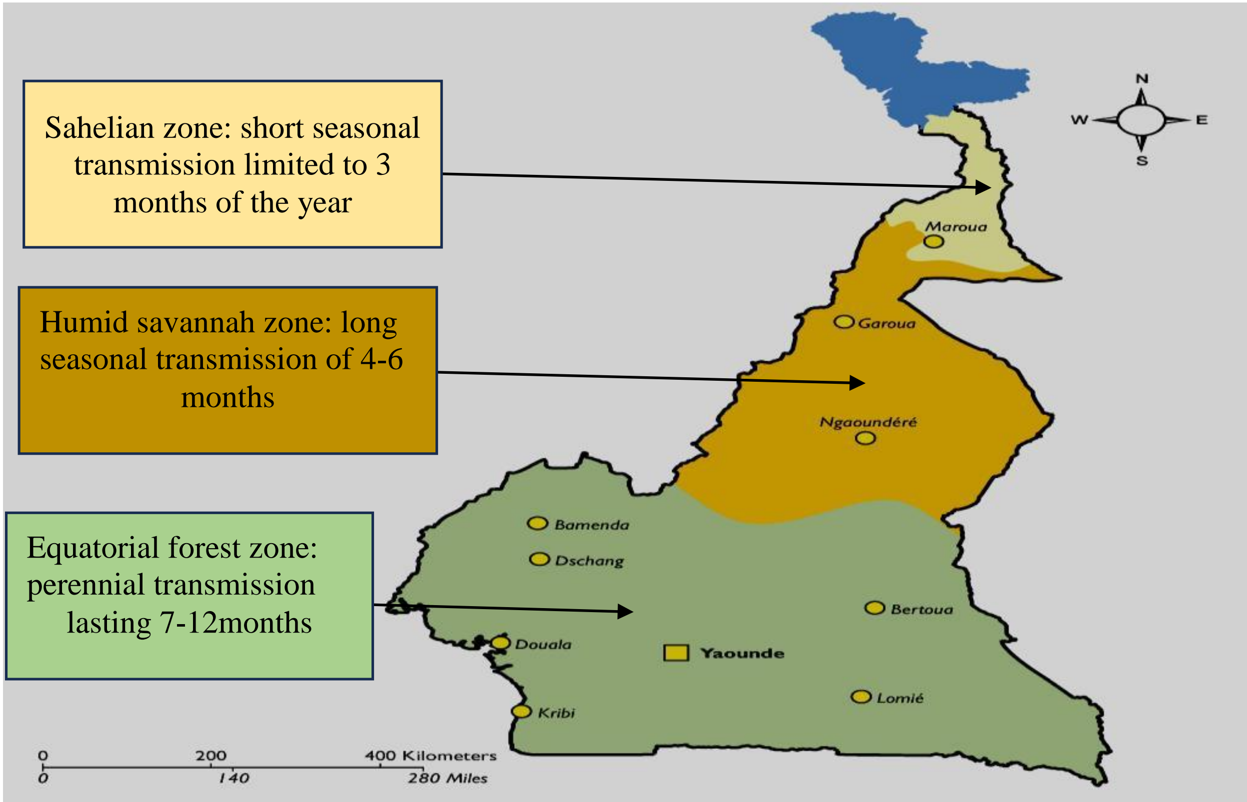


Figure 2: The eco-epidemiological z ones for malaria in Cameroon (NMCP,2010)

I.1.4. PATHOGENIC AGENT

Malaria is caused by a parasite called *Plasmodium*, a protozoan of 1 to 2 micrometer belonging to the animal kingdom and phylum Apicomplexa. May-Grunwag-Giemsa staining shows that it contains a pale blue cytoplasm surrounding a light-coloured nutrient vesicle, and containing a red nucleus and the golden brown or black pigment called hemozoin.

Taxonomy of Malaria parasite:

Phylum	Apicomplexa (Sporozoa)
Class	Haemosporidea (Sporozoea)
Order	Haemosporidia
Genus	Plasmodium
Sub-genus:	Plasmodium, Laverania, Vinckeia

Species: (There are about 120 species, of which 5 infect Man (*P. falciparum, P. vivax, P. ovale, P. malariae, P. knowlesi) (*Source: Srinivas, 2015).

I.1.5. VECTOR AGENT

Female mosquitoes of the genus *Anopheles* are the exclusive vectors of human malaria. There are about 484 species of *Anopheles* (Harbach, 2004), but only about 60 are capable of transmitting human malaria parasites (Bigoga *et al*., 2012). *Anopheles* belong to the *culicidae* family and of order *dipteria.* They require blood meals to mature their eggs, and have a life expectancy of 3 to 12 weeks. Female Anopheles stays close to their place of emergence (less than 300m away) and bite at night between sunset and sunrise. They can live in houses (endophilic) and outside (exophilic), and prefers human (anthropophilic) or animals (zoophilic). Different life stages are observed during growth: egg, larva, nymph, adult or imago.

In Africa, the main malaria vector is An. gambiae s.s (Jean *et al*., 2004), and the *Anopheles* species found in this continent have a relatively long lifespan and a strong preference for humans, which partly explains why over 90% of malaria deaths occur in the sub-Saharan Africa (*Paludisme.Pdf.Crdownload*, n.d.).

In Cameroon, about 52 species of *Anopheles* contribute to disease transmission, with 06 recognised as primary vectors and 10 as secondary vectors. The 06 major vectors are *An.*

Gambiae, An. Coluzzii, An. Arabiensis, An. Funestus, An. Nili and An. moucheti (Antonio-Nkondjio *et al*., 2019).

I.1.6. MODES OF TRANSMISSION

The modes of transmission are varied because of the multitude of factors responsible, including: geographical and climatic factors (temperature, rainfall, altitude, humidity), Socioeconomic factors (poor urbanisation, precarious housing, human activities) and individual factors (age, pregnancy, behaviour). These modes include:

- Infection through the bite of an infested female *Anopheles* mosquito; this is the main mode of malaria transmission
- Transplacental or congenital malaria
- Post transfusional malaria

I.1.7. THE LIFE CYCLE OF PLASMODIUM

Malaria parasite has a complex multi-stage life cycle occurring within two living beings, a vertebrate host and a mosquito vector. The parasites are haploid throughout most of this life cycle, replicating by asexual multiplication twice in a mammalian host: in liver hepatocytes (pre-erythrocytic schizogony) and within red blood cells (blood stage schizogony), and once in the mosquito (sporogony): the cycle is digenic (Guttery *et al*., 2012).

a. Sexual phase in the mosquito host (sporogony)

Mosquitoes are the definitive hosts of malaria parasites where in the sexual life cycle occurs; this phase is called sporogony. When the female *Anopheles* draws a blood meal from an individual infected with malaria, the male and the female gametocytes find their way into the gut of the mosquito. There, they fuse to form zygotes, which subsequently develop into actively moving ookinetes. The ookinetes borrow into the mosquito midgut wall to develop into oocysts. Growth and division of each oocyst produces thousands of active haploid forms called sporozoites (spzs). After the sporogonic phase of 8-15 days, the oocyst bursts and releases spzs into the body cavity of the mosquito; from where they travel to and invade the mosquito saliva glands. When the mosquito thus loaded with spzs takes another blood meal, the spzs get injected from its salivary glands into the human blood stream, causing malaria infection in the human (Cowman *et al*., 2016).

b. Asexual phase in the intermediate host (schizogony)

During the asexual phase, the sporozoites are injected after a blood meal by the anopheles' mosquitos. A small proportion of sporozoites exit the skin dermal layer by migrating to and penetrating a blood vessel within 1-3 hours (Harding, 2020). After an hour, Sporozoites will migrate to liver, pass through Kupffer cells and infect hepatocytes, where the first asexual development occurs (the asexual Exo-erythrocyte cycle) (Cowman *et al*., 2017). Sporozoites will be differentiate into merozoite, from 16–32 (tissue schizogony). In *P. vivax* and *P. ovale*, dormant forms called hypnozoites usually remain in the liver until later in life. *Plasmodium falciparum* does not produce hypnozoites (Cowman *et al.*, 2017). As soon as merozoites leave the liver, an asexual intraerythrocytic cycle begins in the blood. Merozoites (up to 40,000) invade red blood cells and develop into trophozoites. This phenomenon continues until it is controlled by an immune response or chemotherapy, or until the patient dies. After trophozoites transform into schizonts, they generate new merozoites. A schizont is composed of many daughter merozoites (blood schizogony). Red blood cells infected with merozoites lyse, and other red blood cells are also infected. This is a new cycle of schizogony. Duration of each cycle of *P. falciparum* malaria takes about 48 hours. Intermittent fever and seizures are due to the lysis of infected red blood cells.

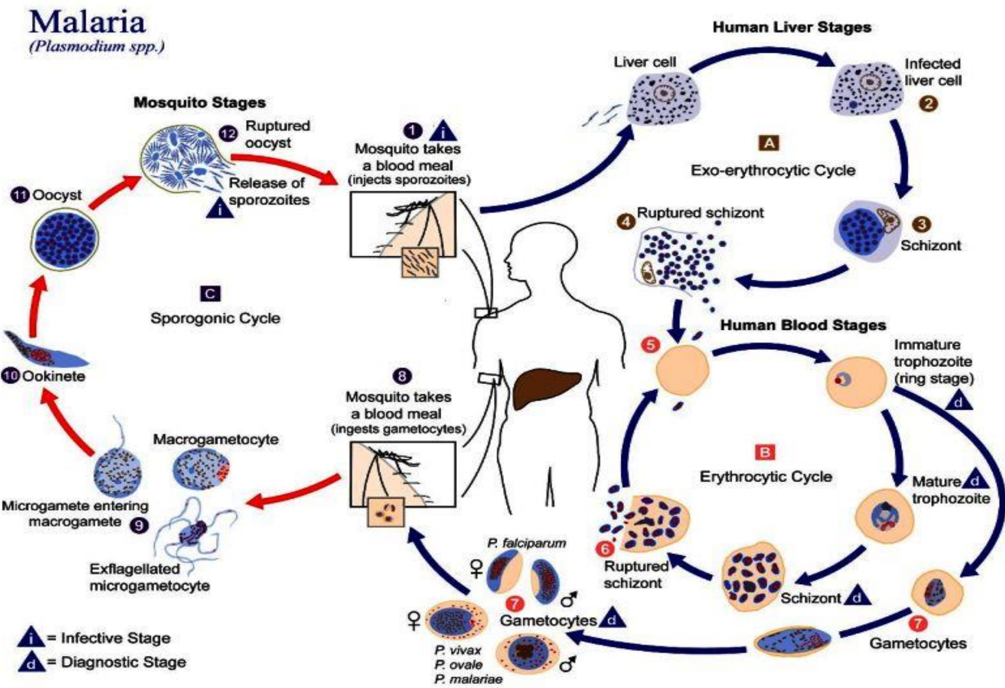


Figure 3: The life cycle of malaria parasites (CDC - DPDx - Malaria, 2024)

I.1.8. SIGNS AND SYMPTOMS

Symptoms vary depending on the type of *Plasmodium* causing the infection, but might include high fever, chills, headache, diarrhea, abdominal pain, fatigue, muscle or joint pain and cough (Shahbodaghi & Rathjen, 2022). In some cases, the illness can progress to severe anemia, kidney and respiratory failure, coma and even death. Some types of malaria cause less severe illness, but if not treated the symptoms can continue for weeks or months with episodes of fever and chills.

I.1.9. DIAGNOSIS OF MALARIA

I.1.9.1. Clinical diagnosis

The clinical diagnosis of malaria involves the identification of symptoms. In cases of uncomplicated malaria, typical symptoms include fever, general fatigue, vomiting, diarrhea, headaches, and muscle pain. It is important to note that this diagnosis is based only on clinical symptoms and should be confirmed with laboratory tests. Diagnosing simple malaria remains challenging as the symptoms observed are common to several other tropical disease, such as yellow fever. It is important to use precise diagnostic tools to differentiate between these diseases. Symptoms such as loss of consciousness (agitation, confusion), respiratory distress, repeated vomiting, hypoglycaemia, jaundice haemorrhage, and hyperthermia are typically observed in case of severe malaria (CDC, 2019).

I.1.9.2. Laboratory diagnosis of malaria

a. Biological diagnosis

- ✓ **Microscopic diagnosis using stained thin and thick peripheral blood smears**

Malaria is diagnosed microscopically by staining thick and thin blood films on a glass slide, to visualize malaria parasites under the microscope. This permits distinction of species and different stages of the parasite life cycle and also to quantify parasitaemia. Microscopy is simple, inexpensive, yet labour-intensive. The sensitivity of detection by microscopy is approximately 10-30 parasites/µl of blood. However, this level of detection is normally not attained in malaria endemic areas where many samples need to be screened in a relatively short time. Thus, incorrect speciation is common and low levels of parasitaemia may be missed (Quakyi *et al*., 2000). To prepare a thick blood film, a blood spot pre-placed on a slide is stirred in a circular motion with the corner of another slide with care being taken not to make the preparation too thick, and allowed to dry without fixative. Since they are unfixed, the red cells

lyse when a water-based stain is applied. Because a larger volume of blood is examined the thick film is more sensitive than the thin film (down to around 40 parasites per µl or 1 parasite per 200 white blood cells) although it requires more expertise to read. There are many methods described for staining blood films for malaria diagnosis, including Giemsa stain (20-30 min), Leishman stain (45 min), and the rapid Field stain method (10 sec).

b. Immunological diagnosis

- ✓ **Rapid diagnostic tests (RDTs)**

RDTs are all based on the same principle. It involves the application of immunological techniques using antibodies (monoclonal) to detect malarial antigens, in blood flowing along a membrane containing specific anti-malaria antibodies by immunochromatography. For malaria diagnosis, 2 important soluble antigens secreted by erythrocytic forms of the parasite in the blood are targeted: Histidine Rich Protein-2 (HRP-2) and Lactate Dehydrogenase (LDH).

RDTs can be performed by individuals with minimal training and requires no electricity or special equipment. If one or more additional bands are visible, the diagnosis is positive and the combination of bands displayed can be used to identify the affected species (Murphy *et al.*, 2013). It offers numerous advantages in malaria diagnosis. They are suitable for field use, cost-effective, easy to use, and require minimal infrastructure.

How ever, the true issue with RDTs remains that false positives and false negatives. This could be explained by their low detection threshold (Bersoza *et al.*, 2018).

c. Molecular diagnostic methods

Recent developments in molecular biological technologies, for example, PCR (polymerase chain reaction), loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, have permitted extensive characterization of the malaria parasite and are generating new strategies for malaria diagnosis.

- ✓ **PCR Techniques**

Developed by Kary Mullis in 1983, the PCR is an in vitro enzymatic amplification of a single or few copies of DNA across several orders of magnitude, generating thousands of copies of a particular DNA sequence of interest (*Kary B. Mullis--Nobel Laureate for procedure to replicate DNA - PubMed*, s. d.). This method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication to

occur. PCR comprises 03(three) main steps: Denaturation which takes place at 92°C, Hybridization/ Annealing which takes place at 54°-55°C, consist of the fixation of the primers on the strands to be amplified and finally Elongation/ Extension using DNA polymerase, which is carried out at 72°C. The principle of PCR involves heating the DNA to denature it into single strands, then cooling it to allow specific primers to anneal to the target sequence, followed by DNA polymerase enzyme extension to replicate the DNA. This cycle of denaturation, annealing and extension is repeated multiples times, resulting in exponential amplification of the target DNA sequence. This method is highly sensitive and specific. It can detect up to 1parasite/µl of blood.

Although this method appears to have overcome the two major problems of malaria diagnosis sensitivity and specificity, its utility is limited by complex methodologies, high cost, and the need for specially trained technicians.

- ✓ **LAMP technique**

It is a new innovative molecular technique that can be used for the detection of *P. falciparum*. Indeed, it is a simple, rapid, specific and inexpensive method of amplification of nucleic acids that was developed by Eiken chemical Co.Ltd. It can use DNA or RNA (with the addition of reverse transcriptase) as a substrate. LAMP takes place in 08 steps and involves 04 primers (FIP, F3, BIP, B3) that can specifically recognize 06 different regions on the target gene. Unlike PCR, this technique does not require initial denaturation, does not use special reagents or sophisticated equipment; the entire reaction takes place at a constant temperature of 61°C (isothermal). It provides high amplification efficiency because of high specificity.

I.1.10. IMMUNITY AGAINST MALARIA

Immunity against malaria starts with the innate response. It requires a lot of efforts to accomplish effective parasite clearance and to avoid collateral damage of the host tissues. (Harding, 2020). In the liver, during the asexual phase, host immune system will recognise infected hepatocytes. Failure to eliminate them will induce the progression of the infection to the blood. Through molecular patterns of the parasite present by dendritic cells, we have the activation of monocytes into macrophages to kill cell free parasites and infected red blood cells: it is phagocytosis. Neutrophils produces toxic substances called Reactive oxygen species (ROS) in respond to plasmodium infection and granules. Dendritics cells and macrophages are antigens presenting cells (APC) and can communicate with the adaptive immune system by presenting some parts of the parasites or by the production of chemical signals that help in the

initiation of an adaptative immune response. The appetitive response is developed specifically to the invading micro-organism. After the infection of blood by merozoites, the complement system is also involved in infection. The immune cells will tag infected cells or pathogens bound with complement components and destroy them. Summarily, the innate system protects the host from plasmodium infection, while the adaptive immune system in necessary for efficient clearance of plasmodium (Harding, 2020).

There is now a known connection between the innate and adaptive immune responses, which begin with monocytes, innate immune cells crucial for protecting the host against malaria (Dobbs *et al*., 2020). After activation, they can differentiate into macrophages, involved in the production of cytokines (Dobbs *et al*., 2017).

It is important to recognize that the immunity against *Plasmodium* during malaria is based on the adaptive immune response involving T-cells and B-cells. T cells are typically categorized into two groups: Tαβ and Tγδ cells (Nugraha *et al*., 2003). Tαβ cells are also divide into two progenitors: Tcd4 and Tcd8. Tcd4 release cytokines and Tcd8 are suppressers cells. Tcd4 is also divided into two: Th1(T helper 2 cells) and Th2 cells. Th1 is implicated in cellular response, destruction of parasites, Th2 in the immoral responses (activation of B cells, secretion of antibodies) (Nugraha *et al*.,2003.). Th1 cells produces IL-1, IL-2, IL-3, IL-6, IFN-γ, TNF-α, IL-12, which are pro-inflammatory cytokines, important against parasites and others and Th-2 cells produces IL-4, IL-5, IL-10, TGF-β, which are anti-inflammatory cytokines involved in the reduction of the inflammatory response during an infection (Leão, 2020; Barkat *et al*., 2019). Excessive inflammation has been linked to severe malaria and death (Barkat *et al*., 2019).

Regarding the development of malaria, an overproduction of pro-inflammatory cytokines can contribute to the severity and pathogenesis of the disease. Severe malaria has been associated with higher pro-inflammatory to regulatory cytokine ratios (Stanisic *et al*., 2014). Thus, a strong inflammatory response followed by an appropriate production of regulatory cytokines are required to control malaria parasite multiplication without inducing major host pathology (Gonçalves *et al*., 2014). A delicate balance is needed between the levels of inflammatory and anti-inflammatory cytokines (Gonçalves *et al.*, 2014)

I.1.11. TREATMENT AND PREVENTION

a. Treatment

It has been strongly recommended by WHO those children and adults with uncomplicated malaria (except women in their first trimester) be treated by artemisinin-based combinations (ACT). Pregnant women in their first trimester should receive quinine and clindamycin. Chloroquine should be used to treat adults and children with uncomplicated *P. vivax, P. ovale, P. malariae* or *P. knowlesi* in areas of chloroquine-susceptible infections while those in chloroquine-resistant areas be treated with ACT. Severe malaria should be treated with intravenous or intramuscular artesunate for all groups for at least 24hours till the patient can tolerate oral therapy (*World Malaria Report 2015*, n.d.).

b. Prevention

Prevention is one of the strategic priorities in the fight against malaria. It is based on three essential strategies;

- **Vector control**

Vector control remains a major asset for prevention, since this method seems to reduce the number of mosquitoes capable of transmitting the disease. The two main techniques are the use of long-lasting insecticide treated mosquito nets (ITNs) and indoor residual spraying. The ITNs form a physical barrier between man and Anopheles, but also a chemical barrier as acting like an insecticide. In addition, indoor residual spraying which is an important means of combatting the development of adult Anopheles involves spraying different rooms in the house with an insecticide with a long duration of action (6 months to 1 year) (*World Malaria Report 2019*, n.d.).

- **Intermittent preventive treatment (IPT)**

In order to reduce the number of cases of malaria in pregnant women, the WHO recommends the administration of IPT with sulfadoxine-pyrimethamine as part of antenatal care to pregnant women. IPT consist of three doses of antimalarial drugs administered at the time of visits.

- **Seasonal chemoprevention**

Seasonal preventive chemotherapy (SPC), formerly known as intermittent preventive treatment is recommended during periods of high seasonal malaria transmission (July to October) throughout the Sahel sub-region. A complete treatment of Amodiaquine and sulfadoxine-

pyrimethamine (AQ+ SP) should be administered to children aged from 3-59months at a regular one-month interval throughout the period of transmission.

I.1.12. MALARIA VACCINES

In October 2021, the World Health Organization (WHO) recommended RTS, S/AS01 (RTS, S), the world's first malaria vaccine, for children at risk in sub-Saharan Africa and in other regions with moderate to high transmission of malaria caused by *Plasmodium falciparum*. RTS, S was created in 1987 by scientists working at GSK laboratories and funded by Gavi (Vaccine Alliance), the Global Fund to Fight AIDS, Tuberculosis, and Malaria. This vaccine directs an immune response against the *Plasmodium falciparum* circum sporozoite protein (*PfCSP*). RTS, S/AS01 is a hybrid vaccine based on recombinant using the recombinant RTS, S antigen. It contains the RTS peptide in which the regions of the *P. falciparum* circum sporozoite protein that induce a humoral immune response (R) or cellular (T) immune response are covalently linked to the hepatitis antigen (S). RTS, S/AS01E was the first malaria vaccine candidate to progress into a phase III clinical trial. In 2011, the first analysis from children (aged 5–17 months) in this trial showed a vaccine efficacy of 55.8% against clinical malaria and 47.3% against severe malaria over 12 months((Stanisic & Good, 2023). RTS, S/AS01 is the first vaccine against any parasitic disease to be recommended for routine use in humans. In Africa, Ghana, Kenya, and Malawi are implementing the malaria vaccine through the Malaria Vaccine Implementation Program. The vaccine has already been administered to 2 million children and has been shown to be safe and effective. Other countries in Africa that have shown interest in this vaccine are Benin, Burkina Faso, Burundi, Cameroon, the Central African Republic, the Democratic Republic of the Congo, Liberia, Niger, Nigeria, Mozambique, Sierra Leone, South Sudan, and Uganda (WHO, 2023).

A second vaccine, named R21/Matrix-M, was announced in October 2023 by the WHO to prevent malaria in children living in high-risk areas. This addition will complement the already existing RTS, S vaccine and will result in a sufficient vaccine supply to benefit children living in areas where malaria is a major public health problem. Either R21/Matrix-M or RTS, S is used for the prevention of *P. falciparum* in children in endemic zones (World Malaria Report, 2023).

I.2. HELMINTHIASIS

I.2.1. DEFINITION AND HISTORY

The word "helminths" comes from Greek meaning worm. The parasites that infect humans can be classified as heirlooms or souvenirs (Al Amin & Wadhwa, 2024). Helminthiasis, also known as worm infection, is any macroparasitic disease of humans and other animals in which a part of the body is infected with parasitic worms, known as helminths. Helminthiasis has been a significant public health issue throughout human history, with evidence of helminth infection dating back thousands of years. In ancient civilizations, such as Mesopotamia and Egypt, there are records of helminths infections and attempts to treat them. The Edwin Smith Papyrus, an ancient Egyptian medical text, describes the presence of worms in the intestines and provides remedies how to treat them. More than a quarter of the world's population, that means approximately 2 billion people are affected by the helminthic parasite, and it is one of the major burdens of developing countries, especially in children (*Soil-Transmitted Helminthiases*, n.d.). The diseases by helminths are neglected tropical diseases because they usually have insidious effects on growth and development. Advances in scientific understanding and medicine in the 19th and 20th centuries led to the development of treatment for helminth infections, anthelminthic drugs were developed to target and eliminate parasitic worms, improving the outcomes for those affected by helminthiases.

I.2.2. EPIDEMIOLOGY

Parasitic helminth infections remain a significant challenge to global health. The last decade has seen a remarkable increase in our understanding of the true disease burden of helminth infections, and there has been increasing momentum on the part of national and non-governmental developmental organizations for prevention and control of these diseases. Soil-transmitted helminth (STH) infections are among the most common infections worldwide with an estimated 1.5 billion infected people or 24% of the world's population (WHO, 2023). These infections affect the poorest and most deprived communities with poor access to clean water, sanitation and hygiene in tropical and subtropical areas, with the highest prevalence reported from sub-Saharan Africa, China, South America and Asia. They are transmitted by eggs present in human faeces, which in turn contaminate soil in areas where sanitation is poor. Over 260 million preschool-age children, 654 million school-age children,108 million adolescent girls and 138.8 million pregnant and lactating women live in areas where these parasites are intensively transmitted, and are in need of treatment and preventive interventions (WHO,

2023). A large part of the world's population is affected with one or more of these soil-transmitted helminths: approximately 807-1,121 million cases with Ascaris, approximately 604-795 million cases with Whipworm and approximately 576-740 million cases with hookworm (CDC, 2024) .

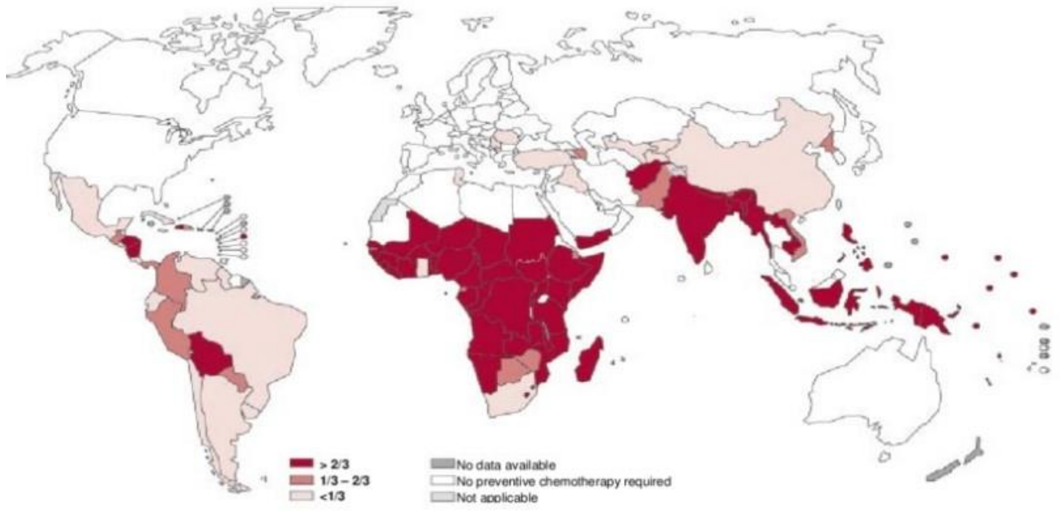


Figure 4: Distribution of soil-transmitted helminthiases and proportion of children (1-14years) in each endemic country (WHO, 2017).

I.2.3. SPECIES INFECTING HUMANS: PATHOGENIC AGENT AND CLASSIFICATION

a. Pathogenic agent

Helminths are a polyphyletic group composed of highly prevalent worms. Depending on the species, helminths may exist as parasites (that affect both human beings and animals) or as free-living organisms that live in nature. There are two major phyla of helminths known as nematodes and platyhelminths. Nematodes are also known as roundworms that include soil-transmitted helminths and the filarial worms that cause lymphatic filariasis (LF) and onchocerciasis. Other phyla platyhelminths also called flatworms, which include flukes(trematodes), schistosomes, and tapeworms (cestodes). The main species that infect people are the round worm *(Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*) and hookworms (*Necatar americanus* and *Ancylostoma duodenale).* These STH species are normally addressed as a group because they need similar diagnostic procedures and respond to same medicines (WHO, 2023).

-Ascaris lumbricoides: is a large parasitic roundworm of the genus *Ascaris*. It is the most common parasitic worm in humans (CDC, 2024) that infects an estimated 772–892 million people globally.

-Trichuris trichiura: is a parasitic roundworm that causes trichuriasis when it infects a human large intestine. It is commonly known as the *whipworm* which refers to the shape of the worm. Globally, whipworms infect an estimated 429 – 508 million people (CDC, 2024).

-Hookworms: are parasitic worms that infect roughly 406 – 480 million people globally. The name comes from the hook-like shape of its head. In humans, infections are caused by two main species of roundworm, belonging to the genus *Ancylostoma* and *Necator* (CDC, 2024).

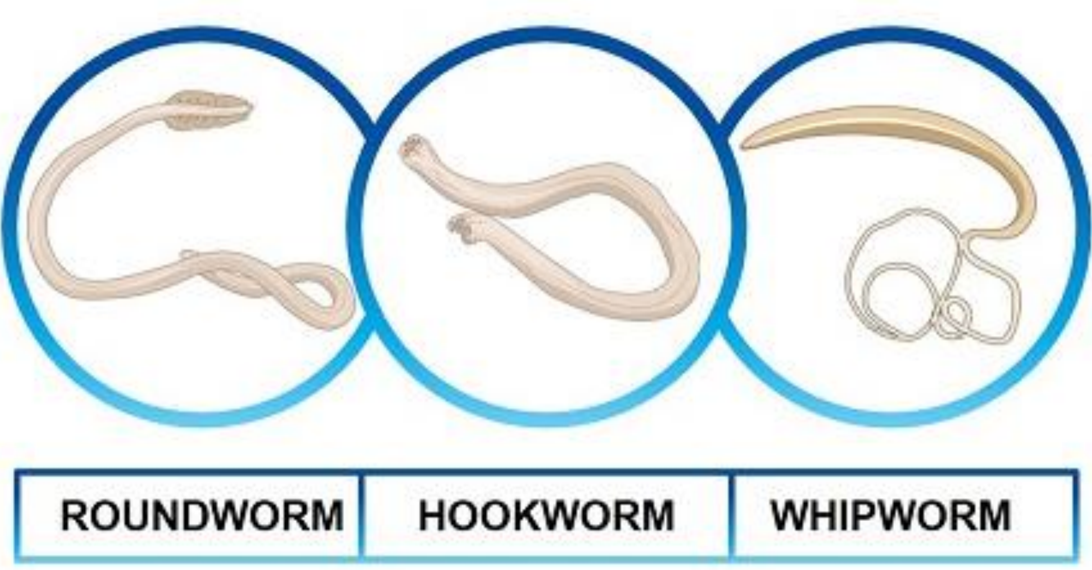


Figure 5: Roundworm, Hookworm and Whipworm

b. Classification of Helminths

Taxonomic classification of helminths

Sub kingdom | Phylum | Class | Genus – examples

Metazoa

Nematodes
Round worms; appear round in cross section, they have body cavities, a straight alimentary canal and an anus

Ascaris (roundworm)
Trichuris (whipworm)
Ancylostoma (hookworm)
Necator (hookworm)
Enterobius (pinworm or threadworm)
Strongyloides

Platyhelminthes
Flat worms; dorsoventrally flattened, no body cavity and, if present, the alimentary canal is blind ending

Cestodes
Adult tapeworms are found in the intestine of their host
They have a head (scolex) with sucking organs, a segmented body but no alimentary canal
Each body segment is hermaphrodite

Taenia (tapeworm)

Trematodes
Non-segmented, usually leaf-shaped, with two suckers but no distinct head
They have an alimentary canal and are usually hermaphrodite and leaf shaped
Schistosomes are the exception. They are thread-like, and have separate sexes

Fasciolopsis (liver fluke)
Schistosoma (not leaf shaped!)

Figure 6: Taxonomic classification of helminth (website: www.arasheedcol.edu.iq)

I.2.4. MODES OF TRANSMISSION

Helminths, or parasitic worms, can infect humans through various mechanisms.

- **Ingestion**: many helminths enter the human body through consumption of contaminated food or water, undercooked meat, unwashed fruits (Harrison *et al*., 2019).

- **Skin penetration:** certain helminth such as hookworms can penetrate the skin and enter the blood stream (Baron, 1996).

- **Vector borne Transmission**: some helminths rely on vectors to complete their lifecycle. For example, filiarial worms causing lymphatic filiariasis are transmitted by mosquitoes (Pilotte *et al*., 2017).

I.2.5. THE PARASITE LIFE CYCLE

Parasitic worms (helminths) have diverse life cycles. Some exhibit a simple (direct) life cycle in which they grow and sexually mature in a single host before releasing eggs or larvae. Many more have complex(indirect) life cycles in which one or more intermediate are infected before transmission to a definitive host (Chubb *et al.*, 2010). Helminths develop through egg, larval(juvenile), and adult stages.

- **Case of *Ascaris lumbricoides***

Ascaris lumbricoides is a nematode round worm that resides like parasites in a human being's small intestine. It is an intestinal worm of the most common helminthic infection in humans worldwide. The life cycle consists of several stages:

1. The egg stage

The adult Ascaris worms lives inside the walls of the small intestine in human's being. The female worm grows up to 35cm in length and lays approximately 20,000 eggs that are passed out of the human stool into the environment. Unfertilized eggs are ingested but are not infective but fertilized eggs are infective and undergo further development in the next stage (*Ascaris lumbricoides Structure and Life Cycle: Introduction, Diagram and Life Cycle, FAQs*, s. d.).

2. The larvae stage

The fertilized eggs grow into a larva. The larva then becomes infective after 18 days to several weeks depending on the environmental conditions like warmth, moist, and soil region. After ingestion, the fertilized eggs hatch and larvae emerge and invade the intestinal mucosa. From there they transport to other parts like lungs. Where they make their way to the throat so that the worms can be swallowed and returned to the intestines so as to reach maturity (*Ascaris lumbricoides Structure and Life Cycle: Introduction, Diagram and Life Cycle, FAQs*, s. d.).

3. The adult stage

When the larvae reach the small intestine, they develop into adult worms. At this stage, the adult worm produces a large number of eggs. Around 2 to 3 months are necessary from ingestion of infective eggs to oviposition by female adult worms. Adult ascaris are able to live for about 1 to 2 years (*Ascaris lumbricoides Structure and Life Cycle: Introduction, Diagram and Life Cycle, FAQs*, s. d.).

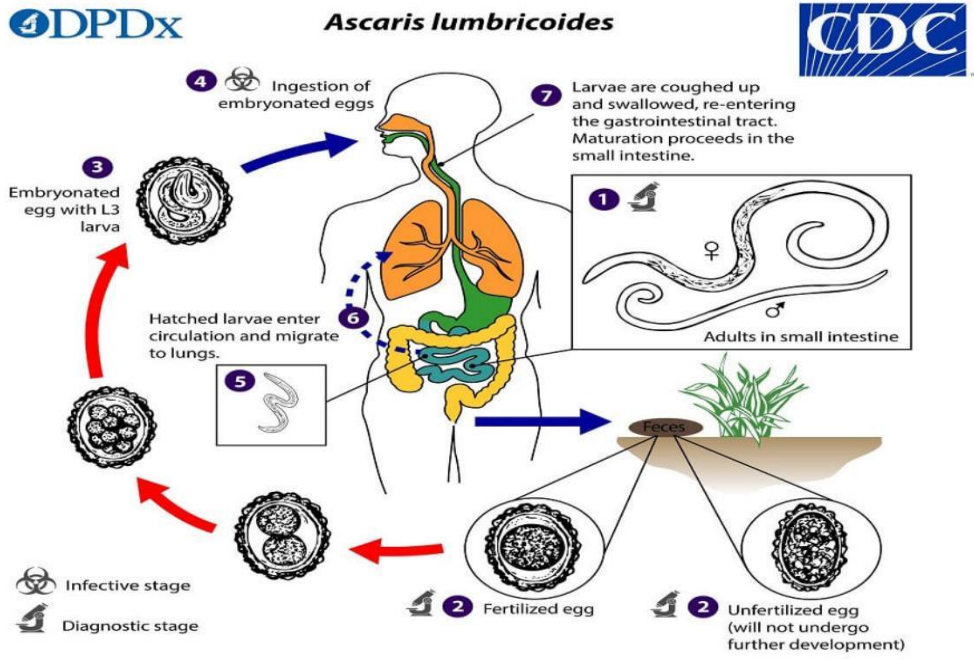


Figure 7: The life cycle of Ascaris lumbricoides (website: https://www.cdc.gov)

I.2.6. SIGNS AND SYMPTOMS

People with mild infection of helminths might not have any symptoms. However, if the infection persist it can lead to more severe symptoms. Helminth infections can cause a range of symptoms, depending on the type of worm involved and the severity of the infection. Some common symptoms include; abdominal pain, diarrhea, nausea, fatigue, weight loss. In severe cases, helminth can cause blood loss leading to anaemia(verywelhealth.com).

I.2.7. DIAGNOSIS OF HELMINTH SPECIES

Helminth species are the major contributor of parasitic infection in the world. Adequate removal techniques are necessary to stop the spreading of these infections, but it can only be successful when detection of these species is done by accurate diagnostic techniques.

I.2.7.1 Clinical Diagnosis

The clinical diagnosis is based on the demonstration of digestive signs characterized by: abdominal pain, acute or chronic diarrhea, constipation, and surgical complications; and extra-digestive signs characterized by a disturbance in sleep, fever, cough and Loeffler's syndrome (ascariasis and trichocephalosis).

I.2.7.2. Laboratory Diagnosis

✓ **Direct Examination**

The direct microscopic examination of faeces is essential to detect parasitic elements such as the larvae of *Strongyloides stercoralis* which are motile, also high concentration of the eggs of helminth infection with *Ascaris lumbricoides*. The main advantage of this method is that it is rapid inexpensive. However, it is only semi-quantitative, and is not often used in the routine analysis. It involves emulsifying a small quantity of fresh tool in one drop of saline on a microscope glass slide. A thin smear preparation is obtained by placing a covered glass on the stool and examined under a light microscope to detect eggs/larvae/trophozoites of the parasites.

✓ **Kato-Katz Technique**

The Kato Katz is the WHO ‘‘gold standard’’ method for detection of STH eggs, and additionally *Schistosoma* eggs. It has several advantages including; high sensitivity, egg quantification, cost effectiveness and requires minimal infrastructure (Mbong Ngwese *et al*., 2020). For the kato-katz technique, the sieved faeces sample (approximately 41.7 mg, 20mg or 50mg depending on the size of the template) is placed in a glass slide. The preparation is covered with a piece of cellophane soaked in glycerol. Subsequently the slide is inverted and gently press down resulting in a thin smear. The added glycerol helps to clear the fecal material around the eggs. The slides should be kept at room temperature. Microscopic examination can best be performed after 24 to 48 hours. The helminthic eggs per gram of stool EPG is calculated as: Number of eggs in smear x 20(for 50mg template) to measure the infection intensity.

✓ **Water Emergence Technique for Detecting Strongyloides larvae in Faeces**

This method requires the use of fresh stool sample. A deep hole is made in the center of the stool specimen and filled with warm water. The incubation period at 35-37 °C in an incubator for up to 3h permits the larvae to migrate out of the feces into the surrounding warm water. Some of the water is pipetted and transferred onto a glass slide and a cover glass is placed over it to make a thin preparation. The preparation is microscopically examined for motile larvae using the 10x objective. This is a cost-effective method suitable to be used in resource-limited settings (Anderson & Schad, 1985).

I.2.7.3. Immunological Diagnosis

- ✓ **Antigen Detection**

The methods described so far are based on the detection of parasitic elements(egg/cyst/larvae) in stool. However, some studies have described the use of coproantigens captured in an ELISA assay. The underlying principle of this assay relies on the capture of parasites excretory/sectory (E/S) proteins using rabbit anti E/S polyclonal antibodies (Bungiro & Cappello, 2005). These methods have been described to be effective in the diagnosis of S. stercoralis and hookworm infections. However, the methods based on antigen detection have not been widely used in STH diagnosis.

I.2.8. IMMUNITY AGAINST HELMINTHS

Studies of the immune response against helminths are of great interest in understanding interactions between the host immune system and parasites. Helminth infections are characterized by an association of Th2-like and Treg responses. Worms are able to persist in the host and are mainly responsible for chronic infection despite a strong immune response developed by the parasitized host. The immune response against helminth infection is primarily mediated by the host's innate and adaptative immune systems. The immune mechanism involved in defense against helminths typically involve a combination of different types of immune cells, such as eosinophils, mast cells, macrophages and T-helper 2 (Th2) cells (Lekki-Jóźwiak & Bąska, 2023).

In response to helminth invasion, the innate immune system recognizes the parasite through pattern recognition receptors (PRRs) and initiate a rapid immune response. Eosinophils and mast cells are key effector cells in the innate immune response against helminth, as they release toxic granules containing enzymes and cytokines that can directly damage the parasite. Macrophages also play a role in clearing helminth infections by phagocytosis and secretion of inflammatory mediators (Peng *et al.*, 2022).

The adaptative immune response against helminths is primarily mediated by Th2 cells, which release cytokines such as interleukin-4 (IL-4), IL-5 and IL-13. These cytokines activate B cells to produce antibodies, particularly immunoglobulin E (IgE), which can bind to the surface of helminths and facilitate their elimination by other effector cells. In addition, Th2 cells can also activate eosinophils and macrophages to enhance the immune response against helminths (Allen und Maizels, 2011).

I.2.9. TREATMENT AND PREVENTION

a. Treatment

Helminths infections can cause significant health issues, but fortunately, there are effective treatment options available. The treatment of helminthiasis aims to eliminate the worms and alleviate symptoms.

1. Albendazole or Mebendazole

The WHO recommended medicines albendazole(400mg) and mebendazole(500mg) are effective, inexpensive and easy to administer by non-medical personnel. These drugs are commonly used to treat infections caused by STH, such as roundworms, whipworms, and hookworms. They help to eliminate the parasitic worms from the body and are usually taken as chewable tablets twice daily for three days (WHO, 2023).

2. Praziquantel

Praziquantel is the drug of choice for treating schistosomiasis caused by body flukes (trematodes) and affect various organs. This drug effectively targets these parasites (Vale *et al*., 2017)

3. Diethylcarbamazine (DEC)

DEC is commonly utilized for infections caused by filarial worms. Filarial worms are responsible for disease like lymphatic filariasis and onchocerciasis (WHO, 2023).

b. Prevention and control

Preventing helminthiasis involves a combination of personal hygiene practices and public health measures including:

- **Good sanitation:** promoting access to clean water and proper disposal of human waste to reduce the risk of contamination.
- **Personal hygiene:** practicing good hand hygiene, including regular handwashing with soap and water.
- **Proper food and water hygiene:** ensuring the consumption of safe and properly cooked food, avoid drinking untreated or contaminated water.
- **Vector control:** taking measures to prevent mosquito bites and reduce mosquito breeding's sites in areas prone to filariasis.

I.3 GENERALITIES ON MALARIA AND HELMINTH CO-INFECTION

I.3.1 DEFINITION AND EPIDEMIOLOGY

Multi-parasitism is the concomitant occurrence of two or more parasites species in a single human host (Afolabi *et al*., 2021). The parasite species broadly categorized into two groups are macro parasites and microparasites. While macro parasites comprise parasitic helminths, microparasites affecting humans are mainly protozoa.

Due to their endemicity in tropical and sub-tropical regions of the world, their degree of association, and the fact that they share the same geographical distribution, helminths and malaria can co-exist (Adio *et al*., 2004). Descriptive studies have shown that parasitic helminths such as STH and schistosomes may co-exist with *plasmodium* protozoa in children living in resource-poor settings in the world (Brooker *et al*., 2007).

Helminthiasis/ plasmodium falciparum co-infections are the most common co-infections because of their dominance in any prevalence study of intestinal helminthiasis and malaria, and their implication in the major damages caused by these parasites in humans (Brooker *et al*., 2007). Prevalence surveys in Africa shows the co-infections *Ancylostoma/Plasmodium falciparum, Ascaris lumbricoides/Plasmodium falciparum* and *Trichuris trichiura/ Plasmodium falciparum* coinfections. The distribution of the co-infection depends mainly on the parasite species, age of the population studied, and the region of study. In Cameroon survey, few data exist on the distribution of this co-infection. According to the studies already carried out, the main co-infections are those of *T. trichiura* and *A. lumbricoides* with *P. falciparum*. Alongside these co-infections, are the mixed infections and mainly that of *T. trichiura* with A*scaris lumbricoides* (Nkuo-Akenji *et al*., 2006). Although the levels of helminth infections are generally low in young children, resulting in few *Plasmodium*–helminth co-infections (Pullan R and Brooker S, 2008), school-aged children, rather than pre-school children or adults, are at greatest risk of co-infections and its consequences ((Mwangi *et al*., 2006); (Brooker *et al*., 2007)).

I.3.2 INTERACTION BETWEEN MALARIA AND HELMINTH

Although the nature of interaction remains uncertain, studies showed that an apparently true biological association exists between *Plasmodium* and helminths when they coexist in a host (Mwangi *et al*., 2006). Hence, the presence of helminth can affect the risk of malaria and severity of the disease; or the occurrence of *Plasmodium* infection may in turn impact the upcoming helminth infections and related morbidities (Nacher, 2011). As a result, disease due

to one of these parasites could be exacerbated or ameliorated due to the co occurrence of the other species resulting in synergistic or antagonistic impacts on the infected host (Degarege & Erko, 2016). Several hypotheses have been set forth to explain the observed (and potential) interactions between malaria and helminths. Most of the evidence points towards helminths infection as having a negative effect on the acquisition of immunity to malaria, but data from Asia suggest that, due to possible modulation of pro-inflammatory and anti-inflammatory cytokines responses, helminths infection might protect against cerebral malaria.Two studies reported that *A. lumbricoides* exerted a protective impact on the severity and patency of malaria clinical infections (Brutus *et al*., 2007), in agreement with the findings of another study (Kirwan *et al*., 2010) which showed that administration of anti-helminth drugs lowered the prevalence and intensity of *A. lumbricoides* infections and also the prevalence of *Plasmodium* infection.

I.3.3. CLINICAL CONSEQUENCES OF MALARIA AND HELMINTH COINFECTION

An important consequence of both malaria and helminth infection is anemia, an important

public health problem in the tropics. It is well recognized that malaria is a significant contributor to anaemia both among young children and pregnant mothers, operating through a number of mechanisms, including hemolysis and phagocytosis (Pullan & Brooker, 2008), while hookworm infection is an acknowledged significant cause of anaemia as a result of intestinal blood loss nutritional theft and impairment of appetite due to immunological factors (Degarege *et al*., 2010). Other clinical consequences may include:

- ✓ **Increased severity of symptoms:** Co-infection of malaria and helminths can lead to more severe symptoms compared to single infections. This can result in more pronounced fever, anaemia, fatigue, and gastrointestinal issues (Degarege *et al*., 2010).
- ✓ **Immune dysregulation**: Helminths infections can modulate the host immune response, leading to altered immune regulation. This can impact the body's ability to fight off the malaria parasite effectively and may result in prolonged or complicated courses of the disease (Nouatin *et al*., 2021)
- ✓ **Impaired treatment outcomes**: Co-infection can interfere with the treatment outcomes of both diseases. The presence of one infection can affect the efficacy of treatment for the other, leading to longer recovery times or reduced response to standard therapies (Afolabi *et al*., 2021).

- ✓ **Impaired cognitive development:** Studies have shown that malaria and helminth coinfection in children can impair cognitive development and educational outcomes due to chronic inflammatory response and anaemia cause by the infections (Nacher *et al*., 2002).
- ✓ **Impaired immune response to vaccines**: Helminth coinfection can also impact the immune response to vaccines, including malaria vaccines. This can reduce the effectiveness of vaccination efforts and may require additional interventions to enhance vaccine efficacy (Hartgers & Yazdanbakhsh, 2006)

I.4. GENETIC DIVERSITY OF Plasmodium falciparum

I.4.1. The plasmodium genome

Plasmodium is haploid for most of its life cycle. It is diploid only at the zygote stage where meiosis and genetic recombination take place. The Plasmodium genome, more specifically that of clone 3D7, was completely sequenced in 2002. It varies in size from 20-25 Mb, contains around 5300 genes and comprises three types of DNA: a nuclear DNA made up of 14 chromosomes that vary considerably in size (0.7Mb for chromosome 1 to 3.3Mb for chromosome 14) as a result of deletions, crossovers and rearrangements; a mitochondrial DNA of 6 Kb and an apicoplastic DNA of 35 Kb (Gardner *et al*., 2002).

Vaccine design for *Plasmodium falciparum* is hindered by polymorphisms in certain vaccine candidate loci. Highly polymorphic regions have been observed in P. *falciparum* antigenic surface proteins, such as the circumsporozoite protein (CSP), the merozoite surface protein 1(MSP-1), the apical membrane antigen 1 (AMA-1), the liver stage antigen (LSA-1) and the thrombospondin related anonymous protein (TRAP). The mechanisms for controlling genetic diversity in the parasite genome are numerous and complex and it has been shown that certain surface antigens display a high degree of polymorphism during the asexual phase of development (Kidima & Nkwengulila, 2015). These include proteins anchored to the glycosylphosphatidylinositol (GPI) of the merozoite membrane, and several families have been identified, including the family of 'Merozoite surface proteins" (MSPs): MSP1, MSP2, MSP4, MSP5, MSP10 (Boyle *et al*., 2014). This genetic polymorphism of *P. falciparum* represents a major obstacle to the development of an effective malaria vaccine candidate because antigenic diversity limits the efficacy of the protective immunity acquired against the disease (Gandhi *et al*., 2014). Asexual blood stage antigens, MSP-1 and MSP-2 are considered prime candidates

for the development of a malaria vaccine and are also suitable markers for the identification of genetically distinct *P. falciparum* parasite sub-populations (Kang *et al*., 2010).

I.4.2. Merozoite Surface Protein 1 (MSP 1)

Merozoite Surface Protein 1 (MSP1) is the most abundant of all GPI-anchored surface proteins in terms of copy number (Gilson *et al*., 2006*)*. MSP1 is synthesized from the onset of schizogony as a 195 kDa precursor that undergoes a series of proteolytic cleavages. This processing modifies the secondary structure of MSP1 so that it can bind spectrin and mediate RBC rupture. Following processing, the MSP1 protein complex consists of four polypeptide fragments of MSP1, the 83 kDa N-terminal fragment ($MSP1_{83}$), two internal 30 and 38 kDa fragments ($MSP1_{30}$ & $MSP1_{38}$) and the GPI- anchored C-terminal 42 kDa fragment ($MSP1_{42}$) along with associating proteins MSP6 and MSP7 (Pachebat *et al*., 2007). It has been suggested that MSP1 has a role in initial contact of the merozoite with the RBC possibly by binding the cysteine rich EGF-like domains of $MSP1_{19}$, via some type of proteoglycan with heparin-like side chains or similar structure (Boyle *et al*., 2010). It has also recently been reported that the N-terminal region of MSP1 interacts with glycophorin A as an essential mediator of invasion (Baldwin *et al*., 2015).

- **Structure of the *Pfmsp-1* gene**

The gene *msp1* is located on chromosome 9 of the *P. falciparum* 3D7 reference genome, between the genomic positions 1,201,305–1,207,576. It is a single-copy gene with no introns within its coding region and is divided into 17 blocks with varying levels of genetic diversity, where 7 blocks are highly polymorphic, 5 blocks are semi-conserved, and 5 blocks are highly conserved. Differences in fragment size and the presence of unique peptide repeats delineate three major block 2 families: RO33, K1, and MAD20. Among these families, K1 and MAD20 alleles are the most diverse since they have varying numbers of unique tri-peptide repeats. RO33 alleles lack these tri-peptide repeats and are monomorphic (Chekol *et al*., 2022).

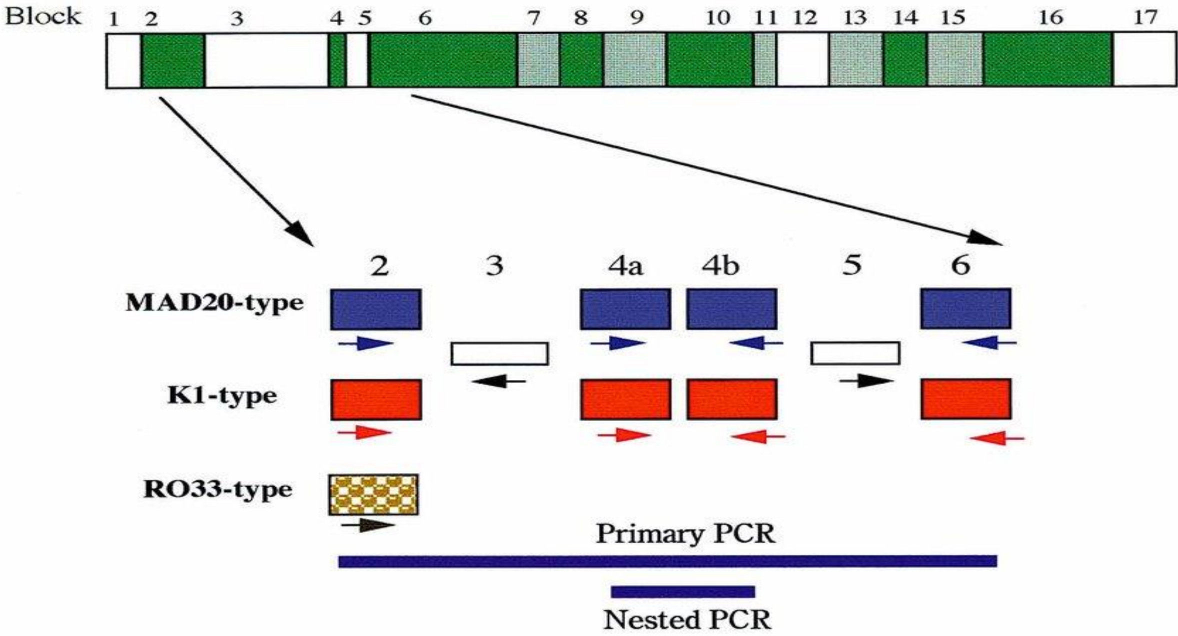


Figure 8: Structure of the P. falciparum merozoite surface protein-1 gene (Tanabe *et al.*, 1987)

I.4.3. Merozoite Surface Protein 2 (MSP2)

After MSP-1, Merozoite surface protein 2 (MSP 2) is the second most abundant protein in terms of copy number anchored to the membrane Glycosylphosphatidylinositol on the surface of the merozoite and has a molecular weight of around 25kDa. There are two main phenotypic forms of MSP 2 resulting from two families of alleles: 3D7/IC and FC27. Both forms of MSP 2 are unstructured, but the entire long recombinant protein forms fibrils under physiological conditions. Fibril formation is mediated by the N-terminal region and this region may also have membrane interaction properties (Beeson *et al.*, 2016). MSP 2 appears to be essential for invasion, is retained on the surface during invasion and is degraded as soon as invasion is complete (Boyle *et al.*, 2014). However, its precise role is unknown and ligand-receptor interactions or binding of MSP 2 to red blood cells have not been described (Beeson *et al.*, 2016).

- **Structure of the *Pfmsp-2* gene**

The MSP 2 protein is encoded by a gene located on chromosome 2 which contains a single open reading frame with conserved, semi-conserved and variable sequences. It is divided into five blocks 1, 2, 3, 4 and 5. The C- and N-terminal regions of blocks 1 and 5 are highly conserved, while blocks 2 and 4 are semi-conserved. Block 3 contains non-repetitive variable sequences incorporating repeat units that differ in length and copy number. These non-repetitive sequences define two allelic families: 3D7/IC and FC27(Figure 7). These alleles

share N-terminal and C-terminal regions, with regions of strain-specific variables (Beeson *et al*., 2016). The strain-specific region is composed of repeat units; the 3D7 family contains a repeat of a motif of four amino acids that are: GGSA. The FC27 family contains 2 copies of a 32-amino-acid motif with sequence ADTIASGSQRSTNSASTSTTNNGESQTTTPTA, followed by a copy of a 12 amino acid motif as follows: ESISPSPPITTT and a final 8 amino acid motif (Boyle *et al*., 2014).

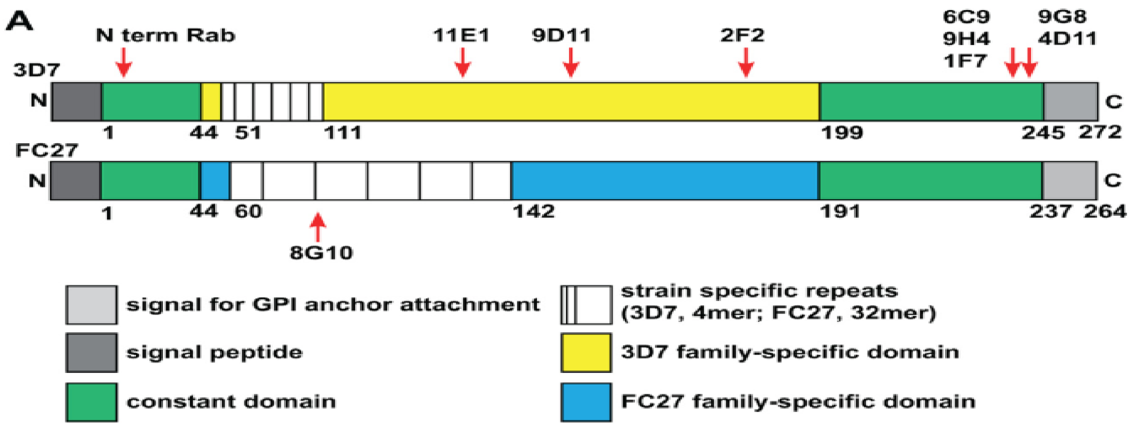


Figure 9: Structure of the two msp-2 allelic families (Boyle *et al*., 2014).

The polymorphic loci of merozoite surface proteins (msp 1 and msp 2) are now well established to assess the genetic diversity of *P. falciparum* population and multiplicity of infection (MOI) which is an indicator of malaria transmission intensity in endemic areas.

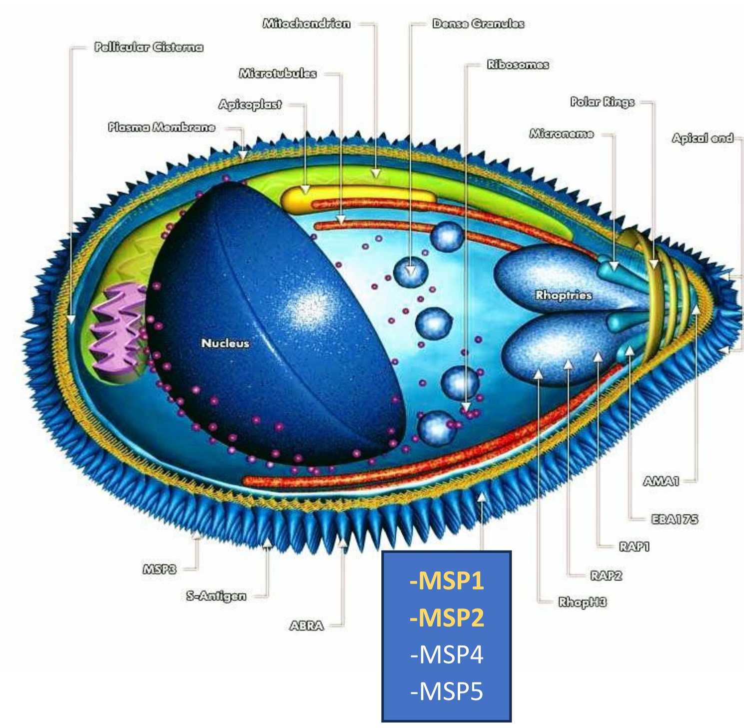


Figure 10: Structure of a merozoite (Beeson *et al*., 2016).

I.5. MARKERS OF ANTI MALARIAL DRUG RESISTANCE

In Cameroon, about 90% of malaria cases are due to *P. falciparum* infections (WHO, 2020), with children younger than 5 years and pregnant women considered the most vulnerable groups. Since 2004, drug policies for the treatment of uncomplicated *P. falciparum* malaria have changed from chloroquine (CQ) therapy to sulfadoxine-pyrimethamine (SP) therapy and then to artemisinin-based combination therapy (ACT). ACTs, based first on the administration of artesunate-amodiaquine and later on the administration of artemether-lumefantrine (AL), (Antonio-Nkondjio *et al*., 2019), were implemented because of the spread of both CQ-resistant parasites and SP-resistant parasites (Basco *et al*., 2006). The recent emergence of *P. falciparum* parasites resistant to artemisinin in Southeast Asia raises the concern that development or spread of such resistance will pose a threat to the malaria control and elimination efforts relying on ACTs in Africa (Ariey *et al*., 2014)**.**While artemisinin resistance has not yet emerged in Africa, *Pfk13*-propeller mutations associated with artemisinin resistance were recently documented in Tanzania, Rwanda, and Uganda ((Bwire *et al*., 2020),(Uwimana *et al*., 2020).

➢ ***Plasmodium falciparum* multidrug resistance 1 (*Pfmdr-1*)**

One important mediator involved in multidrug resistance to ACTs is the ATP-binding cassette (ABC) transporter *P. falciparum* multidrug resistance protein 1 (*PfMDR1*). *PfMDR1* is localized in the membrane of the digestive vacuole (DV) of the parasite and possesses the ability to influx antimalarial drugs toward the lumen of this organelle (Ferreira *et al*., 2011). Sequence analysis shows that PfMDR1 contains two transmembrane domains (TMDs) and two conserved nucleotide-binding domains (NBDs), following a typical TMD-NBD-TMD-NBD arrangement of ABC transporter. Recent structural studies have identified a helical regulatory domain at the N-terminus, which is unusual for AB transporters and may play a role in modulating the protein's activity (Si *et al*., 2023). Mutations in *Pfmdr-1* are associated with reduced influx of diverse anti-malarial drugs reducing their intracellular accumulation (Avcı e*t al*., 2024).Five mutation sites, namely N86Y, Y184F, S1034C, N1042D, and D1246Y, have been identified to be associated with drug resistance in PfMDR1 (Si *et al*., 2023). Codons N86Y, Y184F and D1246Y are uniquely associated with changes in sensitivity to lumefantrine (LF) and amodiaquine (AQ) in sub-Saharan Africa (Adamu *et al*., 2020). While the *Pfmdr-1* 86Y allele was strongly associated with chloroquine (CQ) and amodiaquine (AQ) resistance (Folarin *et al*., 2011), 1246Y alleles were shown to confer resistance to quinine and possess the

capacity to increase the parasite susceptibility to mefloquine (MQ), halofantrine (HF) and artemisinin (Humphreys *et al*., 2007). Most of them are important counterparts in first-line Artemisinin-based Combination Therapy (ACT); thus, *Pfmdr1* has become one of the pivotal factors in malaria resistance to artemisinin combination therapies (Calçada *et al*., 2020).

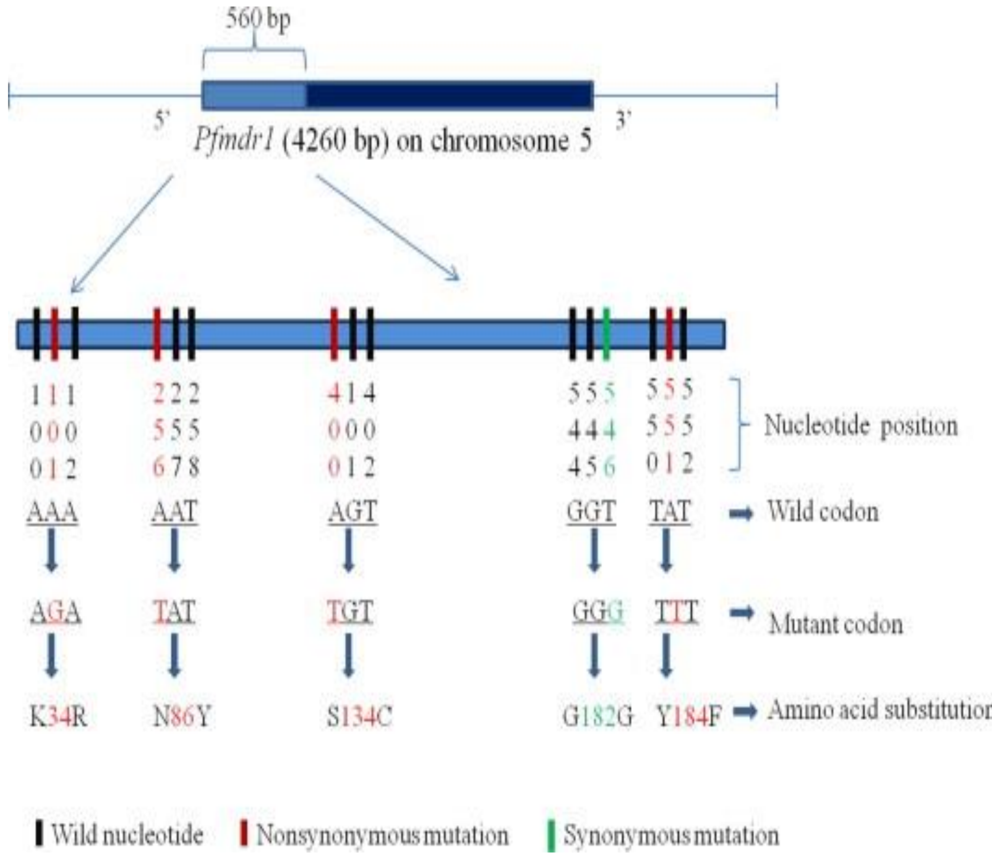


Figure 11: Structure of *Pfmdr-1* gene (Chauhan *et al*., 2014)

CHAPTER II: MATERIAL AND METHODS

CHAPTER II: MATERIAL AND METHODS

II.1. STUDY DESIGN

II.1.1. STUDY TYPE AND DURATION

This was a cross-sectional study carried out between October 2022 to November 2022 in the Mfou Health District. This corresponds to the transition period from the rainy season to the dry season and which corresponds to the pick of malaria transmission in the area.

II.1.2. STUDY SITE

The study was carried out in the Mfou health district. Four neighbouring villages in this health district, namely Nkassomo (3.86º North and 11.51º East), Vian (3.85º North and 11.50º East), Lobe (3.83º North and 11.48º East) and Ndangueng (3.81º North and 11.46º East), were visited to collect blood and stool samples, which were transported directly to the Molecular Parasitology and Disease Vector Research Laboratory at the Biotechnology Centre in Nkolbisson, Yaoundé.

Mfou (3°58 North and 11°56 East) is a district and capital of the Mefou-and-Afamba Division of the Centre Region of Cameroon. Mfou had a population of approximately 6,521 inhabitants with a population density of 1217/Km2 (Mfou, Cameroun - Population et Démographie - CityFacts, n.d.). It is a multi-ethnic community made up of the Ewondo, Bané and Tsinga peoples, whose main source of income is agriculture. Mfou has a typically equatorial climate, with discontinuous seasons: two rainy seasons (March to June and September to November) and two dry seasons (December to February and July to August). The average annual rainfall is 831.7 mm 3 and the temperature varies between 18 - 30°C, with an average of 24°C. The villages of Nkassomo, Mvian, Lobe and Ndangueng are characterized by a lack of drinking water, very close latrines, often attached to households, and very poor sanitary conditions, all of which favour the spread of helminths and mosquito eggs (Zeukeng *et al*., 2014).

II.1.3. STUDY POPULATION

Samples were collected from individuals of 2 years old and above who willingly agreed to participate, provided written and signed informed consent, and had lived in the village for at least six months. A total of 521 participants from four neighbouring villages, aged 2 to 80 years, participated in the study and were divided into six groups as follows: preschool children (<5 yrs), young school children (5-9 yrs), old school children (10-14 yrs), adolescents and young adults (15-24 yrs), adults (25-49 yrs), and the elderly (≥50 yrs). Each participant was clinically

assessed by a medical doctor for febrile malaria symptoms (fever, axial T$\geqslant$37.5^{o} C) or other clinical complaints (headache, abdominal discomfort, etc.), and a well-structured questionnaire was administered to collect other anthropological and clinical parameters related to malaria and helminth infections.

II.2. ETHICAL CONSIDERATION

Ethical clearance used for the study was obtained from the Centre Regional Ethics Committee for Human Health Research (CRERSH-Ce) (CE-No092700/CRERSHCe/2022). Administrative authorizations were obtained from authorities of the Mfou health districts (N^{0}.43/AR/J05.05.01/SP). Participation in the study was voluntary with a written (English and French) informed consents obtained from participants of 21 years and above and parental authorization for participants of less than 21 years. A clinical examination was performed for all eligible participants by a medical doctor. All participants positive for malaria by RDT at the time of blood collection were treated for free by administration of a three-day dose of Coartem (artemether and lumefantrine) and all fever cases received a free dose paracetamol for three consecutive days. However, all participants were systematically dewormed by administration of mebendazole 400mg/kg for free following the national guidelines from the Ministry of Health. All children and elderly persons with mild anemia were given an iron supplement free of charge

Inclusion criteria

- Willing to participate in the study
- Have resided in the locality for the last six (06) months before the survey
- Have voluntarily given a written consent to participate in the study
- Have provided blood and stools samples

Non-inclusion criteria

- Were visitors in the village
- Were unable to provide blood and stool samples

II.3. SAMPLING

A non-probability sampling technique was used in this study. A total of 521 participants were included in the study. Of these, 92 participants who were malaria-positive by PCR were used

to assess the genetic diversity of *Pfmsp-1*, *Pfmsp-2* and *Pfmdr-1* as mono-Infected individuals and 19 participants who were malaria-positive by PCR and helminth-positive by Kato-Katz provided blood and stool samples used to assess the genetic diversity of Pfmsp-1, Pfmsp-2 and Pfmdr-1 as co-infected individuals. Prior to sample collection, a well-structured questionnaire was administered to each volunteer participant to collect anthropological (age, sex; height, education level, etc.) and clinical (temperature, fever, weight, eye colour, etc.) parameters.

II.4. COLLECTION AND ANALYSES OF STOOL SAMPLES

II.4.1. STOOL COLLECTION

Each participant was given a labelled 50 ml screw-cap stool collection container with an internal plastic spoon during the awareness campaign and asked to bring at least a half-full container of faeces after defecation (the morning of the survey). Each participant's faeces container was then labelled with an identification code and date, and kept on ice packs in a medical cooler during the collection period. The cooler was returned to the Molecular Parasitology and Disease Vector Research Laboratory in the evening and stored in a refrigerator at +4°C. The collected faeces were analysed within the next 24 hours using the Kato-Katz technique (Mbong et *al*., 2020).

II.4.2. DIAGNOSIS OF HELMINTHIASIS USING THE KATO-KATZ TECHNIQUE

II.4.2.1. Principle

The kato-Katz technique is based on the violet staining of the membrane of helminth eggs and larvae with green glycerol malachite, which facilitates their identification in stools on the basis of their morphology. It allows the identification of the type of parasite and the determination of parasitaemia, expressed as the number of eggs or larvae per gram of faeces.

II.4.2.2. Preparation of stools on microscopic slides

The Kato-Katz technique which is the WHO "gold standard" was used to assess the prevalence and infection intensity of STHs. For the Kato-Katz technique as described by (Mbong *et al*., 2020), the sieved faeces sample (approximately 41.7 mg) is placed on a glass slide. The preparation is covered with a piece of cellophane soaked in glycerol. Subsequently, the slide is inverted and gently pressed down resulting in a thin smear. The added glycerol serves to 'clear' the faecal material (fat) from around the eggs. Hookworm eggs require about 30 min for this step, while for the other species, the reading of the slide under the microscope can be done after 1 to 24 hours. The eggs are then counted under the microscope and the count expressed in per

gram of faeces. The slides were observed under a light microscope with 10x and 40x objectives by a WHO certified parasitologist to look for helminth eggs and larvae, and to determine parasitaemia. Eggs were then differentiated by size, shape, shell and content (Mbong *et al.*, 2020). Since it was not possible to observe the slides 30mins after collection, it was difficult to observe the hookworms when observed 24hours after collection, hence this may result to a decrease or cancellation of their parasitaemia.

The number of eggs present in all the fields of each slide was determined using a manual counter, scanning one field at the time. Parasitaemia was then determined by dividing the number of eggs obtained by the mass of stool used (41.7mg of stool) as indicated on the template used.

$$N = \frac{\textbf{Number of counted eggs}}{\textbf{41.7mg of faeces}}$$

Where, N = number of counted eggs x 24 (eggs/ g of faeces)

II.5. COLLECTION AND ANALYSIS OF BLOOD

II.5.1. BLOOD COLLECTION

The venipuncture technique was used to obtain blood samples under aseptic conditions. A soft tubular tourniquet was applied to the patient's upper arm while the puncture site was cleaned with an ethyl alcohol swab. Venipuncture was performed with a gauge needle adapted to a suction pump. After puncture, the adapted needle was connected directly to a labelled EDTA tube (code, place, date) and blood was collected. The tourniquet was released and the needle immediately removed. Cotton wool was applied to the puncture sites for one minute to prevent continuous blood flow. RDTs (rapid diagnostic tests) were performed immediately. Then 3µl of blood was used to make thick films and 6µl to make thin films on clean labelled slides and allowed to air dry for at least 30 minutes. The EDTA tubes were then placed on ice packs in a medical cooler during the collection period.

II.5.2. RAPID DIAGNOSTIC TESTS (RDTs) FOR MALARIA

Wondfo one step malaria HRP2/LDH (Pf/Pan) test was the test used during the survey. It is a rapid qualitative, two site sandwich immunoassay test, utilizing whole blood for the detection of *P. falciparum* specific histidine rich protein-2 and Pan specific LDH.

- **Principle**

This is a diagnostic technique based on Immuno-chromatography. Antigens in peripheral blood are captured using either monoclonal or polyclonal antibodies against the parasite antigen targets. Immuno-chromatographic tests can target the histidine-rich protein 2 of *Plasmodium falciparum*, a pan-malarial Plasmodium aldolase, and the parasite-specific lactate dehydrogenase.

Each package was carefully opened and the content removed. The participant's code was written on the cassette before beginning the test. With the aid of a pipette tip, about 5µl of blood was drop on the hole in the cassette, after which 4 drops of buffer were added. The results were noted 15 minutes after. A red line in the test window and red line in the control window was recorded as a positive result; no line in the test window was recorded as a negative result; a red line in the test windows and no line in the control window was noted as invalid; no lines in each window was also noted as an invalid test. The results were then recorded in each participant medical report and questionnaire.

Figure 12: Image of a cassette showing the results of a Rapid Diagnostic Tests (RDT) for malaria. negative (1), positive (2) and invalid (3) results (photographed by Ange KAMGMO on the 20.11.2022)

II.5.3. DETERMINATION OF HAEMOGLOBIN LEVEL AND ANAEMIA

Immediately after intravenous blood collection, a drop of blood (13-15 µl) was collected with a pipette tip and then placed in a microcuvette bearing the participant's code to determine blood haemoglobin using a portable haemoglobinometer, a device based on photometry. This device measures the total concentration of haemoglobin in venous blood. After obtaining the haemoglobin level, the value obtained was recorded in the participant's questionnaire and medical report.

Anaemia was defined as a haemoglobin (Hb) level <11 g/dl and was further classified based on WHO guidelines as severe anaemia: Hb <7 g/dl, moderate anaemia: Hb 7–9.9 g/dl, mild anaemia: Hb 10–10.9 g/dl (WHO, 2021). The patients with anaemia received a tablet of iron.

II.5.4. MALARIA DIAGNOSIS BY MICROSCOPY

The diagnosis of malaria by microscopy is still today the reference method for diagnosing malaria attacks. It allows rapid diagnosis of species, requiring only a microscope and affordable dyes. This classic diagnosis involves three independent and successive steps: performing a thin and/or a thick smear, staining and microscopic observation of the slides.

II.5.4.1. Preparation of the thick and thin blood smear on microscopic slides

On a labelled microscopic slide, 3 µl and 6µl of blood was drop separately with the aid of a pipette tip on a flat and hard surface to prepare a thin and thick smear respectively immediately after blood collection in the field. A second clean, completely degreased microscope slide was brought into contact with the smallest drop of blood (3µl) to spread the blood along the edge of the slide. The second slide was then gently pushed onto the first, maintaining 45° angle and regular contact between the slides during the spreading process. The smear was spread evenly neither too slowly (to avoid the smear been too thin) nor too quickly (to avoid the smear been too short). A corner of the blade used to make the thin smear was used to quickly spread the large drop of blood (6µl) intended for thick smear, so as to make an even thick layer. Spreading consisted of making movement to give the drop a circular shape of about 1cm in diameter. The slides bearing the spreads were air-dried and after, the thin smears were fixed by dipping them in absolute 10% methanol for 3 to 5seconds. The slides were later placed in a microscope dish and by the end of the evening brought back to the laboratory and stored until they were stained.

II.5.4.2 Staining and microscopic examination of slides

On a labelled microscope slide, 3 µl and 6 µl of blood were dropped separately onto a flat, hard surface using a pipette tip to make a thin and thick smear, respectively, immediately after blood collection in the field. A second clean, completely degreased microscope slide was brought into contact with the smallest drop of blood (3µl) to spread the blood around the edge of the slide. The second slide was then gently pushed onto the first, maintaining a 45° angle and regular contact between the slides throughout the spreading process. The smear was spread evenly, neither too slowly (to avoid the smear being too thin) nor too quickly (to avoid the smear being too short). One corner of the blade used to make the thin smear was used to quickly

spread the large drop of blood (6µl) intended for the thick smear, so as to obtain a uniformly thick layer. Spreading consisted of making a movement to give the drop a circular shape about 1 cm in diameter. The slides with the smears were air dried and then the thin smears were fixed by immersion in absolute 10% methanol for 5 seconds. The slides were then placed in a microscope dish and returned to the laboratory at the end of the evening and stored until staining.

Giemsa staining allows blood cells to be differentiated, facilitating the biological examination of different forms and species of Plasmodium. A 10% Giemsa working solution was used for this procedure. This working solution was prepared by placing 5ml of Giemsa stock solution in a 50ml graduated beaker and then adding 45ml of distilled water. Assuming that each slide required 3ml of dye to be stained, the working solution was prepared after each round of 15 slides. The slides were stained with the diluted Giemsa for 20 minutes and then washed with distilled water. The slides were air dried in a vertical position and stored in a slide box before microscopic examination.

A drop of oil immersion was applied to each Giemsa-stained slide and placed on the stage of a light microscope at 100x magnification. We ensured that the selected area had the required staining quality; the final adjustment was made using the fine focus. Each slide was read twice, once by an experienced microscopist and once by myself. If there was a difference of more than 10 parasites per microlitre of blood, a third reading was made by the microscopist. Selected areas of the thick layer were examined for the presence or absence of parasites. If a parasite was identified in a field, the number of white blood cells (WBCs) and parasites were counted across the slide using a manual counter. When the number of WBCs counted reached 200 (minimum), the examination of that slide was terminated. The number of parasites and WBCs were recorded for each slide. Thin sections allow species identification as the appearance of the parasite is best preserved in the preparation.

- **Parasites density determination:**

The number of parasites per microlitre of blood in the thick smear was determined in relation to the number of leucocytes, which was set at 8000 per microlitre of blood. This is a practical method with reasonable and acceptable accuracy. In this method, once a parasite was observed in a field, the parasites were counted in parallel with the leukocytes using a manual counter up to 200 leukocytes (WHO, 2016). This gave the number of parasites per 200 leucocytes, which was extrapolated to the number of parasites per microlitre of blood using the following formula:

$$\text{Parasite /µl of blood} = \frac{\text{Number of parasite counted x 8000}}{\text{Number of WBCs counted}}$$

II.6. DNA EXTRACTION

DNA was extracted from 243 blood samples using the Hot Chelex method.

II.6.1. DNA EXTRACTION BY CHELEX METHOD

- **Imbibition of blood on filter papers**

For blood imbibition, 243 Whatman paper filters of 3mm diameter were used. Using sterile scissors, each paper was cut into 4 equal quadrants. Then, using the same pair of scissors, 04 fingers approximately 1.5 cm long and 1 cm wide were made on the circular part of each section. Each piece of paper was then labelled with the sample code, date and the initials of the technician's name.

Using a 1000 µl micropipette, 100 µl of whole blood was pipetted and gently placed on each finger of the filter paper. This procedure was repeated for each sample and the filter papers containing the blood were left at room temperature for 18 hours, protected from insects and rats, and later placed in zip-lock bags with desiccant to maintain dryness.

- **Principle of DNA extraction using the Chelex method**

This extraction is based on the lysis and chelation at high temperature (100°C) of the metal cations Mg2+ and Zn2+ (DNase cofactor), thus preventing DNA digestion, in order to exchange the affinity of DNA, which is detached from the nitrocellulose membrane (Whatmann paper), to bind to the Chelex 100 in solution and the elimination of contaminants is done by centrifugation.

- **DNA extraction procedure using the Chelex method**

DNA was extracted from blood samples using the Hot Chelex method as described by Plowe *et al* (1995). First, equipment such as scissors and forceps were sterilised in 10% bleach, 70% alcohol and distilled water respectively and then wrung out on sterile tissue paper. Similarly, reagents such as saponin 0.5% (dissolved in PBS 1X) and Chelex 20% (dissolved in PBS 1X) were prepared. The 1.5ml Eppendorf tubes were then labelled with the sample code. Using sterile scissors, one finger of each blood-soaked 3mm Whatman filter paper was cut into the

corresponding 1.5ml Eppendorf tube and 1ml of 0.5% saponin solution was added. The mixture was homogenised and incubated at +4°C for 18 hours to allow haemolysis of the erythrocytes. The supernatant was discarded and 1 ml of PBS 1x was added to each tube. The mixture was then homogenised and incubated at +4°C for 25 min to wash out excess saponin. Using sterile tweezers, the pieces of filter paper were transferred to the second set of Eppendorf tubes (corresponding to each sample) containing 150 µl of sterile distilled water and 50 µl of 20% Chelex solution (previously heated at 100ºC for 10 min to optimise Chelex action). The mixture was vortexed vigorously for 10 seconds, sealed with aluminium foil and then incubated in a water bath at 100ºC for 10 minutes. This procedure was repeated twice. On removal from the water bath, the mixture was again homogenised by vortexing for 10 seconds to allow the DNA to bind to the chelex. Each tube was centrifuged at 14000 rpm for 2 min and the supernatant was collected using clean pipette tips and transferred to a third set of Eppendorf tubes (corresponding to each sample) and centrifuged again at 14000 rpm for 2 min. The supernatant from each tube was collected in fresh tubes and stored at -20ºC for subsequent nested PCR analyses.

II.6.2. MEASURING THE CONCENTRATION AND PURITY OF EXTRACTED DNA USING THE NANODROP

In order the check the reliability of our extractions, the concentrations (in ng/ µl) and purities of extracted DNA from our 243 blood samples were measured using the Nanodrop.

✓ **Principle**

The principle of a nanodrop spectrophotometer is based on the measurement of the absorption of light by a molecule contain in the sample in a small volume. It also gives the purity of extracts. The macromolecules that can be quantified with the nanodrop are: DNA (double and single stranded), RNA and proteins.

➢ **Procedure**

The Nanodrop electrodes were carefully cleaned with 70% alcohol and then the type of DNA to be measured (double stranded) was selected. The instrument electrodes were calibrated using nuclease free water and then the concentration was determined. The concentration and purity of the DNA in each tube was measured by pipetting 1µl of the extracts and carefully placing them on the fixed electrode of the Nanodrop, avoiding bubbles, then covering the whole with

the moving electrode. The measurement was performed by pressing the 'measure' button. After each measurement, the electrodes were cleaned with a sterile tissue and two measurements were taken for each extract and the average was calculated. The ratio of absorbance used to assess the purity of DNA and RNA is 1.7-2.0 (A260/A280).

II.7. MOLECULAR IDENTIFICATION OF *PLASMODIUM* SPECIES IN BLOOD SAMPLE

- **Principle of Nested PCR**

Nested PCR is based on the multiplication of a small region (ssRNA) of the plasmodium genome by temperature variation in a programmable thermocycler, using two sets of primers in two successive PCR runs, and a single enzyme: one Taq polymerase. The one Taq polymerase contained 1.5mM MgCl2, 200µM dNTPs (dATP, dTTP, dCTP and dGTP), PCR buffer (1M Tris-HCL, pH 8.3) and Taq polymerase (5U). Detection of *P. falciparum* DNA in DNA extracts was performed by the nested PCR method using primers for the mitochondrial cytochrome c oxidase III (cox 3) enzyme gene and following the modified protocol of Isozumi et al. (2015).

II.7.1. EXPERIMENTAL PROCEDURE

Table 1: Composition of the master mix reaction for Nest 1 and Nest 2

Order	Reagents	Volume/tube (µl) Nest 1	Volume/tube (µl) Nest 2
1	Nuclease free water	4.5	4.5
2	One Taq polymerase	7.5	7.5
3	Primer 1	0.5	0.5
4	Primer 2	0.5	0.5
5	DNA template	2	2
	Final volume	15	15

- **NEST 1**

The first PCR consisted of amplifying the small subunit ribosomal ribonucleic acid (ssRNA) of the Plasmodium genome using primers rPLU5 and rPLU6, specific for this short genomic sequence. A volume of master mix was prepared according to the number of samples to be amplified (with a margin of error of 10%) and then homogenised. Using a micropipette, 13µl

of this mixture was added to each of the pre-labelled PCR tubes, followed by 2µl of DNA extract, giving a final volume of 15µl. In the negative control tube, the DNA extract was replaced by nuclease free water. The positive control contained all the listed reagents above (Table 1) and a DNA template already known to be positive to *P. falciparum*. The mixture was homogenised, then the tubes were placed in the thermal cycler for amplification under predefined conditions

➢ **NEST 2**

The reaction medium was prepared in three 1.5 ml Eppendorf tubes as shown in Table 1. However, each tube contained a pair of primers specific to the coding region for the 18s subunit of ribosomal DNA of each species (Table 3). The procedure is the same as for NEST-1, in exception that the NEST-1 products (amplicons) were used as DNA template

Table 2: Amplification conditions of Nested PCR (Isozumi *et al*., 2015)

Steps	**Nest 1**	**Number of cycles**	**Nest 2**	**Number of cycles**
Initial denaturation	95ºC for 5mins	1 cycle	95ºC for 80s	1 cycle
Denaturation	94ºC for 1min	25 cycles	95ºC for 40s	30 cycles
Annealing	58ºC for 2mins		50ºC for 30s	
Extension	72ºC for 2mins		72ºC for 30s	
Final extension	72ºC for 5min	1 cycle	72ºC for 5min	1 cycle
End of reaction and conservation	+4ºC for 24hours	1 cycle	+4ºC for 24hours	1 cycle

Table 3: Primer sequences used for species identification by Nested PCR (Snounou *et al*., 1993)

Primers	**Sequences**	**Molecular weight(bp)**	**Specifity of primers**
Nest 1			
rPLUS5 rPLUS6	5'CCTGTTGTTGCCTTAAACTTC3' 5'TTAAAATTGTTGCATTAAAACG3'	1200	Genus Plasmodium
Nest-2			
rFAL1 rFAL2	5'TAAACTGGTTTGGGAAAACCAAATATATT3' 5'ACACAATGAACTCAATCATGACTACCCGTC3'	205	*P.falciparum* specie
rMAL1 rMAL2	5'ATAACATAGTTGTACGTTAAGAATAACCGC3' 5'AAAATTCCCATGCATAAAAAATTATACAAA3'	144	*P. malariae* specie

rOVA1 rOVA2	5’ ATCTCTTTTGCTATTTTTAGTTTGGAGA 3’ 5’GGAAAAGGACACATTAATTGTATCCTAGTG3’	800	*P. ovale* specie

II.7.2. Revelation by agarose gel electrophoresis

Visualisation of the characteristic bands of the different DNA fragments present in the samples after nest 2 was carried out by electrophoresis of a 1.5% agarose gel. Briefly, 0.75 g of agarose powder was weighed on an electronic balance into a 250 ml Erlenmeyer flask, 50 ml Tris borate EDTA 1X (TBE) buffer was added to the Erlenmeyer flask and the mixture was placed in a microwave oven for 2 min to dissolve the agarose. After complete dissolution of the agarose, the mixture was left at room temperature until cooled to around 40-60ºC, then 3µl of ethidium bromide was carefully added to the mixture and the resulting mixture, obtained by rotating the conical flask, was carefully poured into a mould initially containing combs to form the wells. The mould was left at room temperature until the gel had completely solidified. Once the gel had solidified, the combs were removed and the gel was removed from the mould and placed in the electrophoresis tank initially containing the separation buffer (TBE 1X). 5µl of each sample or control (positive and negative) was gently added to the wells, avoiding the first well to which an identical volume of 100bp molecular weight markers was added. Once the samples had been added, the electrophoresis tank was closed and the electrodes connected to a 100V power supply and migrated for 30 minutes. After migration, the gel was removed from the tank and placed in a UV transilluminator to detect the bands representative of the different DNA fragments. The gel was then photographed and the presence of *P. falciparum* was characterized by a band of 205bp for a given sample.

II.8. GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM*

II.8.1. Genotyping of the *Plasmodium falciparum msp-1* gene

Nested PCR was performed to amplify the *msp-1* (block 2) gene and their allelic variants; K1, MAD20 and RO33 families were amplified using allele-specific primers. The conditions for PCR amplification were followed as previously described by (Aubouy *et al*., 2003).

The master mix used for Nest 1 and Nest 2 were each prepared in a 1.5ml Eppendorf tube by multiplying the volume of one tube by the total number of tubes, then respecting the order of introduction of each reagent and their volumes, as summarized below.

Table 4: Composition of the master mix of Nest 1 and 2 of *Pfmsp-1*

Reagents	**Volume/tube (µl)** **Nest 1**	**Volume/tube(µl)** **Nest 2**
Nuclease free water	3.5	3.5
One taq polymerase	7.5	7.5
Forward primer	1	0.5
Reverse primer	1	0.5
DNA template	3	2
Final volume	15	15

- **NEST 1**

A volume of Master mix was prepared according to the number of samples to be amplified (with a margin error of 10%) and then homogenised. Using a micropipette, 12µl of this mix was added to each labelled PCR tube, followed by 3µl of the DNA sample corresponding to each tube for a final volume of 15µl. In the negative control, the DNA extract was replaced by nuclease free water. The overall mixture was homogenised, then the tubes were placed in the thermal cycler for amplification under pre-defined conditions as described in Table 6 using the primer pair M1-OF and M1-OR (Table 5).

- **NEST 2**

A volume of master mix was prepared according to the number of samples (with a margin of error of 10%), then homogenised and 13µl of this mix was pipetted into three different tubes. The amplicons obtained after nest 1 were used as DNA template for nest 2 by pipetting 2µl into the three different tubes to a final volume of 15µl. The total mixture was homogenised and amplified under predefined conditions (Table 6). In each tube, the presence of one of the three allelic families was tested using specific primers: M1-KF + M1-KR specific for the K1 family, M1-MF + M1-MR specific for the MAD20 family and M1-RF + M1-RR specific for the RO33 family.

Table 5: Primer sequence used for *Pfmsp-1* genotyping (Mohammed *et al.*, 2018)

	Primers	Sequence (5'……….3')
Nest-1		
Pfmsp1	M1-OF	CTAGAAGCTTTAGAAGATGCATTG
	M1-OR	CTTAAATAGTATTCTAATTCAAGTGGATCA
Nest-2		
K1	M1-KF	AAATGAAGAAGAAATTACTACAAAAGGTGC
	M1-KR	GCTTGCATCAGCTGGAGGGCTTGCACCAGA
MAD20	M1-MF	AAATGAAGGATTTGTACGTCTTGAATTACC
	M1-MR	ATCTGAAGGATTTGTACGTCTTGAATTACC
RO33	M1-RF	TAAAGGATGGAGCAAATACTCAAGTTGTTG
	M1-RR	CATCTGAAGGATTTGCAGCACCTGGAGATC

Table 6: Amplification conditions for genotyping *Pfmsp-1* gene (Aubouy *et al.*, 2003)

Steps	**Nest 1**	**Number of cycles**	**Steps**	**Nest 2**	**Number of cycles**
Initial denaturation	94°C for 3mins	1 cycle	Initial denaturation	94°C for 3mins	1 cycle
Denaturation	94°C for 30s	29 cycles	Denaturation	94°C for 30s	30 cycles
Annealing	50°C for 45s		Annealing	42°C for 1mins	
Extension	68°C for 2mins		Extension	72°C for 2mins	
Final extension	72°C for 3mins	1 cycle	Final extension	72°C for 3mins	1 cycle
End of reaction and conservation	+4°C for 24hours	1 cycle	End of reaction and conservation	+4°C for 24hours	1cycle

- **Revelation by agarose gel electrophoresis**

The visualization of bands characteristics to the different DNA s fragments present in the samples after Nest 2 was carried out by 1.5% agarose gel electrophoresis as described in section (**II.7.2.**).

II.8.2. Genotyping of the *Plasmodium falciparum msp-2* gene

Nested PCR was performed to amplify the *msp-2* (block 3) gene and their allelic variants; 3D7/IC, and FC27 families were amplified using allele-specific primers. For detection of allelic variants of *Pfmsp*-2, Nested PCR was undertaken according to the protocol by (Dongang *et al.,*2017).

Table 7: composition of the master mix of Nest 1 and 2 of *Pfmsp-2* gene

Reagents	**Volume/tube (µl)** **Nest 1**	**Volume/tube(µl)** **Nest 2**
Nuclease free water	3.5	3.5
One Taq polymerase	7.5	7.5
Forward primer	1	0.5
Reverse primer	1	0.5
DNA template	3	2
Final volume	15	15

- **NEST 1**

A volume of master mix was prepared according to the number of samples to be amplified (with a margin of error of 10%) and then homogenised. Using a micropipette, 12µl of this mix was added to each labelled PCR tube, followed by 3µl of the DNA sample corresponding to each tube, giving a final volume of 15µl. For the negative control, the DNA extract was replaced with nuclease free water. The total mixture was homogenised, then the tubes were placed in the thermal cycler for amplification under predefined conditions as described in Table 9 using the M2-OF and M2-OR primer pair (Table 8).

- **NEST 2**

A volume of master mix was prepared according to the number of samples (with a margin of error of 10%), then homogenised and 13µl of this mix was pipetted into three different tubes. The amplicons obtained after nest 1 were used as DNA template for nest 2 by pipetting 2µl into the three different tubes to a final volume of 15µl. The total mixture was homogenised and amplified under predefined conditions (Table 6). In each tube, the presence of one of the two allelic families was tested using specific primers: M2-ICF + M2-ICR specific for the IC/3D7 family and M2-FCF + M2-FCR specific for the FC27 family.

Table 8: Primer sequence used for *Pfmsp-1* genotyping (Mohammed *et al*., 2018)

	Primers	Sequence (5'……….3')
Nest 1		
Pfmsp2	M2-OF	ATGAAGGTAATTAAAACATTGTCTATTATA
	M2-OR	CTTTGTTACCATCGGTACATTCTT
Nest 2		
IC/3D7	M2-ICF	AGAAGTATGGCAGAAAGTAAkCCTYCTACT
	M2-ICR	GATTGTAATTCGGGGGATTCAGTTTGTTCG
FC27	M2-FCF	AATACTAAGAGTCTAGGTGCARATGCT
	M2-FCR	TTTATTTGGTGCATTGCCAGAACTTG

Table 9: Amplification conditions for genotyping *Pfmsp-2* gene (Dongang *et al*., 2017)

Steps	**Nest 1**	**Number of cycles**	**Steps**	**Nest 2**	**Number of cycles**
Initial denaturation	94°C for 3mins	1 cycle	Initial denaturation	94°C for 3mins	1 cycle
Denaturation	94°C for 30s	30 cycles	Denaturation	94°C for 30s	30 cycles
Annealing	42°C for 1min		Annealing	50°C for 1mins	
Extension	65°C for 2mins		Extension	72°C for 2mins	
Final extension	72°C for 3mins	1 cycle	Final extension	72°C for 3mins	1 cycle
End of reaction and conservation	+4°C for 24hours	1 cycle	End of reaction and conservation	+4°C for 24hours	1cycle

- **Revelation by agarose gel electrophoresis**

Visualization of the characteristic bands of the different DNA fragments present in the samples after Nest 2 was carried out by electrophoresis of a 1.5% agarose gel as described in section

II.9. MARKERS OF ANTI-MALARIAL DRUG RESISTANCE

II.9.1. Genotyping of *Pfmdr-1* gene

The *Pfmdr-1* gene (specifically codon 184) was amplified by nested PCR using sequence-specific primers as described by (She *et al.*, 2020) with minor modifications. The characteristic bands of the different DNA fragments present in the samples after nesting 2 were visualised by electrophoresis on a 1.5% agarose gel

Table 10: composition of the master mix of Nest 1 and 2 of *Pfmdr-1* gene

Reagents	Volume/tube (µl) Nest 1	Volume/tube(µl) Nest 2
Nuclease free water	7.3	7.3
One Taq polymerase	4.7	4.7
Forward primer	0.75	0.75
Reverse primer	0.75	0.75
DNA template	1.5	1.5
Final volume	15	15

- **NEST 1**

A volume of master mix was prepared according to the number of samples to be amplified (with a margin of error of 10%) and then homogenised. Using a micropipette, 13.5µl of this mix was added to each labelled PCR tube, followed by 1.5µl of the DNA sample corresponding to each tube, giving a final volume of 15µl. For the negative control, the DNA extract was replaced with nuclease free water. The total mixture was homogenised, then the tubes were placed in the thermal cycler for amplification under predefined conditions as described in Table 12 using the primer pair MDR 184-1 and MDR 184-2 (Table 10).

- **NEST 2**

A volume of master mix was prepared according to the number of samples to be amplified (with a margin of error of 10%) and then homogenised. The amplicons obtained after nest 1 were used as DNA template for nest 2 by pipetting 1.5µl into the tubes to a final volume of 15µl. The total mixture was homogenised and amplified under predefined conditions (Table 12) using the primer pair MDR 184-3 and MDR 184-4.

Table 11: Primer sequence used for *Pfmdr-1* genotyping (She *et al*., 2020)

Reaction	Target	Primers	Sequences	Product size (bp)
NEST-I	*Pfmdr-1* A region Outer	MDR184-1 MDR184-2	TTAAATGTTTACCTGCACAACATAGAAAATT CTCCACAATAACTTGCAACAGTTCTTA'	612
NEST-2	*Pfmdr-1* A region Inner	MDR184-3 MDR184-4	TGTATGTGCTGTATTATCAGGA CTCTTCTATAATGGACATGGTA	200-600

Table 12: Amplification conditions for genotyping *Pfmdr-1* gene (She *et al*., 2020)

Steps	**Nest 1**	**Number of cycles**	**Steps**	**Nest 2**	**Number of cycles**
Initial denaturation	94ºC for 5mins	1 cycle	Initial denaturation	94ºC for 5mins	1 cycle
Denaturation	94ºC for 30s	40 cycles	Denaturation	94ºC for 30s	40 cycles
Annealing	52ºC for 2min		Annealing	52ºC for 2mins	
Extension	68ºC for 1mins		Extension	68ºC for 1mins	
Final extension	68ºC for 1mins	1 cycle	Final extension	68ºC for 1mins	1 cycle

- **Revelation by agarose gel electrophoresis**

Visualization of the characteristic bands of the different DNA fragments present in the samples after Nest 2 was carried out by electrophoresis of a 1.5% agarose gel as described in section (**II.7.2).**

II.10. STATISTICAL ANALYSIS

Data were entered in Excel and analysed using IBM Statistical Package for the Social Science (SPSS) version 25.0 software (SPSS Inc., Illinois, USA) and Graphpad version 9.0. The Mann-Whitney test was used to compare non-parametric variables, while analysis of variance (ANOVA) tests was used for parametric variables. Chi square test was used to compare proportions. The threshold for statistical significance was set at $P<0.05$.

Multiplicity of infection (MOI) and expected heterozygosity (HE) Multiclonal infections were defined as those having more than one allele in at least one locus out of the loci genotyped. The MOI was determined by calculating the number of different alleles at any one locus detected in the sample; single infections were those with only one allele per locus at all of the genotyped loci. The mean MOI was determined as the quotient of the total number of *P. falciparum* genotypes detected in MSP-1 or MSP-2 by the number of samples positive for either msp-1 or msp-2.

The expected heterozygosity (He) and genetic differentiation (FST) were used to assess the population structure of the parasites. As a measure for genetic diversity, the expected heterozygosity (HE) which represents the probability of being infected by two parasites with different alleles at a given locus and ranging between 0 and 1, was calculated by using the following formula: **He = n/(n-1) (1-ΣPi2)**, where n = sample size, Pi = allele frequency (Jamil *et al*.,2018).

CHAPTER III: RESULTS AND DISCUSSION

CHAPTER III: RESULTS AND DISCUSSION

III.1. RESULTS

III.1.1. SOCIO-DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE STUDY POPULATION

III.1.1.1. Gender distribution

A total of 521 participants from the four villages of Nkassomo, Vian, Lobe and Ndangueng participated in the study, of which 305(58.54%) were females and 216(41.46%) were males. The population sex ratio was 1.41 with a significant predominace of females over men (P= 0.0004). A total of 92, 90, 125 and 214 participants were enrolled from Nkassomo, Vian, Lobe and Ndangueng respectively (Figure 12).

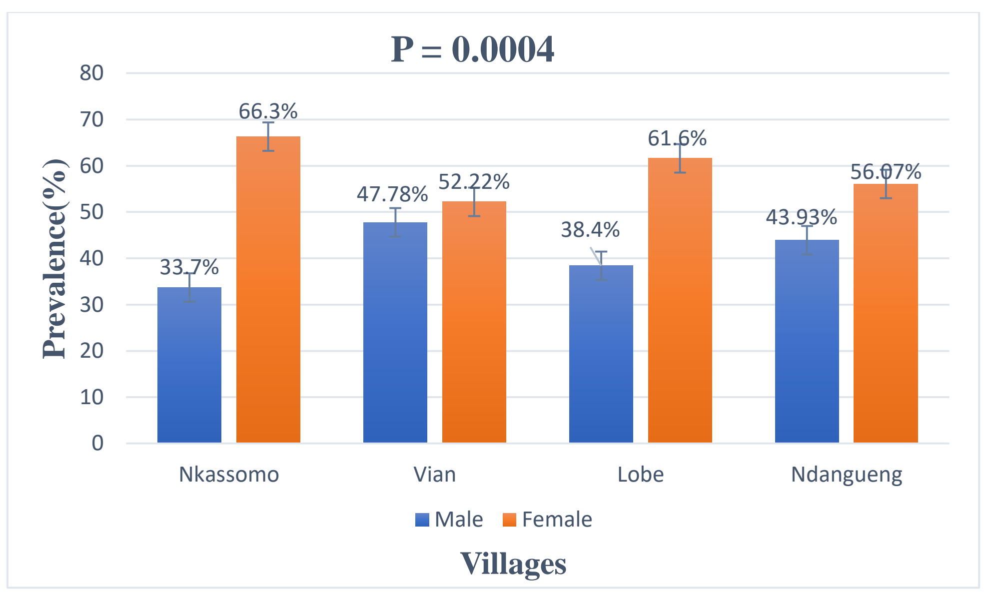


Figure 13: Gender distribution of the study population by villages

III.1.1.2. Age distribution of the study population per village

Individuals of 2 years old and above participated in this study and were divided into 6 age groups. The age of the participants ranged from 2 to 90 years old and the mean age was 22.03 ± 21.50 years. The distribution of the population varies significantly within the age groups with the predominance of people aged above 50-years old (25.72%) (P<0.0001) and a low proportion of individuals aged less than 5-years and school-aged students which are more susceptible to malaria and helminth infections respectively (Figure 13). The mean age was 27.05±14.55 in Nkassomo, 27.42±23.36 in Vian, 8.50±22.40 in Lobe and 33.16±23.23 in Ndangueng.

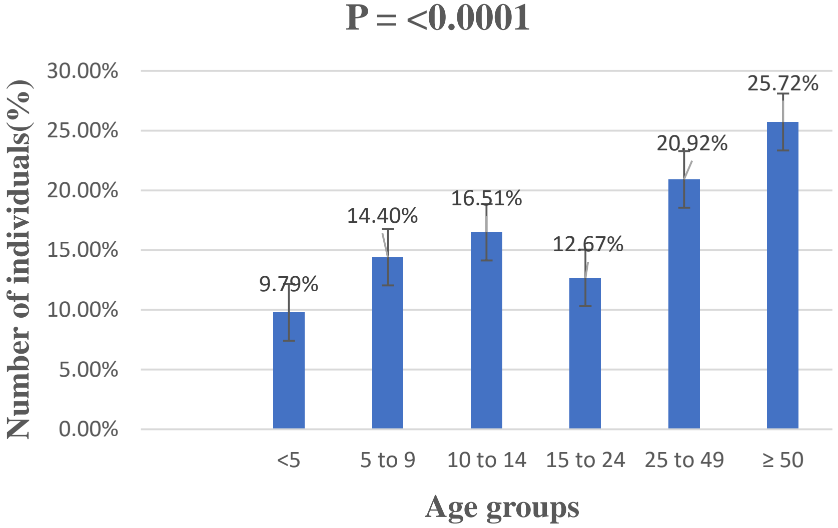


Figure 14: Age distribution of the study population

Of the 521 participants, 31.7% (165) were pupils, 14% (73) were students, 40.3% (210) were unemployed and 14% (73) were employed. In terms of toilet type, 78.5% (409) had a pit latrine, 19% (99) had a non-appointed latrine and 2.5% (13) had a flush latrine

III.1.1.3. Clinical characteristics

Out of a total of 521 participants, 106(20.35%) had fever and were considered symptomatic to malaria while 80 (15.36%) were asymptomatic (all individuals who had no symptoms but were positive by microscopy). Bed net coverage was high in the study population in at least 60.08% of investigated household.

Overall, the mean haemoglobin level was 12.79± 1.90. The prevalence of anaemia was 29.94% (156/521). Anaemia was high in individuals aged 50 years and older and low in those aged 10-14 years.

Table 13: Clinical characteristics of the study population by age groups

Characteristics		Age groups (yrs) (n=521)						Total /mean	P-value
		<5	5-9	10-14	15-24	25-49	>50		
Mean Temp (°C) (M±SD)		36.72 ±0.84	36.95 ±0.53	36.76 ±0.58	36.77 ±0.54	36.72 ±0.74	36.46 ±0.64	36.69 ±0.67	**<0.0001**
Fever, n (%)		06 (11.76%)	11 (14.67%)	09 (10.46%)	13 (19.7%)	13 (11.93%)	26 (19.40%)	80 (15.36%)	0.1962
Malaria clinical statuss	**Symptomatic**	21 (41.17%)	19 (25.33%)	19 (22.09%)	15 (22.73%)	10 (9.17%)	22 16.42%)	106 (20.35%)	**<0.0001**
	Asymptomatic	10 (19.61%)	16 (21.33%)	21 (24.42%)	10 15.15%)	11 (10.09%)	11(8.21%)	79 (15.16%)	0.0005
Mean HB (M±SD)		11.81 ±2.09	12.26 ±1.51	12.89 ±1.53	12.76 ±1.81	13.34 ±2.18	12.95 ±1.86	12.79 ±1.90	**<0.0001**
Anemia, n (%)		23 (34.8%)	33 (55%)	16 (17.8%)	18 (27.7%)	28 (25.5%)	38 (28.8%)	156 (29.94%)	0.0141
Use of mosquito net, n (%)		30 (58.82%)	37 (49.33%)	49 (56.97%)	36 (54.55%)	73 (66.97%)	88 (65.67%)	313 (60.07%)	0.0677

III.1.1.4. Prevalence of malaria

III.1.1.4.1. Prevalence of malaria by diagnostic methods

RDT, microscopy and PCR were performed on all 521 individuals enrolled in the study. The prevalence of malaria varies significantly between the diagnostic tests with 46.64% (243/521) by RDT, 35.7% (186/521) by microscopy and 17.7% (92/521) by PCR was and respectively (Figure 14). Of the 186 microscopy-positive cases, 186 (100%) were due to *P. falciparum* mono-infection. Similarly, of the 92 PCR-positive samples, 92 (100%) were due to mono-infections with *P. falciparum*.

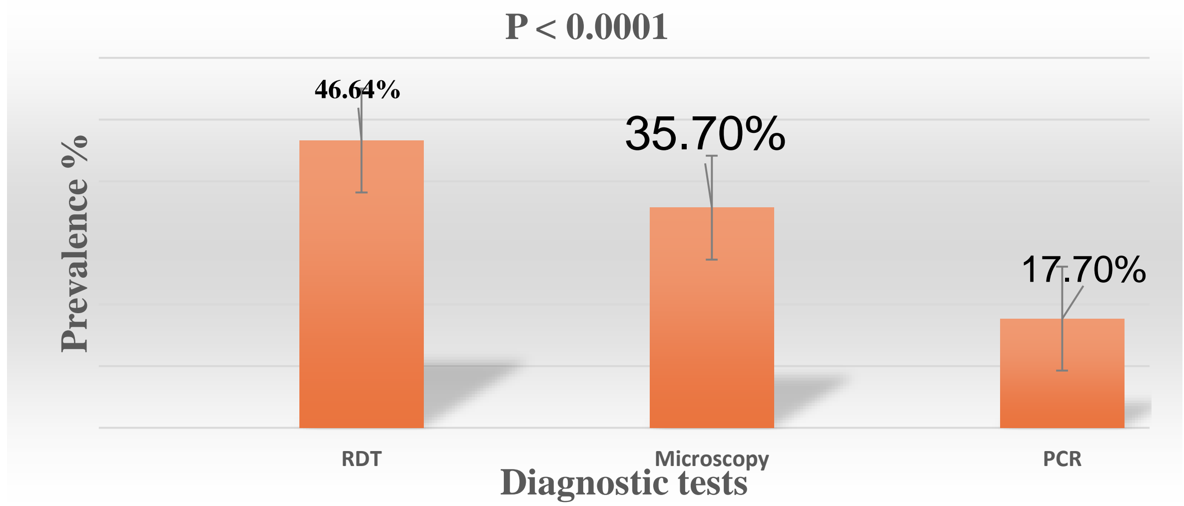


Figure 15: Prevalence of malaria by diagnostic methods

III.1.1.4.2. Prevalence of malaria by age groups

In this study, microscopy-positive participants (n= 186) were considered as microscopic malaria cases. According to age, microscopic malaria was more prevalent in individuals aged less than 5years (60.78%) followed by those aged 5 - 9 years (48%) (Figure 15). The prevalence of malaria decreases with increasing age up to the age group ⩾50 years.

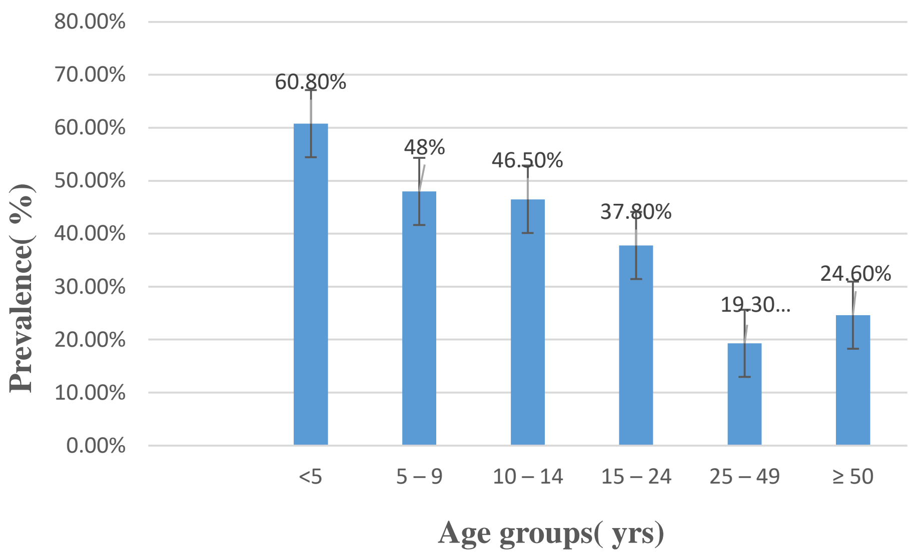


Figure 16: Prevalence of malaria by age groups

III.1.1.4.3. Prevalence of malaria per villages

The prevalence given here was based on microscopy positive samples only. Among the four villages, malaria was more prevalent in Nkassomo with a prevalence of 51.08% (47/92), followed by Ndangueng with a prevalence of 37.38%(80/214), Lobe with a prevalence of 36% (45/125)and finally Vian with a prevalence of 26.67%.(24/90).

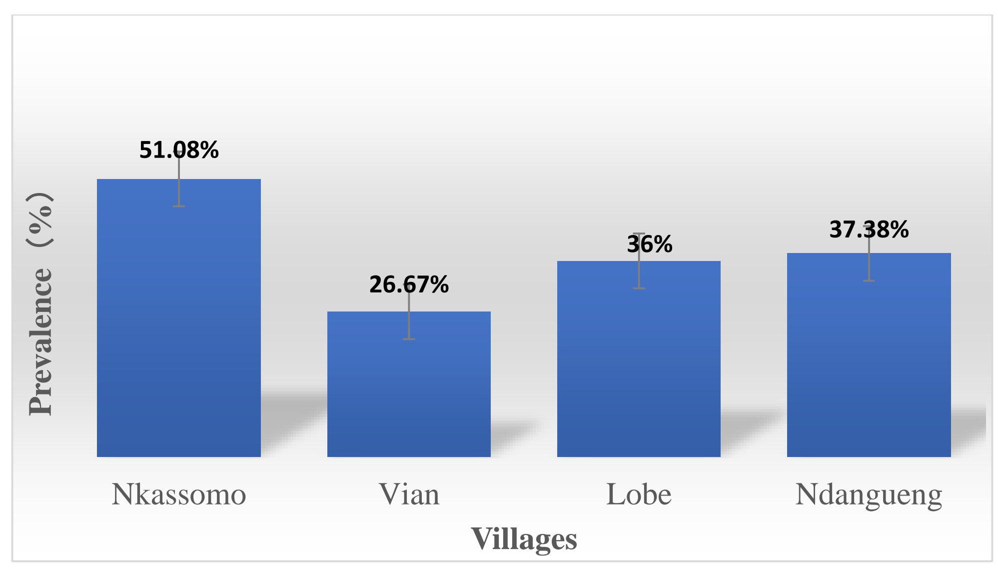


Figure 17: Prevalence of malaria per village

III.1.2. DISTRIBUTION OF MALARIA SPECIES AND PARASITAEMIA

Out of the 521 blood samples collected, *Plasmodium falciparum* constituted the main species observed by the three different diagnostic methods, giving a percentage of 100% with RDT, microscopy and PCR. No other species was observed.

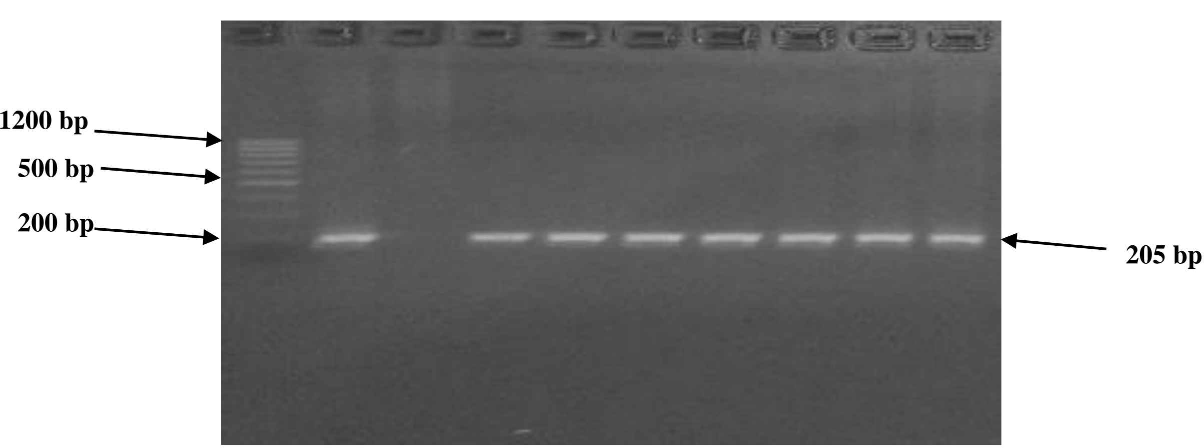


Figure 18: Electropherogram showing *P. falciparum* DNA bands on 1.5% agarose gel

<u>Legend</u>: **MW** = Molecular weight marker, **PC** = Positive control, **NC** = Negative control, **1 – 7** = samples

The mean parasitemia for microscopy-positive malaria cases was 26680 ± 11213.09 p/µL. In this population, 105(56.45%) had a low parasitemia (0 – 499 p/µl), 65(34.95%) had a moderate parasitemia (500 – 4999 p/µl) and 15(8.06%) had a high parasitemia (>5000 p/µl).

According to age, children under 5 years had the highest parasitemia (mean 7568 ± 984.29 p/µl), followed by children between 5 and 9 years (mean 3345.28 ± 2126.98 p/µl). The mean parasitemia for febrile participants was 6947 ± 8061.07 p/µl and that of non-febrile participants was 904.28 ± 8330.62 p/µl (Table 14).

Table 14: Distribution of Parasitemia by age groups

	Parasitemia (p/µL)				
Age(yrs)	**Low 0 – 499**	**Moderate 500 – 4999**	**High >5000**	**Mean**	**P-value**
<5	17.6% (9/51)	29.4% (15/51)	13.7% (7/51)	7568 ±984.29	0.6222
5 – 9	18.7% (14/75)	21.3% (16/75)	08% (6/75)	3345.28 ±2126.98	0.0748
10 – 14	29.1% (25/86)	17.4% (15/86)	0	908.8 ±117.38	**<0.0001**
15 – 24	22.7%	13.6% (09/66)	1.5% (1/66)	963.92	**0.0002**

	(15/66)			±1086.08	
25 – 49	13.8% (15/109)	4.6% (5/109)	0.92% (1/109)	3197 ±11973.19	**0.0001**
⩾ 50	20.1% (27/134)	3.7% (5/134)	0	298.16 ±189.50	**<0.0001**

III.1.3. PREVALENCE OF HELMINTHIASIS IN THE STUDY POPULATION

III.1.3.1. Prevalence of Helminthiasis

III.1.3.1.1. Prevalence of Helminthiasis by villages

From the 521 stool samples collected in the four villages, 56(10.75%) showed the presence of at least one species of soil transmitted helminthiasis (STH). The total prevalence was 3.26%, 6.67%, 0.8% and 21.50% in Nkassomo, Vian, Lobe and Ndangueng respectively. Two types of STH, *Ascaris lumbricoides* (*A. lumbricoides*) and *Trichuris trichiura* (*T. trichiura*) were identified with a prevalence of 6.33% (33/521) for *A. lumbricoides*, 4.4% (23/521) for *T. trichiura* and 2.11% (11/521) co-infections with both species. Overall, *A. lumbricoides* was the most prevalent parasite among the four villages, and statistically significant differences in *A. lumbricoides* infection (P<0.0001) and *T, trichiura* (P = 0.0057) were observed among the four villages. Polyparasitism between these two parasites was rare, with only 11 cases (2.11%) detected.

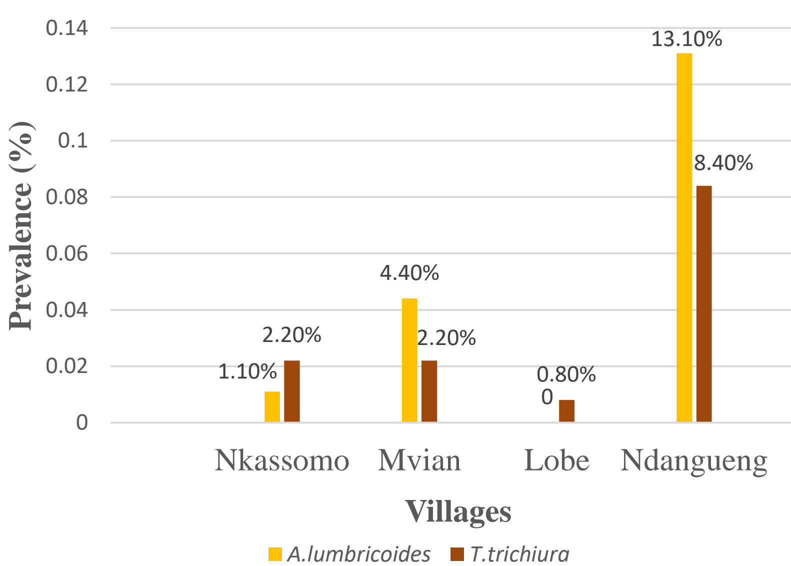


Figure 19: Prevalence of helminthiasis per village

III.1.3.1.2. Prevalence of Helminthiasis by age groups

Among the participants of different age groups included in this study, school-aged children were the most infected including 14(18.67%) and 26(30.23%) participants of 5-9 and 10-14 years old. Less than five-years old children were the least affected with STHs (Figure 20).

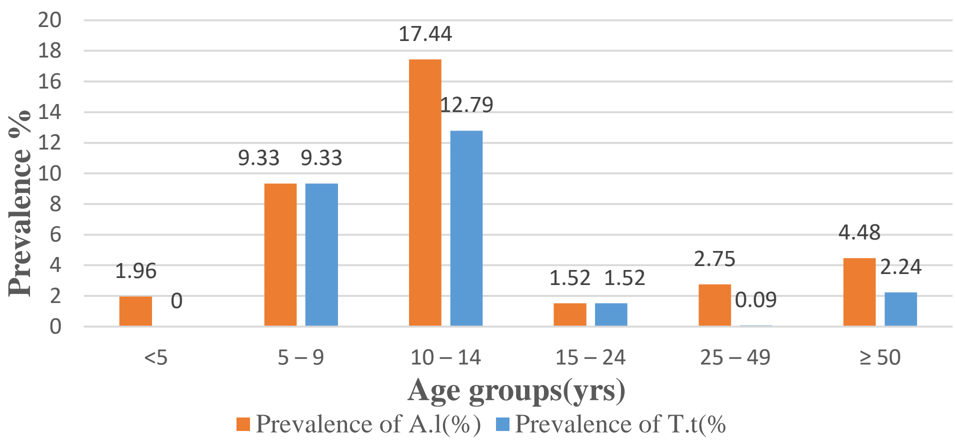


Figure 20: Prevalence of Helminthiasis by age groups

III.1.3.2. Distribution of helminth species and parasite loads

Ascaris lumbricoides, *Trichuris trichiura* were the only helminth parasites detected, among the 10.75% (56/521) participants positive for helminthiasis infection. The geometric mean of the parasite density for each species was 195 ± 46.67 eggs per gram of faeces (epg) for *A. lumbricoides,* and 6.65 ± 6.75 epg for *T. trichiura.* By age, participants aged ⩾50 years had the highest parasitemia (mean average 362.5±683.42 epg), followed by old school-aged children of 10 -14 years (mean average 215.6 ±118.79 epg).

Table 15: Distribution of Helminth species and parasite loads

	Mean parasite loads±SD (epg)	
Age groups (yrs)	***A. lumbricoides***	***T. trichiura***
<5	23	0
5 – 9	127.14±146.40	9.28±9.58
10 – 14	215.6±118.79	0
15 – 24	2	15
25 – 49	43.67±38.84	4

≥ 50	362.5±683.42	7±4.58
P-value	**<0.0001**	#N/A

III.1.4. PREVALENCE OF MALARIA AND HELMINTHIASIS CO-INFECTION

III.1.4.1. Prevalence of Malaria and Helminthiasis coinfection by villages

In total, 19 participants carried at least one STH and *P. falciparum*, giving an overall prevalence of 3.65% (19/521) co-infections. These were two simple co-infections including *A. lumbricoides/ P.falciparum (A.l/P.f)*, *T.trichiura / P. falciparum(T.t/P,f)*, and a mixed infection of *A. lumbricoides/T.trichiura /P.falciparum (A.l/T.t/P.f)* at 2.30%(12/521), 1.92%(10/521) and 0.58%(3/521) respectively. The main interaction was with *A.lumbricoides and P.falciparum,* with the highest prevalence observed in Ndangueng, at 2.11% (11/521) (Table 16).

Table 16: Prevalence of malaria and helminthiasis coinfection by villages

Types of Coinfections	Villages				Total/Mean	P-value
	Nkassomo	Mvian	Lobe	Ndangueng		
A.l/ P.f	0	1.11%(1/90)	0	11(5.14%)	12(2.30%)	**0.0041**
T.t/ P.f	1.08%(1/92)	1.11%(1/90)	01(0.8%)	07(3.27%)	10(1.92%)	0.1454
A.l/ T.t/ P.f	0	0	0	03(1.40%)	03(0.58%)	0.0864
Total	1.08%(1/92)	2.22%(2/90)	01(0.8%)	21(9.81%)	25(4.8%)	0.0003

A.l**:** *Ascaris lumbricoides,* ***T.t*** **:** *Trichuris trichiura,* ***P.f*** **:** *Plasmodium falciparum*

III.1.4.2. Prevalence of Malaria and Helminthiasis coinfection by age groups

Overall, school-aged children were most affected by both malaria and STH infections including 5.33% (4/75) and 13.95% (12/86) of children aged 5-9 and 10-14 years old respectively. Although less than five years old children constitute the most febrile group to malaria, they carried a very low density of STH, and were less susceptible to malaria and STH coinfection in the study communities.

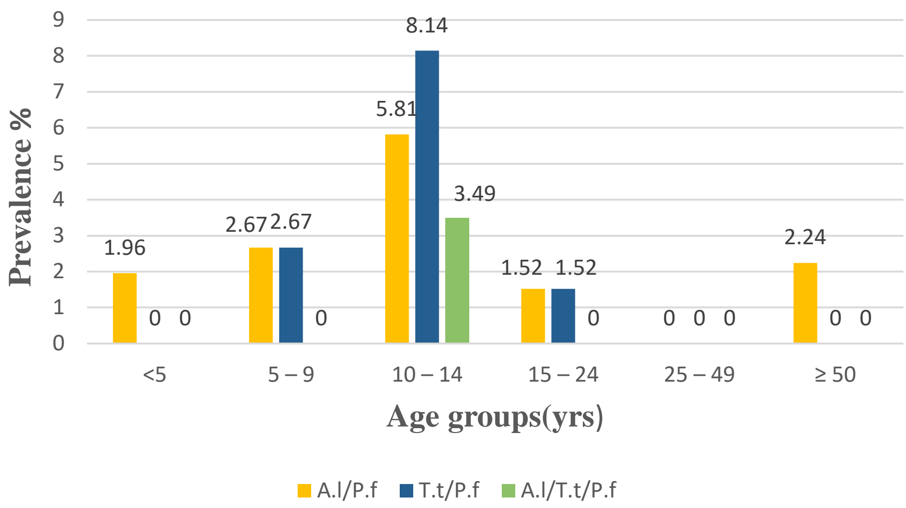


Figure 21: Prevalence of malaria and helminthiasis coinfection by age groups

A.l: *Ascaris lumbricoides,* ***P.f:*** *Plasmodium falciparum,* ***T.t:*** *Trichuris trichiura*

III.1.4.3. Influence of Malaria and Helminth co-infection on parasite densities

In general, there was an increase in the mean parasitaemia of *A. lumbricoides* and *T. trichiura* parasites in their co-infection with *P. falciparum* compared with single infections. The mean parasitaemia of *P. falciparum* decreased with single co-infection with *A. lumbricoides* and *T. trichiura* compared to the mean parasitaemia of a single malaria infection.

Table 17: Influence of malaria and helminth co-infection on parasite densities

Types of infection	**Mean parasitaemia ±SD**		
	A. lumbricoides	***T. trichiura***	***P. falciparum***
P.f	6.45±112.08 epg	0 epg	2688±8330 p/µL
A.l	195.60±117.12 epg	2±2.06 epg	149.18±8753.48 p/µL
T.t	136.82±116.99 epg	6.65±2.06 epg	410.45±2046.65 p/µL
P.f/A.l	198.7±127. 78 epg	0.67±1.96 epg	1865±8753.48 p/µL
P.f/T.t	20.8±136.40 epg	7.4±2.06 epg	451.5±2046.65p/µL
P.f/A.l/T.t	69.33±231.59 epg	2.67±3.61 epg	305.67±652.28 p/µL

III.1.5. GENETIC DIVERSITY OF *Pfmsp-1* AND *Pfmsp-2* GENES IN THE STUDY POPULATION

The total of 92 *P. falciparum* mono-infected samples were successfully amplified for *Pfmsp-1*(100%) and 91 (98.9%) were successfully amplified for *Pfmsp-2*. All of the19 samples co-infected samples with both *P. falciparum* and STHs were successfully genotyped for *Pfmsp-1*(100%, while only 14(73.68%) were genotyped for *Pfmsp-2*.

Table 18: Frequency of *Pfmsp-1* and *Pfmsp-2* allelic families in mono-infected and co-infected populations.

Allelic Family	Mono-infected Population (n = 92)				Co-infected Population (n = 19)			
	Prevalence n (%)	**Fragment Size (bp)**	**No. of Different Alleles**	**Mean Allelic Frequency n (%)**	**Prevalence N (%)**	**Fragment Size (bp)**	**No. of Different Alleles**	**Mean Allelic Frequency n (%)**
Pfmsp-1								
K1	70 (76.1)	160–354	5	20± 11.54	13(68.4)	135–269	6	16.67±18.05
MAD20	66 (71.7)	191–251	3	33.14±19.59	17 (89.5)	224–288	2	50±62.85
R033	62 (67.4)	150	1	100	19 (100)	155	1	100
K1 +MAD20	17 (18.48)	0	0	0	9 (47.37)		0	0
K1+ RO33	13 (14.13)	0	0	0	5 (26.32)	0	0	0
MAD20+RO33	5 (5.43)	0	0	0	5 (26.32)	0	0	0
K1+MAD20 +RO33	36 (39.13)	0	0	0	5 (26.32)	0	0	0
Total			9				9	
Pfmsp-2								
FC27	64(69.6)	288–525	6	16.7±6.50	9 (47.4)	324–1000	9	11.11±5.61
3D7	78(84.8)	436–725	6	16.67±10.94	13 (68.4)	468–617	4	25±15.22
FC27+3D7	58 (63.04)	0	0	0	7 (36.84)	0	0	0
Total			12				13	

III.1.5.1. Genetic diversity of *Pfmsp-1* gene

In *Pfmsp-1*, the K1 allelic family was predominant with a prevalence of 76.1% (70/92), followed by the MAD20 allelic family with 71.7% (66/92) and the R033 allelic family with 67. 4% (62/92) in the mono-infected population, whereas in the coinfected population the R033 allelic family was predominant with a prevalence of 100% (19/19), followed by the MAD20 allelic family with 89.5% (17/19) and finally the K1 allelic family with 68.4% (13/19). In polyclonal infections, the following allelic combinations were observed in the *Pfmsp-1* gene of the mono-infected population K1/MAD20 in 18.48% (n=17) of isolates, K1/RO33 in 14.13% (n=13) of isolates, MAD20/RO33 in 5.43% (n=5) of isolates and K1/MAD20/RO33 (triple allele) in 39.13% (n=36) of isolates. The following allelic combinations were observed in the co-infected population K1/MAD20 in 47.37% (n = 9) isolates, K1/RO33 in 5.44% (n = 5) isolates, MAD20/RO33 in 26.32% (n = 5) isolates and K1/MAD20/RO33 (triple allele) in 26.32% (n = 5) isolates.

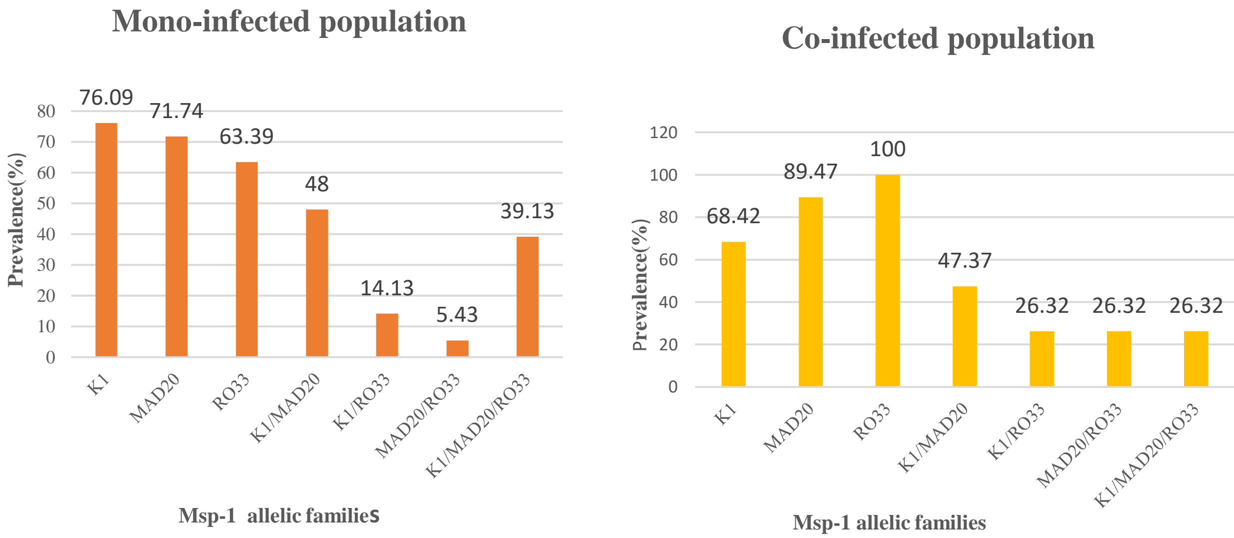


Figure 22: Distribution of *Pfmsp-1* allelic families in the mono-infected and co-infected population

The K1 allelic family in the mono-infected population (Gel A) shows more bands (alleles) than K1 co-infected population (Gel B) meanwhile they are polymorphic in both populations. The MAD20 allelic family shows more bands (alleles) in the mono-infected population (Gel C) than in the co-infected population (Gel D) and they are polymorphic in both populations. The RO33 allelic family are both monomorphic in the mono-infected (Gel E) and co-infected (Gel F) population (Figure 23).

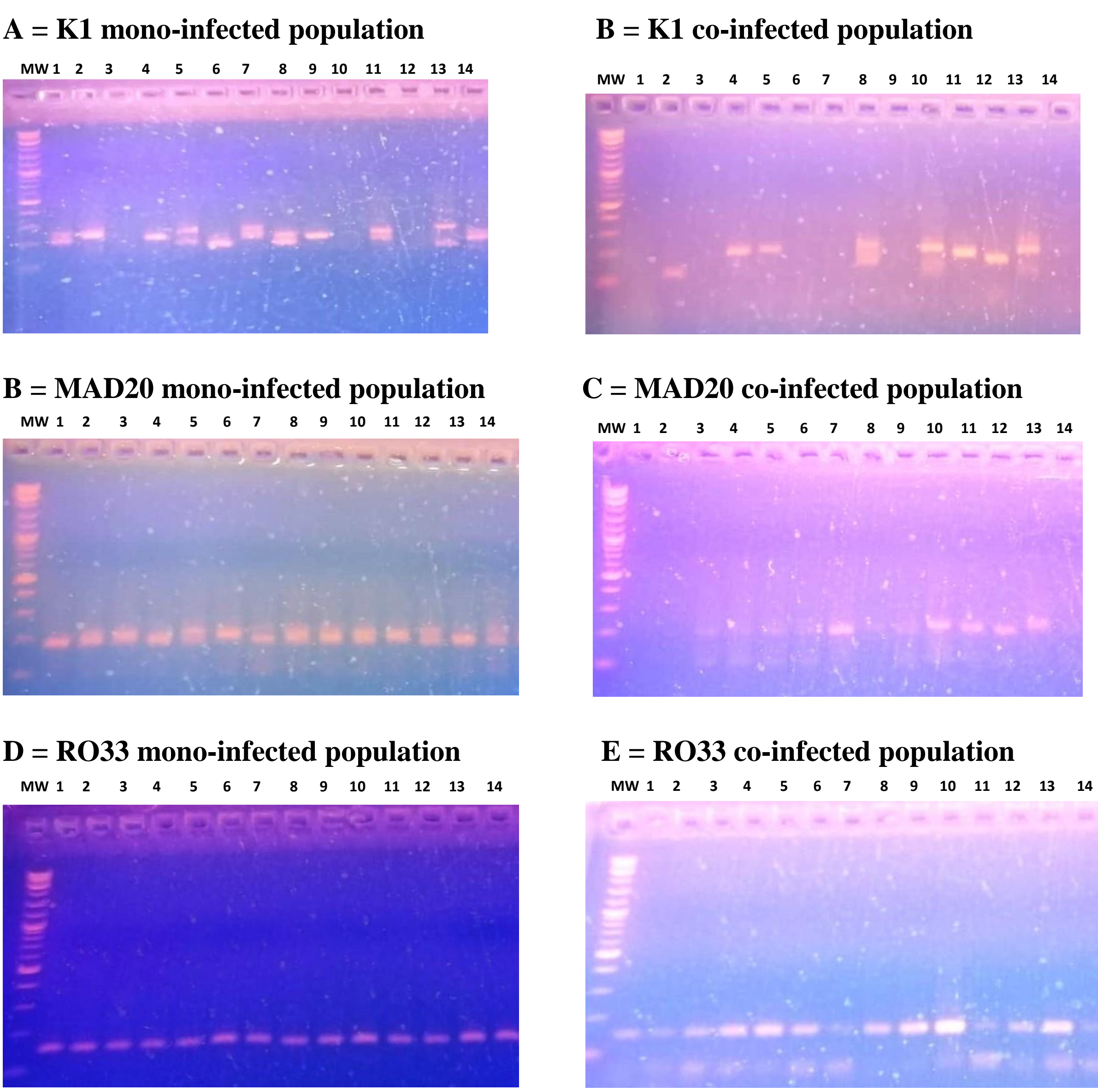
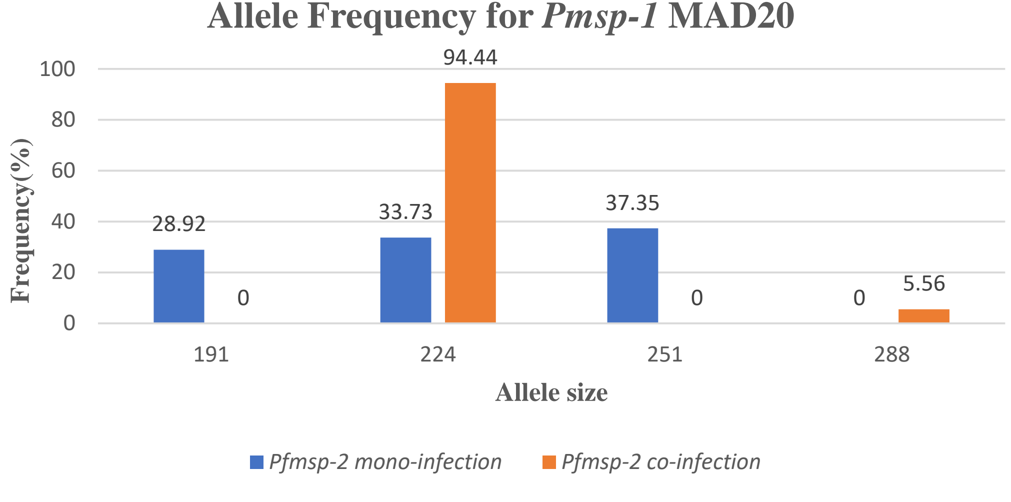
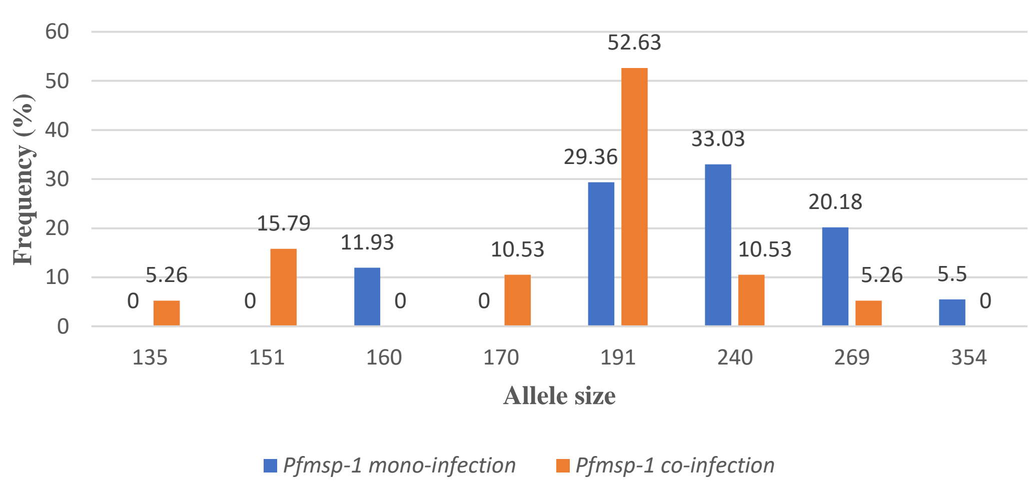


Figure 23: Electrophoregram of *Pfmsp-1* gene alleles in mono and co-infected population

Legend: **MW** = Molecular weight marker, **1 – 14** = samples

III.1.5.1.2. Allelic diversity of *Pfmsp-1* gene





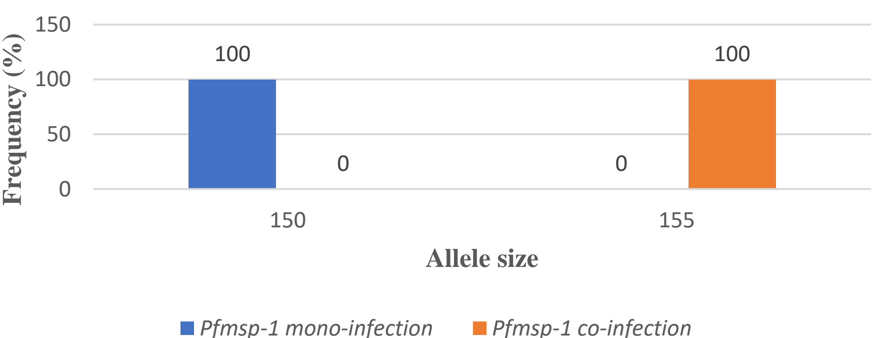


Figure 24: Allelic Frequency for MSP 1

Various alleles of *pfmsp-1* were found in mono- and coinfected individuals with both *P. falciparum* and STHs, suggesting a genetic variation among the *Plasmodium falciparum* populations circulating in the study sites. The differences in allele frequencies between the mono-infected and co-infected populations suggest that the populations may be experiencing different selective pressures or genetic drift due to STHs and leading to parasite genetic differentiation in the hosts. To test this prediction, the Fixation Index (F_ST) statistical analysis was performed to quantify the degree of genetic differentiation between the populations. F_ST values range from 0 to 1, where 0 indicates no genetic differentiation and 1 indicates complete genetic differentiation (Table 20). The Fst values (ranging from 0.068 to 0.368) suggest that there is genetic differentiation among the populations. Specifically, these values indicate moderate to high levels of differentiation, with an average Fst of 0.363. The allelic patterns reveal differences between mono-infected and co-infected populations, with mono-infected individuals showing higher allelic diversity (Na, Ne, I) compared to co-infected populations for *Pfmsp-1* (Figure 24).

Table 19: F-Stastistics and Estimates of Nm overall population for each locus

F-Statistics and Estimates of Nm over All Pops for each Locus								
All Pops.		**MSP1 MAD20**	**MSP1 MAD20**	**MSP1 K1**	**MSP1 K1**	**MSP1 R033**	**Mean**	**SE**
	Fis	1.000	1.000	1.000	1.000	#N/A	1.000	0.000
	Fit	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	Fst	**0.299**	**0.368**	**0.082**	**0.068**	**1.000**	**0.363**	**0.170**
	Nm	0.586	0.429	2.815	3.411	0.000	1.448	0.693

Fis: Correlation between gametes within an individual relative to the entire population, **Fit:** Correlation between gametes within an individual relative to the subpopulation**, Fst:** Genetic differentiation, **Nm:** Gene flow

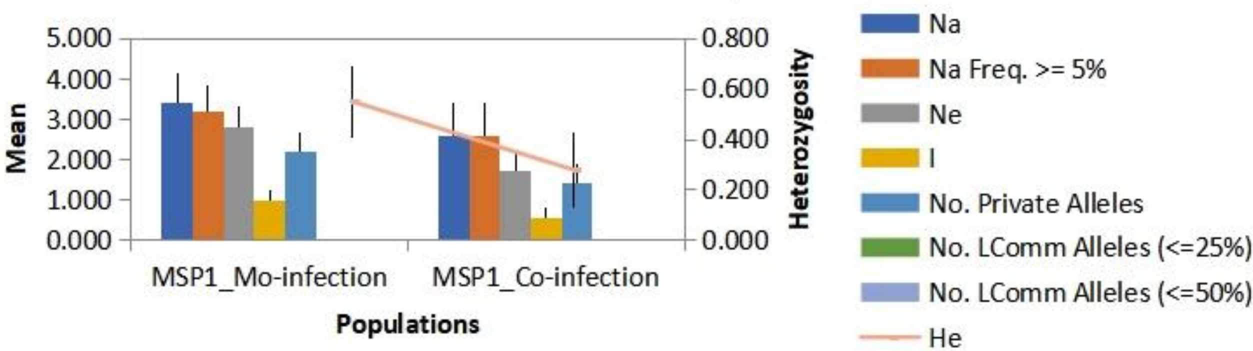
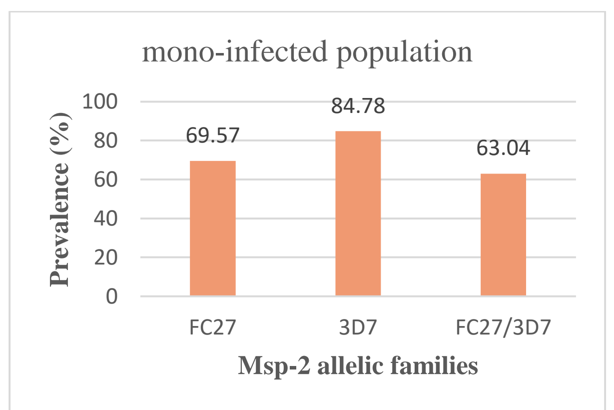


Figure 25: Allelic Patterns across populations

III.1.5.2. Genetic diversity of *Pfmsp-2* gene

For the *Pfmsp-2 gene*, the 3D7 allelic family was the most frequently detected with 84.78% (78/92), followed by the FC27 allelic family with 69.57% (64/92) in the mono-infected population compare to the co-infected population where, the 3D7 allelic family was predominant with 68.42% (13/19), followed by the FC27 allelic family with 47.37% (9/19) (Figure 24). In the polyclonal infections, FC/3D7 allelic combination was observed in 63.04% (n = 58) in the mono-infected population and in 36.84% (n = 9) in the co-infected population.



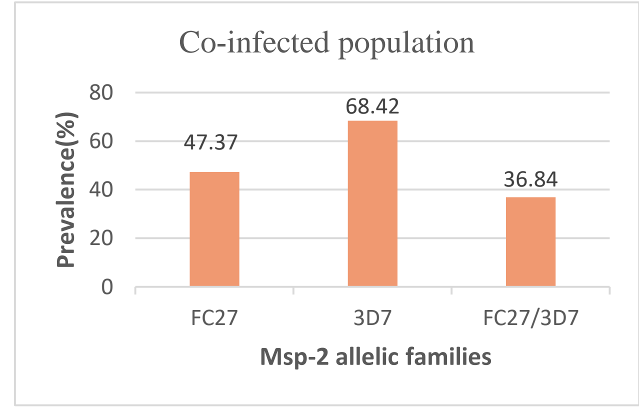


Figure 26: Distribution of *Pfmsp-2* allelic families in the mono-infected and co-infected population

The 3D7 allelic family in the mono-infected population (Gel A) shows more bands (alleles) than 3D7 co-infected population (Gel B) meanwhile the alleles are more diversified in the co-infected population. The FC27 allelic family shows more bands (alleles) in the mono-infected population (Gel C) than in the co-infected population (Gel D) and they are polymorphic in both populations as shown in Figure 27.

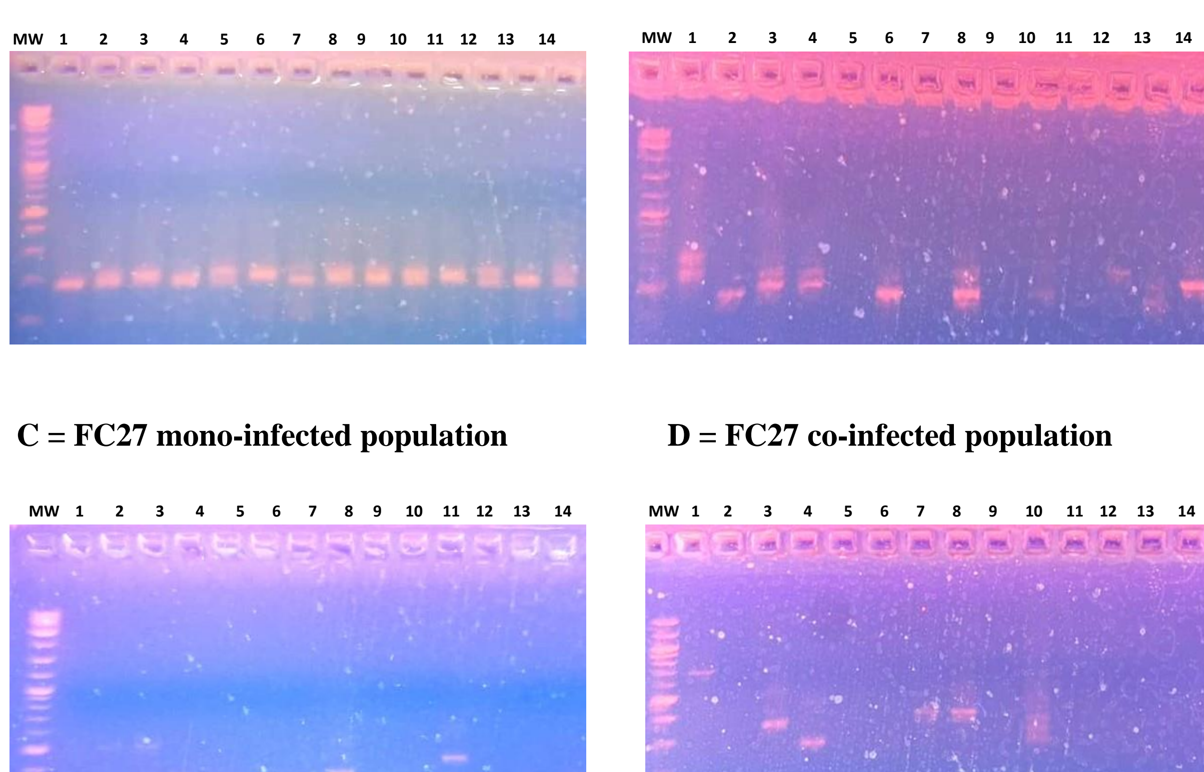
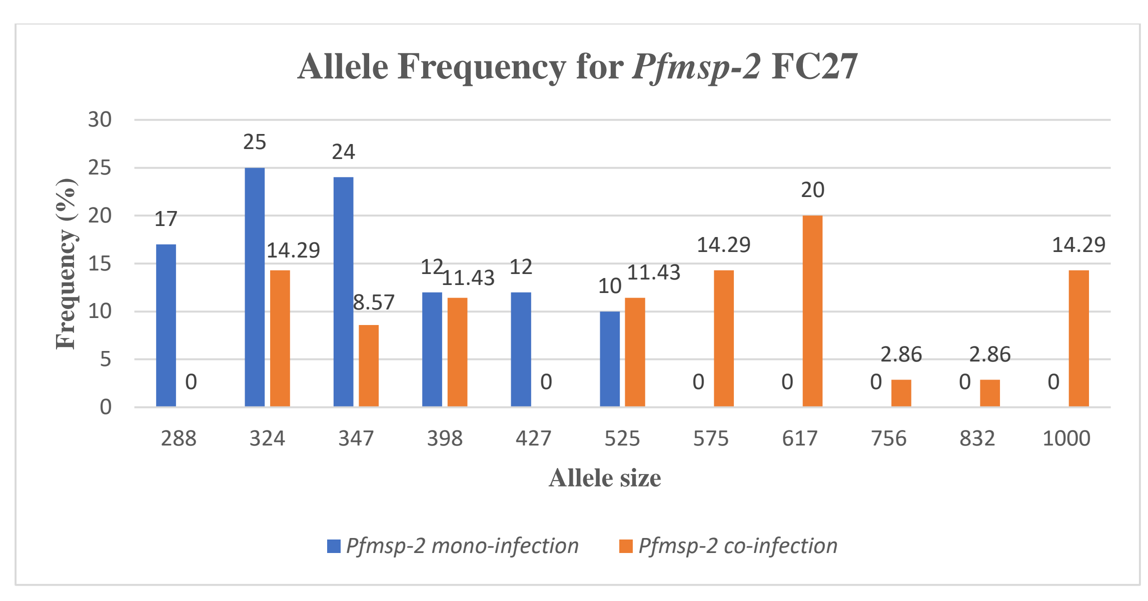


Figure 27: Electrophoregram of *Pfmsp-2* gene alleles in mono and co-infected population

<u>Legend</u>: **MW** = Molecular weight marker, **1 – 14** = samples

III.1.5.2.1. Allelic diversity of *Pfmsp-2* gene

Various alleles of *pfmsp-2* were found in mono- and coinfected individuals with both *P. falciparum* and STHs, suggesting a genetic variation among the *Plasmodium falciparum* populations circulating in the study sites. The differences in allele frequencies between the mono-infected and co-infected populations suggest that the populations may be experiencing different selective pressures or genetic drift, leading to differentiation. To test this prediction, the Fixation Index (F_ST) statistical analysis was performed to quantify the degree of genetic differentiation between the populations (Table 20). F_ST values range from 0 to 1, where 0 indicates no genetic differentiation and 1 indicates complete differentiation. The Fst values (ranging from 0.062 to 0.599) suggest that there is genetic differentiation among the populations. Specifically, these values indicate moderate to high levels of differentiation, with an average Fst of 0.339. The allelic patterns reveal differences between mono-infected and co-infected populations, with mono-infected individuals showing higher allelic diversity (Na, Ne, I) compared to co-infected populations for *Pfmsp-2* (Figure 28).



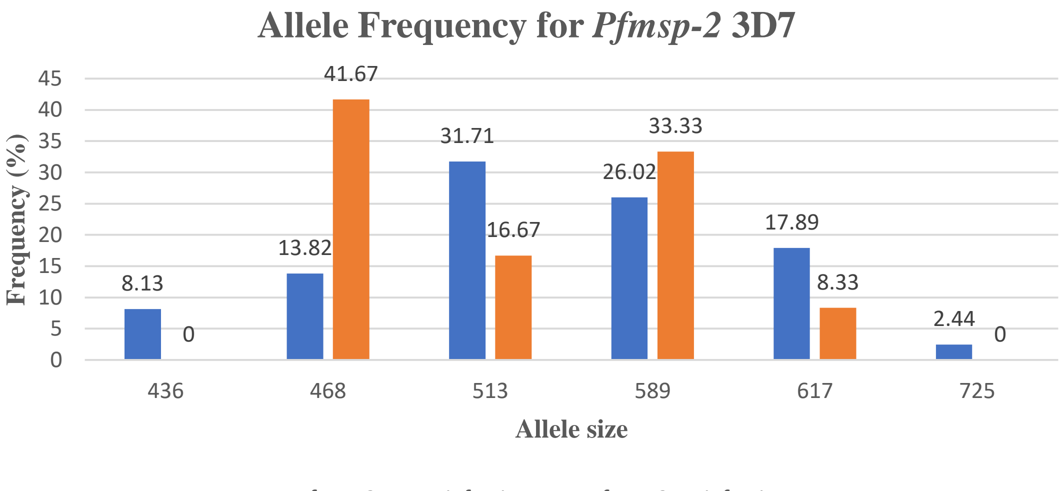


Figure 28: Allelic Frequency for MSP2

Table 20: F-Statistics and Estimates of Nm over All Pops for each Locus

All Pops.		**MSP2 -FC27**	**MSP2 -FC27**	**MSP2 -FC27**	**MSP2-3D7/IC**	**MSP2-3D7/IC**	**MSP2-3D7/IC**	**Mean**	**SE**
a	**Fis**	0.624	1.000	1.000	1.000	1.000	1.000	0.937	0.063
	Fit	0.666	1.000	1.000	1.000	1.000	1.000	0.944	0.056
	Fst	0.112	0.062	0.075	0.599	0.592	0.594	0.339	0.115
	Nm	1.984	3.765	3.065	0.167	0.172	0.171	1.554	0.661

Fis: Correlation between gametes within an individual relative to the entire population, **Fit:** Correlation between gametes within an individual relative to the subpopulation**, Fst:** Genetic differentiation, **Nm:** Gene flow

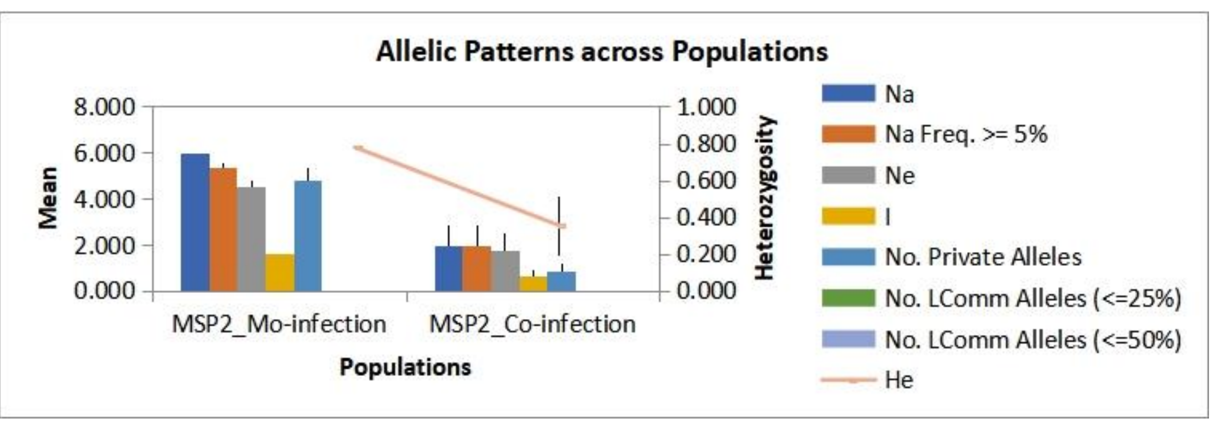


Figure 29: Allelic Patterns for Codominant Data

III.1.5.3. Multiplicity of infection and expected Heterozygosity

The multiplicity of infection (MOI) for the *Pfmsp-1* gene was 1.28 and 1.19 in the mono- and co-infected populations, respectively, whereas for the *Pfmsp-2* gene the MOI was 1.55 and 2.87 in the mono- and co-infected populations, respectively. Heterozygosity (He) at the msp-1 locus was higher in the mono-infected population (0.69) than in the co-infected population (0.38), and was also higher at the msp-2 locus in the mono-infected (0.78) than in the co-infected population (0.71)(Table 21).

Table 21: Multiplicity of infection and heterozygosity of *P. falciparum* populations

Gene	MOI/He	Mono-infected	Co-infected	Independent t-test	P-value
MSP1	MOI	1.28	1.19	0.9974	0.32
MSP2	MOI	1.55	2.87	0.708	**<0.0001**
Overall		2.83	4.06		
MSP 1	He	0.69	0.38	0.832	0.41
MSP 2	He	0.78	0.71	0.967	0.27
Overall		1.47	1,09		

III.1.6. Genetic diversity of *Pfmdr-1* drug resistance marker

Among the 92 samples aalysed as mono-infected samples, the prevalence of the *Pfmdr-1*gene (codon Y184F) was 92.39% and 7.61% for wild-type(Y184) and mutant type (184N), and among the 19 samples analysed as co-infected samples, the prevalence was 68.42% for wild-

type and 31.58% for mutant type. The multiplicity of infection (MOI) for the *Pfmdr-1* gene was 1.11 and 1.38 in the mono- and co-infected populations, respectively.

The *Pfmdr-1* Y184F mono-infected population (Gel A) shows more bands (wild type) than *Pfmdr-1* Y184F co-infected population (Gel B) meanwhile the co-infected population shows more mutant type and the alleles are more diversified (Figure 30).

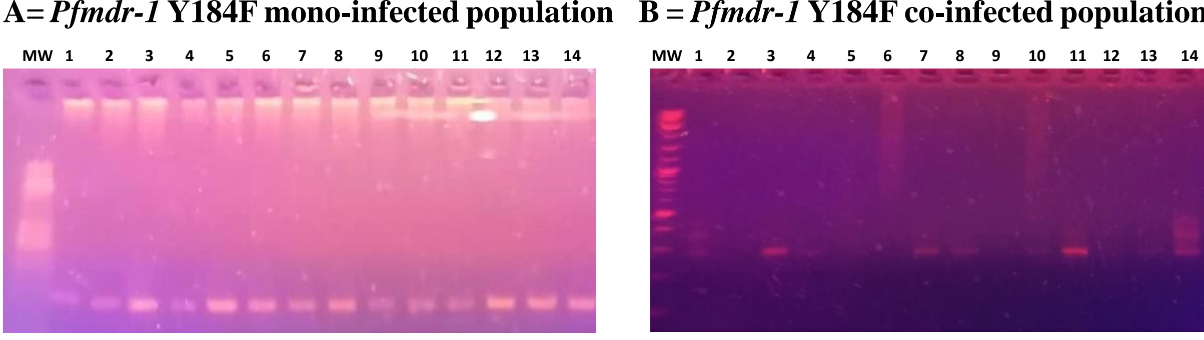


Figure 30: Electropherogram of *Pfmdr-1* Y184F gene in the mono- and co-infected population

Legend: **MW** = Molecular weight marker, **1 – 14** = samples

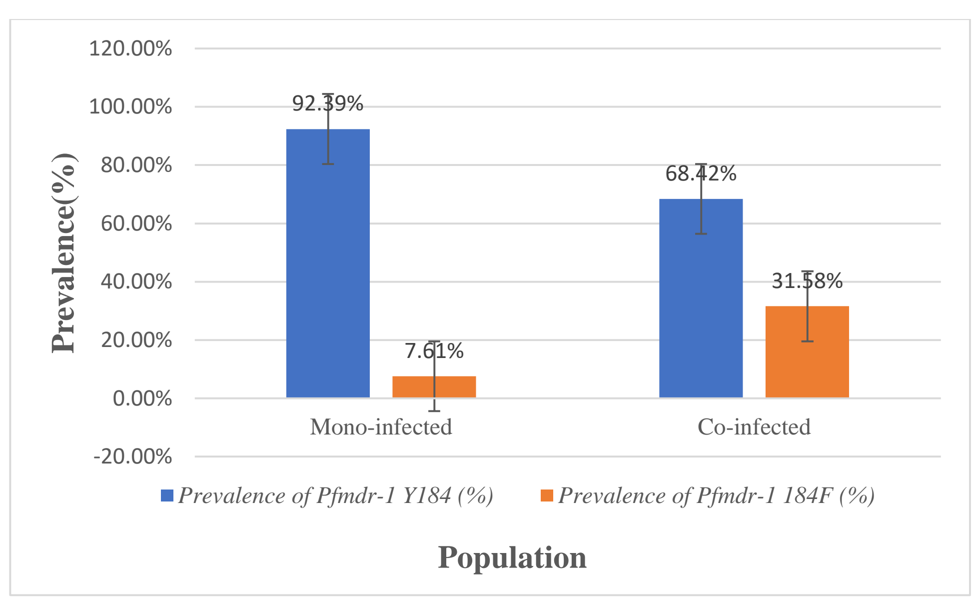


Figure 31: Prevalence of *Pfmdr-1* Y184F in the mono- and co-infected populations

III.2. DISCUSSION

In Cameroon, malaria and helminth infections coexist and are among the leading causes of mortality and morbidity from parasitic diseases. Despite ongoing efforts, the fight against malaria in Cameroon faces many challenges, particularly the increasing resistance of vectors to insecticides and the resistance of parasites to certain antimalarial drugs. This parasite resistance is mainly caused by the constant evolution of the parasite population due to its great genetic diversity, resulting in high polymorphism of certain proteins, including merozoite surface proteins 1 and 2 (MSP-1 and MSP-2), with the emergence of resistance genes such as the *Plasmodium falciparum* multi-drug resistance 1 (*Pfmdr-1*) gene. Genetic diversity in *P. falciparum* is essential for the parasite to adapt to environmental changes, evade host immunity through antigenic variation and evolve resistance. Coinfection with malaria and helminths can affect this diversity. Studies have shown that helminth infections can affect the immune response to malaria by altering selection pressure on the *Pfmsp-1, Pfmsp-2* and *Pfmdr-1* genes, potentially leading to an increase in genetic variation of these genes and increasing the parasite's ability to evade the host immune system.

This was a cross-sectional study conducted on a study population of 521 participants from four neighbouring villages; Nkassomo, Vian, Lobe and Ndangueng in the Mfou Health District, Centre Region of Cameroon. The aim was to assess the effect of malaria and helminth co-infection on the genetic diversity of the *msp-1*, *msp-2* and *mdr-1* genes in *P. falciparum* parasites. The study population consisted of 41.46% males and 58.54% females in six age groups (<5, 5-9, 10-14, 15-24, 24-49 and ⩾50) with the age group ⩾50 years being the most predominant (25.72%). In this study population, 14.97% had fever (>37.5ºC), 20.35% were symptomatic, 15.36% were asymptomatic and 60.08% used a mosquito bed net.

Malaria was diagnosed by RDT, microscopy and PCR with prevalences of 46.64% (243/521), 35.70% (186/521) and 17.65% (92/521) respectively. The discrepancy in prevalence rates between RDT, microscopy and PCR for malaria diagnosis, despite the higher sensitivity of PCR, can be attributed to several factors such as: inhibition in blood samples; substances in the blood can inhibit the PCR reaction, thereby reducing its effectiveness; differences in target parasite load; PCR detects genetic material that may be present in lower quantities than the antigens or parasites detected by RDT and microscopy. This difference may also be due to the challenge that is involved in the implementation of the different techniques. Although all age groups were affected, the peak in malaria prevalence was observed in children under five years

of age and *Plasmodium falciparum* was the only malaria species diagnosed by these tests, similar to the findings of Zeukeng *et al*. (2014) in Mfou. The prevalence of microscopic malaria observed in this study (35.70%) was lower than that reported by Zeukeng *et al*., 2014 (72.2%) in the same health district. This can be explained by the expansion of health education and implementation of malaria control strategies in recent years, particularly the mass distribution of free insecticide-treated bed nets in the study area.

Among the 186 cases of microscopic malaria observed, the mean parasitaemia was 26680 ± 11213.09 p/µL. According to age, the highest parasitaemia was found in children under 5 years of age (mean average 7568±984.29 p/µl), followed by those between 5 to 9years of age (mean average 3345.28 ±2126.98 p/ µl). This can be explained by the fact that, it has been reported that immunological responses vary with age (Alpert *et al*., 2019) thus the immune system in younger children has not yet reached maturity, resulting in their high susceptibility to infection.

Among the 521 stool samples collected in the four villages and analysed using the Kato-katz quantitative diagnostic technique, the prevalence of soil transmitted helminthiasis was 10.75%. This prevalence was almost similar to the findings reported by Flavio *et al*., 2021 (8.87%) in Douala and lower when compared to the findings reported by Zeukeng *et al*., 2014(28.6%) in Mfou. This low prevalence of helminthiasis could be attributed to the National Deworming campaign implemented by the Ministry of Public Health through the National Programme for the Control of Schistosomiasis and Soil Transmitted Helminthiasis (PNLSHI). The helminths detected were *Ascaris lumbricoides (A.l)* and *Trichuris trichuira (T.t*). The most common intestinal helminth was *A. lumbricoides* with a prevalence of 6.33%, followed by *T. trichiura* with 4.41% and co-infection with both species (2.11%). No hookworm infection was observed, which corroborates the findings of Tchinda *et al*., 2012 and Zeukeng *et al.*, 2014 in Mfou health district. The absence of hookworm may be related with the type of soil in the area which may not be favourable for larval development. It may also be due to the time between stool processing and microscopy as hookworms begin to degenerate after about 45 minutes of Kato-Katz preparation. School-aged Children (5-14 years) were the main victims of *A. lumbricoides* and *T. trichiura* infection. This result is similar to the study reported by Zeukeng *et al*.,2014 in Mfou where they reported that children aged 5-11 years were the major victims of these infections. These results show that soil ingestion is higher in young children, which could be explained by the fact that they are generally playing barefooted, and therefore in regular contact with the soil and water, which can be contaminated with helminth eggs.

The overall prevalence of malaria-helminth coinfection obtained from this study was 3.65% which is lower than the 6.8% prevalence obtained by Flavio *et al*., 2021 in Douala; this difference in prevalence may be due to the different environmental conditions in the study area. In addition, there was a difference between the most affected age groups in our two study populations. In fact, we obtained a higher prevalence of malaria-helminth coinfection in children aged 10-14 years, followed by children aged 5-9 years, whereas Flavio *et al.,* 2021 reported a higher prevalence of malaria-helminth coinfection in children aged less than 5 years. However, our findings are similar to the report of Zeukeng *et al.,* 2014 in the Mfou health district, where they reported a high prevalence of malaria-helminth coinfection in children aged 5-14 years. This may be due to the fact that, school-age children often spend a lot of time outdoors, increasing their exposure to mosquito bites, which transmit malaria and to environments where helminths are prevalent. The mean parasitaemia of *A. lumbricoides* and *T. trichiura* parasites increases in their co-infection with *P. falciparum* compared with single infections, which is contradictory to the findings of Zeukeng *et al.,* 2014 where only the mean parasitaemia of *A.lumbricoides* increased in their co-infection. The mean parasitaemia of *P. falciparum* decreased with single co-infection and mixed infection with *A. lumbricoides* and *T. trichiura* compared to the mean parasitaemia of malaria infection only. These results could be due to an increase in anti-malarial immunity by *A. lumbricoides* and *T. trichiura.* The high prevalence of malaria and helminthiases favours their co-infection with risks of exacerbating or reducing the degree of severity of malaria. Anaemia was mostly caused by malaria infection only and was more prevalent in participants of 5-9 years old (55%) and under five years (34.8%). Of the 19 co-infected participants, only two (0.38%) were anaemic. This is probably because the outcome of *Plasmodium*–helminth interactions is beneficial to the host in that the individual negative effect of both infections on the host cancel out as reported by Njua-Yafi *et al.*,2016 in Mutengene.

Out of 92 samples genotyped as mono-infected and 19 samples as co-infected, the prevalence of *Pfmsp-1* and *Pfmsp-2* genes were 100% and 98.9%, respectively, in the mono-infected population and 100% and 73.68%, respectively, for *Pfmsp-1* and *Pfmsp-2* genes in the co-infected population. In this study, for the *Pfmsp-1* gene, we obtained a predominance of the K1 allelic family in the mono-infected population similar to the findings reported by Apinjoh *et al*., 2015 in Mount Cameroon and Ndiaye *et al*., 2019 in Senegal where they both obtained a predominance of the k1 allelic family, while RO33 was the predominant allele type in the co-infected population. Meanwhile, for the *Pfmsp-2* gene, 3D7 was the predominant allelic family

in the mono-infected population similar the findings reported by Ndiaye *et al.*, 2019 in Senegal where 3D7 was the predominant allelic family. 3D7 was also the predominant allelic family in the co-infected population. A total of 09 different allele sizes (150–354 bp) were identified within the three allelic families of *Pfmsp-1* (MAD20, K1, and RO33) in mono-infected population which differ from the 12 different allele size (75-250 bp) reported by Chekol *et al.*, 2022 in Ethiopia while 08 different allele size (135-269 bp) were identified in co-infected population. For the *Pfmsp-2* gene, 12 different allele size (288-725 bp) were identified in the mono-infected population which is almost similar to the 10 different allele size (250-500 bp) reported by Chekol *et al.*, 2022 while 13 different allele size (324-617 bp) were identified in the co-infected population The difference in allele frequencies between the mono- and co-infected populations suggest that the populations may be under different selective pressures, leading to differentiation. STHs can modulate the immune system, potentially affecting the host response to *P. falciparum*. This immune modulation could lead to selective pressure favouring certain alleles in the *P. falciparum* population that are better adapted to the altered immune environment. In *Pfmsp-1* gene, RO33 was found to be monomorphic compared to the polymorphic allelic families K1 and MAD20 while in the *Pfmsp-2* gene, FC27 and 3D7 were found to be polymorphic. Regarding the fixation index value (Fst), a low genetic differentiation (Fst *Pfmsp-1* = 0.363±0.170, Fst *Pfmsp-2* = 0.339±0.115) was found which was higher when compared to the findings reported by Ndiaye *et al.*, 2019 in Senegal (Fst *Pfmsp-1*= 0.011; Fst *Pfmsp-2*= 0.017). This level of differentiation is significant and suggests that the populations are genetically distinct, probably due to genetic drift. These data reveal gene flow between parasite populations, facilitated by extensive human migration events between endemic regions and subsequent displacement of the vector.

The multiplicity of infection (MOI) is an indicator of the level of malaria transmission, as it was found to be higher in areas with high malaria transmission and to decrease as the latter decreases. In the present study, the MOI for both msp-1 (MOI = 1.28) and msp-2 (MOI = 1.55), and the expected He value for msp-1 (He = 0.69) and msp-2 (He = 0.78) differ from the findings reported by Chekol *et al.*, 2022 in Ethiopia (msp-1 (MOI = 1.56, He = 0.23) and msp-2 (MOI = 1.56, He = 0.22)). This difference in MOI and He values may be associated with the difference in study area as the study cities are located under low transmission setting and the difference in the study participants. The overall MOI obtained was 2.83 in the mono-infected population and 4.06 in the co-infected population. These results suggest a higher intensity of malaria transmission in the co-infected population probably due to the fact that helminths can

modulate the host's immune system, often leading to a more tolerant environment for malaria parasites. Overall diversity, expressed as expected heterozygosity (He), was 1.47 in the mono-infected population and 1.09 in the co-infected population. The results indicate a decrease in the genetic diversity of the co-infected population compared to the mono-infected population, likely due to antigenic variation exerted by the dual infection with *P. falciparum* and STHs.

The prevalence of the *Pfmdr*-184 gene in the mono-infected population was 92.39% and 7.61% for the wild type and mutant type respectively which was higher compared to the findings reported by Tarama *et al.*, 2023 in Burkina Faso which had a prevalence of 30.4% and 66.7% for the wild type and mutant type respectively. whereas in the co-infected population we had a prevalence of 68.42% for wild type and 31.58% for mutant type. The results show an increase in the *Pfmdr*-184 mutant type gene in the co-infected population compared to the mono-infected population, probably due to the fact that the presence of helminths can influence the host's immune response, potentially affecting the efficacy of antimalarial drugs. This may create a selective environment that favours different mutations in the *Pfmdr-1* gene, increasing its genetic diversity.

III.3. CONCLUSION

In summary, the effect of malaria and helminthiasis co-infection on the genetic diversity of *Pfmsp-1*, *Pfmsp-2* and *Pfmdr-1* genes was determined from blood and stool samples of participants infected with malaria and helminths among four villages in the Mfou health district, centre region of Cameroon. To this end, it emerged that:

1- Malaria and helminth remain endemic in Mfou health district study area with a prevalence of 35.70% for malaria, 10.75% for helminth and 3.65% for co-infection.

2- The genetic diversity (He) of *Pfmsp-1* and *Pfmsp-2* genes in the mono-infected population was respectively 0.69 and 0.78. The multiplicity of infection (MOI) of *Pfmsp-1* and *Pfmsp-2* gene were 1.28 and 1.55 respectively.

3- Comparing the genetic diversity in the mono-infected and co-infected populations; It has been shown that for the *Pfmsp-1* gene the MOI was higher in mono-infected individuals while for the *Pfmsp-2* gene it was higher in the co-infected individuals. The genetic diversity of *Pfmsp-1* and *Pfmsp-2* genes both decreased in co-infected individuals. For the *Pfmdr-1* gene, the MOI was higher in the co-infected individuals.

III.4. RECOMMENDATIONS

To sustain and further reduce the prevalence of malaria and helminth infections, the National Malaria Control Program (NMCP) and the National Program for the Control of Schistosomiasis and Intestinal Helminthiasis (PNLSHI) can consider the following recommendations:

For the National Malaria Control Program (NMCP):

- ✓ Enhance surveillance to monitor malaria cases and detect any resurgence early.
- ✓ Integrated vector management; continue and expand vector control measures such as insecticides-treated bed nets (ITNs), indoor residual spraying, and environmental management to reduce mosquito breeding sites.
- ✓ Increase community awareness and education programs to promote the use of preventive measures and encourage early treatment-seeking behaviour.
- ✓ Training of individuals in the field of Molecular Biology

For the National Program for the Control of Schistosomiasis and Intestinal Helminthiasis (PNLSHI):

- ✓ Continue regular mass drug administration campaigns to treat at-risk populations, particularly in Mfou, and in other endemic areas.
- ✓ Promote and implement programs to improve sanitation and hygiene, reducing the transmission of helminths.

For the community:

- ✓ Sleep under ITNs every night to protect against mosquito bites
- ✓ Seek for early treatment and wear protective clothing
- ✓ Practice good hygiene and proper sanitation
- ✓ Participate in community deworming programs to reduce the burden of intestinal helminths

III.5. PERSPECTIVES

- ✓ Carry out further research to confirm the observed allelic variations in targeted genes through DNA sequencing.
- ✓ Carry out similar studies in other regions of Cameroon where malaria and helminthiasis transmission are still very high.

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APPENDICES

Appendice I: Ethical clearance

REPUBLIQUE DU CAMEROUN
Paix – Travail - Patrie

MINSTERE DE LA SANTE PUBLIQUE

SECRETARIAT GENERAL

COMITE REGIONAL D'ETHIQUE DE LA RECHERCHE POUR LA SANTE HUMAINE DU CENTRE

Tél : 222 21 20 87/ 677 94 48 89/ 677 75 73 30

REPUBLIC OF CAMEROON
Peace – Work – Fatherland

MINISTRY OF PUBLIC HEALTH

SECRETARIAT GENERAL

CENTRE REGIONAL ETHICS COMMITTEE FOR HUMAN HEALTH RESEARCH

CE N° 09270 /CRERSHC/2022

Yaoundé, the 9 SEPT 2022

ETHICAL CLEARANCE

The Centre Regional Ethics Committee for Human Health Research (CRERSH-Ce) has received the request for an ethical approval for the project entitled: '' **Epidemiological and surveillance studies on malaria and comorbidities with tuberculosis, HIV/AIDS, helminth-NTDs and COVID-19 in selected health facilities in Mfou and Yaoundé**'', submitted by **Mr Jude BIGOGA.**

After evaluation, it appears that the subject is worthy of interest, the objectives are well defined, and the research procedure does not include invasive methods harmful to the participants. In addition, the informed consent form intended for participants is acceptable.

For these reasons, the CRERSH-Ce issued a six (06) months approval for the implementation of the current version of the protocol.

The Principal Investigator is responsible for scrupulous compliance with the protocol and must not make any amendments, however minor, without the favourable approval of the CRERSH-Ce. In addition, the Principal Investigator is required to:

- Collaborate on any descent from the CRERSH-Ce for monitoring the implementation of the approved protocol.
- And submit the final report of the study to the CRERSH-Ce and to the competent authorities concerned by the study.

This clearance may be withdrawn in the event of non-compliance with the regulations in force and the directives mentioned above.

In witness whereof the present Ethical Clearance is issued with the privileges thereunto pertaining. /-

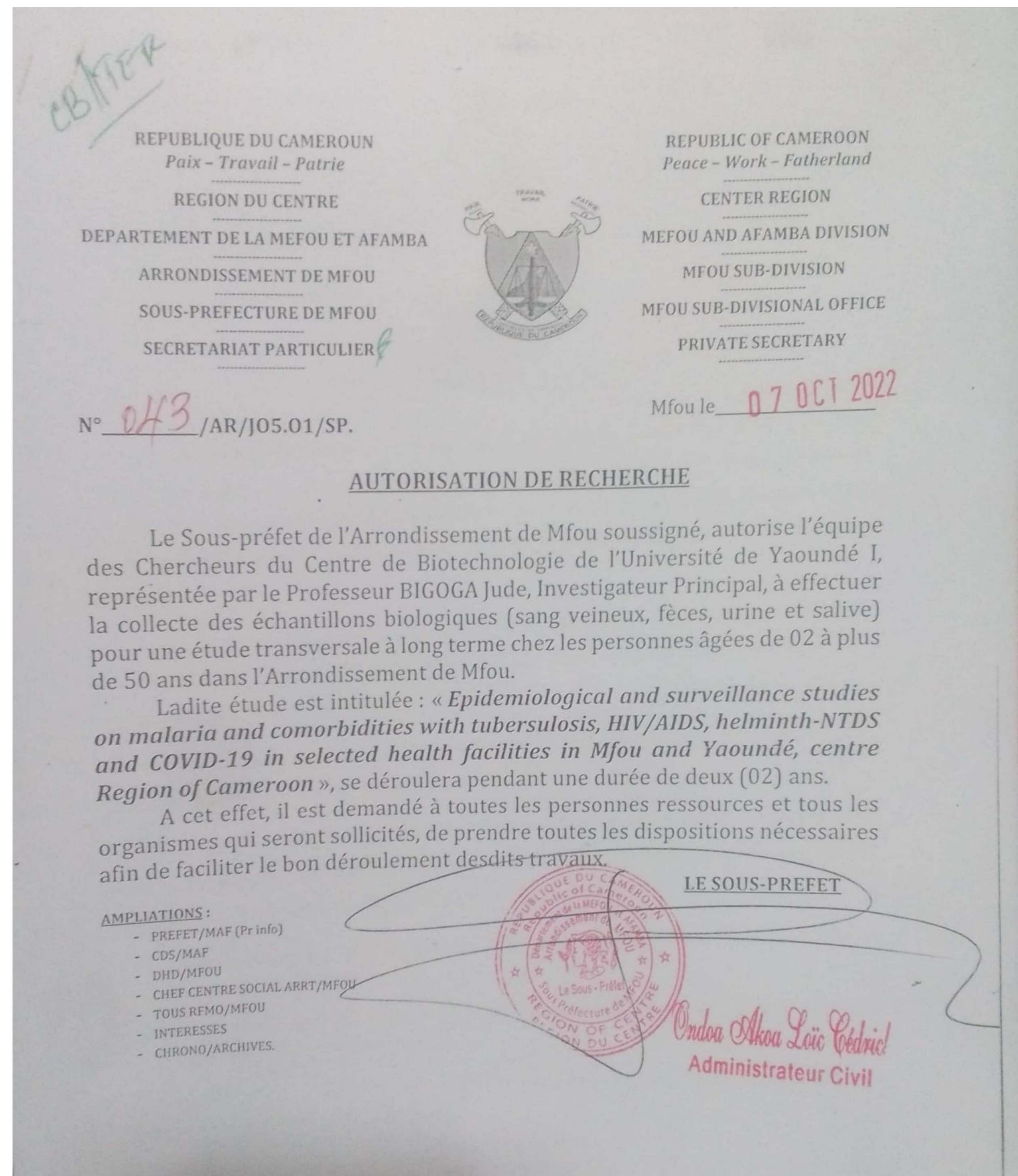
Copy: CNERSH.

THE PRESIDENT

Dr. Dobo Boye Kasimi
Pharmacien

www.minsante.gov.cm

Appendice II: Administrative Authorisation



CB/IER

REPUBLIQUE DU CAMEROUN	REPUBLIC OF CAMEROON
Paix – Travail – Patrie	*Peace – Work – Fatherland*
REGION DU CENTRE	CENTER REGION
DEPARTEMENT DE LA MEFOU ET AFAMBA	MEFOU AND AFAMBA DIVISION
ARRONDISSEMENT DE MFOU	MFOU SUB-DIVISION
SOUS-PREFECTURE DE MFOU	MFOU SUB-DIVISIONAL OFFICE
SECRETARIAT PARTICULIER	PRIVATE SECRETARY

N° 043 /AR/J05.01/SP.

Mfou le 07 OCT 2022

AUTORISATION DE RECHERCHE

Le Sous-préfet de l'Arrondissement de Mfou soussigné, autorise l'équipe des Chercheurs du Centre de Biotechnologie de l'Université de Yaoundé I, représentée par le Professeur BIGOGA Jude, Investigateur Principal, à effectuer la collecte des échantillons biologiques (sang veineux, fèces, urine et salive) pour une étude transversale à long terme chez les personnes âgées de 02 à plus de 50 ans dans l'Arrondissement de Mfou.

Ladite étude est intitulée : « ***Epidemiological and surveillance studies on malaria and comorbidities with tubersulosis, HIV/AIDS, helminth-NTDS and COVID-19 in selected health facilities in Mfou and Yaoundé, centre Region of Cameroon*** », se déroulera pendant une durée de deux (02) ans.

A cet effet, il est demandé à toutes les personnes ressources et tous les organismes qui seront sollicités, de prendre toutes les dispositions nécessaires afin de faciliter le bon déroulement desdits travaux.

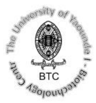
LE SOUS-PREFET

AMPLIATIONS :
- PREFET/MAF (Pr info)
- CDS/MAF
- DHD/MFOU
- CHEF CENTRE SOCIAL ARRT/MFOU
- TOUS RFMO/MFOU
- INTERESSES
- CHRONO/ARCHIVES.

Ondoa Akoa Loïc Cédric
Administrateur Civil

Appendice III: Informed consent form for participants

UNIVERSITE DE YAOUNDE I UNIVERSITY OF YAOUNDE I



FACULTE DES SCIENCES FACULTY OF SCIENCE

DEPARTEMENT DE BIOCHIMIE DEPARTMENT OF BIOCHEMISTRY

THE BIOTECHNOLOGY CENTRE

RESEARCH PARTICIPANT INFORMATION SHEET AND INFORMED CONSENT

Project title: "***Epidemiological and surveillance studies on malaria and comorbidities with tuberculosis, HIV/AIDS, helminth-NTDs and COVID-19 in selected health facilities in Mfou and Yaounde, Centre region of Cameroon***"

NOTICE SHIEST

a) Investigators

1. **Kamgmo Tappe Ange Patricia**, the Biotechnology Centre, University of Yaounde 1
2. **Awanakam Honore**, the Biotechnology Center, University of Yaoundé 1
3. **Prof. Jude Bigoga** (Principal Investigator/PI), the Biotechnology Centre, University of Yaounde 1.
4. **Dr. Francis Zeukeng** (Co-Investigator), the Biotechnology Centre, University of Yaounde 1.

b) Invitation to participate in this research study

Dear Sir/Madam/parent/Legal guardian (for children), we (Lecturers and researchers from the Biotechnology Centre of University of Yaoundé 1, and Members of the CANTAM Consortium), are currently carrying out a study entitled: "***Epidemiological and surveillance studies on malaria, tuberculosis, HIV/AIDS, Helminth-NTDs and COVID-19 in the Centre region of Cameroon***".

In fact, poverty-related diseases such as malaria, tuberculosis, HIV/AIDS, and helminth-NTDs are endemic in Cameroon, and they remain a Public Health problem in the country. Joint together, these infections kill millions of people every year with huge impact on children and pregnant women. As you may know, the harmful consequences of malaria during pregnancy are: severe maternal anaemia, premature deliveries, involuntary abortions, intrauterine growth retardation, low birth weight babies, and even dead of new baby born. You may also be aware about the millions of lives killed by the new coronavirus disease (COVID-19) during the last two years. Several cases of this new coronavirus disease (COVID-19) have been reported in our country by the Ministry of public health, and few information is known about its extend and socio-economic impact across the country. So, it is not good at all for anyone to have one of these infections. To well treat and prevent these diseases, it is important to have a good knowledge on their distribution and the organisms that cause each of the infections. Also, it is important to know how these organisms interact between them in patients carrying more than one type of infection. Therefore, the CANTAM research network, that groups various countries in the Central Africa subregion including Cameroon, is conducting a surveillance study on malaria, tuberculosis, HIV/AIDS, helminth-NTDs, and COVID-19 in order to generate evidence-based data to allow the countries to apply jointly for a large-scale clinical trial, and safe the life of exposed populations. We therefore invite you or your child to participate in this study so that together we can bring our contribution in understanding the epidemiology of emerging infectious diseases in Cameroon.

This is a consent sheet that gives you information about the research study. Read it carefully. We are ready to give additional information concerning the study. So, you should ask any questions you have about this study. You are free to speak to family member of friends before making the decision. If you decide to participate in this research, we will ask you to sign on the Informed Consent Form. However, it is important that you understand why we are conducting this study and what we expect from you before deciding whether or not to participate.

This study received ethical authorization from the Cameroon Regional Ethical Committee (CE-No092700/CRERSHCe/2022), that is in charge of the protection of people who accept to participate in a scientific research study in Cameroon, as well as the administrative and sanitary authorities of your locality.

c) Objectives of the research study

The study will provide up-to-date data on prevalence and incidence of malaria, tuberculosis, HIV/AIDS, helminth-NTDs and COVID-19 in the centre region of Cameroon, as well as the molecular and genetic responses to each infection in the context of mono-infection and comorbidities/coinfections.

d) Research study procedures

We please you to participate or to accept that your child/children take part in this study. If you volunteer to participate in the study, we will start by recording your sociodemographic data and information about the measure(s) you use to protect yourself or your kids against malaria, tuberculosis, HIV/AIDS, helminth-NTDs, and COVID-19. Further each volunteer will be examined by a medical doctor to know about its health-state.

Afterward we will ask you to provide urine and fecal samples for the detection of the organisms causing intestinal infections. Then, we will collect blood samples, saliva sputum, and buccal and nasal swabs, for the detection of the organisms causing malaria, AIDS, tuberculosis, and COVID-19, respectively. These samples will also help to investigate the molecular and genetic interactions among these microbes in patients carrying two or more infection types, as well as your suitability to the new malaria vaccine RST,S/ASO1. Finally, we will harvest mosquitoes' larvae in the vicinity of your household to identify the mosquito's species transmitting malaria in your locality, and their susceptibility to current malaria interventions recommended by the ministry of public health.

e) Voluntary participation and right of withdraw

Taking part in this study is strictly voluntary and you have the right to decide not to participate. If you agree to participate in this study, we will ask you some questions to know you name and first name, age, information's related pregnancy for pregnant women, when you had malaria/helminthiasis/TB/AIDS/COVID-19 for the last time, if you took a treatment against malaria/helminthiasis/TB/AIDS/COVID-19, and so on.

I you decide to participate, and you change your mind afterwards, you will be free to leave and no one will blame you. You will receive the same quality of care normally provided by the hospital/health center.

If you decide not to participate in this research study, you will receive the normal medical care as usual.

f) Risks

The quantity of blood that will be collected after finger pricking (1-2 drops) and venous puncture (3 ml) is not armful for your health and that of your kids. During this process, you will be subjected to some minor risks as mild pain or bruising at the puncture site. The risk of infection is minimal, since only sterile one-time use materials will be used to collect blood. Samples will be collected by experienced personnel trained to take care of highly infectious patients.

Moreover, the collection of the faeces, urine, saliva sputum, and saliva and buccal swabs does not use any invasive method and the collection methods constitute non-armful processes for the populations.

People volunteering for night collection of mosquitoes using the human landing collection method (HLC) will receive anti-malarial prophylaxis as recommended by the national malaria control program (NMCP).

g) Advantages and Benefits

The direct benefits for patients participating in this study include free medical consultations and free laboratory tests to identify the organism causing the targeted infectious diseases (malaria, helminth-NTDs, TB, HIV/AIDS, and COVID-19). Recruited participants will also receive full blood count information as given to them after analyses.

The information collected during this study will allow us to understand up-to-date distribution and burden of infectious diseases (malaria, helminth-NTDs, TB, HIV/AIDS, and COVID-19) in selected health areas in the centre region of Cameroon.

In addition, people (adults aged >20 years old) volunteering for night collection of mosquitoes using the human landing collection method (HLC) will receive anti-malarial prophylaxis as recommended by the national malaria control program (NMCP). This means, they will receive antimalarial drugs prior to mosquitos' collection, and they will be screened for malaria infection after each mosquito's collection period. If infected, they will be treated at free cost. Additional incentives (motivation fees) will also be provided to all collectors.

h) Confidentiality

All participant information will be kept in locked boxes and available only to the members of the study team. The participant's name and address will not be disclosed to the study sponsor. Patient's data/specimens will be identified using the study number and/or initials. Individual patients will not be identified in the resulting publications and presentations from the study. The study will comply with the principles of the Data Protection Act of Cameroon. We will also pay attention to the concept of 'FAIR Guiding Principles for scientific data management and stewardship' which refers to data being Findable, Accessible, Interoperable and Reusable, also considering the period beyond the project. The location of the households will be handled with the same security standards as all other information collected in the project, all data will be encrypted.

i) Compensation and Cost

No composition other than that mentioned above will be provided (see benefits). Also, there are no costs involved in participating in this study. In an unlikely scenario (since only sterile equipment is being used and procedures are carried out by certified phlebotomists) if a participant is injured during venepuncture or the blood collection site is infected, she/he will be reimbursed for all medical expenses according to the government policies.

CONSENT FORM

Statement of consent:

I, Mr/Me/Ms:……………………………………………the undersigned have understood the above information or it has been read and explained to me in the French language. Or the study has been explained to me in my local language (mother tongue) and the answers obtained from my preoccupations were satisfactory.

☐ I voluntary accept to take part in this study.

☐ I voluntary accept that my kid named……………………………………participates in this study.

Signature of participant (parent/guardian)

…………..………………………………………………………………

Name and signature of the witness (for inapt participants)

...…………………………………………………...................................

Name and signature of the research staff

…………..………………………………………………………………

CONSENT FORM FOR USE OF ARCHIVED SAMPLES

Most of the samples we will collect during this research study will be used as described above. However, it is possible that a small amount will be left. In that case, we would like to use it for future studies on malaria, tuberculosis, HIV/AIDS, helminth-NTDs, and COVID-19. If you agree to allow us to use your samples in additional research studies, the results will help improve the health care of others.

☐ Yes, I agree to have my samples used in additional studies other than this one.

☐ No, I do not agree to have my samples used beyond the studies in the original purpose.

Date…………………………………
……

Contacts

For any enquiry concerning this study, fill free to compose any of the following contacts:

Prof. Jude Bigoga, the Biotechnology Centre of the University of Yaounde 1; Tel: 677824730; E-mail:

judebigoga@yahoo.com

UNIVERSITE DE YAOUNDE I UNIVERSITY OF YAOUNDE I

FACULTE DES SCIENCES FACULTY OF SCIENCE

DEPARTEMENT DE BIOCHIMIE DEPARTMENT OF BIOCHEMISTRY

THE BIOTECHNOLOGY CENTRE

NOTICE D'INFORMATION DU PARTICIPANT ET FORMULAIRE DE CONSENTEMENT ECLAIRE

Titre du projet: **" Études épidémiologiques et de surveillance du paludisme et des comorbidités avec la tuberculose, le VIH/SIDA, les helminthiases et les MTN et la COVID-19 dans des établissements de santé sélectionnés à Mfou et Yaoundé, région du Centre du Cameroun** »

NOTICE D'INFORMATION

a) Investigateurs

1. **Kamgmo Tappe Ange Patricia,** Centre de Biotechnologie, Université de Yaoundé 1.
2. **Awanakam Honore,** Centre de Biotechnologie, Université de Yaoundé 1.
3. **Prof. Jude Bigoga** (Investigateur Principal/PI), Centre de Biotechnologie, Université de Yaoundé 1.
4. **Dr. Francis Zeukeng** (Co-Investigateur), Centre de Biotechnologie, Université de Yaoundé 1.

b) Invitation à participer à cette recherche

Cher Monsieur/Madame/parent/tuteur légal, nous (Enseignants et Chercheurs du Centre de Biotechnologie de l'Université de Yaoundé 1, et Membres du réseau CANTAM), menons actuellement une étude intitulée: **" Études épidémiologiques et de surveillance du paludisme et des comorbidités avec la tuberculose, le VIH/SIDA, les helminthiases et les MTN et la COVID-19 dans des établissements de santé sélectionnés à Mfou et Yaoundé, région du Centre du Cameroun »**

En effet, les maladies liées à la pauvreté telles que le paludisme, et le VIH/SIDA sont endémiques au Cameroun et demeure un problème de santé publique dans le pays. Ensemble, ces infections tuent des millions de personnes chaque année avec un impact énorme sur les enfants et les femmes enceintes. Comme vous le savez peut-être, les conséquences néfastes du paludisme pendant la grossesse sont : une anémie maternelle sévère, des accouchements prématurés, des avortements involontaires, un retard de croissance intra-utérin, des bébés de faible poids à la naissance, et même des cas de décès chez des nouveau-nés. Vous êtes peut-être également au courant des effets du VIH qui affaiblit le système immunitaire, rendant une personne vulnérable et exposée à des infections potentiellement mortelles et au cancer. Cela dit, il n'est pas du tout bon pour quiconque d'avoir une de ces infections. Pour bien traiter et prévenir ces maladies, il est important d'avoir une bonne connaissance sur leur distribution et des organismes qui causent chacune de ces maladies. Aussi, il est important de savoir comment ces organismes interagissent entre eux chez les patients porteurs de plus d'un type d'infection. Par conséquent, le réseau de recherche CANTAM, qui regroupe divers pays de la sous-région Afrique centrale dont le Cameroun, mène une étude de surveillance sur le paludisme, la tuberculose, le VIH/sida, les helminthiases et la COVID-19, afin de générer des données factuelles pour permettre aux pays membres de postuler conjointement aux essais cliniques à grande échelle, afin de protéger les populations exposées. Nous vous invitons, vous ou votre enfant, à participer à cette étude afin qu'ensemble nous puissions apporter notre contribution à la compréhension de l'épidémiologie des maladies infectieuses émergentes au Cameroun.
Ceci est une fiche informative qui vous donne des renseignements liés à l'étude. Lisez-la attentivement. Nous sommes prêts à communiquer des informations supplémentaires concernant l'étude. Donc, vous devriez poser toutes les questions que vous avez sur cette étude. Vous êtes libre de parler à un membre

de la famille ou à des amis avant de prendre votre décision. Si vous décidez de participer à cette recherche, nous vous demanderons de signer un formulaire de consentement éclairé. Cependant, il est important que vous compreniez pourquoi nous menons cette étude et ce que nous attendons de vous avant de décider de participer ou pas.
Cette étude a reçu l'autorisation éthique auprès du Comité Régional d'Ethique du Centre (CE-No092700/CRERSHCe/2022) (), qui est chargé de la protection des personnes qui acceptent de participer à une étude de recherche scientifique au Cameroun, ainsi que des autorités administratives et sanitaires de votre localité.

c) Objectifs de l'étude de recherche

L'étude fournira des données actualisées sur la prévalence et l'incidence du paludisme, de la tuberculose, du VIH/sida, en Mfou dans la région du centre Cameroun, ainsi que les réponses immunité et génétiques à impliquées dans chaque infection dans le contexte de mono-infection et de coïnfections.

d) Procédure de recherche

Nous vous prions de participer ou d'accepter que votre/vos enfant(s) participe(nt) à cette étude. Si vous-vous portez volontaire pour participer à l'étude, nous commencerons par enregistrer vos données sociodémographiques et des informations sur les mesures que vous utilisez pour vous protéger ou protéger vos enfants contre le paludisme, et le VIH/SIDA. De plus, chaque volontaire sera examiné par un médecin pour connaître son état de santé.
Par la suite, nous prélèverons des échantillons de sang, pour la détection des organismes responsables du paludisme, et sida. Ces échantillons aideront également à étudier les interactions moléculaires et génétiques entre ces microbes chez les patients porteurs de deux types d'infection ou plus, ainsi que votre susceptibilité au nouveau vaccin antipaludique RST, S/ASO1.

e) Participation volontaire et droit de retrait de l'étude

La participation à cette étude est strictement volontaire et vous avez le droit de décider de ne pas y participer. Si vous acceptez de participer à cette étude, nous vous poserons quelques questions pour connaître votre nom et prénom, votre âge, les informations liées à la grossesse pour les femmes enceintes, la dernière fois que vous avez eu le paludisme/SIDA/, si vous avez pris un traitement contre le paludisme/SIDA, etc.
Si vous décidez de participer et que vous changez d'avis par la suite, vous serez libre de partir et personne ne vous en voudra. Vous recevrez la même qualité de soins fournis par l'hôpital/centre de santé.
Si vous décidez de ne pas participer à cette étude de recherche, vous recevrez les soins médicaux normaux comme d'habitude.

f) Risques

La quantité de sang qui sera prélevée après piqûre au doigt (1 à 2 gouttes) et ponction veineuse (3 ml) n'a pas de risque sur votre santé ou celle de vos enfants. Au cours de ce processus, vous serez soumis à quelques risques mineurs comme une légère douleur ou des ecchymoses au site de piqûre. Le risque d'infection est minime, puisque seuls des matériaux stériles à usage unique seront utilisés pour prélever du sang. Les échantillons seront prélevés par un personnel expérimenté et formé pour prendre en charge les patients hautement contagieux.

g) Avantages et bénéfices

Les avantages directs pour les personnes participant à cette étude comprennent les consultations médicales gratuites et des tests de laboratoire gratuits pour identifier les organismes responsables des maladies infectieuses ciblées (paludisme, et VIH/sida). Les participants recrutés recevront également des informations complètes sur leur statut clinique.
Les informations recueillies au cours de cette étude nous permettront de comprendre la répartition et le fardeau des maladies infectieuses (paludisme, et VIH/sida) dans de santé de la région du centre Mfou Cameroun.
De plus, les personnes (âgées de plus de 20 ans) ayant volontairement accepté de participer à la capture nocturne des moustiques par la technique de capture sur appât humain de l'organisation Mondiale de la Santé (OMS) recevront un traitement préventif contre le paludisme suivant les régulations du programme national de lutte contre le paludisme. Cela signifie qu'elles recevront un antipaludique avant la capture des moustiques et elles subiront un test de diagnostic du paludisme à la fin de chaque période de capture. Et si elles s'avèrent être infectées par le Plasmodium, elles seront soignées gratuitement comme décrit ci-dessus.

Aussi, des motivations additionnelles (frais de capture) seront fournies à tous les collecteurs.

h) Confidentialité

Toutes les informations sur les participants seront conservées dans des boîtes verrouillées et accessibles uniquement aux membres de l'équipe d'étude. Le nom et l'adresse du participant ne seront pas divulgués aux sponsors de l'étude. Les données/échantillons du patient seront identifiés à l'aide du code du patient et/ou de ces initiaux. Les patients individuels ne seront pas identifiés dans les publications et les présentations issues de l'étude. L'étude sera menée conformément aux principes de la loi sur la protection des données du Cameroun. Nous accorderons également une attention particulière au concept de « principes directeurs FAIR pour la gestion des données scientifiques », qui fait référence aux données pouvant être trouvées, accessibles, interopérables et réutilisables, en tenant également compte de la période au-delà du projet. La localisation des ménages sera traitée avec les mêmes normes de sécurité que toutes les autres informations collectées dans le cadre du projet, toutes les données seront cryptées.

i) Rémunération et coût

Aucun autre avantage que ceux mentionnés ci-dessus ne sera fourni. De plus, la participation à cette étude est gratuite. Dans un scénario peu probable si un participant se blesse lors d'une ponction veineuse ou si le site de prélèvement sanguin est infecté, il sera remboursé de tous les frais médicaux selon les politiques gouvernementales.

CONSENTEMENT ECLAIRE

Déclaration de consentement :

Je soussigné M./Mme/Mlle:……………………….…………………… reconnais avoir compris l'étude telle que présentée dans la notice d'information exposée dans la langue française. Ou bien l'étude m'a été bien expliquée dans ma langue maternelle que je comprends le mieux, et les réponses satisfaisantes ont été apportées à mes différentes questions.

- ☐ J'accepte volontairement de participer à cette étude.
  - ☐ J'accepte volontairement que mon enfant nommé................................…..........participe à cette étude.

Signature du participant (parent/tuteur)

…………..…………………………………………………………

Noms et signature des témoins (pour les participants illettrés)

...……………………………………………….....................................

Noms et signature du responsable de l'équipe de recherche

...……………………………………………….....................................

CONSENTEMENT POUR UNE UTILISATION ULTERIEURE DES ECHANTILLONS

Les échantillons collectés au cours de cette étude de recherche seront utilisés comme décrit ci-dessus. Cependant, il est possible qu'il en reste une petite quantité. Dans ce cas nous aimerions les utiliser dans des études ultérieures sur le paludisme, et le VIH/sida. Si vous permettez de le faire les résultats pourraient contribuer à améliorer la santé d'autres personnes.

- ☐ Oui, j'accepte que le reste de mes échantillons soit utilisé dans les études ultérieures autres que celle-ci.
- ☐ Non, je refuse que le reste de mes échantillons soit utilisé dans les études ultérieures autres que celle-ci.

Date………………………………………..

Personnes à contacter si nécessaire

Pour toute demande concernant cette étude, n'hésitez pas à composer l'un des contacts suivants :

Prof. Jude Bigoga, Département de Biochimie, Faculté des Sciences, et Centre de Biotechnologie de l'Université de Yaoundé 1; Tel: 677824730; E-mail: judebigoga@yahoo.com

UNIVERSITE DE YAOUNDE I UNIVERSITY OF YAOUNDE I

FACULTE DES SCIENCES FACULTY OF SCIENCE

DEPARTEMENT DE BIOCHIMIE DEPARTMENT OF BIOCHEMISTRY

THE BIOTECHNOLOGY CENTRE

FORMULAIRE D'ASSENTIMENT (10-20 ANS)

Titre du projet : **"Études épidémiologiques et de surveillance du paludisme et des comorbidités avec la tuberculose, le VIH/SIDA, les helminthiases et les MTN et la COVID-19 dans des établissements de santé sélectionnés à Mfou et Yaoundé, région du Centre du Cameroun"**

Investigateurs

1. **Kamgmo Tappe Ange Patricia** (Investigateur Principal), Centre de Biotechnologie, Université de Yaoundé 1.
2. **Awanakam Honore** (Co-investigateur), Centre de Biotechnologie, Université de Yaoundé 1.
3. **Prof. Jude Bigoga**, Centre de Biotechnologie, Université de Yaoundé 1.
4. **Dr. Francis Zeukeng,** Centre de Biotechnologie, Université de Yaoundé I.

INFORMATION AU PARTICIPANT

Vous êtes invité(e) à participer à cette étude de recherche car vous êtes éligible. Veuillez lire attentivement le formulaire de consentement éclairé qui vous a été remis et en parler avec votre mère, votre père ou votre tuteur avant de décider si vous souhaitez ou non participer à cette étude de recherche. Il leur sera également demandé s'ils vous autorisent à participer à l'étude. Vous ne pouvez pas participer à l'étude si votre mère, votre père ou votre tuteur n'est pas d'accord. S'ils sont d'accord, ils devront signer un formulaire indiquant qu'ils vous autorisent à participer à l'étude. **Si quelque chose ne vous semble pas clair, n'hésitez pas à poser des questions aux membres de l'équipe de l'étude. Vous pouvez également parler de cette étude à qui vous le souhaitez.**

Prenez votre temps avant de décider si vous souhaitez ou non participer à cette étude. Nous voulons nous assurer que la décision de participer à cette étude vient de vous et de vos parents/votre tuteur. Si vous décidez de ne pas participer à l'étude, Si vous ne souhaitez pas participer à cette étude, ce n'est pas un problème. Personne ne vous en voudra et vos droits seront bien respectés.

FORMULAIRE D'ASSENTIMENT – SIGNQTURES REQUISES

Déclaration de consentement	
J'ai lu les informations précédentes et j'ai conscience de ce à quoi je consens. J'ai eu l'occasion de poser des questions et j'ai obtenu des réponses satisfaisantes à toutes mes questions. Je consens de mon plein gré à participer à cette étude de recherche et.	Je confirme que le participant a eu l'occasion de poser des questions sur l'étude et qu'il a reçu des réponses correctes à toutes ses questions. Un test de compréhension oral a démontré la capacité du participant à comprendre l'étude et les principes de la recherche clinique, confirmant ainsi que le consentement a été donné volontairement.
______________________ **Nom du participant (en majuscules)**	______________________ **Nom de la personne recueillant le consentement (en majuscules)**
______________________ **Signature du participant** Date (jour/mois/année) ____________	______________________ **Signature de la personne recueillant le consentement** Date (jour/mois/année) ____________
______________________ **Nom du représentant légal, le cas échéant (en majuscules)** ______________________ **Signature du représentant légal** Date (jour/mois/année) ____________	______________________ **Nom du témoin impartial (en majuscules), dans le cas où le participant ou son représentant légal serait illettré** ______________________ **Signature du témoin impartial** Date (jour/mois/année) ____________

Appendice IV: Structured Questionnaire

"Epidemiological and surveillance studies on malaria and comorbidities with tuberculosis,

HIV/AIDS, helminth-NTDs and COVID-19 in selected health facilities in Mfou and Yaounde, Centre region of Cameroon"

Surveyor ID/Name: _______________________________________ ______/_____/_______

Study site**:** District Hospital Mfou: |____| District Hospital Efoulan: |____|

CMA Ahala: |____| CMA Odza: |____|

District Hospital Cite Verte: |____| District Hospital Etoudi: |____|

1. I. DEMOGRAPHICS

Participant's code : ____________ Names : ____

Date of birth : ____/____/_____ Age: ________Years Sex (M/F):|____| Weight: |___|___|.|___|___|kg

Height: |____|. |____||____|m Marital status: |___|Single |___|Married

Employment status: |___|Student |___|Employed |___|Unemployed

Type of occupation: ____________________ Workplace/school name: ______________________

Residence in past three months: _______________________ Household size: _______persons

Residence type (urban=0, Peri-urban=1, Rural=1): |____|Urban |____|Peri-urban |____|Rural

Contact number: __________________________

CLINICAL PARAMETERS

History of fever in the past 24 hours ☐ Yes ☐ No

Temperature: |____|____|.|____| °C (axillary) Pulse Rate (Heart Rate): |____|____||____| bpm

Blood Pressure: |___|___|___| / |___|___|___| mm/Hg Respiratory Rate: |___|___| bpm

Systolic / Diastolic

Hepatomegaly ☐ Yes ☐ No, if yes, size: |___|___| cm Splenomegaly ☐ Yes ☐ No, if yes, size: |___|___|

cm

II. CLINICAL SIGNS AND SYMPTOMS

☐ Fever	☐ Abdominal Pains	☐ Vomiting	☐ Cough
☐ Weakness	☐ Loss of Appetite	☐ Muscle Pain	☐ Difficult breathing
☐ Headache	☐ Nausea	☐ Diarrhea	☐ Anemia

III. MALARIA INFECTION

1- Did you use a bednet at any time in past 2 weeks? Yes |___| No |___| (**skip to 2**)

If yes, did you use a bednet in last 2 nights? Yes |___| No |___| (**skip to 2**)

Was the bednet impregnated with insecticide? Yes |___| No |___| Don't know |___|

2- Have you taken malaria prophylaxis in past 2 weeks? Yes |___| No |___| (**skip to 3**)

If yes, specify: Name of drug: __________________ Daily dosage _________(mg/day)

Date begun: ___/____/____ Date ended or going to be ended: ___/____/____

3- Have you taken anti-malarials for chemotherapy in past 2 weeks? Yes |___| No|___| (**skip to 4**)
If Yes, specify: Name of drug: __________________ Daily dosage _________(mg/day)

Date begun: ___/____/____ Date ended or going to be ended: ___/____/____

4- Did you use any other preventive practice home in past 2 weeks? Yes |___| No |___| (**skip to 5**)

If yes, did you use any in the last 2 nights? Yes |___| No |___| (skip to 4)

What other preventive practices were utilized? |___|Repellents |___| Herbal repellents |___| Coils

5- Have you taken iron tablets in past 2 weeks? Yes |___| No |___|

If Yes, specify: Name of drug: __________________ Daily dosage _________(mg/day)

Date begun: ___/____/____ Date ended or going to be ended: ___/____/____

6- Laboratory results

1- PCV: _________________ % or Hb: ___________g/dl
2- Slide ID of the thick and thin blood films: ____________
3- Read-out of thick and thin blood films

Tests	**Staff 1 ID**	**Staff 2 ID**	**Staff 3 ID**	**Remarks**
Total WBC/mm³				
Number of parasites/ 200 WBC				
P. falciparum found?	Yes -1 No -2	Yes -1 No -2	Yes -1 No -2	
P. ovale found?	Yes -1 No -2	Yes -1 No -2	Yes -1 No -2	
P. malariae found?	Yes -1 No -2	Yes -1 No -2	Yes -1 No -2	
Gametocyte found?	Yes -1 No -2	Yes -1 No -2	Yes -1 No -2	
Schizont found?	Yes -1 No -2	Yes -1 No -2	Yes -1 No -2	
Microfilaria found?	Yes -1 No -2	Yes -1 No -2	Yes -1 No -2	

HELMINTH INFECTION

1- Which kind of toilet do you use?

|__| Nature/grass |__| Appointed latrines |__| Non-appointed latrines |__| Pullwash latrines |__| Other,

specify ___________________

2- Have you taken Helminthiasis prophylaxis in past 2 weeks? Yes |___| No |___| (skip to 3)

If Yes, specify: Name of drug: __________________ Daily dosage _________(mg/day)

Date begun: ___/____/____ Date ended or going to be ended: ___/____/____

3- Have you taken antihelminth drugs for chemotherapy in past 2 weeks? Yes|___| No|___| (skip to 4) If Yes, specify: Name of drug: __________________ Daily dosage _________(mg/day)

Date begun: ___/____/____ Date ended or going to be ended: ___/____/____

4- Did you use any other preventive practice at home in the past 2 weeks? Yes |___| No |___| (skip to 5)

If yes, did you use any in the last 2 nights? Yes |___| No |___| (skip to 4)

Laboratory results

Tests	Staff 1 ID	Staff 2 ID	Staff 3 ID	Remarks
Total stool used (mg)				
Number of eggs/g of feces				
A lumbricoides	Yes -1 No - 2	Yes -1 No - 2	Yes -1 No - 2	
T. trichiura	Yes -1 No - 2	Yes -1 No - 2	Yes -1 No - 2	
N. americanus	Yes -1 No - 2	Yes -1 No - 2	Yes -1 No - 2	
S. duodenalis	Yes -1 No - 2	Yes -1 No - 2	Yes -1 No - 2	
S. mansoni	Yes -1 No - 2	Yes -1 No - 2	Yes -1 No - 2	
Others	Yes -1 No - 2	Yes -1 No - 2	Yes -1 No - 2	

Appendice V: Differentiation criteria for soil transmitted helminthiasis under the light microscope

STH are distinguished by their stage of evolution in the stool (eggs or larvae). Eggs are differentiated by size, shape, shell and content (Bernazzou, 2010).

-Ascaris lumbricoides: the egg is ovoid with a double shell, the outer shell is brown and nippled, the inner one is smooth and yellowish. It is not embryonated at oviposition and varies in size from 50- 70 x 45-50 um.

-Hookworm: the egg is an ellipsoid with a single, smooth, mice-like shell. dimensions vary from 60-70 um. it contains 2 to 8 blastomeres.

-T.trichuira: the egg is lemon-shaped and has a spur at each pole. the outer shell is smooth and brown, the inner one yellow. it is not embryonated at oviposition and its dimensions vary from 25-30 x 50-60 um.

(a) (b) (c)

Figure 1: STH eggs ; a = *Ascaris lumbricoides* , b = *Trichuris trichuira*, c = *Hookworm* (Bethony *et al*., 2006).

Appendice VI: Identification and stages of the malaria parasites

A- Identification of the malaria parasite

With Giemsa solution, malaria parasites stain in a specific way, whether in the thin ot thick smear. they go through a number of evolutionary stages. however, at all stages, the same elements stain in the same way.

- **Chromatin** part of the parasite nucleus, usually round, stains bright red.
- **Cytolplasm** stains blue; the tone of blue may differ between species and is
- sometimes a differentiatingcharacteristics.
- **Vacuole** retains the shape of the parasite and always remains clear.

B- Stages of the malaria parasites

- **The trophozoite stage**

This is the most commonly seen stage. Often called the ring stage, the 'ring' may appear incomplete in thick films. The trophozoite can vary from small to quite large within the host cell. Usually, there is one chromatin dot; two are common in *P. falciparum.*

- **The schizont stage**

This stage is easily recognized. It begins when the trophozoite has reached its full capacity and the chromatin divides into two. The parasite starts to reproduce asexually, i.e. the cell divides into 'daughter cells' (merozoites) by simple division. Several more divisions of the chromatin follow, which mark the growth of the schizont, until there are many chromatin bodies, each with its accompanying cytoplasm.

- **The gametocyte stage**

The parasite develops into either a male or a female gametocyte in preparation for the sexual phase in the female Anopheles mosquito vector. Gametocytes are round or banana-shaped, depending on the species. The way in which the parasite takes up the stain helps to identify the sex of the parasite in thin films: male (microgametocyte) or female (macrogametocyte). Differentiating between male and female gametocytes is difficult in thick films.

Appendice VII: REAGENT PREPARATION PROTOCOLS

- **Giemsa 10%**

Put 5ml of Giemsa stock solution in a 50ml graduated beaker and then add 45ml of distilled water. Mix gently to homogenize. Store at room temperature

- **EDTA 0.5M (500ml)**

Weigh 93.1g of EDTA (ethylene diamine tetra acetic acid) then add 400ml of distilled water. Add concentrated NaOH to obtain a pH of 8 at which EDTA dissolves. Fill up to 500ml with distilled water and store at ambient temperature.

- **Chelex-100 at 20%**

Weigh 20g of Chelex-100 (powder) and add 50ml of sterilized Phosphate Buffer Solution (PBS). Mix, then let suspension settle. Dispose supernatant then add PBS to 100ml. store at 4°C.

- **1.5% Agarose Gel**

Weigh 0.75g of agarose (powder) then add 50ml of TBE 1X. Boil and leave to cool. Add 3µl of ethidium bromide and mix gently. Poor into the gel mold and allow to solidify.

- **Ethidium Bromide (10ml) (EtBr)**

Weigh 0.15g of EtBr and dissolve in 10ml of doubly distilled water. Conserve at room temperature in an opaque tube sealed with aluminium foil paper.

- **Saponin 0.5%**

Weigh 0.25g of saponin and add 50ml of PBS 1X, then sterilize in autoclave and store at 4°C for 48 hours.

- **10X PBS pH 7.2 (500ml)52**

Weigh 77.75mg of Na2HPO4, 10.2g of NaH2PO4 and 190.84g of NaCl. All are dissolved in 300ml of distilled water and the pH adjusted to 7.2 with HCl 1M. Fill up to 500ml using distilled water. Store at room temperature.

- **1xTBE**

Measure 50 ml of TBE 10X and add 450 ml of distilled water. Mix gently to homogenize. Store at room temperature.

- **Molecular weight marker (200µl)**

Pipette 140µl of buffer-TE 1X, sterilize, then add 20µl of DNA marker. Fill up to 200µl using the loading buffer. Store at -20°C.

- **Loading buffer for electrophoresis**

Weigh 0.125g of bromophenol blue 0.25%; 0.125g of xylene cyanol 0.25%; 7.5g of Ficoll 15%. Mix all in a sterile tube and add 2ml of EDTA 0.5M. Fill up to 50ml using distilled water. Mix and store at room temperature.

- **Dilution of Primers (10%)**

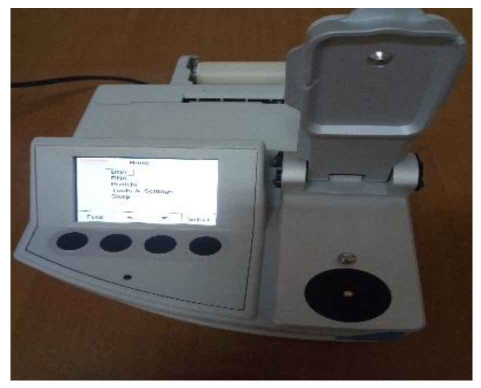
Pipette 10µl of initial concentration of primers and add 90µl of NFW inside an Eppendorf tube and homogenised. Store at -20°C.

Appendice VIII: Laboratory Equipments

Thermocycler

Centrifugator

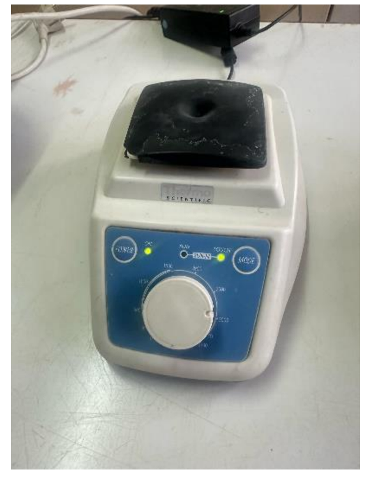
Electrophoretic tank



Nanodrop

Light Microscope

Scale



Pipettes **Vortex**

Transilluminator **Biosafety chamber**