UNIVERSITY OF CAPE COAST

HOST AND PARASITE GENETIC FACTORS THAT AFFECT *PLASMODIUM FALCIPARUM* GAMETOCYTOGENESIS AND MALARIA TRANSMISSION IN SOUTHERN GHANA

BY

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

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Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

Several factors affect gametocyte production and are proposed to vary in different endemic settings and seasons. The current study assessed gametocyte production rates in *P. falciparum* isolates from malaria patients in *ex vivo* assays. Effect of host (G6PD, HBB and ABO blood groups) and parasite (*msp2* and *Pfg377* diversity, and drug resistant strains) factors on gametocyte prevalence were assessed in asymptomatic infections. Study participants were children aged one to twelve years living in Obom and Cape Coast. In an *ex vivo* assay, 54% of the *P. falciparum* isolates produced gametocytes by day three with the mean production rate of less than 1%. High *P. falciparum* prevalence was observed, in up to 60% and 86% of children harbouring the parasites at microscopic and submicroscopic levels respectively. The parasite prevalence in Obom exhibited an extensive seasonal variation ($P < 0.001$) in microscopic and submicroscopic infections. Neither HBB, G6DP variants nor any of the ABO blood groups was associated with gametocyte prevalence; but participants with heterozygous or homozygous HbC had more gametocytes than the other HBB genotypes. Low *P. falciparum* sexual and asexual diversities (MOI<1.5) were observed and gametocyte positivity was significantly ($P = 0.001$) higher in individuals with *msp2* dimorphic infections. Different levels of drug mutant *P. falciparum* strains with *crt* 76T (< 23%), *Pfmdr1* 86Y (< 18%), *dhfr* S108N (< 40%) and *Pfdhps* A437G (> 3%) were observed. Low frequencies (2.1%) of *K13* (C469C and A558S) mutant parasite strains were recorded. *Plasmodium falciparum* infections with both the mutant and wild type drug resistant strains also had *msp2* dimorphic alleles and this will result in the formation of new parasite strains in the *Anopheles* vector for onward transmission.

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DEDICATION

To dearest Winfred, Mawuena and Yewoe for their love and sacrifice!

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LIST OF ABBREVIATIONS

µL	microlitre
µM	micromolar
µg	microgram
ACD	Citrate-dextrose solution
ACT	Artemisinin Combination Therapy
bp	base pairs
BSC	Biosafety cabinet
cDNA	complementary DNA
CPM	Complete Parasite Media
CTs	Threshold Cycle
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
Figure S	Supplementary Figure
G6PD	Glucose-6-Phosphate Dehydrogenase
GHS	Ghana Health Service
Hb	haemoglobin
HBB	beta (ß) haemoglobin
HRP	Histidine Rich Protein
IEF	Isoelectric focusing
IPTp	Intermittent preventive treatment in pregnancy
Kahrp	Knob Associated Histidine Rich Protein gene
KDa	KiloDaltons
M	molar

mL	millilitre
mm	millimetre
MOI	Multiplicity of Infection
MSP	Merozoite Surface Protein
NAG	N-Acetyl-glucosamine
NCBI	National Centre for Biotechnology Information
NTC	No template control
PCR	Polymerase Chain Reaction
Pfcrt	*Plasmodium falciparum* chloroquine transporter
Pfdfhr	*Plasmodium falciparum* dihydrofolate reductase
Pfdfps	*Plasmodium falciparum* dihydropteroate synthase
Pfmdr1	*Plasmodium falciparum* multi drug resistance gene 1
qRT-PCR	Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium
SP	Sulfadoxine-Pyrimethamine
Table S	Supplementary Table
U	Unit
UV	Ultra Violet Light
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

Background of the Study

Malaria still poses a global threat despite years of eradication efforts where almost 3.2 billion people are still at risk of contracting the disease (World Health Organization, 2016a). The most vulnerable groups contracting the disease are children under five years, pregnant women and non-immune travellers to disease-endemic regions (World Health Organization, 2016a). Between 2000 and 2015, a decline in malaria morbidity and mortality was noted in most endemic countries; falling by 37% and 60% respectively (World Health Organization, 2016b). The decline in malaria is attributed to effective vector control and appropriate management of the disease with artemisinin-based combination therapies -ACT (World Health Organization 2014; World Health Organization, 2009). Nonetheless, the *Plasmodium* parasite and the mosquito vector are adapting to humans by altering its genetic or metabolic pathways, resulting in newer and better fitted/resistant strains (Sisowath et al., 2005; Uhlemann et al., 2007; Mwai et al., 2012).

Sub-Saharan Africa still suffers the most burden of malaria with an incidence rate of 90% of all cases and 91% of the disease related deaths universally (World Health Organization, 2016a). Malaria also puts undue pressure on the limited health care facilities and resources in the disease endemic countries. Malaria accounts for 25% to 35% of all outpatient cases, 20% to 45% of hospital admissions, and 15% to 35% of clinically certified deaths in endemic areas (World Health Organization, 2016b). The annual

direct expenditure on malaria is estimated at US$ 2.7 billion and this excludes the loss of man-hours due to the disease (World Health Organization, Global Malaria Programme, & World Health Organization, 2014). Treatment cost of the disease per household income is pegged between 5% to 32% in very poor African settings (Chima, Goodman, & Mills, 2003a). Therefore, malaria costs Africans billions of dollars in direct losses affecting economic growth which further deepens poverty in the region (Roll Back Malaria, 2016).

Ghana spent about US$131.9 million on malaria with an average of US$11.99 per individual treatment cost in 2013 (Sicuri, Vieta, Lindner, Constenla, & Sauboin, 2013; Tawiah et al., 2016), and a treatment of a child was between US$22.53 to US$67.35 (Nonvignon et al., 2016). Most households in Ghana still spend more than half of their income on malaria treatment alone (Asante, Asenso_okyere, 2003; Mia, Begum, Er, Abidin, & Pereira, 2012). The total expenditure on malaria is now higher than two decades ago where the ACTs are ten times more expensive than the previously used drugs. For example, a complete dose of chloroquine was less than US$1 while as ACTs costs more than US$2 (Malik et al., 2006).

Five *Plasmodium* species causes human malaria and four of these, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are found in Africa. *Plasmodium falciparum* causes about 90% of all *Plasmodium* infections in Africa, whereas *P. vivax* accounts for the least percentage due to the absence of the Duffy antigen on the red blood cells (Langhi & Bordin, 2006). *Plasmodium vivax* and *P. knowlesi* uses the Duffy antigen as a receptor to invade human erythrocytes (Chitnis, Chaudhuri, Horuk, Pogo, & Miller, 1996; de Carvalho & de Carvalho, 2011). *Plasmodium knowlesi* is a zoonotic

species (Cox-Singh & Singh, 2008; White, 2008) occurring only in Asia (Singh & Daneshvar, 2013). In many areas outside Africa, infections by *P. vivax* are more common than *falciparum* malaria.

The presence of three out of the five human *Plasmodium* species are reported in Ghana (World Health Organization, 2014). With distribution of species based on clinically diagnosed malaria cases, *P. falciparum* account for 90% of cases. *Plasmodium* malariae is next with about 9% and *P. ovale* infections being less than 1%. *Plasmodium falciparum* is the most virulent, lethal and prevalent species, accounting for a majority of instances where infections lead to morbidity and mortality (World Health Organization, 2016b). Malaria caused by *P. falciparum* remains one of the major threats worldwide due to circulating drug resistant strains contributing to the disease sustainability (World Health Organization, 2015). Other driving forces contributing to the spread of the malaria include poor public health infrastructure, environmental changes (Greenwood & Mutabingwa, 2002) and the greatest being the lack of political will in most endemic countries that contribute to the disease eradicating efforts (World Health Organisation, 2008). In 2011, malaria accounted for 38% of the entire outpatient cases, 27.3% of hospitals admissions and 48.5% of the total certified mortality in children under five years old in Ghana (World Health Organization, 2014). Due to public health interest associated with malaria in endemic zones where almost everyone has suffered or will suffer from the disease and its associated socioeconomic impact, eradication tools are vital to avoid plaguing these zones with poverty.

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An important and effective way to eradicate malaria will be the disruption of the parasite life cycle using Transmission-Blocking Vaccines (TBV). This TBV will target the sexual stage (gametocytes) of the parasite (Kaslow, 1993). Global action on malaria eradication is focusing on the development of new effective drugs and vaccines. This had led to strengthening of research and application of transmission reducing/blocking interventions (The malERA Consultative Group on Health Systems and Operational Research, 2011). These interventions are focusing on eradication tools are targeting the sexual stage of the *Plasmodium* parasite leading to a better understanding of the parasite transmission. Curtailing continuous malaria transmission is crucial however, understanding the developmental biology of gametocyte is complicated. A major challenge to the disruption of the *Plasmodium* life cycle with transmission blocking compounds is also due to the sequestration in the human blood vessels by the immature gametocyte stages (I to III) together with their asexual trophozoites and schizonts during human infections. This poses difficulty in following production and maturation of gametocytes *in vivo*. Notably in the human host, the *Plasmodium* parasite gametocyte exist in peripheral blood as sexually committed rings and matured stage V (Farfour, Charlotte, Settegrana, Miyara, & Buffet, 2012; Miller, Good, & Milon, 1994). Sexual commitment occurs during schizont formation in the erythrocytic parasite cycle preceding the phase and gametocytes are then formed. All merozoites within a single schizont are either committed to the sexual or the asexual pathway (Bruce, Alano, Duthie, & Carter, 1990; Guttery, Roques, Holder, & Tewari, 2015; Josling & Llinás, 2015a). Sexually committed merozoites from a schizont

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develop either into male or female gametocytes (Pietro Alano, 2007; Silvestrini, Alano, & Williams, 2000).

Justification of the Study

The exact mechanism of *Plasmodium* gametocyte production is unknown, but several factors influence gametocytogenesis during an infection. The proposed contributory factors are noted to be either parasite, human host or external environment and these have been tested *in vitro* (Baker, 2010; Bousema & Drakeley, 2011a; Bousema et al., 2011). Environmental factors such as higher parasite densities in parasite-conditioned medium may increase gametocyte production (Bruce et al., 1990; Williams, 1999). Host factors that influence gametocyte production include Red Blood Cell (RBC) surface protein polymorphisms (ABO blood groups), polymorphisms of the red-cell enzyme gene G6PD that causes glucose-6-phosphate dehydrogenase deficiency and haemoglobin disorders such as the sickle-cell trait (Kwiatkowski, 2005; Nacher et al., 2002; Gouagna et al., 2010). Human beta haemoglobin variants are associated with increased gametocyte carriage especially in HbC variant haemoglobin trait in individuals in malaria endemic regions (Gouagna et al., 2010). The ABO blood group and its related susceptibility to malaria and gametocyte prevalence have been reported in different study populations (Grange et al., 2015). These genetic adapted factors can increase gametocyte production in natural infections. Other factors that may influence gametocyte formation could be host immunity, drug treatment and anaemia.

Previous studies have shown varying rates of gametocyte production in different parasite isolates (Graves, Carter, & McNeill, 1984; Abdel-Wahab et al., 2002; Dyer & Day, 2003). Parasite factors including drug resistant strains, multiple parasite species in an infection and variations in certain genes in their phenotypic output (example DNA or RNA methylation) affect gametocyte production (Baker, 2010; Bousema & Drakeley, 2011b). *Plasmodium falciparum* gametocyte prevalence has been associated with the presence of mixed infections with different *Plasmodium* species because of differential life cycle period of the species (Smith et al., 2012). Consequently, commitment to gametocytogenesis may increase or decrease when mixed parasite species are present in an infection. This phenomenon is well demonstrated and mostly in *P. malariae* and *P. falciparum* co-infections where gametocyte production is boosted in the latter species (McKenzie, Jeffery, & Collins, 2002). Within the same *Plasmodium* species, clonal diversity also affects gametocytes production and has an implication on malaria transmission. In most endemic zones, multiple infections are always present with two or more co-infecting clones in an individual infection (Kheir et al., 2010; Morlais et al., 2015).

Gametocytes are found in the blood of patients after treatment because most antimalarial drugs currently in use target only the parasite asexual stages (Butcher, 1997; Dinko, Oguike, Larbi, Bousema, & Sutherland, 2013; Hogh et al., 1998; Tangpukdee, Krudsood, et al., 2008). Gametocytes are usually found in low densities during infections as less than 10% of merozoites differentiate into gametocytes during schizogony but are major contributors to malaria transmission (Baker, 2010; Josling & Llinás, 2015b). This then makes

understanding and targeting the gametocyte biology important and crucial for the eradication of malaria.

Increase in antimalarial resistant parasite is associated with gametocyte carriage in *Plasmodium* infection in Ghanaians after treatment (Dinko et al., 2013; Mockenhaupt et al., 2005), and similar to what pertains in other malaria endemic countries like in Mali and Malaysia (Mwai et al., 2012; Norahmad et al., 2016). The Ghana Antimalarial Drug Policy (ADP) promotes the use of ACTs specifically artesunate amodiaquine, artemether lumefantrine and dihydro-artemisinin piperaquine (Ministry of Health, Ghana, 2009). Artemisinins tolerant parasite could increase gametocyte carriage in asymptomatic carriers of *P. falciparum,* which have the potential to produce gametocytes at low densities. The *Anopheles* mosquito requires only a male and female gametocyte in its three microliters blood meal to spread the malaria disease. These low parasite densities are often missed by microscopists (Schneider et al., 2007). Now that malaria control efforts are moving towards elimination and eventual eradication, it has increasingly been important to develop interventions that have the potential to block the transmission of the disease. Thus, an advanced understanding of the production of gametocyte is required to identify and/or monitor the changes and dynamics of the infectious carriers who are normally asymptomatic.

Statement of the Problem

Co-infecting and drug resistant parasite clones affect *P. falciparum* gametocytogenesis. Emergence of parasite strains developing tolerance to artemisinins in Ghana could increase parasite complexity or presence of sexual

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recombination from multiple and different parasite clone infections. This may lead to an increased rate of sexual recombination that gives rise to parasites with different resistance profiles. Recent studies have reported a number of K13-propeller coding polymorphisms (Taylor et al., 2015) a proposed gene responsible for artemisinins resistance in the parasite. K13 protein belongs to ubiquitin-regulated protein family and the proteins in this group interact and facilitate the degradation and oxidative stress responses. Mutations in the *Plasmodium K13* gene is proposed to protect the parasites from the artemisinin-induced oxidative damage (Adams, Kelso, & Cooley, 2000; Judith Straimer et al., 2015). Even though the presence and distribution of *K13* mutations have not been conclusive for artemisinins resistant phenotypes, it is vital to study their importance as mutations in the parasites genes vary geographically. Sexual commitment of malaria parasites have been shown to be influenced by human genetic factors including β-globin genotype (Gouagna et al., 2010; Bousema & Drakeley, 2011a).

Explanations on why only few number of the parasites are invested into gametocyte production and the role host factors play remain controversial (Morlais et al., 2015; Nassir et al., 2005). This brings to the fore the importance of examining the production of *P. falciparum* gametocytes from infected humans. An *ex vivo* culture system can be used to evaluate the transition of ring stage into gametocyte from *P. falciparum* isolate obtained from infected human. This *ex vivo* system can directly assess production of the *P. falciparum* gametocyte in natural human infections and this is the first time that the method is being used. The population of gametocyte that are produced in each erythrocytic cycle in *P. falciparum* isolate will help to elicit

transmission blocking immunity as it depend on frequency and number of the sexual form an individual is exposed to. Thus, the standard ACT kills immature (I-III) gametocytes, and when there is circulating mature gametocytes the peripheral blood of malaria patient, it is an indication of prolong *Plasmodium* infections. The blood samples collected from malaria patients for RNA and DNA isolation, and tissue culture propagation can help to define gametocyte production rate and distribution in a natural population. An association between gametocyte carriage and certain human genetic traits in malaria endemic countries has been proposed (Gouagna et al., 2010). Therefore, it is imperative to establish the extent to which human haemoglobinopathies, enzymopathies and ABO blood groups can exert influence on gametocyte production in *P. falciparum* infected people in Ghana. This will facilitate the development of effective malaria control measures by developing transmission-blocking interventions such as new drugs and vaccines to curb transmission.

Hypothesis of the study

The study hypothesised that in *P. falciparum*, the rate of gametocyte production at each erythrocytic cycle differs between individuals and parasite isolates. Thus, human blood factors, parasite diversity and drug resistant strains could affect gametocyte carriage.

Purpose of the Study

The study investigated host and parasite factors that contribute to gametocyte production and identification of submicroscopic gametocyte

carriage in human reservoirs. The overall contribution of human host genetic factors (RBC polymorphisms that are sickle cell, G6DP and ABO blood groups), drug resistance markers and parasite diversity to gametocyte production was investigated.

Objectives of the study

The study intended to achieve these objectives:

Objectives of the study 1

To assess *P. falciparum* gametocyte production in the study population

Specific Objectives 1

1.1 To optimize a plate based / high throughput assay for gametocyte development.

1.2 To assess gametocytes commitments and production in-patient samples using *ex vivo* assay.

1.3 To assess gametocyte carriage in the study population by qRT-PCR using mRNA of *Pfs25*

Objectives of the study 2

To determine the effect of host genetic factors on gametocyte prevalence

Specific objectives 2

1.1 To genotype G6PD and HBB, and phenotype the ABO blood groups, in the cohort (asymptomatic) study population

1.2 To compare the genotypes influence on gametocyte prevalence in the study population.

Objectives of the study 3

To determine the impact of the diversity and drug resistant *P. falciparum* on gametocyte prevalence in the study participants between two seasons

Specific Objectives 3

1. To determine the asexual diversity based on the *msp2* genetic diversity and allele frequencies and sexual diversity using polymorphism fragment diversity region 3 of *Pfg377* of *P. falciparum* isolate from the study population and assess whether a particular clone has a higher tendency to produce gametocyte within the seasons.

2. To genotype mutations *Pfcrt, Pfmdr*1, *Pfdhfr*, and *Kelch propeller* (K13) drug resistances markers in the study population.

Delimitation of the study

Plasmodium falciparum isolates used in the gametocyte production rate assay were sampled from children with uncomplicated malaria from Ewim Health Center in Cape Coast. The host and parasite factors were assessed in asymptomatic school children in Obom and Abura, Cape Coast.

Limitation of the study

The study participants were not sampled or followed at short intervals (weekly or fortnightly) and this affected the observation and evaluation of any possible progression of an asymptomatic *P. falciparum* infection into a symptomatic infection in the school children (asymptomatic) group. The identified host genetic variants measured in the study were less than ten percent; it made associated analysis difficult to attain statistical significance with gametocyte positivity. Additional longitudinal studies over a number of

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years will be ideal to confirm the observed distinct seasonal patterns of parasite and gametocyte prevalence in the asymptomatic group.

Definition of Terms

Asexual	not involving the fusion of gamete or non-reproductive stage of the parasite
Asymptomatic	having infections with no physical signs of the disease
Ex vivo	assay done in/on tissue/cell from an organism in an external environment with minimal alteration of natural conditions
Gametocyte	sexual stage of *Plasmodium* parasite
Gametocytogenesis	formation of gametocytes in the vertebrate host during *Plasmodium* infections
Gametogenesis	formation of gametes in the mosquitoes host during *Plasmodium* infections
Genotype	genetic characteristics of an organism
Haemoglobinopathies	abnormal structure of a globin chains
In vitro	assay done in tissue/cell outside their normal organism
In vivo	assay done in tissue/cell in the living organism
Isolates	*Plasmodium* field parasites
Malaria	infection cause by *Plasmodium* parasite
Mutant	alteration in the DNA sequence resulting in genetically different form of the parasite
Prevalence	total number of cases in a population at a particular time
Sexual	reproductive stage of the *Plasmodium* parasite

Symptomatic	showing signs of a disease
Uncomplicated	showing signs of a disease in a mild form
Variant	differ in some aspect from the natural type

CHAPTER TWO

LITERATURE REVIEW

Global Burden of Malaria

In 2015, malaria ranked as the twenty-eighth cause of death in all ages (2018/01/29, http://www.worldlifeexpectancy.com/world-rankings-total-deaths). Global estimate indicates that malaria kills between 1.1 to 2.7 million people each year and a child dies every two minutes in Africa (World Health Organization, 2016). The majority of malaria cases occur in African, then South-East Asia Region (10%) and Eastern Mediterranean Regions accounts for the rest of the 2% (World Health Organization, 2016b). These Figures are under estimated, as not all malaria manifestations are captured since these Figures are obtained only from health facilities whilst in most endemic areas self and traditional medications practices are common (Thandar, Kyaw, Jimba, & Yasuoka, 2015). Threat due to the incidence of malaria puts half of the world's population in danger (World Health Organisation, 2008; 18/03/2018 http://www.who.int/features/factfiles/malaria/en/). Malaria is still a public menace to humanity despite enormous and diverse efforts to control and eradicate the disease (Litsios, 2015).

Economic Burden of Malaria

Malaria is a poverty-associated disease, with most affected people living in the poor social, economic, and environmental conditions. In areas like South-East Asia and Eastern-Mediterranean malaria exist in the poor population where farmers sleep in farmland. This brings them into a constant contact with the vector in contrast to the rich communities or countries where

the settlements are away from farmlands (Parker, Carrara, Pukrittayakamee, McGready, & Nosten, 2015).

Annually, about US$12 billion is estimated to be lost through direct costs involving illness, treatment and prevention from malaria (CDC 27/07/2018, https://www.cdc.gov/malaria/malaria_worldwide/impact.html). Previously, the cost due to malaria was around 1.3% of economic growth lost in countries with intense transmission (Gallup & Sachs, 2011). Thus, 10% reduction in the disease incidence resulted in 0.3% economic growth from farm productions. This is reflected in US$81 to US$480 increase growth per GDP from 2007 to 2012 after malaria incidence and deaths declined during implementations of intervention in some endemic countries (William, 2014).

World Health Organisation estimated that the total cost of malaria to Africa was US$1.8 billion and US$2 billion; this greatly hinders economic and community developmental activities in the region (World Health Organisation, 2008). The cost to governments from the perspective of malaria prevention, treatment, and infrastructures are enormous. Huge foreign exchange losses are attributed to malaria as it reduces opportunities for joint economic partnerships, holiday and leisure industry (CDC, 17/02/2017 https://www.cdc.gov/malaria/malaria_worldwide/impact.html). The WHO in 2015 put the expenditure on just case management of the disease to about US$300 million and between US$2.5 billion to US$8.7 billion of funds would be needed by 2030 for elimination (World Health Organization, 2016a; World Health Organization, 2014). Households in Africa spend between US$2 - US$25 on treatment of malaria and between US$15 - US$20 on prevention of the disease monthly which leads to a drain on the financial resources

(Onwujekwe et al., 2013). This makes resources spent on malaria management and loss of productivity due to loss of working hours significant proportion of the annual income of poor households (Chima, Goodman, & Mills, 2003b; Sachs & Malaney, 2002).

In a study in 1997 conducted in northern Ghana, it was observed that while the cost of malaria care is just 1% of the rich household's income, it was as high as 34% for the poorer households on malaria treatment (Asenso-Okyere & Dzator, 1997;Akazili, Aikins, & Binka, 2007). Other economic assessments have shown that a single episode of the disease results in a loss of one to five working days (Asante & Asenso-Okyere, 2003); which also depends on the severity of the disease (Asenso-Okyere, Chiange, Thangata, Andam, & Mekonnen, 2011) and who is sick (Onwujekwe et al., 2013). The cost of malaria burden on family could be as high as 60% of productivity lost as compared to another family without the disease in the agricultural sector (Onwujekwe et al., 2013).

Determinants of Malaria Transmission

Malaria transmission varies in different regions, countries, within communities and among individuals (Duffy et al., 2015; Mackinnon & Read, 2004). The suitability and availability of ambient temperatures and sufficient rainfalls that favours the mosquito vector breeding and survival are characteristics of malaria endemicity. This guarantees the continuous co-existence or relationship between the human host and the *Plasmodium* parasite. Clearly, the interactions between the *Anopheles* vector, the *Plasmodium* parasite, and the human host, influences malaria transmission in a

given area. This defines the disease endemicity of that particular zone (Hay, Smith, & Snow, 2008). The transmission factors are also dependent on climate and topography of the given area, explaining why the increasing number of malaria cases often coincides with rainy seasons (Hay et al., 2008).

Vectors involved in Transmission

The mosquitoes transmitting human *Plasmodium* parasites are in the genus *Anopheles*, Order Diptera and family Culicidae. Globally, there are about 430 *Anopheles* species of which 40 of them transmit human malaria parasites (https://www.cdc.gov/**malaria**/about/biology/mosquitoes; World Health Organisation, 2008). Malaria transmission dynamics depends on the potentials of the *Anopheles* vectors which differs among species. *Anopheles* species are widely distributed, being found in both the tropics and temperate zones (Caminade et al., 2014; Dhimal, Ahrens, & Kuch, 2014; Hay et al., 2008). The number and type of *Anopheles* species determine the extent of transmission in a given area. *Anopheles gambiae* is known for its leading role in the transmission of *P. falciparum*. Other vectors of malaria are *An. culicifacies* in Southwest Asia, *An. darlingi* in North America and *An. albimanus* in Central America (Sinka et al., 2012).

Anopheles gambiae is a seven species complex with *An. arabiensis* and *An. gambiae s.s* being most efficient vector of transmitting human *Plasmodium* parasites. These are widely distributed in West Africa (Coetzee, Craig, & le Sueur, 2000) but vary geographically (Coluzzi, 1984; Sinka et al., 2016). The distribution of *Anopheles* vectors in Africa are *An. gambiae* in West Africa, *An. funestus* in East Africa (Service, 1980) and *An. pharoensis* in

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Egypt. The most abundant and widespread vectors in Ghana are *An. gambiae*
s.l. and *An. funestus* (Amuzu, Wilson, & Boakye, 2010; Chinery, 1984). The
life cycle of the *Anopheles* in malaria transmission is important for the parasite
survival. The efficiency and potential for malaria transmission have been
linked to the existence of the *Anopheles* vector (Zucker, 1996; Sokhna, Ndiath,
& Rogier, 2013). Clearly, the life cycle of the female *Anopheles* mosquito has
to exceed the developmental duration of the *Plasmodium* sporogonic cycle for
transmission to be completed (Coetzee et al., 2000; R. C. Smith, Vega-
Rodríguez, & Jacobs-Lorena, 2014). Interestingly, the female *Anopheles*
vector is changing its biting pattern by getting blood meal from the human
without barriers like sleeping bed nets (Kabbale, Akol, Kaddu, & Onapa,
2013; Sougoufara et al., 2014). The *Anopheles* populations influences
transmission of *Plasmodium* infection prevalence/intensity as its survival
depends on climatic factors that regulate their development and numbers
(Bayoh & Lindsay, 2003; Dawes, Churcher, Zhuang, Sinden, & Basáñez,
2009). This contribute to the distribution of the paasite and defines
geographical and seasonality in malaria endemicity (Bi, Tong, Donald, Parton,
& Ni, 2003; Thomson et al., 2006).

Parasite contribution to Transmission

As part of the *Plasmodium* fitness strategy, the parasite applies diverse
manipulative mechanism on the *Anopheles* vector and making the mosquitoes
effective as a crucial parameter for transmission of the parasite (Cator, Lynch,
Read, & Thomas, 2012). *Plasmodium* parasites are known to manipulate
various aspects of their vectors' feeding behaviour to increase their survival

(Koella, Sørensen, & Anderson, 1998). This occurs during the completion of the parasites developmental stage in the mosquito, whereby the developed sporozoites induce an increase in the biting frequency influencing the vector and humans contacts (Churcher, Trape, & Cohuet, 2015; Koella et al., 1998). In contrast, at the early developmental stage (oocyst) of the parasite, the *Plasmodium* parasite decreases the feeding rate of the mosquito affecting their biting drive. In a natural human population study, mosquitoes were more attracted to humans infected by the *Plasmodium* transmissible stage the gametocyte than those with the parasites' non-transmissible forms (asexual rings) or uninfected human population (Lacroix, Mukabana, Gouagna, & Koella, 2005). The parasite also has a variety of factors that affect the transmission of the disease in the natural human population in a given area (Bousema et al., 2011; Reece, Drew, & Gardner, 2008).

Human contribution to Transmission

Human behaviour plays a leading role in malaria transmission and found to be causing re-emerging of malaria in previously eradicated communities (Shiff, 2002; Tanner et al., 2015). Thus, human behaviours have a tremendous impact on transmission of malaria, especially on human-vector contacts. Human activities like deforestation change the ecology and correlated to an upsurge of vector population (Pratt-Riccio et al., 2013). Deforestation creates new breeding site affecting the vector's feeding preference. Irrigation systems in farm practices have also created breeding grounds for the vector and thereby increasing malaria transmission. The rearing of domestic animals in most malaria endemic areas have contributed to

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the survival of the disease vector since it provides alternate sources of blood meals which increased the survival of the vector (Smith et al., 2012).

Documented researches have also shown a relationship between malaria transmission and urbanization (Gallup & Sachs, 2011). Urbanization has increased vector diversity in most malaria endemic region. Explosive and unplanned population growth due to urbanization has resulted in increased malaria in areas with previously lower transmission risk (World Health Organization, 2014). This has led to an increase in urban malaria hampering control efforts. Estimates shows that, by 2030, more than 60% of the world's population will live in urban areas and the majority would be in African and Asian countries (United Nations, 2014), and has major implications on malaria epidemiology, eradication and policies. Insights into malaria transmission in sub-Saharan Africa has linked possibility between transmission and emergence of new cities through urbanisation (De Silva & Marshall, 2012; Hay, Guerra, Tatem, Atkinson, & Snow, 2005). This is linked to the reproductive adaptation of the *Anopheles gambiae* vector, which now breeds in both clean and polluted waters. The nominal species, *Anopheles gambiae s.s*, which is the most anthropophilic vector, has also adapted to man and his environment evolving further speciation processes (Shiff, 2002). The development and spread of drug-resistant strains of malaria parasites and resistant vectors to insecticide are key factors in this resurgence of malaria. These pose challenges to malaria control efforts.

Epidemiology of Malaria

The epidemiology of malaria depend on interactions between the human host, the insect vector, the protozoa parasite with the environmental abiotic and biotic factors (Cable et al., 2017). The relationships between these factors determine malaria endemicity in a given area. This, therefore, explains why there is a significant variation in malaria epidemics and the disease severity between and within areas. These variations therefore make it a bit difficult to estimate the true prevalence of *Plasmodium* infections in a given area. Thus, within a kilometre, malaria prevalence can greatly differ in an area (Kelly-Hope & McKenzie, 2009). This affects the disease endemicity characterization and classification (Hay et al., 2008) as estimates of most malaria morbidity and mortality are derived from health facility data with limited accuracy. However in most endemic communities, majority of the populace seek treatment outside the formal health sectors (Foster, 1995; Hastings, 2001; Thandar et al., 2015).

Geographical Distribution and Populations at Risk

Malaria occurs in over 90 countries worldwide with children less than five years and pregnant women being the population facing the highest risk of the disease (World Health Organization, 2014). Children, less than five years are most susceptible to malaria because their immunity to the disease is not fully developed and also most have impaired nutritional status (Bousema et al., 2011). The susceptibility of pregnant women to *Plasmodium* infection is because of the altered levels of hormones, which affects their level of immunity. Pregnant women with malaria suffer increased risks of severe

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anaemia and low birth weight if the baby survives (Cisse et al., 2014), which accounts for an estimated 25 % of all neonatal deaths. Globally, malaria, poverty, and development are interrelated which explains why infections and the greatest burden of the disease occurs in the sub-Saharan Africa (Sachs & Malaney, 2002) and the poorest part of South Eastern Asian countries (WHO, www.who.int/malaria/areas/high_risk_groups/pregnancy/en/ 08/12/2017)

Geographical variations occur in malaria incidence rate, morbidity, and mortality and even within sub-Saharan Africa where the highest incidence of the disease occurs (Hoshen & Morse, 2004). Thus, in Southern Africa, there is relatively lower incidence of malaria in some parts compared to other parts in the region. This variability in the incidence of the disease depends on the mosquito vector present and environment conditions/factors. These interactions correlate with acquisition of immunity and thereby influencing the description of the disease as severe or asymptomatic (Carneiro et al., 2010).

Aetiology of Malaria

Plasmodium species are unicellular eukaryotic protozoan parasites of the blood cells and require two hosts to complete their life cycle. *Plasmodium* parasites infect almost all vertebrates but they are specific in the animal species they infect. About one hundred and six infectious *species* are known (http://parasite.org.au/para-site/text/plasmodium-text.html4/01/2018; www.cdc.gov/dpdx/malaria/03/01/2018).

The protozoan *Plasmodium* uses its complex life cycle as a survival advantage, that requires alternation of stages between the vertebrate host (asexual multiplication), and in the *Anopheles* mosquito (for sexual

reproduction) has made it fitter in the evolutionary train for years (Auld &
Tinsley, 2015). The development of the *Plasmodium* sexual stage in the
mosquito offers it a recombination advantage resulting in progenies' with an
altered biological pathway that are adaptive to ensure transmission despite the
fitness cost the parasites have to pay. Aside from the use of differential host as
a survival adaptation employed by the *Plasmodium* parasite even within a
host, the parasite has distinctive sexual and asexual stages masking itself from
the host immunity (Cowman, Healer, Marapana, & Marsh, 2016). These
adaptive phenotypes can thereby overcome within-host-parasite competitions
and evade drug pressures and host immunity (Talman, Domarle, McKenzie,
Ariey, & Robert, 2004).

Pathogenesis of Malaria

Plasmodium infections cause more morbidity and mortality to their
human hosts. The virulence of the *Plasmodium* parasite during an infection
correlates positively to the parasite transmission rate. This defines the
adaptation of the parasite in a particular area to the changing pressure that is
exerted on it in the natural population thereby leading to well fitted strains in
the population (Mackinnon & Read, 2004). Although all five human
Plasmodium species can cause severe and recurring illnesses that reduce
individual productivity and fitness, *P. falciparum* and *P. vivax* pose the
greatest threat (World Health Organization et al., 2014). However, *P. falciparum* infections are most lethal and responsible for most of the disease
mortality and morbidity that mainly occurs on the African continent.
Similarly, *P. vivax* is more prevalent in Asia (Carlton, Das, & Escalante, 2013;

United Nations, 2014) due to the presence of Duffy binding proteins on the surface of human RBC which the *P. vivax* species use for the RBC invasion (Langhi & Bordin, 2006).

Plasmodium falciparum has the ability to cause infected erythrocytes to adhere to each other resulting in attachment of cells to the endothelial cells leading to sequestration of the parasite in the tissues (Rowe, Claessens, Corrigan, & Arman, 2009). This mechanism enables the parasites to evade host-clearing mechanism by circumventing the passage through the spleen, where the destruction of the parasite occurs (Franke-Fayard, Fonager, Braks, Khan, & Janse, 2010). The sequestration favours parasite growth and reproduction resulting in serious complications to the human host if it occurs in organs like the brain, lungs, and the placenta (Marsh & Snow, 1999). Severe anaemia, coma, pulmonary oedema, and placental compromise in pregnancy are results of such sequestration (Marsh & Snow, 1999).

The main symptom of malaria is fever and the progression of the disease is influenced by factors including the level of host immunity, species of parasite and access to prompt and appropriate/efficacious treatment. Malaria disease can manifest into a broad range from severe to uncomplicated infections. The age of the individual in *Plasmodium* infection is an important predisposing factor in the severity of the disease. Some *Plasmodium* infections develop into severe form of the disease, which can manifest as anaemia, cerebral malaria, hypoglycaemia, and metabolic acidosis presenting respiratory distress (Trampuz, Jereb, Muzlovic, & Prabhu, 2003). Currently, there is no effective vaccine against malaria but eradication of the disease is

by managing infections with antimalarial treatment and controlling of the vector.

Plasmodium Life Cycle

Even though there are differences in geographical distribution, microscopic appearance and clinical features of the five known human *Plasmodium* malaria parasite species, they have some similarities in their life cycle (Singh et al., 2004). Human *Plasmodium* parasite life cycle involves alternation in two hosts, vertebrate host for the asexual stage and invertebrate host for the sexual stage. A bite from an infected *Anopheles* mosquito begins infection in humans where the sporozoites are injected into the human bloodstream. The sporozoites then disappear from the circulatory blood some few minutes after injection and move to the liver kuff cell/hepatocytes. The sporozoites move specifically to the human liver cells as they have ligand proteins (circumsporozoite protein -CSP). The CSPs bind only to receptors on the hepatocytes to enable the entry of sporozoites into liver cells (Bertolino & Bowen, 2015). The sporozoites in the hepatocytes change to feeding trophozoites thereby starting the exo-erythrocytic schizogony (asexual reproduction) phase. The trophozoite then again changes to merozoites depending on the parasite species. The merozoites emerge from the liver after a week to three. *Plasmodium vivax* and *P. ovale* have dormant liver stage parasites, the hypnozoites, which can be reactivated or relapsed to cause malaria several months or years after infection (Imwong et al., 2007). The merozoites in the blood then infect erythrocytes, where they undergo cycles of erythrocyte proliferation, invasion, and multiplication. These infected RBCs

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rupture leading to an increased number in parasite populations. The disease symptoms that normally manifest as recurring fevers, paroxysms, rigors and sweats are attributed to the rupture of erythrocytes and release of new invasive parasites into the bloodstream (Evans, 2002).

The different human *Plasmodium* species cause different fevers at different intervals based on erythrocyte invasion and reinvasion cycles. *Plasmodium falciparum*, *P.* vivax and *P. ovale* reinvade erythrocytes every 48 hours, whereas *P. malariae* does so every 72 hours explaining the tertian and quartan nature of the disease symptoms. Among the human *Plasmodium* infections, the *P. knowlesi* have the shortest asexual life that occurs every 24 hours (Knowles, 1932). In the course of the erythrocytic cycle, a small proportion of the merozoites do not multiply after invading erythrocytes but instead differentiate into gametocytes (Baker, 2010). The gametocytes are ingested by a mosquito in a subsequent blood meal and continue their cycle in the mosquito midgut by maturing into male and female gametes that mate to form a diploid zygote. This diploid stage permits sexual recombination leading to exchange of genetic material that may invariably result in chromosomal gene rearrangement in the offspring (Gregson, 2005). The zygote matures into an ookinetes in the mosquito midgut which then migrates by into the outer gut and forms oocyst which in turn releases sporozoites that migrate to the mosquito salivary glands, completing the life cycle (Figure 1).

Plasmodium falciparum development

The process of *P. falciparum* development depends on so many factors between the asexual rings to the sexual gametocytes in human and the

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sporozoites in mosquito. The parasite has evolved mechanisms to adapt by changing it genetic make-up including antigenic variations (Scherf, Lopez-Rubio, & Riviere, 2008). The survival of the *P. falciparum* in the human population has been possible by the parasite adaptive life cycle by alternating it development in two hosts. More intriguing is how the *Plasmodium* parasite uses it sexual reproduction to ensure survival by using two hosts for this phase (Carter et al., 2013), the gametocyte formation in human is an important player in the *Plasmodium* survival.
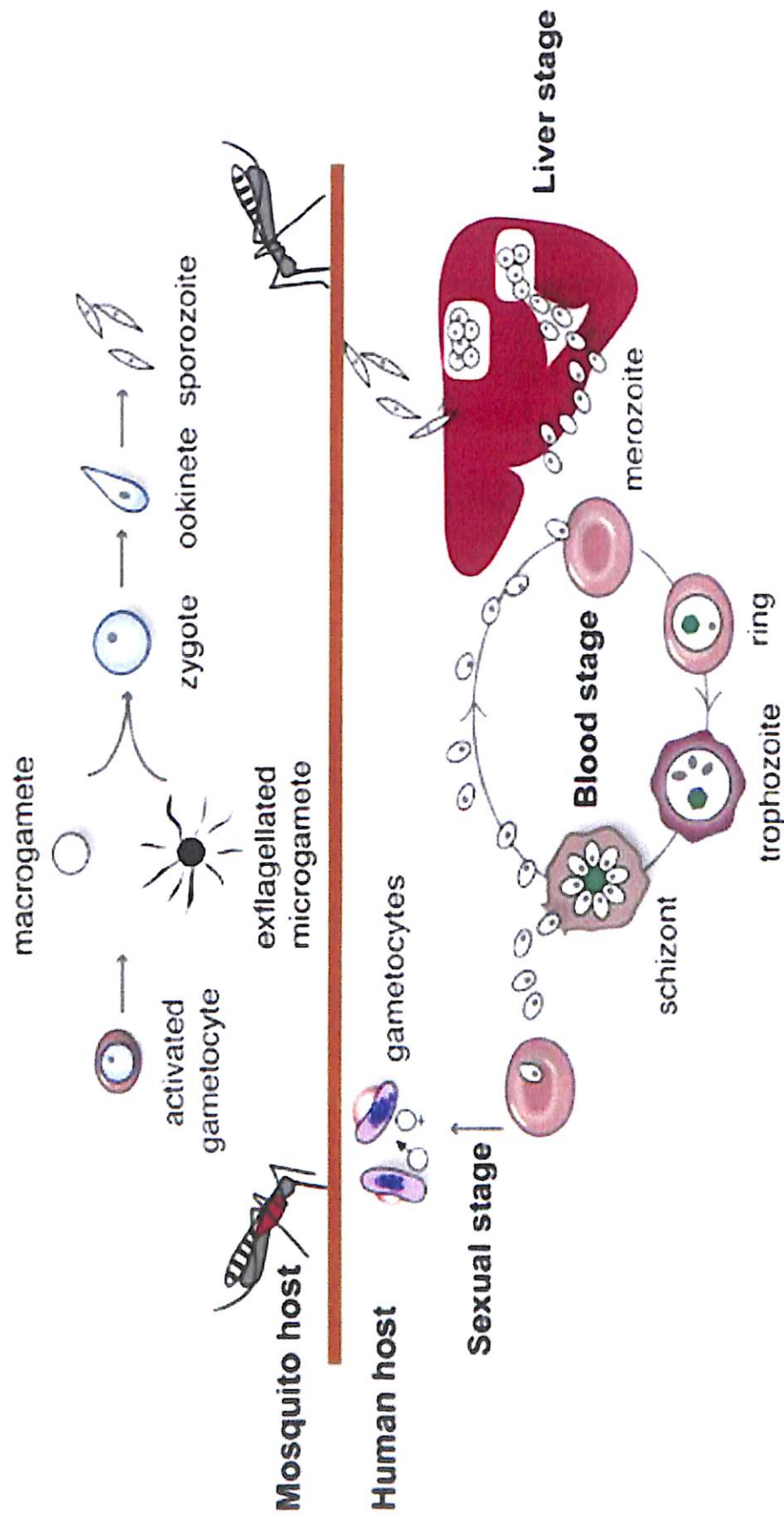
Figure 1: The life cycle of *Plasmodium* parasite (with permission from- https://www.researchgate.net/publication/274259875

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The illustration in Figure 1 shows the *P. falciparum* sporozoites injected into the human blood stream during a mosquito's blood meal. The sporozoites migrate to the human liver within some few minutes from the blood stream (exo-erythrocytic cycle). In the exo-erythrocytic cycle, the sporozoites develop and transform into merozoite (sporogonic cycle) in the liver where they leave to invade red blood cells (erythrocytic stage). The first-generation of merozoite in the erythrocytic stage develops to trophozoite and to schizonts and then to merozoite. During the subsequent erythrocytic cycle, fewer populations of the schizonts get sexually committed (Baker, 2010; Silvestrini et al., 2000). The committed schizonts eventually rupture to release merozoites, which then invade new red blood cells to develop to committed rings that develop into gametocytes. The male and female gametocytes are usually in close proximity to maximize their chances of being picked together by the female *Anopheles* mosquito (Ruecker et al., 2014).

In the mosquito, gametocyte differentiates into gamete within the midgut lumen the male divides into eight microgametes to pair the number of female macrogametes in the population. The male and female gametes mate to form the zygote where the exchange of genetic information occurs. The zygotes then develop and differentiate into ookinetes and migrate into the midgut through the epithelial lining and they develop into the oocyst. The oocyst develops into sporozoites, which migrate into the mosquitos' salivary glands and infect another human host during the mosquitos' blood meal (Aly, Vaughan, & Kappe, 2009).

Biology of *Plasmodium falciparum* Gametocytogenesis

Gametocytes are specialized, distinct and well adapt to human and mosquito transition. This involves many changes occurring in its cell, metabolism, genes expression profile, and proteins to survive. The ratio of gametocytes to asexual forms in *P. falciparum* has been found to be as low as 1:156 (Eichner et al., 2001) to about 1:10 (Chin & Collins, 1980; Eksi et al., 2012). Committing to sexual development and regulation depends on switching and expression of *Pfap2-G* (PFL1085w/PF3D7_1222600) gene which is a transcriptional regulator for gametocytogenesis initiation (Kafsack et al., 2014). The *Plasmodium falciparum* gametocyte development 1 gene (*Pfgdv1*), encoding a peri-nuclear protein is associated with early sexual differentiation with other genes including *Pfs16, mdv1* and *pfpeg4* required for cellular transformations in the parasite (Eksi et al., 2012).

Plasmodium falciparum Committed Merozoite to Gametocyte

In *P. falciparum*, gametocytes arise from erythrocytic asexual stages as opposed to its formation directly from hepatic merozoites as it occurs in other species. The *P. falciparum* sexual differentiation arises from merozoites, where a single schizont developed either into asexual stages or into gametocytes (Silvestrini et al., 2000). Gametocytes from one schizont are all male or all female from the preceding merozoite (Silvestrini et al., 2000). This suggests that the trophozoites prior to asexual generation were already committed to either sexual development or continuous asexual cycle. The sexual forms are sex specific during the committed schizont before stage I gametocyte formation.

Gametocyte formation is normally a female-biased phenomenon (Trager, 2005), and typically the sex ratio is three to five females to a male gametocyte(s) (Delves et al., 2013). The sex bias might be for a fact that during zygote formation a male gamete divides further to 6/8 microgametes (Bousema, Okell, Felger, & Drakeley, 2014) as shown in Figure 2.
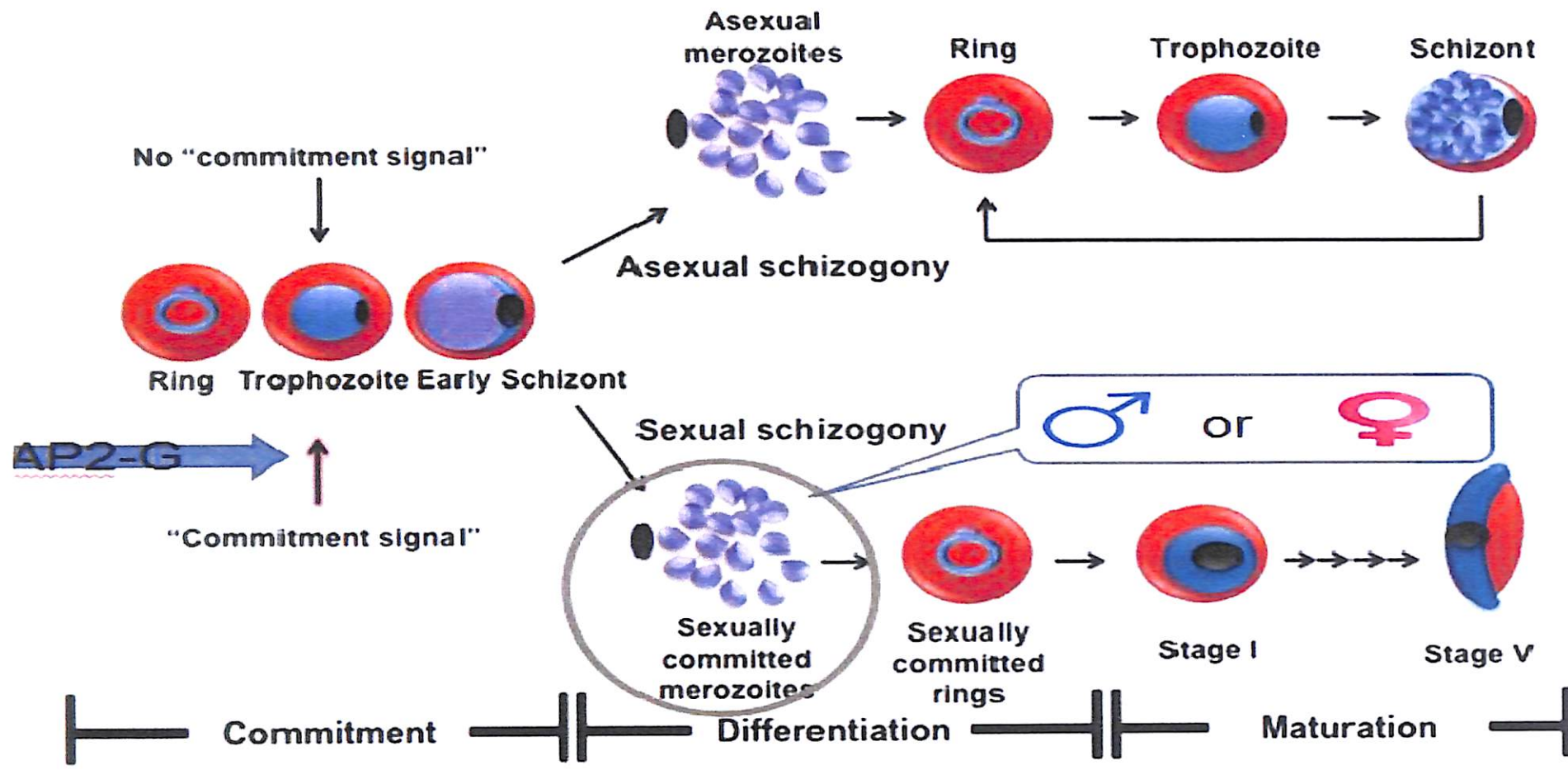
Figure 2: Commitment to gametocytogenesis (Adapted from Ikadai *et al.*, 2013)

Plasmodium falciparum **Gametocytes Development**

Plasmodium falciparum develops into matured gametocyte between 10 to 12 days from committed rings. Gametocytogenesis process is morphologically distinct and categorized into five stages and their locations differ in the human host (Pietro Alano, 2007). Gametocyte development starts from a ring that was part of a committed merozoite (Figure 2). The committed rings are found in the peripheral circulation after bursting from the committed merozoites (Eksi et al., 2012) before developing into stage I gametocytes between 24 to 30 hours post-invasion.

The stage 1 gametocytes take about 48 hours to develop to stage II gametocyte which is D-shaped (Josling & Llinás, 2015b). During the gametocyte stages development, the transitional stages from stage I to II and IV to V takes 48 hours whilst stages I, III and IV requires only 24 hours into their transitions (Ponnudurai, Lensen, Meis, & Meuwissen, 1986). The immature stages I-III and semi-matured stage VI gametocytes sequester in host tissues and bone marrows. Only the stage V gametocyte moves to the peripheral blood due to the absence of CD36 cytoadhesive (Gardiner & Trenholme, 2015). The CD36 cytoadhesive is present in the cells of immature gametocytes (stages I-III) for tissues adhesion. However, the parasites gradually start losing the CD36 cytoadhesive cells from stages III to IV and completely absence in the circulating stage V (Rogers, Hall, Obiero, Targett, & Sutherland, 2000).

Matured stage V gametocytes are crescent, falciform or sickle shaped. There is also sex differentiation where the females are thin, curvy and elongated shaped. The male gametocytes are thicker. Similarly, all gametocyte stages have pellicular complex made up of sub-pellicular membrane vacuole subtended by

an array of microtubules arranged longitudinally (Sinden, 1983; Talman et al., 2004). The pellicular complex strengthens the gametocytes cells retaining their stage specific shapes which are lacking in the asexual amoeboid shaped stage (ring, trophozoite and schizont) parasites (Langreth, Jensen, Reese, & Trager, 1978) but its function is still unknown. The infectious matured gametocytes stay in the peripheral bloodstream for an average of 6.5 days (between 3 to 22 days) for gametocyte formation (Baton & Ranford-Cartwright, 2005; Dixon, Peatey, Gardiner, & Trenholme, 2009; Eichner et al., 2001) and if not picked by mosquitoes then dies off.

Plasmodium falciparum transition from Gametocytes to Gametes

When the fully developed mature stage V gametocytes are picked by mosquito from peripheral blood, the gametocytes move into the midgut of the mosquito, and mature into microgametes and macrogametes in the process called gametogenesis. Gametogenesis involves many factors and enzymes like gametocyte-activating factor xanthurenic acid (XA), for morphological conversion of the crescent shape gametocytes to the round cell-like (rounding-up) gametes essential for fertilization (Trager, 2005).

Genes expressed during Gametocytogenesis in *Plasmodium falciparum*

Numerous stage-specific genes are involved in the gametocyte formation from the sexual commitment phase to the gamete formation phase, and exhibit different expression profiles than in asexual stages. Gene transcript levels and translation to proteins are not constant during developmental stages, and sex specific expressions too occurs (Crabb et al., 1997). Three hundred *Plasmodium* genes have been identified to be sexually specific and exclusively expressed

during sexual development. Morphologically, the five-gametocyte stages are distinct and genes expressed exclusively at different stage and/or sex specific. However, gene expression profiling differs during gametocyte stages development (Bousema & Drakeley, 2011a; Khan et al., 2005; Francesco Silvestrini et al., 2005; Young et al., 2005). Stage and sex specific genes are express and translate into proteins at specific stage or throughout all the developmental stages. Examples of the stage-specific genes include knot associated histidine rich protein (*Kahrp*) asexual genes; *Pfs16* and *Pfge1* early gametocyte surface and developmental genes ; late and sex-specific genes like the *Pfs230* male gamete surface antigen, the *Pfs25* zygote-ookinetes female surface protein and *Pfg377* female osmiophilic body (Bechtsi & Waters, 2017).

Gene expression profiling of some stage and/or sex specific antigens can be used to measure *Plasmodium* transmission (Eksi, Suri, & Williamson, 2008). These differential expressions of genes are also of adaptive function, as the female gametocytes are marked for continuous development and so are most of its genes. The sex specific genes are likely to change its expression profiles in define timing and when needed. The male gametocyte undergoes constant differential development for exflagellation an obligatory for the fertilization in the mosquito. The *Plasmodium* parasite development involves several genes which switches function on define timing (Duraisingh & Horn, 2016). Some of the *Plasmodium* surface specific antigens produced during gametocytogenesis remains in human host where they are found in circulation. These circulating antigens are recognized by the human immune cells and are targets for the formation of specific antibodies by the human immune system.

Compound for Gametocyte Purification

N-Acetyl glucosamine (*N*-acetyl-D-glucosamine/GlcNAc/NAG) has a molar mass of 221.21 g/mol and a monosaccharide that is use for gametocyte purification. The NAG compound is a monomeric unit of the polymer found in most biological systems as in bacterial cell walls, chitin in insects and crustaceans. The role of NAG in gametocyte purification is to prevent the invasion of the erythrocyte by the *Plasmodium* merozoites without affecting the gametocyte maturation (Fivelman et al., 2007). The NAG compound does not affect the parasite's gametocyte, as the sexual stage does not invade new red blood cells. The mode of action of NAG is to compete with the *Plasmodium* parasite for the RBC surface binding ligand/epitomes and thereby blocking erythrocyte invasion by competing with the parasite merozoite (Figure 3). By this, the asexual stages die off and the gametocytes remain. N-Acetyl glucosamine is the commonly preferred compound in the purification of gametocytes in cultures as it is not toxic. The preferred concentration of NAG for gametocyte purification is usually at 50 mM to the parasite culture medium and added on a daily for a minimum of five days to completely prevent merozoites invasion (Fivelman et al., 2007).

RBC receptor (Complement Receptor -CR)
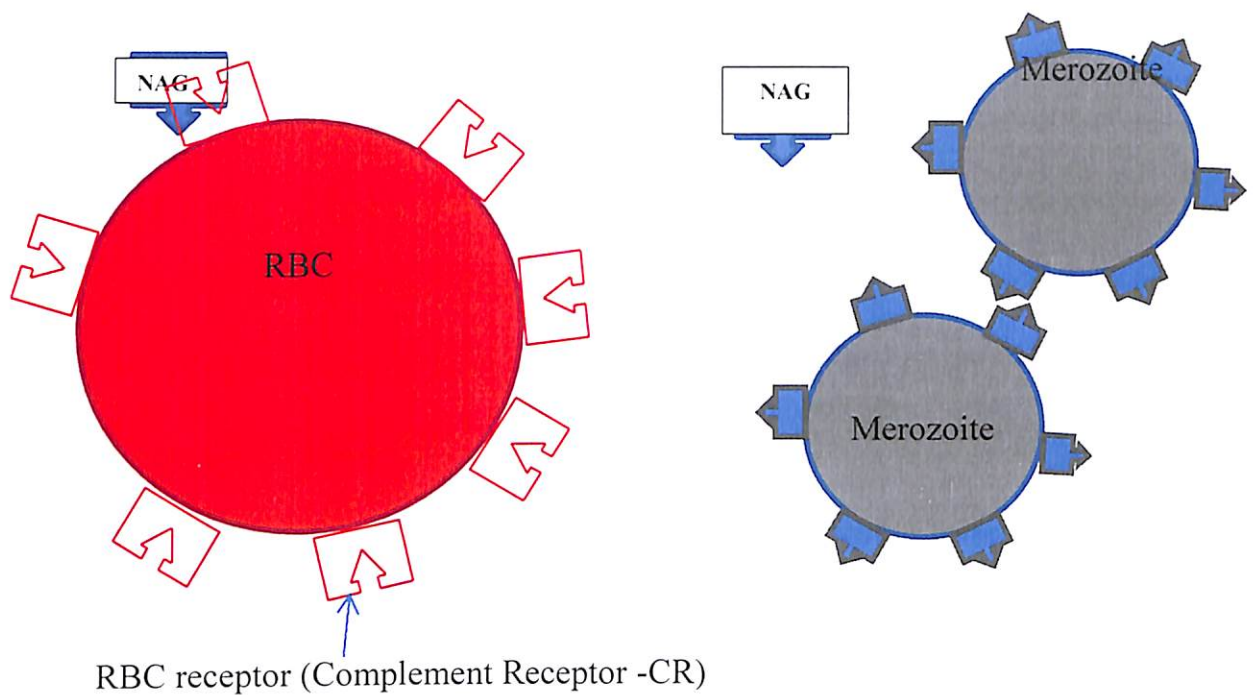
Figure 3: A drawing showing *N*-Acetyl glucosamine molecules and *Plasmodium falciparum* erythrocyte binding proteins competing for receptors on the surface of human erythrocyte.

As most *P. falciparum*, cultures are not hundred percent synchronised *in vitro* or *ex vivo* assays, daily addition of 50 mM NAG to parasite media is used for six or more parasite cycles to clear the asexual parasite (Tanaka et al., 2015). The duration of the NAG in the parasite media in cultures is important for gametocyte purification to ensure continues blocking of the binding epitomes on the RBC surface to prevent merozoites invasion.

Gametocytogenesis in Human Population

From sexual initiation, differentiation and development to matured gametocytes, all the five distinct stages of gametocyte must survive. Two hypothesis have been proposed on gametocytogenesis; with one stating that certain conditions trigger gametocytogenesis (Talman et al., 2004). The other is of the view that as part of the parasite normal life cycle, few of its population

are to undergo gametocytogenesis to ensure the parasite existence in the food chain (Eksi et al., 2012).

Gametocytes arise through a switch from asexual replication in the human host to sexual forms. The exact population of gametocytes produced in each cycle is not certain but it has been proposed to be about 1-10% as observed in previous studies (Chin & Collins, 1980; Eksi et al., 2012). The *P. falciparum* sexual reproduction does not follow the normal evolutionary trend. This is because in *Plasmodium*, the exact number of sexual to asexual ratio in the parasite life cycle is unknown (Carter, Pamba, Duparc, & Waitumbi, 2011; Talman et al., 2004), which is a major challenge in vaccine development and usage in controlling parasitic infections.

Factors influencing Commitment to Sexual development and Dynamics

All forms of *Plasmodium* infections might produce gametocytes at some point during the infection, but with varying frequency and density within and/or among different populations. The limited number of the parasites that are converted to sexual forms during intra-erythrocytic replication is proposed to arise from a number of triggered factors. These factors could be environmental, parasite and host (Reece et al., 2008) and these particular mechanisms needs further clarification. The effects of these three factors influencing gametocytes production are or can interrelate in producing an effect that is usually complex to explain.

Environmental factors affecting Gametocytogenesis

The environmental factors affecting *P. falciparum* gametocytogenesis are host humoral and cellular activities, anaemia, and drug treatment/pressure. Malaria endemic populations have different levels of *Plasmodium* infections and this depend on factors such as parasite densities and the immune status of the population. The immune system has adapted and evolved to protect the host from pathogenic microbes that are evolving in a continuous manner to hid itself and survive the changing environment. The human immune system also has the ability to distinguish self from non-self (González et al., 2011) and this explains why there is immunity to certain diseases after exposure. The host uses both innate and adaptive mechanisms to detect and eliminate pathogenic microbes.

Constant exposure of the host to the same pathogenic epitopes helps the immune system to recognise its antigens over time and builds antibodies for later infections (Chaplin, 2010). However, these antibodies could be short or long-lived. Nonetheless, in most parasitic infections the antibodies are short-lived either by switching off the antigens within or between infections or multi-antigenic exposure during the infection stage (Ferreira, da Silva Nunes, & Wunderlich, 2004). In *Plasmodium* infections, the antibodies are short-lived and the immune system needs periodic exposure of the infection to boost its immunity and by so doing decrease human susceptibility to the disease (Langhorne, Ndungu, Sponaas, & Marsh, 2008).

Effect of Anaemia on Gametocytogenesis

Studies had shown that gametocyte production increases at lower haematocrits or host anaemia (Gbotosho et al., 2011; Trager, 2005). Anaemia in

the host can either be caused be primary and/or secondary factors. The primary

causes of anaemia are normally genetics and in malaria mostly by RBC

polymorphisms (Gouagna et al., 2010) that are evolutionally selected due to the

disease. The secondary factors are triggered by other disease/infections or

nutrition. Anaemia increases gametocyte numbers by signalling for more

parasites to be sexually committed and less RBC affecting merozoites

reinvasion (Ashley et al., 2014). Lower hematocrit and prolonged *Plasmodium*

infections are associated with increased gametocyte detection in peripheral

blood from patients (Ashley et al., 2014).

Effect of Drug Pressure on Gametocytogenesis

Antimalarial chemotherapy or chemoprophylaxis has been the

mainstream in case management and prevention of malaria as the primary

option (Winstanley & Ward, 2006). The most extensively used antimalarials are

the quinolines (quinine and chloroquine) and the antifolate (sulfadoxine and

pyrimethamine) before the introduction of artemisinin combination therapies

(ACTs) in the year 2000. Regrettably, resistance to antimalarial drugs has

proven to be a challenge in malaria control and elimination. The current malaria

control programs are design to reduce the disease burden and eradication, and

without potent vaccine for total elimination, reliability is still on chemotherapy

and/or chemoprophylaxis (Winstanley & Ward, 2006). Hence, selective

pressure is exerted on the parasite populations. Monitoring of drug resistances

in the parasites populations are especially important since the development of

new drugs takes years. The gametocytes of these resistant parasites in the

population need to be genotyped and well characterised geographically for institution of proper elimination tools.

The current drug resistant parasites to the newer drugs are using existing markers the *Pfcrt, Pfmdr1, Pfdhps, Pfdhfr* and the recent *PfK13* markers to adapt to current drugs used in treatment (Norahmad et al., 2016). By using different antimalarial monotherapies treatment in an area, the drugs will mount selective pressure on the parasite population. The drug pressure will wipe out less fitted strains leading to variability in gametocyte carriage and density. Selection promotes creation of new strains, when there is geographical and seasonal variability of gametocytaemia in an area. Increasing antimalarial drug resistance favours gametocyte density and/or prevalence (Barnes & White, 2005). Gametocyte carriage and infectivity are higher in patients infected with drug-resistant parasites. Highly efficacious drugs reduces post-treatment gametocyte prevalence and carriage (Abdul-Ghani, Basco, Beier, & Mahdy, 2015; Barnes & White, 2005).

A regular monitoring of gametocyte prevalence in relation to the parasite's resistance to drugs in a population is a priority to block parasite transmission and spread of resistant parasite strains. This will help in assessing possibility in varying gametocytocidal action of anti-malarial drugs intended to interrupt malaria transmission in specific areas (Abdul-Ghani et al., 2015; Reader et al., 2015). During molecular monitoring, polymorphisms in genes are important precursors providing clues to the emergence of resistant strains. The existence of these polymorphisms in targeted drug genes and their relationship with treatment outcome serve as markers for monitoring antimalarial drug resistance and it distribution.
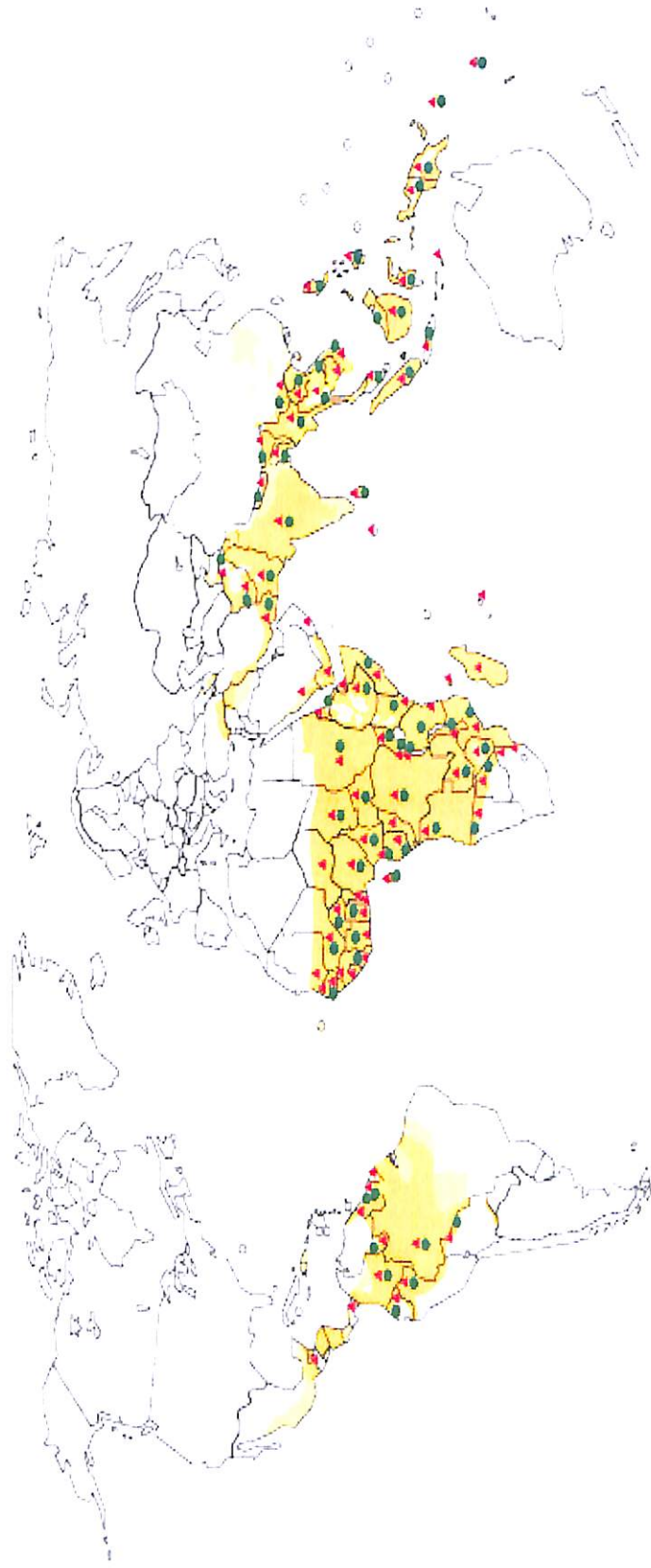
Figure 4: Global distribution of *Plasmodium falciparum* drug resistant parasites (World Malaria Report, 2005)

Areas where malaria transmission occurs (high/low)

Chloroquine resistance

Sulfadoxine pyrimethamine resistance

Mefloquine resistance

Malaria-free areas

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Plasmodium falciparum **genome and gene polymorphisms**

The *P. falciparum* 3D7 nuclear genome is composed of 22.8 mega bases (Mb) distributed among 14 chromosomes ranging in size from approximately 0.643 to 3.29 MB. The overall adenosine and thymine (A + T) composition is 80.6% and rises to approximately 90% in introns and intragenic regions (Gardner et al., 2002). Chromosomes in *P. falciparum* vary considerably in length with most of the variation occurring in the sub-telomeric regions. Parasite isolates from individuals even residing in a single village exhibit extensive size polymorphism (Abdel-Wahab et al., 2002) which arises from recombination between different parasite clones, chromosome breakage and repairing events during meiosis in the mosquito (Bousema et al., 2010).

Plasmodium falciparum **Chloroquine Transporter (*Pfcrt*) Gene**

The *Pfcrt* is located on chromosome seven, and mutations in the gene alters the transport of chloroquine which reduces its influx into or an increased efflux out of the parasite digestive vacuole (Warhurst, 2001), resulting in reduced drug uptake levels. The *Pfcrt* gene may be an anion channel, pumping chloroquine out from the food vacuole. The amino acid changes occurring in the gene encoding this protein, from lysine to threonine at position 76 of the *Pfcrt* gene (K76T) is critically associated with chloroquine resistance in both *in vivo* and *in vitro* assays (Djimdé et al., 2001). Parasites that are highly resistant to chloroquine often have K76T and A220S in *Pfcrt* (Warhurst, 2003). Geographic variations in *Pfcrt* mutation between codons 72 and 76 has been identified in circulating chloroquine-resistant parasite strains.

Plasmodium falciparum has developed an ability to withstand the effects of chloroquine and the widespread of these resistant strains led to the switch from chloroquine to SP (Fansidar®) in many countries.

Plasmodium falciparum Dihydrofolate Reductase and Dihydropteroate Synthase Genes

The combination of sulfadoxine a sulphonamide with the antifolate drug pyrimethamine (SP) inhibits the enzymes dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) (Ferone, 1970) in their synergy antimalarial effect (Chulay, Watkins, & Sixsmith, 1984). *Plasmodia* get their folates *de novo*, and inhibition of *dhps* by these drugs leads to depletion of dTTP decreasing DNA synthesis (Schellenberg & Coatney, 1961). Resistant parasites strains with *dhps* and *dhfr* mutations are still in circulation in most malaria endemic countries including Ghana. Sulphadoxine-pyrimethamine is the current WHO recommended Intermittent Preventive Treatment (IPT) of malaria in pregnancy (IPTp, SP) in all areas with moderate to high malaria transmission. The SP drugs are being used for IPT in pregnancy in Ghana (World Health Organization, 2015). In other countries with high malaria transmission, the SP drugs are used as seasonal chemoprophylaxis in children under five or a partner drug in artemisinin combination therapy (Maiga et al., 2016).

Plasmodium falciparum Multi-drug Resistant Gene 1 *(Pfmdr1)*

Plasmodium falciparum multidrug resistance gene 1 (*Pfmdr1*) is located on chromosome 5 and encodes a putative ATP binding cassette

transporter similar to the multiple drug resistance genes that mediate multidrug resistance in mammalian cell lines (Duraisingh & Cowman, 2005). Polymorphisms in *Pfmdr1* gene are proposed to be responsible for drug resistances in amodiaquine, chloroquine, quinine and modulate artemisinin, halofantrine, and lumefantrine sensitivity. A mutation at N86Y, Y184F, S1034C, N1042D, and D1246Y in *Pfmdr1* gene and/or variations in copy number confers some level of resistances (Sidhu et al., 2006). The *Pfmdr1* haplotype 86N, 184F (Happi et al., 2009; Sisowath et al., 2007; Vinayak et al., 2010), and N86Y (Gómez-Saladín et al., 1999; Nagesha et al., 2003) have been associated with antimalarial treatment failures. The codon 86 mutation is an essential amino acid change because the Asn-to-Tyr substitution at position 86 accounts for the chloroquine-resistant phenotype in *P. falciparum* strains that originated from Asia and Africa independently (Basco, Ndounga, Ngane, & Soula, 2002; Lopes et al., 2002; Taylor et al., 2015). An increase in *Pfmdr1* copy numbers in some instances also causes reduction in parasite susceptibility to certain drugs (Sidhu et al., 2006). A study in Nigeria demonstrated an artemisinin tolerant strain was selects for the *Pfmdr1* N86, F184 and D1246 haplotype in gametocytes (Afoakwah et al., 2014; Happi et al., 2009).

Plasmodium falciparum Propeller Domain of a Kelch Gene (*Pf K13*)

The *K13* (PF3D7_1343700) gene is on chromosome 13 of the propeller domain with 4057 nucleotide base pairs. Artemisinins reduce the parasite burden while the partner drugs with longer half-life are supposed to clear the residual parasites. Unfortunately, most partner drugs in ACT were previously

used for treatment in malaria endemic areas and circulating parasite resistance to them exist. The drugs in ACTs might share the same mode of action, pathways or chemical structure (an example is chloroquine and amodiaquine) thus having the same resistances markers.

The mutant alleles Y493H, R539T, C580Y and I543T, within the kelch repeat motif of the C-terminal K13-propeller domain in parasites lines are associated with delayed or reduced parasite clearance (Ariey et al., 2014). The first three mutations (Y493H, R539T, C580Y) are associated with reduced parasite susceptibility to the artemisinin in Cambodia (Ariey et al., 2014). Point mutations in the propeller domain of *K13* after amino acid position 440 to 700 are associated with artemisinin resistance (Ashley et al., 2014). *In vitro*, artemisinin resistance of parasites from Tanzanian was associated with M476I mutation (Ghorbal et al., 2014). Other studies have not attributed the point mutations in *K13* genes in some parasite isolates to reduced susceptibility to artemisinin drugs (Ariey et al., 2014; Taylor et al., 2015). With different mutations in *K13* gene and artemisinins susceptibility of the parasite, there is geographical importance in the acquisition and distribution of the mutations.

Parasite Factors affecting Gametocytogenesis

The progress of the *Plasmodium* parasites from rings to sporozoites involves different host and cells. The parasite has different invasion mechanisms and antigens to survive the life stages transitions between hosts and stages. The presences of *Plasmodium* sixty *var* genes is an example which the parasite alternate it expression to invade the human immune recognition (Smith et al., 2012). The switching of these *var* genes can occur at each new

erythrocytic cycle. These antigenic variations allow the parasite to modify the antigenic and functional properties of infected erythrocytes to evade host immunity by changing and altering its adhesion capabilities (Smith et al., 1995). Increased gametocytemia has been reported in mixed infections between certain *Plasmodium* species (Bousema et al., 2008).

Infection with different parasite species and strains or clones (as in the multiplicity of infection) results in distinct antigens during infections (De Roode et al., 2005). In malaria endemic areas, human hosts are typically infected with multiple genotype of parasites (Lambrechts, Halbert, Durand, Gouagna, & Koella, 2005). Within-host competition between parasite genotypes is likely to drive transmission success. Sexual reproduction between the parasite with high genetic diversity in a population and/or concurrent infections with polymorphic parasite lines provide genetically favourable progeny. Besides, these off-springs might be well adapted to host immunity and drug pressure (Gurarie & McKenzie, 2006), and could also influence malaria transmission success (Morlais et al., 2015).

Plasmodium falciparum diversity

The number of parasite clones within an infection affects the transmission success, an important role in maintaining *P. falciparum* genetic diversity. Genetic factors are likely to play a role since gametocyte production in the host and vectors varies between parasite lines (Eksi et al., 2012; Lambrechts et al., 2005). Together the dynamics of parasite diversity and gametocyte production might influence human immune status and the spread of multidrug resistant strains of the parasite.

Plasmodium falciparum asexual diversity

Plasmodium falciparum have numerous genes that show extensive genetic polymorphisms. There are available markers to study genetic diversity and multiplicity of infection exhibited by the parasite (Ghanchi et al., 2010). Genotyping the P. falciparum based on genes that express merozoite surface proteins 1 and 2 (mspl and msp2) are useful tools to describe allelic variability and clonal diversity per infection (World Health Organisation, 2008). The mspl gene is located on chromosome 9 with size variation between 185 and 250 KDa. The block 2 of the mspl gene is the most polymorphic region and grouped into three allelic families namely K1, MAD 20, and RO33 type. The msp2 gene consists of FC27 and IC3D7 families (Takala et al., 2006), the gene located on chromosome 2 with varying polymorphic size between 45 and 55 KDa can differentiate P. falciparum clones.

Plasmodium falciparum gametocytes diversity

Determining genotype and diversity of P. falciparum sexual stages in a population depend on the parasite stage and sex specific genes. The gamete surface protein the Pfg377 gene is highly polymorphic and useful in identification of distinct gametocyte producing clones and multiplicity of infection in mixed infections (Alano et al., 1995). The gene encodes seven degenerate amino acid repeats and its alleles, varying in twenty-one base pairs repeats (Alano et al., 1995). Diversity in Pfg377 region 3 is a marker for analysis of parasite diversity (Menegon et al., 2000) and an important tool in mapping malaria transmission.

Effect of Host Genetic factors on Gametocytogenesis

Resistance to malaria is characterised by the development of an immune response by the host and innate features that protect against infections. Features include RBC polymorphisms like haemoglobin (HBB) genotypes (Gouagna et al., 2010; Grange et al., 2015), ovalocytosis (Rosanas-Urgell et al., 2012), ABO blood group phenotypes, Duffy proteins (de Carvalho & de Carvalho, 2011), and the level of G6PD activity in the population (Peters & Noorden, 2009). Gametocyte carriage and malaria transmission potential are related to human genetic variation at the β-globin locus with a higher gametocyte density and/or higher infection rate for individuals with certain haemoglobin genotypes (Gouagna et al., 2010; Grange et al., 2015). Certain haemoglobin variant protects the individual from malaria by impeding invasion and replication of the parasite in the RBC. Red blood cell variants are naturally selected in response to the evolution of *Plasmodium* infections. Thus by the absences of certain RBC surface proteins which the parasite requires for invasion it survival is affected providing protection against severe malaria. These include Duffy binding protein sites, ABO blood group, and other RBC polymorphisms.

Glucose-6-phosphate dehydrogenase and *Plasmodium falciparum* gametocyte prevalence

The gene that codes for G6PD is located on the X chromosome locus q28, it is 18kb long with 13 exons and 12 introns, varying in length between 12 bp and 236 bp (Martini et al., 1986). Over 450 G6PD variants have been differentiated based on enzymatic activity (Ouattara et al., 2014). In malaria

endemic sub-Sahara Africa, the most common mutation is the codon 376 with others at codons 202, 542, 680 and 968 (Ouattara et al., 2014). As erythrocyte grows, the G6PD enzyme activity in the cell decreases and the risk of these older erythrocytes to oxidative stress increases (Peters & Noorden, 2009). Deficiency in G6PD increases the susceptibility of erythrocytes to reactive oxygen species which can lead to hemolytic anaemia (Phillpotts, Tash, & Sen, 2014). The 202A/376G G6PD (A-) has been the most extensively studied variant protecting against severe malaria (Amoah, Opong, Ayanful-Torgby, Abankwa, & Acquah, 2016; Ouattara et al., 2014). The effect of G6PD on gametocyte prevalence is unknown but it is speculated to be its underlining anaemia (Beutler, 2008).

Beta haemoglobin and *Plasmodium falciparum* gametocytes

High frequencies of red blood cell polymorphisms in areas endemic for malaria offer the human population a survival advantage to the disease. The importance of genetic variation in infectious diseases is reported in malaria epidemiological studies (Chapman & Hill, 2012). The most common haemoglobin variants in malaria are the mutations in chromosome 11 at codon 21 on the sixth position of the β- globin from Glutamine to Lysine or Glutamine to Valine which result in haemoglobin C (HbC) and S (HbS), respectively (Forget & Bunn, 2013).

Protective roles of HbC and HbS genetic variants to malaria are due to their resultant abnormal RBC shape (sickle shaped in HbS) and modification of surface proteins on the RBCs in HbC. The attributes of their protective effect is by haemoglobin structure variants being widespread in malaria

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endemic sub-Saharan Africa and Asia (Gouagna et al., 2010). The prevalence of these alleles is around 12 % in many malaria endemic African regions (Flint, Harding, Boyce, & Clegg, 1998; Gouagna et al., 2010; Verra, Mangano, & Modiano, 2009). The HbC and HbS variants causes reduction in the *Plasmodium* parasite cytoadherence leading to lower multiplication (Cholera et al., 2008). The increased removal of parasitized cells by the spleen in abnormal erythrocytes relates to the protection of beta globin variant which reduces the ability of parasites to grow and multiply in the RBCs (Wellems, Hayton, & Fairhurst, 2009; Williams & Weatherall, 2012).

Human Blood groups and *Plasmodium falciparum* gametocyte prevalence

The ABO blood groups are glycol proteins or lipids found on chromosome 9. The presence of glycosyltransferase (GlycosylT) enzyme in this system adds N-acetyl galactosamine galactose, to the H precursor to create the major blood groups A, B and O (Dean, 2005). The major ABO blood group system is divided into the following four major groups depending on the antigen present on the surface of their red blood cells (A, B, AB and O). The presence or absence of the Rhesus factor (Rh) further dividing the classification of ABO into eight sub-groups which are crucial in blood transfusion (Schwarz & Dorner, 2003).

The blood group A is associated with greater risk of severe malarial anaemia and this is when an interactions between *Plasmodium* derived proteins and host RBC surface proteoglycans (such as the blood group A antigen) interact to form rosetting during infection (Rowe et al., 2007). The ABO blood groups and the presence of Rh antigen on them are the major and

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clinically significant in blood donation and in *P. falciparum* infections. High carriage *P. falciparum* gametocyte has been seen in individuals with blood groups B and O (Grange et al., 2015). The antigen on ABO blood group has O-linked glycoprotein that exposes the sugar residues on the surface of the RBCs. The ABO blood group sugar residue is used to determine whether the presence of antigen A or B. As red blood cells with one or both antigens (Anti A and Anti B) are expose to the corresponding antibodies a visible agglutination occurs. Individuals with blood group A have the antigen A on their RBCs surface but anti-B antibodies in their serum. In most blood groups, typing is based on a visual agglutination test. During such test, if agglutination is observed when the blood is sample mixed with Anti A reagent, the individual have blood group A, when blood was mixed with Anti B reagent, then the individual has blood group B. If agglutination is observed in both Anti A and Anti B reagent, then the individual has blood group AB. If no agglutination observed with Anti A and Anti B reagent, then the individual has blood group O. If agglutination is observed after the blood is mixed with Anti Rh D reagent, then the individual is considered positive Rh factor (Mujahid & Dickert, 2015). If no agglutination is observed after mixing the blood with the anti- Rh D reagent, then the individual has a blood group with a negative Rh factor negative (Figure 5 & Table 1).
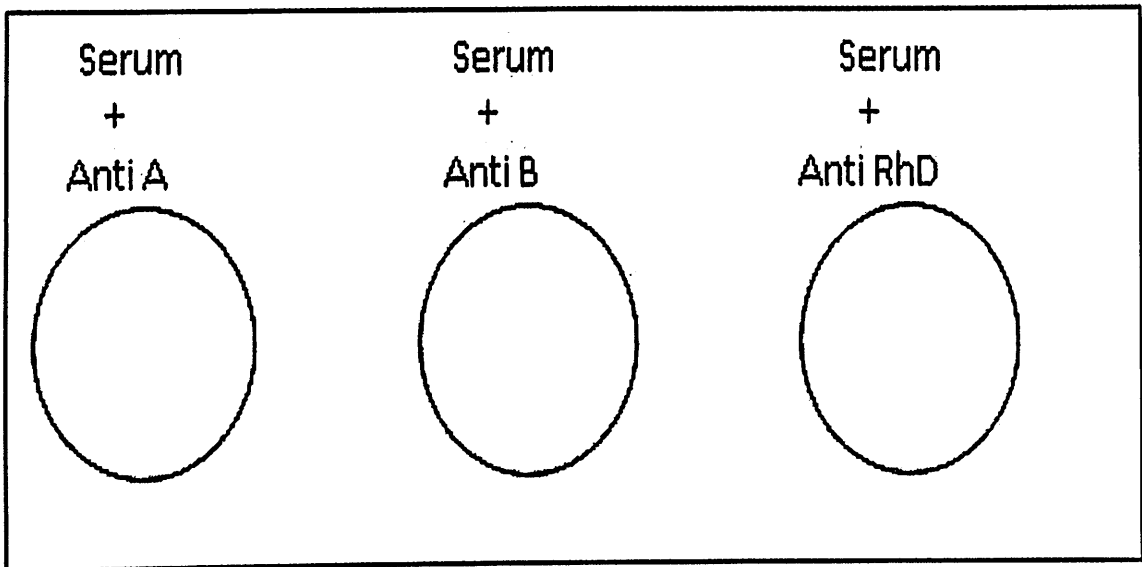
Figure 5: Illustration of the ABO blood group agglutination test plate

Table 1 ABO blood groups phenotype guide

Antigens on the surface of RBCs	Antibodies in the Serum	ABO Blood group phenotype	
		Positive Rhesus factor	Negative Rhesus factor
A	Anti B	A	A-
B	Anti A	B	B-
AB	Neither Anti A or Anti B	AB	AB-
Neither A nor B	Anti A, Anti B, Anti A B	O	O-

CHAPTER THREE

MATERIAL AND METHODS

Study Sites

The *ex vivo* study used *P. falciparum* field samples (isolates) in symptomatic infections which were collected from the Ewim Health Facility in Cape Coast. The longitudinal study was conducted in children from two schools in Obom, a rural town in the Weija district of the Greater Accra Region and Abura in Cape Coast in Central region (Figure 3). Malaria transmission in the study sites is perennial with most of the disease occurring during the major rainy season in May to July (Ghana Statistical Service, 2011; Ghana Urban Malaria Study, 2013). The study sites were selected based on asymptomatic *P. falciparum* prevalence obtained from voluntary exercise and they have similar ecology.

Cape Coast is an urban setting with an estimated population of 227,269 which lies in the Coastal Savannah region. It is about 165 km west of Accra (capital of Ghana) on the Gulf of Guinea (5°06'N 1°15'W) with a land area of 122 sq. km. The major rainy season is between May and July with monthly mean relative humidity varying between 85% and 99%. Obom (5°34' N, 0° 20' W), is also within the Coastal savannah region. The population of the municipality stands at 385,741 from the 2010 Population and Housing Census and the main economic activity is farming. The annual mean rainfall varies between 790mm to 1270mm, along the coast to the extreme north of Ghana. The annual average temperatures range between 25°C in August and 28°C in

January and March, the hottest months with relative humidity of about 75% (Ghana Statistical Service, 2014).
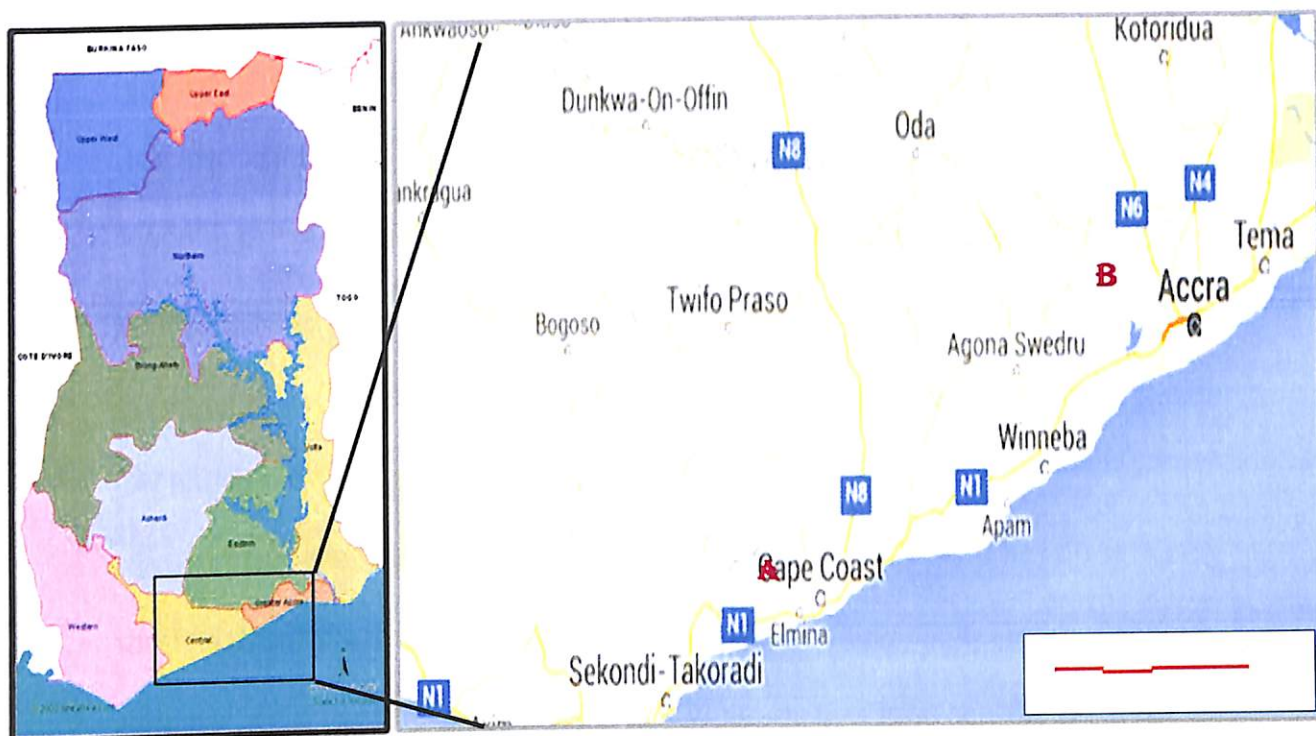
Figure 6: A map of Ghana showing the study sites, A –Cape Coast B-Obom (Adapted from www.ghanaquest.com/map)

Study Design

The research employed a health facility-based cross-sectional and community-based longitudinal studies using convenient sampling. Samples collected in the cross-sectional study were used for the direct assessment of gametocyte production in *ex vivo*. Samples from the longitudinal study were used for the host and parasite factors association.

For the *ex vivo* study, 70 samples were collected from patients aged one to twelve years, who had visited the health centre with uncomplicated malaria between June and September 2015. For the school children survey, 110 study participants aged six to twelve years were selected from 500 pupils.

Study Population

The *ex vivo* group was sampled during the main malaria season and children aged one to twelve years who presented at the health facility with a history of fever were screened for possible enrolment. The inclusion criteria was the presence of *P. falciparum* mono-infection at a density of between 1,000 and 250,000/µl of whole blood in the *ex vivo* group. The parasite densities were estimated using the WHO standardized protocol of the number of parasite in 8000 WBC counts as per microliter of blood (World Health Organisation, 2009; 2016).

The school children were sampled in a longitudinal studies done during the main malaria season, July 2015 (rainy) and the low transmission season in January 2016 (dry) to compare the study parameters between the seasons. To select the study subjects, the pupils were first selected according to those whose parents provided written consent and followed by their age group, putting them in a particular educational level (class 1 to class 5).

Ethical Considerations

The Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR) and Ghana Health Services approved the study (REF: NMIMR IRB00001276 & GHS-ERC02/07/14). Permission was sought from the Ministry of Education Ghana, before recruitment of participants were undertaken in the various schools. The parents/guardians were informed of the study objectives, methods, anticipated benefits, and potential hazards. The parents/guardians were encouraged to ask questions about any aspect of the study that was unclear to them and they were

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informed about their liberty to withdraw their children at any time without penalty. Consent was then sought from the parents/guardians and all-personal information treated confidentially.

Sample Collection

Whole blood samples were collected from children with uncomplicated malaria (symptomatic) for the *ex vivo* study. Again, whole blood was collected from children without signs of malaria for the school/longitudinal study.

Ex vivo (Symptomatic) study Group

From June to August 2015, seventy whole blood samples were collected for the *ex vivo* study from patients who visited the Ewim health centre with uncomplicated malaria by clinical observation and confirmed by thin and thick Giemsa stained blood smears. Prior to drug administration (day zero), 2 mL of venous blood was taken into a citrate-dextrose solution (ACD) vacutainer tubes (Becton Dickinson, USA) for the parasite cultures. In addition to the ACD sample on day zero, 2 mL of venous blood were collected into ethylenediaminetetra acetic acid (EDTA) vacutainer tubes (Becton Dickinson, USA). The EDTA samples were used to measure haemoglobin levels on the haematology analyser (Urit 3000 Plus, China). Immediately after Hb levels reading, the blood was centrifuged for plasma collected and saved for future immunological work. An aliquot of the blood cell was spotted onto a Whatman® 3mm filter paper (Sigma Aldrich, UK). The filter paper blood blots were air-dried and stored at room temperature for DNA extraction. One hundred microliters of the blood cell pellets were stored in 500 μL (1:5 ratios)

of Trizol (Tri Reagent, Invitrogen) for RNA extraction. On the follow-up, seventh day visit (four-day post treatment), 100 µL of pelleted cells were stored for RNA preservation and an aliquot of the pelleted iRBC was spotted onto filter paper as stated above and processed from the 2 mL EDTA whole blood collected from the participants. All the study participants were given a standard curative dose of artemether + lumefantrine (20/120 mg/kg) or artesunate + amodiaquine (4/10 mg/Kg).

Longitudinal (School children) Group

The school children (Sixty from each site) were recruited into a longitudinal study. Samples were collected in rainy (July 2015) and dry (January 2016), seasons. Thin and thick blood film smears and two millilitres of venous blood was taken into an ACD tube. RNA preservation, filter paper blots and plasma samples were handled as previously described in the *ex vivo* study group. Haemoglobin levels (using URIT® 12 haemoglobin test kits, China); body temperature (using the thermometer) and host positivity to *P. falciparum* HRP2 antigens (using rapid diagnostic test -RDT) were measured during the January/dry season visit. Additionally, blood film smears were prepared and parasite density assessed by microscopy during each visit for all the groups. All samples were sent and analyzed at NMIMR.

Optimization of a Plate-based Assay for Gametocyte Development

Gametocyte production assay was optimized for the *ex vivo* assay for easy performance and reproducibility suiting both field and laboratory parasite strain cultivation. The assay for gametocyte production was done using 12-

well tissue culture plates. *Plasmodium falciparum* 3D7 and NF54 parasites strains were used for the optimization where they were cultured and their growth monitored for 14 days. The parasite cultures were cultivated in complete parasite medium (CPM) made of RPMI 1640, 25mg/ml of Hypoxanthine (Sigma Aldrich, UK), 0.5% albumax II (Invitrogen, USA), 10% normal human serum (NHS), and supplemented with 7.5% sodium bicarbonate (Thermo Fisher Scientific, UK). The cultures were gas with 5% CO_2, 5% O_2, and 90% N_2 after media change and incubated at 37°C in an atmosphere. Daily microscopy smears were prepared from the cultures in the 12-well tissue culture plate to monitor parasites growth, assess red cell morphology and parasitaemia estimation.

The assay was set up at ring stages obtained after synchronization. Schizonts synchronized using Nycodenz® (Sigma Aldrich, USA) density barrier gradient method for the isolation and purification as previously described (Carter, Suhrbier, Beckers, & Sinden, 1987). The schizonts were then culture for them to be merozoites where they were sorbitol treated twice within eight hours interval to obtain young rings. Gametocytes were further removed from the rings culture by passing the cells over a magnet separation column (MACs) before the assay set up. The gametocyte production assay was set up with the purified rings at 0.5%, 1%, 2%, 3% and 4% parasitaemia at 3% haematocrit. The two wells were used for the gametocyte production assay that was supplemented with 50 mM of NAG. Culture media were change daily and Giemsa smears prepared for parasite growth monitoring.

Nycodenz (NYCO) Synchronization and Purification of Schizonts

Prior to purification, the parasites were maintained in continuous culture until parasitaemia was about 8.5% and predominantly schizont. The culture was then centrifuged and the supernatant was taken off. Fifteen millilitres of 18% NYCO prepared in 1X SA buffer (10mM Tris, pH 7.3, 170mM NaCl, 10mM glucose) at pH 7.4, was put in a 50 mL centrifuge tube. Ten millilitres of CPM was added to the culture and pipette gently to mix. The culture was gently dispensed on top of the 18% NYCO and then centrifuge at 3,000 rpm for 20 minutes. This separated the cultures into three layers (top – gametocytes; middle – schizonts; and the bottom – uninfected RBC and rings). The top layer was taken out carefully and the second/middle (schizonts) layer was collected into a new 15 mL Eppendorf tube and centrifuged. Smears were prepared to estimate the number of schizonts per microliter of RBCs using a haemocytometer and Giemsa stain smears. The cells were resuspended with 10 mL CPM and were centrifuged at low speed (1000 rpm) for one minute to wash the schizonts. The purified schizonts cultured at 2% parasitaemia and 3% hematocrit with fresh CPM. After 12 hours, a smear was made and culture with 80% young rings was synchronized with 5% sorbitol.

Sorbitol Synchronisation

The culture with about 5% ring stage parasitaemia was centrifuged at 3,000 rpm for 5 minutes and the supernatant (CPM) taken off. Twenty millilitres of 5% sterile sorbitol (Room Temperature -RT) in a ratio of 1:20 of the pelleted culture volume (pelleted volume of 1 mL, 20 mL of 5 % sorbitol) was added to the parasite culture. The cells in the 5% sorbitol and mixed

gently by inverting the tube several times and incubate for 15 minutes at room temperature (RT). The culture was centrifuge for 10 minutes at 3,000 rpm and the supernatant discarded. The cells were resuspended in CPM and smear prepared to estimate the parasitaemia. Gametocyte stages were further remove from the parasite culture by Magnetic-Activated Cell sorting (MACs) column purification method.

Magnetic-Activated Cell sorting (MACs) Column Synchronisation

The MACs column was set with 21 G needle inserted at the tip of the column. The column membrane was washed with two millilitres of CPM and the parasite culture resuspended and pushed through the MACs column through the needle into a 50 mL conical tube. The gametocyte binds to the column whilst the infected rings and uninfected RBC passed through into the tube. Parasitaemia was then determined for setting up the pseudo-patient *in vitro* assay at parasitaemia 0.5%, 1%, 2%, 3% and 4%.

Preparation of Whole Blood Sample for *ex vivo* Parasite Cultures

The participants' whole blood samples were centrifuged for five minutes at 2000 rpm to harvest the plasma and remove the buffy coat. The washed cells were resuspended and with equal volumes of Incomplete Parasite Media (iCPM) and centrifuged at 2000 rpm for five minutes (S 3.4.3). The parasitized cells were then used to set up *ex vivo* parasite assays that were monitored for gametocyte production over an eight-day period. One hundred microliters of the washed cell pellets were cryopreserved with glycerolyte/freezing mix (S 3.4) for follow-up studies when necessary.

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Direct Analysis of Gametocyte Production from the *Plasmodium falciparum* Isolates

Washed iRBCs pellets from the whole blood that was collected from the study participant were suspended in CPM to a final hematocrit of 3%. The assays were set into four wells per sample in tissue culture plate. Cultures were maintain with daily media change and two out of the four wells for each isolate with 50 mM NAG treatment. On the eighth day, cultures were harvested to assess the percentage of parasites that were committed to gametocytes. Controls for the assay were NF54 (high gametocyte producer) and 3D7 (low gametocyte producer) parasite strains. *Ex vivo* parasites' development was monitored by daily Giemsa-stained blood smears and gametocyte production was estimated from 200 microscopic fields count and normalized to 1000 RBCs with an estimation of 40,000 RBCs for the total smear read and each smear read by two microscopists. An average of two hours was used to read a smear for the gametocytecemia estimation in cultures.

Molecular Studies

Molecular tools were used to identify parasite at the submicroscopic level. Host RBC polymorphism and the parasites drug resistance markers and diversity were characterised using extracted gDNA.

Gene amplification and Analysis using qRT-PCR

Total RNA was extracted from trizol-preserved samples and the extraction included a DNaseI treatment step prior to elution from the column.

The extracted and purified RNA was converted to cDNA to check for genomic DNA contamination after RNA extraction, PCR was performed on the RNA and their corresponding cDNA. The cDNA was used to assess asexual (*Pfs18S rRNA*) and matured gametocyte (*Pfs25*) specific transcripts in triplicate using ABI Fast SYBR Green qRT-PCR kit (Thermo Fisher Scientific, USA).

RNA Extraction from Trizol Preserved Samples

Ribonucleic acids were extracted using Rneasy micro RNA extraction kits (Qiagen, USA) by following the manufacturer's protocol. Two hundred microliter of chloroform was added to 500 µL of trizol preserved samples and mixed to homogenize and lyse the red blood cells. The samples were centrifuged at 10,000 rpm for 20 minutes and the plain aqueous phase was remove carefully into a new 1.5 mL micro-centrifuge tube using a p200 pipette (care was taken to avoid collecting the DNA interface). The interphase and organic phase were stored for future DNA and protein extractions. An equal volume of absolute ethanol (1:1) was added to the collected aqueous phase sample and mixed. The aqueous phase mixed with ethanol and was transferred onto the RNeasy minelute spin column (maximum of 700 µL on a column) on a collection tube for RNA binding of the RNA to the column. The column was washed, on-column DNaseI treated for 15 minutes at room temperature. The washing step was repeated and RNA eluted in a total volume of 36 µL at three elutions of 12 µL each (S 3.2). The RNA concentrations were measured with Nano drop and Qubit (S 3.2) followed by cDNA preparation. The remaining RNA samples were stored at -80°C.

Reverse Transcription of RNA to cDNA

The Invitrogen VILO reverse transcriptase kit was used for cDNA preparation for two-step qRT-PCR following manufacturer's protocol. The VILO master mix preparation was supplemented with 5µM of oligo-dT primer for the amplification of the polyA-tailed mRNA and RNase-free water to make it up to a total reaction volume of 20 µL. The reaction mix was incubated at 25°C for 10 minutes, 42°C for 60 minutes, 85°C for 5 minutes, 12°C on hold. The remaining RNA sample was stored at -80°C.

End-point PCR for gDNA Contamination Check

To check for gDNA contamination during RNA extraction or cDNA preparation, PCRs were run on no-template control (NTC), cDNA and their respective RNA samples. The cDNA samples and NTC were diluted to a ratio of 1:10 with RNase and DNase free water. Human (*18S rRNA* or blood group O) and *P. falciparum* (*18S rRNA* or *kahrp*) genes were amplified using GoFlexi Taq (Promega, UK) and Bio Taq polymerase (Bio Tech, Ghana). The total PCR reaction volume was 20 µL using 2 µL of diluted cDNA (1:10) and 2 µL of RNA as templates. The PCR was performed on a T professional thermal cycler (Biometra, Germany). The reaction conditions were initial denaturation at 95°C for 15 minutes, followed by 40 cycles at 95°C for 15 seconds, 52°C (annealing temperature) for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 2 minutes and 11°C on hold. The separation of PCR amplified products was done on a 2% agarose gel composed of three parts of GTG Nui sieve agarose (Sigma Aldrich, USA) and one part of standard agarose in 1X TAE (Sigma Aldrich, US).

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Reverse Transcriptase Quantitative PCR Primers Efficiency Testing

The primers used for the qRT-PCR were first checked for efficiency by plotting a standard curve with a known controls cDNA. The primers were validated for efficiency by amplifying the targeted genes that are important when reporting and comparing assays. The standard curves generated at an expected range of expression for the genes of interest with positive CT ranges of 13-38 (Table S 3.1 & S 3.5).

Two-step Quantitative Real-time PCR (qRT-PCR)

Quantitative two-step real-time PCR (qRT-PCR) was performed on the *P. falciparum* isolated cDNA from asymptomatic children during the rainy and dry seasons. The *Pfs25* matured gametocyte specific transcript, *Kahrp*, *Plasmodium* asexual genes and *Pf18S rRNA* as endogenous control or normalisation genes were assessed. The samples were analysed in triplicate using Fast SYBR Green 1 RT-PCR kit (4385612, Applied BioSystems) to detect submicroscopic gametocyte carriage. cDNA from matured gametocytes (IV & V) and rings from NF54 and 3D7 strains of the *P. falciparum* and malaria naïve human served as controls.

The primers used for the qRT-PCR were validated using cDNA controls and at a concentration of 300 nM. The starting dilution concentrations of the cDNA were either 1.10, 1:20 or 1:100 and subsequent serially diluted in two-folds depending on the expression and abundance of the gene to be assessed. Samples were run in triplicates on Quant Studio 3 Real-Time PCR Systems (Applied BioSystems) using the Fast program cycling conditions at 95°C for 20 sec, 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The data obtained

was used to plot a standard curve and a threshold cycle (CT) selected. The selected positive CTs were above the baseline and below the CTs values of the negative controls. An indicator or estimates for the presence or absence of gametocytes or parasites were analyzed by Quanti v1.3.1 Software (Thermo Fisher Scientific, USA) and as shown in Table S 3.2.

Quantification of Parasite in a qRT-PCR Reaction

Gametocyte and asexual parasite densities were estimated based on serial dilutions of cDNA derived from the controls. The mean CTs from each sample dilution point on a standard curve was used as an estimate of the number of gametocyte or asexual forms per the reaction. Lower and upper limit of the genes were determined by the standard curve obtained from the controls.

The formula for calculating total number of parasite in a qRT-PCR reaction is base on the following assumptions:

The volume of sample used per qRT-PCR volume of 20 µL and calculated by:

Amount of blood sample in one qRT-PCR reaction volume

- Volume of pelleted cells in RNA preservative is typically in 100 µL

- Volume of RNA eluted from column   = (36 µL)

- Volume of RNA used for cDNA   = (12 µL)

$$= \frac{\text{volume of pelleted cells in RNA preservative X volume of RNA used for cDNA}}{\text{Volume of RNA eluted from column}}$$

= (100 x 12)/36 = 33.3 µL

Volume of RNA in qRT-PCR (20 µL) = 2 µL (1.10 dilution) = {(33.3/10)/10}

= 0.33 µL of the original blood sample in RNA preservative.

Gametocyte and asexual parasite densities were estimated per qRT PCR reaction for each gene based on the CT values on the standard curve formula. The mean CTs for each dilution point on a standard curve were equivalent to the number of gametocyte or asexual parasite per reaction. Lower and upper limit of the genes were determined using the controls CT valves on the standard curve. The CT values of the controls for all the genes were determined from the initial cDNA concentration (1:10) of the 0.067 µL of the RNA obtained from preserved whole blood sample.

The mean CTs from the counts gave an estimate of the number of parasite or cells in a qRT-PCR reaction. The purified ring stages from RCM 47 (gametocyte deficient) and C9 (-) strains that produced gametocyte at lower rates and NTC, were used as negative controls for *Pfs25* transcript cut off. A mean CT value equal or above the control mean CTs were consider negative for *Pfs25* transcript. The RCM 47 and C9 (-) rings were positive controls for asexual genes (*Pf18S* and *kahrp* transcripts) with cDNA from whole blood of malaria naïve individuals and NTC used as negative controls. cDNA from purified stage V gametocytes of NF54 strain were used as positive control in measuring the *Pfs25* transcript. The *Pfs25* transcript was amplified to determine submicroscopic gametocyte prevalence in study isolates.

DNA Isolation using Chelex-100 Resin Protocol

Deoxyribonucleic acid was extracted from dried filter paper blood blots using a modified Chelex protocol (Wooden, kyes, & Sibley, 1993). Chelex is an ion chelator that limits destruction of the DNA by inactivating nucleases and chelating heavy metals that may damage the parasite DNA. Two

(3 mm square) pieces of the blood blots from each patient were used. The punch was sterilized in between cuts with 1M hydrochloric acid (HCl) and 70% ethanol. The filter paper blood blots punches were then washed twice with 1 mL of 1X PBS (phosphate buffered saline) by incubating at room temperature for 10 minutes before centrifuging for 2 minutes and discarding the supernatant. One hundred and fifty microliters of sterile double distilled water and 50 μL of 20% Chelex-100 were then added to the above tube containing the washed blots. The mixture was heated to 99°C for 10 minutes followed by centrifugation for 1 minute at 14,000 rpm. One hundred and sixty microliters of the supernatant was transferred into new 1.5 mL tubes, centrifuged for 10 minutes at 13,000 rpm. One hundred and thirty microliter (130 μl) of the supernatant was transferred into new 0.5 mL tubes, to ensure complete removal of Chelex beads. Extracted DNA was stored at -20°C until used.

Typing of Human Red Blood Cell Polymorphisms and G6PD

The participants HBB and G6PD were genotypes; and ABO blood group types were assessed in the study participants.

Haemoglobin Variant Genotyping

The NMIMR sickle-cell unit did haemoglobin typing for the study participant. The HBB typing was performed using RESOLVE® Haemoglobin Kit (PerkinElmer, Finland). The dried blood spot samples were eluted with Hb elution solution in a multi-well plate to inhibit methaemoglobin formation. The eluted samples were loaded into the wells of a precast agarose gel

containing RESOLVE Ampholytes at a pH range of 6 - 8. Haemoglobin bands were staining in 10% trichloroacetic acid (TCA). The gels were incubated in 10% TCA for 10 minutes on a rocking platform. The gels were rinsed after incubation in distilled water, hanged to dry at room temperature. Visual interpretation of the result was done by comparing the band patterns to the controls. The REVOLVE method follows the principle of migration based on molecular weight and the charge on the molecule as in gel electrophoresis.

ABO Blood Groups Typing

The ABO and Rh blood grouping system was done based on agglutination reaction as cited by Karl Landsteiner in a study (Schwarz & Dorner, 2003). Three different drops of 20 µL of plasma from each sample were put on blood grouping disc plate (Figure 5 & Table 1). Antiserum was added to the plasma samples and mix using a pipette. Agglutination was observed in the form of fine red granules within 5 seconds and the individual blood group score by visual observation.

G6DP Genotyping

The characterisation of the G6PD in the host population was by RFLP using a protocol developed by Carter (Carter et al., 2011). The nucleotide 376 mutation was genotyped after which mutant on nucleotide positions 202, 680 and 968 were assessed on samples with position A376G nucleotide polymorphism. Restriction enzyme used for the single nucleotide polymorphism identification were Fast *FokI* enzyme for the of the A376G mutation (G6PD*A) for 10 minutes at 37°C. *NlaIII* for G202A mutation,

BstNI for G680T mutation, and *NciI* for T968C mutation all digested for 40 minutes at 37°C. All the restriction enzymes used were from New England BioLabs (NEB, UK).

The G6PD amplification for detection of the polymorphisms were carried out in a 30 µL reaction volume containing 200 nM dNTP, 1.5 mM MgCl2, 200 nM of each primer and 0.75 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, UK). Four microliters (>20 ng) of gDNA was used as the template for the reaction. The amplification cycling conditions were: initial denaturation at 94°C for 10 minutes, followed by 30 cycles at 94°C for 45 seconds, 58°C (annealing temperature) for 45 seconds and 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. The G6PD 202F & G6PD 202R anneals at 65°C; G6PD 680F & G6PD 680R annealing at 69°C; and G6PD 968F & G6PD 968R anneals at 65 °C following the 376 cycling conditions.

Genotyping *Plasmodium falciparum* Diversity and Drug Resistance Markers

Identification of *P. falciparum* species was done before molecular genotyping of parasite genes. This was important since not all the school children had asymptomatic parasite infections. The parasite diversity defines it clonality or alleles present.

PCR identification of *Plasmodium falciparum* isolates

The nested PCR method for *Plasmodium* species identification based on the amplification of the small subunit ribosomal RNA (*18SrRNA*) gene was

used (Snounou et al., 1999). The initial PCR involved the use of genus-specific rPLU5 and rPLU6 primers to amplify the gDNA fragment or sequence conserved in the four human *Plasmodium* species. The primary amplified product was used as the template for the secondary PCR using species-specific oligonucleotide primer pairs for *Plasmodium* species. The primer pair for *falciparum* was rFAL1 and rFAL2. Genomic DNA from *Plasmodium falciparum* 3D7 strain was used as positive and double-distilled water as negative controls in all amplification reactions. The sequence details of the primers are listed in Table S 3.4.

The gene amplifications were carried out in a 15 µL reaction volume made of 200 nM dNTP, 2 mM MgCl$_2$, 200 nM of each primer, and 0.5 U of One Taq DNA polymerase (New England BioLAB, UK). Four microliters (>20 ng) of gDNA was used as a template for the primary reaction and 1 µL of the product as the template for the secondary reaction. The amplification cycling conditions were initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 58°C annealing temperature for 1 minute, and 72°C for 1 minute with a final extension at 72°C for 5 minutes. All conditions remained the same for the primary and secondary amplifications except the secondary reaction annealing temperature which was 55°C.

Plasmodium falciparum Genetic Diversity and Allele Detection

Genotyping for *P. falciparum* multiplicity of infection in the study population was done by amplifying the polymorphic block 3 region of the *msp2* (PF3D7_0206800) gene which is located on chromosome 2. A nested PCR was performed individually using previously published primer sequences

and conditions (Snounou et al., 1999), that distinguish the two allelic families (FC27 and IC3D7) of the central polymorphic region. The nested *msp2* PCR products were separated using 2% ethidium bromide-stained agarose gels and visualized under UV transilluminator.

All amplifications were carried out in a 15 µL reaction volume, containing 200 nM dNTP, 2 mM MgCl2, 200 nM of each primer, and 1 U of Biotech Taq DNA polymerase (New England BioLAB, UK). Four microliters (>20 ng) of gDNA was used as a template for the primary reaction and 2 µL of the product was used as the template for the secondary reaction. The primers for the inner reactions were S1fw and N5 for IC3D7 whiles M5 was used for FC27. The amplification cycling conditions were; initial denaturation at 94 °C for 5 minutes, 40 cycles at 94°C for 30 seconds, 57°C annealing temperature for 1 minute 30 seconds, and 72°C for 1 minute and final extension at 72°C for 5 minutes. The same cycling conditions were used in both primary and secondary amplifications.

Plasmodium falciparum Gametocyte Diversity

Gametocyte diversity was assessed by amplifying the region 3 of the *P. falciparum Pfg377* gene (PF3D7_1250100), which is the most polymorphic among the four repetitive *Pfg377* sequence regions. Genomic DNA and cDNA were used in the *Pfg377* gene amplification in a nested PCR using published primers (Table S 3.4) and protocols (Menegon et al., 2000).

The amplification was carried out in 20 µL reaction volumes, which contain 200 nM dNTP, 2.5 mM MgCl2, 200 nM of each primer, and 0.5 U of Dream DNA polymerase (Thermo Fisher Scientific, UK). Four microliters

(>20 ng) of gDNA was used as the template for the primary reaction and 1 µL of the product was used as the template for the secondary reaction using primers D1 & R1 and D2 & R2 for the primary and secondary amplifications. The amplification cycling conditions were initial denaturation at 94°C for 5 minutes, 40 cycles at 94°C for 30 seconds, 55°C annealing temperature for 1 minute 30 seconds, and 72°C for 1 minute and a final extension of 72°C for 5 minutes. All conditions remained the same for the primary and secondary amplifications expect the annealing temperature for the secondary reaction which was 50°C. The PCR amplified fragments sizes were scored with GeneRuler 50 bp DNA molecular weight ladder (Thermo Fisher Scientific, UK).

Antimalarial Drug Resistance Markers

The distribution *crt, mdr1, dhfr* and *dhps* and *K13* parasite genes of these markers between the study communities and seasons were assessed using nested PCR. The primer sequences for drug markers are listed in Table S 3.5. The amplification cycling conditions for all the resistance markers were the same for both the primary and secondary reactions. The amplified gene fragments were digested with enzymes to identify the mutation using restriction digestion fragment length polymorphic (RFLP) analysis. A mutant and wild parasite stains were used as controls for codon.

Pfcrt Mutation associated with Chloroquine Resistance

The amplification of *pfcrt* gene (PF3D7_0709000) was done in initial reaction using CRTP1 (P1) and CRTP2 (P2) oligonucleotide primers to

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amplify 559 bp around the codon 72-76 mutations. The secondary reaction consisted of oligonucleotide primer pairs CRTD1 (D1) and CRTD2 (D2) that amplified the 134 bp region using the PCR product from the primary amplification as template.

The amplification reaction was carried out in a 15 μL for the primary and 30 μL secondary volumes. The reaction components were 1X buffer, 200 nM dNTP, 2.5 mM MgCl$_2$, 200 nM of each primer, and 1 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, UK). Four microliters (>20 ng) of gDNA was used as the template for the primary reaction and 1 μL of the product as the template for the secondary reaction. The amplification cycling conditions were initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 56°C annealing temperature for 1 minute 30 seconds, and 72°C for 1 minute with a final extension at 72°C for 5 minutes.

Apo1 restriction enzyme was used for the digest by incubation at 50°C for 50 minutes. The restriction enzyme digested the wild type gene at codon 76 giving fragments sizes of 100 bp and 34 bp. gDNA of 3D7 and Dd2 strains were used as controls.

Pfmdr1 Mutations associated with Multi-drug resistance

A mutation at positions 86 and 184 in the *Pfmdr1* gene (PF3D7_0523000) lead to resistance to chloroquine, 4-amino quinolone, and reduces the parasite susceptibility to artemisinin drugs. For the primary reaction, A1 (f) and A2 (r) oligonucleotide primers were used to amplify the 86-184 gene region to give a product of 639 bp. For the secondary reaction A3 (f) and A4 (r) oligonucleotide primers were used to amplifying a 560 bp

including codons 86 and 186 regions. The PCR product from the primary amplification was used as the template for the secondary reaction.

The amplification reaction was carried out in a 15 µL for the the primary and 30 µL secondary volumes. The reaction components were 1X buffer, 200 nM dNTP, 2.5 mM MgCl$_2$, 200 nM of each primer, and 1 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, UK). Four microliters (>20 ng) of gDNA was used as the template for the primary reaction and 1 µL of the product as the template for the secondary reaction. The amplification conditions were initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, 58 °C (annealing temperature) for 1 minute 30 seconds, and 72 °C for 1 minute with a final extension at 72 °C for 5 minutes.

The *AflII* restriction enzyme digested the codon 86 (N86Y) mutant at 37°C incubation for 1 hour to give fragments of 328 bp and 232 bp. The enzyme did not digest the wild type sequence.

Pfdhfr and *Pfdps* Mutations associated with Sulphadoxine-Pyrimethamine resistance

The *dhfr* gene mutations are associated with pyrimethamine and *dhps* gene mutations to sulphadoxine resistance. Polymorphisms at codons 51 and 108 in the *dhfr* gene and codon 437 in the *dhps* gene were assessed. A nested PCR followed by restriction digest was used to identify these mutations in the isolates as previously described (Duraisingh, Curtis, & Warhurst, 1998).

PCR amplification for the detection of *dhfr* codon S108N mutation

The primary reaction oligonucleotide primers were M1 (f) and M5 (r) to amplify 59 to 164 of the gene to give a 653 bp PCR product size. Oligonucleotide primers F1 and M3 were used for the secondary reaction giving a product of 522 bp.

The amplification reaction was carried out in a 15 µL for the primary and 30 µL secondary volumes. The reaction components were 1X buffer, 200 nM dNTP, 2.5 mM $MgCl_2$, 200 nM of each primer, and 1 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, UK). Four microliters (>20 ng) of gDNA was used as the template for the primary reaction and 1 µL of the product used as the template for the secondary reaction. The amplification cycling conditions were initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 57.5°C (annealing temperature) for 1 minute 30 seconds, and 72°C for 1 minute with a final extension at 72°C for 5 minutes.

For the codon 108 mutation, the 522 bp DNA amplicons from the first primary PCR with M3 and F/ primers were used. B*srI* restriction enzyme (incubated at 65 °C for 1 hour) digested the mutant fragment into 332 bp and 190 bp but did not digest or recognise a site in the wild type fragment. The controls used were K1 or V1/S as mutant and FCR3 as wild type.

PCR amplification for the detection of *dhps* codon A437G mutation

The primary reaction oligonucleotide primers are R1 and R2 to amplify the gene region to give 711 bp products. The secondary reaction used the pairs K and K1 oligonucleotide primer to amplify the 438 bp region.

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The amplification was carried out in a volume of 15 µL for the primary and 30 µL for the secondary reactions. The reaction components were 1X buffer, 200 nM dNTP, 2.5 mM MgCl$_2$, 200 nM of each primer, and 1 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, UK). Four microliters (>20 ng) of gDNA was used as the template for the primary reaction and 1 µL of the product used as the template for the secondary reaction. The amplification cycling conditions were initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 58°C (annealing temperature) for 1 minute 30 seconds, and 72°C for 1 minute with a final extension at 72°C for 5 minutes. The primary and secondary amplification conditions were the same.

For the codon 437 mutation the enzyme *AvaII* (incubated at 37°C for 1 hour) was used to digest and give fragment sizes of 404 bp and 34 bp pattern for the mutant strain but not the wild type strains. The controls used were FCR3 wild type and K1/V1/S as mutant parasite strains.

K13 Mutation associated with Artemisinin Resistance

Mutations in the Kelch propeller (*K13*) gene (PF3D7_1343700) are proposed to be responsible for artemisinins resistance and vary geographically. PCR amplification and Sanger sequencing were performed to identify mutations that spin around the proposed Kelch repeat motif of the C-terminal K13-propeller domain associated with parasite resistance. The amplification of the *K13* genes were based on a hemi-nested protocol described previously (Taylor et al., 2015) with 94 samples. The amplify K13 751 bp region from nucleotides 1279 to 2030 that encodes amino acids from 427 to 676 of K13

gene. The primers used were Artinner F (f) and Artouter R (r) for primary amplification and the secondary reaction with Artinner F and Artinner R to give a 751 bp product size.

The gene amplifications were carried out in a 15 µL for the primary and 50 µL secondary reactions volumes. The component of the reaction were; 1X Buffer II (600 mM Tris-SO$_4$ (pH 8.9), 180 mM (NH$_4$)$_2$SO$_4$, 20 mM MgSO$_4$, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol) and 1 U of Accu Prime High Fidelity polymerase (Thermo Fisher Scientific, USA). Four microliters (>20 ng) of gDNA was used as the template for the primary reaction and 1 µL of the product was used as the template for the secondary reaction. The amplification cycling conditions were, initial denaturation at 98°C for 30 seconds, followed by 30 cycles at 98°C for 10 seconds, 54°C (annealing temperature) for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 5 minutes and gDNA from 3D7 strain was used positive.

All PCR amplifications were performed on T professional Thermal cycler (Biometra, Germany). All amplifications included a positive (laboratory strain parasites) and a negative (water) controls.

DNA Purification and Sanger Sequencing of *K13* Gene Fragment

The amplified *K13* PCR fragments were purified and Sanger sequenced by Macrogen, (Macrogen Rockville, USA) with forward and reverse ArtinnerF and ArtinnerR primers.

K13 gene Fragment Analysis with 3D7 Reference Strain

The 751 bp consensus DNA sequences obtained aligned to reference 3D7 and scored for polymorphisms using multiple alignment software (Mega Clustal W v. 7.0.21). The obtained nucleotide sequences were edited using Chromas v. 2.6.2 software and aligned forward and reversed sequences showing above 80% similarity to generate a consensus sequence using sequence alignment software (Bio Edit v. 7.2.6.1).

Agarose Gel Electrophoresis of PCR Products

Following PCR amplification 10 µl of each PCR product was separated on 1.5%, 2% and 2.5% agarose gels depending on the expected fragment sizes. The gels were made and run with 1X Tris-Acetate-EDTA (TAE) buffer (Thermo Fisher Scientific, UK) stained with 5 mg/mL of ethidium bromide (Sigma Aldrich, UK) for the visualization. DNA molecular weight markers (NEB Quick load, UK) 50 or 100 bp ladder were run in parallel wells for PCR fragment band sizes scoring. The gels were run for 40 to 90 minutes in 1X TAE at 100 volts. The gels were examined using UV transilluminator and photographed. The migrated PCR amplified fragment sizes were estimated in relation to the size of the ladder for all amplified reactions. For the PCR-RFLP, undigested PCR products were run alongside with DNA molecular weight marker on the same gel for scoring.

Data Analysis

All data the obtained were entered into Microsoft Excel, R version 3.4 and GraphPad Prism version 7.0. Descriptive data were analysed into

frequency and percentages and presented in tables and bar charts. Statistical analysis using Mann-Whitney paired two-tailed t-test (GraphPad Prism v 7.0) was used to compare means of continuous variables between groups, such as age, haemoglobin level, and temperature. Two-sample test for equality of proportions with continuality correction (R version 3.4) was performed to predict associations between dependent and independent variables such as parasitaemia and gametocyte production rate; and gametocyte positivity and host genetic factors. The Fisher's exact-test was used to compare variables that differ in proportion. The qRT-PCR data were analyzed with Quanti v1.3.1 Software (Thermo Fisher Scientific, USA). The mean MOI was calculated as a total number of *P. falciparum* genotypes detected per total number of positive samples. All tests were considered statistically significant when p values were ≤ 0.05.

CHAPTER FOUR

RESULTS

Plate Based Assay for *ex vivo* Gametocyte Development

Gametocyte production rate in *P. falciparum* was assessed in an *in vitro* assay using synchronised NF54 ring stage parasites. The assay was optimized successfully in a 12-well tissue culture plate with sufficient RBCs for daily smears and RNA preservation on day eight. Four wells were set for each parasitaemia, with two wells used for gametocyte production assessment. In the wells for gametocyte production, daily parasite culture media was supplemented with or without NAG. Initially, NAG was added to the gametocyte production wells for just three days (Figure 7) which could not clear all the asexual parasites.

Effect of NAG on Parasite Stages

The 50 mM NAG was added to the parasite culture to clear asexual stages in two erythrocyte cycles to inhibit the merozoites from invading new RBCs (Figure 7).
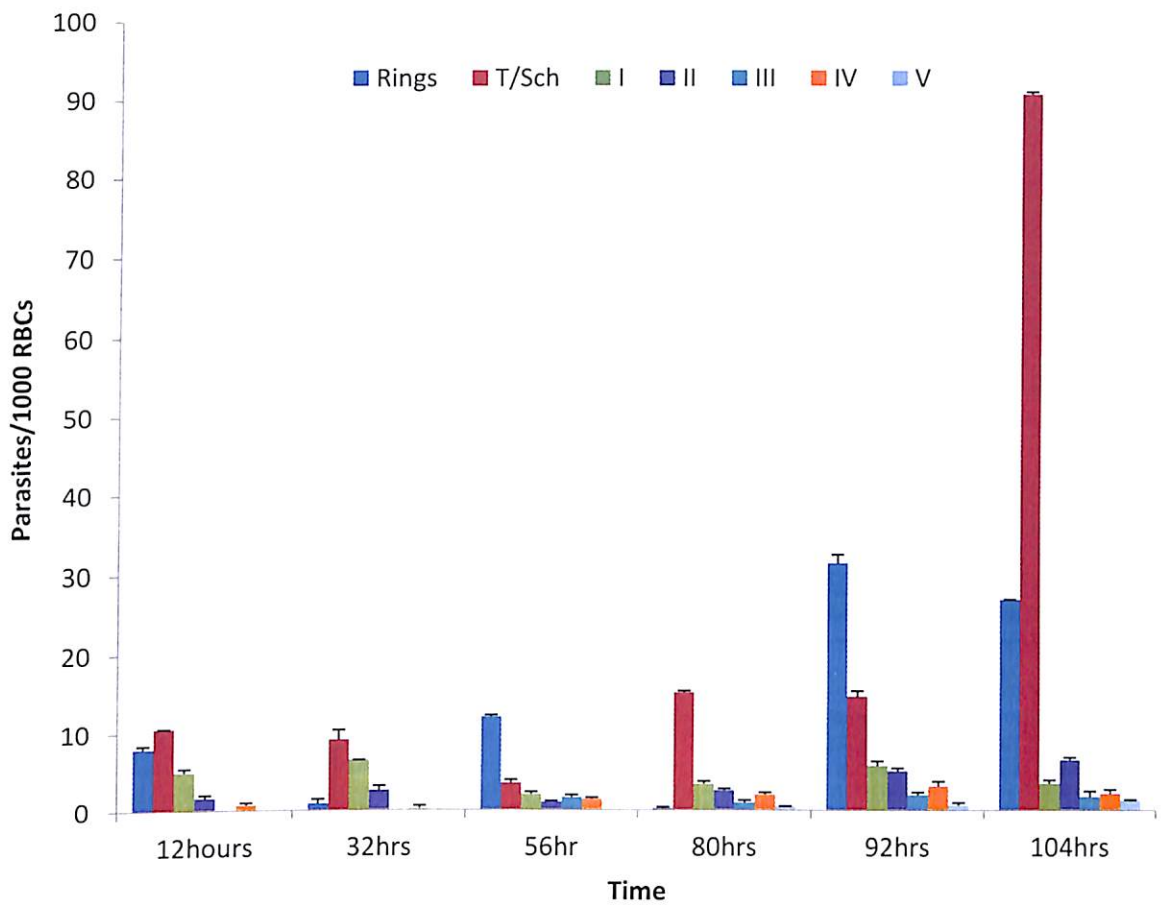
Figure 7: Effect of NAG on *Plasmodium falciparum* NF54 strain stages.
The graph is showing mean ± SD of parasitaemia for parasite stages and the time points. Error bars calculated from three independent readings for the 6 time points and the 50 mM NAG kept on for 72 hrs. Parasite stages were Rings, T/Sch- trophozoite/schizont and I, II, III, IV &V are gametocyte stages.

Adding 50 mM NAG to the assay for three consecutive days (72 hours) affected only one erythrocytic parasite cycle. As shown in Figure 7, the NAG effect on gametocyte production in NF54 parasites with 43-hour erythrocytic cycle showing the reduction of asexual parasites from 12 to 80 hours. This short period of keeping the NAG on the parasite could not effectively clear the asexual stages. Here the fewer merozoites that invaded the RBCs were able to increase the parasite population. During a new erythrocytic cycle, few committed rings were added to the parasite population, which could contribute to the increase of gametocytes observed after 80 hours. A well-controlled

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system that ensured merozoites inability to invade new RBCs was then set up where the daily NAG was kept on the parasites for the entire assay duration (more than six days) which cleared the escaped merozoites in the subsequent cycles.
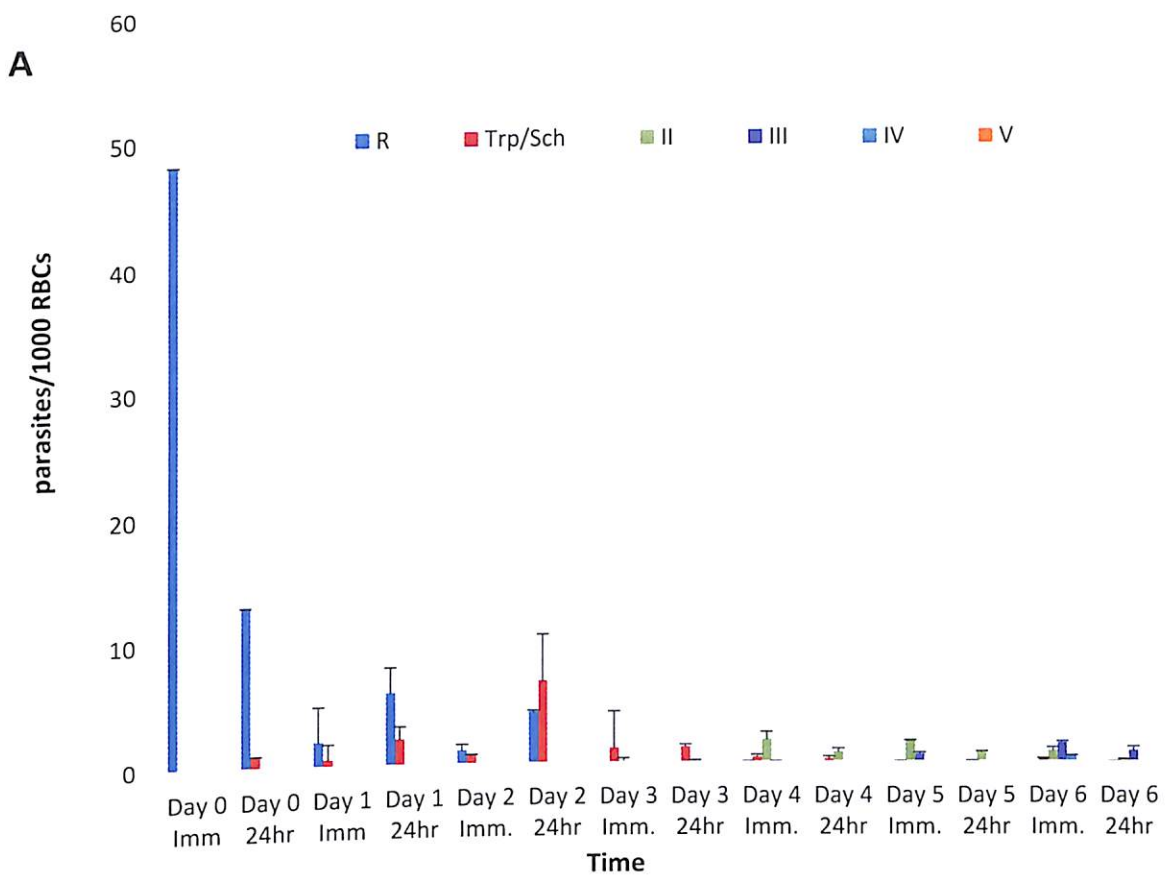
Figure 8 A: Growth pattern of NF54 gametocytes with daily 50 mM NAG for six days. **Imm = Immediate plating, 24hr = plate after 24 hours.** The graph shows mean ± SD of parasitaemia for the parasite stages (rings, Trp/Sch- trophozoites/schizonts, and I-V gametocytes) and on six days *in vivo* cultures. Error bars estimated from three independent microscopy readings.

B
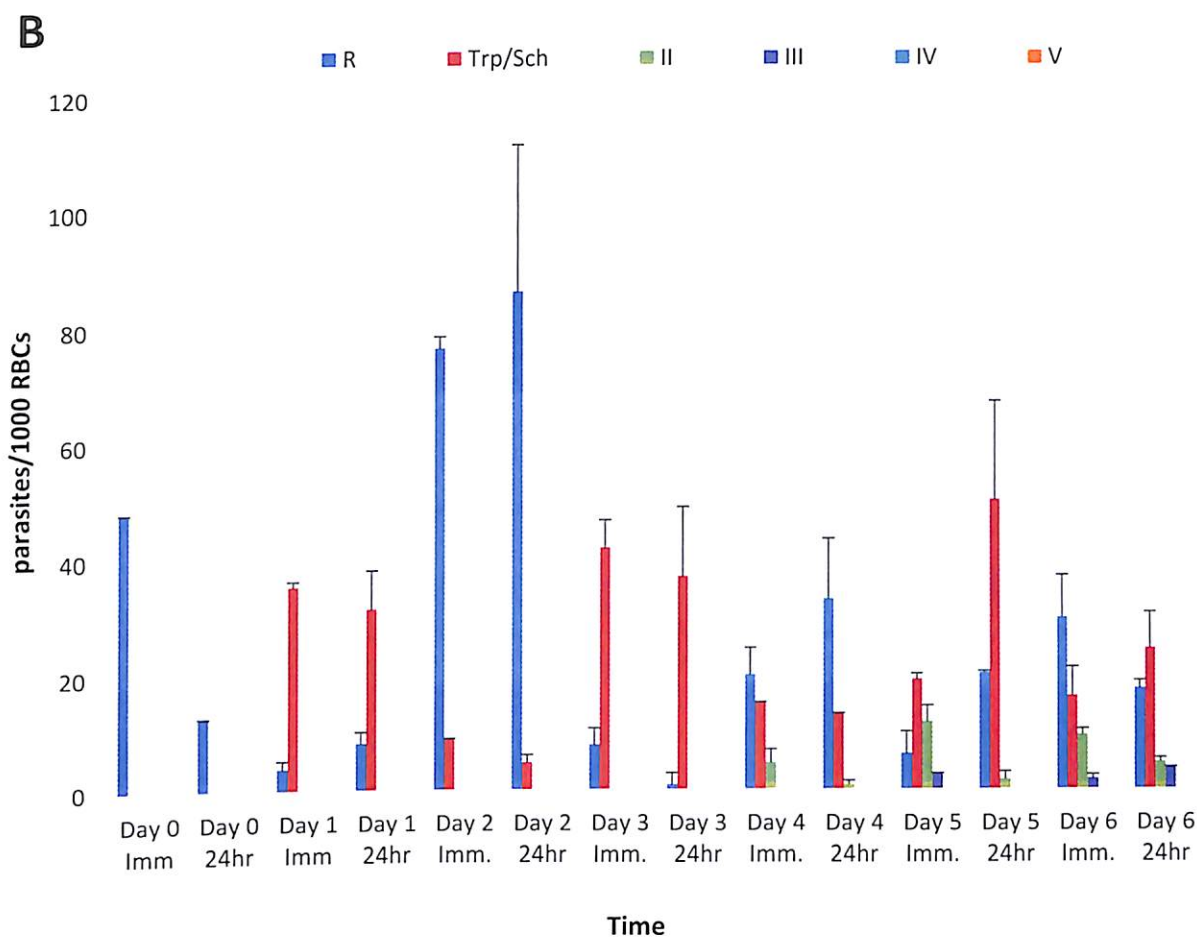
Figure 9 B: Growth pattern of NF54 parasites in the NAG free controls. **Imm = Immediate plating, 24hr = plate after 24 hours.** The graph shows mean ± SD of parasitaemia for the parasite stages (rings, Trp/Sch- trophozoites/schizonts, and I-V gametocytes) and on six days *in vivo* cultures. Error bars estimated from three independent microscopy readings.

The growth pattern in Figure 8 shows parasite stages from the assay with daily NAG media for six days. Time for putting the parasites into culture immediately or at room temperature (RT) for 24 hours after sample collection and the effect on the parasite growth were assessed. The RT overnight incubation period is comparable to the time at which the parasite isolates will get to the laboratory from the study site. From this assay, the parasite growth and stages did not significantly differ (P > 0.05, paired t-test) between the two groups on day six. However, a slight difference was observed in asexual population from day one to four where the estimated parasitaemia in the 24

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hours plating group was slightly lower (P = 0.047, paired t-test) than the immediate (one hour) plating group. The time for adding the NAG after 24 hours incubation where the parasites were mostly late trophozoites could have contributed to this difference in parasitaemia.

The *ex vivo* Assay

The study participants were sex balanced but the sex of three participants were not recorded. During the *ex vivo* assay monitoring, two isolates that did not survive in cultures were excluded on day three by microscopy. In all, sixty-seven (95.7 %) of the isolates were successfully cultured and survived in the eight-day *ex vivo* assay. Four isolates were further excluded as their day zero (D0) Giemsa smears were not available and two participants who were above the 12 years maximuim age limit. Finally, sixty-one isolates were used for the gametocyte production rate analysis.

The mean age of the study participants was 68 months and the mean parasite density was 4.731/μL (log (10)) per microliter of whole blood (84833/μL untransformed). None of the participant had microscopic detectable parasites on day seven after three days of standard antimalarial treatment. The mean white blood cell (WBC) count was 7.3 x10^3 /μL and within the acceptable normal range of 4.5 to 11.0 × 10^3/μL. The mean WBC seen was expected as *Plasmodium* infections do not increase or decrease WBC in the host. Most of the participants had haemoglobin levels (< 11 g/dL) below the acceptable normal range 12 to 18 g/dL and could result from infections with *P. falciparum*. The average temperature for day zero was 38 ^{0}C and much higher than the day seven average temperature (Figure 9).
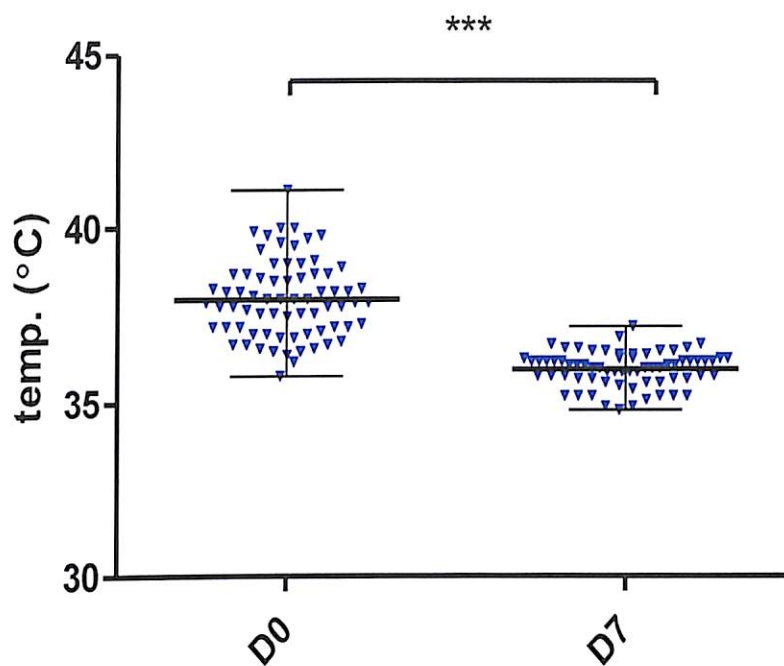
Figure 10: Body temperatures of participants during the clinical visits. Temperature measured before standard ACT treatment (D0) and four days after treatment (D7). Difference in participant body temperature and represented as upper and lower limits of the mean.

The mean body temperatures between the visits differed and were statistically significant (P < 0.001, using paired t-test) as temperature deceased for the day zero and seven visits. The difference in the mean temperature between the visits was 2°C with an average temperature of 38°C recorded on day zero and 36°C for day seven (Figure 9). This decrease in temperature on day seven may have been a result of decreased or clearance of parasite after antimalarial treatment.

Haemoglobin genotype effect on haemoglobin level

Three haemoglobin genotypes were observed in the study participants and the mean haemoglobin (hb) levels were assessed to find the correlation between hb levels or haematocrit with gametocyte densities (Table 2).

Table 2 Average haemoglobin levels within the three β-globin genotypes in the study population

Parameter	AA	AS	AC
Mean	10.60	9.60	11.05
Range	(5.90 -14.10)	(7.80-11.50)	(8.50 -12.80)
n	47	5	6

The average haemoglobin (Hb) level in AC participants was slightly higher than the AA and AS groups. The AS group had the lowest Hb level among the variants but statistically not significant ($P = 0.47$, Kruskal-Wallis One-way ANOVA) among the genotypes. The representations of the AC and AS variants were low among the participants.

The mean haemoglobin levels of the three haemoglobin genotypes among the participant did not statistically differ ($P > 0.05$). However, fewer participants in the HbAA normal genotype group had haemoglobin levels lower (below 6 g/dL, classified as severe anaemia) than in the variant groups.

Gametocyte production rate in *ex vivo* Assay

Mixed infections of *P. falciparum* and *P. malariae* were detected by microscopy in 2.9% (two participants) and by PCR in 5.7% (4 participants) from the isolates population.

Progress of D0 Ring to D8 Gametocyte forms in *ex vivo* assay

The isolates parasitaemia and gametocyte production rate were estimated in the *ex vivo* assay. Growth pattern and stages observed in the *P. falciparum* isolates were similar where the isolates progressing from ring stages on day zero to matured gametocytes (stages IV and V) on day eight in the *ex vivo* assay (Figures 10-12).
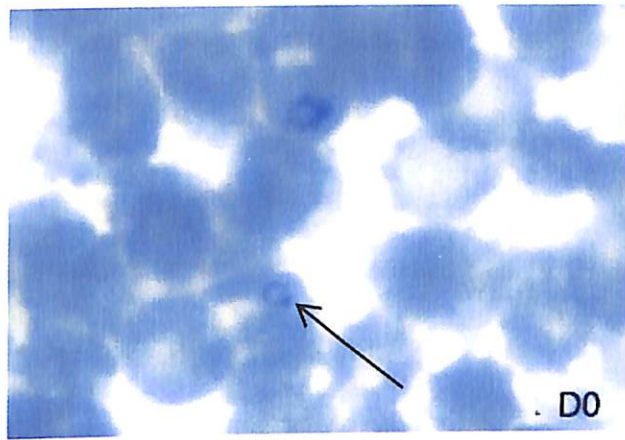
Figure 11: Giemsa stained thin film smear of D0 ring stage parasite for *ex vivo* assay. Microscopic examination of Giemsa stained smears from D0 peripheral blood samples after washing to remove WBCs for parasite cultures. Only ring and matured gametocyte (V) stages were observed showing synchronisation in *P. falciparum* infections in human host.

Collected blood samples were washed for the *ex vivo* assay and all *P. falciparum* isolates stages were rings with six out of the seventy samples having matured gametocytes by thick microscopic smears. The age of the parasite rings among the isolates differed slightly; this is because infections occur at different times and the possibility that different clones infected the individuals. The progress of the parasite isolates as monitored by Giemsa thin smears were comparable to the growth of the isolates reported/expected stages in a previous work (Josling & Llinás, 2015b). For gametocytes growth and the progress monitoring, stage I gametocytes were observed between 24 to 30 hours (day one or early day two) for the assumed 48-hour parasite life cycle. The isolate growths were comparable to the 3D7 strain where the transition from stage I to II and III to V took 48 hours and stages I, III and IV took 24 hours. These were guides for the microscopic monitoring and evaluation of gametocyte production in the *ex vivo* cultures (Figures 11 – 12).

In Figure 11 and on day one (D1), most of the isolates were early trophozoite stages in both NAG and control assays (D1 C & D1 N). Day two

wells had late trophozoite to early schizonts stages in the controls (D3 C). However, in the NAG (D3 N) well the parasites were mainly stage I to II gametocyte and dead merozoites. On day four, the control (D4 C) well had mixed parasite stages with predominant merozoites, rings and few stage II gametocytes. The NAG wells on day four (D4 N) were mainly stage II gametocytes (Figure 11).

In Figure 12, the parasites in the control (D5 C) wells on day five were mainly matured rings (to trophozoites) and schizonts with immature gametocyte stages (I, II and III). In the NAG (D5 N) wells, the parasites were mainly stage II and early III. On day six, the parasites in the control (D6 C) wells had crashed (absence of RBCs for new merozoites to invade) and were mainly merozoites. The D6 N had late stage III and early stage IV gametocytes and on day seven and eight the control (D7 C & D8 C) well had asexual and gametocyte of all stages. The NAG well had stage IV and few V (D7 N & D8 N) parasites. In general, high parasitaemia was observed in the control wells, as expected where NAG prevented the merozoite from invading of new RBCs.
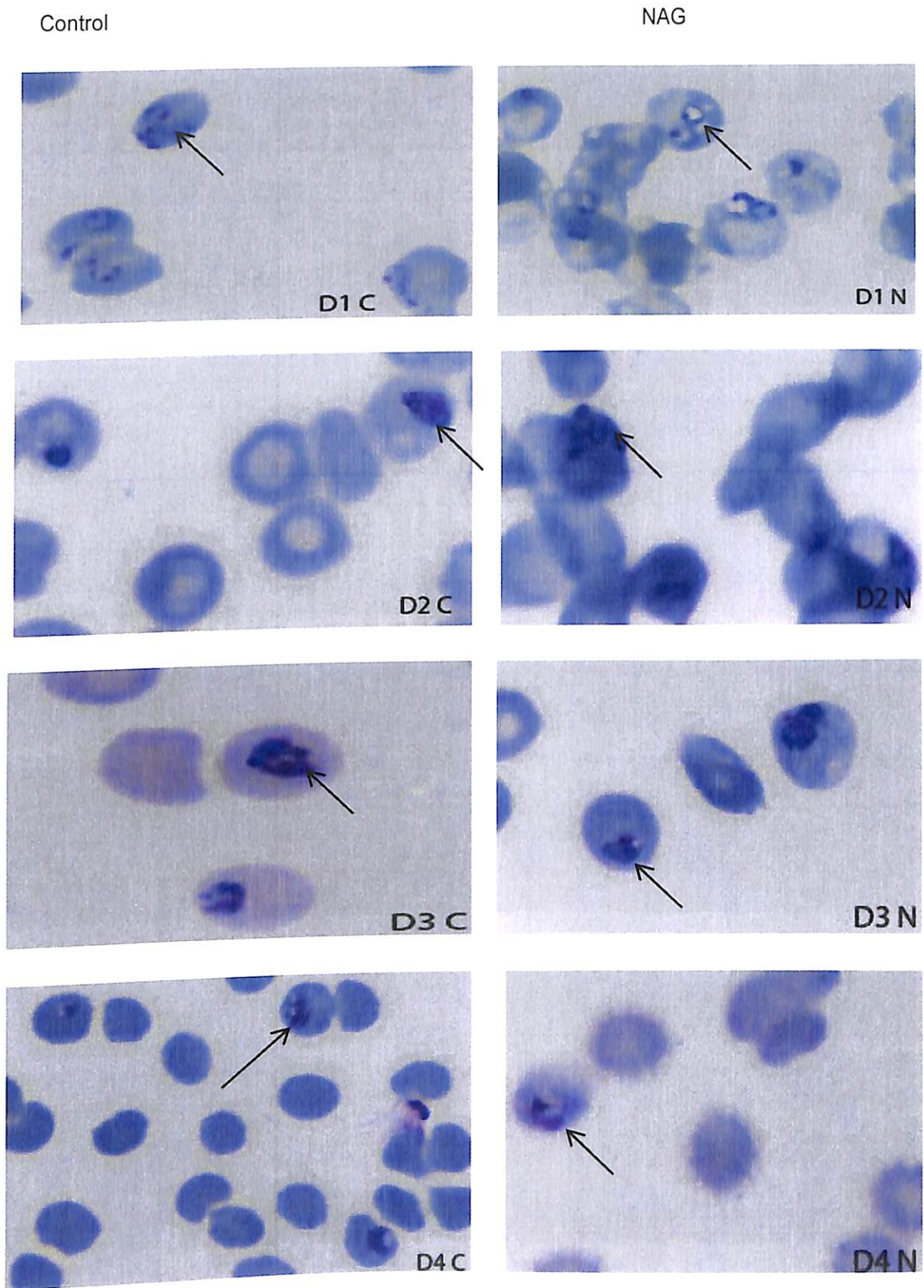
Figure 12: Images of *P. falciparum* isolate growth from day one to four in *ex vivo* cultures. The arrows show the developmental stages of the isolates between the NAG (N) and controls (C). The D4 N gametocytes (stages II) are from the sexual committed rings on day zero.

Control   NAG
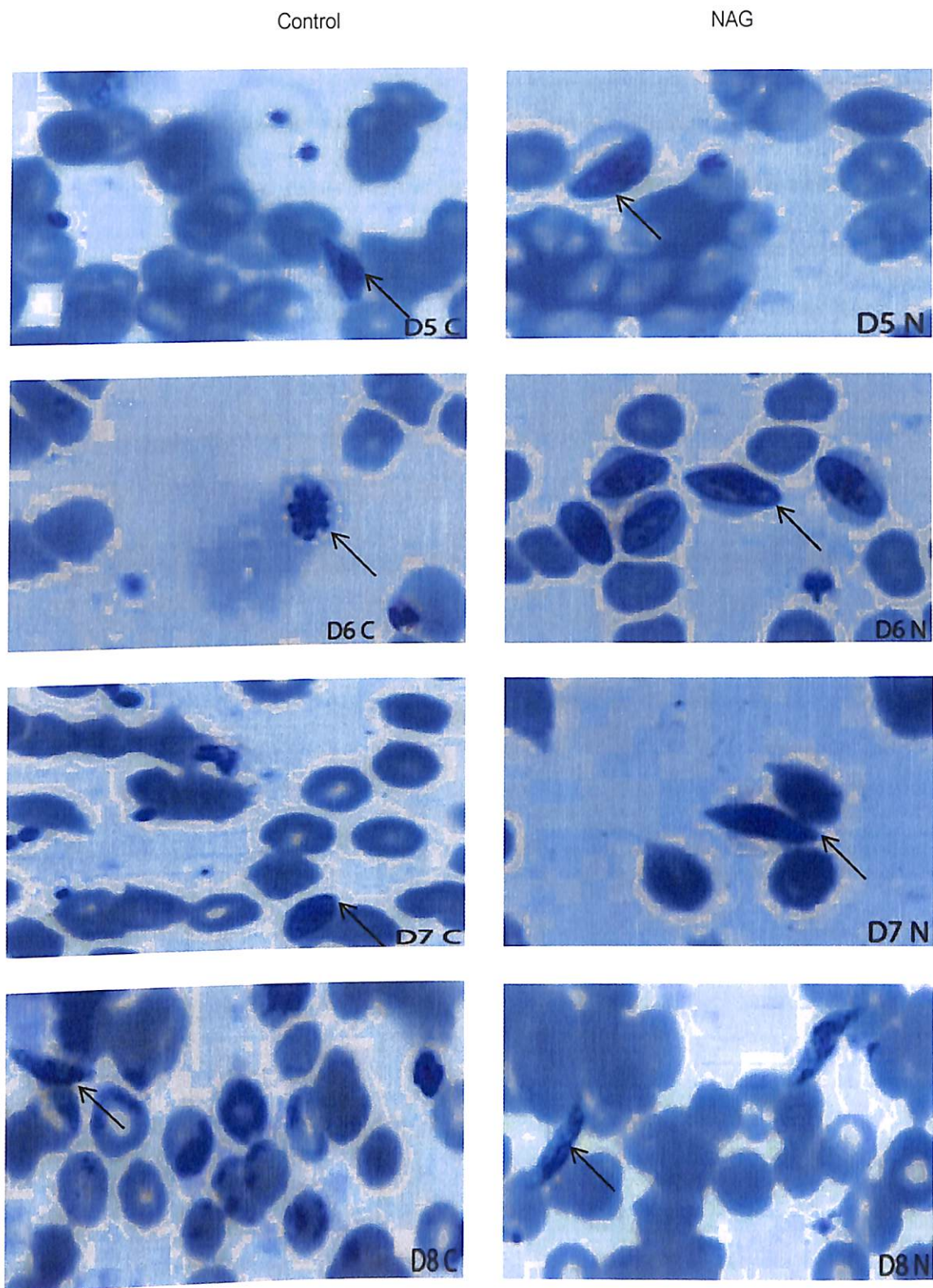
Figure 13: Images of Giemsa stained parasites from day six to eight cultures.
Arrows are showing the developmental stages of the isolates in the NAG (N) and control (C)
wells. The D6 N well have predominant stage III gametocytes, D6 C had high asexual
population and few RBCs. On day 8, the D8 N parasites were mainly stages VI gametocytes
and the D8 C had different population of gametocyte stages and dead asexual parasites.

Gametocyte Production rate in the *Plasmodium falciparum* isolates

Day zero parasitaemia was used to estimate gametocyte production rate daily in the *ex vivo* assay for the isolate (Figures 13 to 14). Gametocyte production rates were compared between the isolates that made gametocyte in the NAG (gametocyte producers) and those that did not (gametocyte non-producers) control assays (Table 3) and among the three haemoglobin genotypes (Table 4).
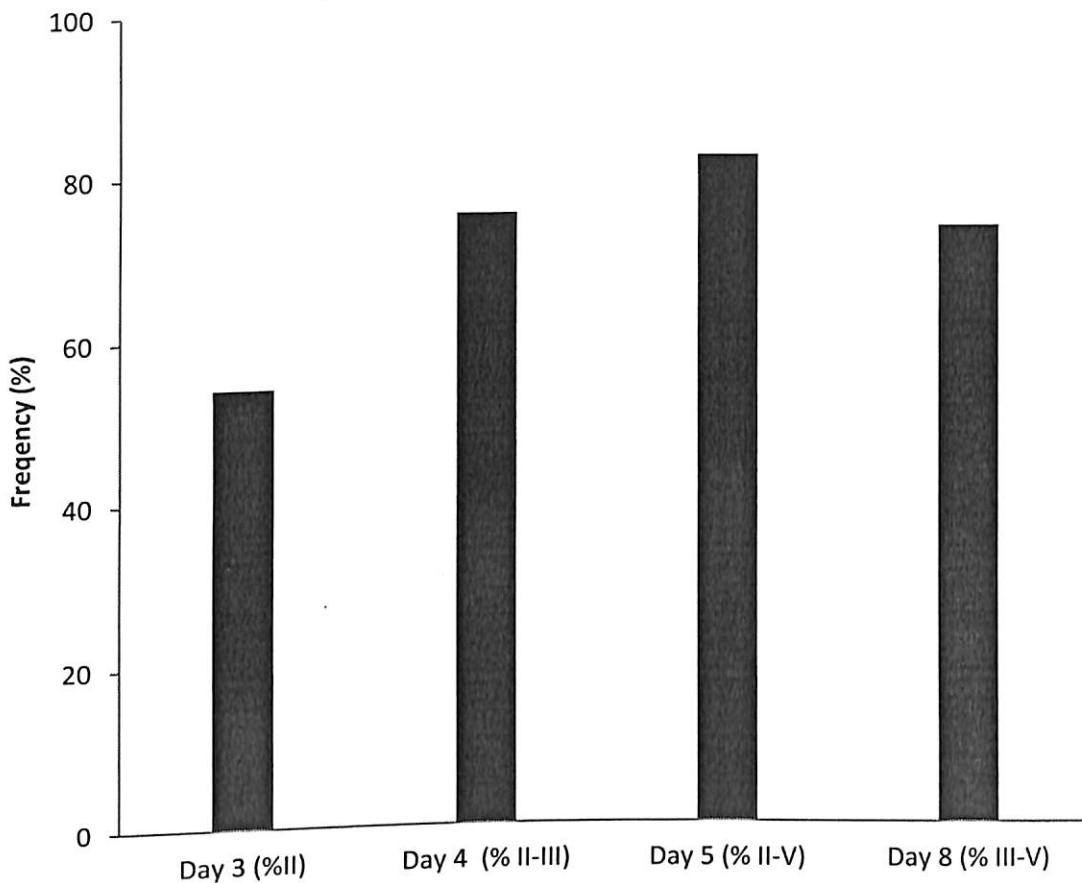
Figure 14: *Ex vivo* rate of gametocyte production in the NAG assay. The graph is showing mean gametocytecemia in four different days, with D5 having the highest numbers. Immature gametocytes (II & III) in D5 to D8 are not from DO committed rings.

The only positive correlation between day zero parasitaemia and gametocyte production rate was observed on day three (P = 0.035 by Spearman r 0.3683 and two-tailed t-test). However, on day three fewer isolates

had gametocyte than day four to eight. The parasite stages were comparable to that observed in the 3D7 and NF54 laboratory strains during the *in vitro* assays. The fewer number of isolates with gametocytes in the estimate NAG assays could result from the high asexual parasitaemia that overshadow the lower gametocytecemia. Also in the NAG assays, dead asexual parasites made it difficult for identification and differentiation of the stages II and III on day three but easy on day eight where the asexual population was less than 3% (Figure 13).

The observed reduction in the gametocyte population on day five to eight could be attributed to the transition of parasite growth stages from III to IV. High numbers of gametocytes were observed on day eight compared to day three and this could result from culture-induced factors such as spent CPM. Nine samples did not make gametocytes in any of the four assessment days. These nine samples without gametocytes in the NAG assay were assessed to verify if the isolates were those that did not make gametocyte at all or late gametocyte producers. The control assays also served to monitor the successful growth of the isolates in the *ex vivo* system (Figure 13).
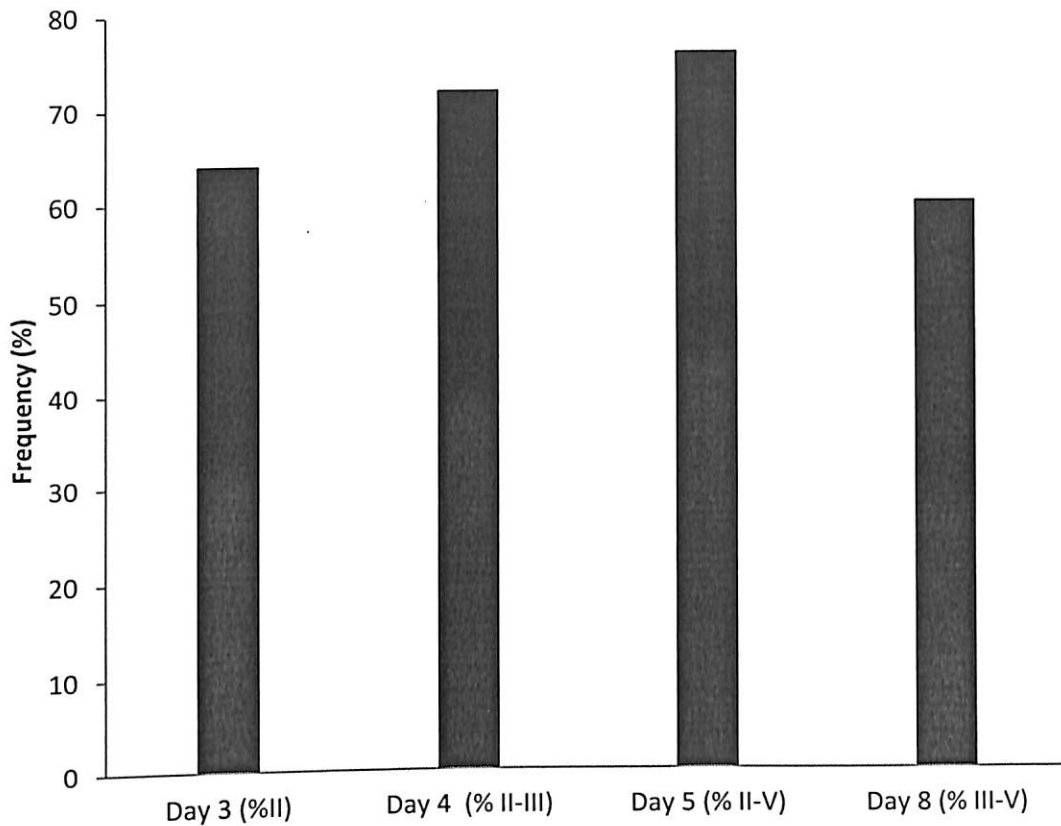
Figure 15: *Ex vivo* rate of gametocyte production in the control assays. The mean gametocytecemia in four different days and D5 having the highest number of isolates with gametocyte.

The counts estimated from the control assay (D3 C, D4 C, D5 C and D8 C) were similar to those assessed in the gametocyte producing isolates (Figure 14). Parasite survival or gametocyte production in *ex vivo* culture settings was confirmed as previously explained. The control assays of the nine non-gametocyte producing isolates had asexual parasites on days two and three as they survived the *ex vivo* conditions. Gametocyte production in the control assay was also similar to the NAG gametocyte assay.

Table 3 Gametocyte production in the non-gametocytes controls and producers NAG assays

Parasite stage	Gametocyte producers NAG			Non-gametocyte Control			
	median	Mean	Range	median	Mean	Range	P-value
D0 % PT	2.8	3.89	(3.81-24.1)	2.4	3.12	(0.3 – 7.6)	0.01
D3 %II	0.02	0.04	(0.002-0.27)	0.3	0.04	(0.002-0.27)	0.74
D4 %II-III	0.06	0.14	(0.002-1.161)	0.04	0.09	(0.002-0.32)	0.47
D5 %II-IV	0.06	0.22	(0.004-2.42)	0.07	0.16	(0.009-0.79)	0.74
D8 %III-V	0.09	0.28	(0.002-1.93)	0.09	0.18	(0.007-0.67)	0.80

The data presented as median and the p- valves as a measure of Fisher exact-test between the two groups.

The Table 3 shows the statistical representation of the gametocyte count between gametocyte producers NAG and non-gametocyte producer control assays (Table 3). There was no significant difference (P = 0.85, df =1 by Fisher exact-test) in the mean gametocyte production between these groups. The only observed difference in the groups was their day zero mean parasitaemia which was statistically significant (P = 0.01). The mean D0 parasitaemia for the NAG gametocyte producers was higher than the non-producing group. However, gametocyte production rate among the groups and days (D3, D4, D5 and D8) were statistically not different (P = 0.3203, df = 1). The mean or median gametocytes production rates in the control were comparable to the gametocyte producing NAG assay. The nine isolates that did not make gametocytes in the NAG assay could be producing gametocytes at much lower rate. The lower gametocytes production rate observed was less than 0.002% when compared to the minimum gametocytes in the gametocyte

producing isolates. Hence, these nine isolates (non-producers) might require more parasite erythrocytic cycles for their gametocytes to be microscopically detectable.

Table 4 Day zero parasite densities and gametocyte production rate in the β-globin genotypes

Parameter Days	AA (n = 47)		AC (n = 6)		AS (n = 5)		P- value
	Mean	Range	Mean	Range	Mean	Range	
D0 PT	3.29	0.34-21.01	5.32	2.15-13.28	6.74	1.34-24.06	0.25
D3 GT	0.63	0.10-4.55	2.38	0.15-12.65	0.41	0.00-2.43	0.42
D4 GT	4.12	0.03-52.07	2.40	0.67-7.88	3.50	0.02-7.46	0.90
D6 GT	5.80	0.66-73.49	3.80	0.70-14.33	3.70	0.65-8.64	0.99
D8 GT	6.39	0.13-49.83	4.17	0.65-14.93	6.14	0.04-21.37	0.93

PT-Parasitaemia, GT-gametocytecemia, Parasitaemia, and gametocytecemia among the three HBB genotypes as estimated by microscopy and the association among the HBB genotypes and gametocyte producing rates did not differ.

The difference in gametocyte production rate among the three main observed haemoglobin genotypes in the study participants were not significant (P > 0.2) using non-parametric one-way ANOVA test (Kruskal-Wallis test). By considering, the small sample numbers in the variant groups it made it difficult to draw meaningful statistical significant conclusion from such associations (Table 4).

End point PCR for qRT PCR

All extracted RNAs were checked for gDNA contamination after cDNA preparation. All the controls and the samples from the symptomatic groups

were checked for parasite by amplifying the 150 bp region of the *P. falciparum kahrp* gene using specific primers and the human *18S* gene (Figure 15) comfirmed that gDNA was not carried over during the RNA isolation.
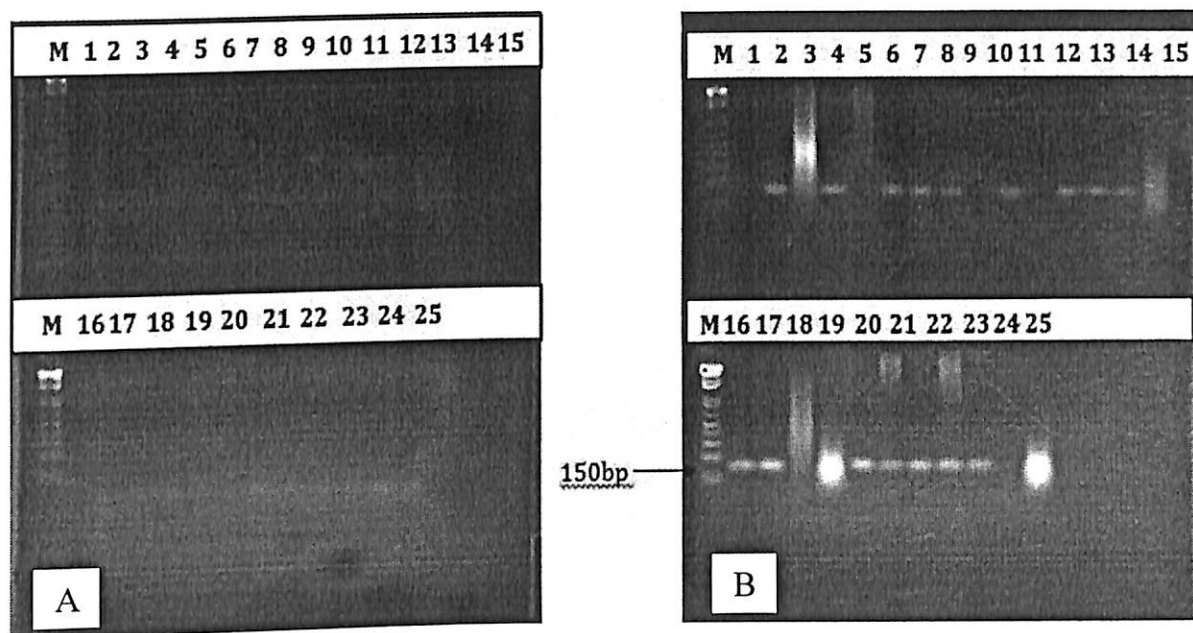
Figure 16: Gel electropherogram of amplified *Pfkahrp* gene fragment
No amplification in the RNA samples show no gDNA contamination during RNA extraction (**A**) and the amplified PCR products in the cDNA samples shows samples with parasite (**B**). The *kahrp* amplified product at 150 bp, M molecular 100 bp DNA ladder, 1-25 are *P. falciparum* clinical isolates on cDNA showing bands and their corresponding RNA.

An amplified PCR product in the RNA template reaction is indication of gDNA contamination during RNA extraction. From Figure 15 A, none of the RNA template reaction had an amplified product and this shows a gDNA-free extracted RNA. Additional validations were carried on cDNA samples that did not amplified. From Figure 15 B, PCR negative samples (1, 3, 5, 9, 11, 15, 18 and 24), the human *18S rRNA* gene was amplified. No amplified product on gel for the *kahrp* and for amplified for the human *18S rRNA* gene is an indication that these individuals do not have *P. falciparum* infections.

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Submicroscopic Gametocyte Carriage in the Symptomatic Population

After the primer optimization, concentration with efficiencies above 90% was used for qRT-PCR amplification (Figure S 3.1 & S 4. 1 - 4). Random hexamers and oligo (dT) primers combined for the cDNA preparation gave the higher amplified yield in the quantification of the ribosomal RNA and poly-A tailed mRNA genes (Figure 16).
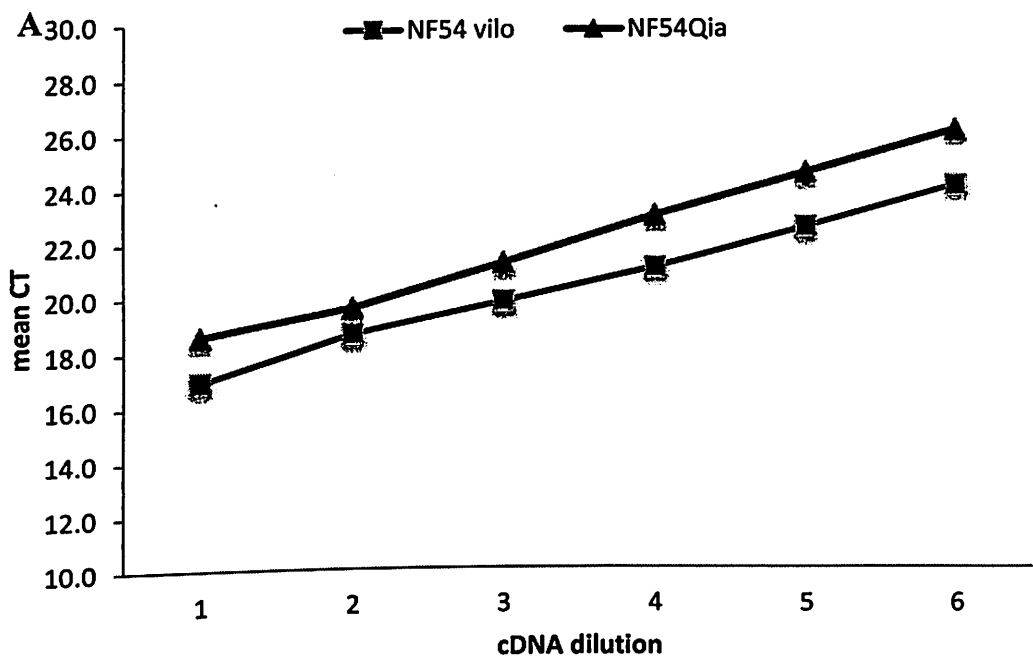
Figure 17: Optimization of reverse transcription kits for cDNA preparation. The mean CTs of the *Pfs25* gene expressions from NF54 matured gametocytes culture. The VILO prep CTs were 2-folds lower than the Qiagen preparation. The CTs are from a six point on standard curve.

From the graph, the mean cycle thresholds (CTs) from the VILO prep were about two CT points lower than the Qiagen preparation. RNA used for this experiment was extracted from matured NF54 gametocytes and the qRT-PCR on 1:10 cDNA dilution on a standard curve plot (Table S 3.5). The VILO kits gave lower mean CTs (16 to 24) than the Qiagen kits (18 to 26) and the VILO kit was ideal for low transcript genes and/or those with fewer copy number of gene (P = 0.002, paired T-test).

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The VILO master mix with random hexamers was supplemented with oligo (dT) to amplify the poly A tail of *Pfs25* mRNA transcript. The cocktail of random hexamers and oligo (dT) ensured the amplification of *18S rRNA* endogenous control.
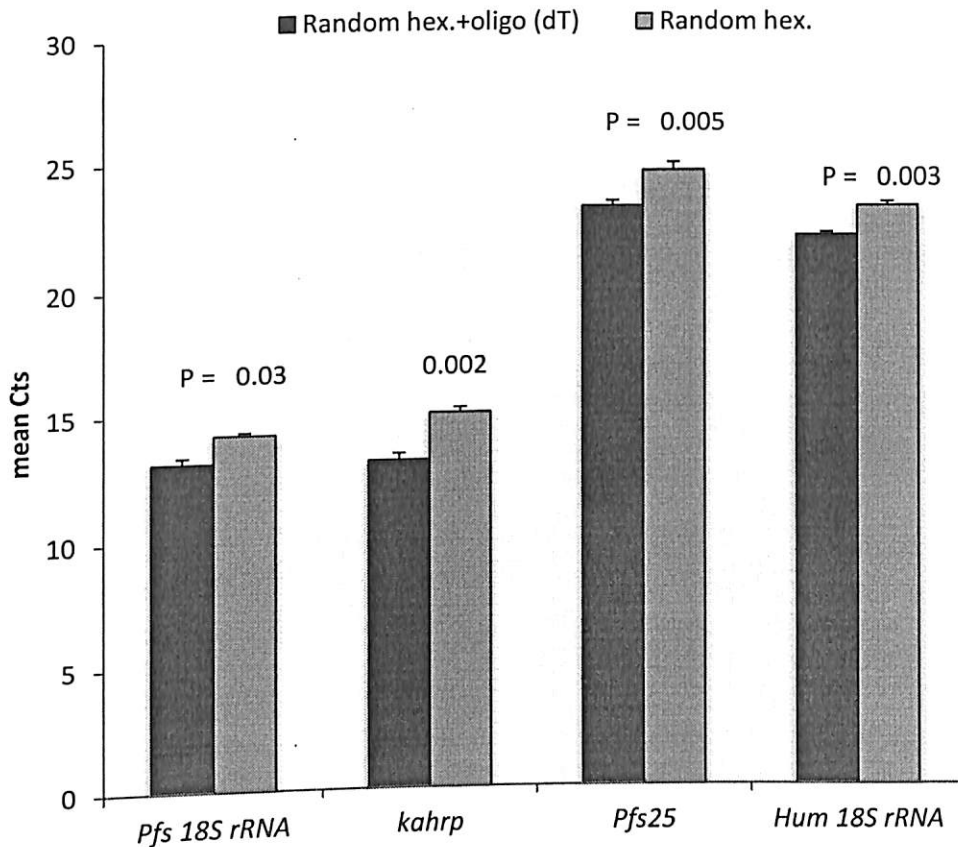
Figure 18: Mean CT for the cDNA priming optimization with parasite and human genes. The mean CTs from the expression profiles with just random hexamers and cocktail (random hexamers + oligo (dT). The gene expression in the single and cocktail of primer(s) used in the cDNA preparation for the amplification of three parasite and human genes.

In Figure 18, the same concentration of RNA extracted from *P. falciparum* C9 strains mainly 90 % ring stages at 3.5% were used for the preparation. Three parasite (*18S rRNA, kahrp* and *Pfs25*) and a human (*18S rRNA*) gene transcript profiles were assessed in each preparation. The amplification CT in the combined primer preparation was lower ($P < 0.04$)

than the random hexamers. The mRNA priming with oligo (dT) is ideal for *Plasmodium* genes since it has numerous polyA-tailed mRNA genes.

Parasite estimation in qRT-PCR

Parasitaemia estimated from Giemsa smears and haemocytometer count for the control parasites were used to estimate the number of parasite in a qRT-PCR. This was calculated or extrapolated from the standard curve on the gene transcripts using cDNA from cultures containing matured gametocytes of *P. falciparum* NF54 strain, NF54 rings, C9 rings, and RCM47 rings reaction (Table 5 & Figure 18)
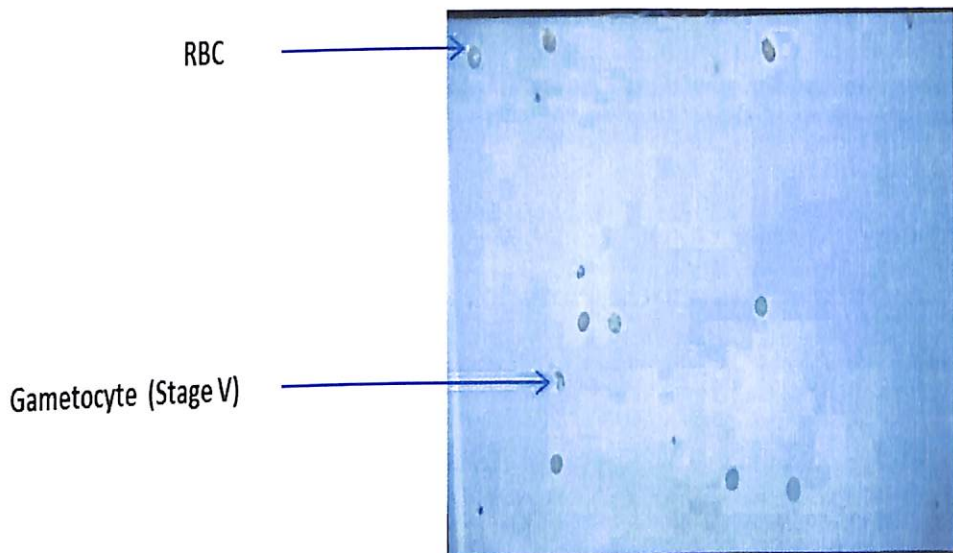
Figure 19: Haemocytometer slide showing RBC and Stage V gametocyte. Total number of cells from haemocytometer will quantify the parasite in a qRT-PCR reaction.

The total cells from each qRT-PCR reaction with parasites (*Pf18S rRNA* and *Pfs25*) or RBCs (RBC from malaria naïve individual as an endogenous control for human *18S rRNA* transcript control) were estimated. The translated CTs to the number of cells in a reaction were affected by the gene expression profile (Tables 5 & S 3.5).

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Table 5 Controls in each gene qRT-PCR reactions and the cells per each reaction

Controls	% PT	Hem./ mL	Total RBC	RBC/mL Trizol	Parasite/ trizol	Parasite/ cDNA	Parasite/ rxn
NF54 Rings	2.6	1.89E+08	4.72E+09	9.44E+08	2.46E+07	8.18E+06	8.18E+04
C9(-) Rings	5.9	3.11E+08	7.77E+09	1.55E+09	9.16E+07	3.05E+07	3.05E+05
RCM47 rings	5.3	3.19E+08	7.96E+09	1.59E+09	8.44E+07	2.81E+07	2.81E+05
Hum. RBC	3.82E+09	3.82E+10	3.82E+10	0	0.00E+00	0.00E+00	
RCM47 rings	1.1	9.28E+08	9.28E+09	4.64E+09	5.10E+07	1.70E+07	1.70E+05
C9(-) Rings	4.8	9.05E+07	9.05E+08	7.24E+08	3.48E+07	1.16E+07	1.16E+05
NF54 gam.	1.9	5.94E+09	5.94E+10	2.97E+10	5.64E+08	1.88E+08	1.88E+06

Hem. –Haemocytometer, culture refers to original cells culture, PT-Parasitaemia by microscopy; gam.-gametocyte. Number of parasite in a qRT-PCR (rxn- Reaction).

Gametocyte Carriage in the Symptomatic Population

The gametocyte carriage in a sample was based on a CT lower than the negative controls (*Pfs25* CTs < 40 and *Pf18 rRNA* CTs < 31). A highly expressed gene was CTs lower than 31, except for *Pfs25* which CTs ≥ 40.
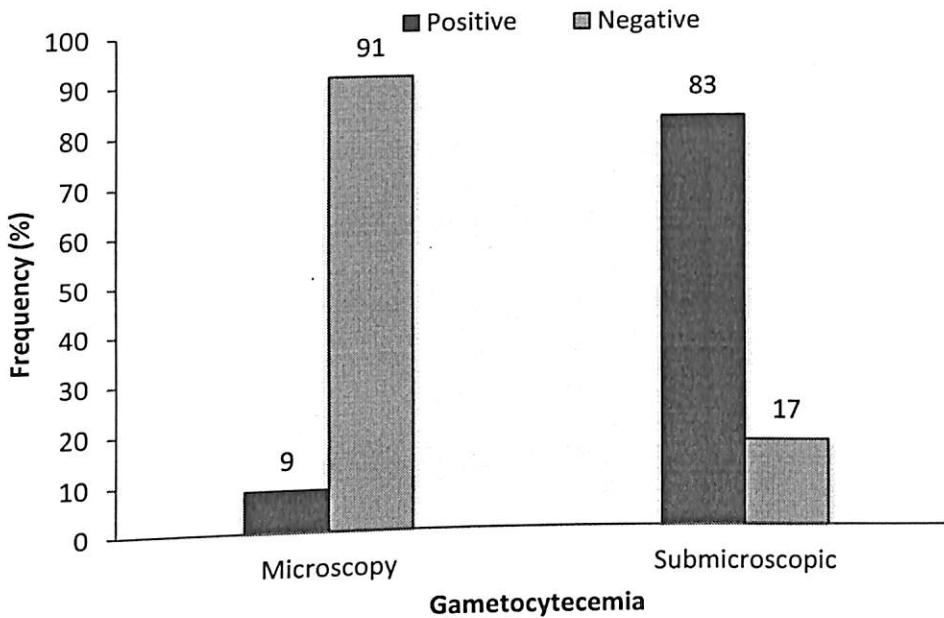
Figure 20: Gametocyte prevalence in the symptomatic group at microscopic and submicroscopic levels. Microscopic prevalence was lower than submicroscopic prevalence.

Before treatment, three samples had gametocytes by microscopy (3/35), but was 30 (n = 35) when assessed by qRT-PCR at the submicroscopic level (using CTs of no template and naïve human whole blood controls). It can be inferred from Figure 19 that most of the isolates (87%, n = 35) had matured submicroscopic gametocytes on day zero. The *Pfs25* is expressed by mature female gametocytes in stage IV and V. The gametocyte prevalence by microscopy and submicroscopic differed statistically (P < 0.0001, df =1).

The School children group

The school children who participated were 61 and 57 from Cape Coast and Obom respectively and sampled during the rainy and dry seasons (Table 6). The difference in the mean body temperature of the participants during visits was not significantly different for both seasons (Rainy: $P = 0.42$; dry: $P = 0.14$ using Mann Whitney two tailed t-test). Within the study sites and seasons, the participants body temperature readings did not differ ($P = 0.56$ and $P = 0.26$). Mean haemoglobin level in participants from Cape Coast was significantly higher ($P = 0.05$) than those from Obom. In the rainy season at Cape Coast, the mean parasite density was 1753 parasites/μL of whole blood ($\log_{10} = 3.49$), which decreased to 530 parasites/μL of whole blood ($\log_{10} = 2.49$) during the dry period. However, in Obom, the mean parasite density was 1015/μL ($\log_{10} = 3.05$) in the rainy season, but increased to 2344 parasites/μL ($\log_{10} = 3.19$) of whole blood in the dry season. The number of participants with *P. falciparum* infections decreased during the dry season in both study communities. Although not significantly different ($P = 0.41$), the mean age of the study participants in Cape Coast (9.2 years) was slightly higher than those from Obom (8.9 years) by Mann-Whitney paired two tailed t-test. In both communities, the females were 57% and 53% in Cape Coast and Obom respectively (Table 5).

Table 6 Characteristics of the study population at enrolment for longitudinal study

Parameter	Cape Coast		Obom	
	Rainy season	Dry season	Rainy season	Dry season
Temperature ^{0}C				
Mean	36.41	36.49	36.54	36.35
SD	0.51	0.46	0.56	0.58
Range	(34.40-37.40)	(34.90-37.40)	(35.20-38.60)	(35.20- 7.20)
n	59	60	54	57
Haemoglobin (g/dL)				
Mean		11.79	11.95	10.47
SD		1.61	1.28	2.15
Range		#(7.50-14.60)	(8.900-15.30)	(4.40^- 4.00)
n		60	52	55
Parasite density/µL of blood				
Mean	1015	530	1753	2344
SD	971	561	1476	2636
Range	(80-3038)	(80-530)	(160-5040)	(560-6920)
Mean(log$_{10}$)	3.49	2.47	3.05	3.19
SD	0.54	0.58	0.46	0.43
Range	(1.90-2.76)	(1.90-2.47)	(2.20 -3.7)	(2.73-3.84)
n positive	4	2	22	11*
n tested	39	36	34	40
HRP2 (RDT) %				
Positive		31.70		64.10
Negative		68.30		35.70
n	^	61	^	57

^ Not done; # A participant had sickle cell disease; n= number of participants tested in the group; *Malaria control intervention was implemented in the community four (4) months before sampling. # Participants with sickle cell disease were included.
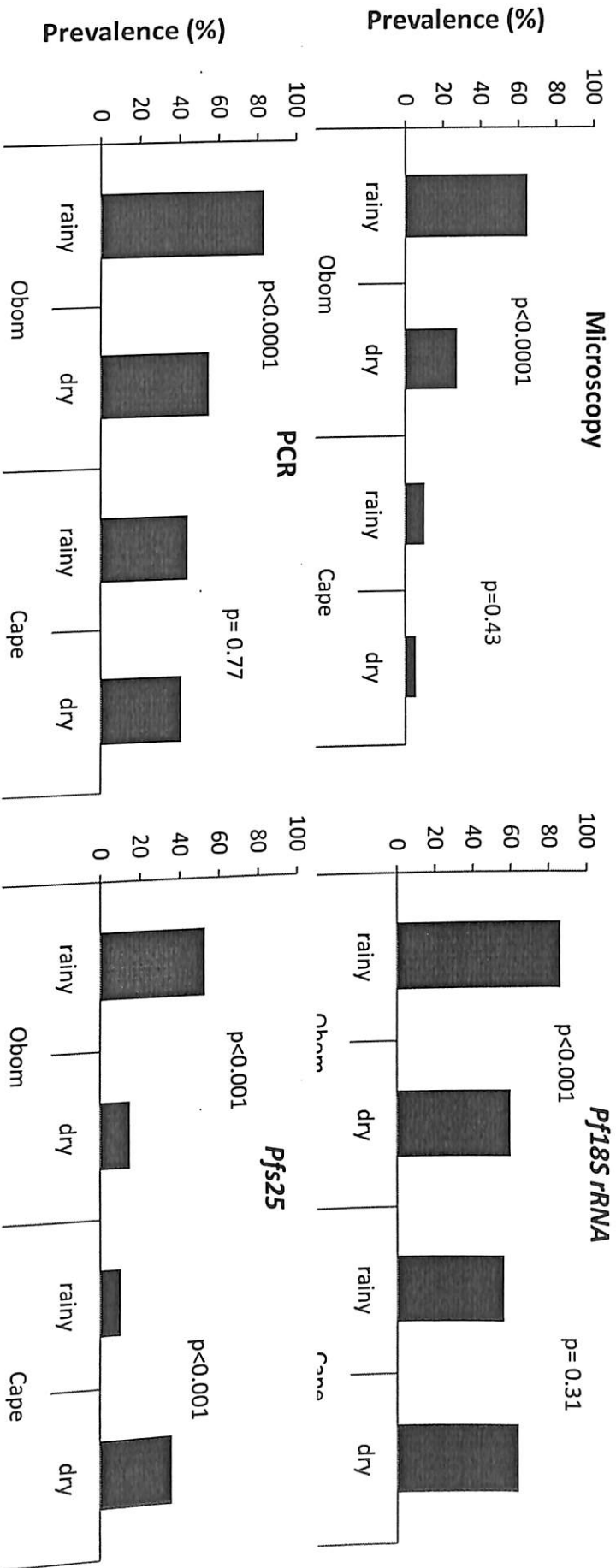
Figure 21: Sexual and asexual parasite prevalence in the school children. Total parasite (sexual and asexual stages) prevalence was estimated by microscopy, PCR, and *Pf18S rRNA* qRT-PCR. The *Pfs25* qRT-PCR detected submicroscopic gametocyte only. Parasite prevalence by PCR used gDNA and by qRT-PCR measures transcripts by mRNA.

Submicroscopic Parasites in the School children

Subsets of 40 samples from each site selected for the assessment of submicroscopic gametocytes gave varied parasite and gametocyte prevalence between the sites and seasons. The extracted DNA from the filter paper blood blots used for conventional PCR amplification and cDNA synthesized from the extracted RNA used for qRT-PCR gave parasite prevalence based on tools sensitivity and specificity (Figure 20).

Infection prevalence among children with *P. falciparum* by microscopy, PCR and qRT-PCR was evaluated in the study communities. Parasite prevalence in Obom was 65% by microscopy in the rainy season and 28% in the dry season, while in Cape Coast it was only 10% and 6% in the rainy and dry seasons, respectively (Figure 20). As anticipated, both PCR and qRT- PCR analysis of *Pf18s rRNA* detected higher parasite infections in participants in the communities, 86% in Obom and 56% in Cape Coast during the rainy season by qRT-PCR. The prevalence of parasite measured by qRT-PCR in Cape Coast during the dry season was higher than the rainy season (64% vs 56%), while in Obom the prevalence decreased from 86% in the rainy season to 60% in the dry season. Gametocyte prevalence of 51% recorded in the rainy season reduced to 16% in the dry season in Obom but increased from 10% in the rainy season to 35% in the dry season in Cape Coast. The submicroscopic parasite prevalence estimated by qRT-PCR was 27% (rainy season) and 56% (dry season) by PCR in Cape Coast. Although submicroscopic parasite prevalence estimated by both PCR and qRT-PCR did not change significantly between the rainy and dry season (p = 0.38), a

pronounced increase was observed among submicroscopic gametocyte prevalence ($P < 0.001$) {Figure 20, Table 7}.

When comparing parasites and gametocytes estimates by PCR and qRT-PCR, a significant difference ($P < 0.05$) was observed between these detection methods. During the rainy season, 41% of children in Cape Coast and 30% of children in Obom harbored asexual parasites without gametocytes. The reverse trend was observed in the dry season where the prevalence of children harbouring only asexual parasites was higher in Obom (41%) than in Cape Coast (28%). This study recorded very high prevalence of asymptomatic parasite carriers among the children from the study communities.

Table 7 Seasonal distribution of *Plasmodium falciparum* prevalence in the study population

Site & season	Microscopy			PCR			Pf18S rRNA			Pf25		
	%	χ2	P value	%	χ2	P value	%	χ2	P value	%	χ2	P value
Cape Coast rainy^	10	0.61	0.43	44	0.08	0.77	56	1.02	0.31	10	17.6	<0.001
Cape Coast dry^	6			41			64			35		
Obom rainy^	65	26.1	<0.0001	83	17.04	<0.0001	86	15.6	<0.0001	51	30.5	<0.001
Obom dry^	28			55			60			16		
Cape Coast rainy*	10	62.2	<0.0001	44	31.2	<0.0001	56	20.4	<0.0001	10	40.9	<0.001
Obom rainy*	65			83			86			51		
Cape Coast dry*	6	15.6	<0.0001	41	3.4	0.066	64	0.19	0.66	35	10.5	0.001
Obom dry*	28			55			60			16		

Parasite prevalence in Obom and Cape Coast during the rainy and dry seasons as measured by microscopy (LM), conventional PCR (*Pf18S rRNA*, DNA amplification) and by qRT-PCR (*Pf18S rRNA*, transcript amplification) and submicroscopic parasite carriage determined using *Pfs25* qRT-PCR. Infection prevalence was analyzed by Two-sample test for equality of proportions (R version 3.4). ^ Compare parasite prevalence within the communities in two seasons. *Compare of parasite prevalence between communities in the two seasons.

Host Factors that affect *Plasmodium falciparum* Gametocyte Prevalence

The 59 (Obom) and 58 (Cape Coast) of the participants' human β-globin, G6DP and ABO blood groups were assessed and their associations with gametocyte prevalence were determined in the school children group. The distribution of the human genes in the sites is summarized in Figures 21 to 23.

Beta haemoglobin Genotypes in the school children group Population

By using haemoglobin migration pattern, six different beta haemoglobin genotypes (AA, AC, AS, CC, SC and SS) were identified in the study population (Figure 21).
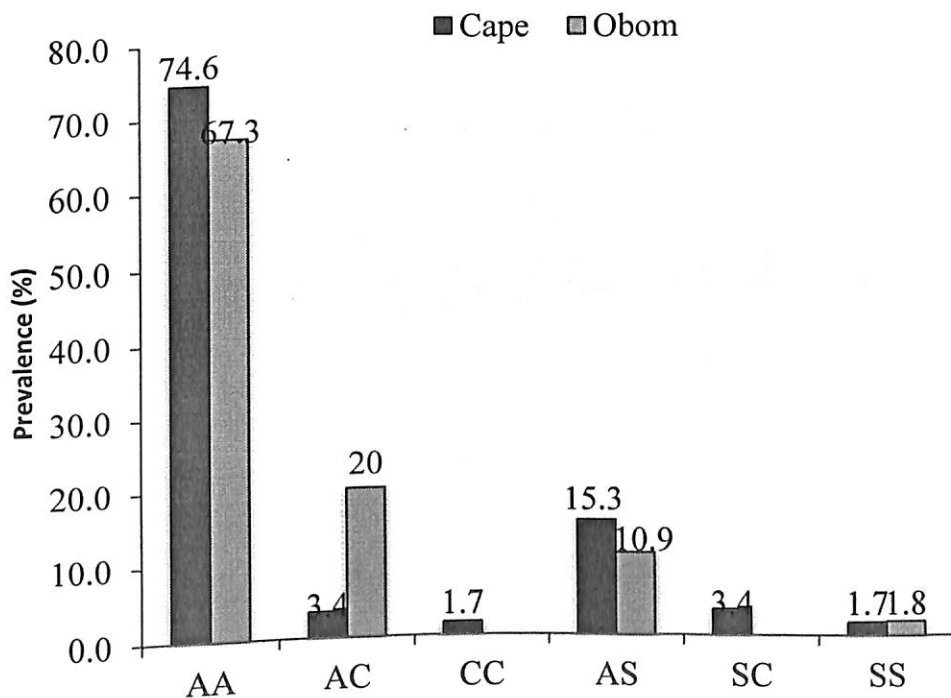
Figure 22: Haemoglobin genotypes observed in study participants.The normal HbAA genotype was higher among the participants in the two study communities. The variant Hb genotypes were least represented.

The prevalence of the normal haemoglobin (Hb) genotype (AA) was 67% and 75% in Obom and Cape Coast respectively. Among the haemoglobin variants, participant with heterozygous C allele were higher, 20% (n = 55) in Obom and only 3.4% (n = 59) in Cape Coast (Figure 21). The heterozygous S was the second dominant variant and was found in 15.3% of the participants from Cape Coast and 11% among participants in Obom. The heterozygous Hb SC (3%) and homozygous Hb CC (2%) variants were observed only among the Cape Coast population. The homozygous Hb C and Hb S were the least found variants in the participants (>2%) from the study communities. By grouping the haemoglobin into normal (Hb AA) and abnormal (heterozygous or homozygous variant) participants from Obom had the highest abnormal variant of 33% (18/55) while 25% (15/59) was recorded from Cape Coast.

ABO Blood groups in the School children Population

All four (A, B, AB and O) common ABO blood group phenotypes were observed in the study. The distribution and frequencies of the blood group phenotypes in the study population are summarized in Figure 22.
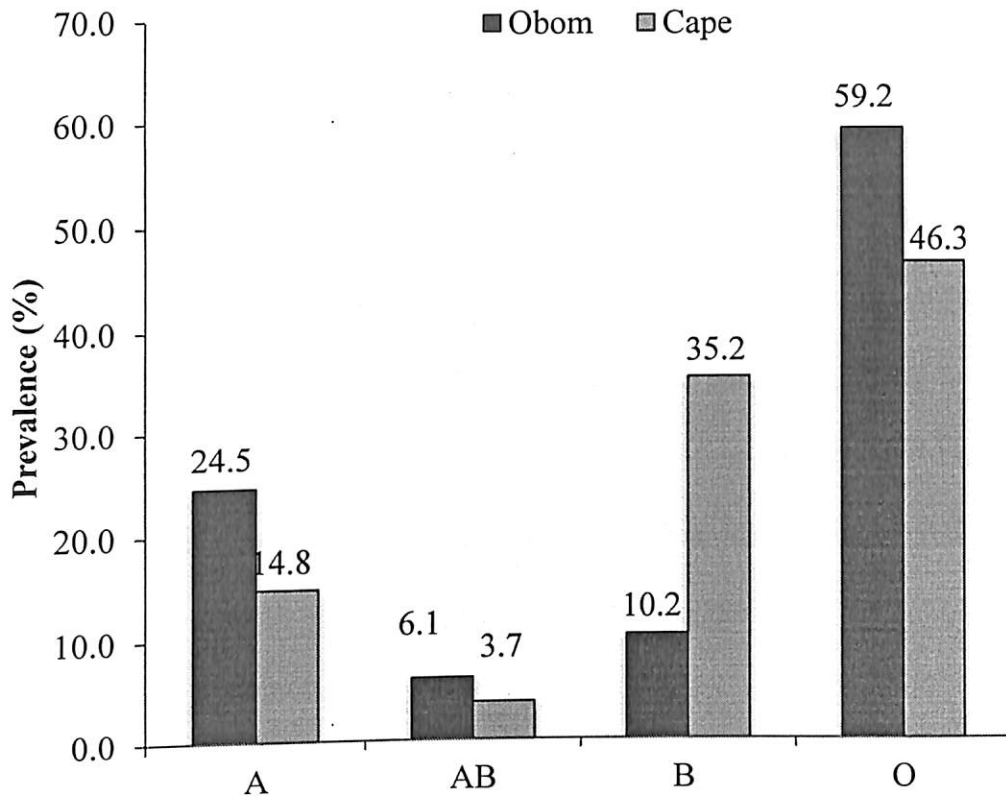
Figure 23: Distribution of the ABO blood groups in the study population.
The blood group O phenotype was higher among the participants from both communities.

There were more blood group O phenotypes among the participants in both communities; the observed frequency was slightly higher in participant from Obom (59.2%) than from Cape Coast (46.3%). On the other hand, the blood group B phenotype was more prevalent in Cape Coast, with 35.2% (19/54) compared to 10.2% in Obom. Blood group A phenotype was also the second highest group in Obom with 24.5% (12/49) and third highest in Cape Coast with 14.8% (8/54) in the population. However, blood group AB was the least phenotype observed in both communities with 6.1% and 3.7% in Obom and Cape Coast respectively.

G6DP Genotypes in the School children Population

In this study, the three prevalent sub-Saharan Africa X-linked G6DP variants were genotyped. These were the wild type B, the A (A376G) mutant variant with about 90% of the enzyme activity and the A- which is A376G variant with one or more of the G202A, G542T, G680T or T968C variants having 8% to 20% of the wild type enzyme activity. The polymorphic frequencies were described based on the following classification; deficient (A- and A-/A-); Heterozygous deficient (AA- or BA-) and the normal enzyme activity variants (A, B, AA, BA, BB).
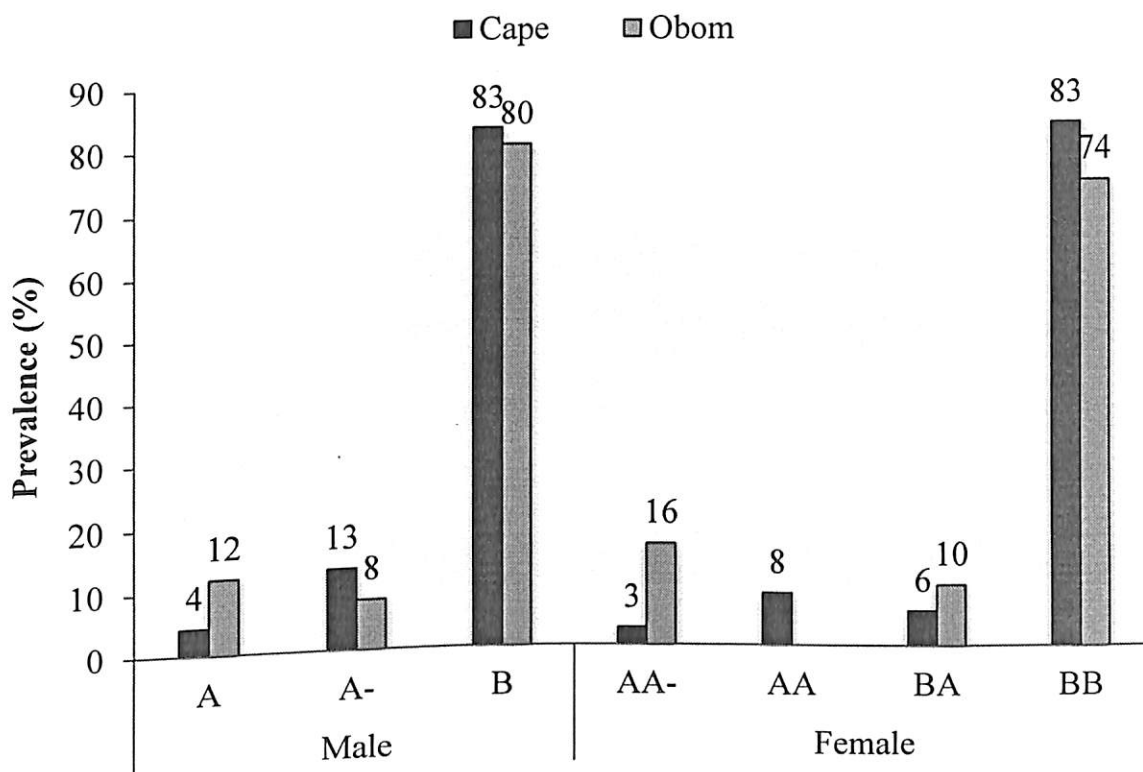
Figure 24: G6DP genotypes in the study population represented among the sex. The normal genotype was higher among the participants from both communities and the variants were less than 14% of the sexes from the communities. The full deficient were found only in the males as the gene is X-sex linked.

The normal G6DP genotype was dominant in the sexes and sites. In the females, 83% and 74% of the participants in Cape Coast and Obom had the

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normal alleles (BB). The major variant found in the females was the AA-, which was more in Obom (6.1%) than in Cape Coast (2.8%). The AA- was the least genotype observed among the others and the AA variant was observed only in the participants (8.3%) in Cape Coast. The female homozygous (A-A-) variant was not observed in the study population. Among the males, the normal allele (B) was the most prevalent (82.6 %) in the Cape Coast and Obom (80%) participants. The dominant male variant found in the Cape Coast was the A- that occurred in 13% (3/23) in the population. In Obom the G6PD A variant, was dominant and present in 12% (3/25) of the male population. Four participants (6.8%) in the Cape Coast population and seven (12.5%) in Obom carried the deficient variant A- and AA- in the males and females.

Red Blood Cells polymorphism and Gametocyte Prevalence

For the assessment of G6PD, haemoglobin genotypes, and submicroscopic gametocyte prevalence association, G6PD was divided into the normal (individual with BB, B, BA, AA and A) and the deficient (individual with AA-, BA-, A- and A-A-) groups. The haemoglobin genotypes were also grouped into the normal (AA) and abnormal (AS, AC, CS, CC, or SS) genotypes. The proportions of these groupings are summarized in Table 8. However, G6PD and ABO blood group was not associated to gametocyte carriage or positivity (P > 0.10) by Two-sample test for equality of proportions. Within the HBB genotypes, gametocyte prevalence was slightly (P = 0.04, Two way ANOVA) associated associated with abnormal haemoglobin (either homozygous or heterozygous).

Table 8 G6PD, haemoglobin genotype, ABO blood groups factors and submicroscopic gametocyte carriage

| Host factor | Pfs25 (%) | | P – value |
	Positive	Negative	
Blood groups (N = 144)			
O (n = 80)	25	75	$\chi2$ = 12
B (n = 39)	38	62	df = 9
A (n = 20)	20	80	P = 0.21
AB (n = 5)^	60	40	
G6PD (N = 150)			
Normal (n = 141)	28	72	$\chi2$ = 0.67
			df = 1
Deficient (n = 9)	22	78	P = 0.41
HBB (N = 151)			
Normal (n = 103)	25	75	$\chi2$ = 1.92
			df = 1
Variants (n = 48)	35	65	P = 0.16

n is the number of participants tested in each group, ^ sample size too small for proper analysis. Two-sample test for equality of proportions was used to determine the association between gametocyte positive and the host factors

Parasite factors that affect Gametocyte Prevalence

Plasmodium falciparum diversity in the school children with asymptomatic infections were determined for sexual and asexual stage genes. The diversity between the parasite stages was slightly different between the two sites. For the asexual stages, DNA-based *msp2* genotyping (after identification of *P. falciparum* species) was used (Figures S 4.2 & S 4.3) whereas the *Pfg377* genotyping by mRNA was used for the sexual stage diversity.

Plasmodium falciparum asexual diversity by *msp2* genotyping

The prevalence of the FC27 and IC3D7 allelic families of the *msp2* marker was genotyped in *P. falciparum* isolates. The parasite *msp2* genotypes distribution varied slightly in the study communities and seasons (Figure 24).
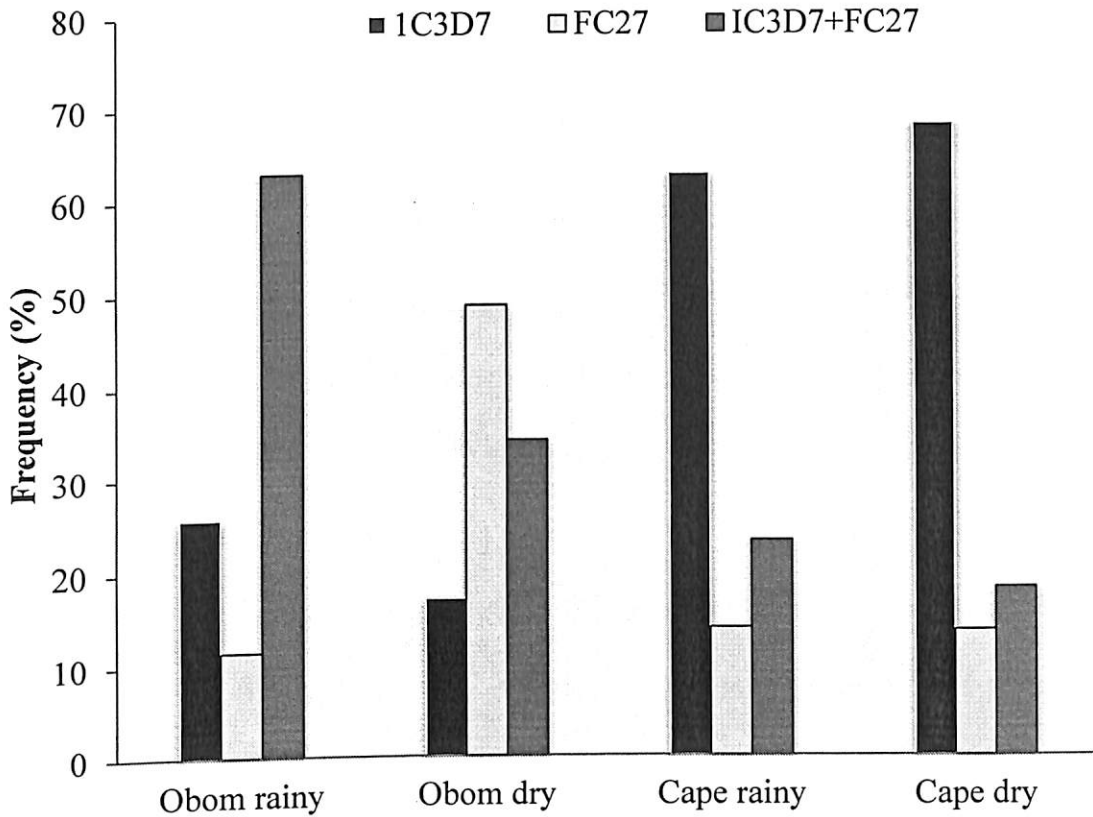
Figure 25: Frequency of *msp2* allelic families in infections. The distribution of parasites within the *msp2* alleles, the prevalence is shown as single (IC3D7 or FC27) or dimorphic (IC3D7 + FC27) clonal infection.

A total of 57 alleles were obtained in Obom from 35 (n = 55) PCR positive samples in the rainy season. The IC3D7 allelic family was more prevalent (9/35) compared with FC27 (4/35). The *msp2* dimorphic infections (both IC3D7 and FC27 alleles) were highest (22/35). In the dry season, 56 alleles were observed in 41 (n = 57) PCR positive samples. The distribution of the alleles was (7/41) for IC3D7 and (20/41) for FC27 families, that increased slightly in the dry season (P = 0.05) and (14/41) for dimorphic infections

(Figure 24). The average multiplicity of infection (MOI) for the *msp2* was 1.6 in the rainy season and decreased slightly to 1.4 in the dry season in Obom.

In Cape Coast during the rainy season, a total of 54 alleles were obtained from 43 (n = 61) PCR positive samples. The IC3D7 allelic family was the most prevalent (27/43) compared to FC27 (6/43) and the dimorphic infections (10/43). In the dry season, IC3D7 allelic family was more prevalent with 16 out of the 31 alleles obtained from the 25 (n = 61) positive isolates, when compared to the FC27 (4/25) and the dimorphic infections (5/25). The IC3D7 infections were more dominant in all infections on both visits. The average *msp2* multiplicity of infection (MOI) observed in the study was 1.3 in the rainy season and did not change much during the dry season visits where the MOI was 1.2.

Generally, the asexual diversity of the *msp2* genotype in the two communities was slightly different. In Obom the dimorphic infections were more prevalent in the rainy season; where the IC3D7 family being higher between the individual clones. This pattern changed during the dry season where the FC27 infections were mostly observed. In Cape Coast, the IC3D7 alleles were more frequent in both rainy (63%) and dry (68%) seasons.

Plasmodium falciparum sexual diversity by *Pfg377* genotyping

Sexual diversity in the parasite isolates was assessed using region 3 polymorphic fragment size of the *Pfg377* gene, a female gametocyte specific marker. Typing the *Pfg377* using mRNA differentiates gametocyte clones while the DNA determines different alleles in the general parasite population (Figure 25).
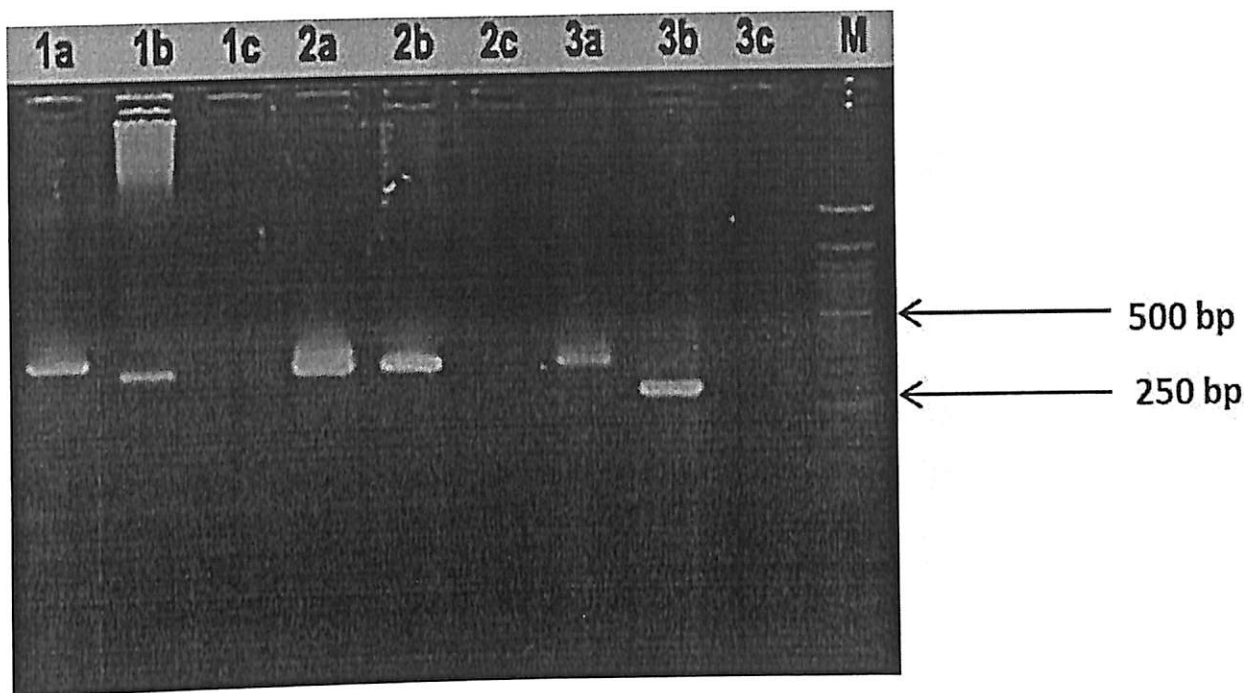
Figure 26: Gel electropherograph of amplified *Pfg377* gene fragment. Lanes: a- gDNA, b- cDNA & c- RNA, M-50 bp DNA ladder. **1, 2 & 3** are samples 1- 3D7 control, **2 & 3** are parasite isolates and **c** for gDNA checks in the RNA. The gametocyte diversity was observed between the base pairs (bp) of **1 b, 2 b & 3 b.**

The amplified PCR product sizes difference as observed on the agarose gel electrophoresis showing diversity among the parasite isolates. The lower PCR product fragment on a gel in the reverse transcriptase (RT) plus mRNA samples confirmed that a transcribe mRNA is amplified. The absence of amplified PCR product on the gel in the **c** columns are indication that gDNA was not carried over during the extraction and purification process in the RNA isolation.

Plasmodium falciparum gametocyte diversity in study population

The *Pfg377* genotyping was able to differentiate the *P. falciparum* asexual from gametocyte stage alleles with introns in the amplified gene region. Five different gametocyte alleles were found in the isolates based on amplified PCR product band sizes on gel, which ranged from 180 bp to 320 bp (Figure 26).

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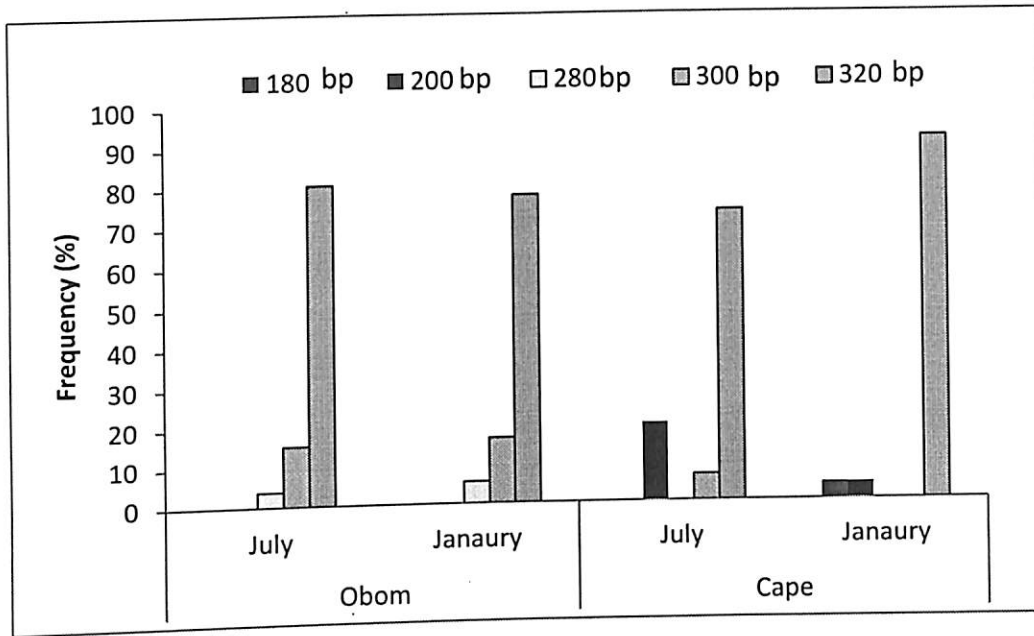
Figure 27: The prevalence of *Pfg377* and diversity in the parasite isolate. Parasite distribution is based on the region three amplified fragments of the *Pfg377* alleles. Prevalence is shown as single distinct alleles, the 300 bp allele was found to be the dominant allele in the two study communities and seasons.

The *Pfg377* genotype was found in 45.6% and 31.6% parasite isolates in the participants from Obom during the rainy and dry seasons respectively. In Cape Coast, *Pfg377* genotypes were detected in 19.7% and 36% of the parasite isolates in rainy and dry seasons. All the *Pfg377* positives samples in Obom had single allele infections during the two seasons. However, in Cape Coast, 25% of the participants had two alleles (200/300) during the rainy season, which decreased to 9% (200/320 & 180/200) in the dry season. Among the five different alleles observed, Obom had three in both seasons whilst Cape Coast had four but the distribution differed between the rainy and dry seasons. Using PCR fragment sizes estimation to determine different sexual alleles, only fewer participants (5/71) had two different gametocyte clones (dimorphic infections). In the parasite population, single allele infections were common and gametocyte MOI in both sites was just low

(<1.5). The presence of different single PCR product band sizes and/or multiple bands in a sample confirms diversity in the gametocytes.

Plasmodium falciparum Drug Resistance

The presence of *Plasmodium* drug resistant isolate has implications on the efficacy of drug treatment and malaria transmission. Genotyping mutations in *Pfcrt, Pfmdr*1, *Pfdhfr*, and *Pfdhps* that are associated with resistance to previously used antimalarial drugs and partner ACT drugs (amodiaquine, SP, mefloquine) were undertaken in this study. The modulation of *P. falciparum* sensitivity to artemisinins is attributeed to any mutation in *K13* gene above 440 to 680 amino acid positions. All PCR amplifications for the drug resistance markers produce single size band as the expected sizes in all cases. All negative controls consistently gave no amplified product before restriction digest or sequencing. The examples of *Pfcrt, Pfmdr*1, *Pfdhfr*, and *Pfdhps* PCR-RFLP electropherogram images are presented in the supplementary data (Figure S 4.2 to S 4.5).
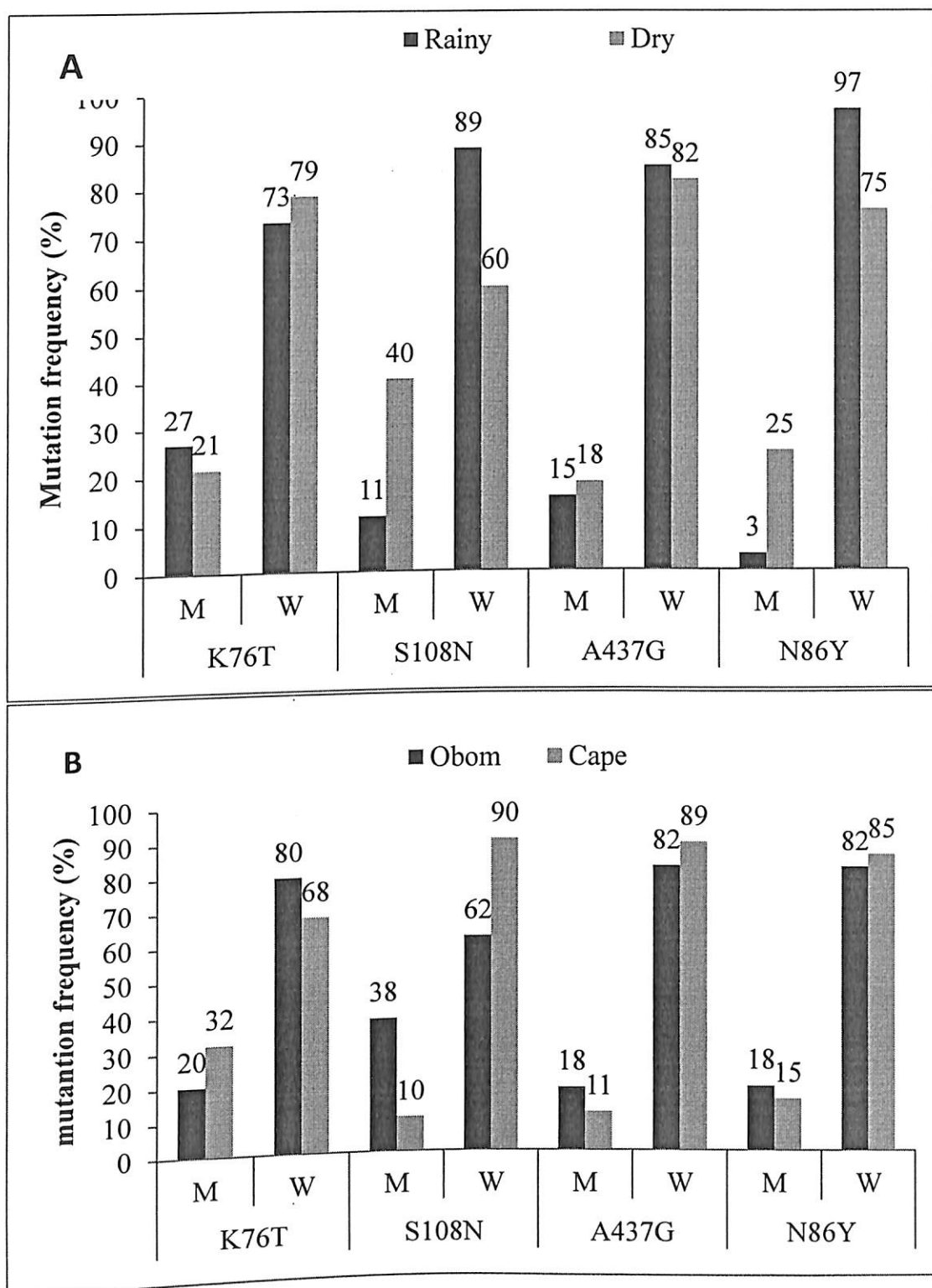
Figure 28: Distribution of the drug resistant strains in the seasons (A) and the study sites (B). **M-** Mutant, **W-** wild type isolates, K76T for *crt*, S108N for *dhfr*, A437G for *dhps* and N86Y *mdr1* drug resistance markers. The number of mutant isolates was higher in the dry season than the rainy season except for K76T *crt* mutants which was the reverse.

The analyses of the drug resistant parasites were grouped into mutant and wild types. Obom had more mutant strains in three out of the four markers

(108N, 437G and 86Y) than Cape Coast. The difference in 108N mutant strains being significant (P < 0.001) between the isolates from the two sites. On the other hand, the 76T mutant strains were more prevalent in Cape Coast than in Obom (Figure 27A). By comparing the mutant strains markers between the dry and rainy seasons, all but 76T was frequent in the dry season (Figure 27B). In the rainy and dry seasons, the distribution of the *dhfr* 108N mutant strain was pronounced (P = 0.001). The frequency of *dhfr* 108N and *crt* 76T mutant isolates were more stable between the transmission seasons and ranging from 3% to 40% in the population.

Mutations in Kelch propeller (*K13*) gene

The hemi-nested PCR amplified the 751 bp regions around nucleotides 1279 to 2030 of the amino acid codons 427 to 676 (> position 440) associated with delayed parasite clearance by WHO. The amplification was successful for the selected 94 parasite isolate cDNA and 3D7 laboratory strain (positive control) purified and sequenced bi-directionally (Macrogen,USA). A PCR negative control was included for gDNA contamination check and this did not have any comparable nucleotides after searching with NCBI nucleotide Blast alignment software.
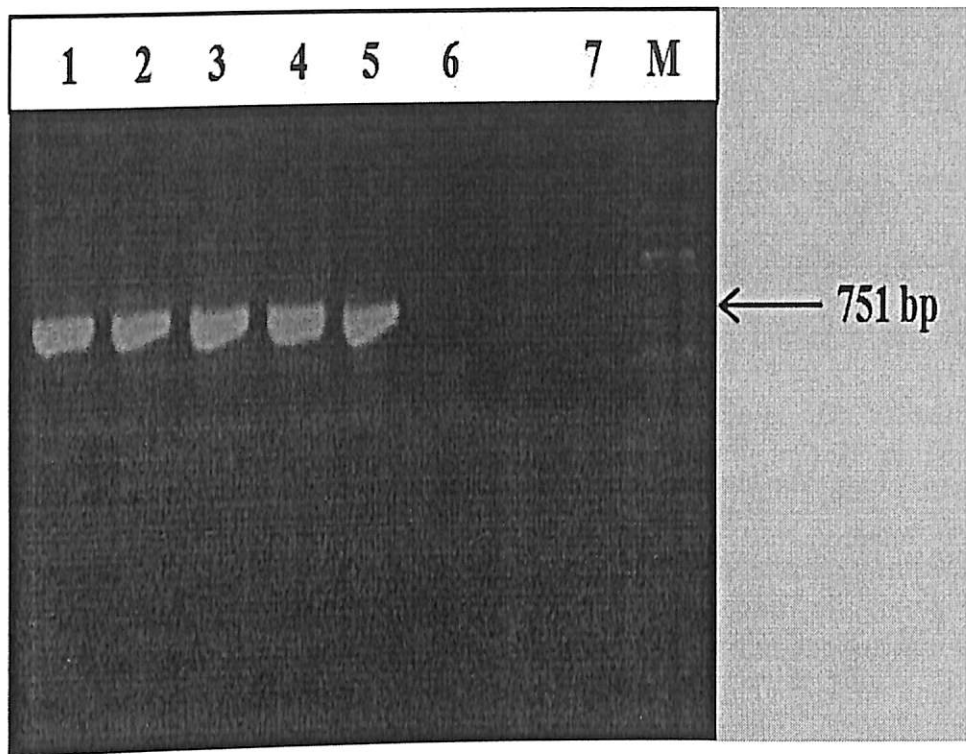
Figure 29: Electropherogram of amplified *K13* fragment
Lanes: 1 = positive control (*P. falciparum* 3D7 wild-type strain), 2-4 = parasite isolates, 5= 3D7, 6 = RT-NTC and 7 = water. Lane M = 100 bp DNA molecular weight marker.

By comparing, the nucleotide sequences obtained with the published 3D7 strain K13 genes spanning around 751 nucleotides from the forward and reverse sequences, two SNPS were identified in the parasite population (2/94) during the dry season in Cape Coast. Sequence analysis of the *K13* gene fragment showed two point mutations in two isolates. A synonymous mutation at position C1407T (C469C) and the other non-synonymous mutation at position G1732T resulting in an amino acid change from alanine to glycine (A557G) were observed after sequence alignment with the reference 3D7 strain sequence. Mutations found in the *K13* gene were validated by repeating a second independent PCR amplification and sequencing. Only two percent of the *P. falciparum* isolates had the *K13* mutations and this shows a low frequency of the mutation in the study population.

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Plasmodium falciparum **factors and Gametocyte prevalence**

From the study, there was no association between having IC3D7 or FC27 infection and gametocyte prevalence. However individuals with the dimorphic (both IC3D7 and FC27) infections had a higher gametocyte prevalence (P = 0.001, df = 2, $\chi2 = 0.51$). Although, between the clones, gametocyte prevalence within FC27 was higher than the IC3D7 infections it was not statistically different (P > 0.05) {Table S 4.4}. Eighty nine percent of the study participants with mutant and wild type strains had *msp2* dimorphic infections. The association between gametocyte clones and prevalence in the study was not done. This was because the gametocyte clonal distribution failed the normality test. Nevertheless, the prevalence of the drug mutant strains was higher in the dry season than the rainy season. Yet still, the gametocyte prevalence distribution in the seasons was the opposite when the data from the two study sites were combined as gametocyte prevalence in Cape Coast was significantly in the dry season than the rainy season.

CHAPTER FIVE

DISCUSSIONS

Discussions

Key to malaria transmission is the ability of the *Plasmodium falciparum* gametocyte to be present, mature and survive human to mosquito transition. To understand the production rate of *P. falciparum* gametocyte and prevalence in an infection or in the population is complicated. This makes it difficult to elucidate the mechanism of naturally acquired transmission blocking immunity to help in developing a vaccine for malaria eradication. In seeking to understand acquisition of transmission blocking immunity, the human host and parasite factors have been shown to play an important role during seasonal malaria transmissions, especially the continuous presence of asymptomatic carriers with gametocytes in an area (Gouagna et al., 2010; Zerihun, Degarege, & Erko, 2011). Here for the first time, this study has established an *ex vivo* assay to estimate *P. falciparum* gametocyte production in natural infections. In addition, host and parasite factors that could possibly be associated with gametocyte prevalence in two communities and seasons in Southern Ghana were assessed.

Mean parasite density observed in the symptomatic study participants per microliter of blood was 84833. High *P. falciparum* densities on day zero were associated with high body temperature (mean 38°C) in the participants. This is due to responses from host immune cytokines to the busting or rupturing of infected erythrocytes during the release of parasite merozoites (Oakley, Gerald, McCutchan, Aravind, & Kumar, 2011). On the seventh day after treating the infection, temperature readings were significantly (P <

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0.0001) lower (mean 36°C) with no microscopic detectable parasites and consistent with a previous report (Ejezie & Ezedinachi, 1992). The observed high density of *P. falciparum* in the infection was associated with anaemia on day zero before treatment. This may be related to the destruction of infected cells at each cycle of merozoite release and reinvasion events, leading to the constant loss of RBCs (Mohandas & An, 2012). Mean haemoglobin levels in the three observed haemoglobin variants in the study participants did not differ significantly (P > 0.20). These findings were similar to earlier reports in Ghana (Kreuels et al., 2010; Tangpukdee, Yew, et al., 2008) and other parts of the world as documented earlier by Gouagna and his colleagues. The mean White Blood Cell (WBC) count in the population was normal and was not associated with *P. falciparum* density as reported before (McKenzie et al., 2005). Two participants had higher levels of WBC above the normal value (>13.8 x 10^3/µL) and that could have resulted from other disease conditions or co-infections with other pathogens (bacterial infections) which were not screened in this study.

To have a well synchronised gametocyte culture, daily usage of NAG maintain for six or more days was recommended as it reduced the asexual population to less than 3%. The gametocyte production rate on day three in the *ex vivo* assays correlated positively (P = 0.001) with day zero parasitaemia (mean 3.8%). The observed gametocyte production in the field isolates by day eight from microscopic count was 0.02% to 1.9%, similar to an *in vitro* gametocyte production which also ranged from 0.93% to 1.18% (Roncalés, Vidal-Mas, Leroy, & Herreros, 2012). In the *ex vivo* assay, more than half (60%) of the isolate made gametocytes from the day three counts and the

number increased to about 80% by day eight. The increase in the isolates with gametocytes is likely due to culture-induced conditions like spent parasite media and changes in incubation temperature (Schuster, 2002). Nine of the *P. falciparum* isolates did not make gametocytes in the NAG assay. Yet, these isolates in their control assay had gametocytes that were comparable to those which made gametocytes in the NAG assay. No gametocyte in the nine 'non-producers' isolates by microscopy may be attributed to the low sensitivity of the method. Thus, the 'non-gametocyte producers' could have submicroscopic gametocytes which were not captured by microscopy. Using qRT-PCR will detect more gametocyte that could have been similar to observations made on day zero. From the *in vivo* gametocyte prevalence, the estimation by qRT-PCR was ten-fold higher than by microscopy (P > 0.001). By determining gametocyte *in vivo,* although *Pfs25* is express by mature female gametocyte, having most of the day zero samples positive for the gene transcript before treatment indicated that infections had occurred early and in asymptomatic forms. Most *Plasmodium* infections in human populations are known to attain a certain threshold (Churcher et al., 2017) before clinical symptoms are observed. In addition, the isolates in this study made gametocytes in the *ex vivo* assay and this has an important implication on malaria transmission.

In the school children group, the participants with asymptomatic infections were exposed to repeated *Plasmodium* infections leading to naturally acquired immunity to the severe form of the disease or high parasite density (Doolan, Dobano, & Baird, 2009). Positivity to *Pfhrp2* was higher among the participants from Obom and almost two-folds higher than in Cape Coast during the dry season. Earlier research has shown that immunity affects

parasite clearance by reducing parasite density and subsequently gametocyte numbers and eventually reducing malaria transmission (Ataide et al., 2017; Hastings, Kay, & Hodel, 2015; McQueen & McKenzie, 2008; White, 2017). The extent and how certain host factors influence malaria transmission has been reported but the associations differ from transmission settings (Driss et al., 2011; Mackinnon, Mwangi, Snow, Marsh, & Williams, 2005; Zerihun et al., 2011). Most children had microscopic infections but at low parasite density (mean 530 - 2344 µL per whole blood for the visits in the sites) and in asyptomatic state. Thus, parasite density below 2000 µL per whole blood is reported not to be associated with fever (Ardiet et al., 2014); and fever in *Plasmodium* infections is common and associated with symptomatic malaria was also not present. At certain points in these asymptomatic participants, an infection could manifest in the individual as uncomplicated or severe form of the disease (McArdle, Turkova, & Cunnington, 2018; Njunda et al., 2015). The study did not follow these asymptomatic children to find out whether these infections will progress into symptomatic form.

The school children had high submicroscopic *P. falciparum* infections in the rainy seasons. The prevalence was as high as 83% and 44% in the seasons for Obom and Cape Coast respectively. At the visits, more than 40% of the children were harbouring submicroscopic *P. falciparum* infections. The observed high prevalence of the infections was comparable to previous studies in the country (Atelu, Duah, & Wilson, 2017; Baiden et al., 2014); and a much higher prevalence have been reported elsewhere in Mali and Indonesia (Ardiet et al., 2014; Pava et al., 2016). These are indications that infections persist but are often in latent forms in most individuals living in malaria endemic areas.

By comparing infections in the two study communities, a decrease (P < 0.001) in parasite prevalence by all the three assessment methods was observed in the dry season. The decrease was pronounced in Obom; and this suggests seasonality in malaria transmission in Obom which is similar to a study in Burkina Faso (Geiger et al., 2013). By contrast, submicroscopic infection prevalence in Cape Coast did not change (P = 0.77) as the difference between the seasonal prevalence was less than five percent, similar to a trend in a previous study in Mali (Ardiet et al., 2014). An unusual increase in the prevalence of submicroscopic gametocytes was observed during the dry season in Cape Coast. This could result from the persistence of parasite strains that have a high longevity. The increase in prevalence of gametocytes in Cape Coast during the dry season was at variance with the findings from a previous study at Burkina Faso (Ouedraogo et al., 2011). This is an indication that a relatively stable transmission of *P. falciparum* might occur year-round in Cape Coast (De Silva & Marshall, 2012; Mabaso, Craig, Ross, & Smith, 2007; Bigoga, Manga, Titanji, Coetzee, & Leke, 2007; Geiger et al., 2013). The reduction in submicroscopic infection observed during the dry season in Obom could result from decrease in vector populations in the dry seasons and/or an intervention which occurred three months prior to the January dry season sampling (Ghana Health Service, National Malaria control Programme, 2016). The intervention in Obom was antimalarial and anthelmintic drug administration, the distribution of insecticide treated bed nets, and these may have reduced parasite prevalence and transmission.

Host genetic factor contributions to gametocyte prevalence have been observed previously (Gouagna et al., 2010; Grange et al., 2015). The effect of

G6PD, Beta globin genotypes and ABO blood groups on gametocyte prevalence did not change by either sites or seasons. The G6PD deficient variant (both partial and full) in the study population was before 13% as reported in our previous study (Amoah et al., 2016). This prevalence is comparable to other reported studies in Ghana where more than 20 % of the study population had G6PD deficient variants (Owusu et al., 2015). The *P. falciparum* prevalence was not associated either with the G6PD status or as a factor for increased gametocyte carriage (P > 0.05). Findings for G6PD protection to *P. falciparum* infections have been conflicting. In a study in Uganda, G6PD variant impact on malaria protection was found only in the heterozygote females (BA-) and seen only in G6PD deficient enzyme activity (Hedrick, 2011; Johnson, Clark, Njama-Meya, Rosenthal, & Parikh, 2009). This study did not measure the enzyme activity but decrease in G6PD enzyme activity can occur in individuals when there is an oxidative stress. Equally, other physiological or pathological conditions not attributed to G6PD genotype can also lead to oxidative stress (Djordjević, Zvezdanović, & Cosić, 2008).

Certain haemoglobin genotypes provide immunity to malaria, enhancing malaria transmission by increasing gametocyte carriage. Among the beta haemoglobin genotypes, HbC was the dominant variant in this study, similar to recent studies in Ghana (Amoako et al., 2014; Piel et al., 2013; Mangano et al., 2015). The recent observation in higher numbers of HbC has been attributed to its increasing clinical relevance (Mangano et al., 2015) and the previous assessment method that were used. Previously, the determination of haemoglobin genotype was solely based on microscopy observation of the

Hb S blood shapes and this characterization could not differentiate the other HBB variants, leaving them undocumented (Wajcman & Moradkhani, 2011). Gametocyte prevalence was significantly (P = 0.04) associated with abnormal haemoglobin (either homozygous or heterozygous) variants. Within the haemoglobin variants, gametocyte prevalence was higher in individuals with either heterozygous or homozygous HbC variants than in the HbS group. The observation made in this study supports those made by Gouagna and colleagues (2010), where there was an association between HbC genotype and gametocyte carriage. The HbC variant might provide protection against severe malaria (Agarwal et al., 2000) possibly by inhibiting the parasite asexual growth and invasion of the RBC to some extent.

The distribution of ABO blood groups at study sites were slightly different, even though the blood group O was high and seen among other African countries (Anifowoshe, Owolodun, Akinseye, Iyiola, & Oyeyemi, 2017; Rowe et al., 2007). The distribution of ABO blood groups was slightly different between the sites, with the distribution of blood group B highly significant between them (P < 0.0001). Further, none of the ABO blood group was associated with gametocyte carriage (P = 0.21). There are also conflicting reports on the association between ABO blood groups with *P. falciparum* susceptibility. A prior study observed an association with blood groups O and B (Drakeley, Secka, Correa, Greenwood, & Targett, 1999), whiles in another, blood groups A, B or AB increased the host susceptibility to *P. falciparum* infections (Zerihun et al., 2011).

In our study, higher gametocyte prevalence was observed in blood group AB isolates but considering the few number of participants in the blood

group AB (n = 5) the error margin associated with this observation was high. Previous studies have found that individuals with blood group B were less infective (with the parasite) to mosquitoes' bite (Drakeley et al., 1999; Tchuinkam et al., 1993), suggesting that infectiousness of an individual may be related to the gametocyte density (Bousema & Drakeley, 2011b). The prevalence of *P. falciparum* gametocyte in the study did not show any significant association with blood types (P > 0.05). The regulatory protein activities of the ABO blood group differ in the individual groups. The exact role of the ABO blood groups in malaria population, distribution between geographical areas makes it difficult to define its role in disease protection (Beiguelman et al., 2003; Zerihun et al., 2011).

On the role of *P. falciparum* diversity on gametocyte carriage, clonality in sexual and asexual parasites at the sites and seasons did not differ much. Infections with multiply clones form the basis of newer and fit parasites during zygote formation in the mosquito (Eksi et al., 2012), an important basis for parasite adaptation to environmental pressure. The multiplicity of infection between the seasons and sites did not differ significantly, likewise the isolates sexual diversity between the study sites and seasons. More than 90% of the infections genotyped were monoclonal. The MOI for *msp2* for both sites and seasons was below 1.6 and lower in the dry season where MOI was 1.4 but similar to previous reports in communities in Ghana between 2010 to 2016 (Agyeman-Budu et al., 2013; Ayanful-Torgby et al., 2016; Nielsen et al., 2002). The MOI of *Plasmodium* infections at the population level is decreasing with declining infections compared with the pre-ACTs era (Agyeman-Budu et al., 2013; Balmer & Tanner, 2011). Comparatively, lower

diversity was observed at the *P. falciparum* sexual stage where only five gametocyte alleles were identified in the entire population. This was far lower than what has been reported in Cameroon (Morlais et al., 2015), though the samples used in the Cameroon study were collected in 2008 when global *P. falciparum* transmission rate and densities in infection were higher than in 2016. This is the first time genotyping of gametocyte alleles in *P. falciparum* isolates has been conducted in Ghana and five alleles were found. The lower allele frequencies observed in the *P. falciparum* diversity and the MOI could explain why unusually high submicroscopic gametocyte prevalence was observed in the Cape Coast infections during the dry season. Explanations to this are related to the fact that *P. falciparum* has a high and efficient capability of clonal inbreeding and outcrossing (Morlais et al., 2015).

Comparing the sexual, asexual MOI and diversities, gametocyte numbers occur far lower than asexual densities (Ouédraogo et al., 2010), a common observation in locations where parasite diversity is low. Other gametocyte clones could be present in the population but at lower frequency and likely to be missed during the amplification and genotyping. The recent decline of *P. falciparum* infection in endemic countries after the introduction of artemisinin combination therapies could account for the decrease in the MOI observed (Gosi et al., 2013; Nassir et al., 2005). This may explain why an association between parasite sexual diversity and gametocyte prevalence could not be established.

Plasmodium falciparum drug resistant isolates increase transmission as the current antimalarials do not affect the circulating mature gametocytes leading to high submicroscopic gametocyte prevalence after treatment

(Ayanful-Torgby et al., 2016; Bousema et al., 2014; Dinko, King, Targett, & Sutherland, 2016; Dunyo et al., 2006). The selection of well adapted *P. falciparum* population after drug treatment have been reported globally (Alam et al., 2011; Griffing et al., 2011). The spread of *P. falciparum* drug resistant isolates to most antimalarials are common in Ghana, obscuring the control and eradication efforts. The present study highlights a high prevalence of *pfcrt* K76T, *mdr*1 N86Y, *dhps* A437G and *dhfr* S108N mutations in population. Mutant parasite strains with these markers in circulation do not safeguard malarial treatment in Ghana. Mutations in the *K13* gene were associated with delayed parasites clearance. The two mutations observed in the parasite isolates in this study have been reported in isolates from Kenya and Congo (Taylor et al., 2015), showing a gradual decrease in the parasite tolerance to artemisinins. Monitoring drug resistant parasites is important in malaria transmission as lessons from the spread of chloroquine resistant parasites in Africa were gradual and independent. Similarly artemisinin resistant strains have markers which are proposed to vary geographically and there role in parasite clearance also differ (Ariey et al., 2014; J. Straimer et al., 2015; Takala et al., 2006; Taylor et al., 2015). Fewer parasites with delayed clearance have been reported in Africa and Ghana but the associated molecular mechanisms are yet to be determined (reference). High prevalence of *dhps* and *dhfr* resistance makers suggests the use of SP in the general population and its usage as prophylaxis in pregnancy results in high selection pressure. The increase in gametocyte prevalence after drug treatment may be attributed to mutations in the parasite to previously used antimalarials.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

Conclusion

The study was able to develop and optimise a plate based *ex vivo* assay to *P. falciparum* rate of gametocyte production. Gametocyte production rate was observed to be less than one percent of the isolates in the *ex vivo* assay and differed among the isolates. Mean parasite densities in the symptomatic infections were three-fold higher than the asymptomatic infections. Variations in host haemoglobin genotypes and blood groups were observed between the two communities. The three RBC polymorphisms genotyped in the study were prevalent and different among the study communities but no association was observed between RBC polymorphisms and gametocyte carriage. Dimorphic infections (FC27 + IC3D7) were the only parasite factor found to be associated with gametocyte prevalence in this study. Low prevalence of *K13* mutations in the *P. falciparum* isolates and the prevalence of *crt, dhfr, dhps* and *mdr1* drug resistant markers are indications of drug pressure on the parasite population. Host immunity may have influenced the parasite transmission as the community with high antibody positivity (to HRP2 antigen) have low frequencies of parasite drug resistant strains. In the high parasite prevalence community (Obom), *P. falciparum* infections showed seasonal prevalence, whereas the pattern in the low parasite prevalence was relatively stable. Thus, communities with varying parasite prevalence exhibit seasonal variations in the prevalence of gametocyte carriers. In this study, high

asymptomatic carriers were observed and they could contribute significantly to the transmission of malaria.

Recommendations

The high prevalence of submicroscopic gametocytes in the participants in the study sites should be assessed through mosquito passage (membrane feeds) to ascertain if they can be transmissible. It is better to assess parasite density using more advanced tools/methods that are sensitive like flow cytometry or qRT-PCR to evaluate gametocytes production at low infection rates that are undetectable by microscopy. The distribution of drug resistant parasite varies geographically and continuous validations are needed to know their effects on malaria transmission in ACTs usage. Additional work to understand underlying factors involved in the complex seasonal pattern observed in this study is needed. Finally, symptomatic and asymptomatic *Plasmodium* infections in older individuals and the role immunity play in human reservoirs need to be investigated.

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APPENDICES

Appendix 1: Supplementary Data

S3.1 DNase on column digests

   a. Mix 70 μL of buffer RDD to 10 μL of stock DNase by pipetting gently.

   b. Add the 80 μL of DNase solution to middle of the column.

   c. Incubate at RT for 15 minutes

S3.2 Qubit® 3.0 fluorometer for RNA concentration

All assays were read in 0.5 mL PCR tube (with kits) and the tubes are labelled only on the lid

All the sample readings were between the standard #2 readings

Standard #2 reading was always higher than standard #1 reading as describe by manufactures and briefly as follows:

S3.2.1 Preparing Qubit working solutions for samples and standards

1. Add   1 μL of RNA HS reagent
2. 199 μL of RNA HS buffer
3. Mix well in an eppendorf tube
4. S3.2.2 Preparation of Qubit standards
5. Add 10 μL of each standard to 190 μL of working solution
6. Vortex briefly by avoiding bubbles and incubates at RT for 2 minutes

   in the dark and read

S3.2.3 Preparation of RNA samples for Qubit reading

1. Add 1 μL of each sample to 199 μL of working solution

2. Vortex briefly by avoiding bubbles and incubates at RT for 30 minutes in the dark and read

3. Vortex/mix all standards and samples briefly by avoiding bubbles

4. S3.2.4 Running of Qubit standards for validation

5. Switch on the Qubit machine

6. Choose assay type. RNA

7. Press Yes to read new standards

8. Insert standard #1 (specific for RNA) and press read when prompt

9. Insert standard #2 (specific for RNA) when prompt after standard #1 reading

Once standards pass the samples can now be run but remember to always enter the right volume and/or concentration of samples

NB: *each samples and standards requires 200ul of working solution, prepare working solution based on the number of samples to be run*

S3.2.5 Preparation of samples for Qubit reading

Add between 1-20ul(x value in calculating final concentration) of samples to 199-180ul of working solution

Concentration of samples (ng/mL) = QF value * 200/x

S.3.3 Designing of qRT-PCR experiment with the Fast StepOne software

1. Open StepOne software v2.3.

2. Click on Design Wizard

3. Name your experiment (preferably with the date included)

4. Instrument 96 well instrument

5. Experiment Type: Quantitation

6. Click Next

7. Define materials and Methods:

8. Quantitation Method: Comparative CT

9. Reagents: SYBR Green

10. Ramp Speed: Fast

11. Template: cDNA

12. Define Targets: Enter the number of Targets (i.e. primer sets) and name them appropriately in the boxes provided.

13. Set up Samples: Enter the number of Samples (i.e. DNA templates) and name them appropriately in the boxes provided.

14. By Default, the program sets up all possible combinations of Targets and Samples in the plate diagram on the right. You can specify a subset by selecting "Specify Target/sample reactions".

15. The plate must be set up EXACTLY as it is displayed on the right side of the screen, because that's how the computer knows which samples is which. If you don't find the setup intuitive, you can clear the auto setup and fill in which primers and templates you want in each well by hand.

16. Run Method: is Auto on Fast

17. Reaction Setup: Fill in your primer concentrations and reaction volumes and it does the math for you. (note: picomole/ul is the same as μM) I usually just do this by hand, so I skip it by hitting next

18. Click finish designing experiment

19. To edit the plate layout, click Edit plate layout, Click assign samples and targets tab. Highlight the wells you want, then use the check boxes next to the targets/samples to assign each well one of each

Remember to include all the sample/target combinations you want and to do them in triplicate

Save the experiment on a USB drive.

S.3. 4 Giemsa Smears Preparation

Plasmodium falciparum parasitaemia i.e. the percentage of infected red blood cells was determined in Giemsa stained blood films (thick/thin). These blood films were made on a frosted standard microscope slide; the smears were air dried and fixed in methanol. Ten percent freshly prepared Giemsa stain was used in staining the blood film smears for 15 minutes. The smears were carefully rinsed with water and air dried in an upright position. The stained smears were examined under a microscope with immersion oil using a 100 X objective lens. The *Plasmodium* parasites were identified, species/stages documented when necessary by species/stage specific morphological features and counted. Parasitaemia was recorded as the percentage of infected RBCs.

S.3.5 *Plasmodium* parasite Cryopreservation

S.3.5.1 Preparation of Deep-freeze solution

1. Dissolve 4.2 g of sorbitol and 0.9 g of NaCl in 100 mL of distilled water.

2. Add 28 mL of glycerol to 72 mL of the above solution.

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3. Filter sterilise. Store @ 4ºC.

S3.5.2 Freezing down cultures

1. Add 100-300 μL of washed parasitized cells in to a labelled cryotube

2. Add equal volume of freeze solution drop wise, slowly to the packed cells at room temperature to allow glycerol to penetrate cells.

3. Freeze immediately in liquid nitrogen.

S3.5.3 Washing of RBC for parasite cultures

Collected venous blood samples were centrifuged for five minutes at 2000 rpm to harvest the plasma and to remove the buffy coat. The red cells pellets were resuspended and washed twice with an equal amount of iCPM at centrifugation of 2000 rpm for five minutes. The wash red cells is then resuspend in an equal volume of CPM (50 % HCT) and stored at 4 ºC usage for a maximum period of two weeks.

S3.6 Preparation of 50 X Tris-Acetate-EDTA (TAE)

1. Dissolve 242 g of Tris base in water

2. Add 57.1 mL glacial acetic acid

3. Add 100 mL of 500 mM EDTA (pH 8.0) solution to the above, and bringing the final volume up to 1 litre.This 50 X stock solution is diluted 50:1 with water to make a 1 X working solution (containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) for the preparation of the agarose gel and running of the gels.

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Table S3.1 The qRT-PCR cDNA dilutions for primers optimisation and

Parasite quantification

Plate Column	10 fold	5 fold	2 fold
A	1	1	1
B	0.1	0.2	0.5
C	0.01	0.04	0.25
D	0.001	0.02	0.125
E	0.0001	0.004	0.0625
F	0.00001	0.0008	0.03125
G	0.000001	0.00016	0.015625
H	NTC	NTC	NTC

Table S 3.2: Primers for qRT-PCR

Gene	Primer sequence
*Pfs*25 F	TCTTTTCCTTTTCATTCAACTTAGCA
R	CCACTCATCTGAATTAAAAATCCTCTT
Pf Kahrp F	CATGGTGCAGGCTATTCAG
Kahrp R	CTTCACCGTCATTTCCTTCATGC
Pf *18S rRNA* F	GCTGACTACTCCTGCCC
Pf *18S rRNA* R	ACAATTCATCATCATATCTTTCAATCGGTA
Hum *18S rRNA* F	TGTGCCGCTAGAGGTGAAATT
Hum 18SrRNA R	TGGCAAATGCTTTCGCTTT

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Table 3. 3: Primers and restriction enzymes for G6PD genotyping

Nucleotide position Gene/primer	Primer sequence	Bp	Restriction enzyme
A376G,			
F	CCCAGGCCACCCCAGAGGAGA	308	FokI
R	CGGCCCCGGACACGCTCATAGCGGCCCGAC ACGCTCATAG-3'		
G202A			
F	CCACCACTGCCCCTGTGACCT	211	NlaIII 215 & 123 A-
R	GGCCCTGACACCACCCACCTT		215 & 123 A- 215
G680T,			
F	ACATGTGGCCCCTGCACCAC	242	BstNI & 123 BA- /AA-
R	GTGACTGGCTCTGCCACCCTG		
T968C			
F	TCCCTGCACCCCAACTCAAC	282	NciI
R	CCAGTTCTGCCTTGCTGGGC		

Only A376G POSITIVE samples were then analyzed for G202A polymorphism.

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Table 3. 4: The *P. falciparum* speciation; diversity of *msp2* and *Pfg377* primers

Gene/primer name	Primer sequence	Bp
Plasmodium Speciation		
PLU5	CCTGTTGTTGCCTTAAACTTC	1200
PLU6	TTAAAATTGTTGCAGTTAAAACG	
rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	
MO1		
msp2		
Outer	M2- OF: ATGAAGGTAATTAAAACATTGTCTATTATA	
	M2- OR: CTTTGTTACCATCGGTACATTCTT	
Inner		
3D7/FC27	S1fw: GCTTATAATATGAGTATAAGGAGAA	
3D7	N5rev: CTGAAGAGGTACTGGTAGA	
FC27	M5rev: GCATTGCCAGAACTTGAA	
Pfg377		
outer	R3D1: GATGAAGGGATATATCACCTCACAATGTG	
	R3R2: GTCATGATTTTCTTCTCCTTCGGATATGG	
	R3D2: CCATAGGAATATTACACCATATCATGTG	
Inner	R3R1: TATGGTGATAAATGAGGAGTGTCCCCTTAC	

Table S 3.5: Primers for amplifying *Pfcrt, Pfmdr1, dhfr, dhps,* and *K13* genes.

Gene/Prim	Primer sequence	Produ	Restriction
Crt codon 76		537	
CRTP1	CCGTTAATAATAAATACACGCAG		
CRTP2	CGGATGTTACAAAACTATAGTTA	134	*ApoI*
CRTD1	TGTGCTCATGTGTTTAAACTT		
CRTD2	CAAAACTATAGTTACCAATTTTG		
Dhfr (51, 59, 108 and 164 alleles)		642	
M5 (r)	AGTATATACATCGCTAACAGA		
M1 (f)	TTTATGATGGAACAAGTCTGC	522	*Bsrl*(108)
M3	TTTATGATGGAACAAGTCTGCGA		
F1	AAATTCTTGATAAACAACGGAAC	326	
M4	TTAATTTCCCAAGTAAAACTATT		
F	GAAATGTAATTCCCTAGATATGG		
DHPS		711	
R2	AACCTAAACGTGCTGTTCAA		
R1	AATTGTGTGATTTGTCCACAA	438	*AvaII* (437)
K	TGCTAGTGTTATAGATATAGGAT		
K/	CTATAACGAGGTATTGCATTTAA		
Pfmdr1 (Region 86, 144 and 184)		639	
A1	TGTTGAAAGATGGGTAAAGAGC		
A2	TACTTTCTTATTACATATGACACC	560	*AflIII* (86)
A3	GTCAAACGTGCATTTTTTATTAA		
A4	AAAGATGGTAACCTCAGTATCAA		
K13 popeller Taylor et al, 2014			
ArtinnerF	GCCTTGTTGAAAGAAGCAGAA	751	
ArtouterR	CGCCATTTTCTCCTCCTGTA		
ArtinnerR	GTGGCAGCTCCAAAATTCAT		

S 3.7 Amplification Conditions for PCR Reactions

Table S 3.7: Amplification reaction condition for characterization of G6PD variants

Reaction components	Conc.
H₂O	1X
10xPCR buffer	1.5 mM
25 mM MgCl₂	0.2 mM
10 µM dNTP	0.2 µM
10 µM Primers G6PD 376/202/680/968F	0.2 µM
G6PD 376/202//680/968R	0.75
Biotech Taq (5U/ µL)	20-50ng
DNA	

Table S 3.8: Reaction condition for *Plasmodium* species identification

Reaction components	Conc.
H₂O	1X
5xPCR buffer (with 1.8 mM)	2.5 mM
25 mM MgCl₂	0.15 mM
10 µM dNTP	0.08 µM
10 µM Primers rPlus F/rFal F	0.08 µM
rPlus R/rFalR	1U
Taq (5U/ µL)	20-50 ng
DNA	

Table S 3. 9: Amplification reaction condition for *msp* 2 genotyping

Reaction components	Conc.
H₂O	1X
10XPCR buffer	2.5 mM
25 mM MgCl₂	0.15 mM
10 µM dNTP	0.08 µM
10 µM Primers M2-OF	0.08 µM
M2-OR	1U
Taq (5U/ µL)	20-50 ng
DNA	

Table S 3. 10: *msp2* semi-nested IC3D7 (N5) and *msp2* semi-nested FC27 (M5)

Reaction components	Conc.
H₂O	
10XPCR buffer	1X
25mM MgCl₂	2.5 mM
10 μM dNTP	0.15 mM
10 μM Primers S1fw	0.1 μmol
N5(3D7)/M5(FC27)	0.1 μmol
Taq (5U/ μL)	1U
DNA	20-50 ng

Table S 3. 11: Amplification reaction condition for *Pf*g377 genotyping

Reaction components	Conc.
H₂O	
10XPCR buffer	1X
25 mM MgCl₂	2.5 mM
10 μM dNTP	0.15 mM
10 μM Primers D1/D2	0.15 μM
R2/R1	0.15 μM
	1U
Taq (5U/ μL)	
cDNA	

Table S 3.12: Amplification reaction condition for identification of mutation in *crt* gene (K76T)

Reaction components	Conc.
H₂O	
5xPCR buffer (with 1.8 mM)	1X
25mM MgCl₂	2.5-mM
10μM dNTP	0.15mM
10μM Primers P1/D1	0.08μM
P2/D2	0.08μM
	1U
Taq (5U/ μL)	
DNA	20-50 ng

Table S 3.13: Amplification reaction condition for identification of mutations in *pfmdr*1 gene

Reaction components	Conc.
H₂O	
5xPCR buffer (with 1.8	1X
25 mM MgCl₂	2.5-mM
10µM dNTP	0.15mM
10µM Primers A1/A3	0.08µM
A2/A4	0.08µM
Taq (5U/ µL)	1U
DNA	20-50 ng

Table S 3.14: Amplification reaction condition for identification of mutations in *dhfr* gene

Reaction components	Conc.
H₂O	
5xPCR buffer (with	1X
25 mM MgCl₂	2.5-mM
10µM dNTP	0.15mM
10µM Primers	0.08µM
	0.08µM
Taq (5U/ µL)	1U
DNA	20-50 ng

Table S 3.15: Amplification reaction condition for identification of mutations in *dhps* gene

Reaction components	Conc.
H₂O	1X
5xPCR buffer	2.5 mM
25 mM MgCl₂	0.15 mM
10 µM dNTP	0.08 µM
10 µM Primers R1/K	0.08 µM
R2/K1	0.5 U
Taq (5U/ µL)	20-50 ng
DNA	

Table S 3.16: *K13* gene amplification reaction condition for Sanger sequencing

| Reaction components		Conc.
H₂O		
10X AccuPrim Hifi reaction mix		1x
10µM Primers	ArtinnerF	0.2
	ArtouterR/ArtinnerR	0.2
		1U
AccuPrim Hifi		20-50
DNA		

S 3.8 Primers validation for qRT PCR amplifications

The validation of the *Pfs25* mRNA, *Pfs18S rRNA*, *Pf kahrp*, and human *18S rRNA* primers was performed on cDNA converted from the control samples using primer concentration of 300 nM. The cDNA was diluted initially in 1:10 for a starting concentration for all the standard curve with subsequent two fold serial dilution until 1: 640 for the high expressed genes 5 fold serial dilution until 1: 156250 for the high transcript genes, the human and parasite *18S rRNA* genes. The controls threshold cycle (CT) was a measure in selected a baseline values with the CT values of the negative controls as an indicator for the presences or absences of parasites or gametocytes. To normalize the qRT-PCR efficiency, a standard curve for each gene was prepared with the dilution of a cDNA positive sample as stated above and resultant slope (>-3.2), the R²(>0.95) and efficiency (between 90-110 %) as an acceptable values. The sequences of the primers, amplification plots, melting curves and standard curves used are shown in Figures S 3.1 & S4. 1 - 4. All qRT-PCR on the samples and controls were run in triplicates with standard deviation less than 0.5 as an acceptable results and those above 0.5 repeated.

S.4.1 Plots from the optimization of primers for qRT-PCR

Human 18SrRNA

Hum 18S F: TGTGCCGCTAGAGGTGAAAT
Hum 18S R: TGGCAAATGCTTTCGCTTT (Kuchipudi et al., 2012)

Standard Curve

Eff% 87%
R^2 0.98
Slope -3.46

Quantity
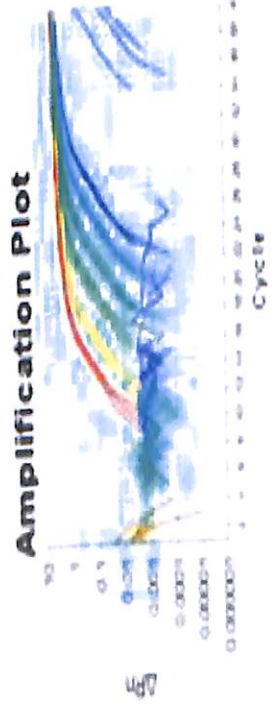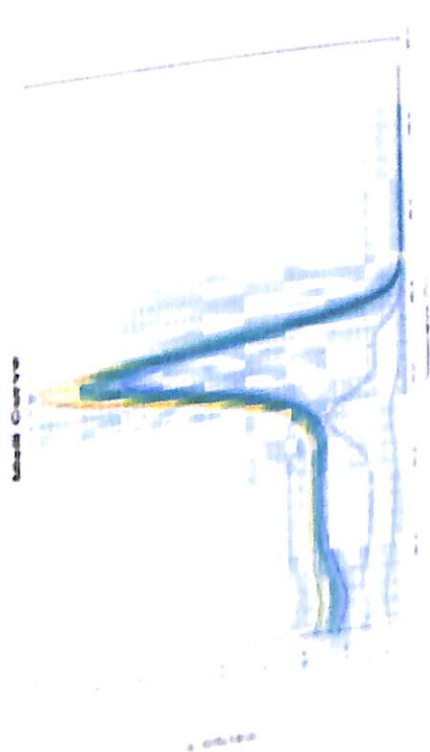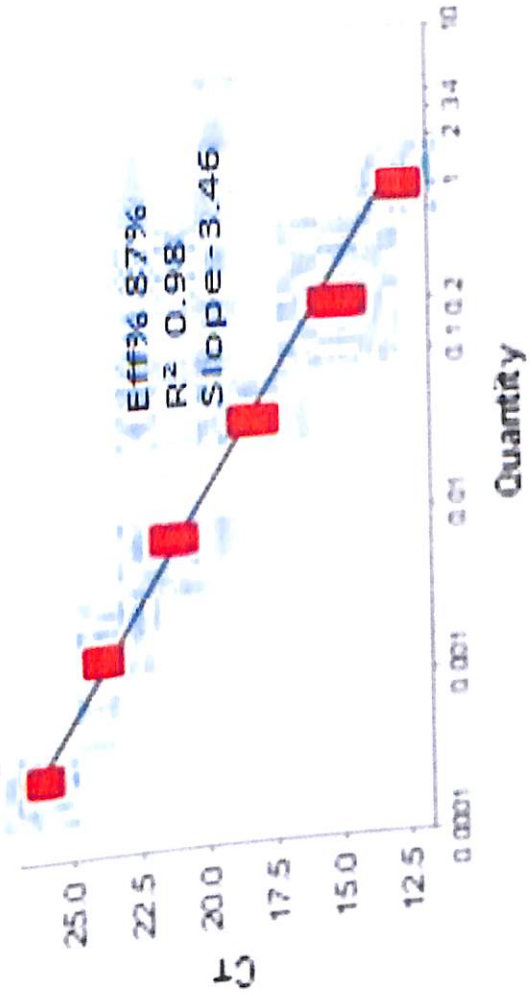
Hum (Kim cDNA)
cDNA Starting concentration 1:20 dilution 1.5
Serial dilution Primer concentration 300 nM

Amplification Plot

PF 18S rRNA

PF 18S F: GCTGACTACTCTCTGCCC
PF 18S R: ACAATTCATCATCAATATCTTTCAAATCGGTA

Asexual |NF54 Rings| cDNA
cDNA Starting concentration 1:10 dilution 1.3 Serial
dilution Primer concentration 300 nM

Standard Curve

Slope: -3.13
r^2: -0.979
Eff%: 178.3

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Asexual (NF54 Rings) cDNA.
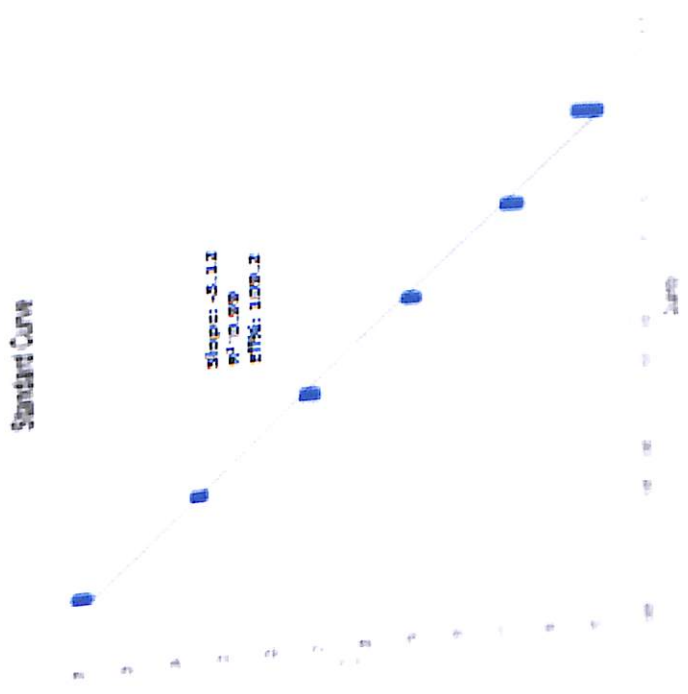cDNA Starting concentration 1:10 dilution 1:3 Serial dilution Primer concentration 300 nM
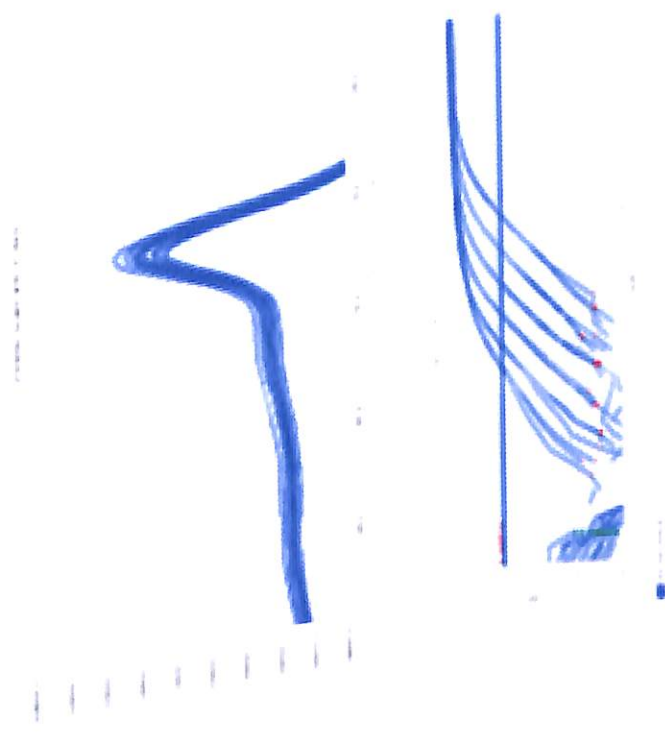
RHARP (PF3D7_0202000)
Kharp F: CATGGTGCAGGCTATTCAG
Kharp R: TTCACCGTCATTCCTTCATGC

Standard Curve

Slope: -3.28
R²: 0.99
Eff%: 101.96

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Standard Curve

Slope: -3.35
R²: 1
Eff%: 98.9

Pfa23 (Barry et al., 2009)
Pfa23 F: TCTTTCCTTTTCATTCAACTAGCA
Pfa23 R: CCACTCCATCTGAATTAAATCCTCTT

NES4 gametocyte 1.5 fold dilution

Melt Curve Plot

Amplification Plot
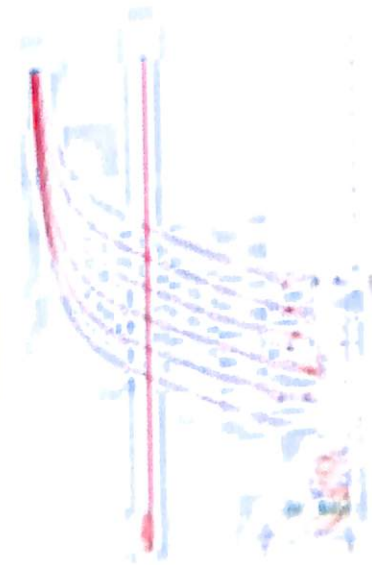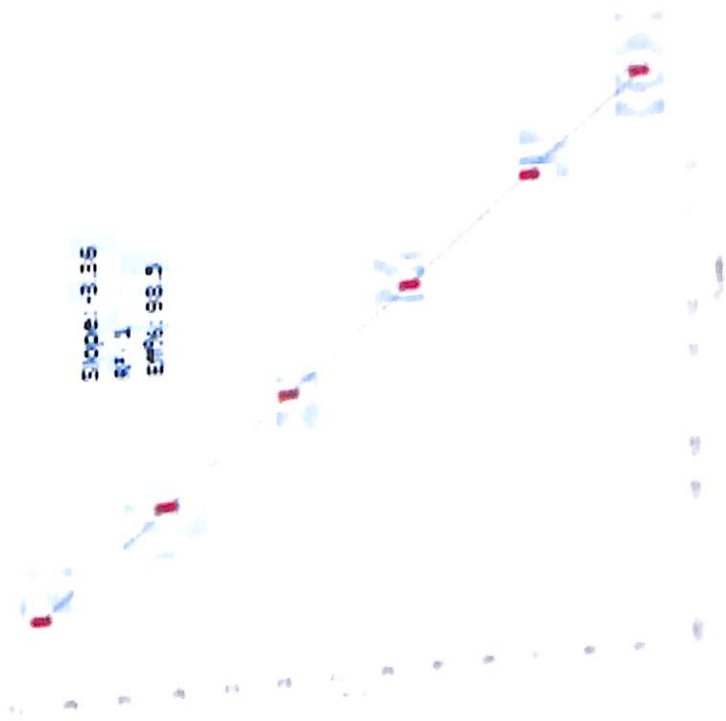
Table S 4.1 The Parasite and human controls in the qRT-PCR cDNA dilutions mean CTs to the gene test measuring expression levels

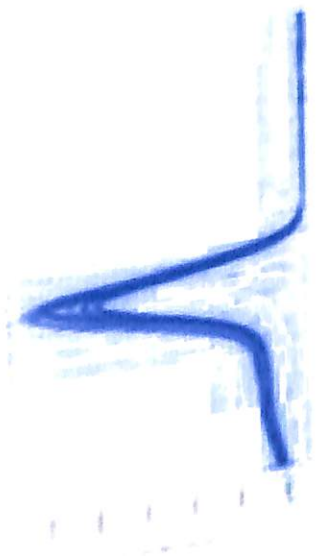| Parameters	Pfs18 rRNA	Hum 18S rRNA	Pfs25	Pfge1	Kahrp
Hum RNA	37.6	28.174	0	37.0	37.2
C9 (-)	13.2	22.792	33.3	21.5	12.9
RCM 47	23.3	34.638	0	35.9	21.8
NF54 Rings	13.6	30.644	0	23.9	ND
NF54 gcyte	13.9	30.6	13.4	18.9	ND
MS gcyte	16.9	ND	23.1	29.0	26.3
NTC	38.7	39.7	0	35.2	ND
Master Mix	38.5	39.2	0	36.4	36.9

The mean CTs from the qRT-PCR amplifications from the genes in the control parasite strains. ND was not measured, Undetermined CT= 0

Table S 4.2 *Plasmodium falciparum* gametocyte prevalence within the β-globin genotypes

parameter	Pfs25 positive	negative	Total isolates
HB CC/AC	47 %	8	9	17
HB SS/AS	19 %	5	21	26
HB AA	26 %	26	75	101

Gametocyte prevalence was measured by *Pfs25* positivity.

The *Plasmodium falciparum* identification
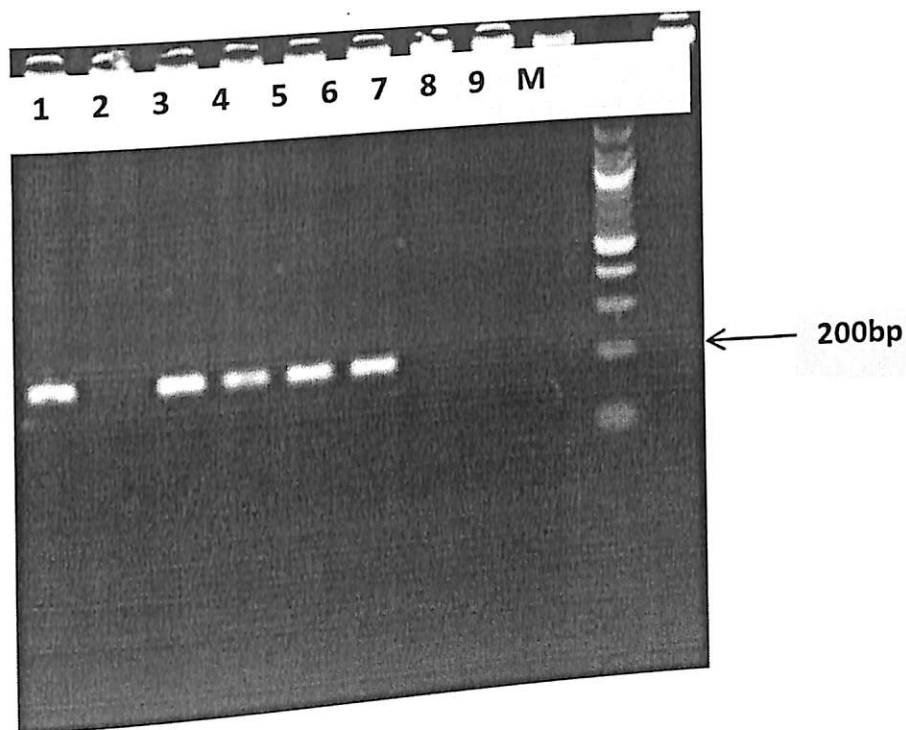
Figure S 4.2: An image of *P. falciparum* species identification in the samples. Lane 1, 3,4,5= samples with *P. falciparum* parasite, Lane 6 = positive control (*P. falciparum* 3D7 strain), 2 = positive control (*P. falciparum* DD2 mutant strain), 2,7,8 = samples without *P. falciparum* parasite, 9 = negative control (water). Lane M = 100 bp molecular weight ladder (Promega). The above shows 2.5 % ethidium bromide-stained agarose gel electrophotography of amplified DNA PCR product fragment of 205bp for *P. falciparum*.

The *Plasmodium falciparum msp2* genotyping
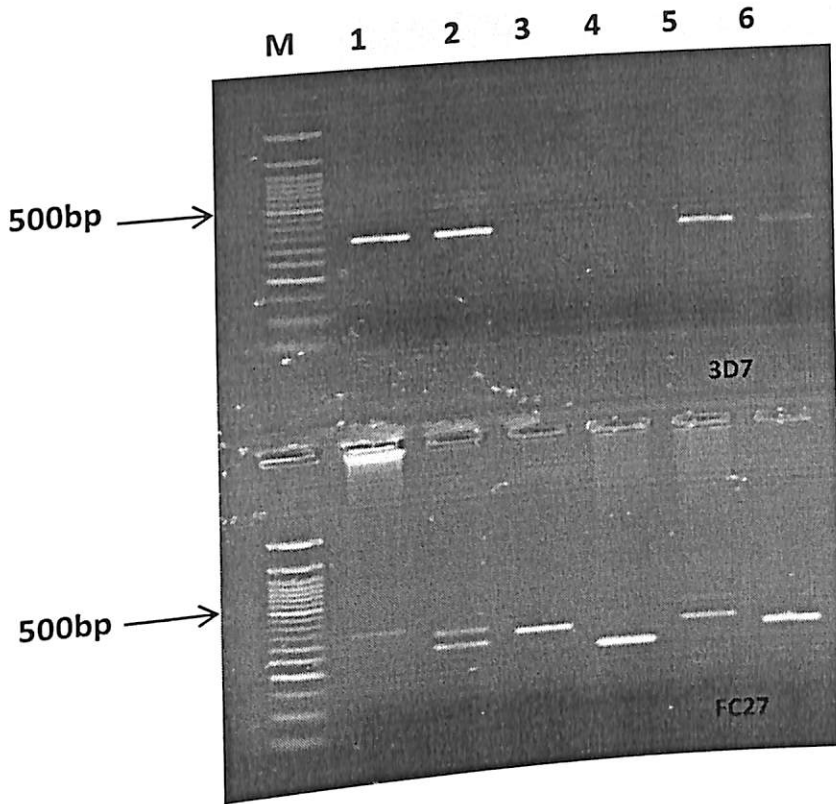
Figure S 4.3: An image of *P. falciparum msp2* gene amplification.
Lane 1-5 samples with *P. falciparum* parasite, Lane 6 = positive controls (*P. falciparum* 3D7 and FC27 strains), Lane M = 50 bp molecular weight ladder (Promega). The above shows 2 % ethidium bromide-stained agarose gel electrophotography of amplified DNA PCR product fragment of *P. falciparum* clones.

The *Pfcrt* K76T mutation
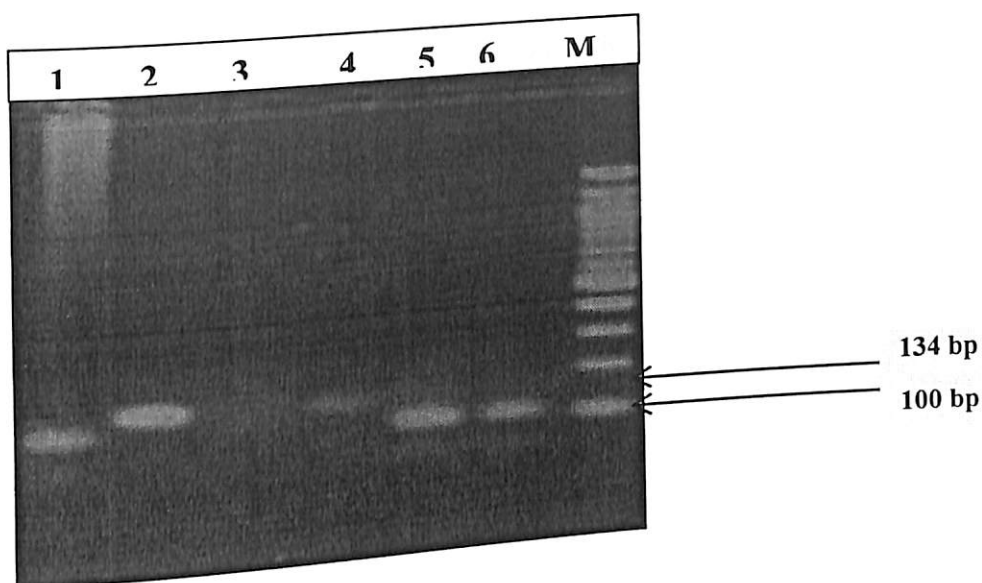
Figure S 4.4: An image of *Pfcrt* gene RFLP from the isolates.
Lane 1 = positive control (*P. falciparum* 3D7 wild-type strain), 2 = positive control (*P. falciparum* DD2 mutant strain), 3 = negative control (water), 4 = undigested PCR product, 5 & 6 parasite isolates. Lane M = 100 bp molecular weight ladder (Promega). The above shows 2.5 % ethidium bromide-stained agarose gel electrophotography of amplified DNA PCR product by the *ApoI* enzyme digest the amplified *crt* product 134 bp and control parasite strains used were 3D7 wild type and Dd2 mutant. The wild-type (K76) digested 134 bp product size to give a fragment of 100 and 34 bp at amino acid position 76 and uncut the mutant (T76).

The *Pfmdr*1 N86Y mutation
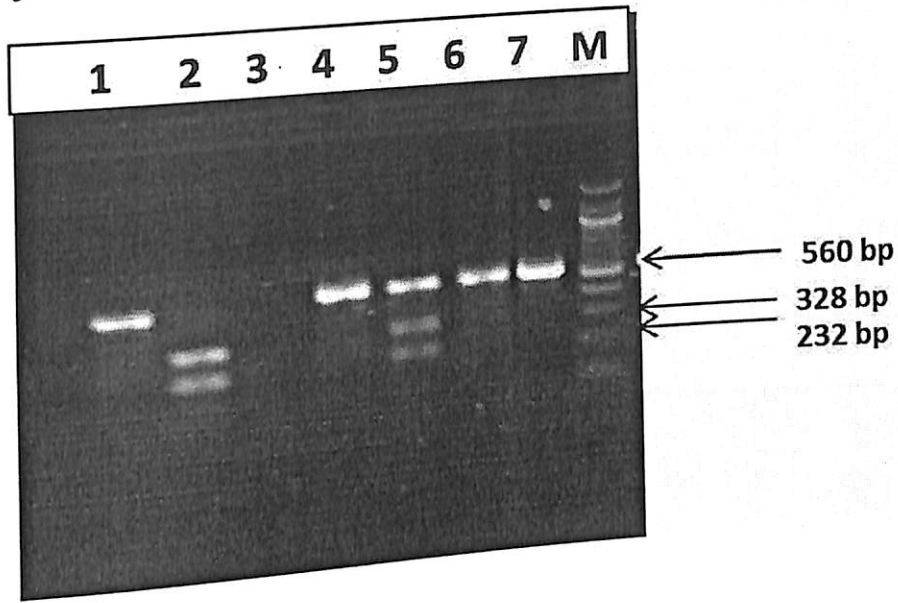
Figure S 4.5: An image of *Pfmdr1* gene RFLP from the isolates in the study Lane 1= 7G8 strain (wild type), 2 = K1 mutant, 3 = Water, 4 = undigested PCR product, 5 = isolates (wild and mutant multi infection in a host), and 6 & 7 mutant isolates. Lane M = 100 bp molecular weight ladder (Sigma-Aldrich, USA).The above shows 2 % ethidium bromide-stained agarose gel electrophoresis of DNA fragments produced by the *Afl1II* enzyme digest of an amplified *mdr1* gene region (560 bp) and control parasites used were 7G8 wild type and K1 mutant. The enzyme digested the wild-type (N86) and cuts the mutant to give a fragment of 328+232 bp at amino acid position 86 (Y86).

The *Pfdhfr* S108N gene mutation
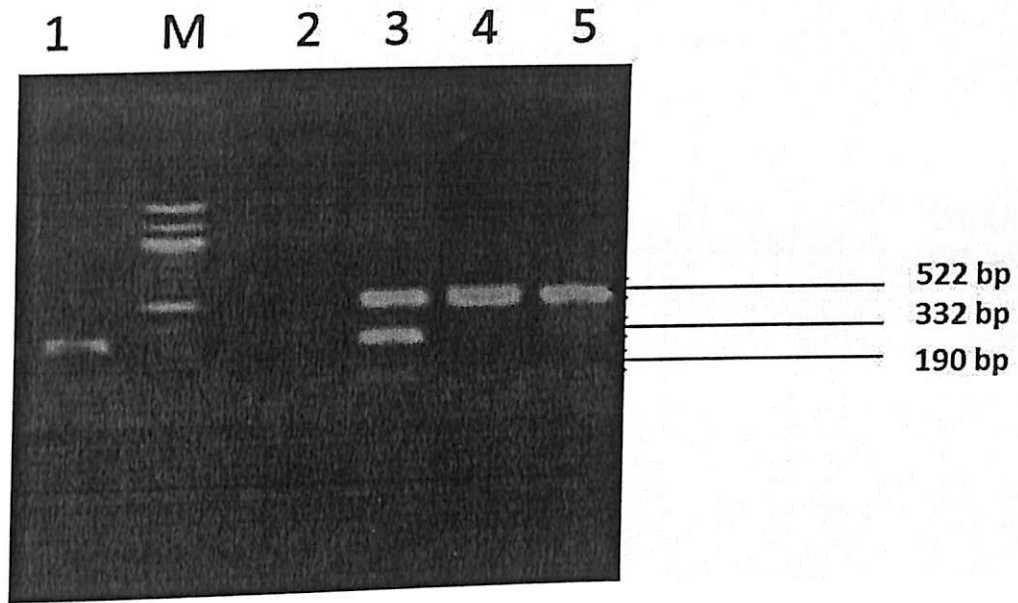
Figure S 4.6: An image of *Pfdhfr* (S108N) gene RFLP from the isolates in the study
Lane 1 = FCR3 strain (wild type), 2 = water (negative control) 3 = isolate (wild and mutant multi infection in a host), 4 & 5 K1 (mutant) control and undigested PCR product respectively. Lane M = 100 bp molecular weight ladder. The above shows a 2 % ethidium bromide-stained agarose gel electrophoresis of DNA fragments produced by the Bsr1 enzyme digest of an amplified *dhfr* gene region (522 bp). The controls are FCR3 for wild type and K1 as mutant. The mutant type is uncut after enzyme digest (S108) and the wild type cuts to 332 + 190 bp fragments at amino acid position 108 (N108).

The *Pfdhps* A437G gene mutation

438 bp
405 bp

Figure S 4. 7: An image of *Pfdhps* (A437G) gene RFLP from the isolates in the study.
Lane 1&2 = isolates (mutants), 3 = K1 mutant, 4 = water (negative control), 5 & 6 = no parasite), 7 = undigested PCR product. Lane M = 100 bp molecular weight ladder (Sigma-Aldrich, USA).The above shows 2 % ethidium bromide-stained agarose gel electrophoregram of DNA fragments produced by the *AvaII* enzyme digestion of an amplified *mdr1* gene region (438 bp) with Controls being FCR3 for wild type and K1 for mutant. The wild type is uncut (A437) and the mutant cuts to 404 + 34 bp fragments (437G).

Table S 4.3 The distribution of drug resistant markers in *Plasmodium falciparum* isolates

codon	Description	Obom Rainy	cape Rainy	Obom +Cape Rainy	Obom Dry	cape Dry	Obom +Cape Dry	Obom +Cape Both seasons
K76T	M	3	2	5	4	2	6	11
	W	33	10	43	41	13	54	97
	MW	8	9	17	8	4	12	29
N108I	M	1	1	2	18	3	21	23
	W	36	27	63	2	33	35	98
	MW	6	1	7	6	2	8	15
N86Y	M	0	0	0	6	0	6	6
	W	20	9	29	27	10	37	66
	MW	0	1	1	6	3	9	10
A437G	M	7	0	7	4	2	6	13
	W	27	12	39	23	4	27	66
	MW	0	0	0	0	0	0	0

M- mutant, W- wild type, MW- mutant & wild type isolates

Table S 4.4 A table showing association between *msp2* genotypes and submicroscopic gametocyte prevalence

	Pfs25 (%)		P value
	positive	negative	
msp2 (N = 97)	17	83	P = 0.74
IC3D7 (n = 36)	22	78	P = 0.62
FC27 (n = 23)	39	61	**P = 0.001**
IC3D7+FC27 (n = 38)			

Appendix 2: Volunteer Agreement Form

Protocol (1) 2014-15   version 1.0   14/8/2014

Parental Consent for children with Malaria

Title: *Plasmodium falciparum* gametocytogenesis

Principal Investigators: Dr Linda Eva Amoah
Address: Department of Immunology, NMIMR, Box LG581, Legon

General Information about Research
Malaria is common in Ghana. When you have malaria, it is important that we treat you and kill all the malaria parasi[tes] in your blood. It is also important to make sure that other people do not get sick with malaria. In order to do this, we want to study how the malaria parasites grow and move from one person to the other. We will take a small drop of blood (100 µL) from your child's finger to see if you have malaria parasites by microscopy. If you do, we will ask fo[r] an additional half a teaspoon (2.5 ml) of venous blood for children under 6 years or one teaspoon (5 ml) of venous blood for children over 6 years will be collected for further analysis. We would also like to know if your child is bett[er] after a week or still have some malaria parasites. So we will request you come back for free screening for malaria in [x] days. In addition to the routine laboratory tests that you do when you go to the hospital with malaria, we will take some of yo[ur] blood back to the NMIMR for more tests including sickling, blood typing and measuring your immune response against malaria parasites

Possible Risks and Discomforts
Your child may experience mild discomfort and bruising is possible at the site where the fingerpick and venous bloo[d] samples will be obtained. This will however resolve within an hour or two.

Possible Benefits
There are no direct benefits to your child. However, your participation may help us develop better malaria treatment[s]

Confidentiality
All information gathered would be treated in strict confidentiality. We will protect information about your child taki[ng] part in this research to the best of our ability. The child will not be named in any reports. However, Dr. Linda Eva Amoah may sometimes look at her research records. If you have any questions, please feel free to ask the clinician i[n] charge.

Compensation
Your child will not be paid for participation in this study but your transportation to the follow up visit will be refund[ed]

Voluntary Participation and Right to Leave the Research
We would like to stress that this study is strictly voluntary. Should your child decide not to participate; it will have n[o] consequences for him/her. Should your child, at any point during the study, decide that he/she does not wish to participate any further, participation will be terminated immediately. Any such decision will be respected without an[y] further discussion. The decision to end participation will not affect the health care your child would normally receiv[e]

Contacts for Additional Information
If you ever have any questions about the research study or study-related problems, you may contact Dr. Linda Amo[ah] of the Noguchi Memorial Institute for Medical Research (0279271632) at any time.

Your rights as a Participant
This research has been reviewed and approved by the Noguchi Memorial Institute for Medical Research Institution[al] Review Board (NMIMR-IRB). If you have any questions about your child's rights as a research participant you ca[n] contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses:
nirb@noguchi.mimcom.org.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title *(Plasmodium falciparum gametocytogenesis)* has been read and explained to me. I have been given an opportunity to have any questions a the research answered to my satisfaction. I agree to allow my child to participate as a volunteer.

_____   _____
Date   Signature or mark of parent or guardian

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered an volunteer has agreed to take part in the research.

_____   _____
Date   Signature of Witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____   _____
Date   Signature of Person Who Obtained Consent

Parental Consent for children with Malaria

Title: *Plasmodium falciparum* gametocytogenesis

Principal Investigators: Dr Linda Eva Amoah
Address: Department of Immunology, NMIMR, Box LG581, Legon

General Information about Research

Malaria is common in Ghana. When you have malaria, it is important that we treat you and kill all the malaria parasite in your blood. It is also important to make sure that other people do not get sick with malaria. Sometimes people have a lot of malaria parasites but are not sick, these people help spread malaria without knowing. We want to understand how people who are not sick help spread malaria. We will take two drops (200µl) of blood from your finger every other week to see if you have malaria parasites by microscopy. If you do, we will ask for an additional quarter teaspoon (1 ml) of venous blood for laboratory analysis. We will take your blood back to the NMIMR for testing, including sickling, blood typing and measuring your immune response against malaria parasites

Possible Risks and Discomforts

You may experience mild discomfort and bruising is possible at the site where the fingerpick and venous blood samples will be obtained. This will however resolve within an hour or two.

Possible Benefits

There are no direct benefits to you. However, your participation may help us develop better malaria treatment.

Confidentiality

All information gathered would be treated in strict confidentiality. We will protect information about you taking part this research to the best of our ability. You will not be named in any reports. However, Dr. Linda Eva Amoah may sometimes look at her research records. If you have any questions, please feel free to ask the clinician in charge.

Compensation

You will not be paid for participation in this study.

Voluntary Participation and Right to Leave the Research

We would like to stress that this study is strictly voluntary. Should you decide not to participate; it will have no consequences for you. Should you, at any point during the study, decide that you does not wish to participate any further, participation will be terminated immediately. Any such decision will be respected without any further discussion. The decision to end participation will not affect the health care you would normally receive.

Contacts for Additional Information

If you ever have any questions about the research study or study-related problems, you may contact Dr. Linda Am of the Noguchi Memorial Institute for Medical Research (0279271632) at any time.

Your rights as a Participant

This research has been reviewed and approved by the Noguchi Memorial Institute for Medical Research Institution Review Board (NMIMR-IRB). If you have any questions about your child's rights as a research participant you c contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title *(Plasmodium falciparum gametocytogenesis)* has been read and explained to me. I have been given an opportunity to have any questions ab the research answered to my satisfaction. I agree to participate as a volunteer.

_____   _____
Date   Signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and volunteer has agreed to take part in the research.

_____   _____
Date   Signature of Witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____   _____
Date   Signature of Person Who Obtained Consent

Appendix 3: Publication

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Seasonal variations in *Plasmodium falciparum* parasite prevalence assessed by varying diagnostic tests in asymptomatic children in southern Ghana

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Abstract

Plasmodium falciparum infections presenting either as symptomatic or asymptomatic may contain sexual stage parasites (gametocytes) that are crucial to malaria transmission. In this study, the prevalence of microscopic and submicroscopic asexual and gametocyte parasite stages were assessed in asymptomatic children from two communities in southern Ghana. Eighty children aged twelve years and below, none of whom exhibited signs of clinical malaria living in Obom and Cape Coast were sampled twice, one during the rainy (July 2015) and subsequently during the dry (January 2016) season. Venous blood was used to prepare thick and thin blood smears, spot a rapid malaria diagnostic test (PfHRP2 RDT) as well as prepare filter paper blood spots. Blood cell pellets were preserved in Trizol for RNA extraction. Polymerase chain reaction (PCR) and semi-quantitative real time reverse transcriptase PCR (qRT-PCR) were used to determine submicroscopic parasite prevalence. In both sites 87% (95% CI: 78–96) of the asymptomatic individuals surveyed were parasites positive during the 6 month study period. The prevalence of asexual and gametocyte stage parasites in the rainy season were both significantly higher in Obom than in Cape Coast (P < 0.001). Submicroscopic gametocyte prevalence was highest in the rainy season in Obom but in the dry season in Cape Coast. Parasite prevalence determined by PCR was similar to that determined by qRT-PCR in Obom but significantly lower than that determined by qRT-PCR in Cape Coast. Communities with varying parasite prevalence exhibit seasonal variations in the prevalence of gametocyte carriers. Submicroscopic asymptomatic parasite and gametocyte carriage is very high in southern Ghana, even during the dry season in communities with low microscopic parasite prevalence and likely to be missed during national surveillance exercises.

Introduction

For malaria elimination and eradication to be possible, control policies for all the various manifestations of malaria, including asymptomatic infections are needed. Currently, the majority of malaria elimination efforts include the use of insecticide treated nets and spray that is vector targeted. Seasonal chemoprophylaxis is also administered predominantly in high risk populations [1]. However, to move towards malaria elimination, an accurate profile of malaria transmission dynamics and infecting parasite clones in different transmission settings are needed to inform the implementation of the aforementioned strategies [2,3]. Inadequate information on malaria transmission intensity in endemic areas is common [4,5], and has resulted in either under or over estimation of the true prevalence of *Plasmodium* infections within different communities [6]. Unfortunately, the true prevalence of *Plasmodium falciparum* (*P. falciparum*) infections in a population is often not available, either because the tools used in the measurements lacked the requisite sensitivity or those specific sites were not included in the study. The results obtained from the sites used in the study are usually generalized for nearby communities [7,8]. The use of molecular methods to monitor parasite prevalence and provide an accurate mapping of transmission intensity will help address the above limitations on inadequate infection prevalence in most malaria endemic area. Although a number of molecular tools have been developed they need to be tested in the field to appreciate their strengths and limitations.

In order to achieve continuous and effective control of malaria to eliminate the burden associated with the disease [9], all the various presentations of *Plasmodium* infections including microscopic or submicroscopic and symptomatic or asymptomatic infections must be targeted. Asymptomatic infections are predominantly submicroscopic but still have the potential to influence malaria transmission [10]. Seasonal malaria chemoprevention (SMC) interventions are usually implemented in high parasite prevalence settings [1,11–13]. This intervention has been successfully deployed in Northern Ghana, where parasite prevalence is high (>40%) and malaria transmission is seasonal [14,15]. However, evidence from other studies show that transmission hotspots exist in areas generally considered as low transmission settings. These hotspots in the low transmission setting have similar high (>40%) parasite prevalence as in high transmission settings but are usually not captured for SMC or other malaria control interventions. These communities are often close to water bodies or have poor sanitation systems [16,17] and harbour transmission reservoirs that sustain the spread of malaria. Recent discussions have suggested an extension of SMC [18] to low transmission settings [19]. Such control measures have the potential to reduce *P. falciparum* parasite infections in communities with varying parasite prevalence, but need to be effectively monitored. Variations in infecting parasite densities and prevalence may influence the effectiveness of implemented interventions which requires careful selection of tools to monitor parasite prevalence amongst other factors [20,21].

As a first step towards accurately mapping parasite prevalence to designing a malaria elimination program we evaluated the relationship between asexual parasite and gametocyte prevalence in asymptomatic children living in two communities with varied malaria transmission patterns in Ghana. We utilized tools with varying sensitivities to assess the seasonal variations in *P. falciparum* parasites prevalence and have evidence to suggest the most appropriate tool to use to assess parasite prevalence in these two distinct settings.

Methods

Ethics, study site, population and sampling

The study had approval from the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR) and Ghana Health Services. Participants were enrolled only after written parental consent had been obtained.

The study randomly enrolled children from public primary schools in Obom and Cape Coast, both in southern Ghana. Obom (05°34′ N, 0° 20′ W), is a rural setting in Greater Accra and also lies in the Coastal savannah region. The mean annual rainfall varies between 790 mm to 1270 mm, along the coast to the extreme north. Relative humidity is about 75% between February and March and the main economic activity in Obom is farming. Malaria transmission is perennial with most of the disease occurring during the major rainy season in June/July [22,23]. Cape Coast (05°05′ N, 01° 15′ W), is an urban setting and lies in the Coastal savannah region on the Gulf of Guinea. The major rainy season is between May and July with mean monthly relative humidity varying between 85 and 99% and the main economic activity of the indigenes is fishing and farming. Malaria transmission in Cape Coast is seasonal with most of the disease occurring during June to July.

Two cross sectional surveys, involving 80 school children (40 from each site) aged between 6 and 12 years were conducted in the peak / rainy season (July, 2015) and during the off peak /dry season (January, 2016) after obtaining written parental consent. On each visit, body temperature was measured using a digital thermometer, after which 2.5 mL of venous blood was collected into EDTA vacutainer tubes and an aliquot used to prepare filter paper (Whatman® 3 mm) blood blots, thick and thin blood smears and spot histidine rich 2 protein malaria rapid diagnostic test (RDT). The rest of the blood sample was immediately separated into plasma and blood cells. One hundred microliters of the blood cells were preserved in 500 μL of Trizol (Invitrogen, UK) and the plasma preserved at -20 °C. During the dry season, haemoglobin levels were assessed using the Urit-12 haemoglobin meter.

Plasmodium falciparum parasite detection

Plasmodium falciparum species identification and parasitaemia were determined using 100X oil immersion microscopy. The thin and thick blood smears were processed for Giemsa staining and evaluated using a WHO protocol [24]. *Plasmodium* species were identified after evaluating the thin films and parasite density was estimated using the thick films. Parasite density was determined as the percent of infected erythrocytes counted per 200 white blood cells (WBC) based on a WHO protocol [24].

Parasite DNA extraction

Genomic DNA was extracted from two 3 mm punched discs of dried filter paper blood blots using the Chelex extraction protocol [25]. Briefly, the two punched discs for each sample were incubated in 1 mL of PBS solution for 10 minutes. The samples were washed twice with 1 mL PBS, followed by centrifugation at 14,000 rpm for 2 minutes. DNA was then extracted from the discs with 100 μL of 10% Chelex (Sigma-Aldrich, USA) in nuclease-free water by heating at 99 °C for 10 minutes, with occasional vortexing. Finally the sample was centrifuges at 14,000 rpm for 1 minute and the supernatant containing the DNA aliquoted into a new tube and stored at 20 °C for PCR.

PCR identification of *Plasmodium falciparum* parasites

Submicroscopic *P. falciparum* parasites were estimated based on the amplification of the *18S rRNA* gene. Nested PCR was performed using genus and species specific primers as described by Singh *et al.* [26]. All reactions were carried out in a 20 μL volume containing 200 nM dNTP, 2 mM MgCl₂, 200 nM of each primer, and 0.5 U of One Taq DNA polymerase (New England BioLAB, UK). Four microliters (>50 ng) of gDNA was used as the template for the primary reaction and 2 μL of the primary reaction product was used a template for the secondary reaction. The reaction cycling conditions were: initial denaturation at 94 °C for 5 minutes,

followed by 30 cycles at 94 °C for 1 minute denaturation; annealing at 50 °C (55 °C for nest 2) for 30 seconds, and 68 °C for 30 seconds; with final extension at 68 °C for 5 minutes. The PCR reaction mixtures were run on a thermal cycler (BioMetra T3000, Germany). Positive and negative controls for the PCR reactions comprising of the 3D7 *P. falciparum* strain and a no template control respectively were included in each set of the reactions. Amplified PCR products were visualized under UV illumination after electrophoretic separation on a 2% ethidium bromide stained agarose gel.

Parasite RNA extraction, purification and RT-PCR analysis

Total RNA was extracted from 80 paired trizol preserved samples collected in the rainy and dry seasons in both sites using the RNeasy micro kit (Qiaqen, USA) following the manufacturer's protocol. Complementary DNA (cDNA) was prepared using ProtoScript® First Strand cDNA Synthesis Kit (New England BioLab, UK) and a mix of 3 µM of random hexamers and 2.5 µM oligo (dT) primers. gDNA contamination check on the extracted RNA was done as previously reported [27]. The cDNA samples were diluted (1:20) before triplicate run with fast SYBR® Green 2X master mix RT-PCR kit on a QuantStudio 3™ Real-Time PCR System (Thermo Scientific, USA). The Fast cycling condition (95 °C for 20 sec, 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec) was used for qRT-PCR amplifications. Real time RT-PCR was carried out on cDNA samples to assess submicroscopic asexual parasite as well as mature gametocyte carriage using *Pf18S rRNA* and *Pfs25* transcript levels respectively. cDNA prepared from ring stage parasites (asexual) as well as matured gametocytes from *P. falciparum* NF54 parasite served as controls for both qRT-PCR reactions and primers were validated as previously described [27].

Data analysis

Statistical analysis was performed using Mann-Whitney paired two tailed t-test (GraphPad Prism v5.0) for the age groups and Two-sample test for equality of proportions with continuity correction (R version 3.4) to determine associations between parasite prevalence within and between participants in the two communities. The qRT-PCR data was analyzed with Quanti v1.3.1 Software (Thermo Scientific, USA). The threshold cycle (CT) cut off based on the negative and the positive controls as previously described [27], where it was used to classified the samples as negative or positive for asexual parasites and gametocytes. Tests were considered statistically significant when P values were < 0.05.

Results

Clinical characteristics of the study participants

The clinical characteristics of the participants in Obom and Cape Coast are shown in Table 1. The mean age of participants in both sites was not significantly different (P = 0.48, Mann Whitney paired two tailed test). In Obom the mean parasite density estimated from the thick blood smears increased from 1753 ± 1476 parasites/µL of whole blood in the 22 smear positive samples out of 34 total samples in the rainy season to 2344 ± 2636 parasites/µL of whole blood in the 11 smear positive samples out of 40 total samples during the dry season, although fewer individuals were infected. In Cape Coast, the mean parasite density was 1015 ± 971 parasites/µL of whole blood in the four smear positive samples out of a total of 39 samples in the rainy season, which decreased during the dry season to 530 ± 561 parasites/µL of whole blood in the two smear positive samples out of a total of 36 samples. The HRP2 RDT test was only performed in the dry season with 65% (95% CI: 46%–80%) positivity rate in Obom, significantly

Table 1. Clinical characteristics of the study participants.

Parameter	Obom		Cape Coast	
	July 2015	January 2016*	July 2015	January 2016
Temperature °C				
Mean	36.51	36.35	36.46	36.49
SD	0.51	0.59	0.53	0.53
Range	(35.20–37.60)	(35.20–37.20)	(34.40–37.40)	(34.90–37.30)
Haemoglobin (g/dL)				
Mean	^	10.59	^	11.87
SD		2.09		1.43
Range		(4.46*–14.00)		(7.50–14.60)
Parasite density/µL of blood				
Mean	1753	2344	1015	530
SD	1476	2636	971	561
Range	(160–5040)	(560–6920)	(80–3038)	(80–530)
Microscopy				
Positive (p/n)	22/34	11/40	4/39	2/36
HRP2 (RDT)				
Positive %	^	65	^	36
95% CI (%)		50–80		20–53

^ Not done;

* A participant had sickle cell disease; p, number that were microscopy positive; n, number of participant tested in the group;

• Malaria control intervention was implemented in the community four (4) months before sampling. There were 32 and 29 children in Obom and Cape Coast respectively who were present at both time points (paired samples).

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higher (P < 0.0001, Two-sample test for equality of proportions) than the 35% (95% CI: 20%–53%) recorded in Cape Coast.

Plasmodium falciparum asexual parasite prevalence

The prevalence of P. falciparum infected children identified by microscopic evaluation of thick blood smears was distinctly different in the two communities. In Obom parasite prevalence by microscopy was significantly higher in the rainy season (65%, 95%CI: 50–80)) than the dry season (28%, 95%CI: 13–42) (P <0.0001), while in Cape Coast, there was no significant difference between the seasons,10% (95%CI: 0–12) and 6% (95%CI: 0–13) in the rainy and dry seasons, respectively (Fig 1). As anticipated, both PCR and qRT-PCR analysis of Pf18s rRNA detected much higher parasite prevalence in both communities, when compared to prevalence by microscopy (Fig 1, Table C in S1 File), reaching a maximum of 86% in Obom and 56% in Cape Coast during the rainy season when measured by qRT-PCR. Surprisingly, the prevalence measured by qRT-PCR in Cape Coast during the dry season was higher than, but not significantly different (P = 0.31) when compared to the rainy season (Fig 1, Table A in S1 File) while in Obom the prevalence decreased significantly (p < 0.0001) from 86% in the rainy season to 60% in the dry season (Fig 1, Table A in S1 File). The reason for this difference is unknown, but could be due to the difference in malaria transmission intensity between the two sites. In Obom, the gametocyte prevalence of 51% in the rainy season reduced to 16% in the dry season, while in Cape Coast it increased from 10% in the rainy season to 35% in the dry season.

In Cape Coast, parasite prevalence estimated by PCR was 34% (rainy) and 35% (dry) higher than microscopy estimates and by qRT-PCR it was 46% (rainy) and 58% (dry) higher than
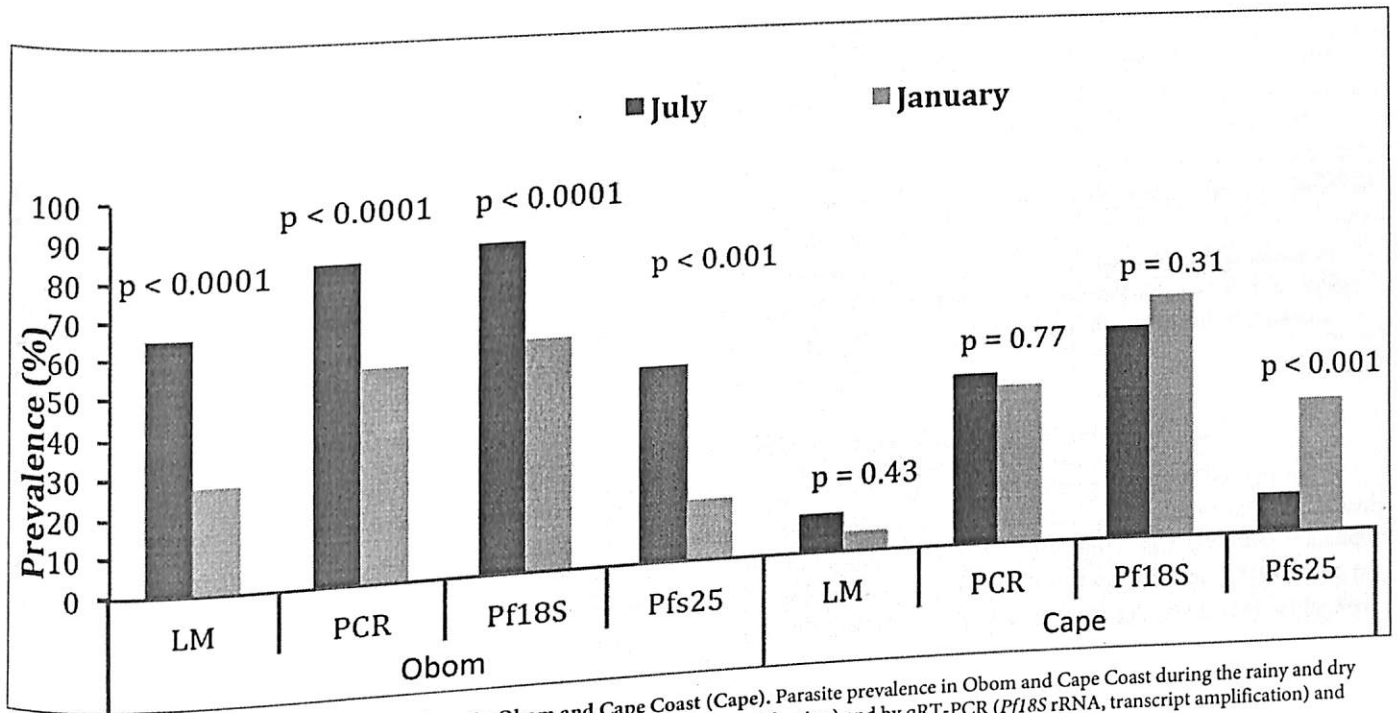
Fig 1. Asexual parasite and gametocyte prevalence in Obom and Cape Coast (Cape). Parasite prevalence in Obom and Cape Coast during the rainy and dry seasons as measured by microscopy (LM), conventional PCR (*Pf18S* rRNA, DNA amplification) and by qRT-PCR (*Pf18S* rRNA, transcript amplification) and submicroscopic parasite carriage determined using *Pfs25* qRT-PCR.

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microscopy (Fig 1, Table C in S1 File). This suggests that for every child that tested positive by microscopy, there were between four to eleven children that had low density infections that were missed. The submicroscopic parasite prevalence estimated by qRT-PCR was 12% (rainy season) and 23% (dry season) greater than PCR in Cape Coast (Table C in S1 File). Although parasite prevalence estimated by both PCR and qRT-PCR did not change appreciably between the rainy and dry season (P = 0.38, Two-sample test for equality of proportions) a pronounced increase by 3.5 fold was observed among submicroscopic gametocyte prevalence (P < 0.001, Two-sample test for equality of proportions) [Fig 1 and Table in S1A Table].

In the rainy season, microscopy underestimated parasite prevalence in Obom by 28% and 32% when compared to estimates determined by PCR and RT-PCR respectively. Although the difference did not seem large, it was significant (P < 0.05, Two-sample test for equality of proportions) [Table A in S1 File]. Similarly, in the dry season, microscopy underestimated parasite prevalence by 49% and 53% when compared to both PCR and qRT-PCR respectively. Parasite prevalence estimated by PCR and RT-PCR were similar at both time points and each reduced by 28% and 26% respectively in the dry season compared to the rainy season. The decrease in submicroscopic gametocyte prevalence in the dry season compared to the rainy season was double that recorded for total parasite prevalence (69%) [Fig 1 and Table A in S1 File].

Malaria transmission is dependent on the prevalence of mature gametocytes in an infection. During the rainy season, 59% of the 39 children in Cape Coast had submicroscopic infections (asexual parasite positive) that contained submicroscopic gametocytes, whilst 70% of the 37 children in Obom harbored submicroscopic both asexual parasites and gametocytes. The reverse trend was observed in the dry season where the prevalence of children harboring asexual parasites with gametocytes was higher in Cape Coast (72%) than in Obom (59%) [Table B in S1 File].

Table 2. Parasite (Asexual and gametocyte) prevalence in paired samples.

Visit	PfI8S (Obom) (% of n)	PfI8S (Cape) (% of n)	P value	Pfs25 (Obom)* (% of 18S)	Pfs25 (Cape)* (% of 18S)	P value
July +/ Jan. +	44	31	0.08	21	0	< 0.0001
July +/ Jan. -	38	17	0.002	67	20	< 0.0001
July -/ Jan. +	13	38	< 0.0001	25	55	< 0.0001
July -/ Jan. -	6	14	0.099	0	0	

July +, positive / infected in July; July -, negative / not infected in July; Jan. +, positive / infected in January; Jan. -, negative / not infected in January; n, total count; PfI8S, total parasite. Pfs25*, total mature gametocyte (qRT-PCR on samples with and without microscopic gametocytes). Paired samples in for each sites (n = 32 for Obom, n = 29 for Cape Coast). PfI8S is reported as a % of the total number of paired children but Pfs25 is reported as a % of 18S positive (parasitaemic) children.

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Paired (children present at both time points) sample analysis

Paired samples from both Obom and Cape Coast revealed how many total children were asymptomatic parasite carriers at either time point. Real time RT-PCR analysis identified only 6% (2/32) of children in Obom and 14% (4/29) of children in Cape Coast to be free of an active infection in both seasons. As high as 44% (14/32) of the children in Obom and 31% (9/29) in Cape Coast were parasitaemic in all seasons (Table 2). Twenty-one percent (3/14) of children from Obom simultaneously harbored mature gametocytes in both the rainy and dry season. None of the children from Cape Coast harbored gametocytes during both the rainy and dry season (Table 2). Sixty seven percent of children who were parasitaemic in the rainy season harbored mature gametocytes in Obom; this was significantly higher but not the dry season harbored mature gametocytes in Obom; this was significantly higher than the 20% of the children in Cape Coast who were parasitaemic only in the rainy (but not the dry season) who harbored mature gametocytes (Table 2). More participants in Cape Coast were parasitaemic in the dry season than in Obom. Six out of 11 (55%) of these parasitaemic children harbored mature gametocytes in Cape Coast, whist only a child (25%) out of the 4 children who were parasitaemic in the dry season in Obom harbored mature gametocytes (Table 2).

Discussion

Microscopy, the gold standard for malaria diagnosis has the limitations of requiring expertise, electricity and has low detection sensitivity, ~50 parasites/µL blood by expert microscopists [28,29]. This low sensitivity can be a major problem in low transmission settings and in detecting asymptomatic infections. The introduction of malaria rapid diagnostic tests (RDTs) revolutionized malaria diagnosis, as they provide quick results without the need for specialized equipment or personnel. The RDTs are widely used in managing malaria in resource constraint settings and also used in mapping *Plasmodium* infections during community surveys [30]. Yet, the relatively low sensitivity (> 100 parasites/µL) of RDT [31], recent identification of parasites which have deletions in the *Plasmodium falciparum histidine rich protein 2* (*pfhrp2*) gene [32,33] and the persistence of the *P. falciparum* Histidine Rich Protein 2 (HRP2) antigen have hampered the usefulness of rapid PfHRP2 based malaria diagnostics. Parasite prevalence can also be determined using molecular tools including polymerase chain reaction (PCR), which has a limit of detection (LOD) of 1-5 parasites/µL of blood, reverse transcriptase PCR (RT-PCR) and real time RT-PCR (qRT-PCR), which both have limits of detection approaching > 0.5 parasites/µL of blood to detect the *Plasmodium* small subunit ribosomal *18S rRNA* gene [26,34,35] or transcript [36], respectively. These molecular tools can also be used to differentiate between the parasite species [28,37–40]. However, these methods are expensive and require technical expertise, electricity, cold storage and specialized reagents.

Gametocyte carriage is an essential component of malaria transmission in any community. However, public health programs do not prioritize monitoring gametocyte densities, due to the need for parasite RNA to differentiate gametocytes from the total parasite population. Historically, gametocytes have been detected using light microscopy, with sensitivities of about 20 mature gametocytes/μL of blood [41,42]. However, in most individuals, gametocyte carriage is usually at submicroscopic densities and requires sensitive molecular tools, to detect stage-specific transcripts that are not as abundant as the *18s rRNA*. The most common tools used to accurately estimate gametocyte carriage include reverse transcriptase PCR (RT-PCR), real time reverse transcriptase PCR (qRT-PCR) and quantitative nucleic acid amplification (QT-NASBA) [41,43–45]. The main transcripts used to monitor gametocyte densities and prevalence include the female mature gametocytes specific transcript *Pfs25* [46,47], and *Pfs230* paralogue, *Pfs230p*, that detects mature male gametocytes [48,49] and *Pfs16*, which is present in all gametocyte stages [50]. Similarly, *Pfg377* a female specific transcript is used to determine gametocyte diversity [51].

Gametocyte production has been suggested to differ in areas with varying microscopic parasite prevalence [43]. Thus, to monitor the infectious reservoir in two communities with different malaria transmission profiles we assessed both the gametocytes as well as the asexual parasitemia to determine the relationship. We also compared three different malaria parasite detection tools to identify the most effective.

Detection tools

Microscopy, was confirmed to have significantly lower sensitivity at detecting *P. falciparum* parasites than PCR and qRT-PCR in both high and low parasite intensity settings (Table S1 Table 1C), as has previously been established [52–54]. Surprisingly, although RDT positivity rate is not a true indicator of parasite prevalence as PfHRP2 antigen persistence and the presence of parasites with deletions in the *Pfhrp2* gene increase the incidence of false positive or negative test results [55,56], in this study it provided a better estimate of parasite prevalence than microscopy in both sites. The RDT positivity rates were significantly higher than parasite prevalence estimated by microscopy but similar to parasite prevalence estimated by PCR in both sites, suggesting that the HRP2 antigen levels detected by the RDT were most likely due to antigens produced by active low level infections that were undetectable by microscopy rather than past exposure to parasites. This similarity between RDT and PCR results suggests that substituting microscopy for the much simpler RDT could provide a more accurate estimation of *P. falciparum* prevalence when screening asymptomatic individuals. In Cape Coast, parasite prevalence estimated by PCR increased by 27% in the wet season and 56% in the dry season when assayed by qRT-PCR, which is consistent with previous reports that real time RT-PCR has much higher sensitivities at detecting malaria parasites compared with PCR [41,42,44]. It is likely that this difference in *P. falciparum* estimation by PCR and qRT-PCR was only observed in Cape Coast, not Obom, because the parasitemias were lower, with some densities below the detection limit of PCR during both seasons. In Cape Coast the average parasitemia was 1015 parasites/μL in the wet season and only 530 parasites/ μL in the dry season compared to Obom where the average parasitemia was above 1750 parasites/ μL in both seasons. More studies with a larger population and in communities with varying malariometric indices are needed to define the cut-off parasitemia for the use of PCR or possibly RDT to assess asymptomatic cohorts.

Seasonal differences in parasite prevalence

Another major difference between Cape Coast and Obom was the seasonal change in both total parasite and gametocyte prevalence. In Obom there was a significant reduction in parasite

prevalence in the dry season by all the three assessment methods as well as a decrease in gametocyte prevalence by *Pfs25* qRT-PCR. In contrast, In Cape Coast although microscopic parasite prevalence decreased by 50%, there was no difference in submicroscopic parasite prevalence in Cape Coast (P > 0.05) between the two seasons. The difference observed by microscopy could be due to the 50% decrease in the average parasitemia in Cape Coast in the dry season, which decreased the number of individuals with parasitemias above the threshold for microscopy but still above the level needed for PCR or RT-PCR. Despite the decrease in total parasite prevalence the gametocyte prevalence significantly increased from 10% in the wet season to 35% in the dry season.

Differences in the dynamics of the mosquito populations in the wet and dry seasons in Obom and Cape Coast could contribute to differences in parasite prevalence. Since parasite prevalence as measured by RT-PCR is similar in both communities in the dry season it is possible that there are similar numbers of circulating mosquitos, but that during the wet season this population only increases significantly in Obom. Obom is a more rural setting with more areas for water to pool creating a seasonal increase in mosquito breeding sites. However, the decrease in Obom could also have been related to the National Malaria Control Program that distributed insecticide treated bed nets in the community four months prior to the January sampling, which was not simultaneously carried out in Cape Coast [57]. Additional longitudinal testing in successive years is needed to evaluate the observed seasonal parasite prevalence patterns.

Gametocyte prevalence

The Control intervention in Obom could also have decreased the gametocyte prevalence in the community, but this does not explain the significant increase in gametocyte prevalence in Cape Coast in the dry season. Even in the wet season, prior to the control intervention the gametocyte prevalence differed in the two communities. During the wet season in Obom 59% of the parasitemic individuals had gametocytes, while in Cape Coast there was lower parasite prevalence and parasitemia and only 17% were gametocytemic. In the dry season the parasite prevalence remained the same in Cape Coast, while the average parasitemia decreased from 1000 parasites/μL to 500 parasites/μL, but the gametocyte prevalence increased so that 52% of the parasite carriers were gametocytemic. The reason for this seasonal increase is unclear and was not observed in Obom, which in the dry season had a similar parasite prevalence to Cape Coast, although in Obom the average parasitemia was much higher, 2300 parasites/μL. It is possible that parasite strains with higher conversion rates are selected for during this time or that conditions in the host during these periods influenced sexual differentiation and maturation allowing continuous transmission as suggested by Okell and colleagues [43]. However, the ratio of gametocyte carriers to parasitemic individuals was even higher in Obom during the high transmission wet season (59%) than it was in Cape Coast during the dry season and is more in line with suggestions that high parasitemia enhances gametocyte production [58]. Reconciling this data requires additional longitudinal studies to confirm the patterns as well as experiments to directly evaluate of the number of gametocyte-committed rings and their in vivo progression to circulating stage V gametocytes 10–12 days later.

Prevalence in paired samples

Using paired samples allowed the direct evaluation of the number of individuals that were parasitemic at one or more collection times. These results indicate that 87% (95% CI: 78–96) of the individuals at both sites were parasitemic at least once within the 6 month study period and that a high number of the participants at both locations harboured an asymptomatic infection at any one time during both the wet and dry season, with the peak season in Cape Coast

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registering the lowest prevalence of 48% (95% CI: 29–68). Such high prevalence asymptomatic infections have previously been reported during the dry season in Northern Ghana, where more than 50% of the participants (children under 12 years) harboured submicroscopic infections [59,60] and suggests the need for considering the expansion of SMC control programs throughout Ghana. Again, the pattern of gametocyte carriage differs between sites. In Cape Coast none of the individuals that were parasite positive at both visits were gametocyte carriers, while in Obom 20% of the individuals that were parasitemic at both collections had gametocytes. Consistent with the higher gametocyte prevalence observed in the wet season in Obom and the dry season in Cape Coast more that 55% of the individual that only had parasites during these collections were gametocytemic. It has been suggested that 20–50% of the overall malaria transmission result from submicroscopic infections [43] therefore these high levels of gametocyte prevalence are likely to be making a substantial contribution to transmission and additional work is needed to understand underlying factors involved in the complex seasonal pattern observed in this study.

Limitation

The absence of active or passive follow up of the study participants prevented the observation of any possible progression of an asymptomatic *P. falciparum* infection into a symptomatic infection. Only a small number of children were sampled in this study. Additional longitudinal studies with a larger sample set over sequential years are needed to confirm the observed distinct seasonal patterns of asexual parasite and gametocyte prevalence.

Conclusion

Parasite carriage in southern Ghana is much higher than previously classified, with majority of the infections presenting as asymptomatic submicroscopic infections. Parasite prevalence estimated by PCR and qRT-PCR is significantly different only in low parasite density settings, thus for an accurate map of parasite carriage in Ghana, PCR or possibly RDT can be used in high parasite density settings and qRT-PCR used in low parasite density settings. Communities with varying transmission patterns also exhibited marked differences in the seasonal pattern of mature gametocyte carriage and the factors contributing to this need to be evaluated further.

Supporting information

S1 File. Table A: Comparison of parasite prevalence between the two seasons in both sites, Table B: Comparison of parasite prevalence between the two sites at each season and Table C: Sensitivities of detection tests at assessing parasite prevalence.
(DOCX)

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