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SCHOOL OF PUBLIC HEALTH

COLLEGE OF HEALTH SCIENCES

UNIVERSITY OF GHANA

ASSESSMENT OF GENETIC DIVERSITY, COMPLEXITY OF INFECTION OF *PLASMODIUM FALCIPARUM* AND RESISTANCE TO ANTIMALARIAL DRUGS IN TWO ECOLOGICAL ZONES IN GHANA

BY

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DECLARATION

I hereby declare that this research work, which was undertaken at the Noguchi Memorial Institute for Medical Research, University of Ghana and the University of Massachusetts Medical School is my own work and where information has been derived from other sources, I confirm that this has been acknowledged in the text or by reference cited. I further declare that no material presented in this work has been submitted for the award of a degree by this or any other University.

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Date ……..

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Prof. Francis Anto	Dr. Anita Ghansah	Dr. Benjamin Abuaku
(Supervisor)	(Supervisor)	(Supervisor)
Date ……	Date ……	Date ……

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ABSTRACT

Introduction: One major challenge to the global agenda for the elimination of malaria is the extensive genetic diversity of the parasite population, resulting in the development of drug resistance and variation in antigens targeted for vaccine development. The aim of this study was to decipher any ecological difference in the evolution of drug resistance and to determine how much the increased use of artemisinin-based combination therapies (ACTs) and other control interventions are shaping the genetic diversity of the *Plasmodium falciparum* population in two ecologically distinct populations in Ghana.

Methods: A total of 803 dried blood spots (DBS) collected on filter paper from symptomatic children, aged 6 months to 14 years, with *P. falciparum* mono-infection in the coastal savanna (Cape-Coast) and the forest (Begoro) zones of Ghana from 2014 to 2017. In addition, a total of 991 *P. falciparum* infected DBS were collected from asymptomatic school children, aged 6 years to 14 years from 2013 to 2017. The study leveraged the high specificity and relatively low-cost of targeted next generation sequencing using molecular inversion probes for targeting and sequencing on the illumina MISEQ platform for sequencing of *P. falciparum* genes (*pfcrt*, *pfdhfr*, *pfdhps*, *pfmdr1* and *pfk13*) implicated in anti-malarial resistance to chloroquine (CQ), sulfadoxine pyrimethamine (SP), lumefantrine, amodiaquine and artemisinin. The *Plasmodium falciparum* Apical Membrane Antigen 1 (*pfama1*) gene was also sequenced for the genetic diversity and complexity of *P. falciparum* infections (COI) analysis. Genetic diversity was compared between the two study populations and the sequences from Ghana were

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compared with sequences from West Africa, East Africa and South East Asia obtained from the gene bank.

Results: The result showed high genetic diversity in *pfama1* in Ghanaian sequences with a total of 164 *pfama1* haplotypes and a haplotype diversity of 0.993. There was no genetic differentiation between the two study populations in Ghana. Parasite isolates from the two ecological zones in Ghana showed a moderate genetic differentiation with sequences from Thailand (Fst=0.054) and low differentiation with sequences from Kenya (Fst=0.004). Seventy three percent (73%) of the infections were monoclonal. The major molecular marker associated with CQ resistance *pfcrt K76T* significantly reduced over the four years of the study ($\chi^2 = 40.57$; $p<0.001$). The rate of re-expansion of chloroquine sensitive strains *pfcrt* K76 was higher in the forest ecological zone compared to the coastal savanna zone. The *pfmdr1* 184F mutant associated with lumefantrine resistance remained high over the years (68% to 83%). The prevalence of the quadruple mutation (**IRNG**K), associated with sulfadoxine-pyrimethamine resistance is almost at fixation, whilst *pfdhps* 540E has remained very low in Ghana. In addition, the study found low prevalence of *pfdhps* 581G mutation associated with sulfadoxine resistance, which has not been previously reported in these parts of Ghana. The South East Asian *pfK13* mutations that confer resistance to artemisinin were not found in these study sites of Ghana

Conclusion: This study provides new data which gives valuable information for developing an effective *pfama1*-based malaria vaccine. The detected of *pfdhps* 581G mutation associated with sulfadoxine resistance and the absence of South East Asian *pfK13* mutations associated with artemisinin resistance in these parts of Ghana provides relevant

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information for the national malaria control programme. The ecological differences observed in the re-expansion of chloroquine sensitive strains provide useful information on hotspots that can be targeted in the design of malaria control strategies in Ghana.

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DEDICATION

I dedicate this work to God Almighty, my family, friends, colleagues and study participants.

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LIST OF ABBREVIATIONS

ACT	Artemisinin-Based Combination Therapy
AIDS	Acquired Immune Deficiency Syndrome
AL	Artemether Lumifantrine
AMA	Apical membrane antigen
ARDS	Acute respiratory distress syndrome
ASAQ	Artesunate Amodiaquine
CI	Confidence Interval
COI	Complexity of Infection
CQ	Chloroquine
CSP	Circumsporozoite Protein
CSZ	Coastal Zone
DAPC	Discriminant Analysis of Principal Components
DBP	Duffy-binding Protein
DBS	Dried blood spots
DHAP	Dihydroartemisinin Piperaquine
DNA	Deoxyribo Nucleic Acid
DVS	Dominant vector species

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EIR	Entomological inoculation rate
FST	F-Statistics
FZ	Forest Zone
G6PD	Glucose-6-Phospate Dehydrogenase
GDHS	Ghana Demographic and Health Survey
GMIS	Ghana Malaria Indicator Survey
Hd	Haplotype diversity
HIV	Human Immune Virus
IPTp	Intermittent Preventive Treatment in pregnancy
IPTc	Intermittent Preventive Treatment in children
IRB	Institutional Review Board
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
K13	Kelch-13
LD	Linkage Disequilibrium
MIM	Multilateral Initiative on Malaria
MIP	Molecular Inversion Probe
MOH	Ministry of Health

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MSP	Merozoite surface protein
NS SNP	Non-Synonymous Single Nucleotide Polymorphism
OPD	Out Patient Department
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PfAMA-1	*Plasmodium falciparum* Apical Membrane Antigen 1
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
pfdhps	*Plasmodium falciparum* dihydropteroate synthase
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
Pfmdr	*Plasmodium falciparum* multi drug resistance
Pgh	P-glycoprotein homologue
PMR	Parasite multiplication rate
RBC	Red Blood Cell
RBM	Roll Back Malaria
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SEA	South-East Asia

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SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-pyrimethamine
TNF	Tumour necrosis factor
TRAP	Thrombospondin related anonymous protein
UMI	Unique molecular identifiers
UMMS	University of Massachusetts Medical School
WBC	White Blood Cell
WGS	Whole Genome Sequencing
WHO	World Health Organization
WWARN	World Wide Antimalarial Resistance Network

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DEFINITION OF TERMS

Complexity of infection (COI): Complexity of infection also known as the multiplicity of infection in some cases is defined as the mean number of parasite genotype of *P. falciparum* natural infections per individual at a particular point in time.

Multi-clonal infection: Multi-clonal infection occurs when an individual is concurrently infected with two or more genetically distinct clones of *P. falciparum* clones.

Clonal infections: Clonal infections occur when an individual is concurrently infected with only one parasite clone or genotype of *P. falciparum*.

***Plasmodium falciparum* mono-infection**: *P. falciparum* mono-infection is defined as an individual infected with only *P. falciparum* without the other *Plasmodium* species.

Symptomatic infections: as defined in this study are children who presented with malaria symptoms (fever) at the health facility and tested positive for *P. falciparum* infection.

Asymptomatic infections: as defined in this study are children who did not have fever (temperature < 37.5 ˚C) but tested positive for *P. falciparum* infection.

Molecular inversion probes: Molecular inversion probes are probes used for targeted capture and resequencing of candidate genes, enabling the ability to assess hundreds of loci in large sample surveys making it cost effective and scalable.

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CHAPTER ONE

1 INTRODUCTION

1.1 BACKGROUND

1.1.1 Trends and distribution of malaria

Malaria is the most deadly parasitic disease of global importance, accounting for 219 million cases and 435,000 deaths in 2017, mainly in African children (WHO, 2018b). Approximately half of the world's population remains at risk of developing malaria and with ongoing transmission in 90 countries. Majority of global malaria cases in 2017 occurred in the World Health Organization (WHO) African Region (approximately 92% of the cases), while 5% occurred in the WHO South-East Asia (SEA) and 2% in the WHO Eastern Mediterranean Region. Although the incidence of malaria reduced by 18% between 2010 and 2017, incidence in some countries increased substantially between 2015 and 2017 (WHO, 2018b).

In Ghana, almost 7 million confirmed cases were recorded in 2017 as stated by the world report, representing approximately 4% of all malaria cases reported worldwide (WHO, 2018b). Malaria accounts for 40% of all out-patient cases in Ghana (Awine, Malm, Bart-Plange, & Silal, 2017) and the principal cause of death in children under 5 years old (IHME, 2016; WHO, 2017). The 2014 Ghana Demographic and Health Survey (GDHS) and the 2016 Ghana Malaria Indicator Survey (GMIS) estimated average parasite prevalence in children aged 6–59 months of age to be 27.0% and 21% respectively (Ghana Statistical Service, 2016).

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Malaria is caused by the protozoan parasites that belong to the genus *Plasmodium.* Out of the five species that infect humans, *Plasmodium falciparum* is the deadliest (White, Pukrittayakamee, Hien, et al., 2014). The other less virulent *Plasmodium* species are *vivax, ovale, malariae, and knowlesi.* Although a milder disease is usually caused by *Plasmodium vivax,* severe disease can also occur after continuous episodes. The severity of the disease by *Plasmodium malariae* and *Plasmodium ovale* is generally similar to the less severe form of *vivax* malaria. *Plasmodium knowlesi* was primarily zoonotic infection prevalent mainly in SEA and can now cause severe malaria in humans (Ashley, Pyae Phyo, & Woodrow, 2018). Almost all deaths from malaria are caused by *P. falciparum* malaria usually in association with severe anaemia or cerebral malaria (White, Pukrittayakamee, Hien, et al., 2014).

1.1.2 Malaria control

The number of deaths from malaria for the past two decades has fallen by about half (Walker, Nadjm, & Whitty, 2018), achieved primarily through augmenting of established interventions like vector control with Indoor Residual Spraying (IRS), Insecticide Treated Nets (ITNs), larval source management and other protective measures such as use of insect repellent and wearing of protective clothing. One of the key successes in the control of malaria is also the use of anti-malarial drugs like artemisinin-based combination therapy (ACT) (Greenwood, 2017). Other control strategies such as Preventive Treatment including Intermittent Preventive Treatment in pregnancy (IPTp) and in children (IPTc) have been used extensively to minimize malaria risk in these high-risk groups (WHO, 2017). Also, chemoprevention is recommended for travelers visiting endemic countries

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(Chen, Wilson, & Schlagenhauf, 2006; White, Pukrittayakamee, Hien, et al., 2014). The only registered malaria vaccine RTS,S/AS01 is still under evaluation and offers only partial protection (WHO, 2016).

The past 17 years has seen an increase in the number of countries progressing to elimination. It is reported that a total of 19 countries attained zero indigenous cases of malaria between 2000 and 2017 (WHO, 2018a). These countries were Algeria, Argentina, Armenia, Azerbaijan, Egypt, Georgia, Iraq, Kazakhstan, Kyrgyzstan, Morocco, Oman, Paraguay, Sri Lanka, Syrian Arab Republic, Tajikistan, Turkey, Turkmenistan, Uzbekistan and United Arab Emirates. Out of these, 7 (United Arab Emirates, Morocco, Armenia, Turkmenistan, Kyrgyzstan, Paraguay, and Sri Lanka) have been certified by WHO as being free of malaria (i.e. 3 consecutive years of zero indigenous cases) (WHO, 2018b). Recent milestones in malaria calls for celebration but the patterns of malaria infection within countries are becoming more heterogeneous. Thus, national malaria control programmes must consider differences in the intensity of malaria infection between regions within a country in designing, implementing and monitoring of interventions as well as the climate (Greenwood, 2017).

1.1.3 Genetic diversity and complexity of *P. falciparum* infections

Genetic diversity in *P. falciparum* as a result of antigenic variation is one of the main hindrances to the development of an effective malaria vaccine. That is, the existence of different genotypes of the parasite, and complexity of infection (COI), which is estimated as the mean count of parasite clones per a single host (Mahajan, Farooq, Dubey, & Malla, 2005; Saha, Ganguly, & Maji, 2016; Thera & Plowe, 2012). Genetic diversity also known

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as genetic variation or polymorphism is usually defined as “the occurrence of two or more alleles at one locus in the same population, each with appreciable allele frequency less than 0.95 (Cavalli-Sforza & Bodmer, 1999; Hartl, Clark, & Clark, 1997; Nei, 1987). The diversity can be due to different molecular mechanisms including base substitution either of deletion or insertion, gene conversion, recombination and single nucleotide polymorphism (SNP). In malaria parasites, the genetic diversity generated in *P. falciparum* populations is a mechanism employed by the parasite to adjust to environmental changes by introducing new forms of drug resistance and diversity in surface antigens for evading the host immune system (Mita & Jombart, 2015). Diversity is associated with mortality, morbidity and malaria control strategies (Tanabe et al., 2015). Molecular epidemiology studies use COI as a measure of the level of the immunity state of an individual (Smith et al., 1999) and malaria transmission in endemic settings (Babiker & Walliker, 1997; Gueye et al., 2018). Therefore, it is important to characterize these patterns and understand the main drivers of *P. falciparum's* genetic diversity and COI to understand the dynamics, distribution, and genetic structure of the parasite population (Mita & Jombart, 2015).

1.1.4 Drug resistance

Drug resistant malaria is a critical public health problem in the sub-tropical and tropical nations of South-East Asia, African and South America, causing serious disease burden in several regions. The general consensus is that drug resistance results from de novo mutations in the parasite, reducing its sensitivity to that particular drug (Chakraborty, 2016). Genetic mutations associated with resistance to antimalarial drugs do occur de novo, independent of drug pressure (Wongsrichanalai, Pickard, Wernsdorfer, & Meshnick,

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2002). However, the presence of drug pressure confers a selective edge of resistant parasites over sensitive strains in the population. Natural selection also affects the survival and spread of resistant strains while slowly destroying the sensitive ones (Chakraborty, 2016; Le Bras & Durand, 2003). Resistance to antimalarial drugs appear to emerge in regions where large populations of parasites encounter specific drug pressure. The situation is very alarming in several regions of Cambodia, Myanmar and Thailand where resistance to both chloroquine and sulfadoxine pyrimethamine emerged from, and in both cases the resistant genes spread to Africa and caused devastating outcomes (White, Pukrittayakamee, Hien, et al., 2014). This has compelled most endemic countries to switch to Artemisinin–based Combination therapy. Resistance to artemisinin has also emerged and is spreading in South East Asia which is of great concern (White, Pukrittayakamee, Hien, et al., 2014), and to date there has been no replacement for artemisinin (Sibley, 2014).

1.2 PROBLEM STATEMENT

Global malaria control strategies have achieved great feats over the past 15 years, with an observed decline in malaria prevalence in many endemic countries, although elimination efforts remain a formidable task. One of the major challenges to achieving the goal of elimination is the extensive genetic variation of the parasite population. This makes it difficult for the development of appropriate antimalarial drugs because, new forms of antimalarial drug resistance are continually emerging. In addition, new forms of antigenic variation hinder the development of effective malaria vaccines (Manske et al., 2012).

The declining malaria prevalence has resulted in the transition of the local malaria epidemiology in several endemic regions, leading to changes such as decreasing and

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unpredictable transmission (Auburn & Barry, 2017; Cotter et al., 2013). The declining malaria transmission can affect genetic diversity patterns and hence the population structure of *P. falciparum*. This has high implication in the emergence and spread of malaria drug resistance and the development of effective vaccines for malaria control (Manske et al., 2012). Therefore, monitoring the genetic diversity and COI of malaria parasites circulating in the population is critical to devising optimal drug- or vaccine-based malaria control strategies (Tanabe et al., 2010).

The current study assessed the genetic diversity, COI of *P. falciparum* and parasite resistance to antimalarial drugs in both symptomatic and asymptomatic infections, which gives the true picture in the population.

1.3 RATIONALE AND JUSTIFICATION

A major set-back to malaria elimination is the extensive genetic variation of the *Plasmodium* parasite population, which makes it difficult to develop appropriate antimalarial drugs and an effective malaria vaccine. Malaria incidence has declined substantially over the past 15 years as a result of improved control measures and research activities such as Roll Back Malaria (RBM) and Multilateral Initiative on Malaria (MIM), as well as more funding from international donor agencies (both private and public). The declining endemicity can result in a split in the genetic structure of the parasite between regions, reduce gene flow and possibly impede the spread of resistance between populations (Dye & Williams, 1997; Pumpaibool et al., 2009). In addition, the population structure in these regions will in the future mimic low transmission settings like SEA, where multiple clones are not common and *P. falciparum* populations show high levels of

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multi locus linkage disequilibrium, lower diversity, and evidence of population structure. It is important to monitor both local and worldwide parasite genetic diversity and COI to reveal how the changes in transmission patterns are shaping the parasite population in terms of the evolution of drug resistance and antigenic variation (Manske et al., 2012; Tanabe et al., 2010).

Monitoring of the parasite population is important for the identification of the most efficient intervention methods to delay the emergence of resistance (Auburn & Barry, 2017). Also, monitoring COI and genetic diversity are key elements in the design and production of a universally potent malaria vaccine, as they give information about the natural dynamics and diversity of vaccine candidate antigens in different endemic settings (Pirahmadi, Zakeri, Mehrizi, & Djadid, 2018; Volkman et al., 2012). There is limited data on the genetic diversity of *P. falciparum* in Ghana and a handful of studies have looked at the effect of declining transmission on the parasite population genetic patterns (i.e. genetic diversity, COI and drug resistance) over a period and more importantly if subtle differences in local levels of endemicity and transmission intensity affect this genetic pattern. The current methods for assessing genetic diversity, COI and drug resistance in malaria only focus on monitoring symptomatic cases neglecting asymptomatic cases, which gives true picture of estimates within the population.

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1.4 RESEARCH QUESTIONS

1 Will the declining transmission of *P. falciparum* affect the genetic diversity of *Plasmodium falciparum* Apical Membrane Antigen 1 (*pfama*1) gene over a period of time?

2 Will the parasite population structure differ temporally (over a period of time) or spatially (by distance—eg, different ecological zones in Ghana and other regions of the world)?

3 Will COI differ between the two ecological zones with different malaria transmission and over a period of time?

4 Will the frequency of resistant markers to antimalarial drugs differ over time between two ecological zones with different transmission patterns?

5 Will genetic changes such as development and spread of resistance to antimalarial drugs differ between symptomatic and asymptomatic cases?

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1.5 STUDY OBJECTIVES

1.5.1 General objective

To investigate the dynamics of *P. falciparum* (including COI, genetic diversity, evidence of selection and drug resistance) infections in two ecological zones of Ghana.

1.5.2 Specific objectives

1. To determine changes in genetic diversity and evidence of selection in *P. falciparum* isolates from two ecological zones (forest and coastal savannah) in Ghana over a period of four years.
2. To determine the genetic structure of *pfama1* gene within the forest and coastal savannah zones of Ghana (temporal and spatial) and other regions of the world.
3. To determine Complexity of Infection in the forest and coastal savanna zones of Ghana over a period of four years.
4. To investigate the prevalence of molecular markers associated with antimalarial drug resistance in both symptomatic and asymptomatic children in the forest and coastal savannah zones of Ghana, over a period of four years
5. To compare prevalence of molecular markers associated with antimalarial drugs in symptomatic and asymptomatic children in Ghana.

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1.6 CONCEPTUAL FRAMEWORK

Figure 1.1 shows how this study of genetic diversity, complexity of infection and drug resistance in two ecological zones in Ghana was conceptualized. The illustrations were adapted to guide my study and literature review. Studies have shown a strong association of transmission intensity with genetic diversity. With unstable transmission of the disease worldwide, there is the need to continually monitor changing dynamics of the parasite genetically over time. Also, within countries, there may be differences in different geographical areas which may affect the genetic diversity of the parasite. Therefore, the study compared the indices of *P. falciparum* genetic diversity in two major ecological zones which differ in transmission intensity of malaria.

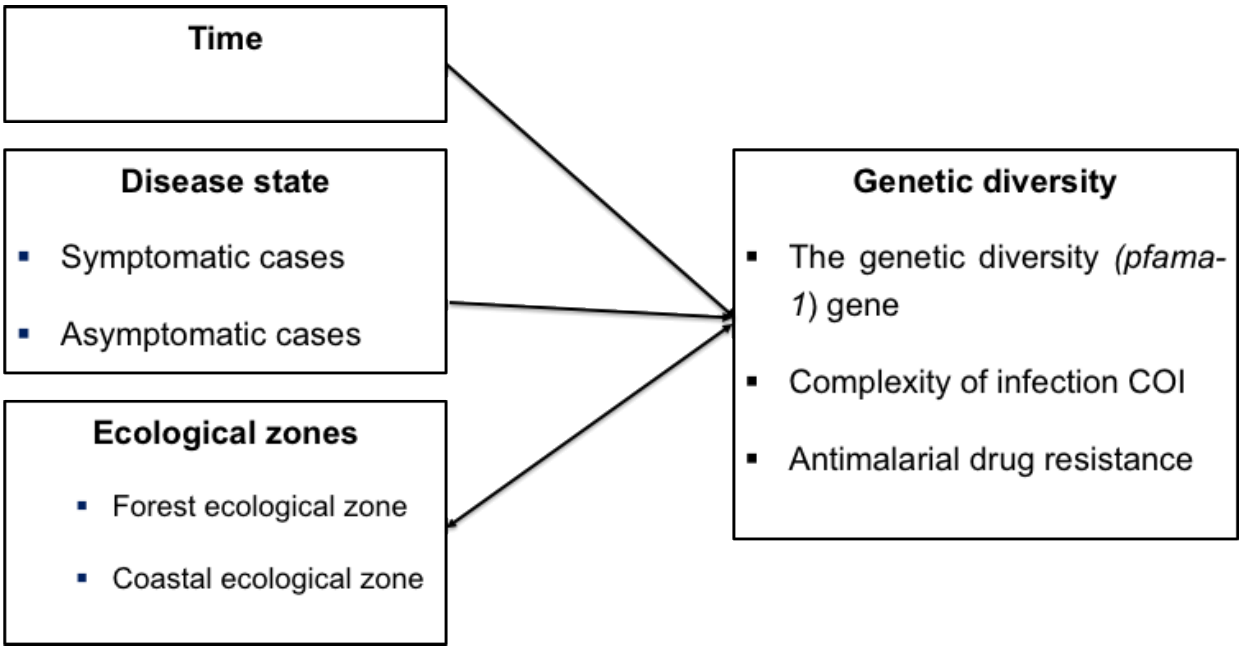

Figure 1.1: The research paradigm illustrating the conceptual framework.

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CHAPTER TWO

2 LITERATURE REVIEW

2.1 BACKGROUND

2.1.1 World malaria trends

Malaria is a serious parasitic disease caused by *Plasmodium* spp. and is transmitted from one human to another through the bite of an infected female *Anopheles* mosquito and rarely through other routes (eg. transfusion or congenital transmission). The disease one of the main causes of morbidity and mortality worldwide. Malaria causes most of the death from infectious diseases worldwide after respiratory infections, HIV/AIDS, diarrheal diseases, and tuberculosis and is the second cause of death (after HIV/AIDS) in Africa (WHO, 2009b). An estimated 219 million new malaria cases occurred in 2017 compared to 239 million cases in 2010 and 217 million cases in 2016. These figures represent 18% reduction in malaria cases between 2010 and 2017 (WHO, 2009b). Although there has been advancement towards reducing malaria over the past decade, data between 2015 and 2017 shows that malaria cases have stalled with an estimated 3.5 million more malaria cases reported in the 10 highest burden African countries compared with the previous year. Despite the stall of progress, several countries have made headway towards elimination. In 2017, 46 endemic countries had reported less than 10,000 cases of malaria compared to 27 in 2010 and 44 in 2016. The WHO African region had most of the malaria cases (92%), then the WHO South-East Asia Region (5% of the cases) and the remaining 2% from

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WHO Eastern Mediterranean Region (WHO, 2018b). Countries with indigenous cases are shown in figure 2.1.

Malaria claimed the lives of about 435, 000 people worldwide in 2017, compared with 607, 000 deaths in 2010. This represents 28% decrease in mortality reported in 2010. Majority of the deaths (93%) were recorded in the WHO African Region where children under five years bear the most brunt. Out of the 435,000 deaths recorded worldwide in 2017, 61% occurred among children under the 5 years. Pregnant women, immuno-compromised people (example patients with HIV), non-immune migrants, mobile populations and travelers are also at considerable higher risk of contracting malaria. About half of the world's population is at risk of developing the disease and active transmission in 2017 occurred in 90 countries. *Plasmodium falciparum* caused almost all the infections in the WHO Africa region (99.7%), as well as most of cases in the WHO regions of Western Pacific (71.9%), the Eastern Mediterranean (69%) and , South-East Asia (62.8%) (WHO, 2018b).

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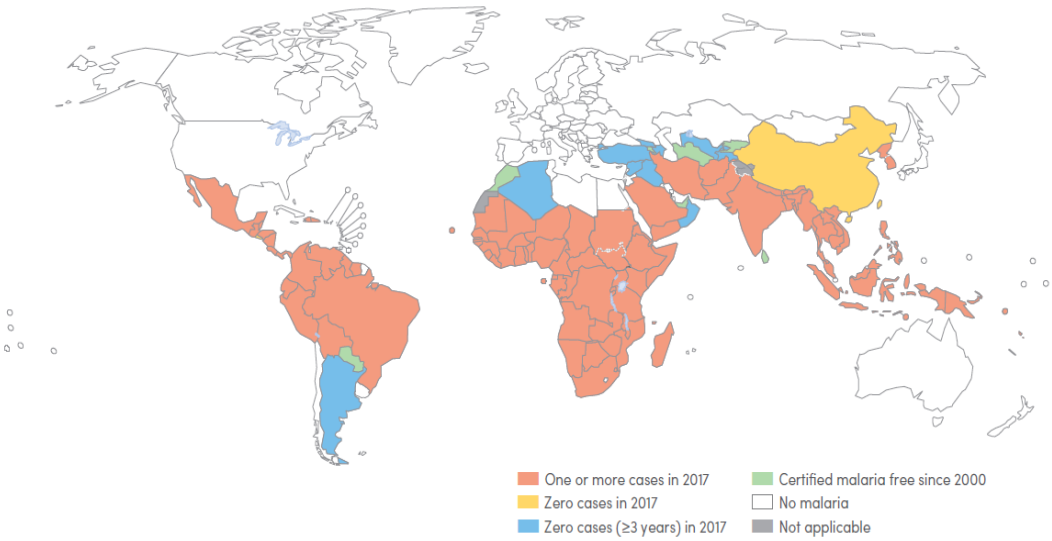

Figure 2.1 Malaria-endemic countries in 2000 and their status by 2017.

Countries certified malaria free were countries that had no case for3 consecutive years. The WHO European Region reported zero malaria cases in 2016 and 2017. China and El Salvador reported no malaria cases in 2017.

(*Source: WHO Malaria report 2018)*

2.1.2 Malaria in Ghana

According to the 2018 world malaria report, Ghana accounted for about 4% of all malaria cases in the world with approximately 7 million reported cases (WHO, 2018b). Malaria is the main cause of morbidity in Ghana, accounting for 40% of all out-patient department (OPD) cases each year (Awine et al., 2017). It is also the foremost cause of death in children under five years (IHME, 2016; WHO, 2017), and can cause a notable number of adult morbidities, and causes economic burden due to workdays lost due to illness. The disease accounted for 11% of all under five deaths in Ghana in 2016. The latest estimates from

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Ghana Malaria Indicator Survey (GMIS, 2016), pegged malaria prevalence in Ghana at about 21% among children aged 6-59 months (Ghana Statistical Service, 2016). This is a decrease from the 2014 Ghana Demographic Health Survey (GDHS) report of 27%.

Malaria is endemic in Ghana and all 29 million Ghanaians are at risk of malaria infection, however, pregnant women and children under 5 years of age are the most venerable group (Awine et al., 2017) due to low immunity. Malaria is the leading disease reported at the OPD thus it burdens most health facilities in Ghana particularly those at the Primary Health Care level. Asante and Asenso-Okyere determined the total cost of malaria control in Ghana to businesses for 2002 to be about US$ 50 million for both direct and indirect costs. The economic burden of malaria on households in 2002 was estimated to be US$13.51 per household (Asante & Asenso-Okyere, 2003). In 2007, the economic burden was estimated at US$10.20 per household for uncomplicated malaria and US$46.62 for severe malaria in 2007 (Abotsi, 2012; Nonvignon et al., 2016).

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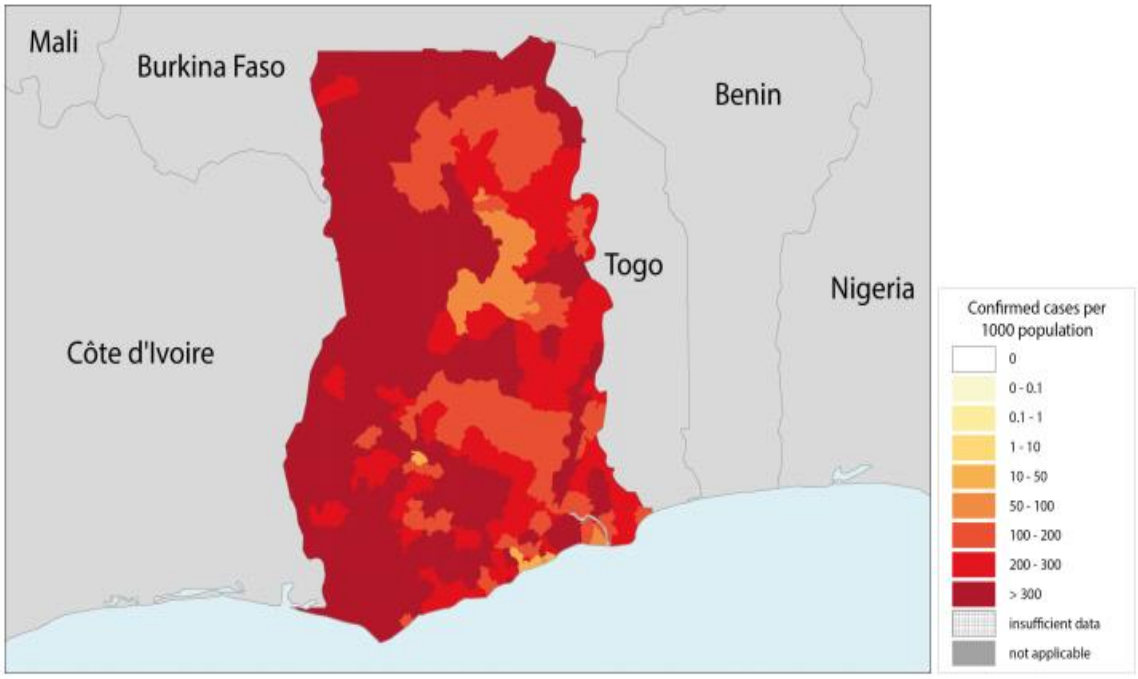

Figure 2.2 Map of Ghana showing the distribution of confirmed malaria cases.

The data shows at least 1 case per 1000 population was recorded throughout the country.

(Source: WHO malaria world report 2018 regional estimates)

2.1.3 Malaria parasites

Malaria is caused by the protozoan parasite called *Plasmodia*—ancient, single-celled protozoan parasite. The five identified parasite species that cause human malaria are *P. falciparum, P. malariae, Plasmodium vivax, P. ovale* and *P. knowlesi (Ashley et al., 2018)*. Two closely related forms of *Plasmodium ovale* have been found, namely *Plasmodium ovale wallikeri* (variant type) and *Plasmodium ovale curtisi* (classic type). *P. vivax and P. falciparum* pose the greatest threat with their distribution by region as shown in figure 2.3. The *P. falciparum* species is the most virulent, accounting for almost all

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deaths, severe disease and most of the cases (WHO, 2018b). It is the predominant species throughout the subtropics and tropics, and also the most prevalent species in most endemic countries, except for South America where vivax is more common. *Plasmodium vivax* has the widest distribution worldwide because of its ability to remain alive at lower temperatures in the mosquito compared to the other four malaria parasites. It is found in temperate areas as well as in subtropical and tropical zones. *Plasmodium ovale* is mainly found in West Africa and benignly infect humans. *Plasmodium malariae* is found mainly in Africa (Cohee & Laufer, 2017). *Plasmodium knowlesi* is mainly found in forested areas of South East Asia, where the natural hosts (pig-tailed macaques) usually lives (Millar & Cox-Singh, 2015; Singh et al., 2004; White, 2008).

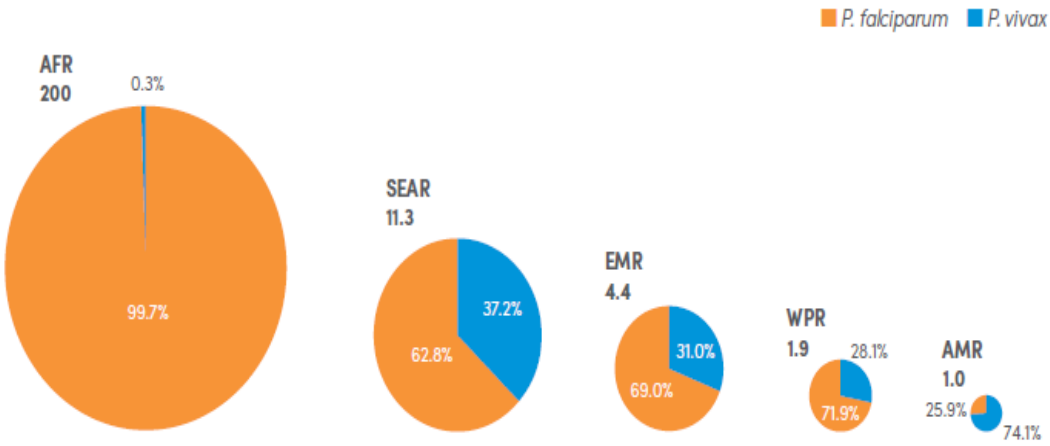

Figure 2.3 Estimates of malaria cases by WHO region and proportions of parasite infectivity by region**.**

Estimated malaria cases are in millions. Orange color denotes *P. falciparum* and blue for *Plasmodium vivax*. EMR: WHO Eastern Mediterranean Region; AMR: WHO American

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Region; AFR: WHO African Region; SEAR: WHO South East Asia Region; AFR: WHO African Region; WPR WHO Western Pacific Region;

(*Source: WHO Malaria report 2018)*

2.1.4 Life cycle of the malaria parasite

Plasmodia parasites have a complicated lifecycle that involves an insect hosts and a vertebrate (Figure 2.4). The vector Anopheline mosquito (insect host) introduces the parasite into humans by injecting approximately 15–20 thread-like forms called sporozoites into the human blood circulation (Ashley, McGready, Proux, & Nosten, 2006). The sporozoites then negotiate their way and move to a few hepatocytes, where they grow and multiply within the parasitophorous vacuoles. In the hepatocytes, one sporozoite can develop into about 10,000–30,000 merozoite containing schizont. Subsequent development takes place for about 5–8 days. The merozoites that develop within the hepatocyte are released from the liver, after multiplication in liver cells and initiate the blood stage of infection.

The product (merozoites) released after the liver stage recognize, attach, and invade the red blood cells (RBCs) and undergo repeated cycles of parasitic development (early, ring stage and mature trophozoites formation) periodically, to produce hundreds of fresh daughter parasites that are released at the end of each cycle. These merozoites then invade more red cells; begin the whole cycle again of infecting red cells, rupturing and release of the new merozoites, which infect more RBCs. The parasites at this stage can either replicate in these cells and rupture resulting in the clinical manifestations of malaria (WHO, 2016) or produce gametocytes which are taken up by insects that feed on the vertebrate host. The

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erythrocytic phase which starts from the merozoite invasion of the red blood cells until schizont rupture takes 48 hours for *P. vivax, P. falciparum* and *P. ovale*, 72 hours for *P. malariae* and 24 hours for *P. knowlesi* (Greenwood et al., 2008).

A small proportion of asexual parasites differentiate into the sexual stage gametocytes. In *P. falciparum* malaria, the differentiation into gametocyte takes about 10 days, while it takes only about 4 days in *P. vivax* malaria. Ingestion of male and female gametocytes by a mosquito is followed by the fusion of the gametocytes in the midgut to produce the zygote. The zygote subsequently develops into ookinetes, which moves to the mosquito midgut and invade the wall to develop into oocysts. The oocysts then undergo meiosis and sexual recombination, and the resulting haploid cells reproduce asexually into the active haploid forms called the sporozoites. Recombinant parasites are produced frequently when different parasites strains are mixed in the mosquito, where cross-fertilization of gametes, zygote formation and meiosis occur. Genetic recombination and meiosis lead to potential generation of novel combination of alleles, which can cause an increase in genetic diversity of the parasite at the population level (Mita & Jombart, 2015). The zygote formation stage is the main diploid stage in the *Plasmodium* life cycle. This stage lasts for a few hours within which meiosis occurs, and the remaining weeks or months of the life cycle consist of haploid forms (Chang et al., 2013; McKenzie, Ferreira, Baird, Snounou, & Bossert, 2001). After maturity, the oocysts burst to release the sporozoites to travel and invade the mosquito's salivary gland. Sporozoites formation completes the lifecycle of the *Plasmodium* parasite. Some of the sporozoites become dormant hypnozoites in the liver, and may relapse of malaria at a later date. In *Plasmodium malariae*, the hypnozoite state

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does not occur, however the parasite may persist in the blood for many years if inadequately treated (Ashley et al., 2006).

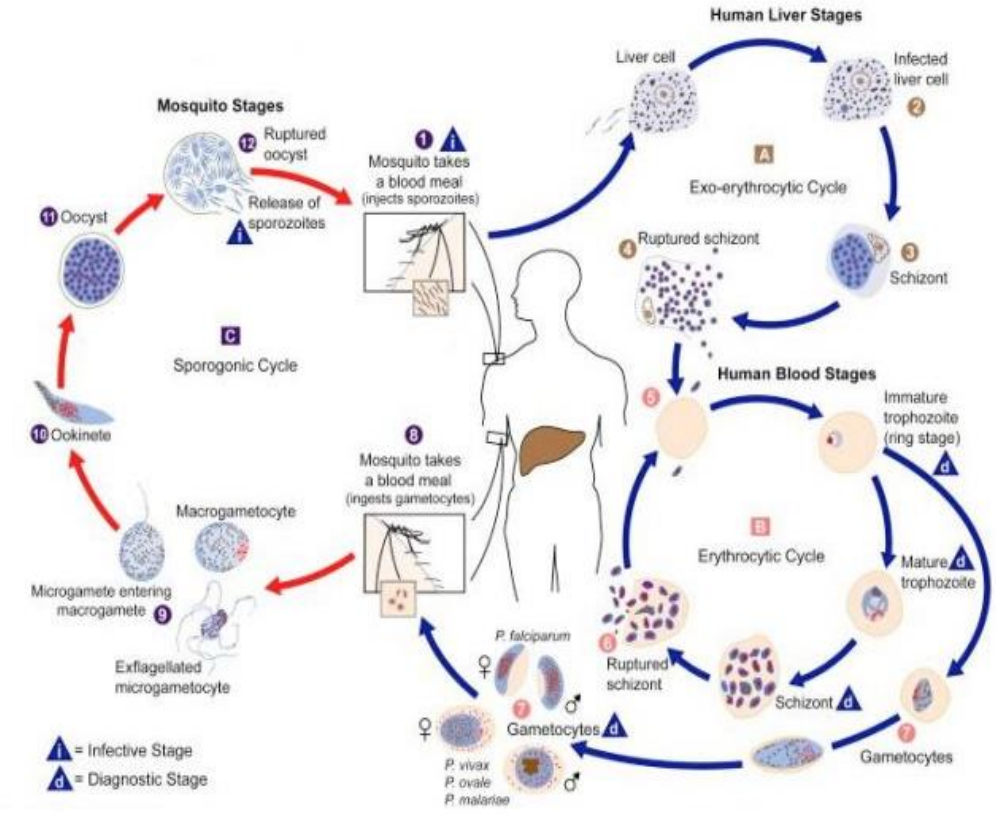

Figure 2.4 Life cycle of the *Plasmodium* spp.

The *Plasmodium* parasite has a complicate, lifecycle involving several stages and occurrs within two living host organisms, the human host and the vector mosquitoe. Upon inoculation of the human dermis by an *Anopheles* mosquito, the sporozoites migrate to establish the first intracellular replicative stage in the liver cells. The proceeds of the pre-erythrocytic stage (merozoites) then invade the erythrocytes (RBC). Severe conditions of

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malaria occur at the erythrocytic stage, some merozoites released during the erythrocytic stage develop into sexual forms (gametocytes), which are taken up by a feeding anopheline mosquito. In the mosquito, the parasite reproduces sexually, to produce the ookinete and then an oocyst in the mosquito gut. The oocyst bursts to release sporozoites, which migrate to the salivary glands where they are injected during the next blood feed.

(Source: https://www.bioscience.com.pk/topics/microbiology/item/147-life-cycle-of-malaria-parasite)

2.1.5 Vector

The malaria parasite in humans is transmitted by the female *Anopheles* mosquitoes. There are about 400 known species of *Anopheles* mosquito; out of these number, about 70 can transmit the parasite to humans (Hay et al., 2010), however, about 30 are major malaria vectors (WHO, 2018b). The distribution of these *Anopheles* species varies in different geographical areas of the world contributing to the varying transmission patterns of the parasite around the word. Africa bears the most burden of malaria morbidity and mortality because it houses the most efficient dominant vector species (DVS) of malaria.

The *A. gambiae* complex is the main vector in Africa, which includes *A. gambiae* with its sibling (s.s and s.l), *A. arabiensis*, which is also of major importance. The coastal adapted species *A. Melas* and *A. merus*, also members of the *A. gambiae* complex are not as effective at transmitting malaria as *A. gambiae* or *A. arabiensis*. The other members of the *A. gambiae* complex are not widely distributed and are restricted (Cuamba & Mendis, 2009; Sinka et al., 2010). Africa is also known to be a center point of other DVS, including, *A. nili*, A. *funestus* and *A. moucheti*, whereby *A. funestus*, in other instances, have a much

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higher effect on malaria transmission compared to *A. gambiae*. *A. freeborni* is the major vector in North America, whereas in the Asian-Pacific region, a highly complex situation persists with multi-species coexistence and variable species dominance (Sinka et al., 2012).

In Ghana, two main malaria vectors; *A. gambiae sensu stricto* and *A. funestus* are responsible for malaria transmission. The geographical distribution of these vectors in Ghana is mainly based on changes in environmental factors, land use and climate. *An. gambiae* (*s.l.*), *An. coustani* and *An. rufipes* are found in urban settings (Mattah et al., 2017). *Anopheles gambiae s.s* and *Anopheles funestus* are found in most parts of the country but in different abundance (Kasasa et al., 2013).

The significant species of the vector bite between dusk and dawn. However, exact biting patterns vary based on type of species (Cohee & Laufer, 2017; WHO, 2018b).

2.1.5.1 Transmission

The female anopheles mosquito transmits the malaria parasite when it seeks blood meal to nature its eggs. In some rare cases, malaria can spread through transfusion of blood from an infected person to a healthy person and also through mother to growing fetus (called congenital malaria). The intensity of transmission depends on a lot of factors associated with the vector, the parasite, the human host and the environment (WHO, 2018b). Varying climate seasons, aquatic habitats and ecological zones are some of the factors that influence the distribution of the malaria vector and thus the transmission of the disease.

Transmission is largely contingent on the distribution and abundance of the mosquito vector. The disease is transmitted more in areas where the mosquito lifespan is longer. A

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longer life span of the vector gives the parasite enough time to complete its lifecycle in the mosquito. The transmission is also high in places that the mosquitoes would rather bite humans than other animals. The mosquito's long lifespan and the habit of biting humans of the African species explains why almost 90% of the malaria cases in the world are in Africa. Also, transmission is reliant on climate conditions like rainfall patterns, humidity and temperature which affect the survival of the vector (WHO, 2018a).

The most important measure of transmission at the moment is the entomological inoculation rate (EIR) because of strong correlation between EIR and malaria endemicity in a population. It measures the level of exposure of an individual to mosquitoes infected with malaria and it is currently the favored measure of evaluating malaria endemicity and transmission intensity (Dery et al., 2010; Drakeley et al., 2003; Hay, Guerra, Tatem, Atkinson, & Snow, 2005). The EIR can be as high as 643 infective bites per person per year (ib/p/y) in high transmission settings (Dery et al., 2010). Table 2.1 below shows the criteria for the classification of transmission intensity proposed by the Worldwide Antimalarial Resistance Network (WWARN).

Within Ghana, malaria transmission is heterogeneous with peaks during the rainy season and differs along varying ecological zones. The disease is transmitted throughout the year mainly in southern parts of Ghana and seasonally in the northern parts (Kasasa et al., 2013). Ghana has six main vegetative/ecological zones namely; Sudan savannah, Guinea savanna, forest-savannah transition, semi-deciduous forest, heavy rain forest and coastal savannah. However, 3 major eco-epidemiological zones (Northern/Guinea savannah, Forest and Coastal savannah zones) were formed based on malaria transmission for control purposes

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by the national malaria control programmes (NMPC). Differences in climate parameters like rainfall patterns, humidity, temperature and also the ecology accounts for these variations.

Malaria transmission in the forest ecological zone is high and perennial with peak transmission occurring after the major rainy season (March to early August). Malaria is quite stable in this region and endemicity is high due to favorable conditions throughout the year for disease transmission. *Plasmodium. falciparum* is the predominant species and the EIR is 21.9 ib/p/y (Dery et al., 2010). The coastal zone has moderate transmission and is also perennial but has low transmission during the dry season with an average EIR of 3.65 ib/p/y. Malaria transmission in the northern savannah zone is seasonal though transmission is intense (EIR = 643 ib/p/y) (Dery *et al*., 2010).

Table 2.1 Classification of malaria transmission

Transmission Region	**MAP**	**Publication [EIR]**	**New Infection Rate**
Low	<5%	EIR < 2 infectious bites/person/year	<15%
Moderate	>=5-<=40%	EIR 2-<25 infectious bites/person/year	>=15%-<=40%
High	>40%	EIR > 25 infectious bites/person/year	>40%

EIR = Entomological Inoculation Rate; MAP= Malaria Atlas Project (MAP) Prevalence estimate

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Source (The WorldWide Antimalarial Resistance Network, 2013)

2.1.6 The Disease (Malaria)

Plasmodium falciparum malaria causes a wide range of disease, which spans from asymptomatic infection to fatal symptoms like severe anemia, cerebral malaria, and multi-organ failure. Factors such as the infected individual's age, genetics and immune response from past malaria infections, and the intensity and seasonality of malaria transmission counts for the varying frequency of episodes of malaria and characteristics of malaria disease (WHO, 2016). The immune status of an individual determines outcomes associated with the disease. Almost all the signs of malaria and clinical symptoms are caused by the asexual forms of the parasite. Disruption of RBCs, localization in tissues and organs by binding to endothelial cells (cyto- adherence), and the release of many pro-inflammatory cytokines are the havocs caused by the asexual stage (Pasvol, 2005). The incubation period which starts from the time of *P. falciparum* infection to initial symptoms ranges from 6 days-1 month for non-immune persons and might be longer in individuals with a degree of immunity or people on prophylaxis (usually with 12 months). Longer period of incubation has been reported for other species of *Plasmodium* (between 6 months to one year). *Plasmodium vivax* and *P. ovale* have the ability to relapse by the development of hepatic hypnozoites which can persist after an extended period of time (Walker et al., 2018).

2.1.6.1 Signs and symptoms

Fever is the most common presentation of the disease. The other symptoms are usually non-specific including headache and vomiting. Common presentations of severe malaria

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in African children are respiratory distress, severe anaemia, convulsion, hypoglycemia and cerebral malaria, but respiratory distress is the most inimical, especially in the presence of other syndromes (Pasvol, 2005; Walker, Nadjm, & Whitty, 2010; Walker et al., 2018). The well-known form of severe malaria is however cerebral malaria. The signs of severe malaria differ between adults and children. In children, severe malaria is commonly presented as respiratory distress, anaemia, convulsions and hypoglycaemia. Symptoms like pulmonary oedema, acute respiratory distress syndrome (ARDS) and acute kidney injury rarely occur in children, but occur mainly in cases of fatal malaria in non-immune adults (Walker et al., 2018).

2.1.6.2 Pathogenesis

Pathogenisis of the disease starts with rapturing and disrupting of erythrocytes infected with merozoites and causes clinical symptoms. In uncomplicated malaria, the parasites invade and cause direct destruction to erythrocytes of all ages thus manifesting symptoms like fever, sweats and chills. In the unique case of *P falciparum*, the erythrocytes containing mature parasites sequester inside small and medium-sized vessels, causing host endothelial cell injury and microvascular obstruction (Ashley et al., 2006). Sequestration involves the cytoadherence of schizont-infected RBC to endothelial cells in post-capillary venules in organs such as the brain. Sequestering parasites presumably cause microvascular obstruction, though the role and extent of this obstruction remains unclear and is the cause of absence of mature parasites from the peripheral circulation. It is the main pathological process leading to sever malaria. It can cause coma in the brain, respiratory failure in the lungs, and placental malaria during pregnancy. Other common symptoms seen in *P.*

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falciparum infections are cerebral malaria, hypoglycemia, metabolic acidosis, and renal failure (Chakravorty, Hughes, & Craig, 2008; White, Pukrittayakamee, Hien, et al., 2014).

2.1.6.3 Diagnosis and Treatment

Diagnostic testing and treatment are valuable components for malaria control and elimination strategies. This contributes to the reduction of the disease and subsequently prevents death. There has been an expansion in malaria diagnosis over the last decades with campaign for diagnostic testing before treatment. Thus contributing to the decline of transmission of the disease in the population. Diagnostic testing meant to detect the erythrocyte stage of the disease. Rapid Diagnostic Tests (RDTs) are universal and commonly used for the diagnosis of malaria in both resource-limited and non-endemic settings but microscopy of thick and thin blood films is the gold standard of diagnosis. Polymerase chain reaction (PCR) offers a more sensitive and specific option compared to the other methods, however, this method is very expensive, limited by speed and unavailability of technology in almost all health facilities in Ghana (Kafai & Odom, 2018)

WHO recommends that suspected malaria should be confirmed using diagnostic testing like rapid diagnostic test or microscopy before treatment. It also recommends that treatment without diagnosis be given only if the patient is unable to be tested within 2 hours of presenting at the facility for treatment (WHO, 2018b). The outcome of parasitological confirmation is usually available within 30 minutes. For treatment, WHO recommends that all uncomplicated *P. falciparum* malaria infection be treated with Artemisine-based combination therapy (ACTs), which combines the fast-clearing artemisinin derivative with a longer-clearing antimalarial partner drug (Kafai & Odom, 2018). In addition, a onetime

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small dose of primaquine should be added to the antimalarial treatment in low transmission areas, to decrease transmission in these settings. In the case of non- falciparum malaria, malaria should be treated with an ACT or chloroquine in places where chloroquine resistant *P. vivax* are not identified. However, in areas with chloroquine resistant *P. vivax*, infections should be treated with an ACT. A full dose of primaquine therapy should be added to prevent relapse from the disease, at doses guided by the patient's G6PD status. Parenteral artesunate (either intramuscular or intravenous) for at least a day is recommended for treatment of severe malaria, followed by a complete 3-day course of an ACT when the patient can take oral medicines (WHO, 2018b). In Ghana, Artesunate Amodiaquine (AA), Artemether-Lumefantrine (AL) and Dihydroartemisinin Piperaquine (DHAP) are the first line drugs for the treatment of uncomplicated malaria (MOH, 2014).

2.1.7 Malaria control

Despite Malaria being treatable, it remains a disease global importance (Raman et al., 2011). There is no one path to enhance malaria control. Such strategies will probably involve several methods like better, pervasive use of known effective methods. A combination of therapeutic treatment and some other potent control method will be advantageous (Greenwood, 2017; Greenwood, Bojang, Whitty, & Targett, 2005; Walker et al., 2018). Malaria control can be divided into two major forms: control of the vector host (Anopheles mosquito) and human host.

Vector control method have been widely used and is an archaic control measure using insecticides including DDT, carbamates, or pyrethroids indoor residual spraying (IRS), the use of Insecticides treated bed nets (ITNS) and environmental control metholds like

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drainage of breeding site, and zoo-prophylaxis (Greenwood et al., 2005). Vector control has substantially improved malaria control, however resistance to chemicals used for vector control and difficulty in achievement of widespread use, reduces the effectiveness of this method of control (Greenwood, 2017). Other setbacks include insufficient financing, weak health infrastructures, limited skilled human capacity, and poor-quality private sector services are posited as barriers to scaling up (Barat, 2006).

Malaria control in the human host involves treatment of malaria cases with antimalarial drugs and prevention of the infection in humans. Prevention involves intermittent preventive treatment of certain populations groups such as pregnant women (IPTp) and children (IPTc) and prophylaxis treatment for non-immune travelers. Antimalarial drugs are essential for malaria control and elimination. The antimalarial drugs are generally classified into four classes of compounds; the quinolines, the antifolates, the artemisinin derivatives and antibacterial agents. They act primarilly to disrupt the erythrocytic stages of malaria parasites responsible for human illness (Cui, Mharakurwa, Ndiaye, Rathod, & Rosenthal, 2015).

Current drugs recommended to be used as first choice for treatment of uncomplicated *P. falciparum* infections is the Artemisinin-based Combination therapy (ACTs) while Chloroquine plus primaquine is recommended for the treatment of *P. vivax* malaria. ACTs usually contains an artemisinin derivative that quickly clears most parasites and a longer acting partner drug to clear the remaining parasites and limits the selection of artemisinin resistance (Nosten & White, 2007; WHO, 2018b). Chloroquine is still the gold standard for treating uncomplicated malaria, which is followed by primaquine to eradicate

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hypnozoites of ovale and vivax. Recommended Drugs used to prevent malaria include atovaquone/proguanil (Malarone), mefloquine, or doxycycline in chemoprophylaxisc regimens for travelers from non-endemic to endemic countries. Sulfadoxine/pyrimethamine is the drug of choice for IPTp in pregnancy and amodiaquine or SP is used for seasonal malaria chemoprophylaxis in the Sahel sub region, where levels of resistance to these drugs are low.

Malaria prevention in humans usually involves avoiding mosquito bites with clothing, insect repellent, sleeping under a treated bed net and use of chemoprophylaxis. The number of deaths from malaria over the past two decades has fallen by about half (Walker et al., 2018), achieved primarily through scaling up of established interventions like vector control with Indoor Residual Spraying (IRS) with insecticides, larva control and protective measures such as the use of Insecticide Treated bed Nets (ITN), insect repellent and wearing of protective clothing. Countries like Turkmenistan (2006), United Arab Emirates (2007), Morocco (2010), Armenia (2011) have achieved elimination in the past 10 years by relying heavily on these strategies. Others, where local transmission no longer occurs, such as Egypt (1998), Mauritius (1998), Oman (2000), Algeria (2005), Syria (2005) are awaiting WHO certification (White, Pukrittayakamee, Phyo, et al., 2014).

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2.2 GENETIC DIVERSITY OR GENETIC VARIATION

Genetic variation or polymorphism is usually defined as "the occurrence of two or more alleles at one locus in the same population, each with appreciable allele frequency less than 0.95 (Cavalli-Sforza & Bodmer, 1999; Hartl et al., 1997; Nei, 1987). Several molecular mechanisms that can cause variation in the parasite genome includes base substitution and recombination (Mita & Jombart, 2015).

The *Plasmodium* parasite uses the diversity they possess in the genome, that enables the parasites to conform quickly to unstable environments (Mita & Jombart, 2015), including antimalarial drugs and immunity of the host, although the mechanism with which they maintain such diversities is unknown. Broadly speaking, genetic variation in the *P. falciparum* can be seen in various ways. One form is antigenic variation which is the ability of infectious organisms to consistently switch its immunogenic epitopes under attack by the immune system of their host. The alteration in the immunogenic epitopes is a mechanism to avoid antibody response and establish a persistent infection. The primary antigen shown on the surface of infected RBCs is a highly variable antigen *P. falciparum* erythrocyte membrane protein one (*Pf*EMP1). The variable (var) gene family controls the different forms of *Pf*EMP1 encoded. There are about 60 var gene in the genome of *P. falciparum*, thus parasites can vary their antigenic signature by changing which var gene to express, and thereby cycling through their repertoire of surface proteins (Kirkman & Deitsch, 2012).

Another form of genetic variation in the *P. falciparum* genome is high polymorphisms in the surface proteins commissioned as vaccine antigens. A major challenge to the

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development of effective malaria vaccines is the high polymorphism in *P. falciparum* especially in genes that encode antigens or membrane-bound proteins. These antigens include prime candidates for the development of malaria like merozoite surface protein 1 and 2 (MSP1&2), Circumsporozoite Protein (CSP), Duffy-binding Protein (DBP), Apical membrane antigen-1 (AMA-1). These surface proteins or antigens are highly polymorphic, due to diversifying selection by the host immune system (Quang et al., 2009). Monitoring of genetic diversity in these malaria vaccine candidate antigens provide ideal vaccine design recommendations and explains results of vaccine efficacy trials. Molecular epidemiological methods are currently being used to determine relevant polymorphism which elicit immune response to vaccine antigens (Farooq, Malla, & Dubey, 2009). Monitoring of antigenic variation in the candidate genes or immune response genes can be used to assess complexity of infection (COI) and to differentiate reinfection from recrudescence during clinical trials. Complexity of infection is the mean number of distinct clones based on polymorphic regions in the genes infecting a single host at a point in time. It has been used in molecular epidemiology to determine the level of immunity in individuals (Tanabe et al., 2015) and malaria transmission levels in endemic settings (Babiker & Walliker, 1997). This can be looked at either within host or at the population level. Another subset of genetic variation is the use of genetic polymorphism to explore its association with the phenotype drug resistance (Campino et al., 2011). This form of genetic variation has been studied extensively to identify mutations in parasites that is linked to treatment failure (Sibley, 2014).

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The gold standard is to look at the genetic variation of the parasite genome-wide. This provides the true diversity in the genetic-makeup as well as reveal the true population structure among a wide geographical area. It also provides insight into genetic differences that cause phenotypes like drug resistance and virulence at the same time. However, this form is very expensive especially in large survey studies of transmission patterns.

Genetic diversity can be affected by both individual and population-level components, which includes recombination, inbreeding, migration, population bottlenecks, and genetic drift as shown in figure 2.5. *Plasmodium falciparum* diversity is usually high in high-endemic regions and low in hypo and meso-malaria endemic areas. This is explained by the level of inbreeding and recombination during the sexual stage in the mosquito (Anderson et al., 2000; Mita & Jombart, 2015). Sexual reproduction occurs after male and female gametocytes ingested by a mosquito. The number of genetically different gametocytes in a mosquito blood feed will determine the degree of inbreeding. The number of distinct gametocytes taken during the mosquito blood meal is also associated with multiple infections in the human host, which is frequent in areas of high transmission intensity, thus increasing the chance of outcrossing of the parasite in the mosquito gut. Whereas, in low transmission settings, low frequency of multiple infection in the human host favors inbreeding (Mita & Jombart, 2015).

Factors that influence the population size also affects within-population diversity strongly. For instance, genetic drift can cause a random change in the allele frequency from one generation to another, and this is usually very obvious in small populations. Rare alleles are easily misplaced and common alleles are more likely be fixed in the case of genetic

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drift, thus resulting in reduced genetic diversity in the population. Another factor that causes a change in the population size is population bottleneck, defined as, instances where a few individuals survive to prolog the existence of a population. Genetic diversity may be lost in consequence of population bottleneck and is usually caused by a number of ecological reasons, particularly in the case of founder effect, where a few individuals move to a new setting and thus forming a new population (Mita & Jombart, 2015).

Earlier attempts at studying the genetic variation focused on phenotypes of traits like the ABO blood grouping, which were heritable. Later, molecular techniques enabled use of genetic markers to determine genetic variation and technology advancement in the last decade has led to increased access to genomes by means of Whole Genome Sequencing (WGS) for assessing genome-wide variation patterns. This recent staggering technological leap, has made available large amounts of genetic markers to investigate the distribution of variation within and between populations (Tsetsos, Drineas, & Paschou, 2018). The diversity of *Plasmodium falciparum* has been explored at the whole genome level and also in highly polymorphic regions like microsatellites, surface antigen proteins (AMA1 and CSP). Genotyping of very polymorphic regions in the genome is cost-effective and provides a granular approach to determine the parasite diversity, population structure and complexity of infection (COI) compared to the whole genome genotyping (Auburn & Barry, 2017).

Describing the genetic diversity and COI of malaria parasites in a geographical setting are relevant for evaluating malaria control interventions (Gueye et al., 2018). Therefore detecting and understanding it's maintenance in the population is important for

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understanding several mechanisms in malaria like identifying drug and vaccine targets (Das, Bajaj, Mohanty, & Swain, 2007).

2.2.1 Antigen diversity of *Plasmodium falciparum*

High polymorphisms have been identified in *P. falciparum* and *P. vivax* antigenic surface proteins such as Circumsporozoite protein (CSP), Merozoite surface protein 1 and 2 (MSP1&2), Apical membrane antigen1 (AMA1) (Basu, Maji, Mitra, & Sengupta, 2013). These surface and membrane-bound proteins form about one tenth the parasite genome Variations in these proteins are evidence of immune selective in both hosts (Bongfen, Laroque, Berghout, & Gros, 2009). These antigenic genes in *P. falciparum* are routinely used for genotyping clinical isolates (Hartl et al., 2002) and genetic diversity.

2.2.1.1 Apical membrane antigen-1 (AMA-1)

Plasmodium falciparum AMA1 (*pfama1)* is a favorable blood stage vaccine prospect under consideration in trials (Dutta, Haynes, Moch, Barbosa, & Lanar, 2003; Kocken et al., 2002; Mitchell et al., 2004; Zhu et al., 2016). This antigen is located in the micronemes of merozoite, an apical organelle where it plays an important role in erythrocyte invasion (Healer et al., 2002; Narum, Thomas, & parasitology, 1994; Peterson et al., 1989; Zhu et al., 2016). The AMA1 antigen is produced during the blood stages of the lifecycle and conserved in apicomplexans. The *pfama1* coding region has an ectodomain and three sub-domains (Domains I, II and III) (Silvie et al., 2004; Zhu et al., 2016), which is highly immunogenic and studies have revealed that antibodies raised against AMA1 prevent merozoite invasion in animal models and *in vitro (Zhu et al., 2016)*. Studies have shown

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Plasmodium AMA-1 sequence diversity among isolates might have occurred due to selective pressure by host immune responses (Zhu et al., 2016). Critical review of data showed a protective association among studies of AMA1 diversity, making AMA1 a leading malaria vaccine candidate and therefore emphasizes the demand for better understanding of genetic diversity of *pfama1* (Fowkes, Richards, Simpson, & Beeson, 2010; Zhu et al., 2016).

2.2.1.2 Methods for measuring` genetic diversity

Genetic measurements including heterozygosity, the number of segregating sites per nucleotide site, the number of alleles, the allelic richness, the proportion of polymorphic loci, and nucleotide diversity are used to estimate genetic diversity in the geographical setting (Mita & Jombart, 2015).

Heterozygosity: Heterozygosity at a locus in malaria parasites is measured commonly using the following formula,

$$h = \frac{n}{n-1}\Sigma_{i=1}^{q}(1 - x_i^2)$$

Where n is the number of infections sampled, x_i is the sample frequency of the i-th allele and q is the number of alleles (Mita & Jombart, 2015; Nei, 1987).

The magnitude of genetic variation in a geographical area is generally estimated by the average gene diversity (H), which is the mean heterozygosity (h) along multiple loci. It is recommended to use as many loci as possible to estimate population diversity because using one locus may generate a biased estimate of within population genetic diversity.

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Therefore, average genetic diversity is measured as the average of this value over r number of loci, shown below:

$$H = \sum_{j=1}^{r} h_j \, / r$$

Where h_j is heterozygosiy at the jth locus number. in this case, the number of samples may be different from the total number of all loci (Mita & Jombart, 2015; Nei, 1987)

Genetic diversity may also be correlated using the variance ($V_s(h)$) proposed by Nei as shown below;

$$V_s(h) = \frac{2}{n(n-1)} [2(n-2)\{\sum x_i^3 - (\sum x_i^2)^2\} + \sum x_i^2 - (\sum x_i^2)^2]$$

Where n is the number of infections sampled and x_i is the sample frequency of the i-th allele. Welch t test is used to test the populations difference (Mita & Jombart, 2015; Nei, 1987).

The proportion of polymorphic loci, number of alleles and allelic diversity:

Population genetic diversity can also be estimated using the proportion of loci, the number of alleles and allelic diversity. Proportion of polymorphic is measured as the mean number of polymorphism sites in the genome. It depends on the number of samples examined and as such caution should be taken when using it (Mita & Jombart, 2015). The average number of alleles at each locus, also known as "allelic diversity" is another method used to measure diversity. It can be affected by sample size and can be rectified by a statistical method known as "rarefaction". Rarefaction estimates the expected allelic diversity as a function

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of sample size and thus enables the ability to compare genetic diversity of populations with different sample sizes (Mita & Jombart, 2015).

Measures of nucleotide variation: Measures such as the number of polymorphic sites, number of singleton polymorphic sites, the average number of pair-wise nucleotide differences per site (θ_π), and the standardized number of polymorphic sites per site (θ_S) have been made possible at the nucleotide level due to the increasing availability of genetic sequences (Tanabe et al., 2013). The number of segregating sites per nucleotide site (Ps), and the nucleotide diversity (π) are further described below (Mita & Jombart, 2015). Segregating sites is the number of nucleotide sites in the sample that have two or more nucleotides. It is estimated using the formula:

$P_s = \frac{S}{n}$

Where N is the total number of sites n and S is the number of segregating nucleotide sites.

The number of segregating sites, which is a strong measure of nucleotide variation can also be influenced by the number of samples. Nucleotide diversity in contrast is not affected by sample size and is the average number of nucleotide differences per site between any two species. Nucleotide diversity is defined as:

$\pi = \sum_y^q x_i \, x_j d_{ij}$

Where x_i is the population frequency,

q is the total number of alleles (different allele sequences), of the i-th allele,

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d_{ij} is the number of nucleotide differences or substitutions per site between i-th and j-th alleles (Mita & Jombart, 2015; Nei, 1987).

F-Statistics: Genetic differentiation among populations can be assessed using F_{ST}, a measures of F-statistics or the fixation index of inter-population variance in allele frequencies (Mita & Jombart, 2015; Tanabe et al., 2013). To measure the genetic divergence among subpopulations, two heterozygosities, expected within population heterozygosity (HS) and expected total heterozygosity (HT) are used in the formular below (Mita & Jombart, 2015; Nei, 1987)

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

It can also be used in the estimation of pairwise distance between the two populations (pairwise FST) and any hierarchical population structure. Figure 2.6 shows estimating genetic differentiation using F_{ST}.

Principal Component Analysis (PCA): Principal Component Analysis is a method used to highlight variation and bring out the important patterns in a dataset by reducing its dimensions. Patterson and colleagues (Patterson, Price, & Reich, 2006) were the first to use PCA to reduce the dimensionality of high throughput genetic data, by developing the EIGENSOFT software suite for the simple application of the method on various popular genomics data formats. PCA methods involved the visualization of components that capture the most difference. In most of the cases, the total genetic variation is due to within population variance (Tsetsos et al., 2018). Discriminant analysis of principal components, DAPC is a multivariate method of clustering data based on prior defined populations. It is

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used to analyze the population structure by assessing how well samples can be reassigned into previously defined populations (Jombart, Devillard, & Balloux, 2010; Kamvar, Larsen, Kanaskie, Hansen, & Grunwald, 2015).

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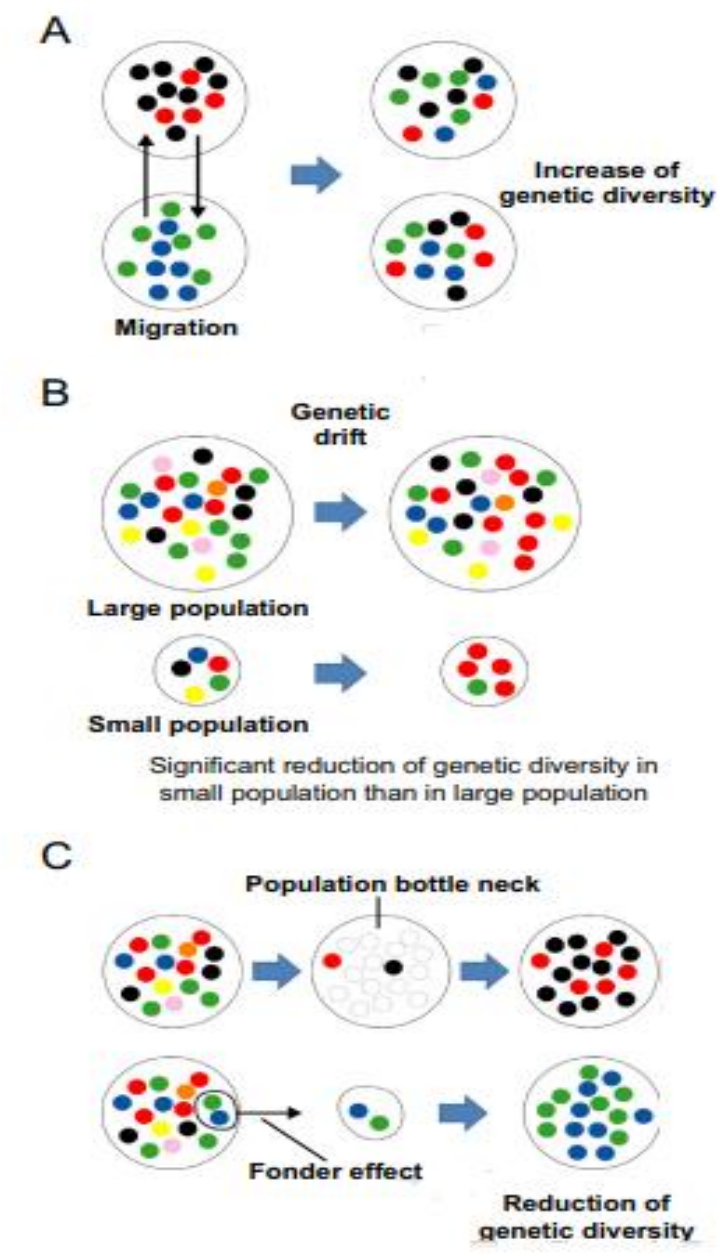

Figure 2.5. Factors that influence genetic diversity of *P. falciparum* population. The big circles are populations of organisms represented as dots.

A show how migration of parasites (movement from one area to another) can change the genetic diversity of the parasite population. **B** shows the eminent effect of genetic drift on

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small populations in contrast to big populations. **C** Shows how population bottleneck occurs a small number of individuals survive out of the lot in a population resulting in loss of genetic diversity in the geographical area (shown in the upper part of C) (Mita & Jombart, 2015). Founder effect occurs when a small group of individuals relocate to another geographic area. This is a form of population bottleneck. Adopted from (Mita & Jombart, 2015).

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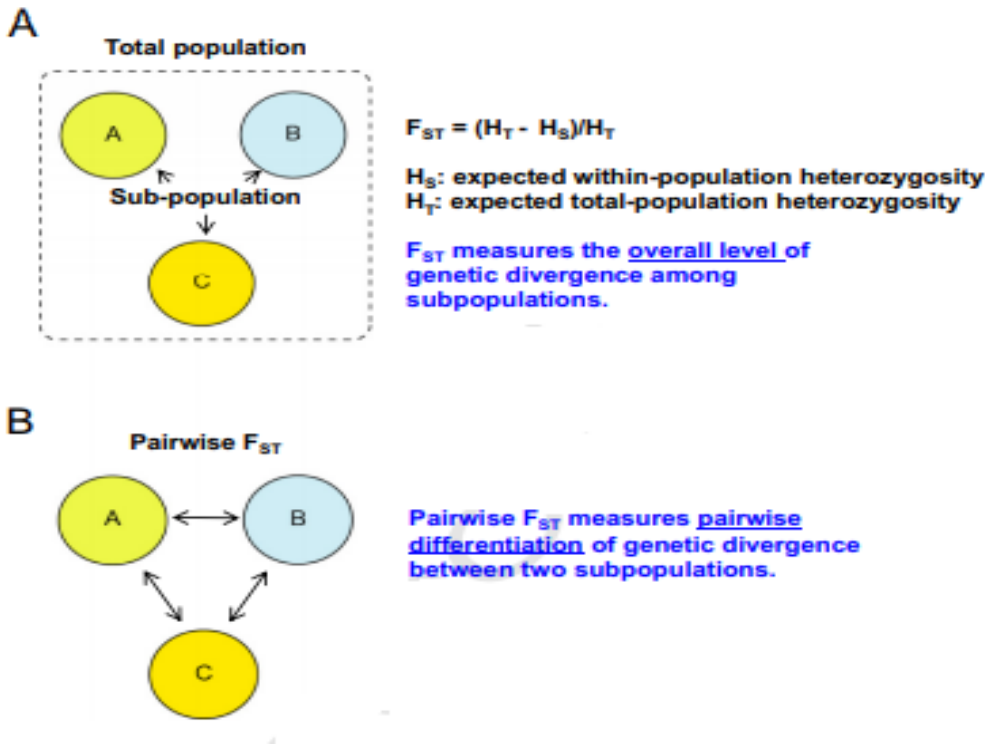

Figure 2.6. Sub-structuring of parasite populations by FST. (A) The total genetic divergence among subpopulations is estimated from the expected within heterozygosity (HS) and expected total heterozygosity (HT) shown in (A). (B) shows how pairwise FST is used to estimate pairwise differentiation between two populations. Adopted from (Mita & Jombart, 2015)

2.2.2 Complexity of infection (COI)

Natural infections with *P. falciparum* in an individual usually comprise of multiple simultaneous genetically distinct parasite clones. These multiple infections occurs usually in areas of high transmission (Pinkevych et al., 2015). The number of concurrent clones in an infection measured as Complexity of infection (COI) but also known as multiplicity of infection (MOI) is associated with the prevalence of cases in the population and the age of

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the individual. Although the determinants of COI are unclear, endemicity and the period of individual infection are relevant. Thus, COI is influenced by the number of infected bites received, the number of new clones transmitted per bite, and the proportion of new clones that survive the liver stage and then initiate blood-stage infection (Pinkevych et al., 2015).

Complexity of infection is an important epidemiological factor in modelling the evolution of resistance. Genetic recombination during the sexual phase in the mosquito is the mechanism underlying the evolution of resistance. After two gametocytes are picked during a mosquito blood meal, they fertilize to form a zygote in the gut. This develops in an oocyst on the gut wall. If the human host carries a single clone and a mosquito picks up gametes during the blood meal, inbreeding occurs. Because, the parasite is haploid for most of its lifecycle, it produces genetically identical gametes from the parent clone, thus no exchange of genetic material occurs during self-fertilization. However, genetic reassortment may occur in the mosquito when its picks up distinct gametes from the human host. Genetic recombination in the parasite life cycle is important in understanding the dynamics of drug resistance because, it is key in the creation or breakdown of markers that resistance (Bloland, 2001; Hastings & D' Alessandro, 2000).

Complexity of infection has been used as a measure of transmission intensity in place of Entomological inoculation rate (EIR) although the relationship between these two is obviously complicated and affected by other confounding factors (Bloland, 2001; Hastings & Mackinnon, 1998; Paul et al., 1998). Some studies have shown that a progressively small increase in COI with increasing EIRs in places of more intense transmission compared to places with lower transmission (Bloland, 2001; Paul et al., 1995).

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2.3 DRUG-RESISTANT MALARIA

2.3.1 Description of drug resistance

Drug resistant malaria is a serious public health problem especially in the tropical and sub-tropical nations of South-East Asia, the African subcontinent and South America, causing disease burden in several regions (Chakraborty, 2016). Drug resistance to an antimalarial drug for uncomplicated malaria can be defined as the reduction in inhibition of parasite multiplication for a given free plasma concentration of the drug (White, 2004). However, the parasite half-life is determined by the innate response of a parasite isolate to drug, and also by its growth stage at the time of treatment and host-related parameters such as pharmacokinetics and immunity (Ariey et al., 2014; Witkowski et al., 2010). Inhibition of the parasite multiplication is important in uncomplicated malaria, to prevent progression to severe disease and to resolve other symptoms. Antimalarial drugs reduce parasite multiplication rate (PMR) to a level lower than the median number of viable merozoites released during destruction of RBCs. This value remains high (ie. Lower killing rate) in the presence of drugs with weak antimalarial activity like tetracycline while low in high killing rates with artemisinin derivatives (White, 2004). WHO defines “drug resistance as the ability of the parasite to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than the recommended dose but within the tolerance of the subject” (Sibley, 2014; WHO, 2010). For a drug to be active against the parasite, it must first obtain entry into the parasite or infected erythrocyte and enough time for its normal action. This definition is improved if the concentration profile of the drug is also taken into consideration. Resistance has developed to all available antimalarial drugs

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including the current drug of choice (Artemisinin-based Combination Therapy), and till date there has been no replacement for Artemisinin (Sibley, 2014). To better understand drug resistance evolution, a look at the history of these drugs and their evolvement is important.

2.3.2 History of antimalarial drug and malaria drug resistance

The pharmaceutical compound Quinine is one of the earliest malaria drugs, however it has not been used widely like the other drugs. Quinine occurs naturally in the bark of a tree native to South America. Cinchona bark was initially used in Europe for treating "the ague" (disease with malaria fever-like symptoms) in the early 17th century. In 1742 Linnaeus named the tree cinchona after the Countess of Chinchon, who was treated with the tree's bark in Peru in the 1600s and credited with the husband for bringing the bark to Spain. Two French Chemists Joseph Pelletier and Jean Biename Caventou isolated the alkaloid quinine from cinchona bark in 1820. The pure quinine was used to treat patients with intermittent fever. It quickly became the therapy of choice for intermittent fever throughout the world and is still an important and effective malaria treatment nearly worldwide (Meshnick, 1998; Meshnick & Dobson, 2001; Wongsrichanalai et al., 2002). First documented resistance of *P. falciparum* to quinine was in human volunteers in Brazil and in Southeast Asia in the1960s (Arrow, Panosian, & Gelband, 2004; Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs; Arrow KJ, 2004).

Determined not to be at the mercy of Allies of Java who controlled valuable quinine stores during World War I, Chloroquine was first synthesized as part of the US World War II military effort in Germany and was later acknowledged as a potent antimalarial in the

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1940s. The formulation of chloroquine arrived too late to help people with malaria in the Sicily following World War II. By 1946, chloroquine had taken over and become the drug of choice for the next 40 years. However, the emergence of chloroquine resistance arose spontaneously from four independent geographic locations: the Thai- Cambodian border around 1957, Venezuela and Magdalena Valley of Colombia around 1960 and Port Moresby, Papua New Guinea in the mid-1970s. Chloroquine resistance *P. falciparum* was first discovered in non-immune travelers to Kenya and Tanzania in 1978 and has since spread to other parts of Africa. Emergence of resistance to chloroquine led to the formulation of other drugs such as sulfadoxine-pyrimethamine, mefloquine, atovaquone-proguanil, and artemisinin derivatives (Malarone) (Arrow et al., 2004; Meshnick, 1998; Wongsrichanalai et al., 2002).

Mefloquine resistance was discovered right after the drug was generally available in 1985 (Hoffman et al., 1985). Sulfadoxine-pyrimethamine (SP) was introduced in Thailand in 1967and resistance to SP was first reported that same year in Thailand (Wernsdorfer & Payne, 1991). SP is the most wildly antifolate combination worldwide. Resistance has developed to all available antimalarial drugs including the current drug of choice (Artemisinin-based Combination Therapy), and till date there has been no replacement for Artemisinin (Sibley, 2014).

2.3.3 Development of resistance to antimalarial drugs

The general consensus is that drug resistance results from a series of de novo mutations that reduces the sensitivity of the parasite to that particular drug. The development of antimalarial drug resistance involves mutations in and variations in the copy number of

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gene encoding for the drug's parasite protein target (White, 2004). The malaria parasite is known for its ability to develop mutations de novo. The mutations occur in where there is high infection and inadequate drug use, and thus reduce the sensitivity of the parasite to that particular drug. Parasite resistance can emerge spontaneously through mutations and gene expression changes in parasite populations in the absence of drug pressure. However, the emergence and subsequent spread of resistance depends on drug selection pressure (Talisuna et al., 2012; Wongsrichanalai et al., 2002). Drug pressure provides a selective edge to resistant parasites. The factors that influence the selection of de novo mutations are listed in Table 2.2. Drugs are particularly vulnerable in cases where a one-point mutation is responsible for a clear reduction in susceptibility. Selection of resistance is influenced by low clearing of the parasite and a shallow concentration–effect relationship.

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Table 2.2: Factors that affect selection of de novo antimalarial drug resistance

(Adapted from (White, 2004))

1. The frequency with which the resistance mechanism arises
2. The fitness cost of the parasite with regards to the resistance mechanism
3. The number of parasites exposed to the drug in the human host
4. The concentrations of drug the parasites are exposed to (This includes the doses used and pharmacokinetic properties of the antimalarial drug or drugs)
5. The pharmacodynamic properties of the antimalarial drug or drugs
6. The phenotypic quantity of resistance (the shift in the concentration-effect relationship) that results from the genetic changes
7. The level of host defence (nonspecific and specific immunity)
8. The simultaneous presence of other antimalarial drugs or substances in the blood that can still kill the parasite if resistance develops to one drug (i.e., the use of combinations)

Many factors contribute to the development and spread of resistance as shown in figure 2.7. The emergence of resistance can be divided in two parts: the de novo mutation that results in the resistant mutant, and selection process under drug pressure results in the subsequent transmission and the spread of resistance. (White & Pongtavornpinyo, 2003). These genetic events which leads to drug resistance are spontaneous, rare and are influenced by drug use, although the selection of resistance strains occurs under drug pressure. Resistance arises mainly during asexual reproduction, and may require only a

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single genetic event in drugs like antifolate or epistasis multiple unrelated events may be vital (epistasis) (Barnes & White, 2005).

The spontaneous emergence of resistance usually occurs in areas of low transmission, reduced immunity, and high parasite load in patients, where most patients tend to be symptomatic and seek treatment. This is the probable explanation why resistance to antimalarial drugs are more likely to develop in SEA (WHO, 2010). South East Asia is characterized by low transmission, low immunity and a long history of haphazard use of antimalarial drugs, which results to high parasitemia in most infections.

In areas of low transmission, most malaria infections become symptomatic, and as such are more likely to receive treatment, which results in selection of resistant strains (site). In areas of high transmission, emergence of drug resistance appears to be low mainly because repeated infections result in acquired immunity which in turn control the infection. This results in asymptomatic infections mostly in adults. Asymptomatic infections and non-use of drugs in these age groups in some cases might decrease the chances of development of resistance (WHO, 2010). Furthermore, many infections in high transmission settings like Africa are polyclonal in semi-immune people and this allow possible outbreeding of multigenic resistance mechanisms or competition between the resistance strains and the sensitive strains in the host (WHO, 2010). Some parts of Africa are transitioning to or have witnessed very low transmission, and thus a hiked chance of spontaneous emergence of resistance on the continent. Factors that influence the de novo selection or emergence, also influence the spread of resistance. These factors include vector and environmental factors, parasite characteristics, characteristics of the drug itself and the interaction of drug-use

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patterns elucidated in table 2.2 and figure 2.7 (Hastings, 2001; Wernsdorfer, 1994; Wongsrichanalai *et al*., 2002).

The spread of antimalarial resistance in malaria parasites occurs because of survival advantage in the presence of the antimalarial and this results in transmission of resistant than for sensitive parasites in most of the cases. Mutations can be associated with fitness disadvantages (i.e., in the absence of the drug they are less fit and multiply less well than their drug-sensitive counterparts) (White, 2004), given that the mutations are not detrimental to the survival or reproduction of the parasite. Other factors, including immunity, host immunity and malaria transmission intensity, affect the spread of resistance, albeit complex.

The spread of drug resistance between populations is helped by the movement of both vectors and people. Human movement plays an important role in this instance. Resistance may also spread through the transport of mosquitoes between countries and across oceans through luggage and vehicles. For several reasons, low and fluctuating transmission favors emergence of resistance (Klein, 2013; Plowe, 2009). This has been demonstrated in molecular epidemiological studies that the patterns of emergence and spread of *P. falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine globally, where it spreads from Asian origin to Africa (Plowe, 2009).

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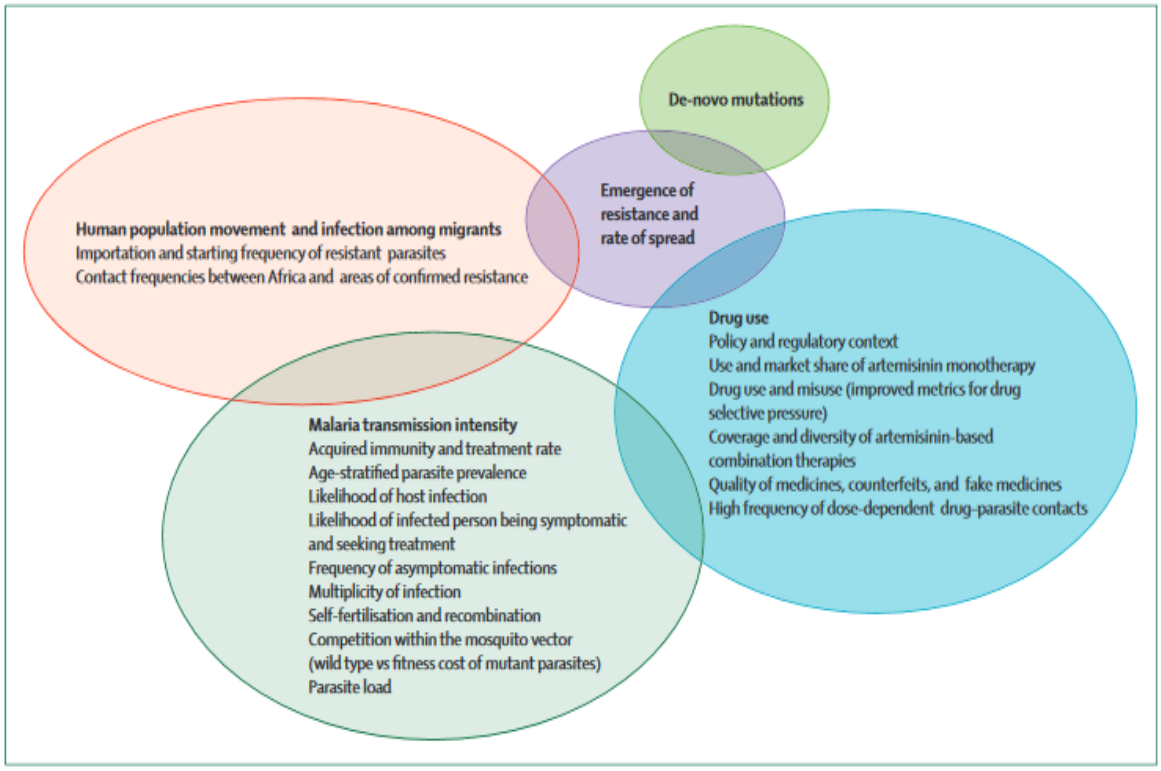

Figure 2.7: Factors that affect the emergence and spread of drug resistance.

Source: (Talisuna *et al*., 2012)

2.3.4 Mechanisms of drug resistance

Many antimalarial drugs have been employed for the treatment of malaria beginning with quinhausu in ancient china (Faurant, 2011) and quinine in the 17th century (Achan *et al*., 2011) and most recently Artemisinin-based Combination Therapy (ACTs). Resistance to all these drugs have been reported. The biochemical mechanism of action and markers associated with resistance for chloroquine, the antifolate combination drugs; sulphadoxine-pyremethamine and artemisinin-based combination therapy, which have been used in Ghana are described below and shown in Table 2.3. The mechanism of drug resistance has

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been extensively studied and understood in cases of chloroquine, the atovaquone and antifolates.

2.3.4.1 Chloroquine resistance

Resistance of the parasite to chloroquine involves an enhanced ability of the parasite to discharge chloroquine at levels that reduces chloroquine levels lower than the levels required for inhibition of haem polymerization (Bloland, 2001). Polymorphisms in two genes of the *P falciparum* genome which encodes for food vacuole membrane explains the molecular basis of chloroquine resistance (Tran & Saier Jr, 2004). *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) has been identified primarily as the main gene associated with CQ resistance in genetic association studies of both sensitive and resistance strains. The *pfcrt* gene encodes a 48.6-kDa protein consisting of 424 amino acids and is located on chromosome 7 (Mita, Tanabe, & Kita, 2009). Among the polymorphic non-synonymous SNPS, mutation at codon 76 which results in an amino acid change from lysine to threonine (K76T), has been shown as the primary mediator of CQ resistance (Fidock *et al*., 2000).

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Table 2.3 Mechanism of action of antimalarial drugs and genetic markers associated with drug resistance

Antimalarial derivatives	**Chemical family**	**Drug name**	**Mechanism of action**	**Genetic markers for drug resistance**
Quinoline derivatives	4-aminoquinolines	Chloroquine	Accumulate in the digestive vacuole of parasite. Inhibition of heme detoxification	Point mutations in *pfcrt, pfmdr1, pfmrp*
		Amodiaquine	Accumulate in the digestive vacuole of parasite. Inhibition of heme detoxification	Point mutations and copy number variation in *pfmdr1*
		Lumefantrine	Accumulate in the digestive vacuole of parasite. Inhibition of heme detoxification	Point mutations and copy number variation in *pfmdr1*
Antifolate derivatives	Sulfa drugs	Sulfadoxine	Inhibits the dihydropteroate synthetase enzyme (pfdhps)	Point mutations in *pfdhps*
	Pyrimethamine	Pyrimethamine	Inhibits the dihydrofolate reductase (pfdhfr)	Point mutations in *pfdhfr*
Artemisinin derivatives	Endoperoxides	Artesunate	Not precisely known	Polymorphism in Kelch 13 Protein
Antibiotics		Artemisinin Artemether	Inhibition the protein synthesis pathway	

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In addition, findings from *in vitro* studies also buttress the association between 76T and chloroquine resistance. Other SNPs at codons 74, 75 and 220 in the *pfcrt* gene always comes with 76 in field isolates, and these are likely to encode compensatory mutations that allows parasites with 76T to maintain adequate fitness, while some also contributes directly to drug resistance phenotypes (Cui et al., 2015; Sidhu, Verdier-Pinard, & Fidock, 2002). Table 2.3 shows molecular markers implicated in the resistance to chloroquine and sulfadoxine pyrimethamine, as well as amodiaquine, lumefantrine and mefloquine, all drugs that are partner drugs in currently used artemisinin combination therapies (ACTs).

pfmdr1 gene located on chromosome 5 and codes for P-glycoprotein homologue 1 (Pgh1), is another gene, which has generated interest in resistance to chloroquine and other antimalarials. Even though the relationship between drug sensitivity and *pfmdr1* polymorphisms are complex, it has been suggested that changes in *pfmdr1* sequence or copy number alter transport of multiple drugs in or out of the parasite food vacuole, with individual polymorphisms leading to opposite effects on different drugs (Cui *et al*., 2015; Koenderink, Kavishe, Rijpma, & Russel, 2010). Mutation at *pfmdr1* N86Y has been associated with chloroquine resistance in some clinical and *in-vitro* studies and other *pfmdr1* polymorphisms, notably Y184F, S1034C, N1042D, and D1246Y, have been identified to be associated with chloroquine resistance at varying degrees. A recent study suggested that *pfmdr1* polymorphisms might modulate susceptibility to chloroquine and other related compounds in parasite transfection experiment (Reed, Saliba, Caruana, Kirk, & Cowman, 2000; Wongsrichanalai *et al*., 2002).

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Table 2.4: Drug resistance markers associated with antimalarial drug treatment failure

(Adapted from (Sibley, 2014))

Antimalarial Drugs	**Alleles with codons association with therapeutic failure**	**Molecular markers**
Sulfadoxine	*pfdhps* 437G and 540E	540E
Pyrimethamine	*pfdhfr* 51I, 59R and108N – triple mutant	Triple mutant
Chloroquine	Many variations of amino acids in 72–76 of *pfcrt* codons CV**IET** and **S**VMN**T** are most important	Allele encoding 76T
	pfmdr1: codons 86Y, Y184 and1246Y	
Amodiaquine	*pfmdr1*: codons 86Y, Y184 and 1246Y	*pfmdr1* 86Y and *pfcrt* 76T
	pfcrt 72–76 SVMNT especially, but also CVIET	
Lumefantrine	*pfmdr1* N86,184F and D1246 or increased copy number	*pfmdr1* N86 and *pfcrt* K76
	pfcrt 72–76 CVMNK	
Mefloquine	Increased copy number of *pfmdr1*	Copy number of *pfmdr1*
Piperaquine	*pfmdr1*: codons 86Y, Y184 and 1246Y	*pfmdr1* 86Y *pfcrt* 76T
	probably *pfcrt* 76T	
Artemisinin derivatives	*PfK13* 493H, 539T, 543T, 580Y	*pfk13* 580Y

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2.3.4.2 Sulphadoxine-Pyrimethamine resistance

Sulfadoxine-pyrimethamine (SP) is the most widely used antifolate antimalarial combination and was introduced in Thailand in 1967 (Arrow *et al*., 2004). Pyrimethamine was initially used alone but resistance evolved within a year of drug use in both *P. falciparum* and *P. vivax.* Soon after, the combination of sulfa drugs and pyrimethamine showed high potency in the laboratory and field isolates. But again, resistance arose in varying degrees around malaria endemic regions (Asia Pacific regions and South America in 1970s and Africa in the 1990s) (Cui *et al*., 2015). Antifolate combination drugs act through sequential and synergistic blockade of two key enzymes in the folate synthesis, dihydrofolate reductase (*dhfr*) domain of the dihydrofolate reductase-thymidylate synthase (dhfr-ts) by pyrimethamine and dihydropteroate synthase (DHPS) by sulfadoxine (Bloland, 2001). Mutations associated with resistance to sulfadoxine (DHPS) and pyremethamine (DHFR) have been determined. Varying degrees of resistance to antifolate combination drugs involves specific combinations of mutations in these genes. Multiple mutations at amino acid codons N51I, C59R, S108N/T, and I164L in *pfdhfr* have been shown to be the sole determinant of pyrimethamine resistance. Whilst, mutation at codon 108 (S108N) is most key for pyrimethamine resistance. Several association studies have reported an increase in the IC_{50} of pyrimethamine by 7–50 fold in S108N mutations than the wild-type. Other mutation(s) in the gene synergistically increase the levels of resistance in pyremethamine. The *pfdhps* gene have been associated with sulfadoxine resistance, specifically, mutations at amino acid positions S436A, A437G, K540E, A581G, and A613S/T have been identified. Mutations at positions 437G and 540E are strong determinants of sulphadoxine resistance while 436A, 581G and 613S enhance the

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association between mutants on codons 437 and 581 with drug resistance (Wernsdorfer & Noedl, 2003). Mutation at codon 437 which results in amino acid change from alanine to glycine (A437G) has been shown to increase IC_{50} to sulfadoxine by 4.8- than the wild-type parasites (Mita et al., 2009).

2.3.4.3 Artemisinin and partner drugs

The Artemisinin-Based Combination Therapy (ACTs) are currently recommended by WHO for treatment of uncomplicated *P. falciparum* infections after chloroquine resistance became widespread. Artemisinin derivatives (eg, dihydroartemisinin, artemether, and artesunate) are used in combination with other partner drugs as shown in Table 2.2. Resistance to the indispensable artemisinin was first observed in the Cambodia–Thailand in SEA (where resistance to drug resistance usually emerges from) before 2008 but treatment failure was documented in 2008 (Cui *et al*., 2015; Dondorp *et al*., 2009; Noedl *et al*., 2008; Phyo *et al*., 2012; Wongsrichanalai & Meshnick, 2008). The emergence of resistance of *Plasmodium falciparum* parasites to artemisinin and its derivatives in Southeast Asia is extremely worrisome because of the risk of artemisinin resistance spreading from Greater Mekong sub region to other areas like Africa, where outcomes can be devastating (Kamau *et al*., 2015). Genome wide association studies done on field samples revealed *pf* kelch protein gene on chromosome 13 (kelch13) to be associated with delayed parasite clearance *in vivo* (Cheeseman *et al*., 2012; Takala-Harrison *et al*., 2013). Further analysis discovered, marker in the propeller domain of the kelch (K13) gene (PF3D7_1343700) to be associated with reduced clearing of parasite following treatmentt with artemisinin in SEA (Ariey *et al*., 2014). Mutations at codons Y493H, R539T, I543T,

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and C580Y, have been delineated with delayed clearance using clinical and molecular data (Ariey *et al*., 2014; Witkowski *et al*., 2010). These mutations are associated with delayed parasite clearance in Cambodia, Thailand, Myanmar, and Vietnam are not common in Africa (Cui *et al*., 2015). Ariey and colleagues demonstrated that of the large number of mutations identified, the C580Y allele accounted for 85% of all mutant alleles observed in Cambodia and most (74%) of the parasites had a single nonsynonymous mutation in the K13-propeller (Ariey *et al*., 2014). Cross-resistance between the 4-aminoquinolines, chloroquine and amodiaquine, has been shown both *in vitro* and *in vivo*. Specifically, there is an interaction between *pfmdr1*and *pfcrt* alleles, which yields different levels of resistance to amodiaquine and chloroquine. The *pfcrt* mutations at codons 72–76 are associated with high levels of amodiaquine resistance in South America, whereas *pfcrt* mutations are linked to greater resistance to chloroquine and moderate resistance to amodiaquine in South-East Asia and Africa. This difference may have been influenced by previous use of amodiaquine in the different regions. *pfmdr1* mutations A86T and 1042 may modulate resistance to Amodiaquine as well (Sa *et al*., 2009; WHO, 2010). On the other hand, the wild-type *pfmdr1* N86 has been shown to be associated with Artemether-Lumefantrine in field studies and as such can be a marker for monitoring its susceptibility (Dokomajilar, Nsobya, Greenhouse, Rosenthal, & Dorsey, 2006; Happi *et al*., 2009; Sisowath *et al*., 2005; WHO, 2010).

2.3.5 Detection and monitoring of antimalarial drug resistance

Measuring and monitoring of antimalarial drug resistance is achieved using four methods in general; in vivo, in vitro, molecular characterization, and animal model studies.

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However, the first three methods are mainly used to detect drug-resistant malaria parasites as recommended by WHO. Each of these methods have their advantages and disadvantages bases on information provided, nature of method, expense and applicability for large surveillance and the measures may not be comparable. These methods complement each other, rather than compete against each other on information of resistance. Malaria elimination strategies require constant surveillance of the parasite population for genetic changes of public health importance, such as new forms of drug resistance (Manske et al., 2012).

2.3.5.1 *In vivo* tests

In vivo test is the gold standard for monitoring of antimalarial drug resistance because it closely reflects actual clinical or epidemiological situations, which involves treatment response of parasites to drugs in use. Symptomatic and parasitaemic individuals are given accepted doses of drug and monitoring of the parasitological and/or clinical response over time. *In vivo* tests provide best information which is close to actual operational conditions. Results from this method explains what can be expected to occur among clinic patients if provider and patient compliance is high. WHO recommends a minimum follow-up period of 28 days for medicines with elimination half-lives of less than 7 days (amodiaquine, artemisinin derivatives, atovaquone–proguanil, chloroquine, halofantrine, lumefantrine, quinine and sulfadoxine–pyrimethamine). For medicines with longer elimination half-lives (mefloquine, piperaquine), longer follow-up periods (42 days) are recommended. (WHO, 2009b). In addition to measuring parasitological and clinical indicators, *in vivo* methodologies allow investigates hematological recovery after malaria therapy because it

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is a major component of malaria (Bloland *et al*., 1993). Although less technically demanding, therapeutic efficacy tests are logistically challenging and imprecise (Greenwood, 2002).

2.3.5.2 *In vitro* tests

In vitro assays consist of the *Plasmodium* parasite exposure to different concentrations of the drugs under controlled environmental conditions observed for inhibition of maturation into schizonts. This method measures the intrinsic sensitivity of *P. falciparum* parasites to antimalarial drugs. Inhibition of maturity to schizoits is measured as "pure" antimalarial drug resistance (WHO, 2010). Being able to perform multiple tests on isolates, simultaneously with several drugs is the major pro of in vitro assays. In vitro assays give more direct information about the sensitivity of the parasite (Greenwood, 2002) but one important con is that the correlation of in vitro response with clinical efficacy in patients is not consistent. Moreover, these tests are technologically more demanding and relatively expensive which makes them potentially more difficult to adapt successfully to routine work in the field (Bloland, 2001). In addition, testing the susceptibility of parasites to drugs *in vitro* can be problematic because the phenotypes of *in vivo* methods do not necessarily correlate with *in vitro* performance, in addition to being technically challenging, laborious, and expensive (Mideo et al., 2013). Inconsistence results with in vitro assays may be because in vivo tests are influenced by the level of immunity of the patient (Wongsrichanalai *et al*., 2002).

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2.3.5.3 Molecular techniques

The use of molecular techniques in the detection and monitoring of antimalarial drug resistance is an evolving field, but offer promising advantages to other methods. Mutations encoding for biological resistance are determined by traditional molecule techniques like PCR and restriction fragment length polymorphism (RFLP) analysis (Duraisingh, Curtis, & Warhurst, 1998), molecular beacons (Durand *et al*., 2000), real-time PCR (Alker *et al*., 2005; Wilson, Alker, & Meshnick, 2005), dot-blot probe hybridization (Ranford-Cartwright, Johnston, Abdel-Muhsin, Khan, & Babiker, 2002), single-nucleotide primer extension (Nair, Brockman, Paiphun, Nosten, & Anderson, 2002), and high throughput Next Generation Sequencing methods (Mideo *et al*., 2013). Traditional methods used to detect resistance struggle to detect rare or low-level resistance.

Advantages that this method has over the others include that only a little amount of genetic material is required, and not dependent on host factors host, less laborious, done in a shorter time and less expense. Disadvantages associated with the use of molecular markers for monitoring resistance include the fact mutations associated with antimalarial drug resistance are known or debated for selected number of drugs and this method requires the use of sophisticated equipment and training. One stumbling block of molecular techniques from becoming the ideal tool for detection of resistance is the inability to confirm associations between mutations and actual drug resistance because resistance may involve more than one locus and multiple mutations. Furthermore, because new forms of resistance mechanisms keep evolving, there can never be an exhaustive list of markers associated drug resistance. Frequency of gene mutations associated with antimalarial drug resistance

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can provide evidence of resistance in that vicinity comparable to data obtained from *in vitro* methods (Bloland, 2001).

2.3.6 Importance of monitoring molecular markers of drug resistance in epidemiology

Characterization and monitoring of molecular markers associated with antimalarial drug resistance is key to understanding drugs resistance mechanisms (WHO, 2010). Large surveillance of drug resistance and studies on population mechanics and dynamics depends solely on resistant markers in epidemiological context because surveillance of these drugs does not only provide information of current resistant situation but is also a way to look out for resistance even before it occurs. The prerequisite is the selection of specific markers for drug resistance and reliable techniques for their identification (Wernsdorfer & Noedl, 2003). Monitoring of molecular markers of resistance has gained substantial attention in the study of epidemiology of resistant malaria over the past three decades. The main reason is the availability of rapid and reliable techniques for their identification and also the advantages it has over in vivo and in vitro tests is that, it is cost effective and scalable and easier method of specimen collection and storage (WHO, 2010). They provide useful information particularly in providing direct evidence of association between treatment failure and the selection of resistant parasite populations.

Markers have been defined for antifolates and chloroquine, however, the inability to confirm associations between mutations and actual drug resistance because resistance may involve more than one locus and multiple mutations poses a great challenge (Bloland, 2001). Thus detection of molecular markers is usually used to confirm if therapeutic failure

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is caused by intrinsic resistance (WHO, 2010). Molecular techniques like, traditional PCR, real- time PCR and next generation sequencing provides a lot of prospects in the use of resistance markers in epidemiology. Differences in the re-expansion *pfcrt* marker patterns open up new opportunities for studying the intercontinental differences of chloroquine resistance. Study of microsatellites flanking these markers provides information about the origin of these markers, which can be explored as a control measure.

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2.4 IMPLICATION OF USE OF SYMPTOMATIC AND ASYMPTOMATIC MALARIA SAMPLES IN THE MOLECULAR SURVEILLANCE OF GENETIC DIVERSITY AND DRUG RESISTANCE

Varying manifestation of malaria infection (symptomatic and asymptomatic) is a function of immunity, which is dependent on age and exposure to the parasites. Thus, while symptomatic infections are often seen in younger children and under low transmission settings, as a result of brief exposure culminating in low acquired immunity to parasites, asymptomatic infection on the other hand are common in older children and usually under high transmission. This is due to the development of acquired immunity gained as a result of frequent and longer exposure to the parasite.

Subsequently, the type of malaria infection developed as a result of the presence or absence of protective immunity influences the level of drug usage in the population and in turn the development and spread of drug resistant parasites. In high transmission settings, the proportion of infections that result in symptomatic infection is comparatively small as compared to those that result in asymptomatic infection, as such resistance to antimalarial drugs develops slowly due to reduced level of drug usage. Under low transmission however, infections are most likely to develop into symptomatic malaria, as such the proportion of the population that are treated will increase. The resulting drug pressure will thus enhance the development and spread of drug resistance parasites (Hastings & D' Alessandro, 2000).

In light of the role malaria infections play in the development of drug resistance, it has become relevant to track drug resistant parasites in both symptomatic and asymptomatic

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infections. This will serve as a form of real-time epidemiology to evaluate the efficacy of antimalarial drugs over time.

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CHAPTER THREE

3 MATERIALS AND METHODS

3.1 STUDY SITE

This study was conducted in two towns in two major eco-epidemiological zones of Ghana, Cape Coast in the Coastal Savanna zone and Begoro in the Forest zone. The Forest zone has high and perennial malaria transmission while the Coastal Savanna has low to moderate and perennial transmission. The locations of the two study sites in Ghana are shown in Figure 3.1.

3.1.1 Begoro

Begoro is the capital of Fanteakwa District in the Eastern Region of Ghana. It is in the forest ecological zone of Ghana and has a population of 108,614, according to the 2010 population census (Census, 2010). The major rainy season in this area lasts from March to early August. The minor rainy season is from September-November. The maximum amount of rainfall per season occurs in June and October each year respectively. The rest of the year is relatively dry. The district has a mean annual rainfall of 1,592 mm (1,224mm to 1,982mm). Begoro has a maximum temperature of about 31.5ºC, minimum temperature of 26.0ºC and mean relative humidity of 74.4%. Malaria is perennial and hyper endemic with high transmission intensity. The most intense transmission in the forest ecological areas occurs in June because of high rainfall (Duah et al., 2007).

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3.1.2 Cape Coast

Cape Coast is located 165 km west of the capital along the Gulf of Guinea (5°06'N, 5.1°N; 1°15'W, 1.25°W). The metropolis is found in the coastal savanna zone of Ghana with a population of 169,894 (Census, 2010). Temperature within the metropolis ranges between 21°C and 36°C all year round, with a maxima rainfall for the two seasons totaling between 750 mm and 1,000 mm. There are two raining seasons where the major season occurs between May and July and the minor rainy season occurs between November and January. The relative humidity in Cape Coast is quite high varying between 85% and 99%. In 2009, the metropolis reported 109, 211 malaria cases compared to 61, 077 cases in 2007. Malaria transmission in the coastal part of Ghana is also perennial and hyper endemic but with low to moderate transmission intensity (Kudom & Mensah, 2010).

3.2 STUDY DESIGN

This study was a cross-sectional study involving symptomatic and asymptomatic malaria infections for four (2014-2017) and five (2013-2017) consecutive years respectively. The study was in two parts, based on whether the enrolled subjects were showing symptoms of malaria or were asymptomatic:

1. A hospital-based study (2014-2017)

2. A school-based study (2013-2015)

Sentinel health care facilities established as part of a surveillance system for monitoring antimalarial therapeutic efficacy in Ghana were used for the hospital survey in Begoro (Forest zone) and Cape Coast (coastal zone). Children presenting with malaria symptoms

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(fever) were screened and those who were positive for *P. falciparum* were enrolled into the study as symptomatic children. For the school-based survey, three basic schools (one from each educational circuit) were randomly selected from each of the two study sites and all children whose parents gave consent to be part of the study were screened. Children who had temperature < 37.5 and all who tested positive for *P. falciparum* were enrolled as asymptomatic infections. The children gave few drops of blood collected on filter paper for molecular analysis. After DNA extraction from the dried blood spot, the study leveraged the high specificity and relatively low-cost of targeted next generation sequencing using molecular inversion probes for targeting and sequencing was done on the illumina MISEQ platform. *Plasmodium falciparum* genes (*pfcrt*, *pfdhfr*, *pfdhps*, *pfmdr1* and *pfk13*) implicated in anti-malarial resistance to chloroquine (CQ), sulfadoxine pyrimethamine (SP), lumefantrine, amodiaquine and artemisinin were sequenced and analysed for both study sites. The *pfama1* gene was sequenced for the genetic diversity and complexity of *P. falciparum* infections (COI) analysis.

3.3 STUDY POPULATION

The symptomatic population consisted of children with uncomplicated *P. falciparum* malaria at the Begoro District Hospital (Begoro) and the Ewim polyclinic (Cape Coast) and aged 6 months -14 years. The asymptomatic population consisted of school children in the study area, aged 6-14 years, presenting with no fever but positive for *P. falciparum* parasitemia.

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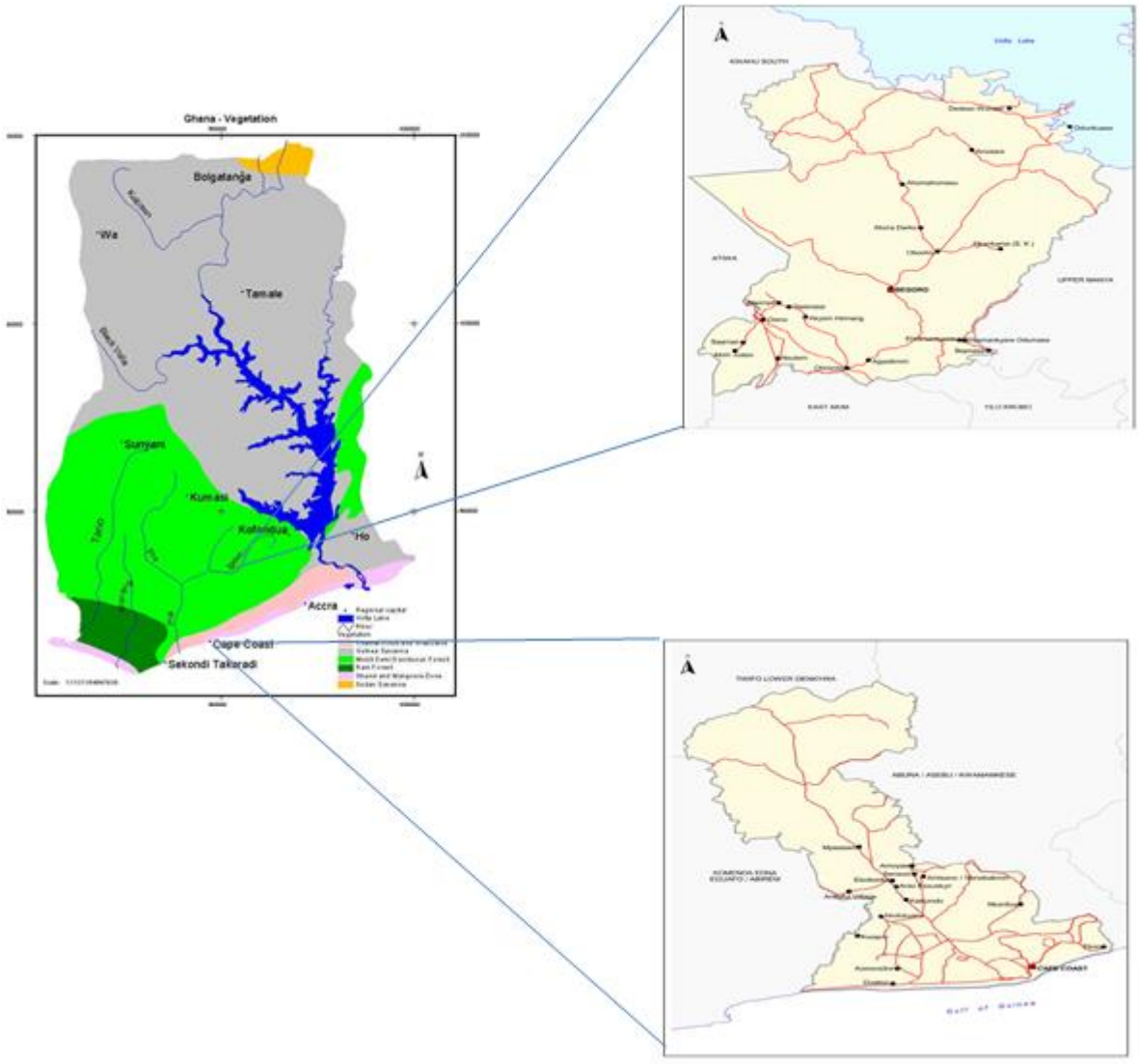

Figure 3.1: Map of Ghana showing selected two sites used for this study. Begoro in the Fanteakwa District is considered a forest zone, which is hyper endemic for malaria with high transmission intensity. Cape Coast in the Cape Coast district is considered a coastal savanna

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3.3.1 Inclusion criteria: hospital-based survey

1. Children aged 6 months to 14 years
2. Axillary temperature ≥ 37.5^{o}C or history of fever during the past 24 hours.
3. Written informed parental consent
4. Child assent for 12 - 14 years
5. *Plasmodium falciparum* mono-infection
6. Asexual parasite density between 1,000 – 250,000/µl

3.3.2 Inclusion criteria: school-based survey

1. Children aged 6 years to 14 years.
2. Written informed parental consent
3. Child assent for 12 - 14 years
4. Axillary temperature < 37.5^{o}C
5. *Plasmodium falciparum* mono-infection < 250,000/µl

3.3.3 Exclusion criteria-hospital and school-based surveys

Individuals were excluded from the survey based on the following criteria:

1. Presence of general danger signs or signs of severe falciparum malaria, mixed or mono-infection with another *Plasmodium* species detected by microscopy.
2. Presence of severe malnutrition (defined as a child whose growth standard is below -2 z-score, has symmetrical oedema involving at least the feet or has a mid-upper circumference < 110 mm).

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3. Presence of febrile conditions due to diseases other than malaria (e.g. measles, acute lower respiratory tract infection, severe diarrhoea with dehydration) or other known underlying chronic or severe diseases (e.g. cardiac, renal and hepatic diseases, HIV/AIDS).

3.4 SAMPLE SIZE AND POWER CONSIDERATION

The sample size was estimated to detect at least 0.2 difference in COI between the coastal savannah zone and forest zone, and between symptomatic and asymptomatic children using the t-test sample size formula (Kadam & Bhalerao, 2010):

$$\boldsymbol{n = \frac{2\left(Z_{\alpha} + Z_{\beta}\right)^{2}}{\left(\frac{\Delta}{\sigma}\right)^{2}}}$$

Where, *n* is the required sample size, Z_{α}, is a constant (set by convention according to the accepted α error and whether it is a one-sided or two-sided effect), Z_{β}, is a constant set by convention according to power of the study, σ is the standard deviation (estimated) and Δ the mean difference (Kadam & Bhalerao, 2010)

Assumptions: α (two-sided) = 0.05; power = 0.80; mean difference = 0.2; σ = 0.50; Z_{α} = 1.96; Z_{β} =0.84.

$$\boldsymbol{n = \frac{2\ (1.96 + \ 100)^{2}}{\left(\frac{0.2}{0.5}\right)^{2}}}$$

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N =100 per site or group

Predicting a 10% genotyping failure rate, a sample of approximately 110 children per site was required. Therefore, a sample size of 110 children per group was required to detect a difference of 0.2 in mean COI between the 2 (independent) ecological zones and symptomatic or asymptomatic with 80% power, using a two-sample t-test and assuming a (two-sided) α of 0.05, effect size of 2.0, and a SD of 0.50.

3.5 STUDY PROCEDURE:

3.5.1 Eligibility assessment and enrollment of study participants

To assess eligibility for enrollment, a simple and structured questionnaire (Appendix I) was administered to each screened child or the parent of the child from both surveys and study sites. The questionnaire gathered information about their age, height, weight, medication for the past two weeks and history of fever. Hemoglobin level was measured using the Hemocue - Hb 201 (Angelhom, Sweden) to exclude anemic children. Blood samples were screened for *P. falciparum* using microscopy on the field. After these procedures, children who tested positive for *P. falciparum,* had Hb greater than 5 g/dl were enrolled into the study and their filter paper blots used for the downstream molecular analyses. Symptomatic children who tested positive for malaria were treated with Artesunate Amodiaquine (AA) at standard dose of 25mg/kg over a period of 3 days.

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3.5.2 Sample collection

At the hospitals, children referred for routine laboratory investigations who met the inclusion criteria were screened as potential study participants. For the school survey, all school children whose parents had given consent were screened. For each potential participant, 400 µl of finger prick blood sample was drawn aseptically for hemoglobin measurement, thick and thin smears used for asexual quantification and species identification; and few drops of blood (50 µl) spotted on filter paper for molecular investigations. The filter paper blood spots were dried and stored individually in Ziploc bags with silica gel at room temperature. The blood films were prepared from fresh blood obtained from pricking the finger of the patient with a lancet. Two drops of blood were used for thin and thick films. The thin films were allowed to air-dry, fixed with methanol (to prevent the RBCs from lysing) and allowed to dry before staining. The thick film was also allowed to dry without fixing before staining. The slides were stained with a 10% Giemsa solution (pH = 7.2) for 15 minutes.

3.5.3 Parasitological examinations

Thin and thick films were examined by two independent Microscopists to allow for the accurate detection of the species of parasite and to determine the parasite density. Light microscopy was used to examine and quantify malaria parasites against 200 WBC.

The parasite density per microliter blood was then calculated assuming 8,000 WBCs per microliter of blood formulas shown below,

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Parasite density (per µL) = (Number of parasite counted x 8000)/ Number of WBCs counted

3.5.4 DNA extraction from filter paper blood blot.

Genomic DNA was extracted from the dried blood spots (DBSs) on filter paper using the QIAamp mini extraction kit (QIAGEN, Hilden Germany) according to the manufacturer's instructions, using 6 (3 mm diameter) punches extracted into 200 µL of eluate and stored at -20˚C until ready for use.

Briefly, 6 (3 mm diameter) punches of dried blood spots from each filter paper were cut out into 1.5 ml microfuge tubes and 180 µL of tissue lysis buffer (ATL) added, after which the tubes were incubated at 85˚C for 10 minutes. Each tube was then briefly centrifuged to remove drops from inside the lids. Twenty microliters (20µL) of proteinase K stock solution was then added to each tube, mixed by vortexing and incubated at 56 ˚C for 1 hour. After the incubation, the tubes were briefly centrifuged again to remove condensation from inside the lid and 200 µL of lysis buffer (AL) added to the samples, mixed thoroughly by vortexing and incubated at 70˚C for 10 minutes. The tubes were briefly centrifuged and 200 µL ethanol (96-100%) added to the samples and mixed thoroughly by vortexing and briefly centrifuged. The resulting mixture was then applied to the QIAamp mini spin columns (in a 2 ml collection tube), and the caps closed and centrifuged at 8,000 rpm for 1 minute. Following this, the tubes containing the filtrate were discarded and the columns placed in clean 2 ml collection tubes. The QIAamp mini spin columns were washed with 500 µl of wash buffer 1 (AW1) and 500 µl of wash buffer 1 (AW2) consecutively to remove any residue of the filtrate from the column. The columns were centrifuged at 8,000 rpm for

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1 minute for the AW1 wash and then at 14 000 rpm for 3 minutes and for the AW2 wash. For each wash, the filtrate was discarded. The tubes were then centrifuged at 14000 rpm for 1 minute to eliminate the chance of possible buffer AW2 carryover. The mini spin columns were then placed in clean 1.5 ml microfuge tubes and the filtrate-containing collection tubes discarded. Finally, 200 µL of AE buffer was added to the spin column and incubated at room temperature (15-25˚C) for 10 minutes. The tubes were then centrifuged at 8,000 rpm for 1 minute to elute the DNA into the 1.5ml microfuge tubes. The isolated DNA was then stored at -20˚C until ready for use.

3.5.5 DNA quantification and detection

The concentration of DNA was estimated using the Qubit 2.0 fluorometer (Qubit, Life Technologies). This fluorometer utilizes specially formulated dyes that bind specifically to DNA or RNA. The Qubit working solution was prepared by mixing 6 µL of Qubit dsDNA high sensitivity (HS) reagent with 11.94 ml Qubit double stranded DNA (dsDNA) buffer to obtain 12 ml working solution. Dilutions of the standard were prepared in 1x TE-buffer. For Qubit DNA quantification of standards, 190 µl of Qubit working solution were mixed with 10 µl of DNA standards (0 and 10 ng/µl provided with the Qubit kit) or diluted DNA standard. For Qubit DNA quantification of individual samples, 199 µl of Qubit working solution were mixed with 1 µl of the DNA sample. Both the standards and the DNA sample mixtures were incubated in the dark for two minutes. Fluorescence intensity of the standards was measured in parallel, followed by the samples on the Qubit 2.0 fluorometer.

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3.5.6 Sequencing and genotyping of *P. falciparum*

In all 803 and 994 *P. falciparum* DNA samples from the hospital-based and school-based surveys respectively, were quantified, Molecular Inversion Probe (MIP) captured, amplified and deep sequenced on Illumina MiSeq platform at the molecular laboratory of University of Massachusetts Medical School (UMMS) (Aydemir et al., 2018). The MIP design, capture, amplification and sequencing were done as previously described (Aydemir et al., 2018).

3.5.6.1 Molecular Inversion Probe (MIP) design

The DNA samples were hybridized with probes designed to capture the region of interest in the drug resistance genes including *pfcrt, pfmdr1*, *pfdhps*, *pfdhfr pfk13* and the *pfama1* gene (Appendix II) as previously described (Aydemir et al., 2018). This study used MIPs targeted amplicon sequencing, which has advantage over existing molecular surveillance platforms. MIPs minimize sequencing errors with unique molecular indexes for error correction (Aydemir et al., 2018). This method can be multiplexed to include hundreds or thousands of additional targets and still be able to obtain good genetic data. In addition, this protocol enables large scale genotyping of number of loci and quantity of samples. Therefore, it provides the means of high-throughput surveillance of molecular markers of public health importance (Aydemir et al., 2018).

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3.5.6.2 MIP Capture

The MIP capture mixture of 10 µl (Table 3.1) containing 0.92µl of Ampligase Buffer (10X), 0.2µl Phusion DNA polymerase (0.0008 units/µl), 0.4µl of Ampligase (0.04 units/µl), 0.4µl of the pooled MIPs (40 nM, total), 0.2µl dNTP (4 µM), 5µl of template DNA (up to 30 ng) were incubated in a preheated thermocycler with the following steps 95°C (10 min), 60°C (1 hr), 4°C hold. Upon hybridization of the MIP arms, the target region (template sequence between the arms) was captured when DNA polymerase was added to the nucleotides complimentary to the target DNA after which DNA ligase sealed remaining gap with the ligations arm forming a covalently closed circular molecule.

Table 3.1 MIP capture reaction mixture

Component	**Volume per sample (µL)**
Water	2.88
10X Ampligase Buffer	0.92
dNTP (0.2 mM)	0.2
MIP pool (40nM)	0.4
Ampligase (1u/µl)	0.4
Q5 polymerase (0.04 u/µl)	0.2
Template DNA	5.0

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3.5.6.3 Exonuclease treatment of MIP Capture Reaction

After capturing the target, all noncircular DNA including unbound probes and template DNA were removed by the exonuclease reaction leaving the circular MIPs by adding 2 µl of exonuclease master mix (Table 3.2) containing 0.2 µl of 1X Ampligase buffer, 0.5µl of 10 units Exonuclease I and 0.5µl of 50 units Exonuclease III and the reactions were performed in a thermocycler with the following steps: 37˚C for 1 hour, followed by 95˚C for two minutes and a final hold step of 4˚C.

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Table 3.2 Exonuclease treatment reaction mixture

Component	Volume per sample (µL)
Water	0.8
10X Ampligase Buffer	0.2
Exonuclease I	0.5
Exonuclease III	0.5
Total volume	2

The two reactions above form the entire capture reaction totaling 12 µl.

3.5.6.4 MIP PCR

The entire capture reaction (12 µl) above was then amplified in a 25 µl PCR reaction containing the following components (Table 3.3): 1X Phusion Polymerase Buffer, 1X Macromolecular Crowding (MMC) solution, 200 nM dNTP, 0.02 units/µl Phusion DNA polymerase, forward and reverse primers (Appendix V), 500 nM each. PCR was performed using a preheated thermocycler with the following steps 98°C 30 s, 21 cycles (98°C 10 s, 63°C 30 s, 68°C 30 s), 68°C 2 min, 4°C hold (Table 3.4).

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Table 3.3 MIP PCR reaction mixture

Component	Volume per sample (µL)
5X Phusion Rxn Buffer	5
5X MMC	5
10 mM dNTP mix	0.5
5 uM f+r primer mix	2.5
Phusion DNA Polymerase	0.25
Total volume	13.25

Table 3.4 MIP PCR cycling conditions

Cycle	Temperature	Time	Number of cycles
Initial Denaturation	98˚C	30 seconds	1
Denaturation	98˚C	10 seconds	
Annealing	63 ˚C	30 seconds	30
Initial Extension	68 ˚C	30 seconds	
Final extension	68 ˚C	2 minutes	1

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3.5.6.5 Library preparation and Sequencing

The amplified products were viewed on 1.5% agarose gel and pools were created by combining 5-10 µl of each PCR product in a single tube and cleaned up to remove the unwanted adapter dimers ~ 200 bp using Ampure XP beads (Beckman Coulter, Catalog #A63881) at 0.8: 1, bead: DNA ratio. If adapter dimers remained after bead clean up, the eluted DNA was electrophoresed on a 1.5% agarose gel and the relevant bands between 500-1000 bp were extracted from the gel using Monarch DNA extraction kit (NEB, catalog # T1020S). The purified amplicons were then sent to the Sequencing Core of UMMS for sequencing on the Illumina MiSeq platform using 250 bp paired end sequencing with dual indexing using MiSeq reagent kit v2 and custom primers (Appendix VI).

3.5.6.6 MIP processing and variant calling

The sequences generated were processed using MIPWrangler software (Hathaway, unpublished) and another software described in Aydemir *et al*. (2018). Briefly, the bc2fastq software was used to demultiplex the sequences and paired end reads stitched together using FLASH (Magoc & Salzberg, 2011). The sequences were then filtered on base quality scores above 30 and less than 70% (Q30 <70%). These filtered sequences were demultiplexed again, this time by target using the extension and ligation arm sequences to generate a file for each target per sample (Appendix II).

Target sequences were corrected using their unique molecular identifiers (UMIs) per sample done by clustering the sequences by UMIs and then creating a consensus sequence

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for each specific UMI. This UMI redundancy removes a significant proportion of PCR errors that occur in late cycles, including polymerase stutter and subsequent sequencing errors. UMI corrected sequences were then further clustered by using the qluster algorithm from SeekDeep allowing accurate detection of single base differences and indels at levels of 1% or less (Hathaway, Parobek, Juliano, & Bailey, 2017). A minimum threshold of ≥ 2 UMIs and ≥ 0.5% relative abundance was set given the variable depth to select a cluster to be included in the final analysis.

Lengths of the captured sequences for each MIP and sample were extracted from the MIPWrangler output. MIPs for each AMA1 gene target was put together for all clonal samples and mapped to the Pf3D7 reference genome. Pairwise alignment was conducted using the LastZ software to determine the difference between the observed sequence and the reference sequence for each probe (Harris, 2007). In addition, sequences for AMA1 gene were obtained from the Genebank for Nigeria, Kenya and Thailand to compare the population genetics of the AMA1 gene.

3.6 DATA MANAGEMENT AND STATISTICAL ANALYSIS

3.6.1 Definition of outcomes

***Estimating prevalence*:** Definitions of outcomes used in this study are as previously described (Okell, Griffin, & Roper, 2017). The prevalence of resistance (Prev) defined as the proportion of infected children carrying at least one resistant parasite clone was calculated as Prev = P(mix) + P(res). Where P(mix) is the proportion of infections with mixed (both wild-type and resistant) parasite clones and P(res) is the proportion of

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infections with only resistant parasite clones. P(mix) was calculated as P (mix) = 1 – (P(res) + P(wt)), where P(wt) is the proportion of infections with all wild-type.

Estimating frequency: The frequency (P) of resistance was defined as the proportion of parasite clones which have a resistance marker and was calculated by combining the relative allele frequencies in each individual sample with equal weighting.

Complexity of infection (COI):

The COI for each *pfama1* MIP was estimated per sample. The overall COI for each individual was estimated as the maximum of the COIs for all the AMA1 target MIPs per sample.

3.6.2 Population genetic analyses of AMA1 gene

In addition to sequences of *pfama1* gene from this study, 207 sequences were downloaded from the Genebank for Nigeria (West Africa) (Spencer D Polley & Conway, 2001), Kenya (East Africa) (Osier et al., 2010) and Thailand (South East Asia) (Polley, Chokejindachai, & Conway, 2003; Zhu et al., 2016).

Multiple alignments of these sequences were performed using MUSCLE in MEGA version x software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) and the data exported to DnaSP version 6.12.01 for analysis (Rozas et al., 2017). Polymorphism and diversity were estimated by estimating the number of haplotypes (h), average pairwise nucleotide diversity (π), the total number of polymorphic sites (S); synonymous (SP) and non-synonymous (NS) SNPs, and haplotype diversity (Hd). Haplotype diversity is estimated using the expected and the observed heterozygosity.

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A sliding window analysis of Tajima's D, Fu and Li's D* and F* tests were estimated using DnaSP to determine departures of the parasite population from neutrality. Negative values for Tajima's D and Fu and Li's D* and F* tests indicate to negative selection, whereas positive values correspond to positive natural selection (Mehrizi, Sepehri, Karimi, Djadid, & Zakeri, 2013). Selection of recent population is characterized by low frequency polymorphisms which gives negative measure of Tajima's, whereas, when there is a balance of both low and high amount of mutations in the population, it indicates a recent population bottleneck thus gives a positive Tajima's D estimate (Alker et al., 2005). Balancing selection maintains alleles at balanced frequencies within populations for evolutionary advantage under immune pressure by conserving the genetic diversity (Arnott et al., 2014). The McDonalt and Kreitman test (McDonald & Kreitman, 1991) was also performed to determine neutrality. The Fisher exact statistic is use to test the significance of the neutrality estimate (NI). A neutrality index of 1 shows neutrality, <1 shows negative selection while >1 shows positive selection (Mehrizi et al., 2013). The recombination parameter (R) and the linkage disequilibrium (LD) indices D' and R^2 were also estimated using the DnaSP software for measures of recombination events in the gene and degree of non-random association between alleles respectively. The LD analysis was done using informative sites, which are positions in a sequence where two-character states occurs two or more times. The Wright's Fst was estimated to assess the proportion of genetic variance due to population subdivision using DnaSP (Zhu et al., 2016). Discriminant analysis of principal components (DAPC) was conducted to look for any signal of population structure between the Ghanaian isolates and those from other endemic countries. DAPC was analyzed using the R package Popr.

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3.6.2.1 Statistical analysis of drug resistant mutations

All Statistical analyses were performed using the statistical software STATA version 15. The significance level was set at $p<0.05$ for all tests. Baseline characteristics (mean age, mean weight, and, mean parasitemia, mean anaemia and mean axillary temperature) were compared across study sites using student's t-test or Wilcoxon-Mann-Whitney test. Prevalence of drug resistant alleles (PrevRes) were estimated and compared between study sites using chi-squares and Fisher exact test. The trends of prevalence over the 4 years were tested using the Cochrane Armitage test trends. Statistical tests for monotonic trend between study sites were performed using Regional Kendall test (Helsel & Frans, 2006). A binomial logistic regression was performed to determine if monitoring drug resistance using asymptomatic samples will differ compared to using symptomatic samples.

3.7 ETHICAL CONSIDERATION

Ethical approval to conduct this study was granted by the Noguchi Memorial Institute for Medical Research Institutional Review Board (IRB) (056/12-13) The participant or their representatives were informed of the purpose of the study, the constraints and potential benefits for the participant/community, possible risks, the expected duration of the subject's participation, the subject's responsibilities, and the number of subjects involved and procedures involved in the research study, including all invasive procedures. Participants or their representative were assured confidentiality and protection of their responses and data collected for the purposes of the study. Written informed consent (Appendix III A.) was obtained from parents or guardians of all children before enrolment.

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In addition, written assent (Appendix III B.) was obtained from all children aged 12 to 17 years.

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CHAPTER FOUR

4 RESULTS

4.1 BASELINE CHARACTERISTICS OF STUDY PARTICIPANT

4.1.1 Baseline characteristics of study participants for hospital survey

Genomic DNA was extracted from 803 dried blood blots on filter paper collected from symptomatic *P. falciparum* infected children over a period of 4 years (from 2014 - 2017); 387 from Begoro and 416 from Cape Coast. There was no difference statistically in clinical and biological baseline characteristics of study participants between the two sites as intended by the study design. The mean age in years of study participants were 5.6 ± 3.6 for Begoro and 5.7 ± 3.6 for Cape Coast. Females accounted for about 49% of all participants in both Begoro and Cape Coast. The mean haemoglobin concentration was low in both sites (10.6 ± 1.8 and 10.8 ± 1.8 g/dl for Begoro and Cape Coast respectively). There was no statistical difference between the geometric mean parasite density in participants from Begoro (44,218/µl) and Cape Coast (45,572/µl). The baseline characteristics are shown in Table 4.1.

4.1.2 Baseline characteristics of study participants for school survey

In all, 5008 school children were screened over the five-year period of the school survey (2013 to 2017). Out of the number of children screened, 991 asymptomatic children were infected with *P. falciparum* and were enrolled into the study. The distribution of enrolled

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study participants by study site and year is shown in Table 4.2. The overall prevalence of malaria was higher in Begoro as expected (32% versus 22% in Cape Coast).

Females accounted for 50.9% of enrolled participants and the three age categories of interest were equally represented in both sites. The geometric mean parasitaemia for Begoro and Cape Coast was 282.3 and 298.9 respectively. Interestingly, the mean hemoglobin of school children in Begoro (12.3) was significantly higher than levels in Cape Coast (10.8) (P value <0.001).

Table 4.1 Baseline characteristics of symptomatic *Plasmodium falciparum* infected participants by study area

		Ecological Zone		
Characteristics	Total (N=803)	Forest N=387	Coastal N=416	P value
Mean Age in years (SD)	5.6 (3.6)	5.6 (3.6)	5.7 (3.6)	0.736
Age Group (years)	**N (%)**	**N (%)**	**N (%)**	
≤ 5	433 (53.9)	207 (53.5)	226 (54.3)	0.812
6 to 14	371 (46.1)	180 (46.5)	190 (45.7)	
Sex				
Female	394 (49.1)	190 (49.1)	204 (49.2)	0.986
Male	408 (50.9)	197 (50.9)	211 (50.8)	
Geometric Mean Density (/µL)	44908	44218	45572	0.668
Range	(461, 681800)	(1049, 681800)	(461, 608600)	
Mean Hemoglobin level in g/dl (SD)	10.7 (1.8)	10.6 (1.8)	10.8 (1.8)	0.187

Table 4.2: Baseline characteristics of asymptomatic *Plasmodium falciparum* infected participants by study area

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Characteristics	Total (N=994)	Ecological Zone		
		Forest (FZ) N=563	Coastal (CSZ) N=431	P value
Prevalence of malaria (%)				
2013	20.5	20.3	20.8	
2014	17.7	24.1	11.3	
2015	25.7	29.0	22.4	
2016	18.9	18.1	19.8	
2017	27.0	32.0	22.0	
Mean Age in years (SD)	10.4 (2.5)	10.3 (3.6)	10.34 (3.6)	0.642
Age Group (years)				
6 - 8	251 (25.3)	114 (25.6)	107 (24.8)	0.914
9 - 11	372 (37.4)	212 (37.7)	160 (37.1)	
12 14	371 (37.3)	207 (36.8)	164 (38.1)	
Sex				
Female	505 (50.9)	299 (53.2)	206 (47.8)	0.09
Male	488 (19.1)	263 (46.8)	225 (52.2)	
Geometric Mean Parasite Density (/µL)	289.4	282.3	298.9	0.6968
Parasite density Range	(13.79, 95400)	(13.79, 95400)	(15.59, 37680)	
Mean Hemoglobin level in g/dl (SD)	12.0 (1.3)	12.3 (1.2)	10.8 (1.8)	<0.001

SD = Standard Deviation

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4.2 GENETIC DIVERSITY OF *PFAMA1* GENE IN TWO ECOLOGICAL ZONES IN GHANA (HOSPITAL SURVEY)

The *pfama1* MIPS were sequenced from the extracted DNA of all 803 DBS collected from symptomatic children in both sites, however 581 samples (72.4%) gave useable sequences after filtering sequences with read counts over 50. Three hundred and thirty samples were found to either have mixed genotypes or did not have all the MIPS of the AMA1 gene and were excluded from further analysis of genetic diversity; hence, the full length of the *pfama1* gene was analyzed in 251 clonal isolates (link to haplotypes in Appendix II). After alignment with the 3D7 sequence, a total of 113 mutations were observed in both sites. The sequences were analyzed using the DnaSP software. A few complex codons could not be resolved by the software and were not analyzed; 523-534, 630-632, 1126-1128 for Begoro and 1246-1248 for Cape Coast.

4.2.1 Prevalence of *pfama1* haplotypes in two ecological zones in Ghana

Of these 113 mutations, 100 mutations occurred in Begoro and 103 mutations occurred in Cape Coast. Based on the nucleotide alignment with 3D7, 164 distinct haplotypes were identified with different frequencies as shown in Figure 4.1; 92 haplotypes from Begoro and 93 haplotypes from Cape Coast. Twenty-one haplotypes were shared in both sites. The sequences of the haplotypes are shown in appendix VI. None of these haplotypes dominated, with haplotype prevalence less than 7% in both sites. Haplotype frequency for Begoro ranged from 1-5% and 1-6% for Cape Coast. The most frequent haplotype was AMA1HAP_7 in both sites with prevalence of about 6%.

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Table 4.3 Indices of genetic diversity of *pfama1* gene and evidence of selection in two ecological zones in Ghana

Diversity Estimates	**Symbol**	**Study Site**			**Year of sample collection**			
		Begoro	Cape Coast	Both sites	2014	2015	2016	2017
Number of samples	n	121	130	251	61	83	42	64
Number of polymorphic sites	S	89	89	99	80	89	74	82
Nucleotide diversity;	π	0.01471	0.01452	0.01464	0.015	0.01431	0.0142	0.0148
Number of non-synonymous single nucleotide polymorphisms (SNPs)	NS	70	66		59	69	53	64
Number of synonymous SNPs	SP	19	24		18	18	16	17
Number of haplotypes	h	92	93	164	55	59	33	56
Haplotype diversity	Hd	0.994	0.991	0.993	0.996	0.986	0.986	0.995
Recombination parameter	R	163	155	162	176	104	183	207
Neutrality tests								
Tajima's D value	D (Ti)	1.37568	1.27842	1.30563	1.14117	0.96897	1.07539	1.13423
Fu and Li's D* value	D* (F&L)	0.63553	1.05831	0.5494	1.10648	0.72308	1.22131	0.69426
Fu and Li's F*value	F*(Fu&Li's)	1.14982	1.38633	1.09255	1.43567	0.98871	1.39271	1.03784

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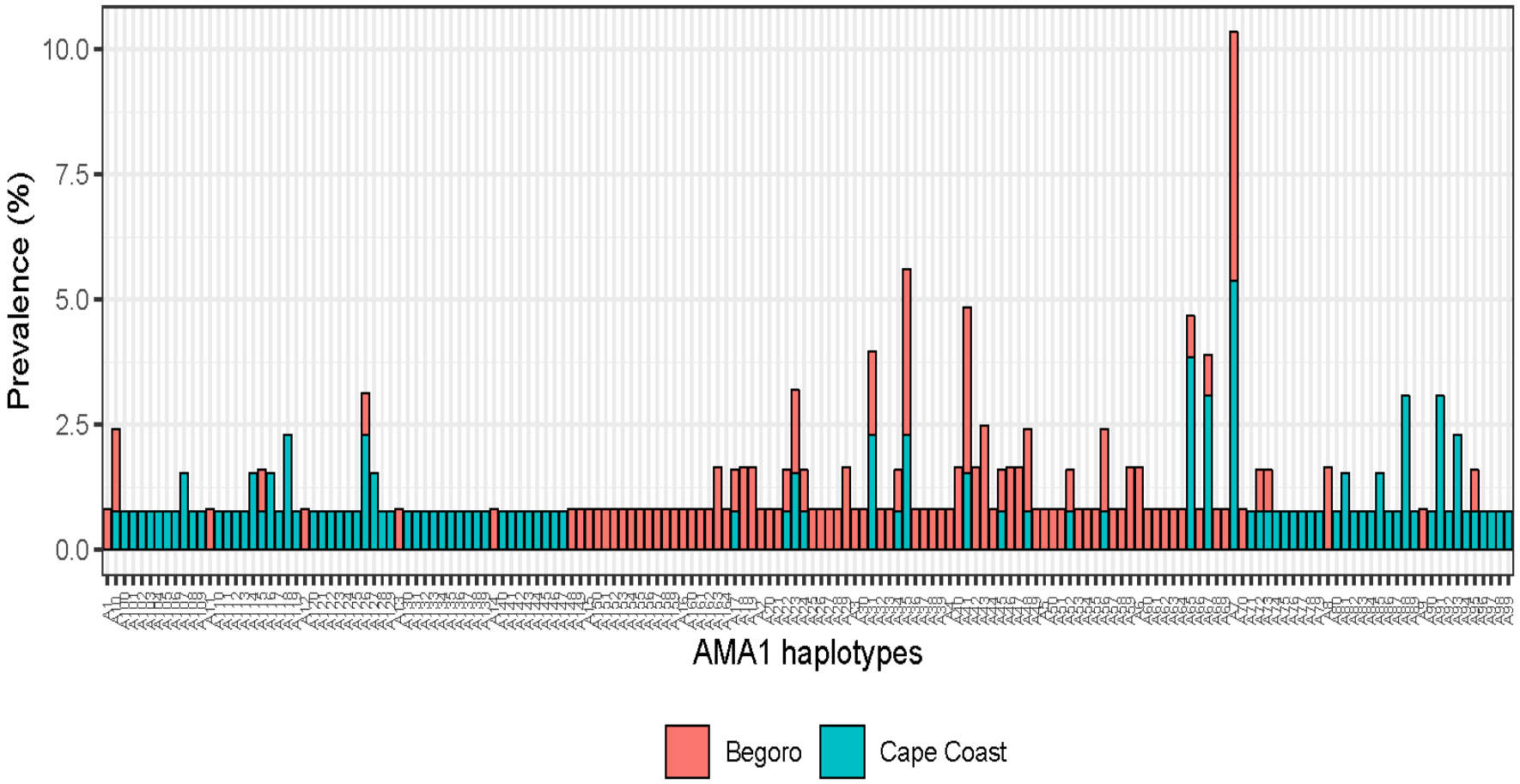

Figure 4.1 Haplotype frequency of the *pfama1* gene comparing haplotypes from the two sites

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4.2.2 Temporal assessment of genetic diversity and evidence of sselection in the *pfama1* gene in two ecological zones in Ghana.

To determine the genetic diversity of both study sites temporally, the haplotype diversity (Hd), number of haplotypes (H), number of segregating sites (S), nucleotide diversity (π), average number of pairwise nucleotide differences within the population (K) was estimated. The total number of polymorphic sites (S) within the sequence region of the *pfama1* was similar for both sites and over time (Table 4.3). It ranged from 74 to 96 polymorphisms over the four-year study period. However, the number of non-synonymous single nucleotide polymorphism, NS SNPs for *pfama1* was slightly higher in Begoro than in Cape coast (70 for Begoro and 66 for Cape Coast). Haplotype diversity was very high for both study sites, with *pfama1* haplotype diversity close to the maximum of 1.0 (the maximum) for both sites; indicating that all haplotypes are rare (Table 4.3). The haplotype diversity was high for the four-year period of the study and did not differ temporally (Hd ranged from 0.96 to 0.985). Similarly, the nucleotide diversity (π) was slightly higher in Begoro than in Cape coast (π = 0.0147 for Begoro and 0.0145 for Cape Coast, Table 4.3). The overall nucleotide diversity (π) in the study sequences was high (0.01464). The highest nucleotide diversity was observed in 2014 and 2017 (0.015 for both years) and ranged from 0.014 to 0.015. The mean number of pairwise nucleotide differences (K) in all sequences was 26.431. There was a slight decline in both nucleotide diversity and haplotype diversity from 2014 to 2016 but increased again in 2017.

To determine whether natural selection contributes to the diversity in the *pfama1* gene in both sites, the Tajima's D as well as Fu and Li's F* and D* statistics was evaluated and the results showed positive in the *pfama1* gene. Tajima's D for Begoro was 1.376 and 1.278

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for cape Coast. D ranged from 0.969 to 1.141 for the four-year period and no trend was observed. There was no fixed difference between species in both sites when McDonald and Kreitman test was performed (Table 4.4).

The amount of recombination and LD were explored to see how much genetic exchange occurred in the *pfama1* locus between the isolates. The recombination parameter, R, was higher in Cape Coast compared to Begoro (Table 4.3). The Linkage disequilibrium analysis showed a decline in LD index (R^2) with an increasing distance between the pairs of nucleotide sites across the entire gene (Figure 4.2).

Table 4.4 McDonald and Kreitman analysis of *pfama1*

	Study Site	
McDonald and Kreitman test	Begoro	Cape Coast
Polymorphic changes within P.falciparum		
Synonymous (Ps)	18	24
Non-synonymous (Pn)	69	66
Fixed differences between species		
Synonymous (Ds)	0	0
Nonsynonymous (Dn)	0	0
Neutrality index	-	-
Fisher's exact test	-	-

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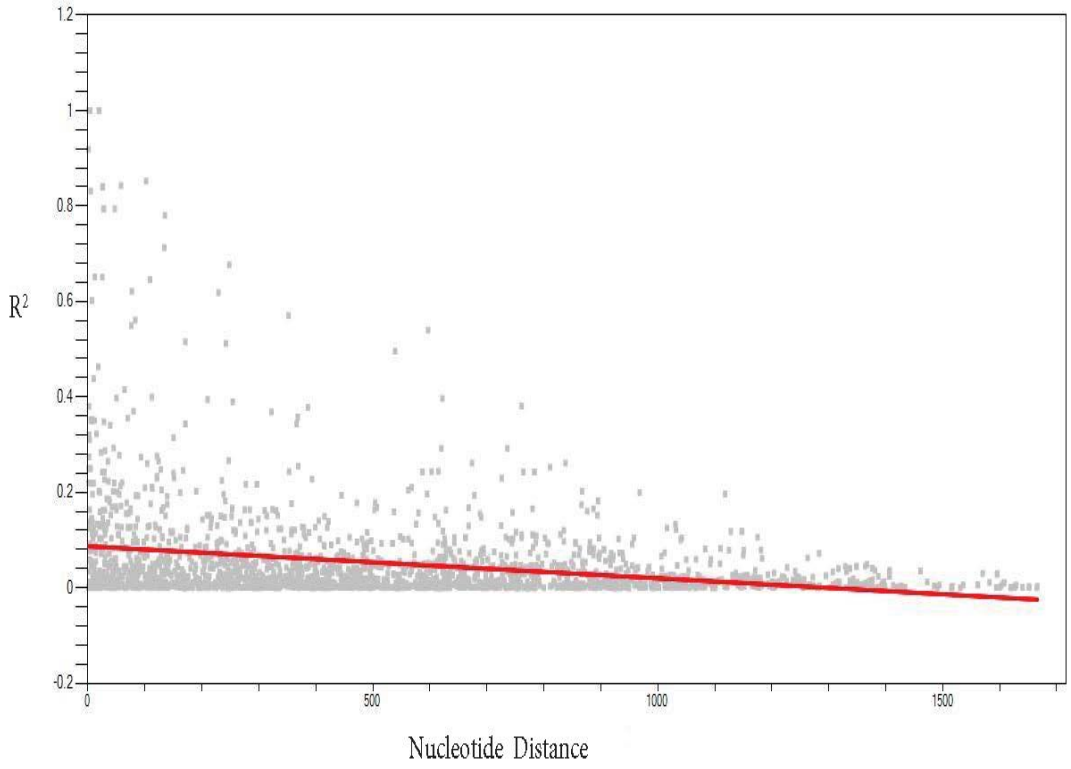

Figure 4.2 Linkage disequilibrium (LD) index ($R^2$by nucleotide distance in the *pfama1* gene from the two ecological zones.

The figure shows a decline in R^2 values as nucleotide distance, showing a high rate of recombination events.

4.2.3 Population differentiations of *pfama1* gene among endemic areas

4.2.3.1 Population genetic analysis in *pfama1* gene

Overall, the 251 Ghanaian *pfama1* sequences were compared with 48 Nigerian sequences, 129 Kenyan sequences and 30 Thailand sequences. The number of segregating sites in the African sequences (ranged from 62 to 77) was higher compared to the Thailand sequences (48). Haplotype diversity was higher in the West African sequences (0.991 for Ghana and 0.994 for Nigeria) than the diversity for East Africa (0.985 for Kenya) and the South East

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Asian sequences (0.871 for Thailand). The nucleotide diversity showed the same pattern with π higher in Ghana than Kenya and Thailand. All the indices of genetic diversity showed a consistent higher diversity in the African populations compared to the South East Asia population of isolates. The results described are shown in Table 4.5.

Table 4.5 Genetic diversity of *pfama1* sequences from Ghana with other endemic countries

Genetic Diversity parameters	**Ghana**	**Nigeria**	**Kenya**	**Thailand**
Number of sequences	251	48	129	30
Number of segregating sites, S	77	62	72	48
Number of haplotypes, h:	138	42	78	9
Haplotype diversity, Hd	0.99073	0.99373	0.98486	0.87126
Average number of nucleotide differences, K	21.9979	21.47872	21.70736	17.86667
Nucleotide diversity, π	0.01682	0.01642	0.0166	0.01366
Nucleotide diversity with JC, PiJC	0.01703	0.01662	0.0168	0.01381

4.2.3.2 Population differentiation between the two Ghanaian ecological zones

The Wright's Fixation index, Fst between different populations was estimated to determine the genetic differentiation between the Ghanaian populations in figure 4.3. The Fst results shows that 99.7% of the variation found in the *pfama1* was within sequences of the same population and not between populations. The 0.4% differentiation observed between the two ecological zones was not statistically different from zero (P value =0.082). This study also compared populations of Ghanaian *pfama1* gene over time and the results showed lower pairwise Fst values (<2%) even though some of the Fst values were significantly

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different from zero (P value<0.05, Table 4.6). But interestingly population differentiation over time was higher than the differentiation between the two ecological zones. This study did not show differentiation of the *pfama1* sequences between the years.

4.2.3.3 Population differentiation among endemic countries

The Wright's Fixation index, *Fst* between different populations was estimated to determine the genetic differentiation between the Ghanaian isolates and other isolates published for West African, East African and South East Asia populations shown in Table 4.5. The Ghanaian *P. falciparum* population was significantly differentiated from both the Kenyan and Thai sequences ($P < 0.0001$) but not with Nigerian isolates ($P > 0.05$) (Table 4.5). The Fst value of the *pfama1* gene comparing Ghanaian isolates and the Nigerian isolates was slightly negative (Table 4.6), suggesting that inter-population differentiation observed was not statistically significant. The West African Isolates showed low inter-population differentiation with East African and South East Asian isolates. The Fst value for comparing the Ghanaian *pfama1* isolates with Kenya and Thailand were positive and statistically significant. The highest positive Fst value was observed between Nigeria and the Thailand isolates (7.6%). The pairwise Fst value for differentiating the Ghanaian sequences from the Thai sequences was 5.2%.

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Table 4.6 Genetic Differentiation *pfama1* isolates between endemic countries

Fst	Population difference by region				Fst	Population difference by year (Ghana)			
	Ghana	**Nigeria**	**Kenya**	**Thailand**		**2014**	**2015**	**2016**	**2017**
Ghana	-	-	-	-	2014	-	-	-	-
Nigeria	0.0007	-	-	-	2015	0.0112*	-	-	-
Kenya	0.0035***	0.0094** *	-	-	2016	0.0042	0.0176*	-	-
Thailand	0.0517***	0.0766***	0.0537** *	-	2017	0.0039	0.0109*	0.0087	-

*** show Fst values with P < 0.0001 and * shows 0.1 < p value <0.05

4.2.4 Population structure analysis of *pfama1*

To determine the distribution of genetic variation of the *pfama1* across different geographical settings with different endemicity, discriminate analysis of principal components (DAPC) was performed to identify the structure of the distribution. Previously published *pfama1* gene sequences from Nigeria, Kenya, and Thailand used to determine whether the Ghanaian isolates were related to West African, East African and South East Asia isolates (ref). The DAPC clustering showed that the first discriminant component separated the Kenyan population from the other regions and the second showed a separation of the Thailand isolates from the African isolates (Ghana, Nigeria and Kenya). The Kenyan *pfama1* sequences also showed a moderate population differentiation with the West African sequences (Ghana and Nigeria) and with the Thai sequences. The Ghanaian and Nigerian isolates clustered together as shown in Figure 4.3, although there were a few outliers.

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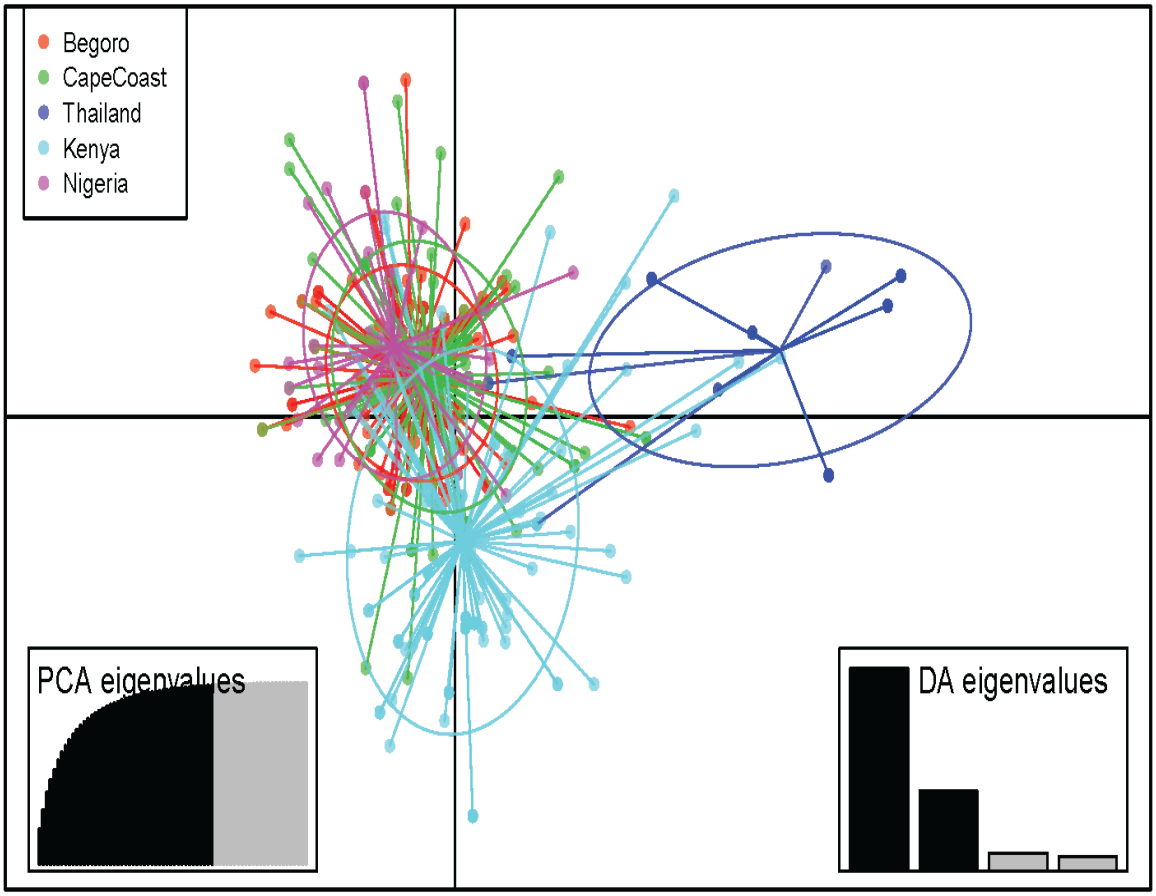

Figure 4.3 Scatterplot from discriminant analysis of principal components of the first two components discriminating *pfama1* gene population by regions.

Points represents individual observations. Lines and shapes represent the different populations. Inertia ellipses represents an analog of 65% confidence interval based on a bivariate normal distribution.

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4.3 COMPLEXITY OF INFECTION OF *PFAMA1* AND ASSOCIATION WITH STUDY SITE, TIME, AGE, HEMOGLOBIN LEVEL AND PARASITE DENSITY.

The mean COI by year and study site for *P. falciparum* isolates collected from symptomatic children as well as the proportion of multiple infections are shown in Table 4.7. The physiological and clinical characteristics of both clonal and mixed infections are shown in Table 4.8. The number of AMA1 genotypes found in *P. falciparum* isolates from the Ghanaian children ranged from 1 to 5 (for both Begoro and Cape coast). The mean COI was 1.27 for Begoro and 1.32 for Cape coast. There was no significant difference between the mean COI between the two sites over the 4-year study period (Table 4.7). The proportion of multiple infections found in the Begoro isolates varied from 20% to 24 % over the study period while the variation was 18 to 36% for Cape coast. However, there was no statistical difference in the proportion of multiple infection between the two study sites (Table 4.7). Multiple infection was higher in children aged between 6 months and 5 years (23.4%) compared to children aged 6 years and above (18.5%), though not statistically significant (P-value = 0.2341, Table 4.8). There was no significant association (P-value = 0.3395) between the hemoglobin level and complexity of infection even though the multiclonal infections had a lower mean hemoglobin level compared to clonal infections (10.8 g/dl compared to 11.0 respectively). Similarly, the study did not show statistically that parasite density had an influence on the complexity of infection (Table 4.8) though, multiclonal infections had a higher parasitaemia compared to clonal infections.

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Table 4.7 Mean COI and proportion of multiple infections of *P. falciparum* genotypes in two ecological zones in Ghana

	Study Site			
Mean COI ± SD (Range)	**All study sites**	**Begoro**	**Cape Coast**	**P-Value**
2014	1.26 ± 0.52 (1-3)	1.24 ± 0.49 (1-3)	1.29 ±0.56 (1-3)	0.681
2015	1.25± 0.51 (1-4)	1.23 ± 0.46 (1-3)	1.26 ± 0.56 (1-4)	0.994
2016	1.27 ± 0.65 (1-5)	1.28 ± 0.59 (1-3)	1.27 ± 0.71 (1-5)	0.719
2017	1.44 ± 0.78 (1-5)	1.37 ± 0.85 (1-5)	1.48 ± 0.73 (1-4)	0.151
Total	1.30 ± 0.61 (1-5)	1.27 ± 0.58 (1-5)	1.32 ± 0.63 (1-5)	0.405
Proportion of mixed infection (n/Ni)				
2014	22 (34/153)	21 (16/76)	23 (18/77)	0.846
2015	22 (41/187)	22 (18/81)	21 (23/106)	1.000
2016	19 (24/124)	20 (12/58)	18 (12/66)	0.821
2017	31 (36/117)	24 (12/51)	36 (24/66)	0.1603
Total	0.23 (135/581)	22 (58/266)	24 (77/315)	0.491

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Table 4.8 Associations of baseline characteristics with complexity of infection

	Complexity of infection		
	Clonal infection	Multiple infection	P-value
Sex			
Female	77.2 (176/228)	22.8 (52/228)	0.4696
Male	80.3 (200/249)	19.7 (49/249)	
Age of children			
Aged 6month - 5years	76.6 (196/256)	23.4 (60/256)	0.2341
Aged 6years to 14 years	81.5 (200/216)	18.5 (49/216)	
Mean parasitaemia (SD)	53624	65249	0.1054
Mean hemoglobin levels (SD)	11.0	10.8 (12/66)	0.3395

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4.4 RESISTANCE MARKERS ASSOCIATED WITH ANTIMALARIAL DRUGS

4.4.1 Hospital survey

4.4.1.1 Prevalence of *Plasmodium falciparum* drug resistance markers

This study used MIPs designed to target known and putative drug resistance mutations to sequence the samples that were microscopically confirmed to have *P. falciparum* mono-infections. After sequencing, 767 samples gave useable sequences out of the 803 samples sequenced and were analysed for the prevalence of known drug resistance mutations in *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps* and putative markers in the *pfk13* gene.

Markers associated with Chloroquine Resistance

The prevalence of individual gene mutations targeted is listed in Figure 4.4. Sequencing of the *pfcrt* showed a steady decline in chloroquine resistance mutant allele *pfcrt*76T and thence the haplotype C**IET** in both sites over the study period. In Begoro, virtually all the parasite isolates had reverted to the wild-type by 2017 (94% of wild-type *pfcrt* 76K and CMNK in 2017) whilst 27% of the Cape-Coast parasite isolates carried chloroquine resistant mutations in 2017. Other mutations such as the *pfcrt* 220S also declined in the two sites, with the forest exhibiting a stronger effect than the coastal savannah zone. The *pfmdr1* 86Y on the other hand remained at low prevalence in the two sites, without any ecological effect. Interestingly, *pfmdr1* 184F prevalence remained high in both sites. The *pfmdr1* double mutant haplotype 86**Y184F,** which is associated with chloroquine treatment failure was maintained at low prevalence, ranging from 10% to 14% and 4% to 18% in Begoro and Cape Coast respectively during the study period (Table 4.12).

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Overall there was a temporal reduction in the prevalence of *pfcrt76T* (χ^2=16, p < 0.001) and *pfmdr186Y* (χ^2=6.6, p=0.010) between 2014 and 2017, although there was a rise in the prevalence of *pfcrt 76T* in Begoro in 2016 and for *pfmdr1 86Y* in 2017 at both sites (figure 4.5). Albeit, *pfmdr1*184F prevalence remained high in both sites, there was no trend observed during the study period. The results showed no statistically significant trend in the other SNPs associated with *P. falciparum* drug resistance over the four-year period of the study (Table 4.11).

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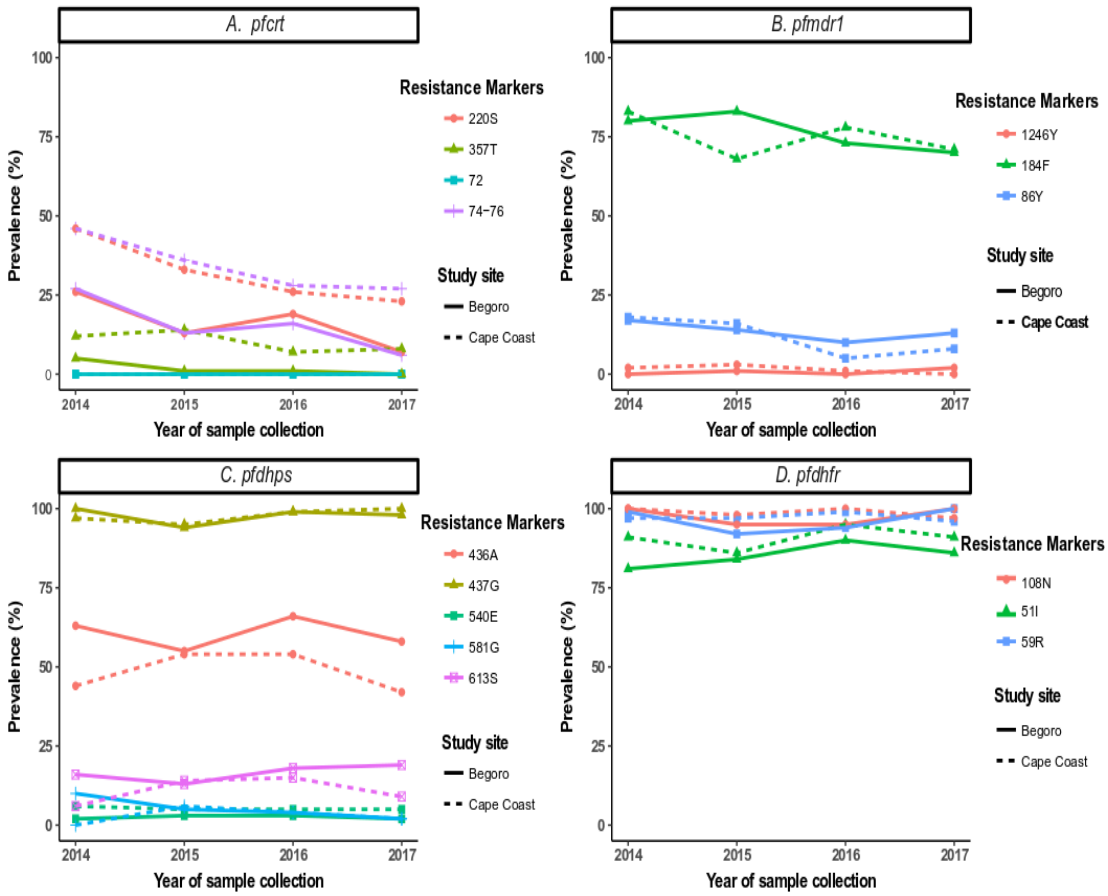

Figure 4.4: Prevalence of mutations linked to *P. falciparum* antimalarial drug resistance in two ecological zones in Ghana over time**.**

Prevalence of mutations associated with resistance in *pfcrt* are in **(A),** *pfmdr1* in **(B)** *pfdhps* in **(C)** and *pfdhfr* in **(D).** Prevalence (Prev) was estimated by dividing the total number of samples with at least one mutant allele by the total number of samples genotyped each year.

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Markers associated with sulfadoxine and pyrimethamine Resistance

Figure 4.4 describes the prevalence of resistance markers with sulfadoxine and pyrimethamine resistance in the two study sites in Ghana. Three (3) of the mutant alleles associated with pyrimethamine resistance (51I, 59R, and 108N) were observed in the study sites, and these were almost at fixation, whilst 164L mutation was absent. Similarly, the sulfadoxine resistance associated *pfdhps* mutation 437G was almost at fixation. However, 540E, which is also a key mutant associated with sulfadoxine resistance remained low (prevalence ranged from 2% to 6%). This study also showed the presence of other *pfdhps* resistance markers, namely S436A, and the highly resistant mutations 581G, and 613S at frequencies that may influence the efficacy of sulfadoxine-pyrimethamine. The prevalence of *pfdhps* mutant 436A ranged from 55% to 66% in Cape-Coast and 42% to 54% in Begoro. The prevalence of 581G and 613S were less prevalent at less than 11% and 20% for Cape-Coast and Begoro respectively. This study also showed the presence of triple mutant *pfdhps* alleles; AGKAS (at 8-13%) in 2017 and quadruple mutant alleles; AGKGS (2%) in 2017 (Table 4.12). The prevalence of the quadruple mutation (**IRNG**K), associated with sulfadoxine-pyrimethamine- ranged between 77% - 86% in the forest zone and 75% - 89% in the coastal town. The prevalence of the quintuple mutation **IRNGE,** which is common in East Africa was very low possibly due to the low prevalence of 540E observed in our study (Table 4.12).

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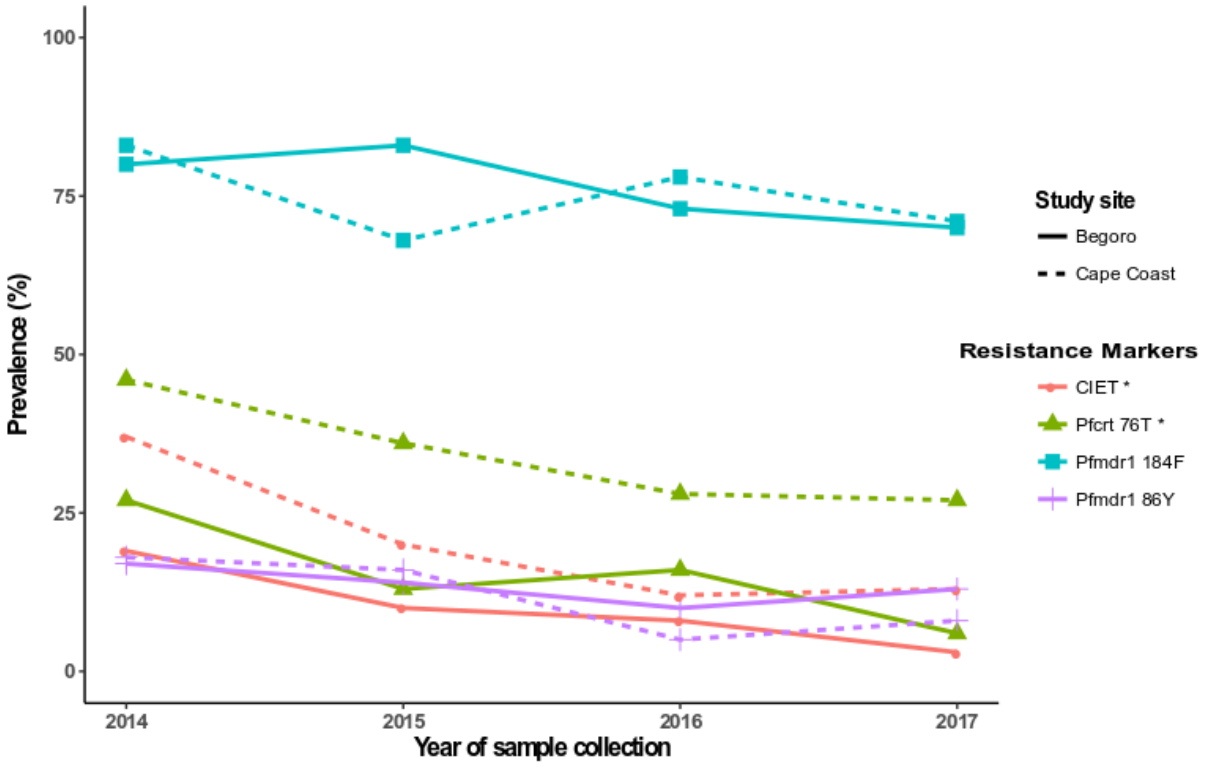

Figure 4.5: Temporal Trends in prevalence of CQ-Associated mutations in two ecological zones in Ghana (*pfcrt* and *pfmdr1*).

Asterisk indicates significant changes over time at both sites ($P < 0.05$ by Cochrane-Armitage test).

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Markers associated with Artemisinin and partner drug resistance

Figure 4.4 and Table 4.12 shows the prevalence of mutations associated with Artemisinin and some partner drugs. A few SNPs were observed in the *pfk13* gene but importantly, none of the Artemisinin associated mutations observed in South East Asia, (M476I, Y493H, R539T, I543T, C580Y) were observed in the study. In addition to the observed reduction in *pfcrt* C**IET** and *pfmdr1* 86Y prevalence over the four-year period, there was also a reduction in *pfmdr1*1246Y polymorphisms (from 3% to 0%, p value =0.5613), albeit not significant. However, prevalence of *pfmdr1*184F (Prevalence > 60%) remained high. When *pfmdr1* codons, N86Y, Y184F and D1246Y were analysed together, the prevalence of the triple mutant haplotype **YYY** was very low (at less than 1% in both sites) as shown in appendix IV Table T3. By convention individuals bearing the N**F**D haplotype were in high frequency in both study sites (65.8% and 62.7% for Begoro and Cape-Coast respectively) whilst about 12% carried the double mutant haplotype **YF**D at both study sites. There was no ecological zone effect on markers associated with amodiaquine and lumefantrine.

Comparison of drug resistance mutations with previous studies in Ghana

Table 4.9 shows a comparison of previous studies on *pfmdr1* and *pfcrt* mutations in Ghana and Table 4.10 for *pfdhps* and *pfdhfr*. Duah and her colleagues (2013) genotyped *pfcrt*, *pfmdr1*, *phdhfr* and *pfdhps* for prevalence of chloroquine resistant parasite isolates in samples collected from the same geographical areas from 2003 to 2010. In the forest zone, *pfcrt* 76T prevalence reduced from 92% in 2005 to 57% in 2010 (6 years after policy change in 2004) and has continued to reduce from 27% in 2014 to 6% in 2017 described in this study. The rate of re-expansion of *pfcrt* K76 was however slower in the coastal town

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compared with the forest region (z=-3.5, p<0.001). Prevalence of *pfcrt* 76T was as high as 95% after 4 years (2008) of policy change (Duah et al., 2012) and was 59.5% eight years after policy change (Afoakwah et al., 2014). This study shows a prevalence of 27% after 13 years since policy change in Cape-Coast. *pfmdr1* mutants, 86Y and 1246Y maintained a steady decline in prevalence over the first eight years (Duah et al., 2012) and the last four years of change in drug policy. But interestingly, *pfmdr1* 184F has remained high over the years in both sites. The *pfdhfr* triple mutation (**IRN**) as well as the *pfdhps* 437G mutation have been almost at fixation during both study times (Table 4.6). However, the quintuple haplotype **IRNGE** has remained less than 20% all these thirteen years of follow-up.

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Table 4.9: Comparison of *P. falciparum* drug resistance alleles prevalence (*pfcrt* 76T and *pfmdr1*alleles) between a previous study conducted at the current study sites and current study.

Site/year	*pfcrt*, 76T	*pfmdr1*, 86Y	*pfmdr1*, 184F	*pfmdr1*, 1034C	*pfmdr1* 1042D	*pfmdr1*, 1246Y	Reference
Begoro							
2005-06	92	67	82	0	36	13	**Duah *et al*, 2013**
	(36/39)	(26/39)	(32/39)	(0/39)	(14/39)	(5/39)	
2007-08	78	60	70	0	0	6	
	(39/50)	(30/50)	(35/50)	(0/50)	(0/50)	(3/50)	
2010	57	27	80	0	73	7	
	(8/14)	(4/15)	(12/15)	(0/15)	(11/15)	(1/15)	
2014	27	17	80	0	0	0	**Current Study**
	(26/95)	(15/90)	(72/90)	(0/89)	(0/89)	(0/85)	
2015	13	14	83	0	0	1	
	(13/98)	(12/87)	(73/87)	(0/90)	(0/90)	(1/85)	
2016	16	10	73	0	0	0	
	(15/91)	(8/79)	(58/80)	(0/79)	(0/79)	(0/74)	
2017	6	13	70	0	0	2	
	(4/63)	(7/55)	(39/56)	(0/53)	(0/53)	(1/54)	
Cape-Coast							
2005-06	95	59	46	0	15	8	**Duah *et al*, 2013**
	(37/39)	(23/39)	(18/39)	(0/39)	(6/39)	(3/39)	
2007-08	95	49	58	0	0	7	
	(40/42)	(21/43)	(25/43)	(0/43)	(0/43)	(3/43)	
2014	46	18	83	0	0	2	**Current Study**
	(42/92)	(16/88)	(73/88)	(0/90)	(0/90)	(2/88)	
2015	36	16	68	0	0	3	
	(48/132)	(17/108)	(73/108)	(0/120)	(0/120)	(3/107)	
2016	28	5	78	0	0	1	
	(27/95)	(4/83)	(66/85)	(0/80)	(0/80)	(1/82)	
2017	27	8	71	0	0	0	
	(19/71)	(5/76)	(48/68)	(0/64)	(0/64)	(0/64)	

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Table 4.10: Comparison of *P. falciparum* drug resistance alleles prevalence (*pfdhps* and *pfdhfr* alleles) between a previous study conducted at the current study sites and current study.

Site	Year	I51	R59	N108	IN	RN	IRN	G437	E540	GE	Reference
Begoro	2005-2006	45	90	83	5	43	33	88	3	3	Duah *et al*., 2012
	2007-2008	56	86	88	4	30	52	92	0	0	
	2010	87	100	93	0	13	80	100	0	0	
Begoro	2014	81	99	100	1	19	80	100	2	2	This Study
	2015	84	92	95	2	11	82	94	3	3	
	2016	90	94	95	1	5	89	100	3	3	
	2017	86	100	100	0	14	86	98	2	2	
Cape Coast	2005-2006	55	75	83	8	23	43	73	0	0	Duah *et al*., 2012
	2007-2008	66	88	88	6	20	60	100	0	0	
	2010	60	85	80	0	20	60	80	5	5	
Cape Coast	2014	91	97	100	3	9	88	97	6	6	This Study
	2015	86	97	98	2	13	84	95	5	5	
	2016	94	99	100	1	5	94	99	5	5	
	2017	90	96	97	1	6	90	100	5	5	

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Table 4.11: Temporal trends in Drug Resistance SNPs prevalence

Gene (chr)	Resistant Markers	Ref. Allele	Alt. Allele	Begoro Prev ,% (n/N)					Cape Coast Prev ,% (n/N)					Over all P-value
				2014	2015	2016	2017	χ^2, P-value	2014	2015	2016	2017	χ^2, P-value	χ^2, P Value
pfcrt				27	13	16		9.92,	46	36	28	27	8.00 ,	16.03,
(chr7)	CRT_M74I	G	T	(26/95)	(13/98)	(15/91)	6 (4/63)	0.0016	(42/92)	(48/132)	(27/95)	(19/71)	0.0047	0.0001
				27	13	16		9.92,	46	36	28	27	8.00 ,	16.03,
	CRT_N75E	A	G	(26/95)	(13/98)	(15/91)	6 (4/63)	0.0016	(42/92)	(48/132)	(27/95)	(19/71)	0.0047	0.0001
				27	13	16		9.92,	46	36	28	27	8.00 ,	16.03,
	CRT_K76T	A	C	(26/95)	(13/98)	(15/91)	6 (4/63)	0.0016	(42/92)	(48/132)	(27/95)	(19/71)	0.0047	0.0001
				26	13	19		6.11,	46	33	26	23	10.28,	14.17,
	CRT_A220S	G	T	(24/93)	(10/77)	(15/80)	7 (4/55)	0.0134	(39/85)	(29/87)	(23/87)	(16/69)	0.0013	0.0002
					1	1		5.21,	12	14			1.54,	
	CRT_I356T	T	C	5 (5/94)	(1/95)	(1/90)	0 (0/59)	0.0224	(11/90)	(17/125)	7 (7/95)	8 (6/71)	0.2146	3.64, 0.0565
pmdr1				17	14	10	13	0.97,	18	16			7.05,	
(chr5)	N86Y	A	T	(15/90)	(12/87)	(8/79)	(7/55)	0.3250	(16/88)	(17/108)	5 (4/83)	8 (5/76)	0.0079	6.66, 0.0099
				80	83	73	70	3.30,	83	68	78	71	1.35,	
	Y184F	A	T	(72/90)	(72/87)	(58/80)	(39/56)	0.0.0694	(73/88)	(73/108)	(66/85)	(48/68)	0.2447	4.43, 0.0353
					1	0		0.79,					1.49,	
	D1246Y	G	T	0 (0/85)	(1/85)	(0/74)	2 (1/54)	0.3734	2 (2/88)	3 (3/107)	1 (1/82)	0 (0/64)	0.2216	0.34, 0.5613

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Gene (chr)	Resistant Markers	Ref. Allele	Alt. Allele	Begoro Prev ,% (n/N)					Cape Coast Prev ,% (n/N)					Over all P-value
				2014	2015	2016	2017	χ^2, P-value	2014	2015	2016	2017	χ^2, P-value	χ^2, P Value
pf dhps				63	55	66	58		44	54	54	42		0.00,
(chr8)	S436 A	T	G	(55/88)	(50/91)	(55/83)	(33/57)		(39/89)	(64/119)	(45/84)	(27/64)		0.9717
				100	94	99	98		97	95 (113/119	99	100		
	A437G	G	C	(88/88)	(86/91)	(82/83)	(56/57)		(86/89)	)	(83/84)	(64/64)		1.27, 0.2589
					3	3								
	A581G	C	G	2 (2/82)	(2/80)	(2/73)	2 (1/53)		6 (5/85)	5 (5/102)	5 (4/81)	5 (3/64)		0.09, 0.7625
				10	5	4								
	A613 S	G	T	(8/82)	(4/80)	(3/73)	2 (1/53)		0 (0/85)	6 (6/102)	4 (3/81)	2 (1/64)		1.85, 0.1740
				16	13	18	19			14	15			
				(13/82)	(10/80)	(13/73)	(10/53)		6 (5/85)	(14/102)	(12/81)	9 (6/64)		1.07, 0.2999
										86				
pf dhfr				81	84	90	86		91	(103/120	95	91		2.85,
(chr4)	N51I	A	T	(76/94)	(77/92)	(75/83)	(52/59)		(82/90)	)	(90/95)	(64/70)		0.0911
										97				
				99	92	94	100		97	(116/120	99	96		0.01,
	C59R	T	C	(93/94)	(85/92)	(78/83)	(59/59)		(87/90)	)	(94/95)	(67/70)		0.9059
										98				
				100	95	95	100		100	(118/120	100	97		
	S108 N	G	A	(94/94)	(87/92)	(79/83)	(59/59)		(90/90)	)	(95/95)	(68/70)		0.78, 0.3774

- Highlighted P. values are statistically significant trends. (P value <0.05)

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Table 4.12: Prevalence of haplotypes associated with *P. falciparum* anti-malarial drug resistance in two ecological zones in Ghana

				2014 Prev (n/N), %			2015 Prev (n/N), %			2016 Prev (n/N), %			2017 Prev (n/N), %		
Drugs	Gene (chr) codons	Genotype	Base Call	Begoro	Cape Coast	P value	Begoro	Cape Coast	P value	Begoro	Cape Coast	P value	Begoro	Cape Coast	P value
Chloroquine, Amodiaquine	***pfcrt* (chr7) codons: 72/74/75/76**														
	Wild type	C-M-N-K	TATA	81 (69/85)	63 (50/80)	0.0124	90 (85/94)	80 (84/105)	0.0638	92 (76/83)	88 (68/77)	0.6731	97 (71/73)	87 (60/69)	0.0278
	Triple Mutation	C-I-E-T	TGAC	19 (16/85)	37 (30/80)		10 (9/94)	20 (21/105)		8 (7/83)	12 (9/77)		3 (2/73)	13 (9/69)	
Chloroquine	**pmdr1 (chr5) codons: 86/184**														
	Wild type	NY	AA	16 (23/69)	22 (15/68)	0.4667	22 (15/68)	41 (33/81)	0.0033	35 (21/60)	26 (18/68)	0.0909	32 (18/55)	40 (23/57)	0.7414
	Single Mutation	Y-Y	TA	1 (1/69)	0 (0/68)		0 (0/68)	1 (1/81)		0 (0/60)	1 (1/68)		0 (0/55)	0 (0/57)	
		N-F	AT	67 (46/69)	61 (42/68)		66 (45/68)	57 (46/81)		60 (36/60)	72 (49/68)		62 (34/55)	54 (31/57)	
	Double Mutations	Y-F	TT	7 (6/69)	16 (11/68)		12 (8/68)	1 (168/81)		5 (3/60)	0(0/68)		5 (3/55)	5 (3/57)	
Lumifantrine, Amodiaquine	**pmdr1 (chr5) codons: 86/184/1246**														
	Wild type	N-Y-D	AAG	26 (16/62)	22 (15/68)	0.5211	22 (14/65)	41 (31/76)	0.014	35 (19/54)	25 (16/65)	0.1171	29 (14/49)	42 (22/53)	0.411
	Single Mutation	Y-Y-D	TAG	2 (1/62)	0 (0/68)		0 (0/65)	1 (1/76)		0 (0/54)	0 (0/65)		0 (0/49)	0 (0/53)	
		N-F-D	ATG	63 (39/62)	62 (42/68)		68 (44/65)	55 (42/76)		61 (33/54)	74 (48/65)		63 (31/49)	53 (28/53)	
	Double Mutations	Y-F-D	TTG	10 (6/62)	16 (11/68)		9 (6/65)	1 (1/76)		4 (2/54)	0(0/65)		6 (3/49)	6 (3/53)	
		N-F-Y	ATT	0 (0/62)	0 (0/68)		2 (1/65)	1 (1/76)		0 (0/54)	0 (0/65)		2 (1/49)	0 (0/53)	
		Y-Y-Y	TAT	0 (0/62)	0 (0/68)		0 (0/65)	1 (1/76)		0(0/54)	1 (1/65)		0 (0/49)	0 (0/53)	

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Pyrimethamine	**pf dhfr (chr4) codons: 51/59/108**														
	Wild type	N-C-S	ATG	0 (0/85)	0 (0/76)	0.1318	6 (5/82)	2 (2/101)	0.4714	6 (4/70))	0 (0/87)	0.1267	0 (0/56)	4 (3/74)	0.1551
	Double Mutations	N-R-N	ACA	21(18/85)	11 (8/76)		11 (9/82)	15 (15/101)		6 (4/70))	6 (5/87)		14 (8/56)	8 (6/74)	
		I-C-N	TTA	1 (1/85)	4 (3/76)		2 (2/82)	2 (2/101)		1 (1/70))	1 (1/87)		0 (0/56)	4 (3/974)	
	Triple Mutations	I-R-N	TCA	78 (66/85)	86 (65/76)		80 (66/82)	81 (82/101)		87 (61/70)	93 (81/87)		86 (48/56)	84 (62/74)	
	pf dhps (chr8) codons: 437/540														
sulfadoxine	Wild type	A-K	CA	0 (0/80)	4 (3/80)	0.2417	5 (4/74)	5 (5/97)	1	0 (0/71)	1 (1/76)	0.4972	2 (1/56)	0 (0/67)	0.7053
	Single Mutation	G-K	GA	99 (79/80)	94 (75/80)		94 (70/74)	93 (90/97)		100 (71/71)	96 (73/76)		98 (50/56)	99 (66/67)	
		A-E	CG	0 (0/80)	0 (0/80)		0 (0/74)	0 (0/97)		0 (0/71)	0 (0/76)		0 (0/56)	0 (0/67)	
		G-E	GG	1 (1/80)	2 (2/80)		1 (1/74)	2 (2/97)		0 (071)	3 (2/76)		0 (0/56)	1 (1/67)	
	pf dhps (chr8) codons: 436/437/540/581/613														
sulfadoxine	Wild type	S-A-K-A-A	GCACG	0 (0/65)	0 (0/72)	0.01679	0 (0/63)	4 (3/77)	0.4292	0 (0/50)	0 (0/63)	0.4128	0 (0/40)	0 (0/48)	0.0587
	Single Mutation	S-G-K-A-A	GGACG	43 (28/65)	57 (41/72)		51 (32/63)	55 (42/77)		44 (22/50)	55 (35/63)		60 (24/40)	81 (39/48)	
		S-A-K-A-S	GCACT	0 (0/65)	1 (1/72)		0 (0/63)	1 (1/77)		0 (0/50)	0 (0/63)		0 (0/40)	0 (0/48)	
		A-A-K-A-A	TCACG	0 (0/65)	3 (2/72)		6 (4/63)	1 (1/77)		0 (0/50)	2 (1/63)		3 (1/40)	0 (0/48)	
	Double Mutations	A-G-K-A-A	TGACG	46 (30/65)	32 (23/72)		33 (21/63)	25 (19/77)		44 (22/50)	30 (19/63)		27 (11/40)	15 (7/48)	
		S-G-E-A-A	GGGCG	0 (0/65)	3 (2/72)		2 (1/63)	3 (2/77)		0 (0/50)	3 (2/63)		0 (0/40)	2 (1/48)	
	Triple Mutations	A-G-K-A-S	GGAGT	3 (2/65)	4 (3/72)		5 (3/63)	6 (5/77)		10 (5/50)	6 (4/63)		10 (4/40)	2 (1/48)	
	Quadruple mutation	A-G-K-G-S	GGACT	8 (5/65)	0 (0/72)		3 (2/63)	5 (477)		2 (1/50)	3 (2/63)		0 (0/40)	0 (0/48)	

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sulfadoxine Pyrimethamine	**pf dhfr /dhps(chr4/8) codons: 51/59/108/437/540**														
	Wild type	N-C-S-A-K	ATGGA	0 (0/72)	0 (0/68)	0.19	0 (0/69)	1 (1/81)	0.6541	0 (0/58)	0 (0/69)	0.3348	0 (0/43)	0 (0/61)	0.6304
	Single Mutation	N-C-S-G-K	ATGCA	0 (0/72)	0 (0/68)		6 (4/69)	1 (1/81)		5 (3/58)	0 (0/69)		0 (0/43)	2 (1/61)	
	Double Mutations	N-R-N-A-K	ACAGA	0 (0/72)	1 (1/68)		3 (2/69)	1 (1/81)		0 (0/58)	0 (0/69)		0 (0/43)	0 (0/61)	
		N-C-S-G-E	ATGCG	0 (0/72)	0 (0/68)		0 (0/69)	0 (0/81)		0 (0/58)	0 (0/69)		0 (0/43)	0 (0/61)	
sulfadoxine Pyrimethamine	Triple Mutations	N-R-N-G-K	ACACA	21 (15/72)	10 (7/68)		9 (6/69)	15 (12/81)		7 (4/58)	4 (3/69)		14 (6/43)	10 (6/61)	
		I-C-N-G-K	TTACA	1 (1/72)	4 (3/68)		1 (1/69)	2 (2/81)		1 (1/58)	1 (1/69)		0 (0/43)	3 (2/61)	
		I-R-N-A-K	TCAGA	0 (0/72)	3 (2/68)		3 (2/69)	4 (3/81)		0 (0/58)	1 (1/69)		2 (1/43)	0 (0/61)	
	Quadruple mutations	I-R-N-G-K	TCACA	76 (55/72)	78 (53/68)		77 (61/69)	73 (59/81)		86 (50/58)	90 (62/69)		84 (36/43)	84 (51/61)	
		I-R-N-A-E	TCAGG	0 (0/72)	0 (0/68)		0 (0/69)	0 (0/81)		0 (0/58)	0 (0/69)		0 (0/43)	0 (0/61)	
		N-R-N-G-E	ACACG	0 (0/72)	0 (0/68)		0 (0/69)	0 (0/81)		0 (0/58)	1 (1/69)		0 (0/43)	0 (0/61)	
	quintuple	I-R-N-G-E	TCACG	1 (1/72)	3 (2/68)		1 (1/69)	2 (2/81)		0 (/58)	1 (1/69)		0 (0/43)	2 (1/61)	

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4.4.2 School survey

4.4.2.1 Prevalence of *Plasmodium falciparum* drug resistance markers

Of the 991 asymptomatic *P. falciparum* samples submitted for sequencing, 103 sample gave useable sequences and were analysed for the prevalence of known drug resistance mutations in *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps* and putative markers in the *pfk13* gene. There was no significant difference in the prevalence of resistance markers associated with antimalarial drugs between Begoro and Cape Coast for the school survey (Table 4.13). The prevalence of *pfcrt* 76T was 11.5 % and 10.7% for Begoro and Cape Coast respectively. Similarly, the prevalence of *pfmdr1* 86Y was low in both sites (prevalence < 20%). However, the prevalence was high in both sites for *pfmdr1* 184F (prevalence > 70%). The *pfmdr1* mutations 1034C, 1042D and 1246Y were not detected in the asymptomatic isolates (Table 4.13). The *pfdhps* 437G mutation is at fixation in both study sites, however, *pfdhps* 540E mutation still remains low (prevalence < 5%) in both sites. Other low prevalent markers *pfdhps* 581G and 613S were also identified in our isolates. The prevalence of *pfdhfr* resistance markers 51I, 59R and 108N was high in both sites (prevalence > 70%), but 164L was not detected in our study isolates. The *pfk13* resistance markers were not detect in the asymptomatic isolates (Table 4.13).

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Table 4.13 Prevalence of point mutations associated with *P. falciparum* anti-malarial drug resistance in two ecological zones in Ghana (school survey)

Gene (chr)	Resistant Markers	Ref. Allele	Alt. Allele	Begoro ,%(N/n)	Cape Coast, %(N/n)	P-value
***pfcrt* (chr7)**	CRT_C72S	**T**	**A**	0 (0/26)	0(0/28)	
	CRT_M74I	G	T	11.5 (3/26)	10.7 (3/28)	1
	CRT_N75D	A	G	11.5 (3/26)	10.7 (3/28)	1
	CRT_K76T	A	C	11.5 (3/26)	10.7 (3/28)	1
	CRT_A220S	G	T	25.0 (3/12)	15.0 (3/20)	0.8151
	CRT_I356T	T	C	0 (0/20)	0 (0/18)	
***pfmdr1* (chr 5)**	N86Y	A	T	15.4 (4/26)	11. 5 (4/22)	1
	Y184F	A	T	76.5 (26/34)	85 (34/40)	0.5249
	S1034C	A	T	0 (0/39)	0(0/52)	
	N1042D	A	G	0 (0/39)	0 (0/52)	
	D1246Y	G	T	0 (0/31)	0 (0/38)	
***pfdhps* (chr 8)**	S436 A	T	G	72.4 (21/29)	44.4 (12/27)	0.06373
	A437G	G	C	100 (29/29)	100 (27/27)	0.605
	K540E	A	G	4.6 (1/22)	4.8 (1/21)	1
	A581G	C	G	2.9 (1/35)	8.3 (3/36)	0.6271
	A613 S	G	A	12.1 (4/33)	11.4 (4/35)	1
***pfdhfr* (chr 4)**	N51I	A	T	78.3 (18/23)	85 (29/34)	0.7414
	C59R	T	C	95.7 (22/23)	97.1 (33/34)	1
	S108 T/N	G	A	100 (24/24)	100 (31/31	
164L		A		0 (0/24)	0 (0/38)	
***pfk13* Kelch (chr 13)**	M476I	C	A	0 (0/29)	0 (0/40)	
	Y493I	A	G	0 (0/22)	0 (0/26)	
	R539T	G	C	0 (0/33)	0 (0/39)	
	I543T	A	G	0 (0/33)	0(0/39)	
	C580Y	G	A	0(0/28)	0 (0/35)	

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4.5 COMPARISON OF DRUG RESISTANCE MUTATIONS BETWEEN SYMPTOMATIC AND ASYMPTOMATIC CHILDREN

To determine if monitoring drug resistance using asymptomatic samples will differ compared to using symptomatic samples, which is normally used, a simple logistic regression was performed to determine the magnitude of effect of using either symptomatic or asymptomatic samples on the prevalence of antimalarial resistance markers. The results showed that using asymptomatic samples revealed lower prevalence of *pfcrt* T76 compared to symptomatic samples. (P-value = 0.023) as shown in Table 4.14. The logistic regression results also showed asymptomatic samples had a lower odds of of resistance in the other *pfcrt* markers, S220 and T356 although, the odds ratio was not statistically significant.

Asymptomatic infections showed non-significant associations with the other markers of antimalarial drug resistance as shown in Table 4.15. It showed a lower odds *pfmdr1* F184 (OR 1.39 95% CI 0.75–1.78; P= 0.3488) and the prevalence of Y86 (OR 1.072; 95% CI 0.39–1.49; P= 1). It also showed an increased odds the prevalence of *pfdhfr1* R59 (OR 1.06 95% CI 0.25–9.45; P= 1) but revealed a reduced in I51 (OR 0.65; 95% CI 0.31–1.49; P= 0.3254). For *pfdhps* markers, asymptomatic infections also showed an increased odds in A436 (OR 1.20 95% CI 0.67–2.19; P= 06103), E540 (OR 1.25 95% CI 0.14–5.33; P= 0.676), G437 (OR 1.41; 95% CI 0.35–4.23; P= 0.5295) and revealed a reduced in S613 (OR 0.85 95% CI 0.34–1.86; P= 0.8116).

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Table 4.14 Prevalence of antimalarial resistance markers comparing symptomatic and asymptomatic samples

Resistance Markers	**Symptomatic children**	**Asymptomatic children**	**p value**
pfcrt			
T76	25.9 (197/770)	11.1 (6/54)	0.02334
S220	24.8 (163/658)	18.7 (6/32)	0.532
T356	6 (50/745)	0 (0/38)	0.1639
pfmdr1			
Y86	12.7 (86/679)	13.5 (7/52)	1
F184	75.4 (519/688)	80.1 (60/74)	0.3488
pfdhfr			
I51	87.9 (638/726)	82.5 (47/57)	0.3254
R59	96.3 (699/726)	96.5 (55/57)	1
N108	98.1 (713/727)	100.0 (55/55)	
pfdhps			
A436	54.4 (280/698)	58.9 (33/56)	0.6103
G437	97.6 (681/698)	100.0 (56/56)	0.6296
E540	3.8 (24/639)	4.7 (2/43)	0.676
G581	4.1 (26/639)	5.6 (4/71)	0.5295
S613	13.6 (87/639)	11.8 (8/68)	0.8116

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Table 4.15 Association between infectivity and prevalence of mutant alleles of antimalarial drugs

Resistance Markers	Asymptomatic children, Odds ratio (CI)	p value
pfcrt		
T76	0.357 (0.123, 0.854)	0.02334
S220	0.701 (0.231, 1.782)	0.532
T356	0 (0.000, 1.472)	0.1639
pfmdr1		
Y86	1.072 (0.395, 2.499)	1
F184	1.395 (0.747, 2.775)	0.3488
pfdhfr		
I51	0.649 (0.309, 1.493)	0.3254
R59	1.062 (0.255, 9.454)	1
N108	∞	0.615
pfdhps		
A436	1.200(0.668, 2.189)	0.6103
G437	∞	0.6296
E540	1.250 (0.138, 5.334)	0.676
G437	1.407 (0.346, 4.233)	0.5295
S613	0.846 (0.338, 1.859)	0.8116

OR, odds ratio; CI, confidence interval.

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CHAPTER FIVE

5 DISCUSSIONS

The study set out to determine the dynamics of *Falciparum* infections in two ecological zones in Ghana by investigating the genetic diversity of AMA1 gene, complexity of infection and drug resistance. Indices of genetic diversity were slightly higher in Begoro in the forest ecological zone compared to Cape coast in the coastal savanna zone, however, there was no statistically significant differentiation between the two ecological zones. The Ghanaian isolates and Nigerian isolates showed moderate genetic differentiation with the East African and South East Asian isolates. Most of the children (73%) carried single *P. falciparum* strains. The rate of re-emergence of Chloroquine sensitive strains after the removal of Chloroquine was accelerated in the forest ecological zone (high to moderate transmission intensity) compared to the coastal savanna zone (moderate to low transmission intensity). The study also found that some mutations associated with resistance to SP have remained very high, near fixation. Resistance markers associated Amodiaquine and Lumefantrine which are used in combination with artemisinin derivatives as first line of treatment were increasing but the South East Asian *pfK13* mutations associated with artemisinin resistance were not found in Ghana. Comparing asymptomatic infections with symptomatic infections revealed that, the prevalence of *pfcrt* 76T was smaller in asymptomatic infections compared to symptomatic infections.

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5.1 GENETIC DIVERSITY, EVIDENCE OF SELECTION AND POPULATION DIFFERENTIATION OF THE PFAMA1 GENE BETWEEN THE TWO ECOLOGICAL ZONES.

An effective vaccine is urgently needed to control the deadly malaria disease, which is a leading cause of mortality and mobility globally. One of the leading antigens for a blood stage malaria vaccine, currently under development, is the *pfama1*; because some studies have shown adequate immune response to the vaccine in animal models (Mehrizi et al., 2013; Takala & Plowe, 2009). However, the extensive genetic diversity of the *pfama1* is a stumbling block, which might interfere with vaccine development (Mehrizi et al., 2013). Understanding of the extent and dynamics of genetic diversity in vaccine antigens is necessary for the guidance in vaccine development (Takala & Plowe, 2009).

In this regard, the *pfama1*, from *P. falciparum* isolates collected from two ecological zones in Ghana, were analyzed and compared with isolates from other endemic countries. This study presents the genetic diversity of the *pfama1* gene and evidence of selection in the gene for the first time, on a large scale, in these ecological zones in Ghana and the first study to compare sequences of *pfama1* between ecologically different populations in Ghana.

The sequence analysis of *pfama1* in this study revealed highly diverse haplotypes with high haplotype diversity (h) and nucleotide diversity (π). Indices of genetic diversity were slightly higher in Begoro in the forest ecological zone compared to Cape coast in the coastal savanna zone. The nucleotide diversity, haplotype diversity and non-synonymous mutations did not show any significant trend over the four-year period of the study. The nucleotide diversity was similar over the study period. These results of genetic diversity

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appear to also correlate with the endemicity in the two ecological zones. *Plasmodium falciparum* diversity is highly associated with malaria transmission and endemicity in a larger sense. This may explain the high genetic diversity observed in both sites (Anderson et al., 2000; Arnott et al., 2014). This study reported a higher prevalence of malaria in Begoro than in Cape Coast from the school survey. Begoro located in the forest ecological zone of Ghana is considered hyper-endemic with high transmission intensity (EIR=21.9 infective bites per person per year (ib/p/y)); while Cape Coast, located in the coastal part of the country is also hyper endemic but with low to moderate transmission intensity (EIR=3.65 ib/p/y) (Dery et al., 2010). The difference in transmission intensity between the two ecological zones may explain why the indices of genetic diversity were slightly higher in Begoro compared to Cape Coast. With scale-up of malaria intervention over the past decade worldwide and in Ghana, it was expected that the observed reduction in malaria cases, as a result of reducing intensity would affect the genetic diversity patterns but this study did not observe any significant difference in the genetic diversity over the four-year period of the study. Reduced malaria parasite prevalence is known to affect the parasite genetic diversity, however, a further sizeable decline in the prevalence of *P. falciparum* in these sites is prerequisite to having an impact on the parasite antigenic diversity (Arnott et al., 2014; Richards & Beeson, 2009; Riley & Stewart, 2013; Takala & Plowe, 2009).

In general, factors like natural selection, the level of endemicity and transmission intensity of malaria are the determinants of the level of sexual outcrossing, population recombination rates and linkage disequilibrium in the population (Eisen, Marshall, Billman-Jacobe, & Coppel, 1999; McKenzie et al., 2001; Mehrizi et al., 2013; Mu et al., 2005). The above observations may explain why high transmission settings in Ghana with high malaria endemicity, moderate prevalence of infection (>20%) and under balancing selection of

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pfama1 is undergoing a high meiotic recombination and linkage disequilibrium is breaking down in both ecological zones.

To identify departures from neutrality, a sliding window analysis of Tajima's D was performed as well as Fu and Li's D* and F*. Positive values of Tajima's D, Fu and Li's D* and F* observed in this study indicates a recent population bottleneck and or balancing selection. This may be explained by the need to retain genetic diversity as an evolutionary advantage under immune pressure (Akey et al., 2004; Arnott et al., 2014). Although the study was not designed to measure the effective population size, it is likely to have reduced as a result of the many interventions introduced in Ghana.

Moreover, available reports show a negative correlation of endemicity with declining LD values in the parasite population, in contrast to positive correlation with recombination (Anderson et al., 2000; Mehrizi et al., 2013; Mu et al., 2005) which explains the higher recombination rate and low LD observed in the Begoro (higher endemicity compare to Cape Coast). The recombination rate of the West African population (Ghana and Nigeria) was higher than the rate in East Africa (Kenya) and South East Asia (Thailand). This may again be explained by the lower endemicity in these regions, which reduces the rate of multiclonal infections and subsequently cross fertilization and recombination in mosquitoes. High recombination rates had frequently been reported previously for *pfama1* sequences from other regions (Osier et al., 2010; Polley et al., 2003; Polley & Conway, 2001; Takala & Plowe, 2009; Tetteh et al., 2009; Zhu et al., 2016).

The coefficient of differentiation between populations also known as the Wright's Fixation statistics (Fst) revealed no clear genetic differentiation of the AMA1 sequences between the two ecological zones of Ghana. Overall, a high within population genetic diversity

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(99.6%) was observed in the Ghanaian isolates with no evidence of clustering between the two ecological zones. All the polymorphic sites in the AMA1 gene were shared indicating there is gene flow between these two study populations. A similar study on the genetic diversity of msp2 over a decade with isolates from these study site revealed high genetic diversity of msp2 and no genetic differentiation between the different eco-epidemiological zones of Ghana (Duah, Matrevi, Quashie, Abuaku, & Koram, 2016). A genome wide sequence analysis in 2015 revealed no differentiation between two ecological zones in Ghana (Duffy et al., 2015). These findings in Ghana are consistent with studies, which have shown similar estimates and demonstrating a pattern in highly endemic West African populations like Mali and Burkina Faso (Manske et al., 2012). The unrestricted gene flow in West Africa and high recombination populations are the plausible explanations for the lack of genetic differentiation of *P. falciparum* in West Africa (Mobegi et al., 2014). Considering the Ghanaian parasite population used in this study with those from other malaria endemic areas, there was no statistically significant differentiation between the Ghanaian sequences from the Nigerian sequences. There was, however, evidence of moderate differentiation between Ghanaian isolates in this study and sequences from Kenya and Thailand. The results observed in this study are consistent with other studies which show evidence of a considerable differentiation of the AMA1 gene between West Africa, East Africa and South East Asia (Manske et al., 2012; Mobegi et al., 2014; Zhu et al., 2016). The population structural difference observed reinforces the fact that adequate response of the AMA1-based vaccine may differ geographically and the need to constantly monitor the genetic diversity within population and differentiation among subpopulations (Mehrizi et al., 2013).

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5.2 COMPLEXITY OF INFECTION

The present study provides current information on the complexity of *P. falciparum* infections in two ecological zones in Ghana, where malaria is marked with low to high transmission and highly seasonal peaks in July and October. The study also provided results on the trend of COI temporally over a four-year period (2014-2017). The total number of genotypes per infection (COI) was determined by the number of genotypes found per patient and this study revealed based on the AMA1 MIP that, 23% of *P. falciparum* infections contained multiple genotypes. Available evidence shows high transmission settings tend to have high genetic diversity and an increased likelihood of carrying multiple genotypes of the parasite. In contrast, low transmission settings have low genetic diversity and clonal infections. (Atroosh et al., 2011; Echeverry et al., 2013; Ghanchi et al., 2010; Mayengue, Ndounga, Malonga, Bitemo, & Ntoumi, 2011; Oyebola et al., 2014). However, there was no statistically significant difference in the mean COI and proportion of genotypes per infection between the two ecological zones used in this study. Similar observations were made by an earlier study conducted in these sites using msp1 and msp2 genes (Duah et al., 2016). The mean COI was 1.64 in 2010 compared to 1.27 in this study for Begoro, and for Cape Coast, the mean COI was 1.13 in 2012-2013 compared to 1.32 in 2017. The mean COI was 3.31 in 2007-2008 (Duah et al., 2016) for Begoro in the forest ecological zone, which has moderate to high transmission intensity. A plausible explanation for no difference between the two ecological zones is that, reducing malaria transmissions as a result of scale-up of interventions is leveling out transmission rate or intensity for these two ecological zones and we might need to redefine these eco-epidemiological zones.

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Complexity of infection did not differ temporally over the four-year period of the study as well. Age and sex were not associated with multiple genotype infections but were associated with an increase in parasitaemia and hemoglobin levels; though not statistically significant. Similarly, there was no significant difference between time points for the study conducted by Duah and colleagues but clearly, there is a huge difference between 3.31 in 2008 (Duah et al., 2016) and 1.27 presently. This indicates that, the effect of reducing transmission on COI may take much longer than four years to be observed.

5.3 RESISTANCE TO ANTIMALARIAL DRUGS

The extensive use of ACTs as first-line treatment is exerting a huge pressure on the parasite population and ecological variations may influence the process. To this end, this study was conducted to monitor the temporal profiles of resistance to antimalarial drugs in two transmission-intensity driven "ecological zones" of Ghana to see the effect of ecology on the evolution of drug resistance. The study observed a further decline in mutations associated with chloroquine resistance in Ghana since the last report in 2012 and 2014 (Afoakwah et al., 2014; Duah et al., 2012). The rate of re-emergence of chloroquine sensitive strains after the removal of chloroquine was accelerated in the forest ecological zone (high to moderate transmission intensity) compared to the coastal savanna zone (moderate to low transmission intensity). The study also found that some mutations associated with resistance to SP have remained very high, near fixation. Resistance markers associated Amodiaquine and Lumefantrine which are used in combination with artemisinin derivatives as first line of treatment were increasing but the South East Asian *pfK13* mutations associated with artemisinin resistance were not found in Ghana.

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Following reports of spread of resistance to chloroquine in sub-Saharan Africa and increasing trends in Ghana, chloroquine was withdrawn in 2004. In consultation and following the recommendations of W.H.O, Ghana changed its anti-malaria drug policy to Artesunate-Amodiaquine (ASAQ) combination as the first line drug for the management of uncomplicated malaria and SP for IPTp in pregnant women, although SP had previously been used as second drug of choice for the treatment of uncomplicated malaria. Around the time, the mutant *pfcrt*76T allele was nearing fixation in Ghana and treatment failure following chloroquine treatment was in the range between 6% and 25% (WHO, 2009a). Subsequent complaints of adverse drug reactions of ASAQ led to the introduction of two more ACTs; Artemether lumifanterine (AL) and Dihydroartemisinin Piperaquine (DHAP) as alternate first line drugs in 2009.

The steady decline in the prevalence of the primary marker associated with chloroquine resistance (*pfcrt* 76T) over the 4-year study period confirms the findings of a study conducted in Malawi where, the removal of chloroquine resulted in the re-expansion of drug sensitive *P. falciparum* strains (Laufer et al., 2010). A similar study conducted by Duah and colleagues (2013) showed a reduction in *pfcrt* 76T prevalence from 92% in 2005-2006 to 57% in 2010 for the forest zone. Another study conducted on samples collected in 2011 by Afoakwah and colleagues (2014). showed a general reduction to about 58.5% in Ghana, which was considered a slow decline after drug policy change compared to other countries. The outcome of the current study after 13 years of chloroquine removal in Begoro especially, is consistent with other studies conducted in some African countries (>90% recovery in Tanzania in 10 years, 100% recovery in Malawi after 13 years and 80% in Mozambique after 5yrs). Cape Coast however, fell short of achieving the levels attained by the Eastern and Southern African countries, with only about 75% recovery, 13 years on

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(Afoakwah et al., 2014; Duah et al., 2013; Kublin et al., 2003; Laufer et al., 2006; Mohammed et al., 2013; Thomsen et al., 2013). On the other hand, data from Cape-Coast agrees with results obtained from studies conducted in Benin (Ogouyèmi-Hounto et al., 2013) and Nigeria (Oladipo, Wellington, & Sutherland, 2015) where the decline occurred at a much slower rate. Reversion to sensitive strains is dependent on multiple factors including but not limited to, time elapsed since the withdrawal of drug, geographical location, illegal drug use and cross-resistance shared between chloroquine and amodiaquine (Ogouyèmi-Hounto et al., 2013). It is quite evident that there was an ecological effect on the resurgence of chloroquine sensitive strains although there are unpublished reports on the illegal use of chloroquine even after withdrawal of the chloroquine in Cape Coast (Afoakwah et al., 2014).

Point mutations in *pfmdr1* have been associated with treatment failure of chloroquine and other antimalarial drugs like amodiaquine and lumefantrine (Elbadry et al., 2013). Previous studies have described *pfmdr1* alleles as modulators of chloroquine resistance. Drug pressure due to lumefantrine (LM) and mefloquine (MQ) has been shown to select for *pfcrt* K76, *pfmdr1* N86, F184 and D1246 (Baraka et al., 2015; Happi et al., 2009; Menard et al., 2012; Price et al., 1999; Wurtz et al., 2014), whiles the selection of *pfcrt* 76T, *pfmdr1* Y86, Y184 and Y1246 have been associated with decreased AQ sensitivity (Danquah et al., 2010; Nsobya et al., 2010; Tinto et al., 2008). This study found low or/and reducing prevalence in N86Y, S1034C, N1042D or D1246Y mutations in *pfmdr1*, which have been shown to be associated with CQ resistance (Duraisingh & Cowman, 2005; Elbadry et al., 2013; Sidhu, Verdier-Pinard, & Fidock, 2002). The relatively lower prevalence of these markers is expected because of removal of chloroquine pressure resulting in the re-expansion of chloroquine sensitive strains (Laufer et al., 2006). The study did now show

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mutations at codons 1034 and 1042 in *pfmdr1* indicating sensitivity by the study parasites to mefloquine (Nkhoma et al., 2009) and AL (Elbadry et al., 2013; Sa et al., 2009). Interestingly, the prevalence of *pfmdr1* 184F remained high for the four-year period of this study and this has previously reported (Duah et al., 2013).

Studies have shown reduced sensitivity to AL in *P. falciparum* with the Y184F mutation in the presence of N86Y and D1246Y (N**F**D) (Elbadry et al., 2013; Nkhoma et al., 2009). The prevalence of *pfmdr1* N86**F**184D1246 haplotype (N**F**D) remained high in both sites. Whereas the prevalence of *pfmdr1* **Y**86Y184**Y**1246 haplotype (**Y**Y**Y**) was found only in Cape Coast at a very low prevalence (1%). **Y**Y**Y** was not observed in both sites in 2017. The relatively lower prevalence of **Y**Y**Y** observed in the study is expected because of cross resistance shared between chloroquine and amodiaquine compared to the single selective force of lumefantrine on N**F**D haplotype. With regards to the lumefantrine (LM) and AQ-associated N**F**D and **Y**Y**Y** haplotypes respectively, similar results and <10% prevalence figures for **Y**Y**Y** respectively were recorded in Ghana, between 2003 and 2010 (Duah et al., 2013). These observations also corroborate with previous reports from Uganda, Kenya, Tanzania and Mozambique (Conrad et al., 2014; Henriques et al., 2014; Humphreys et al., 2007; Malmberg et al., 2013; Raman et al., 2011). These finding suggest that, LM resistant parasites are evolving faster than AQ resistant parasites in Ghana and will likely be an issue to resolve sooner rather than later, thus there is the need for continuous surveillance of these predictive molecular markers for early detection and timely pre-emptive measures.

The accumulation of mutations in the *pfdhps* and *pfdhfr*, the targets of sulphadoxine pyrimethamine (SP), are associated with SP treatment failure (Raman et al., 2011; Roper et al., 2003). Similar to the study reported in 2012 at the same study sites (Duah et al., 2012), the *pfdhfr* triple mutation **IRN** and *pfdhfr/pfdhps* quintuple haplotype **IRGN**K have

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remained high over the years and almost approaching fixation. This suggests that the use of SP as IPTp for pregnant women has over the past 13 years selected for these less resistant haplotypes. This result is consistent with other studies conducted in West Africa (Chang et al., 2013; Ogouyèmi-Hounto et al., 2013) and other African Countries (Bin Dajem et al., 2012; Bouyou-Akotet, Mawili-Mboumba, Tchantchou Tde, & Kombila, 2010; Gesase et al., 2009; Iriemenam et al., 2012; Matondo et al., 2014). SP remains in use in IPTp because the prevalence of the 540E mutation and therefore the quintuple haplotype **IRNGE** required for high treatment failure of SP in South and East Asia (Raman et al., 2011; Roper et al., 2003) is still very low, in Ghana (6%) and in the sub region (Ogouyèmi-Hounto et al., 2013; Tinto et al., 2007). The *pfdhps* triple mutation **AG**KA**S** has previously been reported in Ghana but at a lower frequency (at 1.8-2.6%) (Alam et al., 2011). However, the quadruple mutation **AG**K**GS** is being reported for the first time in our study. In addition, the *pfdhps* 581G mutation has not been previously reported in these parts of Ghana even though one study reported it in one sample in the northern part of Ghana (Mockenhaupt et al., 2005). Evidence indicates the number of mutations is positively correlated with the level of both *in vivo* and *in vitro* SP resistance (Alam et al., 2011; Gregson & Plowe, 2005; Lozovsky et al., 2009; Vinayak et al., 2010).

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5.4 COMPARING ANTIMALARIAL DRUG RESISTANCE ALLELES USING SYMPTOMATIC AND ASYMPTOMATIC ISOLATES

To determine if the prevalence of antimalarial drug resistance alleles will differ using symptomatic or asymptomatic isolates, binomial logistic regression was used to estimate the magnitude of effect of using asymptomatic isolates compared to the symptomatic isolates. This study revealed that asymptomatic infections underestimated the prevalence of *pfcrt* 76T compared to the prevalence in symptomatic infections. It also revealed asymptomatic infections had varying effect on other resistance markers of antimalarial drugs, though not statistically significant.

The underestimation of the prevalence of *pfcrt* 76T using asymptomatic infections is similar to studies that reported that the prevalence of resistant alleles was significantly higher in febrile children compared to afebrile children. It was however, inconsistent with studies conducted in Uganda, Benin and Zanzibar, which reported that prevalence of resistant genotypes was significantly lower in symptomatic children compared to asymptomatic children (Morris et al., 2015; Ogouyèmi-Hounto et al., 2013; Tukwasibwe et al., 2014).

Though the results are inconsistent, its implications to the epidemiology of drug resistant malaria cannot be overlooked. Asymptomatic infections might serve as reservoir for resistant strains thus increasing its selection advantage over the sensitive strains. This is important in the epidemiology of drug resistance (Brown et al., 2012; Morris et al., 2015). To determine the true prevalence of drug resistance in the population, ideally both symptomatic and asymptomatic isolates should be genotyped, however, the poor sensitivity of genotyping tools to low genomic material associated with asymptomatic isolates makes

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it difficult. Moreover, it cost more to sample asymptomatic infections compared to symptomatic infections because with asymptomatic you will need to screen more people to get a few infected cases.

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CHAPTER SIX

6 CONCLUSIONS AND RECOMMENDATION

6.1 CONCLUSIONS

6.1.1 Conclusions

In conclusion, the study has shown that, there is no significant genetic differentiation of the *pfama1* gene between the forest and coastal ecological zones of Ghana. Additionally, *Plasmodium falciparum* populations of West Africa cluster differently from East African and South East Asian populations. The mean COIs for the two ecological zones are similar (1.27 for Forest and 1.32 for the coastal zone) and most of the infections carry single *P. falciparum* clones (73%).

With regards to molecular markers of drug resistance, the study has shown a higher rate of re-emergence of chloroquine sensitive strains in the forest ecological zone compared to the coastal savanna zone. Prevalence of the resistance marker associated with Lumefantrine (*pfmdr1* 184F) remains >50%. Markers that make up the quintuple haplotype **IRNGE** associated with resistance to SP are almost at fixation except for *pfdhps* 540E which has remained low (<5%). In addition, *pfdhps* 581G mutation and *pfdhps* quadruple haplotype **AG**K**GS** associated with sulfadoxine resistance, which have not been previously reported in the two ecological zones, were detected. The South East Asian *pfK13* mutations associated with artemisinin resistance were not found in these study sites of Ghana.

The prevalence of *pfcrt* 76T among asymptomatic individuals was lower compared to symptomatic individuals.

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6.1.2 Public health importance of the findings of this study

1. Since *pfama1* is a favorable vaccine candidate antigen, this study provides current data which gives important information for the development of efficacious *pfama1*-based malaria vaccine

2. This study shows that the antigenic marker AMA1 may be used as genotyping tool that can be applied to other phenotypes of public health importance like distinguishing recrudescence and reinfections during efficacy studies and estimating COI which is used as a measure of impact of control interventions.

3. The ecological differences observed in the re-expansion of chloroquine sensitive strains provides information on hotspots that is important in the design of targeted malaria control strategies in Ghana

4. This study iterates the importance of using both symptomatic and asymptomatic infections in monitoring *P. falciparum* genotypes which gives the true picture in the population and not just of a selected few.

5. This study also leveraged the low cost, specificity and high-throughput of the molecular inversion probes (MIPs) technique (O'Roak et al., 2012) which has been adapted for the AT-rich genome of falciparum (Aydemir et al., 2018) to capture the known mutations the five drug resistance genes in individual infections and sequenced using the Illumina MiSeq platform

6.1.3 Contribution to knowledge

The study revealed low prevalence of *pfdhfr* marker 581G and thus the quadruple mutation AGKGS and these have not been previously reported in these parts of Ghana.

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6.2 LIMITATIONS OF THE STUDY

This study had some inherent drawbacks. One of the major drawbacks was the difficulty in optimizing the MIP protocol to work well on low genomic material (Asymptomatic sample with very low parasitaemia in this case). In addition, in analyzing the genomic diversity of the AMA1 gene, only clonal samples were used, thus much of the analytical power provided by population genetic theory does not apply given that basic assumptions would be violated. With regards to lumefantrine resistance, copy number variation, which is also associated with lumefantrine resistance was not done in this study, thus the results are not conclusive. Other measures such as the use of drugs, which can confound the effect of eco-epidemiological zones on drug resistance was not considered in this study. Four years proved to be insufficient time to detect any subtle changes in measures such as differences in genetic diversity and COI that were targeted in the two ecological zones.

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6.3 RECOMMENDATIONS

Research

1. The persistence of high prevalence of *pfmdr1*184F should be further explored in *in-vitro* and *in-vivo* studies with ACT partner drugs to see the correlation with partner drug resistance in Ghana, if any.
2. In developing asymptomatic survey for sequencing a higher cut off parasitaemia should be used when recruiting participants for the study.
3. More genotyping methods (eg. Amplicon deep sequencing) should be optimized to genotype low quantity genomic DNA from asymptomatic infections with low parasitaemia
4. Continuous pre-emptive monitoring of the complex dynamics of the *P. falciparum* and other species (drug resistance, COI and genetic diversity) for a more effective control and subsequent elimination of malaria in Ghana

NMPC/MOH

1. The subtle ecological impact on the evolution of drug resistance observed in this study can be explored in the design of targeted malaria control strategies in Ghana.

WHO

1. The absence of *pfk13* mutations associated with artemisinin resistance suggest that the artemisinin-based combination therapy remains efficacious in the two ecological zones in Ghana.

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REFERENCES

Abotsi, A. K. (2012). Cost burden of infant malaria treatment on households and health institutions in the upper east region.

Afoakwah, R., Boampong, J. N., Egyir-Yawson, A., Nwaefuna, E. K., Verner, O. N., & Asare, K. K. (2014). High prevalence of PfCRT K76T mutation in Plasmodium falciparum isolates in Ghana. *Acta Trop, 136*, 32-36. doi:10.1016/j.actatropica.2014.03.030

Akey, J. M., Eberle, M. A., Rieder, M. J., Carlson, C. S., Shriver, M. D., Nickerson, D. A., & Kruglyak, L. (2004). Population history and natural selection shape patterns of genetic variation in 132 genes. *PLoS Biol, 2*(10), e286. doi:10.1371/journal.pbio.0020286

Alam, M. T., de Souza, D. K., Vinayak, S., Griffing, S. M., Poe, A. C., Duah, N. O., . . . Koram, K. A. (2011). Selective Sweeps and Genetic Lineages of Plasmodium falciparum Drug -Resistant Alleles in Ghana. *J Infect Dis, 203*(2), 220-227. doi:10.1093/infdis/jiq038

Alker, A. P., Mwapasa, V., Purfield, A., Rogerson, S. J., Molyneux, M. E., Kamwendo, D. D., . . . Meshnick, S. R. (2005). Mutations Associated with Sulfadoxine-Pyrimethamine and Chlorproguanil Resistance in Plasmodium falciparum Isolates from Blantyre, Malawi. *Antimicrob Agents Chemother, 49*(9), 3919-3921. doi:10.1128/aac.49.9.3919-3921.2005

Anderson, T. J., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L., Mollinedo, R., . . . Day, K. P. (2000). Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. *Mol Biol Evol, 17*(10), 1467-1482. doi:10.1093/oxfordjournals.molbev.a026247

University of Ghana http://ugspace.ug.edu.gh

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A. C., Khim, N., . . . Menard, D. (2014). A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature, 505*(7481), 50-55. doi:10.1038/nature12876

Arnott, A., Wapling, J., Mueller, I., Ramsland, P. A., Siba, P. M., Reeder, J. C., & Barry, A. E. (2014). Distinct patterns of diversity, population structure and evolution in the AMA1 genes of sympatric Plasmodium falciparum and Plasmodium vivax populations of Papua New Guinea from an area of similarly high transmission. *Malar J, 13*, 233. doi:10.1186/1475-2875-13-233

Arrow, K., Panosian, C., & Gelband, H. (2004). Saving lives, buying time: Economics of malaria drugs in an age of resistance. . *Committee on the Economics of Antimalarial Drugs, The National Academies Press, Washington, DC*.

Asante, F. A., & Asenso-Okyere, K. (2003). Economic burden of malaria in Ghana.

Ashley, E. A., McGready, R., Proux, S., & Nosten, F. (2006). Malaria. *Travel Med Infect Dis, 4*(3-4), 159-173. doi:10.1016/j.tmaid.2005.06.009

Ashley, E. A., Pyae Phyo, A., & Woodrow, C. J. (2018). Malaria. *The Lancet, 391*(10130), 1608-1621. doi:https://doi.org/10.1016/S0140-6736(18)30324-6

Atroosh, W. M., Al-Mekhlafi, H. M., Mahdy, M. A., Saif-Ali, R., Al-Mekhlafi, A. M., & Surin, J. (2011). Genetic diversity of Plasmodium falciparum isolates from Pahang, Malaysia based on MSP-1 and MSP-2 genes. *Parasit Vectors, 4*, 233. doi:10.1186/1756-3305-4-233

Auburn, S., & Barry, A. E. (2017). Dissecting malaria biology and epidemiology using population genetics and genomics. *Int J Parasitol, 47*(2-3), 77-85. doi:10.1016/j.ijpara.2016.08.006

University of Ghana http://ugspace.ug.edu.gh

Awine, T., Malm, K., Bart-Plange, C., & Silal, S. P. (2017). Towards malaria control and elimination in Ghana: challenges and decision making tools to guide planning. *Global health action, 10*(1), 1381471.

Aydemir, O., Janko, M., Hathaway, N. J., Verity, R., Mwandagalirwa, M. K., Tshefu, A. K., . . . Bailey, J. A. (2018). Drug-Resistance and Population Structure of Plasmodium falciparum Across the Democratic Republic of Congo Using High-Throughput Molecular Inversion Probes. *J Infect Dis, 218*(6), 946-955. doi:10.1093/infdis/jiy223

Babiker, H. A., & Walliker, D. (1997). Current views on the population structure of Plasmodium falciparum: implications for control. *Parasitology Today, 13*(7), 262-267.

Baraka, V., Tinto, H., Valea, I., Fitzhenry, R., Delgado-Ratto, C., Mbonye, M. K., . . . Erhart, A. (2015). In vivo selection of Plasmodium falciparum Pfcrt and Pfmdr1 variants by artemether-lumefantrine and dihydroartemisinin-piperaquine in Burkina Faso. *Antimicrob Agents Chemother, 59*(1), 734-737. doi:10.1128/AAC.03647-14

Barat, L. M. (2006). Four malaria success stories: how malaria burden was successfully reduced in Brazil, Eritrea, India, and Vietnam. *Am J Trop Med Hyg, 74*(1), 12-16.

Basu, M., Maji, A. K., Mitra, M., & Sengupta, S. (2013). Natural selection and population genetic structure of domain-I of Plasmodium falciparum apical membrane antigen-1 in India. *Infect Genet Evol, 18*, 247-256. doi:10.1016/j.meegid.2013.05.015

Bin Dajem, S. M., Al-Farsi, H. M., Al-Hashami, Z. S., Al-Sheikh, A. A., Al-Qahtani, A., & Babiker, H. A. (2012). Distribution of drug resistance genotypes in

University of Ghana http://ugspace.ug.edu.gh

Plasmodium falciparum in an area of limited parasite diversity in Saudi Arabia. *Am J Trop Med Hyg, 86*(5), 782-788. doi:10.4269/ajtmh.2012.11-0520

Bloland, P. B. (2001). Drug resistance in malaria.

Bongfen, S. E., Laroque, A., Berghout, J., & Gros, P. (2009). Genetic and genomic analyses of host-pathogen interactions in malaria. *Trends Parasitol, 25*(9), 417-422. doi:10.1016/j.pt.2009.05.012

Bouyou-Akotet, M. K., Mawili-Mboumba, D. P., Tchantchou Tde, D., & Kombila, M. (2010). High prevalence of sulfadoxine/pyrimethamine-resistant alleles of Plasmodium falciparum isolates in pregnant women at the time of introduction of intermittent preventive treatment with sulfadoxine/pyrimethamine in Gabon. *J Antimicrob Chemother, 65*(3), 438-441. doi:10.1093/jac/dkp467

Brown, T., Smith, L. S., Oo, E. K. S., Shawng, K., Lee, T. J., Sullivan, D., . . . Richards, A. K. (2012). Molecular surveillance for drug-resistant Plasmodium falciparum in clinical and subclinical populations from three border regions of Burma/Myanmar: cross-sectional data and a systematic review of resistance studies. *Malaria Journal, 11*(1), 333. doi:10.1186/1475-2875-11-333

Campino, S., Auburn, S., Kivinen, K., Zongo, I., Ouedraogo, J. B., Mangano, V., . . . Kwiatkowski, D. P. (2011). Population genetic analysis of Plasmodium falciparum parasites using a customized Illumina GoldenGate genotyping assay. *PLoS One, 6*(6), e20251. doi:10.1371/journal.pone.0020251

Cavalli-Sforza, L. L., & Bodmer, W. F. (1999). *The genetics of human populations*: Courier Corporation.

Census. (2010). *2010 Population and Housing Census,[Fanteakwa District]: Eastern Region*: Ghana Statistical Service.

University of Ghana http://ugspace.ug.edu.gh

Chakraborty, A. (2016). Emerging drug resistance in Plasmodium falciparum: A review of well-characterized drug targets for novel antimalarial chemotherapy. *Asian Pacific Journal of Tropical Disease, 6*(7), 581-588. doi:https://doi.org/10.1016/S2222-1808(16)61090-3

Chakravorty, Srabasti J., Hughes, Katie R., & Craig, Alister G. (2008). Host response to cytoadherence in Plasmodium falciparum. *Biochemical Society Transactions, 36*(2), 221-228. doi:10.1042/bst0360221

Chang, H.-H., Moss, E. L., Park, D. J., Ndiaye, D., Mboup, S., Volkman, S. K., . . . Hartl, D. L. (2013). Malaria life cycle intensifies both natural selection and random genetic drift. *Proceedings of the National Academy of Sciences, 110*(50), 20129-20134. doi:10.1073/pnas.1319857110

Chen, L. H., Wilson, M. E., & Schlagenhauf, P. (2006). Prevention of malaria in long-term travelers. *JAMA, 296*(18), 2234-2244. doi:10.1001/jama.296.18.2234

Cohee, L. M., & Laufer, M. K. (2017). Malaria in Children. *Pediatric Clinics of North America, 64*(4), 851-866. doi:https://doi.org/10.1016/j.pcl.2017.03.004

Conrad, M. D., LeClair, N., Arinaitwe, E., Wanzira, H., Kakuru, A., Bigira, V., . . . Rosenthal, P. J. (2014). Comparative impacts over 5 years of artemisinin-based combination therapies on Plasmodium falciparum polymorphisms that modulate drug sensitivity in Ugandan children. *J Infect Dis, 210*(3), 344-353. doi:10.1093/infdis/jiu141

Cotter, C., Sturrock, H. J. W., Hsiang, M. S., Liu, J., Phillips, A. A., Hwang, J., . . . Feachem, R. G. A. (2013). The changing epidemiology of malaria elimination: new strategies for new challenges. *The Lancet, 382*(9895), 900-911. doi:https://doi.org/10.1016/S0140-6736(13)60310-4

University of Ghana http://ugspace.ug.edu.gh

Cuamba, N., & Mendis, C. (2009). The role of Anopheles merus in malaria transmission in an area of southern Mozambique. *J Vector Borne Dis, 46*(2), 157-159.

Cui, L., Mharakurwa, S., Ndiaye, D., Rathod, P. K., & Rosenthal, P. J. (2015). Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network. *Am J Trop Med Hyg, 93*(3 Suppl), 57-68. doi:10.4269/ajtmh.15-0007

Danquah, I., Coulibaly, B., Meissner, P., Petruschke, I., Muller, O., & Mockenhaupt, F. P. (2010). Selection of pfmdr1 and pfcrt alleles in amodiaquine treatment failure in north-western Burkina Faso. *Acta Trop, 114*(1), 63-66. doi:10.1016/j.actatropica.2009.12.008

Das, A., Bajaj, R., Mohanty, S., & Swain, V. (2007). Genetic diversity and evolutionary history of Plasmodium falciparum and P. vivax. *Current Science, 92*(11), 1516-1524.

Dery, D. B., Brown, C., Asante, K. P., Adams, M., Dosoo, D., Amenga-Etego, S., . . . Owusu-Agyei, S. (2010). Patterns and seasonality of malaria transmission in the forest-savannah transitional zones of Ghana. *Malaria Journal, 9*(1), 314. doi:10.1186/1475-2875-9-314

Drakeley, C., Schellenberg, D., Kihonda, J., Sousa, C. A., Arez, A. P., Lopes, D., . . . Alonso, P. (2003). An estimation of the entomological inoculation rate for Ifakara: a semi-urban area in a region of intense malaria transmission in Tanzania. *Trop Med Int Health, 8*(9), 767-774.

Duah, N. O., Matrevi, S. A., de Souza, D. K., Binnah, D. D., Tamakloe, M. M., Opoku, V. S., . . . Koram, K. A. (2013). Increased pfmdr1 gene copy number and the decline in pfcrt and pfmdr1 resistance alleles in Ghanaian Plasmodium falciparum

University of Ghana http://ugspace.ug.edu.gh

isolates after the change of anti-malarial drug treatment policy. *Malar J, 12*, 377. doi:10.1186/1475-2875-12-377

Duah, N. O., Matrevi, S. A., Quashie, N. B., Abuaku, B., & Koram, K. A. (2016). Genetic diversity of Plasmodium falciparum isolates from uncomplicated malaria cases in Ghana over a decade. *Parasit Vectors, 9*(1), 416. doi:10.1186/s13071-016-1692-1

Duah, N. O., Quashie, N. B., Abuaku, B. K., Sebeny, P. J., Kronmann, K. C., & Koram, K. A. (2012). Surveillance of molecular markers of Plasmodium falciparum resistance to sulphadoxine-pyrimethamine 5 years after the change of malaria treatment policy in Ghana. *Am J Trop Med Hyg, 87*(6), 996-1003. doi:10.4269/ajtmh.2012.12-0202

Duah, N. O., Wilson, M. D., Ghansah, A., Abuaku, B., Edoh, D., Quashie, N. B., & Koram, K. A. (2007). Mutations in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance genes, and treatment outcomes in Ghanaian children with uncomplicated malaria. *J Trop Pediatr, 53*(1), 27-31. doi:10.1093/tropej/fml076

Duffy, C. W., Assefa, S. A., Abugri, J., Amoako, N., Owusu-Agyei, S., Anyorigiya, T., . . . Awandare, G. A. (2015). Comparison of genomic signatures of selection on Plasmodium falciparum between different regions of a country with high malaria endemicity. *BMC Genomics, 16*, 527. doi:10.1186/s12864-015-1746-3

Duraisingh, M. T., & Cowman, A. F. (2005). Contribution of the pfmdr1 gene to antimalarial drug-resistance. *Acta Trop, 94*(3), 181-190. doi:10.1016/j.actatropica.2005.04.008

University of Ghana http://ugspace.ug.edu.gh

Duraisingh, M. T., Curtis, J., & Warhurst, D. C. (1998). Plasmodium falciparum:Detection of Polymorphisms in theDihydrofolate ReductaseandDihydropteroate SynthetaseGenes by PCR and Restriction Digestion. *Experimental Parasitology, 89*(1), 1-8. doi:https://doi.org/10.1006/expr.1998.4274

Dutta, S., Haynes, J. D., Moch, J. K., Barbosa, A., & Lanar, D. E. J. P. o. t. N. A. o. S. (2003). Invasion-inhibitory antibodies inhibit proteolytic processing of apical membrane antigen 1 of Plasmodium falciparum merozoites. *100*(21), 12295-12300.

Dye, C., & Williams, B. G. (1997). Multigenic drug resistance among inbred malaria parasites. *Proc Biol Sci, 264*(1378), 61-67. doi:10.1098/rspb.1997.0009

Echeverry, D. F., Nair, S., Osorio, L., Menon, S., Murillo, C., & Anderson, T. J. (2013). Long term persistence of clonal malaria parasite Plasmodium falciparum lineages in the Colombian Pacific region. *BMC Genet, 14*, 2. doi:10.1186/1471-2156-14-2

Eisen, D. P., Marshall, V. M., Billman-Jacobe, H., & Coppel, R. L. (1999). A Plasmodium falciparum apical membrane antigen-1 (AMA-1) gene apparently generated by intragenic recombination. *Mol Biochem Parasitol, 100*(2), 243-246.

Elbadry, M. A., Existe, A., Victor, Y. S., Memnon, G., Fukuda, M., Dame, J. B., . . . Okech, B. A. (2013). Survey of Plasmodium falciparum multidrug resistance-1 and chloroquine resistance transporter alleles in Haiti. *Malar J, 12*, 426. doi:10.1186/1475-2875-12-426

Farooq, U., Malla, N., & Dubey, M. L. (2009). Genetic polymorphism in msp-2, ama-1 and csp genes in Plasmodium falciparum field isolates from north and north-western India. *J Vector Borne Dis, 46*(2), 109-116.

University of Ghana http://ugspace.ug.edu.gh

Fowkes, F. J., Richards, J. S., Simpson, J. A., & Beeson, J. G. J. P. m. (2010). The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis. *7*(1), e1000218.

Gesase, S., Gosling, R. D., Hashim, R., Ord, R., Naidoo, I., Madebe, R., . . . Chandramohan, D. (2009). High resistance of Plasmodium falciparum to sulphadoxine/pyrimethamine in northern Tanzania and the emergence of dhps resistance mutation at Codon 581. *PLoS One, 4*(2), e4569. doi:10.1371/journal.pone.0004569

Ghana Statistical Service, G. (2016). Ghana Malaria Indicator Survey Fact sheet.

Ghanchi, N. K., Martensson, A., Ursing, J., Jafri, S., Bereczky, S., Hussain, R., & Beg, M. A. (2010). Genetic diversity among Plasmodium falciparum field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. *Malar J, 9*, 1. doi:10.1186/1475-2875-9-1

Greenwood, B. M. (2002). The molecular epidemiology of malaria. *Trop Med Int Health, 7*(12), 1012-1021.

Greenwood, B. M. (2017). New tools for malaria control – using them wisely. *Journal of Infection, 74*, S23-S26. doi:https://doi.org/10.1016/S0163-4453(17)30187-1

Greenwood, B. M., Bojang, K., Whitty, C. J., & Targett, G. A. (2005). Malaria. *Lancet, 365*(9469), 1487-1498. doi:10.1016/S0140-6736(05)66420-3

Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H., Alonso, P. L., Collins, F. H., & Duffy, P. E. (2008). Malaria: progress, perils, and prospects for eradication. *J Clin Invest, 118*(4), 1266-1276. doi:10.1172/JCI33996

Gregson, A., & Plowe, C. V. (2005). Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol Rev, 57*(1), 117-145. doi:10.1124/pr.57.1.4

University of Ghana http://ugspace.ug.edu.gh

Gueye, N. S. G., Ntoumi, F., Vouvoungui, C., Kobawila, S. C., Nkombo, M., Mouanga, A. M., . . . Koukouikila-Koussounda, F. (2018). Plasmodium falciparum merozoite protein-1 genetic diversity and multiplicity of infection in isolates from Congolese children consulting in a pediatric hospital in Brazzaville. *Acta Tropica, 183*, 78-83. doi:https://doi.org/10.1016/j.actatropica.2018.04.012

Happi, C. T., Gbotosho, G. O., Folarin, O. A., Sowunmi, A., Hudson, T., O'Neil, M., . . . Oduola, A. M. (2009). Selection of Plasmodium falciparum multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. *Antimicrob Agents Chemother, 53*(3), 888-895. doi:10.1128/aac.00968-08

Harris, R. S. (2007). Improved Pairwise Alignmnet of Genomic DNA.

Hartl, D. L., Clark, A. G., & Clark, A. G. (1997). *Principles of population genetics* (Vol. 116): Sinauer associates Sunderland.

Hartl, D. L., Volkman, S. K., Nielsen, K. M., Barry, A. E., Day, K. P., Wirth, D. F., & Winzeler, E. A. (2002). The paradoxical population genetics of Plasmodium falciparum. *Trends Parasitol, 18*(6), 266-272.

Hastings, I. M., & D' Alessandro, U. (2000). Modeling a predictable disaster: the rise and spread of drug-resistant malaria. *Parasitol Today, 16*. doi:10.1016/S0169-4758(00)01707-5

Hastings, I. M., & Mackinnon, M. J. (1998). The emergence of drug-resistant malaria. *Parasitology, 117 (Pt 5)*, 411-417.

Hathaway, N. J., Parobek, C. M., Juliano, J. J., & Bailey, J. A. (2017). SeekDeep: single-base resolution de novo clustering for amplicon deep sequencing. *Nucleic acids research, 46*(4), e21-e21.

University of Ghana http://ugspace.ug.edu.gh

Hay, S. I., Guerra, C. A., Tatem, A. J., Atkinson, P. M., & Snow, R. W. (2005). Urbanization, malaria transmission and disease burden in Africa. *Nature Reviews Microbiology, 3*, 81. doi:10.1038/nrmicro1069

https://www.nature.com/articles/nrmicro1069#supplementary-information

Hay, S. I., Sinka, M. E., Okara, R. M., Kabaria, C. W., Mbithi, P. M., Tago, C. C., . . . Godfray, H. C. (2010). Developing global maps of the dominant anopheles vectors of human malaria. *PLoS Med, 7*(2), e1000209. doi:10.1371/journal.pmed.1000209

Healer, J., Crawford, S., Ralph, S., McFadden, G., Cowman, A. F. J. I., & immunity. (2002). Independent translocation of two micronemal proteins in developing Plasmodium falciparum merozoites. *70*(10), 5751-5758.

Helsel, D. R., & Frans, L. M. (2006). Regional Kendall test for trend. *Environ Sci Technol, 40*(13), 4066-4073. doi:10.1021/es051650b

Henriques, G., Hallett, R. L., Beshir, K. B., Gadalla, N. B., Johnson, R. E., Burrow, R., . . . Sutherland, C. J. (2014). Directional selection at the pfmdr1, pfcrt, pfubp1, and pfap2mu loci of Plasmodium falciparum in Kenyan children treated with ACT. *J Infect Dis, 210*(12), 2001-2008. doi:10.1093/infdis/jiu358

Hoffman, S. L., Rustama, D., Dimpudus, A. J., Punjabi, N. H., Campbell, J. R., Oetomo, H. S., . . . et al. (1985). RII and RIII type resistance of Plasmodium falciparum to combination of mefloquine and sulfadoxine/pyrimethamine in Indonesia. *Lancet, 2*(8463), 1039-1040.

Humphreys, G. S., Merinopoulos, I., Ahmed, J., Whitty, C. J., Mutabingwa, T. K., Sutherland, C. J., & Hallett, R. L. (2007). Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in

University of Ghana http://ugspace.ug.edu.gh

Tanzanian children treated for uncomplicated malaria. *Antimicrob Agents Chemother, 51*(3), 991-997. doi:10.1128/AAC.00875-06

IHME, I. f. H. M. a. E. (2016). GBD Compare Data Visualization. Retrieved from http://vizhub.healthdata.org/gbd-compare

Institute of Medicine (US) Committee on the Economics of Antimalarial Drugs; Arrow KJ, P. C., Gelband H, editors. (2004). *Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance*. Washington (DC): National Academies Press (US.

Iriemenam, N. C., Shah, M., Gatei, W., van Eijk, A. M., Ayisi, J., Kariuki, S., . . . Shi, Y. P. (2012). Temporal trends of sulphadoxine-pyrimethamine (SP) drug-resistance molecular markers in Plasmodium falciparum parasites from pregnant women in western Kenya. *Malar J, 11*, 134. doi:10.1186/1475-2875-11-134

Jombart, T., Devillard, S., & Balloux, F. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genet, 11*, 94. doi:10.1186/1471-2156-11-94

Kadam, P., & Bhalerao, S. (2010). Sample size calculation. *Int J Ayurveda Res, 1*(1), 55-57. doi:10.4103/0974-7788.59946

Kafai, N. M., & Odom, J. A. R. (2018). Malaria in Children. *Infect Dis Clin North Am, 32*(1), 189-200. doi:10.1016/j.idc.2017.10.008

Kamvar, Z. N., Larsen, M. M., Kanaskie, A. M., Hansen, E. M., & Grunwald, N. J. (2015). Spatial and Temporal Analysis of Populations of the Sudden Oak Death Pathogen in Oregon Forests. *Phytopathology, 105*(7), 982-989. doi:10.1094/PHYTO-12-14-0350-FI

University of Ghana http://ugspace.ug.edu.gh

Kasasa, S., Asoala, V., Gosoniu, L., Anto, F., Adjuik, M., Tindana, C., . . . Vounatsou, P. (2013). Spatio-temporal malaria transmission patterns in Navrongo demographic surveillance site, northern Ghana. *Malaria Journal, 12*(1), 63. doi:10.1186/1475-2875-12-63

Kirkman, L. A., & Deitsch, K. W. (2012). Antigenic variation and the generation of diversity in malaria parasites. *Current opinion in microbiology, 15*(4), 456-462.

Kocken, C. H., Withers-Martinez, C., Dubbeld, M. A., Van Der Wel, A., Hackett, F., Blackman, M. J., . . . immunity. (2002). High-level expression of the malaria blood-stage vaccine candidate Plasmodium falciparum apical membrane antigen 1 and induction of antibodies that inhibit erythrocyte invasion. *70*(8), 4471-4476.

Kublin, J. G., Cortese, J. F., Njunju, E. M., Mukadam, R. A., Wirima, J. J., Kazembe, P. N., . . . Plowe, C. V. (2003). Reemergence of chloroquine-sensitive Plasmodium falciparum malaria after cessation of chloroquine use in Malawi. *J Infect Dis, 187*(12), 1870-1875. doi:10.1086/375419

Kudom, A. A., & Mensah, B. A. (2010). The potential role of the educational system in addressing the effect of inadequate knowledge of mosquitoes on use of insecticide-treated nets in Ghana. *Malar J, 9*, 256. doi:10.1186/1475-2875-9-256

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol, 35*(6), 1547-1549. doi:10.1093/molbev/msy096

Laufer, M. K., Takala-Harrison, S., Dzinjalamala, F. K., Stine, O. C., Taylor, T. E., & Plowe, C. V. (2010). Return of chloroquine-susceptible falciparum malaria in Malawi was a reexpansion of diverse susceptible parasites. *J Infect Dis, 202*(5), 801-808. doi:10.1086/655659

University of Ghana http://ugspace.ug.edu.gh

Laufer, M. K., Thesing, P. C., Eddington, N. D., Masonga, R., Dzinjalamala, F. K., Takala, S. L., . . . Plowe, C. V. (2006). Return of chloroquine antimalarial efficacy in Malawi. *N Engl J Med, 355*(19), 1959-1966. doi:10.1056/NEJMoa062032

Le Bras, J., & Durand, R. (2003). The mechanisms of resistance to antimalarial drugs in Plasmodium falciparum. *Fundamental & clinical pharmacology, 17*(2), 147-153.

Lozovsky, E. R., Chookajorn, T., Brown, K. M., Imwong, M., Shaw, P. J., Kamchonwongpaisan, S., . . . Hartl, D. L. (2009). Stepwise acquisition of pyrimethamine resistance in the malaria parasite. *Proc Natl Acad Sci U S A, 106*(29), 12025-12030. doi:10.1073/pnas.0905922106

Mahajan, R. C., Farooq, U., Dubey, M. L., & Malla, N. (2005). Genetic polymorphism in Plasmodium falciparum vaccine candidate antigens. *Indian Journal of Pathology and Microbiology, 48*(4), 429-438.

Malmberg, M., Ferreira, P. E., Tarning, J., Ursing, J., Ngasala, B., Bjorkman, A., . . . Gil, J. P. (2013). Plasmodium falciparum drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms. *J Infect Dis, 207*(5), 842-847. doi:10.1093/infdis/jis747

Manske, M., Miotto, O., Campino, S., Auburn, S., Almagro-Garcia, J., Maslen, G., . . . Kwiatkowski, D. P. (2012). Analysis of Plasmodium falciparum diversity in natural infections by deep sequencing. *Nature, 487*, 375. doi:10.1038/nature11174 https://www.nature.com/articles/nature11174#supplementary-information

Matondo, S. I., Temba, G. S., Kavishe, A. A., Kauki, J. S., Kalinga, A., van Zwetselaar, M., . . . Kavishe, R. A. (2014). High levels of sulphadoxine-pyrimethamine

University of Ghana http://ugspace.ug.edu.gh

resistance Pfdhfr-Pfdhps quintuple mutations: a cross sectional survey of six regions in Tanzania. *Malar J, 13*, 152. doi:10.1186/1475-2875-13-152

Mattah, P. A. D., Futagbi, G., Amekudzi, L. K., Mattah, M. M., de Souza, D. K., Kartey-Attipoe, W. D., . . . Wilson, M. D. (2017). Diversity in breeding sites and distribution of Anopheles mosquitoes in selected urban areas of southern Ghana. *Parasites & vectors, 10*(1), 25. doi:10.1186/s13071-016-1941-3

Mayengue, P. I., Ndounga, M., Malonga, F. V., Bitemo, M., & Ntoumi, F. (2011). Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in Plasmodium falciparum isolates from Brazzaville, Republic of Congo. *Malar J, 10*, 276. doi:10.1186/1475-2875-10-276

McDonald, J. H., & Kreitman, M. (1991). Adaptive protein evolution at the Adh locus in Drosophila. *Nature, 351*(6328), 652-654. doi:10.1038/351652a0

McKenzie, F. E., Ferreira, M. U., Baird, J. K., Snounou, G., & Bossert, W. H. (2001). Meiotic recombination, cross-reactivity, and persistence in Plasmodium falciparum. *Evolution; international journal of organic evolution, 55*(7), 1299-1307.

Mehrizi, A. A., Sepehri, M., Karimi, F., Djadid, N. D., & Zakeri, S. (2013). Population genetics, sequence diversity and selection in the gene encoding the Plasmodium falciparum apical membrane antigen 1 in clinical isolates from the south-east of Iran. *Infect Genet Evol, 17*, 51-61. doi:10.1016/j.meegid.2013.03.042

Menard, S., Morlais, I., Tahar, R., Sayang, C., Mayengue, P. I., Iriart, X., . . . Berry, A. (2012). Molecular monitoring of Plasmodium falciparum drug susceptibility at the time of the introduction of artemisinin-based combination therapy in Yaounde,

University of Ghana http://ugspace.ug.edu.gh

Cameroon: implications for the future. *Malar J, 11*, 113. doi:10.1186/1475-2875-11-113

Meshnick, S. R. (1998). From quinine to qinghaosu: Historical perspectives. *Malaria: Parasite Biology, Pathogenesis & Protection*, 341-353.

Meshnick, S. R., & Dobson, M. J. (2001). The history of antimalarial drugs. *Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery*, 15-25.

Millar, S. B., & Cox-Singh, J. (2015). Human infections with Plasmodium knowlesi—zoonotic malaria. *Clinical Microbiology and Infection, 21*(7), 640-648. doi:https://doi.org/10.1016/j.cmi.2015.03.017

Mita, T., & Jombart, T. (2015). Patterns and dynamics of genetic diversity in Plasmodium falciparum: What past human migrations tell us about malaria. *Parasitology International, 64*(3), 238-243. doi:https://doi.org/10.1016/j.parint.2014.09.007

Mitchell, G., Thomas, A., Margos, G., Dluzewski, A., Bannister, L. J. I., & immunity. (2004). Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. *72*(1), 154-158.

Mobegi, V. A., Duffy, C. W., Amambua-Ngwa, A., Loua, K. M., Laman, E., Nwakanma, D. C., . . . Conway, D. J. (2014). Genome-Wide Analysis of Selection on the Malaria Parasite Plasmodium falciparum in West African Populations of Differing Infection Endemicity. *Mol Biol Evol, 31*(6), 1490-1499. doi:10.1093/molbev/msu106

University of Ghana http://ugspace.ug.edu.gh

Mockenhaupt, F. P., Teun Bousema, J., Eggelte, T. A., Schreiber, J., Ehrhardt, S., Wassilew, N., . . . Bienzle, U. (2005). Plasmodium falciparum dhfr but not dhps mutations associated with sulphadoxine-pyrimethamine treatment failure and gametocyte carriage in northern Ghana. *Trop Med Int Health, 10*(9), 901-908. doi:10.1111/j.1365-3156.2005.01471.x

MOH. (2014). Guidelines for Case Management of Malaria in Ghana. 3rd ed. Accra: Ministry of Health.

Mohammed, A., Ndaro, A., Kalinga, A., Manjurano, A., Mosha, J. F., Mosha, D. F., . . . Kavishe, R. A. (2013). Trends in chloroquine resistance marker, Pfcrt-K76T mutation ten years after chloroquine withdrawal in Tanzania. *Malar J, 12*, 415. doi:10.1186/1475-2875-12-415

Morris, U., Xu, W., Msellem, M. I., Schwartz, A., Abass, A., Shakely, D., . . . Martensson, A. (2015). Characterising temporal trends in asymptomatic Plasmodium infections and transporter polymorphisms during transition from high to low transmission in Zanzibar, 2005-2013. *Infect Genet Evol, 33*, 110-117. doi:10.1016/j.meegid.2015.04.018

Mu, J., Awadalla, P., Duan, J., McGee, K. M., Joy, D. A., McVean, G. A., & Su, X. Z. (2005). Recombination hotspots and population structure in Plasmodium falciparum. *PLoS Biol, 3*(10), e335. doi:10.1371/journal.pbio.0030335

Narum, D. L., Thomas, A. W. J. M., & parasitology, b. (1994). Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of Plasmodium falciparum merozoites. *67*(1), 59-68.

Nei, M. (1987). *Molecular evolutionary genetics*: Columbia university press.

University of Ghana http://ugspace.ug.edu.gh

Nkhoma, S., Nair, S., Mukaka, M., Molyneux, M. E., Ward, S. A., & Anderson, T. J. (2009). Parasites bearing a single copy of the multi-drug resistance gene (pfmdr-1) with wild-type SNPs predominate amongst Plasmodium falciparum isolates from Malawi. *Acta Trop, 111*(1), 78-81. doi:10.1016/j.actatropica.2009.01.011

Nonvignon, J., Aryeetey, G. C., Malm, K. L., Agyemang, S. A., Aubyn, V. N., Peprah, N. Y., . . . Aikins, M. (2016). Economic burden of malaria on businesses in Ghana: a case for private sector investment in malaria control. *Malaria Journal, 15*(1), 454.

Nosten, F., & White, N. J. (2007). Artemisinin-based combination treatment of falciparum malaria. *Am J Trop Med Hyg, 77*(6 Suppl), 181-192.

Nsobya, S. L., Kiggundu, M., Nanyunja, S., Joloba, M., Greenhouse, B., & Rosenthal, P. J. (2010). In vitro sensitivities of Plasmodium falciparum to different antimalarial drugs in Uganda. *Antimicrob Agents Chemother, 54*(3), 1200-1206. doi:10.1128/AAC.01412-09

O'Roak, B. J., Vives, L., Fu, W., Egertson, J. D., Stanaway, I. B., Phelps, I. G., . . . Shendure, J. (2012). Multiplex Targeted Sequencing Identifies Recurrently Mutated Genes in Autism Spectrum Disorders. *Science, 338*(6114), 1619-1622. doi:10.1126/science.1227764

Ogouyèmi-Hounto, A., Ndam, N. T., Kinde Gazard, D., d'Almeida, S., Koussihoude, L., Ollo, E., . . . Massougbodji, A. (2013). Prevalence of the molecular marker of Plasmodium falciparum resistance to chloroquine and sulphadoxine/pyrimethamine in Benin seven years after the change of malaria treatment policy. *Malaria Journal, 12*, 147-147. doi:10.1186/1475-2875-12-147

University of Ghana http://ugspace.ug.edu.gh

Okell, L. C., Griffin, J. T., & Roper, C. (2017). Mapping sulphadoxine-pyrimethamine-resistant Plasmodium falciparum malaria in infected humans and in parasite populations in Africa. *Sci Rep, 7*(1), 7389. doi:10.1038/s41598-017-06708-9

Oladipo, O. O., Wellington, O. A., & Sutherland, C. J. (2015). Persistence of chloroquine-resistant haplotypes of Plasmodium falciparum in children with uncomplicated Malaria in Lagos, Nigeria, four years after change of chloroquine as first-line antimalarial medicine. *Diagn Pathol, 10*, 41. doi:10.1186/s13000-015-0276-2

Osier, F. H., Weedall, G. D., Verra, F., Murungi, L., Tetteh, K. K., Bull, P., . . . Conway, D. J. (2010). Allelic diversity and naturally acquired allele-specific antibody responses to Plasmodium falciparum apical membrane antigen 1 in Kenya. *Infect Immun, 78*(11), 4625-4633. doi:10.1128/IAI.00576-10

Oyebola, M. K., Idowu, E. T., Olukosi, Y. A., Iwalokun, B. A., Agomo, C. O., Ajibaye, O. O., . . . Otubanjo, A. O. (2014). Genetic diversity and complexity of Plasmodium falciparum infections in Lagos, Nigeria. *Asian Pacific Journal of Tropical Biomedicine, 4*, S87-S91. doi:https://doi.org/10.12980/APJTB.4.2014C1301

Pasvol, G. (2005). Malaria. *Medicine, 33*(8), 39-43. doi:10.1383/medc.2005.33.8.39

Patterson, N., Price, A. L., & Reich, D. (2006). Population structure and eigenanalysis. *PLoS Genet, 2*(12), e190. doi:10.1371/journal.pgen.0020190

Paul, R. E., Hackford, I., Brockman, A., Muller-Graf, C., Price, R., Luxemburger, C., . . . Day, K. P. (1998). Transmission intensity and Plasmodium falciparum diversity on the northwestern border of Thailand. *Am J Trop Med Hyg, 58*(2), 195-203.

University of Ghana http://ugspace.ug.edu.gh

Paul, R. E., Packer, M., Walmsley, M., Lagog, M., Ranford-Cartwright, L., Paru, R., & Day, K. (1995). Mating patterns in malaria parasite populations of Papua New Guinea. *Science, 269*(5231), 1709-1711.

Peterson, M. G., Marshall, V. M., Smythe, J. A., Crewther, P. E., Lew, A., Silva, A., . . . biology, c. (1989). Integral membrane protein located in the apical complex of Plasmodium falciparum. *9*(7), 3151-3154.

Pinkevych, M., Petravic, J., Bereczky, S., Rooth, I., Farnert, A., & Davenport, M. P. (2015). Understanding the relationship between Plasmodium falciparum growth rate and multiplicity of infection. *J Infect Dis, 211*(7), 1121-1127. doi:10.1093/infdis/jiu561

Pirahmadi, S., Zakeri, S., Mehrizi, A. A., & Djadid, N. D. (2018). Analysis of genetic diversity and population structure of gene encoding cell-traversal protein for ookinetes and sporozoites (CelTOS) vaccine candidate antigen in global Plasmodium falciparum populations. *Infection, Genetics and Evolution, 59*, 113-125.

Polley, S. D., Chokejindachai, W., & Conway, D. J. (2003). Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. *Genetics, 165*(2), 555-561.

Polley, S. D., & Conway, D. J. (2001). Strong diversifying selection on domains of the Plasmodium falciparum apical membrane antigen 1 gene. *Genetics, 158*(4), 1505-1512.

Price, R. N., Cassar, C., Brockman, A., Duraisingh, M., van Vugt, M., White, N. J., . . . Krishna, S. (1999). The pfmdr1 gene is associated with a multidrug-resistant

University of Ghana http://ugspace.ug.edu.gh

phenotype in Plasmodium falciparum from the western border of Thailand. *Antimicrob Agents Chemother, 43*(12), 2943-2949.

Pumpaibool, T., Arnathau, C., Durand, P., Kanchanakhan, N., Siripoon, N., Suegorn, A., . . . Harnyuttanakorn, P. (2009). Genetic diversity and population structure of Plasmodium falciparum in Thailand, a low transmission country. *Malaria Journal, 8*(1), 155. doi:10.1186/1475-2875-8-155

Quang, N. D., Hoa, P. T., Tuan, M. S., Viet, N. X., Jalloh, A., & Matsuoka, H. (2009). Polymorphism at the apical membrane antigen 1 gene (AMA1) of the malaria parasite Plasmodium falciparum in a Vietnamese population. *Biochem Genet, 47*(5-6), 370-383. doi:10.1007/s10528-009-9236-4

Raman, J., Mauff, K., Muianga, P., Mussa, A., Maharaj, R., & Barnes, K. I. (2011). Five years of antimalarial resistance marker surveillance in Gaza Province, Mozambique, following artemisinin-based combination therapy roll out. *PLoS One, 6*(10), e25992. doi:10.1371/journal.pone.0025992

Richards, J. S., & Beeson, J. G. (2009). The future for blood-stage vaccines against malaria. *Immunol Cell Biol, 87*(5), 377-390. doi:10.1038/icb.2009.27

Riley, E. M., & Stewart, V. A. (2013). Immune mechanisms in malaria: new insights in vaccine development. *Nat Med, 19*(2), 168-178. doi:10.1038/nm.3083

Roper, C., Pearce, R., Bredenkamp, B., Gumede, J., Drakeley, C., Mosha, F., . . . Sharp, B. (2003). Antifolate antimalarial resistance in southeast Africa: a population-based analysis. *Lancet, 361*(9364), 1174-1181. doi:10.1016/S0140-6736(03)12951-0

Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sanchez-Gracia, A. (2017). DnaSP 6: DNA Sequence

University of Ghana http://ugspace.ug.edu.gh

Polymorphism Analysis of Large Data Sets. *Mol Biol Evol, 34*(12), 3299-3302. doi:10.1093/molbev/msx248

Sa, J. M., Twu, O., Hayton, K., Reyes, S., Fay, M. P., Ringwald, P., & Wellems, T. E. (2009). Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proc Natl Acad Sci U S A, 106*(45), 18883-18889. doi:10.1073/pnas.0911317106

Saha, P., Ganguly, S., & Maji, A. K. (2016). Genetic diversity and multiplicity of infection of Plasmodium falciparum isolates from Kolkata, West Bengal, India. *Infection, Genetics and Evolution, 43*, 239-244. doi:https://doi.org/10.1016/j.meegid.2016.05.038

Sibley, C. H. (2014). Understanding drug resistance in malaria parasites: basic science for public health. *Mol Biochem Parasitol, 195*(2), 107-114. doi:10.1016/j.molbiopara.2014.06.001

Sidhu, A. B. S., Verdier-Pinard, D., & Fidock, D. A. (2002). Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. *Science, 298*(5591), 210-213.

Silvie, O., Franetich, J.-F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., . . . Kocken, C. H. J. J. o. B. C. (2004). A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites. *279*(10), 9490-9496.

Singh, B., Kim Sung, L., Matusop, A., Radhakrishnan, A., Shamsul, S. S., Cox-Singh, J., . . . Conway, D. J. (2004). A large focus of naturally acquired Plasmodium knowlesi infections in human beings. *Lancet, 363*(9414), 1017-1024. doi:10.1016/S0140-6736(04)15836-4

University of Ghana http://ugspace.ug.edu.gh

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., . . . Kabaria, C. W. (2010). The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasites & vectors, 3*(1), 117.

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., . . . Hay, S. I. (2012). A global map of dominant malaria vectors. *Parasites & vectors, 5*(1), 69. doi:10.1186/1756-3305-5-69

Smith, T., Beck, H. P., Kitua, A., Mwankusye, S., Felger, I., Fraser-Hurt, N., . . . Tanner, M. (1999). Age dependence of the multiplicity of Plasmodium falciparum infections and of other malariological indices in an area of high endemicity. *Trans R Soc Trop Med Hyg, 93 Suppl 1*, 15-20. doi:10.1016/s0035-9203(99)90322-x

Takala, S. L., & Plowe, C. V. (2009). Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'. *Parasite Immunol, 31*(9), 560-573. doi:10.1111/j.1365-3024.2009.01138.x

Tanabe, K., Jombart, T., Horibe, S., Palacpac, N. M., Honma, H., Tachibana, S.-I., . . . Mita, T. (2013). Plasmodium falciparum mitochondrial genetic diversity exhibits isolation-by-distance patterns supporting a sub-Saharan African origin. *Mitochondrion, 13*(6), 630-636.

Tanabe, K., Mita, T., Jombart, T., Eriksson, A., Horibe, S., Palacpac, N., . . . Ohmae, H. (2010). Plasmodium falciparum accompanied the human expansion out of Africa. *Current Biology, 20*(14), 1283-1289.

Tanabe, K., Zollner, G., Vaughan, J. A., Sattabongkot, J., Khuntirat, B., Honma, H., . . . Coleman, R. (2015). Plasmodium falciparum: Genetic diversity and complexity of

University of Ghana http://ugspace.ug.edu.gh

infections in an isolated village in western Thailand. *Parasitology International, 64*(3), 260-266. doi:https://doi.org/10.1016/j.parint.2013.09.011

Tetteh, K. K., Stewart, L. B., Ochola, L. I., Amambua-Ngwa, A., Thomas, A. W., Marsh, K., . . . Conway, D. J. (2009). Prospective identification of malaria parasite genes under balancing selection. *PLoS One, 4*(5), e5568. doi:10.1371/journal.pone.0005568

Thera, M. A., & Plowe, C. V. (2012). Vaccines for Malaria: How Close Are We? *Annual Review of Medicine, 63*(1), 345-357. doi:10.1146/annurev-med-022411-192402

Thomsen, T. T., Madsen, L. B., Hansson, H. H., Tomas, E. V., Charlwood, D., Bygbjerg, I. C., & Alifrangis, M. (2013). Rapid selection of Plasmodium falciparum chloroquine resistance transporter gene and multidrug resistance gene-1 haplotypes associated with past chloroquine and present artemether-lumefantrine use in Inhambane District, southern Mozambique. *Am J Trop Med Hyg, 88*(3), 536-541. doi:10.4269/ajtmh.12-0525

Tinto, H., Guekoun, L., Zongo, I., Guiguemde, R. T., D'Alessandro, U., & Ouedraogo, J. B. (2008). Chloroquine-resistance molecular markers (Pfcrt T76 and Pfmdr-1 Y86) and amodiaquine resistance in Burkina Faso. *Trop Med Int Health, 13*(2), 238-240. doi:10.1111/j.1365-3156.2007.01995.x

Tinto, H., Ouedraogo, J. B., Zongo, I., van Overmeir, C., van Marck, E., Guiguemde, T. R., & D'Alessandro, U. (2007). Sulfadoxine-pyrimethamine efficacy and selection of Plasmodium falciparum DHFR mutations in Burkina Faso before its introduction as intermittent preventive treatment for pregnant women. *Am J Trop Med Hyg, 76*(4), 608-613.

University of Ghana http://ugspace.ug.edu.gh

Tsetsos, F., Drineas, P., & Paschou, P. (2018). Genetics and Population Analysis. In S. Ranganathan, M. Gribskov, K. Nakai, & C. Schönbach (Eds.), *Encyclopedia of Bioinformatics and Computational Biology* (pp. 363-378). Oxford: Academic Press.

Tukwasibwe, S., Mugenyi, L., Mbogo, G. W., Nankoberanyi, S., Maiteki-Sebuguzi, C., Joloba, M. L., . . . Rosenthal, P. J. (2014). Differential prevalence of transporter polymorphisms in symptomatic and asymptomatic falciparum malaria infections in Uganda. *J Infect Dis, 210*(1), 154-157.

Vinayak, S., Alam, M. T., Mixson-Hayden, T., McCollum, A. M., Sem, R., Shah, N. K., . . . Udhayakumar, V. (2010). Origin and evolution of sulfadoxine resistant Plasmodium falciparum. *PLoS Pathog, 6*(3), e1000830. doi:10.1371/journal.ppat.1000830

Volkman, S. K., Ndiaye, D., Diakite, M., Koita, O. A., Nwakanma, D., Daniels, R. F., . . . Wirth, D. F. (2012). Application of genomics to field investigations of malaria by the international centers of excellence for malaria research. *Acta Trop, 121*(3), 324-332. doi:10.1016/j.actatropica.2011.12.002

Walker, N. F., Nadjm, B., & Whitty, C. J. M. (2010). Malaria. *Medicine, 38*(1), 41-46. doi:10.1016/j.mpmed.2009.09.017

Walker, N. F., Nadjm, B., & Whitty, C. J. M. (2018). Malaria. *Medicine, 46*(1), 52-58. doi:https://doi.org/10.1016/j.mpmed.2017.10.012

Wernsdorfer, W. H., & Payne, D. (1991). The dynamics of drug resistance in Plasmodium falciparum. *Pharmacol Ther, 50*(1), 95-121.

White, N. J. (2004). Antimalarial drug resistance. *J Clin Invest, 113*(8), 1084-1092.

University of Ghana http://ugspace.ug.edu.gh

White, N. J. (2008). Plasmodium knowlesi: The Fifth Human Malaria Parasite. *Clinical Infectious Diseases, 46*(2), 172-173. doi:10.1086/524889

White, N. J., & Pongtavornpinyo, W. (2003). The de novo selection of drug–resistant malaria parasites. *Proceedings of the Royal Society of London B: Biological Sciences, 270*(1514), 545-554.

White, N. J., Pukrittayakamee, S., Hien, T. T., Faiz, M. A., Mokuolu, O. A., & Dondorp, A. M. (2014). Malaria. *Lancet, 383*(9918), 723-735. doi:10.1016/S0140-6736(13)60024-0

White, N. J., Pukrittayakamee, S., Phyo, A. P., Rueangweerayut, R., Nosten, F., Jittamala, P., . . . Leong, F. J. (2014). Spiroindolone KAE609 for falciparum and vivax malaria. *N Engl J Med, 371*(5), 403-410. doi:10.1056/NEJMoa1315860

WHO. (2009a). Anti-Malaria Drug Policy for Ghana, 2nd Revised Version 2009. Retrieved from http://apps.who.int/medicinedocs/en/d/Js18072en/

WHO. (2009b). Methods for surveillance of antimalarial drug efficacy. Retrieved from https://www.who.int/malaria/publications/atoz/9789241597531/en/

WHO. (2010). *Global report on antimalarial drug efficacy and drug resistance: 2000-2010. WHO.* . Retrieved from http://whqlibdoc.who.int/publications/2010/9789241500470_eng.pdf

WHO. (2016). *Malaria vaccine: WHO position paper – January 2016*. Retrieved from http://www.who.int/wer/2016/wer9104.pdf?ua=1

WHO. (2017). *World Malaria Report*. Retrieved from http://www.who.int/malaria/publications/world-malaria-report-2017/report/en/

WHO. (2018a). Malaria Key Facts. Retrieved from http://www.who.int/news-room/fact-sheets/detail/malaria

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WHO. (2018b). *World Malaria Report*. Retrieved from http://apps.who.int/iris/bitstream/handle/10665/275867/9789241565653-eng.pdf

Witkowski, B., Lelievre, J., Barragan, M. J., Laurent, V., Su, X. Z., Berry, A., & Benoit-Vical, F. (2010). Increased tolerance to artemisinin in Plasmodium falciparum is mediated by a quiescence mechanism. *Antimicrob Agents Chemother*, *54*(5), 1872-1877. doi:10.1128/AAC.01636-09

Wongsrichanalai, C., Pickard, A. L., Wernsdorfer, W. H., & Meshnick, S. R. (2002). Epidemiology of drug-resistant malaria. *Lancet Infect Dis*, *2*(4), 209-218.

Wurtz, N., Fall, B., Pascual, A., Fall, M., Baret, E., Camara, C., . . . Pradines, B. (2014). Role of Pfmdr1 in in vitro Plasmodium falciparum susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, and dihydroartemisinin. *Antimicrob Agents Chemother*, *58*(12), 7032-7040. doi:10.1128/AAC.03494-14

Zhu, X., Zhao, Z., Feng, Y., Li, P., Liu, F., Liu, J., . . . Cui, L. (2016). Genetic diversity of the Plasmodium falciparum apical membrane antigen I gene in parasite population from the China-Myanmar border area. *Infect Genet Evol*, *39*, 155-162. doi:10.1016/j.meegid.2016.01.021

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APPENDIX

APPENDIX I

ANTIMALARIAL DRUG RESISTANCE SURVEILLANCE IN GHANA
(SCHOOL-BASED SURVEY)

STUDY PROFILE		
Site	1. Begoro 2. Cape Coast	
School	Salvation Army Primary	
Participant ID: 01BGR5---		
1. BACKGROUND INFORMATION		
1.1 Name of student:	1.2 Date (dd/mm/yy):	1.3 Enrolment number:
1.4 Age(years):	1.5 Sex: 1. Male 2. Female	
1.6 Weight(kg) :	.1.7 Axillary temperature(°C):	
1.8 Height(cm):		
2. MALARIA SYMPTOMS		
Symptoms	Outcome	Duration if Yes
2.1 Fever	1. Yes » 2. No	
2.2 Headache	1. Yes 2. No	
2.3 Chills and Rigors	1. Yes 2. No	
2.4 Vomiting	1. Yes 2. No	
2.5 Diarrhea	1. Yes 2. No	
2.6 Other l{specify}	1. Yes 2. No	
4.0 PRIOR MEDICATION		
2.7 Other 2{specify)	1. Yes 2. No	
3.0 SREEENING SELECTION CRITERIA		
INCLUSION CRITERIA	YES	NO

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3.1 Aged 6 years - 14 years		
3.2 *P. falciparum* mono infection		
3.3:Haemoglobin ——— or hematocrit ——— Hemoglobin > 5g/dl or Hematocrit > 15%		
3.4 Severe Malnutrition		
3.5 lnformed Consent		

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All prior medication taken within the previous 14 days should be recorded in this section:

Has the student taken any antimalarial medicine? ☐ Yes ☐ No If yes, please specify below.

Medication Name:	Dates:(dd/mm/yy)	Ongoing Yes :√	Total daily dose or unit:(Eg:400)	Route of administration	Indication for use:
	Start: Stop:	☐			
	Start: Stop:	☐			
	Start: Stop:	☐			
	Start: Stop:	☐			
	Start: Stop:	☐			

5.0 Laboratory Form

5.1 Name of student:	5.2 Date: (dd/mm/yy)	5.3 ID number:
5.4 Age (years):	5.5 Sex: 1. Male 2. Female	
5.6 Blood film taken: 1.Yes 2. No	5.7 Filter paper blots: 1.Yes 2. No	
5.8 Hemoglobin: 1.Yes 2. No	5.9 Other:	

5.8.2 Hemoglobin results: _____ *g/dl*

5.10 Parasite species _____ (pf, pm, po, pv)

5.11 Asexual count _____ / _____ wbcs (_____ /ul)

5.12 Presence of *p. f* gametocytes: 1.Yes 2. No

5.13 Comments:

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ANTIMALARIAL DRUG RESISTANCE SURVEILLANCE IN GHANA SCREENING FORM

NAME OF' SITE: BEGORO GOVT. HOSPITAL **SCREENING/ID NUMBER: 07GHR6**

1. Name:	**2. Date:** ***(dd/mm/y})***	**3. Enrolment Number:**
Date of Birth: **Age:_____yrs____mths**	**5. Gender: M _____F _____**	
6. Weight (kg): _____	**7. Axillary temperature (0 C): _____**	

SCREENING SELECTION CRITERIA		
8. Age 6 months – 14 years	**YES**	**NO**
9. Axillary temp. ≥ 37.8°C or history of fever over past 24 hours		
10. *P. falciparum* mono infection		
11. Presence of 1 – 125 parasites/4 – 8 wbcs (i.e. 1,000 – 250, 00/µl)		
12. Hemoglobin _____or hematocrit _____		

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Hemoglobin > 5 g/dl or hematocrit > 15 %		
13. Ability to swallow		
14. Ability and willingness to comply to study protocol/study visit schedule		
15. informed Consent		
ECXCLUSION CRITERIA	**YES**	**NO**
16. Mixed or mono-infection with plasmodium species		
17. Severe malnutrition		
l8. Evidence of other causes of fever Pneumonia/ARI Measles Otitis media Urinary tract infection Gastroenteritis Other:		
19. Presence of chronic disease (e.g. cardiac. renal. hepatic)		

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20. History of allergy to test Medicine		
21. Regular medication which interferes with antimalarial pharmacokinetics (e.g. antihistamines)		
22. Unable to sit or stand (as appropriate for age)		
23. Signs of severe malaria • *Prostration* • *Impaired consciousness* • *Difficulty in breathing (pulmonary oedema, Respiratory Distress Syndrome)* • *multiple convulsions* • *Circulatory collapse* • *Severe anaemia or extreme pallor (i.e Hb < 5gld1 or Ht < 15%)* • *Shock (small pulse, cold extremities)* • *Hypoglycaemia (< 40 mg/dl)* • *Haemoglobinuria (dark coloured urine)* • *Kidney failure (little or no urine in a well-hydrated patient).* • *Jaundice (yellowish coloring of the eyes)* • *Spontaneous bleeding (Disseminated intravascular coagulation)*		

If any of the responses fall within the shaded area, exclude the patient from the study.

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APPENDIX II

Detailed MIP targets, genomic coordinates and sequence information for the drug resistance markers

Gene	MIP	Targets	Chromosome	Extension Start	Extension End	Ligation Start	Ligation End	Capture Start	Capture End	Orientation
dhfr-ts	dhfr-ts_S0_Sub0_mip2	N51,C59,S108	chr4	748552	748526	748140	748165	748166	748525	reverse
dhfr-ts	dhfr-ts_S0_Sub0_mip3	S108,I164,T185	chr4	748341	748365	748748	748715	748366	748714	forward
dhfr-ts	dhfr-ts_S0_Sub0_mip0	V16	chr4	748094	748114	748520	748486	748115	748485	forward
dhps	dhps_S0_Sub0_mip5	A437,S436	chr8	549584	549607	549983	549954	549608	549953	forward
dhps	dhps_S0_Sub0_mip6	K540,A581,A613	chr8	550320	550298	549876	549909	549910	550297	reverse
crt	crt_S0_Sub0_mip1	M74,C72,H97,K76,N75	chr7	403764	403737	403498	403521	403522	403736	reverse

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Gene	MIP	Targets	Chromosome	Extension Start	Extension End	Ligation Start	Ligation End	Capture Start	Capture End	Orientation
crt	crt_S0_Sub0_mip0	M74,C72,H97,K76,N75	chr7	403471	403491	403730	403696	403492	403695	forward
crt	crt_S0_Sub1_mip2	A220	chr7	404262	404294	404606	404578	404295	404577	forward
crt	crt_S0_Sub1_mip3	A220	chr7	404649	404621	404297	404326	404327	404620	reverse
crt	crt_S0_Sub2_mip5	I356,N326	chr7	405329	405352	405633	405604	405353	405603	forward
crt	crt_S0_Sub2_mip4	N326	chr7	405464	405433	405132	405157	405158	405432	reverse
crt	crt_S0_Sub2_mip6	I356	chr7	405796	405763	405535	405569	405570	405762	reverse
mdr1	mdr1_S0_Sub0_mip1	N86,Y184	chr5	958519	958496	958078	958104	958105	958495	reverse
mdr1	mdr1_S0_Sub0_mip3		chr5	959139	959118	958732	958766	958767	959117	reverse
mdr1	mdr1_S0_Sub0_mip2	Y184	chr5	958372	958393	958820	958796	958394	958795	forward
mdr1	mdr1_S0_Sub0_mip13	D1246	chr5	961693	961662	961286	961314	961315	961661	reverse
mdr1	mdr1_S0_Sub0_mip12	S1034,N1042	chr5	960967	960988	961386	961362	960989	961361	forward

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Gene	MIP	Targets	Chromosome	Extension Start	Extension End	Ligation Start	Ligation End	Capture Start	Capture End	Orientation
mdr2	mdr2_S0_Sub0_mip5	T484I	chr14	1956146	1956178	1956566	1956533	1956179	1956532	forward
k13	k13_S0_Sub0_mip1	C580Y	chr13	1724858	1724879	1725296	1725272	1724880	1725271	forward
k13	k13_S0_Sub0_mip0		chr13	1725022	1724999	1724576	1724609	1724610	1724998	reverse
k13	k13_S0_Sub0_mip3	R539T,M476I,I543T,Y493H	chr13	1725320	1725342	1725763	1725736	1725343	1725735	forward
k13	k13_S0_Sub0_mip2	R539T,M476I,I543T,C580Y,Y493H	chr13	1725650	1725623	1725203	1725230	1725231	1725622	reverse
k13	k13_S0_Sub0_mip5		chr13	1725922	1725945	1726370	1726341	1725946	1726340	forward
k13	k13_S0_Sub0_mip4		chr13	1726123	1726097	1725703	1725727	1725728	1726096	reverse
k13	k13_S0_Sub0_mip7		chr13	1726495	1726521	1726916	1726889	1726522	1726888	forward
k13	k13_S0_Sub0_mip6		chr13	1726712	1726680	1726267	1726301	1726302	1726679	reverse
k13	k13_S0_Sub0_mip8		chr13	1727152	1727131	1726730	1726763	1726764	1727130	reverse

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Detailed MIP targets, genomic coordinates and sequence information for *pfama1* gene

Gene	MIP	Probe	Capture Start	Capture End	Orientation
ama1	ama1_S0_Sub0_mip0	ama1_S0_Sub0_mip0_ref	1293915	1294303	forward
ama1	ama1_S0_Sub0_mip1	ama1_S0_Sub0_mip1_ref	1294205	1294593	reverse
ama1	ama1_S0_Sub0_mip2	ama1_S0_Sub0_mip2_ref	1294402	1294796	forward
ama1	ama1_S0_Sub0_mip3	ama1_S0_Sub0_mip3_ref	1294692	1295083	reverse
ama1	ama1_S0_Sub0_mip4	ama1_S0_Sub0_mip4_ref	1294871	1295229	forward
ama1	ama1_S0_Sub0_mip5	ama1_S0_Sub0_mip5_ref	1295159	1295561	reverse
ama1	ama1_S0_Sub0_mip6	ama1_S0_Sub0_mip6_ref	1295333	1295719	forward

Full sequences of probes and capture sequences can be found in the google document link below:

https://docs.google.com/spreadsheets/d/1E2tFZnR6tbkxy5mSDHREdPtfG1to9VWYRkS2w7x2Y-U/edit#gid=319586533

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Link to *pfama1* haplotypes from this study is shown below:

https://docs.google.com/spreadsheets/d/11MF7cY2JZ7tQ8wWn_JQmEJJaofujP4EfGozIit9mN-Y/edit

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APPENDIX III

A. NMIMR-IRB PARENTAL CONSENT FORM

Title: **Impact of Distinct Eco-epidemiology on Malaria Drug Resistance in Ghana**

Principal Investigator: Dr. Anita Ghansah

Address: Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, P.O. Box LG 581, Legon

General Information about Research

We are researchers from the Noguchi Memorial Institute for Medical Research, University of Ghana. We are about to conduct a study into malaria in this community and would like to invite you to take part in it.

Malaria is caused by a *germ* that is passed from one person to the other through the bite of a mosquito that carries the malaria germ. This disease affects all age groups both male and female. In children, the disease can be particularly severe and may cause death.

The spread of malaria *germs* (*Plasmodium falciparum)* that are resistant to the current malaria drugs is a major threat to malaria control, and requires monitoring of the effectiveness of the new artemisinin based combination treatments (ACTs). Monitoring of recently removed drugs like chloroquine is also important because it will tell us whether the malaria *germ* can now be killed again by the removed drugs as the germs may have forgotten how to resist these removed drugs. The goal of this study is to improve current methods for surveying drug sensitivity with new methods, and reveal the importance of the number of types of malaria *germs* present when we are monitoring the effectiveness of the drugs. The study will be conducted in Begoro, in the Forest region and Cape-Coast in the

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Coastal Savannah region of Ghana to see if there were differences in how the malaria *germs* behave in the different regions. This will help the National Malaria Control Programme (NMCP) to decide on the continued use of drugs for malaria treatment. In addition, continuous and effective monitoring will detect the presence malaria *germs* that are becoming resistant to the new drugs and which region it is coming from so that they will be controlled early before such malaria *germs* spread in the population to make the new drugs no longer effective.

Study procedure

The study will be conducted in Begoro, in the Forest region and Cape-Coast in the Coastal Savannah region of Ghana. A total of 200 children/year from the two regions i.e. 100 per region (800 over four years) with mild malaria who meet the inclusion criteria will be enrolled at the district hospitals and treated with Atesunate Amodiaquin, (AA) or Atesunate Lumefantrine (AL) and monitored for 28 days. The study will be conducted during the rainy season, from June-October for 4 years. In addition 100 different school children/region/per year for 4 years (800 in all for the 2 regions) will also be recruited to provide information about the types of malaria *germs* if any, children who are not ill from malaria.

If you agree for your child/ward to participate in this study, you will be asked some few questions about his/her state of health, we will take his/her temperature, weight and take about one tea spoon full of blood from his or her veins before your child is treated with the standard malaria drugs. Your child will also provide about half a tea spoon full of blood on the 3rd and 28th day from a finger prick. We will test for malaria *germs*, how the malaria *germs* respond to the drugs, and to find out the types and number of different types of

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malaria *gems* present in his or her blood. We will also put some of the blood on a special paper for analysis. If your child is recruited from the school, he or she will be pricked in the finger to take about half a teaspoon full of blood, to look at the types and number of different types of malaria germs in the children of the towns. Your child/ward's (recruited from the school) participation in the study will last for about 1 hour and will end today

Possible Risks and Discomforts

When we prick the finger of your child/ward or inject the vein of your child/ward to take blood, he/she will feel a slight pain and there is a small risk of bruising and bleeding that may occur due to the finger prick or injection. However, the procedure will be done with new and clean materials and we will make sure that there is no excessive bleeding from the punctured wound. Any injuries that your child/ward may sustain as a result of his/her participation in the study will be taken care of by the study.

Possible Benefits

Although there are no direct benefits for participating in the study, the knowledge that we will gain will help in the development of new malaria control measures which will be of benefit to people in areas where malaria is a serious problem such as Ghana. If your child/ward recruited from the school is suspected to have malaria on the day of the survey, he/she will receive the recommended treatment at the time of the survey free of charge. However, if the malaria is serious or you are suspected to have other illnesses during the survey, you will be referred for further evaluation and treatment at the hospital.

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Confidentiality

The data that we will collect will be given unique identifiers so that nobody can link the data to the individuals. We will not also share your child/ward's data with a third party without receiving your consent or that of the Noguchi Institutional Review Board, IRB. After working with the data, it will be stored for a period of 10 years in locked cabinets and only the study investigators can access it.

Future Use of Stored Specimens

After the study, the remaining samples will be stored in the Biological specimen repository of the Noguchi memorial Institute for medical Research. The stored samples may be used for future malaria research. If you do not agree to the future use of your sample, the remaining samples will be discarded after this study and we will document the date and method of destroying the samples in our laboratory records.

Voluntary Participation and Right to Leave the Research

Participation in this study is completely voluntary. You can decide not to allow your child/ward participate in this study without giving any reason. If you decide not to allow your child/ward participate in the study, he/she will not lose any benefits that he/she would have otherwise been entitled to. If after you agree to allow your child/ward to participate in the study and you decide later that you want to withdraw him/her, you have the right to do so at anytime without being compelled to continue. If you decide to withdraw your child/ward from the study, it will not affect the medical care that he/she is entitled to.

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Contacts for Additional Information

If you need more information on this study, you may contact, Dr Anita Ghansah, the principal investigator of this project (0271405535), Professor Kwadwo Koram, the Director of Noguchi Memorial Institute and also a co-investigator (02404313130) or Dr Benjamin Abuaku on 0244573235.

Your rights as a Participant

This research has been reviewed, the potential risks and benefits have been evaluated and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: HBaidoo@noguchi.mimcom.org . You may also contact the chairman, Rev. Dr. Ayete-Nyampong through mobile number 0208152360 when necessary. You will be given a copy of the signed informed consent form.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title " Impact of Distinct Eco-epidemiology on Malaria Drug Resistance in Ghana" has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree that my child should participate as a volunteer.

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_____ _____

Date Name and 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 child's parent or guardian. All questions were answered and the child's parent or guardian has agreed that his or her child takes part in the research.

_____ _____

Date Name and 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 Name Signature of Person Who Obtained Consent

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B. NMIMR-IRB CHILD ASSENT FORM

Title: **Impact of Distinct Eco-epidemiology on Malaria Drug Resistance in Ghana**

Principal Investigator: Dr. Anita Ghansah

Address: Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, P.O. Box LG 581, Legon

General Information about Research

We are researchers from the Noguchi Memorial Institute for Medical Research, University of Ghana. We are about to conduct a study into malaria in this community and would like to invite you to take part in it.

Malaria is caused by a *germ* that is passed from one person to the other through the bite of a mosquito that carries the malaria germ. This disease affects all age groups both male and female. In children, the disease can be particularly severe and may cause death.

The spread of malaria *germs* (*Plasmodium falciparum)* that are resistant to the current malaria drugs is a major threat to malaria control, and requires monitoring of the effectiveness of the new artemisinin based combination treatments (ACTs). Monitoring of recently removed drugs like chloroquine is also important because it will tell us whether the malaria *germ* can now be killed again by the removed drugs as the germs may have forgotten how to resist these removed drugs. The goal of this study is to improve current methods for surveying drug sensitivity with new

If you agree to be in this study, you will be pricked in the finger to take about half a teaspoon full of blood, to look at the types and number of different types of malaria germs

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in the children of the towns. Your participation in the study will last for about 1 hour and will end today

Although there are no direct benefits for participating in the study, the knowledge that we will gain will help in the development of new malaria control measures which will be of benefit to people in areas where malaria is a serious problem such as Ghana. You will receive the recommended treatment at the time of the survey free of charge if suspected to have malaria on the day of the survey. However, if the malaria is serious or you are suspected to have other illnesses during the survey, you will be referred for further evaluation and treatment at the hospital.

When we prick your finger to take blood, you will feel a slight pain and there is a small risk of bruising and bleeding that may occur due to the finger prick. However, the procedure will be done with new and clean materials and we will make sure that there is no excessive bleeding from the punctured wound. Any injuries that you may sustain as a result of your participation in the study will be taken care of by the study

You can stop participating at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate.

Your information will be kept confidential. No one will be able to know how you responded to the questions and your information will be anonymous.

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You may ask me any questions about this study. You can call me at any time on 0271405535 or Dr Benjamin Abuaku on 0244573235 or talk to me the next time you see me.

Please talk about this study with your parents before you decide whether or not to participate. I will also ask permission from your parents before you are enrolled into the study. Even if your parents say "yes" you can still decide not to participate.

By signing below, it means that you understand and know the issues concerning this research study. If you do not want to participate in this study, please do not sign this assent form. You and your parents will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled: **Impact of Distinct Eco-epidemiology on Malaria Drug Resistance in Ghana** has been read and or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

Child's Name:……Researcher's Name:……

Child's Signature/Thumbprint:……Researcher's Signature……..

Date: **…….....** Date: **……**

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C. ETHICAL CLEARANCE FOR THE STUDY

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

Phone: +233-302-916438 (Direct)
+233-289-522574
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E-mail: nirb@noguchi.mimcom.org
Telex No: 2556 UGL GH

Post Office Box LG 581
Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

13th July, 2016

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 059/12-13 ***amend. 2016*** **IORG 0000908**

On 13th July 2016, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and amended your protocol titled:

TITLE OF PROTOCOL	:	**Impact of distinct eco-epidemiology on malaria drug resistance in Ghana** ***"Multiplicity of Plasmodium falciparum infection among symptomatic and asymptomatic children in two ecological zones in Ghana"***
PRINCIPAL INVESTIGATOR	:	**Dr. Anita Ghansah**
STUDENT INVESTIGATOR	:	**Ms. Benedicta Ayiedu Mensah**
CO – INVESTIGATORS	:	**Prof. Koram, Dr. Dyann Wirth, Dr. Michael Ofori, Dr. Benjamin Abuaku, Dr. Daniel Neafsey et al**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 12th July, 2017. You are to submit annual reports for continuing review.

Signature of Chair: .....

Mrs. Chris Dadzie
(NMIMR – IRB, Chair)