THE GENETIC DIVERSITY OF PLASMODIUM FALCIPARUM IN GHANA

BY

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DECLARATION

I hereby declare that this research project was duly carried out by me and was undertaken under the supervision and direction of Professor Yaw Asare Afrane and Dr Linda Eva Amoah. The findings have not been presented in whole or part for another degree elsewhere.

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DEDICATION

This thesis is dedicated to my parents, Cynthia Bulley and Kobina Amoah for their wonderful encouragement, endless support and contribution to my education. To my sister, Maame Araba Dawson-Amoah and Tenace Kwaku Setor.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
GHS	Ghana Health Service
NMCP	National Malaria Control Programme
NMIMR	Noguchi Memorial Institute for Medical Research
OPD	Out Patient Department
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
RDT	Rapid Diagnostic Test
Nested PCR	Nested Polymerase Chain Reaction
RNA	Ribonucleic Acid
WHO	World Health Organization
CDC	Centre for Disease Control and Prevention
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
malERA	Malaria Eradication Research Agenda
$MgCl_2$	Magnesium Chloride
ml	Millilitre
^oC	Degree Celsius

ABSTRACT

Background

The population structure of the causative agents of human malaria, *Plasmodium* species including the most serious agent *Plasmodium falciparum*, depends on the local epidemiological and demographic situations, such as the incidence of infected people, the vector transmission intensity and migration of inhabitants (i.e. exchange between sites).One of the major characteristics of malaria parasites is their genetic diversity and an increasing number of studies have reported on the population structure variation of *P. falciparum*. Genetic diversity and population structure of *P. falciparum* is required in predicting the origin and spread of novel variants within a population enabling specific malaria control measures in the era of intensive malaria intervention strategies.

Aim

This study investigated the genetic diversity of *P. falciparum* in sites with different malaria endemicity in Ghana.

Methods

Finger prick blood samples were made from primary school children in five study sites in Ghana. This was carried out during the dry (March) and rainy (July) seasons of 2017. The samples were collected on filter paper and on slides and examined for parasite detection using light microscopy (LM), and molecular diagnosis (18s rRNA nested PCR). The positive *P. falciparum* samples were characterized using microsatellite markers.

Results:

Light microscopy showed the highest prevalence in the Duase during the rainy season with 24.1%. Kpasolgu during the dry season sampling exhibited the lowest prevalence of *P. falciparum* parasites with 7.1%. The southern sites, Anyakpor and Odumase showed an

intermediary prevalence of 12.8% and 16.9% respectively in the rainy season. This was the highest prevalence between the rainy and dry seasons in the southern sites. The sites Pagaza and Kpalsogu found in the northern part of Ghana, the Sahel savannah zone presented 17.7% and 18.2% prevalence respectively. This is according to light microscopy. Nested PCR detected a higher level of parasites in all samples. Duase in the Forest zone showed the highest prevalence, 58.8% in the samples collected in the rainy season. The Forest zone exhibited 9.8% indicating the lowest prevalence among the sites from the samples obtained in the dry season in Duase. This affirms that PCR is a more sensitive tool of detection of parasites in determining the parasite carriage. In determining the variation in diversity, genetic differentiations in the different sites tested were observed. There was a higher genetic diversity evidenced by the highest frequency of heterozygosity in the Northern region.

Conclusion

In this study, little significant differentiation in the genetic diversity was observed between the different ecological zones in Ghana. This reveals a range of population structures within the species of *P. falciparum* and suggests how genetic variation impacts the epidemiology and control measures between different transmission zones in Ghana.

CHAPTER ONE

1.1 Introduction

Malaria is a mosquito-borne disease that has been a major global health risk worldwide for many years. It is common in countries with tropical and subtropical climates. Currently, there are more than 100 countries and territories where the risk of malaria transmission is still a threat. This major global health burden is estimated to have caused 216 million clinical cases in 91 countries and 445,000 deaths worldwide in 2017 (WHO, 2018; Ashley *et al.*, 2018). Among the estimated deaths that occurred worldwide, 80% were in sub-Saharan Africa and 60% affected children under 5 years of age. However, the current integrated strategies and interventions against both the parasite and the vector have led to a reduction in malaria mortality by 47% globally and 37% in the African Region (Hemingway *et al.*, 2016; WHO, 2017).

In humans, malaria is caused by protozoan parasites of the genus *Plasmodium.* There are five species of protozoan parasites of the genus*: P. falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi* and *P. malariae*. Although *P. vivax* is responsible for most malaria infections in the world, the most severe form of malaria is caused by *P falciparum.* The severity of malaria illness depends largely on the immunological status of the person who is infected. Repeated infection over a period of time develops into partial immunity, and without the reoccurrence of infection, immunity will be short-lived (Carpenter *et al*., 1991).

The distinction between infection and disease is particularly significant in regards to malaria since infection does not necessarily result in disease. In malaria-endemic areas, many infected people are asymptomatic; they may harbour large numbers of parasites however, exhibit no outward signs and symptoms of the disease. Notwithstanding, asymptomatic individuals contribute to the transmission of malaria.

P. falciparum infections can result in asymptomatic carriage, uncomplicated or severe malaria (Laishram *et al.*, 2012). The different manifestations of malaria cases are a function of immunity with respect to age and exposure to the parasite (Doolan *et al.*, 2009). Malaria infections that are not detectable with light microscopy are referred to as submicroscopic infections (Okell *et al.*, 2009). Submicroscopic infections occasionally result in acute disease (Rogier *et al.*, 1996), thereby contributing to the transmission of the parasite by transmitting to mosquitoes. The epidemiology of malaria transmission and the severity of the disease vary from region to region, community to community, and even from person to person within a community. This may be due to the different strains of the malaria parasite; the reasons for these differences are however, not fully understood (WHO, 2015).

Information on the nature and extent of genetic diversity within *P. falciparum* is essential in understanding the mechanism underlying the pathology of malaria, the acquisition of immunity, the spread of drug resistance, the condition of transmission and the development of vaccines against the parasite (Ringwald *et al.*, 2000; Basco *et al.*,2004; Yah *et al.*, 2010).

Genetic diversity in the malaria parasite is as a result of genetic recombination from numerous allelic polymorphisms presented at various genetic loci. This has an impact on efforts towards development of vaccines and enhances antimalarial drug resistance (Paul *et al.*, 1998; Hoffman *et al.*, 2001). The level of antigenic diversity resulting in the different infections varies from one malaria endemic region to another and between countries. Thus, the variant forms of the parasite exist at different frequencies in different geographic areas presenting different complexities of infection (Raj *et al.*, 2004). The genetic diversity of parasites has been implicated in evolutionary fitness. Consequently, populations with high diversity have the ability to survive against on-going interventions in malaria endemic areas thereby affecting control efforts (Barry *et al.*, 2013; Dhafer, 2003).

While many responses to detect malaria infections in the form of control and elimination programmes exist, there are challenges such as difficulty to identify mixed infections and/or low levels of parasitemia. There is also no systematic description on how genotyping and molecular studies could contribute towards developing tools to control and manage malaria cases and how these could be included in a surveillance programme. The goal would be to incorporate novel monitoring and malaria evaluation metrics to an accurate mapping of focal points including circulating *Plasmodium* genotypes and human individuals driving transmission (Talundzic *et al.*, 2015).

These focal points can be accurately explored using genetic polymorphisms such as single nucleotide polymorphisms (SNPs) and short tandem repeats (STRs) or microsatellite markers. Genetic polymorphism in *P. falciparum* populations is important as understanding genetic variability may provide the potential for populations to adapt quickly when faced with environmental changes such as introduction of new antimalarial drugs. Microsatellites or single sequence repeats (SSRs), on the other hand, is more suitable for this purpose due to their mutation rate; they gain or lose repeat units by DNA-replication slippage, a mutation mechanism that is specific to tandemly repeated sequences (Schlötterer *et al.*, 2000).

Microsatellites have been suitably used to investigate the population structure of *Plasmodium* (Anderson *et al.*, 1999, Machado *et al.*, 2004; Karunaweera *et al.*, 2008; Van den Eede et *al.*, 2010; Chenet *et al.*, 2012) and optimized protocols for microsatellite genotyping and analysis in *P. falciparum* and *P. vivax* are currently available (Chenet *et al.*, 2012; Orjuela-Sánchez *et al.*, 2013) as well as guidelines for data interpretation according to the specific research question. As a result of the wide range of malaria transmission rates, control programmes are centred on the reduction of the disease burden until it has been maintained at a considerable level to proceed towards the elimination stage. Nonetheless, local heterogeneity in malaria

incidence reduces the efficacy of control strategies. Profiling these cases is crucial to determine the kind of health care services needed (Carter *et al.*, 2000; WHO, 2016). For control programmes to be successful, research on useful and efficient modes of detection, monitoring and response must be undertaken. By understanding the parasite genetic diversity and gene flow dynamics with and between human populations, effective spatial and temporal boundaries for intervention can be implemented. Accordingly, one of the main strategies should focus on characterizing changes in *Plasmodium* population genetics particularly, *P. falciparum* due to the prevalence in Ghana. This is to determine fluctuations in local transmission intensity in the different ecological zones and provide valuable tools for malaria surveillance as well as the evaluation of malaria elimination programmes.

1.2 Problem Statement

In malaria-endemic regions, *P. falciparum* infection is characterized by extensive genetic diversity. Research on the diversity of malaria parasites in Ghana is limited and in the search for an effective vaccine in malaria endemic regions it is crucial to determine genetically, the parasite population in different transmission settings in the country.

Parasite population analysis with neutral microsatellite markers sampled across the genome in asymptomatic malaria individuals have been used to analyse parasites from different parts of the world. The use of these neutral microsatellite markers to describe different transmission or ecological zones are under researched in Ghana. Researchers are also yet to be determine whether, regions of different transmission intensities have parasite populations that are genetically similar or identical with heritable traits in Ghana. This could impact malaria control.

There is an increasingly challenging problem of malaria control in Sub-Saharan Africa due to issues of the development of resistance to commonly used anti-malarial drugs in parasites, to

insecticides in the vector, the parasite and operational problems. Consequently, to develop suitable and novel control strategies against the parasite, it is important to know the extent of genetic diversity existing in the parasite population as well as across different areas of transmission or ecological zones.

The outpatient departments (OPDs) in Ghana, still records a high number of malaria cases in hospitals with 22.3% deaths. The vulnerable groups mostly affected are children and pregnant women. The availability of an effective malaria vaccine is one of the effective ways at combating the prevalence of malaria transmission in the country and the world at large. Genetic diversity of the parasites is still one of the challenges affecting the development of the vaccine (Duah *et al.*, 2016).

1.3 Justification

As malaria control and elimination programmes pursue mass screening and treatment, further studies are needed on the epidemiology of malaria in different eco-epidemiologic landscapes to understand the contribution of submicroscopic infection to the transmission of malaria.

Studies on the genetic diversity of *P. falciparum* will help to adequately monitor and evaluate malaria intervention programmes within the study areas and Ghana as a whole. The surveillance of the gene flow of the parasite across different transmission areas and drug resistant strains will be properly understood.

The *msp-1* and *msp-2* genes have been used in the past in studying the genetic diversity of malaria parasites, however, neutral markers such as microsatellites or single nucleotide polymorphism (SNPs) are better suited for population genetics assessment (Su *et al.*, 2009). These markers can be employed to investigate genetic diversity, multiplicity of infections and parasite carriage. The genotyping technique will also aid to differentiate between several clones of *P. falciparum and*

ultimately provide very useful information on the population genetics of the parasite in different transmission intensities.

This study will provide knowledge on the level of submicroscopic carriage of *P. falciparum* in different malaria settings in Ghana. It will also provide knowledge that will aid future researchers using similar markers to genotype the parasites in different geographical settings in the country. Subsequently, data on the genetic trends of the parasite will assist in establishing a link between the intensity of transmissions in different geographical areas, parasites prevalence and clinical manifestation of *P. falciparum* which is central to the fight against malaria (Balam *et al*., 2014).

1.4 Main objective of the Study

The main objective of the study was to investigate the genetic diversity and population structure of *P. falciparum* in sites with different malaria endemicity in Ghana.

1.5 Specific Objectives

The specific objectives are to:

i. determine the *P. falciparum* carriage in school children in the different malaria transmission zones in Ghana.
ii. determine the genetic diversity and population structure of *P. falciparum* parasites in different malaria transmission zones of Ghana.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria

2.1.1 Pathogenesis of malaria

Malaria is a parasitic disease caused by protozoa of the genus *Plasmodium* and is transmitted through the bite of an infected female *Anopheles* mosquito. It is preventable and curable if diagnosed and treated promptly and correctly (WHO, 2018). In the human body, the parasites multiply in the liver and then infect red blood cells leading to their rupture with subsequent development of complications such as anaemia, depending on severity of the infection. Malaria is most prevalent in tropical and sub-tropical regions. This is due to high rainfall, high temperatures, high humidity and the high presence of stagnant waters where mosquito larvae rapidly mature. The vectors for the transmission of the disease in sub-Saharan Africa are mainly females of the *Anopheles gambiae* species complex.

Malaria can be classified as an uncomplicated or severe disease and may also be asymptomatic. It is classified as uncomplicated when the patient presents with a combination of symptoms such as fever, chills, headache, muscle and joint aches. Other symptoms include tiredness, nausea, vomiting and diarrhoea as well as the presence of parasites in the blood which is usually detected by microscopy .The early symptoms of uncomplicated malaria are non-specific and thus malaria is usually diagnosed on the basis of only symptoms, especially in endemic areas (WHO, 2010). Accurate diagnosis, as in laboratory confirmation of the presence of the parasite, is essential for effective management of the disease in all settings. *P. falciparum* infections that are not promptly treated can quickly progress to severe malaria due to the ability to attain high levels of parasitaemia during its life cycle. The main symptoms of severe malaria generally include coma, severe breathing difficulties, low blood sugar, prostration and severe anaemia. It is diagnosed on the basis of the presence of *P. falciparum* parasites and one of the above symptoms with no other obvious

cause. Children, particularly under five years of age are most affected since they have low immunity against the parasite. Severe malaria can lead to death if prompt treatment is not given.

Asymptomatic malaria refers to the presence of malaria parasites in the blood without symptoms, and is an antecedent to symptomatic malaria (Das *et al.,* 2015). Understanding the impact of the parasite on the human host across the range of severity is important for learning how to improve the management of the disease.

2.2 The Epidemiology of Malaria

Malaria is a febrile illness that remains one of the leading causes of morbidity and mortality in the world. It affects 216 million people all over the world and causes 1-3 million deaths annually (WHO, 2018). It is widespread in tropical and sub-tropical regions of the world particularly; Africa. It is estimated that, in the Tropical African Region, has the heaviest burden of malaria deaths with approximately 90% malaria deaths occurring and 78% malaria deaths occurring in children less than 5 years of age (WHO, 2017). Nevertheless, the incidence rate of malaria is estimated to have decreased by 18% globally, from 76 to 63 cases per 1000 population at risk, between 2010 and 2016 (Figure 2.1) (WHO, 2017).

P. falciparum is the most prevalent malaria parasite in sub-Saharan Africa, accounting for 99% of estimated malaria cases. Outside of Africa, *P. vivax* is the predominant parasite in the WHO Region of the Americas, representing 64% of malaria cases, and is above 30% in the WHO South- East Asia and 40% in the Eastern Mediterranean regions (WHO, 2017). This is due to its ability to develop in the vector at lower temperatures, and also to survive at higher altitudes and in regions of cooler climates (World Malaria Report 2016; 2017).

Sub-Saharan Africa carries a disproportionately high share of the global malaria burden. In 2015, this region was home to 90% of malaria cases and 92% of malaria deaths. The most vulnerable were children under five years of age, accounting for an estimated 70% of all malaria deaths in the region (WHO, 2016).

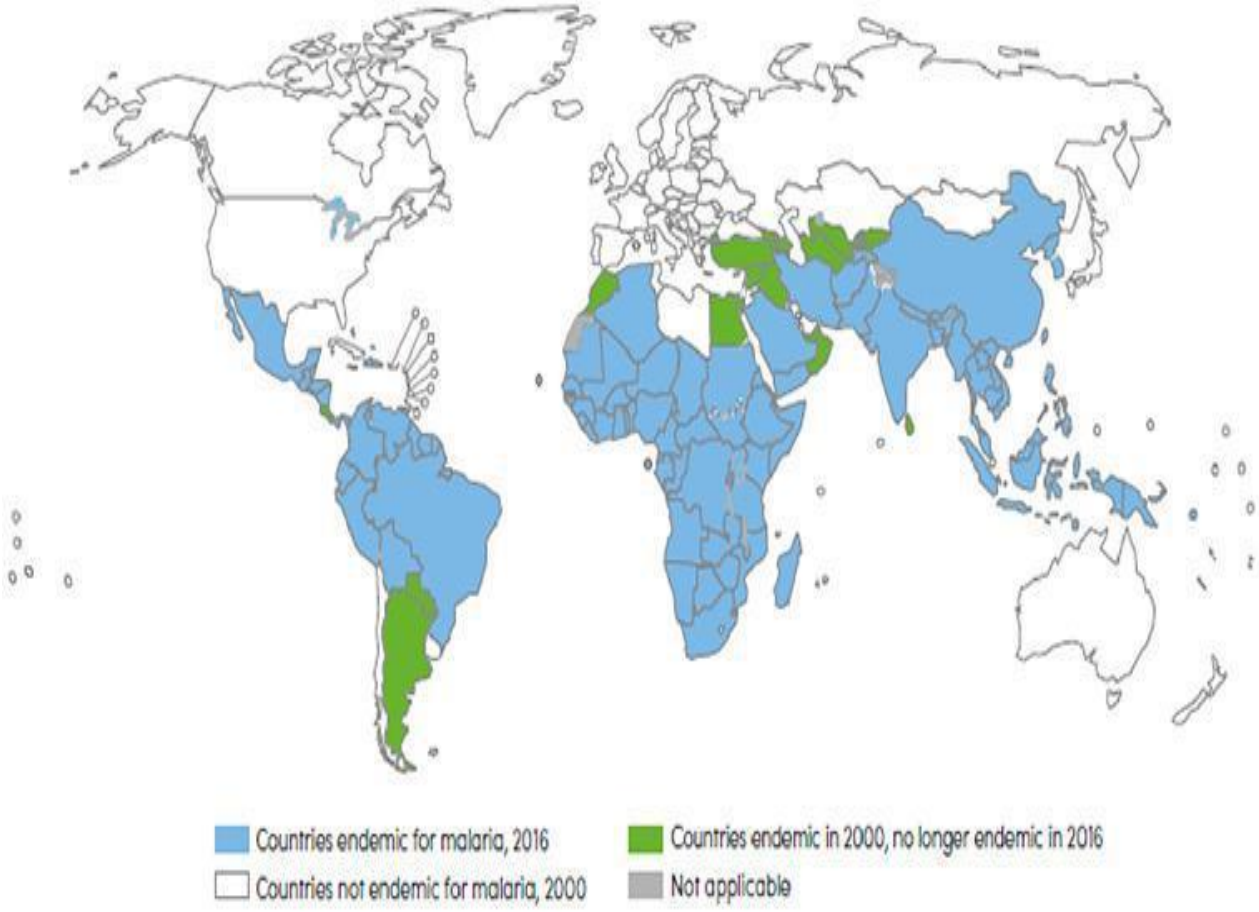

Figure 2.1: Countries Endemic for Malaria

(Source: WHO World Malaria Report, Geneva 2016)

2.3 Malaria Burden in Ghana

Malaria is endemic throughout Ghana and transmission is varied, differing along the different ecological zones. Ghana is made up of three ecological zones; the Sahel savannah found in the northern part of Ghana, the Forest zone in the middle belt of Ghana and the Coastal savannah

zone found in the southern part of the country. Malaria transmission surges during the rainy season with the peak of transmission observed between June-October annually. The Forest and Coastal ecological areas have malaria transmission peaks twice in a year. Transmission levels are lower in the Coastal and Forest zones as transmission is higher in the Sahel savannah zone.

According to the National Malaria Control Programme, about 10.4 million malaria cases are recorded annually resulting in about 26,517 malaria deaths. In 2017, the country recorded approximately 10.2 million suspected malaria cases representing about 34% of OPD cases (National Malaria Control Programme, 2017). Overall, about 19.0% and 2.0% of total admission and total deaths respectively were attributable to malaria. The infection is still a threat to life in children less than five and pregnant woman in Ghana even though some successes have been chalked in recent years in terms of combating the disease in the country (DHIMS, 2018).

2.4 The Life Cycle and Transmission of *Plasmodium* Species

The life cycle of the *Plasmodium* parasite begins in a vertebrate host and ends in an insect vector. Human infection is initiated with a bite from an infected female *Anopheles* mosquito, (Figure 2.2) by injecting sporozoites into the blood stream while taking a blood meal. Approximately 10-100 sporozoites are injected and deposited into the skin of the human host (Doolan *et al.,* 2009; Nkhoma *et al.,* 2012). Sporozoites travel to the liver and invade hepatocytes and multiply asexually to become schizonts that contain tens of thousands of rounded merozoites, per liver cell. This process is known as the pre-erythrocytic cycle or schizogony. It lasts for 6-8 days for *P. falciparum* (Bousema *et al.* 2014). The merozoites are then released into the bloodstream, which initiates the asexual parasite multiplication stage. Some malaria parasite species (*P. vivax*) remain dormant for extended periods in the liver, causing clinical relapses weeks or years later, known as the hypnozoite (Lin *et al*. 2014).

Within the erythrocytes, the parasites feed on haemoglobin and develop into ring-shaped trophozoites.

Repeated cycles of asexual replication within red blood cells (48 hrs for *P. falciparum*) result in thousands of parasite-infected red cells in the host bloodstream, leading to illness and complications of malaria that can last for months if left untreated.

On the other hand, a fraction of the merozoites develop into sexual forms, the gametocytes (micro and macrogametes), which are the only form of the parasite that can be transmitted from human to the vector. *P. falciparum* takes 10-12 days for gametocytes to mature in the bone marrow (Farfour *et al*., 2012) before appearing in peripheral blood where they persist with an estimated mean circulation time of 3.4–6.5 days per gametocyte (Bousema *et al*. 2010). The matured gametocytes circulate in the blood stream until they are picked up by another vector as it takes a blood meal from the infected human. The asexual stage parasites may develop into gametocytes under unfavourable conditions such as stress or the presence of antimalarial drugs (Meibalan *et al.,* 2016). They can be detected in the bloodstream 7 to 15 days from when asexual parasites are spotted (Day *et al.,* 1998). High asexual stage parasitaemia may result in high gametocytemia and subsequently may become more infectious to mosquitoes for transmission (Lin *et al.,* 2014). The density of mature gametocytes in peripheral blood is usually less than 100 gametocytes per µl of blood (Bousema *et al.,* 2014) and the vast majority of these occur at densities not detected by microscopy. Submicroscopic gametocytemia therefore will be expected to be non-infectious to the mosquito; however, studies conducted in Burkina Faso and Kenya showed that children with gametocyte blood film negative could infect comparatively fewer mosquitoes unlike those with microscopic gametocytemia (Ouédraogo *et al.* 2009). The ability of a few mature gametocytes from submicroscopic infections to form gametes that will produce numerous oocytes which could develop into infectious sporozoites cannot be underestimated.

Each gametocyte ingested by a mosquito, forms one female macrogamete or up to eight male microgametes. Within the mosquito mid-gut, male and female gametes fuse to produce a zygote that develops into a motile ookinete, which can penetrate the mid-gut wall and form oocysts. The oocysts undergo sporogony to produce many threadlike sporozoites, which are released when the oocysts busts. The sporozoites then migrate to the mosquito salivary gland, from where they can infect humans during the next blood meal. The presence of mature gametocytes in the peripheral blood is a pre-requisite for transmission and spread of the infection (Webster-Kerr, 2010).

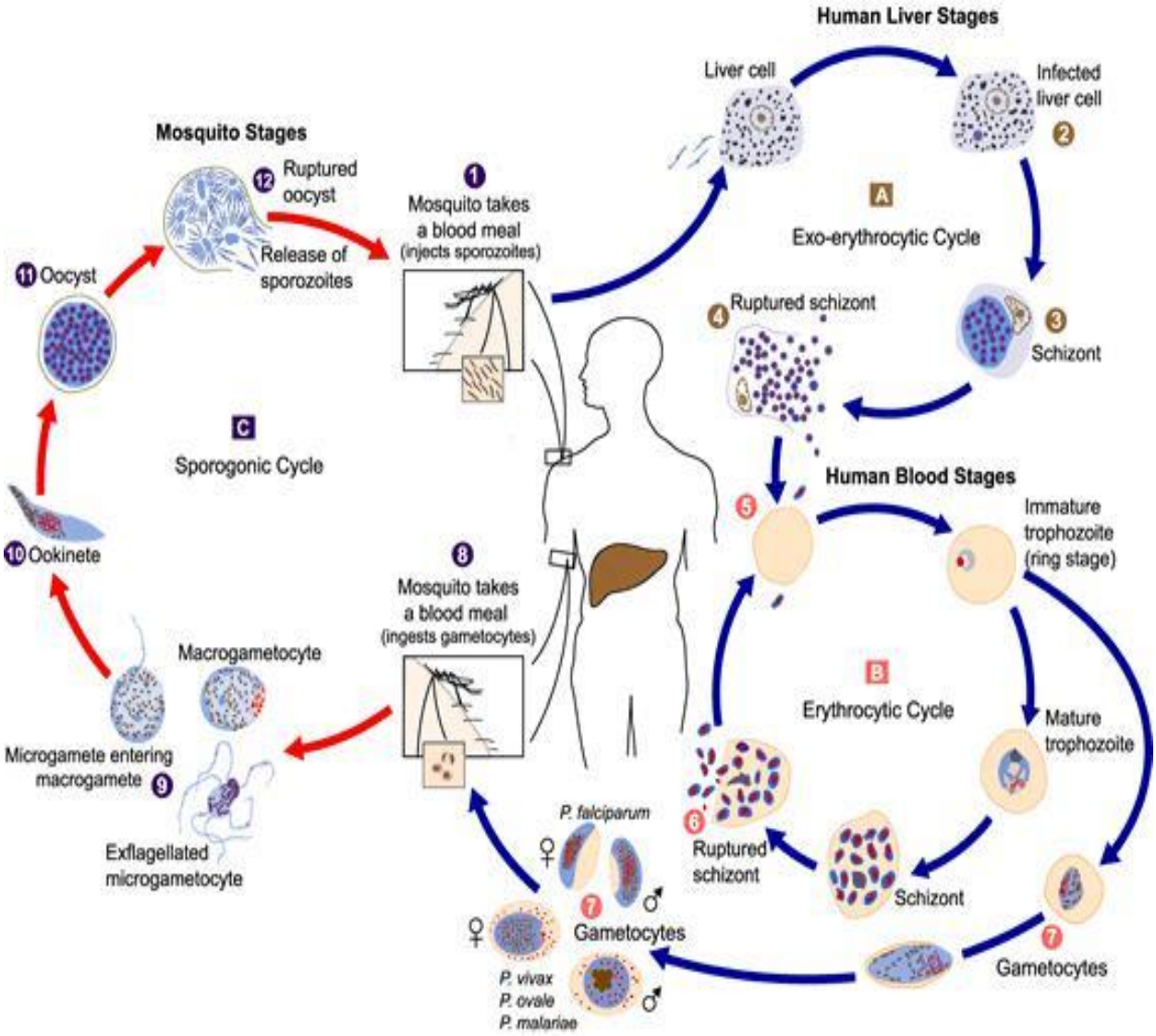

Figure 2.2. The life cycle of malaria parasite

(Source : http://www.cdc.gov/malaria/about/biology/)

2.5 Submicroscopic infection carriage in asymptomatic people

The ability to identify and treat the infection in asymptomatic population, is normally detected by microscopy. However, some asymptomatic infections are undetectable with microscopy and are referred to as submicroscopic or microparasitaemic infections. Submicroscopic infection is defined as low-density *Plasmodium* infection detected only by molecular methods (Hermsen *et al*., 2001). Repeated exposure to malaria may contribute to the build-up of immunity in an individual. This varies widely depending on the intensity of transmission and geographical settings (Njama-Meya *et al.*, 2007) and does not necessarily inhibit infection but can reduce parasite density and symptoms (Tran *et al*., 2013).

Asymptomatic infections are usually not promptly treated, due to the fact that they are not diagnosed early (Lin *et al.*, 2014). However, diagnosing asymptomatic malaria is not straightforward due to the evident lack of clinical manifestations and often subpatent (undetectable by microscopy) level of parasites (Worku *et al.*, 2014). Submicroscopic infection, usually constitutes an important parasite reservoir in asymptomatic populations (Okell *et al*, 2009). Therefore, as malaria transmission burden declines across Sub-Saharan Africa, through control intervention; low-level parasitemia present in asymptomatic individuals continues to maintain malaria transmission in affected populations.

Prompt and accurate diagnosis followed by rapid treatment with an effective antimalarial drug is crucial in malaria eradication and elimination. Nonetheless, the common and standard techniques used for diagnosis of *P. falciparum*, such as microscopy or rapid diagnostic tests (RDTs), are hampered by their low parasite detection limits (malERA, 2011; Ohrt *et al.*, 2008; Moody, 2002). These tools tend to underestimate *P. falciparum* infection when used alone due to their inability to detect low level parasitemia (Bousema *et al.*, 2004; Nkoghe *et al.*, 2011; Geiger *et al.*, 2013; Cook *et al.*, 2015). In recent times, molecular techniques for parasite detection have been introduced to

identify parasite densities in epidemiological studies and correctly estimate submicroscopic infection in many laboratories (Wampfler *et al.,* 2013; Karl *et al.,* 2014; Mwingira *et al.,* 2014; Golassa *et al.,* 2015)

The relevance of asymptomatic infections is now being re-evaluated in the light of malaria elimination initiatives, as malaria control focuses not only on clinical malaria but also on identifying and treating asymptomatic malaria infections (Geiger *et al.,* 2013; Wampfler *et al.,* 2013; Karl *et al.,* 2014; Mwingira *et al.,* 2014; Golassa *et al.,* 2015; Babiker *et al.,* 2013).

2.6 The Control and Elimination of Malaria

Over the last decade, there has been a substantial increase in international funding for malaria control through major international financing agencies such as Bill and Melinda Gates foundation and the US President's Malaria Initiative. This, together with renewed governmental commitment in endemic countries, has resulted in increased coverage of malaria interventions and a reduction in malarial disease and death in several countries, including several in sub-Saharan Africa where the malaria burden is greatest.

Ghana has a rich history of malaria control interventions, dating back to the pre-independence era. Interventions mainly targeted either the parasite in the host (in humans) or the vector (*Anopheles* mosquitoes). Interventions in the form of treatment targeting the parasite in the human host such as amodiaquine pyrimethamine, daraprim or pyrimethamine, primaquine, lapudrine and chloroquine were tested and administered in various ways before independence in the 1950s (National Malaria Control Program, 2013).

In the years between the 1950s and 2000, the use of monotherapies including chloroquine and other medications such as quinine were widespread. As in many endemic countries, the use of chloroquine and other interventions persisted until widespread parasite resistance to chloroquine was reported. This necessitated a policy recommendation for a change (Afari *et*

al., 1992). Thus a treatment policy change was made in 2004 to replace the first-line treatment regime to the artemisinin combination therapies (ACTs), i.e. artesunate-amodiaquine, for treatment of uncomplicated malaria. This treatment policy has since been revised twice, in 2007 and 2009, to include other ACTs, namely artemeter-lumefantrine and dihydroartemisinin-piperaquine (Ministry of Health, Ghana, 2009).

Vector control activities have also been in use since the pre-independence era. Interventions such as indoor residual spraying (IRS) and bed nets have been used before but not on a wide scale (National Malaria Control Program, 2013). These interventions were predominantly deployed in major cities. Insecticide Treated Bed nets (ITNs) were also deployed nationally in 2004, following evidence from field trials of their effectiveness in 1996 in Ghana and elsewhere. A policy was also made to subsidise delivery of ITNs in 2007 (National Malaria Control Program, 2013). Since 2005, IRS activities have been recommended; however, their deployment has been on a limited scale. The coverage levels for ACTs and ITNs have gradually risen to 100%.

Despite the high levels of coverage of the interventions, the level of malaria morbidity in Ghana remains relatively high (Ameme *et al.*, 2014), making it and other West African states unlikely to eliminate local transmission of the disease before 2030 (WHO,2016). Ghana has adopted a strategic action plan with a 2020 target to, among other things; reduce the burden of malaria in the population by 75.0% (National Malaria Control Program, 2016). This requires multidisciplinary research if the ultimate goal of elimination by 2030 is to be attained.

2.7 Genetic diversity of *Plasmodium falciparum*

Genetic diversity is the variation in the genetic composition within or among individuals in a population or species. A major factor in creating genetic diversity arguably is genetic recombination. This is classically defined as the process, by which organisms with novel combinations of genes are produced in crosses between two parent organisms (Conway & McBride, 1991). In *Plasmodium*, this event occurs primarily at the meiotic phase and allows genomic sites of the parasite to classify independently, thus it may act as a diversifying force. This may generate new genetic variants that can spread through the population driven by positive selection (Wootton *et al.* 2002). Genetic divergence varies considerably among the parasite populations and is often associated with local endemicity, transmission rates, geographic isolation levels and migration patterns of human and vector hosts (Anderson *et al*. 2000; Bogreau *et al*. 2006; Pumpaibool *et al*. 2009).

It has been discovered that *P. falciparum* genetic diversity is indicative of the ability of malaria parasites to adapt to their hosts by selection of advantageous traits, such as drug resistance and antigenic variability. In regions of high endemicity of *P. falciparum* such as Sub-Saharan Africa, human hosts often harbour two or more genetically distinct clones of the same parasite (Fraser-Hurt *et al*. 1999) and mosquitoes have high chances of taking up a mixture of genetically distinct gametocytes during a blood meal. Fusion of these two genetically distinct parasites (crossover fertilization) results into the generation of a large repertoire of genetically diverse parasites.

Previous studies established that infection complexity within a host was dependent on exposure (intensity of transmission) and the age of the host (indicative of the level of immunity), and this is negatively associated with the severity of the disease (Babiker *et al.*, 1994; Paul *et al.*, 1995; Ntoumi *et al.*, 1995; Contamin *et al.*, 1996; Al-Yaman *et al.*, 1997; Beck *et al.*, 1997).

Knowledge of the nature and extent of genetic diversity within the species becomes increasingly relevant as control measures become more sophisticated and more selectively targeted towards the molecular components of the parasite. Populations of *P. falciparum* are known to be genetically diverse, even at low levels of endemicity (Paul *et al.,* 1999; Bendixen *et al.,* 2001). This could imply that there is a remarkably high genetic diversity in endemic transmission settings such as Ghana. A study conducted by Jordan *et al.* (2001), found that parasites of major epidemics associated with abnormal weather and the extensions of malaria transmission to other non–malarious areas are also genetically diverse.

Studies have indicated that there is genetic recombination during the sexual phase of the life cycle of the parasite and this enhances the diverse nature of the parasite (Conway *et al.,* 1999; Cano *et al.*, 2007). Hence, malaria control measures must include drug resistance monitoring as well as assessment of parasite genetic diversity as a means of ensuring success of the control programmes. However, the use of only one or two markers, no matter how polymorphic they are, would miss variation at other polymorphic loci, and almost certainly underestimate the magnitude of multiple infections. Thus, incorporating other markers like microsatellite DNAs would give a better picture. Genetic markers like the microsatellite DNAs are powerful tools for researching the population structure and epidemiology of parasites. Nonetheless, the choice of a particular gene marker for typing natural *P. falciparum* clones depends on the question being addressed.

P. falciparum genetic diversity can be evaluated by examining genetic polymorphism of the merozoite surface proteins (*msp-1* and *msp-2*), (Krogstad *et al.,* 1996; Sonden *et al.,* 2015; Bereczky *et al.,* 2006; Auburn *et al.,* 2012). These markers were useful to investigate genetic diversity, multiplicity of infections and parasite carriage. However, the *msp-1* and *msp-2* genes are under pressure and neutral markers such as microsatellites or single nucleotide polymorphism (SNPs) are better suited for assessing population genetics (Su *et al.,* 2009).

2.8 Microsatellite genotyping

Microsatellites are short sequence repeats (SSRs) polymorphic markers, which are found at different loci of the parasite's genome and used extensively to genotype *P. falciparum*. SSRs are tandem repeats of about 2-3 kilo base pairs, which are commonly found in the non-coded regions of the parasite genome (Nyachieo *et al*, 2005). The SSRs may be mononucleotide (CCCC), dinucleotide (ATATAT), trinucleotide (CTACTACTA) and tetranucleotide (GCTAGCTAGCTAGCTA) (Mburu & Hanotte, 2005). SSRs markers show a high level of allelic variation and can be used to differentiate several strains or clones in *P. falciparum* parasites population trends analysis (Anderson *et al*, 1999). Microsatellite repeats vary in number and length of the sequence repeats in various clones or strains of an organism, these differences in the repeats is referred to as short sequence length polymorphism (SSLPs). The diversity in the repeats is essential in population and genomic studies of *P. falciparum* (Doolan, 2002).

Markers developed for amplification of SSRs are polymorphic and highly diverse among parasite isolates due to the variations in length of these repeats sequences, they exist as multiple alleles in the general population and are informative for genetic studies. The rich thymine adenine (A-T) content of *P. falciparum* genome and the general uniqueness of SSRs markers make these markers suitable for linkage map analysis and laying the foundation for genetic characterization of parasites (Ferdig & Su, 2000).

2.9 *Plasmodium falciparum* Population Genetics

P. falciparum genetics has an effect on the population with respect to the nature of alleles and their frequency in the population with time. The structure of the parasite population is important in determining whether the parasite has evolved as a result of local adaptations or other causes that may be due to the distribution in endemic areas of sub-Saharan Africa.

The forces that has an impact on the population genetics of the parasites includes natural selection, mutation, gene flow and migration. The effects of these forces on the genotypes or alleles is the change in its frequency in a population which may turn to be against Hardy-Weinberg principles which states that allelic frequencies will remain the same over a period of time in the absence of evolutionary forces. (Andrews, 2010). Natural selection is the ability of a living organism to adapt to changes in its environment or habitat due to features (traits) in the organism, which has a selective advantage; these features are passed on to the next generation making the organism successful. This is ideal in *P. falciparum* genotypes if some of these traits are diverse and hereditable and to a large extent such variable alleles must reach a point of fixation (a point where only one type of allele remains) relative to other alleles.

Mutation may come about as result of changes in the genome of the parasites as a result of random deletions, insertions or substitution of nitrogenous base(s). This will incorporate new alleles or increase the allelic frequency. Chloroquine confers resistance to *P. falciparum* due to mutations in 2 gene candidates that is; the *P. falciparum* multidrug resistance gene (Pfmdr 1) and *P. falciparum* chloroquine resistance transporter gene (Pfcrt). These mutations were reported in Ghana in the late 1980s (1987) and subsequently the allelic frequency and populations of these mutants increased necessitating a change in policy, in the case management and treatment of malaria in the country (Duah *et al.*, 2006).

Migration is key in the evolution of malaria parasites, it is influenced by the interaction between the human host and the parasites. In 2006, Lion *et al.*, reported that migration and transmission of parasites virulence was closely associated to genes that confer resistance and adaptations to the local setting. It was also discovered that, allelic frequency and population of these genes increases. In the midst of population movement between geographical areas is gene flow, where genetic materials of the parasites are exchanged between populations. Genetic variation is neither created nor eliminated due to the effects of migration and may only support the reshuffling of genetic material of the parasite (Long, 2004). Genetic drift is a mechanism of evolution in which allele frequencies of a population or the frequency of an existing gene variant in a population change over generations due to chance (sampling error). In small populations, the effects of genetic drift is strongest. In between population (intra-populations) genetic diversity increases over a period of time as allelic frequency deviate either from ancestral alleles or within allelic populations (Andrews, 2010).

2.10 Multiplicity and Morbidity of *Plasmodium falciparum* Infections

Multiplicity of infection (MOI) is the number of distinct parasite clones simultaneously infecting an individual. In hyperendemic areas of Africa individuals can harbour up to 5 parasites clones per single infection of malaria in children between 3 to 7 years of age (Henning *et al.*, 2004; Kiwanuka, 2009). Young children less than 3 months of age experience fewer episodes of malaria than older children due to maternal Immunoglobulin G (IgG) immune protection and fetal haemoglobin. These infants (children less than 3 months) have less parasite prevalence and density that can be likened to partially immune adults, but parasite density and prevalence surges and reaches its peak at 4 to 12 months of age in a holoendemic setting (Felger *et al.*, 1999). In 1999, Felger and other researchers reported that morbidity or clinical malaria is associated with MOI, implying that MOI and development of clinical malaria is key to development of immunity against malaria in a

holoendemic area. Thus, increased MOI in children below 1 year may confer protection against clinical malaria. Previous findings however were contrary to what was reported by Fegler *et al*., 1999.

There is strong evidence that morbidity of malaria and MOI are linked, with semi-immune children found to have less incidence of clinical malaria compared to the age group (3-6) years. Henning *et al.* in 2004 reported that MOI was associated with risk of clinical malaria in children below 3 years of age in endemic areas. This suggests that MOI offers protective immunity hence reducing the level of parasitaemia in this age group. Therefore, the risk of developing clinical malaria and subsequent MOI is dependent on age, endemicity of the area and the immune status of the individual (Mayor *et al.*, 2003; Smith *et al.*, 1999).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Sites

The study was carried out in three ecological zones of Ghana. The different ecological zones are the Coastal savannah, the Sahel savannah and the Forest zone (Figure 3.1). The Coastal savannah zone consisted of the sites Ada (Anyakpor) (5°47′N 0°38′E) and Dodowa (Odumase) (5°53′N 0°7′E) whereas the site in the Forest zone was Duase in Konongo (06°37′00″N 01°13′00″W). In the Sahel savannah zone, the sites selected were Tamale (Pagaza) (09°24′27″N 00°51′12″W) and Kumbungu (Kpalsogu) (09°24′27″N 00°51′12″W). In Ghana, malaria transmission is heterogeneous and differs along varying ecological zones. Parasite prevalence is highly seasonal, peaking in a single wet season (June–October) in the Sahel savannah area (Koram *et al.*, 2000; Baird *et al*., 2002; Gyapong *et al*., 2013; Nkrumah *et al*., 2014). However, in both Forest and Coastal ecological areas, malaria parasite prevalence peaks twice in a year (Owusu-Agyei *et al.*, 2012; Gyapong *et al*., 2013; Nkrumah *et al*., 2014).

Anyakpor and Odumase are located in the Greater Accra region of Ghana. Anyakpor is located in Ada, which is bordered to the north and east by the North Tongu District (in the Volta Region), to the south by the Gulf of Guinea, and to the west by Ada West District. According to the 2010 census, the population of the district is 71,671 (Ghana Statistical Service, 2014). Dodowa with has a population of 111,976 residents (Ghana Statistical Service, 2014). Odumase is found in Dodowa with scattered settlements. The vegetation is mainly Coastal savannah; however, one of the sub-districts has dense vegetation. Ada (Anyakpor) and Dodowa (Odumase) experience perennial malaria transmission. The rainfall pattern in the Coastal savannah zone has the major season falling between March and June and the minor in September and October. The mean annual rainfall is 800 mm, which is relatively low (Gyapong *et al.*, 2013).

Duase is a community in Konongo in the Ashanti region of Ghana. Konongo is a gold and manganese mining community that serves as the capital of the Asante Akim Central Municipal. As of 2010, Konongo had a population of 41,238 and is about 53 kilometres (33 miles) from Kumasi, the capital of the Ashanti region. The Forest zone has a major rainy season, which occurs from March to June with, the minor occurring in September or October. The mean annual rainfall measures 1200mm (Ghana Statistical Service, 2014).

The sites in the Sahel savannah zone were Pagaza and Kpalsogu, found in Tamale and Kumbungu. These are located in the Northern Region of Ghana. Kumbungu has a total population of 39,341 (Yidana *et al.,* 2011). It is a farming community (Ghana Statistical Service, 2014; Fuseini *et al.,* 2016). Tamale is the regional capital of the Northern Region of Ghana and doubles as the capital for the Tamale Metropolitan Assembly (TaMA. Mean annual rainfall in the Sahel savannah is 1000-1300 mm (Ghana Statistical Service, 2014). The sites in the Sahel savannah exhibit seasonal transmission settings due to the unimodal rainy season followed by the dry season. The study area has marked seasonal malaria transmission that overlaps with rainfall and vector distribution patterns.

Figure 3.1 Map showing the sites that were sampled

(Source: http://www.maplandia.com/ghana/)

3.2 Study Design and Population

Two cross-sectional studies were conducted in March (dry season) and July (rainy season) of 2017. Children attending kindergarten and primary school (class 1 to class 6) served as the sample population.

3.2.1 Inclusion and Exclusion criteria

Inclusion criteria

Consenting participants who were aged 3-12 who showed no signs and symptoms of malaria (asymptomatic and healthy children) were recruited for the study.

Exclusion criteria

Individuals excluded from the study were those that were non-consenting and showed signs of chronic illness.

Sample size determination

The sample size determination was based on achieving a 90% power of detecting submicroscopic malaria parasite carriers, with 95% confidence that the sample proportions obtained differ from the population proportions by not more than 5%. Using submicroscopic malaria prevalence of 15%, a mean value from previous studies (Diallo *et al.*, 2012) sample size (n) of 196 participants per study site was arrived at using the formula:

$n = Z^2 x\ p\ (1- p)/E^2$ where,

n = required sample size

Z = Confidence level at 95% (standard value of 1.96)

P = Estimated prevalence of submicroscopic malaria carriage.

E = Margin of error at 5% (standard value of 0.05)

Hence, n = 1.962 x 0.15 (1-0.15)/ 0.052

n = 195.92.

For participant's non-response, an estimation of the sample size was 10%.

Thus, the sample size (N) = 196 x 135/100 = 264.6 or 265 per site.

3.3 Sampling Procedure and Sample Collection

Community entry was achieved by contacting the key stakeholders (the Chiefs and the communities at large). Information was also made available to the community at large through community meetings with the community stakeholders. The study was carried out during school hours in the only primary school within the community in all the different sites. Letters with the adequate information were sent to the District Education Service Directorate as well as the headmasters of the schools. Study participants were selected from eligible pupils using the school attendance register as sampling frame. Parents or caretakers and teachers were thoroughly informed about the background and procedures of the study. The selected pupils were recruited into the study after obtaining informed consent from their parents or caretakers. This was carried out by the use of consent forms.

Finger-prick blood samples were collected and used to prepare thick and thin blood smears, which were labelled with the participant's identification number (ID) as well as blotted onto Whatman^{TM} filter paper. The Whatman^{TM} filter paper samples were air dried, placed singly in sealed zip lock bags containing silica gel (to prevent dampness) and labelled (with the sample ID and date). A rapid diagnostic test (RDT) was performed on individuals that showed signs of malaria. The dried filter papers were transported to the laboratory of the Immunology department at the Noguchi Memorial Institute of Medical Research for further molecular analysis.

3.4 Detection of Parasitemia by Microscopy

Laboratory analyses for detecting malaria parasites were carried out at the Department of Medical Microbiology of the University of Ghana under the supervision of highly trained laboratory professionals. The blood smears prepared from the field were air dried, the thin films fixed with absolute methanol and stained with Giemsa stain. For optimum staining reaction, a

10% Giemsa working solution was prepared with buffered deionized water at pH 7.2 and stained for 10 minutes. As a quality control measure, the stained slides were examined by an experienced microscopist who did not have knowledge of the status of the slide analysis. A slide was considered negative only after 200 fields had been examined without seeing a parasite. Any unsettled result was assessed by a third microscopist before the final results were recorded. Thick film stain slides were examined by counting parasites per 200 leucocytes. Parasitaemia was also determined as the proportion of parasites/µL blood = (Number of parasites counted x 8000 white blood cells/µL) / Number of white blood cells counted.

3.5 Molecular analysis for the detection of parasites (PCR)

3.5.1 DNA Extraction

DNA extraction from the dried blood spots on filter paper was carried out using Chelex-100 (Bio-Rad Laboratories CA). A 3 mm disk of filter paper containing the blood spot was excised using a manual puncher (McGill 2 Reach), transferred into a sterile 1.5 ml microfuge tube and incubated overnight at 40 ^{o}C in 1 ml of 0.5% Saponin in 1x phosphate buffer saline (PBS). The Saponin solution was subsequently decanted and the disk washed for 30 minutes in PBS at 40℃ and the PBS was removed after incubation. Thereafter, 50 µl of 20% Chelex- 100 solution to 150 µl of DNase-free water was added directly to the tube and mixed for 30 seconds using the vortex. There was heating at 100˚C for 10 minutes to elute the DNA, and the vortex was used during the incubation. Finally, it was centrifuged at 10,000 rpm for 5 minutes and the supernatant was removed using a fresh tube. The isolated DNA was stored at -20 ^{o}C until it was required for amplification (WWARN, 2007).

3.5.2 Nested PCR

The *P. falciparum* 18sRNA gene was amplified following the recommended standardised protocol from the Worldwide Antimicrobial Resistance Network (WWARN) and World Health Organization (WHO) for the identification of parasite populations (WHO, 2007; WWARN, 2007). The protocol consisted of two rounds of amplification, with the product of the first reaction serving as the template for a second reaction. The primers for both primary and nested PCRs for the detection of the parasite populations are from previously published protocols (Snounou & Singh, 1999; Felger & Snounou, 2008).

NEST 1:

For the first amplification step, 5 µL from the extracted DNA product was added to 10 µL mastermix containing 167 nmol/L dNTP mix, 5X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 25 mM $MgCl_2$, 80 nmol/L fPLU5/rPLU6 primers and 1 µL enzyme *Taq* polymerase). Positive and negative controls comprising of 5 µL 3D7 and 5 µL H_20 respectively were included in the set up.

Cycling Parameters

1. 94 ° C for 4 minutes
2. 94 ° C for 30 seconds
3. 55 ° C for 1 minute
4. Repeat steps 2-4 for a total of 25 cycles
5. 72 ° C for 1 minute
6. 72 ° C for 5 minutes
7. 4 ° C for infinity

NEST 2

For the final amplification step a set of internal primers were used. A volume of 0.5 µl of the PCR1 product serving as template for a nested PCR was added to 14.5 µl of master mix containing 10 mM of dNTP mix, 5X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$) 80 nmol/L fFAL1/rFAL2 primers and 1 µl of Taq polymerase).

Cycling Parameters:

1. 94 ° C for 4 minutes
2. 94 ° C for 30 seconds
3. 58 ° C for 1 minute
4. Repeat steps 2-4 for a total of 35 cycles
5. 72 ° C for 1 minute
6. 68 ° C for 5 minutes
7. 4 ° C for infinity

The primers used for Nest 1 are fPLU5/rPLU6 which are species-specific whereas Nest 2 reactions require *Plasmodium*-specific fFAL1/rFAL2 (see Table 3.1 for primer sequences). These cycling conditions have been determined using a PTC-100 thermal cycler (MJ Research Inc.). Finally, the PCR products were resolved by electrophoresis in 2% agarose gel. The expected sizes of the PCR amplified fragments are 205bp for *P. falciparum* (Snounou & Singh, 1999).

Table 3.1: DNA Sequences of the synthetic oligonucleotide primers for PCR identification of Plasmodium species

Name of Primer	DNA sequence (5' – 3')
r PLU5 (f)	CCT GTT GCC TTA AAC TTC
r PLU6 (r)	TTA AAA TTG TTG CAG TTA AAA CG
rFAL1 (f)	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT
rFAL2 (r)	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC

Source : Snounou *et al.*, 1999

3.5.3 Agarose Gel Electrophoresis

Agarose (Sigma-Aldrich) (2g) was weighed into a 250ml conical flask. A quantity of 100ml of TAE buffer (1X) was added to the agarose and swirled to mix. Depending on the amount of product of the reaction, quantities may change. The mixture was heated in a microwave at maximum power to melt the mixture completely. The agarose mixture was allowed to cool before ethidium bromide (Sigma-Aldrich) was added for visualization. To prepare the gel, the gel tray with a casting apparatus was laid on a level surface. The wells were created by placing the suitable comb in the gel tray. The gel was then poured and allowed to set at room temperature. When the agarose gel was set the comb was removed and the gel was ready for use. Photographs of images of bands formed were taken. The sizes of the PCR products were estimated by comparison with the mobility of a standard of known band sizes.

3.6 Microsatellite Genotyping

Microsatellite genotyping involves PCR amplification of one or several microsatellites (2–6 bp long tandem repeats), followed by fragment sizing by capillary electrophoresis with an automated sequencer and dedicated software (Greenhouse *et al.*, 2006) or a 'lab on a chip' device (Nyachieo *et al.*, 2005).

Six microsatellite loci were genotyped using the primers described by Su and Wellems in 1996 and two round hemi nested PCR (Anderson *et al.*, 1999). The microsatellite markers used were Polyα, TA40, ARA2, TAA87, TAA81, and PfPK2. Their chromosomal locations and fluorescent labelling are detailed in Table 3.2 page.

Table 3.2: Primers used to amplify microsatellites

Locus (Anderson 1999)	**Primer 5' to 3'**	**Fluorescent Label**
Poly α-R	ATCAGATAATTGTTGGTA	FAM
Poly α-F	AAAATATAGACGAACAGA	
Poly α-3(IR)	GAAATTATAACTCTACCA	
PFPK2-3R	CCTCAGACTGAAATGCAT	HEX
PFPK2-F	CTTTCATCGATACTACGA	
PFPK2-R	AAAGAAGGAACAAGCAGA	
TA81-3F	GAAGAAATAAGGGAAGGT	PET
TA81-R	TTTCACACAACACAGGATT	
TAA81-F	TGGACAAATGGGAAAGGATA	
ARA2-3(F)	GTACATATGAATCACCAA	FAM
ARA2-R	GCTTTGAGTATTATTAATA	
ARA2-F	GAATAAACAAAGTATTGCT	
TA87-3F	ATGGGTTAAATGAGGTACA	HEX
TA87-R	ACATGTTCATATTACTCAC	
TA87-F	AATGGCAACACCATTCAAC	
TA40 Rev-1	GAAATTGGCACCACCACA	PET
TA40 For	AAGGGATTGCTGCAAGGT	
TA40 Rev-2	CATCAATAAAATCACTACTA	

Source: CVD Malaria Group, University of Maryland School of Medicine

The protocol (CVD Malaria Group, University of Maryland) was utilized for the genotyping process. For capillary electrophoresis, the second round labelled products were multiplexed and run on an ABI 3100 Genetic Analyser at the Virology Dept. of

the Noguchi Memorial Institute of Medical Research (NMIMR). The length of PCR products was determined with reference to internal size standards using the GeneMapper software (from Applied Biosystems). Negative controls (no DNA template) and positive control *(P. falciparum* 3D7) were included in each run.

The following primer sets were successfully multiplexed in primary PCR reactions:

Multiplex 1: Polyα; PFPK2; and TA81

Multiplex 2: ARA2; TA87; AND TA40

The primary round amplification was performed as a multiplex of all three genotyping markers in a reaction volume of 25 µL containing 3.2 mM $MgCl_2$, 0.24 mM of each dNTP (0.96 mM total dNTPs), 0.2 µM of each primer, and 2.5 µL of 5 × Buffer and 0.625 units of Taq DNA Polymerase with 1 uL of template DNA added. The thermocycling profile was as follows: initial denaturation of 94 ° C for 2 minutes, then 25 cycles of denaturation at 94 ° C for 30 seconds, annealing at 42 ° C for 30 seconds, and extension at 65° C for 40 seconds and a final extension at 65 ° C for 2 minutes.

For the nested secondary round of amplification, each marker was amplified in a separate reaction in a volume of 15 µL using fluorescence labelled reverse primers. The reaction mix contained 3.2 mM $MgCl_2$, 0.16 mM of each dNTP (0.64 mM total dNTPs), 0.2 µM of each locus-specific primer, 2.5 µL of 5× Buffer and Taq DNA polymerase. One microlitre of the first round product was added as template. Both the first and second PCR reactions were carried out in a total volume of 15 µL.

The thermocycling profile was identical to the first round. Both sets of labelled products were diluted and pooled in different wells before being run on an ABI 3100 Genetic Analyser (Anderson *et al.*, 2000; Sue *et al.*, 1999; Anderson *et al.*, 1999).

Alleles were scored manually using Peak Scanner Software (Applied Biosystems) using the height of 100 relative fluorescence units as the minimal peak threshold. The analysis of data files was performed to achieve electropherogram peaks with 100 - 20,000 relative fluorescent unit (rfu) range. The size range of the alleles and the average number of alleles per loci for each population were calculated (Greenhouse *et al.*, 2006).

3.7 Data Management and Statistical analysis

For the first objective, data obtained was first entered into Microsoft Office Excel version 2016 spreadsheets, cleaned, collated and analysed using SPSS version 25 (Nie, Bent & Hull, 1970). The tests used were Kruskal Wallis test and for the post hoc, Dunn's multiple comparison analysis was used to compare prevalence and this helps to identify the pairs with significant differences. For the second objective, samples were evaluated after initial analysis for off-scale data, background fluorescence and sizing errors (100 rfu was set as the minimal peak threshold for allele calling). The allele sizes obtained from the control *P. falciparum* strain, 3D7 were used to correct run to run variation among capillary electrophoresis runs. Microsatellite allele sizes were imported into a Microsoft Excel sheet. Allele peaks that were spaced at intervals corresponding to the trinucleotide repeats in all six loci genotyped were considered as true alleles.

Data obtained after the capillary electrophoresis was analysed using the genetic analysis software GeneAlEx (Peakall & Smouse 2006, 2012). This was used to determine heterozygosity, allele frequency and allelic ranges. The expected heterozygosity (HE) was measured at each locus in each of the study sites as ($HE = [n/(n-1)][1-\Sigma pi^2]$), where pi is the frequency of the i*th* allele. The population genetics software STRUCTURE v2.3.4 (Pritchard, 2000) was also used to investigate the population structure. Shannon Information index (*I*) was used for quantifying genetic diversity in each marker.

Multiplicity of infection is the number of distinct parasites clones in a single infection, which was determined by the ratio of the total number of distinct parasites clones for a gene to that of the number of samples positive for the same gene. *P. falciparum* infections were classified as either monoclonal if it contained only one parasite clone or polyclonal if theinfection contained multiple parasite clones (Razak *et al*., 2016). The only way to describe multiplicity is at a single locus or else it becomes complex. Inferential statistics was the main tool that was employed to analyse the data. Tables and graphical displays were used where necessary and appropriate to summarise data.

3.8 Ethical Consideration

Ethical clearance (CHS-Et/M.S-P1.9/2017-2018) was obtained from the College of Health Sciences Ethical and Protocol Review Committee (EPRC) before blood samples were collected from individual volunteers who had consented to participate in the survey.

CHAPTER FOUR

4 RESULTS

4.1 Demographic characteristics

A total of 1168 participants were enrolled for this study. The study population consisted of 534 (45.7%) males and 634 (54.3%) females. No samples were collected from Odumase in March, 2017. The age of the children ranged from 3 to 13 years, with a mean age of 9 years. Almost 71% (710) of the study participants were between the ages of 6 and 10 years, with the remaining participants being between the ages of 11 and 13 years (30.5%), and 3 and 5 years (7.7%). No significant difference was observed between the ages of the children recruited at all of the five sites (Dunn's multiple comparison, $p>0.05$). Refer to Table 4.1.

Table 4.1: Demographic Characteristics

	Frequency (N=1168)	Percent (%=100)
Gender		
Male	534	45.7
Female	634	54.3
Age		
3-5 years (>5 years)	90	7.7
6-10 years	710	60.8
11-13 years (<10 years)	368	31.5

4.2 Parasitaemia Detected by Microscopy in the Study Sites

Microscopy-based prevalence of asexual *P. falciparum* showed that 188 individuals out of 1168 individuals sampled were positive. This represents 16.1% of the individuals tested. All parasites were identified at the *P. falciparum* ring stage. No samples were collected in Odumase in March, 2017. The highest infection prevalence during the rainy season was in Duase (24.1%) with the lowest occurring in Ada (12.8 %). During the dry season the highest prevalence of infection was found in Pagaza (13.6%) whilst the lowest was in Kpalsogu (7.1%). These are summarised in Table 4.2. Despite the relative differences observed, no significant difference was observed in parasite densities in any site across the different seasons (Dunn's multiple comparison test, $p>0.05$).

Table 4.2: Prevalence rate for Microscopy

MICROSCOPY RESULTS (N = 1168 % = 100)			
Site	**Season**	**N (%)**	**Total**
Ada	Rainy	19 (12.8%)	148
	Dry	14 (12.1%)	116
Dodowa	Rainy	10 (16.9%)	59
Duase	Rainy	52 (24.1%)	216
	Dry	15 (12.8%)	117
Pagaza	Rainy	34 (17.7%)	192
	Dry	14 (13.6%)	103
Kumbungu	Rainy	24 (18.2%)	132
	Dry	6 (7.1%)	85

Table 4.3: Prevalence rate Microscopy per Ecological zone

MICROSCOPY RESULTS (N = 1168 % = 100)			
Site	**Season**	**N (%)**	**Total**
South (Coastal)	Rainy	29 (14.0%)	207
	Dry	14 (12.1%)	116
Middle (Forest)	Rainy	52 (24.1%)	216
	Dry	15 (12.8%)	117
North (Sahel)	Rainy	48 (14.8%)	324
	Dry	20 (10.6%)	188

When considering parasite density according to zones, the northern zone had the highest prevalence of parasites and the middle zone had the least and no significant difference was observed between parasite density values obtained during the dry and wet seasons at each of the three zones (Dunn's multiple comparison, $p>0.05$). Refer to Table 4.3 and Appendix E.

4.3 Prevalence of *Plasmodium falciparum* parasites as detected by Nested PCR

Following microscopy examination of blood smears, all 1168 samples collected from the study sites, were analysed for *P. falciparum* parasites by nested PCR. Out of the total samples tested 461 were positive; this represents an overall PCR-based asymptomatic infection prevalence of

39.5%. The highest prevalence of parasite carriers were found in Duase during the dry season with 59.8% an asymptomatic prevalence. School children living in Kpalsogu had an asymptomatic prevalence of 20.7% in the dry season making it the lowest for the dry season. In the rainy season, the lowest prevalence was found among the Ada population in the rainy season with a prevalence of 17.6%. The highest prevalence was 58.8% in Duase for the rainy season. This can be found in Table 4.4. Significant difference was observed in PCR detected parasite prevalence between the dry and the wet season in Duase (Dunn's multiple comparison test, $p<0.01$), where the prevalence in the wet season was higher than the dry season and in Kpasolgu (Dunn's multiple comparison test, $p<0.01$), where parasite prevalence was higher in the dry season than the wet season. Parasite prevalence between the two seasons in the other sites were not significantly different (Dunn's multiple comparison test, $p>0.05$). Parasite prevalence in the South was significantly lower than in the North and the Middle Zone (Dunn's multiple comparison test, $p>0.05$)

Table 4.4: Prevalence rate for nested PCR

Nested PCR RESULTS (N = 1168 % = 100)			
Site	**Season**	**n (%)**	**Total**
Ada	Rainy	26 (17.6%)	148
	Dry	24 (20.7%)	116
Dodowa	Rainy	18 (30.5%)	59
Duase	Rainy	127 (58.8%)	216
	Dry	70 (9.8%)	117
Pagaza	Rainy	81 (42.2%)	192
	Dry	29(28.2%)	103
Kumbungu	Rainy	65(49.2%)	132
	Dry	21 (24.7%)	85

Table 4.5: Prevalence rate PCR per Ecological zone

NESTED PCR RESULTS (N = 1168 % = 100)			
Site	**Season**	**N (%)**	**Total**
South (Coastal)	Rainy	44 (21.3%)	207
	Dry	24 (20.7%)	116
Middle (Forest)	Rainy	127(58.8%)	216
	Dry	70 (59.8%)	117
North (Sahel)	Rainy	149(46.0%)	324
	Dry	50 (27.3%)	183

All 1168 samples were analysed using microscopy and nested PCR. The children were then categorized into young children 5 years and below, who represented 7.7% (90/1168), children who represented 60.8% (710/1168) and older children who represented 31.5% (368/1168).When taking into account the outcomes for PCR according to zones, the middle zone had the highest prevalence of parasites and the southern zone had the least and no significant difference was observed between PCR values obtained during the dry and wet seasons at each of the three zones (Dunn's multiple comparison, $p>0.05$). Refer to Table 4.5 and Appendix E.

4.4 Comparison of Microscopy and Nested PCR Results for the Detection of *Plasmodium falciparum.*

All 1168 samples were analysed using microscopy and nested PCR. Positive samples for microscopy were 16.3% (188/1168). However, PCR identified more parasite positive individuals as compared to microscopy, 39.5% (461/1168). All samples positive for *P. falciparum* parasites by microscopy were confirmed to be positive by nested PCR.

The asymptomatic infection prevalence (by both microscopy and PCR) was low among children below 5 years but highest in the oldest age group (11-15 years).

The trend is shown in Figure 4.1.

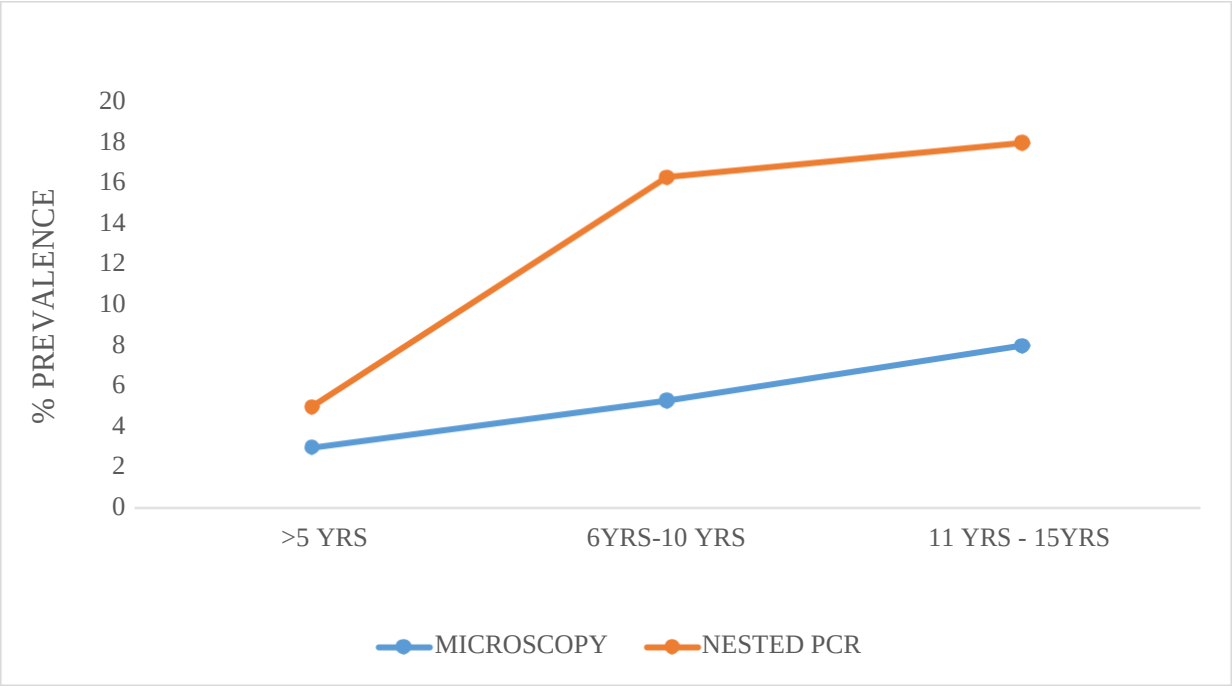

Figure 4.1: Prevalence of *P. falciparum* in age stratified study participants

4.5 Genetic Diversity in the study sites

In this study six microsatellite loci obtained from different chromosomes across the *P. falciparum* genome were analysed. These were adapted from Anderson *et al.*, 1999 namely:

Poly A, TA81, TA40, PFPK2, TA87 and ARA2. The loci assayed were used to determine the allele lengths and to quantify peak heights.

In total, 120 samples; Ada (n=40), Pagaza (n=40), Duase (n=40) were genotyped, parasites were polymorphic at the different loci. The Poly A locus was the most polymorphic with 12.00 alleles in Duase whereas ARA 2 was the least polymorphic loci with allele frequencies of 3.00 found in the Ada and Duase populations.

The Poly A locus had the highest H_E (0.893) whereas ARA 2 had the lowest (0.478). Pagaza parasites showed the highest diversity whereas those from Ada were the least diverse. The details can be found in Table 4.9.4. There was no significant difference (Kruskal Wallis Test, $p > 0.05$) in the average number of alleles of at the 3 study sites where 6 MS loci were genotyped.

Fstatistics computation of expected heterozygosity (FSTAT)

The F-statistics showed the degree of genetic difference across the three populations for each locus, with lower values indicating genetic similarity and higher values indicating a higher degree of genetic diversity. As can be found in Table 4.6.

Table 4.6: Diversity of *P. falciparum* populations from different ecological zones of Ghana

Locus	Fis	Fit	Fst
Polya	0.125	0.159	0.038
Pfpk2	0.220	0.311	0.116
TAA81	0.280	0.327	0.065
Mean	0.208	0.265	0.073

Locus	Fis	Fit	Fst
ARA_2	0.314	0.356	0.062
TA87	0.126	0.168	0.048
TA40	0.421	0.436	0.027
Mean	0.287	0.320	0.045

Sites for each locus: South, North and Central

Table 4.7: The genetic diversity of *P. falciparum* populations from different ecological zones of Ghana

SITE	LOCUS/MARKER	N	NA	NE	HE	ALLELIC RANGE
ADA	**Poly A**	64	9.00	6.03	0.834	135-170
	PFPK2	68	5.00	2.57	0.610	150-170
	TA81	70	10.00	4.06	0.753	112-175
	ARA2	32	3.00	1.89	0.471	60-75
	TA87	22	9.00	4.05	0.753	60-160
	TA40	69	6.00	2.41	0.585	120-225
DUASE	**Poly A**	63	12.00	8.37	0.588	140-170
	PFPK2	66	8.00	5.10	0.681	160-196
	TA81	73	10.00	5.61	0.600	118-170
	ARA2	63	3.00	2.43	0.588	65-75
	TA87	62	6.00	3.13	0.681	90-125
	TA40	64	3.00	2.50	0.600	210-225
PAGAZA	**Poly A**	66	9.00	7.02	0.858	125-175
	PFPK2	67	8.00	5.34	0.813	160-196
	TA81	60	9.00	6.98	0.857	112-160
	ARA2	82	4.00	3.11	0.678	60-75
	TA87	72	6.00	3.54	0.717	90-125
	TA40	62	4.00	3.18	0.685	125-175

**N =sample size Na = number of alleles, Ne= number of effective alleles, H_e= biased expected heterozygosity*

4.5.1 Within host diversity by microsatellites

Samples used were mono or bi-clonal infections with the majority of samples in Pagaza containing more than two clones thus were not included in analyses. Six samples obtained in Ada, marked in asterisk* exhibited clones that are genetically different from each other. Duase found in the central (Forest zone) part of the country were observed to have clones that are genetically similar (Figure 4.2). The North represented the Pagaza samples, whereas the Central area was Duase and South, Ada samples.

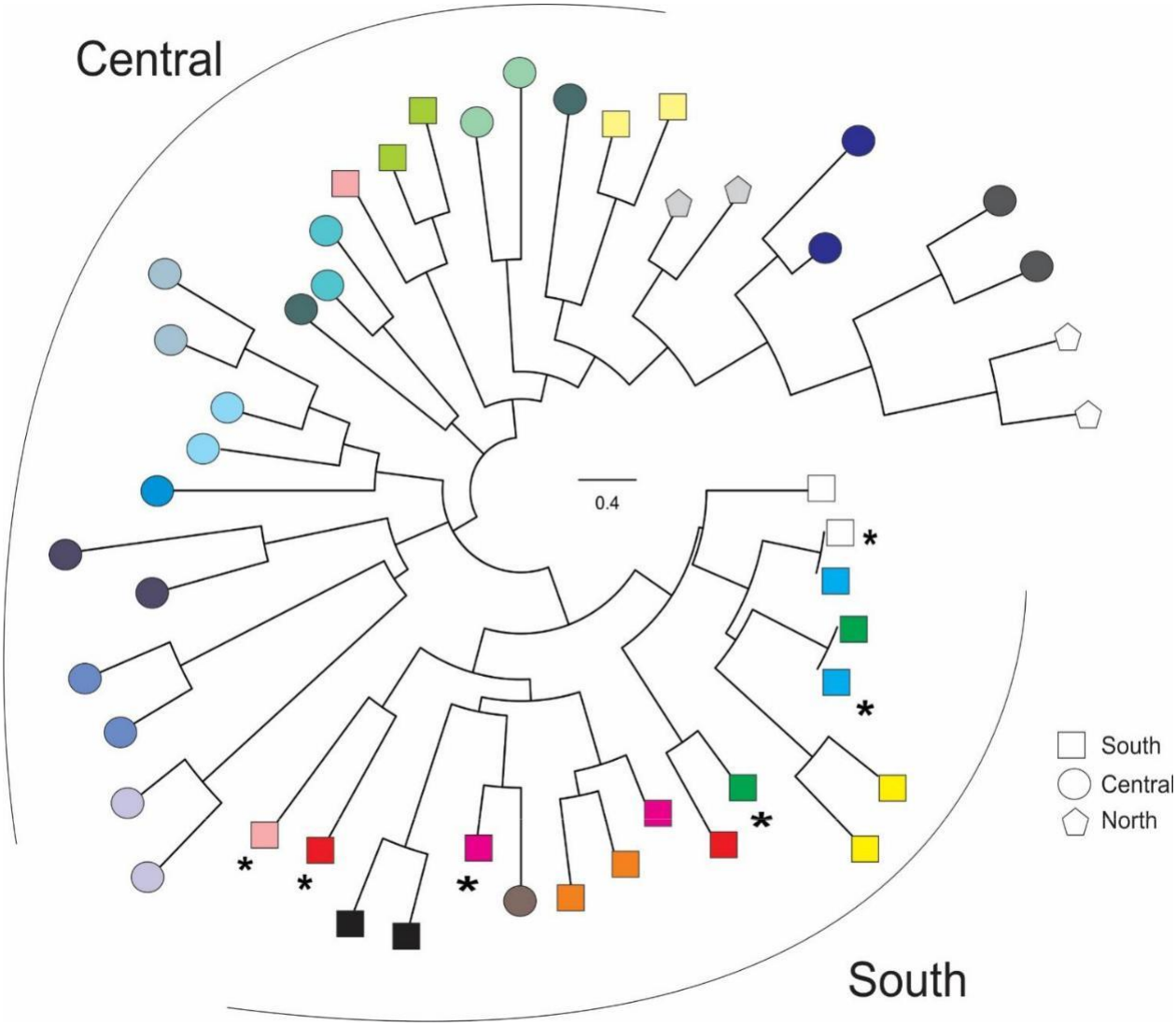

Figure 4.2 Intra-host diversity by microsatellites (North represents Pagaza; the Central, Duase and South, Ada)

4.5.2 Genetic relatedness of *Plasmodium falciparum* samples from different transmission zones

It was also observed that every sample was either a single or multiple parasite strains. Phylogeny was composed on the basis of the presence or absence of an allele. This does not show the genetic identity of individual clones. Parasites in the northern (Pagaza) and central (Duase) parts of the country were observed to be more closely-linked in comparison to those in the southern (Ada) part of the country (Figure 4.3).

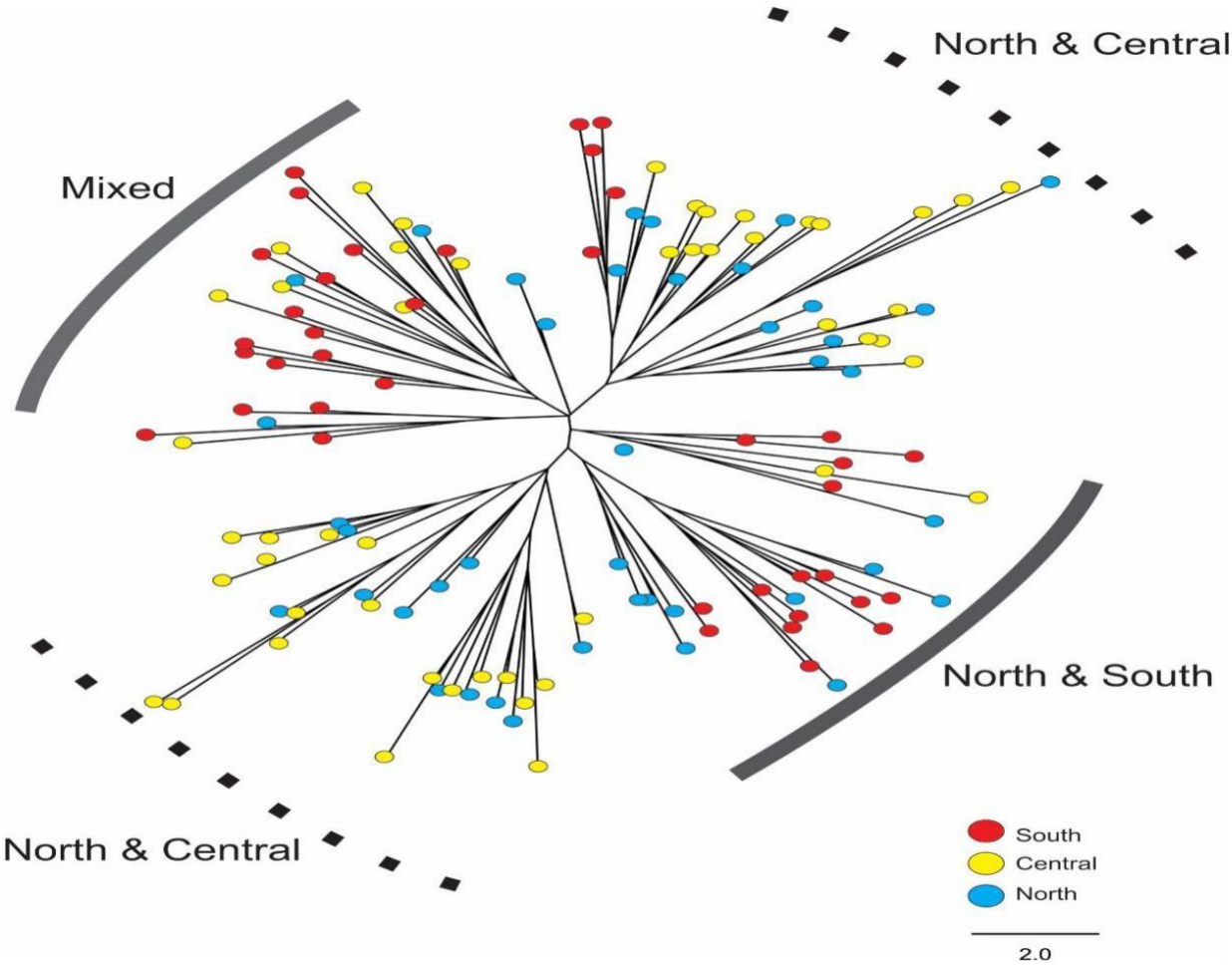

Figure 4.3 Phylogeny of *P. falciparum* samples showing genetic relatedness

4.5.3 Genetic Structure among *Plasmodium falciparum* Samples

Using the basis of the Bayesian inference, two most probable genetic clusters (pink and blue) were determined. The northern (Pagaza) and central (Duase) populations had parasites that mainly shared the blue cluster whereas the southern (Ada) population showed in the pink cluster (Figure 4.4).

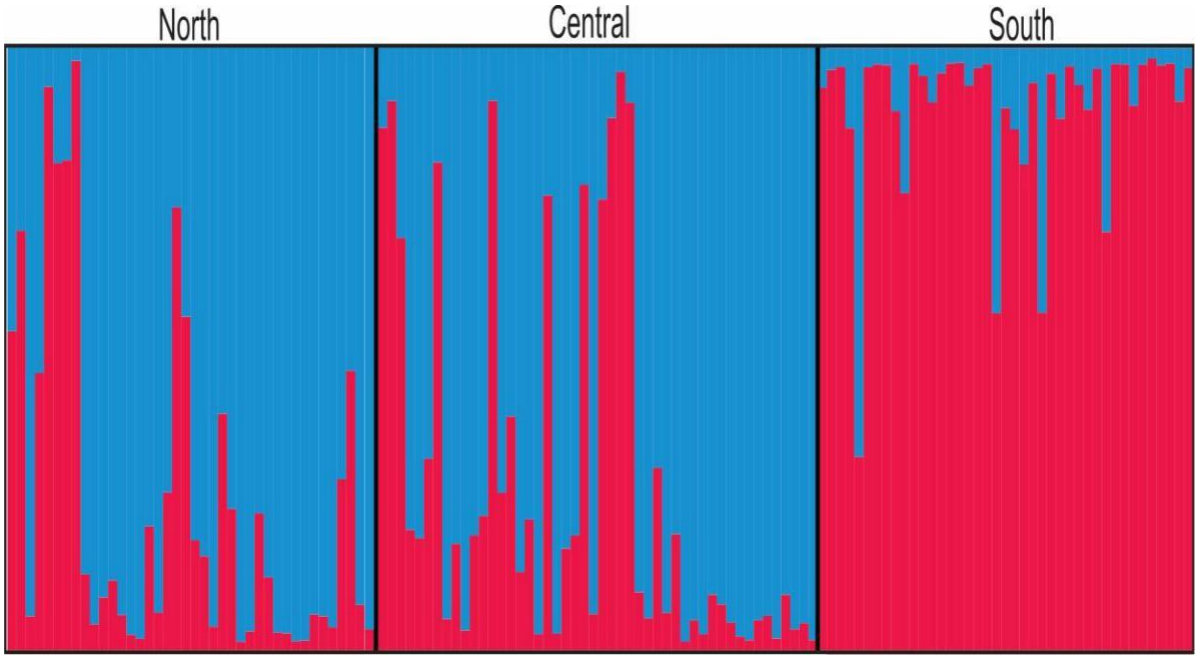

Figure 4.4 Bayesian bar plot showing genotyping structure among samples (North represents Pagaza; the Central, Duase and South, Ada)

4.5.4 Multiplicity of infection (MOI)

The six neutral microsatellites genotyped 120 *P. falciparum* isolates in the sites in the 3 ecological zones. The maximal number of different parasite clones was detected in the Duase community (for TA40) with a MOI range of 1.2 to 1.48 and an average MOI of 1.3. It was much lower in the Pagaza community with a MOI range of 0.98 to 1.2 and an average MOI of 1.1. In the Ada community, the average MOI was 1.2 with a MOI range of 1.0 to 1.3.

CHAPTER FIVE

5.0 DISCUSSION

The study sought to determine the prevalence of asymptomatic *P. falciparum* infections in school children living in the three different ecological zones of Ghana as well as determine the parasite population structure of the isolates. There was also the need to investigate and understand the gene flow of the parasite across these different ecological areas as they could affect malaria intervention measures. The prevalence of asymptomatic carriers was not equally distributed among the three ecological zones and the seasonal prevalence differed with the Forest zone registering the highest and lowest prevalence rates in the rainy season and the dry seasons respectively. The observations made in the different ecological zones suggest that there is greater genetic diversity in the Sahel savannah zone, as well as a higher level of submicroscopic infections and asymptomatics in the Forest zone as compared to the other two zones. This may suggest host parasite immunity or drug resistant strains (Duah *et al.*,2016).

Asymptomatic *P. falciparum* carriage was 16.1%, with 39.5% of these infections identified as carrying submicroscopic infections that are undetected by the routine method of diagnosis, microscopy. Studies conducted in the Gambia and Kenya have shown that about 15-20% of untreated asymptomatic infections developed into transmissible parasites after 4 weeks (Dunyo *et al*., 2006). These malaria infections are a threat to malaria control and elimination efforts because they harbour parasites for long periods undetected, which may be transmitted to others during the transmission season in the community (Bousema *et al.*, 2014).

The highest microscopy-based parasite prevalence was in the rainy season in Duase, while the lowest proportion of asymptomatic children was registered in the peak season in Ada. This showed a higher prevalence of malaria infection which was similar to the observation made by Owusu-Agyei *et al.,* (2009). Results obtained from Duase support the positive link between rainfall and asymptomatic infections, which has been identified in both high and low malaria transmission

settings (Dery *et al.*, 2010) and also the fact that malaria in Duase is seasonal. The rainy season recorded the lowest proportion of asymptomatic infections in Kpalsogu, but was not significantly different from the other time points (P>0.05, Dunn's Multiple comparison test). This is to be expected in perennial transmission settings, where mosquito vectors and malaria transmission only vary slightly between seasons (Elissa *et al.*, 2003).

Detection of malaria parasites by PCR revealed a higher number of positive samples as compared to light microscopy. Out of the total samples tested an overall PCR-based asymptomatic infection prevalence of 39.5% was recorded. The highest PCR-based prevalence of *P. falciparum* parasites was found in Duase with 59.8 % prevalence. According to the seasons, the dry season experienced the highest PCR-based prevalence in Duase, the Forest zone. In comparing the age groups, the age group 11-15 years registered the highest prevalence; this could be as a result of the immunity acquired over the years with many encounters with parasite infections that have kept the parasite population at very low levels. Additionally, prevalence by microscopy was highest among children 11-15 years (8%). The study also showed higher prevalence of submicroscopic infections in the Ada community in the Coastal savannah zone. Similar findings have been reported in a study conducted in Misungwi district located in the north- west Tanzania, where malaria transmission is described as mesoendemic (Mosha *et al.*, 2013). The prevalence of malaria parasites in this study was 8.52% thus submicroscopic infections detected may contribute similar magnitude of transmission in Odumase. These findings could be explained by ecological factors such as ecology and the vegetation of the area that contribute to the transmission of malaria parasites.

Generally, asymptomatic infections are a great threat to malaria elimination efforts and control strategies. Microscopic asymptomatic infections according to this study were observed to be more prevalent during the rainy season than the dry season across all sites in the ecological zones which

is similar to the findings from studies in Nigeria and Mali (Olayemi *et al.*, 2011; Druetz *et al.*, 2018).

Genetic diversity of *P. falciparum* is a selective benefit to the parasite and may be as a result of the genetic recombination of different parasites clones in a single mosquito blood's meal that infects an individual in endemic areas of malaria transmission. Cross mixing events of these different parasites genotypes in the mosquito vector may lead to the parasite's diversity as different parasites clones with unique genetics are combined. This makes the search for an effective vaccine a challenging task (Felger *et al.*, 1994). Microsatellites genotyping was used to assess the diversity of *P. falciparum* in the selected study sites. The application of microsatellites for genotyping *P. falciparum* has become imperative in population and diversity studies because SSRs markers have proven to be stable making microsatellites the markers of choice in recent years for parasite population genetics (Su & Wellems, 1996).

The number of alleles, number of effective alleles and heterozygosity are measures of genetic diversity. The findings for the parameters; heterozygosity (H_E) and number of effective alleles (N_E) were moderate with H_E values ranging from 0.47 to 0.83 while N_E values showed a range of 1.89 to 8.37. There was a higher genetic diversity evidenced by the highest frequency of heterozygosity in the Sahel savannah (Northern region); that is Pagaza. This differs from the expectation that an area with the highest asymptomatic infection prevalence should have the greatest genetic diversity and multiplicity.

The diversity displayed in Duase compared to Pagaza, this could be as a result of the successful nature of the parasite in this geographical area. Many parasites clones may be circulating in the endemic setting due to human immune evasion and the prevalence of drug resistance strains. These resistant strains could be naturally selected hence the frequency of variant strains increases due to

the interactions with the human host, the mosquito vector and the population of the parasite (Duah *et al.,* 2016; Escalante *et al.,* 2004; Osei Tutu *et al.,* 2011).

P. falciparum revealed a relative level of diversity within and among populations. The Heterozygosity (H_e) in the Anyakpor (Ada (0.61) was similar to those reported in less endemic regions such as the Caribbean (H_e = 0.61) (Carter *et al.,* 2015) and Indonesia (H_e = 0.53) (Noviyanti *et al.,* 2015). Higher parasite diversity has negative implications for the success of intervention efforts (Razak *et al.,* 2016). This means that the parasite populations in this study are interconnected and it is unlikely to have major differences in local divergence in allele frequencies. The level of heterozygosity seems to have a profound effect on the performance of the parasite. The more heterozygous the parasite the higher the level of fitness and this could be an adaptive mechanism (Razak *et al.,* 2016).

Parasite diversity shown by the study at the endemic site could be as a result of genetic recombination of different clones at the vector (mosquito) stage, this could disrupt the efforts in the search for antigenic epitopes towards the development of an effective vaccine. However, parasite diversity makes it difficult to develop an effective malaria vaccine (Conway, 2015; Duah *et al.,* 2016). Subdomains with conserved regions, however do have the potential to be used for vaccine development (Dinko & Pradel, 2016).

The patterns of human movement and settlement can determine transmission dynamics of malaria. The observation of the lack of major differences in parasite variants in all three ecological zones may be due to human population movement or migration across the ecological zones. This may be due to migration and emigration of the individuals living in the area as well as the population growth rates. For individuals living in the Sahel savannah ecological zone, migrant workers who move to perennial transmission areas for economic reasons and return to farm their lands before the rains begin may bring in new parasite strains. As such they may become carriers of the malaria

parasite from their ecological zone to other zones and also carry parasites of the other zones back to their areas. These migrating individuals with the parasite infection at low densities may have the parasites mating with the destination malaria parasites. The migrating parasites in the individuals will then contribute gametes carrying alleles that can alter the existing proportion of alleles in the destination population. This may lead to parasite transmission to other members of the community for example children.

Detection of high MOI contributes to knowledge of the genetic diversity of the parasite and is suggested to be influenced by age and transmission intensity (Bendixen *et al.*, 2001; Vafa *et al.*, 2008). . The geometric mean MOI for infections in Pagaza was significantly lower than that determined in Duase (Table 4.1), presumably due a higher incidence of infectious mosquito bites in high transmission settings, that could result in additional inoculum of variant parasites than in lower transmission settings (Das *et al.*, 2017). This discovery supports the general view that low transmission settings are commonly associated with reduced MOI in malaria endemic countries (Ayanful-Torgby *et al.*, 2018; Hoffman *et al.*, 2015). The geometric mean MOI differed significantly across the seasons in Duase (Table 4.1), most likely because the mosquito vector population varied between those time points as is expected for communities in the Forest zone (Das *et al.*, 2017). The low geometric mean MOI identified for Pagaza, the low transmission setting is in line with an earlier report from Ghana where the MOI recorded in areas with low malaria transmission were found to be lower than high transmission areas especially during the peak season (Atrosh *et al.*, 2011). These observations are in contrast to the polyclonal rate of *P. falciparum* in low transmission areas such as Haiti (12.9%) (Carter *et al.*, 2015) and southern China (10–23%) (Wei *et al.*, 2015) that are approaching elimination phase, but similar to endemic countries in West Africa such as Gambia and Senegal (36–50%) (Mobegi *et al.*, 2012; Salgueiro *et al.*, 2016), Papua New Guinea (39–45%) (Schultz *et al.*, 2010), and Southeast Asia such as Cambodia (47%) (Orjuela-Sánchez *et al.*, 2013).

The main aim of most malaria intervention efforts is the reduction in the incidence of malaria in the country but this is affected by the diversity of the parasites which poses a great threat to ongoing interventions aimed at curbing the menace of malaria in the country. In most endemic areas of *P. falciparum* transmissions, control programmes are introduced with little knowledge in the dynamics of parasites diversity. These intended efforts could be derailed by the complexities of multiplicity of infections of the parasites. To achieve the target of such intervention methods (therefore reduction in the prevalence of malaria) in endemic areas such as Duase an improvement in these control methods could achieve the desired targets either than the wholesale nature with which most control programmes are introduced in Ghana (Barry et al., 2013; Felger et al., 1994).

Economic status of the inhabitants of Duase could be another factor for the higher parasite diversity. The inhabitants in these areas may be migrant workers (such as farmers, fishermen, traders and the like), this may affect their ability to take care of the medical needs when infected with malaria may be a challenge, this can lead to persistence of infection and its accompanying complication for the vulnerable groups most especially children. Malaria affects a large majority causing a heavy toll on the poor in Sub – Saharan Africa thereby further impoverishing those affected (Worrall *et al.*, 2002).

CHAPTER SIX

CONCLUSION & RECOMMENDATIONS

6.1 Conclusion

The study examined the prevalence of asymptomatic and submicroscopic infections and the genetic diversity in three ecological zones of Ghana. Asymptomatic infections were highest during the rainy season. In turn, PCR was a better determinant of infections as compared to microscopy. These infections are classified as submicroscopic infections. This shows the limitations of microscopy as a means of diagnosis in the bid to eliminate malaria. This also reaffirms that PCR is a more sensitive tool of diagnosis than microscopy. *P. falciparum* revealed moderate levels of genetic diversity and similar population structure in Ghana. To effectively reduce malaria burden in Ghana, control efforts should focus on the population.

The use of microsatellite markers showed moderate diversity as a whole between the ecological zones as demonstrated with heterozygosity and the number of alleles found in this study. There was moderate genetic diversity in the parasite population in the study sites. The Forest zone showed the highest parasite diversity as compared to the Coastal savannah and Sahel savannah.

6.2 Recommendations

- The Forest area of Duase exhibited the highest parasite infections. It is recommended that control efforts should be targeted in this area to reduce the burden of the carriage of *Plasmodium* parasites by school-going children.
- In order t o determine if the genetic diversity of *P. falciaparum* in different eco-epidemiological landscapes are different, a wider study should be carried out with more sites in the different ecological zones.

- It is also recommended that individuals of all age groups should be included in subsequent studies.
- Future studies should include other species of *Plasmodium* which was not the case in this study.

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APPENDICES

APPENDIX A: ASSENT FORM FOR MINORS

Title: The Genetic diversity of *Plasmodium falciparum* in Ghana.

Principal Investigator: Maame Esi Dawson-Amoah

Address: Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, University of Ghana. P.O. Box LG13, Legon-Accra, Ghana.

General Information about the Research

Malaria continues to be a major public health problem in Ghana and the world at large, claiming several lives annually. It is important to make sure we prevent other people from getting the disease. Recent studies conducted indicate that many people live without signs and symptoms of malaria may harbour malaria parasites at very low levels undetected by microscopy. These infections often persist for months and harbour gametocytes, which results in malaria transmission.

You are invited to participate in the above mentioned research study. The purpose of the study is to determine the proportion of the population that has sub microscopic malaria infections. The study will also investigate the genetic diversity of *Plasmodium falciparum* in different transmission zones in Ghana. This will aid the development of strategies towards the control and elimination of malaria in the country. As a participant, your finger will be pricked to collect about 5 drops (40-50 µL) of blood. The blood will be used to test for malaria by microscopic examination and also PCR for the parasite DNA. Your finger will be cleaned with diluted alcohol as disinfectant and the blood will be taken under aseptic conditions. Participation will take approximately 30 minutes.

Possible Risks and Discomforts

Mild discomfort and bruising in the finger is possible where the blood is obtained. This will however resolve within a day. Sterile techniques and disposable, single-use equipment will be used at all times.

Possible Benefits

There are no direct benefits to the child from this study. However, his/her participation may help those who are at risk of developing malaria. He/she will not be paid for participation in this study. All participants will be given a snack at the end of participation.

Confidentiality

All the information gathered from this study will be kept confidential, and you will not be identified when the report is published.

Voluntary Participation and Right to Leave the Research

Your participation in this research is voluntary. There will be no negative consequences if you or the child decides not to participate in the study. Should you or the volunteer, at any point during the study, decide that he/she do not wish to participate any further, you are free to terminate the participation, effective immediately. Any such decision will be respected without any further discussion.

Contacts for Additional Information: If you ever have any questions about the research study or need further clarification or you encounter any discomfort after participation, you may call Maame Esi Dawson-Amoah, Department of Medical Microbiology, University of Ghana (0208692122) at any time.

Your rights as a Participant

This research has been reviewed and approved by the Ethical and Protocol Review, College of Health Sciences, University of Ghana. If you have any questions about your rights as a research participant you can contact Nana Yaw Abankwah, Administrator at the Research Office of the School of Medicine and Dentistry, Korle-bu between the hours of 8am-5pm.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research titled above 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 to participate as a volunteer.

Signature of the Child _____

Printed Name: _____

Date _____

Signature of the Parent/Guardian_____

Printed Name: _____

Date _____

Signature of the Investigator_____

Printed Name: _____

Date _____

(Each participant will be given a copy of this form)

APPENDIX B: PARTICIPANT INFORMATION SHEET

UNIVERSITY OF GHANA -
DEPARTMENT OF MEDICAL MICROBIOLOGY

ASYMPTOMATIC SCREENING OF MALARIA IN SCHOOL CHILDREN

Name of School_____ Site_____ Date of Screening_____

S. No	PARTICIPANTS NAME	CODE	CLASS	DoB/AGE	Temp ºC	REMARKS
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
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13						
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16						
17						
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20						

APPENDIX C: ETHICAL CLEARANCE CERTIFICATE FROM UNIVERSITY OF GHANA SCHOOL OF MEDICINE AND DENTISTRY

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.: EPRC/MAY/2018 May 23, 2018

Maame Esi Dawson-Amoah
Department of Medical Microbiology
School of Biomedical and Applied Health Sciences
Korle- Bu

ETHICAL CLEARANCE

Protocol Identification Number: ***CHS-Et/M.5 – P1.9/2017-2018***

The College of Health Sciences Ethical and Protocol Review Committee at its meeting on January 25, 2018 reviewed and unanimously approved your research proposal.

Title of Protocol: **"The Genetic Diversity of Plasmodium Falciparum in Ghana"**

Principal Investigator: **Maame Esi Dawson-Amoah**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till May 23, 2019.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: .....
Rev. Dr. Thomas A. Ndanu
For: Chair, Ethical and Protocol Review Committee

Cc: Provost, CHS
Dean, SBAHS
Head, Dept. of Medical Microbiology

• P. O. Box KB 52, Korle Bu, Accra, Ghana. • **Telephone:** +233 (0) 306 665103/4 • **Fax:** +233 (0) 302 660762
• **Email:** admin.chs@ug.edu.gh / provost.chs@ug.edu.gh • **Website:** www.chs.ug.edu.gh

APPENDIX D: CHEMICAL REAGENTS AND EQUIPMENT

I: Reagents for laboratory work

Phosphate buffered Saline (PBS)

Saponin

Chelex® 100 Chelating Resin (BioRad)

dNTPs

$MgCl_2$

Buffer

Nuclease-free water

Taq polymerase

Heat block

Ethidium bromide solution (0.5ug/ml; SIGMA)

100bp molecular weight marker (Gibco BRL)

II Preparation of 50X TAE electrophoresis buffer (1000ml, pH 8.2)

Tris base (SIGMA) 242 g

0.5 M EDTA 100ml

Glacial acetic acid 57.1ml

Deionized water to add up to 1000ml

Add 20ml of 50X TAE buffer stock to 980ml deionized water to obtain 1X TAE.

III: Laboratory equipment

Centrifuge and rotor capable of reaching up to 12,000 × g

Polypropylene micro centrifuge tubes (Eppendorf)

Genetic Analyser (ABI 3100)

APPENDIX E: ANALYSIS ACCORDING TO ECOLOGICAL ZONES

	Zones			
Kruskal-Wallis test				
P value	**< 0.0001**			
Exact or approximate P value?	Gaussian Approximation			
P value summary	****			
Do the medians vary signif. (P < 0.05)	Yes			
Number of groups	6			
Kruskal-Wallis statistic	**70.55**			
Zonal PCR prevalence comparison				
Dunn's Multiple Comparison Test	**Difference in rank sum**	**Significant? P < 0.05?**	**Summary**	
north wet PCR vs north dry PCR	-51.74	No	ns	
north wet PCR vs south wet PCR	98.52	Yes	**	0.01
north wet PCR vs south dry PCR	102.1	Yes	*	0.05
north wet PCR vs middle dry PCR	-12.13	No	ns	
north wet PCR vs middle wet PCR	-143.5	Yes	***	0.001
north dry PCR vs south wet PCR	150.3	Yes	***	0.001
north dry PCR vs south dry PCR	153.8	Yes	***	0.001
north dry PCR vs middle dry PCR	39.61	No	ns	
north dry PCR vs middle wet PCR	-91.79	No	ns	
south wet PCR vs south dry PCR	3.554	No	ns	
south wet PCR vs middle dry PCR	-110.6	Yes	**	0.01
south wet PCR vs middle wet PCR	-242	Yes	***	0.001
south dry PCR vs middle dry PCR	-114.2	Yes	*	0.05
south dry PCR vs middle wet PCR	-245.6	Yes	***	0.001
middle dry PCR vs middle wet PCR	-131.4	Yes	**	0.01

	north wet PD	Pfg	north wet PCR	north wet sex	north wet age
Number of values	277	277	276	277	277
Minimum	0	0	0	1	4
25% Percentile	0	0	0	1	8
Median	0	0	0	2	9
75% Percentile	0	0	1	2	11
Maximum	3254	12	1	2	13
Mean	29.77	0.2491	0.3696	1.505	9.292
Std. Deviation	225.7	1.356	0.4836	0.5009	1.998
Std. Error	13.56	0.0815	0.02911	0.03009	0.12
Lower 95% CI of mean	3.072	0.08866	0.3123	1.446	9.056
Upper 95% CI of mean	56.47	0.4095	0.4269	1.565	9.529
Shapiro-Wilk normality test					
W	0.1096	0.1814	0.6112	0.6364	0.9394
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Passed normality test (alpha=0.05)?	No	No	No	No	No
P value summary	****	****	****	****	****
Geometric mean				1.42	9.05
Lower 95% CI of geo. mean				1.363	8.798
Upper 95% CI of geo. mean				1.479	9.309
Sum	8246	69	102	417	2574

Kruskal-Wallis test				
P value	**0.0096**			
Exact or approximate P value?	Gaussian Approximation			
P value summary	**			
Do the medians vary signif. (P < 0.05)	Yes			
Number of groups	6			
Kruskal-Wallis statistic	**15.19**			
Zonal PD				
Dunn's Multiple Comparison Test	**Difference in rank sum**	**Significant? P < 0.05?**	**Summary**	
north wet PD vs north dry PD	-21.15	No	ns	
north wet PD vs south wet Pd	-0.2637	No	ns	
north wet PD vs south dry Pd	15.33	No	ns	
north wet PD vs middle dry Pd	50.93	No	ns	
north wet PD vs middle wet Pd	11.82	No	ns	
north dry PD vs south wet Pd	20.89	No	ns	
north dry PD vs south dry Pd	36.48	No	ns	
north dry PD vs middle dry Pd	72.08	Yes	**	0.01
north dry PD vs middle wet Pd	32.97	No	ns	
south wet Pd vs south dry Pd	15.59	No	ns	
south wet Pd vs middle dry Pd	51.2	No	ns	
south wet Pd vs middle wet Pd	12.09	No	ns	
south dry Pd vs middle dry Pd	35.61	No	ns	
south dry Pd vs middle wet Pd	-3.505	No	ns	
middle dry Pd vs middle wet Pd	-39.11	No	ns	

	north dry PD	Pfg	north dry PCR	north dry age	DDw wet sex	south wet Pd	Pfg
Number of values	324	324	323	324	59	207	148
Minimum	0	0	0	3	1	0	0
25% Percentile	0	0	0	8	2	0	0
Median	0	0	0	9	2	0	0
75% Percentile	0	0	1	11	2	0	0
Maximum	3254	12	1	13	2	401	32
Mean	29.79	0.2099	0.452	9.228	1.763	13.62	0.223
Std. Deviation	210.9	1.254	0.4985	2.244	0.4291	49.54	2.631
Std. Error	11.72	0.06965	0.02774	0.1247	0.05586	3.443	0.2163
Lower 95% CI of mean	6.74	0.07285	0.3974	8.983	1.651	6.83	-0.2044
Upper 95% CI of mean	52.84	0.3469	0.5066	9.474	1.875	20.41	0.6504
Shapiro-Wilk normality test							
W	0.1179	0.1595	0.6331	0.9486	0.5275	0.3059	0.05854
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Passed normality test (alpha=0.05)?	No	No	No	No	No	No	No
P value summary	****	****	****	****	****	****	****
Geometric mean				8.911	1.697		
Lower 95% CI of geo. mean				8.644	1.57		
Upper 95% CI of geo. mean				9.186	1.833		
Sum	9652	68	146	2990	104	2819	33

Kruskal-Wallis test			
P value	0.683		
Exact or approximate P value?	Gaussian Approximation		
P value summary	ns		
Do the medians vary signif. (P < 0.05)	No		
Number of groups	6		
Kruskal-Wallis statistic	3.11		
AGE			
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
north wet age vs north dry age	2.999	No	ns
north wet age vs south wet age	-18.25	No	ns
north wet age vs south dry age	36.91	No	ns
north wet age vs middle dry age	2.363	No	ns
north wet age vs middle wet age	42.14	No	ns
north dry age vs south wet age	-21.25	No	ns
north dry age vs south dry age	33.91	No	ns
north dry age vs middle dry age	-0.6358	No	ns
north dry age vs middle wet age	39.14	No	ns
south wet age vs south dry age	55.16	No	ns
south wet age vs middle dry age	20.61	No	ns
south wet age vs middle wet age	60.39	No	ns
south dry age vs middle dry age	-34.55	No	ns
south dry age vs middle wet age	5.227	No	ns
middle dry age vs middle wet age	39.78	No	ns

	south wet PCR	south wetsex	south wet age	south dry Pd	Pfg	south dry PCR	south dry age	south dry sex
Number of values	207	148	207	116	116	116	116	116
Minimum	0	1	4	0	0	0	3	1
25% Percentile	0	1	8	0	0	0	7.25	1
Median	0	2	10	0	0	0	9	2
75% Percentile	0	2	11	0	0	0	11	2
Maximum	1	2	12	229	7	1	13	2
Mean	0.2126	1.608	9.382	6.457	0.06897	0.2069	9.043	1.638
Std. Deviation	0.4101	0.4898	1.954	24.79	0.6557	0.4068	2.208	0.4827
Std. Error	0.0285	0.04026	0.1358	2.302	0.06088	0.03777	0.205	0.04482
Lower 95% CI of mean	0.1564	1.529	9.114	1.898	-0.05163	0.1321	8.637	1.549
Upper 95% CI of mean	0.2688	1.688	9.649	11.02	0.1896	0.2817	9.449	1.727
Shapiro-Wilk normality test								
W	0.5031	0.6192	0.9174	0.2776	0.08202	0.4971	0.9456	0.6081
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0001	< 0.0001
Passed normality test (alpha=0.05)?	No	No	No	No	No	No	No	No
P value summary	****	****	****	****	****	****	***	****
Geometric mean		1.524	9.138				8.743	1.556
Lower 95% CI of geo. mean		1.443	8.841				8.317	1.463
Upper 95% CI of geo. mean		1.61	9.446				9.191	1.655
Sum	44	238	1942	749	8	24	1049	190

	middle dry Pd	Pfg	middle dry PCR	middle dry age	middle wet Pd	Pfg	middle wet PCR	middle wet age
Number of values	216	216	216	216	117	117	117	117
Minimum	0	0	0	3	0	0	0	3
25% Percentile	0	0	0	8	0	0	0	8
Median	0	0	0	10	0	0	1	9
75% Percentile	0	0	1	11	0	0	1	11
Maximum	111	0	1	13	61	0	1	12
Mean	2.606	0	0.3889	9.292	4.872	0	0.5983	8.88
Std. Deviation	11.38	0	0.4886	1.966	13.31	0	0.4924	2.475
Std. Error	0.7745	0	0.03325	0.1337	1.231	0	0.04552	0.2288
Lower 95% CI of mean	1.08	0	0.3234	9.028	2.434	0	0.5081	8.427
Upper 95% CI of mean	4.133	0	0.4544	9.555	7.309	0	0.6884	9.333
Shapiro-Wilk normality test								
W	0.2439		0.6182	0.9442	0.4138		0.6222	0.9081
P value	< 0.0001		< 0.0001	< 0.0001	< 0.0001		< 0.0001	< 0.0001
Passed normality test (alpha=0.05)?	No		No	No	No		No	No
P value summary	****		****	****	****		****	****
Geometric mean				9.053				8.431
Lower 95% CI of geo. mean				8.768				7.905
Upper 95% CI of geo. mean				9.347				8.993
Sum	563	0	84	2007	570	0	70	1039