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COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

GENETIC DIVERSITY OF *P. FALCIPARUM* IN LOW AND HIGH TRANSMISSION INTENSITIES IN SOUTHERN GHANA

THIS THESIS IS PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY IN PARTIAL FULFULMENT FOR THE AWARD OF MASTER OF PHILOSOPHY IN BIOCHEMISTRY

BY

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DECLARATION

I declare that this research work was done under the supervision of Dr. S. Pandam Salifu and Dr. Linda E. Amoah, a fellow at the Noguchi Memorial Institute for Medical Research (NMIMR) and that it contains no material previously published by another person or any content accepted for award of any other degree by the University except where references have been cited, this thesis is the results of my research work.

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ABSTRACT

BACKGROUND: Malaria is a public health challenge that is preventable and can be treated but *P. falciparum*, the main parasite agent, has survived in the midst of several intervention programmes aimed at combating the infection especially in Sub - Saharan Africa where malaria is endemic. *P. falciparum* genetic diversity is frequently measured by genotyping the merozoite surface protein (*msp 1, 2)*, however the recent inclusion of Capillary Electrophoresis (CE) based microsatellite markers has enhanced the accuracy of the estimation of multiplicity of infection (MOI) and parasite diversity. This study aims at utilizing 12 unlinked microsatellite markers, *msp -2* and Glutamate-Rich Protein (*glurp)* antigenic marker to determine parasite diversity in different transmission intensities in southern Ghana.

METHODS: Whole blood was collected from asymptomatic volunteers living in Obom (hyper-endemic) and Asutuare (holoendemic). Quick DNA kit was used to extract DNA from whole blood samples. The presence of *P. falciparum* was confirmed by species specific primers Polymerase Chain Reaction (PCR). *P. falciparum* positive samples were then used for genotyping *msp -2* and *glurp* markers. Clonal Parasite positive samples of *msp – 2* were subjected to capillary-based microsatellite genotyping in addition to MSP - 2 genotyping.

Results: 44 (55%) and 24 (30%) samples were positive for N5 (3D7) and M5 (FC27) at Obom with average MOI of 1.30. N5 strain was prevalent at both study sites. Only 8(10%) and 4(5%) of N5 and M5 strains respectively were genotyped at Asutuare with an average MOI of 1.0. To compare the prevalence of N5 and M5, a non parametic t-test of $p < 0.05$ in Obom and $p > 0.05$ was recorded in Asutuare. For *glurp* R II region 55 (68.5%) and 70 (87.5%) were genotyped for Obom and Asutuare sites.

An average of 35 samples were genotyped using microsatellites markers, average number of alleles for both Obom and Asutuare were 10 and 8 respectively. Genetic diversity of microsatellites was low; *He* ranged from 0.00 to 0.577

Conclusion: Parasite diversity was high with *msp – 2* marker compared to *glurp* gene, microsatellites had low genetic diversity of the parasites at both sides but the sensitivity and high resolution makes them the markers of choice for genotyping *P. falciparum.*

Dedication

This research work is dedicated to Mr. and Mrs. Abukari family, my daughter Elham K. Zakaria and son Abdul-Wakeel W. Zakaria

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I give thanks to Almighty Allah for how far He has brought me and for His guidance and protection throughout this research work.

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CHAPTER ONE

1.0 INTRODUCTION

Malaria continues to pose great public health challenge globally despite several interventions to curb its burden with developing countries being the worse affected. Malaria remains one of the most common protozoan parasitic infections of the world with about 3.2 billion people infected in high transmission areas of the world out of which 3 million people die annually. Malaria is caused by a unicellular organism of the genus *Plasmodium* and out of the five main *species* known to affect human *P. falciparum* is the lethal of all because of its virulence and evasion of red blood cells (RBCs) (Josling & Llinás, 2015; Sorontou & Pakpahan, 2015).

In sub-Saharan Africa, malaria is endemic with the peak of transmissions been observed in the raining season. In the last decade in Africa, there has been a decline in mortality cases of the infection from 4.3 million to 2.9 million cases in 2015 especially in children less than five years. The implementation of key intervention programmes has contributed significantly to this positive outlook (Organization, 2015c). Some of these interventions are effective diagnosis and treatments of the infection, distribution of treated bed nets (TBNs) and among others (Agyekum, 2017; USAID, 2016). However parasite genetic diversity of *P. falciparum* continue to frustrate these intervention efforts there by derailing the effort at sustaining long term gain in the fight against malaria in the world (Nkumama *et al*., 2016). In Africa according to Nrumama *et al*., (2016), the population that is increasingly becoming at risk of contracting malaria are teenagers and children under five years thus children of school going age. Sub-Saharan Africa remains the highest area in Africa with increasing cases and deaths from malaria down to southern Africa with least incidence of the infection (Organization, 2011).

Ghana is endemic and perennial for malaria with *P. falciparum* responsible of about 90% of all infections of the *plasmodium* species. *P. malarae*, and *P. ovale* responsible for the remaining 10%, No case of *P. vivax* has been reported in the country. The period of transmission of malaria in Ghana differs from one geographical region to the other with the northern parts of the country having seasonal transmission while southern Ghana records perennial transmission.

Ghana within the first quarter of 2016 recorded 5 million cases of malaria with mortality cases of about 7000 during the same period. Increase access and reporting of suspected malaria cases may be responsible for this hike in the infection at the health facilities. The coming into being of the National Health Insurance Scheme (NHIS), community-based health planning services (CHPs) compound, free distribution of insecticides treated nets to mothers during their first Antenatal visits to hospitals among others has contributed to a reduction in the infection in the same period from 14.1% to 0.6% in children less than five years (MoH, 2016; USAID, 2016).

The life cycle of malaria parasite requires the production of matured gametocytes in humans. At this sexual stage, parasitic agents get into the mosquito vector when it feeds on human blood and alternates between the human host and the vector. (Kiszewski, 2010; Singh & Kumar, 2015). The production of infective male and female gametes (gametocytogenesis) is not only an essential component that ensures propagation but also produces clones or alleles that evade the immune system and generates disease resistant strains. Gametocytogenesis and increased genetic complexity of *P. falciparum* genotypes in addition to the population density of the parasite in different transmission settings ensues pathogenesis and disease progression (Ayanful-Torgby *et al.*, 2016; Baker *et al*., 2010).

Genetic complexity (diversity and density) of *P. falciparum* gametocytes is the number of distinct genomic clones, their recombination or rearrangements with different alleles or polymorphisms showed at different genomic loci and by immune selection (Duah *et al*., 2016). Genomic diversity of *P. falciparum* is an essential indicator to the fitness and adaptations of the parasite's population dynamics in a particular geographical area (Larrañaga *et al*., 2013). Antigenic diversity, allelic polymorphism, chromosomal rearrangements etc are implicated in genetic diversity in *P. falciparum*. These genetic variations are vital in understanding disease transmission, pathogenesis and the development of an effective vaccine (Kidima & Nkwengulila, 2015).

Hypertransmission of *P. falciparum* is associated with low disequilibrium, high genomic variance and high multiplicity of infection (MOI) (Larrañaga *et al*., 2013). In low transmissions areas of malaria, there is low MOI, low genetic diversity (Razak *et al*., 2016). Antigenic variance of *P. falciparum* population dynamics and severity of the infection differs according to age and parasite population densities in varying areas of transmissions, children and pregnant women remain the most vulnerable population group (Larranga *et al*.,2013).

Parasite survival, pathogenesis and immune escape are the result of high level of diversity in surface antigens which serve as markers in *P. falciparum* genomic structure. The gene encoding the merozoites proteins 1, 2 (*msp - 1, 2*) and *glurp* are antigenic markers of *P. falciparum* and are used for the investigations of genetic variation (diversity) of the parasites, the pattern of *P. falciparum* transmission intensities, multiplicity of infection (MOI), host-immune responses against the parasites and population dynamics (Congpuong *et al*, 2014).

The *MSP - 2* is the most diverse antigenic marker which play a vital role in invading red-blood cells (RBCs) during the blood stage transmission of the parasite. *msp 1, 2* and *glurp* antigenic markers are recommended by the World Health Organisation (WHO) (Organization, 2008) and used as indices for differentiating between new infections and failure of treatment (recrudescence) in the course of malaria drug resistance surveillance study in a particular transmission area (Duah *et al,* 2016). Glurp is an antigenic protein marker used to genotype *P. falciparum* and considered by WHO as a vaccine candidate. Glurp is produced during the erythrocytic and merozoite stages in the life of the development of the parasite and recommended by WHO to be used alongside MSP – 2 in genotyping *P. falciparum* from different geographical settings.(Organization, 2008; Pratt-Riccio *et al*., 2013).

MOI from *P. falciparum* as a result of high degree of antigenic variance differs from one parasitic endemic area to another, mutant strains persist and are selected. Gene flow of these strains across different geographical areas ensures parasites survival, pathogenesis, emergence of drug resistant strains and this ultimately derails control efforts aimed at curbing malaria (Duah *et al*, 201 6).

In recent years, molecular tools such as PCR-RFLP, Single Nucleotide Polymorphism (*snps*), microsatellite genotyping using unlinked markers. Molecular typing of the polymorphic regions of *msp 1, 2,* and *glurp* genes are employed in investigating the genetic complexity (diversity) and distribution of *P. falciparum* population and transmission trends in different geographical areas (Kidima & Nkwengulila, 2015). Parasite inbreeding, gene flow, geographical isolation, epidemic expansion can all be successfully determined by these molecular techniques (Razak *et al*., 2016).

Microsatellite genotyping or tandem repeats of 2 or 3 base pairs is a molecular tool used in *P. falciparum* genotyping by unlinked or neutral polymorphic markers. This is used to differentiate strains of malaria parasites and it is vital in investigating parasite population genomic studies (Carlton *et al*., 2015). Short tandem repeats sequences are closely associated with an increased Adenine- Thymine (A-T) content in *P. falciparum* with about 90% repeats, strand-slippage or errors in parasites DNA synthesis is implicated in high repeats (Carlton *et al*., 2015).

Ghana has persistent malaria transmission throughout the year and the infection cuts across all year group but children and pregnant women remain the most vulnerable population. The country is malaria endemic with transmission been classified as either seasonal or perennial depending on one's geographical location. The southern part of the country has coastal savanna vegetation and transmission of malaria is perennial, the forest vegetation characterizes the middle belt of the country with all year transmission in malaria cases while the northern region has guinea savanna vegetation with a seasonal transmission. Peak transmissions are observed in the raining season and drops during the dry season (Duah *et al*, 2016).

Efforts at controlling malaria transmission have been central to the millennium development goals (MDGs). In view of this, the fight against malaria transmission was targeted and prioritised by the United Nations Assembly (UNA) since 2000 and was classified as goal 6 of the MDGs, which aims at reducing the incidence of malaria and by half in 2015. Other indices were adopted to monitor the efforts in curbing malaria related deaths and incidence through cost-effective preventive measures or interventions (Organization, 2015b).

1.1 PROBLEM STATEMENT

Parasite population genetics analysis with microsatellites markers requires Capillary electrophoresis, an area that is still unexploited in Ghana. Assessing parasites genetic diversity with antigenic markers (*msp-2* and *glurp*) presents a huge challenge when agarose gels are used as the discriminatory power of these gels are low compared to CE. (Ferdig & Su, 2000).

P. falciparum genetic diversity is a huge challenge that continues to frustrate intervention efforts at controlling transmission of malaria. Extensive diversity and polymorphism of *msp - 2* gene may be implicated for parasite genetic variation, fitness, and pathogenesis of the condition (malaria) and the emergence of disease resistant strains across different areas of transmission. (Kidima & Nkwengulila, 2015; Singh & Kumar, 2015).

Malaria parasites have developed drug resistant genes over the years and have drawn back efforts at curbing malaria transmission. These strains interfere with intervention and preventive measures in malaria endemic areas. Ghana's baseline treatment and case management for malaria has shifted from chloroquine to Artemisinine- Based Combination Therapy (ACT) in 2004. (MoH, 2009a; Moulds & Diallo, 2012).

The outpatient departments (OPDs) in Ghana still records high number of malaria cases in all hospitals attendance with 22.3% deaths, the vulnerable group (children and pregnant women) are the most affected (Duah *et al*, 2016) Access to health facilities to these vulnerable groups is still a main problem, the situation is even worse in the rural areas where some towns and villages are cut off from the district capitals due to very bad roads and floods in the raining seasons when transmission is at its peak.

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).

1.2 RESEARCH HYPOTHESIS

Genetic diversity in *P. falciparum* is associated with transmission of malaria in different intensities.

1.3 GENERAL OBJECTIVES

The study will investigate the genetic diversity of *P. falciparum* in different transmission areas in Southern Ghana.

1.4 SPECIFIC OBJECTIVES

1. To genotype *P. falciparum* using 12 unlinked microsatellite markers.

2. To determine the antigenic variance in *msp - 2* polymorphic marker.

3. To determine the polymorphism of the R II region of *glurp* gene in field isolates.

4. To determine allelic concordance between *msp - 2* and microsatellites.

1.5 JUSTIFICATION

This current study has the potential of giving more insights into the most polymorphic gene of *P. falciparum*, one of the antigenic variance marker (*msp - 2*) during the blood stage of the parasite's transmission cycle. *msp - 2* is a good candidate for an effective vaccine development against malaria, that can offer huge relieve to the vulnerable population of the world thus pregnant women and children under five years of age especially in developing countries where the rate of transmission is high.

Studies on the genetic diversity of the parasite will aid researchers to properly monitor and evaluate malaria interventions programmes within the study areas and Ghana as a whole. The surveillance of the parasite's mutant gene flow across different transmission areas and drug resistant strain escaping the immune system will be properly understood. Microsatellite genotyping technique will further help 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 research is expected to provide baseline information that will help future researchers using similar markers to genotype the parasites in different geographical settings in the country. Data on genetic trends of the parasite is crucial and may establish a link between 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).

CHAPTER TWO

LITERATURE REVIEW

2.1 GLOBAL CHALLENGE OF MALARIA

The global fight against malaria has seen some significant gains made from 2000 to 2016 according to the WHO but despite these gains the disease is still endemic in many parts of the world. The rate of decline has halted and beginning to reverse in the burden of the disease especially in Africa as indicated in **Figure 2.1** below. The disease still continues to have a devastating effect on the livelihood of infected individuals in these endemic areas of the world (Organisation, 2016: Organisation, 2017).

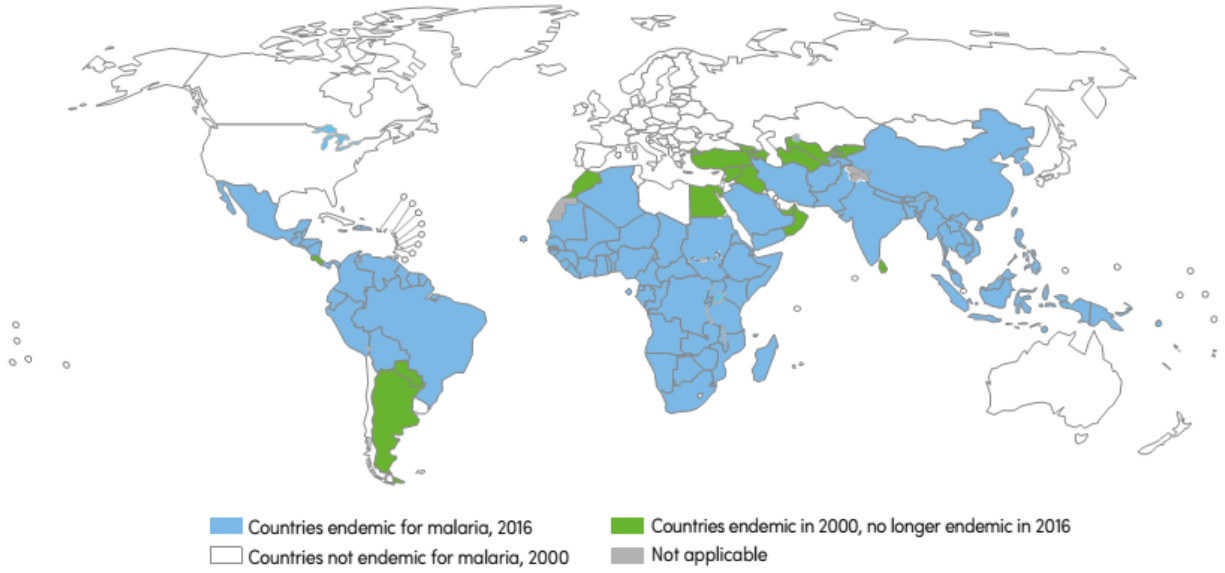

Figure 2. 1: Global malaria endemic countries from 2000 to 2016 (Adopted from *(Organization, 2016a).*

Malaria continues to cause a huge devastating effect on the world's population even though the disease is preventable and can be treated, its drain on peoples' livelihood and health is immense. The percentage of mortality cases attributed to malaria in the developed and developing

countries is 0.2% to 57.9% respectively and that there is a relationship between the endemicity of malaria in a country and its national per capital income. Developing countries of countries of the world are noted for the high prevalence of the disease where the Gross Domestic Product (GDP) is low, so malaria breeds poverty and poverty feeds malaria. According to recent global estimates the incidence and mortality cases attributed to malaria has reduced drastically in the last decade with 21% and 29% case incidence and deaths respectively recorded between 2010 and 2015 (Worrall *et al*., 2002 : Organisation, 2015a).

According to the WHO, about 3 billion persons in the world are at risk of developing malaria with an estimated 198 million cases and 584000 mortalities recorded in 2013. Despite the decline in the number of cases and deaths at 30% and 47% in 2000 respectively, sub-Saharan Africa still contributes to a greater percentage (78%) of which 90% of the mortality cases are in children less than five years of age (Organization, 2015b).

A lot of progress was made in the fight against malaria in 2015 a year set aside for the achievement of the MDG goal 6c to reduce to half and reverse the incidence of malaria and the roll back malaria initiative. 214 million cases was reported with 4380 deaths recorded representing 18% and 48% in reported and mortality cases respectively, a decline in the 2000 figures.(Organization, 2015b). According to WHO MDG goal 6c and the roll back malaria initiative have both been met, for from 2000 to 2015 the percentage decline stood at 37% worldwide with the rate of deaths reduced by 60% (Organization, 2015b).

Poverty and malaria are strongly associated with each other, they feed each other. It is not surprising that in 21st century sub-Saharan Africa is still grappling with issues of malaria with a huge amount of about US$12 billion spent each year in the sub-region for curbing the infection.

It is estimated that the economy of Africa will deep by 1.3% due to the incidence of malaria and the cost of managing the condition. The most deprived areas of Africa continue to bear the brunt of malaria, poor road network, and inadequate health facilities in most rural areas and inadequate qualified health personnel to deal with the changing dynamics of parasite transmission (MoH, 2016; Nonvignon *et al.*, 2016).

2.2 THE BURDEN OF MALARIA IN GHANA

Malaria is endemic in the country all year round with southern Ghana experiencing perennial transmission. The duration of malaria transmission varies from northern to southern Ghana. The northern Ghana has two main seasons, thus the raining and dry season's transmission. Malaria surges during the raining period with the peak of transmission observed June-October each year and deeps in the dry seasons where stagnant water, bushy environments etc generally reduces cutting down on the breeding grounds of the parasites drastically. Southern Ghana is noted for three seasons so transmission of malaria seems to be prolong with the presence of rains been the main architect for breeding and transmission of the parasite (MoH, 2016).

The entire 24 million people in Ghana are at risk of malaria infection but children below five years and pregnant mother are at greater risk of the menace of malaria in the country, Ghana is noted for the tropical climatic conditions a favourable breeding grounds for the mosquito vector. Urbanisation with its accompanied challenges in Ghana has worsened the situation, the major cities in the country are grappling with the springing up of slums, filth and inadequate social amenities among others (Nonvignon *et al*., 2016).

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huge amount of about US$12 billion spent each year in the sub-region for curbing the infection. It is estimated that the economy of Africa will deep by 1.3% due to the incidence of malaria and the cost of managing the condition. The most deprived areas of Africa continue to bear the brunt of malaria, poor road network, and inadequate health facilities in most rural areas and inadequate qualified health personnel to deal with the changing dynamics of parasite transmission (Nonvignon *et al*., 2016: MoH, 2016).

The economic burden of malaria in Ghana is heavy with about US$ 48.73 spent each year at the household level in combating malaria. At the peak of transmission health facilities in the country are overburdened, there is loss of contact hour at schools, productivity has its own share of the challenges of malaria. Malaria is not only dangerous but expensive to manage and treat, it hinders economic growth and opportunities in the country. It is estimated that malaria alone cost the country about 6% of its Gross Domestic Product (GDP) each year as a result the Ghana Poverty Reduction Strategy II (GPRS II) sees introduction of interventions programmes in the country as one area that could lessen the burden of the poor (MoH, 2009). The country spent US$50.05 million and US$66.06 million in 2002 and 2009 respectively on combating malaria and that the economy is expected to grow by national 0.07% each year if the menace of malaria is brought under control (MoH, 2016; Nonvignon *et al*., 2016).

Malaria accounts for about 38% of cases at the outpatient departments (OPDs) and 27% in-patient departments (ward cases) at the health facilities in the country, admissions of children less than 5 years stood at 48.5% in 2015. 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. According to the national malaria control programme (NMCP) out of the 10.4 million suspected cases of malaria recorded in 2016 at the

OPDs of the various health facilities in Ghana, 2137 were admissions (ward cases) and 1264 mortality cases recorded. This according to NMCP represented a decrease in suspected cases and deaths recorded in 2015. The decrease in the number of mortality cases was attained in children less five years from 1037 in 2015 to 590 in 2016 (MoH ,2016).

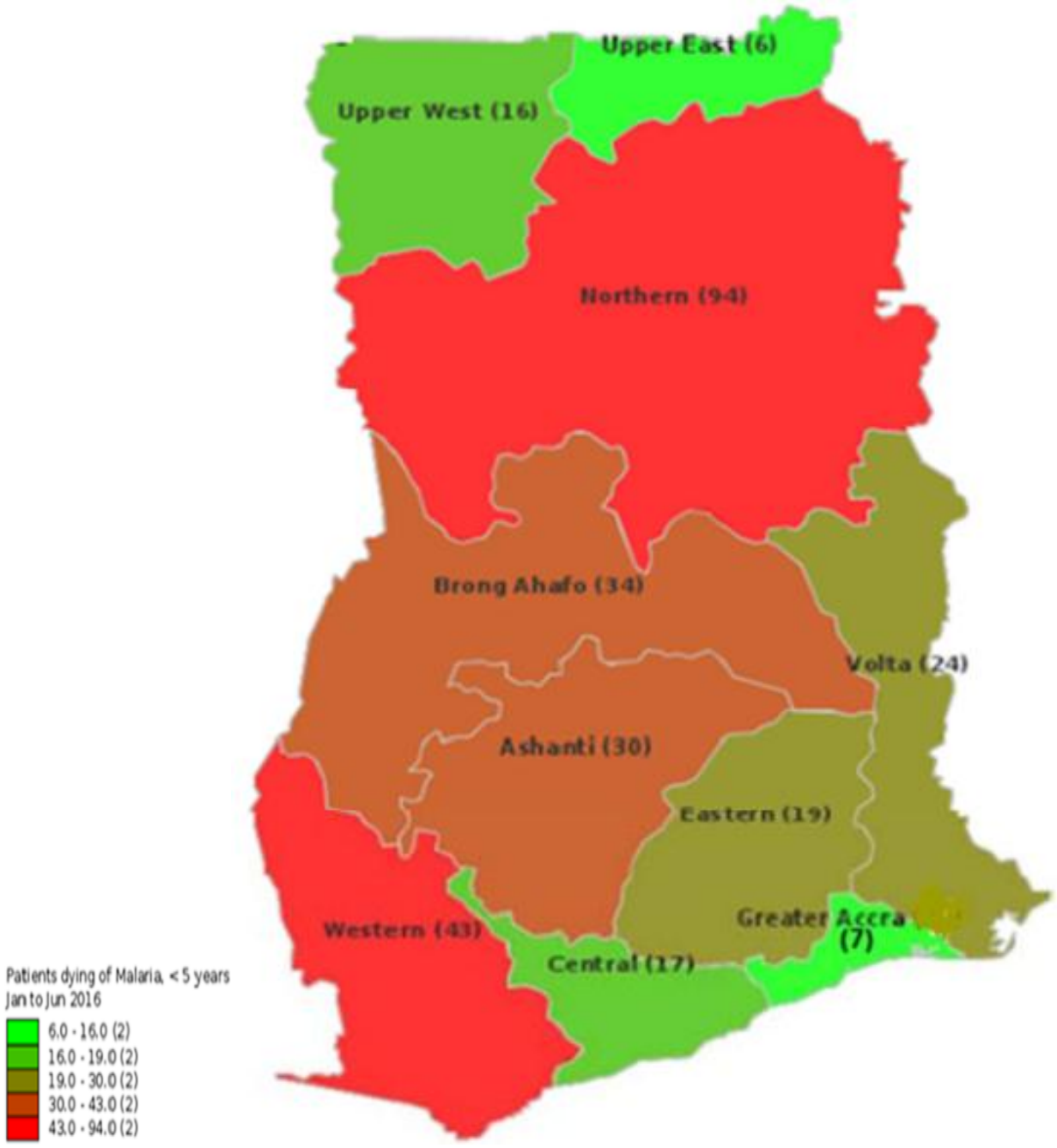


Figure 2.2: Ghana's Regional malaria mortality for children below 5 years of age from January to June 2016 (Adopted from the roll back malaria: GHS, 2016).

Northern region recorded the highest number of deaths (94) in children less than five and Upper East recording the least deaths (6) as shown in **Figure 2.2** above.

The NMCP sets out a target of reducing mortality and morbidity attributed to malaria to about 75% by the year 2020. The report indicates that case mortality declined from 0.51% in 2015 to about 0.32% in 2016 with Ashanti region recording the list mortality figures of 0.13%. Central to the target of reducing mortality is case management with about 12000 health personnel benefiting from a refresher outreach programme that will equip them to better handle malaria cases well. (MoH, 2016).

Appropriate diagnostic of malaria is another area of focus by the NMCP in achieving sustained decline of the condition in the country, to this effect a national protocol for diagnosis of malaria was developed and adopted. Key to this protocol was the diagnosis of malaria by microscopy and rapid diagnostic test (RDT) kit. RDT offered an additional advantage in its usage as it is inexpensive, it does not require expect knowledge for its use etc. The NMCP is also embarking of a routine nationwide monitoring and evaluation of laboratory scientists most especially and other paramedics on quality assurance practices with the use of microscopy in diagnosing malaria as the goal standard but in its absence RDT use is recommended. (MoH, 2016).

As part of the fight against malaria in the sub-region Ghana together with Malawi and Kenya were chosen to pilot the RTS, S malaria vaccine in 2018. RTS,S is a blood stage vaccine that is rolled out in these developing countries to evaluate the protective effects it has on children less than five and to further add up to the already existing intervention programmes endorsed by the World Health Organisation. These countries were chosen because despites several intervention efforts implemented in these areas, malaria is still a huge challenge (https://en.wikipedia.org/wiki/RTS,S; accessed on 15/8/17: (Organization, 2015b).

2.3 CONTROL AND ELIMINATION OF MALARIA IN GHANA: SUCCESSES AND PITFALLS

The global estimates for the prevalence of malaria has declined in recent years even though the population at risk of the menace of the disease stood at 3 billion, sub- Saharan Africa still record a huge number of the mortality cases. Several countries in west African are introducing intervention programmes to fight against the condition (malaria), this according to the 2016 edition of the World malaria report. In Africa Egypt and Morocco have made giant strides in the elimination of malaria in 2000 and 2006 respectively after having reported no malaria cases in three consecutive years.(Awine *et al*., 2017: Organisation, 2016b).

Ghana has is endemic for malaria infection and all age groups can contract malaria, the most vulnerable age brackets are children below 5 years and pregnant women. The call for the fight against malaria in Ghana dates back the colonial (pre-independence) era in which Christopher and Stephenes (1900) did a report and presented their recommendations to the Royal Society's malaria committee. Their findings were based on the research about the breeding sites of mosquitoes, habitation of rural (natives) and expatriate dwellers and the spread of malaria. The report concluded that the main agent for the spread of malaria was indigenous (natives) people and that expatriate dwellers acquire the infection from these natives. They went further to state that efforts should be made to clear all mosquito breeding sites in the communities (Christophers & Stephens, 1900; NMCP, 2013).

Control of malaria during the era of the second world war took a different dimension with the responsibility falling on the military in the Gold Coast. They were tasked to safeguard the Coastal area for personnel who were bound for the European countries, for Accra was a standby point for troops recruited to fight in the second world war in Great Britain and the America.

Malaria related deaths among troops were of major concern to authorities and thus it was a major concern to the wellbeing of the troops (Christophers & Stephens, 1900).

Chemotherapy (drugs) and screening were the main modes of preventive measures adopted during this period but high parasite carriage was a major hindrance to the success of this method of malaria control. This lead to the introduction of insecticides spraying of mosquito breeding areas in the communities subsequent to this an evaluation of this showed a decline in the infection (NMCP, 2013).

The WHO held a malaria conference in Africa (Uganda) in which key decisions were taken to integrate and coordinates all efforts aimed at controlling malaria in the sub-region. Key recommendations reached at the Uganda summit were that all malaria intervention efforts could be integrated to help effectively fight the menace of the infection in Africa. It was also stressed that gains made in the fight against malaria in Africa should be evaluated and that research in malaria should be strengthened and made integral towards combating the infection in the tropics (Christophers & Stephens, 1900).

In the early 1950s chemoprophylaxis field trials were held in Accra among school children where they were given Amodiaquine – Pyrimethamine, this was meant for parasite clearance in addition to Daraprim. An evaluation of the impact of this mode of control showed significant reduction in parasite levels in school children (Christophers & Stephens, 1900; NMCP, 2013).

Control of malaria in the late 1970s and 1990s concentrated on larval control and indoor Residual spraying (IRS) programmes were introduced in the major cities in Ghana which was based on the availability of logistics and personnel. Ghana had no formal agenda to fight malaria in the 1980s hence there were no efforts aimed at controlling malaria in Ghana. Subsequent to

this there were no antimalarial drug policy in the country hence a lot of antimalarial drugs were sold and taken without any regulations and dosing. (Christophers & Stephens, 1900; NMCP, 2013).

In the late 1990 an action plan for the fight against malaria was developed by the MOH in Ghana, this action plan was targeted at reducing the infection such that it would no longer be regarded as a public health issue. Increasing the capacities of the health care providers and provision of equipment as well as focusing on the management of malaria through appropriate diagnosis and treatment (NMCP,2013).

Another initiative at curbing the incidence of malaria and to reduce it to half by the year 2010 was launched in 1998 known as the Roll Back (RBM) malaria initiative. This came at a time when there was a surge in the incidence of the infection in sub – Saharan Africa, the RBM initiative led to the decline in the number of malaria cases especially the vulnerable population in the country (NMCP, 2000).

The national health agenda together poverty reduction policy had been integrated with malaria control programmes and had focused on controlling the mosquito vector and to strengthen the management of cases attributed to malaria during the 2000 to 2015 strategic plan. The major target of the policy was to achieve a decline in malaria cases up to 75% by the year 2015. The strategy went alongside of the policy change in drug (single therapy) to an antimalarial drug combination thus ACTs because the single malaria therapy had failed, artesunate – amodiaquine was initially introduced which was later modified to artemether – lumefunthrine (Koram, 2005: MoH,2009 :NMCP, 2008).

Ghana incorporated malaria control programmes in its national development agenda, a policy framework was developed to ensure prevention and appropriate management/treatment of malaria with the distribution and usage of treated bed nets. The vulnerable group thus children less than five, pregnant women and the aged were treated free of charge of all malaria cases. The NHIS was introduced to address the issue of cash and carry that was generally practiced at the health facilities and made affordability of health care reserved for only the rich and the affluent. Again to ensure that the fight against malaria was not only done at the national level districts and municipal assemblies were directed to use 1% of their allocation of the common fund for malaria control (NMCP,2013).

The success of the intervention methods introduced over the years was to reduce the prevalence of malaria either by targeting the mosquito vector or the *plasmodium* parasite. There was significant progress made towards the fight against malaria through these intervention methods as seen through the reduction in the incidence of the infection in the country. According to the NMCP, (2013) there was a decline of in the number of malaria cases in children less than five years. The report had it that Ghana achieved a parasite clearance rate of 27.5% in children less than five years. The positive outlook in the reduction of malaria cases could be attributed to the introduction of these intervention efforts to help curb the menace of the disease in the country (MoH, 2017).

IRS, distribution of ITNs to pregnant women and children less than five years, appropriate diagnosis of malaria with RDTs and microscopy as the gold standard and case management through chemotherapy whose (drug) policy has changed over the years from a single (monotherapy) to heterotherapy (ACTs) (MoH, 2017).

The NMCP in collaboration with the districts and municipal health directorates have an evaluation and monitoring programmes for health facilities in the country where health personnel involved in diagnosis, treatment/management of malaria are monitored, evaluated and sometimes given in-service training on management of the disease. All the control programmes and strategies have helped in no small measure in reducing the incidence of malaria in Ghana as evident by the WHO malaria report 2015. The RBM initiative has it that the country witnessed a decline in the number of mortality cases of malaria from 379 to 143 in the first quarter of 2017 (MoH, 2017).

The economic burden inflicted on the health facilities by malaria is enormous, significant proportion of income goes into purchasing of essential supplies meant for malaria control programmes. Asante and Asenso-Okyere (2003) suggest that Ghana have a - 0.41% GDP growth due to the cost and burden of malaria on the health delivery systems. At the household level the cost of malaria is put at $ 15.79 (Asante & Asenso-Okyere, 2003).

Resistance developed by the mosquito vector to chemicals used to treat insecticides bed nets and indoor residual spraying method is another huge challenge to health authorities in their quest to win the fight against malaria through prevention methods in the country. Resistance developed by the parasite (*P. falciparum*) necessitated a drug policy change in Ghana in 2004 after widespread parasite resistance was detected and subsequent endorsement of a change from chloroquine to artemither- Lumefantrine, Artesunate Amodiaquin, etc. In Ghana and sub-Saharan Africa, endemicity of malaria is thought to be the consequence of parasites and vector resistance making current intervention and drug treatment very difficult to achieve the intended target (MoH, 2009b; Organization, 2015b).

To tackle the challenges confronting the fight against malaria in the world, a technical plan known as the Global technical strategy or GTS was developed by WHO to target a malaria free world from 2016 to 2030 (Organization, 2015a).

GTS was formulated in line with the Roll Back Malaria initiative and was put into three thematic areas thus to ensure that case management/treatment, intervention and appropriate diagnosis were within the reach of everyone. Control and elimination of malaria (CEM) should be accelerated and surveillance of malaria should be integrated into the main intervention programmes in the country. Successive government should include the CEM in their political agenda (manifesto) , a certain percentage (significant) of the municipal and district assembly common fund should be allocated to the NMCP to effectively roll out intervention programmes towards combating malaria down to the rural areas in the country (Organization, 2015a).

2.4 THE LIFE CYCLE OF *P. FALCIPARUM*

P. falciparum undergoes a complex life cycle that alternating between the human host and the mosquito vector. There are three main stages that the parasites undergo in its life cycle thus: Pre-erythrocytic stage, asexual (ring) stage and sexual (gametocytes) stage as shown in the (**Figure 2.3**).

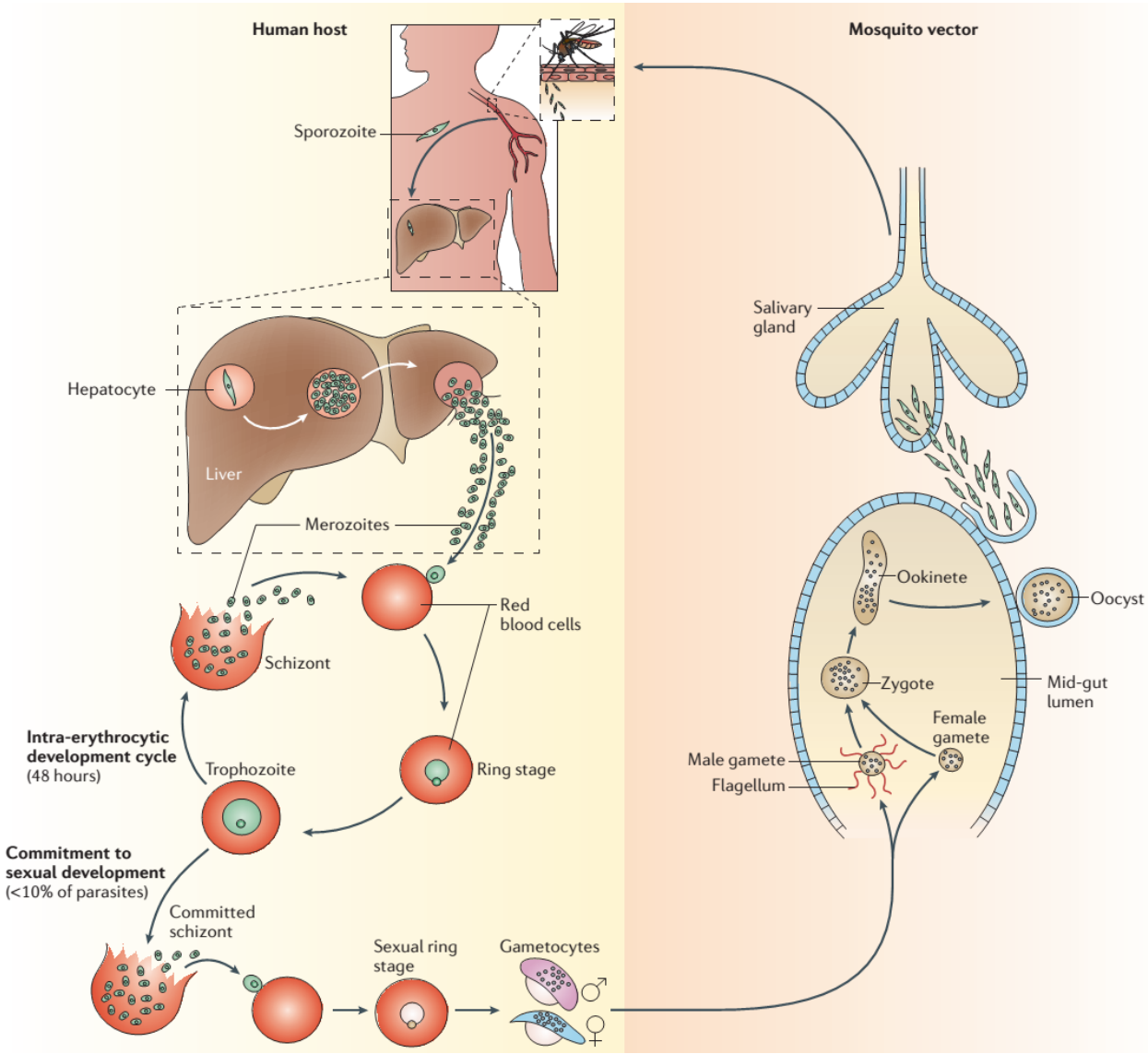


Figure 2.3: Life cycle of *P. falciparum* (Adopted from (Josling & Llinás, 2015).

Plasmodium falciparum undergoes a development that alternating between the host and the mosquito vector. There are three main stages that the parasites undergo in its life cycle thus:

i. Pre-erythrocytic stage

ii. Asexual (Ring) stages

iii. Sexual (Gametocytes) stage

2.4.1 THE PRE-ERYTHROCYTIC STAGE

This stage of the parasite's life cycle starts when the mosquito vector feeds its blood meal from the human host and in the course of this injects several thousands of sporozoites into the bloodstream. The motile sporozoites then evade the liver cells (hepatocytes) and undergo repeated rounds of differentiation or exoerythrocytic schizogony. The sporozoits after a week in the hepatocytes matures and releases merozoites into the bloodstream (peripheral blood). (Nilsson *et al.,* 2015).

At the pre-erythrocytic stage, a 200kDa liver stage antigen 1 (LSA-1) is expressed and plays a role in the parasites survival however, the exact function of this antigen remains unclear. According to Mikolojczak *et al*., (2011), deletions in these proteins presented no effect in the parasites development at the liver and asexual stages of the parasite life cycle.

The circumporozoites protein (CSP) is another molecule that plays an essential role in the infection of hepatocytes. CSP is implicated in the release and attachment of sporozoites to the hepatocytes or the temporal reservoir of the salivary gland of the mosquito vector. CSP is also involved in mediating a protective effect thereby preventing host immune responses like the respiratory bust from reducing the activities at the hepatocytes. At the pre - erythrocytic stage,

there is no evidence of inflammatory responses even though several thousands of merozoites are involved and still present little or no clinical manifestations.

It is significant to note that though the pre-erythrocytic stage of the parasite life cycle does not involve clinical manifestations and disease, it is vital in the proliferation of the hepnozoites and at the end of this stage about 40000 merozoites are released to begin the erythrocytic stage of the parasite life cycle out of a possible inoculation of few parasite by the mosquito vector during its blood meal (Josling & Llinás, 2015).

2.4.2 ASEXUAL ERYTHROCYTIC STAGE

At the asexual stage, merozoites upon release from the liver stage evade the circulatory blood cells (RBCs) and undergo several cycles of repeated infection of uninfected RBCs. In the erythrocytes, the merozoites develop into ring forms trophozoites and then to schizonts that habour about 32 unique merozoites depending on the type of infecting Plasmodium species.

In 2 to 3 days of infection, merozoites undergoes asexual reproduction and then rapture, re-infecting or evading uninfected erythrocytes. The parasites spend approximately 72 hours in the RBCs and during this period the parasite undergoes developmental changes thus from young trophozoites (ring forms) to matures trophozoites and finally schizonts. This stage of the parasite's life cycle is characterized by the formation of the Parasitophorous Vacuolar membrane (PVM) a stage very vital to the parasites in terms of its survival and growth in the RBCs (Normark, 2008).

The repeated intraerythrocytic cycle of rapture and reinfection causes a destruction of RBCs and a drop in haemoglobin level especially in children and pregnant mothers. Parasites toxin released during the rapture of the RBCs is believed to trigger host immune responses thereby leading to

fever, chills, vomiting in the midst of sweats thus clinical manifestation of malaria infections in individuals. The matured erythrocytic stages (trophozoites) of the parasite even though replicative and non-infective in nature, they account for the clinical manifestation witnessed in infected individuals and may also avoid been cleared by the spleen due to stiffness of these asexual stage molecules which stick to vasculature (Nilsson *et al*., 2015).

In view of the complex nature of *P. falciparum's* life cycle, the erythrocytic cycle of the parasite is linked to a variety of antigens thus the *csp, msp-1, 2, 3, glurp* and a host of other merozoites antigens many of which are involved in erythrocytes invasion. Polymorphism and diversity of these blood stage surface proteins makes them good candidates for drug and vaccine targets however, a lot of them are under immune selection pressure presenting a huge challenge for their choice as vaccine or drug targets against malaria (Balam *et al*., 2014).

2.4.3 SEXUAL (GAMETOCYTE) STAGES

At the sexual stage of the parasite life cycle, some of the schizonts in the erythrocytes stage commit to formation of male and female microgametes and macrogametes respectively. It is believed that some parasites' adaptive features may influence sexual parasites formation, thus the number of mature schizonts (asexual parasites) that results in the sexual forms (gametocytes) and the proportion of male and female gametes that develops. It is still unclear the exact point at which asexual parasites decides to commits to sexual forms of the parasites. A gene known as ap-2 is recently implicated for the conversion of asexual to sexual stages of the parasites in *P. falciparum* (Nilsson *et al*., 2015).

The young gametocytes move into the bone marrow where they undergo several developmental stages in about 10 days. At the final stages of the gametocytes development in the bone marrow,

they re-enter the peripherial blood and become matured for infectivity. The male and female gametes are taken up again by the female *Anopheles* mosquito vector during its blood meal and these gametes travel to the midgut of the vector where the male and female gametes develop into microgametes and macrogametes respectively. The macrogametes fertilise with the microgametes to form the zygote then to the Ookinetes and subsequently the Oocytes. The Ooccytes develops into sporozoites which migrate to the salivary gland of the mosquito waiting to be injected into the human host in its next blood meal (Nilsson *et al*., 2015).

2.5.0 *P. FALCIPARUM'S* GENETIC DIVERSITY AND ACQUIRED IMMUNITY

In hyperendemic areas of Africa, individuals are constantly exposed to several episodes of *P. falciparum*, as a result, variable strains of the infection are exposed to such individuals over a period of time. These individuals develop immunity to the parasite with time, clinical manifestations and level of parasitaemia reduces. Infants (up to 3 months) are protected from clinical malaria due to maternal protection passed on to these children, they enjoy this immune protection until they acquire their own protection after 6 years of age. Adolescence and even adults in low transmission areas are prone to *P. falciparum* attack and could develop clinical disease conditions (Hviid, 2010).

Individuals in endemic areas of *P. falciparum* transmission gained acquires immunity to the infection slowly over long period of time, probably years but such immunity may never result in sterile immunity. This means that an individual having acquired immunity to malaria can lose it depending on his exposure to the infection in endemic setting. It is however the belief of many that artificial immunity in the form of a vaccine may be an antidote to these challenges of acquired immunity but diversity of the surface molecules of infected RBCs is thwarting the effort of developing an effective vaccine (Hviid, 2010).

2.5.1 GENETIC DIVERSITY OF *P. FALCIPARUM'S* SURFACE ANTIGENES (PfSAs)

Genetic diversity or antigenic variance is the ability of a pathogen to change its surface protein such that it can develop into strains or clones of the pathogen to outwit host-immune responses In the genome of the parasite antigenic events in DNA rearrangements, variance of *P. falciparum* is very crucial in the parasites pathogenesis and survival in the midst of control measures, immune evasion, emergence of drug resistant strains and the development of an effective vaccine against malaria. *P. falciparum* has a complex genetic make-up making it highly virulent of all the malaria species known. The genetic diversity in the parasite is common in the merozoite surface proteins 1 (MSP1), 2 (MSP2) and glutamate-rich proteins (Glurp) (Joannin, 2010; Kiwanuka, 2009).

In endemic areas, an individual can be infected with several distinct clones of either 3D7 or FC27 genotypes of *P. falciparum,* which may respond differently to immune responses, drug and biochemical markers in the body. Cross- mating of these distinct gametes in the mosquito could be implicated in the nature of diversity and frequency of the different clones in high prevalent area. Diversity of the parasite, frequency and nature of mating in the vector mosquito need to be understood if the fight against malaria is to be achieved (Babiker *et al*., 1994). MSP 1, 2 and Glurp are regarded as antigenic markers making them targets for vaccine development and used for population studies of *P. falciparum*. These markers are used extensively for genotyping the parasites, they are made up of a single copy gene which are stable in the three stages of the parasite's transmission cycle. The allelic family of these antigens is highly variable and can be distinguished easily. Allelic diversity of the parasite may be the result of intragenic repeats within several alleles, the number of copies and length of the repeats (Organization, 2008).

Kemp *et al*., 1990 reports that MSP-1 and 2 are found on chromosome (Chr) 9 and 2, respectively and that meiosis is crucial in the life cycle of the parasite. Host immune evasion is a function of genetic diversity and polymorphism of *P. falciparum.* Fertilization, independent assortments of genes on different chromosomes, crossing – over events between genes on these chromosomes and intragenic recombination are events that results in diversity of antigens of *P .falciparum* (Kidima & Nkwengulila, 2015).

2.6 MEROZOITES SURFACE PROTEIN 2 (MSP- 2)

P. falciparum's polymorphic merozoite surface protein 2 (MSP – 2) is a glycoprotein of about 45kDa, an integral protein, a single copy gene and a well-studied parasites antigen found on the surface of merozoites during the asexual blood stage of *P. falciparum's* life cycle. *msp - 2* gene is anchored to the surface of the merozoite by a Glycophosphatidylinositol (GPI) which is encoded by the carboxylic- terminus (C-terminus) and the amino- terminus (N-terminus) flanking the highly variable non-repetitive regions. The two distinct alleles of MSP-2 have unique variable non-repetitive sequences with their central repeats units varying according to sequence, number and length. Diversity and polymorphism in the merozoite surface antigens, MSP-2 is a selective advantage to the parasite and hence aids it in immune evasion making it one of the successful protozoan parasites ever known in the midst of control efforts. 3D7 has a 4-10 amino acids repeats units while FC27 has a 32 amino acid and a 12 repetitive sequences, the variable repetitive units specific to each of the two alleles give rise to the polymorphic and diverse nature of *P. falciparum's msp* - 2 gene (Felger *et al*., 1994; Irion, 2000; Marshall *et al*., 1991)

msp 2 has ICI/3D7 and FC27 sub-allelic types vary both in number and sequence, according to WHO MSP-2 is a highly polymorphic region of the parasite's genomic structure hence its use by

researchers to genotype the parasite for the study of genetic diversity in different transmission areas. Felger and his colleagues in 1994 reported that the two types of parasite strains to the *msp-2* gene 3D7 shows greater diversity compared to FC27 and that the variations is seen at the 5' end of amplified PCR product. Higher allelic frequencies and conserved motifs were characteristic of 3D7 than FC27 (Duah *et al*, 2016). Kiwanuka 2009, reports that though several studies have genotype *msp-2* gene the use of a single marker to genotype *P. falciparum* might miss closely related strains of the parasite.

In a cross-sectional study by Felger and his colleagues in 1994, they found out that the prevalence of FC27 was higher in adults' subjects with acquired immunity compared to 3D7, which were prevalent in children. According to that study immune evasion by the parasite at a younger age could be a factor for this phenomenon and that the FC27 could be more successful in adults because that strain could outwit the innate immune system than 3D7 strain. While 3D7 was more diverse in terms of size and patterns of amplified product on a gel, FC27 is more associated with clinical malaria and morbidity of the disease. Extensive polymorphism and diversity of *msp-2* exist in natural populations with up to about 8 known alleles for the single-locus marker gene. The diversity of *msp-2* gene is due to the number of copies and variability in the size of the central dimorphic non - repetitive motifs of amino acids which makes *msp-2* gene unique for genotyping field studies of the parasites (Beck *et al*., 2001).

2.7 GLUTAMATE-RICH PROTEIN (GLURP)

A glutamate-rich protein (Glurp) is a soluble asexual stage antigen and produced when schizonts rapture and are said to be present at all stages of *P. falciparum's* life cycle. They are synthesized on new merozoites and during the parasites blood-stage transmission cycle thus pre-RBCs and RBCs stages. High levels of Glurp antigens were found in patients from varying transmission intensities during an immune - epidemiological study. The study further indicates that non-immune patients to *P. falciparum* acquires the Glurp antibody after an infection with the parasite (Pratt-Riccio *et al.,* 2013). Glurp is made up of a non-repetitive (Ro) N-terminal region, an immunodorminant repetitive (R_2) C- terminal and a central repeat. Polymorphism in *glurp* gene is basically sequence variations in the genetic structure of *P. falciparum* in its gene size and the products of proteins (Pratt-Riccio *et al*., 2013).

P. falciparum genetic diversity in the *glurp* gene like the other antigenic markers such as *msp 1* and *2* correlates positively with the endemicity of transmission in a geographical area with up to 2, 3 or 4 alleles observed in a previous study in Honduras, Giyana and Columbia, respectively in low endemicity of the parasites. Similarly up to 8 parasites clones or MOI were observed in sub-Saharan Africa and Asia, a hypertransmission area.(Pratt-Riccio *et al*., 2005; Pratt-Riccio *et al*., 2013).

2.8 MICROSATELLITE GENOTYPING OF *P. FALCIPARUM*

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 to 3 kilo base pairs which are located mostly in the non-coded regions of the parasites genome (Nyachieo *et al*, 2005). SSRs markers show high level of allelic variation and used to differentiate several strains or clones in *P. falciparum* parasites population trends analysis (Anderson *et al*, 1999). The SSRs can be mononucleotide repeats (CCCC), dinucleotide repeats (ATATAT), trinucleotide (CTACTACTA) and tetranucleotide repeats (GCTAGCTAGCTAGCTA) (Mburu and Hanotte 2005) (Applied Biosystems, 2004). Microsatellite repeats vary in number and length of the sequence repeats in various clones/strains of an organism, these differences in the repeats is referred to as short sequence length polymorphism (SSLPs). The diversity in the repeats is vital in population and genomic studies of *P. falciparum* (Doolan, 2002).

Microsatellite genotyping using capillary electrophoresis (CE) provides a more accurate method of distinguishing close related strains of *P. falciparum* that agarose gel based MSP 1, 2 and Glurp antigenic markers may miss. The accuracy of estimation of band size in traditional gel (agarose based gels) has come under criticisms by molecular population genetic researches as some alleles might be wrongly called compared to the high resolution CE based genotyping where correct allelic sizing has greatly improved (Kiwanuka, 2009). SSRs positively correlates with adenine-thymine (A-T) content which is prevalent in *P. falciparum* (Carlton *et al*., 2015). Mutations rates in microsatellites typing is about 10^{-3} to 10^{-4} per locus, which result in strand-slippage, mismatched pairs during DNA replication which are passed on from one generation of the parasite to another. Thus the high mutation rate observed in the SSRs in *P. falciparum*

genome makes them good candidates for identification of parasite isolates and population genetic studies. The rate of mutations (deletions and insertions etc) in SSRs differ within the loci due to the composition of repeats motifs, size and number of repeat sequences, they are mostly accumulated in the non-coded portions of the DNA which has limited significance on genes or protein expression making them show high variability and are generally believed to be selectively unlinked or neutral makers (Carlton *et al* 2015;Nyachieo *et al*,2005)

Linkage mapping can be used to pinpoint DNA sequences that contribute to hereditary phenotypes in organisms that undergo sexual recombination, the relationship between inherited genes variants (specific marker alleles) and the phenotypes can be used to identify chromosomal regions that contain genes affecting traits in either in-bred cross or out-bred cross populations. Genetic mapping uses phenotype-genotype associations to uncover genes linked to diseases (*P. falciparum*) (Ferdig & Su, 2000).

Markers developed for amplification of SSRs are polymorphic and highly diverse among parasites isolates because of variations in length of these repeats sequences, they exhibits multiple alleles in general populations 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 the parasites (Ferdig & Su, 2000).

The application of large (12 or more) number SSRs markers for population studies and parasite typing has increased the accuracy, scoring of parasites clones and better understand transmission dynamics of the parasite. Most research on *P. falciparum* genotyping have used limited number of genes that code for the surface antigens, for instance MSP 1, 2, Glurp and circumsporozoites

proteins (CS). Polymorphisms in this limited numbers of genes cannot fully and accurately reflect the global relationship between parasite isolates and their genomic variations, which sometimes results in over-interpretation of parasite diversity with these antigenic markers which could be under immune selective pressure (Ferdig & Su, 2000).

To improve allelic scoring or sizing, increased discriminatory power and the best possible resolution using end-labeled fluorescent primer with the application of capillary based genotyping, SSRs markers has shown to be more sensitive for purposes of epidemiological and population genetic trend analysis. The automated and rapid nature with which analysis of SSRs typing of the parasites could be done has an added advantage of microsatellites over antigenic marker genotyping of field isolates. Tandem repeats marker-based techniques of typing the parasites for comparing diversity of the parasites in polyclonal infection has been made easily and reproducible. SSRs typing of *P. falciparum* is essential in tracing the ancestry of the parasites, identify drug and vaccine target and understand the function of genes in the pathogenesis of malaria. The shortfall in the application of SSRs markers could be due to allelic scoring that differ by just one sequence repeat and could be scored with similar length (Greenhouse *et al*., 2006; Mayor *et al*., 2003; Organization, 2008).

In a study by Greenhouse and his colleagues in (2006), they reported that using antigenic markers for parasite typing may not be enough to detect alleles associated with failure of treatment (recrudescence) or new infection in hyper-endemic areas mostly Sub-Saharan Africa. The addition of SSRs markers with capillary-based electrophoresis may prove to be the markers of choice and more useful in detecting alleles of the infection.

2.8.1 *P. FALCIPARUM* POPULATION GENETICS

P. falciparum population genetics focuses on the nature of alleles and their frequency in a population with time. Population structure of the parasites is vital in determining whether the parasite has evolved as a result of local adaptations or a fragmented population structure of its distribution in endemic areas of sub-Saharan Africa

Parasite typing using multi-locus markers has become imperative in investigating the population genetics of parasites in endemic areas (Duffy *et al*., 2017). 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 defeat 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).

2.8.2 NATURAL SELECTION

The ability of a living organism to adapt to changes in its environment or habitat because of features (traits) in the organism, which has a selective advantage; these features are passed on to the next generation making the organism successful. Natural selection is favoured in *P. faciparum* 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.

Chloroquine resistant strains to malaria became wide spread and were passed on from one generation to the other in the country few years ago because these resistant strains increased in survival and frequency. The national policy on case management and treatment of malaria had to

shift to artemicinin based combination therapy (ACTs) (Duah *et al*., 2006). Hedrick 2011 reports that human population genetics and geographical distributions appear to overlap extensively and that these factors confer resistance to *P. falciparum* malaria. Variant resistant genes to Glucose 6 Phosphate dehydrogenase (G6PD), α – thalassemia, β- thalassemia and so no present in endemic areas of Africa with high frequency compared to malaria free areas of the world. Positive selective pressure of some diseases resistant to malaria has created a global health challenge with increased frequency in the population such as sickle cell anaemia, chronic inflammatory diseases due to the exertion of pressure on the genome of humans by malaria (Kwiatkowski, 2005).

2.8.3 MUTATION

The changes in the genome of the parasites as a result of random deletions, insertions or substitution of nitrogenous base(s) which incorporates new alleles or increase the allelic frequency. Chloroquine confers resistance to *P. falciparum* due to mutations in 2 gene candidates thus 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 eighty's (1987) and subsequently the allelic frequency and populations of these mutants increased necessitating a policy change in case management and treatment of malaria in the country (Duah *et al*., 2006).

Single Nucleotide polymorphism (SNPs) or point mutation is the main cause of the sickle cell traits in humans due to substitution of valine in the beta sub-unit instead of glutamic acid which is found predominantly in malaria endemic areas of the world – sub-Saharan Africa. Sickle cell traits confer resistance to malaria and are selected positively, the prevalence of this genetic disorder in malaria endemic regions is an indication that evolutionary forces has brought to bear on the genome of *P. falciparum*. (Long, 2004). Mutations of SSRs motifs mostly in the non-

coded regions of the parasite's genome causes phenotypic changes and disease as a result of the loss or gain of a whole repeat unit due to strand slippage (errors) during DNA replication (Su & Wellems, 1996).

2.8.4 MIGRATION

Migration is crucial in the evolution of malaria parasites, which is influenced by the interaction between the human host and the parasites. Lion *et al.*, 2006 reports that migration and transmission of parasites virulence is closely linked to genes that confer resistance and adaptations to the local setting strongly favoured hence 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 favour the reshuffling of genetic material of the parasite (Long, 2004).

In sub-Saharan Africa activities that encourages migration includes mining, conflicts, urbanisation, agricultural activities etc. The consequence of malaria transmission and pathogenesis increases, urbanisation for instance is not properly regulated in most developing countries with its accompanied poor drainage systems, poor housing and sanitation with most parts of the cities engulf in filth providing favourable grounds for breeding mosquitoes. The cumulative effect of these factors is an increase in vector population and allelic frequency of the parasites (Martens & Hall, 2000).

2.8.5 GENE DRIFT

In finite populations due to errors in sampling there are changes in the frequency of allelic of *P. falciparum* population over a period of time. The frequency of alleles becomes fixated (allelic

frequency drift to1) or there is loss of heterozygosity (thus when allelic frequency is 0). Heterozygosity (allelic diversity) within a particular sub-population over a period of time identical alleles becomes fixated, genetic drift causes a loss in genetic diversity. However 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) https://www.google.com.gh/search/evolutionary forces,accessed on 6.8.17).

2.9 MULTIPLICITY AND MORBITITY OF *P. FALCIPARUM* INFECTIONS

Factors that affects *P. falciparum* infection dynamics in endemic areas of sub-Saharan Africa is vital to monitoring intervention efforts and could be used to differentiate between failure of treatment (recrudescence) from new infections.

Multiplicity of infection (moi) is the number of distinct parasite clones simultaneously infecting an individual. In hyperendemic areas of Africa individuals can carry 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 less episodes of malaria than their older age counterparts due to maternal Immunoglobulin G (IgG) immune protection and fetal haemoglobin. Such 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 (Felger *et al*.,1999). Felger and his colleagues in 1999 reports that morbidity or clinical malaria is associated with moi, implying that moi and development of clinical malaria is vital to development of immunity against malaria in a holoendemic area. Thus increased MOI in children less than 1 years may confer protection against clinical malaria, according to previous findings however their reports was to the contrary and that clinical malaria was associated with high MOI in these children.

There is strong evidence that morbidity of malaria and moi correlates, with semi-immune children found to have less incidence of clinical malaria compared to their younger age group (3-6) years. Henning and his colleagues in 2004 reports that moi was associated with risk of clinical malaria in younger children below 3 years of age in endemic areas and that moi offers protective immunity hence reducing the level of parasitaemia in these age group. Thus the risk of developing clinical malaria and subsequent moi is age – depended, endemicity of the area and the immune status of the individual. Cytokine cascade triggers the protective immunity enjoyed by these semi-immune individuals as the anti – parasite immune response causes a decrease in parasite numbers (density) with age (Mayor *et al*., 2003; Smith e*t al.*, 1999).

The surface of infected erythrocytes has a number of *P. falciparum's* encoded variant surface antigens (VSA), typical of these antigens is the erythrocyte membrane protein (EMP 1). A knob -like structure found on the surfaces of infected RBCs which are able to mediate switching of one form of VSA to another and are implicated in the adhessions of the blood stage asexual parasites (ring trophozoites and schizonts) to the vascular endothelium of host tissues (receptors) (Hviid, 2010).

In immune naive individual's clearance of the parasites is a huge challenge because these infected people continue to rapidly produce VSA in severe malaria cases. Severe malaria expressing parasites (SMEP) have conserved domains in which adhesions to vasculature is specific hence immunity builds slowly in such individuals. Variant antigens link to uncomplicated malaria are produced slowly but unlike the severe malaria parasites expressing variant antigens, they are diverse antigenically. This results in chronic malaria infection in partially immune people where case management and treatment is relatively easier but difficult parasite clearance (Hviid, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 ETHICAL CLEARANCE

Archived samples were used for this study, which had already acquired ethical clearance from the Institutional and Review Board of Noguchi Memorial Institute for Medical Research (IRB-NMIM), University of Ghana – Accra.

3.2 STUDY SITE AND SAMPLE COLLECTION

160 archived whole blood samples each from symptomatic and asymptomatic malaria infected persons in Obom and Asutsuare were used for the study. Obom is a high (> 1% - 80%) malaria transmission area with the prevalence of 35%, according to records at the health center in Obom. The main occupation of the inhabitants is small-scale fishing, which is at the Ga south municipality of the Greater Accra region with a population of 1500 people, transmission is perennial with peak transmission in the raining season (June to August) (Anderson *et al* 2000: Amoah *et al*, 2016).

In Asutsuare, transmission of malaria is low (<1%) but increases during the raining season. Asutsuare is located in the Shai- Osudoku district of the Dangbe –West Municipality of Greater Accra (Lo *et al.*, unpublished). The town is located 8km East of Akuse on the river Volta, about 25km from Accra and found on latitude 6.096 and longitude 0.196 (https: sites.google.com/greater-accra/asutsuare; Accessed on 10/10/2016).

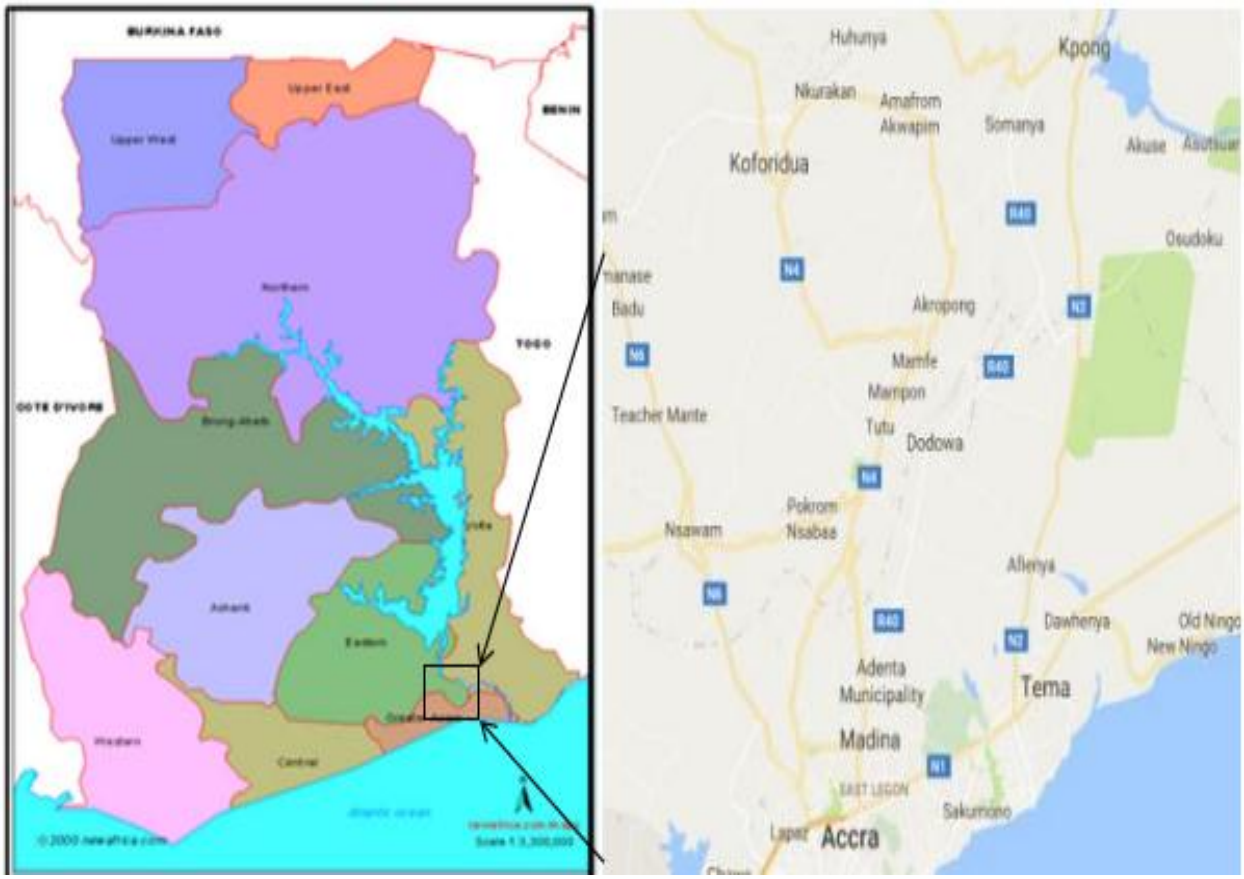


Figure 3.1: Map of study sites, adopted from (Lo *et al*., 2016).

3.3 SAMPLE COLLECTION

The samples used for this research work were archived whole blood, thick and thin smear were prepared on each slide for microscopic examination. 1000 µl blood were used for the extraction of DNA.

3.4 SAMPLE SIZE DETERMINATION

The sample size for the study was determined using: $n = Z^2 * p\ (1\text{-}p)/E^2$, Where n is the sample size, Z is the confidence interval at 95% (1.96), margin of error which is ± 0.05% and P for prevalence of malaria transmission which is about 40%, n = 368.8

3.5 DIAGNOSIS OF MALARIA PARASITES BY MICROSCOPY

The samples were diagnosed for the presence of malaria parasites (mps) after examination on a smear (thin and thick blood film) were made on slides dried in the air and stained for about 10 minutes with 10% Giemsa stain. The slides were viewed by independent microscopiest using a light compound microscope viewed under 100x objective lens with immersion oil. The slides were declared positive by different microscopist if it contained *P. falciparum* or negative if both microscopist recorded no malaria parasites seen (no mps seen), parasite estimation was determined in 200 White Blood Cells (WBC) per field.

3.6 DNA EXTRACTION AND *P. FALCIPARUM* SPECIATION

DNA extraction was done using zymo (Quick- gDNA) extraction kit as contained in the instructional manual with the archives whole blood samples from Obom (hyper-endemic) and Asutsuare (holoendemic)

The genomic DNA (gDNA) isolated were quantified using Nanodrop quantification of DNA and were immediately used for PCR speciation or stored at -20 ^{o}C .

P. falciparum speciation was determined by a nested PCR, the outer amplification was made up of genus specific primers mix of rPlus 5 and rPlu 6 in a final concentration of 10 µM and 0.12 µl per reaction each. The inner nested amplification was done using species specific primer mix of *P. falciparum* in the same final concentration and volume per reaction as the outer (nest 1) reaction. The total volume for both nest 1 and 2 was 15 µl with 0.5U thus 0.05µl per reaction of Biotaq Polymerase used. A sample was considered positive if it contained an amplified product on the gel or negative if no amplified PCR product was present. A 3D7 gDNA strain was used as a positive control with a no template control (NTC) or distilled water (dH_2O) as the negative

control. The Bio - Rad S1000 thermocycler was used to determine the *P. falciparum* PCR speciations of all the samples.

2% agarose gel was prepared using 1x TAE in 0.5ug/ml of ethidium bromide (EtBr) and viewed using a trasilluminator gel doc.

3.7 GENOTYPING OF P. FALCIPARUM WITH MSP-2

The protocol for typing *msp - 2* gene of the parasites was done according to WHO recommended guidelines for genotyping *P. falciparum* with little modifications. Nest 1 involves amplification of the *msp - 2* gene followed by nest 2 reaction which utilizes strain specific primers in a final volume of 15ul. The nest 1 amplification was done using four primer sets; M_1-OF/M_2-OF and M_1 – OR/ M_2 – OR in a final concentration of 133mmol/L. 3µl of the outer reaction was used for the inner (nested) reaction with 200 nM each of either S_1wf /N5 rev for 3D7 strain or S_1wf /M5 rev for FC27 strain. 5 ng/µl of gDNA was used for the nest 1 amplification with 0.04U working concentration per reaction of one Taq Polymerase (www.neb.com/M0480). The amplified products with fragment size of N5 (280-600 base pair) and M5 (380-500 base pairs) were resolved in a 2% agarose gel stained with 0.5 µM/ml of EtBr and visualized in a transilluminator. The above percent of agarose was chosen because it has a good separation in a gel when the fragment size of a PCR product is within 0.2-1 kilobase. The Biometra thermocycler was used for all the amplification of the *msp - 2* PCR reactions. The primer sequence and cycling conditions of *msp 2* is represented below:

Table 3.1 Primer sequence for *msp – 2* and *glurp* genotyping.

Primer	Sequence (5' – 3')	Annealing	Product size
MSP - 2			
Nest 1 M_1-OF	CTAGAAGCTTTAGAAGATGCAGTATTG	54 ^oC	
M_2-OF	ATGAAGGTAATTAAAACATTGTCTATTATA		
M_1-OR	CTTAAATAGTATTCTAATTCAAGTGGATCA		
M2-OR	CTTTGTTACCATCGGTACATTCTT		
Nest 2 SFW	GCTTATAATATGAGTATAAGGAGAA	55 ^oC	
N5rev	GCATTGCCAGAACTTGAA		280 bp
M5rev	CTGAAGAGGTACTGGTAGA		380 bp
Glurp			
Nest1 GF3	ACATGCAAGTGTTGACCTGAAG	54 ^oC	600 bp
GF4	TGTAGGTACCACGGGTTCTTGTGG		
Nest 2 GNF	TGTTCACACTGAACAATTAGATTTAGATCA	54 ^oC	1200 bp

(Falk e*t al.*, 2006; Felger & Snounou, 2008).

These primers are specific to the two strains to which *msp - 2* is group, positive control for N5 was 3D7 and that for the FC27 allelic type is the K1 with nuclease free water as the negative control. The cycling conditions were initial 94^oC for 5 minute followed by 94^oC for 30 seconds and 55^oC I minute annealing with 68 ^oC 1 minute initial extension followed by 68 ^oC for 5 minute and 4 ^oC hold

3.8 MOLECULAR TYPING OF R II REGION OF GLURP OF *P. FALCIPARUM*

The polymorphic R II region of the *glurp* gene was assayed in a nested PCR, both the outer (GF3, GF4) and inner (GF4, GNF) primers were in a working concentration of 50 mM per reaction, with 200 mM dNTP (mix) for each nested reaction. 0.55u/µl of oneTaq polymerase was used in both nested reactions with 3µl of the template DNA used for the amplification of the inner reaction. The total reaction volume for both inner and outer reaction were 15 µl and amplification was done using the Eppendorf thermocycler (nexus gradient)

The resolution of the amplified product was done using 2 % agarose stained with 0.5µM/ml of EtBr and visualized in a trans-illuminator.

3.9 MICROSATELLITES TYPING OF *P. FALCIPARUM*

Molecular genotyping of *P. falciparum's* tandem repeats utilizes unlinked markers at different loci, which has proven to determine multiple clones and population genetics of the parasites. In this study we used 75 samples each from Obom and Asutsuare to identify the polymorphic tri-nucleotide, tetra-nucleotide or penta-nucleotides repeats of the parasites in a two Hemi nested PCR reaction set-up. The twelve-neutral polymorphic SSRs markers used are Poly α, PFPK 2, TA81, ARA2, TA87, TA40, TA42, 2490, TAI, TA60, TA109 and PFG377. The two Hemi nested PCR amplification are Primary and Secondary PCR reaction (Anderson *et al.*,1999).

3.9.1 PRIMARY REACTION SET-UP

This is a multiplexed (more than one target sequence amplified using multiple primer pairs in a single reaction mixture) reaction using unlabeled 3 primers (forward and reverse) sets in a total of 6 primers (thus forward and reverse) for a single reaction of the nest 1 (Hemi 1). Thus for the Hemi 1 amplification, each locus utilizes the first and second primers pair in a final concentration of 0.1µM each as describe by Anderson *et al* (1999) with little modification. The working concentration of one Taq reaction Buffer (5x), $MgCl_2$, and dNTPs were 1x, 2mM, and 200 mmol/L respectively in a total reaction of 10 µl. The 12 unlinked microsatellites used for the Hemi 1 were group into 4 multiplex primer sets as shown below:

Multiplex PCR: 1 - PFPK2 (Chr 12), Polyα (Chr 4), TA81 (Chr 5)

Multiplex PCR: 2 - TA87 (Chr 6), TA40 (Chr 10), ARA 2 (Chr 11)

Multiplex PCR: 3 –TA42 (Chr 5), 2490 (Chr 10), TAI (Chr 6)

Multiplex PCR: 4 – TA109 (Chr 6), TA60 (Chr 13), PFG377 (Chr 12)

There were 24 primer sets in all; each of them had a forward and a reverse primer. The cycling conditions for the Hemi 1 reaction were 94^oC in 2 minutes, followed by 25 cycles of denaturation. 42^oC for 1 minute of annealing temperature the initial extension of 68^oC in 1 minute with 68^oC for 2 minutes final extension and 4^oC hold, all the PCR amplification was done using the Bio-Rad thermocycler. The Hemi 1 amplification was place in a 4^oC while the Hemi 2 reagents were allowed to thaw. The same cycling conditions were used for the Hemi 2 amplification with the only changes been the annealing temperature of 45^oC (Anderson *et al*., 1999; Su α Wellems, 1996).

3.9.2 SECONDARY PCR REACTION (HEMI 2)

The secondary reaction involved light sensitive labeled primers thus 6-FAM, HEX and Atto. They were run on each of the Multiplex hemi 1 primer pairs resulting in three different PCR reactions for each of the four multiplex primers. The second and third primers used for each of the labeled probes as described by Anderson *et al*., (1999) with slight changes.

3 µl of the Hemi 1 product were used as a template for the secondary reaction in a working concentration of one Taq reaction Buffer, $MgCl_2$, dNTPs, were 1x, 2mM, 200mmol/L respectively. 3D7, HB3 and No template control (NTC) were run as positive and negative control respectively in each of the amplification thus Hemi 1 and 2. The forward and reverse primers were in a final concentration of 0.4 µM each. All the 12 SSRs polymorphic markers were optimized with the controls to assess the presence of shutter, companion peaks or signals from the Capillary electrophoresis (CE). The twelve microsatellites primer sequences and that of the probes would be at the appendix (Anderson *et al*., 1999: Greenhouse *et al*., 2006).

In order to prevent photobleaching (breakdown) of the probes, lights were turn out in the PCR reaction area during day.

Table 3.2 Primers sequence of the 12 microsatellites markers of *P. falciparum* in Obom and Asutuare.

Locus	Primer sequence 5'-3'	Probe	Chromosome	SSR linked gene
Poly α-R	ATCAGATAATTGTTGGTA		4	DNA Poly α
Poly α-F	AAAATATAGACGAACAGA			
Poly α-3(IR)	GAAATTATAACTCTACCA	6-FAM		
PFPK2-3R	CCTCAGACTGAAATGCAT		12	Protein Kinase
PFPK2-F	CTTTCATCGATACTACGA			
PFPK2-R	AAAGAAGGAACAAGCAGA	HEX		
TA81-3F	GAAGAAATAAGGGAAGGT		5	
TA81-R	TTTCACACAACACAGGATT			
TAA81-F	TGGACAAATGGGAAAGGATA	Atto 565		
ARA2-3(F)	GTACATATGAATCACCAA		11	Asparagine Rich Protein
ARA2-R	GCTTTGAGTATTATTAATA			
ARA2-F	GAATAAACAAAGTATTGCT	6-FAM		
TA87-3F	ATGGGTTAAATGAGGTACA		6	
TA87-R	ACATGTTCATATTACTCAC			
TA87-F	AATGGCAACACCATTCAAC	HEX		
TA40 Rev-1	GAAATTGGCACCACCACA		10	
TA40 For	AAGGGATTGCTGCAAGGT			
TA40 Rev-2	CATCAATAAAATCACTACTA	Atto 565		
TA42-3F	ACAAAAGGGTGGTGATTCT		5	
TA42-R	GTATTATTACTACTACTAAAGT			

TA42-F	TAGAAACAGGAATGATACG	6 – FAM		
2490-3R	ATGATGTGCAGATGACGA		10	
2490-F	TTCTAAATAGATCCAAAG			
2490-R	TAGAATTATTGAATGCAC	HEX		
TA1-3(F)	CTACATGCCTAATGAGCA		6	
TA1-R	TTTTATCTTCATCCCCAC			
TA1-F	CCGTCATAAGTGCAGAGC	Atto 565		
TA60-F	CTCAAAGAAAAATAATTCA		13	
TA60-R	AAAAAGGAGGATAAATACAT			
TA60-3(IF)	TAGTAACGATGTTGACAA	6 – FAM		
TA109-3F	TAGGGAACATCATAAGGAT		6	
TA109-R	CCTATACCAAACATGCTAAA			
TA109-F	GGTTAAATCAGGACAACAT	HEX		
PFG377-3R	TTATGTTGGTACCGTGTA		12	Gametocyte specific Protein
PFG377-F	GATCTCAACGGAAATTAT			
PFG377-R	TTATCCCTACGATTAACA	Atto 565		

(Anderson *et al* 1999).

Each locus consists of three primer sequence, the first and second sequence in each locus were used for the Hemi 1 (primary) reaction while the second and third sequence (probes) were used for the Hemi 2 (secondary) PCR reaction. The first and second primer sequence at each locus are unlabeled while the third sequence were labeled with a fluorescent dye (Blue, Green and Red).

3.9.3 GEL ELECTROPHORESIS FOR THE AMPLIFIED PRODUCT

In order to confirm the amplification of the PCR product, 1.5% of agarose was used in 1x TAE (Tris Acetate EDTA). This was melted in a microwave at 100 ^{o}C for 5minutes after cooling and 0.5 µg/ml of EtBr added and subsequently poured into the gel tank with the appropriate comb inserted. 10 µl of the amplicon was mix with 2 µl of a 6x loading dye and subsequently loaded into the gel, this was run for 100 volts in 45 minutes and visualized in a UV transilluminator.

3.9.4 CAPILLARY ELECTROPHORESIS OF THE PCR SAMPLE PLATE

Capillary electrophoresis (CE) is an electrokinetic resolution technique of ions thus DNA because of its electrophoretic morbidity and applied voltage, it has an advantage over gel-based electrophoresis because of its high resolution and quick nature with which results are generated. The Hemi 2 amplified PCR product were diluted with deionised water in a ratio of 1:10 but the reaction was modified depending on the strength of the fluorescent probes as described by Anderson *et al*.,1999. 8.5 µl Hi-Di formamide and 0.5 µl of a GeneScan^{TM} 500 LIZ standard per reaction totally 9 ul was made. 1 µl of the pooled amplified PCR product was mixed with 9 µl of the Hi-Di formamide plus size standard and prepared for Capillary electrophoresis. The Hi-Di size standard sample mixture is then denatured at 95 ^{o}C for 3 minutes and chilled with ice before analysis in the ABI 3130 xl analyser. The size standard is made up of 36 single copy DNA fragments of known sizes in the range of 20 - 500 bp which are used to compare the sizes of the unknown samples under study. Samples were run on 5 - dye with the LIZ fluorephore used as the standard. The CE was done in the ABI 3130 xl Genetic Analyser (Anderson *et al*.,1999: Greenmouse *et al*.,2006).

3.9.5 ANALYSIS OF DATA

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 the infection contained multiple parasite clones (Razak *et al*., 2016).

Bar graphs for N5 and M5 strains for both study sites were generated using excel sheet, Graphpad Prism 5 was used to determine the non paramatic t – test (Mann Whitney test) between N5 and M5 strains of *P. falciparum* at each site. $P \leq 0.05$ was considered statistically significant while $P \geq 0.05$ was considered not statistically significant.

Microsatellites analysis was done using the CONVERT 1.31 to convert allelic excel report file into a notepad (text) file for the following Genetic analysis softwares; Arlequin version 3.0, GeneAIEx 6.50. GeneAIEx was used to determine the unbiased expected Heterozygosity; *He* (Genetic variation) with 0 considered as low heterozygosity and 1 high heterozygosity for each locus. *He* was determined using $[n/n-1]\ [1-\sum p_i^2]$, n is the number of *P. falciparum* clones while p_i is the frequency of alleles in each of the SSR locus. Shannon Information index (*I*) was used for quantifying genetic diversity in each marker. (Glaubitz, 2004; Peakall & Smouse, 2006; Razak *et al*., 2016).

CHAPTER FOUR

4.0 RESULTS

4.1 STUDY SUBJECTS AND SCREENING OF *P. FALCIPARUM* IN OBOM AND ASUTUARE

A total of 400 symptomatic and asymptomatic subjects were screened for *P. falciparum* infection, 200 samples per site. 68 samples representing 34% was confirmed by independent microscopies as positive (malaria parasites were present) and 132 negatives (no malaria parasites seen) in Obom. Eight (8) samples representing 4% were positive for malaria and 192 (96%) were negative for malaria parasites in Asutuare.

Species – specific primers were used in a PCR speciation of *P. falciparum*, out of the total number of 200, 112 (56%) samples were positive (thus it showed bands in a gel image) and 88 (44%) were negative (no band in a gel image) for Obom. In Asutuare 105 (52.5%) were positive and 95 (47.5%) were negative by PCR speciation. 3D7 and K1 were used as positive controls for N5 and M5 respectively. All the subjects who were recruited for the two study sites had ages that were between 5 to 30 years and above.

4.2 Genotyping of Block 3 region of *msp – 2* of *P. falciparum* in Obom and Asutuare

MSP – 2 genotyping was used to determine the diversity of the marker in both sites of this study, out of 160 *P. falciparum* isolates 80 samples per site, 76 were positive for the marker. 44 (55%) samples were positive for N5 (3D7) strain with fragment sizes ranging from 280 bp to 500 bp while for M5 (FC27) 24 (30%) were amplified with band size ranging from 300 bp to 700 bp. Four (4) samples were negative for both N5 and M5 at Obom site with average MOI of 1.30 as shown in **Table 4. 2** below. 8(10%) of the samples at this site carried more than one parasite clones (polyclonal)

At Asutuare out of the 80 samples genotyped the *msp – 2* marker, 8 (10%) were positive for N5 strain of the parasite and the band sizes ranges from 280 bp to 500 bp. 4 (5%) were positive for M5 strain with fragment sizes ranging from 380 bp to 500 bp. 68 (85%) samples were negative for both N5 and M5 at this site with average MOI of 1.0.

Table 4.1 Genetic diversity of the polymorphic gene (Block 3) of and *glurp* R II region of Obom and Asutuare

Study area	Marker	N	Fragment R. (bp)	F (%)	MOI
Obom	MSP - 2				
	N5	44	280 – 500	55	1.3
	M5	24	300 – 700	30	
	N5/M5	8		10	
	Negative	4		5	
	Total	80		100	
Asutuare	N5	8	280 – 500	10	1.0
	M5	4	380 – 500	5	
	N5/M5	0		0	
	Negative	68		85	
	Total	80		100	
Obom	Glurp R II				
	Positive	55	600 – 1000	68.5	1.0
	Negative	25		31.5	
	Total	80		100	
Asutuare	Positive	70	600 – 1200	87.5	0.98
	Negative	10		12.5	
	Total	80		100	

Non paramatic t-test (Mann Whitney) was done to compare the prevalence of N5 and M5 strain in Obom which was $p < 0.05$ indicating significant difference between the two strain compared to that in Asutuare whose $p > 0.05$ indicating no significant difference between the two strains

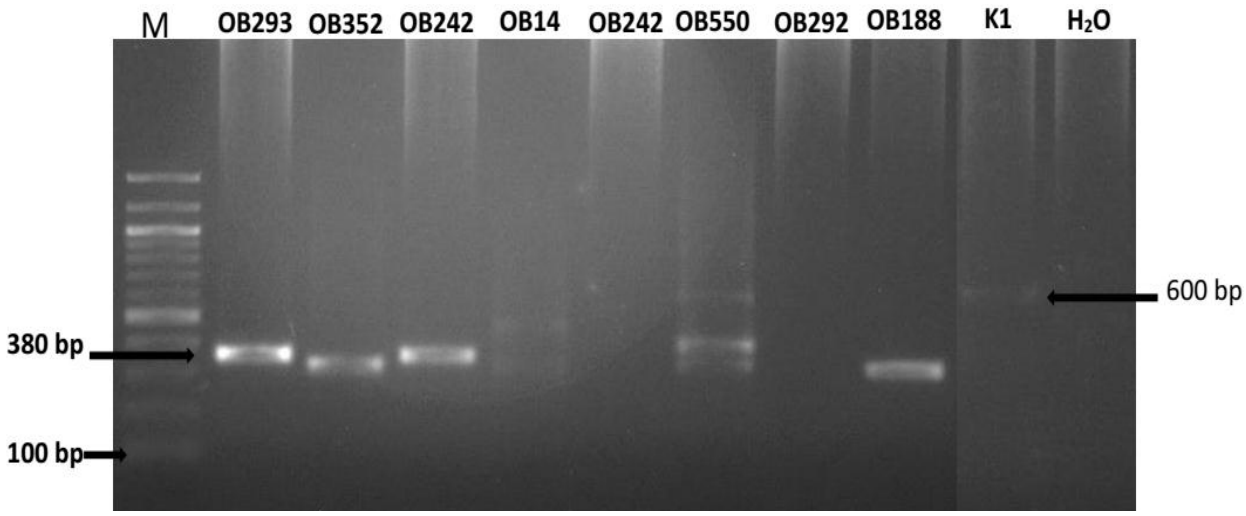


Figure 4.1: Gel image of *msp - 2* genotyping, with samples OB293, OB352, OB14 and OB188 showing clonal strains while OB242, OB550 are multiple strains. Deionised water and K1 were the positive and negative controls respectively. 3 µl of the lather was run together with 5 µl of the samples at 100 volts in 1 and half hour.

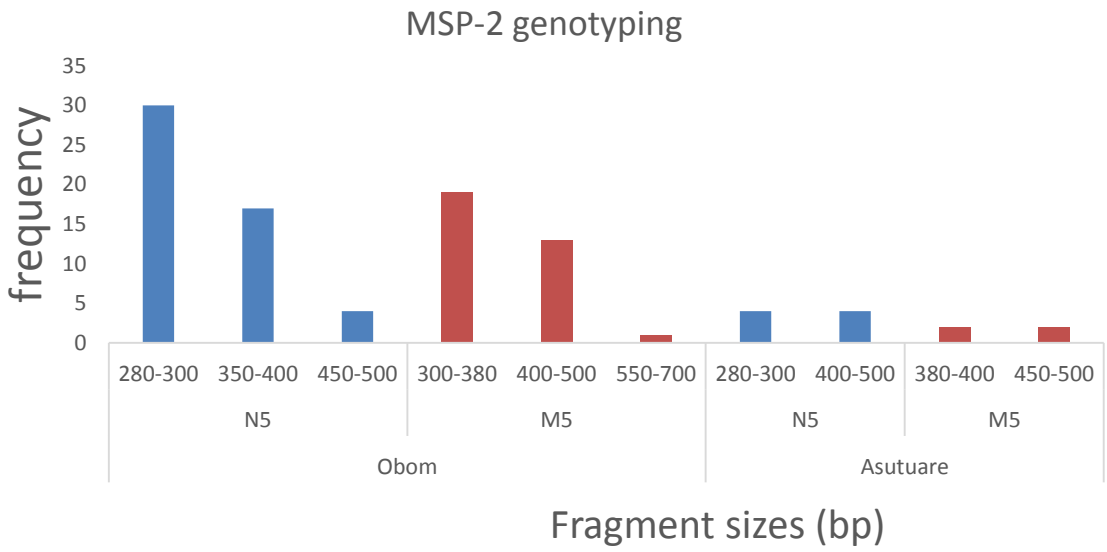


Figure 4.2: Genetic diversity of *msp - 2* in Obom and Asutuare, Blue bars indicates N5 (3D7) strains while red bars represent M5 (FC27) strains.

The prevalence of the number of alleles was compared at both sites in a bar graph as shown in **Figure 4.1** above, N5 had the highest allelic prevalence at fragment range of 280 bp – 300 bp at Obom compared to M5 with least allele at 550 bp to 700 bp at the same site. The same site had wide allelic range of 280 bp to 700 bp for both strains of the parasite indicating higher diversity of the marker at Obom compared to allelic range of 280 bp to 500 bp in Asutuare. At the low parasite prevalence site, *msp – 2* genotyping had the least number of samples positive for this marker

4.2 MOLECULAR TYPING OF THE RII REGION OF *GLURP* GENE IN OBOM AND ASUTUARE

In genotyping the R II polymorphic region of *P. falciparum*, a total 80 field isolates genotyped per site produced variety of fragment ranging from 600 bp to 1000 bp in Obom with 55 (68.5%) and 600 bp to 1200 bp in Asutuare with 70 (87.5 %) number of fragment positive for the *glurp*

gene as shown in **Table 4.1** above. The most predominant fragment was 900 bp with frequency 26 (27.4 %) in Asutuare and the least being 600 bp fragment with a frequency of 1 in Obom as shown in **Figure 4.3** below

In this study, Obom had higher percentage of isolate negative for the *glurp* gene thus 25 (31.5%) compared that of Asutuare of 10 (12.5%). MOI for the *glurp* gene in Obom (MOI = 1.0) was not significantly higher than that in Asutuare (MOI = 0.98) as indicated in **Table 4.1 above**

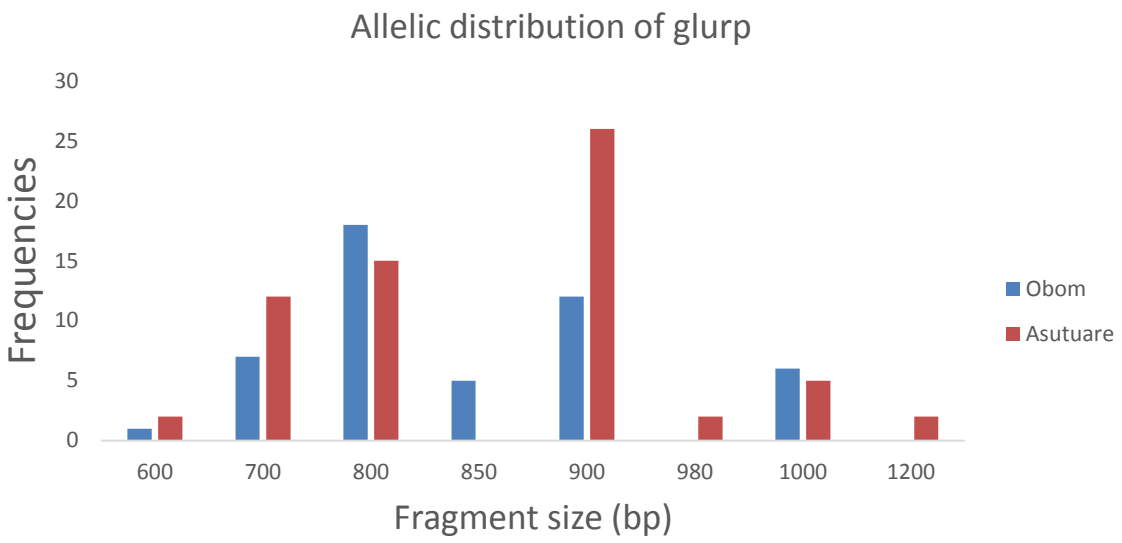


Figure 4.3: Allelic distribution of glurp R II region in Obom and Asutuare, Blue and red bars represent fragment sizes from Obom and Asutuare respesctively

4.3 ANALYSIS OF *P. FALCIPARUM'S* MICROSATELLITES MARKERS IN OBOM AND ASUTUARE

To determine the clonality of *msp – 2* amplified mono-infections, 12 neutral microsatellites markers were used. 48 samples were used to genotype *P. falciparum* in each of the 12 SSR markers in both sites of this study. Poly_a was found to have the highest number of 13 alleles

with fragment sizes of 130 bp to 500 bp in Obom compared to 1 allele with band size of 185 bp to 200 bp in Asutuare as shown in **Table 4.2** below.

Table 4.2 Genetic diversity of 12 neutral microsatellites markers in Obom and Asutuare.

Site	Locus	N	Na	Ne	I	He	Allelic R.
Obom	**Poly_a**	41	13	1.897	1.239	0.479	130 – 500
	PFPK_2	35	10	1.548	0.909	0.359	150 - 250
	TAA81	34	4	1.094	0.229	0.087	75 - 175
	ARA2	29	8	1.450	0.785	0.316	60 - 180
	TA87	39	5	1.306	0.536	0.237	95 - 190
	TA40	31	6	1.223	0.469	0.185	125 - 280
	TA42	34	3	1.061	0.153	0.058	85 - 230
	2490	30	3	1.070	0.169	0.066	85 - 250
	TAI	31	4	1.180	0.365	0.155	85 - 250
	TA60	30	8	1.488	0.824	0.333	80 – 250
	TA109	35	8	2.035	1.159	0.516	95 – 225
	PFG377	40	4	1.881	0.887	0.474	95 – 150
Asutuare	**Poly_a**	41	9	1.367	0.712	0.272	145 - 380
	PFPK_2	36	5	1.223	0.443	0.185	170 - 325
	TAA81	37	4	1.086	0.214	0.080	125 - 200
	ARA2	32	5	1.298	0.534	0.233	75 – 180
	TA87	41	4	1.161	0.336	0.141	95 – 175
	TA40	40	4	1.226	0.399	0.186	125 – 60
	TA42	38	7	1.177	0.418	0.153	180 - 950
	2490	31	1	1.000	0.000	0.000	185 - 200
	TAI	28	2	1.074	0.154	0.070	185 - 200
	TA60	35	3	1.090	0.204	0.084	90 – 175
	TA109	30	3	1.106	0.230	0.098	175 - 200
	PFG377	31	7	2.313	1.181	0.577	95 – 200

The unbiased heterozygosity He was used to determine the genetic diversity of P. falciparum in the two study sites. N is the number of isolates in each marker, Na is the number of alleles, Ne the number of effective alleles I is the Shannon diversity Index (measure of the evenness of alleles), He the unbiased heterozygosity. There was no significant difference in He for both Obom and Asutuare as shown in **Table 4.2** above.

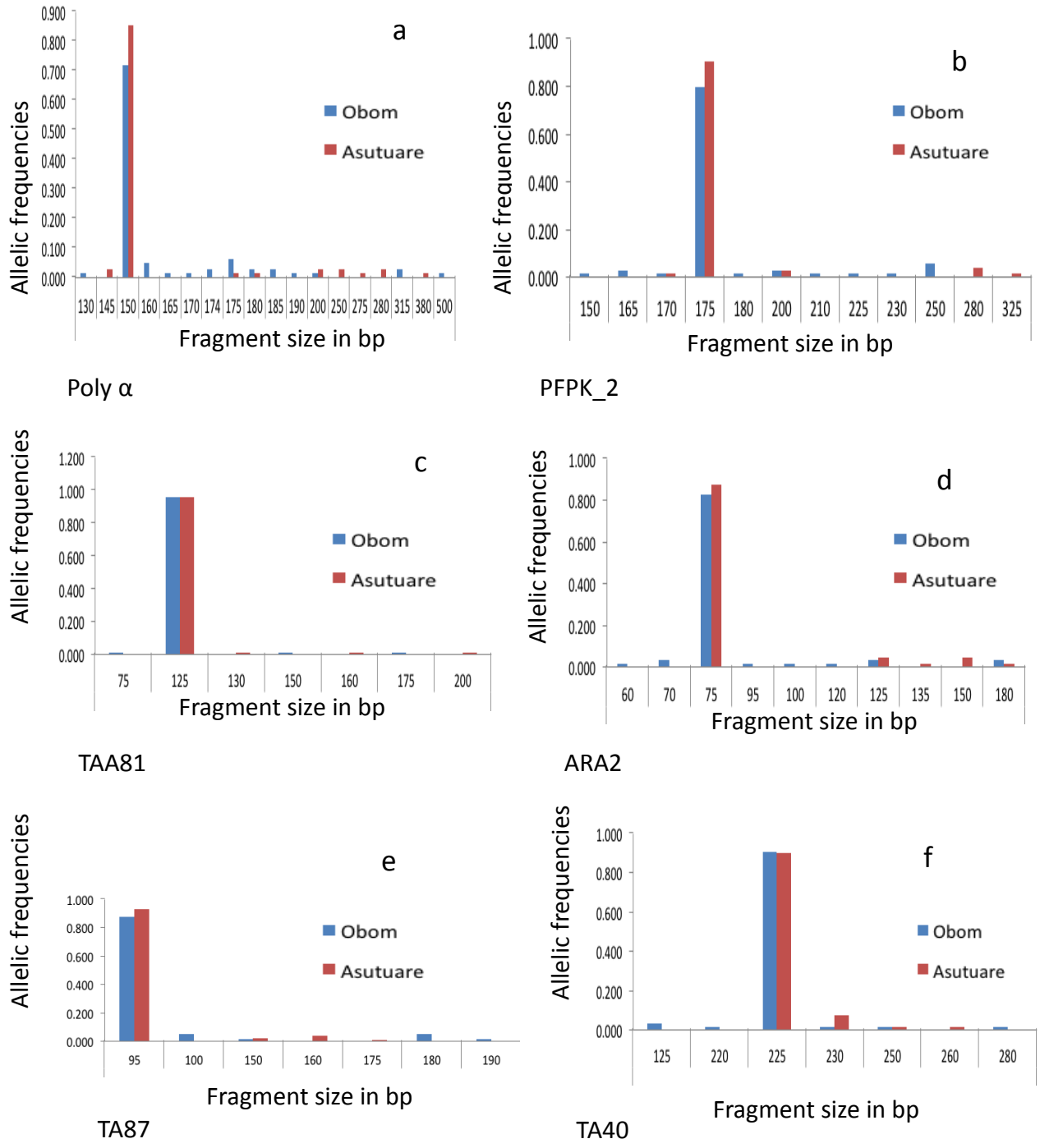
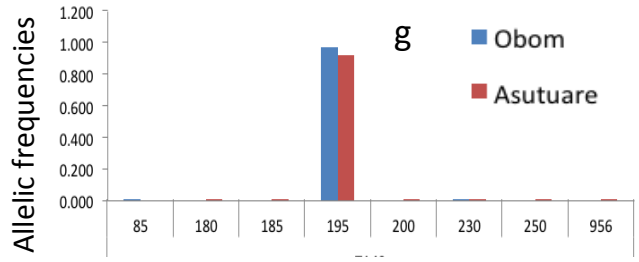
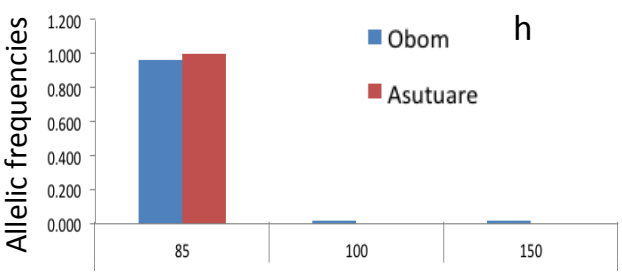


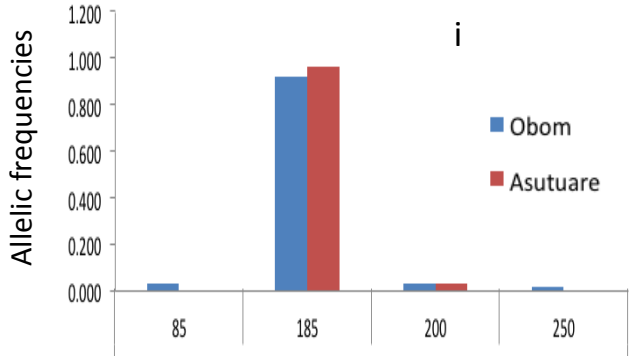
Figure 4.4 a: Panels (a - f) are Allelic distribution of first 6 microsatellites loci of *P. falciparum* in Obom and Asutuare. Allelic frequency is shown on the vertical axis while allelic range (bp) represents the horizontal axis. Blue and red bars represent population of alleles from Obom and Asutuare study sites respectively.



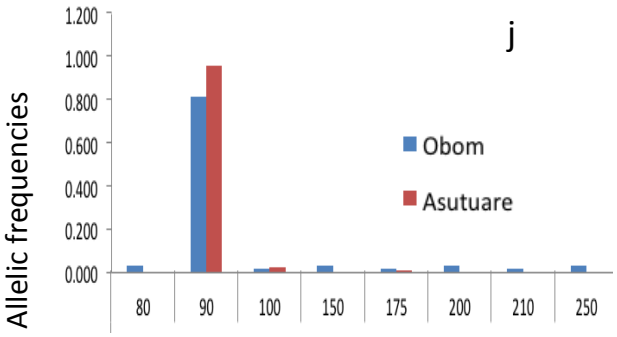
TA42



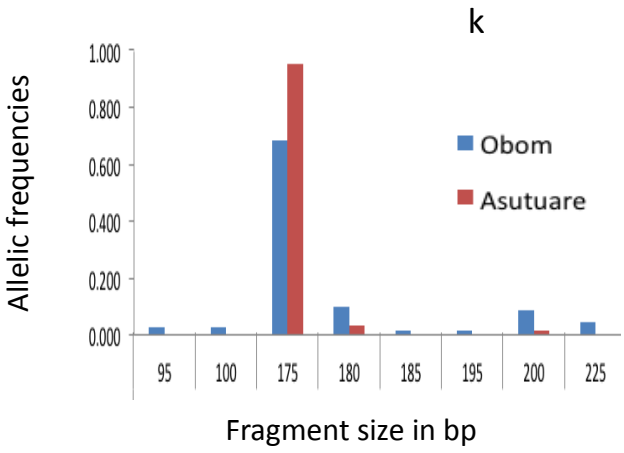
2490



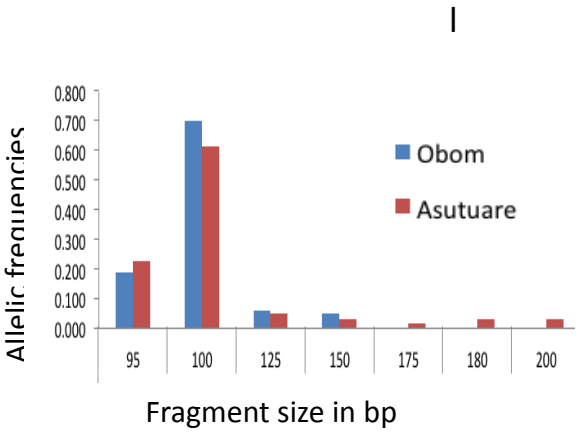
TA1



TA60



TA109



PFG377

Figure 4.4 b: Panel (g – i) are the Allelic distribution of the second 6 microsatellites loci of *P. falciparum* in Obom and Asutuare. Allelic frequency is shown on the vertical axis while allelic range (bp) represents the horizontal axis. Blue and red bars represent population of alleles from Obom and Asutuare study sites respectively.

Poly_α microsatellites marker in Obom was found to have the highest number of bars with diverse fragment ranges as shown in figure 4.8, the least number of allele is seen in marker 2490 which had only I allele at 85 bp in Asutuare

Allelic distribution and hetrozygosity of the loci in the coded region of *P. falciparum* markers (Poly_α, PFPK2, ARA 2 and PFG377) in both study sites were generally higher thus average number of alleles and hetrozygosity of 10 and 0.479 respectively. The non-coded region had average number of alleles and hetrozygosity of 5 and 0.354 respectively as shown in **Table 4.2 above**

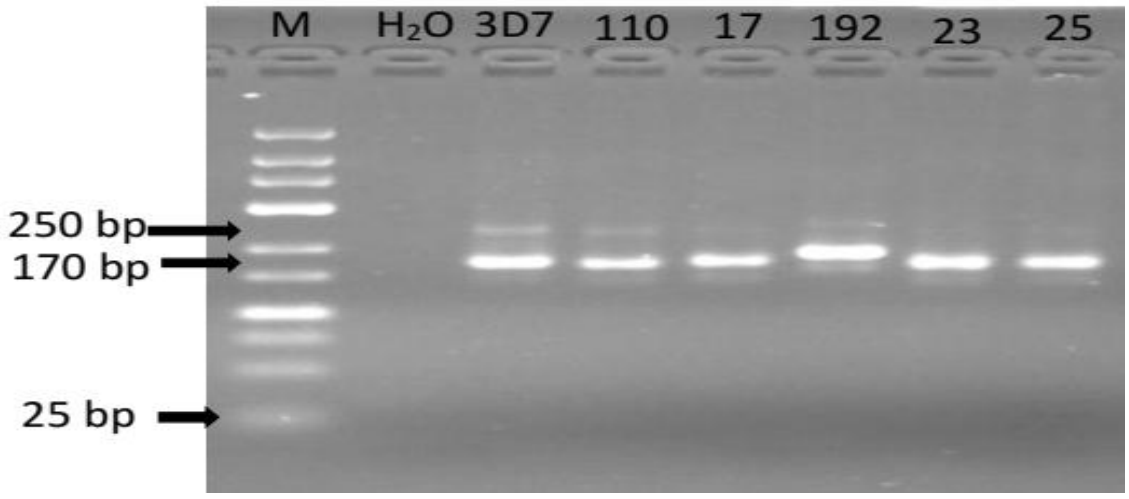


Figure 4.5: TBE Microsatellites gel image, 3D7, 110, and 192 are multi-clonal while 17, 23 and 25 are clonal strains. 3 µl of the ladder (25 bp) and 5 µl of the amplicons were loaded into the TBE agarose gel and run at 80 volts for 2 hours.

To determine allelic concordance or discordance between the microsatellites markers run on TBE agarose gel and samples run on CE with alleles calling by a GeneMapper v 2.6. There was discordance found in two samples as shown above to have double alleles by TBE agarose gel electrophoresis but with CE it was found to be single infection, allelic concordance was found in 3 samples for both TBE gel and CE genotyping. The peak heights for these samples are shown below in figure 4.6.

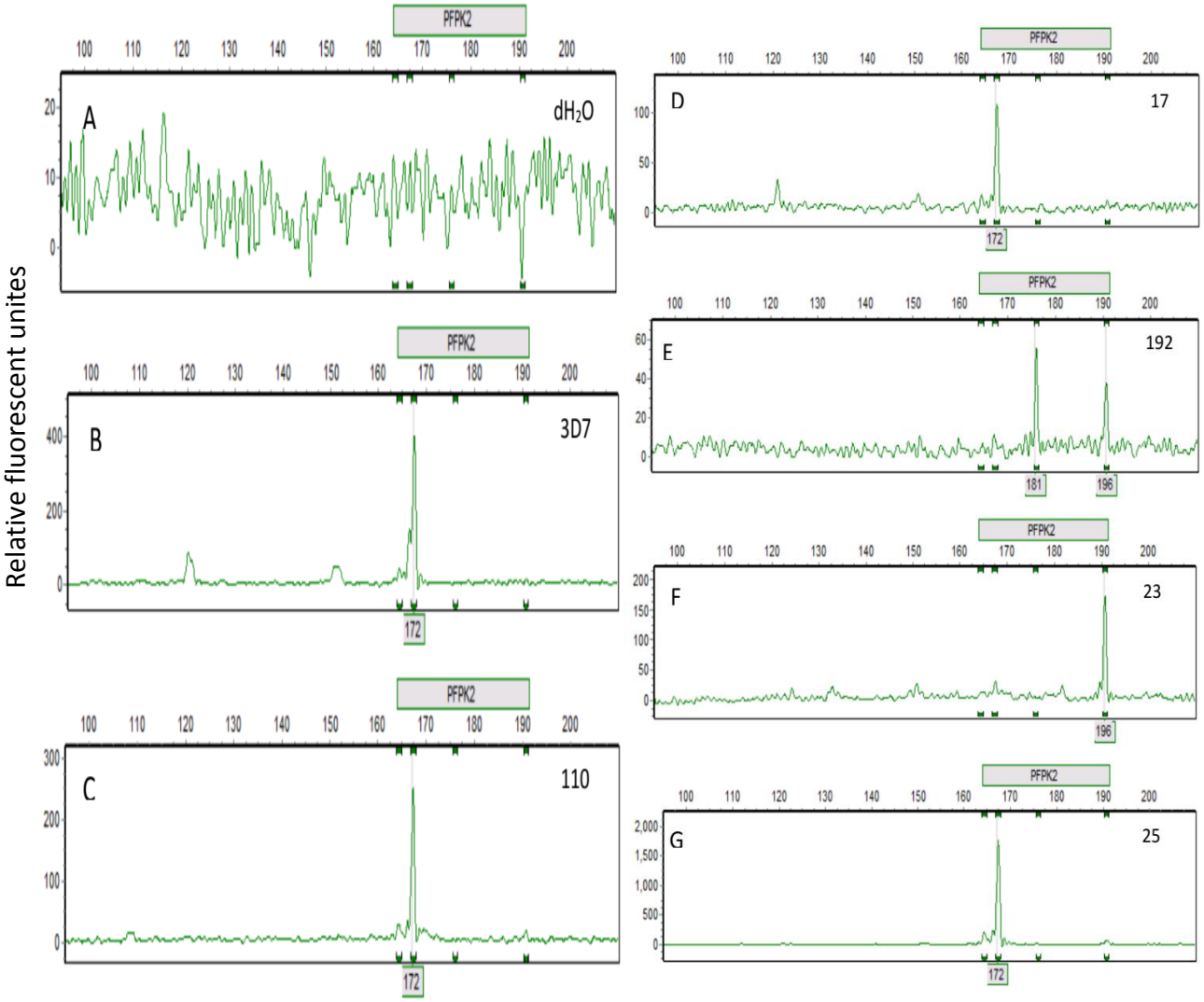


Figure 4. 6: Peak heights of microsatellites CE, the vertical axis represents the relative fluorescent intensity units (RFU) of each allele while the horizontal axis represents the fragment size (bp)

In **Figure 4.6** the peak heights (alleles) were sized or called using the GeneMapper V2.6 software of the ABI 3500XL analyser on POP 7 Polymer programme, 24 capillary tube ejection. Prior to the sample ejection by the Analyser, the samples were mixed with 8.5 µl of Hi-Di formamide (denaturant) and 0.5 LIZ 500 standard per well. 1 µl of a 1:10 dilution of the three PCR amplicons or probes (6-FAM, HEX and PET) mixture was added to 9 µl Hi-Di Formamide / LIZ 500 mixture and denatured at 95 ^{o}C for 3 minutes. The PCR products were optimized to prevent shutter or false positive alleles formation with the microsatellites markers. The samples were covered with Aluminium foil to prevent exposure to light prior to analysis on the analyser since the amplified products contained probes (Fluorescent dyes).

Table 4.3 Microsatellites CE and *msp – 2* genotyping status compared

Site	**Marker**	**N**	**Clonal**	**Mixed Clone**	**Missing Data**
Asutuare					
	Microsatellites	24	21	2	1
	msp – 2	24	10	1	13
Obom	Microsatellites	24	4	0	20
	msp - 2	24	18	3	3

In **Table 4.3,** not only was allelic concordance between MSP – 2 and microsatellites in 3 panels (17, 23 and 25) present but the efficiency of genotyping *P. falciparum* was high in microsatellites markers in Asutuare compared to genotyping *msp – 2* Block 3 regions in the same site. There was disagreement in 2 samples that were multiclone for TBE gel for microsatellites but produced single allele for CE GeneScan (peaks). The number of missing alleles (samples

without bands in a gel image or CE) for *msp – 2* were 14 in Asutuare and 20 for microsatellites in Obom.

4.4 GENETIC DIVERSITY OF THE 3 MARKERS (MSP – 2, GLURP AND MICROSATELLITES)

160 *P. falciparum* isolates were genotyped in both study sites for *msp – 2* and *glurp*, 48 clonal isolates for *msp – 2* were selected for validation by microsatellites markers. 13 different alleles were found in both sites with *msp – 2* with the most prevalent allele of 300 bp with allelic range of 280 bp to 700 bp and the average MOI for both sites were 1.20. The number of isolates positive for the marker at Obom (76) was much higher than that in Asutuare (10) site. The protocol for *msp – 2* typing was repeated for both sides but the results did not change.

Genotyping the R II region of *glurp* produce a total of 9 alleles at both study sites, the most prevalent allele was 900 bp and an average MOI of 0.99. The allelic range for this marker was 600 bp to 1200 bp. We observed that the number of alleles produced with the *glurp* genotyping was higher than *msp – 2* typing for both study sites.

In applying microsatellites genotyping for the 48 field isolates for each of the 12 markers at both sites, the average number of alleles was 13 with 150 bp being the most prevalent allele at Poly_a with allelic range of 60 bp to 500 bp as shown in **Table 4.2** above. The average number of isolates positive for microsatellites were 35 across the 12 loci in both sites of this study. Microsatellites markers were the most diverse of the three with *glurp* being the least diverse marker.

CHAPTER FIVE

5.0 DISCUSSION

P. falciparum isolates were genotyped using *msp – 2* and *glurp* antigenic markers, clonal alleles for *msp – 2* were then subjected to CE based microsatellites typing to determine parasites diversity and populations trends in Obom and Asutuare. Parasites diversity (Polymorphism), allelic frequency for the three markers were assessed in both sites of this study. The addition of CE 12 microsatellites markers has increase the accuracy of genotyping *P. falciparum* in different geographical setting, this study is the first in its kind to the best of our knowledge in genotyping *P. falciparum* in low and high transmission settings with the focus on SSR markers in Ghana (Greenhouse *et al*., 2006).

In genotyping *P. falciparum* in Obom, our data indicates high parasites diversity with *msp – 2* marker compared to glurp gene, this is evidence with MOI (1.30) at Obom being higher than Asutuare (1.0). Allelic distribution of the same marker (*msp – 2)* at Obom was diverse compared to that in Asutuare. We discovered that the prevalence of 3D7 strains in this study was higher than FC27 strain in both study sides, but 3D7 strain was much higher in the endemic parasite setting in Obom than in Asutuare a low endemic area. Polyclonal infection was prevalent in Obom than in Asutuare where no polyclonal infection was recoded. This research reinforces an earlier study done by Osei-Tutu *et al* (2011) and Duah *et al* (2016) (Felger *et al*., 1994) in which high parasite diversity was found to be associated with *msp – 2* typing and that 3D7 were found to be higher than FC27 (Agyeman-Budu *et al*., 2013; Barry *et al*., 2013).

Allelic range for Obom were wider and of higher frequency (280 bp – 700 bp) for both strains of the parasite 68 (80%) compared to a fewer fragment range at Asutuare (380 bp – 500 bp) genotyped thus 12 (15%). Parasite diversity is further buttressed with this finding from the

endemic site compared to the less prevalent site. The implications of the results imply that, there is high diversity of parasites at Obom born from high transmission of *P. falciparum*. This agrees with a similar study done by Congpuong and colleagues in which the diversity of *P. falciparum's* antigenic markers (*msp – 1, 2* and *glurp*) genes were genotyped along the borders of Thailand and Myanmar (Congpuong *et al*., 2014).

The diversity of the *msp – 2* in Obom further suggests that parasites diversity is associated with endemicity (higher transmission intensity) and this study agrees with Agyeman-Badu and his colleagues (2013) where MOI of asymptomatic residence in the middle belt of Ghana found higher 3D7 strains compared to FC27 and that the ratio of these strains genotyped is 2:1 (Agyeman-Budu *et al*., 2013; Arnot, 1998).

Genetic diversity of *P. falciparum* is a selective advantage 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 mating events of these different parasites genotypes in the mosquito vector may lead to parasite's diversity as different parasites clones with unique genetic make – up combined hence making the search for an effective vaccine a difficult task (Felger *et al*., 1994).

The main aim of most malaria intervention efforts is the reduction in the incidence of malaria in the country but this is frustrated 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 only for the intended efforts to be derailed by the complexities of multiplicity of infections of the parasites. To achieve the target of such

intervention methods (thus reduction in the prevalence of malaria) in endemic areas such as Obom a scale up 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).

The Ministry of health has for some years introduced the mass distribution of Insecticides treated Nets (ITNs) to pregnant mothers and children less than five in the country but a sustained reduction in the prevalence of malaria in most endemic area had not been achieved due to the lack of monitoring on the usage of these treated nets and the unsustain nature of the distributions.

The high diversity of *msp – 2* displayed in Obom compared to Assutuare, 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). Genotyping the glurp gene in both sites produced few alleles compared to *msp – 2* and that this could mean that the discriminatory power of *msp – 2* is higher than *glurp* hence the marker *(msp – 2*) will be preferred to *glurp* when typing field isolates form different geographic setting (Duah *et al*., 2016: Organisation, 2008).

Our study reveals significant difference in alleles at Obom compared to Asutuare with the use of MSP – 2 typing as shown in **Figure 4.2** above. The main occupation of the inhabitants of this community is fishing, the movement of people in and out of this area to do buying and selling of fish coupled with the proximity of the town to the river could be a factor that influence parasites

carriage and diversity in the community as well. This finding is consistent with a study done by Worrall and colleagues where there found a strong correlation between the occupation of inhabitants and the prevalence of malaria in the settings. They make a case that people who are mostly Agricultural labours (fishing) are at greater risk of malaria infection, for they live in environment that breeds the parasites all year round (Worrall *et al*., 2002).

Parasite diversity shown by our study at the endemic site could be as a result of genetic recombination of different clones at the vector (mosquito) stage, this could derail the efforts in the search for antigenic epitopes towards the development of an effective vaccine. The immunity acquired by people living in endemic areas of malaria transmission is non-sterile and could be compensated by the presence of an effective malaria vaccine but polymorphism and diversity associated with antigenic markers (*msp – 2*) makes it difficult to develop an effective malaria vaccine (Duah *et al.,* 2016; Conway, 2015).

Pradel and Dinko (2017) suggests that the high polymorphism displayed by *P. falciparum* merozoites antigens makes it difficult to be used as a vaccine candidate thus reinforcing an earlier study done by Conway (1997) and (2015) except those subdomains with conserved regions that has the potential for used for vaccine development (Dinko & Pradel, 2016).

Economic status of the inhabitants of Obom could be another driver for the high parasite diversity. They people in this area are into small scale fishing and other farming activities their ability to take care of the medical needs for those infected with malaria may be a challenge, this can lead to persistence of infection and its accompanying complication for the vulnerable group – children less than five and pregnant mothers. Malaria inflicts the heaviest toll on the poor in Sub - saharan African thereby further impoverishing them (Worrall *et al*., 2002).

Low usage of intervention methods (ITNs) at the community (Obom) could be another factor that drives the endemicity (high transmission) of malaria infection at this setting. There is no research done in the community to the best of our knowledge to assess the impacts of intervention methods in the area.

We observed that several *P. Falciparum* isolates 68 (85%) were negative for *msp – 2* in the low endemic area (Asutuare) compared to similar number of samples which were positive for microsatellites markers in the same site. The number of parasites infecting samples could also account for why such samples with lower number of parasites will be negative for *msp – 2* as compared to microsatellites markers. This result could explain the fact that microsatellites markers were abundantly distributed in the entire 14 chromosomes of *P. falciparum* genome and hence could be easily genotyped compared to *msp – 2* which are located only at the coded regions of the parasites genome. Genotyping fields isolates with the use of SSRs markers could be suitable than antigenic *msp – 2* due to the polymorphic and high discriminatory power shown by these markers (SSRs) (Greenhouse *et al.*, 2006; Su & Wellems, 1996)

The *glurp* antigenic marker was also used to genotyped *P. falciparum* form both Obom and Asutuare, we noticed that diversity of this marker thus allelic pattern, distribution and frequencies were slightly different (little diversity) with this marker (glurp) as compared to *msp - 2*. Thus Asutuare had higher (87.5 %) samples positive compared to Obom (68.5%) positive for the marker. Allelic range for both study sites were not different thus fragment ranges of 600 bp to 1200 bp were recorded. Polyclonal infection was not recorded at both sites of this study with the R II region of *glurp,* MOI in Obom (1.0) was not different from that in Asutuare (0.98). This result implies that genetic diversity of the glurp gene at Obom was not as higher than that in Asutuare as may be the case with *msp – 2* genotyping. Parasites with diverse frequency of alleles

were low for the glurp gene compared to *msp – 2*, in genotyping *P. falciparum* for genetic diversity *msp – 2* could be more informative, high discriminatory power than *glurp* gene. Our results also reveal that in genotyping *P. falciparum, glurp* antigenic marker may not be the best marker in assessing the level of diversity and transmission at a low and high malaria transmission (Gupta *et al*., 2014; Organisation, 2008; Pratt-Riccio *et al* 2013)

The number of alleles for the R II repeats region of *P. falciparum* in both sites of this study was not different as both Obom and Asutuare recorded 5 alleles each. This result implies that the diversity of the *glurp* gene at both sites were not significantly different. The finding agrees with an earlier study in which limited genetic diversity and number of alleles (5) was found in our study areas similar study of the *glurp* gene (Haddad *et al*., 1999; Kumar *et al*., 2014; Pratt-Riccio *et al*., 2013). Polymorphism of the *glurp* gene however increases in other high transmission areas in sub-Saharan Africa (Kumar *et al*., 2014).

Microsatellites genotyping was used to assessed the diversity of *P. falciparum* in Obom and Asutuare after clonal isolates were genotyped by *msp – 2* antigenic marker. The application of Microsatellites for genotyping *P. falciparum* has become imperative in population and diversity studies because SSRs markers have proven to be stable and note under immune selection pressures as compared to antigenic *msp – 2* genotyping, microsatellites has become the markers of choice in recent years for parasite population genetics.(Su & Wellems, 1996).

This study shows higher parasites clones genotyped for the microsatellites compared to *msp – 2* marker especially in Asutuare where the prevalence of *P. falciparum* is low as shown in **Figure 4.4 (a) and (b) above.** This is evident with only 12 isolates genotyped for both N5 and M5 as indicated in **Table 4.1** compared to an average of 35 samples genotyped for microsatellites

markers. This result confirms an earlier study done which shows that microsatellites were stable, reduces unspecific (spurious) bands amplification, and could be genotyped in a single multiplexed PCR amplification. Firdig and Su (2000) reports that microsatellites markers are generally uniqued in which several markers can be assayed in a single reaction with small amount of DNA. The 85% negative *msp – 2 P. falciparum* isolates that were amplified by the microsatellites markers indicated that the SSR markers are polymorphic and distributed abundantly in both the coded and non-coded regions of the parasite's genome (Anderson *et al*., 1999: Su α Wellems 1996).

The number of alleles genotyped in Obom ranged from 3 (2490) to 13 (Poly α) with *He* of 0.66 (2490) to 0.516 (TA 109) at the same study site. The number of alleles in Asutuare ranged from 1 to (2490) to 9 (Poly α), *He* values for the same sites rages from 0.00 (2490) to 0.577 (PFG377) Hetrozygosity is an index used to determine genetic diversity of the microsatellites markers and ranged from 0 (low genetic diversity) to 1 (high genetic diversity). *He* values for both study sites are generally low and could suggest low genetic diversity of these markers in the two study sites. This is in contrast to a similar study done in Mali in which microsatellites markers showed high genetic diversity with an average *He* of about 0.80 and low genetic differentiation of the field isolates (Nabet *et al*., 2016).

Anderson and his colleagues reported that high level of genetic diversity was observed in African with little genetic differentiation and could suggest that people of African descent may be the original reservoirs of the parasites that might have spread to European (South America) (Anderson *et al*.,2000). Another report on microsatellites genotyping was that West African location where the intensity of malaria is high had high diversity of the parasites with microsatellites repeat markers. *He* values in the 8 study sites raged from 0.00 to 0.91. High

number of mixed clones were genotyped in these locations, for instance in Guinea the intensity of malaria is high as opposed to countries in North Africa which recorded limited genetic diversity with the SSR markers. The low endemicity of the infection coupled with an elimination phase in those regions of Africa could explain the low allelic diversity of the markers. Some countries (Egypt and Algeria) in these region are in the elimination phase in the fight against malaria as reported by WHO in 2015 (Mobegi *et al*., 2012; Organization, 2015b).

In determining allelic concordance with TBE agarose gel and microsatellites markers we notice some disagreement in 3D7 and 110 TBE agarose gel and that of CE peak heights. Samples 17, 192, 23 and 25 showed allelic concordance between the TBE agarose gel genotyping and CE peak heights as indicated in **Figure 4.5 and 4.6** above. Microsatellites and *msp – 2* were used to genotyped 24 samples and the sensitivity of the two markers compared, we noticed that out of the 24 samples typed microsatellites clonal positive samples were 21 against 10 for *msp – 2.* 2 samples were polyclonal infection for microsatellites compared to only 1 for MSP – 2. Only 1 sample was declared as missing data for microsatellites as compared to 13 samples for *msp – 2* as shown in **Table 4.3** above for Asutuare. In Obom only 4 samples were positive for microsatellites clonal infection compared to 18 samples for *msp – 2* typing, the number of missing data for the microsatellites were 20 samples compared to only 3, the several SSR samples missing were due to either the samples were not enough for the genetic analyser to be able to run or the samples had dehydrated.

In a similar research that compared the sensitivity of gel-based typing of the parasites to that of CE genotyping noted that the CE could detected field isolates more readily than the traditional gel method of genotyping the parasites. This accordingly has reduced the errors made in the gel-based method of genotyping especially when the purpose of such genotyping method is to

distinguish between new infection and failure in treatment(recrudescence) in anti-malaria trials. Genotyping the parasites in a low prevalent area is equally useful when microsatellites markers are used in such studies than antigenic *msp – 2.* This results confirms the sensitivity of using microsatellites markers for typing the parasites (Gupta *et al*., 2010 : Greenhouse *et al*., 2006) .

The peaks heights electrophoregrams in **Figure 4.6** above, thus in panel A to G shows sharper and less number of shutter peaks. Shutter peaks or false alleles are as a result of inadequate optimisation of the protocol due to unspecific binding or the primers binding to each other or carry forward from a hemi 1 amplification. PCR contamination or slippage in DNA strand during amplification process. This can lead to misrepresentation of data and obviously wrong conclusions.(Greenhouse *et al*., 2006).

CHAPTER SIX

6.0 CONCLUSION

The genetic diversity of *P. falciparum* was assesses using two antigenic markers (*msp – 2, glurp*) and microsatellites markers in two study sites with different transmission intensities. We can conclude that parasite diversity was higher in *msp – 2* genotyping as compared to *glurp* RII region, Obom had higher parasite diversity as compared to Asutuare. Obom has higher parasite prevalence (endemicity) compared to Asutuare, this factor could be responsible for the diversity of the marker (*msp – 2*) and that *msp – 2* has a higher discriminatory power than *glurp* R II region.

The diversity of the parasites with microsatellites markers can be described as low as evident in the *He* values and could be as a result of microsatellites not been under immune selection pressure. In genotyping the parasites, CE has proven to be more sensitive with easy allelic sizing than TBE agarose gel. 85% negative *msp – 2* isolates were all positive for the microsatellites genotyping confirming the fact that the microsatellites repeats were distributed abundantly throughout the genome of the *P. falciparum*

6.1 LIMITATION

The study was done in two transmission areas in southern Ghana, the scope of this study could be widened to the entire country to properly characterise of *P. falciparum* especially for purposes of rolling out intervention programmes.

The microsatellites isolates were not sequenced in this current study so we could not confirm the peaks height generated by the ABI analyser and scored by the GeneMapper.

6.2 RECOMMENDATIONS

Based on this current study, we recommend that a phylogenetic study could be conducted to determine the evolutionary relationship in parasite isolates using microsatellites markers to genotype *P. falciparum* in Ghana.

Microsatellites genotyping could be used in a study that intends to distinguish between new *P. falciparum* infections from recrudescence instead of antigenic markers (msp -2, Glurp etc).

Polyacrylamide could be used in running the gel for the microsatellites instead of TBE, for Polyacrylamide has a better resolution of amplified bands when CE ABI Gene analyser is not available.

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