KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

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Evaluation of Immuno-epidemiological Markers for Assessing Malaria Transmission Intensity in the Hypo-endemic Highlands of Kenya and the Accuracy of Malaria Diagnosis in the Holo-endemic Forest Zone of Ghana

by

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DECLARATION

This thesis is submitted to KNUST, School of Graduate Studies through College of Science, Department of Theoretical and Applied Biology. I declare hereby that this thesis has been composed by myself and has not been accepted in any previous application for a degree here or elsewhere. This thesis presents results of original research undertaken by me. Information taken from other works has been duly acknowledged.

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ABSTRACT

Current tools for measuring malaria transmission intensity have limited sensitivity when transmission is low. Robust surveillance systems are needed to monitor reduced transmission and prevent the rapid reintroduction in areas undergoing elimination. Serologic tools based on the antibody response to parasite and vector antigens are potential tools for transmission measurements. The present study examined the serologic evidence of vector exposure and malaria endemicity in the western Kenyan highland sites along a transmission intensity cline. Total IgG levels to *Plasmodium falciparum* MSP-1_{19} and *Anopheles* salivary gland peptide gSG6-P1 were measured in an age-stratified cohort (< 5, 5-14 and ≥ 15 years) during low and high malaria transmission seasons. Antibody prevalence and level were compared among different localities. Regression analysis was performed to examine the association between antibody prevalence and parasite prevalence. Age-specific MSP-1_{19} seroprevalence data was fitted to a simple reversible catalytic model to investigate the relationship between parasite exposure and age. Higher MSP-1_{19} seroprevalence and density were observed in the valley residents than in the uphill residents. Adults (> 15 years) recorded high and stable immune response in spite of changing seasons. Lower responses were observed in children (≤ 15 years) which, fluctuated with the seasons particularly in the valley residents. In the uphill population, annual seroconversion rate (SCR) was 8.3% and reversion rate was 3.0%, with seroprevalence reaching a plateau of 73.3% by age of 20. Contrary, in the valley bottom population, the annual SCR was 35.8% and the annual seroreversion rate was 3.5%, and seroprevalence in the population had reached 91.2% by age 10. Seroprevalence of gSG6-P1 in the uphill population was 36% while it was 50% in the valley bottom population (χ^2 13.2 *P= 0.0002*). Median gSG6-P1 antibody levels in the Valley bottom were twice as high as that observed in the uphill population [4.50 (1.02) vs. 2.05 (0.92)] and showed seasonal variation. The odds of gSG6-P1 seropositives having MSP-1_{19} antibodies were almost 3 times higher

than the odds of seronegatives [OR =2.87, $P < 0.001$]. The observed parasite prevalence for Kisii, Kakamega and Kombewa were 4%, 19.7% and 44.6% whilst the equivalent gSG6-P1 seroprevalence were 28%, 34% and 54% respectively. The study reveals the micro-geographic variation in malaria endemicity in the highland eco-system; thus validating the usefulness of sero-epidemiological tools in assessing malaria endemicity in the era of decreasing sensitivity to conventional tools. The seroprevalence of IgG to gSG6-P1 was sensitive and robust in distinguishing between hypo, meso and hyper transmission settings and seasonal fluctuations. The study determined the accuracy (sensitivity and specificity) of *Pf*HRP2- based malaria rapid diagnostic test (RDT) in Western Kenya and in the forest zone of Ghana. The performance of RDTs when microscopy is used as the gold standard is reported based on an operational study that took place at the Ghanaian study sites and a field trial conducted based on asymptomatic primary school children in Kenya. Implications for the choice of the RDT in an era of changing malaria epidemiology are discussed.

DEDICATION

This thesis is dedicated to children in Africa, who are bearing the force of the malaria disease burden

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1.02 Chapter Two: Review of relevant literature

The chapter briefly reviews the relevant literature on the biology of malaria disease and current perspectives, the changing epidemiology of malaria, the various malaria transmission measurements and their limitations. Naturally acquired immunity to malaria is briefly introduced; this is followed by the utility of immune-epidemiological markers of exposure to vector bites and parasite infection, and then the review of RDT performance and its utility at low parasite density.

1.03 Chapter Three: General Methodology

The chapter presents the general methodology for the objectives of the study; it presents description of the study area and recruitment procedures, the study design, sample collection, laboratory analysis, quality assurance issues, ethical clearance and the statistical analysis performed.

1.04 Chapter 4: Results

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1.05 Chapter 5 Discussion

This chapter comprehensively discusses the results into details of the main studies and the application of the tools developed and their true value in malaria control and elimination. Principally it discusses the first objective ‘‘Variation in MSP-1_{19} antibody responses to malaria in western Kenyan highlands” and the second objective “variation in exposure to *Anopheles gambiae* salivary gland protein (gSG6-P1) across different malaria transmission settings in the western Kenya highlands”. The last section discusses the accuracy of diagnosis of the malaria rapid diagnostic test kit that is based on the *Pf*HRP2 as it compares with the gold standard microscopic examination of parasites in blood smears.

1.06 Chapter 6 Conclusion and Recommendations

Chapter six highlights the main findings of the study; the applications of the knowledge acquired, recommendations, and limitations. The chapter also suggests further relevant studies that will be needed in the future. This is followed by the reference list and then the Appendices.

CHAPTER ONE: INTRODUCTION

1.1 Background

Globally, there were 2.37 billion people living in areas classified as 'at risk' of *P. falciparum* transmission in 2007 (Guerra *et al.,* 2008). This resulted in 243 million malaria cases in 2008 which became responsible for nearly 863,000 deaths (WHO, 2009). However, during the year 2010, a reduction to 216 million episodes with a consequent 655, 000 deaths were reported, 36 000 lower than the previous year (WHO, 2011). Sub-Saharan Africa shoulders at least 81% of the global malaria burden, where at least 91% of all malaria-associated mortality does occur. Additionally all localities where the prevalence of *P falciparum* exceeds 50% in the population can be found in Africa (Guerra *et al.,* 2008). Disability Adjusted Life Years (DALY) with 85% of the deaths occurs amongst children below the age of five years, where about 40% of health budget is associated with malaria. Thus in Sub Saharan Africa (SSA), the malaria burden is projected to cost USD35.4 million as a loss in annual earnings in Africa (WHO, 2010).

Notwithstanding the overarching burden of malaria in Africa, South of the Sahara there is accumulating evidence that malaria transmission is on the phase of decline in some countries (Rodrigues *et al.,* 2008; O'Meara *et al.,* 2010). Reductions in malaria cases of more than 50% have been observed between 2000 and 2010 in 43 of the 99 countries where malaria transmission is currently ongoing, while decreasing trends of 25%–50% were seen in eight other countries. Countries such as Eritrea, Rwanda, Zanzibar (WHO 2009), Pemba (Jaenisch *et al.,* 2010), Tanzania mainland (Mmbando *et al.,* 2010), Kenya (O'meara *et al.,* 2008), Gambia (Ceesay *et al.,* 2008), and Zambia (Chizema-Kawesha *et al.,* 2010), including Swaziland (Kunene *et al.,* 2011) have reported substantial decline in malaria burden in recent times. Moreover, consistent reductions *Anopheles* population density has been observed

during an 11 year study period, in spite of the absence of planned vector control (Meyrowitsch *et al.*, 2011). Guerra and others recently reported that globally, almost 1 billion people live in areas of extremely low malaria risk and suggested that malaria elimination is epidemiologically feasible in those areas (Guerra *et. al.*, 2008).

The declining infection incidence and by and large the disease burdens as well as decreasing asymptomatic infections are the fall outs of improved quality health systems, including better case management, such as diagnostics and deployment of effective athemisinin –combination therapies. Large scale intervention programs aimed at achieving high coverage of insecticide treated nets (ITNs), indoor residual spraying (IRS) and the advent of intermittent preventive treatment (IPT) in pregnant women, have significantly reduced the malaria burden.

As programs successfully control malaria transmission to near near zero transmission levels, the assessment of malaria-associated morbidity and mortality as a tool for tracking reducing burden will become difficult and less sensitive. One primary indicator of interest will be malaria transmission dynamics (MalERA, 2011). Measuring malaria transmission intensity is one key area where there are substantial knowledge gaps. Thus, the quest for sensitive and robust surveillance tools has become imperative. Such surveillance tools are needed as an intervention to reduce transmission, to measure transmission interruption and maintenance of zero transmission (MalERA, 2011). These should also be useful in mapping the risk of focal residues of transmission (the so called hot spots). However, the existing tools or approaches for measuring malaria transmission intensity have limitations in sensitivity when transmission is approaching pre-elimination and elimination levels.

1.2 Problem statement

The malaria burden in Africa is does not follow a homogeneous distribution. Many parts of the continent are characterized by low transmission intensity (Guerra *et al.*, 2008; Snow *et al.*, 1999). These areas under low transmission are thought to be apposite for intensive malaria control and possible elimination (Hay *et al.*, 2008). As countries scale up malaria control and further reduce transmission burden, there has to be a change in strategies. Malaria surveillance activities need to be changed into effective tools in order to monitor further transmission reduction (Alonso *et al.*, 2011). To accomplish this, activities will need to move from counting number of deaths and symptomatic cases of clinical malaria, to identifying and tracking the risk of human exposure to the *Anopheles* vector and parasite, as well as sensitively detecting actual infections even at very low parasitemia (MalERA, 2011). Against this backdrop of changing malaria epidemiology towards reducing transmission, better monitoring and surveillance approaches, as well as robust tools for assessing malaria burden are essential not only for sustaining the gains of control but also for the enhancement targeted interventions that will maximize the benefits of scarse resources.

The gold standard for assessing malaria transmission intensity is the estimation of the entomological inoculation rate (EIR), which measures the number of infectious bites an individual receives in a given period of time. This process involves collection of mosquitoes in the field, either by Human landing catches or light trap, by pyertrhum spray catch and aspiration (WHO, 1975). This method lacks precision because mosquito distributions are markedly heterogeneous (Drakeley *et al.*, 2003; Mbogo *et al.*, 1995). Sporozoite infection rates are low (below 5%) even in highly endemic areas. Again, this is complicated in areas of low transmission by low total numbers of mosquitoes. Furthermore for practical and ethical reasons, mosquito colection typically uses adult volunteers, however extrapolating the biting

rates and incidence of infections in children has limitations (Smith *et. al.,* 2004). Entomological are labor-intensive, and have become very expensive. Maps based on the combination of malariometric indices with physical geography and climatic data, using geographical information systems (GIS) are available. They reveal large scale variations in malaria transmission intensity across Africa (Craig *et al.,* 1999; Abeku *et al.,* 2004). However, these maps have poor predictive capacity at the community level (Omumbo *et al* 2004).

Estimates of transmission derived from hospital records are unreliable; they overestimate the number of cases that present to health facilities due to over diagnosis (Schellenberg *et al.,* 2004), they also miss large numbers of cases occurring in the community because many people will never show up at the hospital for treatment (Snow *et al.,* 2005). Parasite detection by microscopy requires skilled staff, accuracy and sensitivity; these are very critical in areas of low transmission, where skills are likely to be very weak. The prevalence of parasites found in human blood is influenced by acquired immunity, drug use as well as parasite resistance to drugs. High levels of acquired immunity and rapid clearance of parasites from the circulation could lead to underestimation of transmission at high transmission intensity; drug use and drug resistance can have changeable effects on parasite prevalence in non-immune populations living in areas of low transmission. Routine malaria case incidence allows for 'real-time' assessment of changes making it easy to combine clinical and epidemiological outcomes with other programmatic information that will assist in planning and implementation. This will allow for modification and adjustment of control activities to improve the success of control programs. However, the accuracy of surveillance-based measurements directly depends upon the quality as well as coverage of the system. But in many endemic areas, current surveillance efforts are incomplete and slow. Model-based, cartographic approaches would have been good enough to assess malaria

burden in locations where routine surveillance and quality and coverage are poor. However, since survey and case incidence studies are very expensive to carry out, cartographic models normally utilize information obtained longer period and are thus less suitable for assessing year-to-year variation in malaria burden. the situation is further complicated in areas with poor data coverage, the cartographic models make estimates for larger geographical areas which is unfortunately based on few, conveniently collected data points, this make it increasingly less sensitive and thus unreliable as a means of tracking transmission intensity (Guerra *et al.,* 2008). Furthermore the quality of the data obtained; the sensitivity of the tools used, usually, microscopy and in recent times the rapid diagnostic tests (RDTs), especially the *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2-) based malaria rapid diagnostic test in routine detection of parasitemia presents additional challenges at low parasite densities. Most reported sensitivity variations are observed as false negative tests at relatively low parasitemia <250 parasites/µL (Huong *et al*., 2002; Singh *et al.*, 2003 and Ishengoma *et al*., 2011). In other studies RDTs have been reported to yield false negative results at relatively high parasitemia (1000 P/µL) (Slutsker *et al.*, 1994). Robust and sensitive detection of infection, risk of exposure and, transmission intensity of malaria is thus essential for monitoring, evaluation and surveillance programmes. This will enable linkage of active and prompt detection of infection with a response package to further reduce the reservoir of infection. Therefore, a valid metric, or combination of tools, for detecting low transmission is critical as elimination is approached; however the existing tools for malaria transmission assessments have severe limitations when transmission is very low.

1.3 Rationale

The ideal tool for assessing malaria endemicity would integrate malaria exposure over time, such that it is not a discrete measure or snap shot of the complex continuous process. Measurement of serum antibodies as surrogate for malaria exposure is a valuable way of assessing transmission intensity when the interest is to reveal the longterm transmission potential and to determine the malaria endemicity. This is because anti-malarial antibodies build up after repeated exposures and and also persist for long periods of time after infection (Drakeley *et al.,* 2005). Due to the longer duration of the specific antibody response, seroprevalence represent the collective exposures over time and thus, it not readily affected by seasonality or unstable transmission. Additionally, seroprevalence is a more sensitive measure since it is always higher in a given population than the corresponding parasite rates. This makes immunological markers superior to discrete infections as a marker of exposure in areas under extremely low or unstable malaria transmission (Bousema *et al.,* 2010; Cook *et al.,* 2010). It has also been demonstrated that Age-specific seroconversion rates offer estimates of malaria exposure similar to the entomological innoculatin rates (Corran *et al.,* 2007, Stewart *et al.,* 2009).

The corresponding measure of the human immune response to mosquito salivary proteins would be very convenient (Remoue *et al.,* 2006). This will permit the assessment of vector exposure in children, which ethically is impracticable by human landing collection. The availability of biomarkers of human exposure to the bites *of Anopheles* would be an additional tool that can be used for monitoring of control interventions based on anti-vector measures in low malaria transmission areas (Drame *et al.,* 2010).

Mosquito salivary proteins facilitate blood feeding and their immunological properties modulate the immune response of the human host (Billingsley *et al.,* 2006; Ribeiro *et al.,*

2010). SG6 salivary proteins are immunogenic and elicit specific antibody response (Remoue *et al.,* 2005). A synthetic peptide (gSG6-P1) derived from *Anopheles gambiae* salivary recombinant protein gSG6 is a single immunogenic salivary antigen specific to *Anopheles* genus and highly conserved and thus guarantees high reproducibility for the assay (Poinsignon *et al.,* 2008; Valenzuela *et al.,* 2003; Calvo *et al.,* 2004). The SG6 has been recently reported to have shown promise as a general epidemiological marker of exposure since it shares 99% and 80% identity with A. *arabiensis* and A. *funestus* respectively of which constitutes the main Afro-tropical malaria vectors (Rizzo *et al.,* 2011).

As malaria burden reduces, individuals previously under high malaria transmission are likely to harbor asymptomatic *Plasmodium* infection with low parasitemia that can last for up to 60 days (Baliraine *et al.,* 2009; 2010). Furthermore, detection of asymptomatic malaria cases is hindered by the low sensitivity of parasite-detection-techniques (i.e. microscope and rapid diagnostic tests) in identifying very low parasite densities (Andrade *et al*., 2010). In spite of low density of circulating parasites, asymptomatic carriers can pass on parasites to uninfected *Anopheles* mosquitoes (Alves *et. al.,* 2005). Asymptomatic carriers represent important parasite-reservoirs in malaria endemic areas. For this reason there is a requirement for a more sensitive and accurate diagnostic tool that will be able to detect parasites even at low parasite density. Rapid diagnostic tests (RDTs) offer great potential for the sensitive and accurate diagnosis of malaria, for prompt and appropriate treatment in remote areas. However, there are many reported disparities in their performance at low parasitemia, with no apparent pattern of geographic or quality-related causative factors (Murray *et al.,* 2003; Wongsrichanalai *et al.,* 2007). The WHO recommends a standard of 100% sensitivity at parasite densities of 100/µL (Bell and Peeling, 2006). A highly effective RDT could avert over 100.000 malaria-related deaths and about 400 million unnecessary treatments (Bell and

Peeling, 2006). The sensitivity and specificity of the *Pf*HRP-2 -based RDT relative to microscopy and PCR has not yet been studied in detail in Ghana. There is, thus, an urgent need for a comprehensive study to identify the specific factors responsible for poor sensitivities of RDTs, and point toward possible approaches to improve their diagnostic value.

The success of malaria control in the changing epidemiology of malaria burden will depend on a systematic understanding of the micro-geographic risk of human exposure to parasite and vector bites. This will enable identification of otherwise hidden enclaves of higher risk that constantly seed malaria transmission in the wider community. The current study was carried out to investigate serologic evidence of malaria exposure, risk of vector bites and hence pathogen transmission and the accuracy of diagnosis of malaria rapid diagnostic test at different malaria endemic transmission intensities.

1.4 Research Hypothesis

Under low transmission settings, the entomological inoculation rate (EIR) as a tool for assessing malaria transmission intensity, has a very low sensitivity. However, human-antibody responses to *Pf*MSP-1_{19} and gSG6-P1 will be a more sensitive and robust tool, capable of detecting micro-geographic risk of vector and parasite exposure and hence risk of parasite transmission.

Parasite density play an important role in sensitivity and reliability of *Plasmodium falciparum* histidine rich protein 2 (*Pf* HRP2)-based rapid diagnostic test (RDTs) which is also related to the transmission intensity. Thus parasite density, under different transmission settings will determine the value of the diagnostic tool.

1.5 Aim and objectives

The overarching goal of the study is to evaluate immuno-epidemiological markers as an alternative tool for malaria transmission intensity estimation. This will then be used for vector and parasite surveillance in anticipation of changing malaria epidemiology in Africa.

The combination of these tools will represent sensitive and robust tools for estimating malaria transmission intensity that can be adopted by national malaria control programs (NMCPs).

1.5.1 Specific objectives

The specific objectives were to:

1. Determine the spatio-temporal variation in human antibody response to *Plasmodium falciparum* merozoite surface protein 1_{19} (*Pf*MSP-1_{19}) in an area of differing malaria transmission intensity ecocline as follows:
    a. Determining the age-specific MSP1_{19} seroprevalence and total IgG titers, to generate age-specific seroconversion rates,
    b. Fiting the age-specific MSP-1_{19} seroprevalence data to a reversible catalytic model to examine the link between the force of parasite exposure and age, and
    c. Determining the patterns of MSP-1_{19} antibody responses to malaria in the human populations at different altitudes and seasons.

2. Evaluate host-specific humoral responses to *Anopheles* salivary peptide (gSG6-P1) in residents living in three distinct malaria eco-epidemiological zones as follows:

    a. Determining the correlation between the evolution of immune responses to *Anopheles gambiae* salivary peptide (gSG6-P1) and *Plasmodium falciparum* antigens (MSP1_{19}) in Western Kenya

b. Comparing at the community level, the risk of vector bites and parasite prevalence.

3. Determine the accuracy (sensitivity and specificity) of *Pf*HRP2- based malaria rapid diagnostic test (RDT) in residents from different malaria transmission intensity zones in the holo-endemic forest zone of Ghana and the hypo-endemic highlands of Western Kenya.

   The western Kenyan sitewas considered as very suitable because, in the highlands, for a relatively small spatial scale there are differences in altitude, water accumulation, *Anopheles* population densities and thus hereterogeneities in human exposure to malaria.

CHAPTER 2: LITERATURE REVIEW

2.1. Malaria Disease

2.1.1 Historical perspective of malaria disease

The name malaria came from Italian writers who thought that the disease was caused by foul smelling vapors coming from the Tiberian marshes. The word "malaria" literally means "bad air" in the Italian language. Therefore, the association between the bad air of the marshes (during the rainy season) and the occurrence of periodic fevers formed the basis for the name malaria. The first scientific evidence of malaria transmission was discovered on the 6th November 1880, when Alphonse Laveran saw a gametocyte exflagellating in blood smear from a patient from Algeria (Laveran 1880). This observation pioneered the recognition of *Plasmodia* as the basis of malaria disease (Laveran 1880). Further to this, in 1897, Ronald Ross recognized plasmodial oocysts in the gut epithelium of *Anopheles* fed on parasitemic birds; thereby incriminating *Anopheles* as the vector of malaria (Ross R. 1899). William George Mc-Callum established *Plasmodium* exflagellation as a course of sexual reproduction in 1897 (McCallum 1897) and it was later confirmed that anopheline mosquitoes was the vector of human malaria in 1900. In spite of increased knowledge of the parasite's biology and the availability of advanced technology for malaria research, the parasite has continued to plague man to this day.

2.1.2 Malaria disease and symptoms

Malaria is a mosquito-borne infectious disease of humans and other animals caused by eukaryotic protists of the genus *Plasmodium*. The disease is thus defined by the presence of *Plasmodium* parasites in the blood with associated clinical symptoms of the infection, which are dependent on the plasmodial species, age and immune status of the host. The disease results from the multiplication of *Plasmodium* parasites within red blood cells, the rupture of the infected red blood cells and their subsequent release of toxic substances into the circulation cause chills, headaches and fevers associated with disease in severe cases progressing to coma or death (Warrell and Gilles 2002). The hallmark symptom of malaria is fever, which can be followed by a wide range of other symptoms including headache, chills, diarrhea, lethargy, coughing fits and abdominal or muscular pain (Greenwood *et al.* 2005). Further, more severe manifestations of malaria also vary and include anemia, hypoglycemia, hypotension, intense hemolysis, metabolic acidosis, spontaneous bleeding, hepatitis, acute kidney failure, respiratory distress, convulsion, coma and multiple organ failure (WHO, 2000). The most characteristic clinical signs and symptoms of malaria are fever, chills, sweating and anemia, as well as general symptoms such as headaches, myalgia, malaise and weakness and in severe cases, visceral involvement (splenomegaly and hepatomegaly) (Hickmann 2003).

2.1.3 Plasmodium life cycle

Malaria transmission occurs when the human host is exposed to the bite of an infectious female *Anopheles* mosquito. Mosquitoes on the average inject fewer than 100 sporozoites per bite (Rosenberg *et al.*, 1990; Vanderberg and Frevert 2004); Current studies using intra-vital imaging have observed sporozoites being inoculated by *Anopheles* into the skin, where they are able to stay for up to six hours (Yamauchi *et al.*, 2007). Just about a-third of sporozoites leaving the site of innoculation may enter lymphatic system and drain to the regional lymph nodes; other sporozoites could go into the bloodstream and set out to the liver. It is estimated

that approximately 80% of parasites arrives at the liver but the ability of each of these sporozoites to produce erythrocytic-stage infections might be less (Frevert *et al.,* 2007). Experiments have shown that it takes five infectious bites to establish 100% infection (Verhage 2005). This suggests that greater proportions of sporozoites injected by *Anopheles* do not result in productive infections.

Plasmodium sporozoites travel through Kupffer cells and a number of hepatocytes prior to infecting a hepatocyte (Ishino *et al.,* 2004; Mota *et al.,* 2002). Within the cells of the liver, the uninucleate parasites go through schizogony (asexual amplification) with duration of 2 to 10 days. This depends on the particular species involved (about 5.5 days among plasmodia infecting humans), with one exoerythrocytic schizont enclosing as much as 30,000 merozoites. In *Plasmodium vivax* infection, sporozoites are able to stay within hepatocytes in dormant form described as hypnozoites. This may sponteniously arise into active forms and cause clinical relapses later (Krotoski *et al.,* 1982). Exoerythrocytic schizonts rip apart, and release merozoites into blood-circulatioin where they hurriedly invade erythrocytes (Cowman and Crabb, 2006) initiating the erythrocytic stage of the disease accountable for the clinical malaria symptoms. The invading merozoite takes with it an enveloping membrane acquired from the host cell to the inside of the erythrocyte it has invaded, and then envelopes itself within a parasitophorous vacuole, the merozite now initiates additional asexual development. The parasite matures from ring stage to trophozoites, and then to a schizont, it then goes through three to six mitotic multiplications to result in six to thirty-six merozoites within each erythrocytic schizont. After 48 h, (depending of the species), the schizonts rip apart, egressing merozoites into the blood-circulation. Some of the merozoites attach uninfected erythrocytes and go over the cycle of blood schizongony repeatedly if not disrupted my chemotherapy or immunity. Other morozoites differentiate into sexual forms that circulate in the peripheral blood. *P. falciparum* gametocytes can be seen in perephral blood in about 7 to

15 days after the first invasion of red blood cells (Drakeley *et al.*, 2006). Gametocytes when ingested by a feeding anopheline mosquito differentiate to gametes that fertilize to form a diploid zygote where meiosis occurs. This is illustrated in the Plate 1.0 below.

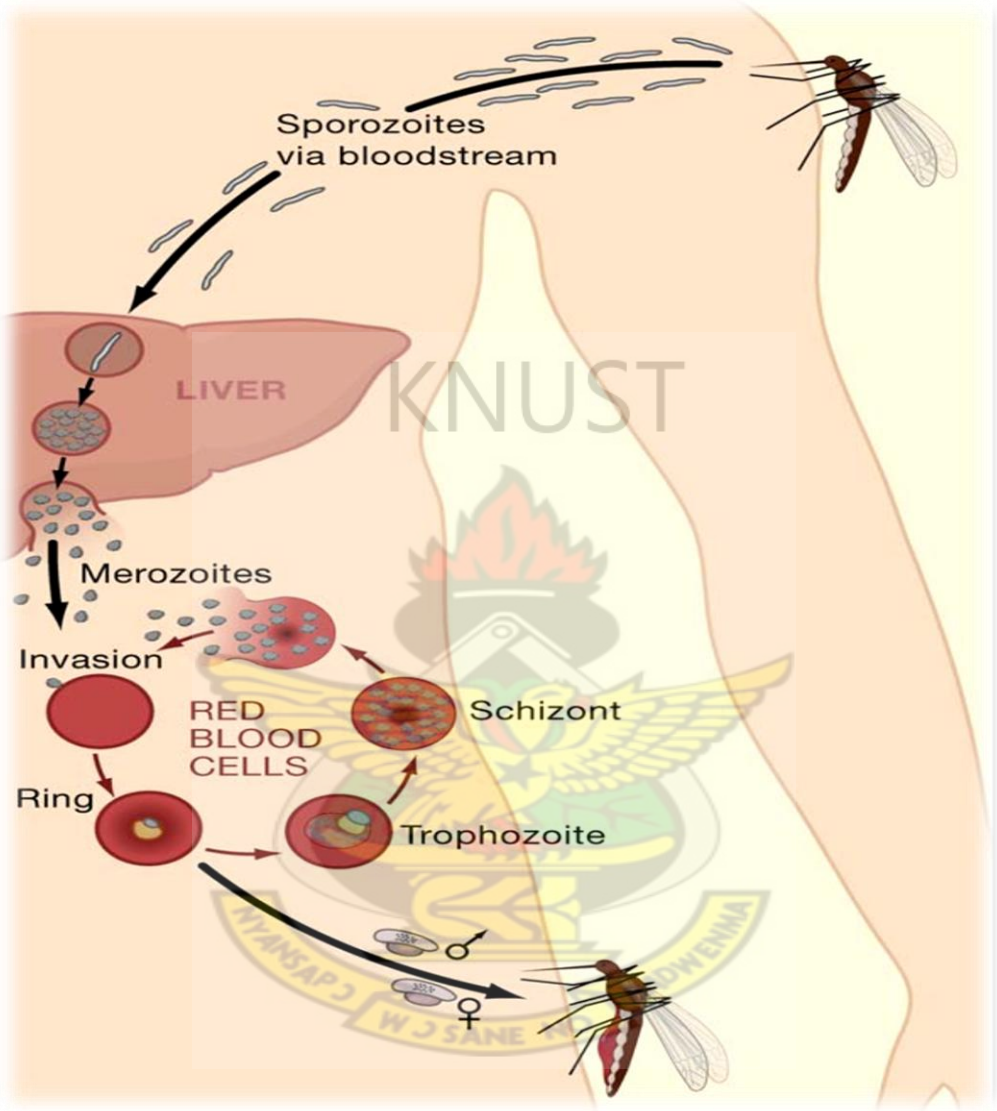

Plate 1.0: ***Plasmodium falciparum*** **cycle in man and mosquito** (Cowman and Crabb, 2006).

The zygote matures into an invasive ookinete that traverse the gut epithelium and attaches to the outer layer of the gut wall. The ookinete differentiates to an oocyst in the hemolymph; schizogony then takes place generating thousands of haploid sporozoites. The

mature oocyst rip apart, and the sporozoites travel actively to the *Anopheles*' salivary glands. The sporozoites break through the glands and rest in the conduit bearing saliva, until it finally have access to a vertebrate host.

2.1.4 Plasmodium species and geographic distribution

There are species of plasmodia that usually infect humans: *P. falciparum, P. vivax, P. malariae,* and *P. ovale*. However, the utmost impact on human health with regards to mortality is by *P. falciparum*.

P. falciparum is well known to cause severe infections more frequently than other *Plasmodium* species because *P. falciparum* exhibits a number of unique characteristics that favor increased disease severity, including high multiplication rates in erythrocytes and reticulocytes, strong cytoadherence to infected erythrocytes (Schofield, 2007) and toxin-induced activation of inflammatory responses. On the other hand, *P. vivax* also represents a significant burden on public health all through the tropical and numerous subtropical or temperate latitudes. Even though less frequently fatal, this infection causes a severely incapacitating disease with recurrent and sometime several episodes of relapse. Moreover, current information indicates that there are cases of severe malaria as a result of *P. vivax* infection (Andrade *et al.* 2010) which displays patterns of inflammation and immunopathology similar to those seen in severe *falciparum* malaria cases. These findings suggest that different *Plasmodium* species can trigger strikingly similar host responses that can result in severe disease with significant risk of severe disease and death caused by the same spectrum of syndromes characteristically associated with *P. falciparum* malaria (Barcus *et al.,* ; 2007). *P. malariae* is also found all through the tropics, but it is likely to show up more in isolated pockets and at comparatively low frequency as *P. falciparum* or *P. vivax*. *P. malariae* resistant to chloroquine has been observed from southern Sumatra, Indonesia

(Maguire *et al.*, 2002). Microscopically confirmed *P. ovale* is is comparatively common in West Africa in relation to eastern Indonesia and New Guinea and the Philippines where it can be described as rare. *Plasmodium vivax* is uncommon in sub Saharan Africa, but common in South Asia and Central America, and is predominant in South America. *Plasmodium ovale* is found mainly in tropical Africa, in West and South Africa, with sporadic reports from other continents, e.g. the South Pacific islands. *Plasmodium malariae* is the least common species of malaria to infect humans, and is infrequent all over the world (Hombhange, 1998). *P. knowlesi;* There is convincing data that an additonal species may in fact regularly infect humans. Cox-Singh and others (2008) confirmed that *P. knowlesi*, which in nature infects macaques in southeastern Asia, was regularly infecting humans living within the vicinity of the macaques, and it was responsible for acute illness and a number of deaths (Cox-Singh *et al*., 2008; Cox-Singh and B. Singh, 2008).

2.2 Epidemiology of Malaria

The global incidence of malaria has reduced by 17% since 2000 and malaria-specific mortality rates by 26%. These rates of decline are lower than internationally agreed targets but nonetheless, represent a major achievement. Current estimates suggest 216 million episodes of malaria occurred in 2010, of which 174 million cases (81%), occurred in the African Region. There were an estimated 655 000 malaria deaths in 2010, of which 91% were in Africa. Approximately 86% of malaria deaths globally were of children under 5 years of age. In 43 out of the 99 countries with ongoing transmission, reductions in reported malaria cases of more than 50% have been recorded between 2000 and 2010, while downward trends of 25%–50% were seen in 8 other countries (WHO, 2011).

2.2.1 Epidemiology and limits of malaria transmission

The epidemiology of malaria is mainly controlled by environmental factors that regulate the vector population of the anopheles mosquitoes. The 1950 WHO expert committee on malaria in Kampala endorsed a classification based on the prevalence of palpable splenomegaly (WHO, 1950). However splenomegaly can result from other chronic parasitic infections, which are highly prevalent in malaria endemic areas. Because of this, the spleen rate has a low specificity in some malaria endemic areas. Metselaar and van Thiel (1959) defined four classes of endemicity using the parasite rates (PR). It is no surprise that there are statistical constraints to measuring PR reliably in low endemic areas and that it is often replaced with measurements of the incidence of malaria from passive or active case detection data to estimate levels of risk (Molineaux and Gramiccia 1988). Despite this, these data remain potentially useful medical intelligence for defining distribution limits simply as contemporary manifestations of local perceived risk (expert opinion). This estimation assumes that the shape of the spleen-rate versus age curve is determined by the frequency of infections to which populations are exposed and by the degree of immunity developed by these populations in response to that number of infections. This approach also assumes that the response of the spleen-rate is the same everywhere. The four classifications based on the parasite rates are as follows: Hypoendemic; if the PR in the age group 2-10 years is as a rule under 10%. It may be higher for part of the year. Areas where there is little transmission and the effects, during the average year, upon the general population, are unimportant. This can be regarded as unstable malaria. Mesoendemic; if the PR in the age group 2-10 years is as rule between 11-50%. It may be higher for part of the year. Typically found among rural communities in sub- tropical zones when wide geographic variations in transmission risk exist. This can also be regarded as unstable malaria in some cases although epidemics are less

severe than in hypoendemic areas. Hyperendemic; if the PR in the age group 2-10 years is constantly over 50% Areas where transmission is intense but seasonal and immunity is insufficient in all age groups. Holoendemic: If the PR is constantly over 75% in the 1-year age group. Perennial, intense transmission resulting in a considerable degree of immunity outside early childhood. Holendemic areas are the areas with the spleen-rate and APR are constantly over 75%. In the holoendemic areas, the absolute parasite rate approaches 100% early in the second year of life but subsequently declines, initially rapidly between the second and fifth years of life and gradually thereafter.

2.2.2 Stable and unstable malaria transmission

Malaria transmission may be broadly categorized as stable or unstable depending on the amount of variation present in malaria transmission over time. For stable malaria, the transmission is high without any marked fluctuation over the years though seasonal fluctuations from year-to-year and within the year do occur. The population in stable endemic areas shows high levels of immunity whatever the seasonal variations. However, in unstable malaria transmission, there is great variability in transmission over space and time and there are low levels of immunity in the populations involved. Areas with stable malaria are said to be endemic and those with unstable malaria as non-endemic areas. Non-endemic areas are prone to epidemics [http://www.Malariasite .com /malaria/definitions_of_malaria.htm]. The site was accessed February 2012.

2.2.3 The relationship between malaria transmission, age of population and distribution of disease

The age pattern, clinical spectrum and total burden of severe malarial morbidity vary considerably with transmission intensity. The level of transmission influences parasite prevalence and shifts the age at which parasitemia peaks. With increasing transmission, the peak parasitemia is attained at a younger age while lower transmission shifts parasite prevalence to an older age bracket (Molineaux and Gramiccia 1980). In malaria endemic areas, both parasitization and severity of infection show age-specific patterns, which reflect acquisition of immunity. In general, children develop clinical immunity over the first five years of life in which they show reduced disease symptoms while retaining high parasite burdens. Distinct differences are observed in the age profile of different manifestations of severe malaria. Studies in the Gambia and Kenya showed that severe anemia peaks at 22 to 27 months of life whereas cerebral malaria peaks later between 40 and 47 months (Marsh and Snow 1997; Bloland *et al.,* 1999). It is still not clear why severe anemia and cerebral malaria vary with age.

2.3 Anopheles Vectors of Human Malaria

Anopheles is the only genus of the family *Culicidae* known to be involved in the transmission of human malaria. There are about 430 known species (Harbach, 2004), 70 of which are known vectors of malaria and of which 40 are considered medically important (Service and Townson 2002). Various attempts to define the distribution of anopheline species have been conducted in Africa (Coetzee 2004; Lindsay and Snow 1998), America (Levine *et al.* 2004b; Rubio-Palis and Zimmerman 1997), Europe (Kuhn *et al.* 2002), and Australia (Sweeney *et al.* 2006) and at the global scale (Kiszewski *et al.* 2004). These are illustrated in the diagram in

Plate 1.2 (page 21). The mapping techniques used in these various studies have been very different, ranging from expert-opinion interpolations to more sophisticated statistical techniques and are, thus, not directly comparable and impossible to synthesize globally. *Anopheles gambiae, An. funestus* and *An. arabiensis* are termed as the most efficient afro tropical vectors in Ghana (Coluzzi M, 1993). The photo below Plate 1.1, shows a typical community or field based survey where all the household members are invited to participate in collection of finger prick blood.

Plate 1.1: Finger pricking during a community survey at Marani- Kenya

The photo above depicts community surveys in the Kenyan highlands and was taken by Kingsley Badu (unpublished photos).

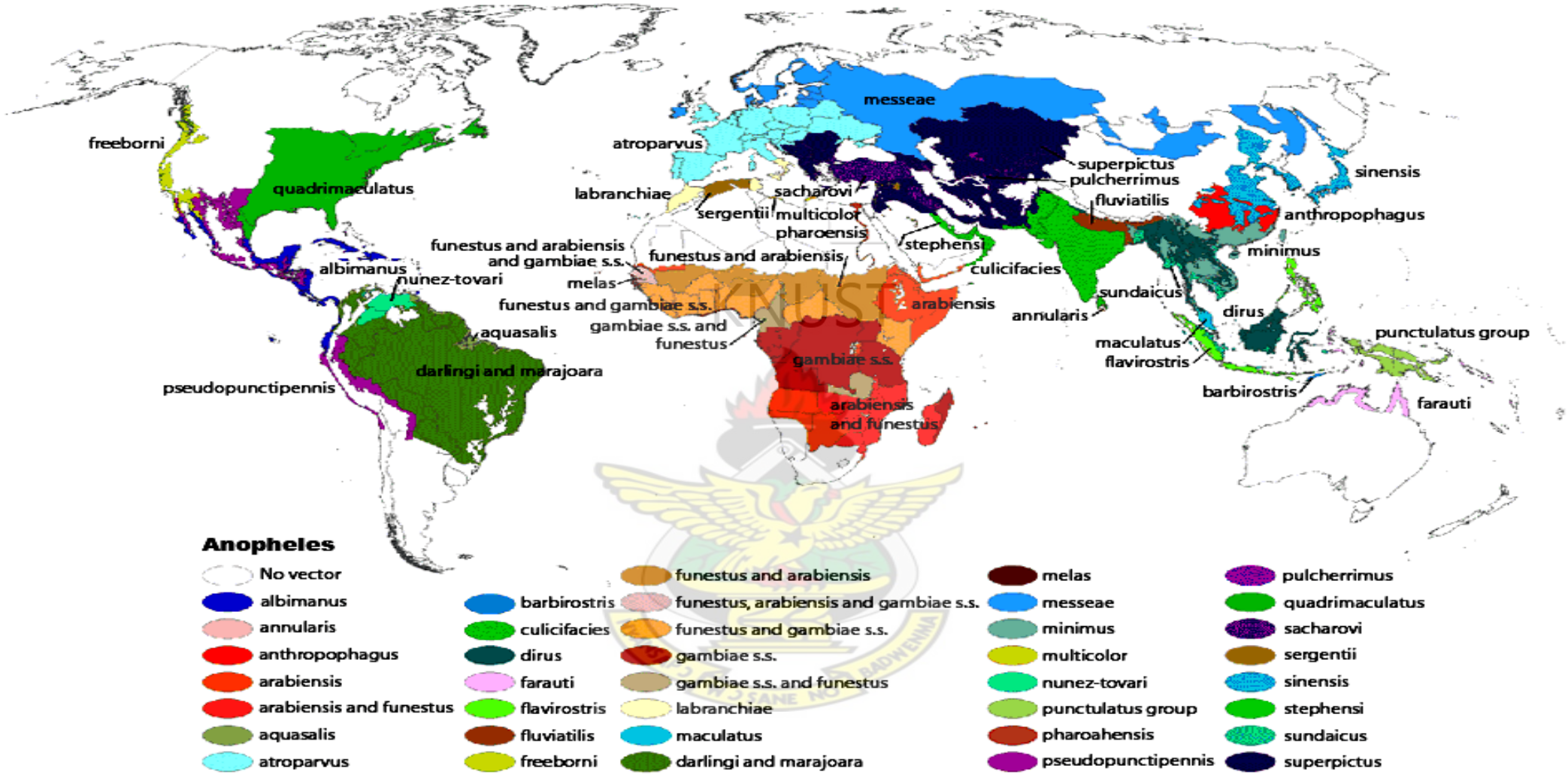

Plate 1.2: Global distribution of *Anopheles* species (Kiszewski *et al.*, 2004)

2.4 Transmission Estimation and Challenges

Entomologic studies have for many years provided the mainstay of transmission pressure measurements through a combination of sporozoite rates, human blood indices, and human-biting rates (Bailey, 1982). However, in areas of low transmission, sampling techniques become insensitive and errors in calculating transmission indices become large. The logistic requirements for intensive entomologic surveillance, reflecting seasonal patterns and marked geographic over-dispersion of vectors are considerable. A full description of the limitations of entomologic measures of *falciparum* challenge has been provided by Dye and Hasibeder (1986).

Entomological inoculation rates (EIR) are derived by multiplying the vector-biting rate with the proportion of mosquitoes infected with sporozoite-stage malaria parasites in order to give a good prediction of transmission level (Beier *et al.,* 1999). Transmission may also be represented by measures of malaria prevalence, incidences of severe disease and mortality.

The entomological inoculation rate lacks precision because mosquito distributions are markedly heterogeneous (Drakeley *et al.,* 2003; Mbogo *et al.,* 1995) and sporozoite compounded in areas of low transmission by low absolute numbers of mosquitoes and infection rates are low (below 5%) even in the most highly endemic areas. Also for practical and ethical reasons, mosquito trapping typically uses adult volunteers, but extrapolation to biting rates and incidence of infections in children has limitations (Smith *et al.,* 2004).

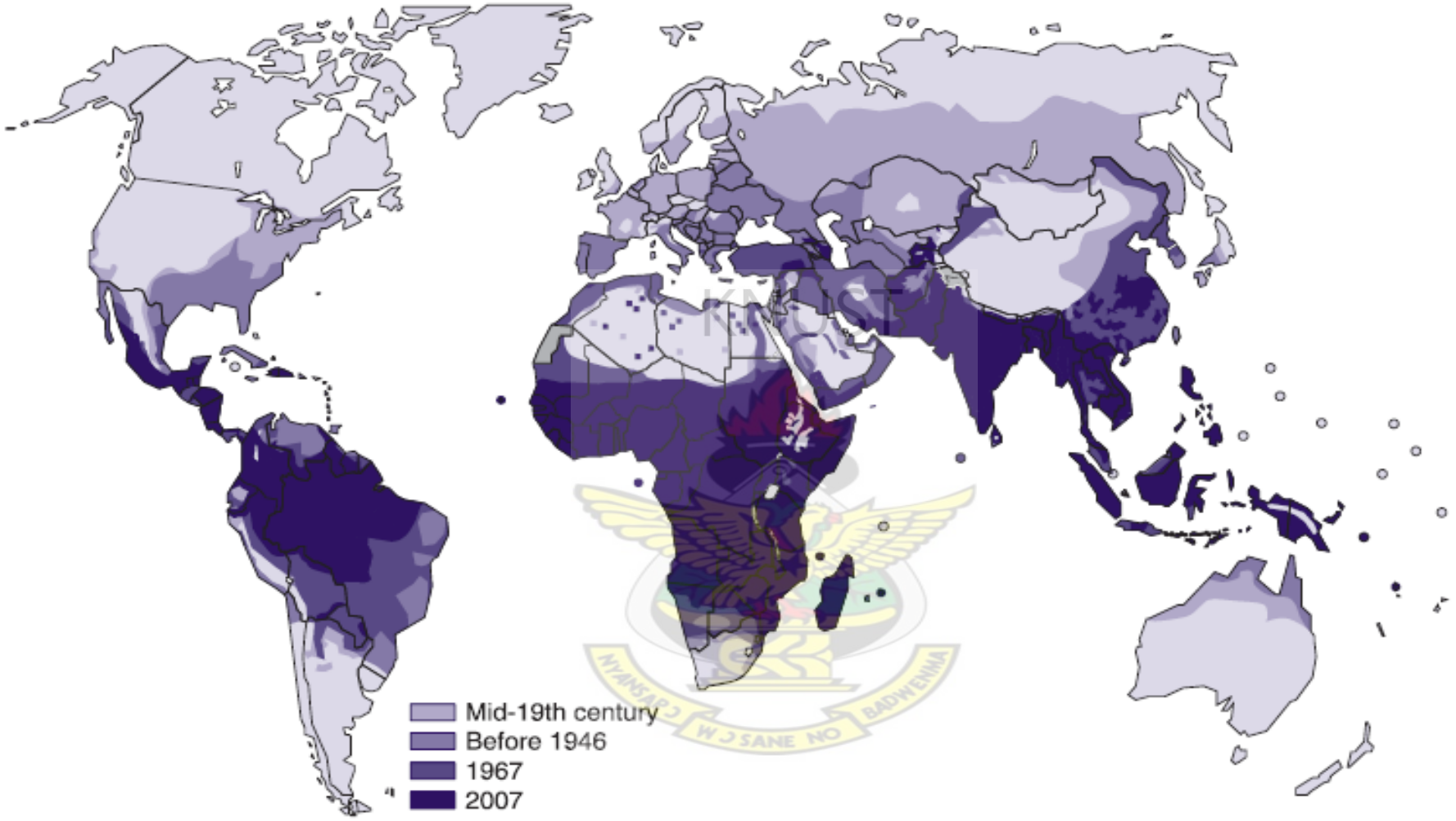

Plate 1.3: Malaria risk areas of the world from mid-19th century to the present, Adapted from WHO, 2009 Control to Elimination (Mendis *et al.*, 2009).

The goal of malaria control is reducing the disease burden to a level at which it is no longer a public health problem. Malaria elimination is interrupting local mosquito-borne malaria transmission in a defined geographical area, i.e. zero incidences of locally contracted cases. Malaria eradication is the permanent reduction to zero of the worldwide incidence of malaria infection caused by a specific agent; i.e. applies to a particular malaria parasite species. The diagram above Plate 1.3 depicts malaria risk areas of the world from the mid-19th century to the present (Mendis *et al.,* 2009).

2.5 Malaria Immunology: Immune Response to Malaria Infection

2.5.1 Innate immune response to malaria

The sporozoite and liver stage represents the first encounter of the host with the parasite, while during the erythrocytic stage; the cyclic rupture of infected erythrocytes produces the clinical symptoms of malaria. The parasite induces a specific immune response, stimulating the release of cytokines from human peripheral blood mononuclear cells (PBMC), (Doolan *et al.,* 1994) which play an important function in activating the host's monocytes, (Esparza *et al.,* 1987) neutrophils, (Djeu *et al.,* 1990) T cells, (Yokota *et al.,* 1988) and natural killer (NK) cells (Ostensen *et al.,* 1987) to react to the subsequent liver and the blood stage parasite. Immunity against the malaria parasite is complex and stage-specific (Holder *et al.,* 1999). Several antigens, specific to the liver stage, have been identified, and it has been suggested that these antigens, along with those brought in with the invading sporozoite, are rapidly processed by the host cell and present on the surface of infected hepatocytes in combination with MHC class I (Weiss *et al.,* 1990). This presentation leads to recognition by cytotoxic T lymphocytes (CTLs) and killing of the infected cell, or stimulation of NK and CD4+ T cells to produce interferon, which can trigger a cascade of immune reactions and can

lead ultimately, to the death of intracellular parasite (Weiss *et al.*, 1990, Wang *et al.*, 1996) Hence, the *Plasmodium* parasite developing within the host hepatocyte is the major target of protective immunity at the extraerythrocytic stage (Kwiatkowski,1995) The CTLs may be directly cytolytic against malaria-infected hepatocytes by releasing perforin and granzyme or by binding to apoptosis-inducing receptors on the infected cells (Kwiatkowski,1995) The merozoite enters the red blood cell by receptor-linked endocytosis. At the time of erythrocyte rupture, parasite antigens are released into the bloodstream, stimulating the release of tumour necrosis factor and other factors (Snounou *et al.*, 2000) Often, merozoites escape the immune reaction and infect other red blood cells, continuing the cycle of the infection and stimulation of the immune system (Riley *et al.*, 1992) Merozoites that survive to the pre-erythocytic stage are responsible for the modification of infected red blood cells in terms of parasite proteins expressed on the cell surface and the concomitant immune response to the *Plasmodium* parasite, resulting in the clinical manifestations of malaria (Riley *et al* 1992 ;Miller *et al.*, 1998). An antibody binding to the surface of the merozoite, and to proteins that are externalized from the apical complex of organelles involved in erythrocyte recognition and invasion, seems to have an important role in immunity to asexual blood stages. This antibody could neutralize parasites, or lead to Fc dependent mechanisms of parasite killing by macrophages (Saul, 1999).

2.5.2 Adaptive immune response to malaria

Both the cell-mediated and the humoral responses play a role in the adaptive immune responses in malaria. In general, cell-mediated immune responses in malaria infection include macrophage activation by NK cells, γδ - T cells or Th1 derived IFNγ for enhanced phagocytosis and killing of parasitized red cells (Fritsche *et al.*, 2001); it also includes the

hindering of parasite growth and development inside hepatocytes by CD8+ cytotoxic T cells and IFNγ-producing T cells (Tsuji and Zavala 2003). Nitric oxides produced by macrophages in response to parasite components and T-cell IFNγ production can also have anti-parasitic effects (Brunet., 2001). The humoral immune responses are effected by antibodies: malaria-specific antibodies mediate a number of anti-parasitic effectors functions including inhibition of cytoadherence; inhibition of red blood cell invasion (Guevara *et al.,* 1997): and antibody dependent cytotoxicity and cellular inhibition (Bouharoun-Tayoun *et al.,* 1995), reviewed in (Artavanis-Tsakonas *et al.,* 2003). Whereas the cell-mediated responses largely dominate the pre-erythrocytic stage of the parasite, the humoral responses dominate the erythrocytic stages.

2.5.3 Persistence of anti-malarial antibodies

The decay rate of antibodies developed as a result of malaria infection has been a bone of contention for some time, though this seems to be largely a result of differences in interpretation between protective immunity and exposure related immunity. These alternative interpretations have been well illustrated by Drakely and Cook (2009). They explain that rapid decline in antibody levels after infection is understood as a loss of protective immunity, whereas exposure is reflected in the longer slower loss of lower antibody levels.

As markers of exposure, antibodies have been shown to persist for several years without re-infection in immigrants to Europe (Bouchaud *et al.,* 2005; Bruce-Chwatt *et al.,* 1972). Antibodies have also been shown to appear very rapidly in individuals re-exposed to malaria during epidemics or naïve transient travelers to malaria endemic areas (Migot *et al.,* 1995, Orlandi Pradines *et al.,* 2007), also in populations from which malaria had been eliminated not too long ago (Kaneko *et al.,* 2000). These data suggest that, a memory antibody response exists in adults that can persist for many years. However, there is evidence that antibody

responses are not as fixed in children as they are in adults, particularly in areas of seasonal malaria (Achtman *et al.,* 2005; Akpogheneta *et al.,* 2008).

2.6 Immuno-epidemiology of Malaria

Immuno-epidemiology is an inter disciplinary applied science that incorporates immunology, parasitology, genetics, epidemiology, ecology, mathematical modeling as well as statistics (Woolhouse *et al.,* 1992) combines individual- and population-oriented approaches to create new perspectives and examines how inter-individual differences in immune responses affect the population dynamics of micro- and macro-parasites to produce the epidemiological patterns of infection observed in heterogeneous host populations (Hellriegel 2001). The term 'Immuno-epidemiology' had traditionally been used in the context of helminths infections but was first associated with malaria by Desowitz in 1966 (Desowitz 1966) and has since been used historically in malaria epidemiology (reviewed in Hellriegel 2001). With a view to surveillance, Immunoepidemiology investigates the influence of population immunity on epidemiological patterns (Zetkin and Schaldach 1992). Monitoring the immune status of a population at appropriate intervals allows estimation of the social, medical and economic importance of infectious diseases, the planning and evaluation of intervention programs. Patterns emerge of innate, natural and artificially acquired immune responses in a population that facilitate predicting the spread of infections. They reveal how immunological differences between individual hosts affect the population biology of the disease agents rather than the incidence of disease itself, and, in genetic terms, how the distribution of different types of immune responses in a host population influences the spread of different serotypes or genotypes in the parasite.

Linking immunological mechanisms to epidemiological patterns takes into account the interrelationship between individual and population levels, and opens up new

perspectives. Translating individual traits to the population level has been attempted in other fields (Sutherland, 1996). The challenge lies in finding individual characteristics that are also relevant at the population level, that is, immunological markers that best indicate different degrees of parasite-specific protection of individuals and that simultaneously have a large quantitative effect on population processes such as parasite transmission. Individual to population clinical surveys and epidemiological field studies often collect cross-sectional data usually consisting of demography, parasitological and serological data in order to address question in immuno-epidemiology.

Serological surveys has been used as the main tool in malaria epidemiology Desowitz (1966) investigated the application of the indirect haemagglutination test in studies on the immuno-epidemiology of human malaria and the immune response in experimental malaria. Suzuki (1991), in a report 'Malaria immuno-epidemiology: a trial to link field study with basic science' where malaria serological assessment was carried out in endemic areas in Haiti, Indonesia, Sudan and in Brazil Amazon, concluded that serological survey was useful in finding latent foci in a controlled area, for the assessment of past epidemics. Kagan *et al.* (1969) conducted series of studies on the serology of malaria by evaluation of the indirect haem-agglutination (IHA) test as an epidemiologic tool.

2.6.1 Seroepidemiology of malaria

The methodology applied in the study of seroepidemiology has evolved over several decades ago, beginning from the very primitive with non-specific antigens that capture broad responses of malaria exposures to current single antigens that measure strain specific exposures.

2.6.2 Evolution of methodologies for measuring antimalarial antibodies

Serological surveys for detecting and estimating malaria transmission are often, by necessity, very large. Therefore in the context of seroepidemiology an ideal tool for measuring anti-malaria antibodies must meet some specific criteria. Over the years there has been an evolution of methodologies with different methodologies meeting some or most of the criteria. The test used must be high throughput that is to say, it must be capable of assaying many samples at once; it must be simple, rapid, and easily interpretable and reproducible so that results in the field can be analyzed quickly. Low cost, to enable widespread standardized use of the assay in the areas where it is needed would be an additional advantage (Drakeley and Cook 2009).

2.6.3 Complement fixation test (CFT)

One of the earliest tests shown to successfully detect malarial antibodies was the CFT (Thomson, 1918). The test is based on competition for complement between the antigen–antibody complex of interest and an indicator system of antigen–antibody complexes (typically sheep red blood cells and corresponding rabbit antibodies) and complement. Complement is used up in the presence of antigen–antibody complexes, and if there is a slight deficiency in complement, the lysis of the red blood cells in the indicator system is reduced. The reduction can be measured photospectronically and will be greater when the quantities of anti-malarial antibody in the serum are greater. This method was widely used primarily as an alternative for the diagnosis of malaria, but fell away with the evolution of the haem-agglutination test, which required much smaller amounts of antigen and was shown to be more sensitive (Wilson *et al*., 1975).

2.6.4 Indirect haemagglutination assay (IHA)

The IHA became a common method of detecting malarial antibodies in the 1960s. It relies on cross-linking between antibodies when they bind antigen, which eventually results in clumping of blood cells. The resulting haemagglutination is measured by eye. The sera are tested by serial dilution with the highest dilution exhibiting agglutination recorded as the titre of antibody present in the serum. Because haemagglutination is measured by the eye, the results become subjective and difficult to standardize with associated reproducibility issues (Lobel *et al.*, 1973). This limits cross comparison of data from different research groups. However, the result remains. However, this simple method has the advantage of being adaptable to micro-titre plates meaning that multiple samples can be tested simultaneously.

2.6.5 Immunofluorescence antibody test (IFAT)

From the 1960s onwards the IFAT became the technique of choice. The technique involves incubating the sera of interest on a glass slide on which the antigen of interest, typically whole parasitized red blood cells, has been fixed. A secondary antibody coupled with a fluorescent compound is then used to detect any bound antibodies from the serum. The fluorescence is examined using a specialized microscope. The key advantage of this method is the relative ease of making IFAT slides, as whole parasites are easier to access and prepare than the extracts of soluble antigens required for the previous techniques. Additionally, the antigenic properties of whole parasites are less variable between batches than those of soluble parasite antigens (Ambroise-Thomas, 1976). Initially, simian malarias were widely used as antigen for the assay with *Plasmodium fieldi* being popular (Voller and Draper, 1982) despite its limited sensitivity for detecting human infections (Draper *et al.*, 1972). *P. vivax* and *P.*

falciparum were originally isolated from infected individuals; though the latter was quickly replaced as *P. falciparum* culture became available as a stable source of antigen. IFAT remained in favour for many years and is still widely used in certain areas (Domarle *et al.*, 2006; Zheng *et al.*, 2008). The main advantage of this method is its higher sensitivity and ability to detect antibodies at a lower concentration than IHA (Draper *et al*., 1972). However, there are various drawbacks to using IFAT. Similarly to the IHA, the test is based on visual examination, thus it is subjective and difficult to standardize. Additionally it is a time-consuming process.

2.6.6 Enzyme-linked immuno-sorbent assay (ELISA)

The ELISA is now widely used for antibody detection to a variety of antigens in laboratories worldwide. The process is similar to the IFAT except that rather than using microscope slides, antigens (most often a single recombinant protein) are coated on to high-binding micro-titer plates. The serum of interest is incubated in the plate following blocking of non-specific sites. Bound antibodies are then detected with a secondary antibody that is linked with an enzyme. The final step involves the addition of an enzymatic substrate that is then converted if there is bound enzyme present in the well, resulting in color change or fluorescence measurable by a spectrophotometer. This process leads to a high throughput, standardizable assay that is relatively cheap and easy to perform, and that generates objective results. One of the first ELISAs used for detecting malarial antibodies was performed by Voller *et al.* in 1975 using antigen obtained from *P. knowlesi*. The ELISA was able to detect antibody in both *P. falciparum* and *P. vivax* parasite-positive individuals. The commercial availability of ELISA components and recombinant proteins enables findings to be compared from different laboratories with robust results (Esposito *et al.*, 1990).

2.7 Application of Seroepidemiological Tools

In an epidemiological context, serological data have been used to assess malaria transmission intensity, to identify areas of focal transmission, to monitor control interventions and to confirm eradication.

2.7.1 Malaria endemicity and risk

Serological measures are a useful alternate measure of transmission intensity in areas of low transmission, where parasite prevalence and EIR are not reliable; they offer a way of accurately assessing endemicity and identifying focal areas of transmission. Different levels of transmission are reflected in the resultant age–seroprevalence curves. In an area of low transmission, development of antibody will be slow and may only be exhibited in adults. But, in areas of heavy transmission, much of the population will be sero-positive, and only young children, who are less likely to have been exposed, will remain sero-negative.

In 1967, a series of sero-epidemiological studies were undertaken in Tanzania at the end of the 1960s evaluating the use of serology for monitoring malaria transmission in areas of different endemicity (Lelijveld, 1972). Surveys were conducted before and after the transmission season in areas classified as holo-endemic, meso-endemic and non-endemic, and antibody responses were measured using IFAT with *P. fieldi* as the antigen. Differences were observed between the mean titers in these seasonal surveys, though antibody prevalence generally remained similar over the year. Antibody prevalence were high in all endemic areas so the mean titer index was deemed more sensitive for establishing differences between transmission areas and changes in exposure over time. The authors concluded that serological data added important information in defining endemicity that other parameters collected (i.e. parasite data and spleen data) did not provide. Several other studies used serology to define malaria endemicity. Prior to the landmark Garki project, surveys were conducted in Kabba

Province, an area of high altitude with low rainfall, and Benue Province, a lowland area with high levels of rainfall, in Nigeria (Voller and Bruce-Chwatt, 1968). A total of 1,082 people were surveyed and antibodies to *P. cynomolgi bastianellii* were detected by IFAT. Parasite prevalence was high in both areas, although parasite densities were noticeably higher in Benue Province, where spleen rates were also higher. The serological data reflected these findings with children under 5 years of age in Benue Province having a similar mean titer as adults in Kabba Province. However, the authors concluded that these differences were due to the timing of the surveys, which took place at peak transmission in Benue Province and in the dry season in Kabba Province. A similar study in Ethiopia in 1967 contrasted two populations in the highlands (Collins *et al.*, 1971). Antibodies to all four human malaria parasites were detected by IFAT in 1,141 people. The authors found 37% overall sero-prevalence in people living at altitudes less than 6,000 feet (1,829 m) but only 4% sero-prevalence in people living higher than 6,300 feet (1,920 m). Positive antibody responses at the higher altitudes appeared to be clustered in one area. The authors suggested that the reason for higher antibody prevalence in this area was due to the presence of a large market drawing people from more malarious areas, compounded by the large population of resident immigrants from endemic areas, and was not due to local transmission. In 1969 in South America, a large-scale evaluation of anti-malarial antibody responses was carried out by the group of Kagan *et al.* (1969), early forerunners of the malaria sero-epidemiology approach. They used blood samples from enrolment of army recruits and tested these for antibodies using IHA with *P. knowlesi* antigen. At the country level, the overall sero-prevalence followed a predictable pattern with prevalences highest in Columbia (range 15–49%), then Brazil (range 10–40%), followed by Argentina (0–30%) and the United States (0–15%). In the West African study, antibody levels to CSP were used to characterize transmission intensity and relate this to the levels of anti-malarial drug consumption (Gardella *et al.*, 2008).

2.7.2 Detecting epidemics and focal areas of transmission

Malaria transmission is notoriously heterogeneous and within single villages some households experience high levels of infection while others appear to be infection and exposure free. There are a variety of factors that influence this heterogeneity leading to foci of infection. Detecting these foci has long been recognized as vital in the struggle to eliminate malaria. Collins *et al.* (1968), sampled villages at different altitudes and at various distances from the Pahang River in Malaysia (Collins *et al.*, 1968). The villages also differed in their ecology, demographics and malaria prevention activities. Antibody prevalence varied widely, ranging from 3% in an area where a successful anti-malarial scheme had been in place to 42% in an area surrounding a health centre that may have influenced the high seroprevalence. However, in two areas there were no positive reactions in children under the age of 5, indicating a lack of recent local transmission. The epidemic in Panama was attributed to emerging resistance of the vector *An. albuminus* to DDT and *P. falciparum* to chloroquine, and was brought under control by prompt effective treatment of cases. In El Salvador, the cause of the epidemic was inferred to be an imported index case with local spread ultimately stopped by the intense dry season limiting vector densities to abrogate further transmission. Comparison of samples from children known to be infected (and sero-positive) in the epidemics and 6 months later showed a complete loss of antibody over this short period. This suggests rapid clearance of the infection with prompt effective treatment in individuals with no pre-existing immunity. Thus, accurate interpretation of serological data not only requires knowledge of the current epidemiological situation but knowledge of treatment history too.

2.7.3 Serological data to monitor malaria control and elimination

Individuals born after successful interruption of transmission will be seronegative (apart from declining maternal antibody in the first year of life), while their older counterparts will remain sero-positive for a time due to their previous exposure. This was elegantly shown in seroprevalence studies by Bruce-Chwatt and colleagues (1973; 1975) several years after malaria elimination campaigns in Mauritius and Greece. The effects of malaria control have also been shown in the shorter term by measuring changes in antibody titers (Cornille-Brogger *et al.,* 1978). The Garki study conducted in Nigeria in the early 1970s remains a landmark assessment of the effect of malaria control on a variety of malariometric, entomological and serological indicators (Molineaux, 1980). The serological survey covered one control village cluster and the two clusters receiving the most intense control approach: indoor residual spraying (IRS) and mass drug administration. There were approximately 1,000 people in the control cluster and 2,000 in the intervention clusters with samples taken at regular intervals over a 5-year period including baseline, intervention and post-intervention periods. Sera were tested for total IgG and IgM and for parasite-specific responses to *P. falciparum* by precipitin, IHA and IFAT and to *P. malariae* by IFAT. These rich results are described by Cornille-Brogger *et al.* (1978). All serological tests showed age-specific increases in both antibody prevalence and titer, consistent with exposure-acquired immunity. The prevalence of antibodies was 100% by the age of 5 years, much quicker than in another rural West African site in The Gambia (McGregor *et al.,* 1965) indicating a much higher rate of exposure in the Garki area. Antibody prevalence was not affected by the intervention but mean titer by age appeared to be sensitive to changes in transmission; both the initial reduction caused by the intervention and the gradual return of transmission to baseline levels after the control programme was stopped. These patterns were similar for each assay although

IFAT appeared to be the most sensitive showing the most marked difference between the protected and unprotected populations. The Pare-Taveta Malaria Scheme in Tanzania was an IRS-based malaria control programme that ran from 1956 to 1959 and caused a significant reduction in transmission (Draper and Smith, 1957; Draper *et al*., 1972). Parasite rates in children aged 2–4- years old dropped from more than 80% in 1955 to less than 5% in the follow-up period in 1959. Serological surveys were carried out some 11 years after the cessation of spraying. During this time there had been a gradual return to pre-intervention levels in the EIR (approximately 50 infective bites/person/ year (ib/p/yr)) though the parasite rate in 2–4- year- olds was, a comparatively low, 30%. In 1970, sera from a cross-sectional survey were tested by *P. falciparum* IFAT. Sero-prevalence with *P. falciparum* was 100% at 2–4- years- of- age. The *P. falciparum* titers showed a step pattern with a steep increase in mean titer from the age of 14 years. This, Draper *et al*. commented, was probably due to this age group still possessing immunity from the pre-intervention period and those below having been subjected to reduced levels of transmission (Draper *et al*., 1972).

2.7.4 Serological data to assess malaria eradication/elimination

The inherent longevity of antibody response makes it the optimal tool to account for the history of malaria exposure in a population. It was reasoned by Bruce-Chwatt and others that persons born after the start of successful elimination programs should be antibody negative and this was tested in a variety of different elimination set-ups. In Mauritius, (1968) was a classic study, the first to use serology to confirm malaria elimination (Bruce-Chwatt *et al.*, 1973). In 1949, an eradication scheme based on DDT spraying was established with a dramatic reduction in cases and parasite rates from 9.5% to 0.5% in just 2 years. However, transmission continued in focal areas. The last case of *P. falciparum* in the country was noted

in 1968. In 1972 Bruce-Chwatt and colleagues took nearly 6,000 blood samples from people living in the Black River district in the south-west of the island, where malaria had been the most persistent and antibodies were detected by IFAT using *P. falciparum* from Aotus monkeys. Younger children were over-sampled in an attempt to confirm elimination by the absence of antibodies in this age group. The low proportion of sero-positive individuals under 20 years of age reflected that these people had been born since initiation of intensive malaria control (post-1949). Sero-positivity in individuals over 35 years of age was much higher as this age group would have experienced high malaria transmission prior to 1949. In this study seroepidemiology was able to gauge past transmission patterns from a single survey and at the same time confirm the virtual absence of recent transmission.

Van der Kaay (1976) used seroepidemiology to study malaria elimination in Surinam. Surinam was neatly divided into three ecological zones: coastal, savannah and jungle, which were in different stages of the malaria elimination process of maintenance, control and attack, respectively. Historically, the coastal areas had always had lower transmission and the jungle interior had the highest malaria morbidity and mortality. The principal reason for this appears to have been the difference in transmission capacity of the vectors with *An. darlingii* inland and *An. aquasalis* on the coast. The issue was further compounded by the higher prevalence of *P. falciparum* in the interior. An elimination programme was started in 1957 based on combinations of IRS, larviciding and medicated salt. By the early 1970s, malaria was all but absent in the coastal belt, had very low prevalence in the savannah belt but remained a major problem in the jungle interior where drug resistance, incomplete IRS and lack of co-operation of villages led to large epidemics in 1972. Surveys were conducted in five areas in 1973 and 1974 to describe the epidemiology in the coastal and savannah areas and assess the effect of targeted control in the high transmission jungle area. IHA was used to measure antibody

prevalence and titer. It was clear from the serological profiles that there was no evidence of recent transmission in the coastal area. Similarly, in the savanna area while seropositives were observed, these were very low titers indicative of no recent transmission and suggesting that recent epidemics had not affected this area. In both these cases, the serological data were an extremely useful adjunct to parasitological data for assigning a phase of elimination. The sero-prevalence profiles also neatly describe the higher transmission areas and show the potential of the serological approach to assess the effect of interventions. This was most apparent when examining the effect of control on the titer of antibody response rather than the prevalence.

2.7.5 The role of serological markers in monitoring malaria transmission

The goal of malaria control is to reduce malaria disease burden to a level at which, it is no longer considered a public health problem. Monitoring and tracking reducing malaria transmission, and evaluating the impact of control strategies, in order to respond to the changing transmission levels, requires reliable predictions of malaria transmission intensity (Deloron and Chougnet 1992). Immuno-epidemiological markers have been recommended as surrogates for malaria transmission dynamics; several investigators have proposed the estimation of the force of infection from parasite in serologic surveys (MacDonald G, 1950; Pull and Grab 1974; Snow *et al.,* 1996). It has long been suggested that anti-malarial antibody prevalence could provide a more accurate estimate of transmission intensity than traditional measures such as parasite prevalence or entomological inoculation rates (Pull and Grab 1974, Snow *et. al.*, 1996).

Theoretically immunological markers present some advantages over parasite prevalence as a means of determining malaria endemicity; this is because antibodies can persist for far longer

duration than any discrete infection, thereby removing the effects of temporal or unstable malaria transmission. As matter of factly, Drakeley *et al.* (2005) reported that seroprevalence rates corresponded to the series of collective malaria exposure over time and was able to, together with parasite prevalence data; deduce changes in transmission intensity over the period. Again age-controlled seroconversion rates have been applied in determining the force of infection (Snow *et. al.,* 1996), where the annual seroconversion of specific antigens have been shown to have a tight correlation with the entomological inoculation rates (Drakeley *et. al.,* 2006; Corran *et. al.,* 2007; Stewart *et. al.,* 2009). This piece of evidence indicated the possibility that serological markers may offer a quicker means of estimating malaria transmission intensity. A number of studies have explored the use of serological markers to evaluate malaria transmission intensity in low transmission settings where parasite and vector indices are no longer sensitive. Additionally immuno-epidemiological markers have been used to identify residual foci of transmission and show an interruption in malaria transmission.

Wilson *et al.,* (2007), investigated the validity of using *Plasmodium falciparum* immunoglobulin 3 (*Pfs*-IgG3) levels in participants as a tool to assess comparative exposure to malaria on a microgeographical scale. The study found that, controlling participants IgG3 levels for age provided spatial patterns that reflected the micro-geographical variations observed for prevalence of parasitemia, and thus, the authors concluded that age-adjusted Pfs-IgG3 levels of participants (school-aged children) were stable, and together with GIS mapping can offer a sensitive tool adequate for the detection of micro-geographical variations in malaria exposure. Stewart *et al.,* (2009) described a rapid way of providing local estimates of malaria transmission intensity based on health centre-based serological survey. The authors measured detectable antibody levels to MSP-1_{19} and AMA-1 specific antigens and using a reversible catalytic conversion model, generated seroconversion rates (SCR) based on

age-specific antibody prevalence. The authors reported that analysis of SCR allowed for the detection of a recent decline transmission intensity which agreed with cross-sectional data collected earlier in the identical communities. The approach thus was sensitive and robust to verify the variabiligy in transmission in a rather limited geographical area and allowed the quantification of the drop in malaria transmission in the region. In north-eastern Tanzania, Bousema *et al.,* (2010a) investigated parasite prevalence rates and age-specific SCRs against *P. falciparum specific* antigens and was able to identify spatial patterns in malaria transmission in the area based on the differences in the SCR estimates. The group thus concluded that the SCR estimates were robustly linked with the local malaria incidence and thus predicted hot spots of malaria transmission with 85% and 95% specificity and sensitivity in respective order. In Somalia, Bousema *et al.* (2010b) in malaria prevalence surveys, found no parasitemia in the population both in season (0/1,128) and out of season (0/1,178) when using microscopy or rapid diagnostic tests for parasite detection. Contrary, the evaluation of immuno-epidemiological markers MSP-1_{19} and AMA-1 seroprevalence data revealed increasing sero-reactivity with age and confirmed variation in malaria exposure within and between study communities. This piece of evidence proved that immuno-epidemiological tools can be applied to elucidate variability in malaria transmission intensity at levels below the detection threshold of conventional malariometric index.

The archipelago of Vanuatu is an area where malaria transmission has been declining variably over the past few decades. Cook *et al.* (2010), applied immuno-epidemiological tools to track the heterogeneity of the malaria transmission in the area. The authors assessed exposure to *P. falciparum* MSP1-$_{19}$ and AMA1 as well as *P. vivax* MSP1-$_{19}$ and AMA1. Using the reversible catalytic model examine age specific SCR, the authors did not only

detect differential dominance of the different plasmodial species but was also able to decipher a change in transmission that happened 30yrs ago.

2.7.6 Modeling force of infection and seroconversion rates

Historically catalytic conversion models have been used widely to estimate force of infection from age-exposure profiles; however it was first used in the context of malaria by MacDonald (1950). Constant risk catalytic conversion model: $X(a) = 1- \varepsilon^{-ha}$ where X(a) denotes the proportion of individuals of age (a) that have been exposed to the parasite, h is the conversion from seropositive to seronegative. That is to say the recovery rate from parasitemia and loss of 1gM antibody and **ε** is the exponenential symbol.

There were two main problems with using this relatively simple technique for the measurement of force of infection of *P. falciparum* in most endemic areas. First, cumulative exposure to *P. falciparum* increases sharply with age, so that a very large proportion of children will have been exposed by the age of one year. A second major problem concerns the choice of a suitable marker of exposure. Strictly, the proportion positive for a given marker is determined by the balance between the force of infection and the rate of reversion to the negative state. Therefore, a constant risk catalytic conversion model can be used to estimate the force of infection from an increase in parasite prevalence with age only by assuming that recovery rates from infection within this period are negligible. Thus, Snow *et al* 1996, Pull and Grab (1974) and Draper and others (1972) all focused on the narrow window of age (infants) in which cumulative exposure increases from zero to very high levels, this effectively avoids the complication of any age-dependent effects such as differential biting by vectors. The authors calculated h using data on parasite prevalence and seroprevalence, on the basis that all infants begin life without parasites and without

antibodies to *P. falciparum* and assumed that recovery rate from parasitemia and loss of *P. falciparum* antibody is negligible or uncommon over the period of their studies.

2.8 Current Perspectives

Currently this model has been improved substantially by Drakeley *et al.* (2005) and Corran *et al.* (2007), they proposed a seroreversion rates and reported an immunological marker that is very sensitive and robust in its ability to differentiate between malaria exposures at different altitudes.

Malaria infection induces rapid and long-lasting antibody responses to whole parasite antigen, as is evident from the prevalence and persistence of antibodies after malaria outbreaks (Collins *et al.*, 1968). However, for single antigens, humoral responses accumulate more slowly (Corran *et al.*, 2007). The simplest interpretation of such distributions proposed by Corran and others (2007) is that, in a given interval of time, all individuals have an equal probability of sero-converting at a constant rate denoted by λ year^{-1} this is a function of the immunogenicity of the antigen and the likelihood of being infected. However, because the proportion of seropositives (P) at equilibrium is less than one for most antigens, either a proportion of the population never become antibody positive or seropositive individuals revert to seronegativity (at a rate ρ year $^{-1}$). In the latter case, P is determined by the relative values of ρ and λ, and these parameters can be fitted by calculating the expected proportions of seropositives at any age using standard maximum likelihood methods based on a binomial error distribution (Riley *et al.*, 1996). *Pt* is the proportion of the population aged at t

$$P_t = \frac{\lambda}{\lambda + \rho}(1 - \exp(-(\lambda + \rho)t)$$

Drakeley *et al.* (2005) fitted an age dependent model to seroprevalence data and proposed that, the simplest model to detect changes in age-linked seroprevalence will be a reversible catalytic conversion model in which the rate of becoming seropositive was variable annually between villages but with a fixed (constant) annual rate of seroconversion.

So they fitted the annual seroconversion rate, λ and the annual rate of reversion to seronegativity, ρ, were estimated by fitting a simple model of the acquisition and loss of antibodies to the age-specific prevalence of the antibodies using maximum likelihood methods assuming a binomial distribution. The equivalent annual entomological inoculation rate (EIR) was then estimated using a calibration curve derived from previously determined values (Corran *et al.,* 2007). Lambda SCR values can be converted to EIR equivalents using a log log regression equation as was reported by Drakeley *et al.* (2005) and Corran *et al.* (2007). The authors based this on data (SCR and EIR) collected from areas of different transmission intensity in Tanzania. Estimates of malaria transmission derived from fitting age-specific malarial antibody prevalence data with a reversible catalytic model have been shown to give excellent correspondence to EIR estimates.

That malariometric and immunological markers give evidence on intensity of malaria exposure over diverse period of time. Detectable antibodies developed against specific malaria antigens accrue in the population at different pace, which is dependent upon their immunogenicity, and corresponds to the force of exposure over a number of years. Thoughtful choice of specific malaria antigens for assessing transmission intensity is very crucial. Antibody responses of all age-groups under unstable transmission to a highly immunogenic antigen or responses in children to a less immunogenic antigen within populations under high transmission may permit the buildup of comprehensive profiles of malaria epidemiology covering extensive geographical area in a rapid and less expensive

way. The immuno-epidemiological approach provides the chance to interrogate variability in malaria endemicity over time as a relevant added constituent.

2.9 Biomarkers to *Anopheles* Salivary Gland Proteins

The malaria parasite is transmitted during a mosquito bite; as the as the mosquito is taking a blood meal, its saliva is injected into the host. The saliva contains several bioactive proteins that are immunogenic to vertebrate hosts. This results in the development of anti-saliva immune response from the host. Human responses to mosquito salivary proteins have been exploited as biomarkers for vector exposure.

2.9.1 Anti-saliva IgG responses as exposure markers to haematophagous arthropod bites

The lack of immune responders to mosquitoe salivary proteins from the sera of children (Reunala *et al.,* 1994) or horses (Wilson *et al.,* 2001) living in Iceland (a country free from insect bites) and with the subsequent immune response after exposure to blood feeding insects (Brummer-Korvenkontio *et al.,* 1994; Peng *et al.,* 1986; Abdel-Naser *et al.,* 2006) are strong evidence indicating that the gaining of an immune response to haematophagous arthropod salivary proteins is exposure driven. The saliva of blood-sucking arthropods is made up of a composite mix of biologically active proteins which may alter hemostatic mechanisms and elicit both cellular and specific humoral response (Billingsley *et al.,* 2006, Ribeiro 1995). Salivary gland extract from mosquitoes was able to elicit immune response in persons living in endemic areas (Remoue *et al.,* 2007; 2006; Waitayakul *et. al.,* 2006) and in visitors briefly exposed to vectors bites. This suggested that salivary proteins could be utilized as immuno-epidemiological markers to assess exposure to mosquito bites. Similarly several studies based on immunological techniques have evaluated individual exposure to a

number of arthropod vector bites as epidemiological markers of vector exposure. To this end, the risk of exposure to vectors such as ticks (Schwartz *et al.*, 1990), *Triatoma* (Nascimento *et al.*, 2001), *Glossina* (Poinsignon *et al.*, 2008) and *Aedes* mosquitoes (Remoue *et al.*, 2007) have all been evaluated.

Schwartz *et al.* (1990) first observed the relationship between insect bites and the level of anti-saliva antibodies in the sera from outdoor workers in New Jersey where the workers had been exposed to the bites of *Ixodes damini* ticks during their forestry activities. Following the absence of tick exposure there was a significant decline in IgG anti-saliva levels. Further to this, a number of immunological analyses verified a correlation between the level of exposure to diverse blood-sucking arthropods and the magnitude of antibody responses to their saliva. Palosuo *et al.* (1997) conducted a kinetic analysis of immune response to *Aedes communis* saliva from the some volunteers from Finnish Lapland across seasonal exposure period and found that in-season exposure to mosquito bites elicited intense response than out-season responses toward salivary antigens.

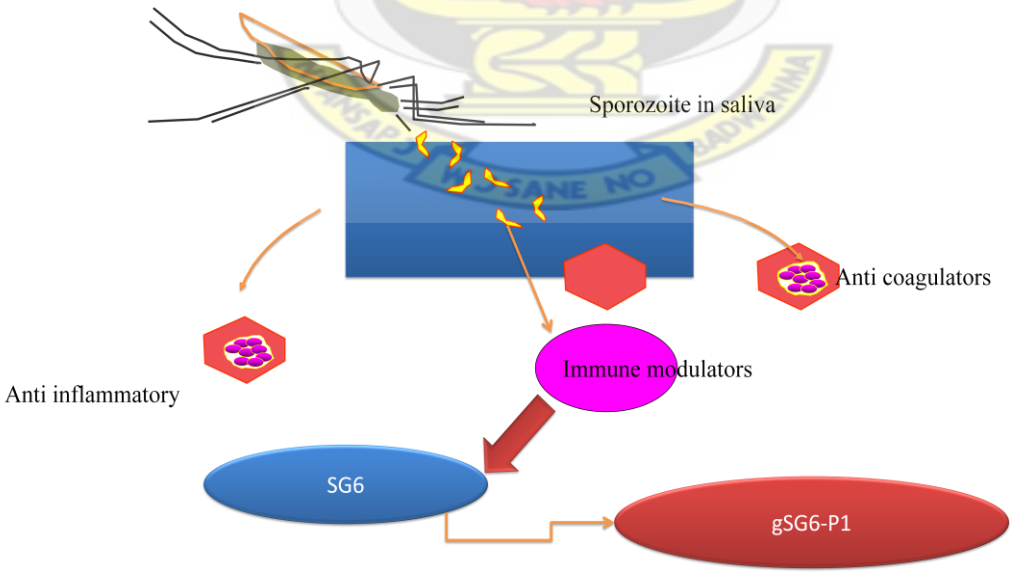

Plate 1.4: Schematic representations of salivary gland proteins being inoculated during mosquito blood feeding.

In the case of malaria, host immune responses against vector saliva can be used as a biomarker of exposure to *Anopheles*. Antibodies against *Anopheles gambiae* saliva have been described in young children from a region in Senegal with seasonal malaria transmission (Remoue *et al.,* 2006). Again, Orlandi-Pradines and colleagues (2007) assessed the effects of a transitory exposure to *An. gambiae* and *Ae. aegypti* in French travelers during a five-month visit to tropical Africa. Travelers transiently exposed to *An. gambiae* bites in endemic areas of Africa developed anti-saliva antibodies (Orlandi-Pradines *et al.,* 2007). In Thailand, antibodies developed against anti-*Anopheles dirus* salivary protein occurred mainly in patients with acute *P. falciparum* or *P. vivax* malaria, persons from non-endemic localities did not have such antibodies (Waitayakul *et al.,* 2006). In the Americas, adult volunteers from villages in the state of Rondônia, Brazil, were tested for antibodies against *Anopheles darlingi* salivary gland sonicates (SGS), as this species dominates as malaria vector in Brazil. Persons who had *P. vivax* infection also had higher titers of anti-SGS antibodies than did non-infected individuals. This test must be potentially useful as an epidemiological tool because antibody levels could discriminate between infected and non-infected with a high likelihood ratio (Andrade *et al.,* 2009). Although no definite data exist on the longevity of immune response to anti-salivary antibodies, some observations suggest a shorter duration compared to blood staged parasite antigens. For example, IgM antibodies developed against the saliva *Triatoma infestans* was detectable just after one day of single exposure to a number of triatomine bugs and decline 18 days after absence of exposure in chickens chickens (Schwarz *et al.,* 2010). Immunoglobulin M responses appeared to be highly sensitive for detecting bug exposures, but the correlation between level of exposure and the level of IgM titers could not be established. However, the evidence so presented point to the pontential utility of short IgM persistence, as an immunoepidemiological marker of transient exposure to insect bites.

Certainly, a simple serological survey could provide evidence of human exposure to the bites of specific vectors of interest and thus complement entomological surveys or eventually replace them when the detection levels are below the detection threashhold human landing catch or other trapping methods (Billingsley *et al.,* 2006). It would be easier and perhaps more appropriate and even cheaper to assess saliva based immuno-epidemiological markers of exposure than conducting clinical trial to calculate vector-borne infectious disease incidence in order to evaluate the impact of anti-vector control interventions within a low risk area (Shililu *et al.,* 1998). Drame *et al.* (2010) validated the practicality of using *An. gambiae* crude saliva extract as an immuno-epidemiological marker for assessing the efficacy of insecticide-treated nets (ITNs) in a hypo-endemic malarious area (Drame *et al.,* 2010). The authors determined the anti-saliva IgG levels, parasite prevalence and mosquito population densities before and after the implementation of ITNs. A significant decline in anti-saliva specific immune response was observed following the implementation of ITNs. This decrease in immune response was correlated with a fall in parasitemia but not with vector population densities as determined by conventional light traps.

2.9.2 Diversity and specificity of salivary components

Current knowledge on the diversity salivary proteins in Arthropoda has come about by the classification of salivary proteins expressed and secreted in a number species of blood-feeding arthropods. The availability of the entire genome sequences of key hematophagous arthropods (Arensburger *et al.,* 2010; Nene *et al.,* 2007; Mongin *et al.,* 2004) had been accompanied by increased transcriptomic and proteomic analysis on salivary glands proteins. Thus, it is possible to identify salivary molecules in a range of blood-feeding arthropods. Currently, the transcription repertoire (named sialotranscriptome) of some 30 species of blood-feeding arthropods has been decoded, revealing several ubiquitous and specific

proteins all through the taxonomic hierarchy (Arca *et al.,* 2007; Calvo *et al.,* 2009; Alves-Silva *et al.,* 2010). A number of research studies have observed varied degrees of cross-reactivity accross diverse vector species, from low to high species-specificity (Pinnas *et al.,* 1986; Volf and Rohouseva 2001; Rohousova *et al.,* 2005). The occurrance of cross-reactivity has often been reported in closely related species indicating that this phenomenon does arise in closely-related salivary proteins. Specificity is a prerequisite for the assessment individual exposure to a specific genus or species of a vector. The independent development of blood-feeding insects and host immune pressure on salivary products brought about diversity of pharmacological molecules in different genera within the same family (Ribeiro *et al.,* 2010; Calvo *et al.,* 2007). This creates the option of having several candidate proteins to assess individual exposure to specific blood feeding arthropods (Ribeiro *et al.,* 2010).

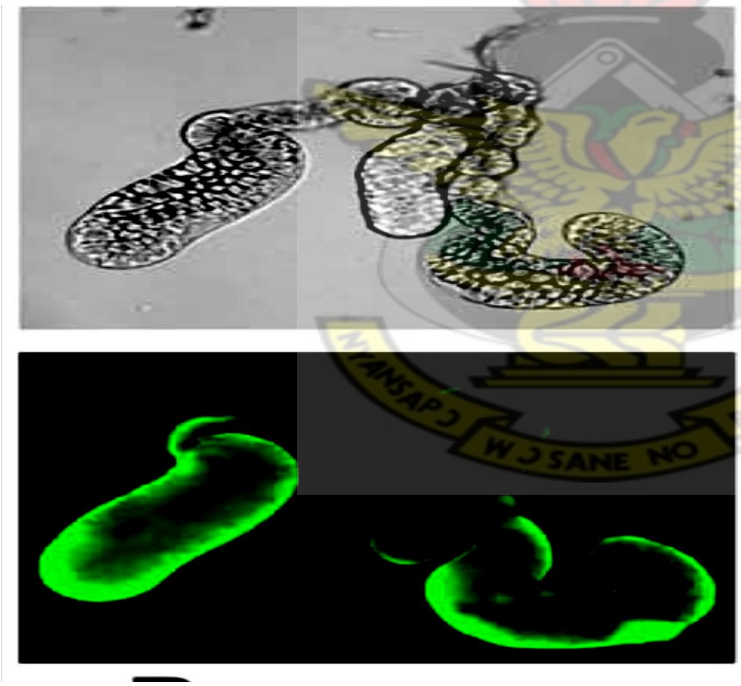

Figure Plate 1.5: gSG6 proteins located in the lateral lobes of salivary glands, via Immuno-staining of *An. gambiae* female salivary glands with the anti-tubulin and the anti-gSG6 antibodies. Adapted from Lombardo *et al.* (2009)

2.9.3 Synthetic salivary components as immunological markers of exposure

The main difficulty in designing a biological test of exposure to hematophagous vector bites are the challenges associated with the collection of salivary extracts and lack of standardized sampling methodology. The salivary protein content can be diverse based on sex, age or diets (Choumet *et al.,* 2007; Prates *et al.,* 2008). Thus, sensitivity, specificity and reproducibility could be achieved by identifying the genus or species-specific immunogenic proteins and expressing them in the recombinant form or synthetic peptides. The recombinant salivary protein 6 (gSG6), of *A. gambiae* which, is a small protein well conserved in the genus *Anopheles* (Lombardo *et al.,* 2009), has been assessed as a biomarker of exposure in children exposed to anopheline bites (Poinsignon *et al.,* 2008). It is known through BLAST analysis that homologous proteins of gSG6 can be found in only eight *Anopheles* species, confirming its specificity to the *Anopheles* genus. This protein has recently been proposed as a serological candidate marker of exposure to Afrotropical malaria vectors (Rizzo *et al.,* 2011; Poinsignon *et al.,* 2010). Using recombinant proteins well conserved between, related vector species may be very useful in evaluating the risk of pathogen transmission. To enhance immunogenicity and optimize anopheline specificity, Poinsignon *et al.* (2008), designed a gSG6-based peptide sequence (gSG6-P1) based on bioinformatic tools (Poinsignon *et al.,* 2008). A positive correlation was observed between immune responders to anti-gSG6-P1 specific antibodies and the exposure levels in exposed individuals. The peptide was detectable in persons exposed to low number of vector bites, and this points to a potential to detect *An. gambiae* exposure under transmission intensity below the sensitivity threashold of entomological methods (i.e. urban areas, highlands, travelers) (Poinsignon *et al.,* 2009). Altogether, these data support the application of immunoepidemiological tools based on salivary components as new tools assessing the risk to vector-borne diseases.

2.10 Malaria Diagnostic Tools

As malaria transmission declines across much of its range, the possibility of elimination is increasingly being considered (O'Meara *et al.,* 2010; WHO, 2009). Many countries are now attaining very low levels of transmission. This presents new challenges that demand improvement in current field diagnostic tools such as microscopy and rapid diagnostic tests [RDTs]. Accurate diagnosis and case identification of malaria parasites in sick patients is essential both to target anti-malarial drugs and to enable effective management of the frequently fatal non-malarial febrile illnesses (Black *et al.,* 2010) that share signs and symptoms with malaria (WHO, 2006; Kallander *et al.,* 2004; Reyburn *et al.,* 2004). A more sensitive diagnostic tool that is Field-ready will be amenable for mass screening and surveillance, and enable targeted control and for monitoring transmission reduction to ensure elimination.

Microscopic examination of blood smears, the gold standard for malaria diagnosis, is inexpensive to perform and has the ability to quantify and differentiate between species. However, it requires trained competent microscopists, rigorous maintenance of functional infrastructure and an effective quality control and quality assurance. The accuracy of microscopy to a large extent depends on the knowledge, skill and experience of the microscopist and the quality of blood smears prepared. This unfortunately varies significantly (Amexo *et al.,* 2004; Ohrt *et al.,* 2007). Critically, good quality microscopy is challenging to keep in isolated-remote areas where malaria usually occurs (Wongsrichanalai *et al.,* 2007). Tests to amplify parasite DNA, typically by PCR are generally available in research settings and are gradually being used in diagnostic laboratories in well-resourced countries. But, such are not routinely available in the field and in the remote localities where malaria is endemic.

2.10.1 Malaria rapid diagnostic test

Rapid diagnostic tests (RDTs) present an opportunity for rapid and precise diagnosis of malaria, leading to timely and proper treatment. They provide parasite-based diagnosis when competent microscopy is lacking and thus can be used by village health workers after as little as an hour of training (Mayxay *et al.,* 2004; Rennie *et al.,* 2007) and as a result lessen disease burden and death in resource-poor malaria endemic settings. Many RDTs currently available are based on rapid immuno-chromogenic tests, in simple kit form. Assays are based on the capture of parasite antigen by monoclonal antibodies incorporated into a test strip. Three types of antigens are targeted: parasite lactate dehydrogenase (pLDH), histidine-rich protein 2 (HRP-2, found in *Plasmodium falciparum* only) and aldolase (pan-malarial antigen, found in all malarial species). So far HRP-2 based RDT is the most sensitive and stable (WHO, 2005). Thus HRP-2 based RDT is designed to capture *Plasmodium falciparum* Histidine rich protein 2 (*Pf*HRP2).

2.10.2 Plasmodium falciparum Histidine-Rich Protein II (PfHRP-2)

*Pf*HRP2 is a 60-105 kD, water-soluble protein synthesized only by *P. falciparum*; it is recognized as a surface-exposed protein in an invaded red blood cells (Wellems and Howard, 1986; Panton *et al.,* 1989; Birku *et al.,* 1999). It can be detected in *in-vitro* culture supernatants of synchronized parasites as early as 2-8 hr after ring development. The amount of *Pf*HRP-2 released *in vitro* continues to increase all through the erythrocytic cycle, with the greater portion being released during schizont rupture (Howard *et al.,* 1986). The gene encoding the protein, *pfhrp2*, is a lone copy subtelomeric gene positioned on chromosome 7. It codes for an amino acid sequence comprising of 34%, 37% and 10% histidine, alanine and aspartic acid respectively (Wellems *et al.,* 1986; Panton *et al.,* 1989; Scherf and Mattei, 1992). Typically, *Pf*HRP-2 has multiple flanking replicates of the sequences AHH and

AHHAAD (Wellems *et al.,* 1986; Panton *et al.,* 1989). Even though the specific function of *Pf*HRP-2 is yet to clarified, recent studies have implicated HRP-2 as an important factor in the detoxification of free heme by converting it to inactive haemozoin (Pandey *et. al.,* 2001, Papalexis *et. al.,* 2001). Others have suggested that HRP-2 may be implicated in modifying the infected erythrocyte cytoskeleton (Bennedetti *et al.,* 2003) and in modulating host immune responses (Bosshart and Heinzelmann, 2003). The extensive diversity in the *pfhrp2* sequence confirms that the role(s) of the molecule is/are not controlled by the sequence varriation in exon 2 and that parasites with one particular *pfhrp2* sequence may not have a robustness or survival benefit. Host immune pressure may add to sequence variability by selecting immunologically diverse types.

2.10.3 Performance of RDTs

Several RDTs in recent times can attain excellent accuracy for *Plasmodium falciparum* at a parasitemia greater than 250 parasites per microlitre (parasites/μL) (Soto *et al.,* 2004; Wongsrichanalai *et al.,* 2007; Endeshaw *et al.,* 2008). At lower parasite density however, variability in sensitivity is more common (Ishengoma *et al.,* 2011; Endeshaw *et al.,* 2008; Broek *et al.,* 2006). Several studies have reported sensitivities and specificities below what is required for operational use (Murray *et al.,* 2003; Wongsrichanalai *et al.,* 2007). Poorer still, based on limited data from performance under routine settings 19%-86% accuracy has been reported (McMorrow *et al.,* 2008).

2.10.4 Parasite factors influencing performance of RDT's

Parasite factors include parasite density, genetic diversity of the *Plasmodium falciparum,* which translates into varriation in the targeted epitopes of the parasite protein as well as the

concentration of HRP-2 proteins in peripheral blood circulation. Mostly, the poor performance of RDT has occurred at relatively low parasite densities ≤500/µLblood (Huong *et al*., 2002; Singh *et al.,* 2003 and Ishengoma *et al*., 2011). However, this level has often resulted in symptomatic malaria in non-immune individuals (Foney *et al.,* 2003). WHO recommends a standard of 100% sensitivity at parasite density of 100/µLof blood (Bell and Peeling 2006), for an RDT to become a useful diagnostic. Perhaps this becomes a more crucial research priority as malaria burden is reducing around the world, because relatively low levels of parasitemia are expected to cause clinical illness even as naturally acquired immunity declines.

Hyper-parasitemia has also been associated with RDT failure**,** (Biswas *et al*., 2005; Gillet *et al*., 2009). The phenomenon 'prozone effect' characterized by false-negative or low signal in immunological response/reactions, owing to an excess of antigens or antibodies (Gillet *et al.,* 2009), has been cited as an explanation (Farcas *et al.,* 2003; Hawkes *et al*., 2007). In the case of hyper-parasitemia, high antigen concentrations block accessible binding sites of the detection as well as capture antibodies, and stop the formation of antigen-antibody-complex with a resultant failure of signal generation. Individuals living in areas of high transmission intensities develop high titers of circulating anti- HRP-2 antibodies that bind to HRP-2 in the plasma to form immune complexes that unlike the free antigen are not detectable in RDT test (Biswas *et al*., 2005).

The vast genetic diversity of *P. falciparum* isolates has also been implicated in RDT failure. Generally, genes positioned within the subtelomeric portions of *Plasmodium* are genetically diverse, since they are highly prone to mutations during recombination events (Figueiredo *et al.,* 2002; Volkman *et al.,* 2002; Ribacke *et al.,* 2007; Scherf, 1996; Anders *et al.,* 1986). Subtelomeric regions from diverse *Plasmodium* species seem to have

undergone rapid evolution, with substantial sequence variability produced in the intricate repeats in these regions (Scherf, 1996). Molecular mechanisms contributing to the creation of tandem-repeated-regions, variability in the length of repeat blocks include slipped strand mis-pairing after DNA replication or repair, disparate mutual combination and gene conversion (Weber, 1988; Dodin and Lovoia., 2005; Hughes, 2004). The different arrangements and diversity in the number of repeats seen in *pfhrp2* gene is because of recurrent recombination of the chromosomes (Baker *et al.,* 2010). This evidence points to a strong correlation between malaria transmission intensity and *pfhrp2* diversity. In high transmission settings, malaria infections are frequently characterized by co-infection of several strains which intensify the likelihood of recombination through sexual reproduction within the vector.

Baker *et al*. (2005) reported a vast variability in *Pf*HRP-2 sequences, at national and regional levels and concluded that 2 types of repeats could predict the sensitivity RDT and only 84% of *P. falciparum* within Asia-Pacific province were likely to be detected at densities <250 parasites/µL of blood. In 2010, however the group failed to associate a correlation between sequence variation and RDT sensitivity at 250 parasites/µL of blood. But the authors conceded that futher insights into the composition of *Pf*HRP-2 and its variability will contribute to the assessment of malaria RDTs, and assist to improve malaria RDTs. Furthermore, a significant differences in the reactivity of HRP2 one specific monoclonal antibody (MAB) to different parasite isolates has been observed; again the binding of four HRP-2 specific MABs evaluated against a single parasite isolate revealed significant differences in reactivity (Lee *et al*., 2006). Gamboa *et al*. (2010) for the first time reported *P. falciparum* natural isolates lacking *pfhrp2* (41%) or *pfhrp3* (70%) or both (21%) genes in the Amazon Peruvian region. This evidence warrants comprehensive research into

sensitivity and accuracy of HRP-2 based malaria RDT in specific countries and epidemiological settings where malaria RDTs are being rolled out in order to ascertain their effectiveness relative to conventional diagnostics. For example, in Madagascar, Mariette *et al.* (2008) based on the observation that high polymorphisms occurred in the *pfhrp2* genes, predicted that on the average 9% (ranging from 6% in the north to as much as 14% in the south) of the country's *P. falciparum* isolates would not be detected at parasite densities less or equal to 250 parasites/µl.

Currently, there is not enough evidence in Ghana and in Kenya on the performance of HRP2-based malaria RDT in the context of low parasitemia. Moreover, as *P. falciparum* infections decline, *P. ovale* and *P. malariae* which are transmitted across a broad geographical range, but at low prevalence, may become relatively more prominent in areas where they were endemic; the information on the effect of the prevalence of other species on the sensitivity of HRP-2 RDT is not existent.

In a high parasite prevalence malaria region in Ghana, it will also be interesting to investigate the operational accuracy of the *Pf*HRP2 based rapid diagnostic test. Several trials of HRP2 accuracy have been reported based on a well supervised testing under research conditions, it will be very interesting to find out in an operational research based on the practice of malaria diagnosis and RDT testing from a rural health care center and an urban hospital to ascertain the accuracy of HRP2 testing using microscopy as a gold standard.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Sites

The study was conducted in hypo-endemic highlands of western Kenya in East Africa and within the holo-endemic areas of the forest zone of Ghana in the West African sub region. The East African highlands of Kenya were chosen particularly because of the need for an unstable malaria transmission region, in order to develop and test seroepidemiological tools for their sensitivity in tracking the changing epidemiology of malaria in that region. In the western Kenyan highlands, malaria control has achieved 90%, reduction in vector population and a further 50–90% reduction in human infections. In some localities malaria transmission has been interrupted, Topography as well as the hydrology within the highland ecosystem has been reported to account for the spatial variability in malaria prevalence and the divergent humoral responses within the human population (reviewed in Githeko *et. al.*, 2012). Vector ecology in the western Kenyan highlands has indicated that transmission is focal creating heterogeneity in human malaria exposure. These characteristics present a unique opportunity for the evaluation of immuno-epidemiological markers for sensitivity and robustness in unstable malaria endemicity. A situation expected to be widespread when countries make progress towards malaria elimination within the context of eradicating malaria.

The study sites in Ghana were chosen to evaluate the diagnostic accuracy associated with the *Pf*HRP2-based rapid malaria test kit. For this study required a population with high parasitaemia and stable malaria transmission endemicity. The whole of Ghana is described as being holoendemic for malaria (WHO, 2010). The forest zone of Ghana particularly been described as having high entomological inoculation rates (Abonuusum *et al.*, 2011) and high parasitemia (Browne *et al.*, 2000) for these reasons Kenya and Ghana were chosen as ideal

geographical sites to test seroepidemiological tools and that the outcome of this study will be relevant for all malaria endemic zones in the world.

3.1.1 Study sites in Kenya

The study was carried out in three sites in western Kenya, including two highland areas and one lowland area that followed dissimilar malaria epidemiological strata with marked differences in malaria transmission intensity. These were Lowland Kombewa (hyper-endemic) in the Kisumu County; and highland areas of Kakamega and Marani Counties which, are known to be meso-endemic and hypo-endemic for malaria transmission respectively (Githeko *et al.,* 2006).

3.1.1.1 Kakamega County

The study areas in the Kakamega County were subdivided into two locations designated as Uphill population and Valley bottom population which describes a hill transect that is approximately 5km apart. Set in the highlands of western Kenya, Iguhu and Lidambiza villages (0°10'N, 34° 44'E, elevation 1,420-1,500 m above sea level (asl), were defined as valley bottom, whereas Sigalagala and Shikondi villages (0° 33'N, 34° 47'E, elevation 1,520-1,600 m (asl) were defined as the uphill sites. The area has a population of about 11,000 people. Kakamega is a somewhat suburban area, which was recently designated a county status; the people in the area predominantly belong to the Luyha tribe of Kenya which is a large group akin to the Akan community in Ghana made of several different subdialects with the predominance of the maragoli dialect in the Iguhu-Litambisa areas where the study took place.

Plate 3.1: A section of the highlands of Marani community

These two locations (uphill and valley bottom) have been variously described as having distinct malaria epidemiology (Githeko *et al.*, 2006; Munyekenye *et al.*, 2005). The topography of the study area displays a medley of hills and valleys interspersed with rivers, and this is typical of the western Kenyan highlands. The hillside is usually speckled with maize plantations. Diverse recurring streams flow in the valleys within the study communities and join the most important *Yala* River, which transcends the area from one side to the order. The inhabitants live in mud-walled houses with thatch or iron sheet roof. The area experiences two main rainy seasons with a mean rainfall of 2,000 mm per annum. The major rainy season typically come about from April to May, with a mean rainfall of 150–260 mm monthly. The minor season typically occurs from September to October, with a slightly

lower magnitude of 165 mm maximum monthly rainfall. The dry season also come about between December and March. The average daily temperature is 20.8°C. Malaria prevalence hit the highest point usually one to two months after the rain. Incriminated vecrors of malaria in the area are *Anopheles gambiae s.s* and *An. funestus* (Githeko *et al.,* 2001*;* Minakawa *et al.,* 2004*).* Majority of mosquito breeding habitats are confined to the riverbanks within the valley bottom area near the streams in both seasons. Adult mosquitoes are clustered in houses near valley bottom (Githeko *et al.,* 2006; Minakawa *et al.,* 2004) and much less at the hilltop. The entomological inoculation rate (EIR) at the valley was 16.6 during 2003- 2004 (Ndenga *et al.,* 2006), while it was 0.04 in the uphill area during 1999-2000 (Githeko *et al.,* 2006; Zhou *et al.,* 2011).

3.1.1.2 Marani

Marani (340^{o} 489E, 00^{o}359S, 1,540–1,740 m asl) is in the Kisii county and has a catchment population of 19,000. It is situated on the highland plateau neighboring the Lake Victoria Basin and distant some 17 km north of the Kisii township. It has a fast flowing River that runs along the shallow gorge. Steep and gently sloping hills and undulating topography characterize the area. The highland area has small patches of forests along the river and streams, which are remnants of a larger forest that has been cleared for cultivation and pasture. The valley is characterized by a good drainage and floods are not common. Marani area is under low and unstable malaria transmission and thus has been described as hypo endemic for malaria (Zhou *et al.,* 2011).

Generally, the landscape of the highland sites is typifyied by valleys and depressions bounded by compactly populated hills. The western Kenyan highlands experience two rainy seasons one being major (March-May) and the other being minor (October -November). The average annual rainfall during this period was approximately 1,900 mm. Peak rainfall occurs between

April and June followed by a shorter rainy season in October and November. The average monthly rainfall is in the range of 150–260 mm in the major season, and the minor in the range of 165 mm. Traditionally the average annual rainfall in the western Kenya highlands has been between 1800–2000 mm with an average annual temperature ranging from 17 to 20 °C (Lalah *et al.,* 2009; Stephens *et al.,* 1992). Daily temperatures range between 13.8 and 28.0°C. The mean annual daily temperature is 20.8°C. *Plasmodium falciparum* is the most important malaria parasite species (Munyekenye *et al.,* 2005) and the principal vectors of malaria are *Anopheles gambiae s.s., An. arabiensis* and *An. funestus* (Minakawa *et al.,* 2002; Ndenga *et al.,* 2006).

3.1.1.3 Kombewa

Kombewa is located on longitude 34°45' E and latitude 0°10' S, with elevation of 150–1,250 m asl. It is in the Kisumu County, which lies in the vicinity of the Lake Victoria basin with a population of 23,000 people. Kombewa is characterized by a rolling terrain bisected by small semi-permanent swampy streams with poor drainage. It lies within the semi-arid lowland and is warmer and drier compared to the two highland sites. Mean monthly rainfall of 120.7 mm; maximum and minimum temperatures of 29.1°C and 18.4°C, respectively, have been reported Malaria is hyper-endemic in this area, with *P. falciparum* accounting for more than 95% of the malaria infections; mosquitoes of the *Anopheles gambiae* complex and *A. funestus* are the major vectors. The EIR in recent times has been estimated to be 31.1 infectious bites per person per year (Ndenga *et. al.,* 2006 and Minakawa *et al.,* 2004).

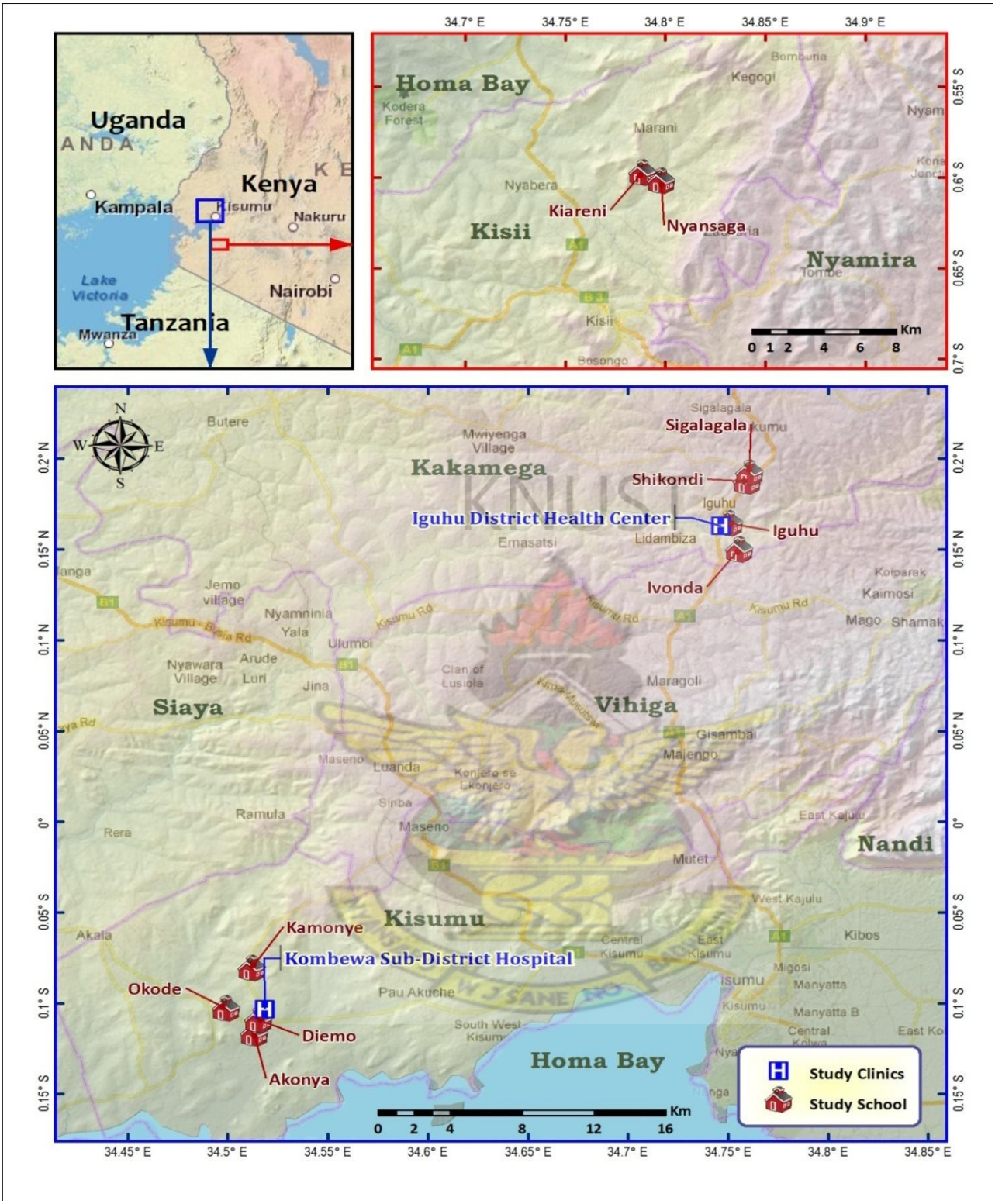

Fig 3.1: Study sites in western Kenya, with study schools in Kisumu, Kakamega and Kisii (Marani) Counties are indicated on the map: Courtesy Ming Chie Lee (2012).

3.1.2 Study sites in Ghana

3.1.2.1 Kumasi, Atonsu-Agogo;

The study was conducted in the Kumasi South District hospital located in the Atonsu-Agogo area, an urban-poor community (Tay *et al.*, 2012) within the city of Kumasi, the capital of the Ashanti Region of Ghana. It is located between latitude 6°30' and 7°00'N and longitude l°30' and 2°00'W. It is the second-largest city in Ghana, and is set within the rainforest zone of West Africa. The estimated population of Kumasi based on the 2002 population and housing census was 1.2 million inhabitants (Ghana Demographic and Health Survey 2003). The peak rainfall is between April and June with an annual rainfall of around 1400mm, and the mean annual temperature is 25.7 ^{o}C with a humidity ranging from 53 to 93%. The climate is said to be wet, semi-humid tropical. The entomological inoculation rates (EIR) in locations in the city with agriculture were estimated to be 9.4 per month in the rainy season and 4.8 in the dry season. Browne *et al.*, (2000) reported that parasite prevalence peaks at 93% in 11-year-old children and declines to a plateau of 20% in adults in the forest zone. Houses within the Atonsu-Agogo (6°64′92″ W 1 °58′17″N); community are made up of brick walls and roofed with corrugated iron sheets. Only a few houses have screened doors and windows. Many houses are clustered together with little spaces between them; there are very few well-planned roads and this gives an impression of a poorly-planned and closed community. Choked gutters and blocked drainage systems are quite common. One end of the community is bounded by thick vegetation made of shrubs and few trees and (it was not surprising that houses) from this area had aggregation of mosquitoes (Tay *et al.*, 2012). The economic activity in this study area is basically small scale trading.

3.1.2.2 Agona

The second community in Ghana was Agona in the Afigya Sekyere District. The District hospital is located in the Agona Township; which is also the district capital. The district spans an area of approximately 714 km^2, and with a total population of 131,658, (Ghana Demographic and Population Census, 2003); it is set in the north-eastern part of the Ashanti Region in the midst of the forest zone of Ghana.

Agona is located 27 km north of Kumasi (Fig 3.2); it has a number of satellite villages. The area is characterized by semi-deciduous forest and farmlands. The population ranges from 1492 inhabitants in the smallest village to 12,877 inhabitants in the largest Population Census data in the year 2000; as reported in Kobbe *et al.* (2006). The altitude variation is between 146m the lowest and 433 m the highest. The temperature variation is 20.4°C–33.5°C, with monthly rainfall between 15 and 214mm. The river Ofin with its tributaries meanders through the district and its reserve, providing pools of water which flood the marshy areas during the rainy season. The catchment area of the hospital covers over 200 km^2, is geographically homogeneous, and is holoendemic for *P. falciparum* malaria, with perennial transmission and seasonal peaks (high transmission from May to October) (Kobbe *et al.*, 2006). Economic activity in the district is limited to subsistence farming and small-scale trading.

The principal malaria vectors are mosquitoes of the *Anopheles gambiae* complex and *Anopheles funestus*. Malaria transmission in the area was found to be intense and perennial in two villages Afamanaso and Kona which are located 3-10km from Agona. The annual biting rate (ABRs) and entomological inoculation rates (AEIRs) for Afamanaso were 11,643 and 866 bites/person/year respectively. Similarly, at Kona the ABR and the AEIR were 5,329 and 490 bites/person/year respectively as observed by Abonuusum *et al.* (2011).

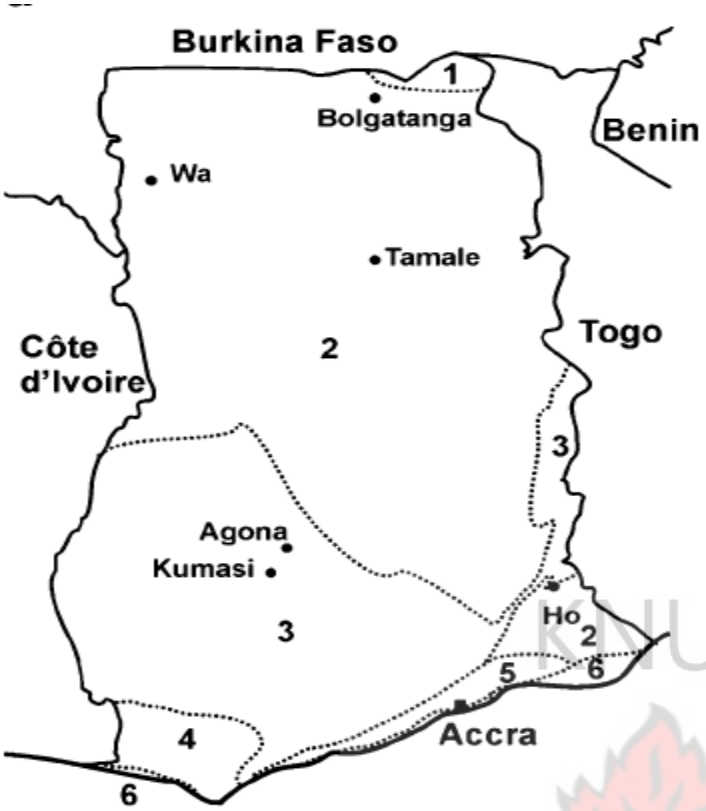

Fig. 3.2: Map of Ghana showing study sites with vegetation zones marked: 1 Sudan savanna, **2** Interior wooded savanna, **3** semi-deciduous forests, **4** rain forests, **5** coastal savannah and **6** strand and mangrove

Choice of study sites for testing accuracy of *Pf*HRP2 rapid test

*Pf*HRP2 is the target protein for many rapid diagnostic tests (RDTs) detecting *P. falciparum*. It has been shown that *pfhrp2* gene exhibits extensive diversity both within and between countries and regions which correlates with malaria transmission intensity (Baker *et. al.,* 2010). Additionally, it is largely reported that, the genetic diversity in the antigen contributes to the variability in RDT sensitivity.

Unlike the highlands of western Kenya which are known to have lower malaria transmission described as hypo- endemic (Githeko *et al.,* 2006), most parts of Ghana on the other hand, are known to be under intense malaria transmission and thus described as holo-endemic. Malaria transmission reported for the Agona area in the forest zone of Ghana, was evidently higher

than that of the city of Kumasi and its peri –urban areas (Abonuusum *et al.*, 2011; Afrane *et. al.*, 2004).

For the comprehensive evaluation of the *Pf*HRP2 - based malaria rapid diagnostic test, there was a need for choosing sites with marked differences in malaria endemicity so that the influence on the test accuracy due to the intensity of malaria transmission and parasites density could be evaluated.

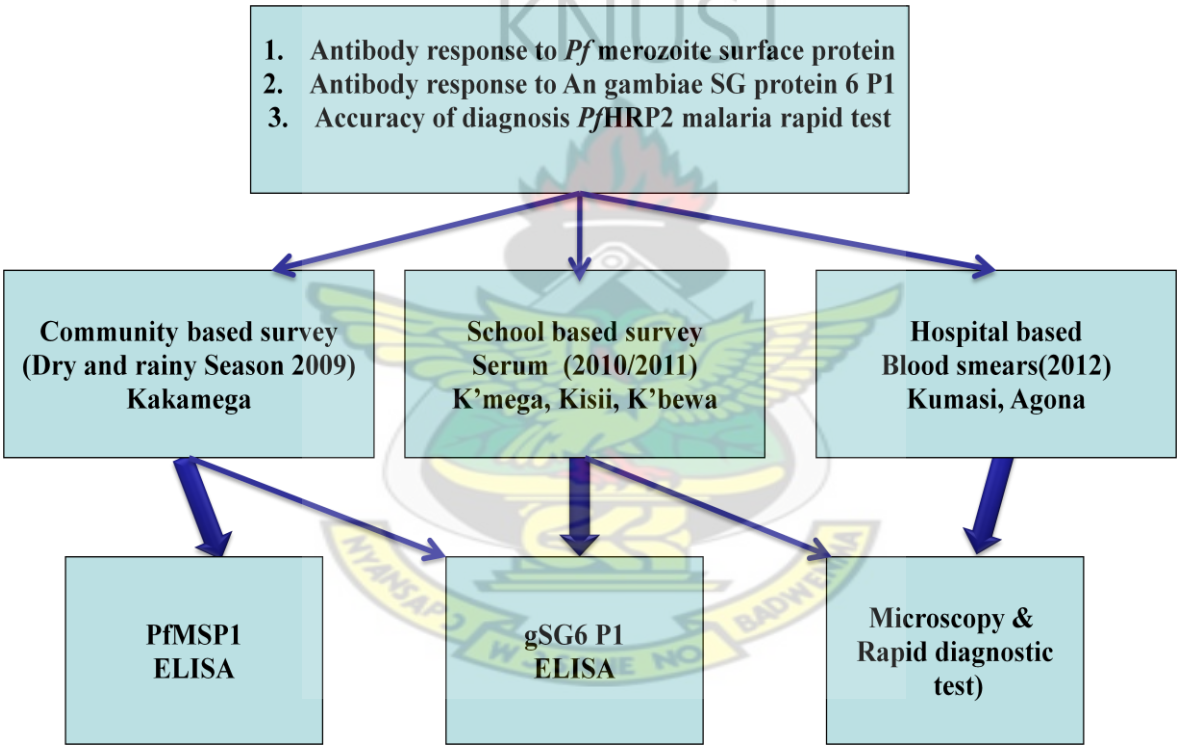

Figure 3.3: Schematic representation of study design

3.2 Study Design

There were three inter–related studies with similar designs (Fig. 3.3). The first study; was to determine the spatio-temporal variation in specific human antibody responses to malaria infection. This consisted of two cross-sectional community based serological surveys, with only one time follow-up after the initial recruitment. Principally, the serologic confirmation of malaria exposure at two highland sites across differing altitudes and transmission intensities were determined. Total IgG levels to *Plasmodium falciparum* MSP-1_{19} WELCOME genotype were determined in an age-stratified cohort from inhabitants of uphill and valley bottom communities. This was conducted during the low and high malaria seasons. Antibody seroprevalence and densities were then compared between different localities. The study did not discriminate participants in terms of age or gender but a representation of all age groups < 5, 5-14 and ≥ 15 age groups. The second study, "antibody responses to *An. gambiae* salivary gland peptide" sought to evaluate specific human antibody responses to *Anopheles gambiae* salivary gland peptide (gSG6- P1), as an immuno-epidemiological marker of mosquito bites, in different transmission settings and seasons. The comparison between anti-MSP-1_{19} seropositives and seropositives that is to say immune responders and non-responders permitted the evaluation of the strength of the gSG6-P1 as a surveillance tool in an area of declining malaria transmission. In this study several cross-sectional surveys were undertaken among primary school children aged between 5 and 16 years among three localities that differed in altitude and malaria transmission intensity (Ndenga *et al.,* 2006; Zhou *et al.,* 2011). This study in addition to the school surveys utilized sera available from the MSP1-$_{19}$ study. The third study, evaluated the accuracy of *Pf*HRP2-based malaria rapid diagnostic test kit. This was an operational (hospital based) study in Ghana together with a field trial in Kenya. Primary school survey in Kenya and hospital

based surveys in Ghana were used for this study. In Ghana the survey was conducted among suspected malaria case cohorts for all age groups among individuals referred to the hospital laboratory for malaria diagnosis.

3.2.1 Study populations

Volunteers were recruited from communities and primary schools in the study sites in Kakamega, Kisii, Kombewa in the western Kenyan highlands; whereas participants in Ghana were recruited from the hospitals in Kumasi and Agona within the forest zone of Ghana. The researcher, field assistants and staff members from the Kenya Medical Research Institute (KEMRI) as well as the hospitals in Ghana were responsible for volunteer recruitment and education programs in the schools, the clinics, or in the community where applicable.

3.3 Sources of Research Material

The first study involved drawing 3 ml of venous blood from the participants to determine antibody responses to malaria infections and *Anopheles gambiae* protein antigens. The second and third studies used finger prick blood samples from participants. Several drops of blood (totaling about 100 µl) were placed on a glass slide (for microscopic examination of malaria infection), also on the RDT test kit (for the *Pf* HRP2-based malaria rapid diagnostic test), and then also on Whatmann filter paper used for *Plasmodium falciparum* species identification using PCR technique respectively.

3.4 Scientific and Ethical Clearance

Scientific and ethical clearance was given by the Ethics Committee of Kenya Medical Research Institute [SCC No. 1382(N)] and the University of California, Irvine (UCI HS#2005-4562]. Additionally for the study conducted in Ghana, the ethical approval was given by the Committee on Human Research, Publication and Ethics at the School of Medical Science at the Kwame Nkrumah University of Science and technology [CHRPE/AP/208/12]. Subject consent procedures were followed, the nature of the study was explained to potential volunteers or their guardians (in the case of children) prior to participant recruitment. Those agreeing to participate were requested to consent to: 1) the nature of the study, 2) the reason for drawing blood, 3) the potential risks involved, and 4) the potential long-term benefits to the community.

3.5 Community Survey

To obtain informed consent for the community survey, individual households were randomly selected; this was followed by visits to the households, where potential volunteers were approached and briefed with the study goals and procedures. They were then invited to enroll in the study according to their free will. All persons consenting to participate either signed consent forms or thumbprinted in the presence of witnesses. Parental consent was sought for children. All participants were taken to the Iguhu district government hospital for venous blood to be taken. For inclusion, all individuals of all age groups were eligible except infants who had not yer attained the age of six months and all persons without any reported chronic infections. For exclusion were those who were less than six months old and those who refused to consent and person with observable chornic infections were not included in the study.

Plate 3.2: Community finger prick blood colletion at a homestead in Kissi, Kenya

3.5.1 School - based Survey

Participants were recruited from the primary schools within the study areas via the primary school's administrators with authorization of the divisional office at the Ministry of Health. Assent was obtained for children (< 18 years of age) for participation with the consent of their parents or guardians. Inclusion criteria included: submission of consent and assent forms and participant had to be at least age five at recruitment, and no reported acute illness except malaria. Exclusion criteria included those who were not willing to participate and thus refused to consent as well as those presenting with acute or chronic illness.

3.5.2 Hospital based survey conducted in Ghana

Permission was granted by the District Director of Health, Ghana Health Service at the two hospitals. Inclusion criteria; Participants consisted of individuals of all age groups with

suspected case of uncomplicated malaria referred to the laboratory for testing, those who were willing to participate, after the purpose of the study had been explained and thus gave their informed written consent. Exclusion criteria: all individuals who refuse to consent were excluded from the study.

3.5.3 Sample size

Sample size for antibody prevalence survey

A pilot study aimed at collecting baseline information on the presence of antibodies at the Kaka mega site in Western Kenya, provided information that 40% of valley residents had detectable antimalarial antibodies. Based on this information, to detect a difference of 20% (i.e. 20% of the hilltop residents have anti-malarial antibodies), using a two-sided test with a type 1 error rate of 0.05 and 80% power, 71 study participants per group were required. Assuming a 10% loss to follow-up in the subsequent season, 80 participants per group were required. Therefore, a total of 960 tests were conducted (80 participants per age group x 3 age groups x 2 seasons x 2 sites = 960).

3.5.4 Sample size calculation: parasite prevalence survey

This was based on binomial distribution assumption to estimate the confidence interval (CI) of parasite prevalence, with an estimated population size, $n = z^2 (pq)/ d^2$, where z is the critical value of standard normal distribution, p is the baseline parasite prevalence and **d** is the level of precision q = 1-p. For 95% CI and precision level of 5% the unadjusted sample size was 384 individuals per site.

$$\eta_0 = \frac{z_\alpha^2 pq}{d^2}$$

Where z^2 is the critical value of standard normal distribution with confidence level of 1 - , p = 1-q is parasite prevalence, and d is the level of precision. Usually, $\alpha = 0.05$ and $d = 0.05$, $z_{0.05} = 1.96$. When p=q=0.5, n_0 has maximum value of 384.

3.6 Parasitological and Immunological Survey

For the first study, two cross-sectional parasitological surveys were carried out during the dry and rainy seasons in February-March, and from June-July 2009. These corresponded to the low and high malaria transmission seasons respectively. Residents in individual, randomly selected households in study communities were contacted, the study objectives were explained to them, and potential volunteers invited for enrollment. All willing individuals after the consent process were taken to the Iguhu District Government Hospital where 3ml venipuncture blood was taken by clinicians and nurses. Demographic data including age and gender were taken, and because altitude was an important aspect of the study, participants were required to validate their usual village of habitation. Sera were seperated and sent to KEMRI research institute at the Kisumu laboratories for storage in freezers at -80°C until further processing. Blood smears were made in accordance to standard procedures described elsewhere (Munyekenye *et al.*, 2005), to determine parasite density and prevalence.

All participants found with fever and any density parasitemia were treated freely by a clinician in accordance with the Kenyan ministry of health guidelines for uncomplicated malaria. Children five years were given insecticide treated nets (ITNs) free of charge as per Ministry of Health policy. A survey on the coverage of ITN was conducted in January 2009, where questionnaires were designed to assess ITN coverage in terms of age, sex and altitude of residential villages of participants. Humoral responses were also evaluated for additional sera available from a previous study (Ogutu *et al.*, 2009) from Kombewa, a lowland (~1200m) high transmission area. The ethical clearance for that study permitted the principal

investigator to investigate the sera with other antigens. This was used to compare the humoral responses found in the highlands as the two areas had very different malaria transmission intensities.

3.7 School - based Serological Survey

To evaluate human exposure to *Anopheles gambiae* salivary gland antigens (gSG6-P1), archived sera available from the previous ($MSP1_{19}$) study was utilized in addition to acquiring new samples. A total of 730 sera were available for testing. Thus, sera were

Plate 3.3: A typical mountain side community in the Western Kenyan Highlands with houses clustered along the slope

available from uphill and valley bottom residents across all age groups and seasons.

However, these sera were available only for the Kakamega site. In addition to the sera available from the Kakamega site, several parasitological surveys were conducted in 2011 from February to April among randomly selected school children aged 6–13 years in all study sites. At least 200 volunteer school children from 2 primary schools were sampled at each

site. Standard finger prick blood (100 µl) was collected into microvettes containing clotting activator. This was later spun and about 50µl of serum was aspirated and stored at -80^{o}c until used.

3.8 Hospital - based Parasitological Survey

For the evaluation of the *Plasmodium falciparum*, Histidine -Rich Protein 2 (*Pf*HRP2) based rapid malaria diagnostic test kit (RDT) a one-time cross-sectional survey was conducted at two hospitals in Ghana. All individuals referred to the laboratory for malaria diagnostic test were approached for informed consent and participation. No more than 100ul of finger prick blood were collected from consenting individuals after a questionnaire had been administered and the volunteer had dully signed or stamped their thumb print. Nurses and laboratory technicians at the hospitals were responsible for obtaining finger prick blood into microvettes provided by the study, this was used to prepare thick and thin blood smears and also for RDT test. The blood smears were brought to the laboratory at KEMRI for another technician blinded to the RDT result to examine the slide by standard microscopy. The finger-prick blood contained in the microvettes were kept in cold boxes at 4^{o}C and brought to the laboratory the same day for storage at -80^{o}C until used.

3.9 Laboratory Procedures

The preferable test for measuring malarial antibodies for a community-wide study needs to be highthroughput, simple, quick and reproducible.

3.9.1 Measurement of humoral responses

The Enzyme linked-immuno-sorbent assay (ELISA) is highthroughput, standardizable assay with low cost, easy to carry out and above all, it produces objective results, it is therefore an

ideas test for measuring antimalarial antibodies. The 19 kDa section of the merozoite surface protein 1 ($MSP1_{19}$) belonging to the *P. falciparum* of the Vietnam Olk strain (*Pf*MSP-FVO 1_{19}) was used as the antigen in this study. The recombinant protein was expressed and purified as described by Darko *et al.* (2005). Total IgG responses to *Pf*MSP-1_{19} protein were determined in serum by indirect Enzyme Linked Immunosorbent Assay (ELISA) (Stewart *et al.,* 2009). Briefly, plates were coated with 0.2µg antigen and incubated overnight at 4^{o}C. After blocking with 0.5% casein, test sera were diluted serially in three corresponding wells from 1:50 to 1:64000. This was then incubated for 2 hours, followed by 100µLper well of the secondary antibody Horseradish Peroxidase-conjugated goat antihuman IgG (KPL, Gaithersburg, MD, USA) for one hour. After addition of ABTS (ABTS (2, 29-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) Peroxidase substrate (Kierkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) 100µL per well, plates were incubated for one hour, and the reaction stopped by addition of 10µL per well of 20% SDS (Sigma, St. Louis, MO, USA). The *SPECTRAMAX* 340pc (Molecular Devices, Sunnyvale, USA) plate reader was used to read all plates at 414nm. After this, the SoftMax Pro v4.1 (Molecular Devices, Sunnyvale, USA) was used to fit a four-parameter curve using analysis based on the serial dilutions. Results were reported in titer values, the endpoint titer was defined as the calculated serum dilution resulting in an optical density of 1.0 in this study.

3.9.2 Salivary peptide antigen gSG6-P1

The *Anopheles gambiae* salivary peptide was designed using bioinformatic tools to enhance its specificity and immunogenicity, as previously described (Poinsignon *et al.,* 2008). It was synthesized and purified (>95%) by Genepep SA ((St-Jean de Vedas, France) and shipped to Kenya in lyophilized form. Peptides were reconstituted in 0.23 mL ultra-filtered laboratory grade water and kept frozen at -80°C until use.

3.9.3 Evaluation of human IgG antibody levels to gSG6-P1 peptide antigen

ELISAs were carried out on the sera to measure total IgG antibody level reacting to the gSG6-P1 antigen. Maxisorp 96-well microtiter plates (NUNC, Roskilde, Denmark) were coated with gSG6-P1 at 20 µg antigen in 100µL in 1xPBS. This was then incubated at 37^{o}C for two and half hours. This was aspirated and washed four times, followed by blocking with 250 µL per well with blocking buffer (0.5% casein, 0.05% Tween 20) for an hour at 22^{0}C. At this stage, the test sera were diluted 1:20 (in blocking buffer) and then incubated at 4^{o}C over night. Human antibody binding specifically to gSG6- P1 was detected by the addition of HRP goat antihuman IgG Ab (1:10,000, Nordic Immunology, Tilburg, Netherlands) and incubated for one hour at 22^{o}C. The Peroxidase substrate was added and plates were incubated for 50 mins, at 22^{o}C. The reaction was then stopped with 10µl of 20% SDS (Sigma, St. Louis, MO). The *SPECTRAMAX* was used to analyze the optical densities at 405 nm. Each test sera were assessed in three replicate wells, ther first two wells (duplicate) and the third well was a blank well containing no antigen (ODn) as control for unspecific reactions in reagents and the plasma. IgG levels were reported as final OD calculated for each serum; as the average OD value with antigen (duplicate wells) minus the OD value that contained no antigen. The co effiecient of variation of standard samples was limited to less or equal to 20%. All assays with variation above this were considered 'failed' and not included in the final analysis. The average OD of negative controls plus 3 standard deviations was used as cut-off for seropositivity.

3.9.4 Microscopic examination

10% Giemsa stain was applied to the thick and thin blood smears and allowed to dry for 15min. This was then examined by two independent competent microscopists for parasite species, prevalence and density. The number of parasites were tallied against 200 leucocytes

and recorded as the number of asexual parasites/µl of blood, with the assumption that 1µl of blood is made up of 8,000 leucocytes (8,000/µl of blood) as previously described by Slutsker *et al.* (1994).

3.9.5 DNA extraction and PCR procedures

Polymerase chain reactions were done to confirm *Plasmomodium falciparum* species identification as determined by microscopy.

3.9.5.1 DNA extraction procedure

The Chelex extraction method as described by Wooden *et. al.* (1993) was used. Approximately 3mm disc of filter paper containing the dried blood spot was punched and placed in a sterile 1.5mL Eppendorf tube; 1mL of 1× PBS containing 0.5% Saponin was added and mixed well. It was then incubated at 4^{o}C overnight. The mixture was centrifuged the next morning at 13,000 rpm for 10 minutes at room temperature. The liquid content was then discarded. This was followed by washing with 1 ml of 1×PBS; it was again centrifuged at 13,000 rpm but for 5 minutes. The PBS was then discarded and 150 µL dd$H_2$0 and 50µL of Chelex 20% was added and then incubated at 95^{o}C for 10 minutes whilst vortexing every two-three minutes to extract the parasite DNA. The sample was then centrifuged at 13,000 rpm for 3 minutes and the supernatant containing the DNA was transferred into a sterile 1.5ml Eppendorf tube. The extracted DNA was then stored at -20^{o}C until used.

Plate 3.4: A study team at work at a homestead in the highlands of Kenya; (a) a field staff takes a participant through consent process and a short interview, (b) finger prick blood being collected into clotting activated microvettes, (c) blood spots blotted on filter.

3.9.5.2 Plasmodium species PCR identification protocol

The nested PCR amplification procedure described by Snounou *et al.* (1993); Singh *et al.*, (1999) for the identification of human *Plasmodium* species was used for the detection and characterization of the infecting *Plasmodium* species. An initial outer amplification (NEST-1) PCR reaction, involved the use of genus-specific primers rPLU5 forward primer 5'-CCTGTTGTTGCCTTAAACTTC-3' (sense) and rPLU6 reverse primer 5'-TTAAAATTGTTGCAGTTAAAACG -3' (anti sense) to amplify DNA sequence targets common to all the malaria species. The product of the NEST1 reaction was then used as template for the second inner (NEST-2) amplification. During the second round of PCR amplification reactions, new primers specifically targeting *P. falciparum* was used. The primers were 5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′ (sense) and 5′-ACACAATGAACTCAATCATGACTACC-CGTC-3′ (antisense). All *P. falciparum* primers used were based on the 18S small subunit ribosomal DNA sequences.

3.9.5.3 PCR reaction volume and concentration

The first PCR was performed in a total volume of 16 µL containing 1 µL of extracted DNA, 14.4µl of 2x AB-gene (Taq, dNTPs and $MgCl_2$): Master Mix (catalog no. AB#0575), and forward and reverse primers rPLU5 25µM, 0.16 µL (final concentration: 0.25 µM) rPLU6 25 µM: 0.16 µl (final concentration: 0.25 µM) respectively and 0.28 µl of H_20.

3.9.6 Cycling conditions

Cycling conditions were same for both PCR cycling procedures, incubation at 94°C for 3 minutes, followed by 39 cycles at 94°C for 30 seconds and 57°C for 30 seconds, 68 ^oC for 45

seconds with a final incubation at 72°C for 10 minutes. Finally, extension temperature was at 72 ^{o}C for 10 min and at 10^{o}c until the PCR machine was stopped. The NEST 2 amplification with *P. falciparum* primers had different annealing and extension temperatures**;** thus 94 ^{o}C for 3 min of initial denaturation was followed by 44 cycles of 94 ^{o}C for 30 sec, 62 ^{o}C for 30 sec, 68 ^{o}C for 45 sec, then followed by 68^{o}C of 10 min extension. The reaction was held at a temperature of 10^{o}C until the PCR machine was stopped.

3.9.7 Controls

The positive control was provided by Malaria Research and Reference Reagents center (MR4) and the negative control was molecular grade water only. The PCR amplified fragments based on the 18S small subunit ribosomal gene of *P. falciparum* corresponds with ~250 bp negative was indicated by no bands.

3.9.8 DNA visualization

After NEST-2 reaction was completed, 1 µL of loading dye was added and run through a 1.5% agarose gel by electrophoresis. The presence of amplification products was detected by staining with GelRed (Phoenix Research Products, UK), (this chemical is not toxic, and the gel can just be discarded in the normal trash). The gel was visualized under a UV light.

3.9.9 RDT data

For the purpose of evaluating the accuracy of malaria diagnosis of the *Plasmodium falciparum* Histidine Rich Protein two (*Pf*HRP-2) based rapid diagnostic test kit, two parasitological surveys were carried out: the first from February 2011 to April 2011 in selected primary schools in Kenya and the second from February to March, 2012 in two hospitals within the forest zone of Ghana.

3.10 Statistical analysis

Seroprevalence was defined as number participants with detectable antibody level above the cut-off value of seropositivity to a specific antigen (MSP-1 $_{19}$ or gSG6-P1) out of the sum of participants tested. Proportion differences in the seroprevalence of MSP-1_{19} and gSG6 P1 between age-stratified cohort within the uphill and valley residents were analyzed using the $\chi 2$ test and any value below 0.05 deemed significant. The Mann–Whitney test was applied to test if medians of seroprevalence between two localities were different from each other. Multinomial logistic regression was applied to study the relationship between MSP- 1_{19} seroprevalence and parasite prevalence while adjusting for age in the study populations. The same test was applied to study the association between gSG6-P1 and MSP-1_{19} seroprevalence also adjusting for age. Linear regression was applied to study the trend of parasite prevalence and age. Further still, age-specific MSP-1_{19} seroprevalence data was applied to a reversible catalytic model that used maximum-likelihood method, assuming a binomial error distribution. The formula below was applied to examine the force of parasite exposure (or infectioin) and age (Drakeley *et al.*, 2005).

$$P_t = \frac{\lambda}{\lambda + \rho}\left(1 - e^{-(\lambda+\rho)t}\right)$$

Where *Pt* is the proportion of seropositives at age *t*, λ lambda is a contant that represents the annual seroconversion rate and ρ (rho) is the annual rate of seroreversion. For *Pf*MSP-1_{19} antibody titer, the statistical software GraphPad Prism software (San Diego, CA, USA) was used in the analysis of the data and the ploting of graphs. The age-seroprevalence curves were modeled with the JMP 9.0 (SAS, Cary, NC 27513, USA).

Table 3.1 2X2 formulae for estimating sensitivity and specificity

		Microscopy			
		Positive	Negative		
RDT	Positive	TP	FP	PPV=	TP/(TP+FP)
	Negative	FN	TN	NPV=	TN/(FN+TN)
		Sensitivity = TP/(TP+FN)	Specificity = TN/(FP+TN)		

Where TP = true positives; FP= false positive; FN= false negatives; TN= true negative

PPV =positive predictive value, NPV = negative predictive value

*3.10.1 Reliability of Pf*HRP *2 - based malaria RDT*

The accuracy (sensitivity and specificity) of the *Pf*HRP2 based Rapid Diagnostic Test Kit (RDT) was determined by the 2X2 Table formulae above. Sensitivity was defined as the true positives divided by the total of true positives and false negatives and specificity is defined as the true negatives divided by the total of false positives and true negatives as show in the table 3.1 above.

CHAPTER FOUR: RESULTS

4.1 Antibody Responses to Malaria Infection

4.1.1 Study population

A total of 1283 people were surveyed in the highland site of Kakamega, including 74 sera that were available from Kombewa a lowland area kindly provided by Dr Ogutu (Oguto *et al.*, 2009). Three hundred and forty eight people out of the total (mainly children and very old people), although gave their consent to participate, it was not possible to obtain venous blood from them. Additionally, a total of 65 sera were not included in the final analysis because the optical densities obtained did not pass the quality assurance test. The quality assurance rule for this assay stated that "any sera whose duplicates had a coefficient of variation (CV) more than 20% will not be included in the analysis. Thus, a total of 870 sera passed the quality assurance criteria and was therefore included in the analysis. Details of the total numbers from specific location and season are shown in the flow chart below (figure 4.1).

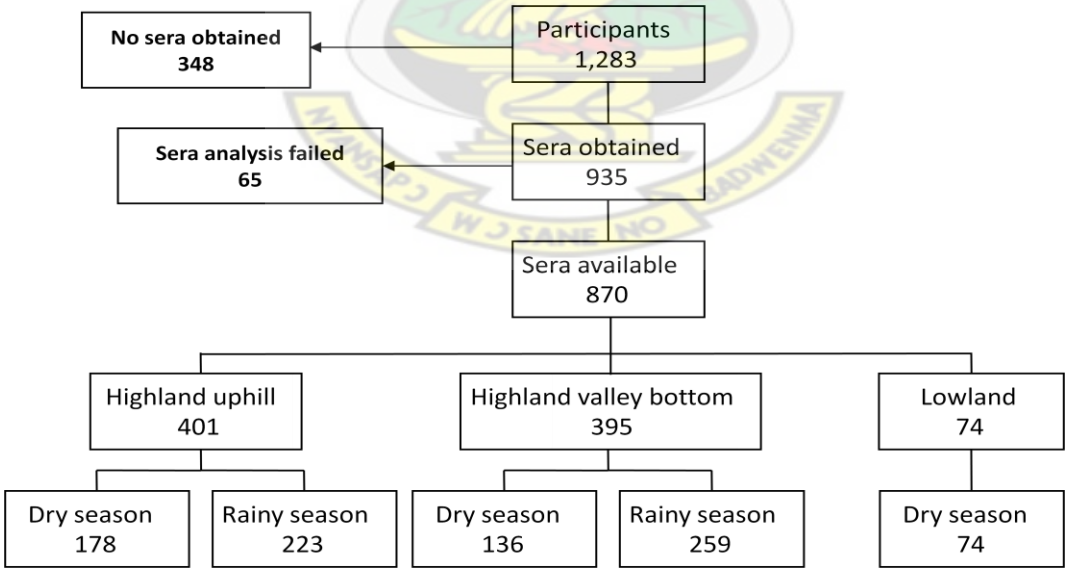

Figure 4.1: A summary of study population partitioning showing numbers of participants per season and locality in Kenya

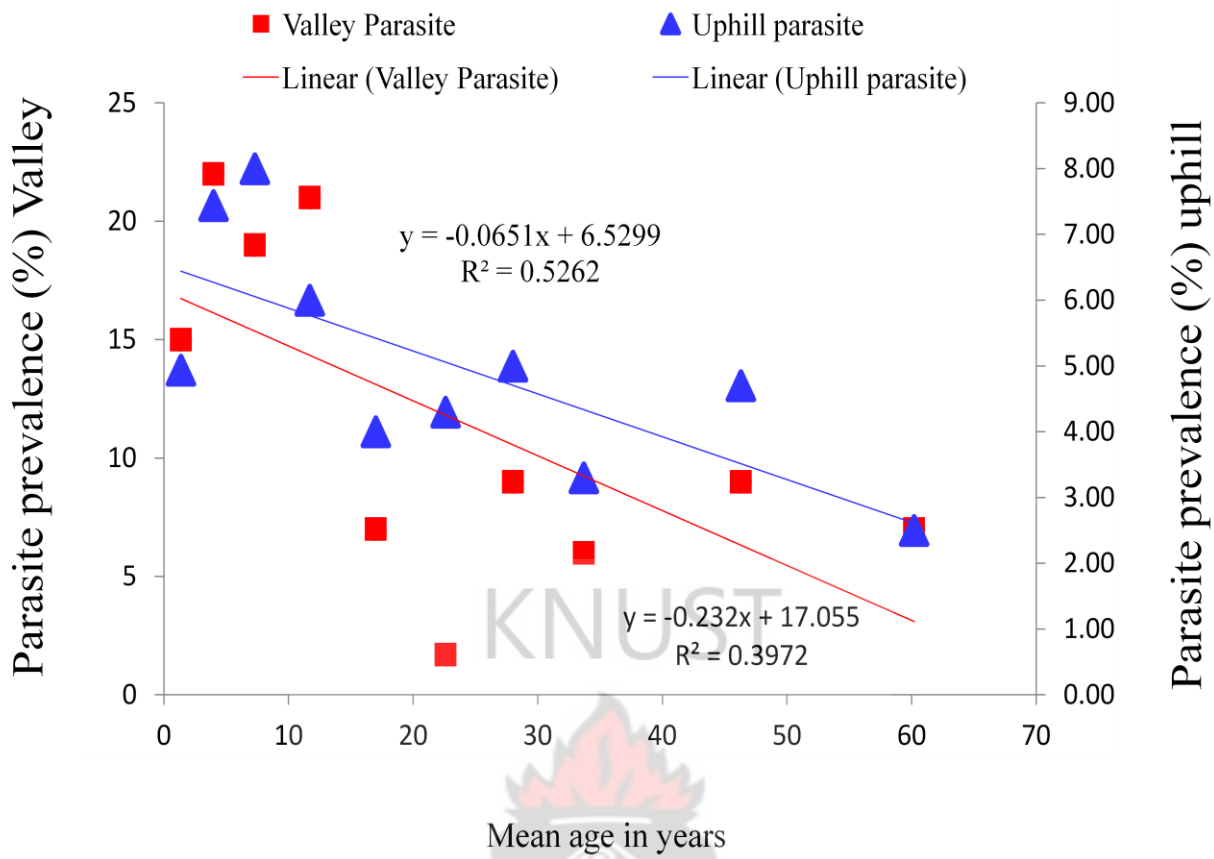

Figure 4.2: Trends in parasite prevalence with age within uphill and valley populations

4.1.2 Parasite prevalence

In Figure 4.2, generally, there was inverse relationship between age and parasite prevalence. Within the uphill residents parasite prevalence decreased with increasing age significantly (R^2 = 0.52, *P* = 0.02). There was a similar trend in the valley area however, this was at the borderline of significance (R^2 = 0.40, *P* = 0.05; Fig. 4.2). Between the uphill and valley residents, parasite prevalence differed markedly being significant in the <5 and 5–14 age-groups, ($\chi^{2\,=}$ 3.93, df = 1, *P* = 0.047) as well as (χ^2 = 9.26, df = 1, *P* = 0.002) in respective order. In these same age-groups, the prevalence of parasite in the valley population was

twice as high as that of the uphill population. However, in the adult group the observed differences were not significant (χ^2 = 1.93, df = 1, *P* = 0.*164).*

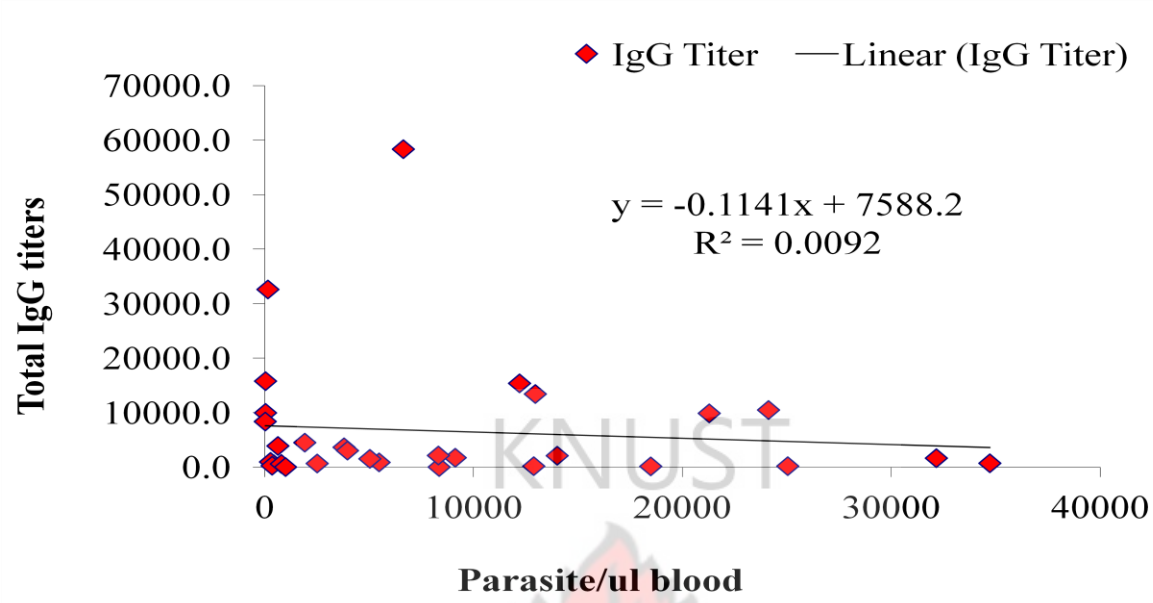

Figure 4.3 Parasite density and *Pf*MSP -1_{19} antibody levels

4.1.3 Trends in anti-MSP-1_{19} antibody levels and parasite loads

Generally, among parasite positive individuals, high anti-MSP-1_{19} antibody levels were associated with low level parasitemia (Fig. 4.3). Majority of parasitemia was associated with low IgG titers (10,000 or below). It was observed that only three individuals had IgG levels above 10.000 in other words over 90% of individuals with any parasitemia of any load had IgG titers relatively low. There was, however, one individual with parasite load of about 65,000 parasites/µl of blood who also had very high IgG titer of about 58.000.

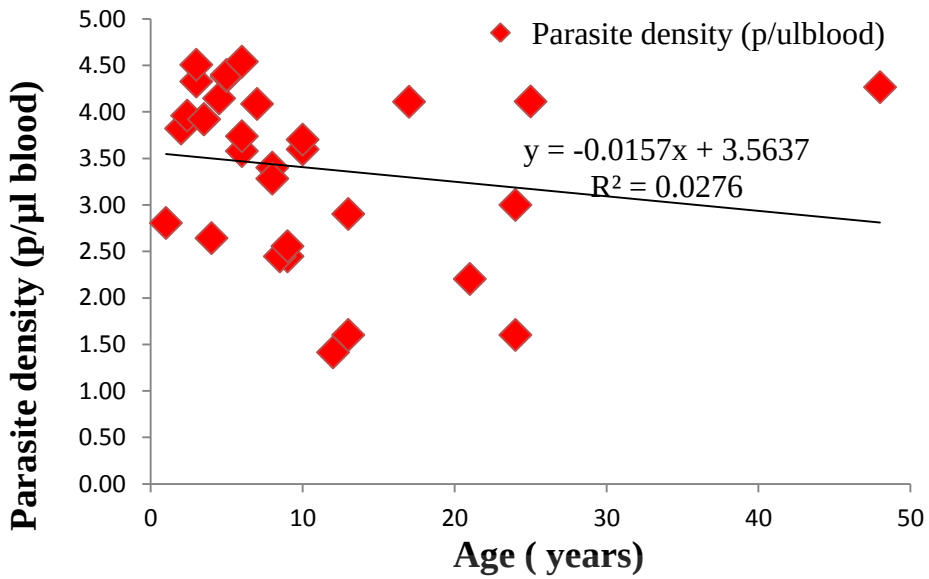

Figure 4.4: Scatter plot showing trends in parasite density in relation to age in *P. falciparum* positive participants

In Fig. 4.4, parasite density plotted against age of study participants, confirmed the earlier finding that parasite density generally decreases with increasing age; however in the highlands it is occasionally observed that individuals above 20 years can still harbour substantial parasite load. In one case a participant had high parasitemia in spite of approaching 50 years of age.

4.2.0 The variation of MSP-1_{19} Seroprevalence with Season, Age, and Altitude

In Table 4.1, the total number of sera tested in each age group (<5, 5-14, and ≥ 15) including the particular season (Dry or Rainy) and locations (uphill or valley) where they were obtained from, are given. Accordingly, the χ^2 analysis revealed that, MSP-1_{19} seroprevalence increased significantly with increasing age. This increase was consistent both in the uphill and in the valley bottom populations, in the dry season ($P < 0.001$) and in the rainy season (uphill; $P < 0.001$, valley bottom; $P = 0.03$).

Highly significant differences in MSP -1 $_{19}$ seroprevalence were found between the uphill and valley bottom residents in both seasons (dry season χ^2 = 33.78, df = 1, P < 0.001, rainy season χ^2 = 69.96, df = 1, P < 0.001). When the data from the two sites (uphill and valley) were pooled (Table 4.1), there was no significant inter-seasonal differences in seroprevalence in <5 and ≥15 years age groups (P = 0. 170 and P = 0.190, in respective order)**.** In contrast, the inter-seasonal variability in seroprevalence was marked within the 5–14 age-group (χ^2 = 10.73, df = 1, P = 0.001), [Table 4.1].

Table 4.1: MSP-1_{19} seroprevalence (%) accross age-groups, season and study sites in western Kenya Highlands

Uphill						Valley				
Season	<5 (n=88)	5−14 (n=174)	≥15 (n=143)	χ^2-value**	*P*	<5 (n=83)	5−14 (n=151)	≥15 (n=160)	χ^2-value**	*P*
Dry	27.50	36.70	75.00	30.34	<0.001	60.00	70.00	98.40	45.59	<0.001
Rainy	25.00	49.10	64.80	17.21	<0.001	71.20	87.10	90.70	11.56	0.003
χ^2-value*	0.067	2.402	1.770			1.007	6.496	3.686		
P	0.794	0.120	0.180			0.315	0.010	0.054		

* Comparison between seasons, df = 1 for all tests

** Comparison among different age groups, df = 2 for all tests

4.2.1 Spatiotemporal variation in total IgG titres

On the whole, increases in MSP-1_{19} seroprevalence resulted in corresponding increases in antibody levels. This was true for both the uphill (R^2 = 0.847, P < 0.001,) and valley residents (R^2 = 0.623, P = 0.011). Controling for age, it was observed by means of multiple logistic regressions, that the odds of having parasites were higher for those with detectable level of MSP1-$_{19}$ antibodies (Table 4.2).

Table 4.2: Association of MSP-1_{19} seroprevalence with parasite prevalence in the different localities

	Parasite prevalence		
MSP-1_{19} seroprevalence			
Locality (n)	***Odds Ratio***	***95% CI***	***P value***
Uphill (401)	2.798	[1.018, 7.693]	0.046
Valley (394)	3.167	[1.196, 8.386]	0.020
Total Uphill and Valley (795)	4.282	[2.200, 8.330]	< 0.001

n = total number of sera analyzed at a particular location

4.2.2 Spatiotemporal heterogeneities in total IgG titres

Total IgG titres observed in the whole population was highly heterogeneous. Temporal and spatial differences as in inter-seasonal and altitudinal variation in the total IgG titers (expressed in titer units) were determined in the uphill and valley residents. Whereas the median IgG titers in the uphill residents was less than 100, that of the valley bottom was above 1000 (Table 4.3). This over 10-fold differences was observed both at the 25^{th} and 75^{th} percentile even before statistical analysis was performed.

Table 4.3: Median Total IgG titres observed from Uphill and Valley residents

Study sites	Uphill	Valley Bottom
No. tested	401	394
Minimum	1.000	1.000
25% Percentile	15.50	184.6
Median	62.80	1102
75% Percentile	378.0	5889
Maximum	36608	117293

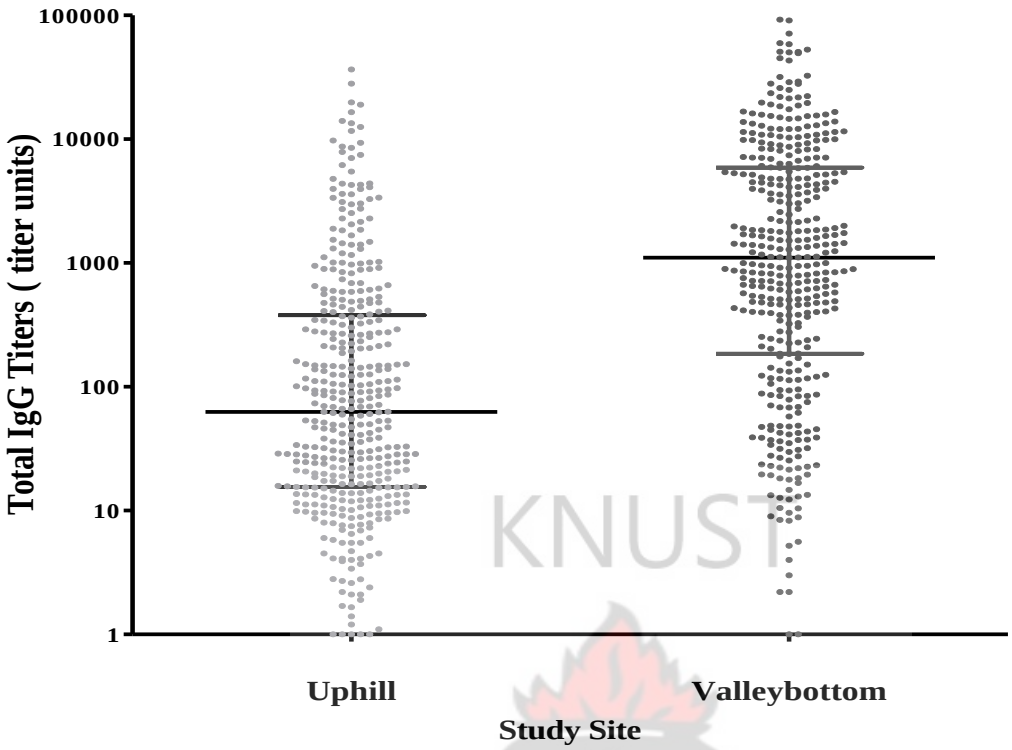

Figure 4.5 scatter plots showing differences in IgG titers between Uphill and valley bottom residents. (Test of significance was by Mann–Whitney, *P* value < 0.001 (Uphill n = 401, Valley n = 394). The error bars show the median and inter-quartile range

It was observed that residents from the valley bottom area had a 13-fold higher titer levels than residents from the uphill population (Mann-Whitney test, z = 13.17, $P < 0.0001$). Age-stratified analysis of IgG titers showed a steady increase with age (Figure 4.6). Post-hoc comparison tests revealed interesting differences: median IgG titers between <5 and 5-14 age groups at both uphill and valley bottom sites were not significantly different from each other ($P > 0.05$). However, the younger age groups (<5 and 5-14) were significantly lower than the older (≥15 yrs) age groups.

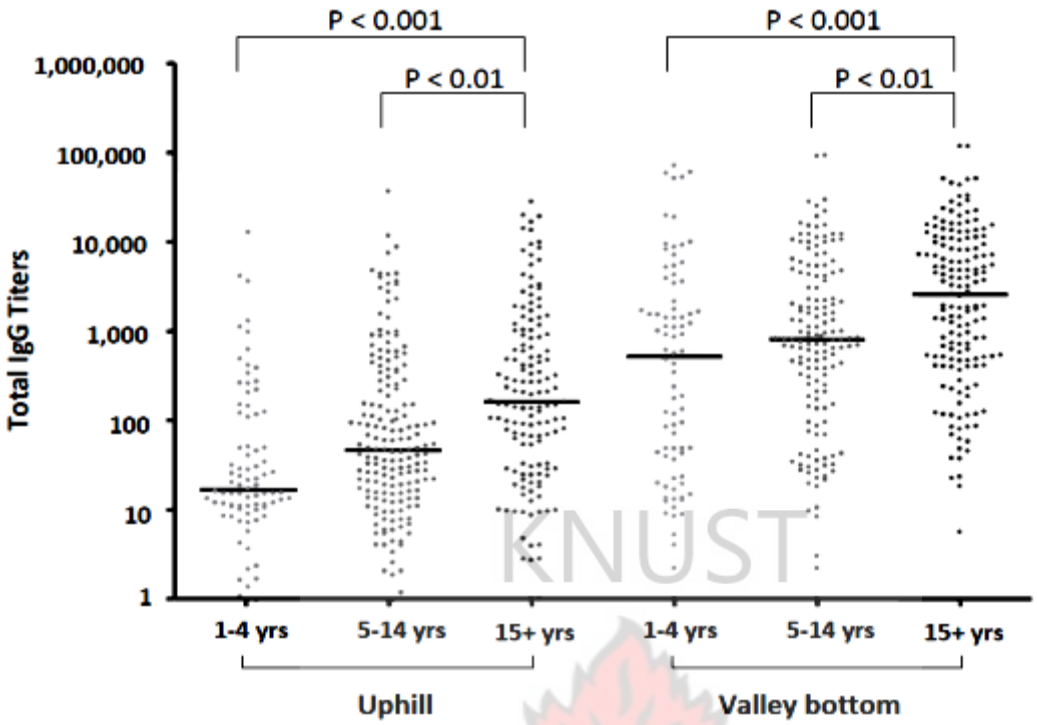

Figure 4.6: Differences in IgG titers between Uphill and Valley Bottom sites, stratified by age. Horizontal bar indicates median value. P value indicates the significance test by Kruskal-Wallis test. Only significant tests are shown.

When MSP-1_{19} seroprevalence in the <5 yrs age groups residing at the three locations; the uphill, the valley and residents of Kombewa, a lowland high transmission area, were compared, it was observed that the uphill population had a significantly lower seroprevalence than both the valley bottom and lowland areas (χ^2 = 24.9, df=2, p = 3.87x 10^{-6}); however, there was no significant difference in seroprevalence between either the high transmission area of lowland and the valley area in the highlands (χ^2 = 2.3, df=1, *p* = 0.13) *p* values are reported in standardized format in the the figure below [Figure 4.7].

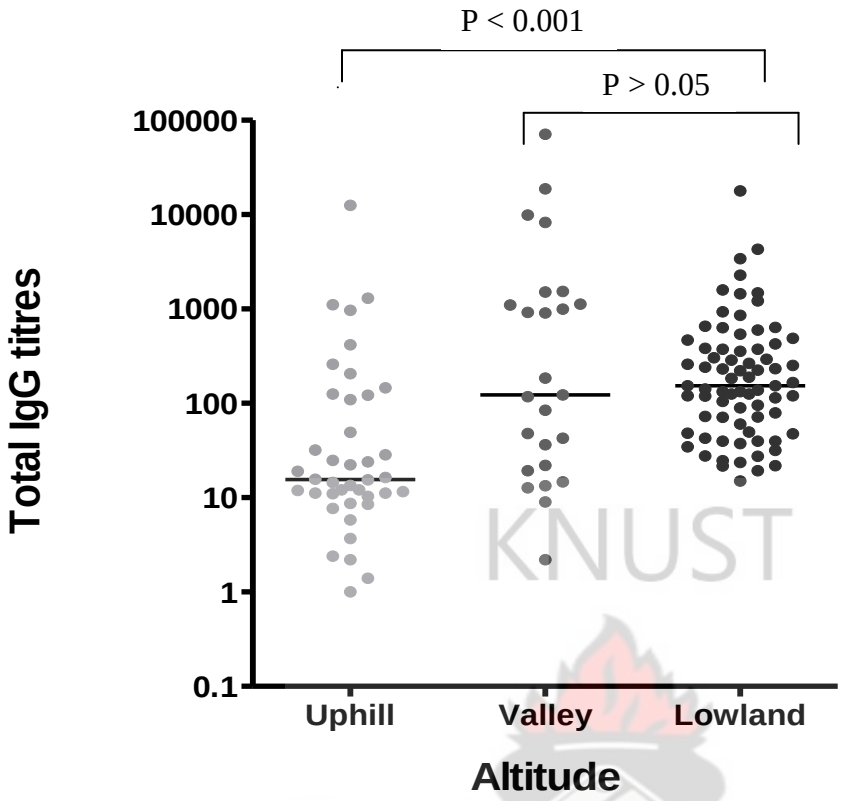

Figure 4.7: Scatter plot of IgG titers of children aged 5 or less across three altitudes - transects during the dry season. *P* value indicates significance by Kruskal-Wallis test. Only the significant tests are shown.

It is noted however, that sera from lowland Kombewa area for this particular assay was available from a previous study (Ogutu *et al.,* 2009) and only the sera taken before vaccines were given to the study participants was used and for that matter only less than 5 year old samples were used for the comparison.

4.2.3 Age-dependent antibody acquisition model

By means of the simple catalytic model applied to examine the force of infection on age, it was observed that seroconversion and seroreversion rates were dependent on age (Fig. 4.8)**.** Annual seroconversion rate of 8.3% and reversion rate of 3.0% were seen in the uphill population with seroprevalence saturating in the population at 73.3% at age 20. However in the valley population, the annual rate of conversion from seronegative to seropositive was 35.8% with 3.5%, reverting to seronegative status annually. By age 10, seroprevalence had reached 91.2% within the valley population (Fig. 4.8).

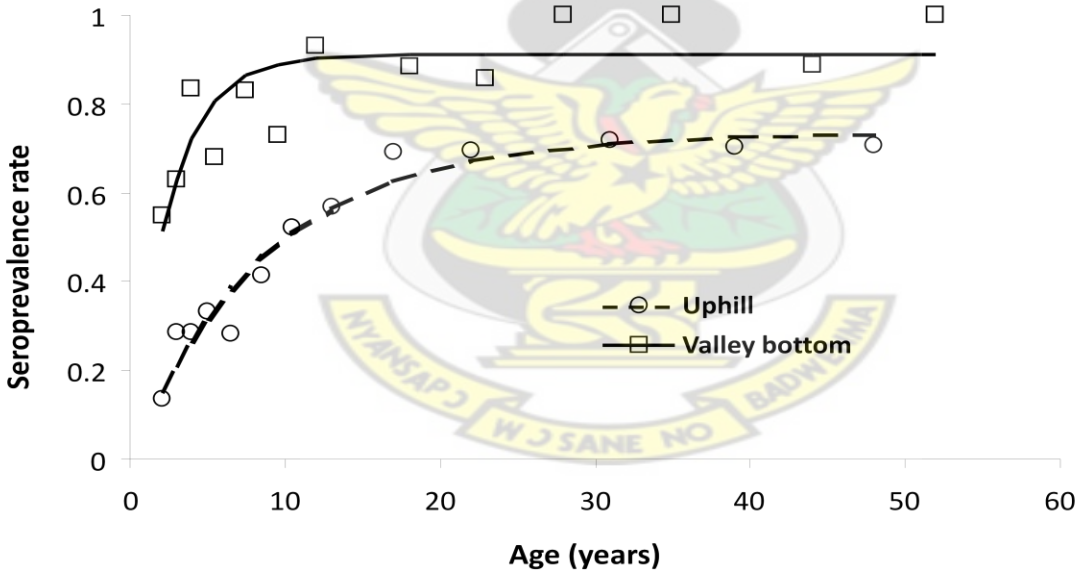

Figure 4.8: Age-dependent seroconversion rates at Uphill and Valley bottom sites. Uphill model: Pt = 0.73(1-e-0.11t), R2 = 0.95, *P* < 0.001; valley bottom model: Pt = 0.91(1-e-0.39t), R2 = 0.67, *P* < 0.001.

4.3 ITN Coverage

A total of 300 households were surveyed for their ITN usage. The overall ITN coverage was 71.0% in the valley bottom and 48.5% in the uphill population (fig 4.9). χ^2 analysis revealed that the coverage in the valley bottom residents was significantly higher than the coverage in the uphill population ($\chi^2 = 27.5$, d.f = 1, $P < 0.001$). ITN coverage varied among age groups but the observed differences were not statistically significant.

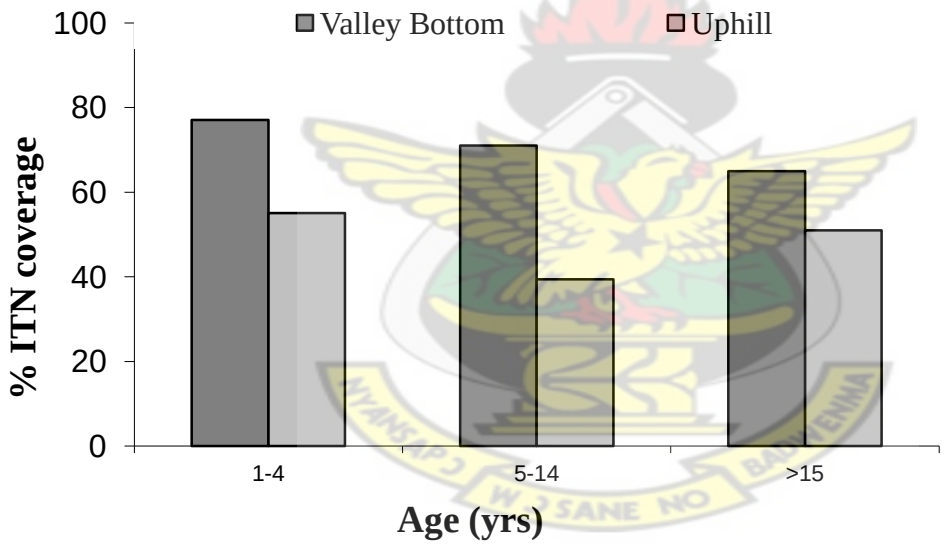

Figure 4.9: ITN coverage by age groups of residents in the uphill and valley bottom study sites

4.4 Antibody Responses to *Anopheles gambiae* Peptide gSG6 P1 in Humans under Varying Transmission Intensities

4.4.1 Study population

All together a total of 1458 sera were available for gSG6-P1 immuno-assay out of which 1366 (93.7%) of them were tested successfully, 92 (6.3%) of them had failed test and thus were not included in data analysis. 744 (54.5%) of tested samples were archived sera obtained from the first (MSP-1 $_{19}$) study, the remaining 622 (46.5%) of them were obtained from the primary school survey conducted in 2011. 607 (81.6%) out of the archived samples had known specific antibody responses to MSP-1_{19}. 322/744 (43.3%) belonged to the uphill community whilst 422/744 (56.7%) were from the valley bottom area. Participants were categorized into three age groups, i.e. <5, 5-14 and ≥ 15 respectively, from the uphill community, 49, 153, and 120 participants were tested. Similarly from the valley bottom population, 79, 159 and 184 belonging to the respective age categories <5, 5-14 and ≥ 15 were tested. A total of 384 (51.6%) were sampled in the dry season whilst 360 (48.4) tested were from the rainy season. The second survey conducted in 2011 sampled a total of 622 participants from three primary schools in three communities that also had different malaria epidemiology. 202 (32.5%) participants were sampled from the Kombewa, 203 (32.6%) from Kakamega area and 217(34.9%) from the Kisii area. All participants were primary school pupils between the ages of 5 and 16. The distribution of the total sera analyzed from Kombewa, Kissi and Kakamega are illustrated in figure 4.10 below.

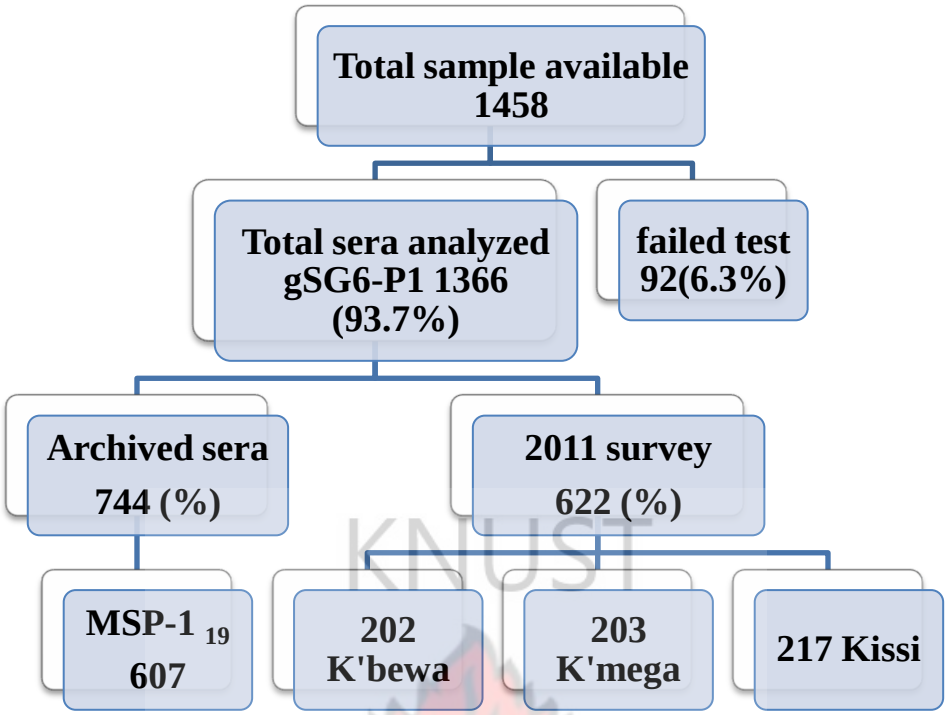

Figure 4.10: A summary of study population partitioning number of sera tested per study site.

4.4.2 Overall IgG levels against gSG6-P1 in valley and uphill residents and MSP-1_{19} seroprevalence

Between the uphill and valley populations, there were significant variability in both total IgG levels and seroprevalence gSG6-P1. In general 36% of uphill residents were seropositive compared with 50% who were seropositive within the valley population (χ^2 13.2 P =0.0002). Antibody levels within the valley population were two-fold higher in comparison to that seen in the uphill population (Mann Whitney; $P < 0.001$; Figure 4.11A). Again significant differences were seen in the antibody levels between the rainy and dry seasons (Mann Whitney; $P = 0.028$; Figure 4.11 B), with higher levels in the rainy season.

A

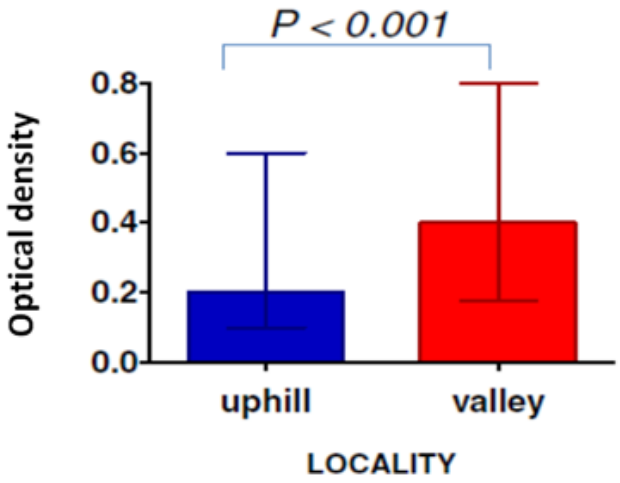

Fig. 4.11A: Median antibody responses to gSG6-P1 in uphill and valley bottom residents; Uphill (n= 322) and Valley (n=422) residents, Mann Whitney: *P*< 0.01 Error Bars indicates interquartile range (25% - 75%).

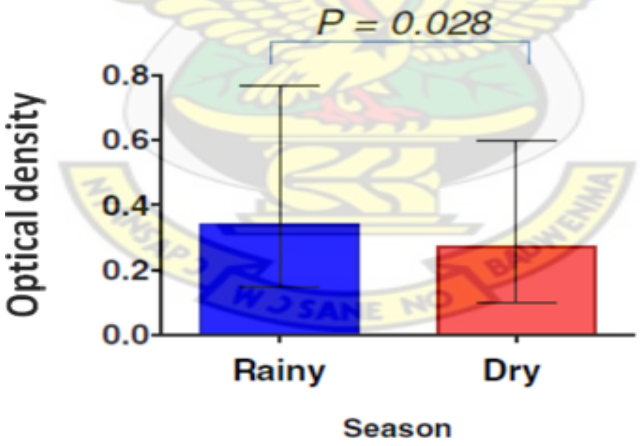

Fig. 4.11B: Median antibody responses in Rainy (n= 360) and Dry (n=384) seasons. Mann Whitney: *P* = 0.028. Error Bars indicates interquartile range (25%-75%).

4.4.3 The association between gSG6 –P1 and MSP -1_{19} exposures

The risk of having detectable MSP-1_{19} specific antibody, following exposure to the parasite was constantly elevated for gSG6-P1 seropositives (Table 4.5). In total the odds of gSG6-P1 seropositives having detectable MSP-1_{19} antibodies were about 3 times higher than unexposed. This was significantly so at both study sites (Table 4.5). Individuals with detectable MSP-1_{19} are more likely to carry gSG6-P1 antibodies than in the case of those individuals who did not have MSP-1_{19} antibodies. The corollary is that individuals with evidence of exposure to mosquito bites more often than not have been exposed to malaria parasites.

Table 4.5: Logistic regression of seroprevalence between gSG6-P1 and MSP-1_{19}

	MSP-1_{19} seroprevalence		
gSG6-P1 seroprevalence			
Study site(n)	***Odds Ratio***	***95% CI***	***P value***
Uphill area (232)	2.168	[1.203, 3.903]	0.010
Valley bottom (375)	2.668	[1.550, 4.592]	< 0.001
Total Uphill and Valley (607)	**2.873**	**[1.977, 4.176]**	**< 0.001**

n = number of study participants in the study area included in the analysis

4.4.3 Age-linked-trends in parasite prevalence and antibody response to gSG6-P1 and MSP-1 19

No particular trend was seen between age and parasite prevalence in the uphill cohort (Fig. 4.12A), but within the valley cohort an inverse relationship was observed that was significant (Fig. 4.12B). This was also the case when the uphill and valley bottom data were combined (Fig 4.12C). Parasite prevalence was generally low when compared with seroprevalence of both antigens at study sites, (Fig 4.12 A-C). Marked variability was seen in the slopes based on the uphill study population when lineer logistic regression was applied (F = 5.216, DFn=2, DFd=21, *P* =0.014), also in the valley population (F = 4.328, DFn=2, DFd=21, *P* =0.026) and when both populations were analyzed together (F = 6.513, DFn = 2, DFd=48, *P* =0.003). MSP-1_{19} seroprevalence correlated well with age at the two study sites and when the data was combined (Fig 4.12). There was however a weak relationship seen between gSG6-P1 and age (R^2 = 0.19), particularly so in the valley population (R^2 = 0.10). But the correlation in the uphill was considerable (R^2 = 0.40) Figure 4.12A.

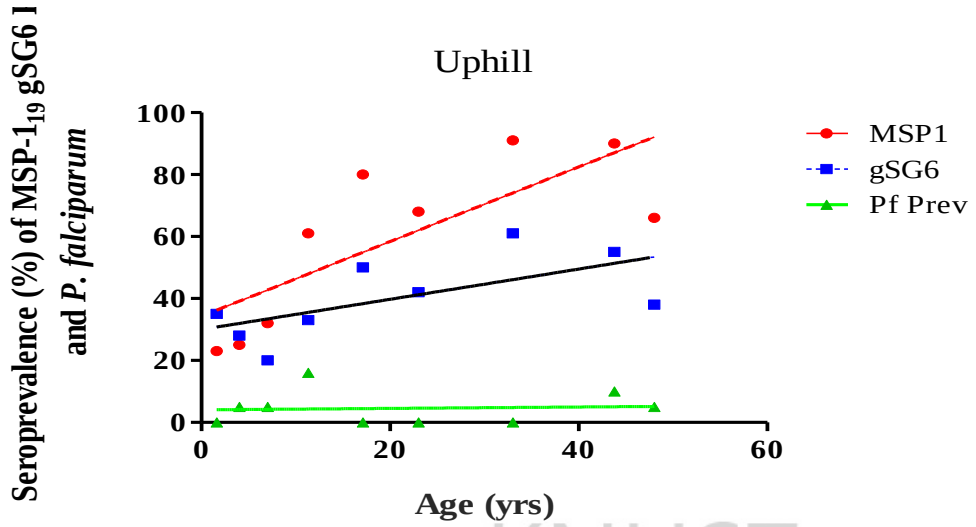

Figure 4.12A: Age trends in gSG6-P1, MSP-1$_{19}$ and parasite prevalence at Uphill (n = 232)

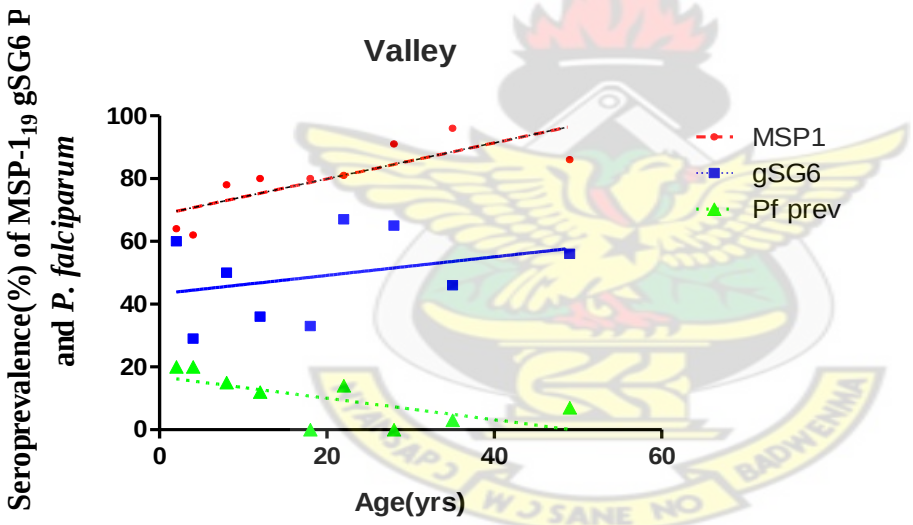

Figure 4.12B: Age trends in gSG6-P1, MSP-1$_{19}$ and parasite prevalence at valley bottom (n=375).

Total population

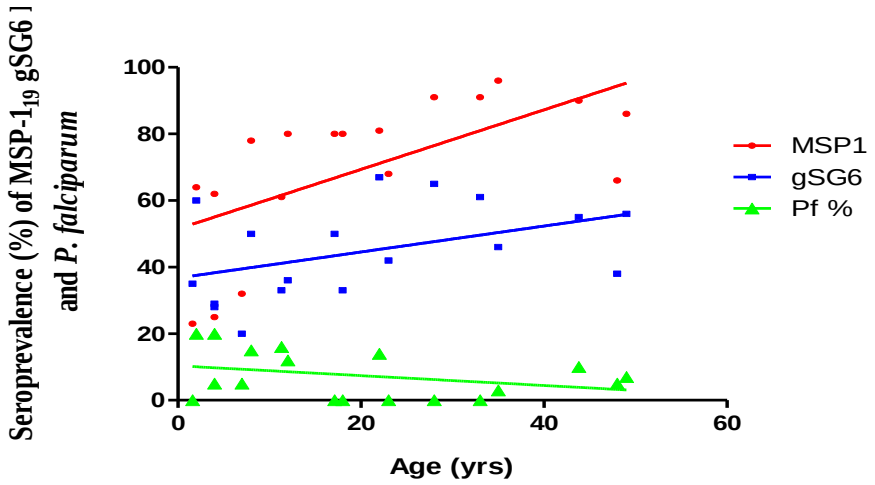

Figure 4.12C: Age trends in gSG6-P1, MSP-1$_{19}$ and parasite prevalence at both sites

The variability in the degree of responses in-between MSP -1$_{19}$ and gSG6-P1 specific antibodies, as shown by the disparities in their intercept were very significant at both study sites and when combined (Table 4.6). However, there were no significant differences in antibody trends as depicted by the slopes.

Table 4.6: Statistical comparisons of age trends (slope) and specific Antibody level (intercept) between gSG6-P1 and MSP-1$_{19}$ at different locations.

Uphill	**F**	**DF n**	**DF d**	***P* value**
Slope	2.717	1	14	0.122
Intercept	6.742	1	15	0.020
Valley				
Slope	0.585	1	14	0.457
Intercept	34.189	1	15	<0.001
Total				
Slope	2.244	1	32	0.144
Intercept	22.191	1	33	<0.001

4.4.4 Antibody responses to gSG6-P1 under different transmission settings and risk of parasite exposure

Significant differences ($P< 0.001$) were observed in parasite prevalence across different transmission settings. The lowest parasite prevalence was seen from the Kisii County which is hypoendemic (4%) followed by 19.7%. prevalence seen in the mesoendemic Kakamega area. The highest parasite prevalence 44.6% was seen in the hyperendemic Kombewa area. The observed parasite prevalence correlated well with increasing gSG6-P1 seroprevalence which also followed the malaria transmission intensity corresponding to Kisii (28%), Kakamega (34%) and Kombewa (54%), (Fig. 4.13).

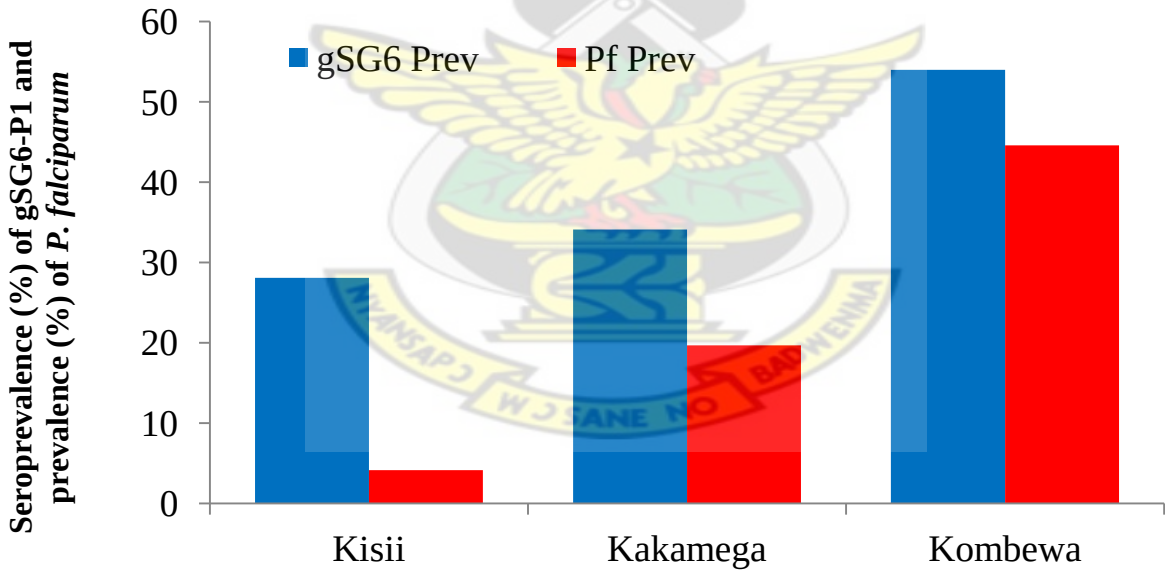

Figure 4.13: A bar graph of gSG6-P1 seroprevalence and the respective parasite prevalence at endemic localities: Kisii (n= 222), Kakamega (n= 203) and Kombewa (n= 202).

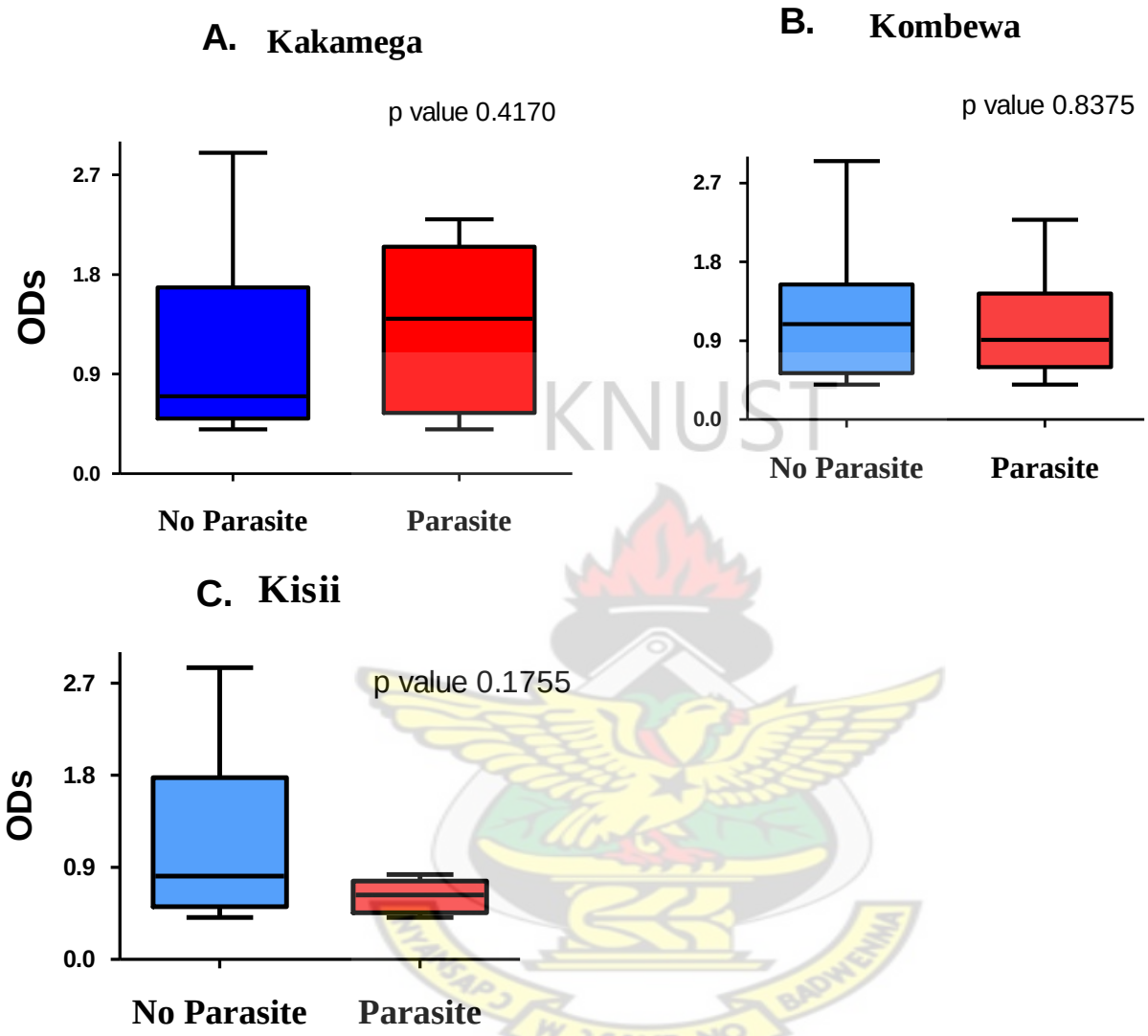

Fig. 4.14: Median antibody responses to gSG6 P1 in individuals with *P. falciparum* infection, and in individuals without infection, at different malaria transmission settings; A Kakamega, B Kombewa and C Kisii. The Box and whiskers: where the whiskers show the range of antibody responses in OD in the population, the box shows the inter-quartile ranges (min 25% max 75%) and the line in the box shows the median antibody responses.

4.4.5 Comparison of gSG6-P1 antibody levels in individuals with *P. falciparum* infections and those without infections

The levels of total IgG to gSG6-P1 measured in optical densities (ODs) were compared in MSP -1_{19} seropositive individuals and seronegative individuals. This was done to determine whether individuals exposed to *P. falciparum* infection (MSP -1_{19} seropositives) had higher levels than unexposed individuals or otherwise. It was observed that MSP-1_{19} seropositives, that is individuals with detectable antibody levels to MSP -1_{19} had significantly higher levels of gSG6 – P1 ($P < 0.0001$) than individuals with no antibodies to MSP – 1_{19} (Fig 4.15)

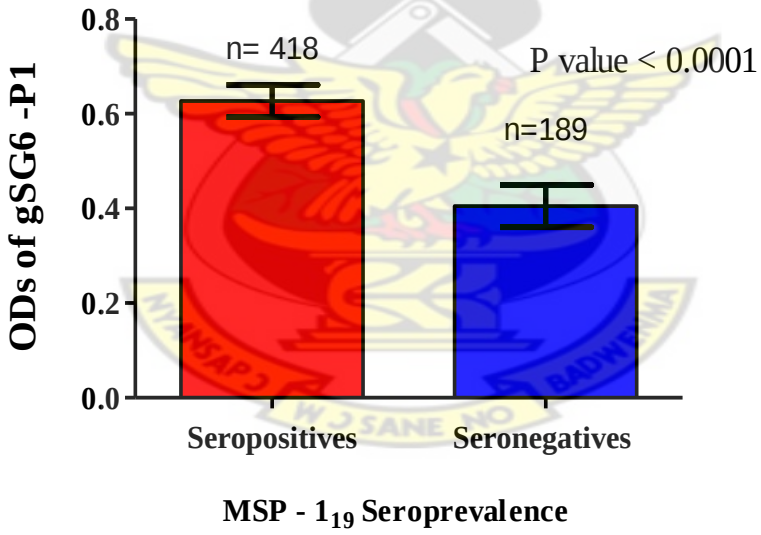

Figure 4.15: Comparison of the Mean total IgG levels to gSG6 P1 in MSP – 1_{19} sero positive and negative individuals. The error bars show standard error of the mean.

4.5.0 Accuracy of diagnosis of the *Pf*HRP-2- based rapid diagnostic test (RDT) for malaria

A total of 859 study subjects participated in this study; 354 from the Ghana site and 505 from the Kenya site. The breakdown of the study population from the specific study sites within the two countries are given in the Fig. 4.16 below. The Ghana site was an operational study, subjects were therefore, recruited from the hospital among patients referred to the laboratory for routine malaria test and thus had no age restrictions. However in Kenya participants were drawn from asymptomatic pupils in the primary schools. The Kenyan study population thus was restricted to the age range between 5 years to 16 years.

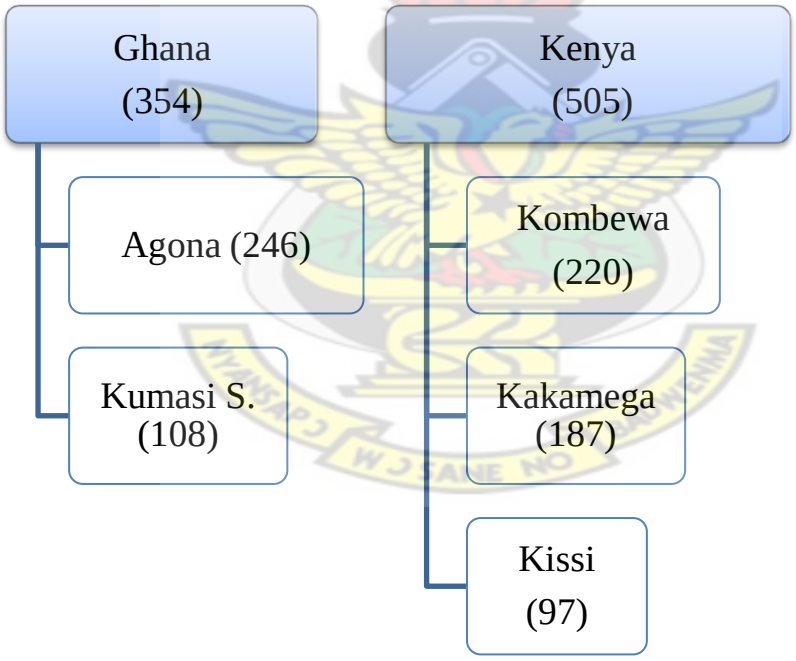

Figure 4.16: Total numbers of study participants in Ghana and Kenya and their respective study locations

4.5.1 Percentage prevalence of P. falciparum at all study sites measured by RDT and microscopy

In the Ghanaian population, participants from the rural area of Agona had high parasite prevalence as determined by microscopy and RDT respectively (Fig. 4.17). The parasite prevalence in the Agona area was higher than that observed in participants from Kumasi South (Atonsu-Agogo) which was respectively determined by microscopy and RDT.

χ^2 analysis revealed that indeed there were no differences between the parasite prevalence determined by microscopy and by RDT in the Agona population (Table 4.15). However, the differences between the parasite prevalence in the Kumasi population as determined by microscopy and the RDT differed significantly by Chi square test (Table 4.15).

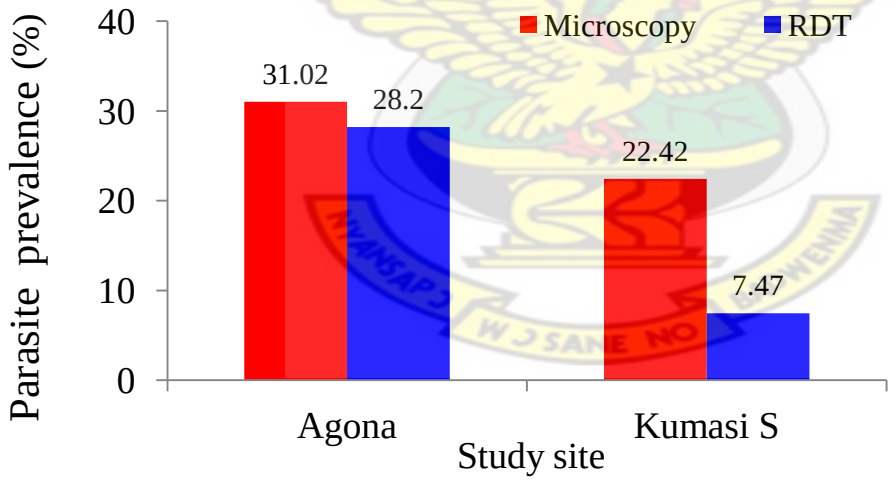

Figure 4.17: Parasite prevalence as determined by RDT test and microscopy from the Ghanaian study locations

Table 4.7: Comparison of the percentage prevalence of *P. falciparum* at Ghanaian and Kenyan study sites as determined by rapid diagnostic test (RDT) and microscopy

	RDT	Microscopy	X^2	P value
Agona	28.2	31.02	0.479	0.488
Kumasi South.	7.47	22.42	9.406	0.002
Kombewa	70.9	65.1	0.668	0.410
Kakamega	34.2	20.3	9.112	0.002
Kissi	5.1	1	2.75	0.097

Asymptomatic *P. falciparum* prevalence in the Kenyan school pupils was high, in Kombewa and Kakamega but lowest in Kisii as determined by microscopy, the corresponding prevalence determined by the rapid diagnostic test (RDT) were also determined (Fig. 4.18).

χ^2 analysis of the differences in parasite prevalence as determined by the RDT and expert microscopist showed similar trends as observed in the Ghanaian study. There were no significant differences between RDT test and microscopy at the Kombewa site with very high prevalence and also the Kissi area with very low parasite prevalence. However there was a significant difference in the parasite prevalence in the Kakamega area with moderately high parasite prevalence (Table 4.7).

4.5.2 Trends in participants' age-related parasite density in the Agona and Kumasi South study in Ghana

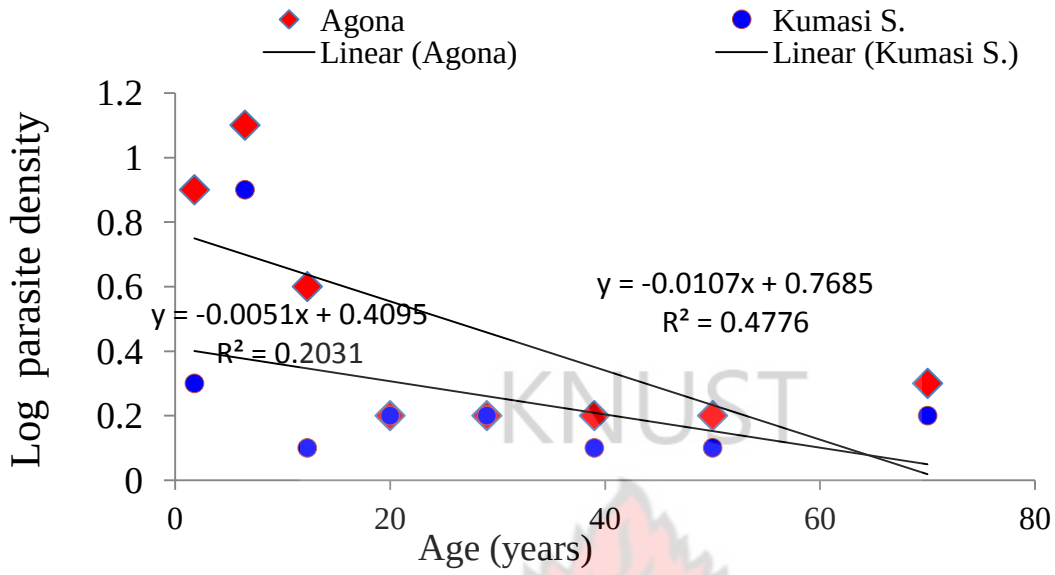

Fig. 4.18 Trends in age of participants and parasite density from the Agona District Hospital and the Kumasi South Hospital.

Parasite density was negatively associated with age of participants; children below the age of 10 had very high parasite densities. Parasite densities declined after the age of 12 and remained stable 20 years up until the age of 50 years, after which there was an apparent rise in parasite density that may be due to immunoscenescence. There was more than a 40-fold difference in the parasite density measured between the rural Agona population and the urban population of Kumasi with the Agona area having substantially higher parasitemia (Fig. 4.18). The differences in parasite density were marked especially in the lower age groups. These differences gradually diminished as the population advanced in age.

4.6.0 Performance of *Pf*HRP2 based malaria RDT

Table 4.8; Sensitivity, specificity, negative and positive predictive values of the RDT test with microscopy as the Gold standard, based on test result from all the study sites except Kisii

	Parasite /µl blood	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
Agona	≤ 250	34.4	88.5	78.4	52.5
	> 250	85.7	72.2	76.4	82.7
Kumasi	≤ 250	33.3	97.6	93.1	60.0
	>250	40.0	ND	100	ND
Kombewa	≤ 250	77.4	33.3	81.5	27.9
	>250	84.4	100	6.25	100
Kakamega	≤ 250	70.0	77.7	97.4	17.5
	>250	85.7	100	20.0	100

NPV = negative predictive value, PPV = positive predictive value, ND = not determined

Generally the RDT test kit performed poorly with microscopy as the Gold standard at the parasite density of ≤250 parasites per micro liter of blood (Table 4.8). This was very much so with the operational study conducted under the hospital routine settings in Ghana. At higher densities of greater than 250 parasites per micro-liter of blood, the RDTs sensitivity improved substantially. Specificity the ability of the test to correctly generate a negative result in the absence of a disease state was generally very good across study sites and parasite densities. The lowest specificity observed 72% this appeared to improve with parasite density, eventually reaching 100% within the Kakamega and Kombewa moderate to high malaria transmission endemic areas.

4.6.1 Polymerase chain reaction (PCR) analysis

For logistic reasons only one hundred randomly selected parasite positive blood by microscopy examinations were tested to confirm the proportion of *Plasmodium falciparum* species among the other species i.e *P. ovale and P. malariae* that may be present. It is common knowledge that, in sub-Saharan Africa, among people of black skin, the *Plasmodium falciparum* constitutes the main parasite species about 95-98%. Although the *Pf*HRP2 was evaluated against only the Gold standard microscopy, it was important to confirm the proportion of *P. falciparum* because the *Pf*HRP2 RDT test kit is only specific *P. falciparum.*

Out of a total of 300 randomly selected parasite positive (*Plasmodium* species) 276 were confirmed by PCR as being *Plasmodium falciparum* which corresponded to approximately 92.0%. The remaining 24 were not amplified since only *P falciparum* specific primers were used. However these 8% may be due to the presence of *P. ovale* and *P. malarie* which are known to be present in the forest zone of Ghana and in the highlands of western Kenya. There is no evidence of the presence of *P. vivax* and *P. knowlesi* in Ghana and in the Kakamega County of western Kenya where the study took place as at the time of field surveys.

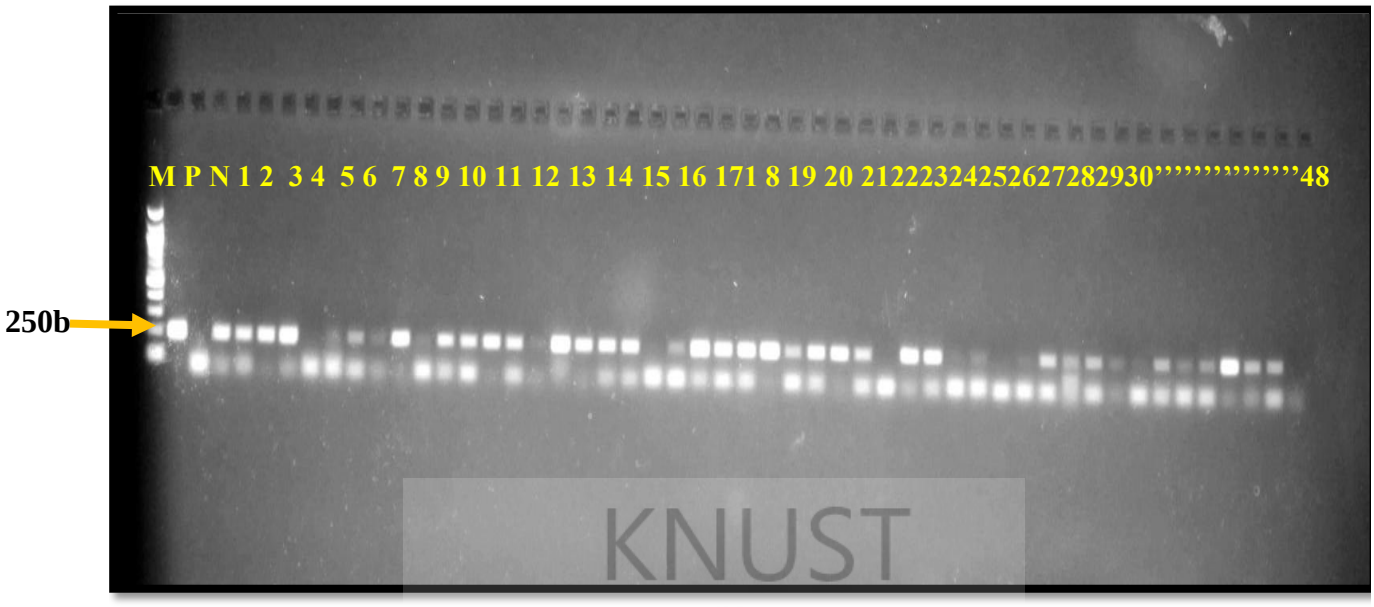

Plate 3.5: Gel electrophoresis of *P. falciparum* amplification products

Gel red stained 1.8% agarose gel electrophoresis of PCR products obtained from the amplification of *Plasmodium falciparum* DNA for species identification. Lanes 1-48 = Amplified DNA *P. falciparum products*, M = 100 bp molecular weight marker, P is a positive controls for *P. falciparum* (plasmids obtained from MR4 at 250bp). N is Negative control containing only molecular grade water and no template DNA.

CHAPTER FIVE: DISCUSSION

5.1 Variation in MSP-1_{19} Responses in the Western Kenyan Highlands

Analysis of age-specific MSP-1_{19} seroprevalence, seroconversion rates (SCR), total IgG titres, and *Plasmodium falciparum* prevalence, showed that, human exposure to malaria parasites in western Kenya is highly variable. Seroprevalence observed within the valley bottom residents was twice as high as that within the uphill residents. This suggests that there is a higher exposure to the risk of pathogen transmission in the valley bottom population than the uphill within the uphill inhabitants. Comparison of the median total IgG titres in the study population revealed even higher heterogeneity in malaria exposure; where the total IgG levels in the valley residents was thirteen-fold higher than that of the uphill residendts. Consistent with these findings are other studies based on entomological and parasitological surveys conducted at the same study area, which have found higher transmission intensity, mosquito spatial aggregation and parasite prevalence at the valley bottom, when compared to the hilltop area (Githeko *et al.*, 2006; Baliraine *et al.*, 2009, Munyekenye *et al.*, 2005). This may be explained by the differences in the accumulation of water between the two study sites. The river Yala which transcends the valley bottom provides permanent breeding sites for the *Anopheles* population responsible for malaria transmission throughout the year. This results in a high risk of exposure to infectious vectors for the valley bottom population. But this is not so for the uphill inhabitants as there is paucity of breeding habitats due to the highly effiecient drainage presented by the highland topography (Githeko *et al.*, 2006, Baliraine *et al.*, 2009; 2010; Munyekenye *et al.*, 2005).

Immune response to malaria is a function of the challenge due to exposure; it develops steadily by means of multiple parasite exposures or the harboring of persistent infection over years. As a

result, seroprevalence so generated represent the collective exposures. It is thus largely unaffected by short-term fluctuations in transmission intensity such as seasonality. This is also due to the fact that MSP1-$_{19}$ specific antibody response persists longer than any discrete infection or even season (Drakeley *et al.,* 2005b; Bousema *et al.,* 2010b). MSP1-$_{19}$ as a biomarker may be limited in tracking discrete seasonal fluctuations in transmission but nonetheless a better indicator of long-term transmission potential. The persistence of antibody response generates seroprevalence that are by far higher than corresponding parasite rates and this makes it a more sensitive marker (Drakeley *et al.,* 2005; Stewart *et al.,* 2009). The high seroprevalence observed in the valley residents may be indicative of highly frequent and or persistent parasite exposure and infection. A previous study at this site, observed that about 38.2% of the valley bottom residents surveyed, carried asymptomatic infections that lasted up to five months, a further 14.2% of them had infections that even persited for longer periods from 6–12 months (Baliraine *et al.,* 2009). Another study based on the persistence of single parasite genotypes observed frequent acquisition and clearance of infections. With the mean infection duration of a particular parasite genotypes lasting for one month and one day, but the longest observed genotype persistence was three months (Baliraine *et al.,* 2010). The presence of any density of parasitemia (exposure); whether persistence (recrudescence) or rapid turnover (acquisition and clearance), have the inherent ability to elicit immune response thus leading to acquisition and maintenance of high levels of anti-malarial antibodies. It is known that persistent of even sub-patent infection is enough to maintain partial immunity which is consistent with the premunition concept (Sergent *et al.,* 1924).

5.1.1 Seroconversion rates and malaria transmission intensity

Seroprevalence within specific age-groupings have been used to calculate seroconversion rates (SCR) as an alternative estimate for malaria transmission intensity. Earlier studies in Tanzania have reported strong correlations of the SCR with entomological index (Corran *et al.,* 2007; Drakeley *et al.,* 2005b; Stewart *et al.,* 2009). Age sero-prevalence curves corresponded to varrying levels of transmission intensity. In a population under low or unstable malaria transmission, the build up of antibodies may be slow, which implies that detectable antibody levels are observed mainly in the adult population. In contrast, an area under high transmission, majority of the population will show detectable antibody levels even at a much tender age (Drakeley and Cook, 2009). This observable fact is clearly confirmed in the age seroprevalence curves in the current study (Fig. 4.8). Within the valley bottom population, a peak of 91.2% seroprevalence is achieved at a tender age of 10. However, in the uphill population one has to reach the age of 20 before the peak of 73.3% seroprevalence can be achieved. These findings bring to the fore the difference in malaria transmission intensity between the two populations, and point to a higher force of infection that the inhabitants of the valley area are subjected to in comparison to the uphill area. These findings agree with other data such as the vector aggregation densities, and the frequency of malaria exposure and asymptomatic infections between valley bottom and uphill as previously reported (Githeko *et al.,* 2006; Baliraine *et al.,* 2009; 2010).

5.1.2 Seroepidemiology; a valuable tool in malaria control and elimination

Under low malaria transmission settings, absolute numbers of mosquito and sporozoite rates are low and blood parasite prevalence are also low. Using entomological inoculation rates in the

determination of malaria transmission intensity under such settings are known to be less sensitive and thus, unreliable (Mbogo *et al.,* 2003). Immuno-epidemiology presents an alternative way of assessing malaria endemicity accurately. It has the potential to identify focal areas of higher transmission (the so called "hotspots') thus enhancing the chances of malaria elimination (Drakeley and Cook, 2009). In the past, a number of studies have applied immuno-epidemiology for the same purpose. For example several serological surveys were conducted in the 1970s to evaluate the impact of elimination and eradication campaigns in several countries. In Tanzania, repetitive cross-sectional sero-epidemiological surveys were conducted among some 1,500 individuals; to accurately differentiate between areas of varying transmission intensity (Lelijveld *et al.,* 1972). Similarly in Surinam**,** in order to support an elimination program**,** about 2,000 participants of all age-groups were serologically assessed and their immune responses to the specific antimalarial antibody corresponded to the malaria burden at the time of sample collection and precisely identified areas which had already eliminated malaria (Sulzer *et al.,* 1975). Immuno-florescent antibody assays were applied to track decreasing seroprevalence over several years during an eradication program in Mauritius and Tunisia until no seropositives were found in children below the ages of five and fifteen. This evidence thus confirmed the success of the eradication campaign at the time (Bruce-Chwatt *et al.,* 1973; Ambroise-Thomas, 1976).

When age-specific IgG titres from the two sites were compared there was a highly significant varriation between the inhabitants of the valley area and that of the uphill, this was indicative of a substantial differences in malaria transmission within that small spatial scale in the highland area. The observed temporal changes in total IgG titers within the 5–14 year age-group pointed to the fact that, their antibody level is changeable with the resultant differential seasonal exposure. This apparent unstable immunity within that age-group tags them as vulnerable and

susceptible to malaria epidemic in an event of hyper-malaria-transmission. This poses a public health threat if they are not specifically targeted for control and preventive interventions. As persons stay seropositive for more than a few years, their serum antibody density (IgG titers) could reflect recent seasonal exposure to malaria parasites. It has been reported previously that antibody titers tend to be higher in persons with active infections but this level declines in tandem with parasite clearance (Drakeley and Cook, 2009). This observation is consistent with the finding in the current study that, the paediatric population (< 15 years age-group) from the valley bottom area also had the highest temporal parasite prevalence. The valley bottom is known for its persistent infections, a single parasite genotype was seen to persist for 3 months (Baliraine *et al.,* 2010), in depth assessment of IgG titre with age discovered that on the average, titre level by age 5 in the valley was comparable to that at age 20 in the uphill site. This was not unexpected because by the age of 10 seroprevalence in the valley bottom area already exceeded 90% (Fig. 4.8).

5.1.3 Declining parasite prevalence in the highlands

The parasite prevalence reported in this study is lower when put side by side with previous reports from the same area (Munyekenye *et al.,* 2005; Baliraine *et al.,* 2009). Munyekenye and co-workers (2005) obtained 47.0% as the average yearly parasite prevalence in children from the age of one to nine years and 9.5% in adults greater than nineteen year olds. Baliraine *et al.* (2009), applying the combined strength of microscopy and PCR parasite detection tools obtained parasite prevalence of 34.4%, 34.1% and 9.1% in five to nine, ten to fourteen and greater than fifteen year groups in respective order. This study carried out two cross-sectional surveys among all age groups, and reports a mean parasite prevalence of 14.0% among the

under-fives and the five to fourteen year olds but only 6.8% in the adult population over the age of fifteen. This result is based on microscopy detection only, which may underestimate parasite prevalence. This is because several people may habor submicroscopic infections below the detection threshold of microscopy. In contrast, the PCR technique or even the nested PCR has demonstrated in some cases the ability to detect 50% parasite prevalence higher than the prevalence determined by microscopy (Ropper *et al.,* 2006). However, all-together, these findings point to the fact that infection prevalence usually declines with increasing age and distance from the valley bottom and that infection prevalence in adults has not gone beyond 10% for a number of years till now. This may be due to the inherent ability of their acquired immunity to clear and repress parasites or with the help of antimalarials for parasite clearance. In general the mean infection prevalence in the valley and the uphill population were 16.3% and 6.3% respectively. Contrary, the parallel seroprevalence in respective order were 79.6% and 46.3% for valley and uphill. This implies using parasite prevalence alone as an indicator for malaria exposure; some 84% and also 94% approximately of in respective order of the valley and that of the uphill residents would be classified as 'unexposed'. Immuno-epidemiology thus offers not only a robust alternative but a much more sensitive tool in characterizing the malaria endemicity under low to moderate transmission intensity when parasite and entomological indexes are not sensitive enough.

A spectrum of MSP-1_{19} levels with wide-ranging titers in the same age-group and study site were observed. Other studies have reported similar inter-individual differences in exposure, susceptibility and disease patterns (Mwangi *et al.,* 2008; Creasey *et al.,* 2004; Bejon *et al.,* 2009). This finding may partly be explained by several factors such as host genetic polymorphism

(Williams, 2006), MSP-1 polymorphism (Tanabe *et al.*, 2007) or antigenic sin (affinity of the immune system to choose to utilize immunological memory based on a prior infectivity when a subsequent slightly different version is is presented). Nonetheless, in a population that primarily belong to a single sub-tribe, differential exposure to *Anopheles* bites will play an important role (Ye *et al.,* 2006). Fundamentally, individual-related factors such as household-roofing structure, use of ITN and other preventive measures (Ter Kuile *et al.,* 2003), as well as the proximity to breeding sites (Oesterholt *et al.,* 2006; Wilson *et al.,* 2007) may all be important determinants of this variation. The second objective of this study examined inter-individual exposure to *An. gambiae* salivary gland peptide (gSG6-P1) previously set as an indicator of mosquito bite exposure (Poinsignon *et al.,* 2008).

5.2 Variation of Host Antibody Responses to *Anopheles gambiae* Salivary Gland Protein

There were substantial variation in both gSG6-P1 seroprevalence and antibody levels when the immune responses within the valley bottom and uphill residents were compared. The antibody levels of gSG6–P1 in the valley population were two-fold higher than that seen in the uphill population. This implies that, there was a higher frequency of vector exposure in the valley population than the uphill population. Seasonal fluctuations in antibody levels were observed unlike the MSP1-$_{19}$ specific responses. The magnitude of gSG6–P1 specific response was higher in the high transmission-rainy season that usualy coincided with high vector exposure. There was a concomitant decrease in antibody levels in the low transmission-dry season, which also normally corresponded to vector densities and invariably low exposure. However this was not the case with the seroprevalence; where proportions of seropositive individuals did not change

significantly with season. Because individuals may remain seropositive for a long period of time, the seroprevalence may not be affected by seasonal variations; however, the levels of antibody response may reflect recent exposure. Antibody levels have a propensity to be higher in individuals who are actively exposed to antigenic agents and declines along with waning levels of the antigens (Drakeley and Cook *et al.*, 2009). Githeko *et al.* (2006) observed that the majority of the breeding habitats of malaria vectors in the highlands comprising 98 % of *An. gambiae s.l.* and 99% of *An. funestus* were restricted within 500m radius in the vicinity of the valley bottom residents. In contrast, only 1% of vectors were found each at the mid-hill and at the hilltop area. This may account for the differences in the seroprevalence and antibody levels to gSG6-P1 between the two sites. This is as a result of the hillside slope providing effective drainage within the valley ecological system and thus restricting mosquito aggregation to the valley bottom. It is well known that the topography and hydrology of the western Kenyan highlands play a critical role the malaria endemicity (Githeko *et al.*, 2006; Munyekenye *et al.*, 2005).

In respect to this evidence it is plausible to conclude that the gSG6-P1 immunoassay is robust and sensitive for the determination of the heterogeneity in malaria transmission risk. This was confirmed when the tool was able to distinguish between the vector population densities of exposure in two different populations that live about 5km apart.

5.2.1 gSG6 P1: a robust tool for assessing risk of parasite transmission?

By means of the multiple logistic regression, it was observed that, the odds of being seropositive for MSP-1 $_{19}$ specific antibodies was significantly higher for individuals with detectable levels

of gSG6-P1 antibodies in their sera. Implying that, the risk pathogen transmission is higher for those who are actively exposed to *Anopheles* bites. This observation was true both for the uphill and valley residents and also for the combined data. Against the background knowledge of different mosquito population densities from the two sites based on previous studies (Githeko *et. al.,* 2006). It is credible to state that, the higher the vector exposure, the higher the risk of pathogen transmission. This conclusion agrees with a well-known phenomenum in malaria epidemiology that malaria is heterogeneous at all levels (Stone *et. al.,* 2012). Others have reported that relatively low proportion of the population (20%) receive a majority (80%) of all infectious bites and disease burden (Smith *et al.,* 2005).

Moreover, seroprevalence of gSG6-P1 among school children in the three study villages in Western Kenya, followed similar patterns as that of parasite prevalence when these were compared. The villages had known entomological inoculation rates that establish the differences in their malaria endemicity. These were Kisii (0.4ib/y), Kakamega (16.6ib/y) and Kombewa (31.1 ib/y) their respective parasite prevalence rates as observed in this study were, in respective order, 4%, 19.7% and 44.6 %. The gSG6-P1 seroprevalence observed were as follows, Kisii 28%, Kakamega 34% and for Kombewa was 54%. For low parasite prevalence, the equivalent gSG6-P1 seroprevalence were higher which indicates its superior sensitivity.

At the population level, it was apparent that gSG6-P1 seroprevalence correlated well with the risk of pathogen transmission. Remoue and others (2006) reported that high anti-saliva antibody levels were predictive marker of clinical malaria. Again, anti-*An. dirus* salivary protein antibodies were found mainly in patients with acute *P. falciparum* or *P. vivax* malaria without occurance in persons from non-endemic areas (Waitayakul *et al.,* 2006). In the state of

Rondônia, Brazil, individuals were tested for antibody levels to *An. darlingi* salivary-gland-sonicates (SGS). Persons infected with *P. vivax* had higher levels of anti-SGS specific antibodies than non-infected persons. Thus, gSG6 immuno assay is a potentially useful epidemiological tool; antibody levels could discriminate between infection and non-infection with a high likelihood ratio (Andrade *et al.,* 2009).

Predictably, MSP-1_{19} Seroprevalence correlated well with age at each site and when the data was combined, the persistence as well as cumulative nature of MSP-1_{19} antibodies have been reported elsewhere (Wipasa *et al.,* 2010). Taken as a whole, the correlation between gSG6-P1 seroprevalence and age was weak, especially in the valley population. The apparent lack of correlation between age and gSG6-P1 seroprevalence as compared to MSP-1_{19} which had a good correlation, constitutes an evidence of the short duration of gSG6-P1 antibodies in the sera of persons who are known to be constantly exposed to *Anopheles* bites. Coupled with the observation of seasonal fluctuations in antibody levels to gSG6-P1, it is convincing to state that antibody to gSG6-P1 does not build up in sera but readily declines in tandem lack of intense exposure. In Burkina Faso, Rizzo *et al*. (2011), observed reported that in hyperendemic areas children had higher responses to salivary proteins while adults had diminished responses. The authors attributed the apparent diminished responses in adults to "desensitization of the adult's immune system to the salivary allergins" (Rizzo *et. al.,* 2011). This actually confirms the non-cumulative nature of gSG6-P1 and thus, the robustness of gSG6-P1 with the inherent ability to detect transient exposure (or seasonal) in the whole population especially under low malaria transmission intensity. This is likely to be the case in the uphill residents as it is the case for children in hyper-endemic areas. This highlights the potential of gSG6-P1 as a more useful tool under low malaria transmission period. A situation that is expected to be widespread as countries

strives towards malaria elimination and eradication. Globally about one billion people currently live under areas of unstable malaria risk (Guerra *et. al.,* 2008); such a tool will be very useful in detecting small scale variation in the risk of mosquito bites under such conditions when entomological indexes are not sensitive or even reliable.

5.2.2 Magnitude of antibody responses of MSP-1_{19} and gSG6-P1

MSP-1_{19} responses were higher than gSG6 responses in the same cohort. The intercepts of the slope indicate the magnitude of antibody responses, the differences further gives ceredibility to the finding that MSP1_{19} humoral response builds up in residends and has a snowballing effect. It is thus more immunogenic than gSG6-P1. The gSG6-P1 is a synthetic peptide which is specially designed to enhance its sensitivity and antigenicity (Poinsignon *et. al.,* 2008). It has reportedly been sensitive (Drame *et al.,* 2010, Poinsignon *et al.*, 2009), with changeable antibody levels in travelers only transiently exposed to *Anpheles* bites. The relative less immunogenicity of gSG6-P1 may be explained that, it is mosquito salivary protein that functions to facilitate blood feeding. It is not intended to invade host tissues and thus, it is only exposed to the immune system for a relatively short time.

5.3 Parasite -based Diagnosis of Malaria; Microscopy or RDTs

Globally, early diagnosis and correct treatment is the fundamental principle of present malaria policy (WHO, 2005). The battle for appropriate treatment through the introduction of more effective, but higher cost, anti-malarial drugs to counter parasite resistance has been won at a public policy level (Ridley and Toure, 2004; WHO, 2006). However, there is a lack of emphasis on identifying the parasitemic cases that would benefit from the administration of these drugs.

Invariably, good-quality microscopy still remains the superior diagnostic method (Gold standard), providing accurate parasite density and species identification, allowing reliable monitoring of the response to therapy and having a role in identifying other etiologies. Microscopy provides lower cost diagnosis (per case) when the case load is high (reviewed in Bell and Peeling, 2006). However, mantaining a high-quality, efficient microscopy service needs a well-ordered health system infrastructure. This includes the assurance of high-quality supplies and reagents, the presence of suitable microscopes, maintenance and technical competence, an ample workplace environment and the skill to make utilizable blood smears. Field microscopy frequently does not meet these necessities. But normally, the sustainability of a microscopy service in remote areas is limited by the capital investment, maintenance and the skill level required (Coleman *et al.*, 2002; O'Meara *et al.*, 2005). It is also obvious that high-quality parasite-based diagnosis has remained unavailable to most patients with tropical febrile illness. The advent of rapid diagnostic tests (RDTs) presented hope for a straightforward, accurate diagnostic test that could be done with ease and simplicity (Premji *et al.*, 1994; Shiff *et al.*, 1994). A remote village health worker could, for the first time, rapidly and accurately distinguish between parasitemic and non-parasitemic febrile illness. Field investigation of histidine-rich-protein 2 (HRP2) RDTs confirmed the accuracy of malaria diagnosis of over 90% for *P. falciparum* malaria at parasitemia levels of greater than 200 parasites per micro liter of blood (Kyabayinze *et al.*, 2008). However, few operational studies have been conducted to ascertain the actual accuracy of diagnosis in the hands of the healthworker. Hospital-based-trials are needed to examine healthcare worker performance and RDT accuracy under 'usual practise' to determine if sensitivity and specificity will remain high and if utilization of the test will decrease over-treatment. Despite their apparent simplicity, the preparation and interpretation of RDTs can

be poor, and an adequate level of training, supervision and development of appropriate instructions are essential for successful implementation (McMorrow *et. al.,* 2008).

5.3.1 Performance of RDTs and the associated challenges

The performance of RDTs in this study was generally poor both in the operational study and the field trial at relatively low parasitaemia. At Agona the sensitivity of the test was 34.4% at parasite density of less or equal to 250 parasites /µL -liter of blood, this means that more than 65% of positive cases with low parasitaemia will pass as false negatives. On the other hand at this same parasite density the specificity was quite high, 88.5% meaning that there is a high likelihood that very few artifacts will pass as positives. At a higher parasite density that is greater than 250 parasites /µL of blood the RDT test performed better with sensitivity rising sharply to 85.7% with specificity of 72% whilst the positive predictive value which instills confidence that positives thus determined are true was also very high 82.7%. It is noteworthy that at higher parasite densities the RDT test perform well with higher sensitivity and specificity and high positive and negative predictive values; these trends were observed at all study sites. The performance observed in the present study was below what has been recommended by the WHO (Bell and Peeling *et al.,* 2006).

In fact, in this setting, none of both RDTs, Paracheck-Pf® reached the sensitivity threshold of 95% for parasitaemia > 100/ µl recommended by the WHO (2000). Such poor performances of the RDT have been observed in Uganda and in Congo DRC (Kyabayinze *et al.,* 2008). There are a number of possible reasons for the observed low sensitivity under operational studies. First, blood smears preparation in the hospital may be an imperfect "Gold standard". Poor staining technique or categorization errors by reference microscopy may have led to false positives blood

smears, reducing RDT accuracy. Again there is the possibility that using the manufacturer's instructions, healthcare worker may have had difficulties in performing the RDT itself and that may have affected measured sensitivity. For example, the loop device for transfering blood to the tes well on the cassette which usually comes with the Paracheck RDT pack, was not easy for health workers to utilize and the resulted effect may have been the inability of the healthcare worker to transfer the right amount of blood to the test well which ofcourse have negative consequences on the test accuracy. This is because, adding too little blood precludes the line from becoming visible, while adding more than necessary will decrease readability because of hemolyzed blood swathing the test strip. This was reported by a similar operational study in Uganda (McMorrow *et al.*, 2008).

Other factors capable of influencing RDT performance are: intra-species diversity of the target antigen (HRP2 protein); the parasite stage-specific expression of the antigen; stability of the antigen *in vivo*; and the biology of the parasite. HRP2 is a protein composed largely of histidine and alanine residues with central repeated units of variable length and type that differ between parasite isolates (Baker *et al.*, 2005). It has been shown recently that some of these variations affect the sensitivity of RDTs that are based on HRP2. A plausible rationalization of the low sensitivity could be due to the difference in the HRP2 genotype of *Plasmodium falciparum*. Low frequency of specific gene sequences have been linked with lower RDT accuracy and sensitivity (Baker *et al.*, 2005) However, this is mostly noted at parasite densities between 200–1,000 parasites per microliter of blood and cannot justify the poor performance observed at relatively higher parasitemia(Baker *et al.*, 2010).

It must be noted that the HRP2 protein is produced only by *Plasmodium falciparum*, which prevalence in the black sub Saharan Africa is anywhere between 90 and 98% with the others *P. ovale* and *P. malariae* having a prevalence between 2-8%. In operational setting like the hospital, microscopy does not differentiate between the species; it is just recorded as "malaria parasite present". The PCR analysis that was done to confirm the presence of *P. falciparum* in the microscopically determined "malaria parasites" confirmed 92% of the malaria parasite being *P. falciparum* species. This implies that about 8% of the parasites present may not be *P. falciparum* and this too will affect the performance of RDT that is solely specific for *P. falciparum.*

5.3.3 Differences in parasite density and malaria prevalence

Between the two tests, i.e. microscopy and RDT, the average parasite prevalence determined for Agona was 30% and that of Kumasi was 15%, (Fig. 4.17), a two-fold difference with participants from the city of Kumasi having significantly lower parasite prevalence than their rural dwellers. Urbanization is known to reduce malaria burden due to many factors such as access to hospitals, better housing and improved personal protection against malaria vectors (reviewed in Hay *et al* 2005). Rural folks on the other hand, may suffer a high malaria burden due to poor housing conditions, restricted access to adequate treatment, lack of personal protection and proximity to breeding sites (Ghebreyesus *et al.,* 1999; Lindsay and Snow, 1998; Ter Kuile *et al.,* 2003). All the odds seem evidently against Agona since the parasite density compared between the two sites showed a 20-fold difference, with study participants from Agona, particularly, the pediatric population having very high parasite densities that may imply higher exposure to malaria transmitting vectors. However this magnitude of parasite density gap between the two

populations closes gradually as individuals advance in years and better able to control their parasitemia (Fig. 4.19).

On the Kenyan side Kombewa had the highest parasite prevalence averaging 68% (the average between microscopy and RDT test). This was not surprising since Kombewa is a lowland area in the Lake Victoria basin; an area that has experienced consistent high malaria prevalence and parasite density which is attributable to high vector densities throughout the year (Beier *et al* 1990). Inhabitants in the region of the Lake Victoria basin, western Kenya, suffered 300 infectious bites pernyear (Beier *et al.*, 1990; Githeko *et al.*, 1993). Indeed, recently Zhou *et al.* (2011), found that whereas malaria prevalence is reducing in the highland area that of Kombewa is actually increasing. In the highland areas of Kakamega and Kissi, vector density and transmission intensity were much lower than the lowland Kombewa area, this may explain the lower parasite prevalence. The differences in malaria endemicity between highlands and lowlands are actually well known phenomena in malaria epidemiology. For example, an altitudinal transect study right from lowland area of 300m (asl) to highland area of 1,700m (asl) in the Usambara Mountains of Tanzania found a greater than 1,000-fold declining malaria transmission intensity between the holoendemic lowland and the hypoendemic highland plateau (Bodker *et al.*, 2003).

It is expected that RDT performance would be better in the areas of high parasitemia and so conscious and vigorous efforts must be focused on improving the design and performance of RDTs for low parasitemia, as the case of low prevalence and parasitemia is expected to be the norm in several countries in the pre-elimination phase of the malaria eradication agenda.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

MSP-1_{19}, a leading vaccine candidate was used as a marker of immune response to parasite exposure and a proxy of malaria transmission intensity between the valley and uphill residents in Kakamega County, Kenya. It was observed that, there were higher immune responses to *Plasmodium falciparum* in the valley residents than in the uphill dwellers indicating a possible exposure to higher infection rates. This implies that, MSP-1_{19} seroprevalence may be used as a reliable epidemiological marker for tracking malaria transmission intensity at deferring altitudes. It was confirmed that anti-malarial antibodies to merozoite surface proteins (MSP-1_{19}) are one of the best immunological markers identified for estimating malaria exposure as a function of transmission intensity across various altitudes. The total IgG titers to the MSP-1_{19} showed 13-fold differences in the magnitude of the response between the two sites that are separated only 5Km from each other, this reveals an important biomarker for distinguishing long term malaria transmission potential irrespective of short-term or seasonal variations in the transmission intensity.

The age-specific MSP-1_{19} seroprevalence was able to show differences in age related risk of exposure; high and stable antibody responses were observed in older residents (more than 15 years of age) at the highland site in spite of changing seasons whilst in children below fifteen (15) years of age, lower responses that also varied with the changing season were observed. If the MSP-1_{19} immune responses could reflect functional immunity, something that was not directly determined in this study and is currently uncertain, then the uphill populations,

particularly children below fifteen years of age, may be at a higher risk of (severe) clinical disease. This hypothesis requires further longitudinal studies.

The second study explored the sensitivity and robustness of the *An. gambiae* salivary gland peptide as an epidemiological marker of human exposure to vector bites. It was observed that both seroprevalence and antibody levels of gSG6-P1 were robust and sensitive, able to detect differences in human exposure to *An. gambiae* bites between two populations living only 5km apart but at different altitudes and vector exposure.

Again it was observed that the antibody responses to gSG6-P1 were sensitive detecting seasonal changes in vector exposure, unlike $MSP1_{19}$ thus, detecting the differences in the vector populations associated with high and low malaria transmission seasons in the rainy and dry seasons respectively. Furthermore, gSG6-P1 seroprevalence correlated with parasite prevalence at the population level. This implies that it could be used as a surrogate tool for monitoring the risk of parasite transmission. The fact that seroprevalence rates of gSG6-P1 were higher than equivalent parasite rates is indicative of higher sensitivity in estimation of malaria transmission and may be a good tool in the era of very low transmission.

At the individual level, it was proven that the likelihood of having detectable anti-$MSP\text{-}1_{19}$ antibodies were higher for gSG6-P1 seropositive individuals. Moreover, in comparison with $MSP\text{-}1_{19}$, gSG6-P1 was found to be less cumulative and thus a better marker of transient exposure. The gSG6 P1 seroprevalence may be exploited as a surrogate epidemiological marker of risk of parasite transmission and a vector surveillance tool across different populations and malaria transmission settings.

Early diagnosis and prompt treatment (EDPT) of malaria with efficient drugs is required for effective malaria control. The emergence of resistance to antimalaria agents, and now the deployment of expensive Artemisinin combination therapy (ACTs) [WHO, 2008] into regions where malaria is highly endemic are increasing the need for rapid, accurate diagnosis of patients who may be infected with malaria. Rapid diagnostic tests (RDTs) offer great potential for the timely and accurate diagnosis of malaria, thereby leading to prompt and appropriate treatment.

Findings from this study have revealed that the actual performance of *Pf*HRP2 depends on two main issues; firstly the parasite density of the blood being tested and secondly the delivery of the test itself. It was observed that the sensitivity and specificity of the kit was generally poor at relatively low parasite densities. However, the sensitivity and specificity rises sharply, with much improved sensitivity and specificity with increasing parasite density. The second finding that possibly affects the performance of the test kit is the delivery itself, the knowledge and experience of the technician or the field worker may play a role in the final outcome of the test.

6.2 Recommendations

Potentially the use of sero-immuno-epidemiological markers of vector and parasite exposure holds the key to sensitive and reliable measurement and monitoring of malaria transmission intensity and its associated control strategies. More data is required for developing MSP 1_{19} and gSG6-P1 immunoassays into tools in malaria surveillance. It is recommended that these markers are tested widely across different epidemiological strata following which national malaria control officers and policy makers should adopt these markers in national programs.

It is recommended that the markers are tested comprehensively at the individual level in the context of longer term community-based longitudinal cohort studies. The addition of geographical information systems in tandem with spatial statistics will enable the identification of spatial clustering of infections and mosquito exposure thereby elucidating malaria transmission 'hot spots'. This will pave a way for targeted control with limited resources for the wider community benefit.

Again, because intense exposure to *Anopheles* mosquito may not of necessity mean a high malaria exposure if the *Anopheles* mosquitoes are not infected (Smith *et al.*, 2004), and since mosquito sporozoite rates may also show spatial disparity (Bousema *et al.*, 2010), exposure to only mosquito bites may not essentially be exposure to parasites and some component of parasite prevalence will always be necessary to support the use of gSG6 P1. It is therefore recommended that more research must be carried out to identify an additional mosquito related marker or *Plasmodium falciparum* sporozoite marker that will indicate exposure to infectious bites.

*Pf*HRP2 RDT must be used with caution in areas under low transmission and especially in children who have less developed immune system. This is because in areas under low malaria transmission there are immunologically naïve people who are more susceptible to malaria even when they have very low parasitemia. If caution is not taken, these people will be denied treatment, since the rapid diagnostic test would most likely give negative results in at least 20% of cases when the parasite density is below 250 parasites/µl of blood. Therefore there should be clear guidelines for treatment of people who turn negative results. It is, therefore, recommended that there should be more studies on the sensitivity of the *Pf*HRP2 test at low parasite densities to improve its performance.

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APPENDICES

Appendix I: Standard Operating Procedures for Laboratory Assays

Standard PCR (AB-gene M'Mix) ***Plasmodium*** **Date: _____**

Sample arrangement on a 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

CYCLING CONDITIONS

94 ^{o}C for 3 minutes

1. 94 ^{o} C for 30 sec
2. 57 ^{o} C for 30sec
3. 68^{o}C for 45 sec
4. 72^{o}C for 10 min
5. 10^{o}C forever

39x

Comments:_____
_____

BLOCKING BUFFER FORM

Reagents

Reagent	Supplier & Catalog #	Preparation Date/Tech/Lot #	Expiration Date
Polyoxyethylene Sorbitan Monolaurate (Tween-20)			
0.5% Boiled Casein Solution	**N/A**		

1. **Procedures** **Time & Initials**

a. Using a serological pipette, add 5mL of Tween-20 to 495mL of 0.5% boiled casein, add a stir bar, and mix on a stir plate until homogenous. _____

b. Write initials and the date the solution was made on the bottle.

This reagent expires 42 hours from the time the solution is made.

3. **Document Revision History**

Version Number	Brief Description of Changes	Effective Date
01	Words "Kondele Serology Laboratory, USAMRU-K" added to title of institution.	December 11, 2007
00	Original	3 July 2007

Technician: _____ Date: _____

DILUENT FORM

1. **Reagents**

Reagents	Supplier & Catalog #	Preparation Date/Tech/Lot #	Expiration Date
Blocking Buffer	**N/A**		
0.5% Boiled Casein Solution	**N/A**		

2. **Procedures** **Time & Initials**

a. Add 12.5mL of blocking buffer to 487.5mL of 0.5% boiled casein in a1L media bottle.

b. Mix by inversion until homogenous.

c. Write initials and the date the solution was made on the bottle.

d. Store in refrigerator at 4°C

_____

This reagent expires 42 hours from the time in which the solution is made.

3. **Document Revision History**

Version Number	Brief Description of Changes	Effective Date
01	Words "Kondele Serology Laboratory, USAMRU-K" added to title of institution.	December 11, 2007
00	Original	4 June 2007

Technician: _____ Date: _____

0.125L Antigen Diluent Form

2. **Reagents**

Reagents	Supplier & Catalog #	Preparation Date/Tech/Lot #	Expiration Date
0.5% Boiled Casein Solution	**N/A**		
10X PBS	**N/A**		
Phenol Red			

3. **Procedures** **Time & Initials**

a. Add 12.5mL of 10X PBS to 112.5mL MQH_2O in a 0.5L media bottle to create 1X PBS. _____

b. Add 50µL of 0.5% Boiled Casein to 125mL of 1X PBS. _____

c. Add 0.001g ± 0.0001g of phenol red, add stir bar to bottle, place on stir plate, and turn stirrer knob to a level sufficient to mix. _____

Actual weight of phenol red added: _____

d. Write initials and the date the solution was made on bottle.

e. Store in refrigerator at 4°C.

This reagent expires 42 hours from the time in which the solution is made.

Technician: _____ Date: _____

1L BOILED CASEIN FORM

Reagents

Reagent	Supplier & Catalog #	Preparation Date/Tech/Lot #	Expiration Date
Casein			
Phenol Red			
10X PBS	N/A		
5N Sodium Hydroxide (NaOH)			
2N Hydrochloric Acid (HCl)			
MQH_2O	N/A	N/A	N/A

Procedures **Time & Initials**

a. Dissolving Boiling Casein:

Add 2mL of 5N NaOH to 125mL MQH_2O in a 1L flask with stir bar. Set the stir plate to 300-400ºC and 300rpm and mix the solution on a stir plate for 20 minutes. Within the first 5 minutes, add 5.00g ± 0.01g of casein to the above solution. _____

Actual weight of casein added: _____

b. Bring to final volume:

Remove the boiled casein from the stir plate and add 100mL of 10X PBS to the flask. QS the boiled casein solution to 1.0L with MQH_2O to create a 0.5% boiled casein solution and mix. _____

c. Cool to room temperature:

Place the 0.5% boiled casein solution into a refrigerator for 40-45min to cool to 25.0°C. _____

Remove the boiled casein solution from the refrigerator and check that the temperature is 25.0± 2.0°C. If the temperature is not in range, cool or reheat until a temperature within range is achieved. _____

d. Add Phenol Red:

Add 0.02g ± 0.001g of phenol red to the boiled casein solution and mix on a stir plate until the phenol red is dissolved and evenly dispersed. _____

Actual weight of phenol red added: _____

e. Adjusting pH: **Time & Initials**

Calibrate the pH meter (UWF-D-E003). Add 3.0mL 2N HCl to

the cooled 0.5% boiled casein solution and continue to mix on a stir plate.

While stirring, adjust the pH of the cooled 0.5% boiled casein solution to

pH 7.40± 0.02 with either 5N NaOH or 2N HCl. _____

Actual pH measurement of solution: _____

Technician: _____ Date: _____

f. Checking pH stability: **Time & Initials**

Allow the pH adjusted 0.5% boiled casein solution to stir for the next 10 minutes and re-measure the pH to reach equilibrium at pH 7.40± 0.02. _____

Final pH measurement of solution: _____

This reagent expires 42 hours from the time the solution is made.

4. **Document Revision History**

Version Number	Brief Description of Changes	Effective Date
01	Words "Kondele Serology Laboratory, USAMRU-K" added to title of institution.	December 11, 2007
00	Original	3 July 2007

Technician: _____ Date: _____

Appendix II - Questionnaire

University of California/Climate and Human health Research Unit /Kenya Medical Research Institute

Parasite factors affecting PfHRP2 based RDTs in Western Kenya

Name of interviewer/

Technician_____ _____ Name of Health Center _____

We would like to you a few questions and request your participation in an investigation that will help find shortcomings in malaria diagnosis and thus help to improve malaria diagnosis in this area. If you agree, you will be required to donate no more than 3mls of blood only one time. You will benefit from free malaria diagnosis and free (standard) treatment if you are found to have uncomplicated malaria

Personal data

Name_____ _____ Age: __ _____ Sex: M [ ] F [ ]

Village _____ Location:_____Code:_____

Malaria related data

Temperature:_____ History of fever in the past 48 hours? Yes [ ] No [ ]

Record any symptom of malaria

_____

Have you travel to an endemic area in the past 2 weeks Yes [ ] No [ ]

If yes, Where _____

Have you taken any antimalarias drug in the past 2 weeks Yes [ ] No [ ]

If yes, what drug did you take? _____

Do you own a bednet? _____ Do you sleep under a bednet_____

RDT related Data

Brand name_____ PfHRP- 2 based. Yes [ ] No [ ]

Date of manufacture: _____Date of expiry:_____batch/lot no. _____

RDT results

RDT reactive Yes [ ] No [ ] RDT non-reactive Yes [ ] No [ ]

Appendix III

Informed Consent and Ethical Approval

Form 1: CONSENT FORM FOR ADULTS

Title of Study: Ecology of African Highland Malaria

Part I: malaria prevalence and human population census

This consent form will be explained and signed by each study participant

Name of Volunteer: _____, Age of Volunteer: _____

1. INVESTIGATOR CONDUCTING THE STUDY:

Dr. Guiyun Yan, Program in Public Health, University of California, Irvine, USA;

Dr. Andrew Githeko, Kenya Medical Research Institute (KEMRI), Kisumu, Kenya;

2. PURPOSE OF STUDY:

The purpose of this project is to determine the effect of landuse on malaria transmission and the effectiveness of mosquito control on malaria prevention.

3. PROCEDURES TO BE FOLLOWED:

A tiny amount of blood will be taken from your finger by pricking it to find out whether you are infected with malaria parasites.

We will ask you some questions about whether you had malaria in the past two weeks, whether you have traveled to malaria endemic areas, whether you have been using bednets and whether

you have taken antimalarial drugs. This information is important for us to determine how active malaria transmission occurs in your village.

We will ask you some questions about childbirth and death and your family income.

4. EXCLUSION CRITERIA:

Residents who intend to relocate during the study period and are unwilling to participate in the study will be excluded. All minors will be excluded from questionnaire survey.

5. DISCOMFORTS AND RISKS:

The finger-prick blood collection method causes slight discomfort. Sterile blood lancets (followed with sterile ethanol) will be used for every single person, the procedures will pose very minimal risk of being infected by other pathogens.

6. BENEFIT TO PARTICIPANTS:

You will not receive financial benefit from your participation, however, if you have a fever or are ill, you will be referred to the local clinic for care.

7. REIMBURSEMENT FOR MEDICAL TREATMENT (This statement is required on all consent forms):

The present project will be responsible for diagnosis of malaria and referral to local clinics for evaluation. Competent staff member of the Kenyan Ministry of Health will perform the evaluation and provide appropriate treatment. The project will only cover the costs of the normal standard treatment of uncomplicated malaria approved by Kenyan Ministry of Health. The study will not attend to other disease unrelated to malaria. If other illness or diseases are identified during the malaria screening, we will provide referral to the appropriate local health authorities. In cases of emergencies, transport, whenever possible, will be provided to the nearest government medical facility.

8. CONFIDENTIALITY:

Information related to you will be treated in strict confidence to the extent provided by law. Your identity will be coded and will not be associated with any published results. Your code

number and identity will be kept in a locked file of the Principal Investigator and Kenyan Investigator.

9. FREEDOM TO WITHDRAW:

Your participation in this study is voluntary and you may discontinue your participation at any time without prejudice and without affecting future health care.

10. CONSENT FORM:

The consent form will be explained to each study participant and signed by the Investigators or the leading scientists conducting the study.

11. NEW FINDINGS:

You will be told of any significant new findings developed during the course of this study.

I HAVE READ AND UNDERSTAND THIS CONSENT FORM,

AND I AM WILLING TO PARTICIPATE IN THE STUDY.

_____ _____

Subject's Name (type or print) Subject's Signature (consent) Date

_____ _____

Witness' Name (type or print) Witness' Signature (consent) Date

_____ _____

Investigator's Name (type or print) Investigator's Signature Date

UNIVERSITY OF CALIFORNIA, IRVINE

Experimental Subject's Bill of Rights

This is a list of the rights for research subject involved in a medical experiment or for those asked to consent on behalf of another:

This list includes, but is not limited to, your right to:

Be informed of the nature and purpose of the experiment.

Be given an explanation of the procedures to be followed in the medical experiment, and any drug or device to be utilized.

Be provided a description of any discomforts and risks reasonably to be expected from the experiment.

Be given an explanation of any benefits reasonably to be expected from the experiment, if applicable.

Be provided a disclosure of any appropriate alternative procedures, drugs or devices that might be advantageous, and their relative risks and benefits.

Be informed of the possible medical treatment, if any, available after the experiment if complications should arise.

Be given an opportunity to ask any questions concerning the experiment or the procedures involved.

Be instructed that consent to participate in the medical experiment may be withdrawn at any time and you may discontinue participation in the medical experiment without prejudice. This decision will not affect any right to receive standard medical treatment.

Be given a copy of the signed and dated written consent form and a copy of this form.

Be given the opportunity to decide to consent or not to consent to a medical experiment without the intervention of any element of force, fraud, deceit, duress, coercion, or undue influence on the subject's decision.

_____ _____

Subject's Signature Date

_____ _____

Parent's/L.A.R.'s Signature Date

Form 2: CONSENT FORM FOR ADULTS

Title of Study: Ecology of African Highland Malaria

Part II: Assessment of Human Exposure to Malaria Infection

This consent form will be explained and signed by each study participant

Name of Volunteer: _____, Age of Volunteer: _____

1. Title of Study: Ecology of African Highland Malaria

2. Investigators:

Dr. Guiyun Yan, Program in Public Health, University of California at Irvine, USA. TEL: 1 949 924 0175;

Dr. Andrew Githeko, Kenya Medical Research Institute, Kisumu, Kenya; Tel: 254 57 20 22923.

3. Purpose: You are being asked to participate in a research study. In this study we hope to determine whether people living uphill and in the valley have different exposure to malaria infection. This information will help us determine how clinical malaria and malaria outbreaks occur in the highlands.

4. Procedures: If you decide to volunteer, you will be asked to allow the physicians from Kenyan Ministry of Health to draw three ml blood. We will test antibody responses to malaria using the blood serum.

5. Risks: The risk that you may be injured during blood drawing is minimal. Sterile syringe (followed with sterile ethanol) will be used for every single person, the procedures will pose very minimal risk of being infected by other pathogens. In the events of you being injured during blood drawing, you will be transported to the government clinics for appropriate treatment.

6. Benefits: You will not receive financial benefit from your participation, however, if you have fever or are ill, you will be transported to the local clinic for care. The project will only cover the costs of the normal standard treatment of uncomplicated malaria approved by Kenyan Ministry of Health.

7. Reimbursement for Medical Treatment (This statement is required on all consent forms): The present project will be responsible for diagnosis of malaria and referral to local clinics for evaluation. Competent staff member of the Kenyan Ministry of Health will perform the evaluation and provide appropriate treatment. The project will only cover the costs of the normal standard treatment of uncomplicated malaria approved by Kenyan Ministry of Health. The study will not attend to other disease unrelated to malaria. If other illness or diseases are identified during the malaria screening, we will provide referral to the appropriate local health authorities. In cases of emergencies, transport, whenever possible will be provided to the nearest government medical facility.

8. Confidentiality: All information associated with this study will remain confidential, and your participation in the study will remain anonymous. Only you and the scientists conducting the study will know the results. Data may be reviewed by representatives of the Ministry of Public Health and the Human Subjects Research Review Board of Investigators' home institutes. Research and clinical findings may be shared with other researchers and the scientific community through lectures or publications. You will not be identified by name.

9. Right to refuse or withdraw: You may refuse to participate in this study. If you decide to participate, you may change your mind about being in the study and quit after the study has started without facing penalties or loss of benefits that you would normally be able to obtain.

10 Consent form: The consent form will be explained to each study participant and signed by the Principal Investigator or the leading scientists conducting the study.

11. Questions: If you have questions relating to the study, please ask Drs. Githeko and Yan. If you have questions concerning the effect participation in this study may have on your rights, please contact Dr. Davy Koech, Director, Kenya Medical Research Institute, Nairobi, Kenya (Tel: 254-2-722541).

I HAVE READ AND UNDERSTAND THIS CONSENT FORM,

AND I AM WILLING TO PARTICIPATE IN THE STUDY.

_____ _____ Date_____

Subject's Name (type or print) Subject's Signature (consent)

_____ _____ Date_____

Witness' Name (type or print) Witness' Signature

_____ _____ Date _____

Investigator's Name (type or print) Investigator's Signature

(Each volunteer will be give a copy of this consent form)

Version 2.3, January 24, 2008

Form 2: ASSENT FORM FOR MINORS

Title of Study: Ecology of African Highland Malaria

Part II: Assessment of Human Exposure to Malaria Infection

This assent form will be explained and signed by each study participant

Name of Volunteer: _____, Age of Volunteer: _____

Who are we?

Our names are Dr. Andrew Githeko, and Dr. Guiyun Yan. Dr. Githeko is a research scientist in the Kenya Medical Research Institute. Dr. Yan is an Associate Professor in the Program in Public Health at the University of California at Irvine.

Why are we meeting with you?

We want to tell you about a study that involves children like yourself. We want to see if you would like to be in this study.

Why are we doing this study?

We are trying to determine whether people living uphill and in the valley have different exposure to malaria infection. This information will help us determine how the malaria outbreaks occur in the highlands.

What will happen to you if you are in the study?

If you decide to be in the study the physicians from Kenya Medical Research Institute will draw 3 ml of blood from you.

What will happen to the blood you provide?

We will use the blood to feed the mosquitoes. You will not be exposed to mosquito feeding. No names will ever be used and data will be kept very confidential. All names will be removed from samples prior to being given to other researchers.

Will any part of the study hurt?

Blood drawing may cause some discomfort. We will use sterile needle and syringe, so you should not be exposed to any pathogens from blood drawing.

Will you get better if you are in the study?

Whether or not you decide to participate in the study, we will transport you to the nearest government clinics for care if you have fever or are ill. We will cover the costs of the normal standard treatment of uncomplicated malaria approved by Kenyan Ministry of Health. In addition, we will spray your house with insecticides to kill mosquitoes at the end of our experiment.

Who will know that you are in the study?

We will not tell anyone that we drawn blood from you. Only you and the scientists conducting the study will know the results about infectiousness of your blood to the mosquitoes.

Do you have to be in the study?

No, you don't. No one will get angry or upset you if you don't want to do this. Just tell us if you don't want to be in the study. And remember, you can change your mind later if you decide you don't want to be in the study anymore.

Do you have any questions?

You can ask questions at any time. You can ask now. You can ask later. You can talk to me or you can talk to someone else at any time during the study.

Andrew Githeko	KEMRI	35-22923
Guiyun Yan	UC Irvine	1-949 824 0175

IF YOU WANT TO BE IN THE STUDY, SIGN YOUR NAME ON THE LINE BELOW.

Signature of the Child: _____

Printed Name: _____

Date: _____

Signature of the Parent/Guardian: _____

Printed Name: _____

Date: _____

Signature of the Investigator: _____

Printed Name: _____

Date: _____

(Each participant will be give a copy of this assent form)

Version 2.3, January 24, 2008

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES / KOMFO ANOKYE TEACHING HOSPITAL

COMMITTEE ON HUMAN RESEARCH, PUBLICATION AND ETHICS

Our Ref: CHRPE/AP/208/12

8th October, 2012.

Mr. Kingsley Badu
Department of Theoretical
and Applied Biology
KNUST

Dear Sir,

LETTER OF APPROVAL

Protocol Title: ***"Assessment of Parasite Factors Affecting Sensitivity of Plasmodium Falciparum Histidine Rich Protein 2 (PfHRP-2) Based Rapid Diagnostic Test (RDT) in Two Malaria Endemic Districts in the Forest Zone of Ghana."***

Proposed Site: ***Department of Theoretical and Applied Biology/KCCR, Sekyere South District Hospital, Agona and the Kumasi South District Hospital, Kumasi.***

Sponsor: ***Principal Investigator.***

Your submission to the Committee on Human Research, Publications and Ethics on the above named protocol refers.

The Committee reviewed the following document:

- A notification letter of 19th March, 2012 from the Sekyere South District Hospital (study site) indicating approval for the conduct of the study in the Hospital.
- A notification letter of 19th May, 2012 from the Kumasi South District Hospital (study site) indicating approval for the conduct of the study in the Hospital.
- A notification letter of 13th January, 2012 from the Kumasi Centre for Collaborative Research in Tropical Medicine (study site) indicating approval for the conduct of the study in the Centre.
- A completed CHRPE Application Form.
- Participant Information Leaflet and Consent Form.
- Research Proposal.
- Questionnaire.

The Committee has considered the ethical merit of your submission and approved the protocol. The approval is for a fixed period of one year, renewable annually thereafter. The Committee may however, suspend or withdraw ethical approval at anytime if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from the Committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee should be notified of the actual start date of the project and would expect a report on your study, annually or at close of the project, whichever one comes first. It should also be informed of any publication arising from the study.

Room 7 Block J, School of Medical Sciences, KNUST, University Post Office, Kumasi, Ghana
Phone: +233 3220 63248 Mobile: +233 20 5453785 Email: chrpe.knust.kath@gmail.com / chrpe@knust.edu.gh

Thank you Sir, for your application.

Yours faithfully,

Osomfuor Prof. Sir J. W. Acheampong MD, FWACP
Chairman

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org; info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **MAY 15, 2008**

FROM: **SECRETARY, KEMRI/National Ethical Review Committee**

THRO': **Dr. J Vulule,**
CENTRE DIRECTOR, CGHR,
KISUMU

TO: **Dr. Andrew K. Githeko (Principal Investigator)**

RE: **SSC No. 1382 (N):** Ecology of African highland malaria **(II)**

FORWARDED
21/5/08
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Sir,

We acknowledge receipt of the revised protocol and ICD (bio-sketches) in your letter dated 2 May 2008. The compromise reached to add the suffix (II) at the end of the title of the study is acceptable in order to accommodate the position of the funding agency and this Committee.

We also note the changes to the ICD to standardize the font size throughout the ICD and the deletion of the section on ownership of DNA which has been overtaken by events.

Due consideration has been given to ethical issues and the study is granted approval from today the 15th May 2008 to 14th May 2009.

Please note that any changes to the research study must be reported to the Scientific Steering Committee and to the Ethical Review Committee prior to implementation. This includes changes to research design, equipment, personnel, funding or procedures that could introduce new or more than minimum risk to research participants.

Respectfully,

R.C. Kithinji

R. C. Kithinji,
For: Secretary,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE