EFFECT OF INSECTICIDE-TREATED BED NETS (ITNs) ON THE GENETIC DIVERSITY OF *P. FALCIPARUM* CIRCUMSPOROZOITE PROTEIN (CSP) IN A MALARIA HOLOENDEMIC AREA OF WESTERN KENYA.

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DECLARATION

I, Muia Anne Ndanu, do hereby declare that this thesis is my original work and has not been presented for a degree in any other University

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To my dear brother, the late Boscow Kivondo.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACTs – Antimalarial combination therapies

ARDS – Acute respiratory distress syndrome

CQ – Chloroquine

CSP – Circumsporozoite Protein

DDT – Dichlorodiphenyltrichloroethane

GoK – Government of Kenya

HIV – Human Immunodeficiency Virus

IPT – Intermittent Preventive Treatment

ITNS – Insecticide-treated bed nets

KEMRI – Kenya Medical Research Institute

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

Pfcs – *Plasmodium falciparum* circumsporozoite gene

RBC – Red blood cells

RBM – Roll Back Malaria

SP – Sulfadoxine-pyrimethamine

Th2R – T helper-2 cell Receptor

Th3R – T helper-3 cell Receptor

WBC – White blood cells

WHO – World Health Organization

ABSTRACT

Malaria continues to place a major burden on health and economic development in the poorest and most marginalized areas of the world. Widespread parasite resistance to antimalarial drugs and vector resistance to insecticides led to renewed global efforts to explore alternative, cost-effective and efficacious malaria control tools. Use of insecticide-treated bed nets (ITNs) has emerged as one of the most cost-effective malaria control strategy prompting initiation of large-scale ITN programmes in many endemic areas. The use of ITNs has been associated with 70- 90% reduction in malaria transmission and significant reductions in morbidity and mortality in vulnerable populations. However, the intensity of malaria transmission determines parasitemia levels, rates of acquisition of immunity in human populations and parasite recombination rates in mosquito vectors. Therefore, we hypothesized that the significant reduction in parasitemia associated with ITN use could have an impact on genetic diversity of polymorphic parasite genes that are under selective pressure, therefore affecting the frequency of circulating haplotypes. To test the hypothesis, we designed the current study to assess the effect of on the genetic diversity of the polymorphic immune epitopes of *Plasmodium falciparum* circumsporozoite protein (CSP), a leading malaria vaccine candidate antigen. Parasite-positive blood samples collected from children enrolled in a series of cross-sectional surveys conducted at baseline, 14, 30 and 46-months post ITN intervention in a malaria holoendemic area were used to isolate DNA. Standard PCR and cycle sequencing were used to determine point mutations in the C-terminal domain of the CSP gene encoding helper T-cell epitopes, Th2R and Th3R. The nature and extent of CSP gene diversity was compared between parasites from children in intervention and control areas, and in the four cross-sectional surveys using haplotype diversity and the π statistic. Differences in the proportion of haplotypes between bed net and control areas and between the different surveys were determined by Chi-square and permutation tests. There were 77 Th2R and 33 Th3R haplotypes detected in this study signifying the high polymorphism of the CSP immune epitopes in natural parasite populations. The most prevalent Th2R and Th3R haplotypes were PSDQHIEKYLKTIQNSLS and NKPKDQLDYEND, 13% and 33.1% respectively. Most of the circulating haplotypes had a very low frequency (< 5 copies). The 3D7-type haplotype had a very low frequency. Although there was no consistency in the prevalence of the haplotypes in ITN and control areas and overtime, there was a trend towards decreased genetic diversity after the first 30 months of ITN use. After 48 months of ITN use the genetic diversity had increased. This has implications on the ongoing field testing of CSP-based malaria vaccines including the RTS,S multi-site trials.

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Malaria continues to be a major public health problem globally and the cause of significant morbidity and mortality annually (WHO, 2003a). The worsening malaria situation has been attributed mainly to the emergence and spread of parasite resistance to available antimalarial drugs and vector resistance to insecticides in areas of high malaria endemicity (Breman, 2001; Trape, 2001; Sharp *et al.,* 2002). In areas of high transmission, the groups most at risk of malaria infection and severe manifestations are young children and pregnant women (WHO, 1996).

The World Health Organization (WHO) through the Roll Back Malaria (RBM) initiative has adapted and advocates wide-scale distribution of insecticide-treated bed nets (ITNs) to vulnerable populations as one of its key technical strategies for malaria control (Nabarro *et al.,* 1998). This is based on the results of several randomized controlled trials in areas of different malaria transmission settings, in Kenya and other parts of the Africa, that have demonstrated that ITNs are an efficacious and cost-effective malaria control tool for the protection of groups most at risk of malaria (D'Alessandro *et al.,* 1995; Binka *et al.,* 1996; Nevill *et al.,* 1996; Habluetzel *et al.,* 1997; Phillips-Howard *et al.,* 2003; ter Kuile *et al.,* 2003; Eisele *et al.,* 2005).

Although ITNs have proved to be an efficacious and cost-effective malaria control tool, concerns have been raised about their impact on malaria

transmission, which could interfere with the development of acquired immunity in children, paradoxically leading to a shift in morbidity and mortality to older age groups (Snow *et al.,* 1996). Several studies have, however, shown that the use of ITNs does not interfere with acquisition of immunity or lead to a shift in morbidity and mortality (Habluetzel *et al.,* 1997; Askjaer *et al.,* 2001; Binka *et al.*, 2002; Kariuki *et al.,* 2003; Lindblade *et al.,* 2004). Nevertheless, there remain unanswered questions on how the reduction in malaria transmission due to ITN use will affect the genetic diversity of malaria parasites, especially the polymorphic parasite surface antigens that are under immune pressure. The current study was designed to investigate the effects of ITNs on temporal changes on polymorphisms in a gene encoding a leading malaria vaccine candidate antigen, the circumsporozoite protein (CSP), in an area of intense and perennial malaria transmission of western Kenya.

Unlike previous studies that investigated the association between transmission intensity and parasite diversity, the current study has several advantages; 1) parasite diversity was compared between intervention and control areas in the same region, thus eliminating biases due to biotic and abiotic factors that could confound data obtained from different geographic regions, 2) the western Kenya ITN trial was extended for two more years beyond the initial two-year efficacy monitoring period, which allows for the assessment of temporal changes in parasite diversity for an extended period, 3) blood samples were obtained from clinically and parasitologically characterized

individuals and from a region where parasite inoculation rates are known, the linkage of this information with the genetic data obtained from this study provides valuable insights on the complexity of malaria infection in relation to malaria transmission, 4) use of polymorphic vaccine candidate gene, which is under immune pressure provides unique and unbiased insight on the parasite diversity and its changes over time in relation to transmission reduction.

1.2 RATIONALE FOR THIS STUDY

A great challenge in vector control has been the unavailability of low-risk, cost-effective and efficacious insecticides as well as increasing vector resistance (Curtis *et al.,* 1994; WHO/ UNICEF, 2005). Although the use of ITNs has shown significant efficacy in protecting both the individuals sleeping under them as well as other community members, an integrated approach involving the use of drugs and vaccines in addition to vector control is necessary to eradicate the remaining malaria infections.

The intensity of malaria transmission has an effect on the general epidemiology of malaria in an area, the severity profiles of the disease in the population, the rates of acquisition of protective immunity and the dynamics of parasite dispersion in mosquito vectors and human hosts (Arnot, 2002). Several studies have investigated changes in the pattern of malaria-specific morbidity and mortality following extended use of ITNs (Gimnig *et al.,* 2003a; Phillips-Howard *et al.,* 2003; ter Kuile *et al.,* 2003; Lindblade *et al.,* 2004). Other studies have investigated the effects of ITNs on naturally

acquired immunity to malaria (Habluetzel *et al.,* 1997; Askjaer *et al.,* 2001; Kariuki *et al.,* 2003). However, there is a paucity of data on the impact of ITNs on the diversity of *Plasmodium falciparum* parasites. There exist information gaps on how the reduced malaria transmission as a consequence of ITN use will affect host-parasite interactions. There is, therefore, need to conduct studies geared towards understanding the effects of transmission reduction due to ITN use on temporal changes on the genetic diversity of malaria parasites and the extent of polymorphisms in leading vaccine candidate genes, which are under host immune pressure. Extensive polymorphisms in the Th2R and Th3R genes could adversely affect the efficacy of a CSP-based malaria vaccine.

1.3 RESEARCH QUESTIONS

i.) Does transmission reduction due to ITN use have an effect on the polymorphisms of the *P. falciparum* CSP immune epitopes, believed to be under immune selection pressure?

ii.) Does transmission reduction affect parasite haplotypes circulating in an area?

iii.) Is there a relationship between reduction in parasite rates in bed net areas and parasite diversity?

iv.) Is there a temporal shift in circulating parasite haplotypes following wide scale deployments of ITNs?

1.4 HYPOTHESES

H_0: The significant reduction in malaria transmission due to ITN use has no effect on polymorphisms of the gene encoding immune epitopes of the *P. falciparum* circumsporozoite protein.

H_A: The significant reduction in malaria transmission due to ITN use has an effect on polymorphisms of the gene encoding immune epitopes of the *P. falciparum* circumsporozoite protein.

1.5 OBJECTIVES

1.5.1 Main Objective

To assess the impact of ITNs on the genetic diversity of the *P. falciparum* circumsporozoite protein in an area of intense and perennial malaria transmission in western Kenya.

1.5.2 Specific Objectives

1. To investigate the nature of CSP gene polymorphisms in parasites from children in ITN and control areas before and after distribution of ITNs
2. To document the CSP gene diversity in parasites before and after introduction of ITNs
3. To compare the CSP haplotypes in parasites from children with or without bed nets

1.6 ANTICIPATED OUTPUT

Comparison of the genetic changes in CSP between *P. falciparum* parasites from bed net and control areas will provide an insight on the long term effects, in parasite diversity, to be expected when the on going ITN use campaigns gain their desired effect of large scale transmission reduction. The current study will provide information on the circulating haplotypes as well as the nature and extent of CSP gene polymorphisms in *P. falciparum* parasites after introduction of ITNs. This will be useful in the on going research, development and clinical trials of CSP-based vaccines.

CHAPTER TWO: LITERATURE REVIEW

2.1 MALARIA BURDEN

Malaria remains a major public health problem in many tropical and sub-tropical regions of the world. Every year, an estimated 350 to 500 million clinical episodes of malaria and 1.2-2.7 million malaria-related deaths are reported worldwide (WHO, 2003a). More than 90% of these deaths occur in sub-Saharan Africa (WHO/UNICEF, 2003). In addition to the direct morbidity and mortality effects, malaria has a devastating impact on income earnings in areas where it is endemic, further impoverishing already poor sectors of society (Sachs and Malaney, 2002). In sub-Saharan Africa, the disease' economic toll in countries with intense transmission causes an estimated 1.3% loss in economic growth annually through loss of working days and wages as well as loss of business and tourism potential (WHO, 2001).

In Kenya, high malaria prevalence rates are found in coastal and lake regions as a consequence of the ecology, which provides ideal conditions for *Anopheles* breeding (Ministry of Health, GoK, 2001). An extremely high malaria prevalence is found around Lake Victoria Basin, which has abundant mosquito breeding sites, favorable temperatures, rural poverty, dense population and poor health infrastructure (Arudo *et al.,* 2003). Anemia is the most common manifestation of severe malaria in the L. Victoria basin region (Bloland *et al.,* 1999) and mostly affects children below two years of age and pregnant women (van Eijk *et al.,* 2007). Prior to distribution of ITNs, the point prevalence of parasitemia in children less than five years old in Asembo bay

was reported to range between 60% and 80% depending on the season (Bloland *et al.,* 1999). In many Kenyan highlands and other semi-arid areas, climatic conditions and topography are conducive for frequent malaria outbreaks of epidemic intensity (Githeko *et al.,* 2001)

The emergence and rapid spread of parasite resistance to widely available, affordable and easy to administer drugs, such as chloroquine, is believed to be the major contributing factor to the resurgence of malaria in many areas of the world and the increase in malaria-specific morbidity and mortality in endemic areas in Africa (Trape, 2001). This is compounded by un-sustainability of vector control measures (Sharp *et al.,* 2002) and changes in climatic conditions that provide ideal breeding conditions for *Anopheles gambiae*, the most efficient and widespread malaria vector in sub-Saharan Africa (Breman, 2001). Additional factors that have contributed to the deteriorating malaria situation include; lack of commitment and effective policies by governments, political instabilities and population movements (Breman, 2001), poor infrastructure resulting in late or delayed presentation to health facilities, lack or inadequate laboratory capacity for adequate diagnosis leading to poor patient management and unavailability or stock-outs of effective antimalarial drugs (WHO/ UNICEF, 2005). The emergence of HIV/AIDS has also increased rates of severe and fatal *P. falciparum* infections in areas where the prevalence of the two infections are high, especially in Africa (Grimwade *et al.,* 2002).

In areas of Africa with stable transmission, malaria has been found to be both a cause and effect of poverty (Sachs and Malaney, 2002). Consequently, a substantially higher prevalence of malaria infection has been found among the poorest population groups (Barat *et al.,* 2003). In addition, malaria-associated mortality rates have been shown to be 39% higher in poor populations (Mwageni, 2002). Several factors could explain these observations. Poor families live in dwellings that offer little protection against mosquitoes and are less able to afford insecticide-treated nets and less likely able to pay either for prompt and effective malaria treatment or for transportation to a health facility with capability for proper diagnosis and treatment of the disease (Akazili, 2002).

2.2 MOST VULNERABLE GROUPS

The severity of malaria in infected populations is determined by several factors including age, pregnancy, genetic make-up, immunity and malaria transmission intensity (WHO, 1996). In areas of high and stable malaria transmission, young children and pregnant women are at high risk for malaria morbidity and mortality, where as in areas of low transmission or epidemic-prone areas, all individuals are at risk (WHO, 1996). People not residing in malaria endemic areas are at increased risk of infection if they move to endemic areas (Askling *et al.,* 2005).

In endemic areas most children experience their first malaria infections during their first or second year of life, when they have not acquired adequate

immunity, making their early years particularly dangerous (WHO, 2003c). In these areas, primigravidae and secundigravidae pregnant women are at high risk of malaria infection (Shulman *et al.,* 2001; WHO, 2003b). The high susceptibility in these groups of pregnant women has been attributed to lack of immunity to unique pregnancy-specific variant surface antigens, predominantly the VAR2CSA protein expressed by parasites that preferentially infect the placenta (Rogerson *et al.,* 2007). However, in cases of inadequate immunity to malaria as a result of living in areas of moderate to low transmission intensity or compromised immunity due to human immunodeficiency virus (HIV) infection, gravidity-dependent acquisition of immunity is affected and multigravidae are also at high risk of severe malaria (Shulman *et al.,* 2001).

2.3 CAUSATIVE AGENTS AND CLINICAL MANIFESTATIONS

There are four main malaria parasites that infect humans: *Plasmodium falciparum, P. malariae, P. ovale* and *P. vivax* (Bruce-Chwatt, 1987). Among the four, *P. falciparum* is the most dangerous and responsible for the majority of infections and deaths in sub-Saharan Africa (Oaks *et al.,* 1991). The major transmission vectors in sub-Saharan Africa are the *Anopheles gambiae* complex and *An. funestus,* with *An. gambiae,* the most efficient malaria vector, being the primary vector in this region (Beier *et al.*, 1994; Afolabi *et al.*, 2006).

The classic description of an individual progressing episodically from shaking chills through intense fevers to drenching sweats is characteristic but not

universal of all *P. falciparum* malaria infections (Oaks *et al.,* 1991). There are several clinical manifestations depending on factors such as age, level of exposure/ level of acquired immunity and transmission intensity (Reyburn *et al.,* 2005). The clinical spectrum ranges from asymptomatic infection to symptomatic infection comprising of fever and acute febrile syndrome, severe malaria and also lethal cases (Miller *et al.,* 2002). Malaria infection in clinically immune individuals is often asymptomatic, or may have symptoms that mimic other diseases, making diagnosis difficult, where as in non-immune individuals there is an increased risk of the infection progressing into severe malaria (Oaks *et al.,* 1991; Sharma *et al.,* 2004). Severe malaria caused by *P. falciparum* manifests itself as severe anemia, jaundice, renal failure, cerebral malaria and acute respiratory distress syndrome (ARDS) (Miller *et al.,* 2002).

Young children may have high blood levels of parasites but relatively mild symptoms, which may lead to misdiagnosis or delayed treatment (Oaks *et al.,* 1991). An acute malaria infection, can then progress in severity and present as seizures or coma which may kill a child directly and rapidly (Steketee *et al.,* 2001). Similarly, repeated malaria infections can lead to chronic infection resulting in the development of severe anemia (Steketee *et al.,* 2001). As a result of chronic malaria or repeated malaria infections, young children are weakened rendering them vulnerable to other common childhood illnesses, such as diarrhea and respiratory infections (Molineaux, 1997). Like malaria, these diseases are responsible for high rates of childhood morbidity and mortality in sub-Saharan Africa (WHO, 2003c)

In pregnant women, malaria infection contributes significantly to maternal and fetal morbidity and mortality (Ross *et al.,* 2006). However, the adverse effects of malaria during pregnancy depend on several factors including levels of pre-pregnancy immunity, genetic factors, HIV co-infection and malaria transmission intensity (Ross *et al.,* 2006). In areas of low transmission or epidemic-prone areas, malaria infection during pregnancy can lead to maternal death and adverse birth outcomes such as spontaneous abortion, stillbirth, premature delivery, low birth weight, and neonatal death (Newman *et al.,* 2003). Conversely, women in areas of high transmission have substantial levels of acquired immunity to malaria, malaria infection during pregnancy can therefore remain asymptomatic and untreated, leading to anemia and adverse birth outcomes such as low birth weight, a major risk factor for infant death in the first few months of life (Desowitz *et al.,* 1992; Steketee *et al.,* 2001).

2.4 IMMUNITY TO MALARIA

People residing in malaria endemic areas acquire immunity through natural repeated exposure to malaria parasites (Gupta *et al.,* 1999). Acquired immunity to malaria can be classified into clinical immunity and anti-parasite immunity (Artavanis-Tsakonas *et al.*, 2003). Clinical immunity is acquired first and protects an individual from acute febrile symptoms of malaria (Artavanis-Tsakonas *et al.,* 2003). Anti-parasite immunity involves development of immune mechanisms to kill the parasites or inhibit their replication (Artavanis-Tsakonas *et al.,* 2003). Immunity is reflected in clinical

disease and profiles of parasite density at different ages following an infection in endemic areas (Artavanis-Tsakonas *et al.,* 2003). In areas of high and perennial malaria transmission, the first infection usually occurs during early infancy, when maternal antibodies and physiological factors provide protection from life threatening illness (Riley *et al.,* 2000). Children who survive malaria during infancy, and have continued exposure to malaria parasites during their childhood, usually gain sufficient malarial immunity to protect them from severe infections later in life (Gupta *et al.,* 1999). Thus, young children in endemic areas have both fewer and less severe episodes of clinical malaria (Artavanis-Tsakonas *et al.,* 2003).

Development of immunity is influenced by malaria transmission intensity (Trape *et al.,* 1994). In areas with high transmission intensity clinical immunity develops at a younger age compared to areas with lower transmission intensity whereby the age at which clinical immunity develops shifts upwards (Sharma *et al.,* 2004). The rate of development of immunity to malaria in endemic areas depends on age; adults develop immunity faster than children (Baird, 1995). Children who have not yet developed protective immunity are at a higher risk of severe malaria infection (Artavanis-Tsakonas *et al.,* 2003). Pregnant women develop immunity to the unique pregnancy-specific variant surface antigens after their second and subsequent pregnancies placing multigravidae pregnant women at a reduced risk of severe malaria infection (Rogerson *et al.,* 2007).

The natural exposure to malaria parasites over a long period results in the development of a diverse repertoire of antibodies (Molineaux, 1996) that protects against many malaria parasite variants (Chattopadhyay *et al.,* 2003). This immunity limits high-density parasitemia and decreases the frequency and severity of clinical malaria episodes in older children and adults despite almost continuous infection (Bull *et al.,* 1998; Marsh, 1992). Unfortunately, sterile immunity is never acquired (Sharma *et al.*, 2004). Repeated administration of anti-malarial drugs has been reported to delay the development of immunity (Issifou *et al.,* 2003; Gatton *et al.,* 2004).

2.5 MALARIA CONTROL METHODS

The WHO has been in the forefront in the fight against malaria since the 1950s. Malaria was successfully eradicated or malaria episodes significantly reduced in regions with temperate climates and seasonal malaria transmission following the launch of the Global Malaria Eradication Programme in 1955 (Malcolm, 2002). This campaign laid emphasis on vector control using dichlorodiphenyltrichloroethane (DDT) for space and residual spraying as well as use of antimalarial drugs and surveillance (Malcolm, 2002). Sub-Saharan Africa did not benefit from the WHO global eradication campaign of the 1950s (Kouznetsov, 1977). This was attributed to several factors including the emergence of insecticide and antimalarial drug resistance, weak infrastructure, high malaria transmission rates and reduced international funding for malaria control and research (Baird, 2000). Malaria control in Africa involved treatment with effective, safe, easily available and affordable antimalarial

drugs such as quinine, chloroquine and sulfadoxine-pyrimethamine (SP) (Haworth, 1988). However, the emergence of antimalarial drug resistance contributed to a high malaria burden in Africa in the early 1990s (Baird, 2000), prompting the WHO to highlight malaria control efforts with the launch of the Roll Back Malaria (RBM) initiative (WHO, 1998). The main objective of RBM is to halve the global malaria burden by 2010 through implementation of four key strategies: insecticide-treated bed nets (ITNs), improved case management, control of malaria in pregnancy, and early warning and containment of epidemics (Nabarro *et al.,* 1998).

2.5.1 Antimalarial drugs

Proper diagnosis as well as availability of both effective and affordable antimalarial drugs is essential for proper malaria disease management. However, there has been development of parasite resistance to widely available, affordable and easy to administer drugs such as chloroquine (CQ) and sulfadoxine–pyrimethamine (SP). The incorrect use of antimalarial drugs, such as over-the-counter dispensation, following self prescription, as well as taking of incorrect dosage of the drugs, has contributed to this resistance. This resistance has led to urgent efforts to look for alternative strategies in malas wellaria control using antimalarial drugs (WHO, 2005). One strategy is to use combination therapy involving two antimalarial drugs with different mechanisms of action to slow down or stop emergence of drug resistance (White, 1999). Following extensive randomized trials, two antimalarial combination therapies (ACTs) have so far been licensed for malaria treatment,

artesunate+amodiaquine and artemether–lumefantrine (Coartem®) (WHO, 2005; Ndayiragije *et al.*, 2004).

Control of malaria in pregnancy in endemic areas has been made possible with the introduction of intermittent preventive treatment (IPT) as part of antenatal care (WHO, 2005). Effective containment of epidemics requires early detection, combined with the availability of control tools such as drugs, indoor residual spraying and ITNs for rapid deployment (WHO, 2005).

2.5.2 Vector control

Since the discovery of *Anopheles* mosquito as the malaria parasite vector in 1897, vector control has been recognized as an important malaria control tool (Trigg and Kondrachine, 1998). Vector control measures can be taken in different steps including; discouraging breeding by denying blood meal and reducing breeding sites, killing larvae and adult mosquitoes by use of larvicidal agents, space sprays and residual sprays (WHO, 2004b). In the early days vector control methods involved elimination of breeding sites by draining stagnant water or layering with oil, later coupled with use of insecticides for space and residual spraying (Trigg and Kondrachine, 1998).

Indoor residual spraying involves application of long-acting insecticide on surfaces such as walls and ceilings of houses to kill mosquitoes as they try to rest after a blood meal (Bouwman *et al.*, 1990). Larvicidal agents are applied at the breeding sites to kill the larvae (WHO, 2004a). Among the 12

insecticides currently recommended for indoor residual spraying, DDT has the longest residual efficacy (WHO, 2007). However, there have been increasing concerns about the risk factors to the environment and individuals, as result of its high toxicity and long half life, involved in the course of using DDT for malaria vector control leading to restriction of its use (United Nations Environment Programme, 2001)..

To improve the cost-effectiveness and sustainability of vector control, a global strategic framework for integrated vector control was developed (WHO, 2004a). This involves the use of a single or a combination of proven vector-control methods tailored according to knowledge of the vector ecology, disease epidemiology and human behavior (WHO, 2004b). Recently, the use of pyrethroids for both indoor space and residual spraying has been adapted as a result of their efficacy and safety compared to DDT (Sharp *et al.,* 1993; Lines, 1996). A more precise and effective use of residual pyrethroids has been by application to bed nets and curtains, to which mosquitoes are attracted in the course of seeking blood meal from bed or house occupants (Curtis and Townson, 1998).

2.5.3 Insecticide-treated bed nets

Wide-scale deployment of insecticide-treated bed nets (ITNs) forms one of the WHO Global Malaria Programme's key technical strategies for malaria control (Nabarro and Taylor, 1998). This adoption follows numerous studies that have shown the effectiveness of ITNs in preventing morbidity and

mortality due to malaria (D'Alessandro *et al.,* 1995; Binka *et al.,* 1996; Nevill *et al.,* 1996; Habluetzel *et al.,* 1997; Phillips-Howard *et al.,* 2003; ter Kuile *et al.,* 2003; Eisele *et al.,* 2005).

Before the demonstration of ITNs effectiveness in malaria control, untreated bed nets had been used for a long time to form a protective barrier around persons using them (Lindsay *et al.,* 1989). However, mosquitoes can feed on people through the nets, nets with even a few small holes provide little, if any, protection (Lindsay *et al.,* 1989). The application of a residual insecticide was greatly found to enhance the protective efficacy of bed nets by killing mosquitoes and other insects, as well as repellent properties that reduce the number of mosquitoes that enter the house and attempt to feed (Snow *et al.,* 1999). In addition, if high community coverage is achieved, the numbers and longevity of mosquitoes will be reduced thereby giving protection to all members of the community, regardless of bed net ownership (Gimnig *et al.,* 2003a).

Randomized controlled trials in areas of different malaria transmission pattern have shown that appropriate use of ITNs can reduce malaria transmission by 70-90% (Gimnig *et al.,* 2003b). Consequently, the reduced transmission has been shown to result in significant reductions in malaria associated under-5 child mortality in several endemic areas. In the Gambia, ITNs were associated with a reduction in child mortality of over 20% (D'Alessandro *et al.,* 1995). A 30% reduction in mortality was recorded in ITN trials both in Kilifi, Kenya

(Nevill *et al.,* 1996) and in Ghana (Binka *et al.,* 1996). In Burkina Faso, ITNs were associated with a 20% reduction in mortality (Habluetzel *et al.,* 1997). In a recent trial conducted in Asembo Bay, western Kenya, an area of high and perennial malaria transmission, ITNs were associated with over 10% reduction in childhood mortality (Phillips-Howard *et al.,* 2003). In addition, the use of ITNs has been shown to result in significant reductions in the adverse effects of malaria during pregnancy (ter Kuile *et al.,* 2003).

However, these results were obtained in a trial setting where nets and insecticide were provided free of charge. There had been doubts whether these results can be replicated in real-life, that is in a situation where ITNs are to be bought, not distributed for free (Curtis *et al.,* 2003). This was addressed by a study involving a large-scale social marketing program in an area of high and perennial malaria transmission in Tanzania (Hanson *et al.,* 2002). Results from this social marketing program showed a rise in ITN coverage of infants from less than 10% at baseline to more than 50% three years later (Abdulla *et al.,* 2001). In addition, the increase in bed net coverage was associated with a 27% increase in survival of children aged 1 to 48 months and a 63% reduction of anemia caused by malaria infection in the same age group (Abdulla *et al.,* 2001). Taken together, the ITN trials and social marketing studies have demonstrated that ITNs are a cost-effective and efficacious malaria control tool for the protection of groups most at risk of malaria, people are wiling to pay for them and large-scale ITN programs are feasible (WHO, 2005).

2.6 MALARIA VACCINES

In addition to the available malaria control tools, extensive efforts have been put in the development of a vaccine against *P. falciparum* malaria to supplement the current methods. Most of the malaria vaccines under development target different stages of the parasite life cycle; pre-erythrocytic vaccines targeting the sporozoite and liver stages, erythrocytic vaccines targeting the blood stages and transmission-blocking vaccines targeting the sexual stages (Jones *et al.,* 1994; Ménard, 2005). Pre-erythrocytic vaccines are some of the most successful malaria vaccines and some have reached phase IIb human trials and were found to be safe and immunogenic (Aponte *et al.,* 2007). These include irradiated sporozoites (Hoffman *et al.,* 2002; Mueller *et al.,* 2005; Kumar *et al.,* 2006), SPf66 which is a subunit vaccine comprising of antigens from the blood stages of malaria linked together with an antigen from the sporozoite stage (Alonso *et al.,* 1994; Nosten *et al.,* 1996; Graves and Gelband, 2006) and RTS,S which is based on 19 NANP repeats and the C-terminus of the CSP protein (Alonso *et al.,* 2005; Bojang *et al.,* 2005; Enosse *et al.,* 2006; Aponte *et al.,* 2007). These vaccines are based or include the circumsporozoite protein, obtained from a cloned parasite line representing a single haplotype as an immunogen (Alonso *et al.,* 1994; Alonso *et al.,* 2005; Kumar *et al.,* 2006). This has raised concerns as to whether such a vaccine would be universally effective against all *P. falciparum* haplotypes circulating in an area (Zevering *et al.*, 1998).

2.7 THE MALARIA LIFE CYCLE

When a female *Anopheles* mosquito feeds on malaria parasite infected blood, gametocytes ingested fertilize in the mosquito's stomach to produce a zygote (Ménard, 2005). The zygote grows into an ookinete which invades the midgut wall of the mosquito where they multiply and develop into an oocyst full of sporozoites (Ménard, 2005). The oocyst grows and rupture to release sporozoites, which make their way to the mosquito's salivary glands (Figure 1).

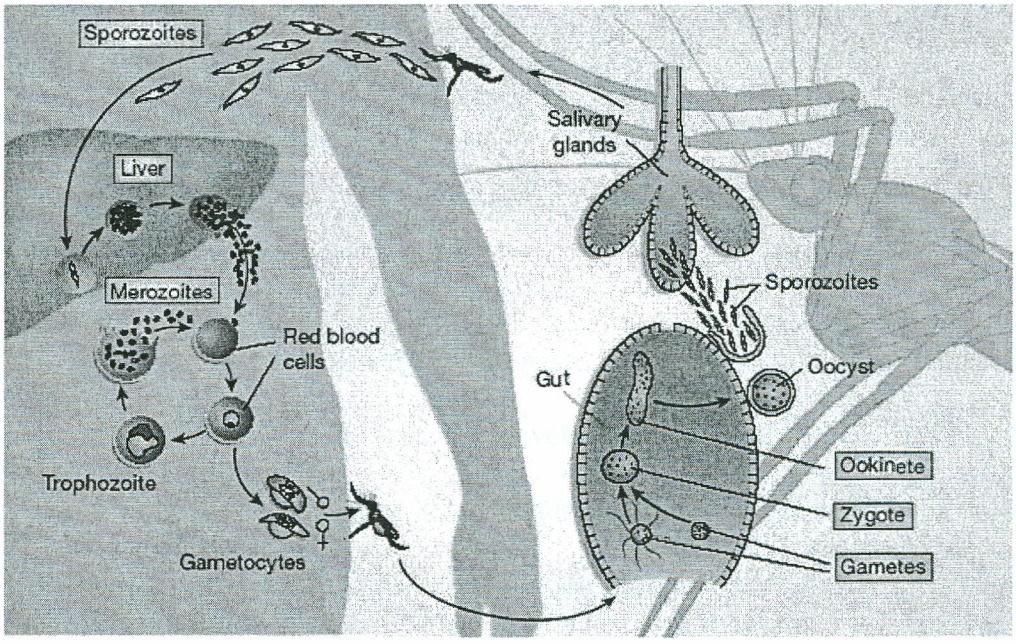

Figure 1: The malaria parasite life cycle showing the pre-erythrocytic stages which include the sporozoites injected by the mosquito and the liver-stage parasites which differentiate into merozoites, the erythrocytic stages which comprise the merozoites and the extracellular stages which include the sexual stages (gametes and zygotes) and ookinetes (Ménard, 2005).

The sporozoites are released from the salivary glands of the infected mosquito during a blood meal into the bloodstream of the human host where they quickly invade hepatocytes (Ménard, 2005). *P. falciparum* liver-stage parasites differentiate and undergo asexual multiplication resulting in merozoites which burst from the hepatocytes and invade erythrocytes (Ménard, 2005).

2.8 THE CIRCUMSPOROZOITE PROTEIN

Parasite surface antigens are good candidates for studying the relationship between transmission intensity and genetic diversity. One such antigen is the circumsporozoite protein (CSP) which is the predominant antigen on the surface of sporozoites (Yoshida *et al.,* 1980). The CSP gene was the first plasmodium gene to be cloned and partially sequenced (Godson *et al.,* 1983). The CSP has been extensively investigated since the 1970s as a candidate antigen for anti-sporozoite vaccine development (Nardin *et al.,* 1998). A vaccine against the sporozoite stage would abrogate the parasite's life cycle before progression to the symptomatic blood stages. The study of the genetic characteristics of the CSP carboxyl terminal goes along way in providing information needed in the design of a CSP based vaccine.

The *P. falciparum* CSP gene, *pfcs*, encodes a protein of approximately 420 amino acid residues with a molecular weight of 58 kDa. The gene, as represented in Figure 2, can be divided into two non-repetitive regions (5' and

3' ends) and a variable central region consisting of central tandem repeats that form immunodominant B-cell epitopes (Rich *et al.,* 1997).

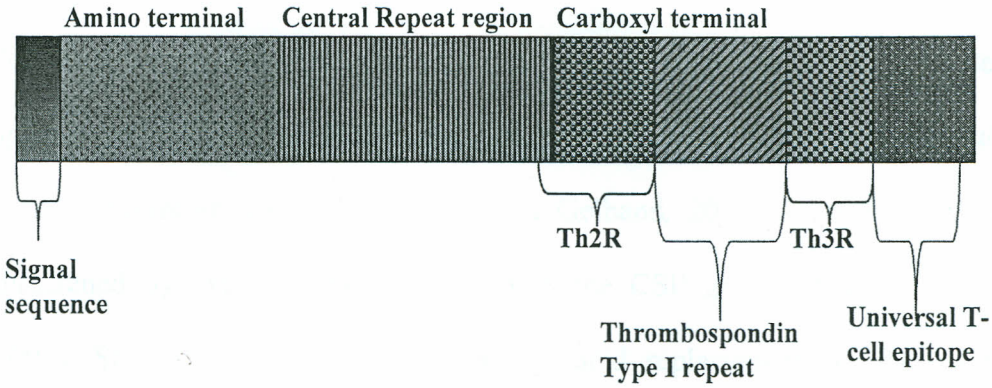

Figure 2: Schematic representation of *P. falciparum* CSP gene. The Amino terminus is preceded by a short signal sequence. The T helper and universal T-cell epitopes are on the carboxyl terminus of the protein.

T-cell epitopes, the immunodominant of which are Th2R and Th3R, occur at the carboxyl terminal of the protein (Lockyer *et al.,* 1989). Th2R occurs to the amino terminal of Th3R and to the carboxyl end of Th3R is found the "Universal T-cell epitope" (Doolan *et al.,* 1992).

Sequencing of the *pfcs* genes or gene fragments from laboratory and field isolates has revealed high levels of genetic polymorphisms at the Th2R and Th3R epitopes and in the repeat allotypes (Escalante *et al.,* 2002). The 5' region (amino terminal end) has less polymorphisms with the 3' end (carboxyl terminal end) accounting for a third of all the genetic diversity of the CSP gene (Escalante *et al.,* 2002) . Sequencing *P. falciparum* from Thailand

isolates revealed that only non-synonymous point mutations occur in the Th2R and Th3R T-cell epitopes and that point mutations outside these epitopes were rare (Kumkhaek *et al.,* 2004).

Although, most of the promising malaria vaccines such as irradiated sporozoites, SPf-66 and RTS,S have been based on the CSP (Bojang *et al.,* 2005; Mueller *et al.,* 2005; Graves and Gelband, 2006), their efficacy is threatened by extensive polymorphism in the CSP gene (Escalante *et al.,* 2002). Studies have reported that amino acid replacements in the T-cell epitopes abrogate their recognition by the immune system of naturally exposed people (Bonelo *et al.,* 2007). The extensive polymorphism would severely limit vaccine efficacy if the vaccine-induced immune responses are allele specific since this could provide the parasite with a means to escape the host protective immune responses (Bonelo *et al.,* 2007).

Several studies conducted in different regions have shown a relationship between malaria transmission intensity and parasite diversity (Escalante *et al.,* 1998; Paul *et al.,* 1998; Anderson *et al.,* 2000; Escalante *et al.,* 2002; Escalante *et al.,* 2004). Transmission intensity is correlated with malaria prevalence in an area and the rates of parasite recombination in mosquito vectors (Rich *et al.,* 2000). Therefore, it is expected that higher parasite rates in humans will maintain higher effective population size where diversifying natural selection could highly contribute to sustaining parasite polymorphisms (Escalante *et al.,* 2002). Transmission reduction as a result of ITN use may,

therefore, have the effect of reducing parasite rates and gene polymorphisms as a consequence of reduced effective population size (Escalante *et al.,* 2002). If this is the case, then it has important implications in malaria vaccine development as it implies that polymorphism, which remains a great impediment in malaria vaccine development can be decreased by transmission reduction. The ITN trial site in western Kenya offers an excellent opportunity to study the relationship between transmission reduction and parasite genetic diversity.

CHAPTER THREE: MATERIALS AND METHODS

3.1 STUDY SITE

The current study used stored red blood cell (RBC) pellets that were collected in a series of cross-sectional surveys conducted by KEMRI/CDC to assess the impact of ITNs on malaria specific morbidity in children less than five years of age in Asembo Bay, Bondo District in western Kenya (Figure 3).

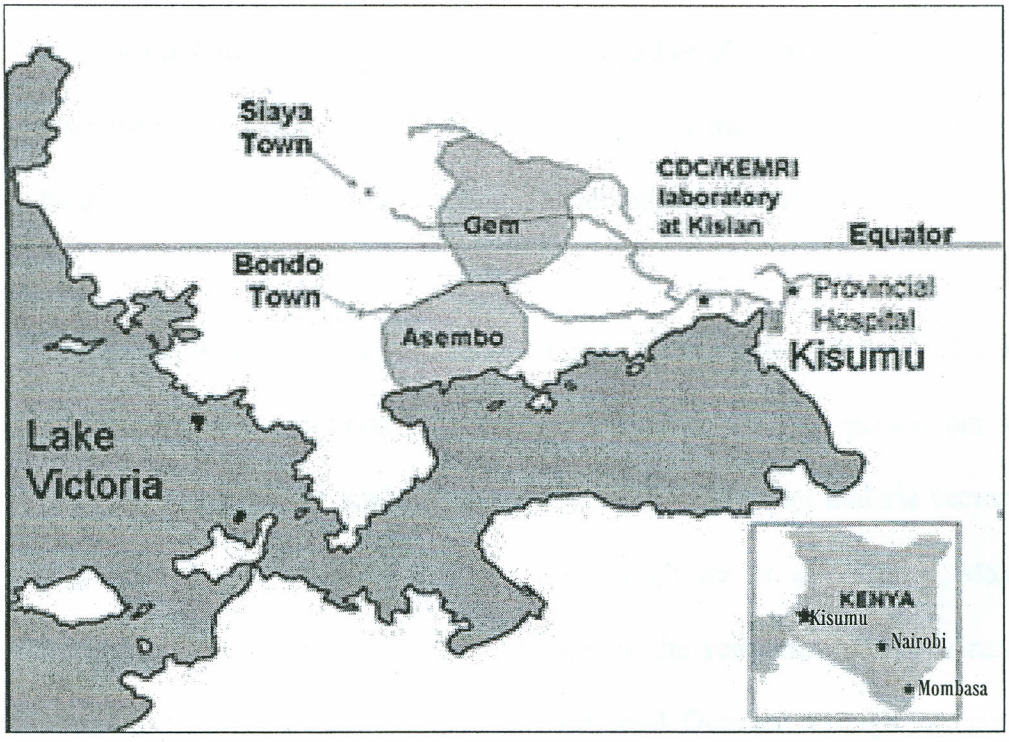

Figure 3: The ITN trial site in western Kenya. Samples used in the current study were collected in cross-sectional surveys conducted only in Asembo.

Asembo Bay is situated approximately 55 kilometers northwest of Kisumu City along the shores of Lake Victoria. More than 95% of the residents belong to the Luo ethnic group. Majority of Asembo Bay residents earn their living

through small-scale farming, fishing and retail businesses. Residents live in scattered homesteads consisting of one or more houses, surrounded by their agricultural plots, resulting in a dispersed settlement pattern (Phillips-Howard *et al.,* 2003). About 65% of the houses are mud-walled and grass thatched. Twenty percent of the houses have a tin roof, while 19% are made of brick with tin roof (Bloland *et al.,* 1999). For most house types, eaves are usually open, allowing unimpeded entrance and exit for mosquitoes. Approximately 20% of the houses have eaves (Phillips-Howard *et al.,* 2003). Sleeping spaces may consist of a bed with frame and mattress or a mat on the floor located in bedrooms, sitting rooms and kitchens (Lindblade *et al.,* 2004).

Before the wide scale deployment of ITNs, malaria transmission in the area was intense and perennial with 60-300 infective bites per person per year (Beier *et al.,* 1994; ter Kuile *et al.,* 2003). The predominant malaria vectors in this area are *A. gambiae* and *A. funestus* (Beier *et al.,* 1994). Malaria transmission in this region occurs through out the year with peaks during and after the rainy seasons, May through July and October through November. Malaria and Human Immunodeficiency Virus are the two most important causes of morbidity and mortality in this population (Phillips-Howard *et al.,* 2003). Malaria illness is the cause of half

of all outpatient hospital visits and hospital deaths in young children in Asembo bay (Phillips-Howard *et al.,* 2003).

3.2 ITN TRIAL

The Kenya Medical Research Institute in collaboration with the Centers for Disease Control and Prevention (KEMRI/CDC) has been conducting multi-disciplinary studies in Asembo Bay since 1992. A large community-based immuno-epidemiologic study had been conducted from 1992 to 1996 in this area (Bloland *et al.,* 1999). The ITN trial had been conducted in Asembo Bay and Gem, an adjoining area to the north of Asembo Bay, from 1996 to 2002 (Phillips-Howard *et al.,* 2003). The current study is restropective of the morbidity cross-sectional surveys that had been conducted from 1996 to 2002 in Asembo Bay only.

Villages in Asembo had been randomly assigned to the intervention or control groups through a public lottery. After a baseline survey in the first quarter of 1996, each household in the intervention villages had received ITNs covering all bed spaces, (Phillips-Howard *et al.,* 2003). This had provided a bed net coverage ratio of 1.5 persons per ITN (Alaii *et al.,* 2003). The bed nets had been pre-treated with permethrin and retreated twice a year by the study team to maintain a target dose of 500mg of permethrin/m^2 (Phillips-Howard *et al.,* 2003). Extensive educational campaign to promote correct use had accompanied distribution of ITNs in the beginning of the trial (Alaii *et al.,* 2003). Adherence throughout the two-year intervention period in children less than five years had been 66% (Alaii *et al.,* 2003).

3.3 CROSS-SECTIONAL SURVEYS

During the 2-year ITN trial period, before the control areas had received ITNs, three cross-sectional surveys had been conducted. The first cross-sectional survey representing the baseline had been conducted in October-November 1996 prior to distribution of ITNs (ter Kuile *et al.,* 2003). The second and third surveys had been conducted in February-March 1998 and June-July 1999, respectively (ter Kuile *et al.,* 2003). After the two year trial period, control villages had been issued with ITNs and an extended surveillance conducted from April 1999 to February 2002. The fourth survey had been conducted during the extended surveillance in May-June 2001. Detailed descriptions of the extended surveillance have been reported elsewhere (Lindblade *et al.,* 2004).

The current study was restropective of the ITN trial and used blood samples collected during the morbidity cross-sectional surveys.

3.4 STUDY DESIGN

In order to acquire parasite diversity data on an approximate yearly basis before and after the ITN intervention, samples for the current study were randomly selected from those collected during cross-sectional surveys conducted at baseline (BX0), 14-months (BX1), 30-months (BX3) and 46-months (BX5) post-bed net intervention.

Table 1

Study Design

Cross-sectional survey	BX0	BX1	BX3	BX5
Months after Intervention	0	14	30	46
Blood sample collection	✓	✓	✓	✓

The current study was approved by the National Ethical Review Committee of KEMRI, Nairobi, Kenya, and the Institutional Review Board of CDC, Atlanta, Ga. Written consent was obtained from parents or legal guardians of children invited to participate in the cross-sectional surveys.

3.5 LABORATORY METHODS

3.5.1 Malaria diagnosis

All laboratory assays were conducted at the KEMRI/CDC laboratories in Kisian, Kisumu, Kenya. Thick and thin blood smears prepared in the field were stained with Giemsa and examined for parasites by microscopy (ter Kuile *et al.,* 2003). Smears were considered negative if no asexual parasites were detected in 100 high-power ocular fields of the thick smear. Parasite densities were counted against 500 white blood cells (WBC) and then expressed per micro liter (mm^3) of blood using an estimated WBC count of 8,000/mm^3 (ter Kuile *et al.,* 2003).

3.5.2 DNA purification

DNA was isolated from stored red blood cell pellets using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, 50 µl of thawed red blood cell pellets was diluted with 150 µl sterile phosphate-buffered saline (PBS) and then transferred to 1.5 ml microcentrifuge tubes containing 20 µl Protease. After the addition of 200 µl of lysis buffer (buffer AL), the contents were mixed thoroughly and incubated at 56°C for 10 minutes to ensure efficient lysis of red blood cells. This was followed by the addition of 200 µl reagent grade ethanol (98%) and the vortexing of the tube. The contents were then transferred to QIAamp spin columns and centrifuged at 6000G for one minute. After removal of the supernatant, the samples were thoroughly washed, first with Buffer AW1 and centrifugation at 6000G for one minute, then with Buffer AW2 and centrifugation at 13200G for three minutes. This was followed by a final centrifugation at 13200G for one minute to completely remove any remaining buffer AW2. The purified parasite DNA was eluted with 200 µl elution buffer (Buffer AE). The DNA was aliquoted into two tubes of 100 µl and stored at -20°C.

3.5.3 Polymerase chain reaction (PCR)

The 3' region of the *falciparum* CSP gene was amplified by standard polymerase chain reaction (PCR). Each 25 µl of amplification reaction mixture contained; 1X TBE buffer, 2.0 mM $MgCl_2$, 250 µM dNTPs, 0.2 µM of each primer and 2.5 U Taq polymerase (Promega Corporation, Madison,

Wisconsin, USA). One set of primers (forward primer; **ACAATCAAGGTAATGGACAAGG** and reverse primer; **AGGATTAATAATGGTATTATCCTTCT**) was used to amplify a 354 base-pair (bp) fragment encompassing the Th2R, Th3R and "the universal T-cell epitope" regions of the CSP gene. The amplification conditions were as follows: 95°C for 5 minutes, 58°C for 2 minutes and 72°C for 2 minutes, one cycle, 94°C for 1 minute, 58°C for 2 minutes and 72°C for 1 minute, 32 cycles followed by a final elongation step at 72°C for 10 minutes using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). For quality control, random amplicons were separated on 1.5% agarose gel and visualized on an UV trans-illuminator, after staining with ethidium bromide.

3.5.4 Cycle sequencing

The PCR amplicon was purified using Centri-Sep columns (Princeton Separations, Adelphia, NJ) to remove excess primers and salts according to manufacturer's instructions. Briefly, 0.8 ml of deionized water was added to the Centri-Sep column, vortexed briefly and left at room temperature for two hours to allow the gel to hydrate. After setting of the gel, interstitial fluid was removed by allowing excess fluid to drain into a wash tube and then spinning at 750 G for two minutes. The sample was processed by transferring 20 µl of PCR product onto the top-center of the gel bed. The column was then placed into a sample collection tube and centrifuged at 750 G for two minutes and the purified amplicon collected at the bottom of the sample collection tube.

The clean PCR product was used for the sequencing reaction using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) as per the manufacturer's instructions. Each 20 µl of the sequencing reaction contained; 2.5X Terminator ready reaction mix, 5X Big Dye sequencing buffer, 0.2 µM of one primer and 200 ng of template. The cycling conditions were as follows; 96 °C for 1 minute, 96°C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes, 25 cycles. The same primers used for the primary PCR were used for the sequencing reactions.

The sequencing products were purified using Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) as described earlier. A speed vacuum (Thermo Electron Corporation, Milford, NY, USA) was used to dry the purified sequencing products to remove all the water. The dry product was then resuspended by adding 10 µl of Hi-Di™ Formamide (Applied Biosystems, Foster City, USA) followed by denaturation at 95 °C for 2 minutes. The samples were then loaded into a 96-Well plate for sequencing using an automated sequencer ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Initial sequence analysis involved base calling to obtain the actual nucleotide sequences. Sequences were manually checked and edited using the ChromasPro software program (Technelysium Pty Ltd, Eden Prairie, MN, USA). Sequences were aligned using the ClustalW software

(http://www.ebi.ac.uk/Tools/clustalw/index.html/) in MEGA version 4 (Tamura *et al.,* 2007). The published sequences; 3D7 and 7G8 were used as reference sequences.

3.6 STATISTICAL ANALYSIS

The characteristics of the study participants; ages, sex ratios, hemoglobin levels and parasite densities in children residing in the ITN and control areas were compared by Fisher's Exact test and non parametric tests. Point mutations at the Th2R and Th3R regions of the CSP gene were explored both separately and combined using the statistic π, which is the average number of nucleotide substitutions between any two sequences (Nei, 1987). Differences in π were compared by randomization methods. Genetic differentiation of the sub-populations was explored using the nearest-neighbor statistic (S_{nn}) which is a measure of how often the "nearest neighbors", in sequence space, of sequences are actually from the same locality in geographic space (Hudson, 2000). Polymorphism at the Th2R and Th3R epitopes was further explored separately using haplotype diversity (Nei, 1987). Differences in the proportion of haplotypes between bed net and control areas and between the different cross-sectional surveys were determined by Chi-square test. To determine the role of recombination on the observed polymorphism, the minimum number of recombination events (Rm) was estimated (Hudson *et al.,* 1985).

The numbers of synonymous and non-synonymous substitutions were estimated using the method of Nei and Gojobori (Nei and Gojobori, 1986)

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The numbers of synonymous and non-synonymous substitutions were estimated using the method of Nei and Gojobori (Nei and Gojobori, 1986)

with the Jukes and Cantor correction (Jukes *et al.,* 1969). The evidence of positive and purifying selection was explored using the Codon-Based Z-test (large sample) (Tamura *et al.,* 2007)

CHAPTER FOUR: RESULTS

4.1 CHARACTERISTICS OF THE STUDY PARTICIPANTS

A total of 663 blood samples that were smear-positive by microscopy were used for DNA purification. The characteristics of the 663 children in all the cross-sectional surveys are shown in Table 2.

Table 2

Characteristics of the study population

Survey¶	Age in months (SE)	Sex ratio, % Female	Pmm^3 (SE)
BX0			
ITN (N = 71)	24.01 (1.78)	36.6	7333 (1103.4)
Control (N = 69)	23.16 (1.84)	39.1	4548.8 (705.9)
P	0.668	0.759	0.0365
BX1			
ITN (N = 70)	20.90 (1.26)	44.3	6885.5 (1262.5)
Control (N = 76)	19.42 (1.22)	47.4	8493.3 (1926.1)
P	0.371	0.709	0.494
BX3			
ITN (N = 92)	29.07 (1.20)	53.3	8029 (2035.4)
Control (N = 89)	29.15 (1.23)	56.2	7500.4 (1497.5)
P	0.900	0.693	0.835
BX5			
ITN (N = 100)	52.23 (1.16)	54.0	5166.7 (918.05)
Control (N = 98)	52.85 (1.23)	53.1	6604.4 (1723.8)
P	0.678	0.895	0.460

N = number of samples tested

¶ Four cross-sectional surveys were conducted: BX0 at baseline, BX1 at 14 months, BX3 at 30 months and BX5 at 46 months.

Pmm^3 = Parasites per microlitre

SE = Standard error of mean

P = P value

In all cross-sectional surveys, there was no significant difference in age and sex ratios between children from intervention and control areas. At baseline, the average parasite density in children randomized to the intervention group was significantly higher than in children randomized to the control group (P = 0.0365, Fisher's test).

4.2 PCR AMPLIFICATION AND SEQUENCING OF THE CSP C-TERMINAL DOMAIN

After PCR amplification of the 354 base pair carboxyl terminal domain of the CSP gene, random sample PCR products were separated on 1.5 % agarose gel to check the success of the PCR reaction (Figure 4).

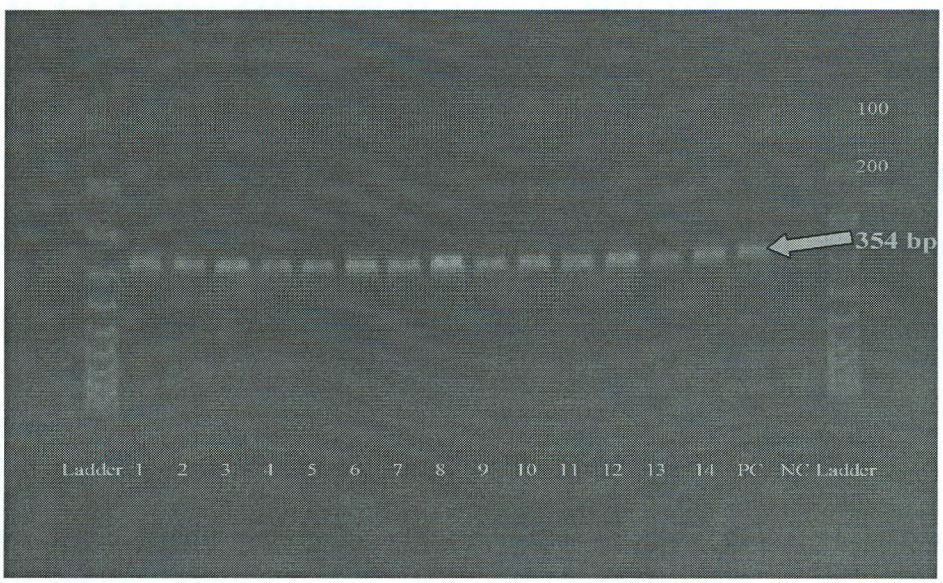

Figure 4: PCR product of the C-terminal domain of CSP gene covering the Th2R, Th3R universal T-cell epitopes (354 base pairs). 3D7 was used as a positive control (PC) and PCR water as a negative control (NC). A 100 base pair molecular weight ladder was used for base pair estimation.

Successfully amplified PCR products were subsequently cleaned and sequenced. Figure 5 is an example of a successful sequencing run.

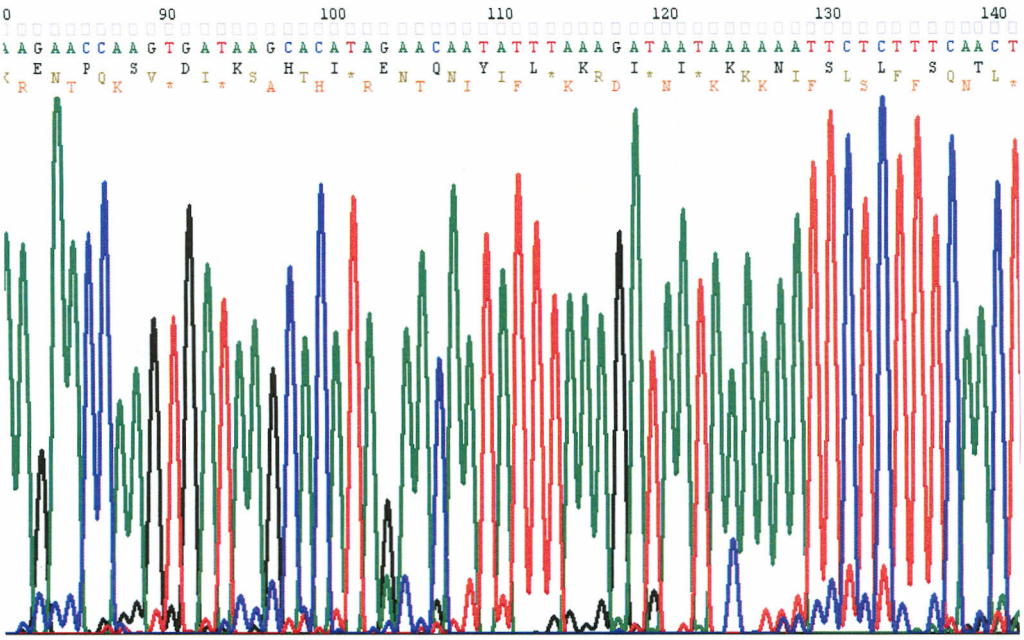

Figure 5: Chromatogram showing sequence data. Each different peak color represents a particular base; green = adenine, black = guanine, blue = cytosine and red = thymine.

Out of a total 663 samples analyzed in the four cross-sectional surveys, 632 samples were successfully sequenced; 138 at baseline (ITN = 70, Control = 68), 144 at survey 1 (ITN = 69, Control = 75), 164 at survey 3 (ITN = 83, Control = 81) and 186 at survey 5 (ITN = 97, Control = 89).

4.3 NATURE OF CSP GENE POLYMORPHISMS

All sequences were aligned based on published results of *P. falciparum* parasite clone 7G8 (Dame *et al.*, 1984).

	329		331		333		335		337		339		342
Allele	K	H	I	E	Q	Y	L	K	K	I	K	N	S	I
7G8	AAG	CAC	ATA	GAA	CAA	TAT	TTA	AAG	AAA	ATA	AAA	AAT	TCT	ATT
1	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
2	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
3	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. C.	. . .	. . .	. . .	. . .	C. .
4	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. T.	. . .	C. .	. . .	. . .	C. .
5	C. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
6	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. C.	. . .	. . .	. . .	. . .	C. .
7	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
8	C. .	. . .	. . .	. . .	A. .	. . .	. . .	C. .	. . .	. . .	C. .	. . .	. . .	C. .
9	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	C	. . .	. . .	. . .	. . .	. . .
10	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. G.	. . .	C. .	. . .	. . .	C. .
11	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. .	C	. . .	. . .	. . .	. . .	C. .
12	. . .	. . .	. . .	A. .	G. .	. . .	. . .	. . .	. T.	. . .	C. .	. . .	. . .	C. .
13	. . .	. . .	. . .	A. .	G. .	. . .	. . .	. .	C	. . .	. . .	C. .	. . .	. . .
14	C. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. C.	. . .	. . .	. . .	. . .	C. .
15	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. T.	. . .	. . .	. . .	. . .	C. .
16	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
17	. . .	. . .	. . .	AC.	G. .	. . .	. . .	. . .	. G.	. . .	C. .	. . .	. . .	C. .
18	C. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. T.	. . .	C. .	. . .	. . .	C. .
19	. . .	. . .	. . .	A. .	G. .	. . .	. . .	. .	C	. . .	C. .	. . .	. . .	C. .
20	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
21	. . .	. . .	. . .	AC.	G. .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
22	C. .	. . .	. . .	A. .	A. .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
23	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. G.	. . .	C. .	. . .	. . .	C. .
24	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. T.	. . .	. . .	. . .	. . .	C. .
25	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
26	. . .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	G. .	. . .	C. .	. . .	. . .	C. .
27	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	C. .
28	C. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
29	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
30	. . .	. . .	. . .	AC.	G. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
31	C. .	. . .	. . .	. . .	. . .	. . .	. . .	. .	C	. . .	. . .	. . .	. . .	C. .
32	C. .	. . .	. . .	A. .	A. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	C. .
33	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. T.	. . .	C. .	. . .	. . .	C. .
34	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. .	C	. . .	. . .	. . .	. . .	. . .
35	C. .	. . .	. . .	A. .	G. .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
36	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. .	C	. . .	. . .	. . .	. . .	C. .
37	C. .	. . .	. . .	A. .	G. .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
38	C. .	. . .	. . .	A. .	A. .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
39	. . .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
40	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
41	. . .	. . .	. . .	A. .	G. .	. . .	. . .	. . .	. C.	. . .	C. .	. . .	. . .	C. .
42	. . .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. . .	. . .	C. .	. . .	. . .	C. .
43	C. .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
44	C. .	. . .	. . .	. . .	A. .	. . .	. . .	. . .	. G.	. . .	C. .	. . .	. . .	C. .
45	. . .	. . .	. . .	A. .	G. .	. . .	. . .	. . .	. G.	. . .	C. .	. . .	. . .	C. .

Allele	329 K	H	331 I	E	333 Q	Y	335 L	K	337 K	I	339 K	N	S	342 I
7G8	AAG	CAC	ATA	GAA	CAA	TAT	TTA	AAG	AAA	ATA	AAA	AAT	TCT	ATT
46	...	...	...	A..	...	...	...	...	...	...	...	...	...	...
47	C..	...	...	A..	...	...	...	...	.T.	...	C..	...	...	C..
48	...	...	...	...	G..	...	...	...	.G.	...	C..	...	...	C..
49	...	...	...	...	G..	...	...	...	...	...	C..	...	...	C..
50	C..	...	...	...	A..	...	...	C..	.C.	...	C..	...	...	C..
51	C..	...	...	AC.	G..	...	...	...	.G.	...	C..	...	...	C..
52	...	...	...	...	G..	...	...	...	...	...	...	...	...	...
53	...	...	...	AC.	G..	...	...	...	.G.	...	C..	...	...	...
54	C..	...	...	...	A..	...	...	C..	.T.	...	C..	...	...	C..
55	...	...	...	...	...	...	...	C..	.C.	...	C..	...	...	C..
56	C..	...	...	...	...	...	...	C..	...	...	C..	...	...	C..
57	...	...	...	A..	...	...	...	...	.T.	...	C..	...	...	C..
58	C..	...	...	A..	A..	...	...	...	.C.	...	...	...	...	C..
59	...	...	...	...	...	...	...	..C	.T.	...	...	...	...	C..
60	...	...	...	...	...	...	...	..C	.C.	...	...	...	...	C..
61	C..	...	...	A..	A..	...	...	...	.T.	...	...	...	...	C..
62	C..	...	...	A..	...	...	...	...	.C.	...	C..	...	...	C..
63	...	...	...	A..	G..	...	...	...	...	...	C..	...	...	C..
64	...	...	...	A..	...	...	...	..C	.T.	...	C..	...	...	C..
65	C..	...	...	...	A..	...	A..	...	...	...	C..	...	...	C..
66	...	...	...	...	...	...	...	..C	...	...	C..	...	...	C..
67	C..	...	...	...	A..	...	.G	...	C..	...	C..	...	...	C..
68	...	...	...	A..	...	...	...	...	.C.	...	...	...	...	C..
69	C..	...	...	...	...	...	...	...	.T.	...	...	...	...	C..
70	C..	...	...	AC.	A..	...	...	...	...	...	...	...	...	...
71	C..	...	...	A..	A..	...	...	...	.G.	...	C..	...	...	C..
72	...	...	...	...	...	...	...	...	.T.	...	CG.	...	...	C..
73	...	...	...	A..	G..	...	...	.C.	...	...	...	...	...	...
74	C..	...	...	...	A..	...	...	..C	.C.	...	...	...	...	C..
75	C..	...	...	...	...	...	...	C..	.C.	...	C..	...	...	C..
76	...	...	...	...	...	...	...	..C	...	...	C..	...	...	...
77	...	...	...	A..	...	...	...	..C	.C.	...	C..	...	...	C..

Figure 6: Nucleotide sequence variation in the Th2R epitope. The 7G8 parasite strain was used as the reference. DNA sequence identity is indicated by a dot and polymorphic nucleotides are shown.

Point mutations (single base pair substitutions) were observed at specific positions, 329, 332, 333, 336, 337, 339 and 342, on the CSP Th2R epitope (Figure 6). Both transitional and transversional replacement of single nucleotides were observed. Most of the substitutions were of a non-

synonymous nature. At the nucleotide level, most mutations were at the first or second position of the codons. One major third codon position substitution at residue 336 led to a K to N amino acid change. Another third codon position substitution observed in some rare Th2R haplotypes at position 335 was either silent or neutral. In most positions there was only one kind of substitution, for example, at position 332 the guanine in the first codon position could only be replaced by an adenine. However, in other positions such as 333 and 337 different types of substitutions were observed such that an amino acid could be replaced by as many as three different residues.

The commonly observed substitutions were of a particular nature. For example, Lysine residue instead of Glutamine at position 333 was almost always accompanied by a Glutamine instead of Lysine at position 339.

Allele	367 N	K	369 P	K	371 D	E	373 L	D	375 Y	E	377 N	378 D
7G8	AAT	AAA	CCT	AAA	GAC	GAA	TTA	GAT	TAT	GAA	AAT	GAT
1	...	...	...	...	...	C..	...	...	...	...	...	...
2	...	...	...	...	...	C..	...	...	...	.C.	...	...
3	...	...	...	...	...	C..	...	A..	...	...	...	...
4	...	...	...	...	...	...	...	...	...	...	...	...
5	G..	...	...	...	A..	C..	...	...	...	...	...	...
6	...	...	...	...	...	...	...	...	...	.C.	...	...
7	G..	...	...	...	...	C..	...	...	...	.C.	...	...
8	G..	...	...	...	...	C..	...	...	...	AT.	...	...
9	...	...	...	...	...	...	...	A..	...	...	...	...
10	GG.	...	T..	...	A..	...	...	...	...	...	...	...
11	GG.	...	...	...	...	...	...	...	...	...	...	...
12	GG.	...	...	...	...	...	...	...	...	.C.	...	...
13	...	...	...	...	...	C..	...	A..	...	.C.	...	...
14	GG.	...	...	...	..A	...	...	A..	...	...	...	...
15	GG.	...	...	...	A..	...	...	...	...	...	...	...
16	G..	...	...	...	...	C..	...	...	...	...	...	...
17	GG.	...	T..	...	...	...	...	...	...	...	...	...
18	...	...	...	...	...	C..	...	...	...	.T.	...	...
19	G..	...	...	...	...	C..	...	A..	...	...	...	...
20	G..	...	...	...	...	C..	...	A..	...	.C.	...	...
21	GG.	...	T..	...	A..	C..	...	...	...	...	...	...
22	G..	...	...	...	A..	...	...	...	...	...	...	...
23	G..	...	...	...	A..	C..	...	...	...	.C.	...	...
24	G..	...	...	...	..A	...	...	A..	...	...	...	...
25	...	...	...	...	...	C..	..G	...	...	.C.	...	...
26	G..	...	...	...	...	...	...	...	...	.C.	...	...
27	G..	...	...	...	...	...	...	...	...	...	...	...
28	...	...	...	...	...	...	...	A..	...	.C.	...	...
29	G..	...	T..	...	...	C..	...	...	...	...	...	...
30	G..	...	...	...	...	...	...	A..	...	...	...	...
31	G..	...	T..	...	A..	...	...	...	...	...	...	...
32	G..	...	...	...	...	C..	...	...	...	.T.	...	...
33	...	...	..G	...	...	C..	...	...	...	.C.	...	...

Figure 7: Nucleotide sequence variation in the Th3R epitope. The 7G8 parasite strain was used as the reference. DNA sequence identity is indicated by a dot and polymorphic nucleotides are shown.

Point mutations at the Th3R epitope were observed at positions 367, 369, 371, 372, 373, 374 and 376 on the Th3R (Figure 7). Transitional and transversional substitutions of a non-synonymous nature were observed. Most of the substitutions were found at the first or second position of the codons. Third

codon position substitutions, that were either silent or neutral, were observed in very rare haplotypes at positions 369, 371 and 373. With the exception of position 376 at which Glutamate was replaced by either Alanine or Isoleucine or Valine, all positions had a single kind of substitution. The most common substitution that occurred at the Th3R region was a cytosine instead of a guanine encoding Glutamine instead of Glutamate at position 372. A cytosine in place of adenine at position 376 led to Alanine instead of Glutamate.

Allele	326	343
7G8	PSDKHIEQYL	KKIKNSIS
Th2R 1	...Q...K..	.T.Q..L.
Th2R 2	....	.T.Q..L.
Th2R 3	....	.T....L.
Th2R 4	....	.T....L.
Th2R 5	...Q......	.T.Q..L.
Th2R 6	...Q...K..	.T....L.
Th2R 7	....Q...K..	...Q..L.
Th2R 8	...Q...K..	Q..Q..L.
Th2R 9	....	N.....
Th2R 10	...Q...K..	.R.Q..L.
Th2R 11	...Q...K..	N.....L.
Th2R 12	......KE..	.I.Q..L.
Th2R 13	......KE..	N..Q....
Th2R 14	...Q......	.T....L.
Th2R 15	....	.I....L.
Th2R 16	....	...Q..L.
Th2R 17	......TE..	.R.Q..L.
Th2R 18	...Q......	.I.Q..L.
Th2R 19	......KE..	N..Q..L.
Th2R 20	......TE..	...Q..L.
Th2R 21	...Q..KK..	.T.Q..L.
Th2R 22	....	.R.Q..L.
Th2R 23	...Q...K..	.I....L.
Th2R 24	......K...	...Q..L.
Th2R 25	.......K..	.E.Q..L.
Th2R 26	....	......L.
Th2R 27	...Q......	...Q..L.
Th2R 28	......K...	.T.Q..L.
Th2R 29	......TE..	....
Th2R 30	...Q......	N.....L.
Th2R 31	...Q..KK..	......L.
Th2R 32	....	.I.Q..L.
Th2R 33	...Q...K..	N.....
Th2R 34	...Q..KE..	...Q..L.
Th2R 35	....	N.....L.
Th2R 36	...Q..KE..	.T.Q..L.
Th2R 37	...Q..KK..	...Q..L.
Th2R 38	.......K..	.T.Q..L.

ES

Allele	326	343
7G8	PSDKHIEQYL	KKIKNSIS
Th2R 39	...Q...K..	....
Th2R 40	......KE..	.T.Q..L.
Th2R 41	.......K..	...Q..L.
Th2R 42	...Q......	....
Th2R 43	...Q...K..	.R.Q..L.
Th2R 44	......KE..	.R.Q..L.
Th2R 45	......K...	....
Th2R 46	...Q..K...	.I.Q..L.
Th2R 47	.......E..	.R.Q..L.
Th2R 48	.......E..	...Q..L.
Th2R 49	...Q...K..	QT.Q..L.
Th2R 50	....	....
Th2R 51	...Q..TE..	.R.Q..L.
Th2R 52	.......E..	....
Th2R 53	......TE..	.R.Q....
Th2R 54	...Q...K..	QI.Q..L.
Th2R 55	....	QT.Q..L.
Th2R 56	...Q......	Q..Q..L.
Th2R 57	......K...	.I.Q..L.
Th2R 58	...Q..KK..	.T....L.
Th2R 59	....	NI....L.
Th2R 60	....	NT....L.
Th2R 61	...Q..KK..	.I....L.
Th2R 62	...Q..K...	.T.Q..L.
Th2R 63	......KE..	...Q..L.
Th2R 64	......K...	NI.Q..L.
Th2R 65	...Q...K.I	...Q..L.
Th2R 66	....	N..Q..L.
Th2R 67	...Q...K..	.T.Q..L.
Th2R 68	......K...	.T....L.
Th2R 69	...Q......	.I....L.
Th2R 70	...Q..TK..	....
Th2R 71	...Q..KK..	.R.Q..L.
Th2R 72	....	.I.R..L.
Th2R 73	......KE..	T.....
Th2R 74	...Q...K..	NT....L.
Th2R 75	...Q......	QT.Q..L.
Th2R 76	....	N..Q....
Th2R 77	......K...	NT.Q..L.

Figure 8: Th2R amino acid sequence variation as compared to the 7G8 strain

Overall, we found 77 Th2R in the 632 samples collected at baseline and at several time points after the introduction of bed nets. The prevalence of the most predominant Th2R haplotypes was low as a result of the high number of haplotypes. The Th2R alleles 1 to 7 (Figure 8) were the most predominant haplotypes appearing in all the cross-sectional surveys in both ITN and control groups, although with a low frequency of less than 13%. Compared to the frequency of Th2R 1 to Th2R 7, the frequency of the other haplotypes was low.

Allelle	367	378
7G8	NKPKDELDYEND	
Th3R 1	.....Q.....	
Th3R 2	.....Q...A..	
Th3R 3	.....Q.N....	
Th3R 4	.....	
Th3R 5	D....Q...A..	
Th3R 6	.......N....	
Th3R 7	G.S.N.....	
Th3R 8	D....Q...I..	
Th3R 9	.........A..	
Th3R 10	G.....	
Th3R 11	D...NQ.....	
Th3R 12	G........A..	
Th3R 13	G...N.....	
Th3R 14	G...E..N....	
Th3R 15	.....Q.N.A..	
Th3R 16	D....Q.....	

Allelle	367	378
7G8	NKPKDELDYEND	
Th3R 17	D....Q.N....	
Th3R 18	G.S.....	
Th3R 19	.....Q...V..	
Th3R 20	D...N.....	
Th3R 21	G.S.NQ.....	
Th3R 22	.....Q...A..	
Th3R 23	D.S.N.....	
Th3R 24	D...E..N....	
Th3R 25	D....Q.N.A..	
Th3R 26	.....Q...A..	
Th3R 27	D........A..	
Th3R 28	D.....	
Th3R 29	.......N.A..	
Th3R 30	D.S..Q.....	
Th3R 31	D......N....	
Th3R 32	D....Q...V..	
Th3R 33	D...NQ...A..	

Figure 9: Th3R amino acid sequence variation as compared to the 7G8 strain

There were 33 Th3R haplotypes overall. The Th3R alleles 1 to 4 were the most predominant and present in all the cross-sectional surveys in both ITN

and control areas (Figure 9). Th3R alleles 1 and 2 were the most prevalent with a frequency greater than 32% in every cross-sectional survey (Figure 9). The frequency of the other variants was low.

The number of Th2R haplotypes was 33 at baseline with a haplotype gene diversity (Hd) of 0.94. The predominant Th2R haplotype was PSDQHIEKYLKTIQNSLS with a relative frequency of 15%.

Fourteen months post-bed net intervention there were 27 (Hd = 0.92) and 28 (Hd = 0.92) Th2R haplotypes in intervention and control areas respectively. The predominant haplotype was PSDQHIEKYLKTIQNSLS in both intervention and control studies with relative frequencies of 21.74% and 20% respectively.

Thirty months after distribution of ITNs in intervention areas, BX3 survey, there were 28 (Hd = 0.94) and 30 (Hd = 0.94) Th2R haplotypes in the intervention and control areas respectively. The most abundant alleles in intervention and control were PSDKHIEQYLKTIKNSLS (relative frequency = 15.66%) and PSDQHIEKYLKTIQNSLS (relative frequency = 16.05%) respectively.

At the BX5 survey, 46 months post-bed net intervention, the number of Th2R haplotypes observed were 35 (Hd = 0.95) and 34 (Hd = 0.96) in intervention and control areas respectively. The novel haplotype

PSDKHIEQYLKTIQNSLS, which has not been reported before, was predominant in both intervention and control areas with a relative frequency of 12.4%.

The overall predominant Th2R haplotype observed in this study was PSDQHIEKYLKTIQNSL. There was no significant difference in the proportion of Th2R haplotypes between intervention and control areas for all the cross-sectional surveys (Figure 10).

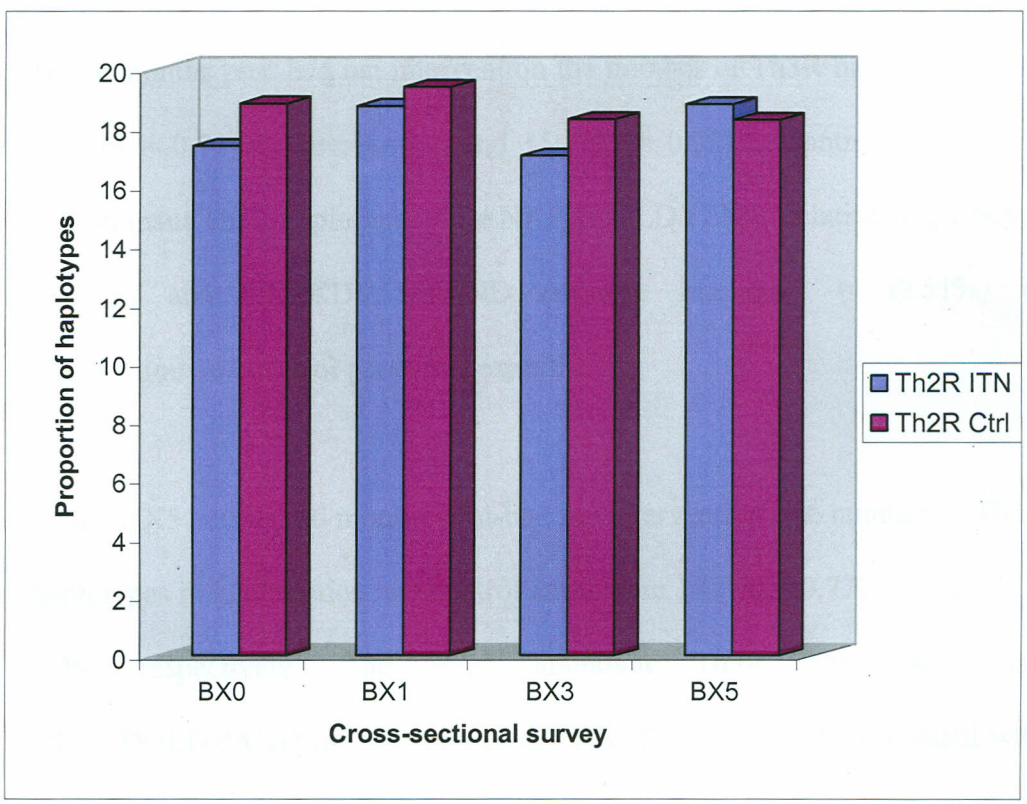

Figure 10: The proportions of Th2R haplotypes in ITN and control areas and across cross-sectional surveys.

At baseline, there were 18 Th3R haplotypes with an Hd of 0.813. The predominant Th3R haplotype was NKPKDQLDYAND with a relative frequency of 32%.

At the BX1 survey, 14 months post-bed net intervention the numbers of Th3R haplotypes were 16 (Hd = 0.80) in intervention and 13 (Hd = 0.79) in control areas. The most predominant were NKPKDQLDYAND (relative frequency = 34.78%) and NKPKDQLDYEND (relative frequency = 37.33%) in intervention and control parasites respectively.

Thirty months post-bed net intervention the number of Th3R haplotypes were 14 (Hd = 0.70) in intervention and 15 (Hd = 0.75) in control areas. The predominant Th3R haplotypes were NKPKDQLDYEND (relative frequency = 42.17%) and NKPKDQLDYAND (relative frequency = 39.51%) in intervention and control parasites respectively.

At the BX5 survey, 46 months post-bed net intervention, the number of Th3R haplotypes in intervention and control areas were 18 (Hd = 0.77) and 13 (Hd = 0.78) respectively. The most abundant Th3R haplotypes were NKPKDQLDYAND in intervention and NKPKDQLDYEND in control with relative frequencies of 36.1% and 37.1% respectively.

The overall predominant Th3R haplotype observed in this study was NKPKDQLDYEND. The proportions of Th3R haplotypes were significantly

higher in ITN areas at BX1 and BX5 but comparable at BX0 and BX3 (Figure 11).

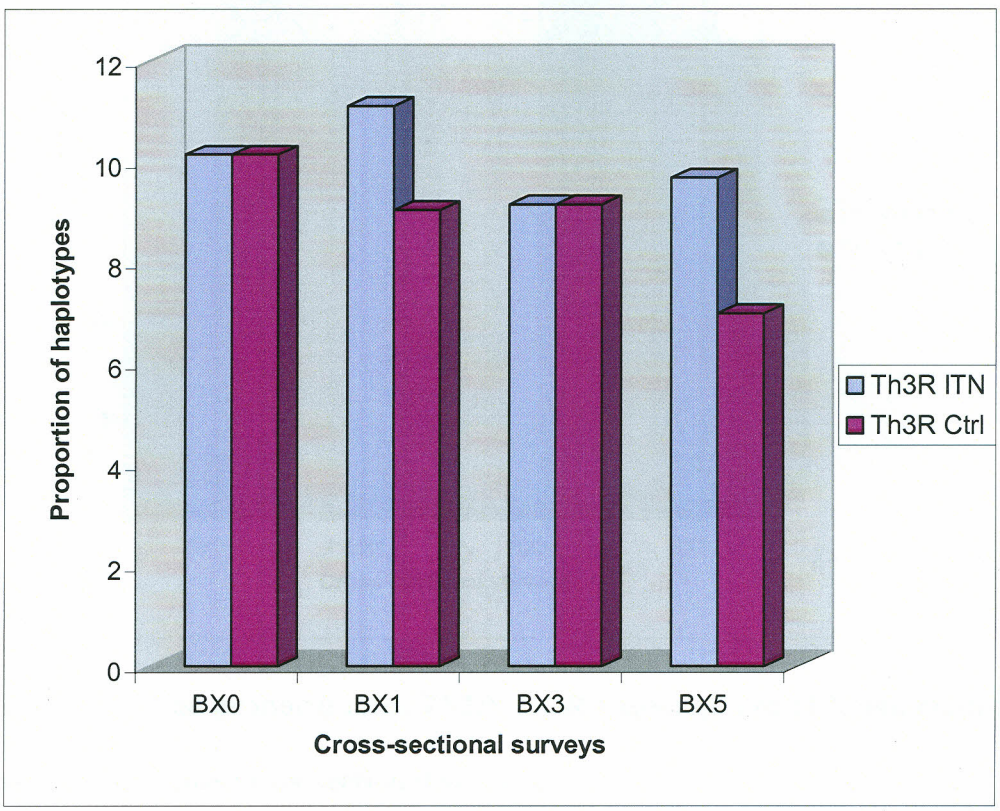

Figure 11: The proportions of Th3R haplotypes in ITN and control areas and across cross-sectional surveys.

Combining Th2R and Th3R regions, the numbers of haplotypes at BX0 were 37 in ITN and 40 in Control; BX1 were 33 in ITN and 36 in Control; BX3 were 36 in ITN and 42 in Control; BX5 were 48 in ITN and 43 in Control. There were no significant differences in Th2R-Th3R haplotype proportions between in ITN and control areas at all the cross-sectional surveys (Figure 12).

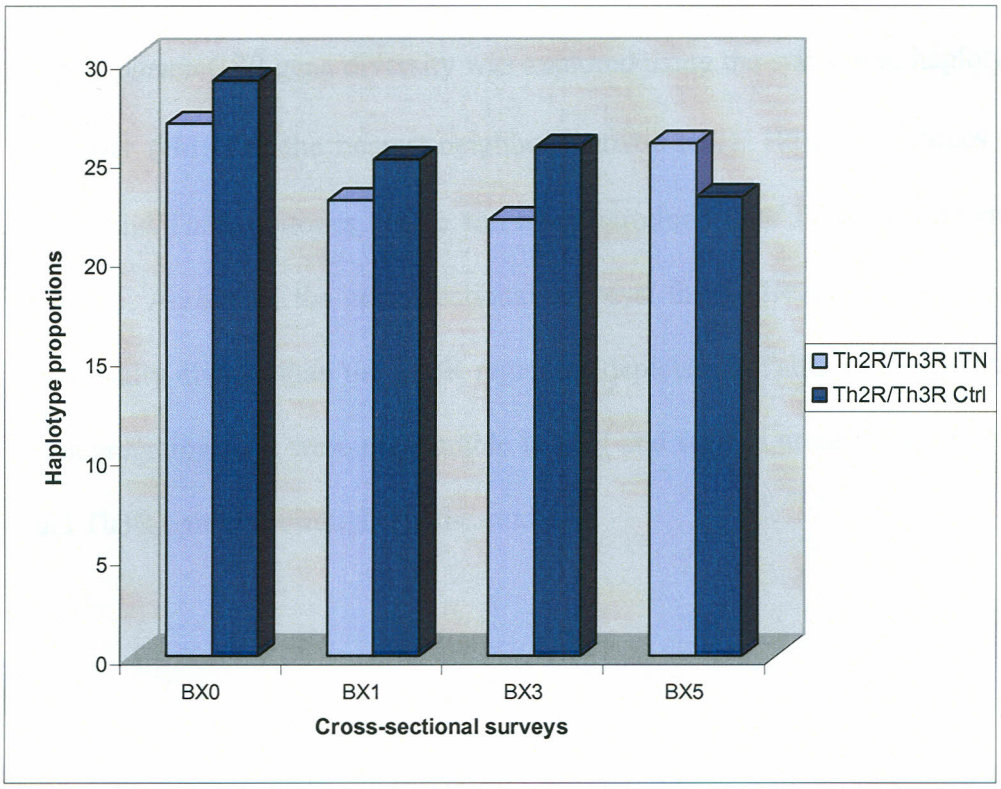

Figure 12: The proportions of Th2R-Th3R haplotypes in ITN and control areas and across cross-sectional surveys.

Using previously described nomenclature (Appendix I and II) and combining both Th2R and Th3R, at baseline there were two equally dominant variants observed CSP-Th2R*06/ CSP-Th3R*02 and CSP-Th2R*U/ CSP-Th3R*08. CSP-Th2R*U (PSDKHIEQYLKTIQNSLS) is a novel haplotype observed that has not been reported before. Many other novel haplotypes were observed but with low prevalence.

4.5 GENETIC DIVERSITY

P. falciparum CSP gene diversity was explored using the statistic π, haplotype diversity (Hd) and the nearest-neighbor statistic (S_{nn}). Hd and π values for Th2R and Th3R epitopes before and after introduction of ITNs are shown in Table 3. Across all the cross-sectional surveys, the Th2R epitope was more genetically diverse than the Th3R region as depicted in Table 3. The numbers of segregating sites were comparable in ITN and control areas for both Th2R and Th3R epitopes through out the study.

Table 3

CSP gene diversity in Th2R and Th3R epitopes in ITN and Control areas

Surve	Region	S	H	Hd	Pi	Rm	D*	F*	D_T
	Th2R ITN	9	24	0.94	0.054	4	0.89	0.88	0.47
BX0	**Ctrl**	10	26	0.94	0.056	4	0.96	0.88	0.33
	Th3R ITN	9	14	0.81	0.048	4	0.07	-0.12	-0.43
	Ctrl	10	14	0.81	0.055	3	0.82	0.48	-0.39
	Th2R ITN	10	27	0.92	0.049	4	1.51	1.15	-0.06
BX1	**Ctrl**	9	28	0.93	0.052	4	1.47	1.31	0.41
	Th3R ITN	9	16	0.80	0.054	3	-0.58	-0.52	-0.17
	Ctrl	8	13	0.79	0.048	3	1.33	0.98	-0.15
	Th2R ITN	10	28	0.94	0.054	4	1.51	1.31	0.35
BX3	**Ctrl**	8	30	0.94	0.051	2	1.43	1.38	0.67
	Th3R ITN	8	14	0.70	0.036	3	-0.13	-0.39	-0.72
	Ctrl	8	15	0.75	0.041	3	-0.29	-0.31	-0.21
	Th2R ITN	11	35	0.95	0.059	4	-0.14	0.13	0.55
BX5	**Ctrl**	11	34	0.96	0.057	4	0.44	0.49	0.36
	Th3R ITN	9	18	0.77	0.052	3	0.67	0.51	-0.03
	Ctrl	7	13	0.78	0.043	3	1.27	0.99	-0.01

S = Number of segregating sites,

H = Number of haplotypes

Hd = Haplotype diversity

Pi = Nucleotide diversity

Rm = Minimum recombination events

D* & **F*** = Fu and Li's D test statistic & F test statistic (Fu *et al.*, 1993)

$\mathbf{D_T}$ = Tajima's statistic

The statistical tests D* and F* were used to test the hypothesis that all mutations are selectively neutral (Kimura, 1983). All the D* and F* values obtained for both the Th2R and Th3R epitopes before and after introduction of ITNs were not statistically significant as shown in Table 3. Evidence of natural selection was explored using the Tajima's D test (D_T). All the D_T values for the Th3R epitope were negative.

The statistic Rm was used to estimate the possible minimum number of recombination events. Nine recombination events were detected in the baseline samples between sites: (329,332) (332,336) (336,339) (339,342) (342,367) (367,369) (369,371) (371,372) (372,374) as depicted in Figure 13.

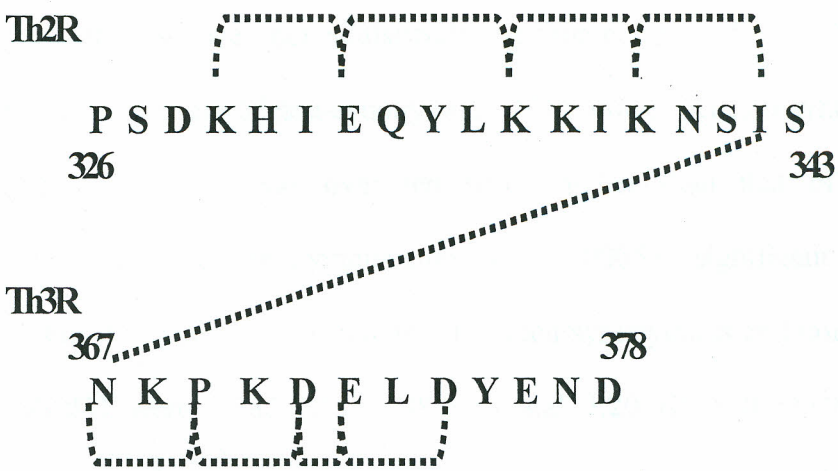

Figure 13: Possible recombination events between Th2R and Th3R at baseline.

At the BX1 survey, eight recombination events were detected in both intervention and control sequences between the following sites: (329,332)

(332,336) (336,339) (339,342) (342,367) (367,371) (371,372) (372,374). At the BX3 survey, eight recombination events were detected in the intervention sequences between sites: (329,332) (332,336) (336,339) (339,342) (342,367) (367,369) (371,372) (372,374). Only six recombination events were detected on the control sequences between sites: (329,332) (332,339) (339,367) (367,372) (372,374) (374,378). At BX5, eight recombination events were detected on both the intervention and control sequences between sites: (329,332) (332,336) (336,339) (339,342) (342,367) (369,371) (371,372) (372,374).

At baseline, as shown in Table 4, parasites from the control area ($\pi = 0.01963$) were more diverse than parasites from the intervention area ($\pi = 0.01825$) but the difference was not statistically significant ($\chi^2 = 51.71$; $P = 0.638$. At baseline, the rate of non-synonymous substitutions per non-synonymous sites (Ka = 0.02165) was over ten times higher than that of synonymous substitutions per synonymous sites (Ks = 0.00053), significant with Ka/Ks = 3.566; $P < 0.001$. The differences between synonymous and non-synonymous (dN-dS) were 3.385 ($P = 0.00048$) and 3.20 ($P = 0.00088$) in parasite sequences from intervention and control areas respectively. A similar trend was observed through out the study (Table 4).

At BX1, there was no significant difference in nucleotide diversity observed between parasites from intervention and control areas with Hd = 0.934, π = 0.01805 and Hd = 0.949, $\pi = 0.01784$ respectively (Table 4).

At the BX3 survey, the control area parasites (Hd = 0.9645, π = 0.0167) were more diverse than parasites from the intervention area (Hd = 0.9442, π = 0.166) (Table 4). However the difference was not statistically significant.

At the BX5 survey, parasites from the intervention area (Hd = 0.9646, π = 0.0203) were more diverse than those from the control area with Hd = 0.9660 and π = 0.0184 (Table 4). The difference was significant with Snn = 0.56; P = 0.014.

Table 4

CSP gene diversity in parasites in ITN and Control areas

Survey	S	H	Hd	Pi	Rm	dN-dS	D*	F*
BX0								
Itn (70)	18	37	0.97	0.0183	9	3.38***	0.62	0.49
Ctrl (68)	20	40	0.96	0.0196	8	3.20***	1.09	0.82
BX1								
Itn (69)	19	33	0.93	0.0181	8	3.19***	0.68	0.46
Ctrl (75)	17	36	0.95	0.0178	8	3.43***	1.74*	1.40
BX3								
Itn (83)	18	36	0.94	0.0166	8	3.23***	0.98	0.68
Ctrl (81)	16	42	0.96	0.0167	6	3.30***	0.82	0.76
BX5								
Itn (97)	20	48	0.96	0.0203*	8	3.36***	0.28	0.36
Ctrl (89)	18	43	0.97	0.0195	8	3.48***	0.97	0.83

S = Number of segregating sites,

dN-dS = Z-test of natural selection

H = Number of haplotypes

Hd = Haplotype diversity

Pi = Nucleotide diversity

Rm = Minimum recombination events

D* & **F*** = Fu and Li's D test statistic & F test statistic (Fu *et al.*, 1993)

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Genetic differentiation between ITN and control areas across the four cross-sectional surveys was further explored using F_{ST} values as shown in Table 5.

Table 5

Comparison of genetic diversity between intervention and control areas using F_{ST} values

	BX0 ITN	**BX0 Ctrl**	**BX1 ITN**	**BX1 Ctrl**	**BX3 ITN**	**BX3 Ctrl**	**BX5 ITN**	**BX5 Ctrl**
BX0 ITN								
BX0 Ctrl	-0.0046							
BX1 ITN	-0.002	-0.005						
BX1 Ctrl	-0.003	0.005	-0.0002					
BX3 ITN	0.002	0.012	0.014	0.009				
BX3 Ctrl	-0.002	0.013	0.015	0.014	-0.004			
BX5 ITN	-0.002	-0.003	0.003	0.007	0.004	0.007		
BX5 Ctrl	-0.006	0.002	0.008	-0.003	0.001	0.003	0.003	

F_{ST} values were derived by the Chi-square test (haplotype data) (Nei 1987; Hudson *et al.*, 1992) and Permutation (randomization) test (Hudson *et al.*,

1992). A high F_{ST} value implies a considerable degree of differentiation (genetically different) between populations. The negative F_{ST} obtained in many of the comparisons between ITN and control sequences implies no differentiation (no genetic difference) between the populations.

Comparisons of genetic diversity between different cross-sectional surveys are as shown in Table 6. Parasites at BX1, $\pi = 0.01794$ were significantly less diverse than parasites at BX0, $\pi = 0.01888$ ($\chi2 = 120.3$; $P = 0.0256$ and Snn = 0.58; $P < 0.0001$). Similarly, parasites from the BX3 survey ($\pi = 0.01658$) were significantly less diverse than parasites from BX0 survey with $\chi2 = 121.48$; $P = 0.025$ and those from BX1 survey with $\chi2 = 116.36$; $P = 0.059$ and Snn = 0.552; $P < 0.01$ (Table 6).

However, parasite genetic diversity observed at BX5 was higher ($\pi = 0.01945$) than the diversity observed at both BX0 (significant with Snn = 0.57; P = 0.025) and BX1 (significant with $\chi2 = 131.04$; $P = 0.0435$). The difference in genetic diversity between parasites from the BX5 survey and those from the BX3 survey was not significant (Table 6).

Table 6

Comparison of genetic diversity between cross-sectional surveys

	BX0 **π = 0.01888**	**BX1** **π = 0.01794**	**BX3** **π = 0.01658**	**BX5** **π = 0.01945**
BX0 **π = 0.01888**				
BX1 **π = 0.01794**	$\aleph^2$ = 120.3; P = 0.026 Snn = 0.58; P < 0.0001			
BX3 **π = 0.01658**	$\aleph^2$ = 121.5; P = 0.025 Snn = 0.56; P = 0.003	$\aleph^2$ = 116.36; P = 0.059 Snn = 0.55; P = 0.006		
BX5 **π = 0.01945**	$\aleph^2$ = 122.844; P = 0.11 Snn = 0.57; P = 0.025	$\aleph^2$ = 131.04; P = 0.04, Snn = 0.57; P < 0.0001	$\aleph^2$ = 15.6; P = 0.27 Snn = 0.51; P = 0.29	

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Different studies have reported the presence of high genetic diversity in regions of high endemicity, especially in Africa, due to the high prevalence of *P. falciparum* infections (Lockyer *et al.,* 1989; Alloueche *et al.,* 2000; Anderson *et al.,* 2000; Escalante *et al.,* 2002). In the context of the ongoing large-scale ITN campaigns, and due to the fact that CSP is one of the most widely characterized malaria vaccine candidate antigens, and that some CSP based malaria vaccines have shown safety and immunogenicity at phase IIb clinical trials (Alonso *et al.,* 2005; Bojang *et al.,* 2005; Enosse *et al.,* 2006; Aponte *et al.,* 2007), it's important to understand the impact of transmission reduction on genetic polymorphisms of the *P. falciparum* CSP gene. This is because transmission intensity is a determinant of malaria prevalence and therefore parasite reproduction rates. Therefore, high transmission would maintain higher effective population size where diversifying natural selection could sustain the parasite polymorphisms (Escalante *et al.,* 2002).

Similar to the previous studies, the current study found extensive genetic diversity on the immune epitopes of the C-terminal domain of the CSP gene. The total genetic diversity observed on the CSP C-terminal for the 632 samples was $\pi = 0.01874$.

High genetic diversity has been associated with intragenic recombination and positive natural selection. Many recombination events were detected both within and between Th2R and Th3R regions. However with the *P. falciparum* CSP carboxyl region, the role of recombination in the observed genetic diversity is not well defined. It has been argued that there is a possibility of convergent substitutions in this region being wrongfully detected recombination events (McCutchan *et al.,* 1992). The role of recombination in genetic diversity would be more reliably appreciated if intragenic recombination was detected in synonymous substitutions (Escalante *et al.,* 2002). This is difficult given the paucity of synonymous substitutions on the *P. falciparum* CSP gene carboxyl terminal. Most of the nucleotide changes observed in this region led to amino acid changes. In addition, the minimum number of recombination events in different cross-sectional surveys did not correlate with the extent of genetic diversity in any of the sub-populations. This leads to the conclusion that most likely the polymorphisms observed at this region are due to mutations maintained by natural selection as a consequence of host immune pressure. Maintenance of non-synonymous mutations by natural selection can be taken as evidence that they confer an advantage to the parasite.

The amino acid replacements, however, seemed to be restricted to certain positions and to the nature of the replacing residue. In majority of the cases, an amino acid was replaced by another with similar chemical characteristics. An amino acid replacement by another with different chemical characteristics was

observed in a single position in the Th3R epitope, where a charged (hydrophilic) amino acid, glutamate at position 376, was replaced by a weakly hydrophobic alanine in some of the predominant haplotypes and by strongly hydrophobic valine or isoleucine in some rare haplotypes. These restrictions in amino acid replacement may mean that residues at these positions are important in the structure and function of the resulting protein. This protein may in turn play a vital role in processes necessary for the survival of the parasite in the host in addition to its role in immune evasion. It is assumed that genes encoding antigenic proteins accumulate mutations that allow the parasite to evade the host immune responses while ensuring its survival in the host (Escalante *et al.,* 2004). This implies that, polymorphism in the *P. falciparum* CSP carboxyl terminal, which encodes antigenic proteins, provides the platform for natural selection to act upon.

Evidence of natural selection was explored using the codon based Z-test and Tajima's D test where as neutrality was tested using Fu and Li's statistics. Mostly positive values were obtained with Tajima's D test for Th2R whereas negative values were obtained for Th3R. This is indicative that Th2R region could be under positive selection whereas the Th3R region may be more under balancing selection. This is supported by the presence of more polymorphisms in the Th2R region as compared to the Th3R region. The values obtained with the Fu and Li's tests were not significant enough to warrant total non-neutrality of the sequences. This reflects the restriction observed in nature of amino acid replacements, and thus, emphasizes on the

dual role (that is, immune evasion and a specific function in the parasite) of the resulting protein.

The overall nucleotide diversity observed in the 138 sequences at baseline, π = 0.01888 was higher than previously reported, π =0.01651(Escalante *et al.*, 2002). One year after distribution of ITNs in intervention villages, there was a significant decrease in *P. falciparum* CSP gene nucleotide diversity. At the BX3 survey, 30 months after the introduction of ITNs in intervention villages, parasites were significantly less diverse than before the introduction of ITNs and 14 months post-ITN intervention. This could be as a consequence of reduced immune pressure. However, there was no significant difference in genetic diversity between parasites from intervention and control areas for both BX1 and BX3 surveys as depicted by the negative F_{ST} values. This could be explained by an earlier observation that ITN use has a "mass effect" on malaria morbidity in villages neighboring areas with high ITN coverage (Gimnig *et al.*, 2003b).

At the BX5 survey, which was conducted 46 months after the introduction of bed nets, parasites from the control area were significantly less diverse than those from the intervention villages. The lower diversity observed in the control villages could be as a consequence of transmission reduction in the control villages whose households had received ITNs, one year prior to the BX5 cross-sectional survey, after the 2-year bed net trial period. Overall, parasites from the BX5 cross-sectional survey were more diverse than

parasites from all the other surveys. This observation may have being by chance. It could also be attributed to increased efficiency of the immune system as a result of reduced parasite burden (Kariuki *et al.,* 2003). In addition, children in the BX5 survey were older compared to the other surveys. They are therefore more likely to have had multiple infections with *P. falciparum* parasites making their immune systems more mature and efficient. This is because in Asembo bay, the use of ITNs has greatly reduced the number of infective bites (transmission), and therefore the parasitemia levels, but has not completely eliminated mosquito bites. Individuals still receive some infective bites when outside their ITNs. The mean ages of children in intervention and control areas were 52.23 and 52.85 months respectively, with the youngest child in the survey being 26.95 months old and the oldest 76.92 months old. These children could have had already acquired a certain degree of immunity against malaria, therefore increasing the immune pressure against the *falciparum* parasites that infected them, consequently accumulation of more mutations by the parasites (Gupta *et al.,* 1999; Kariuki *et al.,* 2003).

The proportions of Th2R haplotypes in the control areas were higher than in ITN areas at BX0, BX1 and BX3 but lower at BX5. A similar trend was observed when considering Th2R and Th3R together. Proportions of Th3R haplotypes were higher in ITN areas at BX1 and BX5 surveys but comparable in ITN and control areas at the BX0 and BX3 surveys. There was no single predominant allele representative observed for either Th2R or Th3R, contrary to reports from studies conducted in regions of low malaria endemicity (Jalloh

et al., 2006). However, some degree of temporal stability was observed especially in the Th3R region with a few circulating allelic types being predominant across the cross-sectional surveys, the frequency of these predominant alleles remained more or less stable over the period of the study. This form of temporal stability was not observed in the Th2R region where the most predominant haplotypes were found in only 22% of the 632 samples. The rare allelic types showed little or no stability. Single novel haplotypes appeared in one cross-sectional survey and not in others. The Th2R epitope was more polymorphic than the Th3R epitope, as reflected both in the number of haplotypes and in the diversity parameters.

The prevalence of the NF54 (3D7) type parasites was very low. There were 8 Th2R and 12 Th3R alleles of the 3D7 type. Combining the Th2R and Th3R, only 5 3D7 type alleles were observed. This implies the action of positive selection that drives the maintenance of point mutations with advantageous traits to the parasites. This observation is further supported by the appearance of new predominant Th2R and Th3R haplotypes, PSDQHIEKYLKTIQNSL and NKPKDQLDYEND respectively. A previous similar study in Asembo bay reported PSDKHIEQYLKKIQNSL and NKPKDELDYEND as the predominant Th2R and Th3R haplotypes using 174 samples (Escalante *et al.,* 2002).

This study had several limitations. The bed net and control areas were in the same locality and the "mass ITN effect' might have masked some differences

in malaria transmission reduction, hence genetic diversity, between ITN and control areas. Similarly, the time between cross-sectional surveys may not be long enough to allow for detection of significant changes in parasite diversity. In addition, the study area has high and perennial malaria transmission and the use of ITNs does not completely eliminate infectious bites. This implies that individuals still receive infective bites that maintain effective immune responses.

5.2 CONCLUSION

In the current study, we found extensive polymorphisms on the C-terminal domain of the CSP gene, resulting in a wide variety of parasite haplotypes. However, the frequency of many of the haplotypes, including the 3D7 haplotype, was low implying that few copies of these haplotypes are circulating in the population. This low frequency of several alleles is comparable to an observation from a similar study done in this region (Escalante *et al.,* 2002). There was a trend towards decreased genetic diversity at the BX1 and BX3 surveys but this trend was reversed at the BX5 survey. There was no consistency in the change in circulating haplotype proportions between ITN and control areas. These observations could mean that if the action of CSP-based vaccines such as the RTS,S is restricted to a few parasite strains then the high number of haplotypes in this region could present a major challenge.

5.3 RECOMMENDATIONS

1. Further studies will be needed to test the trend of parasite genetic diversity as well as the proportion of haplotypes after low transmission has been maintained for a longer period of time.
2. There is a need to conduct similar studies on the impact of ITNs on genetic diversity in areas of low and moderate transmission, where individuals have not acquired much immunity against malaria parasites.
3. It will be interesting to assess whether ITNs have an effect on polymorphism of the CSP helper T-cell epitopes of placental parasites from women of different gravidity.
4. Further studies in different age groups are needed to test the impact of ITNs on the genetic diversity and the association with age.

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6 APPENDICES

Appendix I

	326																342
*CSP-Th2R*01*	P	S	D	K	H	I	E	Q	Y	L	K	K	I	K	N	S	I
(7G8)	cca	agt	gat	aag	cac	ata	gaa	caa	tat	tta	aag	aaa	ata	aaa	aat	tct	att
*CSP-Th2R*02*	-	-	-	Q	-	-	-	K	-	-	-	T	-	Q	-	-	L
(LE5)												-c-		c-			c-
*CSP-Th2R*03*	-	-	-	-	-	-	K	E	-	-	N	-	-	Q	-	-	L
(3D7)							a-	g-			-c			c-			c-
*CSP-Th2R*04*	-	-	-	Q	-	-	-	K	-	-	-	R	-	Q	-	-	L
(BRA 2)				c-				a-				-g-		c-			c-
*CSP-Th2R*05*	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	L
(Well, T9-101, T4R)														c-			c-
*CSP-Th2R*06*	-	-	-	Q	-	-	-	K	-	-	-	T	-	Q	-	-	L
(Gam 366_{1}, Gam 399_{1-10})				c-				a-				-c-		c-			c-
*CSP-Th2R*07*	-	-	-	Q	-	-	-	K	-	-	Q	-	-	Q	-	-	L
(Gam 366_{10})				c-				a-			c-			c-			c-
*CSP-Th2R*08*	-	-	-	Q	-	-	-	K	-	-	Q	-	-	-	-	-	L
(Gam 406_{10}, Gam 419_{1-9})				c-				a-			c-						c-
*CSP-Th2R*09*	-	-	-	-	-	-	-	-	-	-	N	T	-	Q	-	-	L
(Gam 427_{5})											-c	-c-		c-			c-
*CSP-Th2R*10*	-	-	-	Q	-	-	-	K	-	-	Q	-	-	R	-	-	L
(Gam 366_{2-7})				c-				a-			c-			cg-			c-
*CSP-Th2R*11*	-	-	-	Q	-	-	-	K	-	-	-	-	-	Q	Y	-	L
Thai				c-				a-						c-	t-		c-
*CSP-Th2R*12*	-	-	-	-	-	-	T	E	-	-	-	-	-	Q	-	-	L
(Thai $II_{807,836,837}$)							ac-	g-						c-			c-
*CSP-Th2R*13*	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	Y	-	L
(Thai $IV_{827,835a,842}$)														c-	t-		c-
*CSP-Th2R*14*	-	-	-	-	-	-	K	E	-	-	T	-	-	Q	-	-	-
(Thai VII_{835b})							a-	g-			-c-			c-			
*CSP-Th2R*15*	-	-	-	-	-	-	-	E	-	-	-	-	-	Q	-	-	L
(Thai V_{828})								g-						c-			c-

Th2R variation in laboratory and field isolates of *P. falciparum.* Nucleotide and amino acid numbering relate to the 7G8 clone (*CSP-Th2R** 01) (Dame *et al.* 1984). Identity is indicated by a dash and DNA sequence polymorphisms are marked below each amino acid (Alloueche *et al.,* 2000).

Appendix II

	367											378
*CSP-Th3R*01*	N	K	P	K	D	E	L	D	Y	E	N	D
(7G8)	aat	aaa	cct	aaa	gac	gaa	tta	gat	tat	gaa	aat	gat
*CSP-Th3R*02*	-	-	-	-	-	Q	-	-	-	A	-	-
(LE5)						c–				-c-		
*CSP-Th3R*03*	-	-	-	-	-	-	-	-	-	A	-	-
(3D7)										-c-		
*CSP-Th3R*04*	D	-	-	-	-	Q	-	-	-	-	-	-
(Well, FCR3)	g–					c–						
*CSP-Th3R*05*	-	-	-	-	-	Q	-	N	-	-	-	-
(Gam 336b, 406b, 419)						c–		a–				
*CSP-Th3R*06*	-	-	-	R	-	-	-	-	-	A	-	-
(Gam 406c)				cg-						-c-		
*CSP-Th3R*07*	-	-	-	-	-	-	-	-	-	A	D	-
(Gam 406d)										-c-	g–	
*CSP-Th3R*08*	-	-	-	-	-	Q	-	-	-	-	-	-
(AE7, PNG2, Thai VIII)						c–						
*CSP-Th3R*09*	G	-	S	-	-	-	-	-	-	-	-	-
(HB3, D10, X5, BRA1)	gg-		t–									
*CSP-Th3R*10*	-	-	-	-	-	-	-	N	-	-	-	-
(Bra S34, Thai V)								a–				
*CSP-Th3R*11*	D	-	-	-	-	Q	-	-	C	-	S	
(PNG3)	g–					c–			-g-		-g-	
*CSP-Th3R*12*	-	-	-	-	-	-	-	-	-	-	D	-
(Thai VI)											g–	
*CSP-Th3R*13*	G	-	-	-	-	-	-	E	-	-	-	-
(Thai VII)	gg-							–a				

Th3R variation in laboratory and field isolates of *P. falciparum.* Nucleotide and amino acid numbering relate to the 7G8 clone (*CSP-Th3R** 01) (Dame *et al.* 1984). Identity is indicated by a dash and DNA sequence polymorphisms are marked below each amino acid (Alloueche *et al.,* 2000).