EFFECT OF INSECTICIDE-TREATED BED NETS (ITNs) ON MULTIPLICITY OF *PLASMODIUM FALCIPARUM* INFECTIONS IN CHILDREN IN ASEMBO BAY AREA OF WESTERN KENYA

BY

JOHN JACKTON OKEYO

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FACULTY OF SCIENCE

MASENO UNIVERSITY

ABSTRACT

Insecticide-treated bed nets (ITNs) have been shown to significantly reduce malaria transmission in sub-Saharan Africa. Individuals living in areas of high malaria transmission generally harbour multiple parasite strains, which are thought to be responsible for maintaining acquired immunity to malaria. Despite the efficacy of ITNs in reducing bites of infected mosquitoes and parasitaemia, what remains unproven is the impact of ITN on multiple infections of *Plasmodium falciparum* genotypes and the association of multiplicity of infections (MOI) to malaria morbidity indicators. This study used cryopreserved samples collected from children less than 5 years as part a randomized controlled trial of ITNs conducted between October 1996 to February 2001 in Asembo Bay, Rarieda district, western Kenya. Blood samples obtained from 282 malaria-asymptomatic children enrolled at baseline study (BX0) and 5 years after ITN intervention (BX5) had their parasite DNA genotyped. The number of infecting *P. falciparum* genotypes in blood samples was determined by PCR-based genotyping of the merozoite surface proteins 1 and 2 (MSP-1 and MSP-2). The $\chi 2$-test and linear regression analysis were used for data analysis. The results indicated extensive polymorphism of *P. falciparum*, except for R033 family of *msp-1* locus which was poorly polymorphic in Asembo. There was no significant difference in MOI observed among the children in the ITN and control groups, even 5 years of ITN use. Overall, MOI showed lack of association with malaria morbidity indicators, and an inverse relationship of age with the total number of *msp-1* and *msp-2* genotypes. For *msp-2* genotypes, a significant association was shown with auxiliary temperature $\geq 37.5^{\circ}C$ ($p= 0.021$) and parasite density less than 5,000 parasites/µl ($p= 0.014$), and specific families such as R033-type of *msp-1* and FC27-type of *msp-2* with parasite density less than 5,000 parasites/µl ($p= 0.042$ and $p= 0.009$ respectively). This study found that the use of ITN does not reduce multiplicity of infection and association MOI with malaria morbidity indicators could be strain-specific. This suggests that the use of ITN may not compromise the acquisition of protective immunity in high transmission areas that depend on infections with multiple parasites.

CHAPTER ONE

1.0 INTRODUCTION

About 3.3 billion people - half of the world's population - are at risk of malaria and every year, this leads to about 250 million malaria cases and nearly 780,000 deaths (WHO, 2009). People living in the poorest countries are the most vulnerable. Young children less than five years of age and pregnant women residing in malaria endemic areas of sub-Saharan Africa bear the greatest burden of malaria related morbidity and mortality (Sarkar *et al.*, 2009; Marsh *et al.*, 1996). An African child has on average between 1.6 and 5.4 episodes of malaria fever each year. And every 30 seconds a child dies from malaria (WHO, 2009).

The rapid emergence and spread of parasite resistance to antimalarial drugs, vector resistance to insecticides, population movements, environmental degradation, climate change and the rapid spread of human immunodeficiency virus (HIV) have contributed to make the malaria situation worse (WHO, 2005). This has complicated the global malaria control strategies, which have previously laid emphasis on prompt diagnosis and treatment using effective antimalarial drugs and vector control using insecticides (Protopopoff *et al.*, 2007). The other challenge comes from understanding the development of natural immunity and complexity of infection as it relates to protection and pathogenesis.

Over 30% of pediatric admissions to district hospitals in Kenya are due to malaria. The cost to the healthcare system of managing a case of severe malaria is

substantial (Kirigia *et al.*, 1998b). This equatorial community of East Africa has one of the highest rates of malaria in the world, resulting in around a 20% mortality rate in children less than 48 months of age. At any point in time throughout the year, 83% of the children below 36 months of age are carrying malaria parasites in their system. Those children that survive frequent episodes of malaria during early life gradually gain immunity to the disease throughout late childhood and early adolescence. A large study in Kenya (Nevill *et al.*, 1996) demonstrated that over 40% of severe, life threatening malaria cases in childhood could be prevented through the use of ITN, and all-cause childhood mortality could be reduced by 33%.

It is against this background that insecticide-treated bed nets (ITNs) have emerged as an effective malaria control tool. Several randomized trials have demonstrated that appropriate use of ITNs is associated with significant reduction in malaria morbidity and mortality in young children and pregnant women (Lengeler, 2000). Based on this information, the World Health Organization, Global Malaria Program (WHO/GMP) has adopted ITNs as one of its key strategies for malaria control (Protopopoff *et al.*, 2007). Consequently, many malaria endemic countries have implemented wide-scale deployment of ITNs to vulnerable populations as part of the national malaria control programs (Protopopoff *et al.*, 2007).

Although the overall goal of ITN programs is to reduce malaria morbidity and mortality in vulnerable populations, there are information gaps on their impact on host-parasite interactions, especially on multiplicity of parasite infections. The intensity of malaria transmission plays an important role in determining the dynamics of parasite dispersion in the mosquito vector and human host, parasite prevalence rates, the general

epidemiology of malaria in an area and the rates of acquisition of protective immunity (Snow *et al.*, 1994). Several studies have shown that immune effector mechanisms against malaria parasites are variant-specific and infection with many different parasite variants or genotypes is required in order to develop protective immunity against most parasite genotypes circulating in an area (Powell, 1972). This observation has led to hypothesis that reduction of transmission, particularly in high transmission areas, could modify the vector-host-parasite interaction, leading to reduced exposure to many parasite genotypes, ultimately leading to poor development of acquired immunity to malaria and a shift in morbidity and mortality patterns (Marsh & Snow, 1997). So far, studies that have investigated changes in malaria morbidity patterns following extended use of ITNs (Binka *et al.*, 2002) and the effect of ITNs on acquired immunity to malaria (Kariuki *et al.*, 2003) have demonstrated that extended use of ITNs is not associated with a shift in morbidity or mortality patterns or acquisition of immunity to malaria.

However, very limited data exist to demonstrate the effect of ITN use on transmission of multiplicity of infection, prevalence and variation of *P. falciparum* genotypes, and the relationship of *P. falciparum* genotypes with malaria morbidity indicators. These are important points to consider especially in high transmission areas where people are infected with multiple parasite genotypes. The purpose of this study was to measure the effects of ITN use on possible transmission, prevalence and temporal variation of the multiplicity of parasite infections, and the association of *P. falciparum* genotypes with malaria morbidity indicators. Information gained from this study could help in explaining the hypothesis that the impact of bed net in reducing morbidity and mortality lies in its ability to reduce the number of new parasite strains encountered by

reducing high parasite density infections (Genton *et al.*, 1994). This information could be useful in understanding the relationship between malaria transmission and ITN use, and the number of parasite genotypes in an infection and acquisition of immunity to malaria. This information could also be valuable in the rational design and implementation of malaria intervention/prevention programs that reduce malaria transmission.

1.1 Problem Statement

Despite the scale-up of ITNs coverage, prompt diagnosis and treatment with effective antimalarial drugs, malaria in endemic areas of sub-Saharan Africa still remains a major problem. Although numerous epidemiological studies in malaria endemic areas have provided strong evidence that the use of ITNs improves survival and reduces malaria-attributable morbidity and mortality (Habluetzel *et al.*, 1997), little research has been done to analyze the effect of ITNs use on multiplicity of infection (MOI) of *P. falciparum* genotypes, prevalence and variation in parasite genotypes, and the association of *P. falciparum* genotypes with malaria morbidity indicators. These are points of omission particularly when put into consideration that high MOI can be an indicator of transmission level and immune status against malaria. If the issue of transmission of MOI is not fully understood, its overall influence on malaria morbidity and mortality could be underrated and eventually might impact negatively on malaria control strategies. The issue in the long run could make it difficult for malaria endemic regions to integrate their efforts with those of WHO malaria programmes in achieving their overall national goals towards malaria eradication. An understanding of the effect of ITNs use on the dynamics of transmission of multiple infections of *P. falciparum* genotypes could provide insights

in planning and assessing the current and future control strategies that could significantly reduce malaria transmission in the affected areas.

1.2 Justification and Significance of the Study

Individuals in malaria endemic areas are often infected with multiple genotypes of the malaria parasites, which are thought to play a role in the acquisition and maintenance of natural immunity to malaria. The parasites carrying a particular allelic variant may be selected for or against by naturally acquired immunity. From the aforementioned reports, there was need to investigate the effect of ITN use on transmission of multiple parasite infections especially in vulnerable groups, young children, residing in malaria endemic areas, particularly an area with high malaria transmission level such as Asembo, western Kenya. The study was designed to fill the information gap on whether the use of ITN would or would not have effect on multiplicity of infection. The information gained from the study would help in understanding the relationship between multiple parasite infections and ITN use, the effect of *P. falciparum* genotypes on malaria morbidity indicators and in designing malaria control strategies that significantly reduce malaria transmission in endemic areas.

1.3 Alternative Hypothesis (H_1)

The use of ITN reduces the multiple infections of *P. falciparum* genotypes in young children in Asembo Bay, western Kenya.

1.4. Main Objective

To evaluate the effect of ITN use on multiple infections of *P. falciparum* genotypes in young children in Asembo Bay, western Kenya.

1.5 Specific Objectives

i. To determine the effect of ITN on the distribution of *P. falciparum* genotypes infecting children in Asembo, Rarieda district

ii. To compare the prevalence of *P. falciparum* genotypes in children between the ITN users and non-users (control group)

iii. To investigate the temporal variation in *msp-1* and *msp-2* alleles of *P. falciparum* genotypes 5 years after deployment of ITNs

iv. To investigate the association between *P. falciparum* genotypes with malaria morbidity indicators (hemoglobin levels, auxiliary temperature, parasite density, fever lasting 2 weeks) in the young children in Asembo

1.6 Assumptions of the study

i. The use of ITN in reduction of multiplicity of infections of *P. falciparum* genotypes which constitute parasite population infecting individuals still remains unclear

ii. The reports on the association between multiplicity of infections of *P. falciparum* genotypes and malaria clinical outcomes are still variable and need to be resolved

iii. Under carefully controlled ITN trial, children in the ITN group would have reduction in multiplicity of infections and lower prevalence of *P. falciparum* genotypes than those in the control group (non-ITN users)

iv. The loci *msp-1* and *msp-2* are widely used genetic markers in genotyping procedures and in antimalarial drug trials due to their high polymorphic nature and discriminatory powers that have given useful results

1.7 Limitation(s) of the study

i. The method limitation in mind was related to weak DNA bands which despite the fact that the same samples were re-electrophoresed, faint bands were inconsistently visible demonstrating variations in technique's sensitivity. The inborn limitations of the PCR technique to detect minority clones or parasite population dynamics, may be considered as a factor potentially underestimating the multiplicity of infection (Martensson *et al.*, 2007)

ii. The DNA band size was liable to disparity within and between the prepared gels

iii. The role of transmission pressure in modifying the efficacy of ITNs is a factor which is not easy to control since in highest transmission sites, ITN's efficacy is lower

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria burden

Global malaria burden is estimated as 471 million clinical cases of *P. falciparum* (WHO, 2009) resulting in 780,000 deaths per year. Almost this entire burden of morbidity occurred in areas of stable transmission. In endemic areas, children under the age of 5 years and pregnant women, particularly during their first and second pregnancies are most at risk and bear the brunt of the disease (WHO, 2000). About 90 % of all malaria-attributable deaths occur in sub-Saharan Africa (WHO, 1998). This has been largely attributed to two factors: first, the majority of malaria infections in this region are caused by *P. falciparum,* the most lethal among the four human malaria parasites (Snounou, 1993), and secondly widespread distribution of *Anopheles gambiae,* the most efficient malaria vector and the most difficult to control (WHO, 1990). In Kenya, an estimated 20 million people, more than half of the entire population, are at risk of *P. falciparum* infection (Snow *et al.*, 1998), thus calling for urgent attention.

As earlier noted, *P. falciparum* is the most virulent of the four parasites which cause malaria in humans. These malaria parasites are genetically diverse at all levels of endemicity. The inherent variability of *P. falciparum* is particularly prevalent in merozoite surface antigens (MSPs) being targeted for malaria vaccines (Chauhan, 2011). This provides multiple effective evasion and drug resistance mechanisms for the parasite as well as a major challenge to development of an effective malaria vaccine. Thus, the study of multiple infections in malaria parasites is expected to provide new insights into the deployment of new control measures. The genetic complexity of *P. falciparum* and in

particular its ability to generate mutant variants, makes it a successful pathogen. Although information on the frequency of genes conferring, for example, resistance to a certain drug or a vaccine in a given area has obvious relevance to the implementation of control measures using such agents, a critical first step is to obtain information on the genetic polymorphism of the parasites in the human hosts in the community to be investigated. As such, many studies have used the polymorphic regions of merozoite surface proteins 1 and 2 as genetic markers to determine the genetic diversity of *P. falciparum*, multiplicity of infection, assess the level of malaria transmission, as well as investigating the relationship of these factors with acquisition of natural immunity against malaria (Takala *et al.*, 2006). Additionally, MSP-1 and MSP-2 antigens cause immune response in humans and have been identified as potential candidates for blood-stage malaria vaccine (Polley *et al.*, 2006).

A major mechanism for the generation of allelic diversity in the *P. falciparum* MSP-1 gene is meiotic recombination in the *Anopheles* mosquito, which is believed to be dependent on the intensity of transmission. It is suggested that frequent recombination events between MSP-1 alleles intermittently generate novel alleles in high transmission areas 19 (Tanabe *et al.*, 2007). Single nucleotide polymorphisms (SNPs) contribute largely to the variability of *P. falciparum* and provide multiple effective evasion and drug resistance mechanisms for the parasite necessitating the use of molecular techniques to differentiate alleles responsible for recrudescences and re-infections after treatment.

2.2 Immunity to malaria

Protective immunity against malaria develops slowly and requires repeated exposure to the infection in order to be maintained (Elliott *et al.*, 2005). Several studies have indicated that immunity against blood stages of *P. falciparum* infection is mainly antibody mediated (Nebie *et al.*, 2008). Those protective antibodies may act either on their own to mediate antibody-dependent phagocytosis and/or cell-mediated neutralization of parasites (Druilhe & Khusmith, 1987; Kumaratilake & Ferrante, 2000). In areas where malaria is highly endemic, a protective semi-immune status is acquired during the first 10 to 15 years of life (Riley *et al.*, 1989), with majority of malaria related morbidity and mortality occurring in young children (Riley *et al.*, 1989). Epidemiological studies conducted in areas of high malaria transmission in Africa and Southeast Asia indicates age-dependent development of protective immunity against *P. falciparum* malaria (Warsame *et al.*, 1997). It is therefore hypothesized that clinical protection during the first few months of life in high transmission settings allows active immunization to occur and contributes to a reduction in the overall risks of severe malaria throughout childhood (Snow *et al.*, 1998). By the age of five, most children develop a considerable degree of immunity, which reduces the risk of death from *P. falciparum* malaria, provides protection against severe disease, and fewer clinical malaria episodes (Greenwood *et al.*, 1992). Naturally acquired protective immunity builds up with repeated exposure to malaria, which is manifested by lower parasite densities and fewer clinical malaria episodes in older children and adults. In areas with lower transmission intensity, the age at which clinical immunity develops shifts upwards (Snow *et al.*, 1995). In areas with very low malaria transmission rates, immunity develops so slowly that

individuals in all age groups are equally susceptible to clinical malaria and severe disease (Luxemburger *et al.*, 1997).

Several target antigens for antibody-mediated inhibition of parasite growth or invasion have been identified. The molecular markers used in many of the previous studies belong to genes encoding polymorphic surface antigens such as the merozoite surface protein 1 and 2 (MSP-1 & 2) (Babiker & Walliker, 1997). The genes *msp-1* and *msp-2* are polymorphic and their allelic forms differ in their ability to abrogate recognition by the host's immune response (Babiker & Walliker, 1997).

2.3 Implications of genetic diversity of *P. falciparum*

Genetic diversity provides *P. falciparum* has the potential capacity of avoiding the immune response, and possibly supporting the selection of drug or vaccine resistant parasites (Meyer *et al.*, 2002; Schreiber *et al.*, 2002). The acquisition of drug resistance by *P. falciparum* has severely curtailed global efforts to control malaria. Strategies to prevent the rapid spread of parasites resistant to novel drugs or vaccines require an understanding of the population structure of the parasites. It is suggested that multiplicity affects the prevalence of genes involved in resistance to antimalarial drugs (Jelinek *et al.*, 1999). Drug resistance seems to spread faster in higher transmission areas, regardless of drug pressure; and in low transmission areas, drug pressure seems to be the critical factor (Le Bras & Durand, 2003). In a study (Aubouy *et al.*, 2003), variation of the alleles of *msp-1* and *msp-2* with age was observed with parasite prevalence decreased in the children aged < 7 years after 7 months of undetected transmission. In a cohort study in Ndiop (Zwetyenga *et al.*, 1999) changes in *msp-1* and *msp-2* allelic distribution pattern was being influenced by season. The substantial seasonal fluctuations in the genotypes

reflect complex dynamics of multiple-clone infections during chronic asymptomatic parasite carriage. The parasite prevalence decreased markedly in children aged < 7 years after 7 months of undetected transmission. (Konate *et al.*, 1999) reported differential distribution of allelic families of *msp-1* and *msp-2* loci in Ndiop and Dielmo in a combined genotyping of *msp-1* and *msp-2* loci and whose results showed that 90% of the isolates contained more than one parasite genotype. This high percentage of multiple parasite fragments was similar in both Dielmo surveys but twice that observed in Ndiop. One main limitation to the development of a vaccine against *Plasmodium falciparum* is the antigenic diversity related to *P. falciparum* polymorphism (Aubouy *et al.*, 2003).

Merozoite surface proteins: MSP-1 and MSP-2 are two proteins causing immune response in humans (Cavanagh *et al.*, 1998) and are prime vaccine candidates. In a study (Beck, 2007), polymorphic region from *P. falciparum*: The loci: *msp-1*, *msp-2* and *glurp* (glutamate rich protein) have been reported for their selection as markers for parasite genotyping in antimalarial drug trials and efficacy studies. Perhaps as part of an immune evasion mechanism, $msp\text{-}1_{19}$ and *msp-2* DNA sequences include variable blocks generating antigenically diverse forms (Smythe *et al.*, 1990). The polymorphism at these two loci is mainly assessed by the number of repeats (Snewin *et al.*, 1991), which can be used to distinguish by size the different alleles after PCR amplification. The phenomenon of clone fluctuation, which consists of kinetic changes in the relative load of each parasite subpopulation present in blood (Farnert *et al.*, 1997), is important to consider when studying *P. falciparum* diversity. Strain-specific immunity is largely responsible for protective immunity to malaria parasites (Jouin *et al.*, 2001). In malaria endemic areas, infections due to multiple parasite clones are frequent (Farnert *et al.*, 1999), and clone

fluctuation may be a logical strategy to slow down the appearance of strain-specific responses. High polymorphism in *msp-1*, *msp-2* and *glurp* genes in different geographical locations in malaria endemic areas and clone fluctuations: disappreance and reappearance of alleles of *msp-1* and *msp-2* following treatment (Aubouy *et al.*, 2003) have been reported, suggesting difficulties encountered in assessing polymorphism and distinguishing reinfection and recrudescence. The extensive genetic polymorphism of several *P. falciparum* genes is exploited for genetic finger printing and assessing parasite population dynamics. For instance, numerous clinical drug trials have applied genotyping approach of the three loci: *msp-1*, *msp-2* and *glurp* of *P. falciparum* genotypes to correct the outcomes of drug efficacy studies (Mugittu *et al.*, 2006).

2.4 Multiplicity of infection and morbidity

Parameters describing the infection dynamics of *P. falciparum* are important determinants of the potential impact of interventions and are potential outcome measurements for malaria intervention trials. Low parasite densities, periodic sequestration of parasites, and the presence of multiple concurrent infections make it essential to use molecular techniques to estimate the force of infection and duration of infections in endemic areas. To date, a high degree of polymorphism has been demonstrated at both the *msp-1* and *msp-2* loci in parasites from areas of stable malaria transmission. As a consequence, in such areas it is rare to find parasites of the same two-locus genotype in more than one subject. Some surveys have documented *P. falciparum* multiplicity of up to nine different parasite clones at a given time in a single asymptomatic host (Engelbrecht *et al.*, 2000). In the last decade, emphasis in several

studies has been placed on investigating, if any relationship exists between multiplicity and variables like parasite density, age and infection outcomes. Studies in Tanzania (Beck *et al.*, 1997) and in Papua New Guinea (al-Yaman *et al.*, 1997) suggested that in individuals with substantial previous exposure to malaria, co-infection with multiple clones of *P. falciparum* can protect against subsequent clinical malaria attacks. However, other studies, mainly of individuals with little previous exposure such as in infants, multiplicity was positively associated with parasite density and risk of clinical morbidity (Mayor *et al.*, 2003; Felger *et al.*, 1999) contrary to the suggestion from many studies that high multiplicity is protective against clinical malaria. This finding probably reflects the immune status of these very young children; the absence of premunition in infants may be a major factor contributing to their great vulnerability to clinical malaria (Smith *et al.*, 1999c). These observations imply that in highly endemic areas MOI are not directly correlated with exposure to *P. falciparum* (Engelbrecht *et al.*, 2000). As with parasite density, the relationship between multiplicity and age is still unclear—some studies have reported decrease in multiplicity with age (Owusu-Agyei *et al.*, 2002), others have observed a positive correlation in infants and children but not in older individuals (Smith *et al.*, 1999b). Some reports have indicated decrease of MOI during adulthood to the levels found in infants (Mayor *et al.*, 2003) while others did not observe any relationship between the two parameters (Zwetyenga *et al.*, 1998). This suggests that the mechanisms controlling multiplicity of infection and parasite densities follow different profiles and so are different. Several studies, among them, (Ariey *et al.*, 2001) have investigated the association of specific *P. falciparum* genotypes with the clinical disease and virulence to find out why severe malaria is seen to occur in only a small percentage of patients but no

definitive results are currently available. However, there are some indications that parasites differ in their virulence. .Some studies have shown an over expression (Ofosu-Okyere *et al.*, 2001; Felger *et al.*, 1999; Ranjit *et al.*, 2005) or complete absence (IE *et al.*, 2007) of one of the allelic types of either *msp-1* or *msp-2* in severe malaria. Others did not see any association between any MSP-1 or MSP-2 genotype and clinical status (Shigidi *et al.*, 2004).

Genetic diversity of *P. falciparum* plays a major role in the natural acquisition of immunity to malaria infections and is also a concern to the development and deployment of control measures. In humans living in malaria endemic regions, immunity to *P. falciparum* is acquired as a result of natural exposure to multiple infections over many years. In holo- or hyper-endemic areas, immunity develops at a younger age than in areas where transmission is less intense (Zwetyenga *et al.*, 1998). It is usually considered that immunity to *P. falciparum* has two components: an anti-disease immunity, which develops rapidly; and an anti-parasite immunity, which is acquired slowly and leads to a marked decrease in parasite densities. Acquired anti-*P. falciparum* immunity reduces parasite density, limits the number of parasite genotypes infecting an individual at any given time, and controls parasites against which a strong immune response has been mounted (Zwetyenga *et al.*, 1998). It has been suggested that asymptomatic infections protect against developing clinical malaria and that such protection is enhanced by the diversity of infecting strains (Owusu-Agyei *et al.*, 2002). This fact makes the hope of developing effective malaria vaccines a realistic goal but is being constrained by the antigenic diversity of *P. falciparum*. Nevertheless, a coherent theoretical framework of how protective immunity to *P. falciparum* malaria is acquired (Basco *et al.*, 2004)

following natural exposure to the parasites is beginning to emerge, as a result of combined clinical and epidemiological data with basic immunological research. This is based on the fact that IgG antibodies against the most frequent subtypes of block 2 of *msp-1* are important in acquired antimalaria immunity (Conway *et al.*, 2000).

2.5 Malaria epidemiology and control strategies

The global success efforts to develop effective treatment by use of antimalarials, and vector control program using insecticides have been hampered, among them, by insecticide resistance in mosquito (Good *et al.*, 2004); drug resistance in the parasite ((Bjorkman, 2002), and lack of funding and commitment in malaria endemic countries to invest appropriate resources needed for prevention and control of malaria infections (Mills *et al.*, 2008). A successful malaria control and prevention program would require the coordinated use of several strategies, including the use of ITNs and potential malaria vaccines whose effectiveness faces stiff challenge from extensive genetic diversity in *P. falciparum* (Graves & Gelband, 2003).

A large study in Kenya by (Nevill *et al.*, 1996)) demonstrated that over 40% of severe, life threatening malaria cases in childhood could be prevented through the use of ITN, and all-cause childhood mortality could be reduced by 33%. Over 30% of pediatric admissions to district hospitals in Kenya are due to malaria. The cost to the healthcare system of managing a case of severe malaria is substantial (Kirigia *et al.*, 1998a). The other challenge comes from the understanding the development of natural immunity and complexity of infection as it relates to protection and pathogenesis. The antigens MSP-1,

MSP-2 and GLURP are the most commonly used molecular markers (Snounou & Beck, 1998) since they have been widely used in drug trials and have given useful results.

2.6 The use of ITNs and transmission of *P. falciparum* genotypes

In malaria endemic areas, multiplicity of infections (MOI) can be a useful indicator of transmission. Data suggest that the average number of different malaria parasite strains in an individual is well correlated to transmission level (Babiker & Walliker, 1997). Blood samples obtained in a bed net study showed a significant decrease of 16.4% in microscopically determined *P. falciparum* prevalence in children in the ITN group at the end of the trial in Asembo (Fraser-Hurt *et al.*, 1999). However, no significant difference was observed in parasite density or multiplicity of infection determined by PCR-RFLP among infected children with or without ITN (Fraser-Hurt *et al.*, 1999). It was assumed that chronic infections formed a large proportion of all infections. Hence limited reduction in exposure would not have much impact on multiplicity and premunition might still be established (Smith *et al.*, 1999c). The number of multiple infections with *P. falciparum* might be an important indicator of the degree of acquired immunity against malaria, since multiplicity of infections increases with age over the first few years of life in parallel with the increase in clinical immunity (Smith *et al.*, 1999a). In addition, persisting infections have been proposed to provide protection against clinical disease when infected with new parasite (Beck *et al.*, 1997; al-Yaman *et al.*, 1997; Farnert *et al.*, 1999). (Beck *et al.*, 1997) found out that multiple concurrent *Plasmodium falciparum* infections with different falciparum genotypes are associated with reduced risk of clinical malaria. The effect of insecticide treated bednets on the

dynamics of multiple *P. falciparum* infections was investigated (Smith *et al.*, 1999b) using PCR-RFLP genotyping data from *msp-2*. The report indicated that for FC27-type of *msp-2* genotypes, there was no significant difference in multiplicity of infections detectable in children aged initially 5 to 24 months with or without sleeping under a bed net.

Differential distributions of classes of *msp-1* and *msp-2* genotypes according to clinical status have been observed in other studies. (Ekala *et al.*, 2002) found a high frequency of K1-types of MSP-1 in all *P. falciparum* isolates from samples studied in Gabonese residents in Gabon, West Africa. The result also corroborates findings from a different site in Gabon where K1-type was the major allelic family found in isolates from symptomatic cases (Kun *et al.*, 1998), and whose presence together with MAD20-type was significantly associated with asymptomatic malaria and consequently a reduced risk of developing the symptomatic disease (Amodu *et al.*, 2005). However, in other studies, the presence of MAD20-type were found to be strongly associated with symptomatic disease (al-Yaman *et al.*, 1997; Ofosu-Okyere *et al.*, 2001). (Beck *et al.*, 1997) reported a significant negative correlation between MOI (as indicated by the number of infecting *msp-2* genotypes/subject) and morbidity. (Amodu *et al.*, 2008) also reported that monoclonal-infection appeared to put a child at relatively high risk of severe malaria (both cerebral malaria and severe anemia malaria). (Ofosu-Okyere *et al.*, 2001) found, however, that the probability of having asymptomatic malaria episode was positively associated with a relatively high MOI. They also reported that the probability of having asymptomatic malaria episode was increased by the presence of FC27-types of *msp-1*, which according to the study (Amodu *et al.*, 2008) it was the absence of (or lack of

detectable) FC27-type that was found to be significantly associated with a 5-fold increased risk of uncomplicated malaria with the asymptomatic children as the reference category. This implies that the study found out that it was the presence of FC27-types that was significantly associated with asymptomatic malarial infection and consequently, a reduced risk of developing the milder (uncomplicated) form of the symptomatic disease. In Papua New Guinea, however, (al-Yaman *et al.*, 1997) found an association between severe malaria and FC27-types, indicating that this allele may be linked to virulence.

In a study of complexity of the *msp-2* locus and the severity of childhood malaria in south-western Nigeria, (Amodu *et al.*, 2008) reported that 3D7-types were found to be strongly associated with uncomplicated malaria, whereas those of the FC27-types were strongly associated with asymptomatic infection. In a study in Gabon in West Africa, (Aubouy *et al.*, 2003) reported commonly observed multiclonal *P. falciparum* infections with MOI of 4.0, but recorded very little polymorphism in the RO33-type of *msp-1*. In another study in Orissa in India, (Ranjit *et al.*, 2005) found that a 550-bp allele of the 3D7 family was over-expressed in cases of severe malaria (who had a mean age of 30 years). Similarly, (Sahu *et al.*, 2008) in their study in the same state of Orissa, reported that 3D7-type varying in size from 400-820 bp were detected in most children who developed cerebral malaria, severe anaemia or both. Although, (Ranjit *et al.*, 2005) also found a 200-bp of MAD20-type to be over-expressed in the cases of severe malaria, MAD20-type of 150-300 bp was only detected in $\leq$ 50% of the children who developed cerebral malaria severe and/or severe anaemia. In Sundergarh, (Joshi *et al.*, 2007) also reported common appearance of 3D7-types not only in isolates from hospitalized children who

developed severe malaria, but also among field isolates from smear-positive subjects of unspecified ages, presumably, with generally mild or asymptomatic malarial infections.

Some studies have shown that *msp-1* genotypes are associated with the development of clinical disease, and in some case severe disease. (Ofosu-Okyere *et al.*, 2001) found that the presence of the MAD20-subtype of *msp-1* and FC27-type of *msp-2* were positively associated with the development of the clinical disease. Studies in malaria-endemic villages in Orissa, eastern India by (Vijay Kumar *et al.*, 2005) also clearly demonstrated that MAD20-type predominates among the parasite *msp-1* isolates from different geographical locations in Indian population.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area and population

The current study used blood samples collected in cross-sectional studies conducted by KEMRI/CDC to determine the impact of ITNs on malaria morbidity in children less than five years of age in Asembo Bay, western Kenya (ter Kuile *et al.*, 2003a). Asembo is located 200 km^2 along the shores of Lake Victoria in Nyanza Province, western Kenya (Figure 1). The population in Asembo at the time of survey was approximately 60,000 living in 79 villages. According to the bi-annual census conducted as part of the ITN project, approximately 3,300 children less than three years of age lived in this area at the time of the ITN trial. The population is ethnically homogeneous and more than 95% belongs to the Luo ethnic group (ter Kuile *et al.*, 2003a). Except for the market centers, the population of each village is highly sparse and the majority of inhabitants practice subsistence farming and live in family compounds surrounded by their fields. The majority of the people earn their living from fishing and small-scale farming and retail businesses.

Malaria and human immune-deficiency virus (HIV) are the two most common causes of morbidity in this population (Phillips-Howard *et al.*, 2003). Asembo is an area of intense perennial malaria transmission that occurs throughout the year, with two peaks during and after the long rains from March through May, and the short rains from October to December (Bloland *et al.*, 1999). Approximately 90% of the malaria infections are due to *P. falciparum*, 7% are mixed infections with *P. malariae*, and 1% is mixed with *P. ovale* (ter Kuile *et al.*, 2003b). Single infections with *P. malariae* or *P.*

ovale make up the remainder. Severe anaemia is the major clinical manifestation of malaria in young children, pregnant women and in HIV-malaria co-infected individuals.

Entomological studies indicate that three mosquito species transmit malaria in this region, with *Anopheles gambiae complex* and *An. funestus* responsible for more than 90% of the transmission while *An. arabiensis* is responsible for the remaining (ter Kuile *et al.*, 2003b). Both *An. gambiae complex* and *An. funestus* bite primarily indoors late at night and their biting is diminished by use of ITNs (McElroy *et al.*, 2001). Estimated entomological inoculation rates calculated as a crude yearly average, vary tremendously at an individual household level, but range from 60 and 300 infectious bites per person per year in Asembo Bay (Beier *et al.*, 1990; Beier *et al.*, 1994). The malaria parasite prevalence in children younger than 5 years was approximately 75%, while the incidence of clinical malaria was approximately 33.9 per 100 person-months (Bloland *et al.*, 1999).

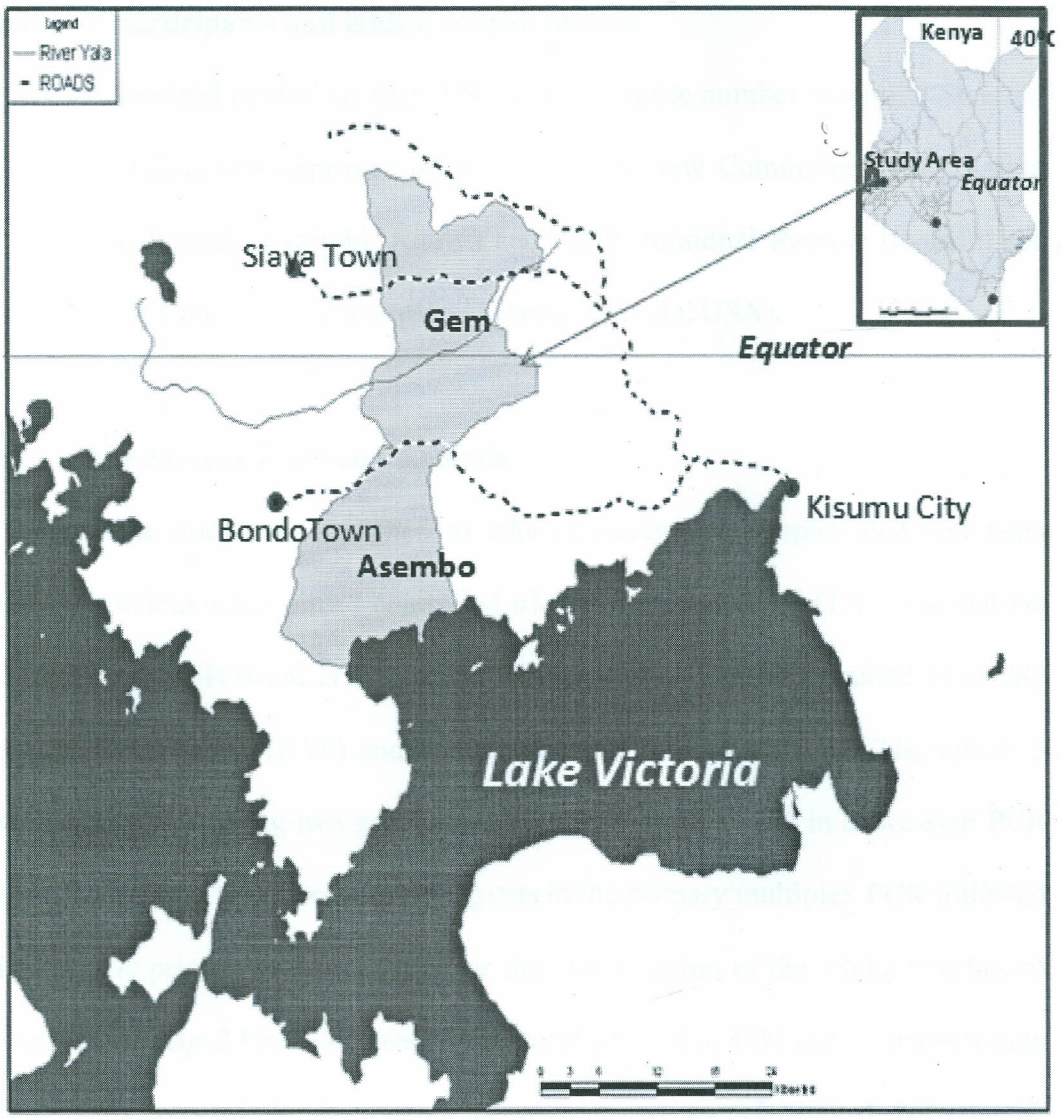

Figure.1. Map of Kenya showing the ITN trial sites in Asembo and Gem, greater Siaya district, western Kenya

3.2 Selection of participants and ethical considerations

The randomized controlled trial ITN study, reference number Scientific Steering Committee (SCC) 381, was approved by the Ethical Review Committee of the Kenya Medical Research Institute (Nairobi, Kenya) and the Institutional Review Board of the Centers for Disease Control and Prevention (Atlanta, Georgia, USA).

3.3. Design of the current study and methods

The current study was designed to take advantage of samples that had been collected in a previous randomized controlled trial of the efficacy of ITNs in a malaria endemic area (Phillips-Howard *et al.*, 2003). Cryopreserved isolates obtained from 282 children at baseline survey (BX0) and 5 years after the introduction of ITNs, survey 5 (BX5) were genotyped, using two genetic markers: *msp-1* and *msp-2* in a two-step PCR technique using primers for the conserved regions in the primary multiplex PCR followed by family-specific primers in nested PCR for the identification of the allelic families of *msp-1* block 2 and *msp-2* block 3 genes to analyze the effect of ITN use on transmission of multiple infections of *P. falciparum* genotypes and association of between *P. falciparum* genotypes with malaria morbidity indicators.

3.4 Sample size determination

A total of 282 blood samples from study participants from each of the two cross-sectional surveys: BX0 and BX5 were used in this study for genotyping analysis of *msp-1* and *msp-2* gene polymorphisms. The sample size was calculated at 95% confidence level, an alpha level of 0.5 and at 80% power to detect a 25% of the proportion of the

population with attributes (malaria) and 75% without from children residing in ITN and control groups. The children from the intervention (ITN) and control groups were also matched for gender and age.

In order to compare the outcomes of the two treatment groups, the formula provided below was used to estimate the sample size needed to achieve the specified level of significance (Hollis & Robinson, 1964).

$$N = \frac{Z^2 PQ}{E^2} \qquad \text{i.e. } N = \frac{(1.96)^2 \times (0.25 \times 0.75)}{(0.05)^2}$$

=288 (rounded off to 282 because 6 samples could not be tracked during data cleaning)

N = sample size

Z^2 =standard deviation from the mean used in other similar studies

P = proportion of the population with attributes (malaria)

Q = proportion of the population without the attributes (malaria)

E = standard error allowable and significant at 0.05 level in similar studies

3.5 Isolation of *P. falciparum* parasite DNA

Parasite DNA was extracted from parasitized red blood cells (pRBC) using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. In brief, DNA was extracted from parasitized red blood cells by adding 20µl lysis buffer (500mM Trip pH 8, 5mM EDTA pH 8, 100mM NaCl, and 1% SDS) and then followed by addition of 20µl Proteinase K (Boehringer Mannheim, Germany). The contents were thoroughly mixed by swirling the tube. The reaction mixture in a 1.5 ml

microcentrifuge tube was incubated at 56 °C for 10 min. DNA pellet from each sample was precipitated by grade ethanol (96 – 100%), washed and briefly centrifuged at 8,000 rpm before elution into 1.5 ml microcentrifuge tube. The DNA lysate was then stored at 70 °C for later use.

3.6 PCR amplification of *P. falciparum msp-1* and *msp-2* genes

The amplification of *msp-1* and *msp-2* genes was done by nested PCR (Gene Amp® PCR System 9700, A & B Applied Biosystem Foster City CA) in a 25 µl reaction mixture that made use of the products of the multiplex PCR. The 25 µl reaction mixture of the multiplex primary PCR contained 1µl DNA, 1X TBE buffer, 2mM $MgCl_2$, 200 µM dNTPs, 100 nM of the right primer set, 1.25 units/µl Hot Start Taq DNA polymerase (Gibco BRL Life Technologies) and 15.75 µl PCR water. The multiplex PCR conditions were as follows: initial denaturing at 94 °C for 2 min, followed by 30 cycles of denaturing at 94°C for 30 sec; annealing at 56°C for 1min and extension at 72°C for 1min. There was a final elongation of 72°C for 5 min, and then held at 4°C until further processing. The multiplex primary PCR (pPCR) products for *msp-1* and *msp-2* genes were diluted 1:10 and 1:20 respectively for use in the secondary PCR. After the completion of the multiplex pPCR amplification then dilution of the products, the diluents of the field isolates were amplified separately for *msp-1* and *msp-2* genes using family-specific nested PCR according to WHO recommended genotyping procedures (RGPs), (WHO, 2007).

Nested PCR for *msp-1* and *msp-2* contained essentially similar reagents as for the multiplex primary PCR except for the primers, the volume of DNA and PCR water, and

PCR conditions that varied. For nPCR of *msp-1*, the amplification conditions were as follows: initial denaturing at 94 °C for 2 min, followed by 30 cycles of denaturing at 94°C for 30 sec; annealing at 59°C for 1min and extension at 72°C for 1 min. There was a final elongation of 72°C for 5 min, and then held at 4°C. For *msp-2*, the conditions were: initial denaturing at 94 °C for 2 min, followed by 30 cycles of denaturing at 94°C for 30 sec; annealing at 58°C for 30 sec and extension at 72°C for 1 min 30 sec. There was a final elongation of 72°C for 5 min, and then held at 4°C. Positive and negative controls were systematically incorporated in each PCR run. The size of the nested PCR product for *MSP-1* considered as positive ranged from 125-250 bp while for *msp-2* ranged from 250-450 bp. Oligonucleotide primers (Gene Amp PCR System 9700 PE Biosystems, USA) used in nested PCR amplifications are shown in Table I.

Table I: Sequences of oligonucleotide primers used to amplify polymorphic regions of *msp-1* and *msp-2* genes in *P. falciparum* isolates from Asembo.

Reaction Multiplex	Primers	Sequence	product size (bp)
1° PCR	*msp-1* M1-OF	5′CACATGAAAGTTATCAAGAACTTGTC3′	
	M1-OR	5′GTACGTCTAATTCATTTGCACG3′	
	msp-2 M2-OF	5′GCAGTATTGACAGGTTATGG3′	
	M2-OR	5′GATTGAAAGGTATTTGAC3′	
2° PCR	*msp-1* K1		125-250
	M1-KF	5′5′AAATGAAGAAGAAATTACTACAAAAGGTGC3′	
	M1-KR	5′GCTTGCATCAGCTGGAGGGCTTGCACCAGA3′	
	*msp-1*MAD20		125-250
	M1-MF	5′AAATGAAGGAACAAGTGGAACAGCTGTTAC3′	
	M1-MR	5′ATCTGAAGGATTTGTACGTCTTGAATTACC3′	
	msp-1 R033		125-250
	M1-RF	5′TAAAGGATGGAGCCAATACTCAAGTTGTTG3′	
	R033-R2	5′CAAGTAATTTTGAACTCTATGTTTTAAATCAGCGTA3′	
2° PCR	*msp-2* FC27		250-450
	S1-fw	5′GCTTATAATATGAGTATAAGGAGAA3′	
	M5-rev	5′GCATTGCCAGAACTTGAA3′	
	msp-2 3D7		250-450
	S1-fw	5′GCTTATAATATGAGTATAAGGAGAA3′	
	N5-rev	5′CTGAAGAGGTACTGGTAGA3′	

Legend: primers for *msp-1* M1-OF and M1-OR (conserved); *msp-2* M2-OF and M2-OR (conserved); *msp-1* M1-KF and M1-KR (K1 family-specific); M1-MF and M1-MR (MAD20 family-specific); M1-RF and R033-R2 (R033 family-specific); *msp-2* S1-fw and M5-rev (FC27 family-specific) and, S1-fw and N5-rev (3D7 family-specific).

3.7 Electrophoresis and visualization of *msp-1* and *msp-2* PCR products

After the nested PCR amplification, the amplified PCR products for each of the *msp-1* and *msp-2* loci were separately resolved on 3 % and 2 % agarose gel (Sigma Chemicals, USA) for each family-specific nested PCR respectively (Gibco BRL Technologies, Basel, and CH). Agarose gel was stained with 2µl ethidium bromide. During sample loading, 4 µl of the nested PCR product of each sample was mixed several times with 1 µl blue juice (x6 loading dye) before loading into each well according to the designed loading pattern. The samples were loaded on separate wells then electrophoresed at 120 voltages for 1 hour 30 min. The 100 bp molecular weight ladder (Invitrogen Life Technologies, California, and USA) was used to obtain the optimum comparison in the identity of the base pair size differences in the DNA fragments for *MSP*-1 and 2 genotypes present in the blood samples of the participants enrolled in BX0 and BX5 cross-sectional study surveys. Positive and negative controls were systematically incorporated in each PCR run. The gel was then visualized and captured, using a UV transilluminator (UVP Bioimaging Systems, USA). Labworks™ Software (Version 4.0 for Windows UVP Inc, CA 91786) was used to score the distinct bands (family-specific types) of *msp-1* and *msp-2* loci that fell within the expected product size in base pairs. According to product qualification, PCR product was regarded positive when the band fell within the cut-off range of 125-250 bp for *msp-1* family-specific genotypes (K1, MAD20, RO33) and 250-450 bp for *msp-2* family-specific genotypes (3D7 and FC27). Twelve (12) samples that were unresolved were repeated and this time

around with higher amount of DNA (5 µl) during primary multiplex PCR amplification. The primary PCR products were not diluted for subsequent nested PCR amplification. Amplification conditions for the repeat samples remained the same as before. For the nested PCR results (distinct DNA bands), a positive test was defined as the detection of at least a single band of the family-specific genotypes for each of the two genes: *msp-1* and *msp-2* assayed. The gel images which resulted from electrophoresis of nested PCR products of *msp-1* and *msp-2* genes are shown in Figures 2 and 3.

3.8 Data management, processing and statistical analyses

All the data from clinical status, parasitological investigations and PCR genotyping were entered, screened and cross-checked for errors before they were merged and entered into Excel (Microsoft) Spreadsheets. The data analyzes were performed by version 12.0 of the Statistical Package for the Social Sciences (SPSS) for Windows software (SPSS Inc, Chicago, IL). The Chi-square test was used to find out the differences in frequencies and distribution of *msp-1* and *msp-2* genotypes, and their family-specific subtypes between the ITN and control groups. The two-tailed Chi-square test was used to compare the differences between groups. Proportions of binary outcomes were compared using non-parametric tests. Pearson correlation Chi-square test was calculated to assess association between qualitative and quantitative variables, such as between MOI and malaria morbidity indicators. For all tests, statistical significance was defined as a ρ-value ≤ 0.05.

CHAPTER FOUR

4.0 RESULTS

4.1 Baseline characteristics of the study population in BX0 and BX5 surveys.

The results for the baseline characteristics of the study population in BX0 and BX5 surveys are shown in Table II for the within-survey analysis. A total of 282 blood samples were used for genotyping of *msp-1* and *msp-2* loci of *P. falciparum* parasite. The male to female ratio was 1:1 and 1.1:1 in ITN and control groups, respectively. The results of Chi-square analysis indicate insignificant difference in mean age, mean haemoglobin level, fever lasting 2 weeks, mean temperature and mean parasitaemia density between ITN and control groups in each survey. The children in the ITN and control villages in BX0 were younger: mean age 24.3 months [95% confidence interval (CI), 21.2–27.3] compared to those older children in BX5 with mean age 52.4 months [95% confidence interval (CI), 50.6–54.3]. For example, the age difference was statistically insignificant between the ITN and control groups for each survey ($p= 0.483$ for BXO and $p= 0.443$ for BX5; Chi-square analysis). The results in Table II further indicate a significant increase of 31.7% in mean haemoglobin values and a significant decrease of 25.8% in microscopically determined *P. falciparum* prevalence when children in the ITN group are compared between BX0 and BX5 surveys. Overall, older children in BX5 survey demonstrated reduced parasite density and higher haemoglobin levels compared to those younger children in BX0. However, the results for baseline characteristics of study participants in BX0 and BX5 surveys are shown in Table III. The between-survey results indicate significant difference in gender ($p= 0.012$; Chi-square analysis), mean haemoglobin level ($p< 0.0001$), mean fever lasting 2 weeks ($p< 0.0001$),

mean temperature ($p< 0.002$) all by t-test analysis. On the other hand, t-test analysis revealed insignificant difference in mean age in months ($p= 0.281$) and mean parasitaemia density ($p= 0.898$) in the children for the between-survey analysis (BX0 and BX5).

Table II: Baseline characteristics of the study population in BXO and BX5 surveys

	BXO (N=101)			BX5 (N=181)		
	ITN	Control	p-value	ITN	Control	p-value
N= 282	41	60		85	96	
Males (%)	27(66)	35(58)	0.446	38(45)	45(47)	0.770
Females (%)	14(34)	25(42)		47(55)	51(53)	
Mean age, months	24.0	23.7	0.483	52.2	52.1	0.443
Mean Hb (g/dL)	8.2	8.2	0.717	10.7	10.9	0.131
Mean fever lasting 2 weeks	25(61)	33(55)	0.551	66(78)	78(81)	0.549
Mean temperature (^{0}C)	36.7	36.7	0.185	36.9	37.0	0.495
Mean parasitaemia (p/mm^3)	7314	4910	0.401	5426	6658	0.520

Legend: N= number of children; ITN and control= treatment arms within a survey; P= significance level set at 0.05; BX0= baseline survey; BX5= survey 5 (conducted in the 5th year of the study); Hb= haemoglobin values expressed in grams per deciliter (g/dL); % is put in bracket.

Table III: Baseline characteristics of study participants in BX0 and BX5 surveys

	BX0	BX5	p-value
N= 282	N=101	N=181	
Males (%)	62(61)	83(46)	0.012
Females (%)	39(39)	98(54)	
Mean age (months)	23.8	52.4	0.281
Mean Hb (g/dL)	8.2	10.8	< 0.0001
Mean fever lasting 2 weeks	1.4	1.2	< 0.0001
Mean temperature (^{0}C)	36.7	36.9	0.006
Mean parasitaemia (p/mm^3)	5886	6079	0.898

Legend: N= number of children; P= significance level set at 0.05; BX0= baseline survey; BX5= survey 5 (conducted in the 5th year of the study); Hb= haemoglobin value expressed in grams per deciliter (g/dL); % is put in bracket.

4.2. The effect of ITN on the distribution of *P. falciparum* genotypes infecting children population in Asembo, Rarieda district

The results showing the distribution of *P. falciparum* genotypes infecting children in Asembo are summarized in Tables IV, V and VI; supported by Figures 2 and 3. This study found extensive diversity of family distribution of *msp-1* and *msp-2* alleles of *P. falciparum* genotypes shown in Table IV. Overall, the family distribution of *msp-1* presented 22 K1-types, 18 MAD20-types and 19 R033-types comprising 59% of all the total alleles. However, *msp-2* gene demonstrated 21 3D7-types and 20 FC27-type making up 41% of the total alleles. Of all the samples run, K1-types were 251/282 (89%), MAD20-types 213/282 (76%) and R033-types 223/687 (79%) within the base pairs range

from 125 to 250 base pairs; whereas there were 233/282 (49%) FC27-types and 242/282 (51%) 3D7-types of *msp-2* within the range of 250 to 450 base pairs.

In the same Table IV, locus family distribution was as follows: K1-types 22/59 (37.3%), MAD20-types 18/59 (30.5%) and R033-type s 19/59 (32.2%) at *msp-1* locus, while at *MSP*-2 locus, 3D7-types were 21/41 (51.2%) and FC27-types 20/41 (48.8 %). The difference in family distribution of *P. falciparum* genotypes between BX0 and BX5 was not statistically significant at *msp1* locus: K1-types (p= 0.558) and R033-types (p= 0.052), but was statistically significant for MAD20-types (p= 0.005). The difference in the distribution of *P. falciparum* genotypes infecting children between BX0 and BX5 was statistically insignificant at *msp-2* locus as indicated by 3D7-type (p= 0.785) and FC27-type (p= 0.172) using Pearson Chi-square analysis. The parasite genotypes totaled to 100 in the population, with K1-type of *msp-1* the predominant type and FC27-type and 3D7 type of *msp-2* appeared in almost equal proportion. Apart from a nearly equal family distribution of *P. falciparum* genotypes at locus level, the current study also observed lack of significant statistical difference in the distribution of allelic families between ITN users and nonusers (p= 0.532; p= 0.814, p= 0.908; p= 0.871; and p= 0.251 for K1, MAD20, R033, FC27 and 3D7-types respectively using Chi-square analysis). However, the study noted very rich polymorphism in *msp-1* and *msp-2* loci with the majority of genotypes having three or more variant forms, except for R033-type of *msp-1* locus that was the least polymorphic. Of majority of the samples genotyped, large percentage 271/282 (94%) contained at least 2 distinct parasite lines (or variants) at *msp-1* and *msp-2* loci compared to R033-type of *msp-1* that indicated only 1 parasite line in this study area, supported by Figures 2 & 3. Genotyping detection remained high 271/282 (96.1%),

combined detection of *msp-1* and *msp-2* genotypes in the children population (not tabulated).

Distribution of multiplicity of infection (MOI) in *P. falciparum* is summarized in Tables V for BX0 and BX5 surveys and in Table VI for ITN and control groups for comparison purposes. The values obtained for each category were first converted to percentages for uniformity using Ms Excel for windows version 4.0. These percentages were finally converted to decimals to obtain mean MOI and then bar graphs drawn using Ms Excel for windows version 4.0. For the survey results, MOI was higher in BX5 than in BXO. A similar trend of MOI distribution was observed in both the surveys and treatment groups. The distribution of MOI was generally higher in the survey BX5 than BX0 (Table V), with a similar pattern of distribution reflected for the treatment groups: being higher in the control than in the ITN group (Table VI). MOI did not show any significant statistical difference between the treatment groups (P= 0.115; Pearson Chi-square analysis not tabulated). However, a significant statistical difference in MOI was observed only between the survey groups for combinations of K1/R033-types and K1/MAD20/R033-types (p= 0.030 and p= 0.028 respectively; Chi-square analysis). Generally, the distribution of the mean of MOI of KI/MAD20/R033 of *msp-1* and 3D7/FC27 of *msp-2* was quite high (> 0.6) in both surveys and treatment groups compared to very low mean MOI (≤ 0.1) for K1/MAD20, K1/R033 and MAD20/R033 combinations of *msp-1* shown in Figures 4 and 5 respectively.

Table IV. The effect of ITN on the distribution of *P. falciparum* genotypes infecting children in Asembo, Rarieda district

	msp-1			*msp-2*		
	K1	MAD20	R033	FC27	3D7	Total
No. of variants	3	4	1	3	4	15
Total (%) sample genotypes	251(89)	213(76)	223(79)	233(83)	242(86)	-
No of genotypes	22	18	19	20	21	-
Total number of genotypes	-	59	-	41	-	100
P value in BX0	0.558	0.005	0.052	0.172	0.785	
P value in BX5	0.558	0.005	0.052	0.172	0.875	
P value ITN/Control	0.523	0.814	0.908	0.871	0.251	
Amplicon size (bp)	125-250	125-250	125-250	250-450	250-450	

Legend: *msp-1*= merozoite surface protein-1, *msp-2*= merozoite surface protein-2; BX0= baseline survey; BX5= survey 5 (5years after completion of the study); K1; MAD20 and R033 are families of *msp-1*; 3D7 and FC27 are families of *msp-2*; P= significance level set at 0.05; ITN/Control= ITN users & nonusers, and bp= base pairs

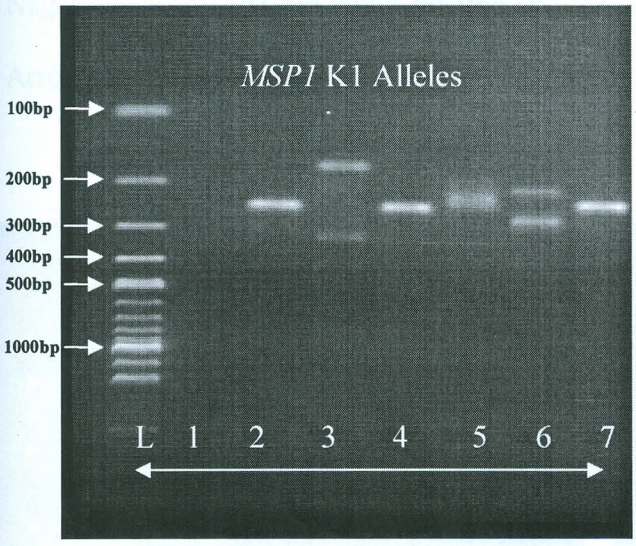

Fig.2a. *msp-1* K1 allele

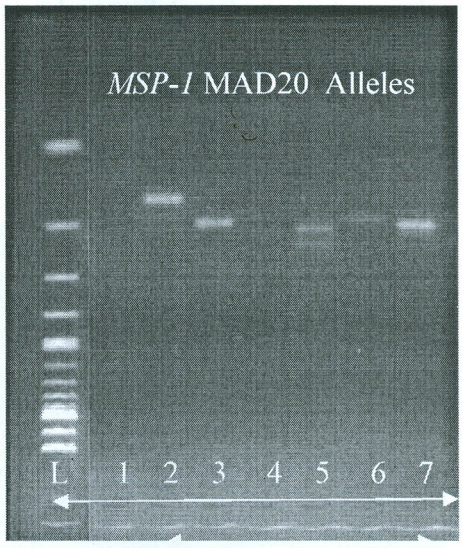

Fig.2b. *msp-1* MAD20 allele

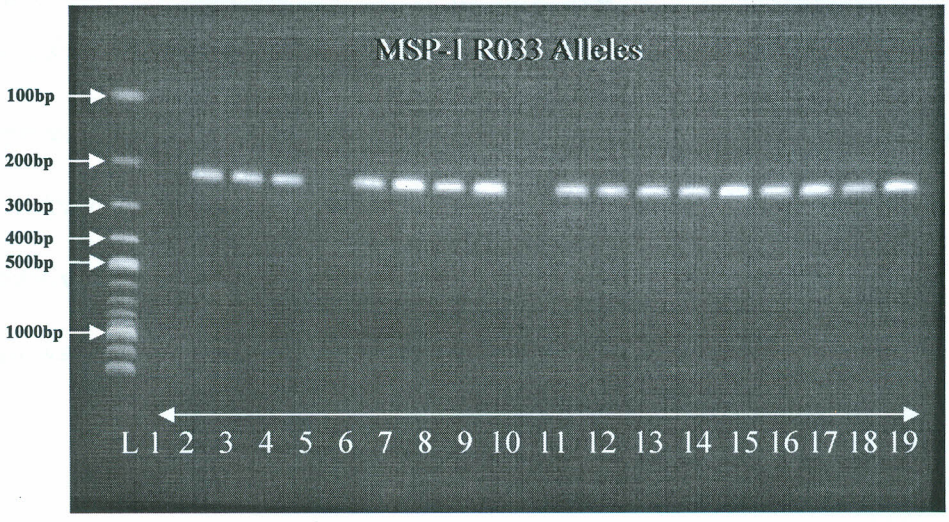

Fig.2c. *msp-1* R033 allele

Figure.2a-c. Gel images of nested PCR products showing *msp-1* gene polymorphism in *P. falciparum* isolates in children in Asembo: L-Molecular marker (100 bp), Lane 1-

Negative control (NC), Lane 2-Postive control (PC) and Lanes 3 to 19 are test samples. Arrows indicate the size of the molecular marker

Fig.3a. *msp-2* 3D7 allele

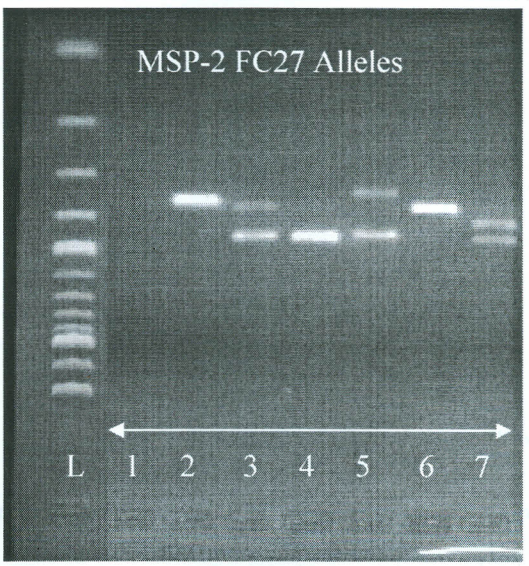

Fig.3b. *msp-2* FC27 allele

Figure.3a-b. Gel images of nested PCR products showing *msp-2* gene polymorphism in *P. falciparum* isolates in children in Asembo: L-Molecular marker (100 bp), Lane 1-Negative control (NC), Lane 2-Postive control (PC) and Lanes 3 to 7 are test samples. Arrows indicate the size of the molecular marker

Table V. The mean distribution of multiplicity of infections (MOIs) of *msp-1* and *msp2* alleles of *P. falciparum* genotypes between BX0 and BX5 surveys

MOI	BXO (N=101)	BX5 (N=181)	p-value
msp-1 K1/MAD20	9	11	0.374
K1/R033	4	21	0.030
MAD20/R033	0	2	0.289
K1/MAD20/R033	76	113	0.028
msp-2 3D7/FC27	72	141	0.215

Legend: MOI (multiplicity of infection); N (number of children); ITN and control (treatments arms); *msp-1* (merozoite surface protein-1); *msp-2* (merozoite surface protein-2); P (significance level set at 0.05); BXO and BX5 (cross-sectional surveys); K1, MAD20 and R033 are allelic families of *msp-1* and 3D7 and FC27 allelic families of *msp-2*

Table VI. The mean distribution of multiplicity of infections of *msp-1* and *msp-2* alleles of *P. falciparum* genotypes between ITN and control group

MOI	ITN (N=126)	Control (N=156)	p-value
msp-1 K1/MAD20	7	13	0.366
K1/R033	10	15	0.662
MAD20/R033	0	2	0.202
K1/MAD20/R033	83	106	0.712
msp-2 3D7/FC27	98	115	0.430

Legend: MOI (multiplicity of infection); N (number of children); ITN and control (treatment arms); *msp-1* (merozoite surface protein-1); *msp-2* (merozoite surface protein-2); P (significance level set at 0.05); K1, MAD20 and R033 are allelic families of *msp-1* and 3D7 and FC27 allelic families of *msp-2*

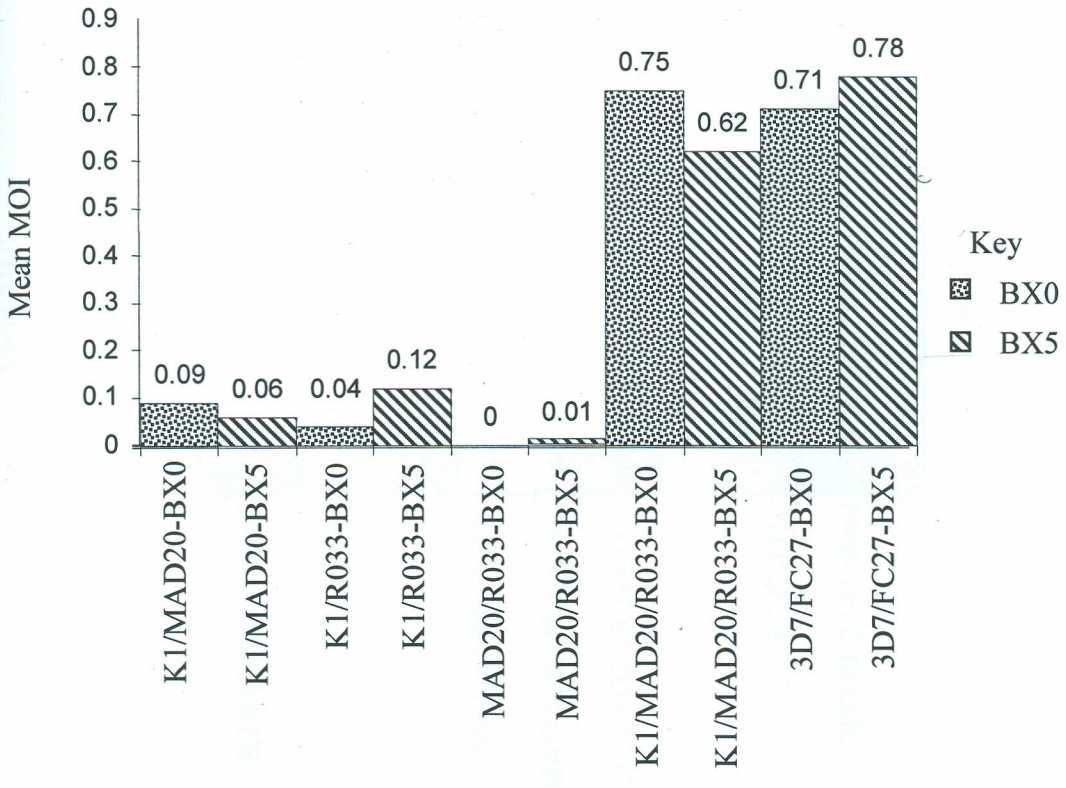

Figure.4. Comparison of mean multiplicity of infections in BX0 and BX5 surveys

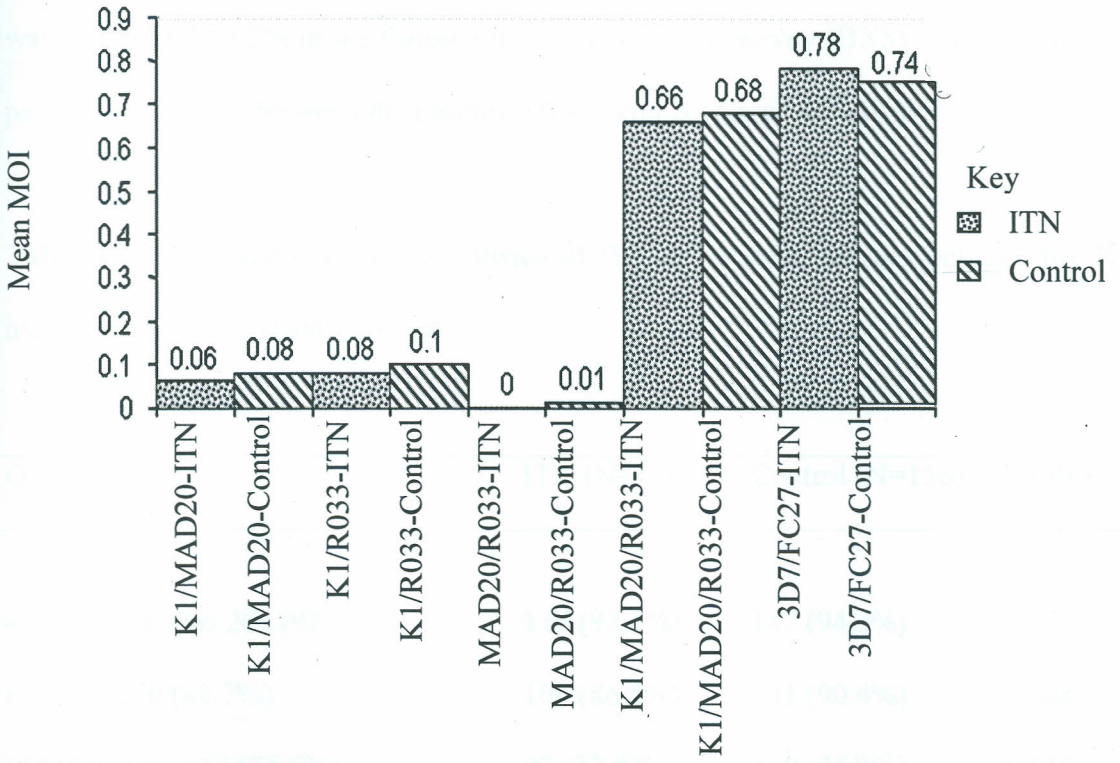

Figure.5. Comparison of mean multiplicity of infections in ITN and Control groups

4.2.1. Comparison of the prevalence of *P. falciparum* genotypes between the ITN users and non-users (control group)

The results of the comparison of the prevalence of *P. falciparum* genotypes in children between the ITN and control groups are shown in Table VII. In this study, the overall parasite prevalence of *P. falciparum* genotypes was over 90% in malaria asymptomatic children enrolled in the BX0 and BX5 surveys. The prevalence of *P. falciparum* genotypes detected by PCR remained high in both treatment groups, with average prevalence of 44.7% in the ITN group and 55.3% in the control group. The difference in the overall parasite prevalence between the two groups was statistically

insignificant (p= 0.236; Chi-square analysis). The parasite prevalence at baseline (BX0) was 35.8% and 64.2% in the follow-up survey (BX5-up survey (BX5). The difference in parasite prevalence between the baseline (BX0) and BX5 was statistically

Table VII. Comparison of the prevalence of *P. falciparum* genotypes between the ITN users and non-users (control group)

Overall (n=282)	ITN (N=126)	Control (N=156)	P value
msp-1 genotypes 263 (93.3%)	116 (92.1%)	147 (94.2%)	0.470
K1-type 250 (88.7%)	109 (86.5%)	141 (90.4%)	0.308
MAD20-type 213 (75.5%)	93 (73.8%)	120 (76.9%)	0.545
R033-type 223 (79.1%)	100 (79.4%)	123 (78.8%)	0.915
msp-2 genotypes 262 (92.9%)	121 (96.0%)	141 (90.4%)	0.066
3D7-type 242 (85.8%)	112 (88.9%)	130 (83.3%)	0.184
FC27-type 233 (82.6%)	107 (84.9%)	126 (80.8%)	0.360
msp-1 & *2* genotypes 271 (96.1%)	123 (97.6%)	148 (94.9%)	0.236

Legend: N (number of children); ITN and control (treatment arms); *msp-1* (merozoite surface protein-1); *msp-2* (merozoite surface protein-2); P (significance level set at 0.05); K1, MAD20 and R033 are allelic families of *msp-1* and 3D7 and FC27 allelic families of *msp-2*.

4.2.2. Temporal variation in *msp-1* and *msp-2* alleles of *P. falciparum* genotypes 5 years after deployment of ITNs

The results of the temporal variation in *msp*-1 and *msp*-2 alleles of *P. falciparum* genotypes 5 years after deployment of ITNs are summarized in Table VIII. There was insignificant difference in the variation of *msp-1* and *msp-2* alleles of *P. falciparum* genotypes over time, except for MAD20-type of *msp-1* that demonstrated a significant statistical difference (P= 0.005; Chi-square analysis) between the baseline (BX0) and the follow-up survey (BX5). However, there was no significant statistical difference in the parasite variation for the remaining allelic families of *msp-1* between the two surveys as demonstrated by K1-type (p= 0.558; Chi-square analysis) and R033-type (p= 0.052; Chi-square analysis). Allelic families at *msp-2* locus also demonstrated lack of significant statistical difference in parasite variation over observational period as evidenced by 3D7-type and FC27-type (p= 0.785 and p= 0.172 respectively; Chi-square analysis).

Table VIII. Temporal variation in *msp-1* and *msp-2* alleles of *P. falciparum* genotypes 5 years after deployment of ITNs

Genotypes/Allelic family	BX0 (N= 101)	BX5 (N=181)	p-value
msp-1 genotypes	97 (96%)	166 (92%)	0.134
K1-type	93 (92%)	157 (87%)	0.558
MAD20-type	84 (83%)	129 (71%)	0.005
R033-type	83 (82%)	140 (77%)	0.052
msp-2 genotypes	94 (93%)	168 (93%)	0.573
3D7-ype	86 (85%)	156 (86%)	0.785
FC27-type	80 (79%)	153 (85%)	0.172

Legend: N= number of children; BX0= baseline survey; BX5= 5 years after deployment of ITNs; P= probability or significance level at 0.05; K1, MAD20 and R033 are allelic families of *msp-1* and 3D7 and FC27 allelic families of *msp-2* .and % is put in bracket.

4.2.3. Association between *P. falciparum* genotypes with malaria morbidity indicators (haemoglobin levels, auxiliary temperature, parasite density and fever lasting 2 weeks) including age.

The results for the association between *P. falciparum* genotypes with malaria morbidity indicators as determined by Pearson correlation Chi-square test are summarized in Table VIII. Haemoglobn levels (< 5g/dL, < 7g/dL, < 11g/dL), auxiliary temperature (<37.5^{0}C and ≥ 37.5^{0}C), parasite density (<5,000 parasites/µl and ≥5,000 parasites/µl) and fever lasting 2 weeks were considered as the clinical outcomes of the disease. The overall mean multiplicity of *msp-1* and *msp-2* genotypes per infection were 3.53 (95% CI: 3.37-3.69) and 3.21(95% CI: 3.03-3.39) respectively. The majority of infections were multiple (polyclonal) 263/282 (93%) and few, 19/282 (7%) were monoinfection of *P. falciparum* genotypes (not tabulated).

The data analysis by Pearson correlation Chi-square test showed that age, in the range of 2 to 77 months in this study, was not significantly correlated with multiplicity of infections (MOI) (p= 0.403; not tabulated). There was no significant association between MOI with age category and clinical outcomes, for example, with age category: <37months and ≥37months (p= 0.334 and p= 0.463 respectively), haemoglobin level < 5g/dL (p= 0.285), <7g/dL (p= 0.415), <11g/dL (p= 0.935); fever lasting 2 weeks (p=

0.357); auxiliary temperature: <37.5^{0}C (p= 0.983), ≥37.5^{0}C (p= 0.410) and parasite density <5,000 p/µl (p= 0.131 and ≥5,000 p/µl (p= 0.304).

Regarding the association between *msp*-1 genotypes with age category and clinical outcomes, the genotypes showed insignificant correlation with age category: <37months and ≥37months (p= 0.449 and p= 0.316 respectively), haemoglobin level < 5g/dL (p= 0.263), <7g/dL (p= 0.238), <11g/dL (p= 0.704); fever lasting 2 weeks (p= 0.153); auxiliary temperature: <37.5^{0}C (p= 0.760), ≥37.5^{0}C (p= 0.996) and parasite density <5,000 p/µl (p= 0.951) and ≥5,000 p/µl (p= 0.660). However, *msp*-2 genotypes demonstrated significant association with mean auxiliary temperature ≥37.5^{0}C (p= 0.021) and parasite density <5,000 p/µl (p= 0.014), but insignificant association with age category: <37months and ≥37months (p= 0.356 and p= 0.878 respectively), haemoglobin level < 5g/dL (p= 0.263), <7g/dL (p= 0.372), <11g/dL (p= 0.825); fever lasting 2 weeks (p= 0.616); mean auxiliary temperature: <37.5^{0}C (p= 0.703) and parasite density ≥5,000 p/µl (p= 0.466).

The result of the variation of age with total *msp-1* and *msp-2* genotypes using regression analysis in Figure 6 shows inverse relationship between age and the total number of infecting *msp-1* and *msp-2* genotypes which was insignificant (F .044; p= 0.826^a, where "a" is constant predictor such as age in months). The parasite density < 5,000 parasites/ µl was found to be significantly correlated with R033 (p= 0.042) of *msp-1*, FC27 (p= 0.009) of *msp-2* and even total *msp-2* genotypes (P= 0.014) using Pearson correlation Chi-square test (not tabulated).

Table IX. Association between *msp-1* and *msp-2* genotypes with malaria morbidity indicators (haemoglobin levels, auxiliary temperature, parasite density and fever lasting 2 weeks) including age

Variables	Age (months)		Hb level (g/dL)			2 week's fever	Auxil temp. (37.5^0C)		Parasitaemia density (p/µl)	
	<37	≥37	<5	<7	<11		<37.5	≥37.5	<5,000	≥5,000
N	102	180	6	27	91	281	251	31	156	86
P of *msp-1*	0.449	0 .316	0.263	0.238	0.704	0.153	0.760	0.996	0.951	0 .660
P of *msp-2*	0.356	0 .878	0.263	0.372	0.825	0 .616	0.703	0.021	0.014	0.466
P of MOI	0.334	0.463	0.285	0.415	0.935	0.357	0.983	0.410	0.131	0 .304

Legend: Parasitaemia= *P. falciparum*-positive blood smear; N= number of children; Hb= haemolgobin levels g/dL; p/µl= parasites per microlitre of blood; auxil= auxiliary temperature; *msp-1* and *msp-2* are *P. falciparum* genotypes and P= the probability or significance level at 0.05. The data are presented as proportions in the various categories. Statistical significance was determined by χ^2 – test.

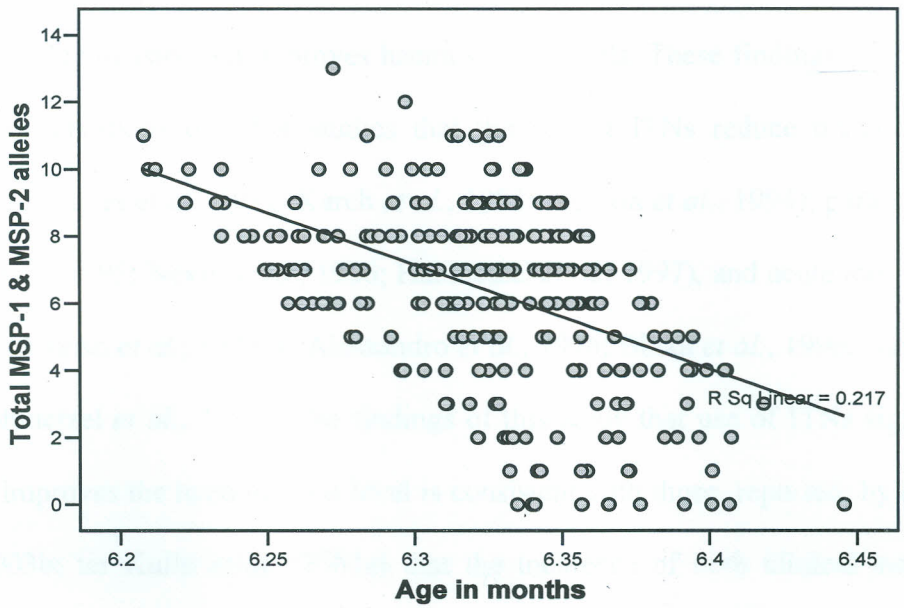

Figure 6. The relationship between age in months and the total number of infecting *msp-1* and *msp-2* genotypes in the children population

CHAPTER FIVE

5.0 DISCUSSION

5.1 Baseline Characteristics of the children population in BX0 and BX5 Surveys

The results of the baseline characteristics in this study confirm that the use of ITN reduces parasite density and improves haemoglobin levels. These findings are consistent with the observations of other studies that the use of ITNs reduce malaria parasite prevalence (Graves *et al.*, 1987; Karch *et al.*, 1993; Jaenson *et al.*, 1994); parasite density (Premji *et al.*, 1995; Nevill *et al.*, 1996; Habluetzel *et al.*, 1997), and acute morbidity and mortality (Alonso *et al.*, 1993; D'Alessandro *et al.*, 1996; Binka *et al.*, 1996; Nevill *et al.*, 1996; Habluetzel *et al.*, 1997). The findings of this study that use of ITNs significantly ($p<.001$) improves the haemoglobin level is consistent with those reported by (ter Kuile *et al.*, 2003b; ter Kuile *et al.*, 2003a) that the incidence of both clinical malaria and moderate-severe anemia (hemoglobin level <7 g/dL) were reduced by 60% ($P < 0.001$ for both). The finding of this study also reaffirms reported protective efficacy of ITNs in young children less than five years who are yet to develop immune competence against malaria compared to older children. The use of ITNs essentially reduces exposure to malaria by preventing mosquito bites. In a study by (ter Kuile *et al.*, 2003a), ITNs substantially reduce exposure to malaria and subsequent malaria-associated morbidity in children less than 24 months old in areas of intense perennial malaria transmission, and that reduced malaria exposure during infancy did not result in increased malaria morbidity in one-year-old children with continued use of ITNs. Although reduced exposure to *P. falciparum* malaria parasites due to ITN use reduces parasite density, there was lack of evidence to prove that a similar situation would reduce the transmission of multiple

infections of *P. falciparum* genotypes, possibly attributable to chronicity of infections- an important immune protection against malaria. The mean of fever in weeks attributable to malaria was lower in older children in BX5, possibly as a sign of developing sufficient physiologically stable anti-parasite in-built mechanisms to ward off infection compared to younger children in BX0 whose protective immunity is insufficiently developed.

5.2. The effect of ITN on the distribution of *P. falciparum* genotypes infecting children in Asembo, Rarieda district

This study found a more-or-less even family distribution of *P. falciaprum* genotypes in the children population with regard to *msp-1* and *msp-2* loci. The distribution of *msp-1* infections comprised K1-type (37%), MAD20-type (31%) and R033-type (32%), compared to that of *msp-2* which consisted of 3D7-type (59%) and FC27-type (41%). This study also found a very rich polymorphism of *msp-1* and *msp-2* loci, each composed of at least 18 different allelic types, with the least family-type not less than 31% of all the parasite strains at each locus. The observed high family diversity at *msp-1* and *msp-2* loci was consistent with the report by (Aubouy *et al.*, 2003). This genotype heterogeneity encountered in the study area, which may differ according to malaria endemicity, geographical location, transmission intensity, seasonal sampling and clinical status, could be suggestive of the functional relationship of the two loci with the disease outcome in this population. This study found *msp-2* locus to be usually highly polymorphic compared to *msp-1* locus in this area probably due to its functional adaptability. A nearly equal family distribution of *msp-1* and *msp-2* allelic families observed in the study area is consistent with reports of other studies (Eisen *et al.*, 1998)

and also consistent with some studies (Eisen *et al.*, 1998; Aubouy *et al.*, 2003) of a slightly higher distribution of FC27 and 3D7 allelic families at *msp-2* locus in the field isolates. It is hereby postulated that the FC27 in synergy with 3D7 could provide protective immunity against malaria.

This study further observed R033-type to be least (poorly) polymorphic, with only 1 detectable parasite line frequently detected at 160 bp. This finding corroborated with some reports (Zwetyenga *et al.*, 1999; Sallenave-Sales *et al.*, 2000; Heidari *et al.*, 2007) but differed from other reports (Peyerl-Hoffmann *et al.*, 2001) in west Uganda and (Aubouy *et al.*, 2003) in Gabon who reported at least 3 detectable allelic strains of R033. The general lack of polymorphism in R033-type suggests its genetic stability with regard to natural selection. This could be an evolutionary advantage in malaria endemic regions to the asymptomatics. The lack of genetic diversity of R033-type despite selection pressure may also provide a clue to immune protection against severe malaria or may be more relatively important in the pathogenesis of *P. falciparum* infection in clinical presentations. This study also demonstrated that K1-type was the most frequent (predominant) at *msp-1* locus and overall. This finding is consistent with the reports by (Aubouy *et al.*, 2003; Ekala *et al.*, 2002; Kun *et al.*, 1998) that K1-type was the allelic family present in high frequency in all isolates from samples studied. However, these finding contrasted other studies (Ghanchi, 2010) that placed K1-type in second position after MAD20 at *msp-1* locus on symptomatic patients of all ages, and (Robert *et al.*, 1996) repoted R033-type as the most frequent family at the *msp-1* locus in isolates from asymptomatic subjects (aged between 6 months to 10 years in Dienga village, Gabon).

The exposure of the children in Asembo to all the three allelic variants of *msp-1* and two of *msp-2* loci either singly or in varying combinations of K1/R033 and KI/MAD20/R033 co-infections could be associated with acquired immunity and certain clinical outcomes. For example, at *msp-2* locus, 213 (75.5%) were 3D7/FC27 combination, while at *msp-1* locus, 189 (67%) were K1/MAD20/R033 combination, 25 (8.9%) K1/R033 combination, 20 (7.1%) K1/MAD20 combination, with only 2 (0.7%) MAD20/R033 combination between the two surveys and treatment groups. Even though these results show lack of significant statistical relationship between the treatment groups with respect to the distribution of MOI, significant statistical relationship between BX0 and BX5 surveys was observed with respect to K1/R033 and K1/MAD20/R033 combinations ($p= 0.030$ and $p= 0.028$ respectively; Chi-square analysis). This finding shows that use of ITNs did not affect the distribution of MOI since multiple infections in malaria endemic areas suggest significant development of sterilizing immunity against malaria. MAD20/R033 and K1/MAD20/R033 co-infections could provide effective immune protection against malaria in children in Asembo. This finding corroborated the study from (Anong, 2010) that reported the association of R033/K1 co-infections with fever and was particularly common among the infected children aged 3 to <6 years.

Majority of the isolates (97%) had multiple *msp-1* and *msp-2* parasite genotype infections, containing at least 2 distinct parasite lines, with majority of double-strain infections belonging to the K1 family and single clonal infection to R033 family. This complexity of infection in young children in Asembo may reflect their strong antiparasite immunity, and extensive distribution of *msp-1* and *msp-2* allelic families among the children suggests heterogeneity of *P. falciparum* infections. The allelic heterogeneity

may differ according to several factors and may suggest that concomitant immunity to reinfection by several parasite lines similar to those initially present would be very unlikely due to genetic recombination.

5.2.1. Comparison of the prevalence of *P. falciparum* genotypes in children between ITN users and non-users (control group)

From the results of the comparison of the prevalence of *P. falciparum* genotypes in children between the ITN and control groups are shown in Table VII, this study did not find significant difference in parasite prevalence of *P. falciparum* genotypes between ITN users and non-users (control group). There was insignificant decrease of the genotypes in the ITN (44.7%) and equally insignificant increase in the control group (55.3%). The finding is consistent with a study (Smith *et al.*, 1999b) that reported no statistically significant difference in parasite prevalence between users and nonusers despite reduction of infection multiplicity by 17% in ITN users. Lack of statistically significant difference between users and non-users in observed genotype prevalence for individual alleles suggests that, in areas of high *P. falciparum* endemicity, ITNs have little effect on the establishment of chronic malaria infection. This suggests that, in areas of high *P. falciparum* endemicity, ITNs have little effect on the establishment of chronic malaria infection and does not cause significant reduction in the prevalence of *P. falciparum* genotypes. The infections once established, may become chronic and last for a long time at low-density infections. If this may be the case, then limited reduction in exposure by ITN use would not have much effect on the prevalence of multiplicity of infections. The

higher prevalence of the *P. falciparum* genotypes in the control group may be due to exposure to more infective mosquito bites than in the ITN group.

There was also no significant statistical difference in the parasite prevalence between the baseline survey (BX0) which stood at 35.8% and the follow-up survey (BX5) at 64.2% (p= 0.750; Pearson Chi-square analysis, not tabulated). The insignificant difference in parasite prevalence may be due to age-related prevalence in burden of *P. falciparum* infections, the influence of parasite population dynamics, establishment of chronic infections, micro-geographical factors or a combination of these factors.

5.2.2 Temporal variation in *msp-1* and *msp-2* alleles of *P. falciparum* genotypes 5 years after deployment of ITNs

From the results of this study (see table VII), there was variability in circulating genotype populations- a decline in the parasite population during the follow up survey (BX5) for *msp-1* allelic families, with the decline in MAD20 being statistically significant (p= 0.005) suggesting its role in immune protection or a stable malaria infection in the area. However, *msp-2* genotypes demonstrated an increase in percent genotypes overtime, with 3D7 family showing a marginal increase. However, these changes in values are statistically insignificant. These observed differences were difficult to interpret in view of any antimalarial drug administration during the study period. It is quite likely that *msp-2* could be playing a functional role in malaria immunogenicity, while MSP-1 genotypes involved in malaria pathogenesis since they are reduced with time with acquisition of immune competence. The other reason for the differences in

findings could be that some of these participants may have received anti-malarial treatment prior to enrolment in the study, such a treatment was likely to reduce the number of genotypes in an infected individual, and therefore *P. falciparum* genotypes identified in these participants may not be representative for the parasite population in the study population. In a sense, the temporal change of parasite genotypes shown in this study could be interpreted as a natural selection either by vaccination or chemoprophylaxis, with strain-specific immunity providing a selective advantage for parasites expressing "novel" alleles at the later time point (BX5). However, the proportion of polyclonal infections (not tabulated) did differ between the time points: higher in these asymptomatic children 5 years after deployment of ITN than before. This contrasted the findings of studies (Fraser-Hurt *et al.*, 1999) and (Smith *et al.*, 1999b) that reported lack of statistically significant difference in multiplicity of infections between ITN users and nonusers. This insignificant difference in multiplicity of *P. falciparum* infections between ITN users and nonusers suggested that in areas of high *P. falciparum* endemicity, ITN use would have little or no effect on the establishment of chronic malaria infection and would not interfere with the development of multi-strain immunity in the population.

5.2.3 Association between *P. falciparum* genotypes with malaria morbidity indicators (haemoglobin levels, auxiliary temperature, parasite density and fever lasting 2 weeks) including age.

The results of the association between *P. falciparum* genotypes with malaria morbidity indicators are summarized in Table VIII. This study did not find the association between MOI and age ($p= 0.334$ and $p= 0.463$ for age categories < 37 months and ≥ 37 months respectively in the age range 2 to 77 months). However, lack of correlation between MOI and age in the present study needs to be confirmed in a study population with broader age range. This finding is consistent with the report that found a decrease of MOI with age in asymptomatic subjects >15 years (Ntoumi *et al.*, 1995; Bendixen *et al.*, 2001). The finding, as shown in Figure 6, is also consistent with other studies (Aubouy *et al.*, 2003; Heidari *et al.*, 2007; Vafa *et al.*, 2008). In a study (Eliades *et al.*, 2006), the overall prevalence of malaria parasitaemia would increase with age up to age of 5 years, ranging from 69.4% to 73.0%. This so because older children would have sufficient immunity to malaria compared to younger individuals. Some studies so far conducted in holo- or hyper-endemic areas indicate that immunity develops faster and at younger age than in areas with less intense transmission by (Baird, 1995). The finding of this study is further supported by other studies by (Farnert *et al.*, 1999; Zwetyenga *et al.*, 1998)) that achieved the same result with symptomatic subjects. From the studies so far conducted, age cannot be used as a predictor of multiplicity of infections since in older children protective immunity is more mature. Very high MOI may also be reflective of the clinical and immunological status of the patients even though conflicting results are present in the literature.

Reports regarding the relation of MOI and malaria morbidity are contradictory (Vafa *et al.*, 2008). Some studies have shown positive correlation between MOI and presentations of clinical malaria. (Branch *et al.*, 2001) and (Ofosu-Okyere *et al.*, 2001) showed association of malaria morbidity with high MOI. However, several studies have shown inverse association (Farnert *et al.*, 1999; Muller *et al.*, 2001). That this study demonstrates no correlation between MOI and malaria morbidity indicators is evidenced by lack of significant association between MOI and haemoglobin levels: <5g/dL (p= 0.263), <7g/dL (p= 0.238) and <11g/dL (p= 0.704) fever lasting 2 weeks (p= 0.357); auxiliary temperature: $<37.5^0$C (p= 0.983), $\geq 37.5^0$C (p= 0.410) and parasite density <5,000 p/µl (p= 0.131 and ≥5,000 p/µl (p= 0.304). A number of studies have suggested that high MOI may confer protection from subsequent clinical malaria (Muller *et al.*, 2001; Farnert *et al.*, 1999). Since multiplicity of infection shows insignificant statistical association with clinical malaria in the asymptomatic children, it did not appear to have protective effect. Haemoglobin levels cannot be used to predict the outcome of multiplicity of infections since the haemoglobin level depends on age, immune status and other conditions of the body. Alternatively, immune responses, e.g. cytokine production, varying with different parasite strains may contribute to the unequal impact of distinct genotypes on Hb levels. However, in asymptomatic children, MOI is considered to reflect acquired immunity or premunition (Smith *et al.*, 1999a; Smith *et al.*, 1999c) and to reduce the risk of subsequent clinical malaria (Farnert *et al.*, 1999).

In this study, age was not related to the number of genotypes, in agreement with several studies achieved with symptomatic or asymptomatic subjects (Farnert *et al.*, 1999; Zwetyenga *et al.*, 1998). This study further showed no association between *msp-1*

and *msp-2* genotypes with malaria morbidity indicators, except for *msp-2* with auxiliary temperature ≥37.5^{0}C (p= 0.021) and parasitaemia density <5,000 parasites/µl (p= 0.014) considered to reflect acquired immunity or premunition in asymptomatic children in this malaria holoendemic area. The findings were also consistent with (Farnert *et al.*, 1997) who reported a significant negative correlation between MOI (as indicated by the number of infecting *msp-2* genotypes/subject) and morbidity. Moreover, these results underline the fact that parasite characteristics are certainly not the only ones involved in malaria morbidity. Host characteristics such as genetic polymorphisms, immune status, or endemicity could also play a key role. Since multiplicity of infection showed insignificant statistical occurrence in the asymptomatic children, it did not appear to have protective effect. In a prospective study in an endemic area of Papua New Guinea (al-Yaman *et al.*, 1997) individuals with single or no infection had higher risk of falling ill with malaria subsequently than individuals with multiple clone infections. (Farnert *et al.*, 1999) showed that multiple concurrent *P. falciparum* infections, with different genotypes, are associated with reduced risk of clinical malaria in older children, probably due to the development of premunition (al-Yaman *et al.*, 1997; Beck *et al.*, 1997).

In conclusion, infection complexity (infection with more than one parasite line) in Asembo was very high (97%) of all the genotyped isolates. This multiple *msp-1* and *msp-2* parasite lines in asymptomatic children in Asembo reflect the accumulation of strain-specific immunity overtime, since naturally-acquired immunity consists of a series of 'strain'-specific responses, with each individual host capable of mounting his or her own repertoire of responses and thus resulting in a mosaic human population with a heterogeneous success rate for each individual strain.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

i. There was extensive diversity in the distribution of *P. falciparum* genotypes infecting children in Asembo, showing allele heterogeneity associated with protective immunity in the area. K1-type was the predominant allele overall. There existed rich polymorphism at *msp-1* and *msp-2* loci in the area, except for R033-type that was poorly polymorphic. The difference in the distribution of allelic combinations such as K1/R033 and K1/MAD20/R033 was statistically significant between the baseline and follow-up studies, linking the presence of high MOI with protective immunity in malaria endemic areas. (Ofosu-Okyere *et al.*, 2001) in their study found that the probability of having asymptomatic malaria episode was positively associated with a relatively high MOI of which this study has confirmed.

ii. The prevalence of *P. falciparum* genotypes in children between the ITN and non-users (control group) detected by PCR remained relatively high in both the ITN and control groups. According to (Ekala *et al.*, 2002) it was found that there is a high frequency of K1-types of *msp-1* in all *P. falciparum* isolates from samples studied in Gabonese residents in Gabon, West Africa. Age-dependent prevalence of genotypes could be indicative of the influence of age on the presentation of asymptomatic malaria infections in a holoendemic area.

iii. On the temporal variation of the genotypes, more-or-less similar results were obtained in *msp-1* and *msp-2* alleles of *P. falciparum* genotypes over the study period, except for MAD20-type of *msp-1* that registered significant change in parasite population over time. Lack of change in parasite population over time could suggest weak selective pressure for parasite genotypes thus leading to a stable human population in the area in the absence of frequent migrations. However, if the selective pressure is strong enough for the genotypes, possibly as the case with MAD20-type, then this causes change in the parasite genotypes.

iv. There was lack of association between *P. falciparum* genotypes with most malaria morbidity indicators; except for *msp-2* which showed strong association with auxiliary temperature $\geq 37^0$C and parasite density < 5,000 parasites/µl, supported by reports that the relation of multiplicity of infections (MOI) and malaria morbidity are contradictory (Vafa *et al.*, 2008), hence further studies be conducted to resolve these contradictory reports. These results underline the fact that parasite characteristics are certainly not the only ones involved in malaria morbidity.

6.2 Conclusion

This study shows that parasite isolates in Asembo Bay area of western Kenya are highly diverse with respect to *msp-1* block 2 and *msp-2* block 3 allelic families. In the current study, the use of ITN in malaria control was not associated with a reduction of multiple *P. falciparum* infections. The impact of ITN use on multiplicity of infection (MOI) still remains unchanged and unresolved. The study also did not find any relation between MOI and malaria morbidity indicators. There was lack of significant difference

in the prevalence *P. falciparum* genotypes between ITN users and non users, as well as the temporal variation of the parasite genotypes over time. Even though the results of this study may reflect a significant underestimation of the genetic diversity of the *P.falciparum* population in Asembo Bay area, the study provides an attempt to gain a thorough picture of *P. falciparum* molecular epidemiology in Asembo Bay area, essential information for health policy makers since the genetic diversity and allelic-family distribution found in this study is similar to reports from areas sharing high malaria endemicity.

6.3 Recommendations

Further studies should:

1. Find out if variation in infecting *msp-1* and *msp-2* genotypes can be used as a predictor in malaria disease outcomes (morbidity and mortality) in a longitudinal study across different age profiles.
2. Investigate whether the multiplicity of infections of *P. falciparum* parasite is a human host or vector characteristic under strict adherence to bed net use.
3. Use of several gene loci to find out the relation of parasite genotypes with malaria morbidity indicators across different age profiles and geographical settings.
4. Carry out genome-wide analysis of *msp-1* and *msp-2* loci from different geographical locations in relation to immune responses to understand strain-specific immunity.

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