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ABSTRACT

The complexity of *Plasmodium falciparum* infections in children in western Kenya

To investigate the allelic complexity of infection (COI) of *Plasmodium falciparum* infections in children living in Kisumu, western Kenya, samples from three studies conducted from June, 2003 through May, 2006 were analyzed: a longitudinal cohort, a phase 1 field trial and a phase 2 field trial. Samples from the studies were analyzed using nested PCR of the highly polymorphic *msp1* block 2 to observe potential selective effects of the vaccine, a MSP1 formulation.

The longitudinal cohort, used to determine the baseline COI, followed 60 children 1-4 years old for 13 months who donated scheduled samples monthly and additional samples when ill with clinical malaria. Results revealed a COI that was dependent on age, parasite density, illness, village location and bed net use. Nearly all infections were with multiple genotypes. Fluctuations of the three examined alleles of *msp1*, K1, MAD20 and RO33, were rapid and random within individual children, as well as the entire study group, indicating a highly diverse parasite population. Parasite density was found to be directly correlated with COI in those children with clinical illness. As the density increased, the contribution of the K1 allele proportionately increased while the contribution of the RO33 allele decreased. Presence of the invariable RO33 allele was also found to be mildly protective against clinical illness. For the first time, bed net use was found to decrease COI in 1-2 year old children who were both asymptomatic carriers of parasites and ill with clinical malaria; the RO33 allele was again most associated with the decrease in COI. In the phase 1 dose-escalation trial with 135 children, those

participants who received the full vaccine dose had a decrease in COI following vaccine administration. In that group of children, the RO33 allele was identified in much greater prevalence following vaccine administration. Samples that were RO33 positive were also predominately chloroquine sensitive. The phase 2 vaccine trial with 400 participants is still currently blinded; initial analysis showed an increased COI in ill patients, a finding that contrasts with previous reports. Combined, the three studies provided evidence of the rapidly evolving immunity to malaria, even within the limited 1-4 year age range of the study participants.

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The complexity of *Plasmodium falciparum* infections in children in western Kenya

by

Ardath White Grills

Dissertation submitted to the Faculty of the Emerging Infectious Diseases Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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This dissertation is dedicated to my family in recognition of thirty-plus years of unwavering support for whatever dream I choose to chase, and my husband, George for being by my side when I'm chasing them.

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CHAPTER ONE: INTRODUCTION

Background

Malaria claims more than 1 million lives every year [1] and places an enormous burden on those exposed to the disease; new estimates now indicate that there are 515 million clinical episodes annually of *Plasmodium falciparum* [2], the most virulent form of the disease. One major focus of research is the development of an effective vaccine. These efforts are complicated by the lack of development of sterile immunity even in populations with constant, high exposure. In those areas, surveys reveal that most individuals are chronically, asymptomatically infected with one or more types of malaria parasite.

The measure of concurrent infections, known as the complexity of infection (COI), is a useful epidemiologic tool to examine the efficacy of a vaccine or drug, as well as serving as a marker for the immune status of an individual, and in population-based studies as an indication of malaria transmission dynamics [3]. In vaccine field trials, the participants usually have ongoing malaria infections complicating the interpretation of results. Traditional measures of effectiveness such as the presence of parasites or incidence of clinical illness may obscure some findings of benefit of the candidate vaccine. For example, the malaria vaccine examined in this work was designed in the 3D7 clone of the NF54 strain of falciparum malaria [4]. In the field, numerous different allelic types circulate that are not the same as 3D7. It is essential to determine if the vaccine only affects the strains for which it is specific, known as homologous protection, or if there is an heterologous or cross-protection against parasites that encode alternative alleles. The use of parasitologic and/or genotypic analysis such as COI often provides

more useful data for this type of analysis because it can determine the identity of the infecting strains and make comparisons between them (Table 1).

Table 1. Benefits of molecular epidemiology analyses in vaccine trials

Laboratory outcomes		**Value added by molecular epidemiology**	Refs
Clinical and/or parasitological	Genotyping		
Time to parasite positivity (prepatent period)	PCR determined prepatent period	More sensitive outcome	[5, 6, 7]
Incidence of clinical episodes Time to first clinical episode Parasite density	Detection of new genotypes Duration of infection with individual clone	Identify parasite dynamics	[7, 8, 9]
Hemoglobin concentration Rate of anemia and/or severe anemia Incidence of severe malaria	Relationship to specific genotype	Identification of allelic type associated with the episode Comparison to vaccine components Examine potential selective pressure	[7, 10]

Adapted from Felger et al. *Trends Parasitology*. 2003; 19:60-63.

Vaccine interventions could affect the rate of acquisition and/or loss of malaria strains. Genotyping studies are currently the only molecular means available to assess these selective effects. Since correlates of protection for malaria have not been identified, COI may be useful as a surrogate marker. Changes in COI observed following vaccine administration provide evidence that the intervention had a biological effect, although the interpretation of the significant difference is difficult. While COI clearly reflects the immune status of malaria-exposed individuals, the currently available data does not agree on the exact relationship, and the immunological processes involved are not fully understood.

Development of complex infections

Complex infections arise when one or more female *Anopheles* mosquitoes feed and inject more than one strain of parasites into a human host. These parasite differences

arise from multiple allelic differences and antigens of both the sexual and asexual stages of its life cycle. In the human host, *P. falciparum* undergoes several rounds of asexual replication; the parasites undergo schizogony and a small minority differentiate into male and female stages. When the mosquito feeds and takes in the male and female gameteocytes, exflaggelation occurs, and the gametes fuse and form short-lived diploid zygotes. These undergo meiotic division, creating haploid cells that after further development and asexual replication to become infective sporozoites. The motile sporozoites migrate to the mosquito salivary glands and once again can infect humans during blood feeding.

No effective recombination results when there is fusion of male and female gametes from the same clone, known as selfing. Outcrossing, the fusion of gametes from different strains, results in recombination. Regular outcrossing in the field results in high recombination rates, a phenomena known as panmixia [11], that is a hallmark of holoendemic transmission areas that have an exceptionally high prevalence of falciparum malaria. The majority of the population in holoendemic regions is infected with more than one type of *P. falciparum* parasite, termed a multi-allelic or multiclonal infection. Regions with lower transmission, such as South America and southeast Asia, have a lower effective recombination rate and higher inbreeding of parasites resulting in a lower diversity in the human population [12, 13, 14].

Immunologic effects related to COI

The number of alleles that circulates in a host is a function of both the transmission intensity and the immune status of the individual. *P. falciparum* COI has

been found to be affected by many factors such as age, parasitemia, pregnancy, drug treatment and resistance, experimental vaccines and the presence of other human malarias. In holoendemic areas, several of these factors follow a pattern of decrease from a high in infancy to a low during adulthood. Clinical malaria incidence [15], mean parasite density [15, 16, 17] and COI in clinical malaria [17, 18, 19] all follow a similar pattern of decrease over time in holoendemic areas. Similarly, there is a direct correlation identified between COI and parasite density in infancy; those with high parasite density typically have a high COI. With increased age, this relationship is no longer apparent [15]. In contrast, the COI identified in asymptomatic infections has been found to increase from a low in infancy to a peak in adolescence followed by a subtle decline in adulthood [20]. For this investigation, several different factors were evaluated: age, clinical disease incidence, parasite density, bed net use and village location.

Age The effect that age has on COI is varied. Many infections are asymptomatic and/or are cleared by the individual without developing a high level of parasitemia. This effect is seen even in very young infants [18], and as the child ages, both the COI and duration of infection increases [21]. For example, in a study of two Tanzanian villages with different transmission dynamics [19], the village with the highest number of infective bites also had an age-dependent COI that increased in older children. Meanwhile, adults (>15 years) in the study had significantly lower detectable COI than children. In a similar study of two Senegalese villages [22], the area of intense transmission had age-dependent COIs that had twice as many alleles as a village with lower transmission.

Clinical illness Contamin et al. [23] conducted a narrow longitudinal survey of 10 children in a holoendemic transmission area of Senegal over a period of four months. This study revealed that the genetic characteristics of the parasite population during a clinical episode differed substantially from the population carried asymptomatically. While they found that the allelic distribution could be either more or less complex depending on the patient, the trend was toward a less complex infection being associated with clinical disease.

A prospective study found that the risk of developing clinical disease was much lower in children with multiple strains compared to aparasitemic children or children with single infections [24, 25]. In infants (<1 year old), the opposite result was found: in clinical cases the COI was higher than in asymptomatic carriers and with every additional infecting genotype, the risk of fever doubled. This may indicate that in the early, naive stages of the immune response almost all new infections cause disease [26].

In some studies with varying levels of malaria endemicity, infection complexity correlated with more severe illness, although there is disagreement as to the correct association. Conway et al. [27] found that an increased COI correlated with more severe malaria in areas with mesoendemic or seasonal transmission in The Gambia. This finding has been refuted by several other researchers who found a significantly lower number of alleles in those with clinical and/or severe malaria [20, 24]. Al-Yaman et al. [24] found that in their prospective study in an holoendemic region of Papua New Guinea, clinical malaria was significantly attributed to a lower COI. This supported the interpretation that multiple infections protected against clinical manifestations. In a hypoendemic

transmission area of Senegal, Robert et al. [20] compared severe and mild malaria cases and found a significantly lower COI in those with the more severe clinical symptoms.

Parasite density In holoendemic areas, there is a direct correlation between COI and parasite density identified in infants; this relationship does not exist in older children or adults [25, 28]. Branch et al. [29] found that infants 6 months to 1 year of age had lower COIs and parasitemias than children over 2 years old who had a reduced ability to resist higher parasitemias. Other researchers have found that despite a lower COI, patients with severe malaria had a much higher parasitemia [20]. In symptomatic cases, independent of parasitemia, a recent study found that a higher COI was associated with low hemoglobin concentrations and severe anemia [30].

Bed Nets Information on the effect of insecticide impregnated bed nets on COI is rather limited, but all agree that use of bed nets does not affect COI [31, 32, 33]. The reason for this appears to be related to transmission: because mosquitoes carry multiple genotypes a slight reduction in mosquito bites as is afforded by bed net use does not directly translate to a reduction in COI.

COI in Vaccine studies

Previous studies The effect that vaccines have on COI is varied depending on the vaccine formulation and the subjects tested. For example, two previous studies were performed in Tanzania and in The Gambia in conjunction with randomized trials of the Spf66 malaria vaccine [8, 9]. A significant decrease in COI was seen in asymptomatic vaccine recipients when compared to asymptomatic placebo recipients. In symptomatic children, a slight trend toward increased COI was seen in the vaccine recipients. Overall,

there was no reduction in the prevalence of blood-stage infection and no significant overall reduction in malaria prevalence in the trial. Beck et al. [9] concluded that the reduced COI in asymptomatic infections was a consequence of an increased rate of elimination of individual parasite strains. The authors postulated that because the immune system is stimulated in high-density infections, Spf66 may induce a response sufficient to control high-density infections and prevent morbidity, but not fully eliminate the low-density infections.

The second vaccine study to use genotyping methods was the phase 1-2 trial of Combination B, a trivalent recombinant blood stage formulation [7, 34, 35]. The researchers examined the COI of one of the genes used in the vaccine and found a significant decrease in parasite density and COI among the vaccine recipients. The genotyping data further allowed them to conclude that Combination B provided only homologous protection and failed to protect against heterologous strains that did not match the vaccine formulation [7].

The most recent report on a vaccine study that examined COI of infecting genotypes following vaccine administration used RTS,S/AS02A in a pediatric population of southern Mozambique [36]. The results were similar to those found with Spf66: in asymptomatic children there was a significant reduction in COI among vaccine recipients in comparison to controls. Among those admitted to the hospital with a clinical episode of malaria, there was no reduction in COI compared to controls. In a study of RTS,S/AS02A among adults in The Gambia [37], there was a vaccine-related delay to first infection, indicating a level of vaccine efficacy, but no difference in COI among vaccine recipients and controls.

Present vaccine The antigen used in the vaccine under study here is derived from the merozoite surface protein 1 (MSP1) a polymorphic protein of approximately 190 kDa that is the major surface protein of the invasive merozoite stage of *P.falciparum*. The MSP1 precursor molecule undergoes post-translational proteolytic processing to produce fragments of 83, 28, 38 and 42 kDa that are covalently linked as a complex on the surface of the mature merozoite (Figure 2) [38]. Tanabe et al. [39] aligned sequences of *msp1* genes and grouped the molecule into 17 blocks, consisting of conserved, semi-conserved or variable sequences. When different sequences of strains and isolates were aligned, the blocks, with the exception of block 2, were found to be dimorphic, classified either as MAD20 (3D7)-like or Thai-K1-Wellcome (FVO)-like families [40]. Block 2 is trimorphic, containing not only the K1 and MAD20 families found throughout the rest of the gene, but also the RO33 allele which is unique to the block 2 region. K1 and MAD20 are highly variable allelic families with various repeat units while RO33 is a conserved non-variant allele.

Because the intervention that is the focus of this investigation is the FMP1 vaccine formulation that uses MSP1 as an antigen, using *msp1* to determine COI allows for the assessment of the selection pressure exerted by the vaccine on the gene.

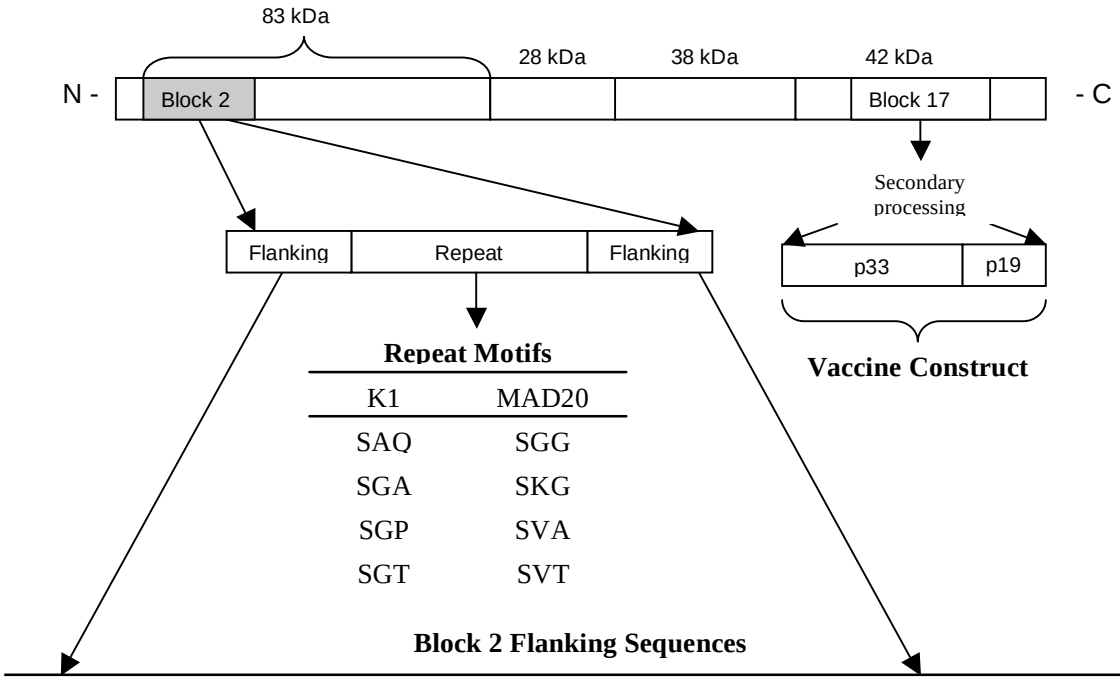


Figure 1. Schematic representation of MSP1. The block 2 repeat patterns and flanking sequences of K1 and MAD20 are shown. The full RO33 block 2 sequence is also shown for comparison, with the frequent codon 64 substitution in bold.

K1 In block 2, K1 and MAD20 type alleles contain different tripeptide repeats with serine at the first position (Figure 1). K1 repeats are the most variable in size and variety [41]. For example, Branch et al. [29] found 23 polymorphic repeat regions during a 3-year study of 60 children in Asembo Bay, Kenya. In Ghana, 31 different repeat regions were identified from 375 samples [42]. Despite this variety, there are discernable patterns among the tripeptide repeats. The repeat motif begins with a uniform string of the amino acids SAQSGT. The repeat strings consist of the amino acid serine in the

primary position, followed by glycine, threonine, alanine, or glutamine in one of the following patterns: SGT, SGP or SAQ. The repeats end with the motif SGPSGT. The highest repeat number diversity was seen with SGT and SGP [40, 41, 43].

MAD20 The MAD20 allele is less variable than K1 with 8 - 14 tripeptide repeat units [41]. Similar to K1, the repeat strings begin with serine, however the following amino acids are different. Combinations of SGG, SVA, SVT, SKG are found, usually starting with SKG or SGG SVT but always ending with SVA SGG [41, 44].

RO33 Of the many isolates examined, RO33 is nearly always identical; a single non-synonymous nucleotide replacement is frequently seen in codon 64 [44]. This results in aspartic acid being replaced by glycine. There is also a rare substitution at codon 104 whereby G was replaced with D [41, 44]. Several researchers in Africa have reported finding more than one size of RO33, though no one has yet presented further explanation of the observation or determined the sequence of these alternate forms [17, 42, 45].

In addition to the three parental families, recombinants have also been identified. Contamin et al. [23] identified K1-RO33 recombinant forms in Senegalese children. A recently identified MAD20-RO33 recombinant was found to occur in about one-fourth of samples genotyped from the Asembo Bay Cohort in western Kenya [46]. The field observations of recombinant block 2 alleles are supported by laboratory cross studies in which intragenic recombination was clearly demonstrated. Recombination between the entire 5' variable region of *msp1*, blocks 2-6, was demonstrated in both laboratory conditions and field isolates [47, 48, 49]. However, the presence of recombinants in heavily mixed field isolates are difficult to interpret; in vitro recombination during PCR

is not infrequent and the use of nested PCR, a common procedure in field studies, increases the chance that false recombinants may be detected [50].

II. Hypotheses and Research Approaches

Hypothesis 1:

In a prospective longitudinal cohort of children 1-4 years old in a holoendemic area of western Kenya, I hypothesize that genotypes will vary as a consequence of transmission intensity and acquired immunity. Complexity of infection is predicted to be age-dependent even within the small age range of the study, demonstrating the rapid acquisition of immunity that occurs in a high transmission area. Additionally, we hypothesize that genotypes in symptomatic clinical cases are predicted to be the result of increased parasite density of a single strain and a strong correlation between decreased alleles and increased risk of developing clinical disease.

Research Approach:

Samples collected as part of a longitudinal cohort study were used to determine the natural dynamics of allelic complexity in children living in or near Kisumu, Kenya. Participants donated finger-prick blood samples monthly and when clinically ill over a 13-month period. The narrow focus on children 1-4 years old allowed for refinement of the findings regarding the acquisition of partial immunity, particularly the age-dependent nature of COI, the relationship between clinical illness and the number of circulating strains.

The focus of the research was to identify the relationship of COI with age, disease incidence and parasite density. Various different demographic and behavioral factors were considered to explain the relationship with COI, such as village location, gender and bed net use.

The final focus of this portion of the research was to standardize the methods used to determine COI and to improve upon shortcomings identified in previous work. The methods presented here are the result of those efforts.

Hypothesis 2:

The literature regarding the effect of COI is contradictory, thus evaluating the effect that a vaccine formulation, such as the FMP1/AS02A candidate vaccine analyzed here, may have on COI is difficult. However, I hypothesize that an efficacious vaccine formulation will increase the COI among recipients. While this may seem counter-intuitive, in young children, an increased COI would indicate the acquisition of protective immunity similar to that of a naturally exposed older child. The end result would be a young vaccine recipient that is able to maintain an increased number of asymptomatic falciparum infections without developing clinical illness.

Research Approach:

Samples from the phase 1 and 2 field trials of FMP1 vaccine in Kisumu, Kenya were used to test this hypothesis. Analysis was performed on the block 2 of *msp1* to assess the COI. This information provided a secondary measure of vaccine efficacy and a more complete picture of any selective effects of the vaccine and its ability to influence the acquisition of immunity to falciparum malaria.

CHAPTER 2: METHODS

Introduction

Traditionally, blood smears have been used to determine the presence of infection and parasite density. While adequate in the field, this method fails to provide all the information necessary to adequately assess the efficacy of a vaccine candidate. Multiple, complex infections cannot be assessed by blood smear because there are no unique morphological traits among different infecting falciparum strains. Thus, the use of molecular methods, especially those using polymerase chain reaction (PCR) amplification of target genes, provide more detailed information than microscopy on secondary effects such as genetic selection.

The most commonly used method to determine the COI of malaria is to analyze highly polymorphic genes that differ from parasite to parasite. *P. falciparum* COI studies usually focus on three genes: *msp1*, the gene for merozoite surface protein 1, *msp2*, and *glurp*, the gene encoding glutamate-rich protein. These three genes share the common feature of being single copy in the haploid blood stages of the parasite life cycle and having highly variable regions with insertion/deletion regions. When examined using DNA amplification, the presence of more than one allele represents a multi-clonal infection.

Several techniques have been applied to determine COI, but each method has serious deficiencies. The basis of each technique is the PCR which is used to amplify the sample DNA to a detectable concentration. PCR has made it possible to identify many different parasite strains; detection is further improved for samples with low DNA concentrations by using a two-stage nested PCR. In addition, the use of family-specific

oligonucleotide primers for MAD20, K1, and RO33 allows for the easy classification of allelic variants and improves the sensitivity of detecting minor variants in mixed infections. There is a tradeoff in PCR methodologies between detection and quantitation. Detection is optimized in order to identify all the alleles in a sample, making absolute quantitation of the alleles in the sample impossible.

The nested PCR may also have the unintended consequence of amplifying samples that have a high DNA concentration to the point where spurious bands are created on a gel (Figure 2). The bands are usually the result of overloading DNA in a sample well that results in "laddering". This ladder appearance is a likely occurrence given that the parasite density of the field samples is unknown. These bands are identical to those from real strains and make interpretation difficult. Dilution usually makes these bands disappear, but it is then unclear if the disappearance was because they were false bands or because they were the true representation of a minor clone diluted beyond the point of detection.

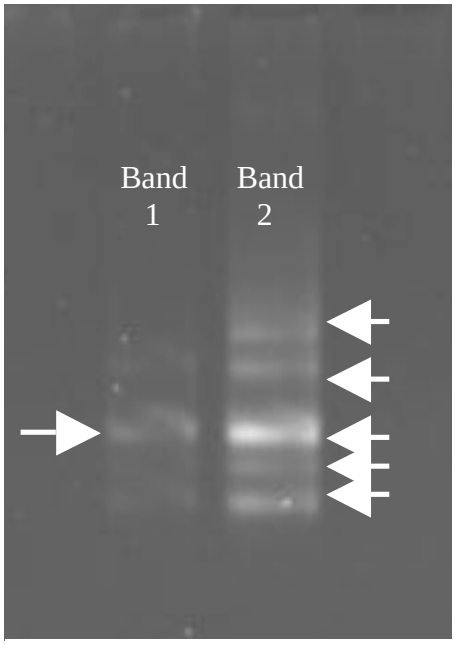


Figure 2. Example of a difficult to interpret gel. Band 2 has at least 5 visible bands (arrows) but it is not clear whether they are true or a spurious ladder from overloading. Band 1 is a 1:10 dilution of Band 2; the single band is indicated with the arrow.

When tracing individual alleles in longitudinal sets of blood samples, the average duration of a single genotype can be calculated. This analysis is complicated if there are failures to detect all infections present in the host at any one time, due to parasite sequestration or an extremely low parasite concentration. When three or more serial

samples from the same individual are genotyped, patterns of alternating presence and absence of specific genotypes may indicate the failure of PCR to detect all infections present [3]. The relative frequency of such patterns allows estimation of the sensitivity of the PCR assay as well as the clearance rate and the duration of infections.

Mayor et al. [51] and others [52, 53] proposed that the detection of lower density infections may be hindered by the density-dependent constraints of PCR whereby the minor populations are present but cannot amplify because the dominant parasite with the most DNA will consume the majority of reagents. This assay-dependent competition was variable at a level of less than 100 parasites/microliter and unreliable at levels less than 10 parasites/microliter [53].

No matter the technique used to obtain the COI, the value is usually an underestimate. It is likely that some parasite strains are sequestered on the day of collection and are unrepresented in the blood sample [54] or at frequencies too low to detect in the sample. Viriyakosol et al. [55] reported that when collected blood samples were established in culture, a substantially greater proportion of isolates could be detected after cultivation that were not detected in the originally analyzed blood sample.

The assays used to determine COI all share one shortcoming: reliance on subjective interpretation of the presence and number of genotypes from electrophoretic gels. The interpretations are unreliable from one researcher and one lab to the next (Figure 2). In their comparison of seven respected genotyping laboratories, Farnert et al. [53] further supported the idea of intra-assay competition by demonstrating that when known concentrations of laboratory isolates were mixed, the strains at low concentration were more difficult to detect than when analyzed as a single infection, even when the

same number of parasites were used to create the sample. Overall, the authors found that in their study of the procedures of seven different well-known laboratories, the reports of variations in parasite-population structure in diverse geographical areas, or over periods of time, might actually be due to technical differences between the laboratories rather than true epidemiological differences [53].

Methodologies other than gel electrophoresis were examined prior to deciding upon the methods used here so that the analysis of the PCR amplification product would be improved. The ideal method was one that met the following criteria: (1) robust and sensitive to detect all strains in a sample, (2) specific to exclude spurious results, (3) reliable upon repeated measurements, (4) high-throughput to process thousands of samples in a short period of time and (5) semi-quantitative to reveal the relative concentrations of the strains in a sample. Because the assay design relies on the use of a nested PCR, which may preferentially amplify some DNA over others in a sample, measurements can only be thought of as semi-quantitative. The amount of DNA in each sample will also vary depending on parasite density, sample degradation, or intra-assay competition for limiting reagents and this will limit the ability to compare quantitative measures from sample to sample. After comparing different systems, microfluidics gel electrophoresis was identified as the superior method to analyze the samples.

The microfluidics system (Agilent 2100 bioanalyzer, Agilent Technologies, Palo Alto, CA USA) was chosen because it met the criteria identified for an ideal system. It uses very small quantities of reagents and sample that are run under pressure on an analysis chip (Caliper DNA 500 LabChip®, Caliper Technologies, Hopkinton, MA). The samples are separated electorphoretically as with a traditional gel and then fluorescently

detected to yield quantitative measurements. The major advantage of this system is the ease of set-up and use; it requires very little optimization in comparison to other methods. Another advantage is that it uses only one microliter of sample so repeated assays can be run from the same PCR product. One limitation of the system is the potential for run-to-run variation. The microfluidics LabChip® can hold only twelve samples per assay, so quality control will need to be established by re-running a percentage of samples to ensure comparability. The Agilent system provides a quantitative measurement of the sample; this is compared to internal markers that run with every sample to ensure consistency. While the true quantitative measurement cannot be used, peak height-to-peak height measurements can be compared to yield a semi-quantitative proportionate measurement of the strains in a sample.

Study Site and Design

The basis of this study was three separate clinical investigations conducted in children in western Kenya, an area of holoendemic transmission. The transmission level varies during the year with two peaks following the “long rains” from April through June and the “short rains” from August through October. The entomological inoculation rate (EIR), a measure of the transmission intensity, is estimated to be 0.75 infective bites/day [56]. The first was a longitudinal cohort of children aged 1-4 years old. Study participants donated samples monthly for 13 months and when clinically ill. Clinical illness in all studies was defined as fever $\geq$ 37.5 °C and a positive malaria blood film. A smaller case-controlled study using additional samples from the longitudinal cohort was also conducted. Samples from asymptomatic children were paired with a sample from an

episode of clinical illness and matched for parasite density, age in months, month the sample was taken and village location. Second, a phase 1 field trial of FMP1 was conducted with 135 children. Samples from the day of first vaccination and day 90 (30 days after final vaccination) were used to determine COI and optimize the assay. Finally, a phase 2 field trial of FMP1 was conducted; samples were collected on the day of vaccination, day 85 (one month after third vaccination) following vaccination and when the children were clinically ill with malaria. This trial consisted of a cohort of 400 children aged 1-4 who received either three doses of vaccine or a non-malaria vaccine comparator (rabies vaccine).

DNA Extraction

Samples from the vaccine trials were whole blood from which DNA was extracted using Qiaqen DNA Blood MiniKit (Qiagen Inc., Valencia, CA) using either the spin or manifold protocol and stored at -20° C. Samples used for the epidemiological study were filter paper blood dots. To extract the DNA from the blood dot, a 4mm punch was taken from the sample and processed using the Generation extraction kit (Gentra Systems, Inc., Minneapolis, MN) with a modified protocol. Briefly, the punched sample was placed in a 0.2 ml tube and washed three times with 150 µl of Purification solution. The sample was incubated for 15 minutes at room temperature, the solution was mixed three times with a pipettor up and down to wash the filter paper, and the solution was then discarded. Next, 150 µl of the Elution solution was added to the tube and allowed to incubate at 4° C overnight. Following the incubation period, the Elution solution was pipetted up and down once to rinse the filter paper and was then discarded. The DNA

remained bound to the filter paper through this step. If the filter paper was still dark in color after the overnight incubation in the Elution solution and rinse step, 150 µl of Elution solution was again added to the tube for an additional 2 hour incubation at room temperature. Following incubation, the Elution solution was pipetted up and down once to rinse the filter paper and then discarded.

To elute the DNA, 150 µl of the Elution solution was added to tubes, tubes were incubated for 15 minutes at 99° C to release the DNA from the filter paper and then cooled to room temperature. The final step was to pipette the solution up and down 15 times gently with a wide-tipped pipette to remove all DNA from the filter paper. The 150 µl of the Elution solution containing the extracted DNA was placed in a clean tube and stored at -20° C.

Nested PCR

PuReTaq Ready-to-Go PCR beads (GE Healthcare Biosciences Corporation, Piscataway, NJ) in a 96-well format were used to perform the reactions. One µl of extracted DNA was added for the primary PCR to a solution of water and primers for a total volume of 25 µl per sample tube. For the secondary PCR, 1 µl of the primary amplified product was used as the DNA template for MAD20 and RO33 while 1.5 µl was used for K1. Primer sequences, concentrations for primer pairs, volumes added and optimized conditions are listed in Table 2.

PCR conditions were as follows: initial denaturation at 94° C for 5 min; then 94° C 15 sec, 56° C 30 sec, 72° C extension (see time in Table 2) repeated 35 times for the primary and 30 times for the secondary; followed by a final extension at 72° C for 5 min.

Table 2. Primer sequences and optimized conditions for block 2.

Reaction	Sequence	Conc. (pm)	Vol. (µl)	Extension (sec)
Primary		7.5		60
Forward	AAG CTT TAG AAG ATG CAG TAT TGA C		1.5	0
Reverse	CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA		1.5	
K1		10		30
Forward	TGA AGA AGA AAT TAC TAC AAA AGG TGC		2	
Reverse	AA TGA AGT ATT TGA ACG AGG TAA AGT G		2	
MAD20		7.5		30
Forward	GCT GTT ACA ACT AGT ACA CC		1.5	
Reverse	TGA ATT ATC TGA AGG ATT TGT ACG TCT TGA		1.5	
RO33		2.5		30
Forward	GCA AAT ACT CAA GTT GTT GCA AAG C		1	
Reverse	AGG ATT TGC AGC ACC TGG AGA TCT		1	

Analysis of Amplified Product

To determine the presence of the RO33 allele, 1% agarose gels stained with ethidium bromide were used for visualization. Band size was compared to a 50-2500bp ladder.

The size and number of K1 and MAD20 alleles were analyzed using two methods. For the phase 1 vaccine trial samples a 4% high-resolution agarose gel (MetaPhor agarose, Cambrex Bio Science Rockland, ME) in chilled 1X TBE buffer was used. Gels were poured using the standard protocol; 10 µl of sample was loaded with 2 µl of Orange G loading dye. The two outer lanes of the gel were loaded with 50 bp ladder (Invitrogen Corporation, Carlsbad, CA) for band size determination. Gels were run at a slow voltage at 4°C and then stained with ethidium bromide. The number of alleles was determined visually; this was confirmed using LabWorks 4.0 software that identified bands and calculated band size (UVP, Inc., Upland, CA).

For the phase 2 vaccine trial and MEPI, the K1 and MAD20 alleles were analyzed with the microfluidics bioanalyzer (Agilent 2100, Agilent Corporation, CA) using a 500

bp DNA LabChip® (Caliper Technologies, Hopkinton, MA). To process samples, a gel matrix containing DMSO DNA dye was added to one well of the 16-well LabChip. The LabChip was then pressurized to force the gel matrix into a capillary bed under the sample wells. Following pressurization, 1 µl of a standard ladder was added to a well with additional gel matrix. The 12 sample wells were loaded with a 15 bp and 600 bp marker that standardized each well and 1 µl of sample.

The 2100 Expert software (version B.02.02) provided with the Agilent 2100 was used for analysis of the samples. The software has a set cut-off limit of 1.0 ng/µl of sample, which is the equivalent of 20 FUs, or fluorescent units, the arbitrary unit for light fluorescence measured by the software. The minimum valid reading as defined by the Agilent Corporation was 2.5 times the background fluorescence detected by the machine for each assay. This value was significantly lower than 20 FUs for the assay performed here. For this reason, the 1.0 ng/µl cut-off value was considered a conservative estimate to use that would not cause the mischaracterization of a spurious peak as a true allele. However, as a conservative estimate, there was the possibility that true alleles would not be detected. An analysis of 200 samples with a cut-off of 0.5 ng/µl revealed that only 6 additional bands would be detected. Therefore, there was a 3% chance that additional alleles may be identified in a sample.

Chloroquine resistance assay (PfCRT)

For the phase 1 vaccine trial, samples were also analyzed to determine whether the parasites were sensitive or resistant to the drug chloroquine. The assay was designed to differentiate between four point mutations within the chloroquine resistance transporter

gene located on chromosome 7. Sensitive strains contain the amino acid sequence MNK in position 74-76 of the Pfcrt protein. The chloroquine resistant strain haplotype detected by this assay is the most common in Africa and has the amino acid sequence IET in place of MNK. The PCR-based assay used an initial amplification to increase DNA, followed by real-time PCR amplification using TaqMan probes (Applied Biosystems, Foster City, CA) on an Opticon II real-time PCR thermal cycler (MJ Research / Bio-Rad Laboratories, Hercules, CA). Primer and probe sequences are presented in Table 3 with the detected mutations noted.

The primary reaction consisted of 1 µl DNA, 1 µl of each primer and 23 µl water added to PuReTaq Ready-to-Go PCR beads (GE Healthcare Biosciences Corporation, Piscataway, NJ). Amplification conditions were initial denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C denaturation for 30 seconds, 52°C annealing for 30 seconds and 72°C extension for 30 seconds. A terminal extension at 72°C for five minutes was followed by holding at 4°C.

For the second reaction, the master mix consisted of 1 µl DNA product from the first reaction, 2.5 µl 10X Buffer, 2 µl dNTPs, 6 µl of 6mM MgCl, 2 µl probe, 1 µl of each primer, 0.125 µl AmpliTaq Gold, and 9.375 µl water for a total 25 µl volume. Conditions for amplification were 95°C denaturation for 10 minutes, followed by 40 cycles of 95°C denaturation for 15 seconds and 60°C combined annealing and extension for 1 minute. After each cycle, a plate reading was taken.

Table 3. Primer sequences for PfCRT. The point mutations detected by the probes are highlighted in blue.

Reaction	Sequence	Conc. (pm)
Primary		20
Forward	AAT GAC GAG CGT TAT AGA G	
Reverse	TGA TTG GAT ATT TCC AGC AG	
Secondary		20
Forward	GCT CAT GTG TTT AAA CTT ATT TTT AAA GAG ATT AAG GAT AAT	
Reverse	AAG TTG TGA AGT TTC GGA TGT TAC AAA AC	
Probes		4
Sensitive	TGT ATG TGT AAT **GA**A **T**A**A** AAT TT	
Resistant	TAT GTG TAA T**TG** A**A**A **C**AA TTT TT	

Statistical Analysis

Statistical analysis was hypothesis-driven to prevent the creation of spurious significant results. Factors associated with COI were first analyzed followed by examinations of the individual allele families. Relationships between COI and the allele families to factors of age, illness status, village location, bed net use and/or vaccine status were then assessed. Statistical significance for studies was considered to be a *p* value less than 0.05.

Analyses were performed using Statistical Analysis Software (SAS) 9.1 for Windows (Cary, NC). Poisson distribution was used as it is optimal for non-normally distributed data such as the COI and because the values that were measured were primarily event counts. In SAS, the PROC GENMOD program with Poisson distribution was used for COI, K1 and MAD20 analyses. When RO33 and PfCRT status was examined, the binomial nature of the values required logit distribution. For the MEPI longitudinal analyses, the generalized estimating equations (GEE) correction was used to relax the assumptions of independence and account for the repeated measures within each child. Data were analyzed using two different methods of dependence: compound

symmetry (CS) which assumes equal correlation for all pairs of dates examined, and first-order auto regressive correction (AR) in which adjacent dates are more likely to be similar than those further apart in time. There was no difference identified between the two methods, so AR was chosen. Additional descriptive statistics such as means and frequency distributions as well as non-parametric analyses were calculated using SPSS 12.0.1 for Windows (Chicago, IL).

CHAPTER THREE: MEPI

Introduction

This study was a longitudinal cohort, referred to as MEPI (Malaria EPIdemiology), conducted from May, 2003 – June, 2004. Study participants, children between 1-4 years of age, donated finger-prick blood samples monthly for 13 months and when clinically ill. The initial analysis, presented here, focused on 60 children.

The 60 children that formed the study population were drawn randomly from the reference population of 277 children by first excluding all children that did not have at least 10 of the 13 monthly scheduled samples. The remaining experimental population was then 199 children. Twenty children from each age group of 12-23 months, 24-35 months and 36-47 months of age at time of study enrollment were randomly chosen (Research Randomizer, http://www.randomizer.org). The study population was compared to the experimental population and found to be identical with regard to age in months, gender, reported bed net usage, number of scheduled visits and number of episodes of clinical illness (Table 4).

A second, smaller case-control study was also conducted with the MEPI samples, termed the parasite density study. Ill cases were matched to an asymptomatic control from the same village on the basis of sample date, age in months and parasite density in three categories. The low category was defined as <500 parasites/μl, medium was ten-fold higher at 5,000 – 6,000 parasites/ μl and high was >40,000 parasites/μl.

Table 4. Summary of demographic information of sample population of 60 children compared to experimental population.

A. 1 year olds

Volunteer ID	Gender	Age (months)	Village	Bed net use	# Clinical ill episodes	# Scheduled visits	Total # visits
35	male	21	JIMO	no	3	15	18
164	male	19	WEST OTHANY	yes	3	14	17
166	female	18	WEST OTHANY	no	4	13	17
194	male	18	SOUTH CENTRAL SEME	no	4	13	17
207	female	21	SOUTH WEST SEME	no	7	12	19
219	male	22	SOUTH CENTRAL SEME	no	3	10	13
254	male	22	WEST NGERE	no	6	10	16
293	female	22	EAST RERU	yes	5	13	18
320	male	16	EAST NGERE	yes	0	14	14
322	male	18	EAST RERU	no	5	14	19
323	male	20	WEST KADINGA	yes	3	13	16
329	male	18	EAST KADINGA	yes	2	16	18
330	male	18	SOUTH WEST SEME	yes	5	10	15
354	male	16	EAST OTHANY	yes	1	16	17
379	female	16	SOUTH WEST SEME	yes	3	17	20
385	male	13	EAST KADINGA	no	1	10	11
471	male	16	JIMO	no	4	12	16
477	female	20	EAST OTHANY	no	0	14	14
505	male	18	EAST RERU	no	2	11	13
537	male	21	UPPER KOMBEWA	yes	4	10	14
Sample mean	**0.75**	**18.65**		**0.45**	**3.85**	**12.85**	**16.70**
Population mean	**0.63**	**17.90**		**0.45**	**3.48**	**13.02**	**16.50**
P - value	**0.35**[1]	**0.32**		**1.00**[2]	**0.51**	**0.76**	**0.73**

B. 2 year olds

Volunteer ID	Gender	Age (months)	Village	Bed net use	# Clinical ill episodes	# Scheduled visits	Total # visits
137	female	26	SOUTH WEST SEME	no	9	14	23
285	female	26	EAST NGERE	yes	3	16	19
401	male	27	EAST OTHANY	no	2	15	17
415	male	27	EAST OTHANY	no	5	13	18
102	female	28	SOUTH CENTRAL SEME	no	3	12	15
103	female	28	EAST OTHANY	yes	5	15	20
163	female	28	WEST OTHANY	no	7	10	17
221	female	28	WEST OTHANY	no	0	14	14
411	male	29	SOUTH WEST SEME	yes	3	12	15
305	female	30	EAST RERU	no	1	14	15
314	female	31	WEST KADINGA	no	0	14	14
70	female	32	WEST NGERE	yes	3	13	16
76	male	33	SOUTH WEST SEME	yes	2	10	12
193	female	33	EAST OTHANY	no	3	10	13
306	female	33	WEST SEME	no	3	12	15
316	female	33	EAST RERU	no	3	14	17
410	female	33	WEST OTHANY	no	1	15	16
138	male	34	EAST KADINGA	no	3	11	14
196	male	35	SOUTH WEST SEME	yes	4	13	17
318	female	35	WEST SEME	no	2	11	13
Sample mean	**0.30**	**30.45**		**0.30**	**3.20**	**12.90**	**16.10**
Population mean	**0.42**	**30.11**		**0.28**	**3.51**	**13.49**	**17.00**
P - value	**0.36**[1]	**0.70**		**0.84**[2]	**0.64**	**0.28**	**0.28**

C. 3 year olds

Volunteer ID	Gender	Age (months)	Village	Bed net use	# Clinical ill episodes	# Scheduled visits	Total # visits
29	male	36	WEST SEME	yes	4	13	17
121	male	36	WEST OTHANY	no	3	17	20
448	male	36	WEST NGERE	no	2	15	17
6	male	37	EAST RERU	yes	1	13	14
170	female	39	EAST OTHANY	yes	3	17	20
474	female	40	EAST OTHANY	yes	3	11	14
412	female	41	SOUTH WEST SEME	yes	1	13	14
319	male	42	SOUTH WEST SEME	no	3	16	19
368	male	42	WEST OTHANY	no	0	15	15
383	female	42	SOUTH WEST SEME	yes	0	15	15
428	female	42	WEST OTHANY	no	5	9	14
292	male	43	EAST RERU	yes	2	11	13
9	male	44	KODERO WEST	yes	2	15	17
222	female	44	EAST OTHANY	no	2	15	17
270	male	44	WEST NGERE	no	1	16	17
303	male	44	WEST KADINGA	no	3	14	17
452	female	44	WEST SEME	yes	2	17	19
181	male	45	SOUTH WEST SEME	no	3	17	20
447	male	45	WEST SEME	no	2	13	15
131	female	46	SOUTH CENTRAL SEME	no	0	15	15
Sample mean	**0.60**	**41.60**		**0.45**	**2.20**	**14.35**	**16.55**
Population mean	**0.58**	**41.86**		**0.32**	**2.28**	**14.00**	**16.28**
P - value	**0.89**[1]	**0.76**		**0.28**[2]	**0.85**	**0.53**	**0.68**

[1] Calculated based on male being assigned dummy value “1” and female assigned value “0”

[2] Calculated based on yes being assigned dummy value “1” and no assigned value “0”

Results

A total of 851 samples were analyzed. Of those, 308 were PCR positive for malaria (Table 5). During symptomatic visits, older children had a significantly ($p = 0.02$) higher percentage of samples positive for malaria as detected by PCR. The graphical distribution of COI values (Figure 3) revealed that most samples collected during the regularly scheduled monthly visit were negative among the three age categories with a fairly normal Poisson distribution for the positive values. Among the sick children, there is an equal distribution of COI of 0-2 in the 1 and 2 year old children while the 3 year olds are predominantly parasitemic with one or more alleles.

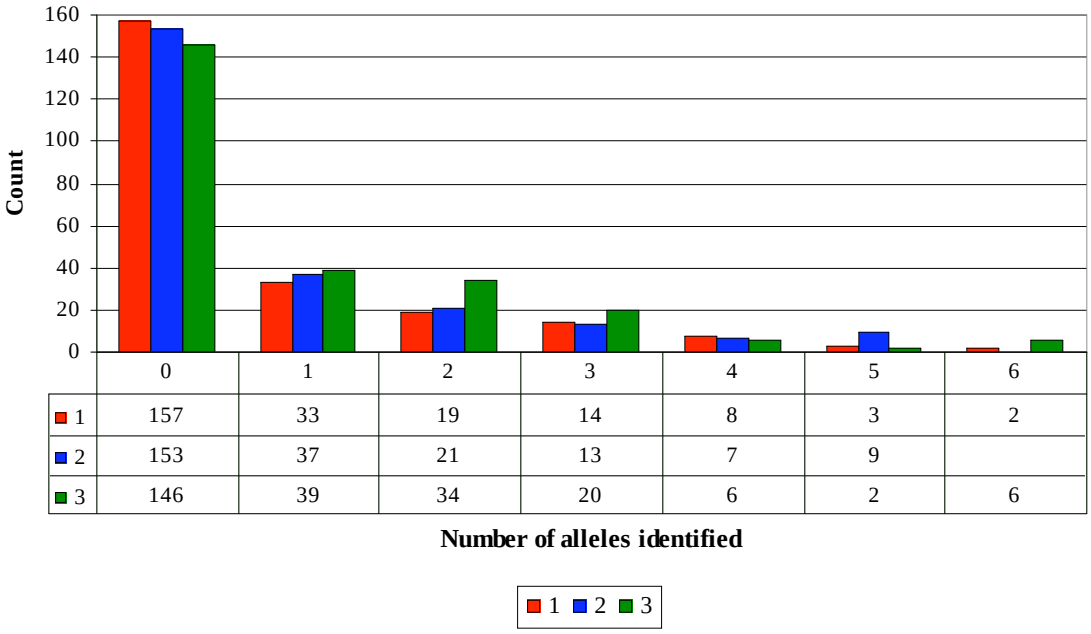
Table 5. Summary of samples analyzed for COI

		# Samples positive by PCR (% total)	
Age (years)	# Samples analyzed	Scheduled	Symptomatic
1	282	78 (33.1)	34 (73.9)
2	288	86 (36.1)	37 (74.0)
3	281	103 (41.4)	30 (93.8) *
Total	851	267 (36.9)	101 (78.9)

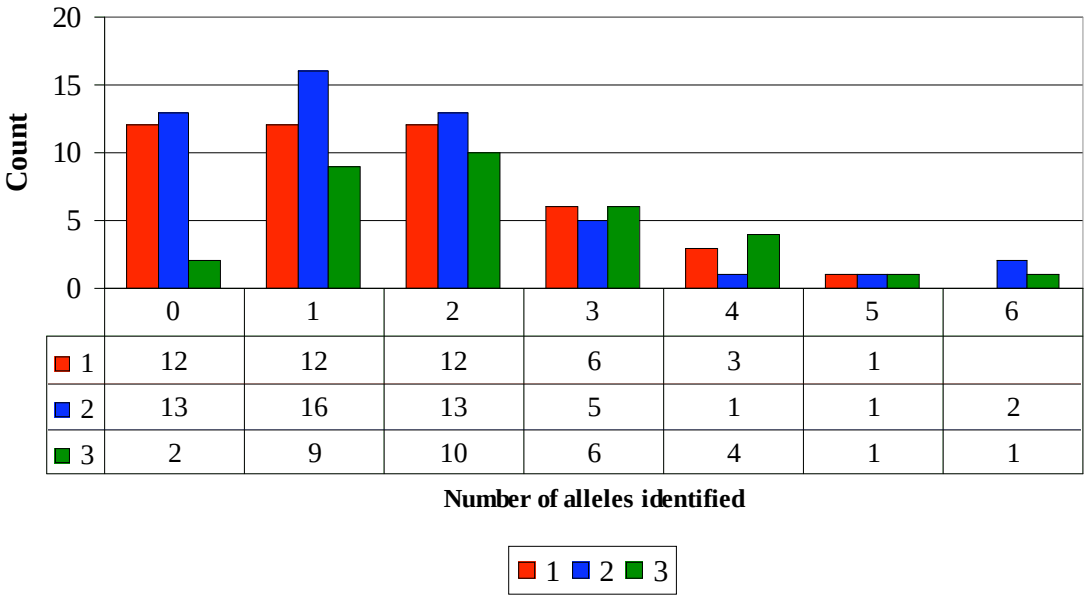
* Proportion of positive samples detected in symptomatic 3 year olds was significantly higher than that found in younger children ($p = 0.02$).

With both K1 and MAD20, distributions were calculated using clusters of 10 base pairs. When the data was initially explored, no specific pattern of clustering was apparent that would disagree with this clustering scheme. The clustering was done primarily because each potential repeat region is 9 base pairs in length. Also, the analysis equipment used to measure base pair size has an accuracy of ± 6 base pairs. Overall, there were 395 K1 alleles identified representing 126 size variations (Figure 4). When clustered, this resulted in 23 K1 repeat segments for the scheduled visits and 17 K1 repeat

segments for the samples from clinically ill children. The MAD20 allelic family, as expected, was less variable than the K1 family, with 230 alleles identified covering 57 different base pair sizes (Figure 5). For both scheduled visits and samples from the clinically ill children, MAD20 had 18 different repeat regions identified. Finally, the single RO33 allele was identified in 204 samples making it the most prevalent single allele, and the second most prevalent allelic family, in the sampled individuals.



	0	1	2	3	4	5	6
1	157	33	19	14	8	3	2
2	153	37	21	13	7	9	
3	146	39	34	20	6	2	6



	0	1	2	3	4	5	6
1	12	12	12	6	3	1	
2	13	16	13	5	1	1	2
3	2	9	10	6	4	1	1

Figure 3. Distribution of the number of identified alleles arranged by participant age in samples collected during scheduled visits (top) and clinical illness (bottom).

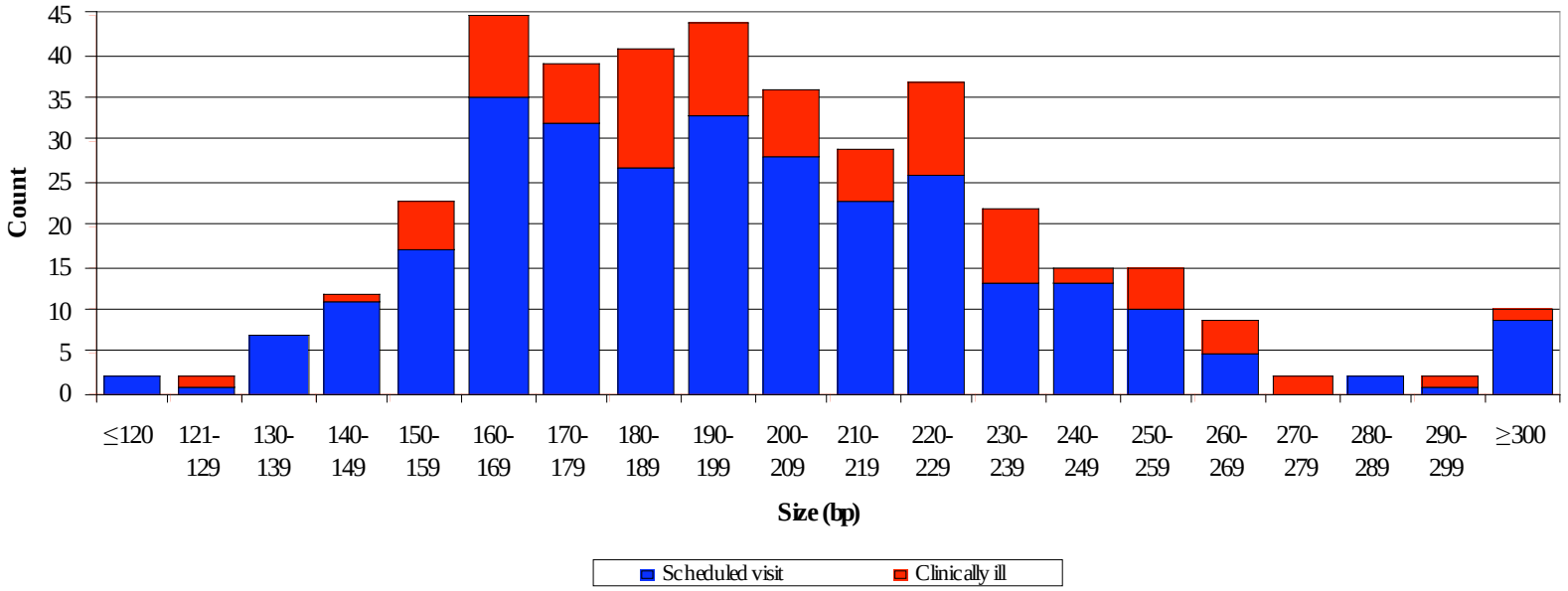


Figure 4. Distribution of K1 alleles identified during scheduled blood draws or clinical illness.

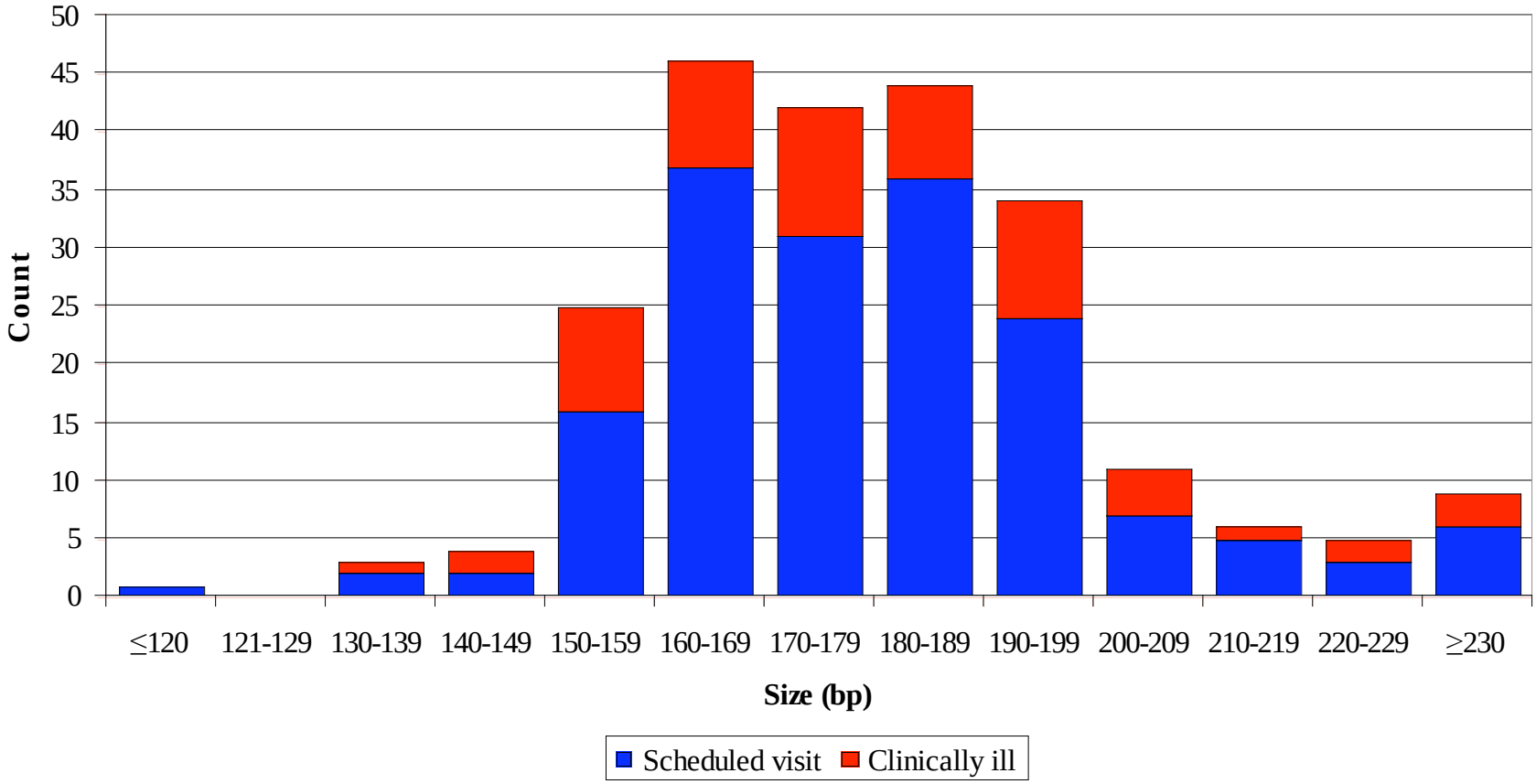


Figure 5. Distribution of MAD20 alleles identified during scheduled blood draws or clinical illness.

As a longitudinal study, all data points, including negatives, were included for analysis. The inclusion of negative samples for analysis differs in methodology from prior studies which considered only positive samples for analysis. When only positive values were used to determine the overall mean COI regardless of time, the age – complexity relationship that is normally seen was eliminated; all of the values for COI were approximately 2.33 in samples collected during a scheduled visit (Table 6). In addition, the mean COI of the asymptomatic infections was higher than that seen in children that are ill when only positive values were used. However, when negative values are included, the COI of ill children is increased over the asymptomatic values.

Table 6. Comparison of COI using only positive values and all values

	Scheduled visit		Clinically ill	
Age	Positive values	All values	Positive values	All values
1	2.32	0.73	2.12	1.54
2	2.32	0.80	1.83	1.53
3	2.33	0.94	2.48	2.41

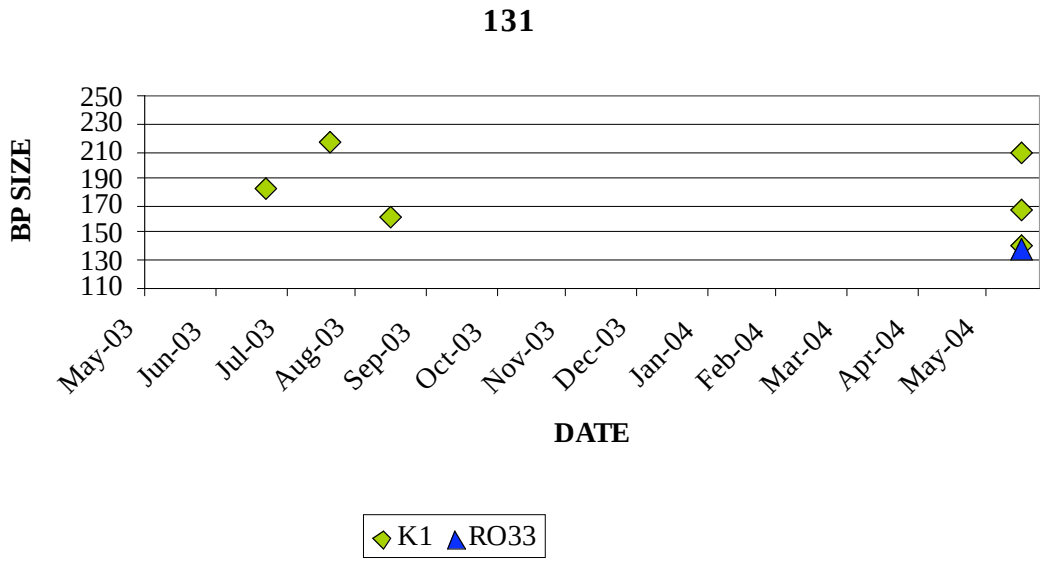
Several patterns were identified with regard to infection and illness in the children; representative samples of the patterns are depicted (Figure 6). The first pattern was those children who had no malaria infections throughout the study period (n = 3). The next pattern, shown as A in Figure 6, included children who had no episodes of clinical illness and varying levels of asymptomatic parasitemia and COI (n = 10). The next pattern, B, was the most prevalent: the children who had detectable parasitemias for most of the study, but had only 1 or 2 episodes of clinical illness over the 13 month observation period (n = 32). Pattern C were those who had equal distribution between asymptomatic infection and clinical illness episodes (n = 10). Finally, there is a small

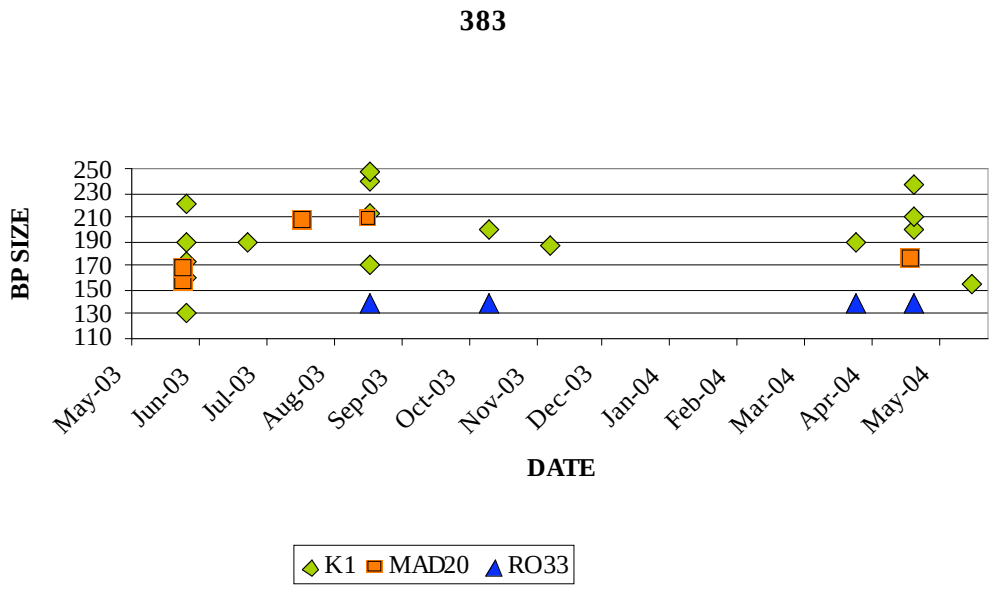
group of children, pattern D, (n = 5) who rarely had alleles present but were clinically ill whenever samples were identified as positive.

The statistical model for the MEPI study initially considered several epidemiologic factors, including village, illness status, age category, log parasite density, and use of bed net. Among those, the age category ($p < 0.001$) and log parasite density ($p < 0.0001$) were highly significant, while bed net use was barely significant ($p = 0.0499$). This indicated that the three factors were predictors of the COI; in particular, log parasite density was a far better predictor of COI than any other factor identified.

Figure 6. Representative individuals of the different identified patterns of infection and clinical illness. Identification number correlates to those used in Table 2. Clinical illness is indicated by boxed areas.

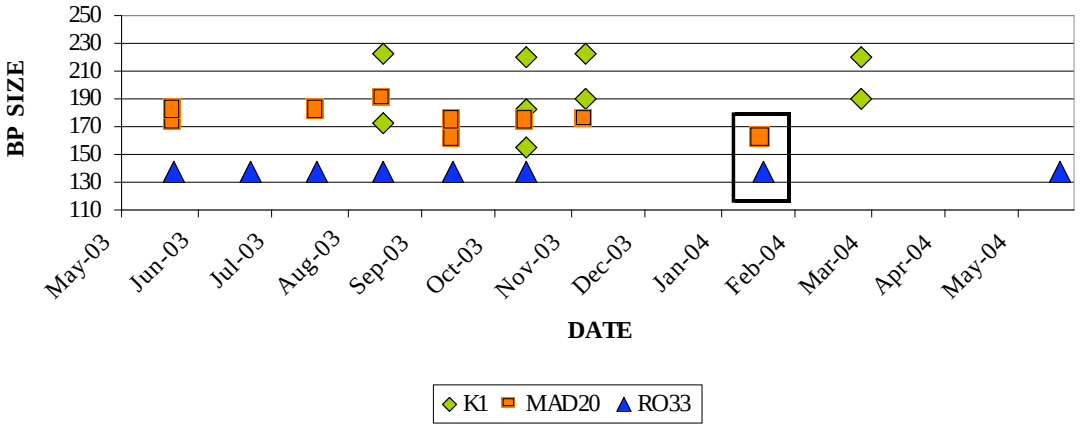
A. Absence of illness, with varying levels of infection complexity and asymptomatic parasitemia.





B. Prevalent asymptomatic parasitemia with very little clinical illness.

270



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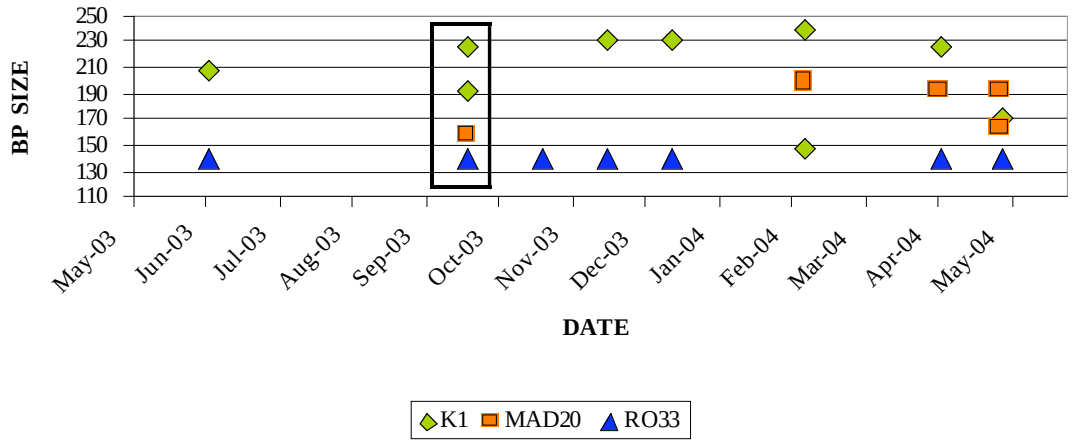
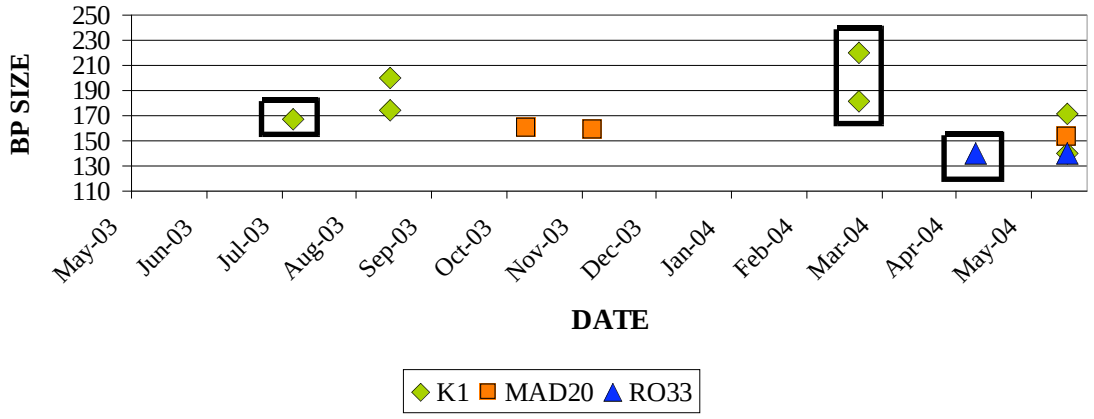


C. Equal mix of asymptomatic parasitemia and clinical illness.

102



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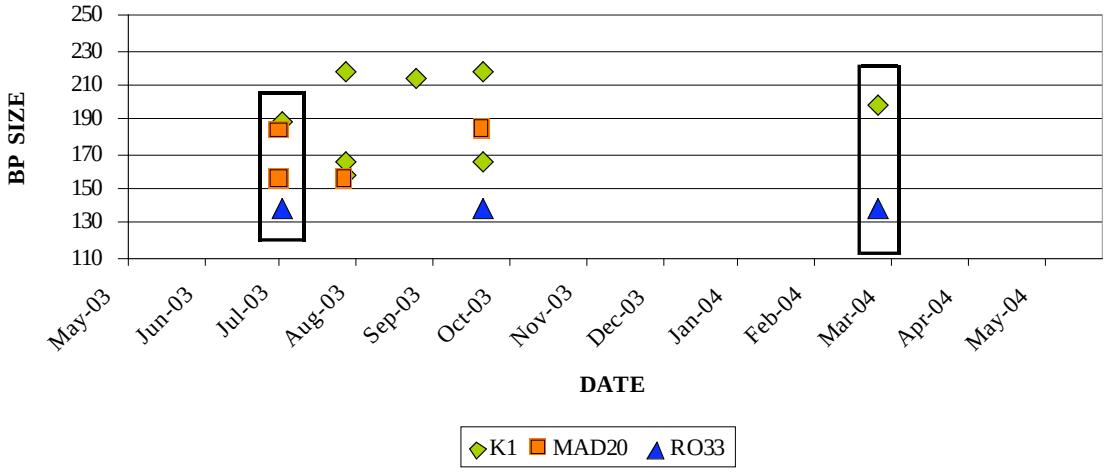
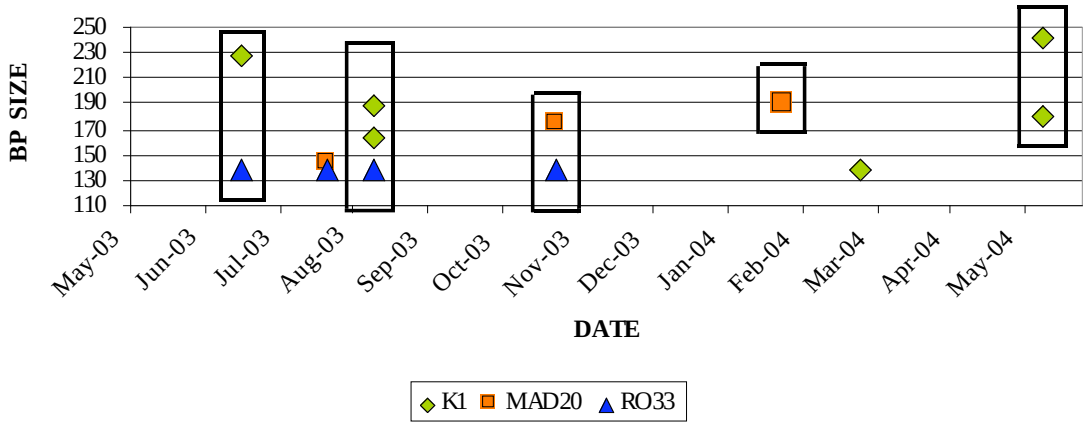


D. Majority of detected alleles were associated with clinical illness.

103



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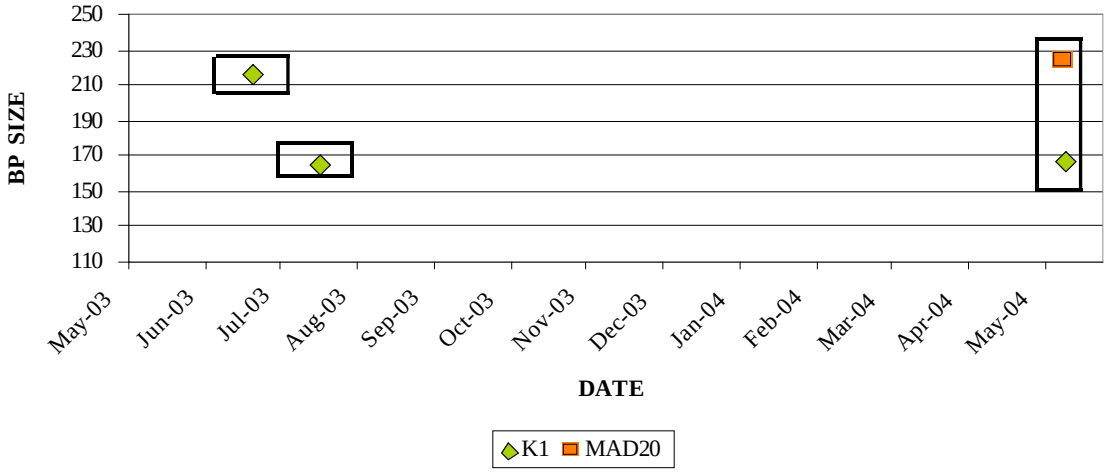


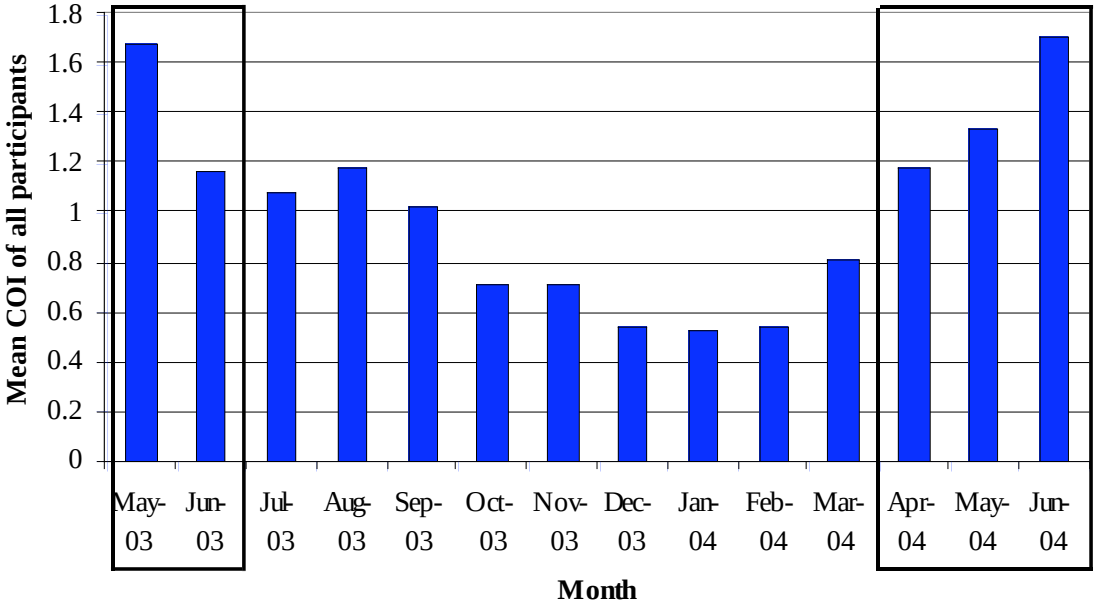


Figure 7. COI measured over study period, May 2003 – June 2004. The period following the "long rains" of March - May are associated with a period of significantly increased COI (boxed areas).

Seasonal variation. The first analysis compared COI by month and year to identify a difference that would be related to the two seasonal periods of rain. For the study period, the period of rainy weather that usually occurs in Nov-Dec, termed the "short" rains, did not occur. Therefore, only two seasons were clearly evident: the high season of transmission during the "long rains" (April – June), when COI was consistently increased, and the intervening months from July – March (Figure 7).When date was considered without regard for season there was no difference in the distribution of COI or the individual alleles. However, when season was considered as a contributing factor to the fit of the model, the COI was 1.55 times higher following the "long rains" than throughout the rest of the year ($p < 0.0001$, $z = 0.439$). While all alleles were significantly increased during this high transmission season, there was no over-representation of any single allele or genotype. Because of the strong relationship

between season and COI in this study, further analyses for other variables controlled for the seasonal effect.

Age. The effect of age on COI in children and adolescents has been well-described in other geographical areas as directly increasing with age. In this analysis, age was explored in two separate ways: (1) the age category of the child at the time of study enrollment, and (2) the age of the child at the time the sample was taken.
When the categorical age was used, the COI was found to be significantly related (p = 0.0059) and increased with the older children. The analysis was then repeated using the specific age of the children in months at the time a sample was drawn. For every additional month of age, there was a 2.5% increase in COI (p = 0.0008). For the individual alleles, there was no difference identified with K1, however for the MAD20 and RO33 alleles revealed a 3% increase (p = 0.0066) and 5% increase (p = 0.0003), respectively was noted as the age increased each month.

Illness. When illness was examined, the model excluded log parasite density because it was a consistently better predictor of COI than illness and confounded the data. Children who were ill had a COI 2.3 times higher ($p < 0.0001$) than children who were asymptomatic and donating their monthly scheduled sample. When the ill children were compared to each other by age category, the 1 and 2 year old children had a significantly reduced COI (p = 0.036) compared to 3 year olds.

Each allele was examined to determine if there was a relationship with illness. It was found that the presence of the RO33 allele produced a slight decrease in the rate of illness (RR = 0.98). If RO33 was not present, there was a 1.6 fold increase in illness for

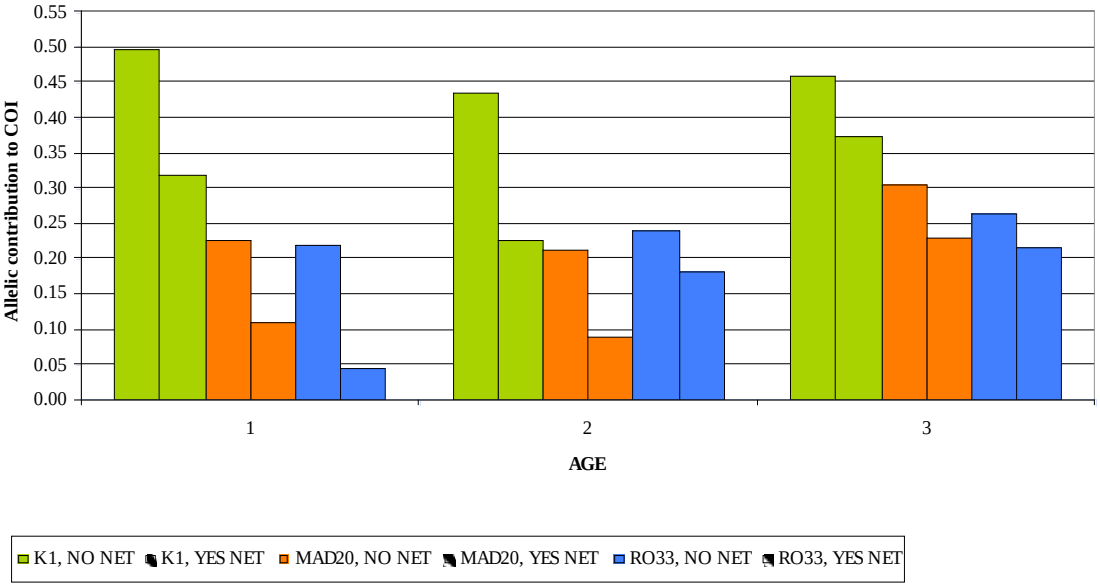
each additional K1 allele. For MAD20, each additional allele in the absence of RO33, resulted in a 2.8 fold increased rate of illness.

Village. The effect of village location on COI was explored using effect coding, a method to compare each village to the average value for all villages. By doing so, those villages that were above or below the overall mean were apparent. These analyses also took into consideration season and the age of the child at the time of sample, but because the sampling from each village was small, the interpretation of findings has been limited. East Reru (n children = 7), Jimo (n = 2) and Kodero West (n = 1) were consistently high. Because only three children accounted for the elevation seen in two of the villages, the differences are most likely related to differences within the individual children related more to their immune status than to location or other controlled factors. South Central Seme (n = 4) and East Othany (n = 9) had lower COI values throughout the study period. When individual alleles were examined, the same profiles were seen with two exceptions: West Kadinga (n = 3) had much fewer K1 alleles that trended toward significance (p = 0.06) and South West Seme (n = 11) had fewer MAD20 alleles.

Bed Net Use. The study design did not allow for confirmation of bed net use by visiting the homes, therefore the values relied on reported bed net usage by mothers. Since only 40% of study participants had reported bet net use, there appeared to be no strong societal pressure that may cause an individual to misrepresent use of the bed nets; therefore the results are considered valid.

Samples from 1 and 2 year old asymptomatic children with bed net use had a reduced COI compared to those that did not use a net. Among the clinically ill children, the 3 year olds had a significant reduction in COI while the 1 and 2 year olds did not.

When the alleles were examined individually, no differences were identified in the K1 alleles between children with no reported bed net use and those with bed net use in either asymptomatic or clinically ill children. Fewer MAD20 alleles were identified among the 2 year old asymptomatic bed net users ($p = 0.028$) while the clinically ill 3 year olds also had fewer alleles ($p = 0.014$). The finding in 3 year olds was also found with the RO33 allele; among those that were clinically ill, those that used a bed net were less likely to have the RO33 allele ($p = 0.086$). For children who lacked RO33 and did not report bed net use, there was a 4.3 fold increased risk of clinical illness ($p < 0.0001$).In the asymptomatic children, the RO33 allele was reduced by 50% in the 1 year olds who used a bed net ($p = 0.002$).



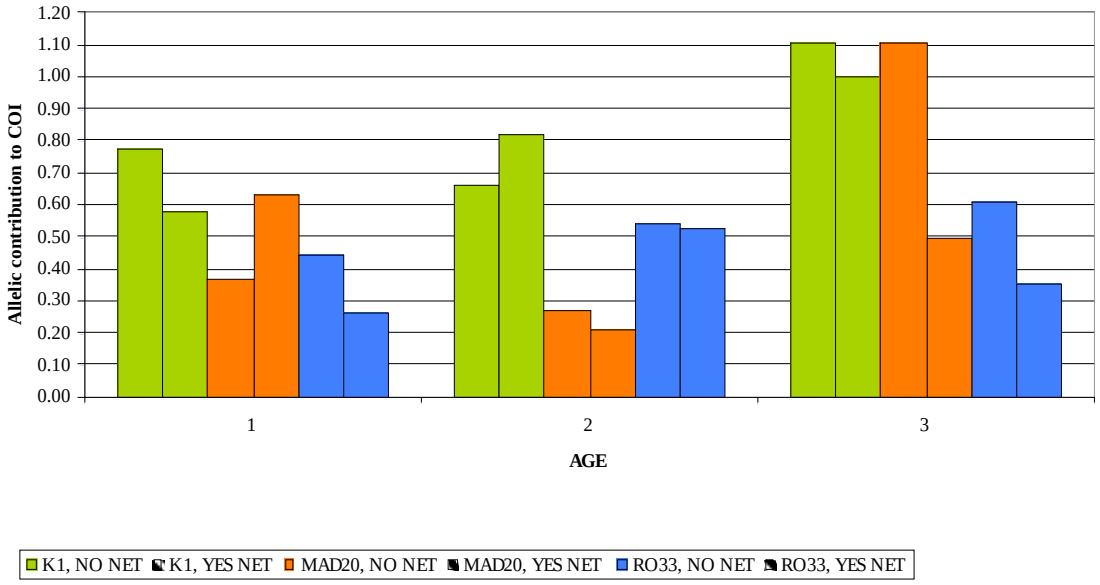


Figure 8. Distribution of alleles in samples from asymptomatic (top) and ill (participants categorized by bed net use and age.

Results: Parasite density study

A total of 115 samples were included in the study that matched an ill case to an asymptomatic carrier with equivalent parasite density on the basis of age, date of sample and village. Some of the villages were too small to provide a 1:1 match for cases. In those instances, there were multiple ill cases matched to a single control. This was particularly evident in the lowest parasite density category (<500 parasites/μl) in which there were 32 ill cases matched to 20 asymptomatic controls. For the medium parasite density category (5,000 – 6,000 parasites/μl), there were 26 ill cases and 24 controls. The high parasite density category (.40,000 parasites/μl) was comprised of 6 ill cases matched to 7 asymptomatic controls.

The data was explored using two different methods: first, the three parasite density categories were used to analyze COI using non-parametric methods. After finding significance with those methods, dummy variables were used to replace the categorical parasite density ranking to analyze the data using more stringent parametric methods.

Using the non-parametric Kruskal-Wallis test, a significant association was found between parasite density and COI ($p < 0.001$). This was followed by the Dunn post-hoc procedure to determine the direction of the association. It revealed that the difference between the medium density and low density and the difference between the high density and low density were significant.

The transformation of parasite density categories into dummy variables allowed analysis using linear regression. Parasite density explained nearly 14% of the variation seen in COI ($R^2 = 0.136$). While highly significant ($p < 0.0005$), the model's fit increased

even further when illness status was considered. Among the clinically ill children, parasite density accounted for 23.4% of the variation ($R^2 = 0.234$, $p < 0.0005$) while in asymptomatic children the variation explained by parasite density decreased to 19.5% ($R^2 = 0.195$, $p < 0.005$). In all of these analyses, age did not have a significant impact on the relationship between parasite density and COI.

COI in clinical vs. asymptomatic malaria. Among the children that were not clinically ill, those with a medium parasite density had a significantly higher COI than those children with a low parasite density ($R^2 = 0.195$, $p = 0.001$). Those children with a high parasite density had a decreased COI compared to the medium parasite density group from 3.29 to 2.57. The difference between those children with a high parasite density was not significantly different from those with a low parasite density ($p = 0.206$) (Figure 9).

In contrast to the findings from the children that were asymptomatic, among those that were clinically ill, there was a positive direct relationship between parasite density and COI where COI was greatest in those with the highest parasite density and smallest in those with low parasite density. This difference was significant between the children with high parasite density compared to those with low parasite density ($p < 0.001$) but was not significant among the children with medium parasite density and low parasite density or from high parasite density and medium parasite density.

In children with clinical malaria, there was a decrease in the contribution of RO33 to the total COI as the parasite density increased. Likewise, there was an increase in the contribution of K1 to the total COI as the parasite density increased. The differences in

contribution between RO33 and K1 was nearly equal; thus as K1 increased, there was a subsequent equal decrease in the RO33 allele (Table 7).

Table 7. Percent contribution of each allele to parasite density.

Illness Status	Parasite Density	K1 (%)	MAD20 (%)	RO33 (%)
Not Ill	Low	63.6	21.2	15.2
	Medium	54.4	26.8	19.1
	High	66.5	22.2	11.3
Ill	Low	41.8	27.3	30.4
	Medium	59.7	24.4	16.0
	High	64.2	25.1	10.7

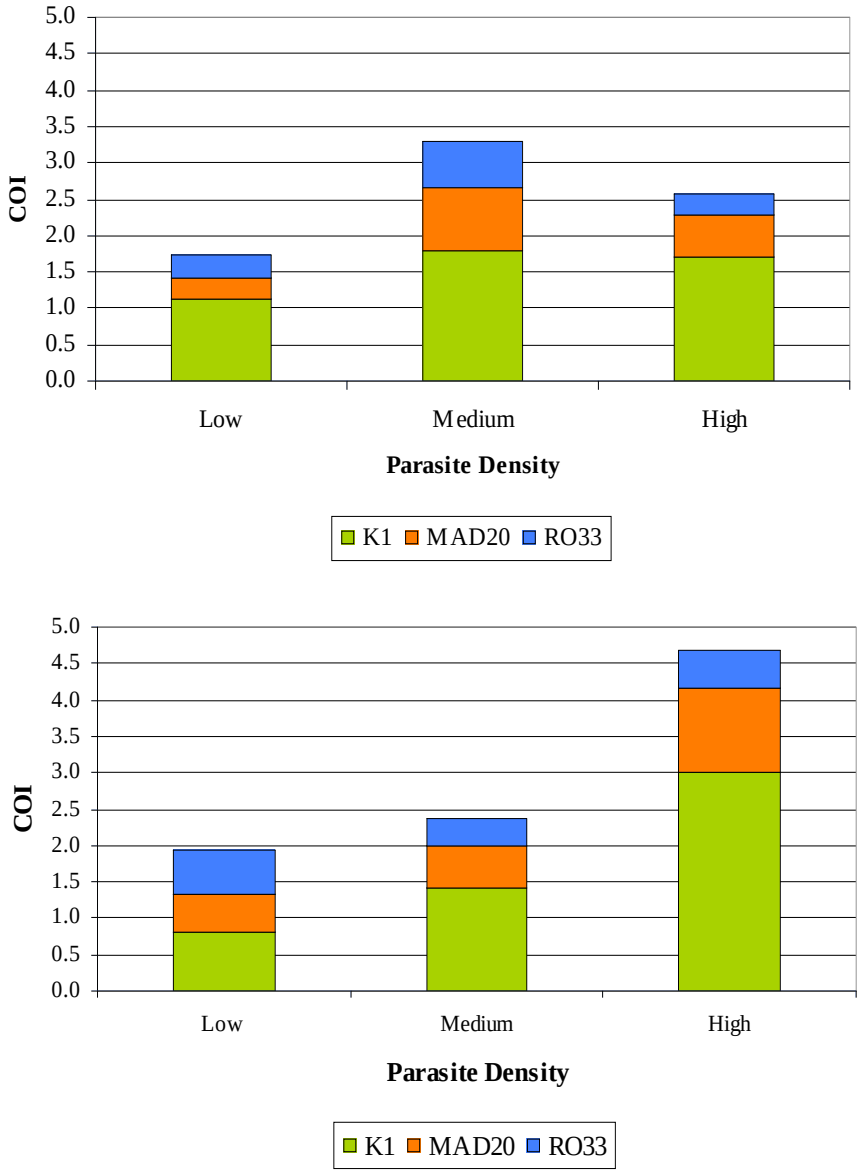


Figure 9. Relationship between parasite density category and COI among asymptomatic (top) and clinically ill children (bottom).

Discussion

The results from this longitudinal study are an in-depth look at the dynamics of malaria infections over time in young Kenyan children. It provided the opportunity to explore COI as a function of age, parasitemia and epidemiologic factors, as well as examine the gradual development of immune response.

The first aim of the study was to determine COI and identify any allelic patterns that may result over time within the children. In agreement with previous work in Africa, the MEPI data revealed that the K1 allele was the most frequently identified, followed by RO33 and finally MAD20 which is less variable and identified least [19, 57, 58]. This occurs despite differing endemicities, and reflects a pattern seen throughout the continent [17, 29, 59].

The calculated COI as well as the variation seen within the individual children was quite different than what has been previously reported. For example, Magesa et al. [60] analyzed blood samples taken daily for a month from 34 children 1-5 years old living in a holoendemic area of Tanzania. They found that the COI for asymptomatic children averaged 4.9 genotypes/isolate; in symptomatic children it was 2.7 genotypes/isolate. The mean minimum number of genotypes for the asymptomatic children was 10, demonstrating that there was rapid turnover of alleles in the short, one-month period of study. Over the 31 day period, they identified the RO33 allele in 339 samples, as well as 931 K1 alleles and 541 MAD20 alleles. In contrast, the data reported here from western Kenya included nearly twice the number of children, measured for 13 months, but had half as many MAD20 and RO33 alleles and one-third fewer K1 alleles. This influenced the overall COI as well which was much lower in this study than was

calculated in the study in Tanzania. While the low COI was not fully expected, a recent report from Asembo Bay in western Kenya also found a lower COI than expected [29]. Another report from a holoendemic area of Gabon [61] had a COI of 1.73 among children, while only 30% of the samples taken were PCR positive for malaria. This variability, even among areas that are all described as being holoendemic and would be categorized as having equal transmission characteristics, hints at the limitations of applying COI findings among other geographic regions. The findings discussed here are generalizable to the holoendemic areas of western Kenya, but reflect environmental, behavioral and perhaps genetic factors that would limit their applicability in other situations.

The improvements to the methodology used to measure COI are another explanation for the lower COI seen here. The data for the polymorphic K1 and MAD20 allele families was generated using the more accurate microfluidics electrophoresis system and did not rely on the interpretation of electrophoretic gels. This prevented the possibility of misinterpreting spurious bands as true allele types and allowed for the easy discrimination of amplicons that differed by only nine base pairs in size. The instrument specifications allow for detection of DNA fragments that are in greater quantity than 2.5 times the background fluorescence reading. The background fluorescence on the assay used here was 0.8 – 1.2 units. The baseline for positive readings would therefore be 3 fluorescence units. However, for interpretation of the results here, a cutoff of 15 fluorescence units was used to provide a more stringent interpretation of COI.

The relationship between age and COI has been well-described in the literature; the COI typically increases with increased age [19, 21]. MEPI afforded the opportunity to

explore this relationship on a much smaller scale within a very tight age range. The results revealed that for every additional month of age, there was an increase in COI that translated into a 30% increase in COI for every additional year of age among study participants.

One aspect of MEPI findings differed from the published literature. Bed net use had previously not been found to affect COI [31, 32, 33]. The most recent study by Bolad et al. examined children 3-6 years old in a holoendemic area of Burkina Faso. In our results a reduction in COI was only seen among asymptomatic 1 and 2 year old children and the 3 year old clinically ill children, therefore the disparity between our results and those of Bolad et al. were not surprising. In fact, all previous work on this topic focused either on older populations or broad age categories in which the number of participants of age 1 or 2 was limited.

The data revealed that by age 3, the use of a bed net did not impact COI in asymptomatic infections. Beyond the explanations related to development of immune regulation to parasites, there is a behavioral explanation that also should be considered. The older children may be more active than the younger children during the peak hours when mosquitoes feed. If the 3 year olds are not inside their bed net during those hours, they would be expected to receive the same number of mosquito bites as those children who did not have bed nets. Similarly, by three years of age, if the bed nets are not maintained adequately or replaced, the efficacy of the device may be compromised by holes and tears which would increase the exposure to mosquitoes.

Unlike in most previous work, the COI measured during illness was increased over that seen during asymptomatic parasitemia. This can be explained first by the nature

of the longitudinal analyses. Because all values were considered, not just positive ones, there would be a much lower average COI among those who were asymptomatic because the value includes those who also had no detectable parasitemia at that time point. Children with the symptoms of clinical illness are more likely to have a readily measurable COI. Therefore, when negative values are excluded, those values would most likely be found in children who are asymptomatic. The resulting COI would then be higher, making it appear that the COI in ill children was lower.

A second explanation can be found with the data from the case-control study. As parasite density increased among the clinically ill children, RO33 contributed significantly less to the overall composition of COI while there was an equal increase in K1 alleles. Since K1 has the most variability of the identified alleles, there is a potential for an increase in K1 to result in an overall increase in COI. Another explanation for our result is related to the limitations of the assay. Because these samples were collected as filter paper blood dots, the total amount of blood was low. More strains may be detected in samples with higher parasite density because the strains would be above the detectable limits of the test; therefore, those samples from asymptomatic children with low parasite densities would be an underestimate of the true COI.

Two different trends have been previously noted with parasite density. One finding was that COI decreased with increased parasite density [20] in individuals who were clinically ill. That study was conducted in a hypoendemic region of Senegal. In MEPI, the exact opposite was found. Our results tend to agree with a report from a holoendemic region of Tanzania in which there was a direct relationship between COI and parasite density [15].

Changes to individual alleles were explored in addition to COI to determine if any differences could be ascribed to a single allele. While no single clonal variant of either K1 or MAD20 was found to influence COI or have a relationship to age, illness, bed net use or village (data not shown), a few patterns did emerge with the allelic families. It was determined that the less variable alleles, in this case MAD20 and RO33, were more likely to be affected by outside factors as well as the immune system controls. For instance, MAD20 and RO33 were both significantly reduced by bed net use, while there was no effect on K1. During clinical illness, as parasite density increased, the prevalence of MAD20 remained the same, RO33 decreased and K1 increased. RO33 appeared to have a greater effect on COI than either K1 or MAD20. For every additional month of age, there was a 5% increase in RO33, the greatest gain among the three allelic families. As has also been reported previously [24, 29, 61], in this study RO33 was found to be mildly protective against illness and the absence of it was more likely to result in illness among children with K1 or MAD20. Children with reported bed net use and detectable RO33 allele were significantly protected from illness.

The reasons why the MAD20 and RO33 alleles appear to be affected by variables such as bed net use while the K1 allele is not are speculative. Since K1 is the most variable of the alleles in Kenya, the lack of notable changes in COI hints at difficulties with immune regulation of this allelic family. This idea underlies the theory of direct diversity, the idea that more complex infections have more antigens and are therefore harder to control [51]. In support of the model of direct diversity is data from various studies of antibody response to malaria that concluded that the host humoral responses which reduces parasite density below detectable levels is specific for a single allele type

and not related to clearance of other clonal types [62, 63, 64]. This supports the hypothesis that partial immunity to falciparum malaria is predominantly species and genotype specific and builds over time based on the number of exposures an individual receives. Several studies have reported that an increase in the number of *msp1* block 2 antigens recognized by an individual was associated with a reduced risk for clinical malaria episodes and the acquisition of partial immunity [65, 66, 67].

In addition to the theory of direct diversity, two other theories have been proposed for the relationship between COI and immunity. The first of these, premunition, is the idea that chronic multiclonal infections provide protection against superinfection [28]. Ideally an individual must be able to mount an immune response which controls parasite densities sufficiently to prevent clinical attacks but also allow the original infection to remain at low density. One argument for the existence of premunition was found with the children that remained asymptomatic throughout the 13-month study while carrying a varying number of alleles. In contrast, other children had an episode of clinical illness nearly every time they were detected with parasites. According to the theory of premunition, the lack of controlled asymptomatic parasitemia may have made them susceptible to illness. To apply the theory of premunition, it must be argued that protection cannot be highly specific for any one individual parasite strain. Because of the high level of panmixia in the field, strains that are identical for all polymorphic antigens would be exceptionally rare. If protection were against only homologous infections, it would be very limited. It is more likely that protective immune responses are directed against several polymorphic antigens with some degree of heterologous cross-protection to prevent clinical illness.

A final theory of the connections between COI and immunity is that of immunological antagonism, commonly thought of as a “smoke screen” defense. In this model, concurrently infecting parasite strains facilitate each others’ survival by interfering with the primary T cell response. By misdirecting the immune response, the parasite is able to effectively perpetuate a population of naïve hosts it can repeatedly infect [68]. One example of the immune evasion mechanism is altered peptide ligand antagonism [69] in which the concurrent presentation of closely related antigens prevents effector functions such as cytotoxicity. The smoke screen theory may be typified by those children who carried alleles throughout most of the study and got several clinical illnesses. Because of the sample interval, in most instances it was not possible to determine if infection was the result of a newly introduced allele or one that was able to overrun the immune system controls, but either way the pattern is indicative of a continuously susceptible host.

Because none of the three theories of direct diversity, premunition or smoke screen defense are fully applicable to the multitude of patterns of infection seen with the MEPI participants, a separate, simpler explanation is forwarded. That is the idea of micro-heterogeneity. Heterogeneity on a small scale may result from a number of factors from genetic differences to behavioral, epidemiologic and geographic factors [70] that translates to a small group of children, even within the same village, being at a much greater or reduced risk for exposure and subsequent clinical illness.

Clonal variants were found to appear and disappear over time in the absence of antimalarial treatment, which also influenced the different patterns of infection that were identified among the children. These occurred in all three age categories without any

discernable relationship to gender, bed net use or village location. The patterns may be a reflection of a demographic characteristic that was not measured. This may be a reflection of low parasitemia or sequestration [40] especially considering the small volume of blood analyzed. However, it is equally likely that the immune system is ultimately able to clear the infection or reduce it below the detectable limit of the assay, particularly because the clonal variants tended not to reappear in later samples.

While the MEPI study was able to answer some questions regarding the dynamics of COI in children in Kisumu, it still leaves many questions unanswered. What causes some infections to progress to clinical illness when others are carried asymptomatically? Since neither the age of the child nor COI nor specific alleles seem to influence this progression, what are the critical factors of parasite or host that result in illness versus asymptomatic parasitemia?

In conclusion, the MEPI epidemiologic cohort provided more detailed information about the COI of infections in children 1-4 years old, the age in which rapid acquisition of clinical immunity is occurring. COI was an adequate marker to measure the development of immunity. Using it, it was found that age, parasite density, bed nets and the village of residence all influenced COI, but that no overall discernable pattern of developing immunity generalizable to all patients was identified.

CHAPTER FOUR: MAL031

Introduction

FMP1, or Falciparum Merozoite Protein-1, is a leading vaccine antigen against erythrocytic malaria parasites [4]. It is an antigenic formulation of the p42 fragment of the gene expressed in *Escherichia coli* in combination with GlaxoSmithKline Biologicals' proprietary adjuvant AS02A. The dimorphic, semi-conserved p42 region of the gene was known to induce antibodies in animal trials [71] and small scale human trials [72] prior to being used in field trials in Kenya.

The p19, which is the only remaining fragment of the MSP1 protein upon merozoite invasion of the red blood cell, results from the proteolytic processing of the 42 kDa C-terminus into two fragments of 33 and 19 kDa respectively (Figure 1). The gene encoding the 19 kDa region is highly conserved, with only four single nucleotide polymorphisms (SNPs). The strain used for the vaccine construction, 3D7, is differentiated from the other predominant strain type, FVO, by identifying the SNPs within the p19 region.

A phase 1 field trial, designated MAL031, has been conducted in Kombewa Province near Kisumu, Kenya. It was a randomized, double-blind, dose-escalation trial of FMP1 in adjuvant AS02A versus Imovax® rabies vaccine as a comparator control in 135 children. The children were divided so that there were 45 in each of three age cohorts (1 = 12 to 23 months, 2 = 24 to 35 months, and 3 = 36 to 47 months). Within the cohorts, subjects were randomized so that 30 received the FMP1 in one of three doses (Cohort A, 10 µg; Cohort B, 25 µg; Cohort C, 50 µg) and 15 received Imovax® (Figure 10). The

start date for each cohort was staggered by 14 days. The study was conducted from August – November, 2003, with participants receiving vaccines at day 0, day 30, and day 60. Samples analyzed were from day 0 and day 90.

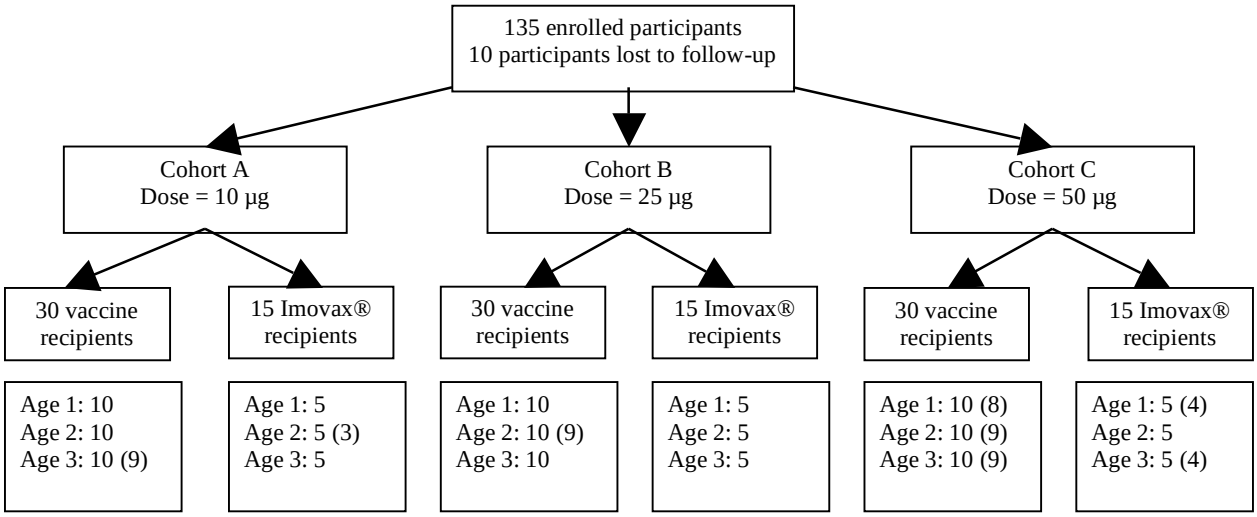


Figure 10. Design of MAL031 study. Numbers in parentheses indicate the final number of participants included in analysis.

An additional assay was used in MAL031 to explore any effects that the vaccine may have on the dynamics of drug resistant *P. falciparum* strains. In Africa, Malawi successfully eliminated chloroquine as a treatment regimen in 1993 and replaced it with alternative chemotherapeutic regimens [73]. Kublin et al. found that the prevalence of resistance dropped from 85% in 1992 to 13% in 2000 following the 1993 replacement of the drug with alternatives [74].

Results

To increase the statistical power of the comparisons, an analysis was done to determine if the Imovax® comparator control recipients from all three arms of the study could be combined into a single group. The analysis focused on the comparability of the COI in the controls at day 0 and day 90. The controls from the three cohorts were found

to be comparable with respect to their COI at both day 0 ($p = 0.42$) and day 90($p = 0.58$). There was also no difference related to the different ages of the children within the comparator controls. Therefore, the Imovax® recipients from the three cohorts were combined into one group ($n = 41$), termed comparator controls (CC). Next, the effect of age was explored across the three cohorts and CC; it was found to not be significant within any of the groups. Finally, the COI at the different dates for day 0 and day 90 were explored to see if there was a difference in the distribution with time. Again, the results were not significant and controlling for this factor was not necessary.

Table 8. Summary of COI and block 2 allele values for each cohort divided by age group.

A. Cohort A

Day 0 Vaccine					**Day 90 Vaccine**				
	# Alleles					# Alleles			
Age Group	K1	MAD20	RO33	COI	Age Group	K1	MAD20	RO33	COI
1	4	4	5	1.30	1	2	1	3	0.60
2	6	3	6	1.50	2	4	1	1	0.60
3	5	4	4	1.30	3	5	0	4	1.00
Day 0 Control					**Day 90 Control**				
	# Alleles					# Alleles			
Age Group	K1	MAD20	RO33	COI	Age Group	K1	MAD20	RO33	COI
1	11	0	1	2.40	1	5	0	2	1.40
2	7	3	3	4.33	2	1	0	0	0.33
3	5	1	3	1.80	3	0	1	1	0.40

B. Cohort B

Day 0 Vaccine					**Day 90 Vaccine**				
	# Alleles					# Alleles			
Age Group	K1	MAD20	RO33	COI	Age Group	K1	MAD20	RO33	COI
1	4	1	3	0.80	1	5	2	1	0.89
2	4	4	6	1.40	2	2	3	3	0.80
3	9	5	6	2.00	3	7	2	1	1.00
Day 0 Control					**Day 90 Control**				
	# Alleles					# Alleles			
Age Group	K1	MAD20	RO33	COI	Age Group	K1	MAD20	RO33	COI
1	6	4	3	2.60	1	1	2	3	1.20
2	2	0	1	0.60	2	0	2	0	0.40
3	3	3	4	2.00	3	2	1	2	1.00

C. Cohort C

Day 0 Vaccine					**Day 90 Vaccine**				
	# Alleles					# Alleles			
Age Group	K1	MAD20	RO33	COI	Age Group	K1	MAD20	RO33	COI
1	3	1	0	0.50	1	2	1	7	1.25
2	3	4	2	1.00	2	10	3	8	2.33
3	10	8	6	2.67	3	3	2	9	1.56
Day 0 Control					**Day 90 Control**				
	# Alleles					# Alleles			
Age Group	K1	MAD20	RO33	COI	Age Group	K1	MAD20	RO33	COI
1	0	2	1	0.75	1	0	0	4	1.00
2	1	1	2	0.80	2	0	0	5	1.00
3	5	3	1	2.25	3	0	2	4	1.50

Vaccine effect. The effect of the vaccine on the distribution and abundance of block 2 alleles was explored by comparing the COI at day 90 with the baseline day 0 measurements. First, the total number of positive samples was examined (Table 9). There was a clear pattern with cohort C having all samples positive at day 90 while the other two cohorts had fewer positive samples at day 90 than day 0. Cohort A and B were not statistically significant from the control; but cohort C, which received the full vaccine dose, had a COI 1.9 fold higher at day 90 than day 0.

Table 9. Summary of samples analyzed for MAL031

		Day 0	Day 90
	Total participants	# samples positive (%)	
Cohort A	29	20 (68.9)	12 (41.3)
Cohort B	29	16 (55.2)	11 (37.9)
Cohort C	26	13 (50.0)	26 (100.0)
CC	41	24 (58.5)	24 (58.5)

The individual allelic families were explored to determine which were most associated with this significant increase (Figure 11). Values for the MAD20 allele were found to be equal at day 0 and day 90. The proportion of K1 alleles was increased at day 90, and while there was not a significant difference between the vaccine cohorts and CC there was a trend ($p = .083$). There was a clear pattern of increased K1 alleles with increased vaccine dose: A increased 1.82-fold, B increased 2.29-fold and C increased 2.73-fold.

The RO33 allele was found to contribute most to the identified difference between COI at day 0 and day 90. In particular, there was no difference among the vaccine recipients or controls at day 0; by day 90 there was a significant reduction in the RO33 allele in Cohort A by 67% ($p = 0.041$) and in Cohort B by 82% ($p = 0.0042$), but a 145% increase in the RO33 allele in C ($p < 0.0001$).

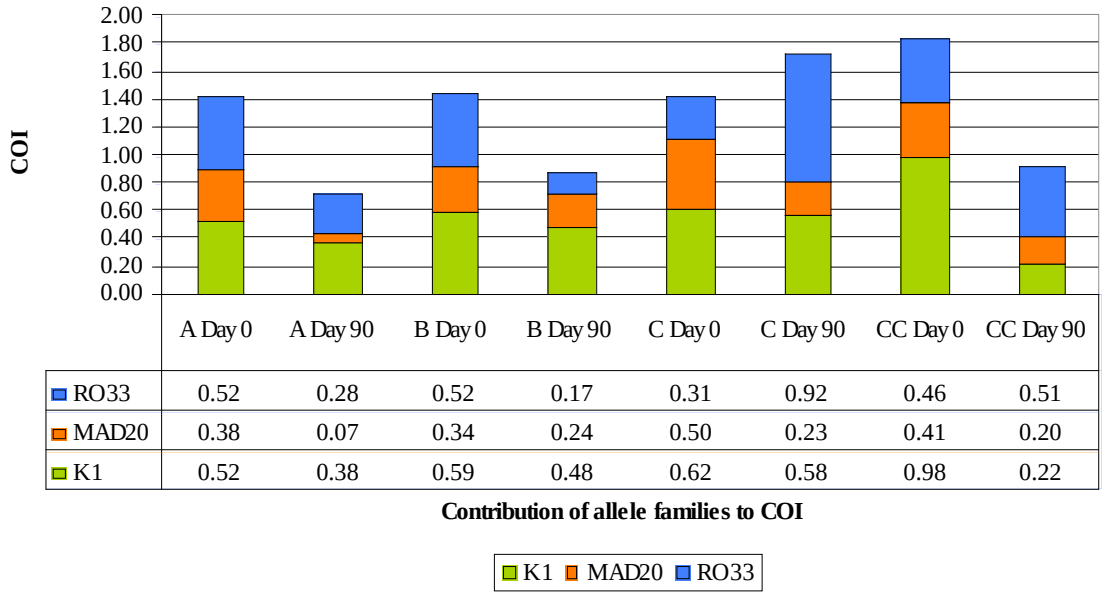


Figure 11. Contribution of block 2 alleles to COI at day 0 and day 90 for cohorts A, B, C and CC.

PfCRT The analysis focused on the distribution of the chloroquine sensitive allele (CS) and the chloroquine resistant allele (CR). The PCR was performed on the total DNA extract so the samples had the potential to have multiple parasite populations. Therefore, an individual sample may contain CS, CR, both or neither. Discordant samples, those in which a COI was measured but CS or CR was negative, accounted for 7% of the day 0 samples and 6% of the day 90 samples.

CS and CR were first explored to determine if a baseline day 0 correction was necessary for any cohort that may have a distribution of the alleles that differed significantly from the others. Cohort A was found to have significantly fewer CS than the other cohorts or CC and was controlled for in the analysis. There was no difference in distribution of CS by age, but for CR age was a significant factor ($p = 0.032$) and was controlled for in the model.

On day 90, there was a large difference between the distribution of CS among the cohorts in comparison to CC ($p < 0.0001$): cohort A had 43% fewer CS alleles than CC, cohort B had 92% fewer, while cohort C had a 2 fold increase in CS alleles over CC. CR had a similar pattern of distribution that did not differ among the cohorts and CC but did differ within the cohorts. Cohort A had 15% fewer CR alleles compared to CC, cohort B had 52% fewer ($p = 0.024$) and cohort C again had nearly a 2 fold increase ($p = 0.0049$) compared to CC.

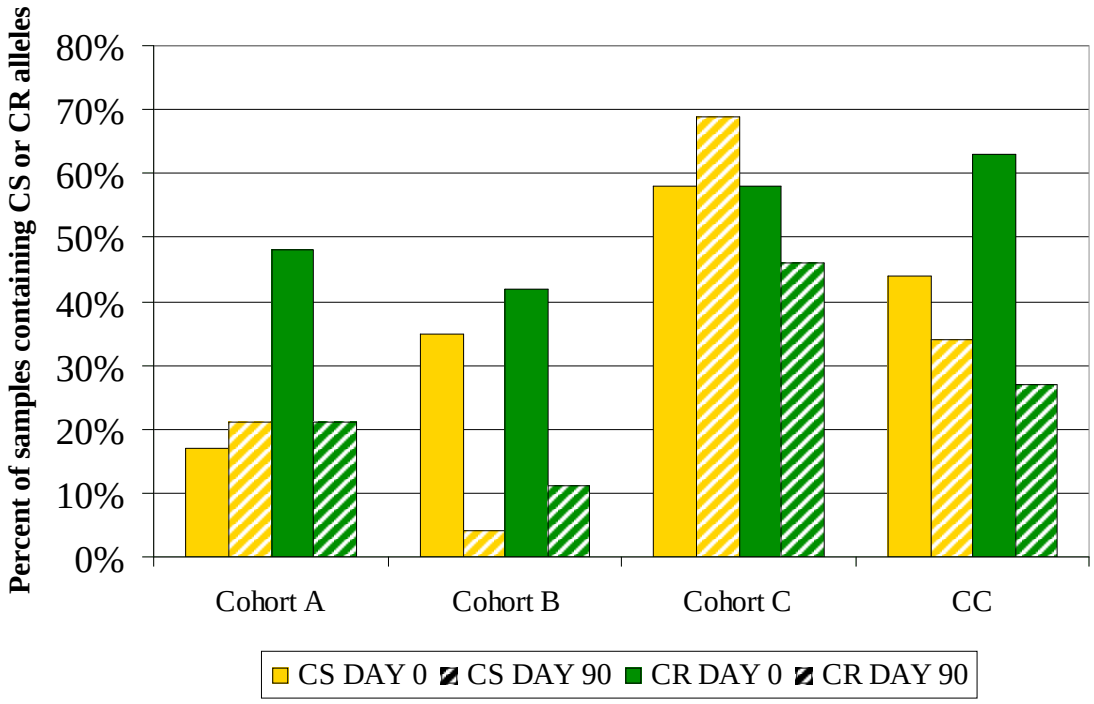


Figure 12. Average value of CS and CR alleles at day 0 and day 90 for three cohorts and CC.

Discussion

As with the MEPI study, all COI values were included in the analysis of MAL031. This was done because values of zero, especially at day 90, may indicate a vaccine effect that would have otherwise been missed if only positive values were considered. When only non-zero values are considered, the cohorts A and B and CC, had a decrease in value over time. Cohort C, when analyzed this way, had an increase in the number of samples that were positive at day 90. Because most of the samples from Cohort C participants had only the single RO33 allele, the COI calculated with only positive samples is lower. By using all values, there is an increase in the COI that better represented the results from that cohort.

From day 0 to day 90 there were changes in COI that may be attributable to the vaccine among the three recipient cohorts. The proportion of K1 alleles increased with increasing vaccine dose. While this did not significantly raise the COI, there was a clear trend toward that outcome. Our hypothesis was that vaccine administration would result in an increased COI. The effective outcome would then be that the vaccine recipients would acquire an immune response similar to that of older children who are able to remain asymptomatic with numerous infections.

The PfCRT assay had startling changes associated with the vaccine cohorts at day 90. The reduction of both CS and CR alleles identified in participants in cohorts A and B was related to the overall reduction in COI following vaccine administration. However, since there was a greater reduction in CS than in CR, the proportion of CS to CR was changed. The final result was an increase in absolute and relative CR infections. Conversely, cohort C participants had an increase in total CS infections at day 90 and the

ratio of CS to CR which was nearly equal at day 0 shifted such that CS was over 40% higher at day 90 than CR.

The discordant samples, those that were positive for COI but negative for the PfCRT assay most likely are attributable to DNA degradation. The study was conducted 6 months after the other two assays so damage during frozen storage or during thawing of the samples may explain the results. The PfCRT assay was rerun on those samples a second time and continued to be negative. The results from the COI assay perfectly matched those from a separate assay examining the C-terminal end of *msp1* and were considered accurate with regard to being either positive or negative for detectable parasites.

The use of the chloroquine resistance assay for samples from a vaccine trial was a novel idea. While it is incorrect to conclude that a vaccine could impact drug resistance directly, the vaccine may reduce or eliminate certain strains that encode for either drug resistance or sensitivity. This would be a chance occurrence, unless the vaccine antigen was linked to the *pfcrt* gene and also located on chromosome 7. Despite the limitations to conclusions that may be drawn from its use here, the assay did provide more information when analyzed in combination with COI. Knowing the number of infections within a sample provides a more robust picture than knowing merely that there are both CS and CR alleles within that sample. Likewise, if the PfCRT assay is coupled with analysis of COI at several different loci, it helps to build a more complete phenotype for the individual parasite strains.

The results from cohort C, the group that received the full vaccine dose, may indicate a very unusual vaccine effect. At day 90, the participants in cohort C were

overwhelmingly RO33 positive; this extended to the comparator controls as well. Along with the unusual distribution of RO33, was the interesting finding that most of the infections were CS as well. Unusual relationships between RO33 and vaccine use have been reported previously with the Spf66 vaccine, where there was a lack of the vaccine effect on RO33-type alleles [8]. Alternate explanations for the findings were explored: the children did not live in the same village or geographic area and their history did not indicate any other reason for this finding.

However, these findings are not necessarily attributable to the vaccine because the Imovax® recipients in cohort C also had the same profile. Possible assay difficulties were explored. The DNA from these samples was amplified three separate times and analyzed by two different researchers to account for potential bias or technical difficulties. Confirmation of the results comes also from the PfCRT assay as well as a third independently conducted assay examining single nucleotide polymorphisms of the p19 C-terminal end. All three assays confirm the identical profile of the parasites infecting the children in cohort C. Because these assays were all run on the same samples of extracted DNA and no original blood samples remained to redo this procedure, cross-contamination of the samples cannot be ruled out as a possible explanation.

Another explanation for the cohort C results may be attributable to micro-heterogeneity as discussed with the MEPI trial. The participants of cohort C began the trial two weeks after the start date for cohort B and 4 weeks following cohort A. This staggered time course may have introduced micro-heterogeneity in the form of a “mini epidemic” of RO33 allele-carrying parasite strains that infected the cohort C participants at day 90.

The MAL031 phase 1 field trial represented the first use of the block 2 of *msp1* as a tool to assess the selective pressure of a vaccine formulation of the same gene. With this genotypic tool, an unusual distribution of the RO33 allele was seen in the cohort that received the full vaccine dose. The vaccine may also have affected the distribution of parasites that are sensitive to chloroquine. Because of the unique findings associated with the cohort that received the full vaccine dose, these findings will need further confirmation from the larger phase 2 field trial to ensure their veracity.

CHAPTER FIVE: MAL036

Introduction

The phase 2 field trial (MAL036) was a randomized, double-blind trial of 50 μg FMP1 in adjuvant AS02A versus Imovax® rabies vaccine in 400 children aged 1-4 years. The study was conducted from April, 2005 – April, 2006, with participants receiving vaccines at day 0, day 30, and day 60. Samples from four time points were analyzed: day 0, children with clinical malaria illness between day 0 and day 85, day 85, and the first 500 episodes of clinical malaria illness following day 85. Table 10 summarizes the number of samples analyzed at the respective time points.

Table 10. Summary of samples analyzed for MAL036.

Time point	# Samples analyzed	# Samples positive (%)
Day 0	400	244 (61.0)
Ill, pre-Day 85	549	529 (96.4)
Day 85	386	151 (39.1)
Ill, post Day 85	508	463 (91.1)
TOTAL	1843	1387 (76.0)

Results

The study was conducted as a double-blind clinical trial. The analysis presented here was performed on data that was still blinded; therefore no conclusions on effects of the vaccine can be drawn. Of the 400 enrolled participants, 14 were lost to follow-up by day 85; only the data from the 386 participants that completed day 85 was included in analysis.

Table 11. Summary of alleles identified in MAL036.

	K1		MAD20		RO33
Time point	# Samples positive	# Alleles identified	# Samples positive	# Alleles identified	# Samples positive
Day 0	221	484	174	298	82
Ill, pre-Day 85	527	1140	275	504	386
Day 85	141	328	111	176	89
Ill, post Day 85	381	984	247	334	252

As seen previously, K1 was again the most prevalent allele family identified in the samples. In the ill samples, RO33 was the second most prevalent, followed by MAD20. In the day 0 and day 85 samples, this was reversed and RO33 was not as frequently identified as MAD20 (Table 11).

As a surrogate for knowing who received either the vaccine or the comparator control, ELISA values from study participants were used. These values were measured at day 0 and day 85; the difference in response between the two time points was categorized by high, medium or low. The high category contained those children with an increase in ELISA titer from day 0 to day 85 of >20 μg/ml (n = 147), medium were those values between 5.1 and 20 μg/ml (n = 91), while low are those ≤5 μg/ml (n = 148). For the analyses here, the participants grouped in the medium category were disregarded because their titer value did not allow them to be categorized as a clear responder or non-responder.

Allele Distribution. The distribution of the allele sizes was explored after normalizing the data. The normalization was performed because of the differences in the number of samples analyzed at each time point. The distribution presented in Figures 13 and 14 is the number of samples possessing an allele size divided by the total number of

samples positive for the particular allele at that time point. The K1 allele family was normally distributed throughout the study with a few identified exceptions. The 180 - 189 bp genotype had a 6% decrease in prevalence from the initial day 0 samples and the samples from ill patients from the period after day 0 to the day 85 samples and the samples from ill patients after day 85. The 220 - 229 bp K1 genotype was identified in over 24% of the ill patients after day 85, an increase over the previous time points. Finally, the 270 – 279 bp genotype had an increase in prevalence of over 8 % at the day 85 time point and in ill patients after day 85.

Two genotypes of MAD20 were identified as being unusually distributed. The first was the 160 – 169 bp genotype that decreased by half from day 0 to the remaining time points. The second genotype was a particularly dominant one identified as 180 – 189 bp. Logistic regression was performed to explore the distribution of the 180 – 189 bp MAD20 genotype that was clearly over-represented in samples (Figure 14) early in the study period but declined ($p = 0.05$) by study end. This genotype was particularly prevalent in those individuals with clinical malaria infection ($p < 0.0001$) prior to day 85; after that time point it was still likely to be associated with clinical illness, but not significantly ($p = 0.06$). The children in the high ELISA category tended not to have the 180 – 189 bp genotype when ill, but this was not statistically significant ($p = 0.09$).

The statistical analysis of MAL036 began by exploring possible seasonal variation in COI values. Using the same criteria of increased COI identified during the MEPI study, which was the months of April-June and November, the samples were divided and COI examined for any significant patterns or differences. Changes in COI were not related to season so no correction was necessary for future analyses. Sample

date was also explored initially because the day 0 and day 85 time points were staggered over the course of three weeks. No differences were identified with COI related to specific date so no correction to the model was made.

COI. In general, there was a decrease in COI between the day 0 and day 85 samples ($p = 0.0003$). To determine if the decrease in COI was attributable to an allele, the contribution of K1, MAD20 and RO33 was measured; no association was found.

The COI was analyzed with relation to ELISA category to determine if that provided an explanation for the decrease between day 0 and day 85. To do so, the log value of the COI was used in the Poisson regression to correct for the few higher COI values within the dataset and allow for a more equitable comparison. There was no significant difference identified between the low and high ELISA categories at any time point; the values across time points were also equivalent. The mean COI of samples among the clinically ill children was significantly higher than those from day 0 and day 85 ($p < 0.0001$), but there was no variation in COI related to ELISA category or any difference in COI between the two illness time periods (Table 12). When each allele was explored individually to determine if one allele accounted for a disproportionate change in values, there was no difference between the high and low ELISA categories.

Table 12. Mean COI at different time points divided by ELISA response category.

	ELISA response category	
Time Point	**Low**	**High**
Day 0	1.6	1.6
Ill, pre-Day 85	3.4	3.2
Day 85	1.1	1.3
Ill, post Day 85	3.4	3.4

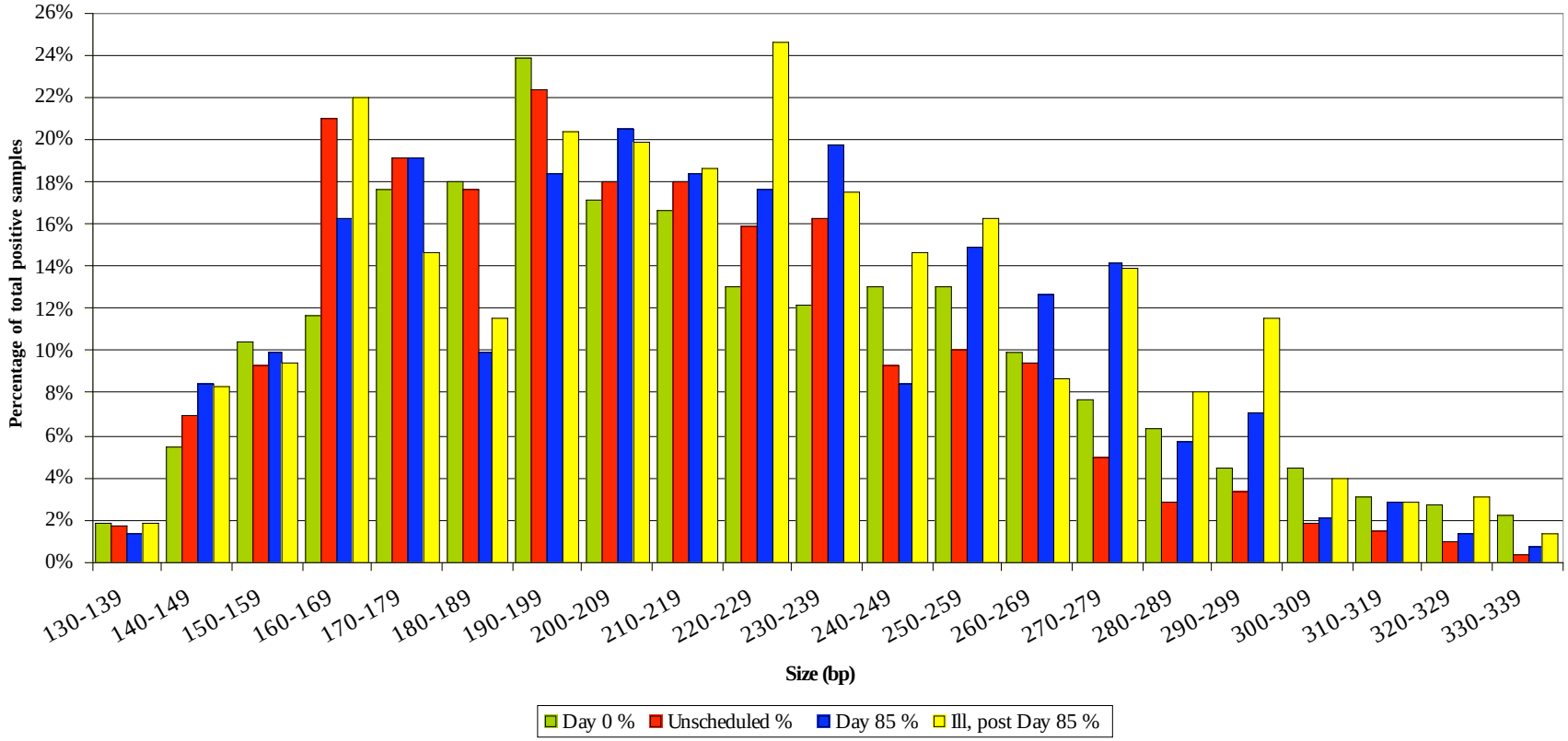


Figure 13. Normalized distribution frequency of K1 samples. Values obtained represent the percent of total positive samples at that time point.

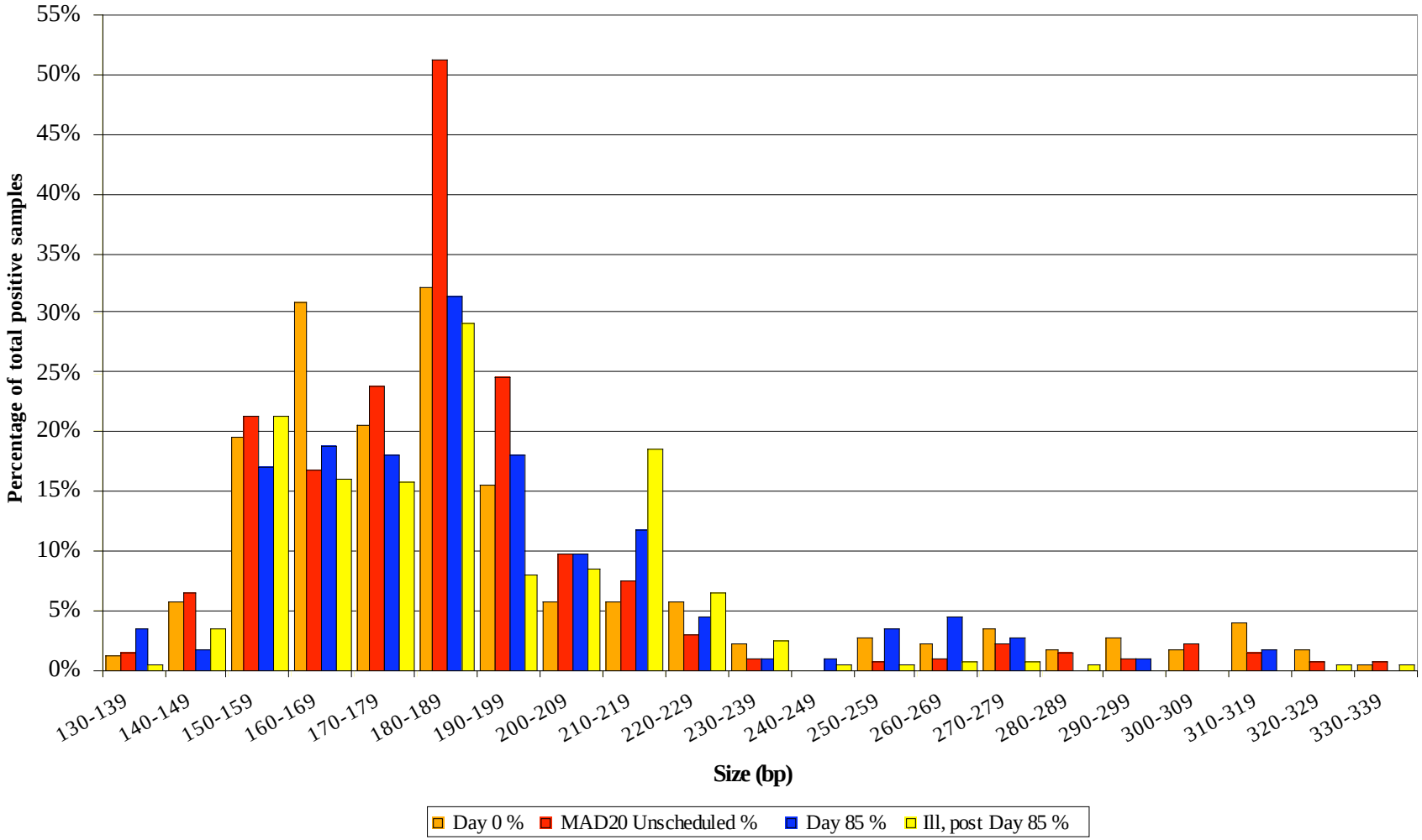


Figure 14. Normalized distribution frequency of MAD20 samples. Values obtained represent the percent of total positive samples at that time point.

Discussion

Although the identity of the participants was blinded, the use of ELISA values allowed for the categorization of individuals at day 0 and day 85. There were no noted differences among those who had a high ELISA response compared to those who did not; however, there was a decrease in COI between day 0 and day 85. Interestingly, the same difference in time did not affect the COI in ill children. In those children, COI was equal among all groups and time points.

As with the MEPI study, the COI calculated from clinically ill samples was significantly higher than that identified in asymptomatic infection. Even when only positive COI values are used to calculate values for day 0 and day 85, the difference is still significant (data not shown). This is a finding that differs from other studies of COI. It suggests that infection is not caused by a newly introduced novel strain that is able to cause infection. If this were the case, one would expect to find a reduced COI in illness, since illness would be the result of a single allele causing illness. Rather the model that is applicable with the data here, is that of density-dependent parasite regulation [75, 76]. In density-dependent parasite regulation, many strains are present and circulating. The different strains do not behave independently and overall density is dependent upon regulation by partially indiscriminate mechanisms [28]. Illness results when one parasite population is able to increase in density causing illness and subsequent immune system inhibition of all strains. By this model, the same number of strains would be expected with both asymptomatic samples and those from the clinically ill. However, because of

the increased parasite density in ill samples, DNA amplification may detect more strains from a sample during illness [51, 52, 53]; this would account for the increased COI during clinical illness.

The findings from this study again reinforce conclusions drawn from both the MEPI epidemiologic cohort and the MAL031 phase 1 study with regard to the distribution of allele families. K1 has definitely been established as the dominant allele in the study area; samples spanning the three study periods from June 2003 – February 2006 highlight this fact. In MAL036, there was an interesting reversal in the prevalence of the MAD20 and RO33 alleles. Whereas the other two studies had RO33 as the second most prevalent, in MAL036, this was found only to be the case in the samples obtained during clinical illness. At day 0 and day 85, MAD20 was far more predominant than RO33.

Another difference noticed in MAL036 in comparison to the MEPI study, was the prevalence of RO33 in the ill patients. This increased prevalence may actually point to a reversal of the findings that RO33 was mildly protective against illness during the MEPI study. The results from MAL036 may agree with a study from Robert et al. [20] in which RO33 was associated with severe malaria more frequently than the other alleles. In clear contrast, other researchers found that RO33 had a protective effect against future symptomatic infections [24, 29] and was more likely to present as a mild disease [59]. This association with RO33 will be evaluated further when the data are unblinded to determine if there is indeed an association with illness or protection from it. Another explanation for the increased prevalence of RO33 may be tied to the MAL031 study. The increase in the RO33 allele may indicate a similar distribution of RO33 as was seen with

the phase 1 study; that is an association with the allele and the cohort that received the vaccine. A clear determination of this will not be possible until the results are unblinded.

The increases and decreases observed in different genotypes of K1 and MAD20 appear to be chance fluctuations. The identification of the 180 – 189 bp MAD20 genotype in over 50% of the samples from ill children prior to day 85 may indicate that it is a more virulent parasite strain. However, it may also be representative of a particular parasite strain that increased in high number during the peak transmission season in a type of “mini-epidemic”. Since we do not have samples from asymptomatic carriers between the day 0 and day 85 time points, it is impossible to compile a true longitudinal history of the genotypes to determine whether these identified fluctuations are chance occurrences or indicators of something more significant.

In summary, the MAL036 phase 2 field trial provided limited information about the use of COI in vaccine trials, because of the blinded data. However, it strengthened observations made during the MEPI trial regarding COI during illness and once again hinted at unusual findings associated with the RO33 allele. Perhaps most interesting, it allowed the tracking of an exceptionally fit clone of MAD20, and demonstrated how prevalent a single genotype of the falciparum parasite can be even in a large study population.

CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSIONS

Malaria as a re-emerging infectious disease

Infectious diseases account for 26 % of deaths globally, second only to cardiovascular diseases [77]. The emerging infectious diseases, those that have been recognized as new etiologic agents, such as HIV/AIDS, *E. coli* O157:H7 and hepatitis C, account for a large proportion of those deaths [78]. In the shadows of the emerging infectious diseases are those that both historically and currently continue to cause death and disease in unchecked numbers. These re-emerging infectious diseases rarely get the attention of the developed world, except when an outbreak directly threatens them. However, they are just as important for global health.

Re-emerging infectious diseases are defined as those that are resurging in a different form or different location. Examples include monkeypox in the United States, vancomycin-resistant *Staphylococcus aureus*, multi-drug resistant tuberculosis and malaria. The burden of these diseases varies from small, focal outbreaks that disappear rapidly, to ancient epidemic scourges. In the latter category is malaria, most notably that caused by *P. falciparum*. Every 30 seconds a child under the age of five dies from falciparum malaria [1]. This adds up to 1-2 million deaths annually and nearly a half-billion clinical episodes [2]; prior to the advent of anti-malarial drugs and transmission-blocking preventatives, the toll was far worse on a proportional basis [79]. Evidence suggests that malaria is the strongest single selective pressure in the recent history of the human genome [80].

Recent advances in malaria research may hold promise for reducing or eliminating malaria. The sequencing of both the *P. falciparum* [81] and *Anopheles gambiae* [82] genomes will hopefully lead to new discoveries about the parasite and one of its most important mosquito vectors. The main thrusts of research in malaria appear to be the development of new anti-malarial drugs, the development of control mechanisms such as pesticides and the development of an effective vaccine. A vaccine could not only enhance current programs of control, but if highly effective may ultimately be able to eliminate the disease.

Studies on new vaccines have shown some promise in the field. Most notably, RTS,S/AS02A has shown short-term efficacy of 34 % in a study of men in The Gambia. Following a booster one year later, the efficacy was 47 % [83]. In children tested with RTS,S/AS02A in Mozambique, the vaccine regimen showed 30 % reduction in clinical disease, a delay to first infection and a 60 % prevention of severe disease [84]. Over 18 months of follow-up, the total vaccine efficacy was determined to be 35 % [85]. Because of its promise, RTS,S/AS02A will be the first malaria vaccine candidate that will be tested in phase 3 trials [86]. Although noteworthy for its status now, RTS,S/AS02A vaccine may not be alone for long; Malkin et al. cite 14 different candidate vaccines that are currently in phase 1 or phase 2 testing [86].

Significance of present research and future directions

The research presented here is one small component in the effort to develop an effective malaria vaccine. The complexity of infection was explored in three different

studies: an epidemiologic cohort and two vaccine studies, a phase 1 and phase 2 field trial. The purpose, ultimately, was to assess COI as a marker of immune function and a tool to expand knowledge about the acquisition of immunity in a population susceptible to malaria illness and to identify the selective pressure of a candidate vaccine formulation.

The MEPI study, detailed in Chapter Three, provided information about the dynamics of COI in a population without intervention other than treatment of clinical episodes. Intriguing results showed for the first time that the use of bed nets by 1-2 year old children significantly reduced COI, particularly affecting the RO33 allele. In cases of clinical illness, those children who slept under a bed net had far fewer MAD20 and RO33 alleles.

In the MAL031 phase 1 field trial, further findings related to RO33. In cohort C, the group of children that received the full dose of vaccine, samples at day 90 were overwhelmingly positive for a falciparum clone that was both RO33 positive and chloroquine sensitive. Whether this was the result of the vaccine or not cannot be confirmed, but other researchers have noticed the unusual allele.

As discussed in Chapter Four, results from a previous vaccine trial indicated that the formulation had no effect on the RO33 allele [8]. This also appeared to be the case with MAL031. Branch et al. speculated that RO33 may be linked to a particular p19 C-terminal allele [29]. Evidence for this linkage was found with MAL031 data; the majority of samples from cohort C participants had an identical p19 genotype, but there is no indication that the p19 genotype would be less affected by the vaccine.

Branch et al. suggested that the RO33 allele may induce a more effective immune response. Other researchers have suggested that RO33 may have a role unrelated to immune evasion [87, 88], the presumed function of the repeat regions of the K1 and MAD20 alleles. Instead, it seems that the RO33 allele may be the marker for a more benign strain of *P. falciparum*, one that effectively evades immune activation. Evidence for this includes; (1) the allele was more likely to be chloroquine sensitive, (2) bed net use disproportionately affected RO33, (3) with increasing parasite density in illness, RO33 decreased in proportion to the other two alleles, and (4) the presence of the RO33 allele was associated with protection from illness in the MEPI study. Further evidence comes from the RO33 sequence: it is dramatically different from that of the other two block 2 alleles, and contains no repeat region [40]. Further research into the identity of the RO33 allele and its role in malaria immunity is clearly needed.

The MEPI epidemiologic cohort clearly demonstrated that parasite density and COI are directly correlated and in fact, the parasite density fluctuations confounded COI and were a better explanation for variations seen during illness and with age than COI. This finding has two implications. The first is that analysis of COI may not be a necessary or even worthwhile use of resources for most studies. Given the lack of comparability of COI among researchers and laboratories [53] and the differences associated with malaria transmission, the validity of previous work cannot truly be compared to anything else. Improvements and standardization of the methods of determining COI may improve this aspect. Newer studies have begun employing alternative techniques such as automated capillary electrophoresis systems [42] comparable to the microfluidics system employed in this work. Advanced techniques

need to be adopted and further refined so that epidemiologic studies of complexity of infection can be compared among different time points, regions and researchers.

However, even with improved analytic methods, parasite density remains a superior measure and one that confounds COI, in this study as well as others [51]. The second implication is that parasite density is still a limited tool that does not provide additional information about how many parasite strains are infecting an individual or the identity of those strains. Therefore, in vaccine trials when the goal is to assess selective pressure, parasite density is not necessarily ideal. The ultimate answer may lie in the development of a new molecular epidemiology tool that will be an improvement on measuring both parasite density and COI.

One means to improve the study of COI is to use microsatellite markers, the marker of choice for most population genetic studies [89]. Microsatellites are simple tandem repeats, usually dinucleotide, found within the genomic sequence [90] of every eukaryote studied to date [91]. Because they are neutral markers, unlike msp1 which is under selective pressure, microsatellites are used to make inferences about populations or transmission of parasites [92]. Mu et al. have recently created a complete genetic map for *P. falciparum* that can be used to pinpoint DNA sequences that contribute to heritable phenotypes such as virulence or drug resistance [93]. Recent work has used microsatellite markers in combination with *msp1* to learn more about the full genotype of a clone [94, 95]. By using this combined method, the researchers are able to identify differences in a clonal type even when the *msp1* type is identical. This type of approach would allow researchers to assess the selective pressure of a vaccine on a gene such as *msp1*; but then relate that information to the parasite population as a whole.

Summary

The work presented in this dissertation demonstrated the application of one molecular epidemiology tool used to study malaria. While useful for reaching limited conclusions, in the end, we are left with more questions regarding the complexity of infection. Improved techniques such as microsatellite markers may provide additional pieces to the puzzle. However, we still do not have full understanding of the inter-connected relationship between the parasite, its vectors and the human response to the disease that will be needed to eradicate malaria. That understanding will come from the joining of the different pieces of data from molecular biology, immunology, genetics, entomology, parasitology and epidemiology into a coherent whole.

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