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Abstract

The Molecular Epidemiology of Malaria in Western Kenya

Malaria epidemiology reflects a complex web of inter-related factors: host, parasite, vector and environment. The nature, duration and severity of malaria infection depend upon these fixed and changing factors, and are complicated by varying levels of acquired immunity in individuals. The present research used molecular biology tools to examine three distinct topics related to malaria epidemiology: 1) the distribution of TNF-α, IFN-γ, IL-6, TGF-β, and IL-10 gene polymorphisms; 2) the rate and pattern of mixed *Plasmodium* species infections; and 3) *Plasmodium falciparum* growth dynamics. The first two research topics were examined in a cohort of 248 males recruited from three highly endemic villages in western Kenya where severe malaria anemia is common. The third topic was investigated among 22 volunteers during the post-challenge phase of a malaria vaccine trial sponsored by Walter Reed Army Institute of Research. Individuals in the Kenya cohort were found to have a marked bias toward genotypes associated with low expression of IFN-γ and IL-6, cytokines that, at high plasma levels, have been previously implicated in anemia and poor malaria outcomes. By contrast, the frequency of the TNFα –238A allele, which has been associated with severe malarial anemia, was found to be similar to frequencies reported in a number of diverse populations. Over the course of three malaria transmission seasons, non-falciparum malaria was consistently seen in a minority (5-10%) of volunteers, most commonly as a dual infection with *P. falciparum*. In contrast to previous reports of long-term stable parasite density among

individuals with mixed species infections, in 80% of cases, multi-species infections were detected when individuals had their highest rate of parasite density. Among clinical trial volunteers, a highly sensitive real-time PCR assay found a ~48 hour periodicity in parasite density and a relatively wide range in parasite multiplication rate. Four individuals were identified as having some degree of resolution of infection, and an additional five volunteers were found to have a delayed pre-patent period. These results demonstrate the potential of molecular epidemiology and illustrate the subtle and complex relationship developed between humans and malaria parasites over millions of years of co-evolution.

Key words: malaria, epidemiology, molecular epidemiology, Kenya, cytokines, PCR, real-time PCR, molecular beacon probes, vaccine

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The Molecular Epidemiology of Malaria in Western Kenya

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Acronyms

ABO	Blood type
BP	Base Pair
Ct	Threshold Cycle
EIR	Entomological Inoculation Rate
ELISA	Enzyme linked immunosorbent assay
DHEAS	Dehydroepiandrosterone sulfate
G6PD	Glucose 6 phosphate dehydrogenase
Hb	Hemoglobin
HbC	Hemoglobin C
HbS	Hemoglobin S (sickle cell trait)
HLA	Human Leukocyte Antigen
IL	Interleukin
IRB	Institutional Review Board
LPS	Lipopolysaccharide
LSA	Liver Stage Antigen
Mg++	Magnesium
MHC	Major histocompatability complex
N1	Nest 1
N2	Nest 2
OR	Odds Ratio
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
P.f.	*Plasmodium falciparum*
P.m.	*Plasmodium malariae*
P.o.	*Plasmodium ovale*
P.v.	*Plasmodium vivax*
PMR	Parasite multiplication rate
RBC	Red blood cell
RR	Relative risk
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
ssurRNA	Small subunit ribosomal RNA
TNF	Tumor necrosis factor
WRAIR	Walter Reed Army Institute of Research
USAMMC	United States Army Medical Materials Command
USFDA	United States Food and Drug Administration
USUHS	Uniformed Services University of the Health Sciences

Chapter One: Introduction

Introduction

In hyperendemic regions of malaria infection, almost all individuals are exposed to the malaria parasite. However, not all individuals become infected, nor do they develop identical densities of parasites or suffer similar clinical syndromes. This chapter will present an introduction of the general the factors related to susceptibility to malaria, and more specifically, three specific factors which influence malaria epidemiology: 1) human cytokine gene polymorphisms; 2) concurrent multi-species malaria infection; and 3) immune regulation and parasite growth.

General factors related to malaria susceptibility

Parasitologists have traditionally measured human susceptibility to malaria parasites based upon three biological outcomes:

1) Patent parasitemia (infection)
2) Parasite density; and
3) Clinical malaria.

It has long been understood that these outcomes are influenced by a range of factors, including age, prior exposure to malaria, immune response, and genetic background of the host. While the above outcomes generally represent a single spectrum of possible result (i.e., one leads to the next), each presents a useful, and in part, independent, measure of host response to infection.

Age is an important factor related to infection. Newborn infants are thought to be protected from infection by transplacental transfer of maternal antibody and fetal

hemoglobin. However, with time, children rapidly become susceptible to infection. Despite this susceptibility, some degree of innate and/or acquired immunity to malaria is maintained, or rapidly established, in children in endemic regions. For example, in northern Ghana, with entomological inoculation rate (EIR) above 300/year (i.e., more than 300 bites of a malaria-infected mosquito per year per person), and on average 2-10 episodes of acute infection/year, malaria mortality affects 5% of children aged ≤ 7 years (1). Parasite prevalence was found to be highest in the oldest children (5-7 years), but parasite density, rate of febrile illness, and anemia, were highest in those 6-11 months old. This general finding -- that children first develop a partial immunity to malaria parasite density and morbidity, and later develop a partial immunity to malaria parasite infection -- has become a cornerstone of our understanding of malaria immunology. Neither immunity is totally effective however, with millions of adults suffering malaria infection and morbidity annually, and 100-fold or greater fluctuations in parasite density within 6-hour periods possible (2).

Most research has focused upon the parasite's antigenic polymorphisms as the most important reason for this delayed and incomplete development of acquired immunity. Researchers postulate that the multiple strains of malaria parasites present, and the on-going process of recombination during sexual reproduction cycles in the mosquito, produce a significant challenge to the immune system, and that the acquisition of partial immunity is a consequence of exposure to a range of immunodominant strains (3).

Alternatively, evidence of dependence upon immune system development has been recently suggested. Baird (4) has shown that adult migrants to hyperendemic Irian Jaya, Indonesia were more rapidly able to control incidence and high-density parasitemia than

their children, despite initially being more severely affected by infection. Similarly, Duffy *et al.* (5) reported that measures of physical maturation and hormonal changes (DHEAS and testosterone) explained differences in parasite density within individuals in the same age strata.

The combination of prior exposure and of immune system maturation may provide some explanation for the diverse syndromes of malaria complications seen in different parts of the world. For example, it has commonly been observed that in regions with high levels of malaria endemicity, cerebral malaria is less common than severe malaria anemia, and in regions with lower levels of malaria endemicity the reverse is true (6). However, it is possible that genetic variations in host and/or parasite populations are responsible for these observations.

Several genetic factors have been shown to protect individuals from the outcomes previously mentioned (infection, parasite growth and reproduction (parasite density), and severe clinical outcomes). The most commonly known (and earliest identified) genetic factors are the inherited disorders of hemoglobin (sickle cell and thalassemia). Individuals that are homozygous for the HbS variant of hemoglobin suffer the consequences of sickle-cell disease, but heterozygosity at this locus is strongly protective against severe malaria. The greatest impact of HbS seems to be to protect against death and severe disease, with less effect on infection (7). These protective effects are probably due to impaired entry into, and growth of parasites in, affected erythrocytes (8).

The mechanism of protection of α and β thalassemia against malaria is less clear. One study in Vanuatu, found that an increased susceptibility to mild *Plasmodium vivax* and *P. falciparum* infections was observed among young children with α–thalassemia (9).

The authors have proposed that more frequent infections of immature red blood cells might protect them against later life-threatening illness.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency hinders the capacity of red blood cells to deal with oxidative stress. Males that are hemizygous, and females that are heterozygous (G6PD is an X-linked trait), were found to be protected against severe malaria in both East and West Africa (10). This benefit might result from impaired parasite growth in the erythrocytes (11), or from more efficient phagocytosis of parasitized red blood cells at an early stage of parasite maturation (12). The absence of Duffy receptors (the result of a single nucleotide polymorphism (SNP) within the promoter region of the Duffy chemokine receptor gene), by contrast, prevents mediated entry of *P. vivax* into erythrocytes (13). Other genetic factors which have been identified as influencing malaria infection, growth or clinical outcomes include: ovalocytosis, HLA variants HLA-B53 and haplotype HLA-DRB1*1032-HLA-DQB1*0501 (14). Still more, as yet undiscovered, genetic factors are likely to play a subtler role in regulating infection, or preventing the severity of clinical syndromes.

Jepson *et al*. (15) attempted to quantify the genetic component of malaria susceptibility in African children with a longitudinal study of 257 Gambian twins. The comparison of concordance rates among monozygotic and dizygotic twins indicated a significant genetic influence on susceptibility to malaria fever episodes, but not on the level of parasitemia. By contrast, reports from Cameroon and Burkina Faso (16-17) have reported a genetic influence on parasitemia based upon a recessive determinant with a complex mode of inheritance. Other studies in Burkina Faso (18-19) found different rates of susceptibility to both parasitemia and disease in three different ethnic groups living

within the same community, a finding previously reported in terms of susceptibility to malaria-related splenomegaly in The Gambia (20). Although it is impossible to rule out sociocultural factors that may have influenced susceptibility, these studies present strong evidence of a genetic impact working at the population level.

While studies of repeated exposure to homologous strain sporozoites have shown that short-term sterilizing immunity is possible (21), in field settings there is no widespread development of sterile immunity to infection. Evidence to support the concept of partial immunity to infection (for example, to antigenically similar strains previously seen) is mixed, and is likely confounded by the variability in experimental approaches and the poor sensitivity of diagnostic techniques. A recently published study by Owusu-Agyei *et al.* (22) conducted in Ghana found adult and infant rates of reinfection following radical clearance of malaria parasites to be identical, with the vast majority of infections for both groups occurring between week 5 and week 10 post clearance.

Nonetheless, the majority of research in the field of malaria immunology has concentrated on the identification of immunodominant epitopes for the development of a sterilizing malaria vaccine. The present research, by contrast, will examine a more comprehensive set of questions related to the molecular epidemiology of malaria – looking at host genetic factors, at parasite-parasite interaction and immune regulation.

Specific factors related to malaria susceptibility

Host genetic factors – cytokine promoter polymorphisms

With the increasing ease, and decreasing expense of genetic and molecular biologic tools, more and more attention has been paid to host genetic factors that might explain differences in susceptibility or clinical outcomes related to malaria infection. Based upon our understanding of the pivotal role of cytokines in the immune and pathologic response to malaria and other infectious diseases, increasing focus has naturally been placed upon examining cytokine genes.

Recently, polymorphisms have been described for several cytokine genes, including tumor necrosis factor alpha (TNF- α), interleukin-10 (IL-10), transforming growth factor beta (TGF-β), interleukin-6 (IL-6), and interferon gamma (INF-γ) (see Table 1).

Table 1. Polymorphic Sites of Cytokine Genes

Gene	**Polymorphic Sites Identified**	**Reference**
TNF- α	+70, -163, -238, -244, -308, -376, -574, -856, -862, and –1031	23-28
IL10	-592, - 819, -851 and –1082	29-32
TGF-β	–509, -800, -869, -915 and –988	33-35
IL6	–174, -572 and –597	36-39
IFN-γ	+874 and –333	34, 40

Note: Site is relative to the transcription start site of the gene

The specific relationship between cytokine polymorphisms and the level of cytokine expression is often unclear, and can be controversial. While studies of TNF- α production have shown stable interindividual variations (of up to tenfold differences) (41-43), analyses of specific mutations have given conflicting results. Studies of TNF-α -1031 polymorphisms found increased expression (28) as did studies of polymorphisms at sites –238, -308, -856, and –862 (26, 28, 44-48), however other studies of these latter

sites and +70, -376, and –574 found no effect (49-54). One study of TNF-α –862 found a 31% decrease in TNF expression (55). A recent study by Bayley *et al.* (56) examined the function of polymorphisms at –376, -308, -244, and –238 sites via mutant constructs transiently transfecting the T cell line Jurkat, the B cell line Raji, and the onocytic cell line U937. The study found no change in transcriptional activity from mutants introduced at –308 and –376 sites and small increases in basal activity in the U937 cell line from mutants introduced at –244 and –238. It is important to note however that TNF transcriptional regulation is known to be cell type- and stimulus-specific (57). Results of these and studies of other cytokine gene polymorphisms are summarized in Table 2, below.

Table 2. Effect of Genotype and Haplotype on Expression

Gene	**Effect**	**Polymorphic Sites Identified**	**Reference**
TNF- α	Increased	-238, -308, -856, -862, –1031	26, 28, 44-48
	No Effect	+70, -376, -574, -238, -308, -856, -862, –1031	49-54
	Decreased	-862	55
IL10	Increased	–1082G, -819C, –592C	30, 58-59
	Decreased	Haplotypes: -1082A, -819C, –592C and –1082A, -819T, –592A	
TGF-β	Increased	+869T, +915 G and +869T/C, +915 G	34
	Intermediate	+869T/C, +915 G/C and +869C, +915G	
	Decreased	+869T/C, +915CC and +869C, +915G/C	
IL6	Increased	–597G, -572G, -373A_9/T_{11}, -174G, –174GG or –174GA	60
	Decreased	–597A, -572G, -373A_8/T_{12}, -174G	
IFN-γ	Increased	+874TT	61-62
	Intermediate	+874T/A	
	Decreased	+874AA	

Despite the inconclusive results of the association between cytokine polymorphism and expression, more than one hundred studies have found associations between cytokine polymorphism and a range of infectious, chronic and autoimmune related diseases (summarized in 63).

Studies of cytokine polymorphisms and malaria have focused exclusively upon the –238, -308 and –376 site of the TNF-α gene. Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine with a wide range of physiological effects including a central role in inflammation (64). Excessive production of TNF-α has been implicated in the pathogenesis of malaria as well as a number of other infectious and autoimmune diseases (65).

TNF-α is released after schizont rupture, and very high concentrations can result from pulse release in the paroxysms of synchronous parasites (63). Elevated TNF-α levels (as well as other inflammatory cytokines such as IL6 and IL1 β) have been specifically implicated in relation to severe malaria (particularly cerebral malaria) in a number of studies (67-69).

Polymorphism at the –238 site (G→A) of the TNF promoter region has been correlated with severe anemia (OR=2.5, $p < .001$) in one study in The Gambia (70). This study also found the –238A allele to be protective against cerebral malaria, a finding replicated in another study in individuals from Kenya (OR=0.2) (71), but not in individuals from The Gambia (OR=1.0) (71).

The TNF-α –308 site has been examined in a larger number of case-control studies conducted in The Gambia, Kenya, Tanzania, and Sri Lanka. Presence of the –

308A allele was associated with cerebral malaria (OR=4.1, p=.02) in The Gambia (72) and with severe disease (of both malaria and non-malaria origin) in Sri Lanka (OR=2.65, p=.021 for severe and complicated malaria and OR=2.68, p=.04 for severe non-malaria infection) (73). Individuals with homologous (-308AA) polymorphisms were found to have higher risks of cerebral malaria in both Kenya and The Gambia (RR=3.3, p=0.16 in Kenya, RR=3.6, p=0.04 in The Gambia) (71). No correlation was seen between –308A allele presence and measures of parasite density, prevalence, or multi-clonal infection in a study in Tanzania (74).

The only study to examine the association between the –376 TNF-α polymorphism and cerebral malaria found a greater than fourfold risk in individuals with the –376A allele in both The Gambia and in Kenya (71). These relationships are summarized in Table 3, below.

Table 3. Reported associations between TNF-α polymorphisms and malaria

Site	Association	Strength	Location	Reference
-308	A = cerebral malaria A ≠ severe anemia	RR=4.1	Gambia	72
	AA = cerebral malaria	OR=3.3 p=.16	Kenya	71
	AA = cerebral malaria	OR=3.6 p=.04	Gambia	71
	A ≠ parasite density, prevalence, multiplicity	N/A	Tanzania	74
	AA= pre-term birth	RR = 7.3 p=.002	Kenya	75
	A = severe disease (malaria or other infectious dx)	OR= 2-3	Sri Lanka	73
-238	A = severe anemia A ≠ cerebral malaria	OR=2.5	Gambia	70
	A ≠ cerebral malaria	OR=0.2	Kenya	71
	No association	OR=1.0	Gambia	71
	A = IL10/TNF <1 (cerebral + severe anemia)	p<.01	Gabon	76
-376	A = cerebral malaria	OR=4.3	Gambia	71
	A = cerebral malaria	OR=4.6	Kenya	71

Interestingly TNF-376A is in negative linkage disequilibrium with –308A (although both associate with cerebral malaria), and positive linkage with –238A (71).

Although other cytokines have been extensively examined in terms of malaria susceptibility and pathogenicity, no research has been published examining the potential association between cytokine polymorphisms other than TNF-α and malaria.

Interaction of multiple species malaria infections

While host genetics are one factor which influences malaria epidemiology, many individuals theorize that the parasite may also have some direct or indirect means to influence malaria parasite density and/or clinical disease. Field studies measuring the cross-sectional prevalence of malarial species often record fewer mixed infections than expected by chance, suggesting that one parasite has excluded another, or suppressed its parasitemia to undetectable levels (summarized in 77; 78). Longitudinal studies have shown the possibility that malarial parasites may favorably affect the host environment for each other, as shown by the recrudescence of a latent malarial species immediately before or after the parasitic wave of another species (77).

Studies conducted in the 1930s on neurosyphilitic patients examined the dynamics of simultaneous multispecies malaria co-infections in nonimmune adults, and found limited evidence of sequential episodes of infection (79-80), indicating some degree of within-host interactions between species.

While a number of studies of avian, murine and simian malaria parasites have shown evidence for cross-species immunity, little research has been conducted among human malaria species. One study in Vanuatu (78), an area endemic for both *Plasmodium*

vivax and *Plasmodium falciparum*, found fewer multiple infections than expected, and distinct seasonal peaks in *P. falciparum* and *P. vivax* prevalence, which the authors attributed to cross-species interactions. Research in a region of low endemicity in Thailand (EIR of less than 1 (81)) found high rates of cryptic infections (revealed by PCR) following microscopic diagnosis of *P. vivax* (82) or treatment for either *P. falciparum* or *P. vivax* infection (83-85).

A more recent report by Bruce *et al.* (86), described a longitudinal analysis of the dynamics of multiple, co-infecting *Plasmodium* spp. and genotypes in infected but clinically asymptomatic children in a region of intense malaria transmission in Papua New Guinea. The study site had a reported EIR of approximately 365. Although the parasite density of each species or genotype fluctuated over time, sometimes quite significantly, the total parasitemia stayed more or less constant at approximately 1000 parasites per microliter of blood (or roughly .02% parasitemia), for periods longer than any single infection episode. One hypothesis emerging from the research is that a mechanism of density-dependent regulation is triggered by the growth of the majority parasite population above a threshold density, resulting in the inhibition of minority co-infections. When antibody responses specific for the majority population start to circulate (87-88), total density will fall beneath the threshold and density-dependent regulation will cease; minority populations could then expand until limited again by the density-dependent mechanism.

While Bruce *et al.* (86) examined asymptomatic children, an unresolved question is the impact of multiple species on clinical outcomes. This was examined in part in a study by May *et al.* (89) that examined the impact of subpatent multi-species and multi-

clonal plasmodial infections in relation to anemia in children from Nigeria. The authors found that the prevalence of anemia was more severe with increasing numbers of *Plasmodium* species detected by species-specific PCR ($p<0.0001$). Examining age-adjusted Hb levels and RBC counts, the authors found a larger impact from multi-species than multi-clonal infections. By contrast, research in Thailand (90) found that severe malaria was 4.2 times (95% CI 2.3-7.9) *less* likely in patients with (blood smear detected) mixed *P. vivax* plus *P. falciparum* infections versus *P. falciparum* infections alone. Other research conducted in Papau New Guinea (91) failed to show any interaction.

Parasite growth dynamics

An examination of parasite growth dynamics combines both previously discussed factors – host immune regulation and parasite population dynamics. However, this complexity makes it a particularly difficult area to study. In malaria endemic regions individuals have varying degrees of both past and present exposure, limiting our ability to effectively analyze parasite growth dynamics. By contrast, malaria therapy (previously used for treatment of neurosyphilis) or a clinical vaccine trial is an ideal experimental design for modeling parasite growth dynamics.

The majority of parasite growth models proposed to date (92-94) are based on the model by Anderson *et al*., (95) which uses a set of four coupled differential equations representing the rates of change in numbers of: 1) uninfected erythrocytes; 2) parasitized erythrocytes; 3) merozoites; and 4) immune effectors, over time. Molineaux and Dietz (96) have noted, however, that these models have little similarity to observed, natural infections. Instead, Molineaux *et al*. (97) proposed a mathematical model which focuses

on the factors controlling infection and incorporates three putative control mechanisms: 1) the innate immune response; 2) an acquired variant-specific immune response; and 3) an acquired variant-transcending immune response. Data simulated from this model were compared to observed asexual parasite density data of 35 malaria therapy patients who made a spontaneous recovery after primary inoculation, and found to more closely resemble observed measures than previous models.

Simpson *et al.* (98) examined a larger number of the same malaria therapy patients, focusing specifically upon the expansion phase of the infection (prior to the induced immune response). Their model was designed empirically based upon the observed: 1) parasite multiplication rate (PMR); and 2) length of parasite life-cycle (period), and found a PMR of approximately 8, and a period of 55h, slightly longer than the expected 2-day cycle.

Understanding parasite growth dynamics, particularly during the initial phase of infection, provides particular insight to better understand the development of acute malaria in a non-immune individual and for the evaluation of asexual blood stage malaria vaccines. In the past decade, a number of prototype vaccines that target the asexual blood stage of malaria have been developed and are undergoing testing (99-101). Although these vaccines have been chosen for their ability to elicit an anti-parasite response, no practical and sensitive clinical trial assay has been developed to measure their impact on parasite growth. The current 'gold standard,' thick blood smear detection of parasites, has a threshold for microscopic detection (between .000025% to .0002% infected red blood cells) higher than that needed for critical evaluation of partially effective vaccines. The development of a sensitive assay that can improve detection of low level infection and

elucidate the degree of partial protection (illustrated by delayed patent parasitemia or lower parasite multiplication rate) would be of considerable usefulness. The evaluation of combination vaccines that include both erythrocytic and pre-erythrocytic components would be easier to evaluate as well.

Specific hypotheses and research approaches

Hypothesis 1:

(a) Cytokine allele frequencies play an important role in the manifestation of malaria-related disease. (b) Populations in areas of stable malaria transmission may have profound biases in allele frequency based on selection.

Research approach:

(a) Examine the association between cytokine genotypes and haplotypes and malaria parasitemia, parasite density, and clinical outcomes. (b) Examine and compare the gene frequencies of cytokine promoter polymorphisms in endemic and non-endemic populations.

Hypothesis 2:

Multi-species malaria parasite infection has an important influence on parasite density and clinical outcome that is underestimated by traditional blood slide diagnostic assays.

Research approach:

Examine the incidence of multi-species malaria infection using a sensitive quantitative PCR assay and assess the influence of multi-species infection on parasite density and clinical outcomes.

Hypothesis 3:

Current malaria vaccines which target blood stage antigens may provide a degree of partial efficacy not currently measured using traditional blood slide methods. A sensitive quantitative PCR assay can identify differences in parasite growth dynamics during the expansion phase of infection and improve the evaluation of vaccine candidates.

Research approach:

Examine parasite growth dynamics among immunologically naïve volunteers exposed to *P. falciparum* challenge using quantitative PCR.

Chapter 2: Methods

Introduction

One factor complicating our understanding and evaluation of factors affecting malaria outcomes (such as parasite incidence and density) is the difficulty determining new infections versus persistent infections. In highly endemic malaria regions with partially immune populations, concurrent parasite clearance and reinfection is common.

Measuring infection and parasite density has traditionally been done via the low-tech and field applicable thick and thin blood smears. However, as molecular techniques have improved our understanding of parasite polymorphisms and low, persistent infections, it has become increasingly obvious that a true understanding of the epidemiology of malaria requires the use of molecular measures. Thin and thick blood smear do not allow for the determination of strain specificity, and commonly undercount species multiplicity as well. Studies have found as many as six to eight different *P. falciparum* parasite strains in individuals at one time (102-105). Because of the difficulty in morphologically distinguishing parasite species, particularly when there is a mixed infection with a preponderance of one species over others, microscopy techniques generally misclassify or underestimate mixed infections.

Molecular techniques allow for a much greater sensitivity and specificity of detection than traditional microscopy. Using nested PCR it is possible to detect a single copy of a target sequence (106), limiting sensitivity only to the total volume of blood

analyzed in the amplification assay. PCR product detection such as ethidium bromide staining reveals as little as 10 ng of DNA, and hybridization techniques with labeled primers/probes or high pressure liquid chromatography quantification can increase this threshold further.

In terms of specificity, molecular techniques provide the ability to distinguish differences in malaria strains/species based upon the presence of specific sequences or gene alleles. However, minor differences in length and position of PCR primers are known to affect the efficiency of amplification. Sensitivity and specificity of PCR is also dependent upon the means and conditions used to hybridize primers and probes, which is sensitive to a range of conditions including Mg++, temperature and pH. The following section will present the specific protocols used for my dissertation research.

Specific protocols for dissertation research

Study subjects – Kenyan cohort

Analysis of host genotype and multi-species malaria infection came from blood donated by a cohort of volunteers in a study led by Dr. Patrick Duffy, WRAIR. Volunteers were recruited from the villages of Wangarot, Riwa Ojelo, and Waringa, Rarieda Division, Nyanza Province, in western Kenya, 10 km north of Lake Victoria. The region has a high burden of malaria infection with a reported entomological inoculation rate in excess of 300 infectious bites per person per year (107).

The study was conducted within the defined protocol approved by ethical review boards of the Walter Reed Army Institute of Research and the Kenya Medical Research Institute. The USUHS IRB reviewed the prior approvals and provided an exemption from

USUHS review. All volunteers gave signed informed consent prior to entry into the study.

After the exclusion of individuals with abnormal hemograms or evidence of chronic disease on physical examination, 248 males aged 12-35 years entered the study at the beginning of the high transmission season in April 1996. To initiate the study, volunteers were simultaneously treated with three days of quinine sulfate (10mg/kg twice daily) and seven days of doxycycline (100 mg twice daily) to eradicate current malaria infections. Five volunteers were removed from first season analysis because they did not complete the eradication treatment; the remaining 243 volunteers became aparasitemic during the week following treatment with quinine and doxycycline.

Thick and thin bloodsmears were obtained from each volunteer prior to initial treatment with quinine and doxycycline and then weekly for 16 weeks after initial treatment. In addition, blood was spotted on Whatman 3M filter paper and stored at room temperature for subsequent DNA extraction. Specific outcomes included time to reappearance of parasitemia, mean parasitemia on all bloodsmears taken during the season, and frequency of parasitemia. Analyses using mean parasitemia of only positive bloodsmears produced similar results as those using mean parasitemia on all bloodsmears, and therefore only the latter results were used.

In August 1996, and again in April 1997, the original volunteers were re-enrolled, again treated with quinine and doxycycline, and followed for 16-18 weeks in the same manner as previously described. Overall, 143 volunteers participated in all three seasons and were the basis for the analysis of cytokine gene polymorphisms and multi-species infections.

Study subjects – Naïve volunteers

The influence of immune regulation, parasite growth and clearance, was examined using blood from 22 malaria naïve volunteers enrolled in WRAIR MAL019, a double-blind, experimental malaria Phase I/IIa vaccine study. Subjects ranged in age from 18-45 and had no prior exposure to malaria.

The trial had four main treatment arms (in addition to infectivity control subjects who received no vaccine) with single or combination vaccines targeting circumsporozoite, merozoite or both malaria stages. Individuals were given vaccine on a 0, 1, and 3 months immunization schedule followed (2 weeks post final vaccine dose) by challenge via exposure to the bite of five laboratory-reared *P. falciparum* (3D7 strain)-infected mosquitoes.

Subjects were examined daily between days 5-20 post challenge and approximately 2 mL whole venous blood was drawn for malaria smear and spotting on Gentra capture card (Gentra systems, Milwaukee, WI). Daily blood collection was continued in the absence of a positive diagnosis or until three consecutive daily blood films were found to be negative following diagnosis and treatment.

The study was conducted within the defined protocol approved by ethical review boards of the U.S. F.D.A., Walter Reed Army Institute of Research, and the Office of the Surgeon General, U.S. AMMC. The USUHS IRB reviewed the prior approvals and provided an exemption from USUHS review. All volunteers gave signed informed consent prior to entry into the study.

Prior Analysis

Prior to the current research, several analyses were previously undertaken with the Kenyan cohort. Several measures of clinical, antigen, lymphocyte and cytokine function were conducted contemporaneously to blood collection as part of a separate research protocol. Results from these analyses will be used as part of the statistical analysis of the influence of host genotype and multi-species infection on parasitologic and clinical outcomes of this study, and therefore relevant methods are presented in brief in this section.

In each season, hemograms were performed on heparinized blood using a Coulter cell counter model T-890 (Coulter Corp., Hialeah, FL). ABO blood group and hemoglobin phenotype were determined on 154 volunteers with commercially available reagents (Sigma, St. Louis, MO).

Two recombinant LSA-1 polypeptides were expressed with a thioredoxin fusion partner. The LSA-1 N terminal polypeptide (LSA N) contained amino acids 28-150 and the C terminal polypeptide (LSA C) contained amino acids 1630-1909, based on the sequence of LSA-1 from parasite strain NF-54 (108). These recombinant proteins flank the central repeat region of LSA-1. Fusion proteins were purified by metal chelate chromatography. Thioredoxin alone was purified under identical conditions and used as negative control for all assays. LSA N and LSA C were used at a concentration of 10 μg/ml and thioredoxin was used at an equivalent molar concentration. All assays were performed using a single lot of each recombinant protein. Recombinant proteins were greater than 95% pure as determined by Coomassie Blue stained SDS-PAGE gel. Lipopolysaccharide (LPS) in the LSA C preparation was measured to be less than 100

EU/mg protein by gel clot LAL method.

PBMCs were thawed, washed twice in RPMI 1640, and resuspended in RPMI 1640 supplemented with 10% human AB sera at 0.5 x 10^6/ml. PBMCs were used at 50,000 cells per well in round bottom microtiter plates. Stimulants were added to a final volume of 200 µl. Cells were incubated for 5 days in a humidified incubator at 37 °C and 5% CO_2. On the 5th day, 125 µl samples of cell free culture supernatant were harvested from each well and stored at -70°C for subsequent cytokine analyses. In the second season, assays were performed with LSA N, LSA C and PHA. In the third season, assays were performed with LSA N and LSA C.

Cytokine analyses on culture supernatants were performed in duplicate on 50 µl samples by sandwich ELISA. Paired antibodies and standards for IFN-γ (MabTech, Nacka, Sweden), TNF-α (Genzyme, Cambridge, MA), and IL-10 (PharMingen, San Diego, CA) were used as previously described (109). Standard curves for each cytokine were linear to at least 10 pg/ml. To calculate the LSA-1-specific cytokine response, the background cytokine concentration measured in thioredoxin-stimulated wells was subtracted from the cytokine concentration measured in wells stimulated with LSA N or LSA C.

Genotyping for the TNF-α –308, TGF-β, IFN-γ, IL-10 and IL-6 cytokine gene polymorphisms was carried out using a commercially available kit (OneLambda, CA) which utilizes sequence specific priming polymerase chain reaction (SSP-PCR). The kit resolves the –308 G/A alleles in the promoter region of TNF-α, the +869 (codon 10) C/T and +915 (codon 25) G/C alleles in the leader sequence of TGF-β, the –1082 G/A, -819 C/T and –592 C/A alleles in the promoter region of IL-10, the –174 G/C alleles in the

promoter region of IL-6, and the +874 A/T alleles in intron one of the IFN-γ gene.

DNA extraction

For the present research, DNA was extracted from blood spots dried on filter paper using three different approaches: 1) Chelex (InstaGene); 2) a commercial kit (Gentra systems); and 3) a phenol-chloroform technique, all of which use a multi-step process of cell lysis and DNA recovery. Comparisons of all three techniques found that yields of DNA were comparable, with the Gentra extraction providing the most efficient approach. The recommended protocol was modified after further optimization tests to include three purification washes and an overnight incubation.

DNA amplification

PCR amplification of two genes was conducted: the host TNF-α gene and the *Plasmodium* small sub-unit ribosomal RNA gene. For the amplification of the TNF-α gene, a nested PCR methodology was designed that first amplified a 650 nucleotide segment located toward the 5' end of the gene [reverse primer was based upon McGuire *et al.* (70)]. Next, an allele specific nested forward primer set was designed that selectively amplified the 238A or 238G [modified from McGuire *et al.* (70)] and 376A or 376G [modified from May *et al.* (76)] alleles. These primers were designed to end with the specific nucleotide at the 3' terminus to reduce the possibility of false priming. Primers were supplied by Oligos Etc (Wilsonville, OR). Primers and amplified segment are shown in Table 4, below.

Table 4. TNF-α gene PCR primers and amplicon

Name	Size	F + R Primer	Amplicon
N1 TNF-α	650	F - ctcaacggactcagctttc R – gcactgaaagcatgatccgg	ctcaacggactcagctttctgaagcccctcccagttctagtt ctatctttttcctgcatcctgtctggaa(g/a)ttagaagg aaacagaccacagacctggtccccaaaagaaatggaggc aataggttttgaggggcatggggacggggttcagcctccag ggtcctacacacaaatcagtcagtggcccagaagacccccc tcggaatc(g/a)gagcagggaggatggggagtgtgaggggt atccttgatgcttgtgtgtccccaactttccaaatccccgccccc gcgatggagaagaaaccgagacagaaggtgcagggccca ctaccgcttcctccagatgagctcatgggtttctccaccaagg aagttttccgctggttgaatgattctttccccgccctcctctcgc cccagggacatataaaggcagttgttggcacacccagccag cagacgctccctcagcaaggacagcagaggaccagctaag agggagagaagcaactacagacccccctgaaaacaaccc tcagacgccacatcccctgacaagctgccaggcaggttctct tcctctcacatactgacccacggcttcaccctctctccctgga aaggacaccatgagcactgaaagcatgatccgg
N2 TNF-α 238 allele	459	F - agacccccctcggaatcg/a R - gcactgaaagcatgatccgg	agacccccctcggaatc(g/a)gagcagggaggatggggag tgtgaggggtatccttgatgcttgtgtgtccccaactttccaaa tccccgcccccgcgatggagaagaaaccgagacagaaggt gcagggcccactaccgcttcctccagatgagctcatgggtttc tccaccaaggaagttttccgctggttgaatgattctttccccgcc ctcctctcgccccagggacatataaaggcagttgttggcacac ccagccagcagacgctccctcagcaaggacagcagaggac cagctaagagggagagaagcaactacagacccccccctgaa aacaaccctcagacgccacatcccctgacaagctgccaggc aggttctcttcctctcacatactgacccacggcttcaccctctct cccctggaaaggacaccatgagcactgaaagcatgatccgg
N2 TNF-α 376 allele	599	F - tcctgcatcctgtctggaag/a R – gcactgaaagcatgatccgg	tcctgcatcctgtctggaa(g/a)ttagaaggaaacagacca cagacctggtccccaaaagaaatggaggcaataggtttt gaggggcatggggacggggttcagcctccagggtccta cacacaaatcagtcagtggcccagaagacccccctcgga atcggagcagggaggatggggagtgtgaggggtatcctt gatgcttgtgtgtccccaactttccaaatccccgcccccgcg atggagaagaaaccgagacagaaggtgcagggcccact accgcttcctccagatgagctcatgggtttctccaccaagga agttttccgctggttgaatgattctttccccgccctcctctcgc cccagggacatataaaggcagttgttggcacacccagcca gcagacgctccctcagcaaggacagcagaggaccagcta agagggagagaagcaactacagacccccccctgaaaacaa ccctcagacgccacatcccctgacaagctgccaggcaggt tctcttcctctcacatactgacccacggcttcaccctctctccc ctggaaaggacaccatgagcactgaaagcatgatccgg

After optimizing each nest 2 (N2) reaction individually, a duplex reaction was designed, where each N2 amplification would include one of two sets of primers: 1) 238G + 376A; or 2) 238A + 376G. Providing for a competing reaction in each sample also allows for the reduction of false priming. As the G allele for both sites is dominant,

and previous authors have cited a positive linkage between the 238A and 376A alleles, it was expected that at least one reaction would be positive for each. Reaction conditions were 95 x 5m; 45 cycles of 95 x 30s, 48 x 45s, 72 x 60s; and 72 x 4m for the N1 reaction, and 95 x 5m, 32 cycles of 95 x 30s, 54 x 45s, 72 x 60s; and 72 x 4m for the N2 reaction on a MJ Research PTC-100 Programmable Thermal Cycler (Waltham, MA).

For the analysis of *Plasmodium* multi-species interactions and immune regulation, growth and clearance, a region towards the 5' end of the small sub-unit ribosomal RNA gene was selected. A nested PCR approach was used to increase the sensitivity of the assay, with the first PCR reaction designed to amplify an approximately 500 nucleotide segment, using genus specific primers designed to amplify a conserved region of the gene. A second, nested, reaction again used genus-specific primers to amplify an internal segment of 133-136 (depending on species) nucleotides. This site was selected to have a central region with species-specific variability. The conserved primers were:

```
Nest 1:

Forward primer (N1plF): 5′ – GCC TGA GAA ATA GCT ACC ACA T – 3′
Reverse primer (N1plR): 5′ – GTT GTT CAA TTT TGT TAT TCC ATG CT – 3′

Nest 2:

Forward primer (N2plF): 5′ – GCG CGT AAA TTA CCC AAT TCT – 3′
Reverse primer (N2plR): 5′ – CCA GAC TTG CCC TCC AAT T – 3′
```

Reaction conditions for nest 1 were 95C x 120s, 35 cycles of 95C x 10s, 54C x 20s, 72C x 30s; and for nest 2 were 95C x 120s, 35 cycles of 95C x 10s, 54C x 20s, 72C x 10s, using a Cepheid SmartCycler (Sunnyvale, CA) with fluorescent imaging at 54C. Using genus-specific primers allows for the amplification of multiple species of malaria maintaining the ratio of starting template. As a control, a 121 nucleotide segment of

homologous human small subunit ribosomal RNA gene was also amplified, using specific primers:

```
Forward primer (HssuF): 5′ – CGC AAA TTA CCC ACT CCC – 3′
Reverse primer (HssuR): 5′ – CCT CCA ATG GAT CCT CGT T – 3′
```

Reaction conditions were identical to the previous reaction stated. The resulting amplified segments are shown in Table 5.

Table 5. Small subunit ribosomal RNA gene PCR amplicon

Name	Size	Amplicon
Pv	133	gcgcgtaaattacccaattctaaagaagagaggtagtgacaagaaataacaatacaaggccaatctggctttgtaattgg aatgatgggaatttaaatccttcccataatacaattggagggcaagtctggtg
Po	135	gcgcgtaaattacccaattctaaagaagagaggtagtgacaagaaataacaatacaaggccatttcatggttttgtaatt ggaatgatgggaatttaaaaccttcccaaaattcaattggagggcaagtctggtg
Pm	135	gcgcgtaaattacccaattctaaagaagagaggtagtgacaagaaataacaatgcaaggccaaattttggttttgcaatt ggaatgatgggaatttaaaaccttcccagaaggcaattggagggcaagtctggtg
Pf	136	gcgcgtaaattacccaattctaaagaagagaggtagtgacaagaaataacaatgcaaggccaatttttggttttgtaatt ggaatggtgggaatttaaaaccttcccagagtaacaattggagggcaagtctggtg
H	121	cgcaaattacccactcccgacccggggaggtagtgacgaaaaataacaatacaggactctttcgaggccc tgtaattggaatgagtccactttaaatcctttaacgaggatccattggagg

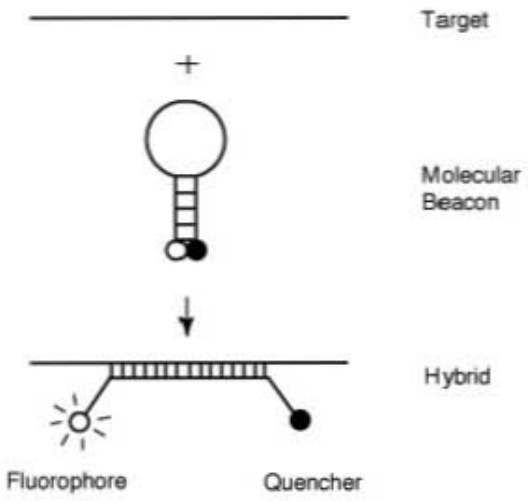
DNA Genotyping

Results from the cytokine gene analysis were examined using gel electrophoresis. A 1.5% agarose gel (Sigma, St. Louis, MO) was used to differentiate amplified products. Presence or absence of 459 and 599 base pair bands were scored compared to a ϕX174 DNA/*Hae* III Marker (Promega, Madison, WI) with 11 defined bands ranging in size from 72 base pairs (bp) to 1,353 bp, including a marker at 603 bp.

For the multiple species *Plasmodium* infection analysis, molecular beacon probes were used to quantitatively measure the presence (and ratio) of each malaria species, as well as human template present. Molecular beacon probes are hairpin-shaped molecules with covalently linked fluorophores. When the molecule is self-annealed the

configuration requires that the fluorophore is in close proximity to a quencher which thus limits background fluorescence. The loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem hybrid and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, and the fluorescence to be detected by the machine.

Figure 1. Hybridization of Molecular Beacon Probe



The variable region, located at the approximate midpoint of the amplified segment, (position 61-78 of the consensus sequence), shows a range of nucleotide substitutions and deletions:

```
Pf     5′- CAA TTT TTG GTT TTG TAA -3′
Pm     5′- CAA ATT TTG GTT TTG CAA -3′
Po     5′- CAT TTC ATG GTT TTG TAA -3′
Pv     5′- CAA TC— -TG GCT TTG TAA -3′
H      5′- TCT TTC GAG GCC CTG TAA -3′
```

Pairwise comparisons of the different species find 2 (Pf vs. Pm), 3 (Pf vs. Po), 4 (Pf vs. Pv) or 5 (Pm vs. Po; Pm vs. Pv; Po vs. Pv) nucleotide differences in this region. The homologous region of hypervariability in the human ssurRNA, has more than five nucleotide difference in comparison to each of the *Plasmodium* genes. Using this region of variability, specific probes were designed for each species present:

```
Pf: 5′-CCT(GCC AAT TTT TGG TTT TGT AAT TGG AAT GGT GG)C AGG
Pm: 5′-C(CA AGG CCA AAT TTT GGT TTT GCA ATT GGA ATG) CCT TGG
Po: 5′-C(CA AGG CCA TTT CAT GGT TTT GTA ATT GGA ATG) CCT TGG
Pv: 5′-CT(A GGC CAA TCT GGC TTT GTA ATT GGA ATG ATG G)CC TAG
H:  5′-GCG ACA (TCT TTC GAG GCC CTG TAA TTG GAA TG)T CGC
```

Sequences within parentheses represent hybridizing sequences. Probes were synthesized by MWG Biotech (High Point, NC) and Sigma Genosys (Woodlands, TX). Predicted confirmations (using the Zucker Mfold program, http://bioinfo.math.rpi.edu/) in the quenched, or self-annealing form and arm sequences are shown in Table 6, below.

Table 6. Predicted structures of the probes

Probe	Predicted structure
Pf	ATTTTTGGTTTT CCTGCCA \ GGACGGT G GGTAAGGTTAAT
Pm	CAAATTTTGGTT CCAAGGC \ GGTTCCG T TAAGGTTAACGT
Po	CATTTCATGGTT CCAAGGC \ GGTTCCG T TAAGGTTAATGT
Pv	ATCTGGCTTTG CTAGGCCA \ GATCCGGT T AGTAAGGTTAA

Validation of TNF-α results

In addition to the repetition of the assay to ensure reproducible results, validation of the results from the cytokine gene analysis was undertaken using two separate techniques: molecular beacon probes for the –238 site and restriction enzyme analysis for the –376 site.

For the –238 polymorphic site, a N2 reaction amplified an 84 nucleotide segment centering on the –238 position. Specific molecular beacon probes were designed for each allele. Reaction conditions were 95C x 120s, and 35 cycles of 95C x 10s, 52C x 30s, 72C x 15s using a Cepheid SmartCycler. Specific sequences were:

238G: TET -(aga)ctcggaatcggagcagggag(tct)-DABCYL
238A: FAM-(aga)ctcggaatcagagcagggag(tct)-DABCYL

For the G allele, the predicted structure of the probe was:

GGAATC
AGACTC G
TCTGAG G
GGACGA

The A allele probe is identical, with the exception of the A nucleotide corresponding to the SNP(*):

GGAATC
AGACTC A*
TCTGAG G
GGACGA

For the –376 allele, restriction enzyme analysis was used. Maintaining the original N1 reaction, a new set of N2 primers were designed to amplify a 627 bp segment. Reaction conditions were 95C x 5m, 40 cycles of 95C x 30s, 52C x 45s, 72C x 60s; and

72C x 4m using a MJ Research PTC-100 Programmable Thermal Cycler (Waltham, MA). By choosing conserved primers downstream from the polymorphism selection bias is neutralized.

The presence of the "A" allele in position –376 creates the sequence "aatt" which is recognized by the restriction enzyme Tsp509I (New England Biolabs, Beverly, MA) which cuts double stranded DNA prior to this sequence (/aatt) when incubated at 65 degrees for ≥ 1 hour. The presence of the "G" allele in this position precludes the DNA from being cut. The cut sequence would be 581 bp in length and can be distinguished from the 627 bp uncut sequence using 1.5% gel electrophoresis (Sigma, St. Louis, MO). The presence of a single band indicated a homozygous genotype (at either position) whereas two bands indicated a heterozygous genotype. One quarter of samples were validated using these techniques. Specific primers and amplicon for the validation at the --238 and –376 sites is summarized in Table 7.

Table 7. TNF-α gene validation

Name	Size	Primers	Amplicon
TNFα -238	84	F – cagtcagtggcccagaag R – cttgatgcttgtgtgtcc	cagtcagtggcccagaagacccccctcggaatc(g/a)gagcagggaggatggggagtgtgaggggtatccttgatgcttgtgtgtcc
TNFα -376	627	F – gcccctcccagttctagttctatct R – gcactgaaagcatgatccgg	gcccctcccagttctagttctatctttttcctgcatcctgtctggaa (g or a) ttagaaggaaacagaccacagacctggtccccaaaagaaatggaggcaataggtttgaggggcatggggacggggttcagcctccagggtcctacacacaaatcagtcagtggcccagaagacccccctcggaatcggagcagggaggatggggagtggagggtatccttgatgcttgtgtgtccccaactttccaaatccccgcccccgcgatggagaagaaaccgagacagaaggtgcagggcccactaccgcttcctccagatgagctcatgggtttctccaccaaggaagttttccgctggttgaatgattctttccccgccctcctctcgcccccagggacatataaaggcagttgttggcacacccagccagcagacgctccctcagcaaggacagcagaggaccagctaagagggagagaagcaactacagacccccctgaaaacaaccctcagacgccacatcccctgacaagctgccaggcaggttctcttcctctcacatactgaccacggcttcaccctctctcccctggaaaggacaccatgagcactgaaagcatgatccgg

Validation of ssurRNA gene amplification

Results from the multiple species and the immune regulation and parasite growth analyses were validated through several methods. Positive control samples were developed for *Plasmodium falciparum* and *Plasmodium vivax* species by spotting calculated quantities of parasite from culture (*P.f.*) and from infected monkeys (*P.v.*) in uninfected blood. In addition a range of positive and negative samples of *P. falciparum* from culture were provided by Dr. Ted Hall, WRAIR, in a blinded test of the sensitivity of the assay. Each species was also cloned using a Promega MiniPrep and pGEM-T Easy Vector cloning kit (Promega, Madison, WI). Clones were sequenced to confirm identity and the cloned plasmids were serially diluted from 10^7 to 10^0 copies per sample to develop a standard curve defining the threshold for detection versus the initial quantity of DNA template.

All samples were tested twice in the presence of positive controls (of both cultured *P. falciparum* added to blood in known concentrations, and ssu rRNA clones) and negative controls (both no-template controls and non-infected blood controls).

Amplified products were also separated by agarose gel electrophoresis (stained with ethidium bromide) and compared to DNA products of standard length to determine if the products were of appropriate size. Additionally, a subset of samples was monitored across a temperature gradient to examine melting point peaks for the main product present. Samples showed the anticipated size (based upon primer design) and specific melting temperature.

Statistical analyses

A range of parametric and non-parametric statistical analyses techniques was used to analyze results. The relationships between cytokine genotype and several measures of resistance to *P. falciparum*, (including time to reappearance of parasitemia, mean parasitemia, and frequency of detectable parasitemia) was first examined. Time to reappearance of parasitemia was examined with Kaplan-Meier models (group differences evaluated with log rank test). Mean parasitemia and frequency of parasitemia were evaluated with Student's t-test for dichotomous phenotypes (TNF-α and IL-6) and ANOVA for phenotypes with three levels (IL-10, IFN-γ and TGF-β). Regression analysis was used for the examination of multiple genotypes.

The relationship between cytokine phenotype and cytokine production in response to LSA-1 polypeptides and PHA was examined using Student's t-test for dichotomous genotypes and ANOVA for genotypes with three levels. The distribution of cytokine phenotypes in the cohort of Kenyan males was compared to the phenotype distribution of other populations reported in the literature by X^2 analyses and each genotype was measured to assess Hardy-Weinberg equilibrium.

Cytokine responses, mean parasitemia and frequency of parasitemia required $\log_e$ transformation (ln(value+1)) to obtain normal distributions. Potential confounding of immunologic variables and cytokine phenotypes by age, ABO blood group, or hemoglobin phenotype was explored with contingency table analyses, ANOVA, and multivariate linear regression where appropriate.

Analyses were performed using blood smears taken over the entire season of follow-up (raw data) or using only those blood smears taken before first treatment with

quinine and doxycycline (adjusted data). Analyses of both adjusted and raw data yielded similar results; therefore, only results for the adjusted data are presented here. All analyses were performed with SPSS 10.0 for Windows.

Quantitative values for the multiple species infections were calculated based upon a comparison to standard curves developed for the cloned species. Adjustments were made for the variable quantity of blood spotted and the variable efficiencies of DNA extraction by using the human small subunit ribosomal RNA gene as an internal standard.

Chapter 3: Cytokine Promoter Polymorphisms

Introduction

Multiple human genes which affect susceptibility to *Plasmodium* spp. infection, growth and clinical outcomes have been identified over the last fifty years (7, 70-72, 110-117), starting with the discovery of sickle cell anemia by Haldane (118). Specific genes identified include those that encode hemoglobin and red cell variants, (such as thalassemias (119-120) and sickle hemoglobin (HbS) (121), hemoglobin (Hb) E (122-123), HbC (124), glucose-6-phosphate dehydrogenase (G6PD) (10), and erythrocyte Band $3^{400\text{-}408}$ responsible for Southeast Asian ovalocytosis (110-111)). Other human genes identified include those of the MHC encoding HLA which play a critical role in the function of the immune system, including: HLA-DRB1*1302 and HLA-B53, tumor necrosis factor (TNF-α)(72), as well as intercellular adhesion molecule 1 (ICAM-1, CD54) (114-116), and inducible nitric oxide synthase (iNOS) (112-113, 125).

The inclusion of TNF-α among this list is unsurprising given the evidence of the role of a range of cytokines (including TNF-α, IL10, and IL6), in severe malarial anemia and/or cerebral malaria. Although circulating levels of a number of cytokines have been examined for their associations with malaria susceptibility, no research has been published investigating the potential association between malaria outcomes and cytokine alleles other than those of TNF-α.

Results

Allele frequencies

Genotyping data from volunteers was compared to other populations for single nucleotide polymorphisms in TNF-α (Table 8-10), IFN-γ (Table 11), IL-6 (Table 12) genes, and haplotypes of TGF-β (Table 13) and IL-10 (Table 14) genes. The distribution of genotypes or putative cytokine expression phenotypes (i.e., expression levels predicted by genotypes) in this Kenyan cohort differed from the distributions reported in other populations for all cytokines except TNF-α –238 (Table 8).

Fewer than 1% of subjects were found to be homozygous, and 14% of subjects were found to be heterozygous for the 'A' allele at the –308 TNF-α site (Table 9). This frequency was found to be similar to African-American populations, and Kenyan, Tanzanian, and Gabonese (mild malaria) infants, but different from Gabonese (ill, not malaria) and Gambian children. Compared to Caucasian populations, a significant ($p=.001$) difference was noted overall, with an increasing frequency of the 'A' allele associated with northern European populations.

For the TNF-α –376 genotype, again less than 1% of volunteers were found to be homozygous, and 10.6% were found to be heterozygous for the 'A' allele (Table 10). This frequency was significantly different from Kenyan, Gabonese and Gambian populations surveyed ($p<.02$), as well as Caucasian populations ($p<.001$), all of whom reported lower frequencies.

Insufficient data was available for comparisons to African populations in malaria endemic regions for the remaining genes (Tables 11 – 14), however, data was compared with previously reported frequencies from US or European black, white, and Indian

populations.

For the IFN-γ +874 polymorphic site, the frequency of homozygous 'T' genotype among volunteers was 2% and the frequency of heterozygous 'A/T' genotype was 20.8% (Table 11). This frequency was significantly ($p<.001$) lower than both USA black or USA or UK white populations. An even more significant bias was seen in IL-6 genotypes with a frequency of the homozygous 'C' genotype among volunteers (at 0%) and the frequency of heterozygous 'C/G' genotype (1.3%) (Table 12). These frequencies were significantly different from all populations identified, including: UK Afro-Caribbean ($p=.03$), USA black ($p=.002$), and UK Indian ($p<.001$) populations; and UK and USA white populations ($p<.001$).

Frequencies for TGF-β and IL-10 haplotypes also found differences between the present study and reported rates for USA black ($p=.02$, for TGF-β) and UK white ($p<.001$, for IL10) (Table 13 and 14). The frequency of high TGF-β phenotypes in the present study was found to be 71% compared to 64% among USA blacks, and approximately 76% among USA and UK white populations. The frequency of low IL-10 haplotypes was found to be 36% in the present study compared to 37% in USA blacks, and 28% in USA and UK white populations.

Overall, differences in genotype or haplotype frequency were greatest in comparison to Caucasian populations. Differences were also significant in comparison to populations of West African ancestry, such as Afro-Caribbeans and African Americans, for IFN-γ, IL-6, and TGF-β, but not for TNF –308 and IL-10. African Americans in eastern seaboard urban areas are commonly of West African ancestry with ~30% Caucasian admixture [128].

Hardy-Weinberg analysis was conducted to assess the degree of equilibrium among genotypes. All genetic polymorphisms were found to be in equilibrium.

Table 8. Frequency (%) of TNF-α –238 genotypes and A allele in the present study and in comparison populations.

		Seasonal/ intermediate Transmission						No modern transmission		
Genotype or allele	This study n=148	Kenya child [71] n=311	Gabon child– mild malaria [76] N=87	Gambia child– ill, not malaria [70] n=371	Gambia child– mild malaria [70] n=349	Vietnam adult– health [126] n=76	Vietnam adult– ill, not malaria [126] n=206	Spain white adult– healthy [127] n=324	Holland adult– healthy [23] n=116	Holland white adult– healthy [51] n=403
G/G	88.5	NR	88.5	87.2	88.5	89.5	90.8	84.3	90.5	91.8
G/A	10.8	NR	11.5	12.1	10.9	10.5	9.2	14.5	9.5	8.2
A/A	0.7	NR	0.0	0.8	0.6	0.0	0.0	1.2	0.0	0.0
A	6.1	7.4	5.6	6.9	6.0	5.3	4.6	8.5	4.7	4.1

NR –not reported.

Table 9. Frequency (%) of TNF-α –308 genotypes and A allele in the present study and in comparison populations.

			Perennial/high transmission		Seasonal/ intermediate transmission[a]					No modern transmission[b]			
Genotype or allele	This study n=150	USA black adult– healthy [128] n=64	Kenya infant [75] n=1048	Tanzania infant [129] n=204	Gabon child– mild malaria [76] n=87	Gambia child– ill, not malaria [72] n=325	Gambia child– mild malaria [72] n=332	Gambia adult – healthy [130] N=141	Gambia adult – ill, trachoma [130] n=141	Spain white adult– healthy [127] n=324	Germany white adult [131] n=153	Holland white adult– healthy [132] n=54	Holland adult– healthy [23] n=116
G/G	85.3	85.9	82.9	82.8	85.3	69.0	71.4	81.6	71.6	75.6	67.3	57.4	56.6
G/A	14.0	12.5	15.3	16.2	14.7	29.8	26.8	17.0	24.1	23.1	28.1	35.1	38.6
A/A	0.7	1.6	1.8	1.0	0	1.2	1.8	1.4	4.2	1.2	4.6	7.4	4.5
A	7.7	7.8	9.5	9.1	7.4	16.1	15.2	9.9	16.3	12.3	18.7	25.0	23.8

[a]p=.046, weighted frequency A allele of seasonal/intermediate transmission vs. present study.
[b]p=.001, weighted frequency A allele of no modern transmission vs. present study.

Table 10. Frequency (%) of TNF-α –376 genotypes and A allele in the present study and in comparison populations.

		Seasonal/intermediate transmission[a]					No modern transmission[b]		
Genotype or allele	This study n=150	Kenya child [71] n=311	Gabon child–mild malaria [76] n=87	Gambia child–ill, not malaria [71] n=371	Gambia child–mild malaria [71] n=349	Gambia adult–healthy [133] n=239	Spain white adult–healthy [127] n=324	Holland adult–healthy [23] n=116	Holland white adult–healthy [51] N=403
G/G	88.7	NR	90.8	97.3	96.0	95.0	91.0	97.8	99.0
G/A	10.6	NR	9.2	2.4	3.7	5.0	8.3	2.2	1.0
A/A	0.7	NR	0.0	0.3	0.3	0.0	0.5	0.0	0.0
A	6.0	4.2	4.6	1.5	2.2	2.5	4.7	1.1	0.5

NOTE. NR – not reported

[a]$p<.02$, weighted average frequency vs. present study.

[b]$p<.001$, weighted average frequency vs. present study.

Table 11. Frequency (%) of IFN-γ +874 genotypes and T allele in the present study and in comparison populations.

Genotype or allele	This study n=149	USA black– healthy and renal disease [134] n=43	USA white– healthy and renal disease [134] n=102	UK white– healthy and cadaveric donors [35] n=164
T/T	2.0	7.0	20.6	20.7
A/T	20.8	55.8	53.9	54.3
A/A	77.2	37.2	25.5	25
T	12.4	34.9[a]	47.6[a]	47.9[a]

[a] p<.001 compared to present study.

Table 12. Frequency (%) of IL-6 –174 genotypes and C allele in the present study and in comparison populations.

Genotype or allele	This study n=149	UK Afro-Caribbean [38] n=101	USA black– healthy and renal disease [134] n=43	UK Gujarati Indian [38] n=115	UK white [135] n=120	USA white– healthy and renal disease [134] n=102	UK white– healthy and cadaveric donors [38] n=383
G/G	98.7	91.1	81.3	73.9	33.3	45.1	37.6
C/G	1.3	8.9	18.6	24.3	42.5	39.2	44.1
C/C	0.0	0	0.0	1.7	24.2	15.7	18.2
C	0.7	4.5[a]	9.3[b]	13.9[c]	45.5[c]	35.3[c]	40.3[c]

[a] p=.03 compared to the present study.
[b] p=.002 compared to the present study.
[c] p<.001 compared to the present study.

Table 13. Frequency (%) of TGF-β haplotypes in the present study and in comparison populations.

Haplotype	Putative phenotype	This study n=150		USA black [a]– healthy and renal disease [134] n=45		USA white– healthy and renal disease [134] n=102		UK white– healthy and cadaveric donors [35] n=107	
TG/TG	High	32.0	71.3	31.1	64.4	26.5	75.5	41.1	76.6
TG/CG		39.3		33.3		49.0		35.5	
TG/CC	Intermediate	4.7	23.4	6.7	20.0	8.8	18.6	12.1	16.8
CG/CG		18.7		13.3		9.8		4.7	
TG/TC		0.0		0.0		0.0		0	
CG/CC	Low	4.7	5.4	11.1	15.5	4.9	5.9	5.6	6.5
CC/CC		0.0		0.0		1.0		0.9	
TC/TC		0.0		4.4		0.0		0	
TC/CC		0.7		0.0		0.0		0	

[a] p=.024 compared to the present study.

Table 14. Frequency (%) of IL10 haplotypes in the present study and in comparison populations.

Haplotype	Putative phenotype	This study n=150		USA black– healthy and renal disease [134] n=41		USA white– healthy and renal disease [134] n=101		UK white [a]– healthy and cadaveric donors [35] n=330	
GCC/GCC	High	10.7	10.7	2.4	2.4	16.8	16.8	30.0	30.0
GCC/ACC	Intermediate	20.0	53.3	17.0	61.0	29.7	54.5	20.6	41.8
GCC/ATA		33.3		43.9		24.8		21.2	
ACC/ACC	Low	6.0	36.0	7.3	36.6	5.9	28.6	8.2	28.2
ACC/ATA		15.3		19.5		16.8		12.4	
ATA/ATA		14.7		9.8		5.9		7.6	

[a] p<.001 compared to the present study.

Linkage analysis

In addition to examining frequencies of individual cytokine gene polymorphisms, the association of specific genotypes was assessed through linkage analysis. Pairwise analysis of linkage between alleles found that only TNF –238A and TNF –376A were significantly associated ($p<.001$). This finding has previously been reported as one of complete linkage, with –376A only occurring in a subset of individuals who possess –238A (72).

Additionally, the most common genotype, found among 94 of 149 subjects (63%), was TNF –308GG, IFN-γ +874AA, and IL-6 –174GG. Individuals with this genotype were significantly more likely to have an IL-10 genotype associated with high expression than individuals without this genotype (15% vs. 4%, $p=.03$).

Association with *in vitro*, parasite and clinical outcomes

The presence of cytokine gene polymorphisms was compared to a wide range of previously collected measures of cytokine production, parasitemia, and clinical outcomes. No association was found between cytokine production by PBMCs and putative expression phenotypes of TNF-α, IL-10 or IFN-γ.

In the third season, individuals with the TNF –308A allele (n=17) had a 40% increased frequency of parasitemia (40% vs. 29%; $p=0.02$), and became reinfected more rapidly after treatment ($p=0.014$) than individuals without this allele (n=99). This association was not observed in the other two transmission seasons. Individuals with the TNF-α –308GG/IFN-γ +874AA/IL-6 –174GG genotype were found to be infected less frequently but to have higher mean parasite density each season, although this trend did not achieve significance in all seasons. No relationship between other cytokine alleles and measures of parasitemia or anemia

(hemoglobin and ferritin) was detected.

Discussion

Better understanding the pathogenesis of severe disease due to malaria is crucial for the development of novel therapies and control strategies. By examining the frequency of cytokine genotypes in a cohort of young Kenyan men from an area of intense malaria endemicity, a comparison can be made to other populations from both malaria endemic and non-endemic regions to explore the potential influence of specific host genotype factors and malaria susceptibility.

In comparison to Caucasian populations, the frequencies of all cytokine alleles in the study population were significantly different except those of TGF-β and TNF –238. Because the TNF –238A allele has been associated with severe malarial anemia, it was surprising that this allele was not biased in a population for which severe anemia is a major cause of childhood mortality. On the one hand, an increased frequency might have implicated TNF –238A as a factor in the extremely high rates of severe anemia observed among young children in this study area. On the other hand, the mortality ensuing from severe anemia in this area of high malaria transmission would be expected to select against an allele contributing to susceptibility. All cytokine gene polymorphisms including TNF –238 alleles were in Hardy-Weinberg equilibrium and therefore did not provide evidence for selective mortality in the current cohort, although small deviations might not be detected in this sample of 150 young men.

Frequencies of TNF –308 and –376 alleles in this cohort differed significantly from some but not all West African and European comparison populations. For example, the frequency in

Kenyan populations of the –308A allele previously associated with cerebral malaria was similar to its frequency in healthy Gambian adults and Gabonese children with mild malaria, but significantly lower than European populations and Gambian populations with malaria or other illnesses. Conversely, the frequency of TNF –376A allele previously associated with cerebral malaria was higher in this population than all other African and European populations previously examined, but the differences were not consistently significant in pairwise comparisons. Further, TNF –376A allele frequencies did not vary in a fashion that distinguished East and West Africans.

As found in previous studies (75, 129), there was no consistent relationship between polymorphisms and measures of malaria infection. This is perhaps unsurprising, as this population of young men has acquired substantial immunity that controls most episodes of parasitemia. On the other hand, if cytokine promoter polymorphisms modulate disease rather than parasitemia, as has been observed with non-immunologic resistance genes such as hemoglobin S (34), a difference in clinical outcomes would be expected, although again the effect may be limited due to the age and exposure of the cohort (136).

There were dramatic differences in frequency between the present study and comparison populations for IL-6 and IFN-γ alleles. Polymorphisms in these cytokine genes have not been previously examined for associations with malaria outcomes. The striking bias favoring the IL-6 –174G allele over –174C made it difficult to conduct any statistical testing related to malaria outcomes. Elevated plasma IL-6 levels have been associated with severe disease and death due to malaria (68, 137), and the IL-6 –174C allele has been associated with increased in vivo expression of IL-6 in neonates (138) and adults (139) during acute phase reactions. Selection against the IL-6 –174C allele could result from malaria-related mortality early in life, although

disequilibrium suggesting selection was not detected by Hardy-Weinberg analysis in this young adult population.

Compared to Caucasian and African-American groups, this study population was also profoundly skewed toward an IFN-γ genotype previously associated with low cytokine expression (61). High plasma levels of IFN-γ have been associated with severe malaria (68) and with pregnancy malaria-related low birthweight (109). Thus, an allele that decreases IFN-γ expression could be favorably selected as a consequence of both pregnancy malaria and childhood malaria.

In particular, IL-6 and IFN-γ may play roles in the development of severe anemia in malaria-exposed populations. In a study of *in vitro* cytokine expression by immunocytes collected from an endemic area of Malawi, chronic iron deficiency was most strongly associated with elevated production of IL-6 and IFN-γ (140). IL-6 causes transferrin receptor density on hepatocytes to increase (141-142), ferritin synthesis to increase, and transferrin synthesis to decrease (141). In patients with anemia resulting from multiple organ dysfunction syndrome, the reticulocyte response to erythropoietin is inversely correlated with IL-6 levels (143). IFN-γ increases ferritin H-chain gene transcription (144), and also inhibits the growth of erythroid precursors in vitro (145-146). Together, IL-6 and interferon-γ may inhibit erythropoiesis either directly or by diversion of iron from erythroblasts to other cellular stores.

One constraint of this study is the limitation of examining polymorphisms in exclusion of other genetic information. Additional cytokine gene polymorphisms (for example IL-6 –597, –572, and –373) could not be genotyped. Another constraint was the absence of data on IL-6 and IFN-γ allele frequencies from a wider range of exposed populations.

Although no association was found between any cytokine genotype and cytokine

production in response to stimulation with malaria antigens, the lack of concordance between putative expression phenotypes and *in vitro* production may reflect difficulty in determining cytokine levels at only two sampling times, variable intercurrent illness in the host, or strong relationships between cytokine levels and differential exposure to *P. falciparum*.

Conclusions

In summary, this study examined cytokine genotype frequencies in a young adult cohort from an area where severe malarial anemia is frequent and cerebral malaria is uncommon during childhood. The TNF –238A allele previously associated with severe malarial anemia occurred at a frequency in this population that is similar to its frequency in West African and Caucasian populations. Profound biases exist in the distribution of IL-6 and IFN-γ genotypes in comparison to populations of European and West African ancestry, and these genotypes have been previously associated with low levels of expression for both cytokines. Further work in different geographic areas is needed to confirm whether these biases in IL-6 or IFN-γ allele frequencies contribute to the relative frequency of anemia or cerebral malaria in different populations.

Chapter 4: Multi-species interactions

Introduction

The four human malaria species differ greatly in their clinical manifestations, transmission potential and the biological and immune responses elicited with the human host. Niche differences among *Plasmodium* species suggest that interactions have been important in shaping the evolution and ecology of this genus. For example, two species preferentially invades reticulocytes (*P.o.*, *P.v.*), while one prefers mature cells (*P.m.*). One has large exoerythrocytic schizonts and multiplies rapidly in the blood (*P.f.*) and another multiplies more slowly (*P.m.*). Two species have latent liver forms (*P.o.*, *P.v.*) while two do not (*P.f.*, *P.m.*). Despite these significant differences, and evidence of the interactive effects of mixed malaria infections, the epidemiology and influence of mixed species infections in sub-Saharan Africa is poorly characterized and incompletely understood. Interactions between different parasites simultaneously infecting the same individual could therefore be imagined to produce significant changes in the course of the infection, and the potential to produce disease.

Field studies measuring the cross-sectional prevalence of malarial species often record fewer mixed infections than expected by chance, suggesting that one parasite has excluded another, or suppressed its parasitemia to undetectable levels (summarized in 77; 78). Longitudinal studies have shown the possibility that malarial parasites may inhibit or favorably affect the host environment for each other, as shown by the recrudescence of a latent malarial species immediately before or after the parasitic wave of another species (77).

Despite the potential importance of better understanding mixed species parasite interactions in terms of both basic biology and preventive approaches, research into the pattern of mixed-species infections and the relationship between mixed-species infection and clinical outcomes has been limited and contradictory. Few reports of the degree of mixed species infections and their impact on the course of infection or clinical outcomes have been reported from sub-Saharan Africa. In addition, differences in experimental approaches and human and vector population characteristics have limited the comparative value of what results have been reported, as have the technical challenges of measuring low-level mixed infections (either microscopically or by PCR).

Results

Summary of results from blood smear data:

An average of approximately 350 male subjects aged 12-35 years were followed over three consecutive malaria seasons in three rural villages in a highly endemic region of western Kenya (Table 15).

Table 15. Description of cohort

Season	**Year**	**Month**	**Transmission Intensity**	**# Subjects**	**# Observations**	**# weeks follow-up**
1	1996	April – July	High	310	3348	10.8
2	1996	Aug. – Nov.	Low	453	5755	12.7
3	1997	April – July	High	289	4546	15.7

At the start of each season, prior to treatment of all subjects, nearly one-half were found to be positive for *Plasmodium falciparum*, less than 1% were found to be positive for *Plasmodium ovale*, and a range of 1-3% were found to be positive for *Plasmodium malariae*. No cases of *Plasmodium vivax* were reported (Table 16). Over the course of the follow-up

period, cumulative incidence of *Plasmodium* spp. infections were calculated: nearly 95% were found to have had at least one *Plasmodium falciparum* positive blood smear in the 'high' seasons of season 1 and 3, and nearly 8 in 10 were found to have had at least one *Plasmodium falciparum* positive blood smear in the 'low' season classified as season 2. By contrast, rates of *Plasmodium ovale* infection ranged from 2-5% across the three seasons, while rates of *Plasmodium malariae* infection varied from 2-10%.

Table 16. Blood smear results

Baseline	**% *P.f.* + (n)**	**% *P.o.* + (n)**	**% *P.m.* + (n)**	**N**
S1	48.1 (149)	0.0 (0)	1.0 (3)	310
S2	46.2 (207)	0.4 (2)	2.0 (9)	448
S3	44.7 (127)	0.7 (2)	3.2 (9)	284
Cumulative	**% *P.f.* + (n)**	**% *P.o.* + (n)**	**% *P.m.* + (n)**	**N***
S1	93.4 (227)	2.1 (5)	2.1 (5)	243
S2	78.7 (280)	2.5 (9)	5.1 (18)	356
S3	95.5 (253)	4.9 (13)	9.8 (26)	265

* Analysis of % positive parasitemia for the cumulative time period was restricted to individuals with a minimum of 4 post-treatment follow-up visits (blood smears).

Presence of mixed infections

A small number of individuals were found to have simultaneous or sequential mixed species malaria infections (Table 17). Across each season, and with each type of mixed infection, it was more common to find simultaneous mixed species infections, than sequentially positive individual infections.

The most common mixed infection was *P.f.*+/*P.m.*+, although *P.f.*+/*P.o.*+ was also common in the third observed season. No dual infections with *P.o.*+/*P.m.*+ were found, and only one triply infected blood smear was noted.

Table 17. Number of individuals with evidence of simultaneous and sequential mixed-species malaria positive blood smears, by type.

Classification	**Season**	***P.f.*+/*P.o*+**	***P.f.*+/*P.m.*+**	***P.o.*+/*P.m.*+**	***P.f.*+/*P.o.*+/*P.m.*+**	**Total**
Simultaneous	S1	3	5	0	1	243
	S2	3	14	0	0	356
	S3	11	19	0	0	265
Sequential	**Season**	***P.f.*+/*P.o*+**	***P.f.*+/*P.m.*+**	***P.o.*+/*P.m.*+**	***P.f.*+/*P.o.*+/*P.m.*+**	**Total**
	S1	1 (*P.f.*→ *P.o.*)	0	0	0	243
	S2	3 (2 *P.f.* →*P.o.*) (1 *P.o.*→*P.f.*)	1 (*P.f.*→ *P.m.*)	0	0	356
	S3	2 (*P.f.* → *P.o.*)	9 (*P.f.* → *P.m.*)	0	1 (*P.f./P.o*→*P.f./P.m.*)	265

Note: Some individuals reported mixed infections at more than one time-point, however the above table reflects only the number of individuals, not the number of timepoints.

Timing/pattern of mixed infections

In all but one case of sequential infection with different malaria species, patent *Plasmodium falciparum* infection was seen first. In the second season, three individuals were found to have only *Plasmodium malariae* infection throughout the season, and one individual was found to have only *Plasmodium ovale*.

For those individuals with simultaneous mixed infections, the timing of the positive blood smear is described in Table 18 below. As this table illustrates, mixed species infections were more commonly seen after week five. Few individuals with simultaneous infections were found to have such infections across multiple seasons. An individual found to have either *P.m.* or *P.o.* in one season was no more likely to have *P.m.* or *P.o.* in an additional season than any one else ($p>0.05$). However, within a specific season, multiple occurrences of mixed infections were seen. For example, in season three, four individuals were found to be *P.f.*+/*P.m.*+ on more than one occasion. In the same season, one individual was found to be *P.f.*+/*P.o.*+ on two occasions.

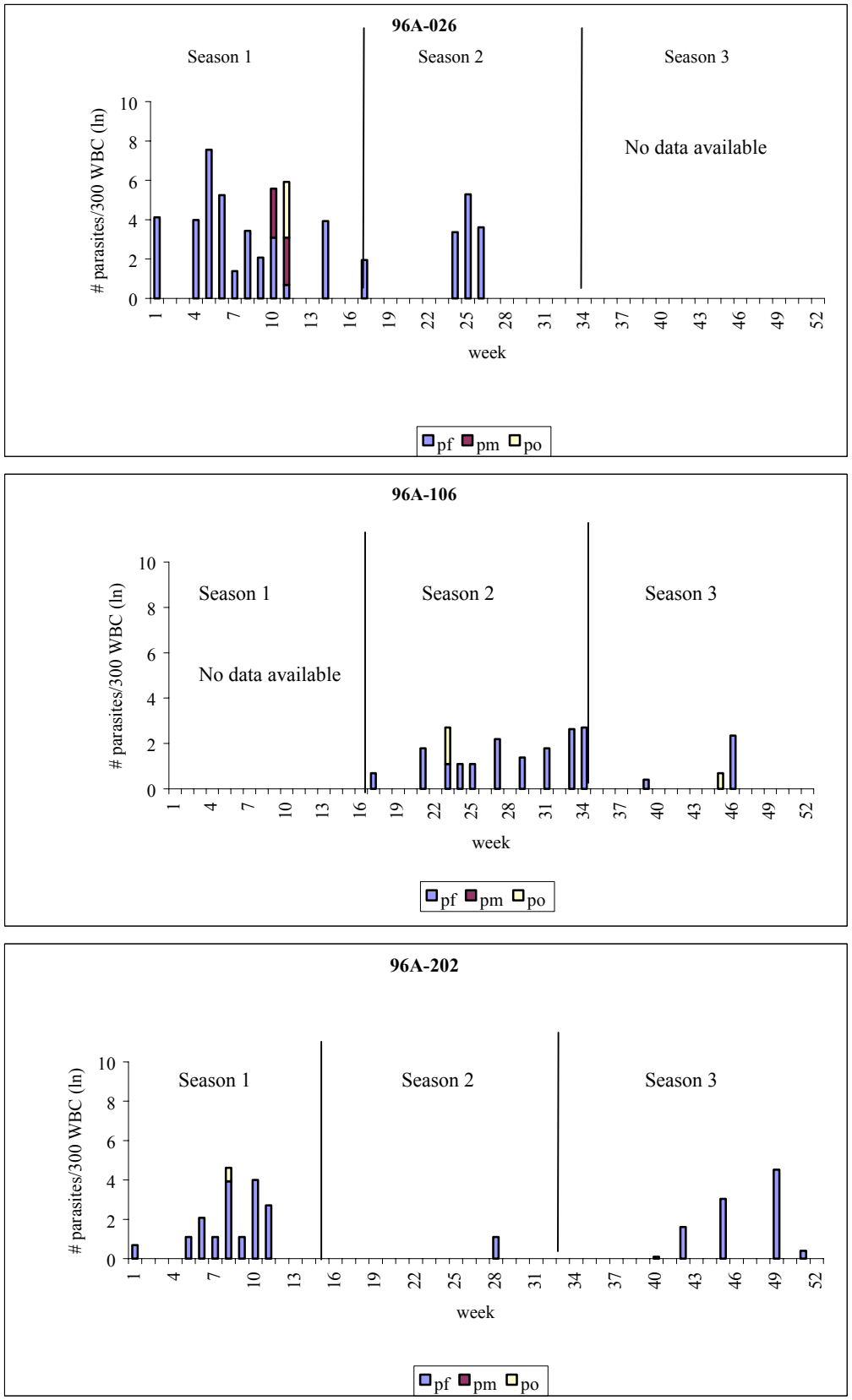
Figure 2 provides a more in-depth examination of the patterns of infections in those individuals (n=11) who acquired either *P.m.* or *P.o.* infection and had data from at least two seasons. Two general observations can be noted:

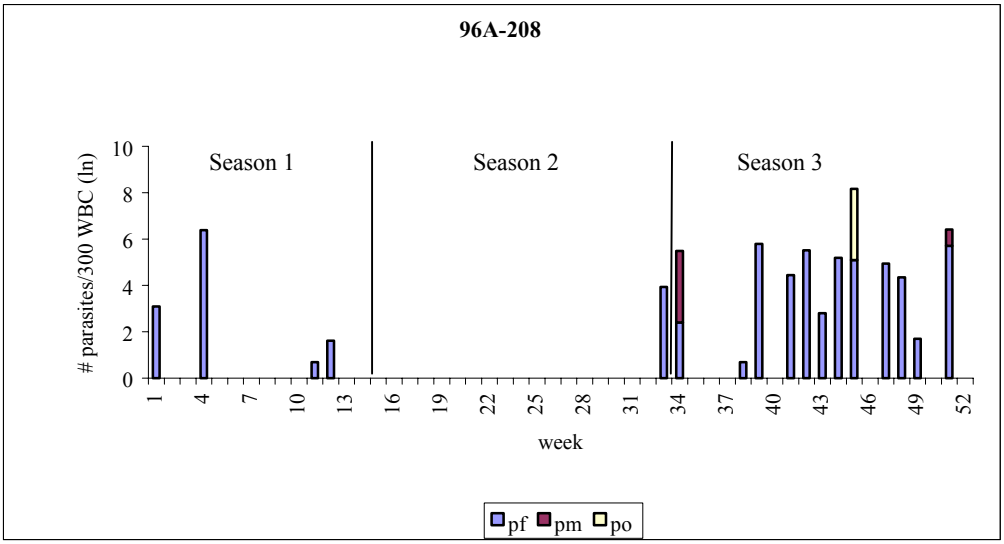
- In most cases (80%), the blood smear with a mixed infection represents the highest rate of parasitemia for the individual for that season.
- Three patterns of mixed infections are noted, each nearly equally represented: 1) those that emerge immediately after multiple positive *P. falciparum* blood smears; 2) those seen either immediately or some weeks following a single *P. falciparum* infections; and 3) those seen as the first positive blood smear in a season.

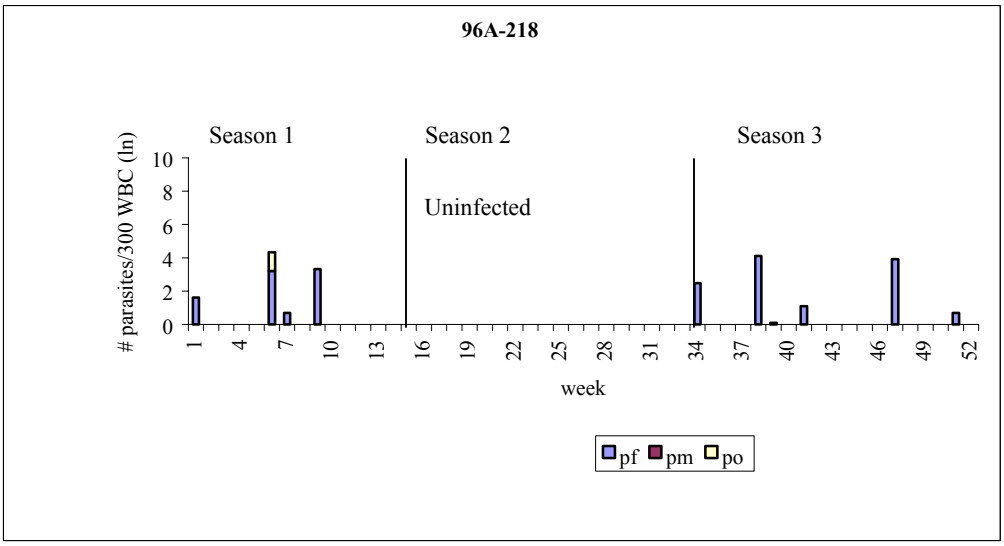
Table 18. Mixed malaria infectious by season and week follow-up.

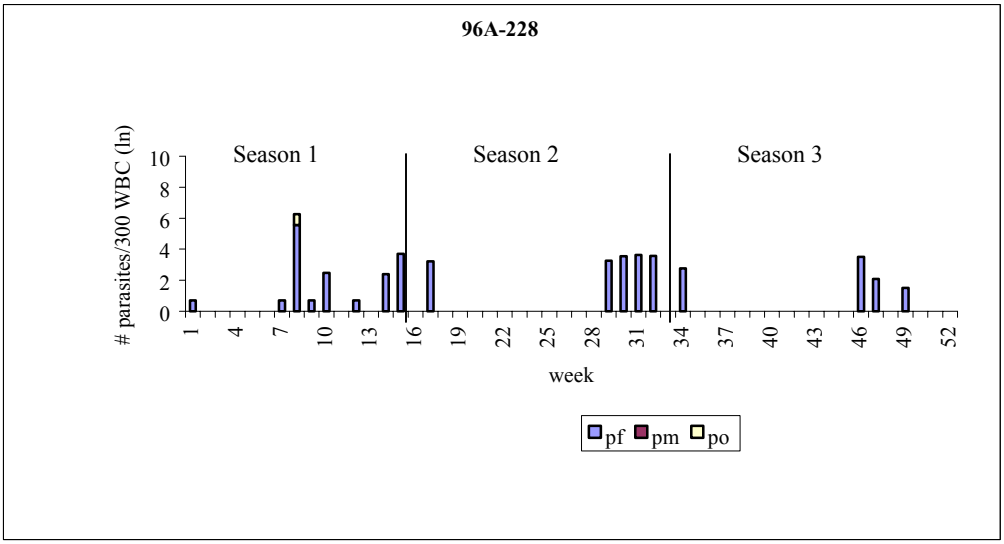
Week Number	1996 (Season 1: 96A cohort only)				1996 (Season 2: 96A and 96B cohorts)				1997 (Season 1: 96A and 96B cohorts)			
	P.f./P.m./P.o.	*P.f./P.m.*	*P.f./P.o.*	*P.m./P.o.*	*P.f./P.m./P.o.*	*P.f./P.m.*	*P.f./P.o.*	*P.o./P.m.*	*P.f./P.m./P.o.*	*P.f./P.m.*	*P.f./P.o.*	*P.o./P.m.*
0		96A-005 96A-085 96A-133				96A-274 96B-003 96B-031 96B-036 96B-057 96B-128 96B-228 96B-249	96A-163			96A-021 96A-106 96A-120 96A-130 96B-078 96A-208	96B-030 96A-163	
1												
2										96A-074		
3												
4												
5		96A-288	96A-218									
6							96A-106					
7			96A-202 96A-228								96A-274 96B-106	
8										96B-120	96A-086 96B-255	
9		96A-026				96B-144 96B-224 96B-252						
10	96A-026						96A-297			96A-135 96B-175 96B-120	96A-139 96A-240	
11						96B-138 96B-193				96B-246	96A-208	
12						96B-056				96A-164		
13										96B-052 96B-057 96A-164		
14										96A-034	96A-283	
15										96A-164	96A-163	
16												
17										96B-082 96A-208	96B-120	
18										96A-133 96A-134 96B-205 96B-246 96A-164		

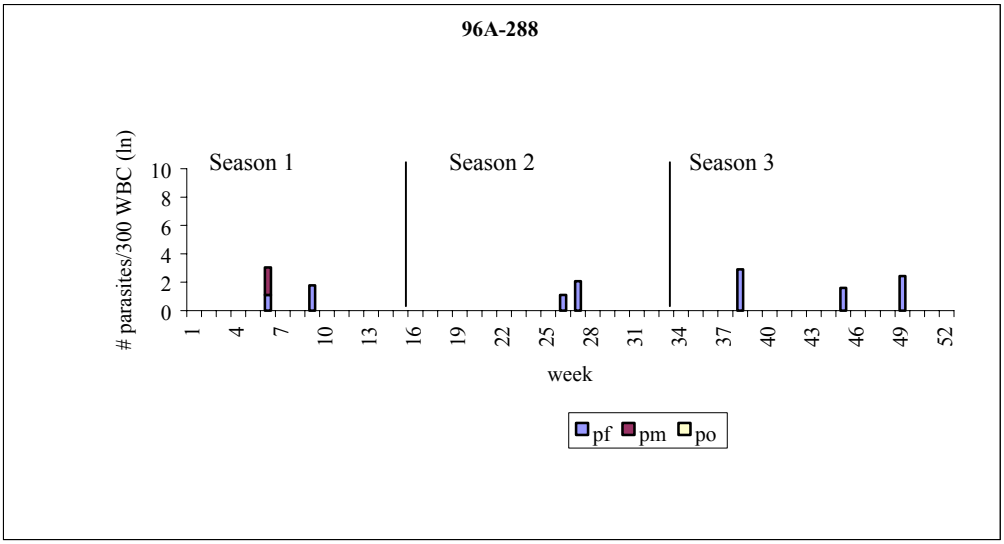
Figure 2. Dynamics of mixed species malaria infection in 11 individuals.

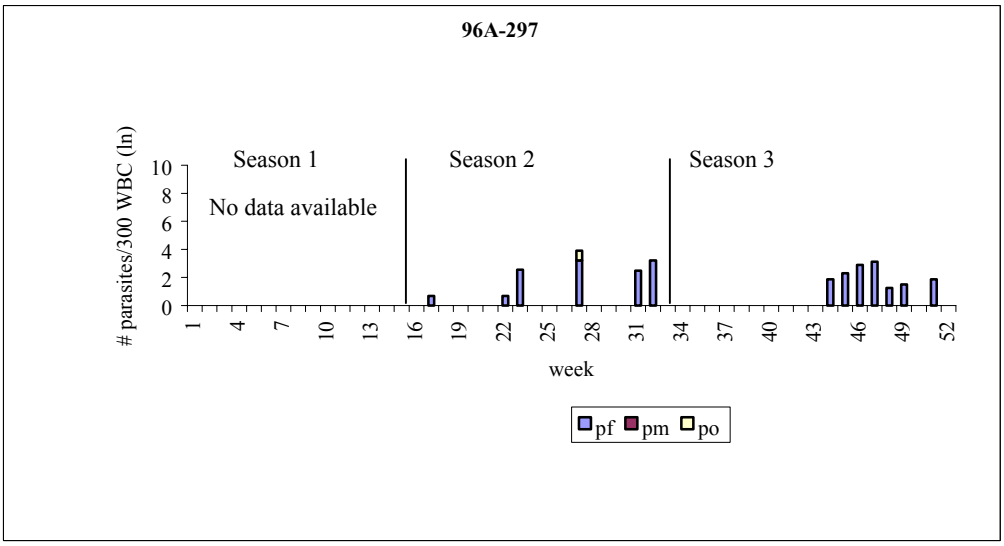


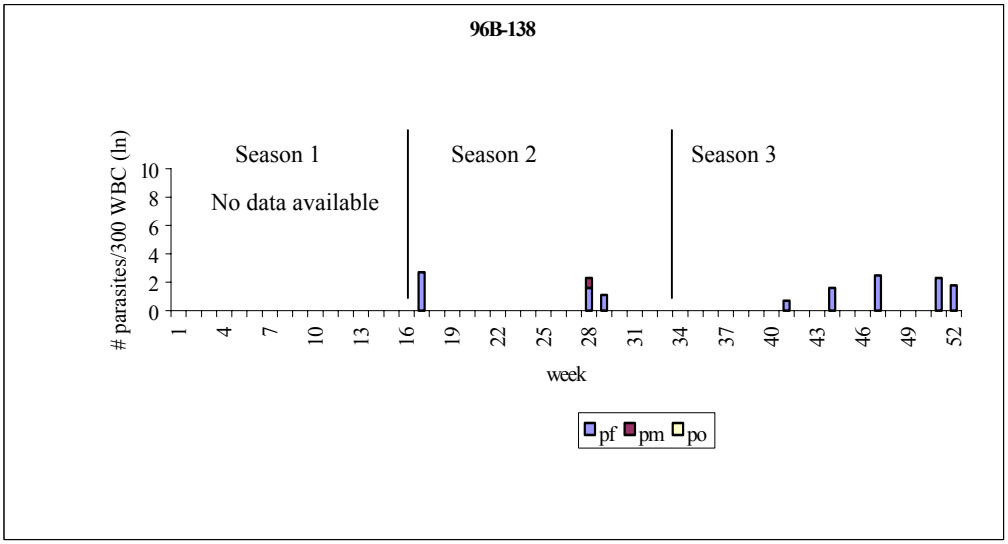

96A-208
Season 1
Season 2
Season 3
parasites/300 WBC (ln)
week
pf pm po

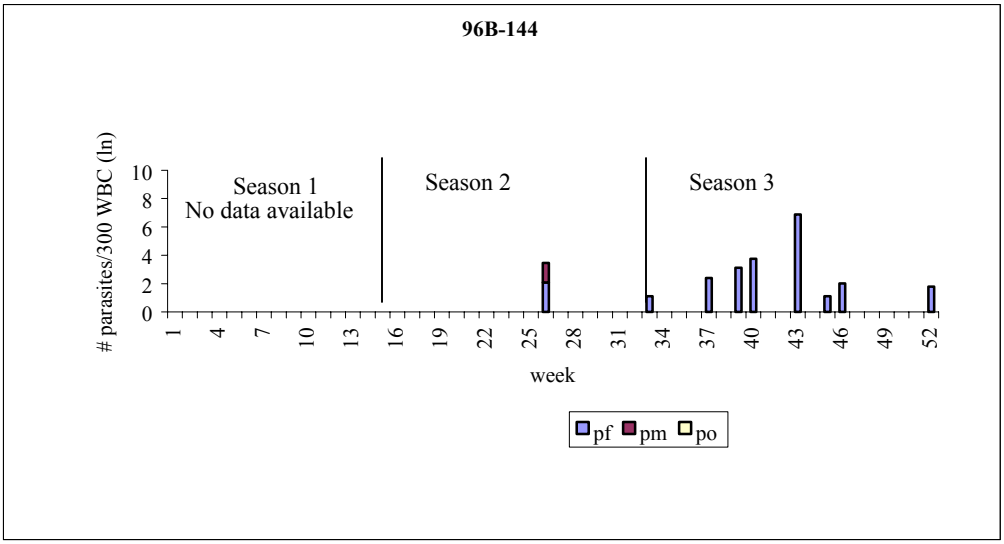

96A-218
Season 1
Season 2
Season 3
Uninfected
parasites/300 WBC (ln)
week
pf pm po

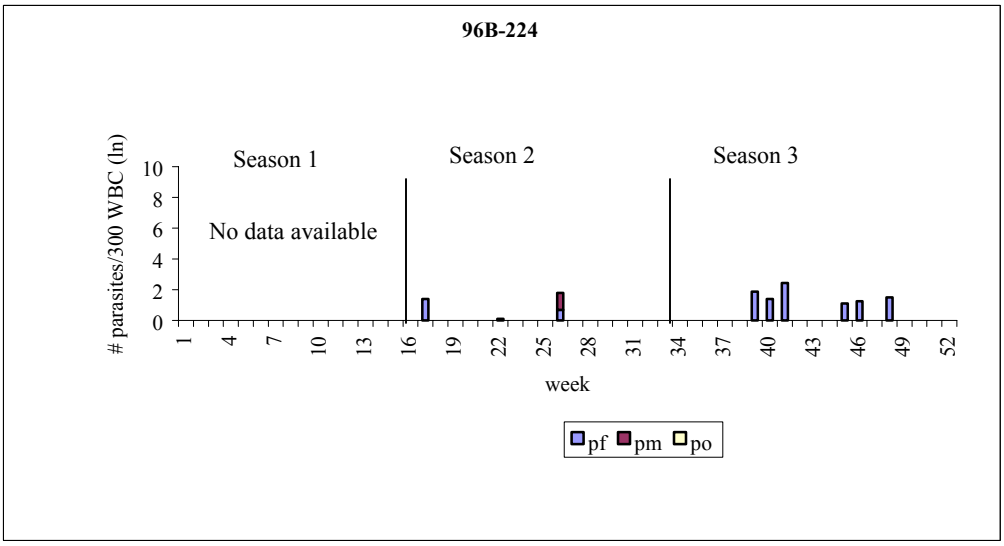

96A-228
Season 1
Season 2
Season 3
parasites/300 WBC (ln)
week
pf pm po


96A-288
Season 1
Season 2
Season 3
parasites/300 WBC (ln)
week
pf pm po


96A-297
Season 1
No data available
Season 2
Season 3
parasites/300 WBC (ln)
week
pf pm po


96B-138
Season 1
No data available
Season 2
Season 3
parasites/300 WBC (ln)
week
pf pm po


96B-144
parasites/300 WBC (ln)
Season 1
No data available
Season 2
Season 3
week
pf pm po

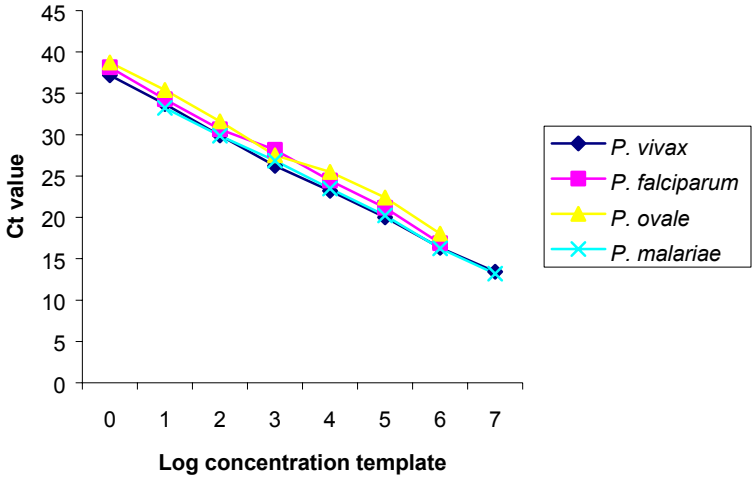

96B-224
parasites/300 WBC (ln)
Season 1
No data available
Season 2
Season 3
week
pf pm po

Summary of results from real-time PCR analysis

To assess low-level *Plasmodium falciparum* and mixed species infections more sensitively, a real-time PCR assay was developed using genus-specific primers and a species-specific molecular beacon probe (see Chapter 2, Methods). The range of detection of the assay was assessed via both serially diluted samples of known concentrations of plasmid clones, and through dilutions of known concentrations of *Plasmodium falciparum* parasites grown in culture and added to uninfected blood.

Figure 3 below demonstrates the range of detection and the correlation between initial template quantity (or copy number) and cycle threshold (CT) value for detection using a Cepheid SmartCycler real-time PCR machine. Using initial quantities ranging from 10^0 to 10^7 plasmid copies, the assay was able to detect presence of a specific *Plasmodium* spp. DNA at the 38th to the 12th cycle, with little variability seen between species.

Figure 3. Log concentration versus Ct value for plasmid clones of ssurRNA gene from 4 human malaria species



Similarly, using samples of known *P. falciparum* concentration from culture, the sensitivity of the assay was evaluated. Detection to a level of one parasite per 25 microliters of blood, or approximately 0.00001% parasitemia was detected and differentiated from negative control specimens, during an investigator-blinded evaluation (data not shown).

The assay was used to evaluate the degree of low-level mixed infections in a cross-section of samples from the Kenya cohort in season one. Samples from week 15 or 16 post-clearance were selected to increase the likelihood of exposure to the less common *Plasmodium* spp. One hundred subjects meeting the stated criteria were randomly selected and tested using the assay. Despite the high level of sensitivity demonstrated previously, results from PCR screening of field specimens found fewer overall infections than blood smear results (Table 19). However, the assay did detect a greater number of mixed species infections and single infections with *Plasmodium ovale* and *Plasmodium malariae*. PCR results found four *P.f.* infections (compared to 71 from blood slides) and one *P.f.*+/*P.m.*+ mixed infection (compared to four). PCR results also found two *P.o.* infections, two *P.m.* infections, and 10 *P.f.*+/*P.o.*+ infections (compared to zero for all types in blood smear results). No *Plasmodium vivax* infections were found.

Table 19. Comparison of blood smear and PCR results.

Malaria species	**% positive (bs)**	**% positive (PCR)**
Plasmodium falciparum	71	4
Plasmodium ovale	0	2
Plasmodium malariae	0	2
P.f.+/*P.m.*+	4	1
P.f.+/*P.o.*+	0	10
P.o.+/*P.m.*+	0	0
N	100	100

Note: All species were screen for the presence of *P. vivax*, however, no samples were found to be positive.

Correlation of mixed species infections and clinical outcomes

Due to the low levels of mixed species infections in either blood smear or PCR analyses, evaluation of the effect of mixed species infection to clinical outcomes were limited by small sample sizes. Nonetheless, three measures of anemia (red blood cell count, mean packed cell volume, and hemoglobin were measured in groups reporting mixed infections versus no mixed infections. No significant differences were seen (data not shown).

Discussion

Few recent reports of the prevalence of mixed species malaria infections have been published from East Africa. One exception, a cross-sectional study of pregnant women in Msambweni (Coast Province), Kenya, found rates of mixed infection of 7% for *P.f.*+/*P.m.*+, 2% for *P.f.*+/*P.o*+, 4% for *P.o.*+/*P.m.*+, and 11% for *P.f.*+/*P.o.*+/*P.m.*+ (24% overall) using PCR (147). Estimates of expected frequencies of mixed infections based upon random distribution of infection within the cohort found lower than expected frequencies of *P. malariae* and *P. ovale* single infections and of *P.f.*+/*P.o.*+ double infections. Higher than expected frequencies of no infections (47%) and of triple infections (11%) were found.

Two studies in West and Central Africa found similar overall rates of mixed infections. In a community based cross-sectional study in Equatorial Guinea, Rubio *et al* found 7% of subjects to have dual infections by microscopy (90% *P.f.*+/*P.m.*+ and 10% *P.f.*+/*P.o.*+), and 29% of subjects to have dual infections by PCR (81% *P.f.*+/*P.m.*+, 6% *P.f.*+/*P.o.*+, 4% *P.m.*+/*P.v.*+, 6% *P.m.*+/*P.o.*+, 4% *P.f.*+/*P.m.*+/*P.o.*+) (148). In Nigeria, a

cross-sectional survey of 228 children without overt signs of malaria (aged 1-11 years) found 28% had either a double or triple infection according to PCR (no specific information is given on associations) (89).

Older reports found strikingly similar patterns of mixed species infections. For example, a report from DB Wilson in Tanganyika Territory (now Tanzania) in 1936 found low levels of *P. malariae* and *P. ovale/vivax* (the report does not distinguish the two) single infections (3% and 1% respectively), and more frequent *P.f.*+/*P.m.*+ mixed infections (ranging from 29% to 2%, depending on age) than *P.f.*+/*P.o.(v.)*+ infections (2% overall) or *P.m.*+/*P.o.(v.)*+ infections (<1% overall) (149).

It is interesting therefore that despite significant differences in study populations, ages, and transmission intensities between these studies and the present study, the pattern of mixed infections appear substantially similar, with 6.5% of individuals reporting mixed infections (on average across all three seasons), and a ratio of 1.7 *P.f.*+/*P.m.*+ infections to *P.f.*+/*P.o.*+ infections in high transmission seasons, and 4.5 *P.f.*+/*P.m.*+ infections to *P.f.*+/*P.o.*+ infections in low transmission seasons (according to microscopy). The absence of any *P.o.*+/*P.m.*+ mixed infections is also similar to the low frequency in previous reports of this combination.

The differences in the ratio of *P.f.*+/*P.m.*+ to *P.f.*+/*P.o.*+ in high versus low malaria transmission season may reflect differences in transmission potential, as previously hypothesized by Boyd and Aris in 1929 (cited by 77). The researchers argued that *P. malariae* does better when transmission and prevalence are lower due to the parasite's superior ability to establish a chronic infection, especially if it is freed from the suppressive influence of *P. falciparum*. A practical illustration was described by Bruce-

Chwatt (cited in 77) who described how the prevalence of *P. malariae* had doubled in Tanzania when a malaria control program reduced *P. falciparum* prevalence.

The lack of a larger number of sequential mixed infections in the present results was surprising though, and contradicts previous reports from cross-sectional and longitudinal studies (82, 106, 147, 150). Several factors may explain this result. First, the radical clearance of parasitemia at the baseline of each transmission season most likely changed the pattern of susceptibility to new infection[*]; secondly, the time period observed was relatively short; and thirdly, previous reports of sequential infections likely included recrudescence and relapse, a problem likely avoided in the present analysis. The incidence of sequential mixed infections may have been greater if the results from PCR analysis had been considered. PCR results in the subset tested found higher rates of *P. ovale* infection, but overall considerably lower rates than would be expected based upon the blood smear results. The most likely explanation is a loss of sensitivity of detection due to low efficiency of DNA extraction from filter paper samples stored for 5-6 years.

Previous studies have reported high sensitivity of PCR detection of malaria parasites from filter papers when compared to microscopy readings (151-152). In addition, reports have analyzed the efficiency of DNA extraction from filter paper stored for shorter periods of time, such as 1 month (153) to 1 year (T. Hollings, personal communication), and found little loss of sensitivity. Important variables to maintaining sensitivity were storage at room temperature or cooler. Factors found to result in loss of sensitivity, included: storage at high temperature (30C) and humidity (60%), and lengthy drying indoors at room temperature (153). Significant differences were also seen in the

[*] A similar finding is reported by Snounou *et al.* (150) who found that immunologically naïve travelers to Sao Tome e Principe were much more likely to be infected with mixed infections than residents.

type of filter paper used (153; T. Hollings, personal communication). A more recent study of two filter paper blood collection systems [IsoCode STIX (Schleicher & Schuell) and FTA Gene Guard card (Gibco BRL)] found high sensitivity for single-species malaria infection (>96%), but low sensitivity to mixed (*P.f.* and *P.v.*) infections (42% and 63% sensitive, respectively) (154). Analysis of *P. falciparum* and *P. vivax* positive control samples blotted on filter papers and stored over a two-year period for the present study found marked (10^3) decreases in sensitivity over that time period (data not shown).

The findings of high levels of parasitemia in concert with mixed infections, and nearly equally likely patterns of mixed infection following sequential *P. falciparum* infection, as the first patent infection, or following a single earlier *P. falciparum* infection, raise several interesting issues in regard to the interaction of *Plasmodium* spp. and host regulation of infection. Unlike Bruce *et al*, which found remarkably stable levels of parasitemia despite changing parasite species dynamics (86), the present results indicate a lack of density-dependent regulation. Any conclusions should be viewed in light of the low overall sample size. Similar to the current study, Molineaux *et al.* (155) found that individuals in Nigeria with higher density *P. falciparum* parasitemia were more likely to also be positive for *P. malariae* than those with lower parasitemias.

Previous researchers have reported a pattern of mixed infection following consecutive *P. falciparum* infections. For example, Hill *et al* (156) examined 22 children in Portugal on a weekly basis for 42 weeks. *P. vivax* was more likely to present immediately following three or more examinations (over six weeks) positive for *P. falciparum*.

Molineaux *et al.* (155) did not provide specific details on the pattern of mixed infections, but did find certain relationships that suggested that two separate processes were at work in terms of balancing mixed species infections. First, host heterogeneity could lead to an uneven distribution of parasites in certain susceptible hosts, and secondly, the presence of one species could lead to the suppression of another. This could explain the higher than expected frequency of certain mixed infections (such as *P.f.*+/*P.m.*+) with the lower than expected frequency of others (*P.f.*+/*P.o.*+ or *P.m.*+/*P.o.*+).

Although the PCR assay was unable to improve the overall detection of mixed species infections, the assay demonstrates a highly sensitive method for measuring low-level infections for future studies. The use of real-time PCR and molecular beacon probes allowed for the detection of all four human malaria species at levels 50 times lower than standard PCR methods and greater than 100-fold lower than microscopy.

Conclusions

The present results contribute to an understanding of the prevalence and association between different malaria species in Western Kenya, and demonstrate a new approach to sensitively measure low-level mixed infections using real-time PCR and species-specific molecular beacon probes. In the present study, multi-species infections were found to be associated with seasonal peaks in parasite density, and to be more commonly *P.f.*+/*P.m.*+ than *P.f.*+/*P.o.*+ or *P.m.*+/*P.o.*+. Further work is needed to better understand the level of mixed species infections in sub-Saharan Africa, and the influence of density-dependent or cross-species immune regulatory mechanisms. The identification of robust, field-applicable, methods for the collection and storage of samples prior to PCR analysis is an important step for future research efforts.

Chapter 5: Parasite growth dynamics

Introduction

Understanding parasite growth dynamics, particularly during the initial phase of infection, provides insight to better understand the development of acute malaria. The study of initial phase parasite growth dynamics in partially immune individuals in endemic areas is confounded however by the heterogeneity of exposure, partial immune protection, and parasite strains. In non-immune individuals, parasite growth dynamics can provide considerable insight into the mechanisms of both parasite and human regulation of infection. This approach, however, is ethically untenable. By contrast, measuring parasite growth dynamics in non-immune individuals provided vaccination by candidate malaria vaccines allows for both the examination of parasite growth dynamics in those unprotected, and for the evaluation of the complete or partial effectiveness of the proposed vaccine.

Vaccine testing of asexual malaria vaccine candidates is a costly and time-consuming process. Because the microscopic detection limit is close to the disease threshold, volunteers face potential risk and considerable discomfort. Field trials relying on natural challenges are also expensive, requiring large sample sizes and often difficult to measure (and interpret) endpoints. In the past decade, a number of prototype vaccines have been developed that target the asexual blood stage of malaria (99-101). Although these vaccines have been chosen for their ability to elicit an anti-parasite response, no

practical and sensitive clinical trial assay has been developed to measure their impact on parasite growth. The current 'gold standard', thick blood smear detection of parasites, has a threshold for microscopic detection (between .000025% to .0002% infected red blood cells) higher than that needed for critical evaluation of partially effective vaccines. The development of a sensitive assay that can improve detection of low level infection and elucidate the degree of partial protection would be of considerable importance. The evaluation of combination vaccines that include both erythrocytic and pre-erythrocytic components would be easier to evaluate as well.

Results

Incidence of infection

A subset of twenty-two volunteers among forty-six enrolled in a WRAIR sponsored experimental malaria vaccine trial were examined daily for positive blood smears starting 5 days post *P. falciparum* sporozoite challenge. Among the 22 volunteers, 14 were found positive by thick blood smear. Positive blood smears were detected between day 9 – 19 post challenge, with a majority of individuals found between days 12 to 15. PCR results by contrast found 16 individuals positive following challenge, varying in day of detection from day 5 to 14. The majority of individuals found positive were detected between day 5 and 6 (Table 20).

Table 20. PCR results of blood samples from 5 to 19 days post challenge.

Pt/DPC	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
3															
4															
5								X							
6															
7							X								
12								X							
13											X				
16											X				
20															
21															
25										X					
26					X										
35															
36										X					
39															
40															
42										X					
49															X
55												X			
58													X		
67					X										
69						X									

Note: Diagonally slanted striped boxes indicate PCR negative results; shaded boxes indicate PCR positive results. X indicates day of blood smear positive result. DPC – day post challenge. Pt. – patient number.

Parasite growth characteristics

Five volunteers who had positive PCR samples six days prior to blood smear detection were included in a subset analysis of parasite growth characteristics. Using cloned *P. falciparum* samples of known quantity serially diluted to define a standard curve linking the threshold cycle for PCR detection with the Log_{10} parasite density, growth characteristics were estimated.

All individuals showed an approximate 48 hour periodicity, however two individuals (5 and 7), synchronous with one another were out of synch by 24 hours

respective of the other volunteers (Figure 4). Parasite multiplication rate (PMR) was calculated using the equation:

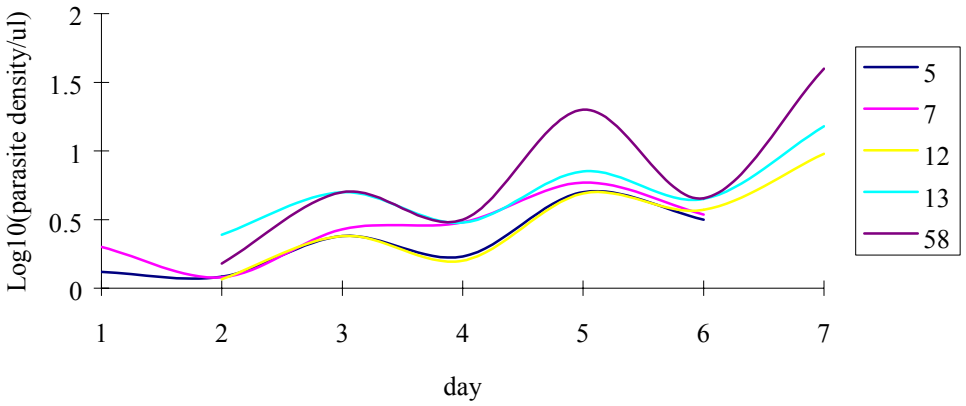
(1) $dY/dt = bY$,

Where Y = parasite density (parasitized red blood cells/ul), t = time (days), b = growth rate constant of the parasite (/day) and:

(2) PMR (for 48h period) = 10^{2b} (98)

Three patterns among the five volunteers could be described according to the PMR. The rate for volunteer 7 and 13 was found to be 6.3, the rate for volunteers 5 and 12 was 12.8, and the rate for volunteer 58 was 16.3.

Figure 4. Parasite Growth Dynamics



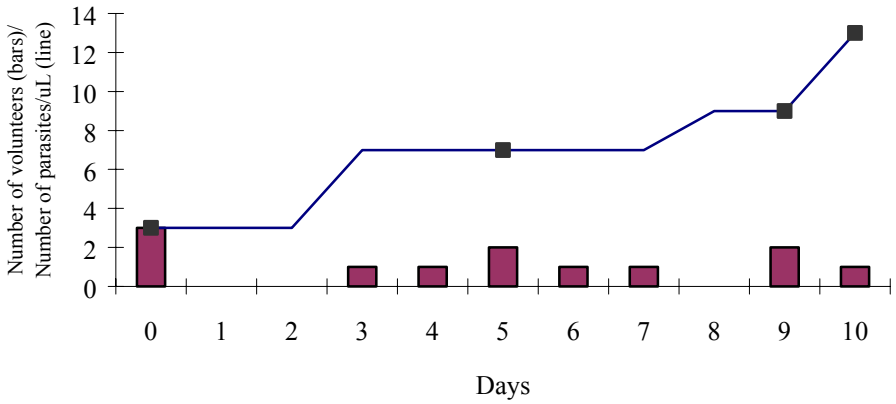
Correlation between parasite density and detection

The number of days prior to the blood smear detection that PCR was able to identify a positive individual is plotted in Figure 5, below. In three cases, there was no difference between the date of blood smear detection and the date of PCR detection. In

six cases, there were between 3 and 7 days difference, and in three cases there were 9 or 10 days difference.

The parasite density at the time of blood smear detection was found to be positively correlated with the number of days prior to blood smear that real-time PCR was able to detect parasitemia (Figure 5, line). Higher levels of parasite density at the time of blood smear detection were associated with earlier detection by real-time PCR, and conversely, lower levels of parasite density at the time of blood smear detection were associated with a shorter interval between real-time PCR detection and blood smear detection.

Figure 5. Number of days real-time PCR positive prior to blood smear positive and parasite density of positive blood smear



Note: The weighted average value for samples from days 3-7 is provided above day 5.

Discussion

Better understanding the initial phase growth dynamics of *P. falciparum* infections in immunologically naïve populations is crucial for improving the evaluation of asexual stage vaccines. Partially effective vaccines, which may influence parasite growth but not impact the specific incidence of infection, cannot currently be effectively measured.

Malaria parasitemia in the initial period of infection prior to the initiation of an immune response increases exponentially then achieves a transient steady state, modulated by strong oscillations with a period of approximately 2 days. In the present study, real-time PCR analysis found two volunteers who were not identified as infected by blood slide detection that showed low level, apparently self-resolving infections. Two other volunteers were found to have positive samples, followed by several (3 and 4) consecutive days of negative results before becoming positive again and being detected by blood smear. Other individuals (13, 16, 49, 55 and 58) were found to have significantly delayed (> 3 standard deviations from the day of detection of infectivity control volunteers) dates of patency, marked by prolonged periods between PCR detection of malaria infection and blood slide detection. These patterns indicate the possibility of partially effective blood stage vaccines at work.

To explore patterns of initial phase growth, quantitative levels of parasitemia were calculated for five volunteers who had positive PCR samples at least six days prior to blood smear detection. It was anticipated that this analysis would show low rates of parasite multiplication, hindered by the acquisition of partial immunity stemming from previous vaccine administration. However, PMRs for these individuals were calculated at between 6.3 and 16.3, similar to previously reported rates (7.7 with a 95% confidence interval of 4.7 to 12.6) among 328 malariotherapy patients (98).

The PMR is believed to be associated with the severity of infection (157-158), with those malaria strains with intrinsically higher multiplication rates causing more severe malaria than those strains with lower rates. This association can be explained by the fact that rapidly expanding infections would be more likely to reach dangerous parasite

burdens before host immunity or anti-malaria treatment was effective. In the current study, the finding of three different PMR among five naïve volunteers receiving identical strains of malaria parasites may indicate the importance of host factors in addition to parasite genotype in defining PMR. A degree of individual variation was previously noted among malaria therapy patients in a previous study (98).

The PMR was expected to correlate with the day of detection (or a specific threshold of parasite density): individuals with high parasite multiplication rates were expected to be identified as parasitemic before individuals with lower PMR, with the delay potentially reflective of the partial efficacy of the asexual vaccine. This is not what was observed. All individuals had a similar day of first PCR detection (day 5 – 7). The volunteer (58) with the latest date of blood smear detection (day 17) had the highest PMR. One possible explanation for this is the variability in thick smear blood slide reading accuracy.

Another important finding of the PCR results was the identification of a clear 48h periodicity with oscillating levels of parasitemia noted for all five volunteers examined. Fluctuations in parasite density are caused by cycles of sequestration and ring form clearance followed by schizont rupture and merozoite release. If the parasite age distribution is synchronous and from a single ‘brood’, as in the present study, then the observed parasite density should increase with a rising sine wave pattern (whereas a completely asynchronous infections would produce a rising straight line on a semi-log plot). Individuals with very large amplitudes in their parasite density-time profiles have highly synchronous infections whereas less synchronous infections have amplitudes that approach zero.

The cause of synchrony and periodicity of malaria infections is unclear. Mathematical models (95) which assume that all merozoites emerge from the liver during a limited time-window still fail to predict synchrony. One proposed explanation for this observation has been that fever (caused by bursting erythrocytes) modulates the stage-specific survival rate of the parasites (159). The present study results however demonstrate synchrony in the absence of fever and at very low levels of parasitemia. Differences in amplitude in the current study could indicate that factors affecting synchronicity are to a certain degree influenced by host genetic factors and/or the success of partial immunity of the vaccine.

Data from several immunization studies conducted in animals (160-162) show a clear positive correlation between antibody level, the degree of protection, and initial parasite growth rates. This suggests that the measurement of the initial parasite growth rate would be a useful indicator of vaccine efficacy. While a reduction in parasite growth may not fully prevent disease, it could provide a measure that could be included in a defined selection criterion for further vaccine development.

Several limitations to this analysis must be mentioned. Firstly, the study was conducted as a double-blind clinical trial with the specific vaccine regimes unknown to investigators, and has yet to be unblinded. This limits the extent to which the effect of pre-erythrocytic or erythrocytic vaccines effects can be measured and compared. Secondly, the small number of volunteers in the vaccine trial and particularly in the subset analysis of initial parasite growth dynamics limits the conclusions which can be drawn.

Conclusions

The development of a highly sensitive real-time PCR assay allowed for the measurement of initial phase parasite growth dynamics in volunteers participating in an experimental malaria vaccine trial. Evidence was found in nine individuals of some degree of immune regulation of infection: in four individuals partial or complete resolution of infection was seen, and in five individuals a delayed pre-patent period was noted. The results also demonstrate that parasites, even at very low levels of infection, have marked periodicity. Several additional steps are needed to further develop the assay and understand the effectiveness of the tested vaccine. First, parasite multiplication rates and the apparent resolution of infection must be correlated with vaccine type when the assignment of volunteers to treatment arms becomes unblinded. Second, a larger number of volunteers is needed to understand better the normal range of PMR for the *P. falciparum* 3D7 clone used for challenge. Third, additional research must be done to find appropriate trial endpoints given the increased sensitivity of the PCR approach and the possibility of resolution of infection or modulation of parasite growth. The optimal balance between monitoring individuals PCR positive for malaria infection and treating prior to sufficient levels of parasitemia associated with disease must be found to protect volunteers but allow for thorough evaluation. Achieving this balance will accelerate the testing of vaccine candidates by reducing the expense and potential harm to volunteers while eliciting more specific information on levels of success short of complete protection.

Chapter 6: General discussion and conclusions

Introduction

Malaria epidemiology is a complex web of inter-related factors: host genetics, parasite diversity, mosquito dynamics and environmental conditions. The nature, duration and severity of malaria infection depend upon these fixed and changing factors and are complicated by varying levels of acquired immunity in individuals and result in varying impacts upon communities.

Malaria is a major cause of childhood mortality in sub-Saharan Africa despite the fact that most (~99%) cases are uncomplicated, or 'mild', with very low mortality. Potential reasons for this selectivity – and the lack of definitive explanations – were addressed a decade ago by Brian Greenwood and colleagues in an article entitled, "*Why do some African children develop severe malaria?*" (163) The same year, the National Academies of Science published a report, *Malaria: Obstacles and Opportunities* (164) which recommended (among other findings) that future research efforts focus upon 1) the determination of epidemiologic risk factors for severe and complicated malaria; and 2) characterizing parasite and human variability as they relate to the development of immunity, and the evolution of clinical disease (p. 234).

After more than a decade of intensified research on malaria (and increasing impact worldwide), what more is now known about malaria epidemiology and the risk

factors for severe disease? What added information and tools have molecular epidemiology provided in the effort to better understand parasite and human variability and to develop effective control strategies? This final chapter will briefly address key advances, and highlight continuing uncertainties, in our understanding of malaria epidemiology, and then discuss the contribution of the present research towards a better understanding of malaria epidemiology and control.

Recent advances in malaria epidemiology

Since 1991 it has become increasingly clear that genetic factors significantly contribute to whether or not a child develops severe malaria. More genetic loci have been shown to affect malaria infection and clinical outcomes than any other disease of humans (165). Genetic loci identified have been linked primarily to either polymorphism of red blood cells (directly influencing parasite invasion and nutrition) or immune regulation (including MHC genes and promoter regions for TNF, iNOS2, and other cytokine genes). The characterization of these loci, and their inclusion as variables in subsequent case-control studies, have allowed for an estimation of their protective efficacies (including estimations of different levels of protection against severe versus mild malaria (166)) in a range of populations in different endemic regions. Nonetheless, the effort to understand the impact and interaction between these protective loci has likely only begun. Fundamental questions, such as the magnitude of the overall genetic effect, are still largely unknown. Although the interaction of the Duffy blood group (and, more recently, α-thalassemia) and *P. vivax* are an exception, relatively little information on potential

genetic influence on non-falciparum malaria species is known, and a number of specific interactions are likely to be as yet uncovered.

Over the last decade, it has become increasingly evident that heterogeneity in *parasite* genetics contributes to the wide spectrum of disease severity observed as well. *P. falciparum* has a 25-30 megabase genome containing 14 nuclear chromosomes (167-168). Molecular karyotyping of different *P. falciparum* isolates has demonstrated frequent and considerable size polymorphism among homologous chromosomes. A genetic linkage map using microsatellite markers has been developed with more than 900 specific markers in 14 interfering linkage groups (169). Several full-length restriction maps and gene assembly have been determined by using shotgun optical mapping (170-171). More significantly, the sequencing of the entire *P. falciparum* genome is nearing completion (172). Building upon this knowledge, advances in DNA microarray technology can now allow for the examination of the interplay of gene expression levels at different parasite life stages or between different isolates (173).

Underscoring this evidence of genetic diversity in *Plasmodium* populations, a variety of parasite phenotypes have been proposed with a range of putative virulence factors. These include the ability of certain parasite strains to bind infected to uninfected erythrocytes and the capacity of parasites to induce variable amounts of TNF production by host macrophages. Changes in the antigenic phenotype of infected RBCs have been associated with changes in adhesive properties, and these properties may vary greatly between individuals and between infections. Furthermore, the antigenic and adhesive properties of circulating parasites may be quite different to those sequestered at a particular site. These features have made it difficult to reliably identify parasite virulence

factors associated with specific clinical syndromes. Numerous host molecules have been identified that can act as receptors for the adhesion of infected RBCs (including CD36, ICAM-1, VCAM-1, CSA, HA, rosetting receptors, etc.), and different populations of malaria parasites may use different combinations of these receptors. However, the specific role of each of these receptors in the pathogenesis of malarial disease remains largely unclear.

The potential influence of mixed species and multi-clonal infections on infection and disease severity has received relatively less attention in the past decade. The few studies reported have found conflicting evidence of the impact of mixed *Plasmodium* species infections on such clinical outcomes as anemia (89) and severe malaria (90), and although a larger number of studies have examined the impact of multi-clonal infection, results in this area have also been inconsistent (174-177).

By contrast, an area that has seen substantial increases in research activity, if not in our knowledge of malaria biology or epidemiology, is malaria vaccine development. The first report of a field trial of a malaria vaccine in an endemic area was in 1990 in Burkina Faso (178). Fourteen field trials have been reported subsequently (reviewed in 179), targeting a range of malaria proteins, and using a variety of approaches (protein, peptide, DNA, recombinant viral, etc.). Despite this increase in the number of candidate vaccines, advances in the techniques required for the evaluation of vaccine trials, both at Phase II and Phase IIb/III stages, have been lacking. The continuing difficultly in evaluating vaccines and defining appropriate study endpoints is of increasing importance and an impediment to rational decision-making in deciding which candidates to advance to further (and larger and more expensive) trials, and which to set aside.

In response to the need to evaluate and compare multiple (phase IIa) malaria vaccine trials, WRAIR developed a standardized challenge model using well-characterized parasite isolates and mosquitoes, providing a high probability of infection (and small variation in pre-patent period) in naïve control subjects. Validation of infection is done using thick blood smear microscopy, with treatment immediately upon positive identification of two parasites. Identification of partially protective blood-stage vaccines using this model is difficult however, in that it requires an estimation of delayed patency that may be beyond the level of precision provided by microscopic diagnosis. In addition, vaccine protection against high parasite density (i.e. protection against disease rather than infection) would be difficult to evaluate using this approach as early treatment precludes assessment of the impact on parasite multiplication rate or clinical effects. Another limitation of this model in evaluating blood stage or multi-stage vaccines is the loss of specific information related to protection at different stages of infection – specifically, it cannot identify which stage was effective, or exclude the possibility that protection was achieved or lost through some unanticipated cross-reactivity with the pre-erythrocytic stages of the parasite.

An alternative challenge model has been proposed (180), which involves the induction of infection by inoculation of infected blood, and the measurement of parasite multiplication in volunteers using a semi-quantitative PCR. The model assumes that, in the presence of immunity to the blood stage of the parasite, the rate of multiplication will be delayed, even at low parasite densities. One limitation shared with the WRAIR model is the inability to detect a protective effect against high parasitemia (i.e. density dependent regulation). In addition, the added risk of blood inoculation (allowing the

possibility of transmission of an unidentified pathogen) makes this model more difficult to endorse. The next decade will see continued attempts to develop an effective malaria vaccine, and the need for more informative vaccine evaluations requires the development of a system that can use elements from both of these models.

Significance of present research and future directions

The research presented in the previous chapters examines three distinct topics related to malaria epidemiology and control, attempting to provide a more complete understanding of host and parasite variability. Specifically, research was conducted related to host genetics (chapter 3), parasite interactions (chapter 4), and parasite growth dynamics and vaccine evaluation (chapter 5).

In Chapter three, research is presented from a cohort of young adults living in Western Kenya, a region where severe malarial anemia is frequent and cerebral malaria is uncommon. The research examines the frequency of different genetic alleles in the promoter region of a range of cytokine genes (TNF-α, IFN-γ, IL-6, TGF-β and IL-10). Although the TNFα –238A allele was previously associated with severe malarial anemia, in the present study it was found to occur at a frequency similar to populations from West Africa and Europe/North America. By contrast, profound biases were found in the distribution of IL-6 and IFN-γ genotypes compared to these same populations. These genotypes have been previously associated with low levels of cytokine expression. High plasma levels of both cytokines have been associated with severe disease and death (68, 137) and poor pregnancy outcomes (109) due to malaria. High levels of both have also been related to severe anemia in malaria-exposed populations (140).

Further work in different geographic areas is needed to examine the relative frequencies of IL-6 or IFN-γ polymorphisms, and the extent to which they contribute or protect against the risk of severe anemia in different populations. Ideally, additional research could examine allele frequencies among pregnant women and their infants, and follow them longitudinally for the impact of the different alleles. Additionally, more sophisticated studies should include a wider range of genetic loci to examine in more depth the potential interactive effects of multi-gene haplotypes on the development of severe anemia among children in malaria endemic regions.

Chapter four presents information from the same cohort on the rate and pattern of mixed *Plasmodium* species infections over three malaria seasons. Individuals were cleared of all malaria infections at the start of each successive season and followed for 16-18 weeks. Non-falciparum malaria was seen in a minority (5-10%) of volunteers in each malaria season, most commonly as dual infections in association with *P. falciparum*. In most cases (80%), mixed infections were detected when individuals reported their highest rate of parasite density for that season. Consistent with what has previously been reported in a number of regions of sub-Saharan Africa, the most common dual infection seen was *P. falciparum* with *P. malariae*. In high transmission seasons, this co-infection was seen nearly twice as often, and in the low transmission season it was seen nearly five times as often, as dual infection of *P. falciparum* and *P. ovale*. Chapter four also demonstrates a real-time PCR assay 50 times more sensitive than standard PCR methods, and greater than 100-times more sensitive than microscopy. The assay is capable of detecting each of the four human malaria species using molecular beacon probes specific to conserved regions of the small subunit ribosomal RNA gene, and reduces the difficulty

commonly seen in microscopy, of detecting low level infections of one species in the presence of high level infections of another.

The mechanisms involved in the cross-species immune regulation of multiple infections are still unclear, but could provide potentially significant insights for vaccine development and malaria control interventions. Recent studies suggest that the maintenance of asymptomatic infections play a part in preventing disease (181-183), and that some mechanism of species-transcending immune regulation (such as antibodies against parasite toxins or iNOS) may play a role in maintaining parasite density below clinical symptom thresholds (86). Further research is needed to better understand these phenomenon, and the epidemiology and dynamics of multi-clonal infection of a single malaria species. Although PCR analysis of polymorphic antigen genes has allowed for some insight into infection complexity of *P. falciparum* and *P. vivax* infection, standard PCR does not allow information on the density of individual genotypes of a species. It is conceivable, albeit technically challenging, that a real-time PCR assay could be developed to address this question as well.

In Chapter five, results from an analysis of parasite growth dynamics during the post-challenge phase of a malaria vaccine trial are presented. Twenty-two volunteers enrolled in a WRAIR-sponsored experimental malaria vaccine trial were examined by both thick blood smear and real-time PCR for up to 20 days post-sporozoite challenge. Positive blood smears were detected in 14 individuals (between day 9 – 19 post-challenge), with a majority of individuals found to be positive between days 12 to 15. PCR results by contrast, found 16 individuals positive, varying in day of detection from day 5 to 14. The majority of individuals were detected between day 5 and 6. Two

individuals were found to have positive samples, followed by several consecutive days of negative results before becoming positive again and being detected by blood smear. Five individuals were found to have significantly delayed (>3 s.d. from the day of detection of control volunteers) dates of patency, marked by prolonged periods between PCR detection of malaria infection and blood slide detection. A subset analysis of five volunteers found an approximate 48-hour periodicity in parasite density, and a relatively wide range in the parasite multiplication rate (from 6 to 16).

The use of real-time PCR to evaluate post-challenge vaccine provided several results unavailable from traditional thick smear microscopy. First, evidence was found in four individuals of some degree of resolution of infection (in two cases complete, in two cases partial), and in an additional five individuals of immune regulation of parasite growth (resulting in delayed patency). The PCR assay also demonstrated that parasites, even at very low levels of infection, demonstrate marked periodicity. Finally, the results illustrate a means for distinguishing differences in pre-erythrocytic and erythrocytic control of infection in multi-antigen vaccines by the use of parasite multiplication rate in combination with measures of pre-patent period.

Additional research is needed to further test the assay in the evaluation of malaria vaccine candidates. While the present results suggest possibly important effects of malaria vaccine protection and immune regulation of parasite growth, the results are limited by the fact that the assignment of volunteers to the different vaccine arms of the study has not yet been unblinded to investigators, and that the overall sample size is fairly low. The application of the assay to the evaluation of phase IIb/III field trials could

provide further information on the effect of parasite growth rate and density-dependent regulation.

Summary

It is not surprising, given the long interaction between humans and malaria parasites, and the powerful selective evolutionary pressures, that the coevolution of host and parasite polymorphisms result in complex and subtle interactions. Analyzing this relationship in the same population study is a potentially powerful, yet challenging approach, which has been underutilized. A few examples of this type of study demonstrate the potential for significant results. For example, research examining HLA type among African children found a specific influence on the strain of malaria parasite present (184). Another example is the observation of a possible parasite allelic association with the sickle hemoglobin variant (185). Similarly, the frequency of allelic polymorphism in the promoter region of TNF-α, IL-6, and INF-γ genes (as well as other cytokine genes) likely reflect an evolutionary balance between the beneficial and deleterious effects of different levels of circulating cytokines.

As more and more genomic sequences from both humans and *Plasmodium* spp. become available, functional genomics will lead to a greater understanding of host-parasite interactions. Completion of the Malaria Genome Project and the availability of new technologies for genome-wide comparison of genomes and expression profiling will also help identify key targets in biochemical pathways that are parasite-specific. The information generated from this post-genomic area will advance knowledge of parasite and human genes which influence the pathogenesis and manifestations of malaria, and

can help guide the development of effective drugs and vaccines. Innovative research approaches are needed to continue to advance the field of malaria epidemiology. For example, despite interesting findings from one small twin study that examined susceptibility to malaria fever (15) and a study of siblings in nine families in Gabon (186), no large scale studies of genetic markers and susceptibility to severe disease among siblings have been published. Many questions concerning malaria epidemiology remain unanswered. The increasing burden of malaria morbidity and mortality, now affecting 40% of the world's population in more than 100 countries, requires on-going dedication and commitment to finding more answers.

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