VALIDATION OF A CYTOMETRIC MULTIPLEX ASSAY AND EXAMINATION OF ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM* ANTIGENS IN THE HIGHLANDS OF WESTERN KENYA DURING A PERIOD OF LOW MALARIA TRANSMISSION

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ABSTRACT

Malaria is one of the world's most deadly diseases. Even though it is highly preventable and treatable, it is a leading cause of morbidity and mortality in the tropics. As the campaign for malaria eradication widens, more areas will achieve low transmission of *Plasmodium falciparum*. However, little data exists on how absence of transmission will affect antibody responses to malaria antigens. The current knowledge of immunological responses to malaria, a major tropical disease, is insufficient, and a better understanding of these responses would support vaccine development. The aim of this study was to validate and standardize a cytometric multiplex assay for simultaneous detection of antibodies to malaria vaccine candidates and examine antibody responses to *P. falciparum* antigens in the highland regions of western Kenya. Antibody frequencies and levels to *P. falciparum* vaccine candidate antigens and a VCA-p18, non-malarial antigen were measured, just before interruption of transmission and fifteen months later. One thousand randomly selected individuals from two study sites (Kipsamoite and Kapsisiywa) prone to epidemics in western Kenya were tested for antibody reactivity. The degree of association between median fluorescence intensities (MFI) or optical densities (OD) was assessed using Pearson's correlation (r). Chi-square analysis was used to compare the antibody frequencies at the two sites while paired antibody frequencies were evaluated by McNemar test. Differences in antibody levels between the two sites were compared using Mann Whitney test. Optimal amounts for CBA antibody testing differed according to antigen. Results for monoplex CBA testing correlated strongly with multiplex testing for all antigens ($r = 0.88$-0.99, $P<0.0001$ - 0.004), and antibodies to variants of the same antigen were accurately distinguished within a multiplex reaction. MFI values were essentially identical for all antigens tested at 1000 beads/analyte/well versus 5000 beads/analyte/well (all $r \geq 0.99$, all $P < 0.0001$). Correlations between CBA and ELISA were strong (AMA-1-3D7 $r > 0.55$, AMA-1-FVO $r > 0.37$, EBA-175 $r > 0.8$, MSP-1-19 $r > 0.92$, MSP-1-42 $r > 0.89$, MSP-3 $r > 0.70$, GLURP-R0 $r > 0.82$, GLURP-R2 $r > 0.90$ ($P < 0.05$) for all antigens except AMA-1-FVO. Antibody levels and frequencies at both sites increased across age groups ($P < 0.0001$). IgG levels to all *P. falciparum* antigens decreased at both study sites over the fifteen-month period but not for Epstein Barr Virus VCA-p18 antigen. IgG levels for most antigens correlated significantly with each other for both sites during the first and second surveys. These results show that CBA may be the preferred method of testing for antibodies to *P. falciparum* antigens, as CBA can test for antibodies to multiple recombinant antigens from a single plasma sample, and has greater range of values in positive samples and lower background readings for blank samples than ELISA. Further, the rapid decline in median antibody levels to all vaccine candidates indicates that these populations will be at risk of epidemics. This study gives us an opportunity to further understand correlates of humoral immunity in an epidemiological context that informs vaccine development strategy in unstable seasonal transmission areas experiencing zero transmission.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria in human beings is caused by five *Plasmodium* species: *P. falciparum, P. ovale, P. malariae, P. vivax* and *P. knowlesei* (Breman, 2009). It is transmitted by female *Anopheles* mosquitoes. Among the causative agents of malaria, *P. falciparum* is the most pathogenic (Hay *et al.*, 2004). Malaria continues to be a serious public health problem and an impediment to economic growth and development in tropical countries over 600,000 deaths each year, mainly young African children (WHO. 2012). The frequency and severity of *P. falciparum* malaria generally decreases over successive malaria exposure, suggesting that immunity is exposure related (Langhorne *et al.*, 2008). Vector-control programmes have proved to be ineffective of eradicating malaria. Drugs to kill the parasite are not very effective and cases of drug resistance developing have been reported. Some of the most effective interventions currently being implemented for reducing the burden of malaria infection and disease include use of insecticide treated bed-nets (ITNs), insecticide residual spraying, and artemisinin combination therapies (ACT); and intermittent preventive therapy (IPT) is being studied as an intervention to reduce morbidity in intermittent preventive therapy-infants (IPTi), intermittent preventive therapy-children (IPTc) and intermittent preventive therapy pregnant women (IPTp) programs (Fegan *et al.*, 2007; Ghani *et al.*, 2009).

The widespread implementation of insecticide-treated nets (ITNs) is a major intervention strategy likely to significantly reduce morbidity and mortality from malaria across Africa (WHO, 2008). Development of an effective malaria vaccine is considered a public health priority. To generate vaccine-mediated protection is a complex challenge. Currently available vaccines have largely been developed empirically, with little or no understanding on how they activate the immune system (Crompton *et al.*, 2010).

Transmission of *Plasmodium falciparum* in high-altitude communities is limited by low ambient temperature. Small changes in climate may therefore provide transiently suitable conditions for unstable transmission in populations that have acquired little functional immunity (Hay *et al.*, 2004).

Antibody responses to malaria antigens have traditionally been determined using enzyme-linked immunosorbent assay (ELISA). This technique requires each antigen to be tested individually and involves relatively long incubation periods, making the technique to be labour-intensive and time consuming as well as requiring significant amounts of antigen and plasma. This partly explains why up to date studies of vaccine development and immune responses analyses have focused on analyzing either one or a few immunodominant antigens at a time. Development of techniques that measures antibodies to multiple antigens simultaneously would be greatly advantageous. Recent advances in bead-based flow cytometry have made multiplex assay an attractive alternative to ELISA for *P. falciparum* antibody testing (Cham *et al.*, 2008). The beads in multiplex assay anchor the antigens as opposed to ELISA where the surfaces of the wells of microtiter plate anchor the antigen. However, development and validation of multiplex assay to

measure *P. falciparum* antibodies had not been studied which was one of the objectives of this thesis research.

Examining antibody responses to multiple *P. falciparum* antigens in a cohort study in a highland area of western Kenya during an extended period of low malaria transmission and after a documented interruption of malaria due to intervention strategies is necessary to enable malaria immunologists to assess the impact of low malaria transmission on humoral responses to *P. falciparum* antigens. Studies that evaluate changes in *P. falciparum* antibody frequencies during interrupted malaria transmission are lacking. Hence this study addressed this particular gap in knowledge which will aid in our understanding of immune responses to malaria antigens.

Inadequate understanding of the mechanisms of naturally acquired clinical immunity against plasmodia may be an important factor contributing to the failure to develop a practical vaccine. Antibody-dependent mechanisms are presumed to play an important role in protection and a wide range of antigen-specific antibodies as well as polyclonal antibody production have been implicated (Bereczky *et al.*, 2004; Lucchi *et al.*, 2008). Evidence exists from the passive transfer of antibodies between immune and non-immune individuals that this immunity can be antibody mediated (Cohen *et al.*, 1961). However, changes in *P. falciparum* antibody levels during absence of malaria transmission which would inform vaccine design strategies had not been studied, which was also another objective of this thesis research.

Identifying which antigens of *P. falciparum* contain the epitopes that are the targets of naturally acquired immunity is logically important for the design of a vaccine to elicit these responses in

naive individuals. There was a need to investigate whether human antibodies to *P. falciparum* antigens diminish when malaria transmission is interrupted.

1.2 Statement of the Problem

Malaria is one of the most devastating infectious diseases that threaten humankind. There is no effective therapeutic control. The increasing incidence of antimalarial drug and insecticide resistance is making it urgent the need to discover alternative control measures. Development of a malaria vaccine is considered a good alternative and therefore a public health priority. A major problem in developing a malaria vaccine is the difficulty in pinpointing the responses involved in immunity to malaria and their target antigens. Work on identifying which antigens of *P. falciparum* contain the epitopes that are the targets of naturally acquired immunity is scanty therefore the design of a vaccine to elicit protective responses in naive individuals is dragging. Measurement of antibody titers in serum samples from individuals living in malaria-prone areas to determine antigens that could be included in an effective malaria vaccine in immune-epidemiological studies is unclear. Identification and validation of reliable immunologic correlates of protection against infection and morbidity are important goals of malaria vaccine research that this study is targeting.

1.3 Justification of the study

New tools and methods are needed for the study of adaptive immunity to malaria. The number of identified malaria antigens represents only a small portion of about 5,000 predicted proteins (Vaughan *et al.*, 2009). In addition, the development of standardized protocols that permit the measurement of antibody responses would allow more definitive human immunological studies.

Further the improved methods for studying the human immune system are also needed to allow more definitive and comprehensive analyses and confirmation of results. Knowledge about the adaptive immune response to malaria infection is broad but somewhat superficial at present. Further, knowledge on rates of seroreversion of malaria antigens may be important in shedding light on the optimal vaccination schedules for vaccine trials in different ages in areas of low transmission.

1.4 Objectives of the Study

1.4.1 General Objective

To validate a cytometric multiplex assay and examine antibody responses to *Plasmodium falciparum* antigens in two sites (Kapsisiywa and Kipsamoite) in the highlands of western Kenya during a period of low malaria transmission.

1.4.2 Specific Objectives

1. To determine optimal parameters for cytometric multiplex bead assay for analysis of antibody responses against *P. falciparum* antigens.

2. To evaluate the effect of transmission intensity and age over time on antibody frequencies to *P. falciparum* antigens in two sites (Kapsisiywa and Kipsamoite) in the highland region of western Kenya.

3. To investigate the effect of transmission intensity and age over time on antibody levels to *P. falciparum* antigens in two sites (Kapsisiywa and Kipsamoite) in the highland regions of western Kenya.

4. To assess the relationship between antibodies over time to *P. falciparum* antigens and antibody levels to VCA-p18 in the highland regions of western Kenya, and

5. To determine rates of antibody seroreversion across age for different malarial antigens in the highland regions of western Kenya.

1.4.3 Null Hypotheses

1. Multiplex assay is not more sensitive and reproducible than ELISA for measurement of antibodies against malaria.

2. Antibody frequencies in highland regions of western Kenya do not decrease overtime.

3. Antibody levels in highland regions of western Kenya do not decrease over time.

4. There is no relationship between antibodies over time to *P. falciparum* and levels to VCA-p18 in highland regions of western Kenya.

5. Antibody seroreversion rates are not different by age and *P. falciparum* antigen in highland regions of western Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria remains one of the leading causes of morbidity and mortality in the world affecting approximately 219 million people worldwide and causing about 660,000 deaths each year (WHO. 2012), majority of which occur among children under five years (Breman, 2009; Schumacher & Spinelli, 2012). In spite of increased knowledge of the parasite's biology and the availability of advanced technology for malaria research, there has been little success in eradicating malaria (Greenwood, 2009). Indeed, the hopes of complete eradication have given way to a more pragmatic goal of controlling malaria disease (Greenwood, 2008). Although malaria is a treatable and preventable disease, the wide-spread occurrence of drug-resistant parasites and the emergence of insecticide resistant mosquito vectors have remarkably hindered effective disease control (Guerin *et al.*, 2002). Therefore, an effective vaccine would be a better strategy to reduce the burden of malaria (Andre, 2003; Bairwa *et al.*, 2012). However, development of an effective malaria vaccine requires identification and incorporation of target immunogens derived from various stages of parasites. There is also need to circumvent antigenic polymorphism among major vaccine candidates (Moorthy *et al.*, 2004).

Chemotherapy is the mainstay of malaria control, but it is being undermined by the rapid emergence and spread of parasite resistance to the currently available drugs. Currently, there is widespread resistance to chloroquine and sulphadoxine/pyrimethamine in many malaria-endemic

areas (Rogers *et al.*, 2009). Whereas a vaccine might provide the best alternative to drugs, the search for a malaria vaccine has proved to be frustrating. Malaria parasites employ a diverse array of adaptive strategies against the host immune system and even where immunity is achieved, it is incomplete (Langhorne *et al.*, 2008).

2.2 Malaria Parasite and Mosquito Vector

2.2.1 Causative agent and the life cycle of malaria

Malaria is caused by protozoan parasites of the genus *Plasmodium* which are transmitted by female *Anopheles* mosquitoes. There are four main *Plasmodium* parasites that infect humans: *P. falciparum*, *P. vivax, P. malariae*, and *P. ovale* (Breman, 2009). However, recent findings demonstrated the zoonotic transference to humans of *Plasmodium knowlesei* (Breman, 2009; Jongwutiwes *et al.*, 2004), a malaria parasite that was previously observed in primates. All the five species of *Plasmodium* that naturally infect humans are transmitted by the mosquito vector.

Malaria has a complex life cycle that involves two stages of replication occurring in the vertebrate host and insect vector (Figure 1). Sexual replication occurs in the mid-gut of the mosquito vector and asexual reproduction in the human host. The vertebrate stage of the lifecycle is made up of exo-erythrocytic and erythrocytic parts. Infection in humans begins when sporozoites injected by the biting mosquito enter the bloodstream within an hour (Amino *et al.*, 2006; Yamauchi *et al.*, 2007) and infects hepatocytes (Baer *et al.*, 2007; Frevert *et al.*, 2005; Mota & Rodriguez, 2002). The sporozoites are transferred via the bloodstream to the liver, where they begin a period of asexual reproduction as liver schizonts. Within 14 days they are released at once into the circulation; destroying the host hepatocytes. Invasion of erythrocytes is thought

to occur soon after merozoite bursts from hepatocytes which marks the beginning of the erythrocytic stage. Within the erythrocyte, the merozoite differentiates into trophozoites. By 38-40 hours post invasion, the trophozoite has further differentiated into a schizont which divides to give 16-20 daughter merozoites (White & Kilbey, 1996).

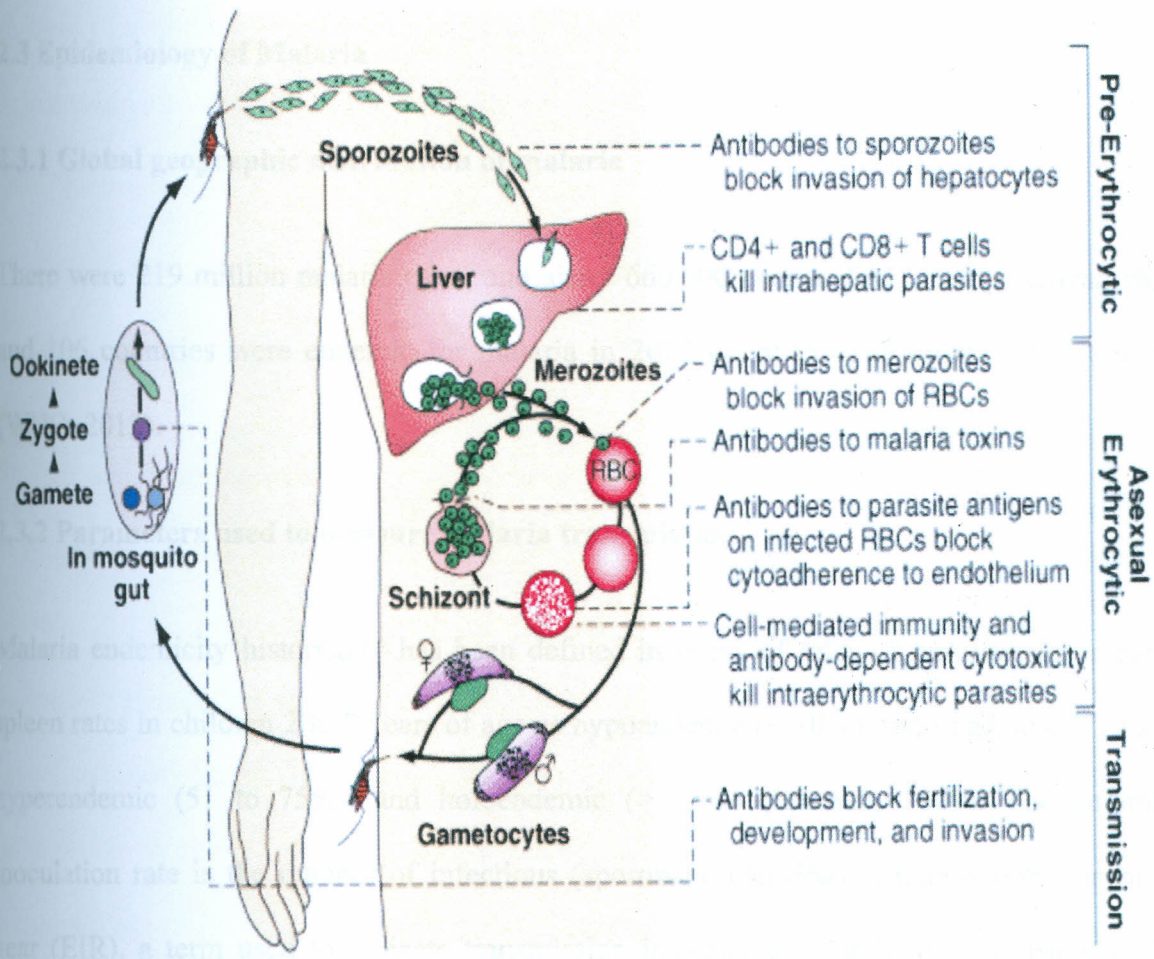

Figure 1. Life cycle of *Plasmodium* parasite showing the various stages that can be targeted by host immune responses (Breman, 2009).

Approximately 48 hours post invasion, the distorted erythrocyte bursts and releases the merozoites into the circulation to continue the erythrocytic cycle. It is during the asexual part of

the life cycle that the symptoms of malaria manifest. During this repeated cycle, some merozoites differentiate into male and female gametocytes, which can be taken up by mosquitoes during a blood meal; maturation of the parasite occurs within the mosquito gut and sporozoites migrate to the *Anopheles* salivary glands (Wipasa *et al.*, 2002).

2.3 Epidemiology of Malaria

2.3.1 Global geographic distribution of malaria

There were 219 million malaria cases and about 660,000 people died, mostly African children, and 106 countries were endemic for malaria in 2010 mainly in sub-saharan Africa and Asia (WHO, 2012).

2.3.2 Parameters used to measure malaria transmission

Malaria endemicity historically has been defined in terms of rates of parasitemia or palpable-spleen rates in children 2 to 9 years of age as hypoendemic (< 10%), mesoendemic (11 to 50%), hyperendemic (51 to 75%), and holoendemic (> 75%) (Breman, 2009). The entomologic inoculation rate is the number of infectious (sporozoite carrying) female anopheline bites per year (EIR), a term used to indicate transmission intensity of a defined area (Breman, 2009). While there are seasonal and geographic differences between areas, an EIR of <10 per year is a low transmission area, 10 – 49 per year is intermediate transmission, and > 50 year is high transmission (Kelly-Hope & Mckenzie, 2009). Constant, frequent, year-round infection is termed stable transmission, generally in areas with EIRs of >100 per year. In stable transmission areas, most adults experience malarial infections that are asymptomatic, while in low or sporadic transmission areas, complete protective immunity is not acquired and symptomatic disease may

occur at all ages that may result in epidemics in such areas (Breman, 2009). An epidemic can develop when there are changes in environmental, economic, or social conditions, such as heavy rains following drought or migrations (usually of refugees or internally displaced people (IDPs) (Hay *et al.*, 2004) from a non-malarious region to an area of high transmission; a breakdown in malaria control and prevention services can intensify epidemic conditions which usually results in considerable morbidity and mortality among all age groups (Breman, 2009; Kiszewski & Teklehaimanot, 2004).

2.3.3 Malaria in Africa

Present epidemiologic findings show that Africa is undergoing a reduction in malaria transmission which has been attributed to effective large scale malaria control programmes (WHO, 2012). Interventions such as the use of effective anti-malarial therapeutics and insecticide treated bed nets (ITNs) have resulted in reducing both the burden of malaria and its associated mortality in Africa. For instance, widespread use of ITNs in Kenya resulted in a 44% reduction in mortality in children below 5 years over a two year period (Fegan *et al.*, 2007). The combined use of ITNs and artemisinin-based combination therapy in Zanzibar reduced mortality by 52% in under-fives over a two year period (Bhattarai *et al.*, 2007). Thus, in Africa, the problem of malaria continues to be a huge shadow over the socioeconomic development of the continent. In the East African highlands, there is an estimated 34 million people at risk of *P. falciparum* malaria (Ernst *et al.*, 2006).

2.4 Malaria Control Strategies

2.4.1 Vector control

Currently, vector control is focused on the use of insecticide treated bed-nets (ITNs). Although ITNs have proved efficacious in reducing severe malaria morbidity and mortality among children, there are concerns over their sustainability and long-term effects on the development of malaria immunity (Snow *et al.*, 1994; Snow *et al.*, 1995; Trape & Rogier, 1996).

2.4.2 Chemotherapy of malaria

Antimalarial drugs are designed to prevent or cure malaria. Two types of antimalarial drugs are to be distinguished, those taken as preventive called prophylactic drugs, and those that are taken once the person is already infected, called therapeutic drugs (Ogutu, 2013). Currently recommended treatment regimens in Kenya are artemisinin-based combination therapies, (Watsierah *et al.*, 2012). Antimalarial combination chemotherapy is widely advocated for delaying the development of resistance to the remaining armory of effective drugs. The concept of combination therapy is based on the synergistic or additive potential of two or more drugs, with independent modes of action and different biochemical targets in the parasite (Martinelli *et al.*, 2008).

2.5 Antigens in Malaria Vaccine Development

2.5.1 Circumsporozoite protein (CSP)

Circumsporozite protein (CSP) forms a dense coat on the parasite surface and has been hypothesized to mediate many of the initial interactions between the sporozoite and its two hosts (Menard, 2000; Sinnis & Nardin, 2002). CSPs are comprised of a central repeat region that is diverse across *Plasmodium* species, and flanking the repeats are two conserved domains: region I, a 5-amino acid sequence at the N terminus of the repeats, and a known cell-adhesive motif C-terminal to the repeats termed the type I thrombospondin repeat (TSR). Studies with recombinant CSP, peptides representing portions of CSP, and sporozoites expressing heterologous CSP suggest that this protein targets sporozoites to both mosquito salivary glands and the mammalian liver (Rathore *et al.*, 2005; Tewari *et al.*, 2005).

2.5.2 Thrombospondin-related anonymous protein (TRAP)

Thrombospondin-related anonymous protein (TRAP) is present in the sporozoite secretory invasive organelles involved in sporozoite motility and infectivity of liver cells (Sultan *et al.*, 1997). *Plasmodium* TRAP is the most extensively studied transmembrane protein. TRAP is stored within the micronemes of sporozoites, released onto the cell surface at the anterior tip upon contact with a host cell and translocated to the posterior pole of the sporozoite along its surface during penetration. It's essential role is thought to link the actin–myosin motor through its cytoplasmic domain while binding to hepatocytes via its extracellular portion (Bhanot *et al.*, 2003).

2.5.3 Liver stage antigen-1 (LSA-1)

Liver stage antigen-1 (LSA-1) is a 230 kDa protein characterized by a central repeat region containing 86 repeats of the 17-amino-acid sequence EQQSDLEQERLAKEKLQ or minor variations thereof (Fidock *et al.*, 1994). Flanking these repeats are a non-repetitive 154 residue N- terminal region and a 280 residue C-terminal region (Fidock *et al.*, 1994), known to contain B cell and CD4+ and CD8+ T cell epitopes (Cummings *et al.*, 2010). The sequence of LSA-1 repeat and non-repeat regions is highly conserved across strains of *P. falciparum* suggesting a crucial role during liver schizogony (Fidock *et al.*, 1994). LSA-1 is a surface protein which is solely expressed in infected hepatocytes and is believed to play a role in liver schizogony and the release of merozoites (Guerin-Marchand *et al.*, 1987; Hollingdale *et al.*, 1990). PfLSA-1 induced specific humoral, cellular, and cytokine immune responses in infected individuals (Connelly *et al.*, 1997) and have been considered a vaccine candidate for *P. falciparum*, due to their antigenic and protection-including immunogenic properties (Kurtis *et al.*, 2001; Taylor-Robinson, 2003). Three reasons support its development as a vaccine antigen. First, it is highly conserved suggesting that a candidate vaccine based on the 3D7 strain might elicit immune responses that cross-react with all other strains of *P. falciparum* (Fidock *et al.*, 1994). Secondly, LSA-1 is abundantly expressed from early through late schizogony, presumably allowing time for both circulating and memory-recall effector cells to infiltrate the liver and exert their effector function (Cummings *et al.*, 2010). Third, it is possible that high titer antibody could act upon the cloud of flocculent liver stage antigen enveloping hepatic merozoites to impede the latter's emergence and subsequent invasion of erythrocytes (Hollingdale *et al.*, 1990).

2.5.4 Apical membrane antigen-1 (AMA-1)

Apical membrane antigen-1 (AMA-1) is an 83kDa polymorphic membrane protein expressed in both sporozoites and merozoites. The protein is located in the micronemes, rhoptry organelles of merozoites (Crewther *et al.*, 1990) and is involved in the reorientation and formation of tight-junction that is necessary for invasion of red blood cells by merozoites. Evidence suggests that AMA-1 is a potential vaccine candidate based on studies that observed that mice and monkeys are protected from parasitemia upon vaccination with recombinant AMA-1 from *P. falciparum* (Anders *et al.*, 1998; Stowers *et al.*, 2002). Also protection has been shown to be acquired in rodents after immunization with recombinant AMA-1 against *P. chabaudi* and *P. yoelii* infections (Narum *et al.*, 2002). Studies in humans using antibodies to the full length AMA-1 have also been associated with protection (Polley *et al.*, 2004) prior to a malaria transmission season. Further studies have also shown that polyclonal anti-AMA-1 antibodies inhibit *in-vitro* merozoite invasion and in this way interfere with the processing of the antigen (Narum *et al.*, 2002).

2.5.5 Merozoite surface protein-1 (MSP-1)

Merozoite Surface Protein-1 (MSP-1) is the most abundant surface component of the merozoite stage of the parasite life cycle, totaling up to 40% of the GPI-anchored merozoite surface protein coat (Sanders *et al.*, 2005). MSP-1 is synthesized as a high molecular weight precursor (195kDa), membrane anchored, which undergoes proteolytic processing to yield fragments of several sizes 83, 42, 36, 28 – 30, and 19 kDa (Cooper, 1993; Holder, 1994; Holder & Blackman, 1994; Wipasa *et al.*, 2002). MSP-1 plays a role in the binding to and invasion of erythrocytes by

merozoites (Cowman *et al.*, 2000). Secondary proteolytic processing of MSP-1_{42} results in a final conserved C-terminal membrane-anchored moiety MSP-1_{19}. Accessibility of the merozoites to the host immune system occurs between schizont rapture and the invasion of a new red cell making this stage to be a possible target for protective immunity (Mcbride & Heidrich, 1987). Immune responses to the 83 kDa and 42 kDa fragments have been associated with protection against natural infections in west African children (Riley *et al.*, 1992; Tolle *et al.*, 1993). Antibodies to MSP-1_{19} have been found abundantly in naturally exposed people from endemic areas (Cavanagh *et al.*, 2004; John *et al.*, 2004). *In vitro*, antibodies against MSP-1_{19} fragment have been associated with inhibition of merozoite invasion of red cells (Egan *et al.*, 1999) while immunization of mice with the *P. yoelii* equivalent of this region protected them against challenge infections by the same species (Daly & Long, 1993). Seroepidemiological studies indicate that levels of anti-MSP-1_{19} antibodies are strongly correlated with protection against clinical malaria among Gambian children (Riley *et al.*, 1992) and also among Kenyan children and pregnant women (Branch *et al.*, 1998).

2.5.6 Merozoite surface protein-3 (MSP-3)

Merozoite surface protein-3 (MSP-3) is a 48-kDa protein (Oeuvray *et al.*, 1994). It is a polymorphic parasite antigen that may have a role in parasite invasion, which is evidenced by the finding that truncation of the MSP-3 gene reduces parasite invasion of erythrocytes (Mills *et al.*, 2002). Antibodies to the conserved portion of MSP-3 have been shown to mediate antibody dependent cellular inhibition (ADCI) of parasite growth in cooperation with monocytes *in vitro* (Oeuvray *et al.*, 1994). In immunization/challenge studies with MSP-3, *Saimiri sciureus* (Carvalho *et al.*, 2005) monkeys were protected from lethal challenge with malaria.

2.5.7 Erythrocyte-binding antigen-175 (EBA-175)

Erythrocyte-binding antigen (EBA) is a 175 kDa merozoite expressed protein located in the micronemes; it mediates sialic acid-dependent invasion of red blood cells (RBC) (Sim *et al.*, 1994). It has also been shown to elicit potentially protective antibody responses (Okenu *et al.*, 2000). EBA-175 was the first member of the erythrocyte-binding ligand family characterized and shown to bind to the major glycoprotein found on human erythrocytes, glycophorin A, during invasion (Narum *et al.*, 2002). Recombinant fragments of EBA-175 are recognized by human sera from malaria-endemic areas (Daugherty *et al.*, 1997; Mccarra *et al.*, 2011). Additionally, IgG1 antibodies to EBA-175 peptide 4 are associated with protection against clinical malaria (Toure *et al.*, 2006).

2.5.8 Glutamate-rich protein (GLURP)

Glutamate-rich protein (GLURP) is a 220-kDa protein expressed in the preerythrocytic stage, in schizonts, and on the surface of newly released merozoites (Borre *et al.*, 1991). The antigen contains an amino-terminal nonrepeat region R0 (GLURP 94 – 489), a central repeat region (GLURP 489 – 705) (R1) and a carboxy – terminal (GLURP 705 – 1178) (R2) repeat region (Dodoo *et al.*, 2000). GLURP is an antigen expressed in all stages of the parasite life cycle in humans, including on the surface of newly released merozoites (Borre *et al.*, 1991). Antibodies against GLURP were found to react with the asexual, hepatic, and gametocyte stages of the parasite (Borre *et al.*, 1991), suggesting that GLURP is synthesized throughout the entire life cycle of *P. falciparum* in the vertebrate host. It is highly antigenic and the gene encoding

GLURP shows little polymorphism in geographically different *P. falciparum* isolates (De Stricker *et al.*, 2000).

2.6 Development of Multiplex Assays

Most studies that have determined antibody levels in human plasma samples have been measured through the use of enzyme linked immunorsorbent assay (ELISA) (Connelly *et al.*, 1997; Noland *et al.*, 2008), though a number of studies have also used multiplex assay for antibody determination (Cham *et al.*, 2009; Lal *et al.*, 2005). Comparison studies of antibody measurements through multiplex assay and traditional uniplex ELISA assay have shown a high correlation (Fouda *et al.*, 2006). The availability of the multiplex assay necessitates its validation, standardization and application as a suitable alternative for measurement of immune molecules that need to be determined as essential correlates of immunity. Cytokine levels have also been measured through use of both ELISA (Chelimo *et al.*, 2003) and multiplex assays (Carson & Vignali, 1999; De Jager *et al.*, 2003). Development and application of multiplex assay will result in abundant information being available from a single individual being tested.

The bioplex^{100} system can simultaneously quantitate up to 100 different proteins, peptides, DNA fragments and RNA fragments from a single drop of sample in a well of a microtiter plate (Lal *et al.*, 2005). The multiplex assay is a bead format assay in which each bead set is internally color coded with different ratio of red to infrared dyes that results in a unique bead set that can be classified separately by the bioplex machine. The beads in multiplex assay anchor the antigens as opposed to ELISA where the surfaces of the wells of microtiter plate anchor the antigen. The bioplex machine has two lasers; one laser beam excites the internal colored dyes for classification of the bead sets while the other laser excites the reporter fluorochrome

phycoerythrin (PE) (Cham *et al.*, 2008). Through classification of the bead set, various bead sets are distinguished which correspond to up to 100 different analytes that the machine could quantitate, while the amount of analyte present in the plasma, serum or supernatant is quantified by excitation of the reporter fluorochrome (Giavedoni, 2005). To maximize the benefits of the multiplex assay there is need to understand some of the technical, scientific and optimal parameters for the development and validation of this technique.

2.7 Antibody-mediated Responses to *Plasmodium* Antigens

B cells and antibodies are largely involved in immunity to malaria. Experiments with antibodies purified from the sera of African adults who were clinically immune to malaria and given by passive transfer to susceptible children have shown that immunoglobulin G is at least a main component of defense against the asexual blood stage of *P. falciparum* (Bouharoun-Tayoun *et al.*, 1990; Cohen *et al.*, 1961; Druilhe & Perignon, 1994) and also the transfer of antibodies from immune adults living in endemic areas but not from non-immune adults to children with severe clinical malaria and high parasitemia resulted in significant reduction in both disease symptoms and parasite levels (Bouharoun-Tayoun *et al.*, 1990). Antibodies may act in different ways, by preventing merozoite invasion of red blood cells (Dent *et al.*, 2008; Egan *et al.*, 1999), by attacking infected RBCs and facilitating phagocytosis, or by preventing cytoadhesion of infected RBCs (Miller & Hoffman, 1998; Mo *et al.*, 2008).

Mice that lacked B cells were unable to clear *P. chabaudi chabaudi* infection which progressed to chronic parasitaemia (Von Der Weid *et al.*, 1996). The degree of protective immunity in humans (Braga *et al.*, 2002; Piper *et al.*, 1999) and monkeys (Egan *et al.*, 2000) has been

reported to correlate with levels of antibodies against blood stage antigens. Further, antigen-specific *P. falciparum* antibodies have been implicated to play a crucial role in controlling parasitemia through antibody dependent cellular inhibition (ADCI) (Bouharoun-Tayoun *et al.*, 1995; Wipasa *et al.*, 2002). IgG1 and IgG3 subclasses are cytophilic antibodies involved in protection against *P. falciparum* malaria in humans (Jafarshad *et al.*, 2007). They mediate opsonization and antibody dependent cellular inhibition together with monocytes and macrophages. Correlation of antibodies against merozoites and blood stage parasites with protection has been observed as a reduction in morbidity, high density parasitaemia and symptomatic malaria (Bull *et al.*, 1998; Iriemenam *et al.*, 2009; Kinyanjui *et al.*, 2007; Ofori *et al.*, 2002; Stanisic *et al.*, 2009).

Selection of antigens capable of eliciting strong and harmless antibody responses is a prerequisite for the development of a malaria vaccine (Garraud *et al.*, 2003). Early studies showed that purified IgG from malaria-immune adults, when transferred to children acutely ill with malaria resulted in reduced fever and decrease in malaria (Cohen *et al.*, 1961). Most antibody-based analyses of protection are tethered on seropositivity (usually defined as the mean plus 3 standard deviations of non-malaria exposed sera). This indicated that antibodies against *P. falciparum* proteins play a critical role in controlling blood stage infection. However, which of the 5,400 possible *P. falciparum* proteins elicits the production of protective antibodies is unclear (Gardner *et al.*, 2002). Few studies have examined the antibody responses against multiple malaria antigens (John *et al.*, 2008) and whether these might be persistent or diminish when malaria transmission is low. The completion of *P. falciparum* genome sequencing has identified numerous new (and old) parasite antigens that are being characterized. Development of high-

throughput assays employing suspension array technology (Fouda *et al.*, 2006) can allow for simultaneous analysis of antibodies to multiple antigens using minimal amounts of sera.

Some studies of malaria in areas of low transmission support the view that the risk of uncomplicated malaria is similar in young and old individuals, although severe disease and death from malaria in these areas occurs frequently in young children (Carneiro *et al.*, 2010; Okiro *et al.*, 2009). Studies on age-related risk of malaria in highland areas, which have unstable, seasonal, and low levels of malaria transmission, have produced conflicting results (Guerra *et al.*, 2008).

CHAPTER THREE

METHODOLOGY

3.1 Study Area

This study was conducted in two sites: Kapsisiywa and Kipsamoite. Kapsisiywa is characterized by seasonal *P. falciparum* malaria transmission pattern while Kipsamoite has always had fewer cases of malaria compared to Kapsisiywa (John *et al.*, 2009). Both study sites are located in Nandi County in western part of Kenya, a highland area prone to epidemics, with an estimated entomological inoculation rate of <1 infectious bite per person per year (Noland *et al.*, 2008). These sites have previously experienced an interrupted malaria transmission due to the wide scale malaria control interventions (John *et al.*, 2009). The sites lies between 0°16'55.64° N to 0°21'52.40° N latitude and 34°59'7.17" E to 35°5'19.90" E longitude. Appendix 1 shows map of Kenya with the two study sites. Kipsamoite covers an area of approximately 16 km^2 (Ernst *et al.*, 2009). Elevation in Kapsisiywa ranges from 1,887m to 1,982m, while Kipsamoite varies between 1,950m and 2,100m (Cohen *et al.*, 2008). Kipsamoite's western side is bordered by a forest which is hilly and rocky. A swamp borders the eastern part of the study area (Ernst *et al.*, 2006). Some sections of the swamp have been drained for farming purposes but in other areas they are undrained.

Major occupation of the individuals living in this study area is subsistence crops farming (maize, beans and vegetables), cash crop farming (sugar cane and tea) and animal husbandry (chickens, cattle, sheep and goats). *Anopheles gambiae s.l* is the most abundant vector (97.5%) and *Anopheles funestus* is at 2.5% (Ernst *et al.*, 2006). *P. falciparum* is the predominant malaria

species. In 2006, both sites were targeted by the Ministry of Health for indoor residual spraying and distribution of ITNs to pregnant women and children <5 years (John *et al.*, 2009).

3.2 Study Population

The population at the study sites was about 3,787 for Kapsisiywa and 4,180 for Kipsamoite individuals, most of these people were of Kalenjin tribe (Noland *et al.*, 2012).

3.3 Sample Size Calculation

The sample size was calculated with the following assumptions: 80% power to detect a 40% decrease in antibodies.

Sample size = effect size (0.4) power (.8) prevalence (0.5)/significance (0.05) 2007/2008 (5700/1700) rate of return (90%).

2007 - Number of individuals whose plasma was collected in 2007

2008 - Number of individuals whose plasma was collected in 2008

= 0.4x0.8x0.5x5700x90/0.05x1700

= 965 individuals (Rounded off to 1000)

(Noland *et al.*, 2012; Noland *et al.*, 2008).

Since these samples were from a site wide survey, a random selection of 1,000 individuals was done from both sites and those selected must have had their blood samples collected in both surveys. 1,000 randomly selected individuals from the two study sites (Kipsamoite, n = 457;

Kapsisiywa, n = 543) were tested for antibodies to a panel of antigens. In Kipsamoite, they were grouped as 0 - 5 yr (n = 109); >6 –15 yr (n = 174); >16 – 40 yr (n = 114) and >40 yr (n = 60). In Kapsisiywa, they were grouped as 0 - 5 yr (n = 128); > 6 –15 yr (n = 177); >16 – 40 yr (n = 150) and >40 yr (n = 88). By gender distribution 480 were males and 520 females.

Positive controls were obtained by mixing plasma samples from 30 individuals who live in an area endemic of malaria. The assumption was that in lowland area these individuals must have been exposed to malaria hence positive humoral immune responses could be detected.

3.4 Inclusion Criteria

Individuals of any age who live within the boundaries of the two study sites.

3.5 Exclusion Criteria

Declining to participate in the study.

3.6 Experimental Design

Two site-wide surveys were conducted in May 2007 and July 2008. Blood samples were collected from one thousand individuals at both time points (Kapsisiywa, n = 543 and Kipamoite, n = 457). Blood (about 500µl) was collected by finger prick into heparinized tubes. Also, consenting individuals provided drops of blood for preparation of thin and thick blood smears for malaria microscopy. Plasma was separated at the two field laboratories by centrifugation at 1,100 x g for 5 min. Separated plasma and blood pellet were transported on ice to Kenya Medical Research Institute (KEMRI), Kisumu, Kenya, for storage −20 °C until use.

One thousand randomly selected paired individuals from the two study sites (Kapisisiywa, n = 543; Kipsamoite, n = 457) were tested for antibodies to a panel of *P. falciparum* antigens. A subset of randomly selected individuals (Kapsisiywa, n = 296; Kipsamoite, n = 231) were also tested for antibodies to VCA-P18 antigen (an Epstein Barr Virus antigen). Every participant sample was given a unique study identification number, which included information on site of residence within the study area, village of residency, household and member status of the participant. Data was double-entered and verified using File Maker Pro database software.

3.7 Microscopy for Parasite Detection and Parasite Density Counts

Thick and thin blood films were made for malaria microscopy. The blood smears were stained using 5% Giemsa solution at pH 7.2. Parasites in the thick smears were counted against 200 white blood cells (WBCs) and the counts recorded for *Plasmodium* spp asexual forms and gametocytes. Two microscopists read the slides to determine if a slide was positive or negative for quality control. In case of discrepancies between the two microscopists, a third microscopist was sought. Individuals diagnosed with malaria were referred for treatment at the local health centers. Parasite density was estimated by counting the number of parasites per 200 leukocytes in an oil-immersion thick blood film at 100× objective lens magnification. Parasite counts were converted to parasites per lμl assuming an average of 8,000 leukocytes/μl for each sample.

3.8 *Plasmodium falciparum* Recombinant and Peptide Antigens

Recombinant proteins to the *P. falciparum* antigens: apical membrane antigen-1 (AMA-1, full length ectodomain, 3D7 and FVO strains), erythrocyte-binding antigen (EBA-175, non-

glycosylated region II), glutamate rich protein (GLURP, conserved non-repeat N-terminal region, amino acids 25–514, R0; and repeat C-terminal region, amino acids 705–1178, R2, 3D7 strain), merozoite surface protein-1 (MSP-1_{19}, E-KNG variant; MSP-1_{42}, 3D7, FUP and FVO strains), merozoite surface protein-3 (MSP-3, C-terminus, FVO strain), LSA-1 (C-terminal region, amino acids 1628 to 1909, 3D7 strain) were used for testing. Recombinant AMA-1 and LSA-1 previously expressed in *E. coli* and provided by David Lanar, Walter Reed Army Institute for Research, MA, USA; recombinant MSP-1_{42} and MSP-3 previously expressed in *E. coli*, and recombinant EBA-175 previously expressed in *Pichia pastoris*, and provided by David Narum, National Institutes of Health, USA; recombinant GLURP previously expressed in *E. coli* and provided by Michael Theisen, Statens Seruminstitut, Copenhagen, Denmark; recombinant MSP-1_{19} previously expressed in *Saccharomyces cerevisiae* and provided by the Malaria Research and Reference Reagent Resource Center (Manassas, VA) originally deposited there by David Kaslow, were also used for testing. For circumsporozoite protein, the $(NANP)_5$ repeat peptide was used, and *P. falciparum* parasites from the 3D7 parasite clone were cultured in the preparation of schizont crude extract (SE) antigen used in ELISA assays. LSA-1 antigen, the central amino acid repeat sequence (LAKEKLQGQQSDLEQERLAKEKLQ-EQQSDLEQERLAKEKLQ, LSA-1 Rep) was used. Blank samples consisted of the plasma diluent alone.

3.9 Control Antigen

EBV-specific antibody measurements were detected using synthetic immunodominant peptide epitope of viral capsid antigen (VCA-p18) provided by Jaap Middeldorp (Vrije Universiteit Medical Center, Amsterdam, The Netherlands). VCA-p18 antigen served as an internal control

to the malaria antigens. This enabled the determination of whether changes in antibody levels to malaria antigens could specifically be attributed to *Plasmodium* antigens and related to the period of absent parasite exposure. Optical densities were used as a proxy indicator of antibody levels due to lack of a realistic criteria of categorizing individuals as either responders or non-responders.

3.10 Coupling of Proteins to Microspheres

Microspheres were purchased from Luminex Corporation (Austin, TX). The bead stock was resuspended by gentle inversion for 1min. An aliquot of 612,500 beads was removed and centrifuged at 16,000g (Labnet Wood bridge, NJ) for 3min. The supernatant was removed and 100µl of distilled water added and centrifuged at 16,000g for 3min. Beads were resuspended in 80µl of activation buffer, 100mM monobasic sodium phosphate pH 6.2 (Sigma, S3139), by vortexing (Scientific Industries Bohemia, NY) and sonication for 20sec each.

10µl of 50mg/ml N- hydroxysulfosuccinimide sodium salt (Sigma, 56485) was added and the beads mixed by vortexing for 10sec at moderate speed to activate the beads for cross-linking to proteins. Next, 10µl of 50mg/ml N-[3-dimethylaminopropyl] – N`- ethylcarbodiimide hydrochloride (Sigma, E1769) was added and the beads mixed again by vortexing for 10sec at moderate speed. All incubations of beads were performed in the dark (covered with foil). The bead mixture was rotated on a rotary shaker (Labnet Edison, NJ) at room temperature for 20min and vortexed for 10sec at 10min and at 20min, both at moderate vortexing speed. Beads were pelleted by centrifuging at 16,000g for 5min. Beads were then washed twice with 250 µl of 100 mM morpholineethanesulfonic acid (MES) pH 6.0 (Sigma, M2933), buffer (Adapted from

Luminex coupling protocol). Beads were again pelleted by centrifugation at 16,000g for 5min. To coat the beads with antigens, pelleted beads were resuspended with the relevant antigen and the volume adjusted to 500µl per reaction by addition of coupling buffer (100 mM MES pH 6.0). Beads were conjugated to 0.5, 1, 2 and 5, 10 and 1000µg of different antigens. The antigen and activated beads mixture was incubated on a rotary shaker for 2hr at room temperature in the dark to allow bead coupling to occur. After being coated with proteins, beads were centrifuged at 16,000g for 3min and washed twice with 250µl of PBS-TBN and resuspended in 200µl of PBS-TBN. To determine the percentage recovery after the coupling procedure, coupled beads were counted on a hemocytometer (Hausser Scientific Horsham, PA).

3.11 Quantification of Antibodies Specific to Malaria Antigens

The volume of working solution (50µl/ well) was calculated together with the number of beads that would result in 1,000 beads/region/well or 5,000 beads/region/well. Bead stocks were then combined in a 15ml amber conical tube and diluted with PBNT to result in 100 microspheres/µl or 20 microspheres/µl. 96 well-millipore microtiter plates (MABVN 1250, Millipore corporation, Billerica, MA) were pre-wetted with 100µl of PBNT/well and aspirated using a millipore vacuum manifold and 50µl of working bead solution was transferred to it. Plasma samples were thawed at room temperature, mixed and centrifuged at 16,000g for 3min. Plasma was diluted through a series of concentrations: 1:100, 1:200, 1:400, 1:1000, 1:2000 and 1:4000. These serial dilutions were taken arbitrarily from information in previous studies. Plasma samples were diluted in either buffer A (1xPBS, 0.1% BSA, 0.05% Polysorbate 20, and 0.05% sodium azide) or buffer B (1xPBS, 1% BSA, 0.05% Polysorbate 20, 0.05% sodium azide, 0.5% polyvinylalcohol, and 0.8% polyvinylpyrrolidone). Buffer A is the standard buffer used by other

studies of multiplex CBA for *P. falciparum* antigens (Cham *et al.*, 2009), while buffer B has been used for multiplex antibody testing to other antigens and found to decrease background reactivity (Waterboer *et al.*, 2006).

Fifty µl of diluted plasma was added to each of the well of the microtiter plate. The plasma was mixed with the beads three times by pipetting up and down. The plates were incubated in the dark on a shaking microplate shaker (IKA® MTS, Wilmington, NC) at 600 rpm for 30sec, followed by 300 rpm for 30min. Plates were aspirated using a millipore vacuum manifold and washed twice with 100 µl/well of PBNT, and beads were resuspended in 50µl PBNT by pipetting. 50µl of diluted 1: 1000 goat antihuman IgG (gamma- chain specific, F(ab`)2 fragment-R-phycoerythrin (Sigma, P-8047 St. Louis, MO) in PBNT was added to each well, and incubated in the dark with shaking at 600 rpm for 30sec, followed by 300 rpm for 30min. Plates were aspirated using a millipore vacuum manifold and washed twice with 100 µl/well PBNT. The beads were resuspended in 100µl PBNT by mixing and analyzed on bioplex^{200} machine (Hercules, CA). The reader was set to read a minimum of 100 beads with of unique fluorescent signature/region and the results expressed as median fluorescence intensity (MFI). Table 1 shows the parameters that were optimized and tested in the standardization and validation of the multiplex assay. Appendix 2 shows the multiplex assay protocol.

Table 1. Parameters optimized in the multiplex cytometric bead based assay with a set of variables investigated, description of samples used and standardized values that gave optimal results.

Experimental condition tested	**Values tested**	**Type and number of plasma samples used**	**Outcome**
Amount of antigen	0.5, 1, 2, 5, 10 and 1000µg	Positive pool (see Methods) in duplicate wells per antigen amount	Different antigens had different optimal amounts (see Results)
Plasma buffer component BSA Polyvinylalcohol Polyvinylpyrrolidone	BSA, polyvinyl alcohol, polyvinylpyrrolidone concentrations Buffer A (0.1%, 0%, 0%) vs. Buffer B (1%, 0.5%, 0.8%)	North American (non-malaria exposed) control pool and positive plasma pool	Buffer B had similar MFI values to Buffer A for positive plasma pool samples, but lower MFI values than Buffer A for NA controls
Plasma dilution	1:100, 1:200, 1:400, 1:1,000, 1:2,000 and 1:4,000	30 samples from persons from a malaria endemic area, 7 North American control samples and duplicate positive pool plasma samples	Optimal 1:100 or 1:200
Assay format	Monoplex (each Ag) Multiplex (10 different Ags)	8 malaria endemic plasma samples	Multiplex and monoplex gave similar values
Number of microspheres per reaction	5000 beads/analyte/well, 1000 beads/analyte/well	3 North American, 16 malaria endemic samples	1000 and 5000 beads/analyte/well gave similar values
Reproducibility	0 day 7 days later	Positive pool, 2 North American and 3 malaria endemic samples	Highly reproducible results

3.12 Total IgG ELISA Assay

Similar plasma samples were tested for IgG antibodies to the same *P. falciparum* antigens by enzyme-linked immunosorbent assay (ELISA) in order to validate the multiplex assay. Recombinant antigens were dissolved in 0.01 M PBS to concentrations: 0.1 µg/ml for AMA-1 3D7, AMA-1 FVO, EBA-175 and GLURP-R2, 0.2 µg/ml for MSP-1_{42} FVO, MSP-1_{42} 3D7, and MSP-1_{42} FUP, 0.5 µg/ml for GLURP-R0, MSP-1_{19} and MSP-3 FVO. CSP, LSA-1, schizont extract and VCA-p18 peptides were dissolved in 0.05 M sodium bicarbonate (pH 9.6) to a concentration of 10 µg/ml. Fiftymicroliters of antigen solution was added to Immulon-4 plates (Dynex Technologies,Chantilly, VA). Following overnight incubation at 4°C, washing with PBS-0.05% Polysorbate 20, and blocking in 5% (wt/vol) nonfat powdered milk in PBS, duplicate 50µl samples of serum diluted through a series of concentrations ranging from 1:100, 1:200, 1:500, 1:1000, and 1:2000 in 5% powdered milk were added to wells and incubated for 2hr at room temperature. After washing with PBS-0.05% Polysorbate 20, 50µl of alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000 in 5% powdered milk was added and incubated for 1hr. After extensive washing with PBS-0.05% Polysorbate 20, *p*-nitrophenylphosphate was added in accordance with the manufacturer's instructions (Sigma, S0942 St. Louis, MO). The optical density (OD) was measured at 405 nm (Molecular Devices, Sunnyvale, CA). Each ELISA and multiplex plate coated with individual peptide contained blanks, negative and positive controls. Antibody levels were expressed in arbitrary units (AU), which were calculated by dividing the OD generated by the test sample by the mean OD plus 3 SD generated by samples from 40 North Americans never exposed to

malaria. Study participants with values > 1.0 AU were considered responders. Appendix 3 shows the total IgG ELISA standard operating procedure.

3.13 Statistical Analysis

The degree of association between median fluorescence intensities (MFI) or optical densities (OD) values was assessed using Pearson's correlations (r). Comparison of buffers in multiplex assay was determined using student's t-test. Differences in the proportion of individuals with antibodies to various *P. falciparum* antigens were evaluated by chi-square test. Antibody frequencies of paired samples were evaluated by the McNemar test. Mann Whitney U test was used to test differences in antibody levels between the two sites. Antibody levels of paired samples were evaluated by Wilcoxon-matched pair sign test. Spearman rank correlation was used for correlating antibodies to antigens that had various forms. The independent variable considered was age. Correlations between continuous variables, e.g., antibody levels and age, were assessed by Spearman's rank correlation. All statistical test were 2-sided and a *P* value of less than or equal to 0.05 was considered to be statistically significant for all comparisons. Analyses were conducted with Stata software version 10.0 (Stata Corporation, College Station, Tx).

3.14 Ethical Considerations

Ethical approval for the study was obtained from Kenya Medical Research Institute National Ethical Review Committee (Appendix 4) and the Institutional Review Boards of Makerere University and University of Minnesota. Informed consent was obtained from study individuals or, in the case of minors, from their parent or guardian (Appendix 5).

CHAPTER FOUR

RESULTS

4.1 Determination of Optimal Parameters for the Development of Cytometric Multiplex Bead Assay

4.1.1 Optimal amount of antigen for multiplex CBA assay

The optimal antigen amount was the amount that yielded the highest average MFI value. The optimal amount for MSP- 1_{42} FUP, MSP- 1_{42} 3D7 and GLURP-R0 was 0.5µg; for AMA-1 3D7, MSP-1_{42} FVO, MSP-3 FVO was 1µg; for AMA-1 FVO and EBA-175 was 2µg; and for MSP-1_{19} was 10µg. The optimal amount for GLURP-R2 was not tested separately, and the same amount as for GLURP-R0 (0.5µg) was used in multiplex testing.

4.1.2 Monoplex and multiplex CBA formats

Results showed that MFI values for monoplex and multiplex were similar and statistically significant correlations between the two formats for all antigens was observed, with a Pearson's correlation coefficient (r) ranging from 0.88 to 0.99, and all P values <0.0001 except for MSP-1_{42} FUP, P= 0.004 and MSP-1_{42} 3D7, P= 0.0003 (Figure 2 A-J for AMA-13D7, AMA-1 FVO, EBA-175, MSP-1_{19}, MSP-1_{42}3D7, MSP-1_{42}FUP, MSP-1_{42}FVO, MSP-3, GLURP-R0 and GLURP-R2, respectively).

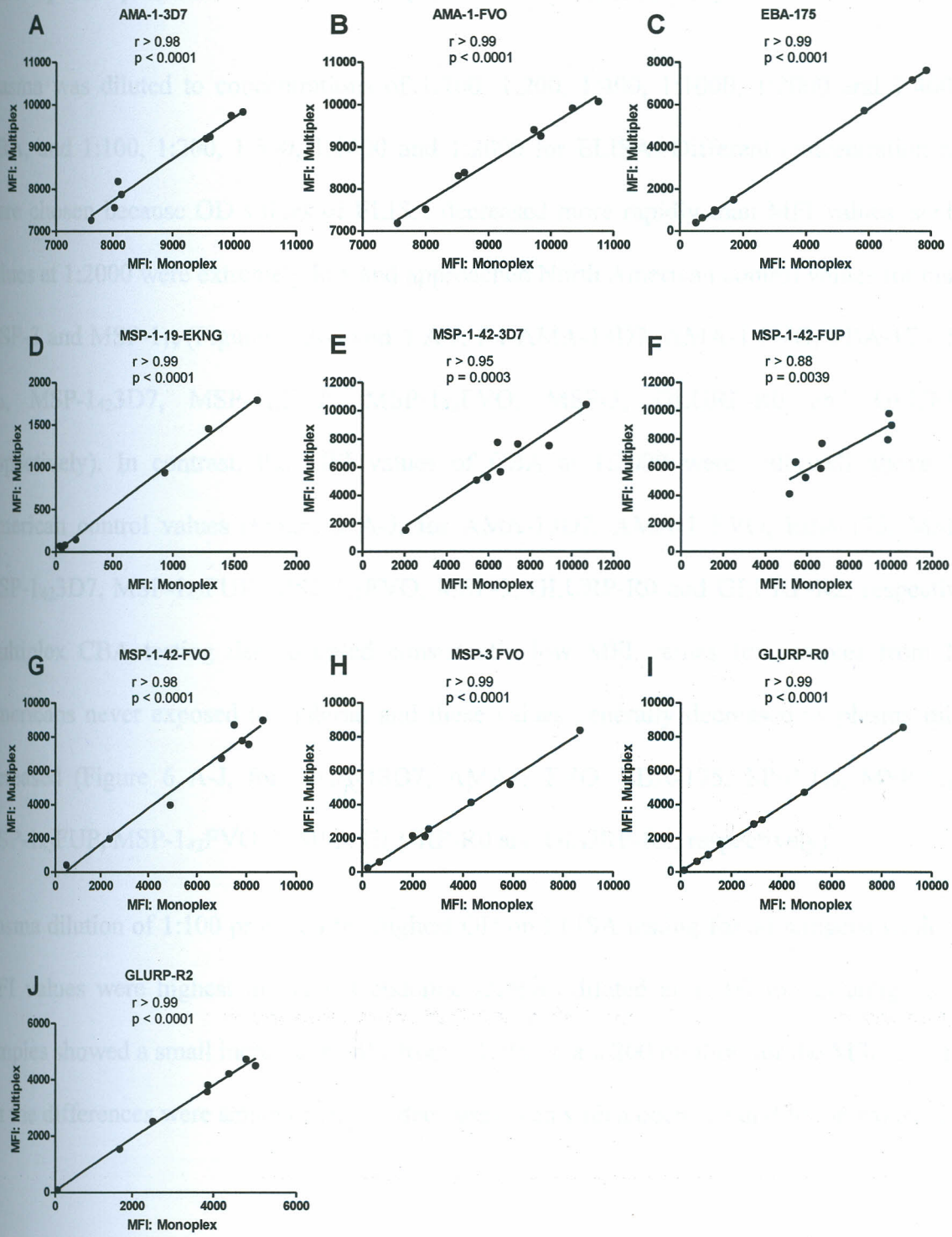

Figure 2 A-J. Correlation of data between monoplex and 10-plex CBA in testing plasma samples by antigen, plasma from individuals in an malaria endemic area, (n = 8).

4.1.3 Optimal plasma dilution for multiplex CBA and ELISA assays

Plasma was diluted to concentrations of 1:100, 1:200, 1:400, 1:1000, 1:2000 and 1:4000 for CBA, and 1:100, 1:200, 1:500, 1:1000 and 1:2000 for ELISA. Different concentration ranges were chosen because OD values of ELISA decreased more rapidly than MFI values, such that values at 1:2000 were extremely low and approached North American control values for instance MSP-3 and MSP-1_{19} (Figures 3 A-J and 4 A-J, for AMA-13D7, AMA-1 FVO, EBA-175, MSP-1_{19}, MSP-1_{42}3D7, MSP-1_{42}FUP, MSP-1_{42}FVO, MSP-3, GLURP-R0 and GLURP-R2, respectively). In contrast, the MFI values of CBA at 1:2000 were still well above North American control values (Figure 5 A-J, for AMA-13D7, AMA-1 FVO, EBA-175, MSP-1_{19}, MSP-1_{42}3D7, MSP-1_{42}FUP, MSP-1_{42}FVO, MSP-3, GLURP-R0 and GLURP-R2, respectively). Multiplex CBA testing demonstrated consistently low MFI values for samples from North Americans never exposed to malaria, and these values generally decreased as plasma dilution increased (Figure 6 A-J, for AMA-13D7, AMA-1 FVO, EBA-175, MSP-1_{19}, MSP-1_{42}3D7, MSP-1_{42}FUP, MSP-1_{42}FVO, MSP-3, GLURP-R0 and GLURP-R2, respectively).

Plasma dilution of 1:100 provided the highest OD on ELISA testing for all antigens while mean MFI values were highest in malaria endemic samples diluted at 1:100 for all antigens. Nine samples showed a small increase in MFI from a 1:100 to a 1:200 dilution for the MSP-1_{42} alleles, but the differences were smaller than the decreases seen with a decrease in dilution from 1:100 to

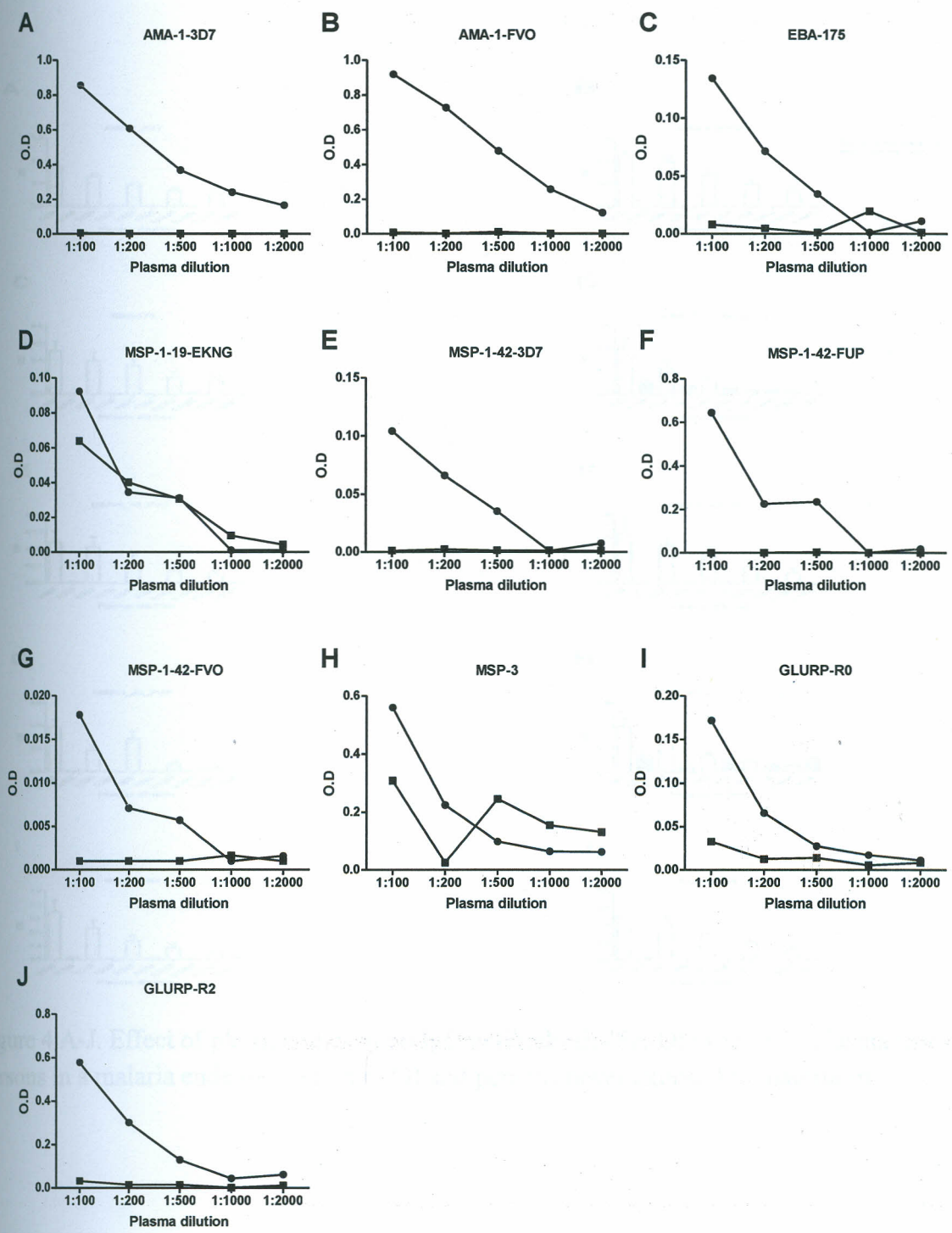

Figure 3 A-J. Effect of plasma dilution on IgG antibody ELISA OD values. Individuals from a malaria endemic area (filled circles, n = 30) vs. North Americans (filled squares, n = 7). OD values on Y-axis differ by antigen.

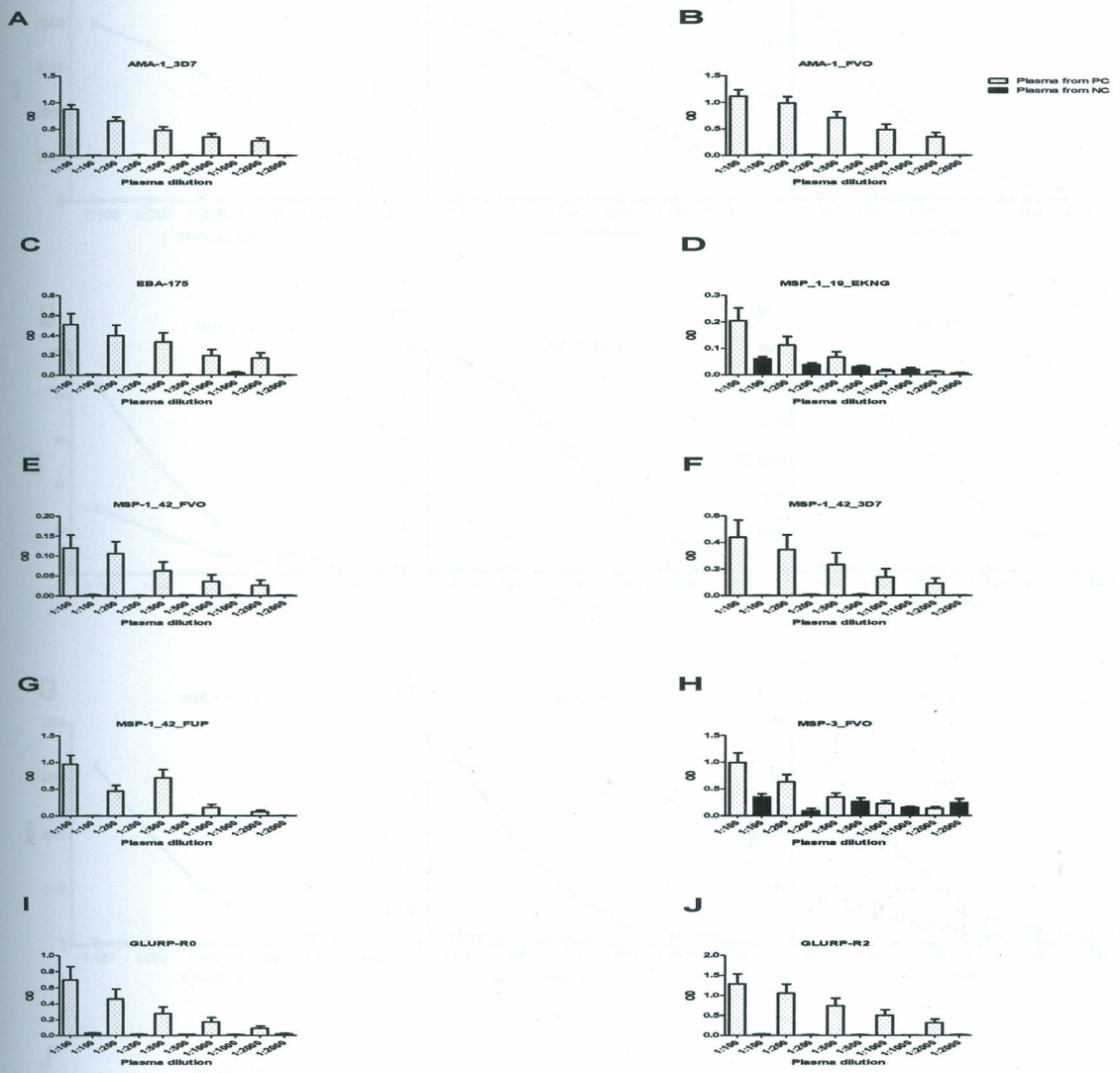

Figure 4 A-J. Effect of plasma dilution on IgG antibody ELISA OD values of plasma from persons in a malaria endemic area (n = 30) and persons never exposed to malaria (n = 7).

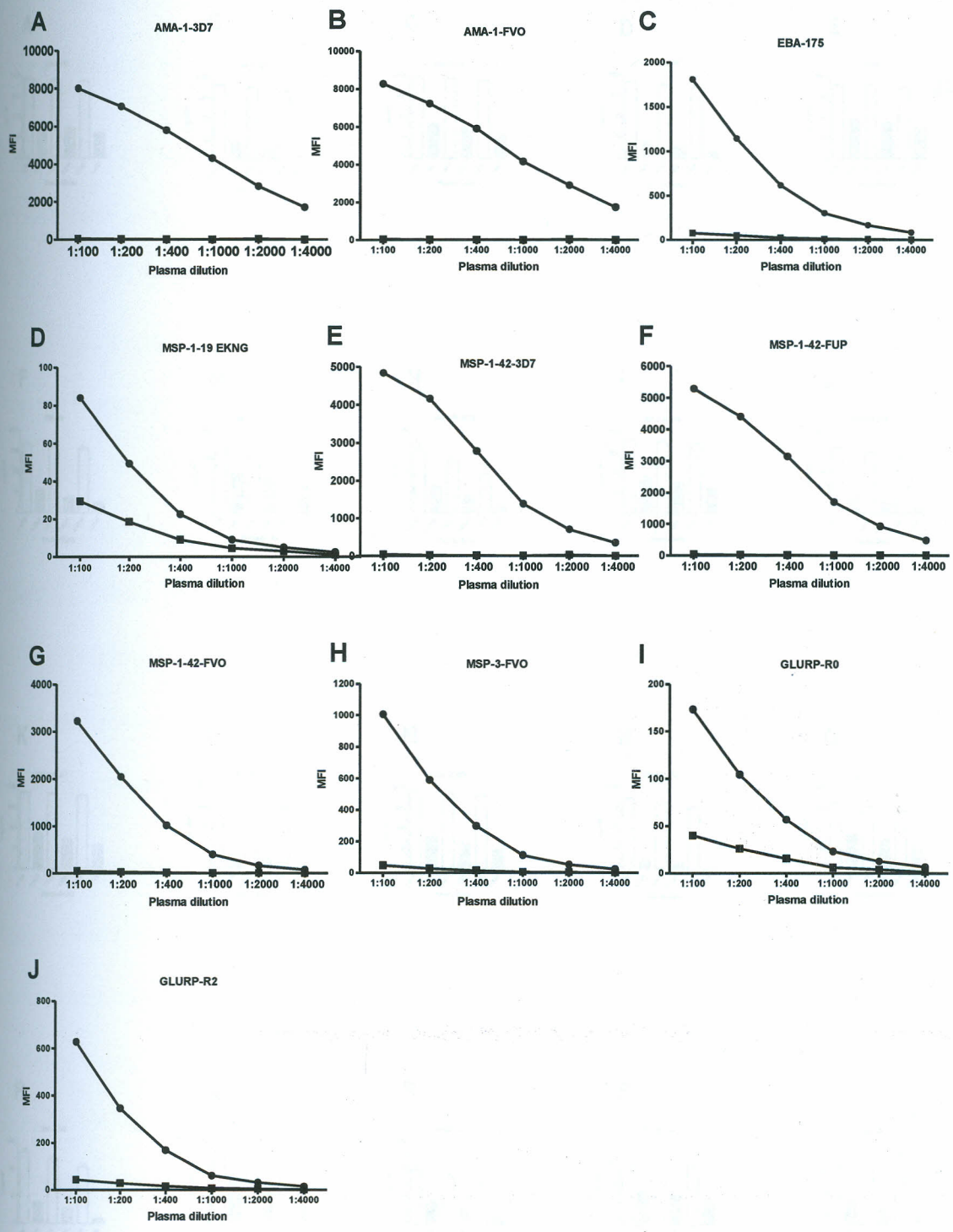

Figure 5 A-J. Effect of plasma dilution on IgG antibody MFI values. Individuals from a malaria endemic area (filled circles, n = 30) vs. North Americans control participants never exposed to malaria (filled squares, n =7).

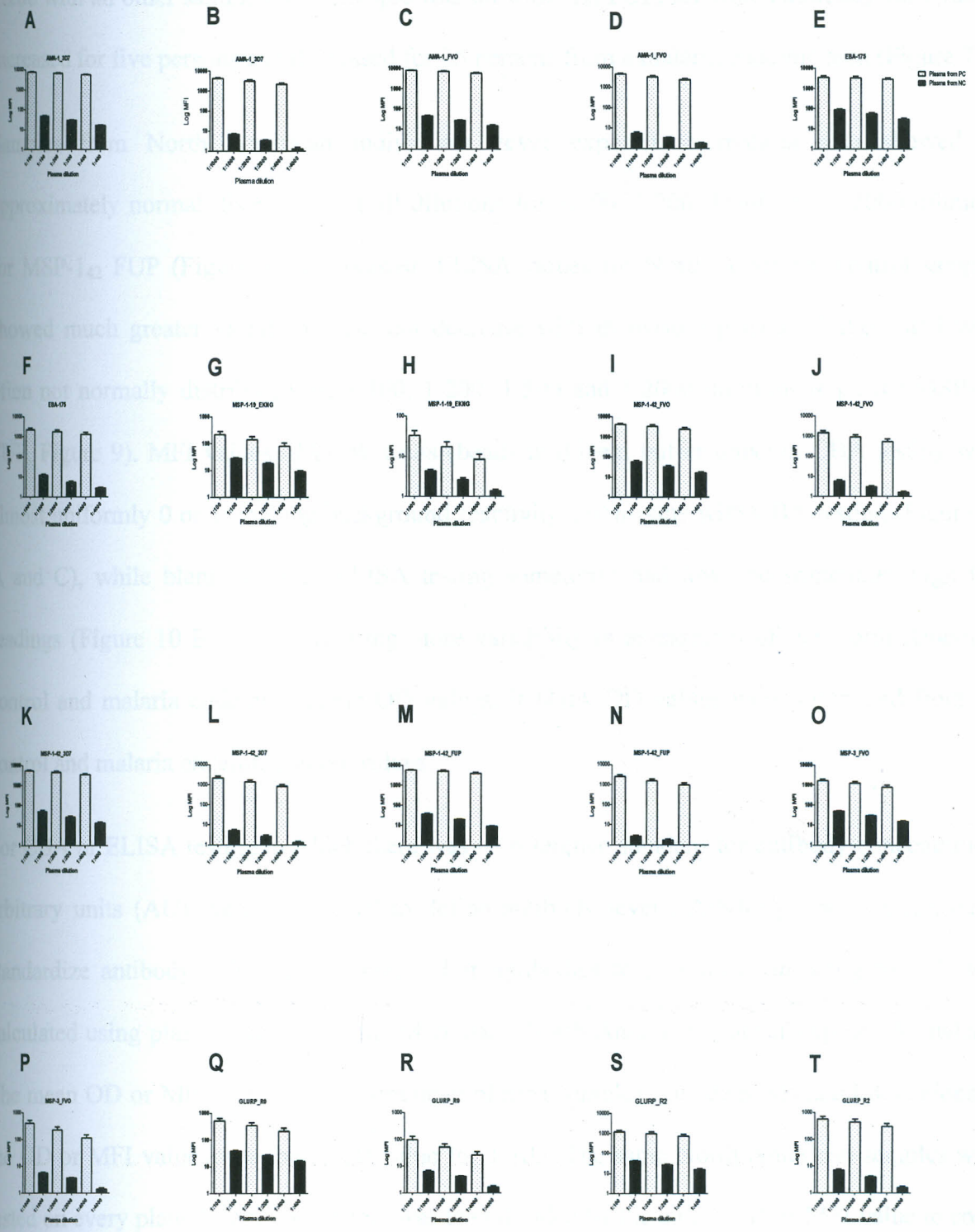

Figure 6 A-J. Effect of plasma dilution on IgG antibody CBA MFI values of plasma from persons in a malaria endemic area, (n = 30) and persons never exposed to malaria, (n = 7).

1:200 with all other samples. An example was for MSP-1_{42} FUP, for which antibody MFI values increased for five persons and decreased for 25 persons from a malaria endemic area (Figure 7).

Samples from North American individuals never exposed to malaria also showed an approximately normal distribution at all dilutions for 1:100, 1:200, 1:400 and 1:2000 dilutions for MSP-1_{42} FUP (Figure 8). In contrast, ELISA values for North American control samples showed much greater variability, did not decrease with increasing plasma dilution, and were often not normally distributed for 1:100, 1:200, 1:500 and 1:2000 dilutions (e.g., for MSP-1_{42} FUP, Figure 9). MFI values of blank wells (beads in diluent buffer only) in CBA testing were almost uniformly 0 or 1, making background reactivity a non-issue with CBA testing (Figure 10 A and C), while blank wells in ELISA testing sometimes had low and sometimes high OD readings (Figure 10 B and D), creating more variability in assessment of the North American control and malaria endemic sample OD values, if blank OD values were subtracted from the control and malaria endemic sample values.

For CBA or ELISA testing in which there was no reference standard for antibody concentration, arbitrary units (AU) were often used to define antibody levels. Arbitrary units were used to standardize antibody values across plates. For antibodies to *P. falciparum* antigens, AU was calculated using plasma samples from individuals (North Americans) never exposed to malaria. The mean OD or MFI of the North American plasma samples plus three standard deviations of the OD or MFI values was the cutoff value for 1 AU. The same North American samples were tested on every plate. Every test sample was then divided by the cutoff OD or MFI value to come up with an AU value, and a sample was categorized as "positive" by either multiplex or ELISA if

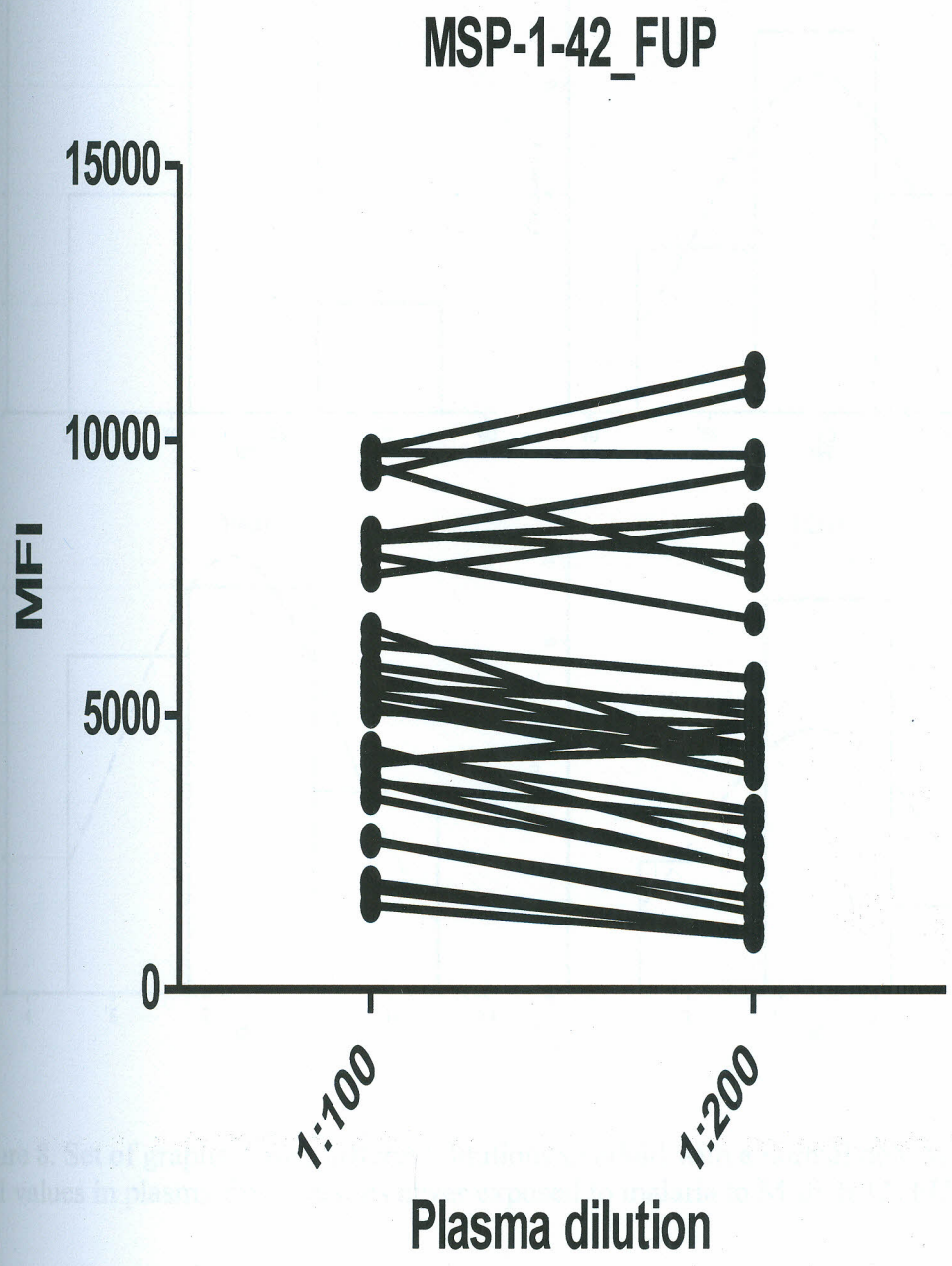

Figure 7. MFI values for antibodies to MSP-1 (42) FUP in individuals from a malaria endemic area (n = 30) at 1: 100 and 1: 200 plasma dilutions. Each dot represents an individual.

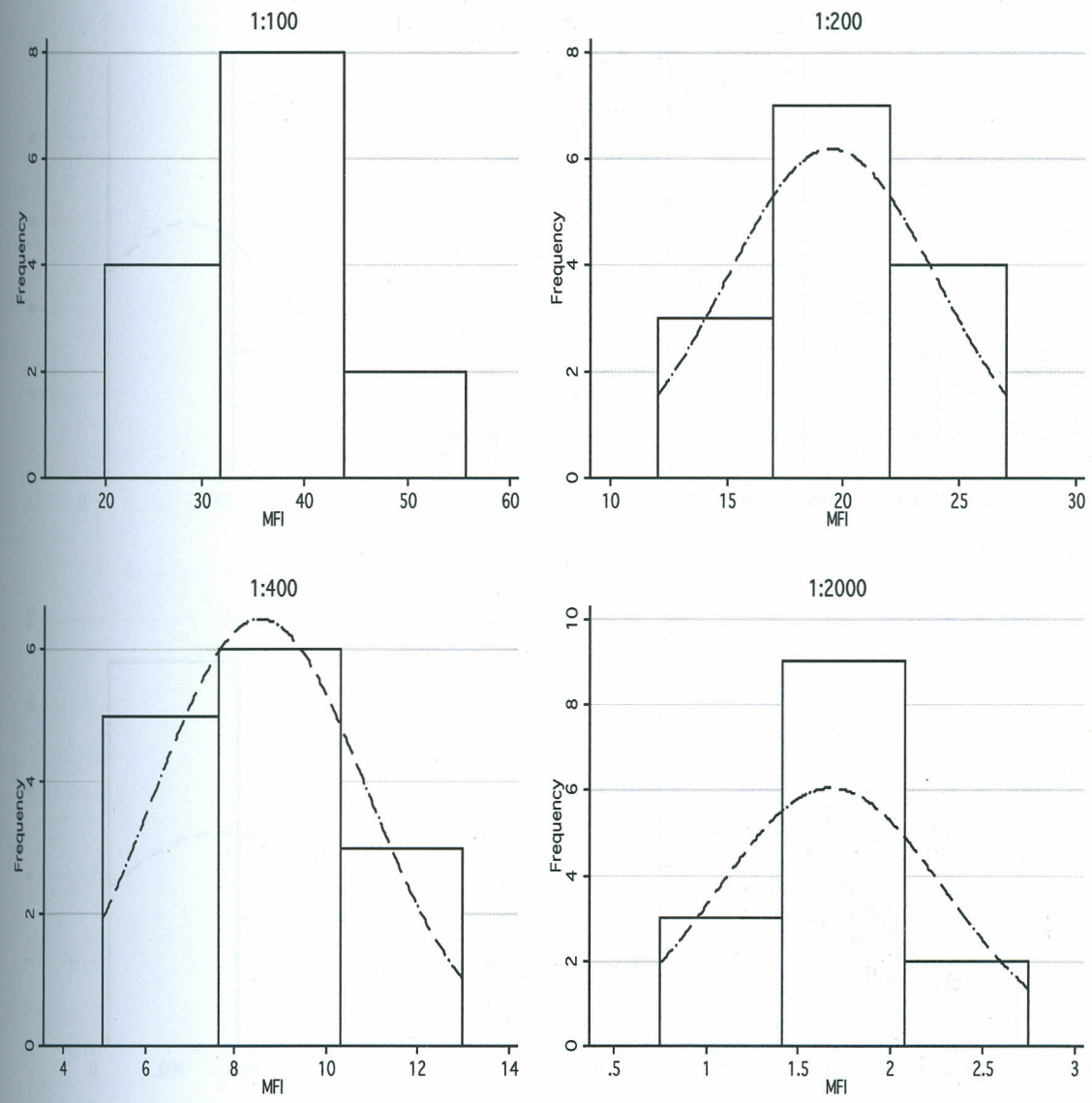

Figure 8. Set of graphs across different dilutions overlaid with a normal density curve of CBA MFI values in plasma from persons never exposed to malaria to MSP-1(42) FUP (n = 7).

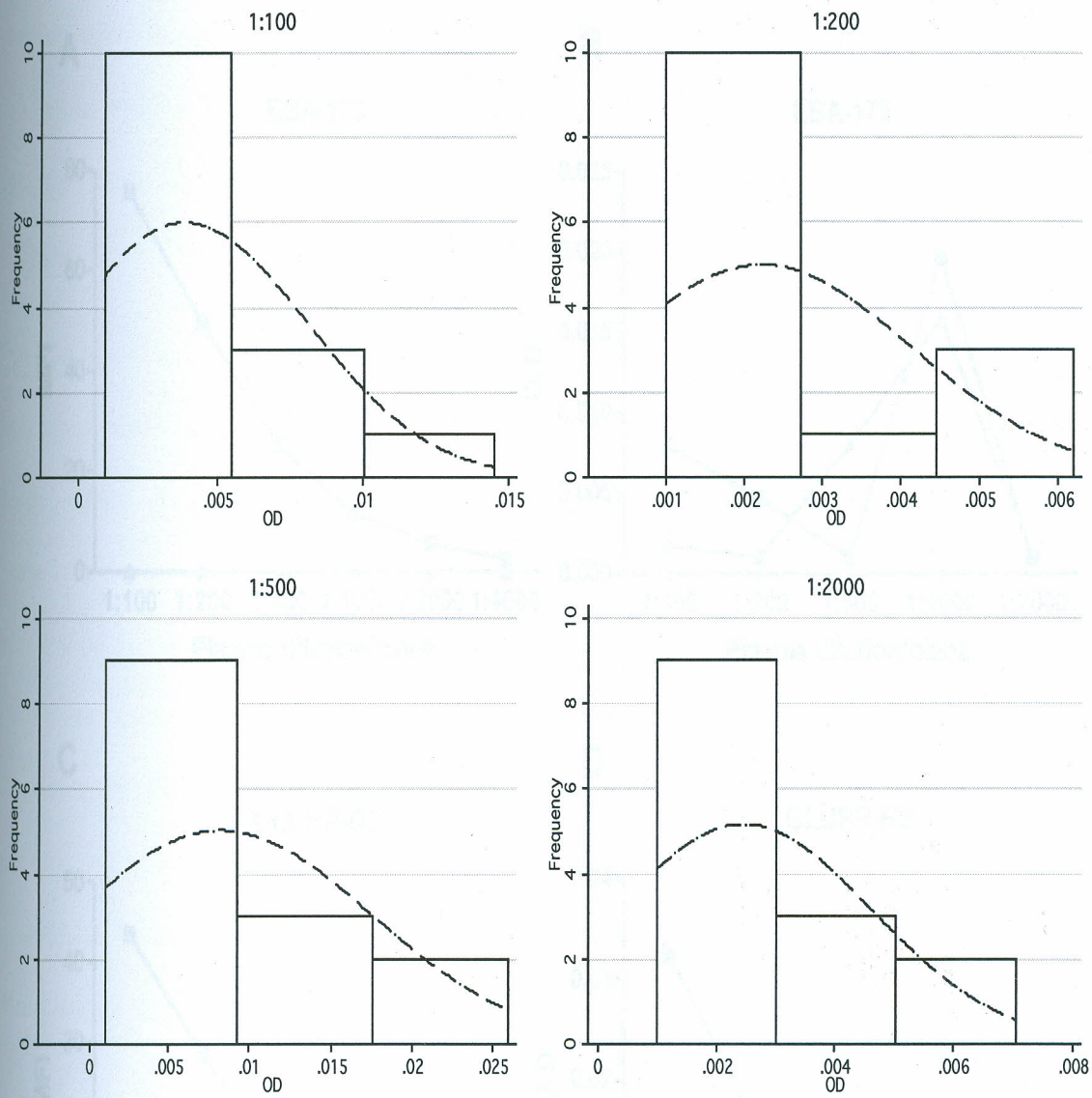

Figure 9. Set of graphs across different dilutions overlaid with a normal density curve of ELISA OD values in plasma from persons never exposed to malaria to MSP-1(42) FUP (n = 7).

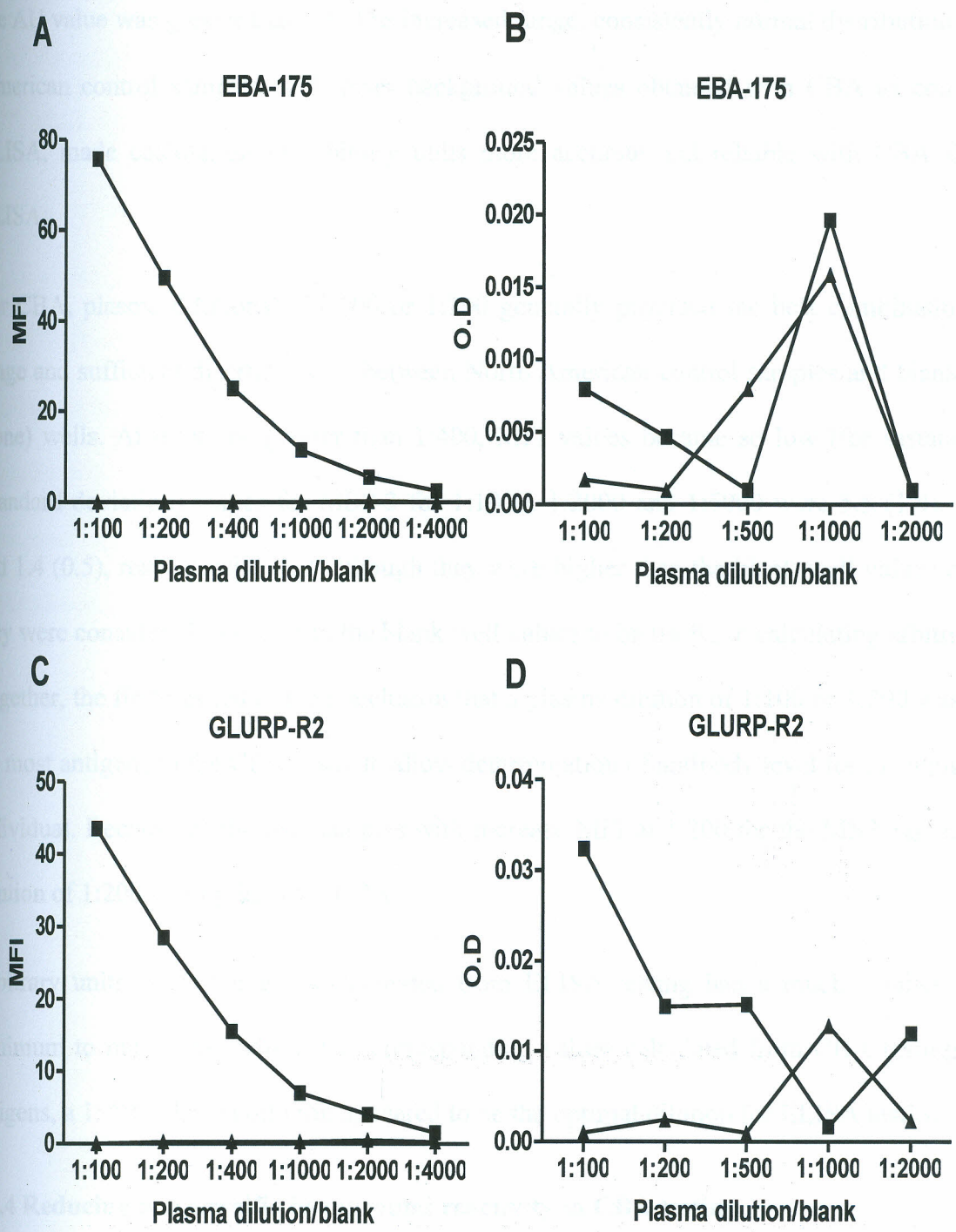

Figure 10 A-D. CBA and ELISA values of North Americans and blank samples for *P. falciparum* antigens. North American control plasma (filled squares, n = 7) or PBS filled (filled triangles). Beads or well coated with EBA or GLURP-R2 for CBA and ELISA.

the AU value was greater than ≥1. The increased range, consistently normal distribution of North American control samples, and lower background values obtained with CBA as compared to ELISA, made calculation of arbitrary units more accurate and reliable with CBA than with ELISA.

For CBA, plasma dilutions of 1:100 or 1:200 generally provided the best combination of AU range and sufficient discrimination between North American control samples and blank (diluent alone) wells. At dilutions greater than 1:400, MFI values became so low (for instance, mean (standard deviation) values for MSP-3 for 1:1000, 1:2000 and 1:5000 were 5.5 (1.4), 3.6 (0.9) and 1.4 (0.5), respectively) that although they were higher than the blank well values of 0 or 1, they were considered too close to the blank well values to be useful in calculating arbitrary units. Together, the findings led to the conclusion that a plasma dilution of 1:100 or 1:200 was optimal for most antigens in the CBA assay to allow determination of antibody level for the antigen in an individual. Because of the few samples with increase MFI at 1:200 for the MSP-1_{42} antigens, a dilution of 1:200 was optimal for CBA.

Arbitrary units (AU) values as calculated from ELISA testing had a much smaller range of minimum to maximum values than corresponding values calculated from CBA testing. For all antigens, a 1: 100 plasma dilution appeared to be the optimal dilution for ELISA testing.

4.1.4 Reducing non-specific background reactivity in CBA testing

To investigate reduction of non-specific background reactivity, two buffers were compared with plasma pooled from North American individuals (a mixture of 7 North American plasma samples) and positive pooled samples. Pooled samples of each type were tested in quadruplicate

for each buffer at a dilution of 1:200. Buffer A was compared to Buffer B; (see Study Design and Methods section for rationale). The two buffers were compared using a six-plex CBA (AMA-1 3D7, AMA-1 FVO, EBA-175, MSP-1_{42} 3D7, MSP-1_{42} FUPand MSP-1_{42} FVO).

The two buffers yielded similar MFI values for the positive pool samples for all antigens (Figure 11), with statistically significant differences only for EBA-175 (buffer B > buffer A) and MSP-1_{42} FUP (buffer A> buffer B). In contrast, MFI values for the non-malaria exposed North American plasma pool were significantly lower for buffer B for all six antigens (Figure 12). The pooled positive plasma samples also had reduced bead aggregation when buffer B was used compared to buffer A. Buffer B thus provided greater differentiation in MFI values between plasma samples from malaria-exposed as compared to non-exposed individuals.

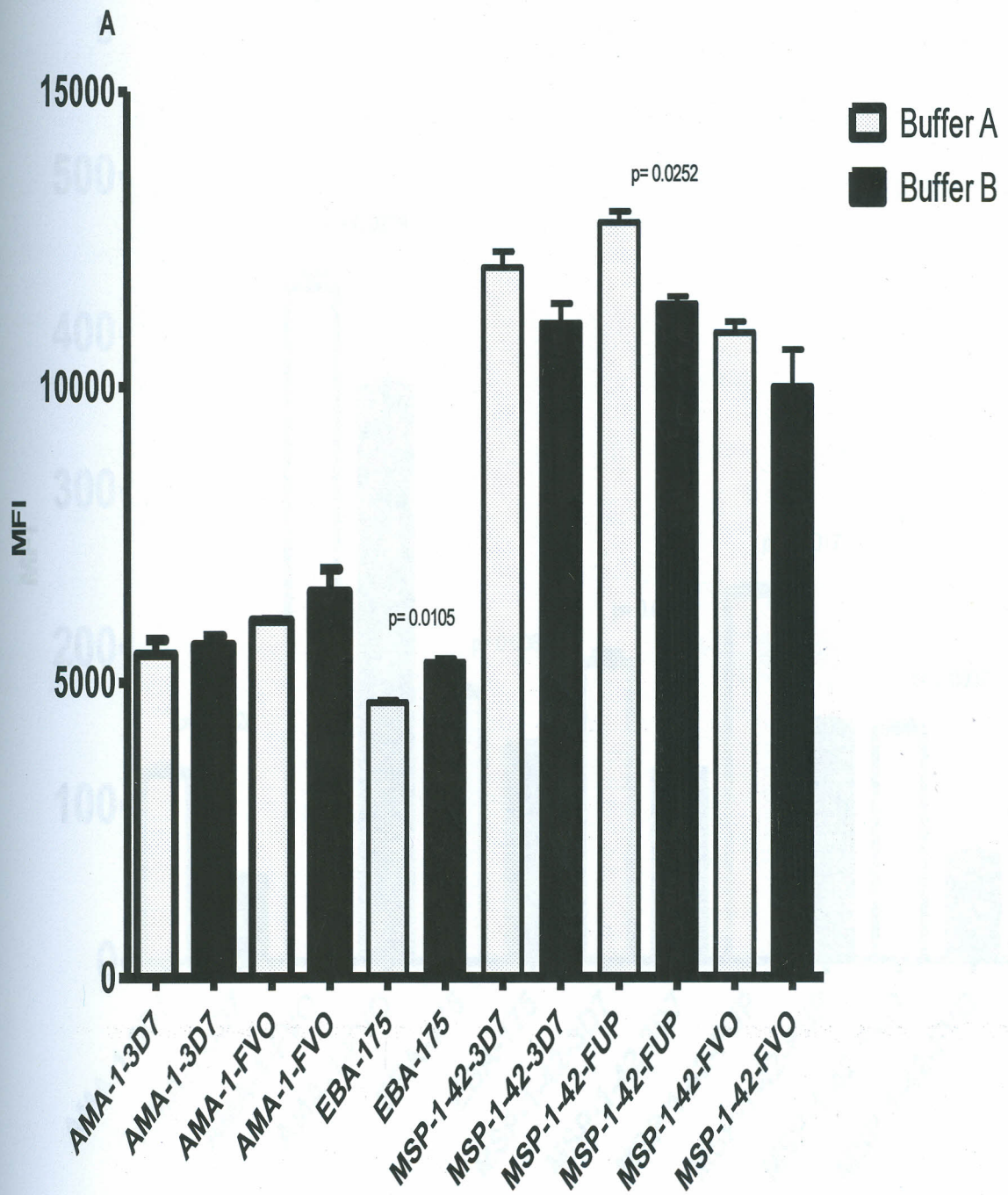

Figure 11. Comparison of CBA MFI values to *P. falciparum* antigens using Buffer A vs Buffer B with malaria endemic positive plasma pool.

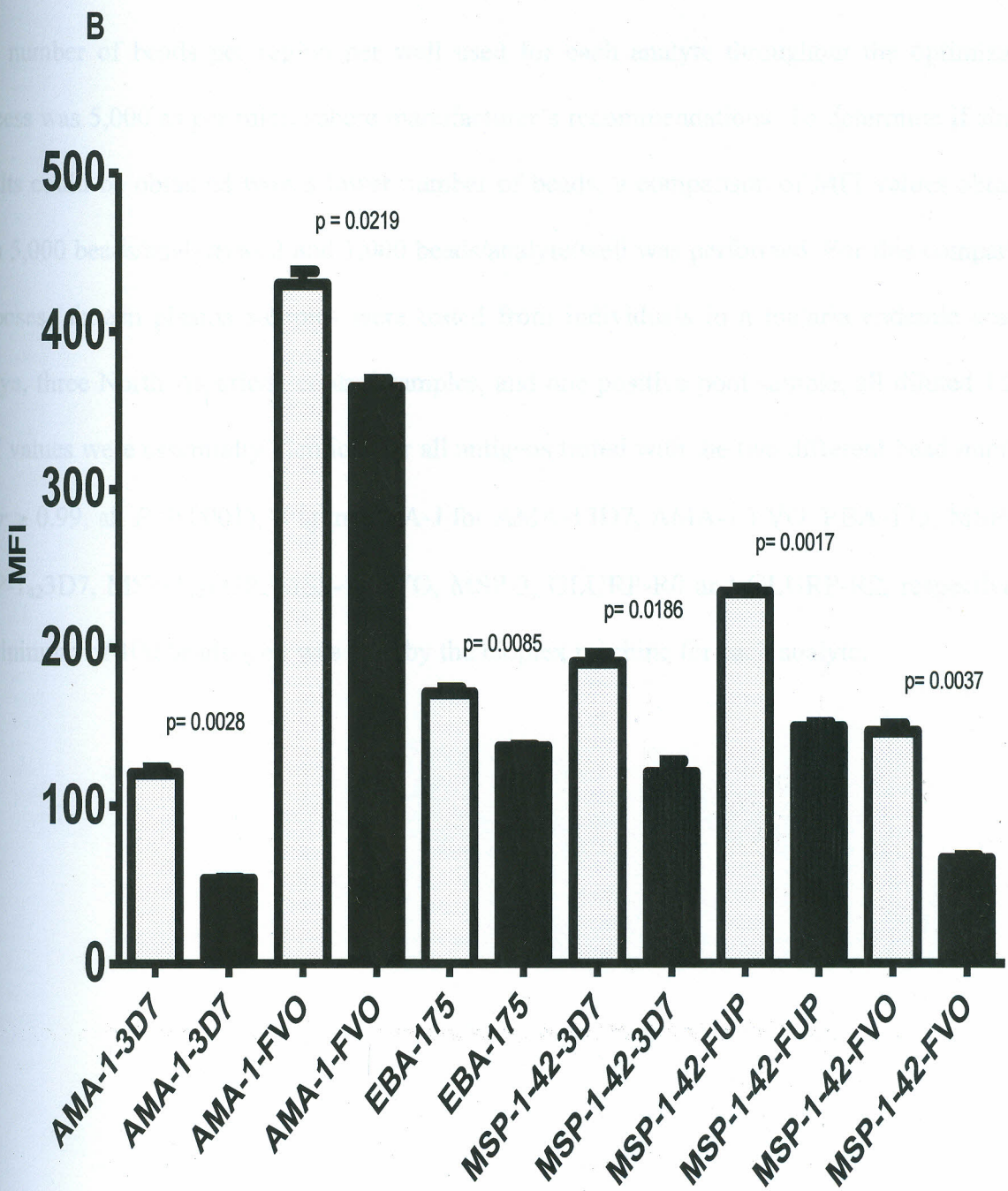

Figure 12. Comparison of CBA MFI values to *P. falciparum* antigens using Buffer A vs Buffer B with North American pool.

4.1.5 Optimizing the number of beads per CBA analyte per well

The number of beads per region per well used for each analyte throughout the optimization process was 5,000 as per microsphere manufacturer's recommendations. To determine if similar results could be obtained with a lower number of beads, a comparison of MFI values obtained with 5,000 beads/analyte/well and 1,000 beads/analyte/well was performed. For this comparison purposes, sixteen plasma samples were tested from individuals in a malaria endemic area of Kenya, three North American control samples, and one positive pool sample, all diluted 1:200. MFI values were essentially identical for all antigens tested with the two different bead numbers (all $r \geq 0.99$, all $P<0.0001$), (Figure 13 A-J for AMA-13D7, AMA-1 FVO, EBA-175, MSP-1_{19}, MSP-1_{42}3D7, MSP-1_{42}FUP, MSP-1_{42}FVO, MSP-3, GLURP-R0 and GLURP-R2, respectively). A minimum of 100 beads/well was read by the bioplex machine for each analyte.

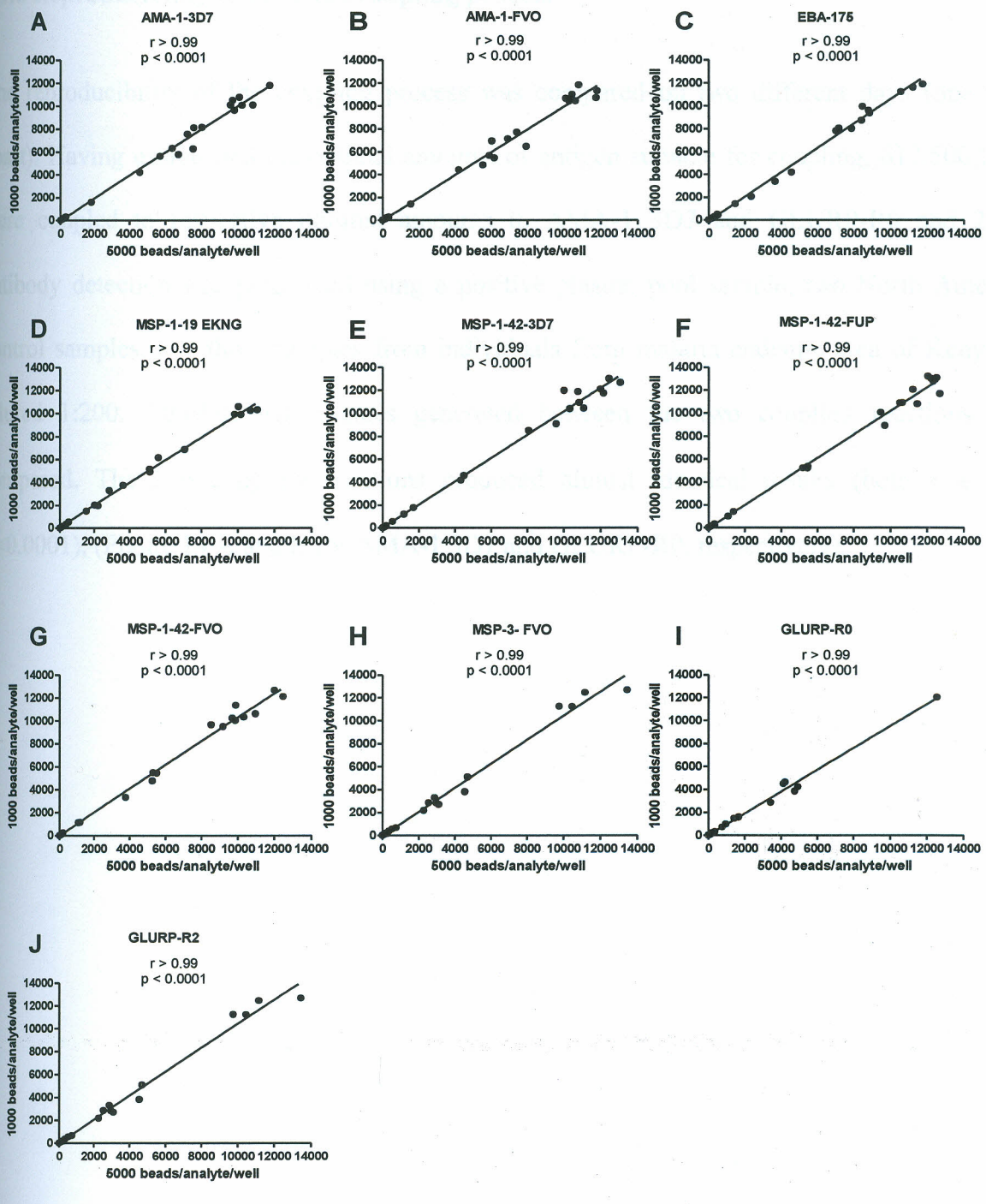

Figure 13 A-J. Correlation of data from CBA testing with 5,000 beads/analyte/well vs. 1,000 beads/analyte/well. Plasma samples from individuals from a malaria endemic area (n = 16), North American control plasma (n = 3) and a positive pool sample by antigen.

4.1.6 Reproducibility of the CBA coupling process

The reproducibility of the coupling process was compared on two different days (one week apart). Having established the optimal amounts of antigen suitable for coupling, 612,500 beads were coupled to respective optimal amounts to AMA-1 3D7 and GLURP-R0 and 2-plex antibody detection was performed using a positive plasma pool sample, two North American control samples, and three samples from individuals from malaria endemic area of Kenya, all diluted 1:200. Absolute MFI values generated between the two coupling reactions were compared. The two coupling reactions produced almost identical results (both $r \geq 0.99$, $P<0.0001$), (Figure 14 A and B for AMA-1 3D7 and GLURP-R0, respectively).

4.1.7 Concordance between multiplex CBA and ELISA assay

Optical density values by ELISA and MFI values by CBA were compared for samples from 20 individuals from malaria endemic area of Kenya and North Americans. Correlations between CBA and ELISA were strong (r >0.7) and highly significant (P<0.001) for all antigens except AMA-1, (Figure 15 A-J for AMA-13D7, AMA-1 FVO, EBA-175, MSP-1_{19}, MSP-1_{42}3D7, MSP-1_{42}FUP, MSP-1_{42}FVO, MSP-3, GLURP-R0 and GLURP-R2, respectively).

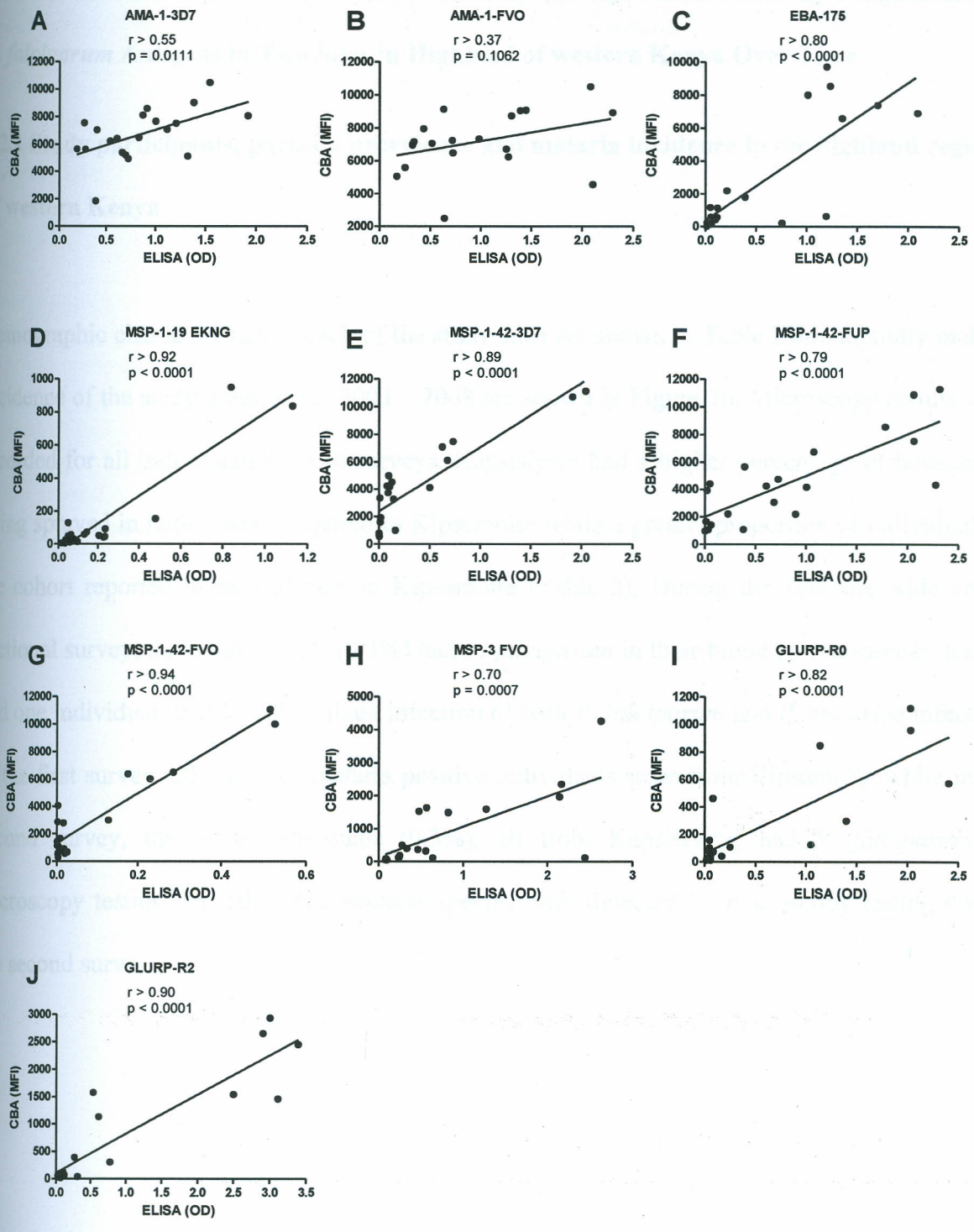

Figure 15 A-J. Concordance between multiplex CBA and ELISA. Correlation of CBA MFI values and ELISA OD values for antibodies to *P. falciparum* antigens in individuals from a malaria endemic area (n = 20).

4.2 Determination of How Transmission Intensity and Age Affect Antibody Frequencies to *P. falciparum* Antigens in Two Sites in Highland of western Kenya Over Time

4.2.1 Study participants, parasite prevalence and malaria incidence in the highland region of western Kenya

Demographic characteristics of each of the study sites are shown in Table 2 and monthly malaria incidence of the study areas, since 2003 – 2008 are shown in Figure 16. Microscopy results were recorded for all individuals in both surveys. Kapsisiywa had a higher percentage of households being sprayed in both years compared to Kipsamoite while a greater proportion of individuals in the cohort reported using bed nets in Kipsamoite (Table 2). During the first site wide cross-sectional survey, three individuals (0.3%) had *P. falciparum* in their blood by microscopy testing and one individual (0.1%) had a mixed infection of both *P. falciparum* and *P. malariae* infection. In the first survey, all the three malaria positive individuals were from Kipsamoite while in the second survey, the three individuals (0.3%), all from Kapsisiywa, had *P. falciparum* by microscopy testing. No other *Plasmodium* species were detected by microscopy testing during the second survey.

Table 2. Characteristics of the study sites and individuals whose antibody responses were examined.

Site	Kipsamoite	Kapsisiywa
Total number of people	457	543
Median age, years	11.76 (0.5 – 90.5)	13.29 (0.3 – 108.4)
Male: Female ratio	0.936	0.912
% house sprayed in 2007	70.5	95.1
% bednet usage in 2007	27	23
% bednet usage in 2008	17	9

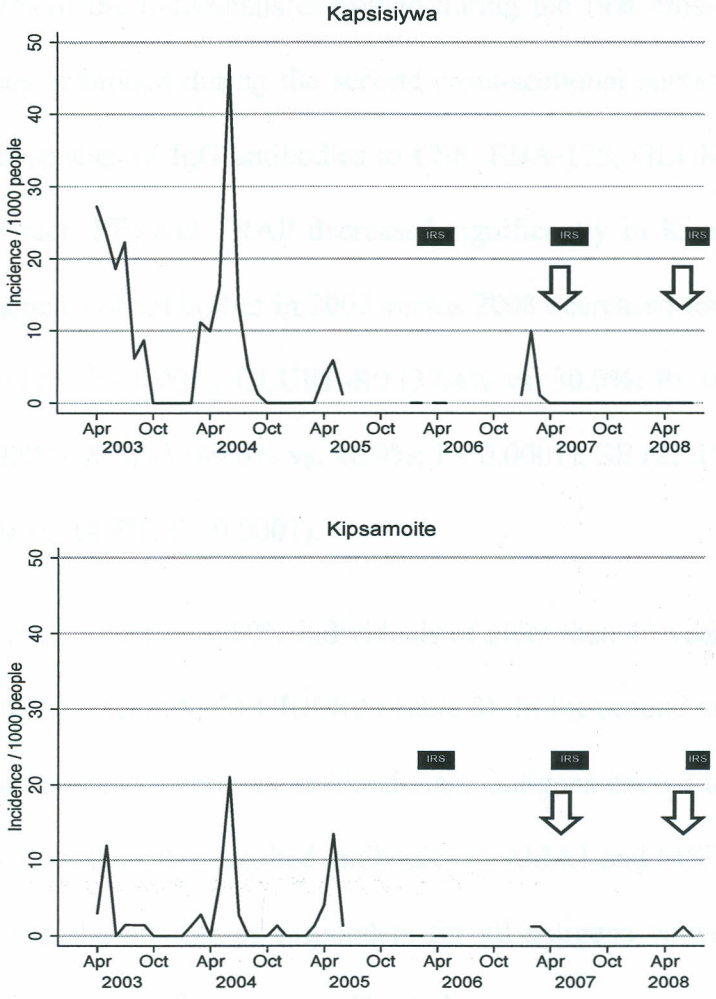

Figure 16. Malaria incidence since 2003-2007. Arrows indicate when indoor residual spraying was conducted.

4.2.2 Antibody frequencies in Kipsamoite

Immunoglobulin G antibodies to pre-erythrocytic antigens were present in >30.6% of the individuals during the first cross-sectional survey and in >22.1% during the second cross-sectional survey as shown in Figure 17A. IgG antibodies to blood stage malaria antigens were detected in >47.7% of the individuals examined during the first cross-sectional survey and in >40.9% individuals examined during the second cross-sectional survey except for GLURP-R0 (Figure 17B). Frequencies of IgG antibodies to CSP, EBA-175, GLURP-R0, MSP-1_{19}, MSP-3, schizont crude extract (SE) and TRAP decreased significantly in Kipsamoite from 2007-2008 (Figure17). Frequencies of antibodies in 2007 versus 2008 decreased for CSP (30.6% vs. 22.1%; P= 0.0006), EBA-175 (P= 0.003), GLURP-R0 (37.4% vs. 30.0%; P< 0.0001), MSP-1_{19} (62.8% vs. 58.2%; P= 0.0003), MSP-3 (49.0% vs. 40.9%; P< 0.0001), SE (22.1% vs. 11.6%; P< 0.0001) and TRAP (22.3% vs. 14.7%; P< 0.0001).

During the first survey, more than 80% individuals of older than 40 years had positive responses to blood stage antigens except to GLURP-R0 (Table 3). In the second survey, more than 73% of the individuals had positive responses to blood stage antigens except to GLURP-R2 (Table 3). 100% individuals older than 40 years had antibodies to AMA1 and MSP1_{42}. There was a general trend of reduction in the number of responders for all antigens between the first and second cross-sectional survey across all age groups (Table 3).

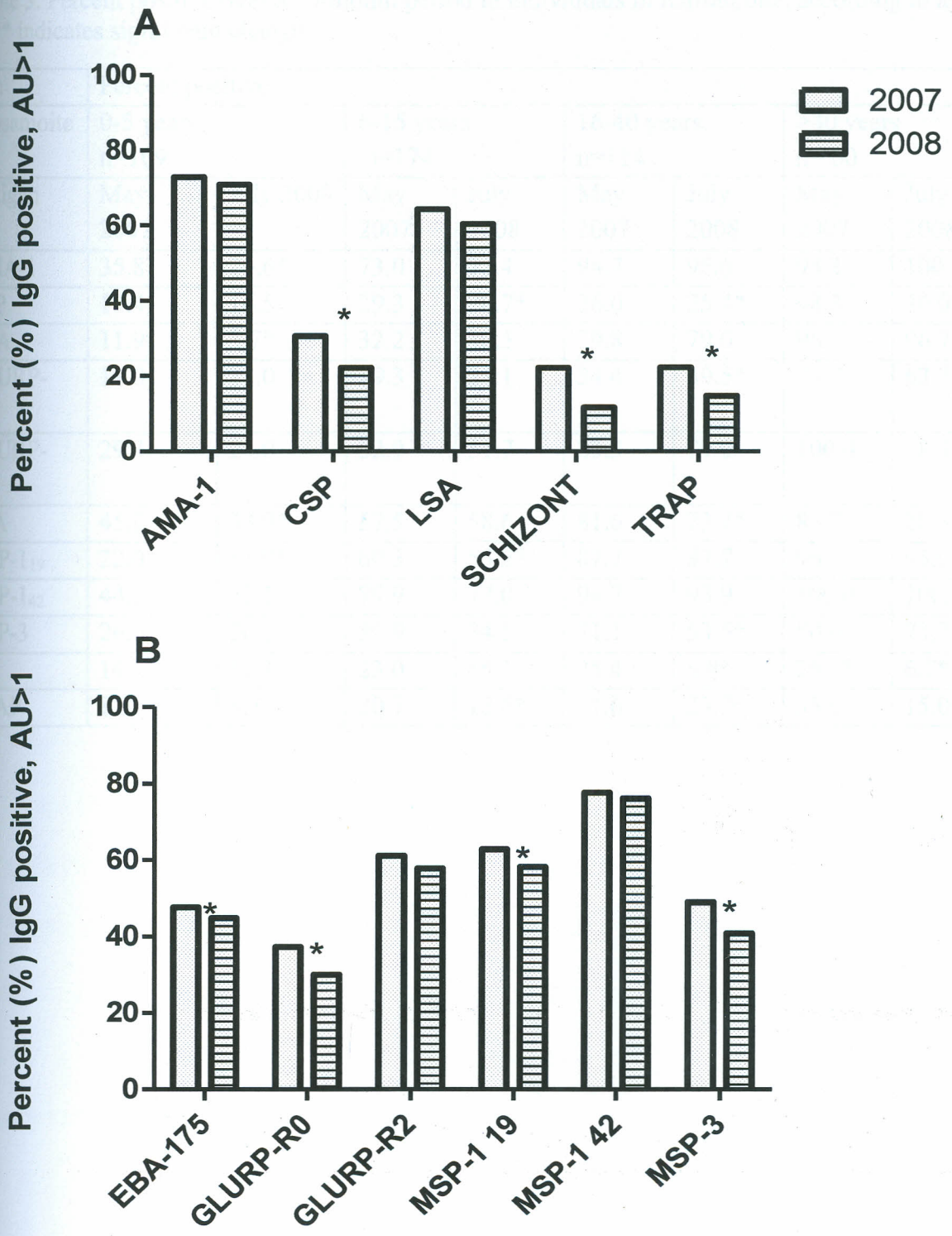

Figure 17. Antibody frequencies of (A) pre-erythrocytic and (B) blood stage antigens in Kipsamoite 2007 and 2008. An * indicates statistical significance.

Table 3. Percent positive over a 15-month period in individuals in Kipsamoite, according to age. An * indicates significant change.

Site	Percent positive							
Kipsamoite	0-5 years n=109		6-15 years n=174		16-40 years n=114		>40 years n= 60	
Antigen	May 2007	July 2008	May 2007	July 2008	May 2007	July 2008	May 2007	July 2008
AMA-1	35.8	26.6*	73.0	72.4	94.7	95.6	98.3	100
CSP	17.4	16.5	29.3	20.7*	36.0	25.4*	48.3	30.0*
EBA	11.9	3.7*	32.2	30.5	79.8	79.0	96.7	96.7
GLURP-R0	11.0	11.0	29.3	24.1	54.4	39.5*	76.7	63.3*
GLURP-R2	29.4	23.0	52.9	51.7	83.3	79.0	100.0	98.3
LSA	45.0	33.9*	57.5	58.6	81.6	73.7*	86.7	88.3
$MSP\text{-}1_{19}$	22.0	11.9*	60.3	55.2*	87.7	87.7	96.7	95.0
$MSP\text{-}1_{42}$	44.0	43.1	79.9	77.0	94.7	93.9	100.0	100.0
MSP-3	26.6	20.2	37.9	34.5	71.1	53.5*	80.0	73.3*
SE	14.7	10.1	23.0	16.1	25.4	8.8*	26.7*	6.7*
TRAP	8.3	8.3	20.7	12.6*	31.6	23.7*	35.0	15.0*

For all the antibodies, their seroprevalence increased with age (P< 0.0001) (Table 3). Among the cohort, 29 (6.3%) and 7 (1.5%) individuals were seropositive to the eleven *P. falciparum* antigens SE during the first and second surveys, respectively. On the contrary, 26 (5.7%) and 32 (7.0%) individuals were seronegative to the eleven *P. falciparum* antigens in the first and second survey, respectively.

4.2.3 Antibody frequencies in Kapsisiywa

Antibodies to AMA-1 and CSP decreased from 80.7% to 78.1%; 38.9% to 24.3%, (P= 0.0164, P< 0.0001, respectively) while antibodies to LSA and TRAP decreased from 71.6% to 65.6%; 22.8% to 19.5% (P< 0.0001, P= 0.0003, respectively) as shown in Figure 18A. Antibodies to MSP-$1_{19}$and MSP-3 decreased from 75.1% to 72.7%; 61.7% to 53.2% (P= 0.028, P< 0.0001, respectively) as shown in Figure 18B. Antibody frequencies to GLURP-R0 decreased from 48.1% to 44.2% (P = 0.0140) as shown in Figure 18B. Antibody frequencies increased across age groups (P<0.0001) as shown in Tables 4 in both surveys. Antibody frequencies were higher in Kapsisiywa than in Kipsamoite for both surveys in each of the age groups. Among the cohort, 65 (12.0%) and 39 (7.2%) individuals were seropositive to the eleven *P. falciparum* antigens (excluding *P. falciparum* SE) tested during the first and second surveys, respectively. On the contrary, 23 (4.2%) and 40 (7.4%) individuals were seronegative to the eleven *P. falciparum* antigens in the first and second surveys, respectively.

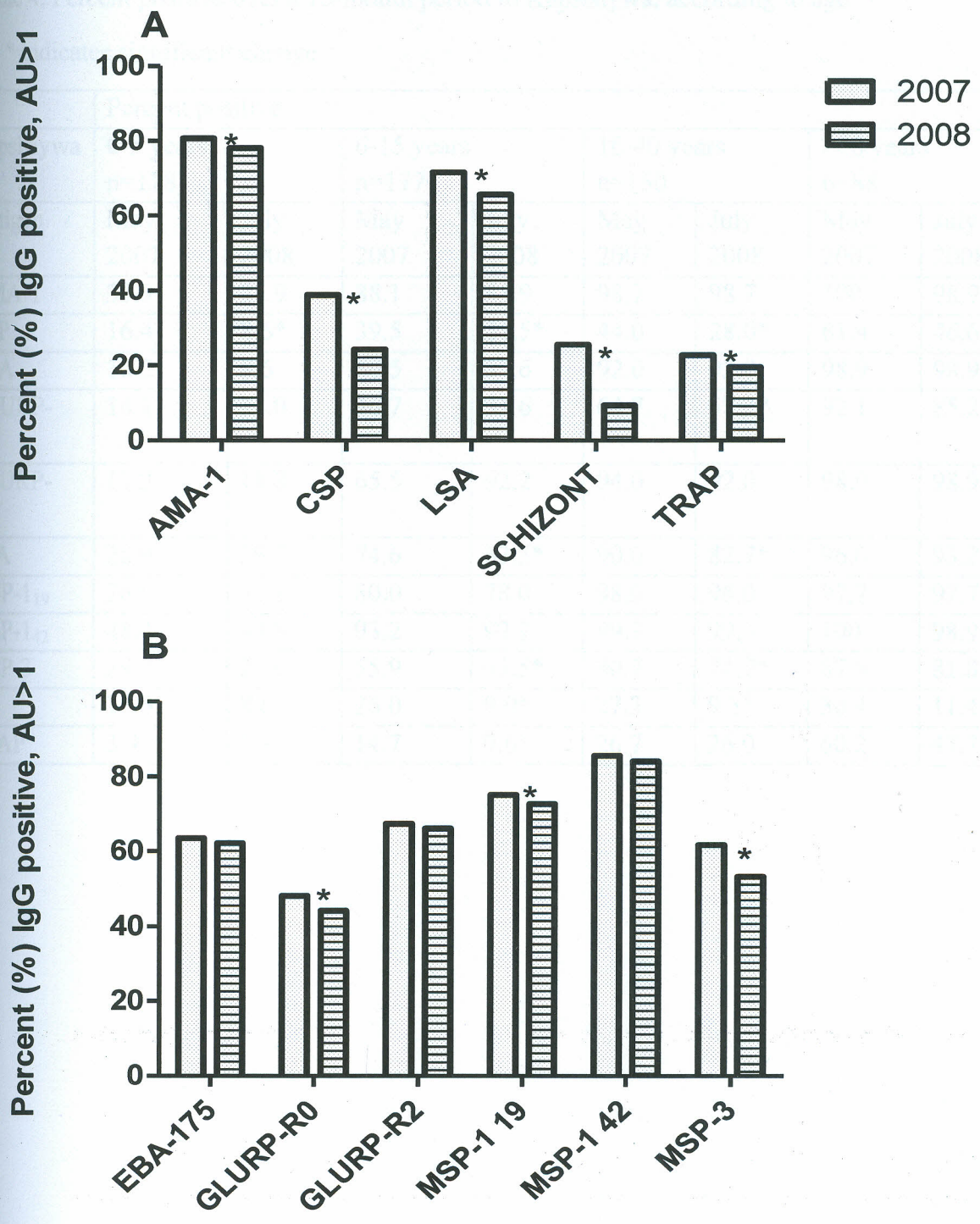

Figure 18. Antibody frequencies of (A) pre-erythrocytic and (B) blood stage antigens in Kapsisiywa in 2007 and 2008. An * indicates statistical significance.

Table 4. Percent positive over a 15-month period in Kapsisiywa, according to age.

An * indicates significant change.

	Percent positive							
Kapsisiywa	0-5 years n=128		6-15 years n=177		16-40 years n=150		>40 years n=88	
Antigen	May 2007	July 2008	May 2007	July 2008	May 2007	July 2008	May 2007	July 2008
AMA-1	35.9	28.9	88.1	85.9	98.7	98.7	100	98.9
CSP	16.4	8.6*	39.5	21.5*	44.0	28.0*	61.4	46.6*
EBA	10.2	8.6	60.5	57.6	92.0	92.0	98.9	98.9
GLURP-R0	14.1	18.0	36.7	31.6	64.7	57.3*	92.1	85.2*
GLURP-R2	17.2	18.8	65.5	62.2	94.0	92.0	98.9	98.9
LSA	28.9	29.7	74.6	63.3*	90.0	82.7*	96.6	93.2
MSP-1_{19}	26.8	21.1	80.0	78.0	98.0	96.0	97.7	97.7
MSP-1_{42}	48.4	43.8	93.2	92.7	99.3	99.3	100	98.9
MSP-3	29.7	21.9	55.9	43.5*	80.7	74.7*	87.5	81.8*
SE	15.6	8.6	26.0	9.0*	27.3	9.3*	36.4	11.4*
TRAP	3.9	6.3	14.7	9.6*	26.7	26.0	60.2	47.7*

4.3 Determination of How Transmission Intensity and Age Affect Antibody Levels to *P. falciparum* Antigens in Two Sites in Highland of Western Kenya Over Time

4.3.1 Antibody levels in Kipsamoite

Levels for all antibodies increased with age ($P < 0.0001$) (Table5). Across the two time points, antibody levels decreased significantly, ($P < 0.0001$). Generally, blood stage antigens had higher antibody levels compared to pre-erythrocytic antigens in both surveys (Table 5).

Table 5. Median antibody levels for the year 2007 and 2008 for *P. falciparum* antigens in Kipsamoite, by age. An * indicates significant change.

Site	0-5 years n=109		6-15 Years n=174		16-40years n=114		>40 years n=60	
Kip	May 07	July 08	May 07	July 08	May 07	July 08	May 07	July 08
AMA-1	0.56(0.1-55.1)	0.46(0.1-55.9)*	5.82(0.1-68.9)	3.95(0.1-67.9)*	32.64(0.3-73.0)	27.63(0.2-71.9)*	42.46(0.7-74.3)	39.33(1.0-79.8)
CSP	0.49(0.1-11.1)	0.44(0.1-5.8)	0.67(0.1-12.9)	0.49(0.1-6.2)*	0.74(0.1-7.8)	0.51(0.1-10.4)*	0.99(0.2-10.2)	0.64(0.1-4.4)*
EBA-175	0.25(0.1-11.3)	0.30(0.0-4.8)	0.52(0.1-64.6)	0.52(0.0-62.1)*	14.58(0.2-67.8)	8.90(0.1-68.9)*	36.35(0.1-67.5)	37.85(0.2-68.3)
GLURP-R0	0.18(0.0-4.9)	0.17(0.0-7.7)	0.38(0.0-43.8)	0.32(0.0-13.7)*	1.18(0.1-25.0)	0.59(0.0-15.0)*	3.72(0.1-37.3)	1.71(0.1-32.3)*
GLURP-R2	0.33(0.0-40.5)	0.25(0.0-35.2)*	1.13(0.0-92.8)	1.05(0.0-81.4)*	6.14(0.1-87.7)	3.86(0.0-85.9)*	20.77(1.3-98.8)	17.16(0.6-84.5)*
LSA	0.80(0.1-25.3)	0.69(0.1-29.8)	1.33(0.1-28.1)	1.41(0.0-26.5)*	3.78(0.3-44.0)	2.06(0.2-24.5)*	4.30(0.3-44.8)	3.58(0.3-30.0)*
MSP-1_{19}	0.32(0.1-37.0)	0.30(0.0-50.4)*	2.10(0.0-171.0)	1.41(0.0-151.6)*	16.26(0.2-172.5)	11.33(0.0-174.5)*	77.14(0.4-162.8)	65.74(0.4-180.6)*
MSP-1_{42}	0.82(0.1-169.6)	0.70(0.1-259.6)*	14.00(0.2-255.9)	9.22(0.1-2300.1)*	89.72(0.4-279.7)	64.75(0.2-278.0)*	139.62(2.3-275.8)	132.57(2.6-318.8)
MSP-3	0.44(0.0-12.3)	0.31(0.0-9.8)*	0.61(0.0-52.8)	0.48(0.0-18.4)*	2.21(0.1-34.0)	1.27(0.0-22.2)*	5.67(0.1-53.2)	3.53(0.0-34.5)*
SE	0.54(0.1-5.9)	0.29(0.0-2.3)*	0.62(0.1-5.1)	0.42(0.0-4.1)*	0.69(0.3-6.3)	0.30(0.0-2.4)*	0.71(0.2-3.5)	0.35(0.0-2.3)*
TRAP	0.18(0.0-12.9)	0.15(0.0-16.6)	0.26(0.0-15.1)	0.25(0.0-11.8)*	0.41(0.1-10.7)	0.32(0.0-13.8)*	0.58(0.1-9.6)	0.36(0.0-10.8)

*Statistical significance

4.3.2 Antibody levels in Kapsisiywa

Multiple antibody levels decreased significantly over time as shown in Table 6. Antibody levels increased across age groups ($P < 0.0001$) in both surveys. Antibody levels were higher in Kapsisiywa than in Kipsamoite for both surveys in each of the age groups.

Table 6. Median antibody levels for the year 2007 and 2008 for *P. falciparum* antigens in Kapsisiywa, by age. An * indicates significant decrease.

	0-5 years n=128		6-15 Years n=177		16-40years n=150		>40 years n=88	
Kap	May 07	July 08	May 07	July 08	May 07	July 08	May 07	July 08
AMA-1	0.55(0.1-69.5)	0.51(0.0-60.9)*	24.46(0.1-77.0)	18.49(0.0-80.5)*	42.14(0.3-78.3)	42.55(0.2-75.6)	41.06(1.1-73.5)	45.45(0.3-72.0)
CSP	0.49(0.0-6.0)	0.35(0.0-5.9)*	0.75(0.2-10.9)	0.52(0.0-13.9)*	0.89(0.2-14.4)	0.67(0.1-7.5)*	1.56(0.3-24.2)	0.88(0.1-4.7)*
EBA-175	0.25(0.1-38.8)	0.25(0.0-51.6)	2.53(0.1-72.2)	1.68(0.0-69.0)*	36.08(0.3-75.8)	35.28(0.2-70.3)	40.14(0.9-71.8)	39.14(0.5-70.9)*
GLURP-R2	0.29(0.0-77.9)	0.27(0.0-45.1)	2.60(0.0-93.2)	2.04(0.0-97.8)*	21.47(0.1-94.6)	16.57(0.1-99.1)*	37.40(1.0-102.7)	30.81(0.3-102.6)*
GLURP-R0	0.21(0.0-33.2)	0.16(0.0-53.6)*	0.48(0.0-44.0)	0.40(0.0-45.9)*	2.23(0.1-57.1)	1.57(0.0-62.3)*	7.85(0.1-58.5)	4.5(0.0-52.7)*
LSA	0.56(0.1-15.1)	0.43(0.0-19.0)	2.23(0.1-44.0)	1.45(0.0-37.5)*	5.66(0.3-71.6)	4.28(0.1-51.5)*	9.81(0.2-45.1)	7.32(0.1-55.3)*
$MSP\text{-}1_{19}$	0.38(0.1-143.1)	0.27(0.0-100.3)*	11.67(0.0-189.3)	7.13(0.0-168.3)*	49.09(0.2-182.9)	45.94(0.2-177.1)*	109.96(0.5-180.2)	100.26(0.0-188.8)*
$MSP\text{-}1_{42}$	0.93(0.1-233.1)	0.87(0.0-251.4)	63.42(0.3-285.6)	45.92(0.0-283.4)	139.04(0.8-280.1)	135.61(0.5-303.3)*	152.10(1.7-302.5)	175.76(0.4-283.4)
MSP-3	0.42(0.0-19.6)	0.32(0.0-7.0)*	1.31(0.0-43.7)	0.77(0.0-40.4)*	5.14(0.1-35.1)	3.68(0.1-27.8)*	7.48(0.1-57.6)	6.04(0.1-48.4)*
SE	0.60(0.1-3.0)	0.29(0.0-4.9)*	0.66(0.1-6.1)	0.41(0.0-2.1)*	0.75(0.2-7.0)	0.46(0.0-4.8)*	0.91(0.2-16.3)	0.47(0.1-4.7)*
TRAP	0.17 (0.0 -2.3)	0.15(0.0 – 3.0)	0.23 (0.0 – 36.6)	0.20 (0.0 -15.9)*	0.49 (0.1-18.0)	0.36 (0.0 -11.7)*	1.35 (0.1 – 18.6)	0.90(0.1-19.5)*

*Statistical significance

4.4 Determination of Antibody Correlations to *P. falciparum* Antigens Over Time and Antibody Levels to VCA-p18 in Two Sites in Highland Region of Western Kenya

4.4.1 Antibody correlations in Kipsamoite

Immunoglobulin G levels to all antigens correlated significantly to each other during the first survey (Table 7) while in the second survey, levels of IgG correlated except for antibodies to schizont extract (Table 8). Levels of IgG antibody to LSA-1 were significantly correlated with levels of IgG antibody to CSP, TRAP, EBA-175, and MSP-1. In addition to LSA-1, levels of IgG antibody to EBA-175 were also significantly correlated with levels of antibodies to TRAP and AMA-1.

Table 7. Antibody correlations between *P. falciparum* antigens for all individuals (n = 457) in Kipsamoite, 2007. An * indicates statistical significance.

Antigen	MSP-3	AMA-1	MSP-1_{42}	MSP-1_{19}	EBA-175	GLU-R0	GLU-R2	LSA	TRAP	SCH	CSP
MSP-3											
AMA-1	0.5250*										
MSP-1_{42}	0.4760*	0.7960*									
MSP-1_{19}	0.5065*	0.7759*	0.9516*								
EBA-175	0.5562*	0.8186*	0.7394*	0.7630*							
GLU-R0	0.5179*	0.5302*	0.5499*	0.5885*	0.6115*						
GLU-R2	0.5602*	0.6777*	0.7307*	0.7592*	0.7309*	0.6233*					
LSA	0.4200*	0.5077*	0.4973*	0.5263*	0.5454*	0.4951*	0.4952*				
TRAP	0.3375*	0.3596*	0.3649*	0.4090*	0.3963*	0.4343*	0.4064*	0.3758*			
SE	0.3016*	0.2463*	0.2710*	0.2890*	0.2945*	0.3357*	0.2529*	0.2531*	0.2376*		
CSP	0.2487*	0.3065*	0.3241*	0.3418*	0.3559*	0.3063*	0.2997*	0.2798*	0.2076*	0.5225*	

*Statistical significance

Table 8. Antibody correlations between *P. falciparum* antigens for all individuals (n = 457) in Kipsamoite, 2008. An * indicates statistical significance.

Antigen	MSP-3	AMA-1	MSP-1_{42}	MSP-1_{19}	EBA-175	GLU-R0	GLU-R2	LSA	TRAP	SCH	CSP
MSP-3											
AMA-1	0.4891*										
MSP-1_{42}	0.4249*	0.7937*									
MSP-1_{19}	0.4760*	0.7706*	0.9494*								
EBA-175	0.5311*	0.8066*	0.6949*	0.7237*							
GLU-R0	0.4624*	0.4818*	0.4871*	0.5190*	0.5384*						
GLU-R2	0.5226*	0.6800*	0.6910*	0.7244*	0.7372*	0.5437*					
LSA	0.3538*	0.4366*	0.4287*	0.4479*	0.4752*	0.4075*	0.4908*				
TRAP	0.2708*	0.3025*	0.2977*	0.3599*	0.3648*	0.3267*	0.3704*	0.3301*			
SE	0.0969*	0.0706	0.0583	0.0887	0.0979	0.1124	0.1091	0.1616	0.2010		
CSP	0.1879*	0.1299	0.1584*	0.1933*	0.1981*	0.1879*	0.1992*	0.1534*	0.1636*	0.3887*	

*Statistical significance

4.4.2 Antibody correlations in Kapsisiywa

Levels of total IgG correlated significantly to each other in both 2007 and 2008 surveys as shown in Tables 9 and 10, respectively.

Table 9. Antibody correlations between *P. falciparum* antigens for all individuals (n = 543) in Kapsisiywa, 2007. An * indicates statistical significance.

Antigen	MSP-3	AMA-1	MSP-1_{42}	MSP-1_{19}	EBA-175	GLU-R0	GLU-R2	LSA	TRAP	SE	CSP
MSP-3											
AMA-1	0.5219*										
MSP-1_{42}	0.5892*	0.7538*									
MSP-1_{19}	0.6191*	0.6989*	0.8660*								
EBA-175	0.6368*	0.8242*	0.7579*	0.7629*							
GLU-R0	0.6091*	0.5198*	0.5877*	0.6951*	0.6287*						
GLU-R2	0.6963*	0.6927*	0.7373*	0.8339*	0.7749*	0.7606*					
LSA	0.5910*	0.5876*	0.6239*	0.6932*	0.6588*	0.6327*	0.7258*				
TRAP	0.5390*	0.4612*	0.5400*	0.6130*	0.5787*	0.6112*	0.6237*	0.5890*			
SE	0.2673*	0.2843*	0.3269*	0.3980*	0.3394*	0.3653*	0.3763*	0.3577*	0.3750*		
CSP	0.3977*	0.3850*	0.4014*	0.4787*	0.4569*	0.4600*	0.5004*	0.4720*	0.4285*	0.5700*	

*Statistical significance

Table 10. Antibody correlations between *P. falciparum* antigens for all individuals (n = 543) in Kapsisiywa, 2008. An * indicates statistical significance.

Antigen	MSP-3	AMA-1	MSP-1_{42}	MSP-1_{19}	EBA-175	GLU-R0	GLU-R2	LSA	TRAP	SE	CSP
MSP-3											
AMA-1	0.5683*										
MSP-1_{42}	0.5599*	0.7743*									
MSP-1_{19}	0.6234*	0.7357*	0.9067*								
EBA-175	0.6758*	0.8489*	0.7737*	0.7705*							
GLU-R0	0.6159*	0.5211*	0.5922*	0.6773*	0.6111*						
GLU-R2	0.6981*	0.7239*	0.7521*	0.8133*	0.7801*	0.7323*					
LSA	0.5639*	0.6014*	0.6366*	0.6827*	0.6596*	0.6125*	0.7027*				
TRAP	0.4882*	0.4276*	0.5110*	0.5725*	0.5248*	0.5714*	0.5644*	0.5148*			
SE	0.2556*	0.2415*	0.2349*	0.2733*	0.3054*	0.2460*	0.2864*	0.2467*	0.1614*		
CSP	0.2814*	0.3395*	0.3614*	0.3902*	0.3875*	0.3538*	0.3765*	0.3892*	0.2881*	0.3899*	

*Statistical significance

4.4.3Antibody levels to VCA-p18 antigen as a stable indicator of antibody responses over time in both Kipsamoite and Kapsisiywa

In both Kapsisiywa and Kipsamoite, median antibody levels were stable and steadily maintained over time (Kapsisiywa, p= 0.8544; Kipsamoite, p= 0.4392) as shown in Table 11. Antibody levels in both sites were similar across all age groups in Kipsamoite (P= 0.2850, P= 0.5480); Kapsisiywa (P= 0.4110, P= 0.8000) for 2007 and 2008, respectively. In both sites, a similar trend was observed in that children under the age of five years had a higher level of VCA-p18 antibodies which contracted to lower levels in the 6-15 and 16-40 age groups but adults >40 years had the highest levels of VCA-p18 antibodies.

Table 11. Optical density (OD) of VCA p18 in Kipsamoite and Kapsisiywa in 2007 and 2008.

Site	Kipsamoite (n = 231)			Kapsisiywa (n = 296)		
Antigen	2007 OD	2008 OD	P	2007 OD	2008 OD	P
VCA-p18	1.37 (0.1 - 3.1)	1.43(0.1-3.1)	0.4392	1.43 (0.1 - 3.3)	1.42(0.1-3.0)	0.8544

4.5 Determination of Antibody Seroreversion Rates by Age for *Plasmodium falciparum* Antigens in Two Sites in Highland of Western Kenya Over Time

4.5.1 Persistence of the IgG antibody responses over time

Persistence of the antibody responses was determined by categorizing individuals into four groups: Maintained, Lost, Gained and No-response. "Maintained" were seropositive at both surveys while "No-response" were seronegative at both surveys. "Lost" were those that seroconverted to become seronegative in the second survey and "Gained" were those that seroconverted to become seropositive in the second survey. MSP-1_{42} was the most immunogenic malaria antigen in both Kipsamoite as shown in Table 12 and Kapsisiywa as shown in Table 13 in 2007. More than 54% and >73% of the individuals were nonresponsive to CSP and TRAP, respectively, in both study sites (Tables 12 and 13). Results showed that older individuals had a higher percentage of persistence than the young for all the antibodies in both study sites (Tables 14 and 15) with AMA-1 and MSP-1_{42} having the highest percentage persistence. Kapsisiywa had higher percentage of persistence of antibody responses to all antigens except antibodies to SE. Further, a greater proportion of individuals in Kipsamoite had no response to all antigens as compared to Kapsisiywa.

Table 12. Percent individuals with a no response, maintained, lost and gained antibody responses to *P. falciparum* antigens in Kipsamoite between 2007 and 2008.

Percent with No response, Maintained, Lost or Gained over the 15 months period in Kipsamoite (n = 457)				
Antigen	(%)No response	(%) Maintained	(%) Lost	(%) Gained
AMA-1	24.3	68.1	4.8	2.8
CSP	59.5	12.3	18.4	9.8
EBA-175	49.5	42.0	5.7	2.8
GLURP-R0	58.9	26.3	11.2	3.7
GLURP-R2	33.3	52.1	9.0	5.7
LSA	27.6	52.3	12.0	8.1
MSP-1_{19}	35.9	56.9	5.9	1.3
MSP-1_{42}	18.8	72.6	5.0	3.5
MSP-3	46.6	36.5	12.5	4.4
SE	71.1	4.8	17.3	6.8
TRAP	75.9	12.9	9.4	1.8

Table 13. Percent individuals with no response, maintained, lost and gained antibody responses to *P. falciparum* antigens in Kapsisiywa between 2007 and 2008.

Percent with No response, Maintained, Lost or Gained over the 15 months period in Kapsisiywa (n = 543)				
Antigen	(%)No response	(%)Maintained	(%) Lost	(%) Gained
AMA-1	17.5	76.2	4.4	1.8
CSP	54.1	17.5	21.5	6.8
EBA-175	34.1	59.9	3.7	2.4
GLURP-R0	47.2	39.4	8.7	4.8
GLURP-R2	28.7	62.2	5.2	3.9
LSA	23.6	60.8	10.9	4.8
$MSP\text{-}1_{19}$	22.8	70.7	4.4	2.0
$MSP\text{-}1_{42}$	12.0	81.4	4.1	2.6
MSP-3	35.2	50.1	11.3	3.1
SE	68.9	3.9	21.7	5.5
TRAP	73.5	15.8	7.0	3.7

Table 14. Percent positive individuals with persistent and nonresponsive antibody responses to *P. falciparum* antigens in Kipsamoite, according to age between 2007 and 2008.

	Percent positive							
Kip	0-5 years n=109		6-15 years n=174		16-40 years n=114		>40 years n= 60	
Antigen	Persistent	*Nonres	Persistent	*Nonres	Persistent	*Nonres	Persistent	*Nonres
AMA-1	22.0	59.6	69.5	24.1	93.8	3.5	98.3	0.0
CSP	6.4	72.4	11.4	61.4	14.9	53.5	20.0	41.6
EBA	1.8	86.2	25.8	63.2	76.3	17.5	96.6	3.3
GLURP-R0	5.0	83.4	19.5	66.0	36.8	42.9	63.3	23.3
GLURP-R2	12.0	59.6	44.8	40.2	77.1	14.9	98.3	0.0
LSA	24.7	45.8	48.8	32.7	68.4	13.1	81.6	6.6
MSP-1_{19}	11.0	77.0	52.8	37.3	86.8	11.4	95.0	3.3
MSP-1_{42}	33.0	45.8	74.7	17.8	92.9	4.3	100.0	0.0
MSP-3	11.9	65.1	29.3	56.9	51.7	27.1	73.3	20.0
SE	1.8	77.0	8.0	68.9	4.3	70.1	1.6	68.3
TRAP	5.5	88.9	10.9	77.5	23.6	68.4	11.6	61.6

*Nonres = Nonresponsive

Table 15. Percent positive individuals with persistent and nonresponsive antibody responses to *P. falciparum* antigens in Kapsisiywa, according to age between 2007 and 2008.

	Percent positive							
Kap	0-5 years n=128		6-15 years n=177		16-40 years n=150		>40 years n=88	
Antigen	Persistent	*Nonres	Persistent	*Nonres	Persistent	*Nonres	Persistent	*Nonres
AMA-1	23.4	58.5	84.7	10.7	98.0	0.6	98.8	0.0
CSP	3 .9	78.9	14.1	53.1	20.6	48.0	38.6	30.6
EBA	5.4	86.7	53.6	35.5	90.6	6.6	98.8	1.1
GLURP-R0	9.4	77.3	27.2	58.8	53.3	31.3	84.1	6.8
GLURP-R2	8.5	72.6	58.7	31.0	91.3	5.3	97.7	0.0
LSA	16.4	57.8	59.3	21.4	81.3	8.6	93.1	3.4
MSP-1_{19}	17.9	71.0	74.5	16.3	95.3	1.3	97.7	2.2
MSP-1_{42}	35.9	43.8	90.4	4.5	99.3	0.7	98.9	0.0
MSP-3	14.0	62.5	41.8	42.3	72.0	16.6	81.8	12.5
SE	2.3	78.1	4.5	69.4	3.3	66.6	5.6	57.9
TRAP	2.3	92.1	7.9	83.6	19.3	66.6	45.4	37.5

*Nonres = Nonresponsive

MSP-1_{42} was the most immunogenic malaria antigen, with more than 33% of children under 5 years in both study sites being seropositive in both years (Tables 14 and 15). GLURP-R2, LSA, MSP-1_{19}, MSP-3 showed intermediate levels of immunogenicity, while EBA-175 and TRAP were markedly less immunogenic than any of the malaria antigens; both the prevalence and median antibody levels of these antigens were much lower in children under 5 years. Antibody persistent prevalence to TRAP in children under 5 years old was less than < 6 % in both sites as shown in Tables 14 and 15.

CHAPTER FIVE

DISCUSSIONS

5.1 Determination of Optimal Parameters for the Development of Cytometric Multiplex Bead Assay

A number of recent papers have used multiplex CBA testing to assess for the presence of antibodies to *P. falciparum* antigens (Cham *et al.*, 2010; Cham *et al.*, 2009). In this study, a comprehensive assessment of experimental parameters to optimize for multiplex cytometric bead assay (CBA) testing of antibodies to *P. falciparum* antigens is provided. A recent paper, (Ambrosino *et al.*, 2010) characterized optimization of plasma dilution and amount of antigen for CBA malaria antibody testing and focused on testing antibodies to peptides of various *P. falciparum* antigens. The present study to the findings of this and prior papers by assessing optimal plasma dilution and antigen amount for several recombinant antigens not previously tested, investigating antigenic variants in the same multiplex testing, establishing optimal diluent buffer for the lowest background reactivity, and demonstrating that the testing can be performed accurately with significantly less beads than have previously been used in malaria studies. The present study also demonstrates the specific advantages CBA may have over ELISA in antibody testing when there is no absolute reference standard, and relative units must be devised using samples from individuals without exposure to the pathogen to compare measurements of exposed populations. The decreased background activity, increased range of positive values and better differentiation of North American control samples from background and from each other with CBA as compared to ELISA, all make the CBA a better assay for calculation of "arbitrary units".

Previous studies have found CBA to be similar to or better than ELISA for measurement of antibodies to *P. falciparum* or *P. vivax* antigens in malaria endemic populations (Fernandez-Becerra *et al.*, 2010; Fouda *et al.*, 2006), and the present study concurs with these findings. Studies of antibodies to other infectious organisms have similarly shown that CBA compares well to established reference assays for these organisms (Reder *et al.*, 2008; Shoma *et al.*, 2011; Wagner *et al.*, 2011). Advantages noted over ELISA include specimen conservation, the ability to measure multiple analytes simultaneously, and reduction in sample processing time due to kinetics of the assay and the cost minimization.

The present study adds to this literature by demonstrating optimization of antigen concentration, plasma dilution, buffer solution, and number of beads. The study demonstrates that antibodies to antigenic variants can be tested in the same well (multiplex) and results are virtually identical to results of testing any variant alone by CBA (monoplex). It also provides evidence for the superiority of this testing as compared to ELISA testing in terms of providing less background, better differentiation of "positive" responses, and a broader dynamic range of antibody level values. These finding together support CBA as a precise, accurate and more efficient alternative to ELISA for testing antibodies to multiple *P. falciparum* antigens, including multiple variants of the same antigen.

Determining appropriate working amounts of antigens, is important in controlling surface density of the malaria antigens on the bead surface. Coupling small amounts may result in sub-optimal coating and low activity (less antibody binding), while too much antigen could result in precipitation and aggregation of the beads on the bottom of the microtiter well surface and this may impair the surface suspension antibody binding. Additionally, one would like to the use the

lowest amount of antigen that will provide the desired binding of antigens. Reduction of background activity will enable malaria immunologists to measure accurately low frequent antibodies especially in travelers (Jelinek *et al.*, 1998) and in individuals under medication or individuals who are in recovery phase since antibodies have been shown to decline after malaria episodes (Achtman *et al.*, 2005; Kinyanjui *et al.*, 2007).

Comparison between the 10-plex CBA and individual ELISA assays showed strong and highly significant correlations for all antigens except AMA-1 (Figures 4.1, 14A-J). The reasons for this discrepancy with AMA-1 are not clear. AMA-1 is among the most immunogenic *P. falciparum* antigens, and average MFI values for both AMA-1 variants were much higher than for other antigens, while OD values showed a similar distribution to that seen for other antigens. Low values were seen for both assays with North American controls, so it is not clear which assay more accurately reflected the range of AMA-1 values in this populations. As expected, the variations between the assays are likely explained by the different detection principles of the assays, namely: fluorescence in CBA and colorimetric enzymatic color change in ELISA. The enhanced dynamic range and the very low background noise signal of CBA make it a preferred method of testing for antibodies when compared to ELISA. Multiplex assays and other high throughput assays are likely to be the future methods of testing for assessment of immunity in malaria endemic areas. The present study has documented findings not seen by other groups testing CBA (Cham *et al.*, 2008), such as an increase in MFI for a few individuals at plasma dilutions lower than 1:200, showing evidence of a modest prozone phenomenon in these individuals. These findings demonstrate that some degree of compromise is required in decisions on a final dilution, as one dilution may not be the ideal for all antigens.

In the present study, long-term storage of multiplex beads, to see how long they could be used was not tested. Other studies have found that they provide slightly reduced MFI values after 7 and 12 months after initial bead coupling, if stored at 4^0C (Cham *et al.*, 2008; Lal *et al.*, 2005) and assessment of longer storage periods should be done in future studies. Lyophilization of coupled bead sets, as used in a recent study (Cham *et al.*, 2009), may provide an attractive alternative for long-term use of the same bead sets on different samples over time.

5.2 Determination of How Transmission Intensity and Age Affect Antibody Frequencies to *P. falciparum* Antigens in Two Sites in Highland of Western Kenya Over Time

This study has used a panel of immunologically well-characterized recombinant proteins and peptides to measure naturally acquired antibody responses to *P. falciparum* antigens in asymptomatic individuals living in a highland area in western Kenya where malaria incidence has recently been reported as interrupted (John *et al.*, 2009). This study illustrates the importance of identifying malaria antigens that induce long lasting antibodies when transmission is absent. Determining the dynamics of antibody responses to various malaria antigens during periods of very low/absent malaria transmission is critical in vaccine studies, since such studies are likely to distinguish between antibody responses that are long lasting and those that are short lived. These would provide knowledge on longevity of antibody responses of the suitable vaccine candidates and when these responses need to be initiated and when best to administer boosting responses. Understanding these correlates of immunity is critical before vaccine trials are initiated in areas of unstable seasonal malaria transmission (Moormann *et al.*, 2006).

This study has shown that during periods of absent malaria transmission in asymptomatic individual's frequencies of antibodies to CSP, GLURP-R0, MSP-1_{19}, MSP-3, TRAP, and to heterogeneous schizont crude extract decrease dramatically in areas with unstable seasonal malaria transmission. This probably suggests that antibodies induced by these antigens lasts for a shorter duration compared to other malaria vaccine candidates in these areas. This relatively fast rate of decline for these antibodies has implications on the vaccine trials in areas that experience

unstable malaria in the light that antibodies to these antigens have been implicated in protection against high level parasitemia and disease.

These findings are also in agreement with the idea that antibodies to CSP and schizont extract serve as an indicator(s) of recent infection (Cobelens *et al.*, 1998; Druilhe *et al.*, 1986; Webster *et al.*, 1992; Wipasa *et al.*, 2010). Clinical trials of *P. falciparum* CSP-based vaccines have shown that it induces both humoral and cellular immune responses (Hutchings *et al.*, 2007) and as the most advanced malaria vaccine candidate, it has entered phase III clinical trial (Casares *et al.*, 2010). The generation of relevant epidemiologic and immunologic data for CSP is of crucial significance, but such data are lacking, particularly for regions characterized with unstable seasonal malaria. Whether this rapid waning of frequencies of CSP and TRAP antibodies observed in this study and in early studies (Noland *et al.*, 2008) that observed lower frequencies of pre-erthrocytic antibodies is a result of insufficient T cell help or lack of enough contact time (30 mins) between the sporozoite antigens and immune cells that would culminate in an effective immune activation to occur, needs to be ascertained. Further, possible explanations for the low antibody frequencies or high nonresponsiveness could be that antibody responses to pre-erythrocytic antigens in this highland population and or North Americans are more susceptible to genetical control, and this possibly results from the monoethnic study population.

The low antibody frequencies can be mediated by HLA dependent restriction of immune responses (Good *et al.*, 1988). A likely explanation for low antibody frequencies, found in the present study, could also be that individuals immune system are generally predestined to have a lower frequency of naive B cells with specificity to these epitopes expressed by pre-erythrocytic

antigens. This finding suggests that malarial antigens whose frequencies decrease over time would require that vaccine trials being performed in unstable areas based on these antigens, need periodic regular booster doses (Kinyanjui *et al.*, 2009). The booster doses administered will facilitate a long-lived protection to be achieved or with the possibility of incorporating a suitable adjuvant to enhance the immune responses against this antigen.

5.3 Determination of How Transmission Intensity and Age Affect Antibody Levels to *P. falciparum* Antigens in Two Sites in Highland of Western Kenya Over Time

This study results show that antibody levels to all the leading malaria vaccine antigens decreased when parasite exposure was very low or absent but a steady-state level of VCA-p18 antibodies was maintained over time. This finding is important in the light that antibodies to these antigens have been associated with protection against clinical disease (Dodoo *et al.*, 2000; Egan *et al.*, 1996; Oeuvray *et al.*, 2000; Osier *et al.*, 2007; Perraut *et al.*, 2005; Stanisic *et al.*, 2009), in a combined model (Osier *et al.*, 2007), and in preclinical and clinical studies that suggest that responses to many blood stage antigens are involved in protection against clinical malaria (Gray *et al.*, 2007). Also, antibodies to MSP-3 and GLURP have been implicated to play roles in inhibiting parasite neutralization that is dependent on their interaction with Fcγ receptors on monocytes or other effector cells (Oeuvray *et al.*, 2000; Soe *et al.*, 2004). Coupled with the dramatic decline of antibody levels to these antigens, individuals in unstable seasonal areas may have a reduced capacity to have efficient effector responses that these antibodies have been implicated to facilitate.

The present study measured antibodies to a wider panel of malaria antigens as compared to other antibody based studies that measured one or relatively few *P. falciparum* targets (Nebie *et al.*, 2008; Noland *et al.*, 2008; Nwuba *et al.*, 2002; Polley *et al.*, 2004). Antibody responses directed against sporozoite stage may function to provide pre-erythrocytic immunity against infection by sporozoites (Wipasa *et al.*, 2002). The rapid decline of anti-CSP and anti-TRAP responses during periods of low malaria transmission resulted in low levels of anti-CSP IgG and anti-TRAP IgG, respectively, in many individuals at the start of the second cross-sectional survey which may

predispose the individuals to malaria infection, since in seasonal and unstable areas almost all infections are followed by disease (Iriemenam *et al.*, 2009). Vaccination trials with sporozoite antigens will need to involve multiple booster doses to maintain higher levels of these antibodies. The ultimate goal would be to induce higher levels of CSP antibodies (Kubler-Kielb *et al.*, 2010). Antibody levels to all malaria antigens tested in this cohort decreased, in agreement with other studies (Akpogheneta *et al.*, 2008; Fonjungo *et al.*, 1999; Kinyanjui *et al.*, 2007), suggesting that the proportion of antibodies responsible for neutralization, opsonization and other effector functions might have decreased substantially over time with lack of parasite exposure. It is likely that short-lived antibody secreting cells (ASC) may have generated most of these malarial antibodies (Manz *et al.*, 2005; Manz & Radbruch, 2002) since longevity of antibody responses may provide a clue on the type of plasma cells responsible for antibody production. This finding suggests that retention of malaria antibody levels is poor and homogenous across all age groups in unstable seasonal malaria transmission areas.

5.4 Determination of Antibody Correlations to *P. falciparum* Antigens Over Time and Antibody Levels to VCA-p18 in Two Sites in Highland Region of Western Kenya

Antibody correlations were high in both study sites in 2007 and 2008. Previously, it has been shown in the same study area that individuals with IgG antibodies to multiple antigens had a significant predictor of protection than individuals with a limited number of antibodies (John *et al.*, 2008). Thus, it can be suggested that individuals living in unstable, seasonal areas of malaria are likely to have differences in malaria vulnerability since responders to all antigens were fewer in Kipsamoite as compared to Kapsisiywa in 2007 and 2008. The data presented here also shows that antibodies to schizont crude extract may be a good immunoepidemiological indicator to present the prevailing malaria transmission in unstable seasonal areas.

By comparing antibodies to malaria vaccine leading antigens with VCA-p18, which is acquired very early in infancy life through mother-child contact, the current study showed that levels of all malaria specific antibodies decrease over time while antibodies to VCA-p18 are maintained over time. Malaria specific antibodies increased with age as compared to VCA-p18 antibodies which did not, suggesting that a single exposure would probably drive the acquisition of VCA-p18 antibodies. Further, the current findings support the notion that malaria results in activation of short-lived *P. falciparum* specific plasma cells (Langhorne *et al.*, 2008) but is in contrast to an earlier study that showed that a significant proportion of individuals in Thailand acquired B cell memory responses to *Plasmodium* antigens (Wipasa *et al.*, 2010). Probably this subset of short-lived plasma cells activated by exposure to malaria infection is responsible for the dramatic decline of antibody levels to *P. falciparum* antigens. It can be speculated that stable antibody levels to VCA-p18 were due to stable frequencies of either VCA-specific memory B cells or

long lived plasma cells (LLPC) in these individuals. The steady state of VCA-p18 antibodies maintained across all age groups is an indication that the frequency of cells driving the secretion of these VCA-p18 antibodies might also be stably maintained across age groups.

5.5. Determination of Antibody Seroreversion Rates by Age for *Plasmodium falciparum* Antigens in Two Sites in Highlands of Western Kenya Over Time

In this study, three different individuals were positive by microscopy for *Plasmodium* during first and second survey. These few malaria cases demonstrate interruption of malaria in this unstable area and the existing incidences provide a near perfect model for studies on longevity of antibody responses to *P. falciparum* antigens. Less persistent antibody responses were observed for antibodies to GLURP-R0, LSA and TRAP than to AMA-1, EBA-175, GLURP-R2 and MSP-1_{42} in the cohort study. The lack of antibody responses (no response) in some of the individuals, particularly for TRAP (>70%) maybe explained with the idea that the individuals' immune system might have been stimulated by the antigens but made no detectable antibody responses. As expected, Kapsisiywa had a lower persistence of antibodies to schizont extract and may be due to the high success rates of IRS spraying that was conducted in 2007 by Ministry of Health, Government of Kenya, compared to the percentage coverage in Kipsamoite. An age-wise persistence of blood stage antibodies was also reported in this thesis research probably due to higher percentage of long-lived plasma cells commonly in older individuals as compared to young individuals in agreement with past findings (Akpogheneta *et al.*, 2008).

A question of central importance is how antibody responses are maintained in individuals residing in unstable transmission areas. Indeed, immunity in life-long residents of holoendemic areas can wane if the individual is removed from the malarious area. Previous work has suggested that sustained humoral response to LSA-1 (Zhou *et al.*, 2002) and MSP-1 (Drakeley *et al.*, 2005) can be achieved with limited parasite exposure, whereas high-level antibody response to other antigens may require frequent re-exposure. Other studies in a nearby highland area of

seasonal malaria transmission revealed that CSP- and TRAP-specific responses declined significantly during the dry season (John *et al.*, 2003). However, the fact that the decline was more dramatic in children versus adults, suggests that cumulative exposure can eventually induce long-lasting immunity. Several studies have attempted to characterize memory B cell populations in malaria patients. One study from coastal Kenya observed a deficiency in malaria-specific memory B cells (MBC) (Dorfman *et al.*, 2005) while a more recent study has described the expansion of a hyporesponsive MBC in *P. falciparum*-infected versus healthy Malian children (Weiss *et al.*, 2009).

The decrease in antibody frequencies and levels to schizont extract crude antigen may likely be as a result of the successful IRS carried out in May 2007 (John *et al.*, 2009) as shown in Figure 2 and its humoral effect were seen in the subsequent year which had a more pronounced effect in Kapsisiywa (the site with higher household IRS coverage). This justifies why antibody responses to schizont extract could be a proxy measure to ascertain the level of malaria transmission. Lastly and most important, this thesis reports maintenance of stable frequencies of MSP-1_{42} and GLURP-R2 antibodies in both study areas.

CHAPTER SIX

SUMMARY, CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

6.1 Summary

Cytometric bead assay can test antibodies to multiple recombinant *P. falciparum* antigens from a single plasma sample, and has greater range of values in positive samples and lower background readings for blank samples than ELISA. The study demonstrates low frequencies of antibodies to preerythrocytic antigens (CSP, LSA-1 and TRAP) compared to blood stage antigens (MSP-1(42), MSP-1(19) and EBA-175) in areas of unstable transmission. IgG levels to all *P. falciparum* antigens decreased at both study sites over the fifteen-month period but not for Epstein Barr Virus VCA-p18 antigen. IgG levels for most antigens correlated significantly with each other for both sites during the first and second surveys. Differences in age, type of *P. falciparum* antigen are some of factors that account for differences in rates of seroconversion.

6.2 Conclusions

1. Improved multiplex cytometric bead assay testing, with low background reactivity, optimal antigen concentration and plasma dilution, and a lower number of beads has been validated to be used by several malaria researchers.

2. Antibody frequencies to CSP, GLURP-R0, MSP-1_{19}, MSP-3, SE and TRAP decrease dramatically in unstable, areas during periods of low malaria transmission. The study demonstrates low frequencies of antibodies to preerythrocytic antigens (CSP, LSA-1 and TRAP) in areas of unstable transmission.

3. There was decline in antibody levels to all malaria antigens except to VCA-p18 when malaria is interrupted in unstable transmission areas. Antibody correlations were high in both study sites in each of the years.

4. High correlation of antibodies to the different antigen variants was also observed in the study sites.

5. Antibody seroreversion differed by age and by the type of *P. falciparum* antigen.

6.3. Recommendations

1. Cytrometric bead assay can be used in the simultaneous measurement of antibodies to *P. falciparum* antigens

2. Malaria vaccine trials in highland region of western Kenya will need to involve multiple frequent boosting for humoral immunity to be maintained.

3. Frequent boosting with vaccine antigens will result in maintenance of levels even when transmission is low.

4. Malaria vaccine based on the tested antigens will not require combination of subunit vaccines as antibody responses were correlated.

5. Antibody seroreversion are higher in young children hence they will require more boosters and adjuvants when vaccine trials is initiated.

6.4 Suggestions for Future Research

1. Future studies should address immunological analyses of IgG subclasses to the panel of recombinant antigens used in this study especially from CSP, TRAP, MSP-1_{19}, MSP-3 and

GLURP antigens to determine which among the IgG subclasses decreased, and a comparison of avidity of antibodies across age groups and over time in unstable seasonal areas.

2. Assessment of MSP-1_{42} and GLURP-R2 IgG subclass antibodies would also be vital for determination of which subclasses were stable over time.

3. Further research is needed for development of multiplex assay to measure antibody functional capacity.

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