

**Antigen Specific IgG Subclass Antibody Responses To
Plasmodium falciparum Antigens: A Comparison Of Antibody
Concentrations And Antigen Binding- Affinities Between The
Fulani And Two Other Sympatric Tribes.**

ABSTRACT

Parasitological, clinical and Immunological responses to *Plasmodium Falciparum* malaria have been show to vary remarkably between three sympatric ethnic groups in Burkina Faso, West Africa. The Fulani despite similar exposure and no difference in protective measures against malaria infection have been found to have higher antibody responses than the Mossi and the Rimaibé. This study Corroborates and expands on the humoral responses of the Fulani in comparison to the non-Fulani. The level of Immunoglobulin (Ig) isotypes are measured in each ethnic group. Overall type of subclass response does not vary between ethnic groups however the quantity and quality is significantly different between ethnic groups. IgG1 and IgG3 are the dominant antibody isotypes detected with higher levels found in the Fulani than in the Mossi and Rimaibé. Further analysis into the mechanism of protection by determining the avidity of antibodies as a measure of antibody affinity showed the Fulani to have highly avid antibodies than the non-Fulani. In conclusion this study highlights findings not previously reported showing the Fulani have higher antibody isotypes that are also more avid than the other ethic groups.

ANTIGEN SPECIFIC IGG SUBCLASS ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM* ANTIGENS: A COMPARISON OF ANTIBODY CONCENTRATIONS AND ANTIGEN BINDING-AFFINITIES BETWEEN THE FULANI AND TWO OTHER SYMPATRIC TRIBES. 1

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LIST OF ABBREVIATIONS.

ADCI	Antibody dependent cellular inhibition
AI	Avidity index
AMA-1	Apical membrane antigen-1
CD	Cluster of differentiation
CI	Confidence interval
CSP	Circumsporozoite protein
CTLA4	Cytotoxic T-lymphocyte antigen-4
ELISA	Enzyme linked immunosorbent assay
GuHCL	Guanidine Hydrochloride
IgG	Immunoglobulin
LSA	Liver stage antigen
MSP	Merozoite surface antigen
Obs	Observations
OD	Optical density
OPD	O-phenylenediamine
PCR	Polymerase chain reaction
RBCs	Red blood cells
RESA	Ring infected erythrocyte surface antigen
SP	Sulphadoxine- Pyrimethamine
STRAP	Sporozoite threonine-asparagine-rich protein
TGF β	Tumour growth factor beta
TRAP	Thrombospondin related protein
Tregs	T regulatory cells
HbS	Haemoglobin S

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Study Objectives.

Past studies reported various differences in the magnitude of response to *P. falciparum* antigens between the Fulani and other ethnic groups living in the same malaria endemic area of Burkina Faso, West Africa. These differences cannot be explained by environmental factors as these groups are exposed to similar levels of exposure to *P. falciparum* infections.

The objective of this project is to investigate whether there are qualitative differences in the serum antibody response to various *P. Falciparum* antigens between three sympatric groups, Fulani; Rimaibé and Mossi, living in Burkina Faso, West Africa. More specifically, the study quantified amounts and affinities of *P. Falciparum* specific antibodies of different isotypes, and then analysed for inter-ethnic differences in these measures among the three groups.

1.0 Introduction

Malaria was described early in the 1700s by an Italian Francesco Tori¹. He associated it with ‘foul air’ in the marshy regions. Thus the term malaria is from the Italian mal’ aria, which means bad air. Later in the 19th century Charles Louis Alphonso Laveran identified the presence of parasites in the blood of a patient suffering malaria.

As the world and human civilisation evolved, climate changes and improved sanitation reduced the habitats necessary for mosquito breeding, thus reducing malaria transmission while the use of pesticides such as DDT, assisted in its eradication in Europe and the United states of America. However, malaria is still endemic in many parts of the world such as Africa, India, South East Asia, and South America.

The exact mortality rate of malaria is unknown although globally, over one million deaths are caused directly by malaria annually². Children less than five years of age, pregnant mothers

and non-immune individuals account for the majority of malaria associated deaths. In Sub Saharan Africa alone, nearly one million children below five died of malaria in 2000³.

Malaria parasites are transmitted by the bite of the female *Anopheles* mosquito in the process of taking a blood-meal. There are four main Plasmodium parasites that infect humans, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. However, recent findings demonstrated the zoonotic transference to humans of *Plasmodium knowlesi*, a malaria parasite that was previously only observed in primates⁴. Further investigations with the use of advanced diagnostic methods such as nested Polymerase chain reaction (PCR) have found regular cross-infections and misdiagnosis between *P. malariae* and *P. knowlesi* in Malaysia⁵

These four species of *P falciparum* associated with human malaria differ in their geographical distribution, induction of immunological responses, morphology and drug susceptibility. *Plasmodium falciparum* is the most virulent causing severe disease and is responsible for the high mortality rate in African children^{2, 6}.

1.1 Malaria life cycle.

The *P. falciparum* cycle begins when sporozoites gain entry into the human (mammalian) host from an *Anopheles* mosquito bite (*figure1*). Once in the blood stream, they are transported to the liver where they migrate through several kupffer cells before invading one⁷,⁸. This stage is referred to as the pre-erythrocytic stage and no clinical symptoms are observed at this stage. Once in the liver cell, the sporozoite grows and differentiates into a liver-schizont and ruptures releasing 20,000-30,000 merozoites into the hepatic venous circulation that invade erythrocytes to begin the erythrocytic cycle. The merozoites entry into the erythrocytes involves complex interactions with various receptors⁹. Once inside, the parasites feed on haemoglobin while developing inside the parasitophorous vacuole. The

early erythrocytic stage, the ring stage can be observed through a thick or thin smear preparation and forms the basis for a positive diagnosis of clinical malaria in association with various symptoms¹⁰. Within 48 hours, the parasite undergoes further development from the ring stage to trophozoites then to a schizont¹⁰. Thereafter, the schizont ruptures releasing approximately 20 merozoites from each cell that will then invade other erythrocytes repeating the cycle¹¹. A small proportion of asexual parasites develop into gametocytes. These are ingested by the female mosquito during a blood meal. This commences the sexual stage of the parasite's lifecycle in the vector. In the mosquito, mating occurs and the resultant oocytes undergo mitotic divisions and growth into over 10,000 sporozoites from a single oocyst¹². The sporozoites reside in the mosquito salivary glands and during a blood meal are released into the human host.

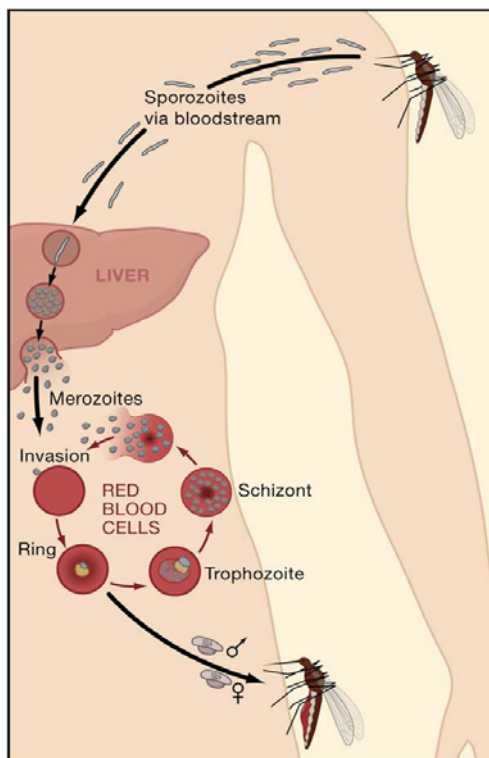


Figure 1: Malaria life cycle. Adapted from invasion of the red blood cell by malaria parasites⁹

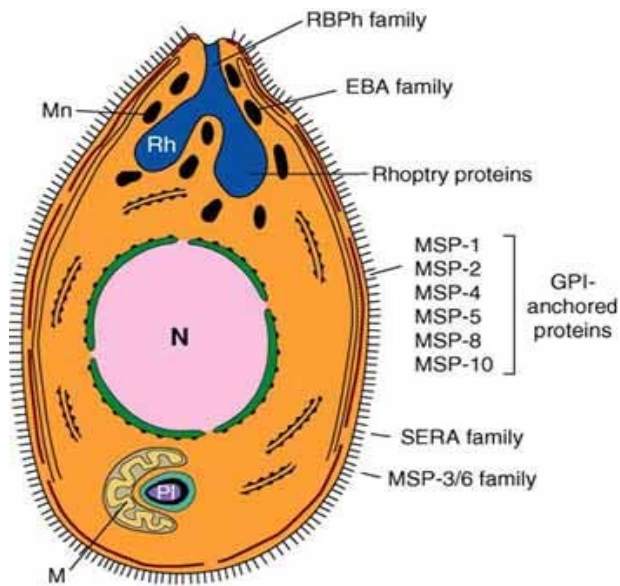


Figure 2: Schematic diagram of a merozoite. Adapted from the Walter and Eliza institute website (www.wehi.edu.au/facweb/indexresearch.php?id=53)

1.2 Malaria control and treatment.

Malaria control and treatment involves many factors due to the diverse breeding and feeding nature of the vector and the parasite life cycle. Common methods include the use of insecticide treated nets and curtains, residual wall spraying, clearing of breeding sites and improved diagnosis and drug treatment.

The use of personal protective methods such as long sleeved clothing, mosquito repellents including lotions and burning incense containing repellents (mosquito coils) reduces the number of bites thus parasite transmission. This is however subject to personal discretion, often deterred by warm humid weather conditions and in some cases lotions and incense repellents cause reactions. The use of Insecticide treated nets has been found to reduce mortality by 44% in African children¹³. Insecticide treated nets are a feasible, affordable and sustainable method unfortunately they do not prevent transmission during other periods.

Regular upgrade training of staff, diagnosis and monitoring of malaria in developing countries has contributed to reducing clinical disease¹⁴. Consistency however is hampered by poor infrastructure in developing countries and unstable governments.

Combination therapies are the current recommendations by the world health organisation. This is due to the malaria parasite's ability to develop resistance to monotherapy treatments. Until the 1990s, Chloroquine was successfully used in the treatment of malaria before resistance was observed that was associated with an increase in malaria morbidity and mortality^{15, 16}. Artemisinin combination therapy studies in Africa found them more efficacious in treatment than previous first line treatments such as chloroquine and sulphadoxine-pyrimethamine¹⁷. The current combination therapy in use is Lumefantrine-artemether (Co-artem) which is more efficacious than Sulphadoxine- Pyrimethamine (sp)¹⁸ although more expensive.

1.3. Malaria and the immune system.

Immunoglobulin from malaria exposed adults has been shown to provide immunity through passive transfer to non-immune individuals^{19, 20}. Immunity to malaria is naturally developed with continuous exposure to malaria antigen^{21, 22}. Natural immunity however wanes if the intensity, length and duration of exposure to malaria antigens decreases²³. Genetic factors such as Hb variants and thalassaemias have also been implicated in the acquisition of immunity to *P Falciparum* malaria^{24, 25}. These traits are widely distributed in malaria endemic regions²⁶. Their protective mechanism is not known though HbS and β -thalassemia have been shown to enhance phagocytosis of parasitized erythrocytes²⁷.

For this study we focus on the IgG antibody responses to four malaria antigens, Apical membrane antigen 1 (AMA-1), Merozoite surface protein 1₁₉ (MSP-1₁₉), Merozoite surface protein 2 (MSP-2) and Circumsporozoite (CSP).

1.3.1. Circumsporozoite protein (CSP). One of the early vaccine designs used irradiated sporozoites which conferred protection against challenge with laboratory bred mosquitoes²⁸,²⁹. Currently the leading vaccine in clinical trials RTS, S/AS02 is derived from the Circumsporozoite antigen expressed on the surface of the sporozoites found in the pre-erythrocytic stage. CSP consists of a tetrapeptide region (Asn-Ala-Asn-Pro) that is conserved between parasite variants making it a suitable marker for vaccine design and measurable in different populations. Various studies have found immunization with CSP to induce immune protection in monkeys³⁰, rodent³¹ and humans^{28,32}. Apart from CSP, other pre-erythrocytic proteins can also stimulate an immune response and are in vaccine development these include Liver stage antigen-1 and 3 (LSA1 and 3)³³, Thrombospondin related adhesion protein (TRAP)³⁴, sporozoite and liver stage antigen (SALSA)³⁵ and sporozoite threonine-asparagine-rich antigen (STRAP)³⁶. Antibodies generated against pre-erythrocytic antigens protect by blocking binding and invasion of erythrocytes however, they have a short half life but are less polymorphic than blood stage antigens³⁷.

1.3.2. Merozoite surface protein 1₁₉ (MSP1₁₉). Merozoite surface proteins 1₁₉ (MSP1₁₉), Merozoite surface proteins-2 (MSP-2), Apical membrane antigen-1 (AMA-1) and glutamate rich protein (GLURP) are expressed on the surface of the merozoite in the erythrocytic stage (*figure 2*) and are currently possible candidates for blood stage vaccines. Merozoites surface protein 1₁₉ is a fragment portion of MSP1₄₂. It contains two cysteine rich epidermal growth factor (EGF)- like domains that are conserved in all species³⁸. MSP 1₁₉ has been shown to elicit antibody immune responses that are protective in non-human primates³⁹ and rodent⁴⁰. Further work in mice highlighted the importance of antibody responses to MSP1₁₉ when B cell knockout mice immunized with MSP1₁₉ could not resolve a *Plasmodium yoelii* infection⁴⁰. Furthermore, passive transfer of purified IgG sera specific for MSP1₁₉ protected naïve mice⁴⁰.

1.3.3. Apical membrane antigen-1 (AMA-1). Apical membrane antigen-1 is highly polymorphic⁴¹⁻⁴³. In rodent and non-human primate studies it has been found to generate species specific immunity^{44, 45}. AMA-1 combined with MSP-1₁₉ in rabbits and primates was shown to stimulate an antibody immune response that inhibited parasite growth in vitro⁴⁶. Clinical trial with a combination AMA-1 vaccine stimulated the production of allele specific antibody responses that were found to be protective⁴⁷.

1.3.4. Merozoite surface protein-2 (MSP-2). MSP-2 is highly polymorphic with a series of variable repeat motifs^{48, 49}. In a combination vaccine (MSP-1, RESA and MSP-2 3D7 allele) trial, MSP-2 was associated with reducing allele specific malaria parasite density in the population⁵⁰. Further studies demonstrated that antibodies against MSP-2 reduced the risk of clinical malaria⁵¹.

Although antigens expressed on the sporozoites and merozoites in the blood are exposed for only short periods, antibodies generated are shown to block invasion of RBCs by merozoites, participate in possible anti-parasite mechanisms including opsonization and antibody dependent cellular inhibition⁵²⁻⁵⁶.

1.3.5. Adaptive immunity. Adaptive immunity is made up of humoral and cell mediated responses. In this project we quantify and analyse the affinity of humoral response from natural exposure to malaria. The immune system to infections is a complex interaction between the innate and adaptive system. B cells secrete antibodies that engage in immune responses to extra cellular pathogens whereas T cells target intracellular pathogens. B cell responses can be independent or dependant on T cell interactions. Independent B cell interactions are activated by polysaccharide and toll-like receptor antigens such as bacteria that stimulate plasma cells that are short-lived^{57, 58}. T cell dependant antibodies are generated through linked recognition, when a primed helper T cell recognises the specific epitope on

an antigen presenting B cell in the presence of appropriate costimulation^{57, 59}. The activated B cell can then undergo differentiation into short-lived plasma cells which form the extracellular foci in the secondary lymphoid organ and secrete antibodies of low affinity and then die within days⁶⁰ or migrate to B cell areas and form germinal centres from where they undergo proliferation and differentiation and into memory B cells, and long lived plasma cells that secrete high affinity antibodies⁶¹.

Apart from the generation and regulation of immune responses with B cells, T cells also release cytokines that stimulate the innate immune systems and influence the pathology and magnitude of immune responses in different disease and animal models⁶². A subset of T cells, T regulatory (Tregs)^{63, 64} cells and Follicular T cells among others have recently been identified⁶⁵. Follicular T cells were shown to contribute to the proliferation and development of high affinity antibodies in germinal centres⁶⁶. The function of Tregs is still yet to be fully comprehended and varies dependant on the phenotype and experimental model. Depletion of regulatory T cells in rodent models shows loss of disease resistance, pathogenesis and result in death^{67, 68} while studies in malaria naïve vaccinated subjects, showed the presence of Tregs (CD4⁺CD25⁺FOXP3⁺) and Tumour growth factor-beta (TGFβ) to be associated with increased parasite growth in vivo. In the Fulani, Tregs (CD4⁺CD25⁺) were found to be lower than in the Mossi and Rimaibé this however did not affect their immune responses to malaria antigen⁶⁹.

1.4 Malaria among the Fulani, Rimaibé and Mossi.

The Fulani, Mossi and Rimaibé live in a hyper- endemic malaria region northeast of Ouagadougou, Burkina Faso in West Africa. The origins of the Fulani are genetically distinct from the Mossi and Rimaibe⁷⁰. The Fulani are nomadic pastoralists of Caucasoid origin whereas the Mossi and Rimaibé are long established sedentary farmers in the sub- Saharan savannahs of Sudanese negroid origin^{71, 72}. The Fulani over time have adopted sedentary

farming and sociocultural habits between the three ethnic groups have amalgamated providing interethnic comparisons in syntopic conditions.

The Fulani in various parasitological, immunological and clinical studies have been shown to exhibit lower parasite loads, less morbidity and varied antibody prevalence and levels to various malaria antigens (CSP, RESA, TRAP and crude antigen) ⁷³⁻⁷⁵. In addition, the reduced morbidity and mortality among the Fulani was correlated with higher seroprevalence and higher antibody titres regardless of the conserved or polymorphic nature of the antigen (CSP) ^{73, 74}. Thus in spite of being exposed to comparable inoculation rates of malaria and having similar protective measures, the Fulani, unlike the Rimaibé and Mossi, encounter less disease and this resistance is associated with higher prevalence and titres of malaria specific antibody⁷⁶. These differences were also observed in other West African countries including Mali⁷⁷, Nigeria⁷⁸ and the Gambia⁷⁹⁻⁸¹ with various genetic, humoral and physiological features (Splenomegaly) found distinctly in the Fulani and not in the Rimaibé and Mossi. Further genetic comparative studies showed a higher IL4-524 allele frequency which was associated with increased antibody levels against malaria antigens among the Fulani than in the Rimaibé and Mossi⁸². Interleukin-4(IL4) cytokine is essential in inducing IgG class switching from IgM/IgG to IgE and enhances antigen presentation to B lymphocytes⁸³. Conversely, the Fulani in comparison to the Rimaibé and Mossi had lower genes associated with T regulatory cell function (CTLA4 and FOXP3⁺) ⁶⁹ and lower malarial resistance genes in comparison to the other ethnic groups⁸⁴.

Other studies have tried to explain the difference in immune responses in the Fulani through cultural studies, noting that the Fulani have a strong cultural identity and conduct their affairs with great diligence and their attitude towards illness may affect their immune responses ⁸⁵. Immune response variation has highlighted essential factors in the application of intervention and vaccine trials and measures of outcome⁸⁶. Many studies have highlighted the difference

between the Fulani, Mossi and Rimaibé immunological responses however the basis for differential responses to malaria is still unclear.

Most studies comparing the interethnic humoral responses measure the total IgG response with few measuring antibodies of other isotypes. Measuring total IgG antibody levels was found not to be a good indicator of reduced risk of clinical malaria⁸⁷ whereas IgG1 and IgG3 antibodies through opsonization mediates phagocytosis⁸⁸ and in conjunction with monocytes through antibody dependant cellular inhibition (ADCI)⁵³ reduces the risk of clinical malaria. This study compares the antibody isotype responses in the Fulani, Mossi and Rimaibé in different age groups.

2.0 Materials and Methods.

2.1 Study site and population.

Archived samples from an immuno-epidemiological study conducted in two rural villages 35 Km north east of Ouagadougou in Burkina Faso, West Africa were used. A longitudinal cohort was established from August 1994 to March 1995. Serum samples were collected during the high and low transmission season providing both pre- exposure and post exposure antibody profiles to malaria antigens. Several detailed studies conducted in the area highlight the high transmission seasons to be in June to August^{89, 90} with up to one infective mosquito bite per person per night.

Over 500 sera samples were collected after obtaining consent from two villages where the Mossi, Rimaibé and Fulani ethnic groups reside. The cohort consisted of both male and females aged 1 to 80years old. Pregnant women were excluded from the study. Thick and thin blood smears were examined for Plasmodium Falciparum parasites. A parasite count of ≥ 5000 parasites/ μ l and a temperature $\geq 37^{\circ}\text{C}$ were used in the clinical diagnosis of malaria.

Blood collected in the field was spun in the laboratory to obtain the plasma and samples catalogued and stored at -80°C.

2.2 Antigens.

Recombinant P. Falciparum merozoite surface proteins-1₁₉⁹¹ (Wellcome sequence^{92, 93}) and merozoite surface protein-2⁹⁴ (full length 3D7 sequence) were produced as described previously in *Escherichia coli* as Glutathione S-transferase fusion proteins. Apical membrane antigen -1 (3D7 sequence) were produced in *Escherichia coli* as a Hexa- His tagged fusion protein as previously described⁴⁵. Circumsporozoite protein (Gift from Eric Tongren, CDC). These antigens were selected for use in this study due to responses from a previous study showing that the Fulani had significantly higher responses than the other ethnic groups (Cook, J. PHD work in progress). The samples were picked from the August 1994 samples and had been previously shown to have a high total IgG response for these antigens.

Furthermore, these antigens were selected due to their potential as vaccine candidates and the association of their respective antibodies to clinical protection from malaria in field studies^{45, 95, 96}.

2.3 ELISA (Enzyme linked immunosorbent assay) antibody subclass assays.

Samples were screened for antibody subclasses against Merozoites surface protein 1₁₉ (MSP1₁₉), Apical membrane antigen 1 (AMA-1), Merozoites surface protein 2 (MSP-2) and Circumsporozoite protein (CSP) using ELISA assays. Immulon -4- microtitre plates (Dynatech, Billingshurst, UK) were coated overnight at 4 °C with 50µl/ well of antigen in 0.1M sodium carbonate/bicarbonate (pH9.6). Each antigen was coated at concentrations optimised in earlier studies. AMA-1, CSP and MSP1₁₉ were coated at a concentration of 1:1000 and MSP-2 1:800. The following morning plates were washed three times (3X) in Phosphate buffer saline Tween 0.05% (PBS-T) (Sigma, St-Louis, MO, USA) and blocked with PBS Tween 0.05% 1% skimmed milk (PBS-T-milk) for three hours and incubated at

room temperature. Plates were then washed 3X and prior sera stored in PBS 0.05% Tween, 0.01% Azide was diluted. Serum dilution (50µl/ well) varied with each antigen. MSP1₁₉ was used at 1:1000, MSP-2 1:1000, 1:800, AMA-1 1:2000 and CSP 1:200 diluted in PBS-T-milk. Plates were incubated overnight at 4 °C. Subsequently plates were washed 6X with PBS-T and incubated with 50µl/ well of sheep anti-human immunoglobulin subclasses conjugated to horseradish peroxidase (The binding site ltd, Birmingham, UK) for 3 hours at room temperature. Antibody subclasses IgG2, IgG3, IgG4 and Total IgG were diluted at 1:10,000 in PBS- T- milk while IgG1 was diluted at 1:5000. These concentrations were chosen after titrations were performed. Following a further six washes in PBS-T, 100µl/well of O-phenylenediamine substrate (OPD) (Sigma, St-Louis, MO, USA) in 0.1M citric acid and 0.2M dibasic sodium phosphate was used for detection. After a 15 minute incubation period the reaction was stopped with 2M sulphuric acid (H₂SO₄) and optical density (OD) measured at 492nm using a Dynex reader. Data was converted using Dynex revolution software (Dynatech, Billingshurst, UK). All concentrations used were previously optimized for each antibody subclass and antigen. To control for day to day and inter- assay variation, specific antibody isotypes were directly coated on the plates in a three fold serial dilution at a starting concentration of 1000µg/ml, 50µl/well. OD values generated from the plates were converted into antibody units (µg/ml) using the standard curves generated from the serial dilution of each antibody isotype. If the co-efficient of variation between duplicate samples was greater than 15% samples were disregarded. If the serial dilution of the specific antibody isotype on the plate assayed or the PBS control blanks were faulty, the plate absorbance readings were disregarded and samples repeated.

2.4 Avidity ELISA.

An optical density (OD) reading of greater than 0.4 obtained using ELISA isotype experiments and median value responses to each antigen were used to group individuals into high and low titres responders. Samples with high titres were then measured for their antibody affinity by determining their avidity. Low sample responders were not used in this experiment due to our inability to detect avidity when initial OD readings are low.

Using a similar protocol as the ELISA assay, 96 well Immunol -4- microtitre plates (Dynatech, Billingshurst, UK) were coated with antigen at 50 μ l/ well in 0.1M sodium carbonate/bicarbonate (pH9.6) and incubated overnight at 4 °C. Plates were washed using PBS -T (Sigma, St-Louis, MO, USA) and blocked with PBS Tween 0.05% 1% skimmed milk (PBS-T-milk) for 3 hours at room temperature. 50 μ l of sera samples diluted in PBS-T-milk (similar dilutions as ELISA protocol) were aliquoted into each well and stored overnight in 4 °C after washing each plate 3X in PBS-T. The next day after three washes in PBS-T, 75 μ l/well 4M Guanidine Hydrochloride (GuHCL) was aliquoted into each well in duplicate and 75 μ l/well of PBS into coinciding control wells. Following a 10 minute incubation plates were washed 6X in PBS-T and 50 μ l/well of sheep anti-human IgG-HRP conjugate subclass antibodies (The binding site ltd, Birmingham, UK) added and incubated for 3 hours at room temperature. Plates were then washed 6X in PBS-T and developed with 100 μ l/well of OPD substrate solution (Sigma, St-Louis, MO, USA). 25 μ l/well of 2M H₂SO₄ stopped the reaction and the optical density was read at 492nm using a Dynex reader. No avidity assays were run on antibodies recognising MSP2 antigen due to the chelating agent having a direct effect on the rMSP antigen (Drakeley, C. Manuscript in preparation).

2.5. Statistical analysis.

Optical density(OD) was converted into $\mu\text{g/ml}$ using antibody isotype standard curves generated for each run that were cross referenced to a statistically generated curve. Previous experiments have successfully used the statistical model (designed by Corran, .P. London School of Hygiene and Tropical medicine) to estimate malaria transmission from serological data. Assays against CSP antigen were limited by the high sample dilution (1:200) and only 40 samples had sufficient volumes and were high responders.

The ethnic groups were initially analysed individually then grouped into two categories of Fulani and non-Fulani this enabled the comparison of responses in each individual ethnic group and between the two categories. Grouping was done due to the small sample size (40) in the Rimaibé ethnic group.

Titres were log transformed and age categorised in to five groups of roughly equal numbers for statistical analysis.

Antibody subclass responses to each antigen in the ethnic groups were then tabulated and T test used to asses any association between ethnic groups. Antibody isotypes were then evaluated and further analysis on the prominent antibody isotype, IgG1 or IgG3 responses to malaria antigens compared in each ethnic group and investigated for significance using Chi squared analysis. Linear regression analysis between antibody isotype responses, age and ethnicity were used in evaluating the association. The final regression model included the effect of ethnicity, age and initial antibody response.

Avidity of each sera sample was calculated as an avidity index (AI). This is obtained by dividing the OD with chelating agent (+GuHCL) over the OD without chelating agent (-GuHCL) represented as $AI = \frac{+GuHCL}{-GuHCL}$.

Using a cut off point defined by median responses to antigen, sample responses were grouped into low and high avidity antibodies. Percentage of responses with high AI were analysed using chi square analysis between the Fulani and non- Fulani. ANOVA was then used in the analysis of means between ethnic groups. To analyse the association of antibody titres and avidity indices logistic regression models were used. The final analysis examined the effect of ethnicity, age and antibody concentrations on the AI of antibodies using logistic regression.

Data analysis was limited by sample numbers due to low sample volumes and low responders. All statistics were performed using Stata TM (Stata Corp, Texas, USA).

3.0 Results.

3.1 IgG isotype response to malaria antigens. A total of 301 samples were assayed and had varied IgG isotype antibody concentrations to malaria antigens. The distribution in ethnicity of the 301 samples consisted of 125 Fulani, 46 Rimaibé and 130 Mossi highlighting higher responses in the Fulani.

Data was log transformed as original data did not form a normal distribution.

Consistent with previous studies,^{97,98} low IgG2 and IgG4 antibody levels were detected (Appendix 5.2) across all three ethnic groups. Therefore further analysis was restricted to Total IgG, IgG1 and IgG3. Only samples with detected IgG antibodies were included in the calculations for the median antibody concentration ($\mu\text{g/ml}$) of each IgG isotype against malaria antigen (*Table 1*).

AMA-1 was found to be the most immunogenic antigen with high IgG1 and IgG3 prevalence (268/301 responders) and high median titres ($696.8\mu\text{g/ml}$. *Table 1*). MSP-2 and MSP1₁₉ showed intermediate levels of immunogenicity while CSP showed low immunogenic

responses with low titres (64.2µg/ml IgG3). Conversely, only 40 samples were assayed for CSP with an equal distribution between ethnic groups, Fulani 14, Mossi14 and Rimaibé 12.

Box plot representation of the data (*figures 1 to 4*) highlights antibody subclass responses to each antigen in each ethnic group. The Fulani overall had higher median concentrations of antibody specific (*table 2*) to all antigens compared to the Mossi and Rimaibé. Median antibody concentrations were significantly higher among the Fulani than the other ethnic groups in response to AMA-1, MSP1₁₉ and MSP-2 for both IgG1 and IgG3 isotypes (*figures 1 to 4*).

Antibody	AMA-1		MSP1-19		MSP-2		CSP	
	No. of Obs	Median (µg/ml) (IQ)	No. of Obs	Median (µg/ml) (IQ)	No. of Obs	Median (µg/ml) (IQ)	No. of Obs	Median (µg/ml) (IQ)
IgG1	268/301	696.8 (284.9 -1262.6)	217/301	242.9 (103.8 -491.3)	217/301	298.9 (69.7-656.6)	40/40	132.7 (85.5-173.4)
IgG3	143/301	144.6 (45.9 -334.8)	108/301	123.5 (15.8-299.2)	261/301	545.4 (283.4-875.0)	38/40	64.2 (30.6-148.8)
Total IgG	267/301	2175.0 (657.0 -3161.2)	280/301	861 (0.13-1634)	274/301	1365.0 (859.7-1989.1)	40/40	457.7 (343.5-486.3)

Table 1: Summary of the IgG isotype antibody responses to each malaria antigen. Median (µg/ml). No. of Obs represents number of observations to each antibody isotype. Included in the median concentration calculation are only samples with IgG isotypes against malaria antigens.

The Fulani antibody isotypes responses compared to the Non-Fulani were significantly higher ($p < 0.0005$ *table 2*) in IgG1 isotype responses to AMA-1 and MSP1₁₉ antigen and in IgG3 isotype responses to MSP1₁₉ and MSP-2. Other isotype antibody responses were not found to be significantly different between ethnic groups although the Fulani antibody isotype responses to each antigen were consistently higher than non-Fulani.

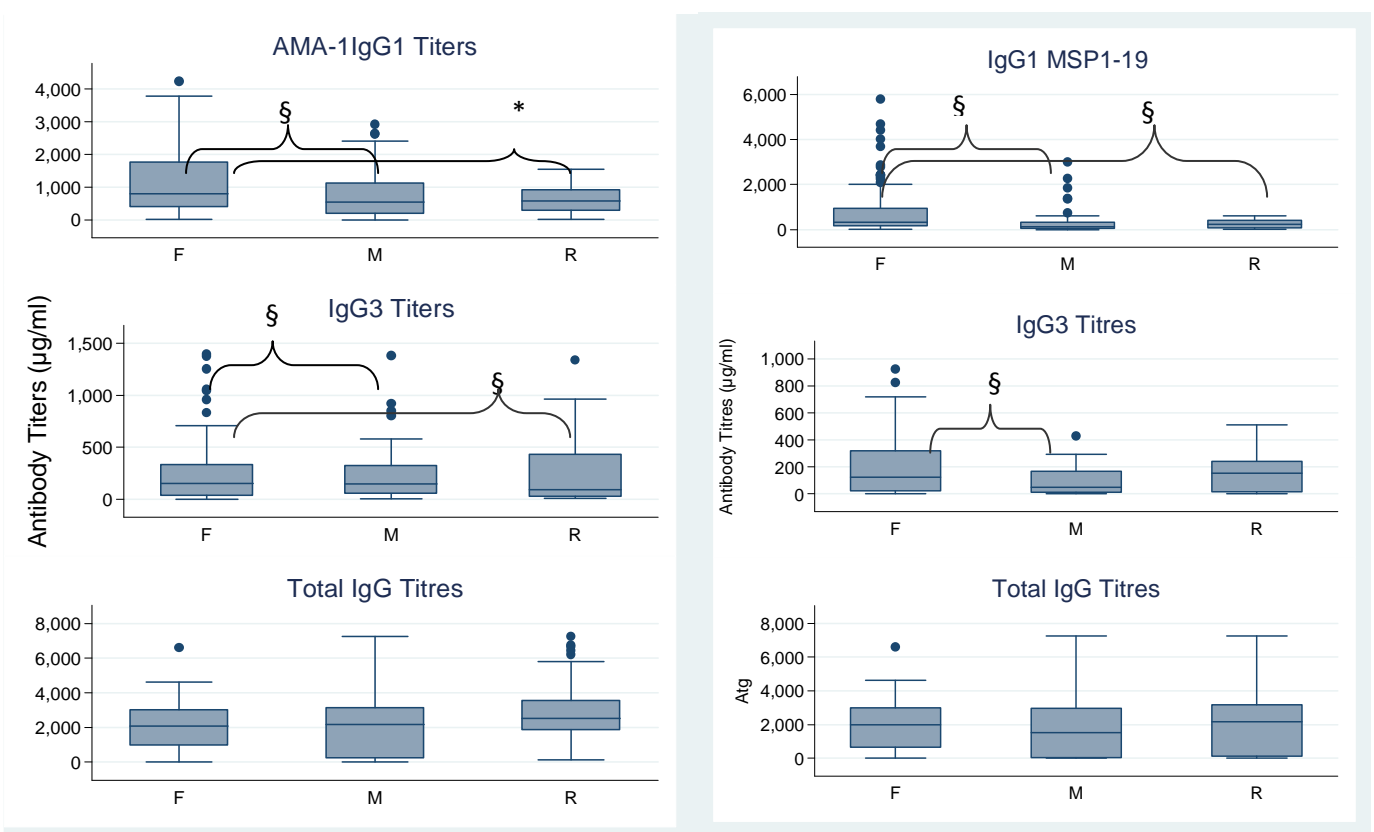


Figure 1 and 2: Antibody subclass responses recognising AMA-1 and MSP1₁₉ antigen in each ethnic group. F- Fulani, M-Mossi and R- Rimaibé. Significant differences in antibody titres observed between the Fulani, Mossi and Rimaibé. *= P<0.05 and § = P<0.0005.

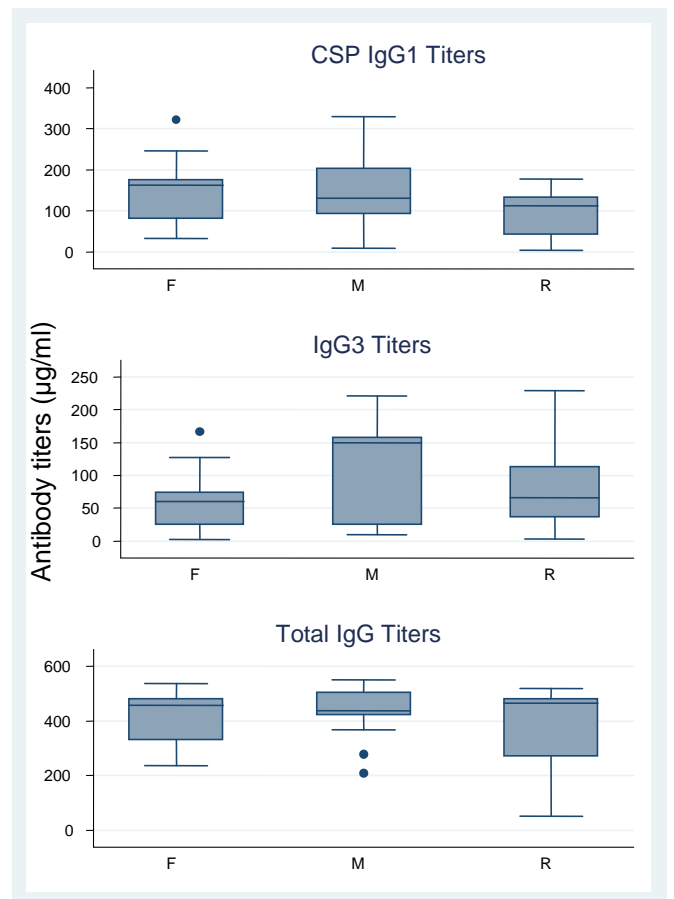
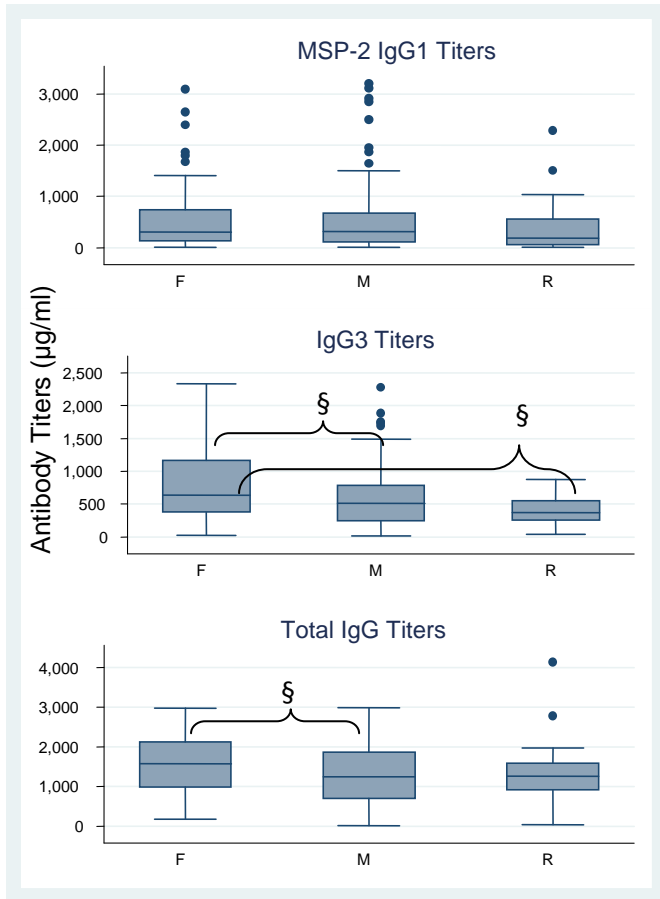


Table 2 and 3: Antibody isotype responses recognising MSP-2 and CSP in each ethnic group.

F-Fulani, M-Mossi and R- Rimaibé. Significant differences observed in antibody titres

between the Fulani Mossi and Rimaibé ethnic groups *= P<0.05 and § = P<0.0005.

Antibody subclass	Ethnicity	Number of responders	Mean (µg/ml) (Confidence interval)	Risk Ratio (RR)	P value (Likelihood ratio)	
AMA-1	IgG1	Fulani	125	6.6 (6.4-6.7)	-0.61 (-0.91 to -0.32)	0.0001 [§]
		Non-Fulani	143	5.9 (5.7-6.2)		
	IgG3	Fulani	77	4.7 (4.4-5.1)	0.04 (-0.44 to 0.51)	0.88
		Non-Fulani	66	4.7 (4.4-5.1)		
	Total IgG1	Fulani	124	6.5 (6.0-6.9)	0.25 (-0.35 to 0.84)	0.41
		Non-Fulani	143	6.7 (6.3-7.1)		
MSP1 ₁₉	IgG1	Fulani	117	5.8 (5.6-6.1)	-1.08 (-1.45 to -0.69)	<0.0001 [§]
		Non-Fulani	100	4.8 (4.5-5.1)		
	IgG3	Fulani	62	4.9(4.3-5.5)	-1.35 (-2.21 to -0.48)	0.002 [§]
		Non-Fulani	46	3.6 (2.9-4.2)		
	Total IgG1	Fulani	110	6.6 (6.1-7.0)	-0.16 (-0.65 to 0.33)	0.52
		Non-Fulani	104	6.4 (6.2-6.6)		
MSP-2	IgG1	Fulani	119	5.4 (5.2-5.7)	-0.08 (-0.46 to 0.30)	0.68
		Non-Fulani	125	5.4(5.1-5.6)		
	IgG3	Fulani	120	6.3 (6.1-6.5)	-0.45 (-0.68 to -0.21)	0.0003 [§]
		Non-Fulani	141	5.9 (5.7-6.1)		
	Total IgG1	Fulani	124	7.2 (7.1-7.3)	-0.41 (-0.63 to -0.19)	0.0003 [§]
		Non-Fulani	150	6.8 (6.6-6.9)		
CSP	IgG1	Fulani	14	4.8 (4.5-5.2)	-0.37 (-0.98 to 0.23)	0.22
		Non-Fulani	26	4.5 (4.1-4.9)		
	IgG3	Fulani	13	3.7 (3.1-4.4)	0.38 (-0.38 to 1.14)	0.32
		Non-Fulani	25	4.1 (3.6-4.6)		
	Total IgG1	Fulani	14	6 (5.8-6.1)	-0.10 (-0.38 to 0.19)	0.49
		Non-Fulani	26	5.9 (5.7-6.1)		

Table 2: Comparison of the antigen specific IgG isotype concentrations between Fulani and non-Fulani showing the non-Fulani have approximately 0.6 chance of being positive for IgG1 AMA-1 isotype antibodies. Mean concentration is in µg/ml and the confidence intervals are in brackets. * = P<0.05 and § = P<0.0005.

3.2 IgG1 and IgG3 isotype responses in ethnic groups. Previous studies have shown that IgG1 and IgG3 antibodies are associated with protection from clinical malaria either on

their own or in association with monocytes^{53, 88}. In this study antibody isotype responses to IgG1 and IgG3 were assayed in all 301 samples. The ratio of IgG1 to IgG3 responses for each antigen was derived in each ethnic group (Table 3). Isotype ratios were determined in each ethnic group to examine if isotype polarisation varied in individual groups. The Fulani were found to have more IgG1 than IgG3 antibody responses for AMA-1, MSP1₁₉ and CSP than the Non-Fulani. AMA-1 had the most number of responders with Fulani having 122 individuals with higher IgG1 than IgG3 responses and 8 individuals with higher IgG3 than IgG1 responses (Table 3). The ratio of IgG1 to IgG3 was detected to be higher in response to all antigens in the Fulani with an exception to MSP-2. Despite a higher IgG1 polarisation and high IgG1:IgG3 ratio in the Fulani than non-Fulani, a comparison between ethnic groups was of borderline significance. IgG3 was detected at a ratio higher than IgG1 in response to MSP-2 with Fulani having 91/301 individuals polarised to an IgG3 response in comparison to 32/301 individuals polarised to an IgG1 response (Table 3)

	Fulani	Mossi	Rimaibé	P Value
AMA-1 IgG1>IgG3	98% (125)	93% (111)	89 (35)	0.07
MSP1 ₁₉ IgG1>IgG3	81% (120)	75% (80)	79% (33)	0.62
MSP-2 IgG1>IgG3	26% (123)	40% (111)	37% (35)	0.07
CSP IgG1>IgG3	86% (13)	57% (14)	67% (12)	0.25

Table 3: IgG1:IgG3 ratio responses to each malaria antigen observed in each ethnic group.

Comparison between individual ethnic groups showed an IgG1 polarisation in all ethnic groups.

3.3 IgG isotype responses to malaria antigens in relation to age. Acquisition of antibodies that provide immunity to malaria has been associated with the increase in age⁹⁹ and with increased exposure to a varied repertoire of the malaria antigen^{100, 101} that occurs over many years.

Using linear regression analysis to investigate the relationship between age and isotype subclass responses, increase in age was significantly associated with an increase in antibody isotype titres for AMA-1 IgG1 (RR 0.009, CI -0.017 to -0.001, $P < 0.02$), AMA-1 IgG3 (RR 0.01, CI 0.003-0.027, $P < 0.0124$). Highly significantly associated with MSP-2 IgG1 (RR -0.02, CI -0.02 to -0.01, $P < 0.0001$) and significantly associated with CSP IgG1 and IgG3 (RR 0.01, CI 0.01-0.04, $P < 0.0387$ and RR 0.02, CI 0.001-0.04, $P < 0.03$ respectively).

3.4 IgG isotype responses to malaria antigens in relation to ethnicity. Univariate regression analysis of the Antibody titres evaluated in the Fulani compared to the non-Fulani showed a decreased association in;

- AMA-1 IgG1 (RR -1.07, CI -0.9 to -0.31, $P < 0.0001$)
- MSP1₁₉ IgG1 subclass responses (RR -1.07, CI -1.46 to -0.69, $P < 0.0001$), IgG3 isotype responses (RR 1.34, CI -2.21 to -0.47, $P < 0.002$) and in
- MSP-2 IgG3 responses (RR -0.44, CI -0.68 to -0.20, $P < 0.0003$) with change in ethnic group from Fulani to Non- Fulani.

3.5 Antibody isotype responses in relation to ethnicity and age. Data generated thus far showed the Fulani ethnic group generally had higher antibody titres and more responders in comparison to the Mossi and Rimaibé. These results confirm findings observed in other studies in the same area⁷⁶ and in Mali¹⁰² with IgG1, IgG3 and IgM subclass antibody responses found to be significantly higher in the Fulani both in Burkina Faso and Mali. To discern the effect age had on the amount of antibody generated in each ethnic group, logistic regression analyses were used. Ethnicity and age were found not to have a confounding effect in the different ethnic groups on the generation of antibody titres (Table 4). Ethnicity had a negative effect on the IgG isotype concentration, with a shift from the Fulani to the

non-Fulani resulting in decreased IgG1 isotype antibody concentrations to AMA-1 , MSP 1₁₉ and MSP-2. Age was found to not have a significant effect. This may be attributed to the sample selection, limiting the ages sampled to ones that already had saturated antibody isotype responses to malaria antigens.

	AMA-1				MSP 1- 19			
	IgG1 RR (95% CI)	P value	IgG3 RR (95% CI)	P value	IgG1 RR (95% CI)	P value	IgG3 RR (95% CI)	P value
Ethnicity	-0.58 (-0.88 to -0.28)	<0.0001	-0.15 (-0.64 to 0.33)	0.54	-1.05 (-1.45 to -.67)	<0.0001	-1.64 (--2.54 to - 0.74)	<0.0001
Age (Years)	-0.01 (-0.02 to 0.00)	0.062	0.01 (0.00 to 0.03)	0.01	-0.004 (-0.14 to 0.01)	0.43	0.024 (0.00 to0.04)	0.03

	MSP-2				CSP			
	IgG1 RR (95% CI)	P value	IgG3 RR (95% CI)	P value	IgG1 RR (95% CI)	P value	IgG3 RR(95% CI)	P value
Ethnicity	0.014 (-0.36 to 0.38)	0.94	-0.42 (-0.67to -0.18)	0.001	-0.50 (-1.08 to 0.07)	0.09	0.21 (-0.54 to 0.96)	0.57
Age (Years)	-0.02 (-0.03 to -0.01)	<0.0001	-0.004 (-0.1 to 0.00)	0.18	0.02 (0.00 to 0.03)	0.02	0.02 (-0.00 to 0.04)	0.05

Table 4: Association of ethnicity (Fulani Vs Non-Fulani) and age (years) to antibody isotype responses. Ethnicity had an effect on the antibody isotype concentrations while age did not seem to have an effect. .

The results were not affected by analysis of ethnicity in individual ethnic groups of Fulani , Mossi and Rimaibé or in the two categories of Fulani and non-Fulani.

3.6 Avidity indices of antibody isotypes in each ethnic group. Having identified higher antibody titres in the Fulani, I examined the affinity of the isotype antibodies by measuring the avidity. Avidity index was calculated for each isotype response to each antigen. All avidity index data was not logged.

Antibody avidity responses were divided into high and low responders. The cut off point was generated based on the median responses to each antigen (*table 5*).To determine if the avidity

of isotype antibodies varied between ethnic groups, Wilcoxon rank sum test was used to compare the responses in the Fulani and non-Fulani. IgG3 AMA-1 antibodies were significantly ($P < 0.0023$) more avid in the Fulani than the non-Fulani and showed higher concentrations of IgG1.

Furthermore, the Fulani had more avid antibodies towards MSP1₁₉ and MSP-2 although data was not found to be significant, this could be attributed to the limited responses from a small sample size.

Antigen		Fulani	Non-Fulani		P value
AMA-1	IgG1(n)	65% (57)	37% (67)	9.38	0.002
	Mean AI (95% CI)	0.26 (0.22- 0.32)	0.19 (0.15- 0.23)	5.58	0.02
	IgG3 (n)	67% (6)	0% (2)	2.67	0.10
	Mean AI (95% CI)	0.11 (0.04 - 0.18)	0.05 (-0.53 to 0.63)	1.32	0.3
	Total IgG (n)	54% (74)	43% (54)	2.1	0.15
	Mean AI (95% CI)	0.27 (0.22 - 0.31)	0.22 (0.19- 0.27)	1.4	0.24
MSP119	IgG1(n)	48% (25)	54%(13)	0.12	0.73
	Mean AI (95% CI)	0.51 (0.42 - 0.63)	0.58 (0.47 - 0.68)	0.53	0.47
	IgG3 (n)	50% (2)	50% (2)	-	-
	Mean AI (95% CI)	-	-	-	-
	Total IgG (n)	51% (50)	45%(31)	1.0	0.66
	Mean AI (95% CI)	0.44 (.037 - 0.51)	0.42 (0.34 - 0.50)	0.15	0.70
CSP	IgG1(n)	100% (2)	33% (3)	2.22	0.14
	Mean AI (95% CI)	0.14 (-0.01 to 0.28)	0.08 (-0.02 to 0.19)	2.9	0.19
	IgG3 (n)	33% (3)	33% (3)	-	-
	Mean AI (95% CI)	-	-	-	-
	Total IgG (n)	50% (4)	50% (10)	0.00	1.00
	Mean AI (95% CI)	0.14 (0.07 - 0.21)	0.15 (0.10 - 0.2)	0.05	0.82

Table 5: Summary of the percentage of High AI isotype responses and the mean AI against malaria antigens in each ethnic group. N represents the number of individuals with a high isotype AI. Chi squared analysis was used to evaluate the proportional difference in the number of responders in the Fulani and non-Fulani while ANOVA was used to evaluate the mean comparison.

3.7 Effect of antibody titres on the avidity index of antibody.

Having established that the IgG1 and IgG3 isotype responses to malaria antigens were higher among the Fulani than the non-Fulani, I examined whether the higher responses in the Fulani were associated with higher affinities. Lack of a positive association would suggest that the

higher antibody concentrations among the Fulani are due to more antibodies rather than improved binding strength of antibodies.

linear regression was used to evaluate the influence antibody titres had on the avidity of antibodies, applied. The results showed that AMA-1 IgG1 antibody titres had a significant effect on the AI of antibodies with an Odds ratio of 1.78 (1.01-3.14) and P value <0.041. This suggests that the higher avidity indices in the Fulani are due to higher binding of antibody isotypes and not higher antibody isotype concentrations.

Other antibody titre responses to malaria antigen showed a positive association between high antibody titres and high AI however, these was not found to be significant.

3.8 Association between antibody titres, age and avidity of antibody isotypes. A

recent study has found a significant positive association between the magnitude of antibody responses and AI (Drakeley C. Manuscript in preparation). To discern the effect antibody titres, ethnicity and age had on the development of avid antibodies, data was analysed with logistic regression. Complete analysis on each isotypes were not feasible in CSP and MSP1₁₉ antigen due to a low number of responders (*table 5*).

The odds of a high AI was found to be 66% (RR 1.6 P <0.0001 CI 0.15 - 0.77) in the Fulani relative to the non-Fulani for IgG1 AMA-1 adjusted for age and antibody titres without chelating agent.

Whereas the odds of high AI (P <0.005 CI 0.29 - 1.3) for AMA-1 IgG3 in the Fulani relative to the non – Fulani adjusted for age and antibody titres without chelating agent was found to be 40% (*table 6*).

		OR (95% CI)			P value
		AI (-GuHCL)	Ethnicity	Age (yrs)	
AMA-1	IgG1	19.4 (4.1- 92)	0.34 (0.2 - 0.8)	1.01 (1 – 1.0)	<0.0001
	IgG3	420 (1.1 ⁻⁷ - 1.7 ⁺¹²)	—	1.4 (0.4 – 5.6)	0.27
	Total IgG	6.3 (1.8- 22)	0.6 (0.3-1.3)	1.0 (1.0-1.0)	0.006
MSP1₁₉					
MSP1₁₉	IgG1	8.4 (0.4 - 166)	1.3 (0.3 - 5.3)	1.0 (0.9-1)	0.4
	IgG3	—	—	—	—
	Total IgG	3.1 (0.5-20)	0.8 (0.3-2)	1.0 (1.00-1.02)	0.6
CSP					
CSP	IgG1	—	—	—	—
	IgG3	—	—	—	—
	Total IgG	7.1	1	1	0.9

Table 6: Effect of antibody isotype concentrations without chelating agent (-GuHCL), age and ethnicity on the AI. Results were not generated for CSP and MSP1₁₉ due to a small sample size.

4.0 Discussion

The influence of ethnicity on immune status in three sympatric ethnic groups naturally exposed to *P falciparum* malaria was examined in this study. The study found high antibody responses to malaria antigens to be higher among the Fulani than in the Mossi and Rimaibé. These results are in agreement with results from various other studies in the same malaria endemic region that found antibody responses higher in the Fulani than other ethnic groups^{73, 76, 77}.

4.1 Antibody isotype responses in three sympatric ethnic groups.

The magnitudes of antibody responses and their isotypes varied between antigens. IgG1 and IgG3 antibody isotypes were readily detected in response to all malaria antigens while IgG2 and IgG4 was low and almost undetectable. This was observed regardless of ethnicity and has previously been reported in sera from other malaria endemic regions⁹⁸.

The ratio of isotypes showed a polarisation towards IgG1 than IgG3 with dominant IgG1 detected towards MSP1₁₉, CSP and AMA-1 while high IgG3 antibodies responses were observed towards MSP-2. This confirmed previous experiments that found naturally acquired human antibody responses to MSP-2 to be predominantly of the IgG3 subclass and associated with less clinical disease^{97, 103, 104}.

IgG isotype responses were most immunogenic in response to AMA-1 antigen consistent with previous studies⁹⁵.

The study found a marked association between age and antibody isotype titres, with an increase in antibody as age in years increased. Hence the two variables age and ethnicity influence levels of antibody responses independent of each other. However it is difficult to disentangle the effects from levels of exposure. Earlier studies showed that with an increase

in age and exposure, the acquisition of a wider array of antibodies to the malaria antigen contributes to protection¹⁰¹. However, antibodies have been shown to vary with the type of malaria antigen and level of exposure¹⁰⁵ evident in our results showing high AMA-1 and MSP-2 antibody responses, with specific polarisation of isotypes to different antigens similar to earlier studies that showed IgG1 isotype polarisation in MSP1₁₉^{106, 107}. protection against malaria is often a contribution of many factors including age, level of transmission and genetic factors amongst many others thus the higher antibody titres and affinity in the Fulani would require further investigation.

4.2 Avidity of antibodies generated from natural exposure in three sympatric

ethnic groups. Studies on immunological responses to malaria antigens have shown diverse factors contribute to the acquisition, antibody isotype switching and preservation of antibodies against malaria antigens¹⁰⁸.

Affinity in immunology is used to describe the strength of a single bond between antigen-antibody complexes while avidity is the overall strength between many binding sites such as IgM.

In this study avidity measured as a gauge of antibody affinity found high AI in the Fulani among the three sympatric groups.

Highly avid antibodies were detected in the Fulani irrespective of age and starting concentration of antibody titres. However avid antibody detection was limited by small sample size and low responses. This could be explained by several factors found in previous studies that show that

- naturally acquired antibodies to *P Falciparum* can decrease to low levels more rapidly than to other infections or immunization¹⁰⁹.

- perhaps the large quantities and variation of the malaria antigen interfere with avidity maturation of antibodies¹¹⁰ shown in human and rodent¹¹¹ studies.
- And also long-lived plasma cells occupy limited niches in the liver and spleen competing for survival, contributing to the lower populations of long lived cells¹¹².

These further highlights the challenge in vaccine designed to stimulate avid long-lived antibodies.

4.3 Strengths and weaknesses of the study.

This study confirms and extends previous findings regarding humoral responses in three sympatric ethnic groups in Burkina Faso. It highlights total IgG , specific IgG isotype responses with corresponding data on the avidity of the antibodies from natural exposure to malaria. It examines these responses in the pre and erythrocytic stages of malaria infection in all three sympatric groups. These observations are analysed in all ages generating results associated with ethnicity and age.

However, the demonstration of disparities between the Fulani, Mossi and Rimaibé is limited by the bias in sample selection, sample volumes and size thus responses to some antigen were not observed and the associations in the study was restricted.

4.4 Conclusion

In conclusion the study demonstrated that the Fulani mount higher IgG isotype responses than the Mossi and Rimaibé who are ethnically distinct although living in sympatry. These responses are predominantly of IgG1 and IgG3 isotypes and highly avid in nature. The elevated avid antibody responses suggest a protective role against malaria in the population.

4.5 Prospective work.

Although the result illustrate a distinction in the titre and avidity of antibodies between the three sympatric ethnic groups, a replicate study with a larger sample size of proportional distribution between ethnic groups would provide more evidence into the degree of variability in immune responses.

Further analysis in the longitudinal cohort (August to March 1995) on individuals with high AI responses would provide more information into the duration and avid nature of antibodies in association to ethnicity and OD.

Also further immuno-serological investigation using micro arrays and other *P falciparum* antigens such as P Falciparum erythrocyte membrane protein 1 (pfemp1) involving the parasitological and clinical data in the Burkina Faso longitudinal cohort may highlight a full range of antibody isotype responses in the three sympatric ethnic groups and their association with protection.

APPENDICES

5.1 Sample selection and initial assays.

The August 1994 Burkina Faso data set used for this study consisted of 526 samples found to have IgG antibodies against MSP-2, MSP1₁₉ and AMA-1. These consisted of 204 Mossi, 175 Rimaibé and 146 Fulani.

190 Samples positive for all three antigens were selected for this study.

140 Samples not positive but had shown high cytokine activity in a previous study (Verra, F ongoing work) were added to the data set for further analysis.

Previous experiments had found 99 samples (Fulani 43, Mossi 33 and Rimaibé 23) positive for total IgG against CSP antigen. These were further tested for antibody subclasses.

Each antibody subclass was optimised against monoclonal conjugated antibody before any ELISA assays were commenced.

All serum dilutions were prepared from frozen aliquots. Only sufficient amounts to run assays were diluted to minimise on result variation and maximise on serum quality.

To obtain the avidity indices of samples, 743 AI were generated from the samples selected these number are higher than the ELISA samples run because each sample that was positive according to the cut off was assayed against 3 antibody subclasses (Total IgG, IgG1 and IgG3) and against three antigens (MSP1₁₉, AMA-1 and CSP).

5.2 IgG2 and IgG4 antibody responses.

Initial experiments using 99 samples detected low IgG2 and IgG4 levels (range 0-526µg/ml) across all ethnic groups towards all malaria antigens. Thus to minimise on sample volumes no further assays to detect IgG2 and IgG4 were done.

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