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Parasite and Host Factors That Drive Heterogeneity in Human Malaria

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Declaration

I declare that, except for where noted, all work contained in this thesis was performed and composed by myself. Where others have contributed to elements of the work, this is clearly acknowledged in the text. No material presented in this thesis has been submitted to any other university or for any other degree or professional qualification. Parts of this work has been published in the *Malaria Journal* (2016) and a copy is included in the appendix.

The blood samples used for the experimental work considered in this thesis were obtained from immuno-epidemiological studies in Zimbabwe as part of a larger project investigating malaria and schistosomiasis coinfection involving collaboration between groups from the University of Zimbabwe, the National Institute of Health Research in Zimbabwe (formerly the Blair Research Institute) and the University of Edinburgh, as well as an eleven year malaria epidemiological study in Daraweesh, eastern Sudan. I was not involved in the blood sample collections from either study sites.

Laboratory-based work, including the serological work was conducted by myself. Blood collection and processing of parasite cultures for the data in Chapter 6 was done by me. I have undertaken all the analysis (except where specified) presented in this thesis.

Seth Appiah Amanfo
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Abstract

Malaria affects over half of the world's population and causes half a million deaths annually, especially in Sub-Saharan Africa. Four species of the apicomplexan *Plasmodium* parasite (*P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*) are responsible for malaria in Africa. Both parasite and host factors contribute to heterogeneity in the risk of developing malaria, clinical manifestation of the disease as well as the number of treatments required to clear parasites. The epidemiology of the different species, and the role of exposure to mixed-species *Plasmodium* co-infections in generating heterogeneity remains poorly studied. Being an obligate intracellular parasite the blood-stage life cycle of the *Plasmodium* parasite takes place in the erythrocytes of the human host. The surfaces of these erythrocytes are the medically important ABO blood group antigens that have been reported to influence the susceptibility or otherwise of an individual developing severe malaria. In this thesis I have considered the contributions of the species of *Plasmodium* parasites and the ABO blood group of the host in driving heterogeneity in human malaria.

The aims of this thesis were to determine:

(i) the seroepidemiology of the different *Plasmodium* species in two mesoendemic African populations (Zimbabwe and Sudan);

(ii) to determine if heterogeneity in clinical presentations of malaria (history of fever, body temperature and parasitaemia) and response to drug treatment is related to exposure to single *vs*. mixed-*Plasmodium* species infection;

(iii) the spatial and temporal dynamics of malaria prevalence and *Plasmodium* species distribution in a mesoendemic village in eastern Sudan;

(iv) gene expression changes in 3D7 *P. falciparum* parasites as they infect erythrocytes of different ABO blood group donors.

For aims (i to iii) I developed an enzyme-linked immunosorbent assay using antigens derived from *Plasmodium* merozoite surface protein 1, also known as MSP-1_{19}, to detect IgG antibodies to all four malaria parasite species in Zimbabwean and Sudanese

populations. In the Zimbabwean study, plasma samples from 100 individuals each (aged 5-18 years) from three villages (Burma Valley, Mutoko and Chiredzi) were screened for exposure to *Plasmodium* parasites. In Daraweesh, Sudan, plasma samples from 333 individuals (aged 1-74 years) who had experienced a first malaria episode between 1990 and 2000 were recruited into the study. For study aim (iv) I cultured a single clone of 3D7 *P. falciparum* parasite using erythrocytes of individuals of different ABO blood group types, harvested parasite RNA and sequenced it to determine gene expression changes in the different hosts.

I showed that human IgG antibodies to MSP-1_{19} antigens of the four *Plasmodium* species are species-specific and do not cross-react. In both study populations almost all antibody responses involved *P. falciparum*, and single-species responses were almost exclusively directed against *P. falciparum* antigens. Mixed-species responses accounted for more than a third of responses, and were associated with chloroquine treatment failure, with significantly high proportion of individuals with mixed-species infections requiring repeated treatment with chloroquine/sulfadoxine-pyrimethamine for parasite clearance. This finding highlights the need for a sensitive method for detecting mixed-species malaria infections to enable the assessment of the true prevalence and magnitude of the disease burden caused by the non-*falciparum* species in endemic populations. Drug treatment failures associated with mixed species infections have significant impact on malaria morbidity and mortality. Treatment failure or partial parasite clearance has the potential to allow dormant liver stages of *P. vivax* and *P. ovale* to become a source of parasite reservoir for onward transmission. Furthermore, untreated low-grade chronic infections caused by *P. malariae* have been reported to cause systemic diseases many years after the primary infection. Spatial analysis of malaria epidemiology showed that malaria parasite transmission in Daraweesh was focal, and that infections are not randomly distributed in the village. Two space-time clusters of significantly increased malaria risk were identified (1993-1999, and 1998-1999) with marked variations between households, but little or no variation in the species of *Plasmodium* over time. Similarly, multiple significant clusters were identified for the parasite species; three for *P. falciparum*, two for *P. vivax* and *P. malariae*, and one for *P. ovale*. These clusters had overlapping time

frames, with some of the species significantly infecting the same households. This suggests that even in a small geographic area malaria transmission shows heterogeneity, and that such data can provide useful information to guide malaria control efforts. Finally, I demonstrated that 3D7 *P. falciparum* parasite growth was similar in the erythrocytes of different blood group donors, and provide preliminary data to show that the non-coding RNA gene, PF3D7_1370800, is differentially expressed in blood group A donors relative to blood groups B and O donors. Further research is needed to better understand the role of this gene in malaria pathology.

All together, these findings will aid malaria researchers and other stakeholders in making informed choices about tools for diagnosing *Plasmodium* species, and control programmes targeting eradication of malaria caused by all *Plasmodium* species, as is the case of incorporating these findings into current malaria research in Sudan.

Lay summary

This thesis focussed on malaria, a parasitic disease caused by *Plasmodium* parasites. The disease affects over half of the world's population, most especially in Sub-Saharan Africa. Four species of *Plasmodium* (*P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*) are responsible for malaria in Africa. Individuals can be exposed to or infected with more than one species of the *Plasmodium* parasite (defined as mixed-species infections) either at the same time or sequentially. However, in clinical diagnosis, mixed-species infections are often not recognised by microscopy, which is the most widely used laboratory method for diagnosing the disease. In view of this the prevalence and magnitude of disease burden caused by the non-*falciparum* species in endemic population are not well documented. The *Plasmodium* parasite is an obligate intracellular parasite, meaning it must continuously live in a living cell. Its blood-stage life cycle in humans takes place within the red blood cells. On the surfaces of these red blood cells are the ABO blood group antigens that have been associated with an individual's likelihood of being susceptible or resistant to developing severe malaria. However, there has been no study on the gene expression changes when a single clone of *Plasmodium* parasite infects individuals of different blood groups.

In this thesis, I assessed ways of improving the diagnosis of the species of malaria parasites using proteins derived from the surface of the *Plasmodium* parasite called merozoite surface proteins (also known as MSP-1_{19}). I further investigated the contribution of these species to the clinical and treatment outcomes, and determined the spatial and temporal dynamics of malaria. Finally, I determined gene expression changes in *P. falciparum* parasites as they infect red blood cells of different ABO blood groups. The blood samples used for the laboratory work in this thesis were obtained from studies carried out in three villages in Zimbabwe (Burma Valley, Mutoko and Chiredzi), Daraweesh in eastern Sudan, and Edinburgh in the UK.

I found that the MSP-1_{19} antigens are very sensitive and capable of detecting the specific type of *Plasmodium* species an individual has recently or previously been exposed to. Although most people were usually infected with or exposed to *P. falciparum*, more than a third of the population studied always carried mixed-species

infections involving *P. falciparum* and one or more of the other species. Majority of these people with mixed-species infections failed to clear the parasites with the standard single course of chloroquine treatment and required repeated treatment with either chloroquine and/or sulfadoxine-pyrimethamine. Analysis of the space-time dynamics of malaria in the small village of Daraweesh in Sudan showed that malaria parasite transmission was very focal and localised in certain parts of the village at certain transmission seasons. I identified two significantly increased clusters of malaria risk (1993-1999, and 1998-1999) which varied very much between households. Similarly, multiple significant clusters were identified for the four parasite species; three for *P. falciparum*, two for *P. vivax* and *P. malariae*, and one for *P. ovale*. These clusters had overlapping time frames, with some of the species significantly infecting the same households. Parasite growth in the red blood cells of different ABO blood group donors was similar. The non-coding RNA gene, PF3D7_1370800, for which not much is currently known was found to be expressed more in blood group A donors relative to blood groups B and O donors.

This thesis highlights the need to explore MSP-1_{19} antigens as a diagnostic tool for malaria diagnosis. This will enable a better assessment of the prevalence and magnitude of the disease burden caused by all *Plasmodium* species in malaria endemic population. Drug treatment failure associated with mixed-species infections has major implications on morbidity and mortality of disease. There is therefore the need for healthcare providers to identify the specific type of *Plasmodium* parasite(s) an individual is infected with so as to administer appropriate medication, and monitor treatment to prevent the development of drug resistant parasites. The findings of the space-time analysis suggests that even in a small geographic area malaria parasite transmission shows heterogeneity, and that such data can provide useful information to guide malaria control efforts. The findings presented in this thesis will aid malaria researchers and other stakeholders in making informed choices about intervention tools for control programmes targeting eradication of malaria caused by all *Plasmodium* species. These findings are currently being incorporated into future malaria research in Sudan.

Acknowledgements

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I would also like to thank my family, my wife Josephine and children, Ohenewaa, Andrew and Benjamin. There were times I had to be home to help with childcare but had to stay longer in the laboratory in order to be able to get this piece of work together, but you were understanding. To all my fellow Ghanaian and African colleagues at the University of Edinburgh thank you for your support and friendship.

Finally, all thanksgiving and praise be to God for how far He has brought me.

Abbreviations

^{o}C	Degree Celsius
ACD	Acid citrate dextrose
ADCC	Antibody dependent cellular cytotoxicity
ACTs	Artemisinin combination therapy
AMA-1	Apical membrane protein 1
ANOVA	Analysis of variance
APS	Ammonium persulphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDRs	Complementarity determining regions
CSP	Circumsporozoite protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DW	Daraweesh
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EZERB	University of Zimbabwe's Ethics Review Board
Fab	Fragment antibody binding
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
Fc	Fragment crystallizing
FR	Framework regions
GPI	Glycosylphospatidylinositol
GST	Glutathione S transferase
H_2O_2	Hydrogen peroxide
H_2SO_4	Dihydrogen (iv) sulphite
HEPES	4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid

HIV	Human Immunodeficiency Virus
HRB	Horse radish blood
HRP-II	Histidine-rich protein II
IF	Interferon
IFA	Immunofluorescent antibody assay
Ig	Immunoglobulin
IL	Interleukin
IMC	Inner membrane complex
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton
LB	Luria broth
LLR	Log likelihood ratio
MAHRP1	Membrane-associated histidine-rich protein 1
MDA	Mass drug administration
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
ncRNA	Non-coding Ribonucleic acid
OD	Optical density
OPD	o-Phenylenediamine dihydrochloride
OR	Odds ratio
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate-buffered saline Tween
PCR	Polymerase chain reaction
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfSUB	*P. falciparum* subtilisin
pLDH	Parasite lactate dehydrogenase
RDTs	Rapid diagnostic tests
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
RR	Relative risk

SD	Standard deviation
SDS	Sodium dodecyl sulfate
SP	Sulfadoxine-pyrimethamine
TEMED	Tetramethylethylenediamine
TNF	Tumor necrosis factor
WHO	World Health Organization

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Chapter 1. General introduction

Malaria is a vector-borne disease caused by unicellular protozoan parasite of the genus *Plasmodium*. It is one of the most important public health diseases, with both economic and health implications in tropical and Sub-Saharan Africa (WHO 2015). The disease remains a major cause of death and morbidity worldwide, affecting the lives of people in over a 100 countries and is responsible for the annual worldwide death of approximately half a million people, mostly in Sub-Saharan Africa (WHO 2015, WHO 2014).

In malaria endemic areas children under the age of five years and pregnant women carry the heaviest burden of disease (Milner 2017). Early disease diagnosis, treatment and close monitoring/management are crucial in preventing the development of severe or life-threatening malaria (Bhatt *et al.* 2015). Severe malaria is a rare clinical outcome, and is mostly seen in children below the age of five years; it is caused predominantly by *Plasmodium falciparum* (Goncalves *et al.* 2014). However, the non-*falciparum* species are equally capable of causing chronic or fatal disease when they are undetected by conventional diagnostic tools, or parasitaemia caused by them becomes sub-clinical without full cure (Vinetz *et al.* 1998). For example, acute febrile episodes of *P. vivax* infections are associated with anaemia, intrauterine growth retardation (McGready *et al.* 2012), miscarriage, and severe and fatal disease (Baird 2013, Rodriguez-Morales *et al.* 2008, Tjitra *et al.* 2008, Barcus *et al.* 2007). Both parasite and host factors play key roles in the clinical outcomes and the heterogeneity that arise following infection with *Plasmodium* parasites (Vignali *et al.* 2011, Gupta *et al.* 1994). Parasite factors include the species and genotype of the *Plasmodium* causing disease (Ariey *et al.* 2001), parasite genes expressed during infection (Vignali *et al.* 2011), and polymorphism in parasite antigens on infected red cells (Smith *et al.* 2013). Host factors may include age, genetics (Driss *et al.* 2011, Luzzatto 1974), and red cell surface antigens such as the ABO blood group antigens (Cooling 2015, Gupta and Chowdhuri 1980). In this thesis I have considered the contribution of the species of *Plasmodium* parasite and the ABO blood group system to heterogeneity in clinical presentation observed in human malaria.

Malaria in Sub-Saharan Africa is caused mainly by four species of the *Plasmodium* parasite, (*P. falciparum, P. vivax, P. malariae* and *P. ovale*). In the forest regions of South-East Asia, *P. knowlesi* is increasingly being reported in human populations as causing zoonotic disease (Cox-Singh and Singh 2008, Cox-Singh *et al.* 2008). In terms of research, drug and vaccine developments, attention has been heavily devoted to *P. falciparum* because it is the species associated with increased mortality (PATH 2011, Oliveira-Ferreira *et al.* 2010). In several countries currently implementing malaria control programmes, the control strategies are mainly directed toward malaria caused by *P. falciparum* (Abeyasinghe *et al.* 2012, Oliveira-Ferreira *et al.* 2010). Further evidence demonstrating the neglect of the non-*falciparum* species is seen in worldwide malaria endemicity surveys that are only focussed on *P. falciparum* mortality owing to limited availability of data on the other species (Gething *et al.* 2016, Hay *et al.* 2009). This attention on one *Plasmodium* species poses a danger as knowledge on the non-*P. falciparum* species and their clinical and treatment profiles in endemic areas is not well understood. The health implication of focusing on one species is seen in the relative rise in the prevalence and the overall malaria burden caused by the non-*falciparum* species in areas where progress has been made at controlling *P. falciparum* malaria (Abeyasinghe *et al.* 2012, Oliveira-Ferreira *et al.* 2010).

The diagnosis of malaria has long been achieved by microscopy of blood films stained by Giemsa (Fleischer 2004, Giemsa 1904). This method is considered the 'gold standard' and is based on detailed examination of stained blood films for characteristic *Plasmodium* species blood-stage morphological features such as the size and shape of asexual blood-stage parasites, pigment granules, presence of cell inclusions, as well as the size and shape of the infected erythrocyte (Obare *et al.* 2013, Payne 1988). Although microscopy is the reference method, it has some limitations, including the need for highly experienced technicians, variability in smear quality, the inability to determine malaria species at low parasitaemia, and the loss of slide quality with time (Barber *et al.* 2013, Payne 1988). In view of this, the presence or absence of blood stage parasites is frequently reported rather than determining the species of *Plasmodium* causing malaria (Obare *et al.* 2013, Ohrt *et al.* 2002).

In malaria endemic areas, humans may be infected and harbour mixed-*Plasmodium* infections in various combinations and there are several reports of deficit of mixed infection possibly owing to undiagnosed cases or underestimation of mixed-infections (Mayxay *et al.* 2004, Bruce and Day 2002, Bruce *et al.* 2000). Mixed-species infections are therefore far more common than is generally reported (Mayxay *et al.* 2004, Mehlotra *et al.* 2000). *P. malariae* and *P. ovale* infections have been underestimated or under researched, and morbidity and mortality associated with these species, either as mono- or in mixed-infection are still poorly defined and quantified (Collins and Jeffery 2007, Collins and Jeffery 2005). In recent years, however, there has been a greater focus on malaria caused by *P. malariae* and *P. ovale*, with a view to understanding their long-term contribution to disease and pathology (Daniels *et al.* 2017, Sutherland 2016, Rayner 2015). These recent developments in malaria research have heightened the need for a clear knowledge of the epidemiology of these species and their associated clinical outcomes and treatment requirements.

On the surface of human erythrocytes are antigens belonging to the various blood group systems (Cooling 2015, Fung 2014, Reid 2012, Daniels 2002). Antigens of the ABO blood group system are the most important of all blood group antigens, owing to their importance in ABO compatibility and transfusion medicine (Fung 2014). The *Plasmodium* parasite's life cycle in the human host (discussed in Section 1.1.1 on page 5) takes place inside the erythrocytes (White *et al.* 2013a), the surfaces of which are found these blood group antigens. In malaria, the ABO blood group plays a role in the susceptibility or resistance of individuals to developing severe malaria (Rowe *et al.* 2009b, Rowe *et al.* 2007). This finding is further strengthened by the worldwide distribution patterns of ABO blood group types and malaria endemicity. Blood group O individuals are more prevalent in malaria endemic regions compared to higher prevalence of blood group A individuals in regions where malaria is rare (Reid 2012, Cserti and Dzik 2007). In spite of the relationship between ABO blood group antigens and malaria, gene expression changes that occur when the same parasite clone infects host erythrocytes of different blood groups have not been characterised.

This thesis is divided into two components: a seroepidemiological study (Chapters 3-5) and gene expression study (Chapter 6). In the seroepidemiology work, I have

determined human IgG antibody responses to the four *Plasmodium* species in two African populations (Zimbabwe and Sudan). I used an enzyme-linked immunosorbent assay (ELISA) of *Plasmodium* merozoite surface proteins (discussed in more detail in Section 1.7) from all four human parasite species as a diagnostic tool. Subsequently, I characterised the outcomes of some observable clinical features (fever and parasitaemia) and treatment requirement associated with malaria caused by single *P. falciparum versus* mixed-*Plasmodium* species. Furthermore, the spatio-temporal dynamics of malaria based on malaria epidemiology in Daraweesh, a village in eastern Sudan has been characterised.

For the gene expression study, I cultured a single clone of *P. falciparum* 3D7 parasite in the erythrocytes of individuals belonging to three different ABO blood groups (Groups A, B and O), harvested and sequenced parasite RNA, and determined the differential parasite gene expression changes that occurred between the different blood group types.

The findings of the seroepidemiological work highlight the higher prevalence of mixed-species *Plasmodium* infections in Africa and their associated drug treatment failures, and provide important information that could be incorporated into new malaria research and malaria elimination programmes by African governments and policy makers, as is the case in Sudan. The gene expression study provides some preliminary data that could be investigated further to better understand the role of ABO blood groups in malaria epidemiology.

1.1 *Plasmodium* species and malaria

1.1.1 The biology of *Plasmodium* parasites

Plasmodium parasites have a complex life cycle (summarised in Figure 1.1.1) that takes place in both the invertebrate definitive host, the female *Anopheles* mosquito, and the vertebrate intermediate human host. The exogenous sexual cycle, sporogony, takes place in the mosquito, while the asexual cycle, schizogony, occurs in the human host (White *et al.* 2013a). The cycle begins with the inoculation of between 10-100 motile sporozoites from the salivary duct of an infected female *Anopheles* mosquito into a susceptible host's subcutaneous tissues during blood meal by the vector (Prudencio *et al.* 2006). The most prevalent of these mosquito species in Africa are *Anopheles gambiae sensu stricto*, *An. funestus*, and *An. arabiensis* (Sinka *et al.* 2010). In the human host the *Plasmodium* paraites' life cycle occurs in the liver and the blood. The sporozoites enter the host's hepatocytes, undergo nuclear divisions, multiply and produce between 10,000 and up to 30,000 daughter merozoites in 5·5–16 days (depending on the species of *Plasmodium*) within a hepatocyte (Bruce and Day 2002, Bruce *et al.* 2000, Murphy and Oldfield 1996, Garnham 1988b). Some biological differences in the life cycle of the four human *Plasmodium* species that may affect the growth potential of the parasites in the human host are summarised in Table 1.1.1 (Bruce and Day 2002).

In *P. vivax* and *P. ovale* infections, some intrahepatic forms remain dormant as hypnozoites for between two weeks to more than a year, and these may serve as reservoir responsible for the relapses that are characteristic of infections caused by these two species (Markus 2011b, White 2011, Douglas *et al.* 2011, Krotoski *et al.* 1980). When the hepatic schizont ruptures, merozoites are liberated that invade new erythrocytes (Miller *et al.* 2002, Weatherall *et al.* 2002). On the surface of these merozoites are merozoite surface protein 1 (MSP-1) (Holder 1988, Holder *et al.* 1985, Holder and Freeman 1982) that I have used for the serological work described in this thesis (MSP-1 is discussed later in more detail in Section 1.7). These merozoites replicate, rupture the erythrocytes, and release between 8 and 32 merozoites per

erythrocyte, depending on the species (Bruce *et al.* 2000), that in turn invade new erythrocytes to propagate the cycle.

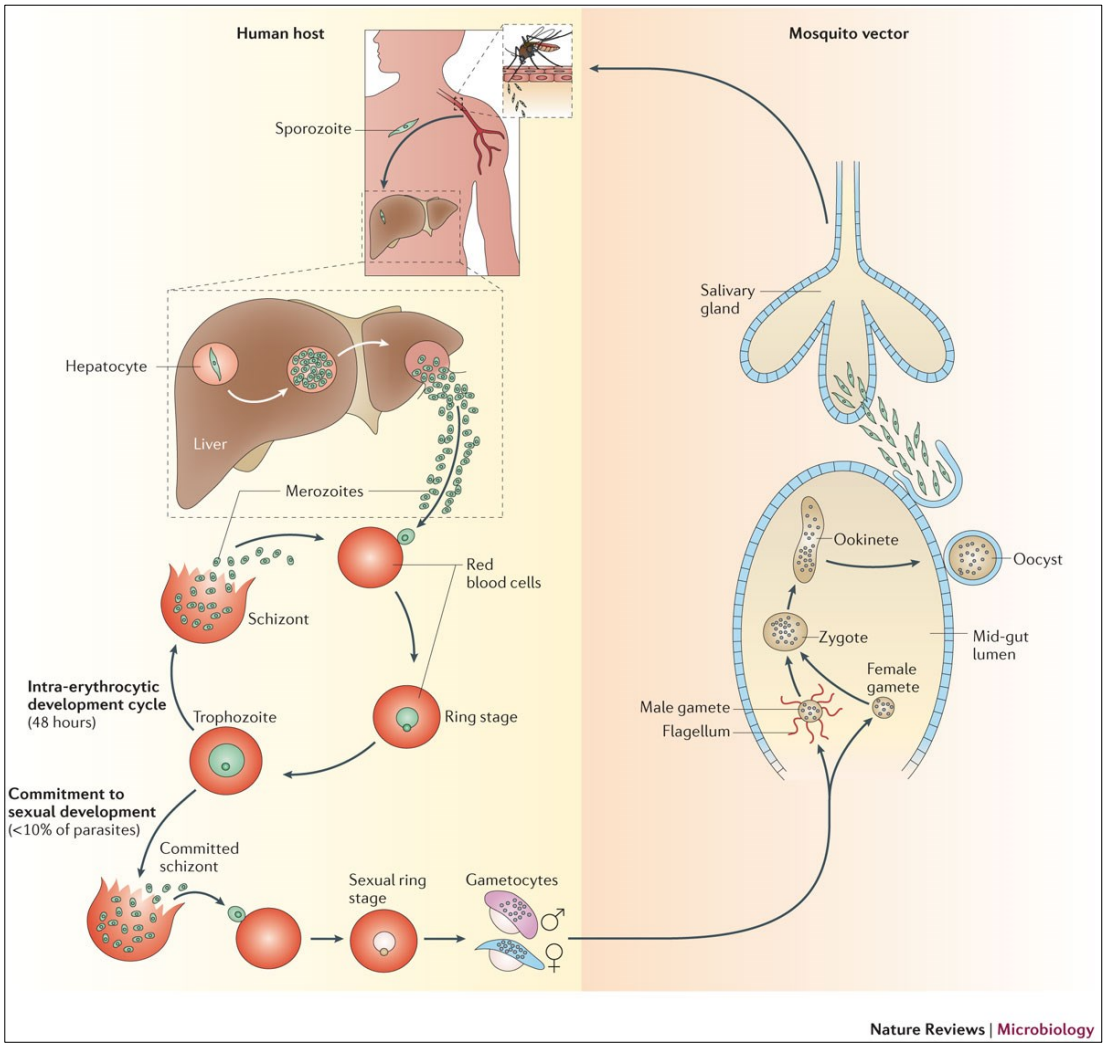


Figure 1.1.1: Lifecycle of *Plasmodium* parasite in the mosquito and human hosts. Sporozoites from the salivary duct of an infected female *Anopheles* mosquito are injected into a host's subcutaneous tissues during blood meal, that travel to the liver. After the liver stage development, tens of thousands of merozoites are released into the blood that invade red blood cells. The parasites then undergo repeated rounds of asexual multiplication. In each cycle, a small proportion of parasites commit into the development of the sexual form of the parasite, the gametocyte. The merozoite surface protein 1 (MSP-1) (discussed in more detail in Section 1.7) which is the most abundant protein on the merozoite surface (Holder *et al.* 1992) and the ABO blood group antigens on red blood cells (Fung 2014) are the focus of the data presented in this thesis. Figure was taken from (Josling and Llinas 2015).

Table 1.1.1: The different *Plasmodium* species show phenotypic differences in their life cycles in the human host.

Plasmodium species	Duration of pre-erythrocytic Cycle (days)	Merozoites in liver schizont	Duration of erythrocytic cycle (hours)	Merozoites in erythrocytic schizont	Sequester	Latent liver stages	RBC preference
P. falciparum	5.5–7	30 000	48	16-24	Yes	No	All types
P. vivax	6-8	10 000	48	14-20	No	Yes	Reticulocytes only
P. malariae	14-16	15 000	72	8-10	No	No	Older RBC
P. ovale	9	15 000	50	6-12	No	Yes	Reticulocytes only

Table adapted from (Bruce and Day 2002).

The asexual cycle in the erythrocytes takes approximately 48 h for *P. falciparum*, *P. vivax*, and *P. ovale*, and 72 h for *P. malariae* (White *et al.* 2013a, Bruce and Day 2003, Biggs and Brown 2001, Gilles 1993, Garnham 1988b, Shute 1988). This blood stage asexual replication cycle is associated with exponential growth in parasite numbers, such that in a susceptible individual, the parasite population can expand by between six and 20 times per cycle (Simpson *et al.* 2002). The work in Chapter 6 of this thesis discusses the gene expression patterns when a single clone of *P. falciparum* grown in the erythrocytes of different ABO blood group individuals

At a parasitaemia of approximately 50 parasites/μL of blood, *Plasmodium* blood-stage parasites and parasite antigens are respectively detectable by microscopy and rapid diagnostic tests (White *et al.* 2013a). The period of time from sporozoite innoculation by the mosquito and the first appearance of trophozoites in the erythrocytes is called the "prepatent period" (Murphy and Oldfield 1996). This period is species-dependent and ranges from 9 days in *P. falciparum*, to 15 days in *P. malariae* (White *et al.* 2013a). It is at this stage that the symptomatic manifestation of clinical complications such as chills, fever, anaemia, and multiple organ failure associated with malaria become evident (McQueen and McKenzie 2006, Mackintosh *et al.* 2004, Ramasamy 1998). It has long been suggested that the fever and chills associated with malaria are a consequence of paraite waste products such as haemozoin, a malaria pigment, released from the degradation of haemoglobin in the erythrocytes by the actively growing merozoites (Golgi 1889). Nutrient importation and disposal of toxic waste are brought about by the intraerythrocytic merozoites' ability to modify the cell membrane (White *et al.* 2013a).

All four human *Plasmodium* species are capable of rosette formation (Lowe *et al.* 1998, Angus *et al.* 1996, Udomsanpetch *et al.* 1995). Rosetting is a phenonemon in which *Plasmodium*-infected erythrocytes adhere to non-infected erythrocytes with pathological consequences (Newbold *et al.* 1999, Newbold *et al.* 1997, Rowe *et al.* 1995). After several cycles of erythrocyte invasion, release and re-invasion, some blood-stage parasites develop into long-lived sexual forms, male microgametocytes and female macrogametocytes. These sexual forms can be ingested by competent female *Anopheles* mosquitoes during a blood meal for the start of the sexual

development of the parasites in the vector (Biggs and Brown 2001). In the stomach of the mosquito, fusion of micro- and macrogametes occurs by meiosis leading to the formation of a zygote, which is able to penetrate the mosquito's midgut and develops into an oocyst. Asexual division of the mature oocyst leads to the formation of sporozoites that migrate to the salivary glands in readiness to be injected into a new susceptible human host (Gilles 1993).

1.1.2 Global burden, distribution and epidemiology of malaria

Malaria is estimated to affect the lives of nearly 3 billion people in over a 100 countries (WHO 2015), the distribution of which is illustrated in Figure 1.1.2 on page 10. Malaria is responsible for the annual worldwide death of more than half a million people (WHO 2015, WHO 2014, WHO 2013). The geographical distribution of malaria parasites depends on the ecology and the vectorial capacity of the *Anopheles* mosquito host (Hay *et al.* 2004, Murray and Lopez 1997).

P. falciparum is found in almost every malaria endemic region of the world and causes the heaviest burden of disease, both in terms of morbidity and mortality (White *et al.* 2013a). This burden is predominantly highest in children under the age of five years and pregnant women in Sub-Saharan Africa (WHO 2015, WHO 2013).

P. vivax has the widest geographic area of all the human malaria parasites, extending into temperate climates (Howes *et al.* 2016, Howes *et al.* 2015). Although mortality associated with this species is lower than *P. falciparum*, morbidity associated with it is high (Poespoprodjo *et al.* 2009). *P. vivax* accounts for 6% of all global malaria cases, and outside of Sub-Saharan Africa, it contributes to more than half of all malaria cases (Howes *et al.* 2016, WHO 2014). Intracellular dormant liver stages of this species serve as a parasite reservoir and are protected from host immune response. *P. vivax* requires the Duffy antigen for merozoite invasion of red blood cells. Most populations in Sub-Saharan Africa lack these Duffy antigens and are considered to be refractory to *P. vivax* infection (Howes *et al.* 2011, Carter and Mendis 2002). Recent evidence from epidemiological studies from Africa, however, suggests the contrary (Fru-Cho *et al.* 2014, Menard *et al.* 2010), with *P. vivax* infections now being diagnosed in Duffy negative populations.

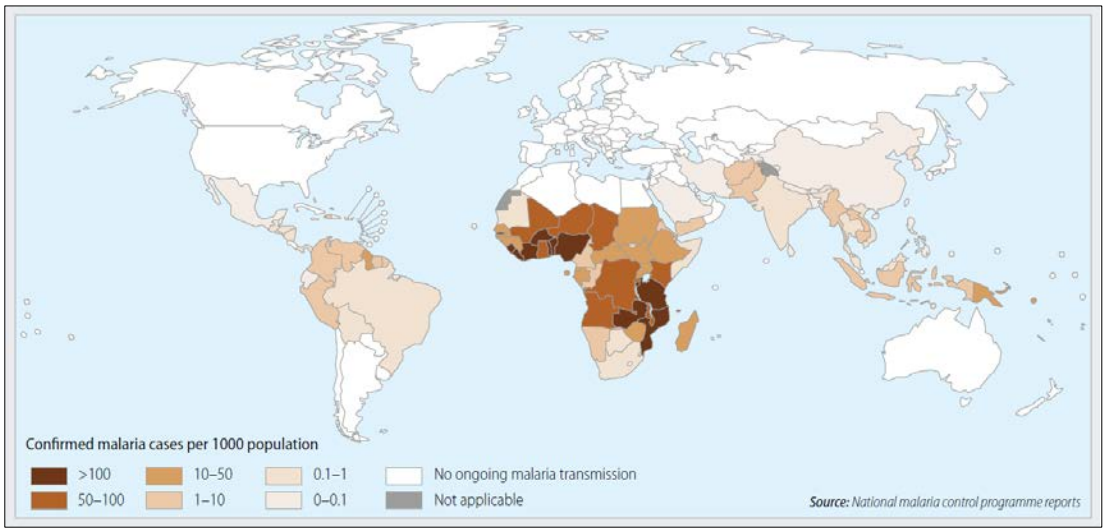


Figure 1.1.2: Worldwide distribution of malaria endemic areas according to high, moderate and low prevalence areas in 2014.

Sourced from the World Health Organization online resource: http://www.who.int/malaria/publications/world_malaria_report_2014/wmr-2014-no-profiles.pdf [Accessed 20/09/2016].

P. malariae is found throughout the tropical world from Africa to South America, and remains the most understudied species among the four human malaria parasite (Collins and Jeffery 2007). This is partly because it causes low-grade chronic infections and is often seen in mixed infections with *P. falciparum* rather than as a monospecies infection, and therefore is frequently undiagnosed (Rayner 2015, Dinko *et al.* 2013, Mueller *et al.* 2007, McKenzie and Bossert 1999, Black *et al.* 1994). The worldwide distribution of *P. malariae* overlaps with that of *P. falciparum* (Collins and Jeffery 2007). Malaria caused by this species is mostly diagnosed only when molecular techniques such as polymerase chain reaction (PCR) are employed (Snounou *et al.* 1993), as they are frequently missed by microscopy owing to the low-level parasitaemia associated with it (Collins and Jeffery 2007). *P. malariae* is morphologically identical to *P. rhodaini* in African apes, and *P. brasilianum* in New World monkeys in South America (Coatney *et al.* 1971). Recent evidence of cross-species transmission of *P. brasilianum* from monkey to man (Lalremruata *et al.* 2015) has highlighted the possible danger of *P. malariae* being transmittable as a zoonotic

infection (Rayner 2015). The few epidemiological studies focusing on this species are largely on hospital-based case studies and in returnees who have recently or previously visited a malaria-endemic country (Sutherland 2016, Savargaonkar *et al.* 2014, Badiane *et al.* 2014, Senn *et al.* 2014, Hommel *et al.* 2013).

P. ovale has a much more limited distribution, and can be found in tropical Africa, New Guinea, the eastern parts of Indonesia and the Philippines (Collins and Jeffery 2005, Lysenko and Beljaev 1969), as well as in the Middle East (Al-Maktari *et al.* 2003) and in parts of India (Jambulingam *et al.* 1989). Much of the epidemiological studies on this species are confined to studies in highly endemic areas in Africa and the Southwest Pacific, with not many documented studies from South America and Asia (Mueller *et al.* 2007). Similar to *P. vivax*, this species also forms dormant liver stage parasite that can persist in the host for a long time (Markus 2015, Markus 2011a, Markus 2011b).

1.2 Clinical manifestations of malaria

The species and genotype of the infecting *Plasmodium* parasite, and the immunological status of the infected individual contribute to the clinical outcomes associated with malaria (Milner 2017, Miller *et al.* 2013). The pathology of malaria arises as a consequence of the release of parasite toxins into the bloodstream (Miller *et al.* 2013), destruction of infected erythrocytes (Buffet *et al.* 2011), host immune response to parasite antigens (Clark *et al.* 2006) and sequestration of infected erythrocytes by *P. falciparum* (White *et al.* 2013b). Fever, though non-specific to malaria, and undifferentiated from that caused by other pathogens is a classical hallmark of malaria (Gazzinelli *et al.* 2014).

Fever is defined as an increase or a rise in body temperature caused by the actions of thermoregulatory pyrogens on the hypothalamus (Walter *et al.* 2016, Oakley *et al.* 2011). The American College of Critical Care Medicine, the International Statistical Classification of Diseases, and the Infectious Diseases Society of America, all define fever as a core body temperature of 38.3°C (Walter *et al.* 2016, O'Grady *et al.* 2008). In malaria epidemiological studies patients with body temperature of 37.5^{o}C and above

are generally defined as having fever (Seyoum *et al.* 2017, McKenzie *et al.* 2006, Creasey *et al.* 2004). In 1886, Camillo Golgi recognized that malaria fever coincides with the cyclical release of merozoites during schizont rupture of the infected erythrocyte (Golgi 1889). Such fevers occur every 48 h (defined as tertian fever) in *P. falciparum, P. ovale and P. vivax* infections, and every 72 h (defined as quartan fever) in *P. malariae* infections (Bruce and Day 2002, Gilles 1993, Garnham 1988b). Fever may be is beneficial to the host and plays important roles such as inhibition of *Plasmodium* parasite growth (Kwiatkowski 1990), and the enhancement of both the innate and adaptive immune response against pathogens through higher maturation rates of immune cells (Tomiyama *et al.* 2015, Tournier *et al.* 2003).

The WHO recommends all cases of suspected malaria to be confirmed by parasitological diagnosis before treatment (referred to as "test, treat and track") (WHO 2015, WHO 2014). However, in resource-poor malaria endemic areas, presumptive treatment of malaria especially in children under the age of five years is based on fever presentation (Singh and Sharma 2014) and the clinical judgement of health care providers, without laboratory confirmation (Kyabayinze *et al.* 2010, Chandler *et al.* 2008). This usually leads to overdiagnosis and overestimation of the malaria burden (Bhatt *et al.* 2015), since all fevers are not a consequence of malaria.

For example, a study in Kenya found between 7 and 57.2% of children who were presumptively diagnosed and treated with antimalarial drugs to be negative for malaria upon examination of blood for the presence of parasites (Onchiri *et al.* 2014). Similarly, in Angola, overdiagnosis of malaria cases based on clinical presentations was found to have an average error rate of 85% after microscopic examination of blood smears (Manguin *et al.* 2017). Given that other bacterial and viral diseases may present with fever that are undifferentiated from that caused by malaria (Kallander *et al.* 2004, Genton *et al.* 1994, O'Dempsey *et al.* 1993), treatment of every childhood fever with antimalarial drugs without laboratory confirmation might contribute to drug misuse and wastage (Manguin *et al.* 2017), and promotes the emergence of drug-resistant strains of *Plasmodium* parasites (WHO 2015). The total malaria burden might also be

overestimated in such settings, while other causes of fever may remain undiagnosed (Manguin *et al.* 2017, Chandler *et al.* 2008).

Other characteristic symptoms associated with malaria include headache, nausea, abdominal discomfort and vomiting, as well as muscular and joint aches (Gazzinelli *et al.* 2014). While prompt diagnosis and treatment will ensure parasite clearance and avert any complications (Singh and Sharma 2014, Lambrechts *et al.* 1999), untreated cases of malaria may lead to severe malaria characterised by metabolic acidosis (Greenwood *et al.* 2005), respiratory distress (Schellenberg *et al.* 1999), prostration (Demissie and Ketema 2016), severe anaemia (haemoglobin concentration below 5 g/dl) due to erythrocyte destruction and impaired erythropoiesis (Haldar and Mohandas 2009), repeated convulsions, hypoglycaemia (blood glucose <2.2 mmol/l), coma and death (WHO 2014).

Of the four species of human *Plasmodium* parasites, *P. falciparum* is responsible for life-threatening disease and complications such as severe anaemia (Haldar and Mohandas 2009), cerebral malaria (Schofield and Grau 2005), renal failure and hepatic dysfunction (Badiane *et al.* 2014). *P. vivax and P. ovale* infections may result in the development of relapse many months or years after the primary infection, due to reactivation of the dormant liver-stage forms, the hypnozoites (Markus 2011b, Krotoski *et al.* 1980). *P. malariae* although causes very low morbidity, is capable of causing long-term chronicity that can result in nephrotic complications (Badiane *et al.* 2014, Gilles and Hendrickse 1960) and splenomegaly (Vinetz *et al.* 1998). This species has also been associated with drug treatment failures in undiagnosed mixed-species infections by unknown mechanisms (Smith *et al.* 2011).

1.3 Malaria diagnosis

Early and effective diagnosis of malaria is key to the treatment and management of disease, thereby reducing associated complications and mortality (McKenzie *et al.* 2003, Lambrechts *et al.* 1999). Ideal diagnosis of malaria involves a combination of physical examination for clinical signs and symptoms of the disease and confirmation of the presence of *Plasmodium* parasites in the blood with laboratory tests (WHO 2000,

Bain *et al.* 1997). Clinical signs and symptoms of malaria such as fever, headache, chills and abdominal pain (WHO 1990), are however non-specific, as these are also common with other parasitic, viral and bacterial infections (Kallander *et al.* 2004, O'Dempsey *et al.* 1993). In view of this, clinical diagnoses of malaria alone is often imprecise (Manguin *et al.* 2017, Onchiri *et al.* 2014), although it is the basis of therapeutic care for the majority of febrile patients in resource-poor malaria endemic areas, where laboratory confirmations are unavailable (Manguin *et al.* 2017, Roucher *et al.* 2012).

Confirmatory diagnosis of malaria is accomplished by identifying asexual blood-stage parasites by microscopy (WHO 2000, Bain *et al.* 1997, Payne 1988), detection of parasite antigens and antibodies in blood using rapid diagnostic tests (RDTs) (Wilson 2012, Moody and Chiodini 2002) and the application of molecular techniques such as the polymerase chain reaction (Snounou *et al.* 1993).

1.3.1 Microscopy of Giemsa-stained blood films

Conventional laboratory diagnosis in clinical settings is by microscopic examination of Giemsa-stained thick and thin peripheral blood smears for parasite identification and species differentiation respectively of the asexual blood stages of the *Plasmodium* parasites (Bain *et al.* 1997). This involves identifying morphologic characteristics such as the size of the infected red blood cell, cytoplasmic inclusions (e.g. Schuffner's dots found in *P. vivax* and *P. ovale*) and features of the parasite blood stages (McKenzie *et al.* 2003, Bain *et al.* 1997). Microscopy has the advantage of parasite identification, species differentiation (when performed by expert microscopists), quantitative enumeration of parasite density and pigment identification (Bronzan *et al.* 2008, Chotivanich *et al.* 2007).

In spite of the above advantages, microscopy has many challenges. It is influenced by the type of species, endemicity and parasitaemia, host immunity, sequestration, and observer factors (Obare *et al.* 2013). In most African countries where malaria is endemic, laboratory facilities for the confirmation of the disease at the periphery of the health care system are inadequate or unavailable (Cibulskis *et al.* 2011), owing to lack of personnel and technical requirements of microscopy (Cibulskis *et al.* 2011, WHO

2000). Additionally, the processes from blood sampling, slide preparation, staining, and examination are time consuming (Barber *et al.* 2013). This often results in clinical decisions being taken without laboratory confirmation, especially for children under five years old (WHO 2000). Microscopy has the added limitation of an observer's inability to differentiate between the blood-stages of the different *Plasmodium* species (Barber *et al.* 2013), as the morphological differences between these species are very subtle. Examples of these morphological differences among the four *Plasmodium* species are summarised and illustrated in in Table 1.3.1 and Figure 1.3.1 on page 16 and 17 respectively.

Table 1.3.1: Morphological differences exist in the blood-stage parasites of human *Plasmodium* Species.

***Plasmodium* spp.**	**Rings**	**Trophozoites**	**Schizonts**	**Gametocytes**	**Appearance of ring-infected RBC**
P. falciparum	Delicate cytoplasm; 1-2 chromatin dots, occasional appliqué (accolé) forms	Seldom seen in peripheral blood; compact cytoplasm; dark pigment	Seldom seen in peripheral blood; dark pigment clumped in one mass	Crescent or sausage shape; chromatin in a single mass or diffused; dark pigment mass	Normal size; multiple infection common
P. vivax	Large cytoplasm; large chromatin dot	Large amoeboid cytoplasm; large chromatin dot; yellowish-brown pigment	Large, may almost fill RBC; yellowish-brown, coalesced pigment	Round to oval; may almost fill RBC; chromatin compact, eccentric or diffused; scattered brown pigment	Normal to 1.25x nRBC; occasionally fine Schuffner's dots
P. malariae	Sturdy cytoplasm; large chromatin dot	Compact cytoplasm; large chromatin dot; dark brown pigment	Large nuclei, clustered around mass of coarse; dark-brown pigment	Round to oval; may almost fill RBC; chromatin compact, eccentric or diffused; scattered brown pigment	Normal to 0.75x nRBC
P. ovale	Sturdy cytoplasm; large chromatin dot	Compact with large chromatin; dark-brown pigment	Large nuclei, clustered around mass of coarse; dark-brown pigment	Round to oval; may almost fill RBC; chromatin compact, eccentric or diffused; scattered brown pigment	Normal to 1.25x nRBC; round to oval; occasionally Schuffner's dots

Adapted from Centers for Disease Control and Prevention (CDC) (accessed https://www.cdc.gov/dpdx/malaria/dx.html, on 15/05/2017). nRBC: normal red blood cell.

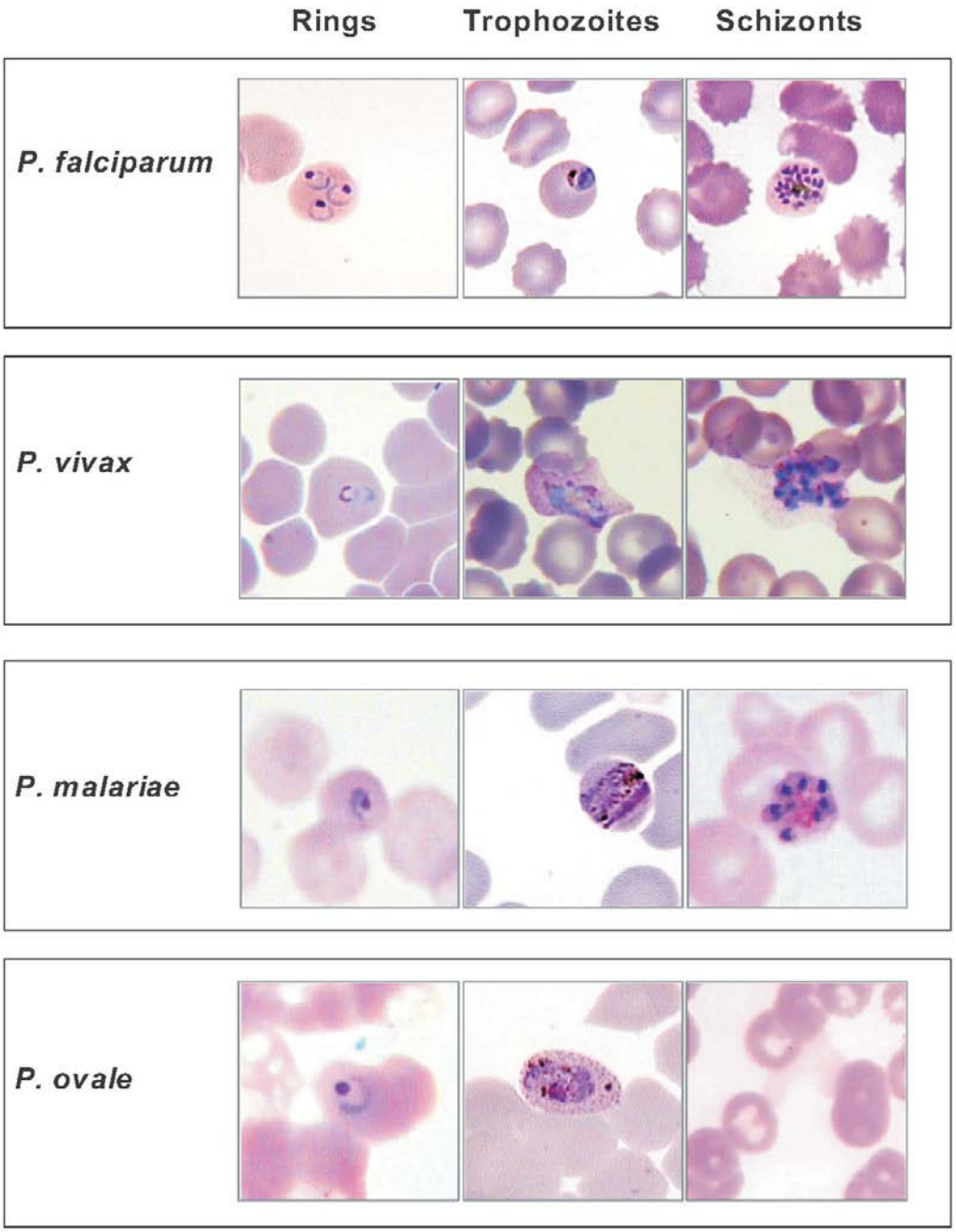


Figure 1.3.1: Morphological differences exist in the blood-stage parasites of human *Plasmodium* Species. Giemsa-stained thin blood film of asexual blood-stage parasite development (ring, trophozoite and schizont) of *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Figure adapted from (Chotivanich *et al.* 2007).

The difficulty in differentiating the morphology of the different species may lead to misdiagnosis of the type of *Plasmodium* species and in cases of mixed-species infections, result in underestimation (Obare *et al.* 2013, Harris *et al.* 2010, Mayxay *et al.* 2004). This is often seen even when expert research microscopists are involved in malaria diagnosis by microscopy (Obare *et al.* 2013, McKenzie *et al.* 2006), as detection levels of parasites by microscopy is 40 parasites per µL of blood (Bruce *et al.* 2008, Zimmerman *et al.* 2004).

In very low transmission regions, low parasitaemia of <5 infected erythrocytes per microliter of blood can make species identification by microscopy very difficult, if not impossible (Zimmerman *et al.* 2004). Moreover, microscopists who are not frequently 'exposed' to examining the different *Plasmodium* species often misdiagnose mixed-species infections or report the presence of parasites without speciation (Obare *et al.* 2013). The 'non reporting' of the species of *Plasmodium* an individual is infected with owing to the above outlined limitations of microscopy is addressed in this thesis by the application of a sensitive diagnostic tool based on *Plasmodium* merozoites surface proteins (discussed in more detail in Section 1.7 and Chapters 3 and 4).

1.3.2 **Rapid diagnostic tests (RDTs)**

Rapid diagnostic tests (RDTs) are based on immunochromatographic methods for the detection of antigens derived from *Plasmodium* parasites in lysed blood (Moody and Chiodini 2002, Shiff *et al.* 1994). Most RDTs employ a dipstick or test strip with monoclonal antibodies incorporated and directed against the target parasite antigens (Wilson 2012).

Three parasite antigens commonly targeted in commercial RDTs kits include histidine-rich protein II (HRP-II) (Shiff *et al.* 1993), parasite lactate dehydrogenase (pLDH) and aldolase (Palmer *et al.* 1998). HRP-II is a water-soluble protein produced by trophozoites and young gametocytes of *P. falciparum* and abundantly expressed on red blood cell membrane (Rock *et al.* 1987, Howard *et al.* 1986). *Plasmodium* parasite LDH is an enzyme produced by both the asexual and sexual stages of *Plasmodium* parasites (Makler and Hinrichs 1993). Both pLDH and aldolase are enzymes essential in the parasite's glycolytic pathway. Some RDTs detect *P. falciparum*-specific and

pan-specific antigens such as aldolase, or pan-*Plasmodium* pLDH (Wilson 2012, Tangpukdee *et al.* 2009, Meier *et al.* 1992).

RDTs offer the advantage of being simpler to perform and to interpret, requiring no electricity or special equipment or training and thereby making it possible for peripheral health workers to be trained on its usage (Harvey *et al.* 2008).

There are currently over 86 varieties of RDTs on the market, examples of which include Paracheck, ParaHit-F, ParaSight-F test, SD Bioline, ParaScreen, and OptiMAL test (Tangpukdee *et al.* 2009, Palmer *et al.* 1998, Shiff *et al.* 1994, Shiff *et al.* 1993). Test kits currently available that report detection of pLDH from all four *Plasmodium* species that infect humans are able to distinguish *P. falciparum* from *P. vivax,* but not between *P. vivax, P. ovale* and *P. malariae* infections (Hemingway *et al.* 2016, Graves *et al.* 2015, WHO 2013, Wilson 2012, WHO 2000). Such test kits although claim to detect all four *Plasmodium* species make mention of only *P. falciparum* and *P. vivax* in their brand name or marketing material (WHO 2013). At very low parasitaemia, most RDTs are less sensitive for the detection of *P. vivax* (White *et al.* 2013a). This is an essential shortcoming of these RDTs as they are unable to detect mixed *Plasmodium* species, especially of *P. malariae* and *P. ovale*. RDTs have the added disadvantage of high cost, making them not readily available at all peripheral health facilities. Sensitivity of >90% is usually reported for RDTs in the detection of *P. falciparum* at densities above 100 parasites per μL of blood, however, sensitivity decreases markedly below this parasite threshold (Wilson 2012, WHO 2000).

Table 1.3.2 on the next page shows examples of the most commonly used RDTs or commercial kits available on the market, the target antigens incorporated and the *Plasmodium* species reported to be detectable.

Table 1.3.2: Commonly available RDTs in current use.

RDT/commercial kit	Manufacturer	Target antigen	Species detected
Para Sight F	Becton-Dickson, USA	HRP-II	*P. falciparum*
Paracheck	Orchid Biochemical, India	HRP-II	*P. falciparum*
NOW P. f/P. v	Binax Inc, USA	HRP-II	*P. falciparum* *P. ovale* *P. vivax* *P. malaria*
OptiMal	Flow Inc, USA	pLDH	*P. falciparum* *P. ovale* *P. vivax* *P. malaria*
Malaria EIA	Trinity Biotech's Captia, USA	MSP-1_{19} of *P. falciparum* and *P. vivax*, and *P. falciparum*-specific MSP-2A and 2B serogroups	*P. falciparum* *P. ovale* *P. vivax* *P. malaria*

RDT: Rapid diagnostic test, HRP-II: Histidine Rich Protein II, pLDH: parasite lactate dehydrogenase, MSP: merozoite surface protein, EIA: enzyme immunoassay

1.3.3 Immunofluorescence and molecular techniques

In epidemiological surveys and screening of blood donors in most developed countries immunofluorescent antibody assay (IFA) which detects antibodies against asexual blood stage malaria parasites are employed (She *et al.* 2007). The use of IFA is simple and sensitive, but time-consuming (Tangpukdee *et al.* 2009). In most research laboratories, diagnosis of malaria is based on molecular techniques such as polymerise chain reaction (PCR), microarray and flow cytometry.

Molecular techniques such as PCR amplifies a selected region of the malarial genome, and offer the advantages of species identification, detection of parasites as low as 5 parasites/μL of blood (Snounou *et al.* 1993) and its application in identifying the development of drug-resistance parasites. However, PCR is of limited use in clinical settings in most developing countries, except in research laboratories, because it is expensive and requires a sophisticated laboratory manned with well-trained staff (Daniels *et al.* 2017, Nkumama *et al.* 2016).

1.4 Treatment of malaria

Historically, treatment of uncomplicated malaria had been based on monotherapy (i.e. the administration of a single drug over the course of a number of days) with the administration of either chloroquine or amodiaquine until the emergence of chloroquine-resistant *Plasmodium* species in the mid-1980s (Wernsdorfer *et al.* 1995). Following two guidelines by the WHO in 2006 and 2010 most malaria endemic countries have moved away from chloroquine to artemisinin combination therapies (ACTs), which is the recommended treatment regime for uncomplicated malaria in children and adults (Dondorp *et al.* 2010, Baird 2008, Bosman and Mendis 2007).The exception is in pregnant women who are in their first trimester in which a seven day course of quinine with clindamycin is recommended (WHO 2015, Cui and Su 2009).

ACT is a combination of artemisinin derivative which has a shorter elimination half-life of about 1 hr (Cui and Su 2009, Nosten and White 2007) and a partner drug with a longer half-life (Chang 2016, White 1999a). The artemisinin component is effective against both asexual and sexual blood-stages of the *Plasmodium* parasites (Bousema *et al.* 2010b, Skinner *et al.* 1996), but has limited or no effect on hepatocytic parasites such as the hypnozoite forms of *P. ovale* and *P. vivax* (Chang 2016). The partner drug is subsequently able to clear any remaining parasites and also delays the development of resistance to artemisinin (Cui and Su 2009, White 1999a, White 1999b). Recommended ACTs combinations include artemether with lumefantrine, artesunate with either amodiaquine or mefloquine, dihydroartemisinin with piperaquine, and artesunate with sulfadoxine-pyrimethamine (WHO 2015, Nosten and White 2007).

ACTs are administered based on patient age and body weight (Nosten and White 2007). In addition to ACTs being generally tolerable by patients, they are generally very effective and safe (Cui and Su 2009, Nosten and White 2007). In malaria patients in which the species of *Plasmodium* is unknown it is recommended to treat as uncomplicated *P. falciparum* infection (WHO 2015). In areas with both chloroquine-resistant and chloroquine-susceptible infections, the recommendation is to treat adults and children with confirmed *P. vivax*, *P. ovale*, or *P. malariae* malaria with the standard ACTs (Cui and Su 2009, Nosten and White 2007). Intravenous or intramuscular administration of artesunate in the first 24 hours is strongly recommended for severe malaria cases until such a time that patients can tolerate oral medication (WHO 2015, Cui and Su 2009).

1.5 Antimalarial drug resistance

Global malaria control and elimination efforts are hampered by the emergence and spread of antimalarial drug resistance parasite strains that follow the development of new drugs. This cyclical historic development has plagued malaria therapeutics for more than six decades. The first effective drug for treating malaria was the alkaloid-type of compound, quinine. This was isolated in 1820 from the bark of cinchona tree by the French pharmacists, Pierre-Joseph Pelletier and Joseph-Bienaimé Caventou (Kaufman and Ruveda 2005). Quinine was the drug of choice widely used as prophylaxis or for the treatment of malaria until World War II (Chang 2016, Packard 2014, Peters 1990). The mechanism of action of quinine is poorly understood, but it is generally believed to act on the asexual intra-erythrocytic stages of the four *Plasmodium* species (Peters 1990, Peters 1987a). The drug interferes with the parasite's ability to dissolve and metabolize haemoglobin (Peters 1970). This enables the free and toxic haem components to accumulate in the erythrocytes at higher concentrations and eventually kill the *Plasmodium* parasites. The first reported resistance of *Plasmodium* parasites to quinine was in 1910 in Thailand (Peters 1982). Resistance to this drug was however not widespread; and that even in the era of increased use of artemisinin-based combination therapies, quinine remains a first-line drug in the treatment of severe malaria in some parts of the world (WHO 2006).

In 1934, German chemists developed a new antimalarial quinine derivative, chloroquine (Coatney 1963), which became the most widely used synthetic antimalarial drug during the 1950s to the 1970s. However, within a decade, chloroquine-resistant *P. falciparum* strains emerged (Eyles *et al.* 1963), notably in Colombia and at the Cambodia-Thailand border (Payne 1987, Peters 1970), an area that has become a historical site of emerging antimalarial-drug resistance. The reasons often cited for this high prevalence of drug resistance at the Cambodia-Thailand border include the way the drugs are used and the social and economic conditions prevailing at this location (Packard 2014). Resistant strains from the Cambodia-Thailand foci spread steadily in the 1960s and 1970s through South America, Southeast Asia, and India (Peters 1987a) (see map on Figure 1.5.1 on page 27). In Africa the first reported case of chloroquine-resistant *P. falciparum* was detected in Kenya in 1979 (Fogh *et al.* 1979); followed by a wave of resistant *P. falciparum* across the continent within a decade (Peters 1987a, Mutambu *et al.* 1986, Ekue *et al.* 1983). The insurgence of chloroquine-resistant *P. falciparum* contributed to increased malaria-associated morbidity and mortality, notably among children in Africa (Anderson and Roper 2005, Trape *et al.* 1998, Greenberg *et al.* 1989).

Chloroquine is a diprotic weak base and, at physiological pH (7.4), can be found in three forms, the uncharged, mono-protonated and di-protonated. The uncharged chloroquine is the only membrane permeable form of the drug and it freely diffuses into the erythrocyte up to the lysosomal isolated acidic compartment known as the digestive vacuole. In the digestive vacuole, the drug molecules become charged and accumulates into the acidic digestive vacuole, since the membranes are not permeable to charged species (Yayon *et al.* 1984, Homewood *et al.* 1972). It has been known that *P. falciparum* strains that are chloroquine sensitive accumulate much more chloroquine in the digestive vacuole than strains that are resistant to the drug (Saliba *et al.* 1998, Yayon *et al.* 1984). Chloroquine resistant is as a result of point mutations in the gene encoding for the *P. falciparum* chloroquine resistance transporter (PfCRT) protein (Djimde *et al.* 2001, Fidock *et al.* 2000, Wellems *et al.* 1991). However, there is evidence to show that another mutation, S163R, is capable of restoring the

chloroquine sensitivity of parasites that carry the PfCRT mutation (Wellems 2004, Johnson *et al.* 2004).

Chloroquine is still the first-line treatment for *P. vivax* malaria in areas where this species is endemic (Seifu *et al.* 2017). When chloroquine is given with primaquine, the combination is highly effective against both the acute infection and in preventing relapses caused by hepatic hypnozoite forms. Although *P. vivax* accounts for nearly as many cases of malaria as *P. falciparum* in areas where these two species are co-endemic, and is exposed to similar high levels of chloroquine pressure, resistances to this species were not reported until 1989 in Papua New Guinea (Whitby *et al.* 1989), i.e. about three decades after chloroquine-resistant *P. falciparum* strains were first reported. Chloroquine-resistant *P. vivax* is now widespread across most countries endemic for the disease, especially in the regions of Southeast Asia (Baird *et al.* 1997) and in South America (Whitby 1997). In a systematic review and meta-analysis of the global extent of chloroquine-resistant *P. vivax*, Price *et al*., 2014 found that of the 129 eligible clinical trials involving 21,694 patients at 179 study sites, and 26 case reports describing 54 patients, chloroquine resistance was present in 53% of 113 assessable study sites (Price *et al.* 2014). These were spread across most countries that are endemic for *P. vivax*. Clearance of parasitaemia assessed by microscopy in 95% of patients by day 2, or all patients by day 3, was 100% predictive of chloroquine sensitivity (Price *et al.* 2014).

In an effort to combat resistant strains, a number of alternative synthetic antimalarial drugs were developed, including, sulfadoxine–pyrimethamine (commonly called Fansidar) and mefloquine. Sulfadoxine–pyrimethamine interferes with the synthesis of folic acid and by extension, the synthesis of nucleotides required for the parasite's DNA synthesis. Sulfadoxine–pyrimethamine is often used as a second line drug in areas where chloroquine-resistant *P. falciparum* species are present (Creasey *et al.* 2004). The drug's resistance is conferred by a single mutation in the gene encoding the enzyme dihydrofolate reductase (Wu *et al.* 1996). Unlike chloroquine resistance to *P. falciparum* that took many years to develop in a limited number of foci, resistance to sulfadoxine–pyrimethamine was reported to have arose rapidly on many independent occasions (Peters 1987b).

Since the year 2001 about forty countries have adopted the WHO's recommended artemisinin-based combination therapy for treating uncomplicated malaria caused by *P. falciparum* (WHO 2006). About half of these countries have adopted the artemether-lumefantrine combination (commonly known as Coartem) as their first- or second-line treatment. The introduction of ACT therapy has resulted in the reduction of global malaria-related morbidity and mortality (Bhattarai *et al.* 2007, Carrara *et al.* 2006, Barnes *et al.* 2005). Artemisinin and its derivatives are very potent antimalarial drugs and are capable of reducing parasite biomass to the order of ∼1/10 000 per cycle (White *et al.* 1999). Artemisinin works by accelerating parasite clearance (White 2008) of young, circulating, ring-stage parasites and preventing the further maturation and sequestration of these parasites (ter Kuile *et al.* 1993). Artemisinin resistance parasite strains have been reported in Southeast Asia (Ashley *et al.* 2014, Dondorp *et al.* 2009, Noedl *et al.* 2008). This resistance has been shown to be associated with polymorphisms in the *P. falciparum* kelch protein gene located on chromosome 13 (kelch13) (Ashley *et al.* 2014). In spite of its effectiveness and excellent safety and efficacy profile (White 2008), a decline in the parasite clearance rate by artemisinin has been documented in the last decade among patients near the Thai–Cambodian border (Dondorp *et al.* 2009). This area is most notable as resistances to other antimalarial drugs in the previous decades had originated from (Verdrager 1986). In this same area, increased numbers of copies of the mefloquine resistance gene PfMDR1 (Price *et al.* 2004) has been associated with high failure rates with artesunate–mefloquine therapy (Wongsrichanalai and Meshnick 2008, Alker *et al.* 2007). The first reported indigenous artemisinin resistant *P. falciparum* strain from Africa was contracted from Equatorial Guinea by a Chinese returnee who had no previous clinical history of malaria (Lu *et al.* 2017).

In parts of Africa, the median parasite clearance half-life by artemisinin is faster (1.9 hours) compared to that seen along the Thailand–Cambodia border (7 hours) (Ashley *et al.* 2014). These slow clearing infections (parasite clearance half-life >5 hours) are strongly associated with kelch13 mutations (Ashley *et al.* 2014). There are also social and biologic dynamics to this burden of antimalarial-drug resistance (Packard 2014).

Generally, the emergence of antimalarial drug resistant *Plasmodium* species are believed to be the consequence of widespread use and the drug's availability over the counter in many countries where malaria is endemic (White 2004). For example, the high emergence of antimalarial drug resistance seen in Cambodia is often attributed to the migration of mine workers to the Pailin province from neighbouring regions of Cambodia, Thailand, Vietnam, and Myanmar, as well as from Bangladesh (Packard 2014). It is estimated that about 1000 to 1200 migrant workers (80% with no immunity to malaria) arrive in Pailin each month (Verdrager 1995). The mining activities create hundreds of shafts, which collect water from seepage and rains. These shafts become a good breeding sites for *Anopheles dirus* population that are very highly efficient in transmitting *Plasmodium* parasites. These miners are said to usually stay for 3 to 4 months, live in the open air and sleep under very rudimentary shelters, exposing them to multiple infective bites (Packard 2014). This cycle of drug development and the development of resistant parasite strains threatens worldwide initiatives to control and eliminate malaria. This development calls for a global concerted efforts to curb the spread of resistant parasites, and also support the development of the next generation of antimalarial drugs.

Figure 1.5.1 on page 27 illustrates the timeline of the development of chloroquine-resistance *P. falciparum* species from the Worldwide Antimalarial Resistance Network. I have also summarised the historic discovery of the major antimalarial drugs, their mode of actions, and the years and countries where resistance to these drugs were first reported Table 1.5.1 on page 28.

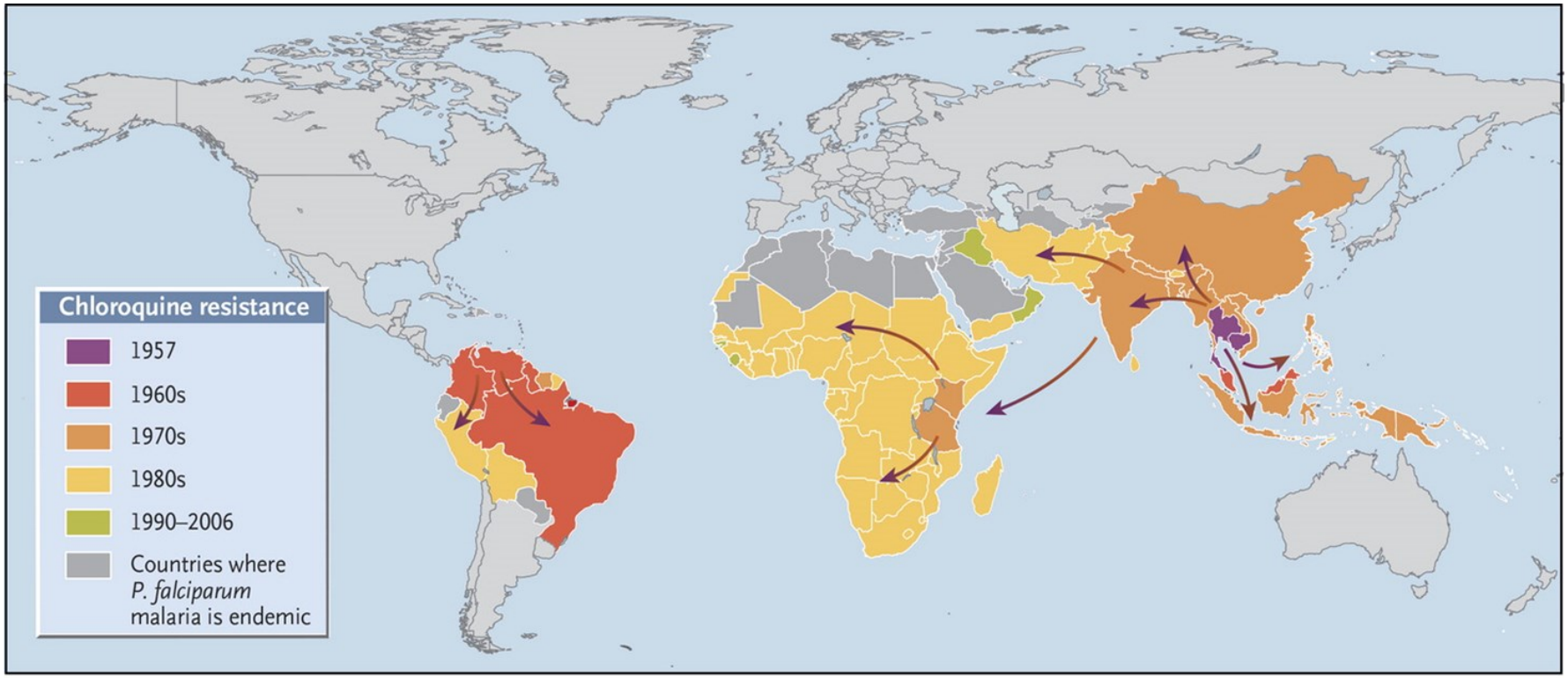


Figure 1.5.1: Chloroquine-resistance *P. falciparum* species first reported in 1957 at the Thai-Cambodia border and spread across continents. The arrows represent the spread of the drug resistance across continents. Reproduced with permission from Packard 2014. Data are from the WorldWide Antimalarial Resistance Network.

Table 1.5.1: Antimalarial drugs, targets, mode of action and the development of resistance.

Antimalarial drug	Date discovered	Target parasite stage	Mode of drug action	Year of first reported resistance	Country where resistance first reported
[1]Quinine	1820	Asexual blood stages and gametocytes	Interferes with the parasite's ability to dissolve and metabolize haemoglobin	1910	Thailand
[2]Chloroquine	1934	Asexual blood stages	Inhibits haemozoin production	1950s	Colombia and at the Cambodia-Thailand border
[3]Sulfadoxine/ Pyrimethamine	1940s	Asexual blood stages	Act synergistically to inhibit dihydrofolate reductase and dihydropteroate synthetase	1970s	Border areas of Thailand, Cambodia, and Myanmar
[4]Artemisinin	1970s	Asexual blood stages and gametocytes	Haem-mediated decomposition of the endoperoxide bridge to produce carbon-centred free radicals	2013	Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam

Note: numerical superscripts are respectively referenced on the next page.

[1](David and Jacoby 2005, Pukrittayakamee *et al.* 1994, Peters 1982), [2] (Wellems 2002, Wernsdorfer *et al.* 1995, Bjorkman and Phillips-Howard 1990b, Bjorkman and Phillips-Howard 1990a, Eyles *et al.* 1963), [3](Sibley *et al.* 2001, Hurwitz *et al.* 1981), [4](Dondorp *et al.* 2009, Cui and Su 2009, Noedl *et al.* 2008).

1.6 Mixed-*Plasmodium* species infection, why do we need to know more?

In malaria-endemic regions, infections involving more than one *Plasmodium* species are common and yet the epidemiology of the different species, especially the non-*falciparum* species has received very little attention (Sutherland 2016, Rayner 2015). Over a decade ago, Zimmerman *et al.* reviewed the importance of knowing more about mixed-*Plasmodium* species infections as knowledge about the dynamics of mixed infection was limited (Zimmerman *et al.* 2004). One of the key knowledge gaps highlighted in that review was the lack of diagnostic methods that could best detect all *Plasmodium* species commonly found in malaria-endemic areas, especially in very low transmission settings where low-level infections are common. As previously discussed, there are still no RDTs on the market capable of detecting all four human *Plasmodium* species in Sub-Saharan Africa. I believe the antigens derived from *Plasmodium* merozoite surface used in this thesis for the seroepidemiological work provide key answers for their development into RDTs applicable in malaria endemic settings, which can detect recent exposure to infections caused by the different *Plasmodium* species.

The focus of malaria research since Zimmerman's review is still concentrated on *P. falciparum* as this species is the one that causes life-threatening disease. Adequate knowledge of the species of *Plasmodium* will enable accurate quantification of the prevalence of the different species and the magnitude of the disease burden associated with each species in malaria-endemic populations. It is essential to know the species of *Plasmodium* causing malaria in order to ensure targeted treatment of infecting parasites and to improve the management of patients with malaria. Misdiagnosing mixed-*Plasmodium* infections could lead to a prolonged parasite clearance time, anaemia and drug resistance (de Roode *et al.* 2004). Non clearance of the non-*P.*

falciparum species poses a danger of relapse by *P. vivax* and *P. ovale* (Coldren *et al.* 2007, Collins and Jeffery 2005), whiles low-grade parasitaemia of *P. malariae* has potential long term health effects (Badiane *et al.* 2014, Hase *et al.* 2013, Hommel *et al.* 2013, Collins and Jeffery 2007).

As malaria control and elimination efforts are intensified, it is important to focus attention on all parasite species so that the non-*P. falciparum* species which can persist in the host for many months and years (Badiane *et al.* 2014, Collins and Jeffery 2007) do not serve as reservoirs for future malaria epidemics even when *P. falciparum* is eliminated.

1.7 *Plasmodium* merozoite surface proteins

The *Plasmodium* asexual life cycle in human involves both parasite and infected host proteins that are essential for the survival and continuity of the parasite's development. Proteins on the surface of merozoite elicit immune response in infected persons and contribute to protective immunity in malaria (Beeson *et al.* 2016). Important parasite proteins on the surface of the merozoite include apical membrane protein 1 (Peterson *et al.* 1989), merozoite surface proteins (Holder *et al.* 1992, Holder 1988) and erythrocyte binding antigen 175 (Camus and Hadley 1985). All these antigens are expressed as asexual blood stage antigens, and have been shown to be key targets of protective immunity mediated by antibodies (Fowkes *et al.* 2010, Osier *et al.* 2008, Polley *et al.* 2006, Polley *et al.* 2004). In spite of their antigenicity, high rates of antigenic polymorphism exhibited in these proteins have hindered their use as malaria vaccine candidates (Thera *et al.* 2011).

In this thesis I have extensively used antigens derived from merozoite surface protein 1 (also known as MSP-1_{19}) of the four human *Plasmodium* species as well as *P. falciparum*-specific MSP-1 Block 2 and MSP-2 serogroups A and B for my seroepidemiological work. These antigens have been described in more details in Section 1.7. The choice of MSP-1_{19} antigens is based on evidence that these antigens are immunogenic (Holder *et al.* 1999, Cavanagh *et al.* 1998, Holder *et al.* 1992), and that the antigens of *P. falciparum* and *P. vivax* are currently incorporated in one commercial kit that is used extensively for the screening of blood donors in many European blood banks (Seed *et al.* 2005a, Kitchen *et al.* 2004).

Since the recent isolation and characterisation of *P. malariae* and *P. ovale* MSP-1_{19} antigens (Birkenmeyer *et al.* 2010) with limited characterization of the responses to these parasite proteins in human population (Muerhoff *et al.* 2010), there has been no human study utilising all four parasite MSP-1_{19} antigens for seroepidemiological studies. The *P. falciparum*-specific MSP-1 Block 2 and MSP-2 serogroups A and B are also immunogenic and have been extensively used in studies exploring the dynamics of malaria transmission intensity and protective immunity to *Plasmodium* infection (Cavanagh *et al.* 2004, Metzger *et al.* 2003, Cavanagh *et al.* 2001, Cavanagh and McBride 1997).

1.7.1 Merozoite structure and erythrocyte invasion

The merozoites are one of three invasive stages of the *Plasmodium* parasite's life cycle. Although *Plasmodium* parasites are obligate intracellular pathogens, merozoites spend a brief extracellular life prior to host erythrocyte invasion (Bannister and Mitchell 2009, Bannister and Mitchell 2003). Merozoites are unicellular and are the smallest cells of all the *Plasmodium* life cycle stages and appear as ovoid-shaped cells, measuring ~1.6 μm long and 1.0 μm wide, and sufficiently equipped for invasion and life in the host erythrocytes (Bannister and Mitchell 2009, Bannister and Mitchell 2003, Bannister *et al.* 2000). The structure of *P. falciparum* merozoite is shown in Figure 1.7.1 on page 32.

Merozoites are covered by a proteinaceous fibrillar coat that is 15-20 nm thick (Garcia *et al.* 2008, Bannister *et al.* 1986, Langreth *et al.* 1978). Merozoites have a unique ultrastructural organization of organelles within their apical membrane, characteristic of which are the three sets of secretory vesicles: the rhoptries, the micronemes, and the dense granules (Bannister and Mitchell 2003). These apical organelles play key roles in merozoite attachment to, and invasion of host erythrocytes, as the molecules necessary for erythrocyte invasion are stored within them (Bannister and Mitchell 2003, Bannister *et al.* 2000).

The micronemes are smaller organelles, but more abundant elongated vesicles that stain densely and are clustered apically (Garcia *et al.* 2008, Bannister and Mitchell 2003). It is suggested that the microneme subpopulation may be more than one, and that this ensures sequential secretion of the different micronemal contents during the

different phases of merozoite invasion of host erythrocytes (Cowman *et al.* 2012, Singh *et al.* 2007).

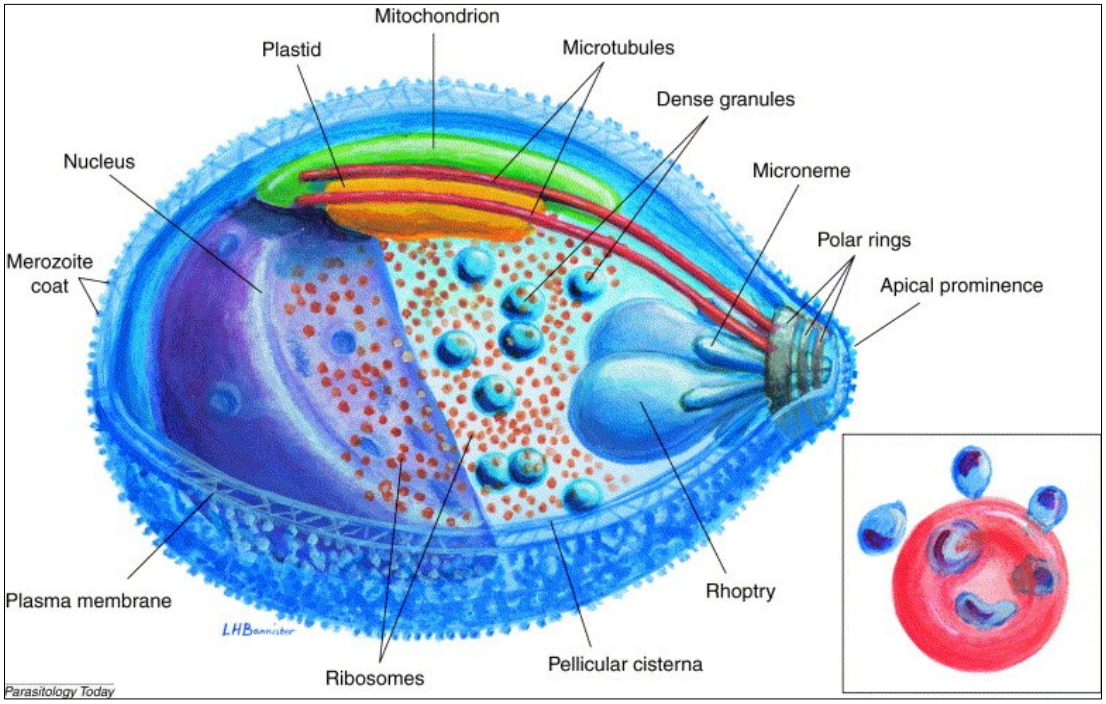


Figure 1.7.1: Structure of *Plasmodium falciparum* merozoite. Three-dimensional organization of a *Plasmodium falciparum* merozoite, with the pellicle partly cut away to show the internal structure. Inset: relative sizes of merozoites and the host erythrocyte being invaded. Figure reproduced with permission, from (Bannister *et al.* 2000).

The rhoptry are twin pear-shaped organelles, measuring 650 nm long and 300 nm wide, and hence much larger than micronemes, and have their apical ends converging on the centre of the apical prominence (Kats *et al.* 2006, Bannister and Mitchell 2003, Bannister *et al.* 2000). Rhoptries are functionally subdivided into the rhoptry neck and the rhoptry bulb, having distinct contents that are released at different time points during host erythrocyte invasion (Cowman *et al.* 2012, Kats *et al.* 2006). The release of the rhoptry neck's content has been shown to precede that of the rhoptry bulb (Kats *et al.* 2006).

The dense granules appear as small rounded vesicles scattered nearer the apical half of the merozoite (Garcia *et al.* 2008). At the apical end of the merozoites are the three polar rings that define the site for rhoptry and microneme secretion during erythrocyte

invasion by merozoites (Garcia *et al.* 2008). At the posterior-basal end are located a single nucleus, a mitochondrion, and a plastid called apicoplast that are responsible for genetic and metabolic processes (Bannister and Mitchell 2009).

In the mature merozoite both the rough endoplasmic reticulum and Golgi complex are either absent or residual (Garcia *et al.* 2008). Beneath the plasma membrane are two additional membranes, the pellicular cisterna, that form the inner membrane complex (IMC) (Cowman *et al.* 2012, Bannister *et al.* 2000). The IMC functions to provide anchorage to several accessory proteins and by extension support the actin-myosin motor within the merozoite (Farrow *et al.* 2011). The IMC together with the plasma membrane, form the merozoite pellicle that lines the whole cell with the exception of the apical end of the structure (Garcia *et al.* 2008, Bannister and Mitchell 2003).

The sequence of event leading to merozoite invasion of host erythrocytes is described below. The merozoite attaches to uninfected host erythrocytes in a process that involves binding between molecules, usually proteins, on host and parasite surfaces (depicted in the insert of Figure 1.7.1 on page 32) (Bannister and Mitchell 2009, Bannister *et al.* 2000). The parasite then reorients itself so that its apical end is in close proximity to the host cell membrane; this triggers secretion from the apical organelles (Wright and Rayner 2014, Bannister and Mitchell 2009, Bannister and Mitchell 2003, Bannister *et al.* 2000). Although the sequence of secretion is not well characterised in *Plasmodium*, in *Toxoplasma*, another apicomplexan, it is known that micronemes are the first organelle to secrete their contents, followed by the rhoptries and finally the dense granules (Carruthers and Sibley 1997). Using the actomyosin motor and a host of invasion-related molecules, merozoite are able to invade the host erythrocyte and forms a parasitophorous vacuole (Kats *et al.* 2006).

The two most essential merozoite surface proteins in terms of their relative abundance, immunogenicity and the extensive research in human malaria studies are the MSP-1 and *P. falciparum*-specific MSP-2. These antigens are the focus of the seroepidemiology work conducted in this thesis. I will discuss these antigens in more detail below with reference to the structure, location and human antibody responses to these antigens.

1.7.2 **Merozoite surface protein 1 (MSP-1) and processing**

The merozoite surface protein 1 (MSP-1) was the first surface protein to be described among the families of MSP antigens, and has been previously described as a leading malaria vaccine candidate (Holder 1988, Holder and Freeman 1982). MSP-1 has been variously referred to as gp195, p190, polymorphic schizont antigen (PSA), precursor to the major merozoite surface antigen (PMMSA), merozoite surface antigen 1 (MSA-1), and MSP-1. It is the product of a single-copy gene of about 5 kbp and the most abundant parasite protein on the surface of the asexual blood stage merozoite, constituting about 31% of the glycosylphospatidylinositol-anchored (GPI-anchored) protein coat (Gilson *et al.* 2006, Blackman *et al.* 1990, McBride and Heidrich 1987, Holder *et al.* 1985). MSP-1 is present in all four human *Plasmodium* species (Birkenmeyer *et al.* 2010, Pizarro *et al.* 2003).

Because of its abundance and presence on the merozoite surface, it is a target of antibody mediated immune responses (Cavanagh *et al.* 1998, Cavanagh and McBride 1997, Tolle *et al.* 1993). MSP-1 has an approximate molecular weight of 195 kDa and is synthesised as a precursor protein by the intracellular schizonts of both the hepatocytes and blood stage asexual cycle in the human host (Suhrbier *et al.* 1989, Holder 1988). The exact function of the protein has yet to be fully elucidated, but it is generally believed to be essential for merozoite invasion of erythrocyte, based on evidence of its surface location, relative abundance and the limited diversity in the C-terminal fragment (Beeson *et al.* 2016). In recent years, a role of MSP-1 processing in maintaining the viability of *P. falciparum* has been proposed, based on evidence that parasites with an inefficiently processed MSP1 mutant often exhibit delayed egress, while merozoites that lack surface-bound MSP1 display a severe egress defect (Das *et al.* 2015).

In *P. falciparum*, MSP-1 protein undergoes two proteolytic cleavages; a primary processing by *P. falciparum* subtilisin 1 (PfSUB1) that yields four polypeptide fragments of different molecular weights 83, 28, 38 and 42 kDa (McBride and Heidrich 1987, Holder *et al.* 1987, Holder *et al.* 1985). These fragments are held together in a non-covalent complex (Holder *et al.* 1992, McBride and Heidrich 1987) with two other proteins MSP 6 (Trucco *et al.* 2001) and MSP 7 (Kadekoppala and Holder 2010, Pachebat *et al.* 2007). Just before merozoite invasion of erythrocytes,

there is a secondary processing of the 42-kDa C-terminus fragment by PfSUB2 that yields a 33-kDa N-terminal and a 19 kDa C-terminal fragment (MSP-1_{19}) (Harris *et al.* 2005). The MSP-1_{19} fragment is retained on the surface of the merozoite during its invasion of erythrocytes (Blackman *et al.* 1990). All other parasite proteins as well as the MSP 6 and MSP 7 are shed off just before or during erythrocyte invasion (Holder *et al.* 1992). These events are shown in Figure 1.7.2 on page 36. Immunization with whole MSP 1 elicited complete protection to monkeys challenged with a homologous parasite strain (Siddiqui *et al.* 1987); however, when these monkeys were challenged with a heterologous strain, the observed protection was only partial (Hall *et al.* 1984).

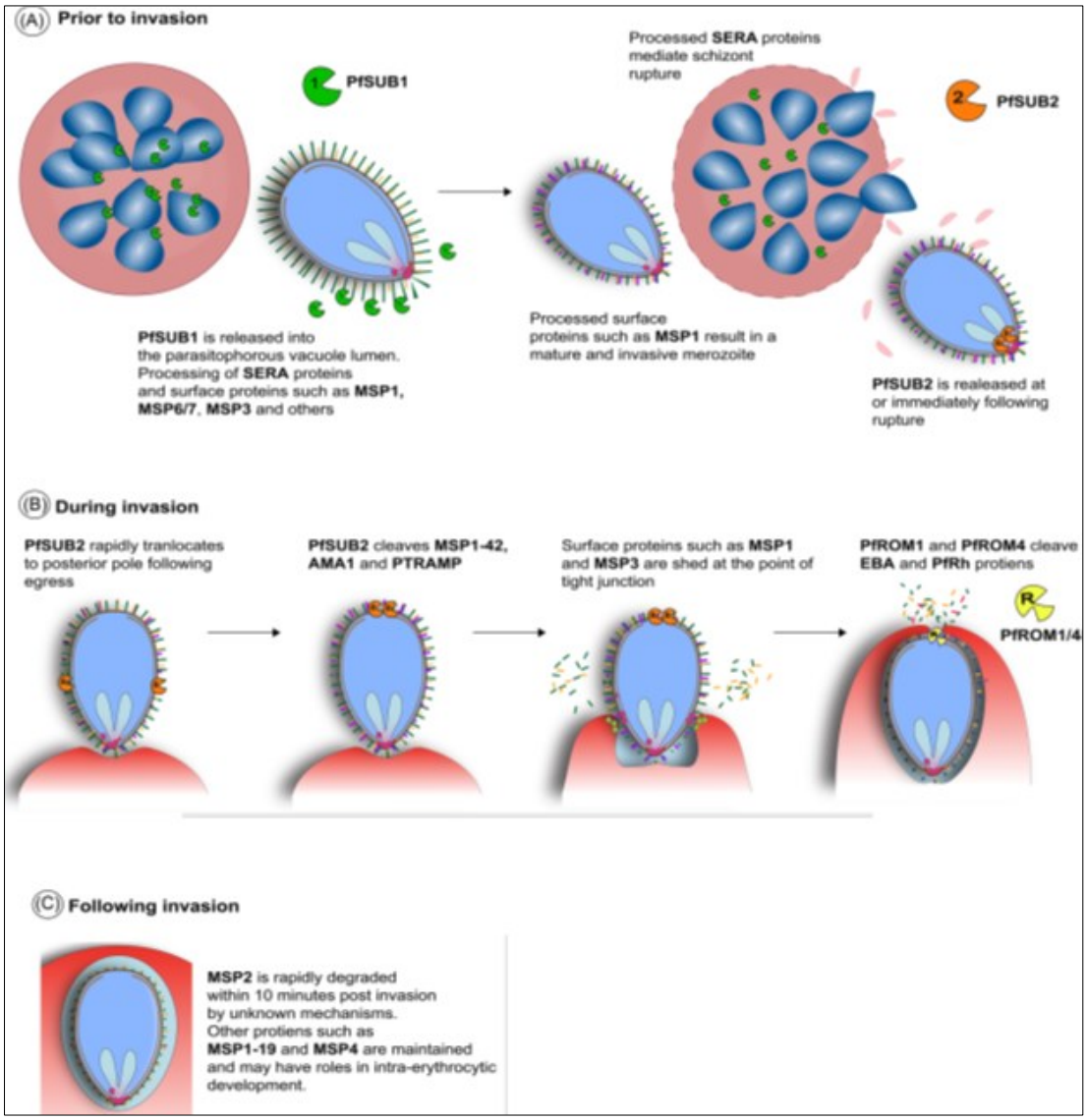


Figure 1.7.2: Processing of *Plasmodium* merozoite proteins before, during, and after erythrocyte invasion. (A) Prior to invasion, PfSUB1 is released from the merozoite into the parasitophorous vacuole lumen where it processes SERA proteins and a number of MSP. (B) Around the time of rupture, PfSUB2 is released and translocates to the apex of the merozoite. PfSUB2 cleaves MSP1-42, AMA1 and PTRAMP. During invasion, cleaved and peripherally-associated surface proteins are shed at the point of tight junction, while other proteins such as MSP2 and MSP4 are internalized during invasion. (C) Following invasion, MSP2 and other proteins are rapidly degraded, whereas $MSP1_{19}$ and MSP4, are maintained post-invasion and may have roles in intraerythrocytic parasite development. Figure taken from (Beeson *et al.* 2016).

1.7.3 **Sub divisions of MSP-1 gene**

The MSP-1 protein can be divided into 17 blocks, with sequence analysis revealing that the *P. falciparum* MSP-1 gene is made up of highly conserved (91- 96% homology), semi-conserved (65-75% homology) and polymorphic sites (10-38% homology) (Tanabe *et al.* 1989, Tanabe *et al.* 1987), as illustrated in Figure 1.7.3. For the purpose of the seroepidemiology work in this thesis, the MSP-1 Block 2, and Block 17 which contains the 19 kDa fragment, MSP-1_{19}, are of the most interest and further discussed.

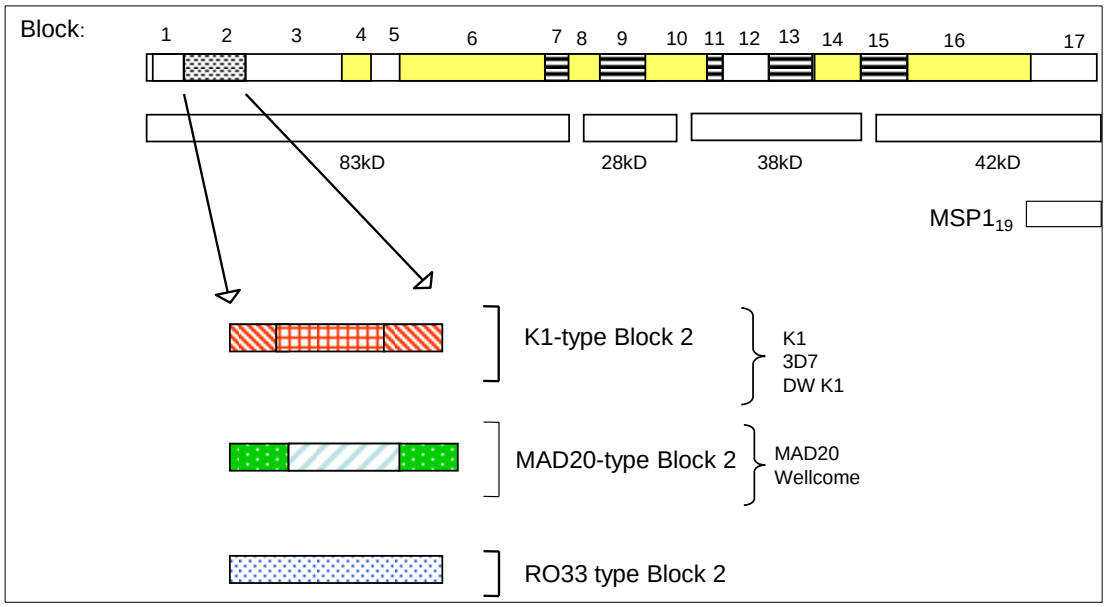


Figure 1.7.3: Schematic diagram of *P. falciparum* msp-1 gene divided into 17 Blocks according to the degree of polymorphism. Conserved (open boxes), dimorphic (full), semi-conserved (hatched boxes), polymorphic (speckled box). The four fragments produced by the primary enzymatic cleavage are illustrated below the Blocks with their respective sizes. The structures of the three allelic types of Block 2 are enlarged and examples of each type are given (Cavanagh *et al.* 1998, Tanabe *et al.* 1987).

The MSP-1 Block 1 and Block 2 antigens have been shown to respectively possess conserved and type-specific epitopes, with sera from malaria endemic areas capable of recognising these type-specific epitopes (Cavanagh *et al.* 1998). The N-terminus Block 1 is relatively conserved and contains the sequence YSLFQKEKMVL, which

is incorporated in the Spf66 vaccine (Masinde *et al.* 1998). The Block 1 has T-cell epitopes within it and at its junction with the Block 2 and is capable of inducing cellular and humoral responses (Parra *et al.* 2000, Quakyi *et al.* 1994). In a longitudinal study in eastern Sudan (Cavanagh *et al.* 1998) and a cross-sectional study in Gabon (Mawili-Mboumba *et al.* 2003), antibody responses to the conserved MSP-1 Block 1 region were rarely observed.

The most polymorphic region of the MSP-1 gene is the Block 2 which contains over a 100 variants and is found in the 83kDa N-terminus region of the MSP-1 gene (shown previously in Figure 1.7.3). The Block 2 sequences fall into three basic serotypes, named as K1, MAD20 and RO33, after representative clones in which they were first described (Certa *et al.* 1987, Tanabe *et al.* 1987, Holder *et al.* 1985). Among these three serotypes, the RO33 is largely conserved with few point mutations and without repetitive sequences (Jiang *et al.* 2000, Certa *et al.* 1987). The K1 and MAD20 serotypes have centrally polymorphic repetitive sequences comprising of tripeptide repeat patterns that are unique to each serotype; these are flanked by semi-conserved sequences (Jiang *et al.* 2000, Cavanagh and McBride 1997). The repetitive sequences in both K1 and MAD20 vary with respect to length but of approximate size of about 90 amino acid residues (Tetteh *et al.* 2005).

Unlike the Block 1 and its junction with Block 2 (Parra *et al.* 2000, Quakyi *et al.* 1994), the Block 2 region has been suggested to lack T-cell epitopes (Cowan *et al.* 2011). In both Africa and Southeast Asia, in terms of allelic frequency, Block 2 has been identified as the locus with the lowest inter-population variability, with serum IgG antibodies against the K1 and MAD20 serotypes reported to be strongly associated with protection from *P. falciparum* malaria (Conway *et al.* 2000).

The allele frequency of Block 2 varies in different geographical settings. In Africa, the K1-type predominates followed by the MAD20-type and RO33-type (Mwingira *et al.* 2011). A recent study in Mauritania showed that about 90% of *P. falciparum* isolates were of the K1 family, either carried alone or in association with the MAD20 and RO33 alleles (Salem *et al.* 2014). The allelic frequencies in Africa contrasts with that observed in Asia. For example, in Malaysia, 80% of isolates were found to be of the RO33-type, with K1-type being the least frequent, while in other Asian countries the

MAD20-type predominates followed by the K1-type and RO33-type (Atroosh *et al.* 2011).

Antibody responses to MSP-1 Block 2 are short-lived (Cavanagh *et al.* 1998), predominantly of the IgG3 subclass type followed by IgG1 in African population (Cavanagh *et al.* 2004, Cavanagh *et al.* 2001). This is contrary to higher prevalence of IgG1 to IgG3 ratios reported in South American populations (Da Silveira *et al.* 1999). A synthetic MSP-1 hybrid comprising the Block 1 and Block 2 variants has been characterised and found to be immunogenic in mice (Cowan *et al.* 2011). When antibody reactivity from Africans naturally exposed to malaria parasites were compared between individual Block 2 serotypes and the hybrid, it was observed that reactivity to MSP-1 hybrid was stronger than with the individual Block 2 serotypes with an observed association to reduced incidence of clinical malaria (Cowan *et al.* 2011).

The C-terminus MSP-1 Blocks 15-17 contain the 42 kDa fragment whose secondary cleavage product is the MSP-1_{19} that is unique in all four *Plasmodium* species (Birkenmeyer *et al.* 2010). The MSP-1_{19} contains approximately 100 amino acids, the majority of which are conserved in *P. falciparum* isolates from different malaria endemic countries (Kang and Long 1995). Generally, antibody responses to MSP-1_{19} antigens are short-lived following drug treatment of malaria and in the absence of repeated exposure (Akpogheneta *et al.* 2008, Cavanagh *et al.* 1998), and are predominantly of the IgG1 subclass (Tongren *et al.* 2006, Cavanagh *et al.* 2001, Egan *et al.* 1995). Higher levels of IgG1 in children positively correlate with reduced parasitaemia (Shi *et al.* 1996). IgG3 response against MSP-1_{19} antigens is the second most prevalent subclass observed (Tongren *et al.* 2006, John *et al.* 2004b). IgG2 and IgG4 responses against MSP-1_{19} antigens are detectable in only a minority of African populations (9.4-31%) (John *et al.* 2004b, Shi *et al.* 1996, Egan *et al.* 1995).

These observations in Africa contrasts that of studies in Brazil, in which higher frequencies of IgG2 and IgG4 subclasses against MSP-1_{19} antigens have been reported (Scopel *et al.* 2006, Scopel *et al.* 2005, Ferreira *et al.* 1998). Moreover, IgG subclass responses to MSP-1_{19} antigens appear to be unaffected by the clinical malaria state of the patient, as both asymptomatic and acutely ill individuals respond similarly (Scopel

et al. 2005). Furthermore, the age of an individual, and the seasonal variation in malaria transmission do not affect responses, as sera from all age groups usually show a similar pattern of response to these IgG subclasses (Cavanagh *et al.* 2001, Branch *et al.* 2000, Egan *et al.* 1996, Egan *et al.* 1995).

A rise in IgG response to either the Block 2 or the MSP-1_{19} is mostly observed only during or after a documented clinical malaria episode (Cavanagh *et al.* 1998). Similar to the Block 2 antigens, the overall frequency of antibody responses to the conserved C-terminal MSP-1 region (including MSP-1_{19}) also varies geographically. These frequencies have been reported to be from 45 to 60% in The Gambia (Riley *et al.* 1992), over 75% in Kenya, and about 90% in Sudan (Cavanagh *et al.* 1998). In view of this, IgG response to MSP-1_{19} antigens is a good marker for detection of exposure to malaria parasites. This is the reason for using the MSP-1_{19} antigens from all four African human *Plasmodium* parasites to investigate the seroepidemiology of human IgG antibody responses to these antigens in the two mesoendemic African populations (Zimbabwe and Sudan) investigated in this thesis. Details of the study populations are given in Chapter 2.

1.7.4 **Merozoite surface protein 2 (MSP-2)**

Merozoite surface protein 2 (MSP-2) (Figure 1.7.4 on page 41) is a 45-55 kDa protein present on the surface of the asexual blood stages of the ring, mature schizonts and merozoites of *P. falciparum* of which there is no known homologue in the other three human *Plasmodium* species (Smythe *et al.* 1988). It is the second most abundance protein on the surface of the *Plasmodium* merozoite accounting for about 21% of all GPI-anchored proteins (Gilson *et al.* 2006). Similar to the MSP-1, this protein is anchored to the cell membrane by a GPI domain and is shed from the merozoite surface prior to erythrocyte invasion by merozoites (Smythe *et al.* 1991, Smythe *et al.* 1988). MSP-2 is highly polymorphic and exhibits sequence and antigenic variation between isolates (Smythe *et al.* 1991). The protein has a central polymorphic region which constitutes over 60% of the approximately 220-residue mature polypeptide chain (Gilson *et al.* 2006, Smythe *et al.* 1991).

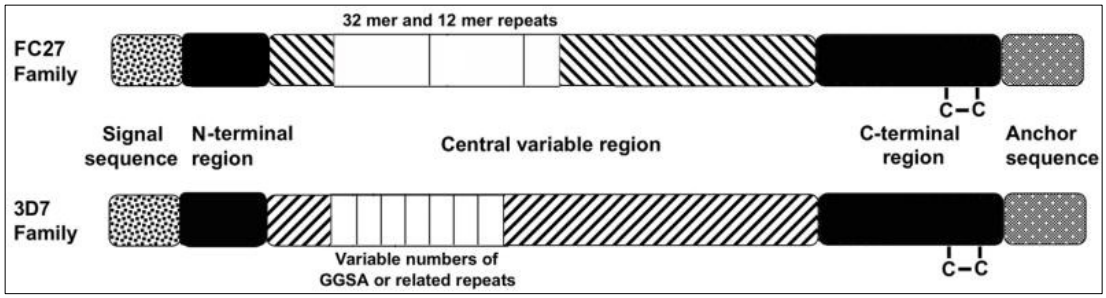


Figure 1.7.4: The structural layout of *Plasmodium* msp-2 gene. The conserved (black), repeat (white), and family specific dimorphic (hatched) regions. Adapted from (Zhang *et al.* 2008).

The MSP-2 protein exhibits variable number, length and sequence repeats and is flanked by non-repetitive motifs and by the relatively conserved N- and C-terminal domains (Smythe *et al.* 1991, Fenton *et al.* 1991). Based on the non-repetitive sequences, the different alleles of MSP-2 are grouped into two major serogroups (Fenton *et al.* 1991, Smythe *et al.* 1991), namely serogroup A which includes the strains T9/96, Camp, 3D7, and IC1, and serogroup B which includes the strains FCQ-27, K1, and Dd2 (Taylor *et al.* 1995). The extensive polymorphism in MSP-2 has been suggested to be accounted for by selection pressure of protective host immune responses (Conway and Polley 2002).

MSP-2 is highly immunogenic and induces an IgG response in persons exposed to *P. falciparum* parasites (Taylor *et al.* 1995). Antibody responses to the full-length MSP-2 variants are age dependent (al-Yaman *et al.* 1994). Allelic family-specific antibody responses are detectable in naturally immuned individuals and this is seen in the ability of sera from semi-immune individuals to react strongly to a significant number of recombinant MSP 2 antigens compared to malaria naïve individuals, who show no or weaker reactivity to varying numbers of antigens (Felger *et al.* 2003). High prevalence levels of antibody response to MSP-2 serogroups are generally observed in malaria endemic regions (Polley *et al.* 2006, Taylor *et al.* 1995). In a study of adults from The Gambia, seventy-nine percent of sera tested showed reactivity to the full length MSP-2A and 2B serogroups (Taylor *et al.* 1995), and in Kenya, as high as 100% reactivity was observed (Polley *et al.* 2006). In other studies from Papua New Guinea, antibody responses to both the MSP-2A and 2B serogroups from adults with malaria accounted

for more than 90% (al-Yaman *et al.* 1994). These antibody responses to MSP-2 antigens have been associated with protection (al-Yaman *et al.* 1994), reduced risk of severe malaria (Polley *et al.* 2006), and recognise epitopes within the dimorphic and polymorphic regions of the protein (Taylor *et al.* 1995). In the Gambian study above, the authors found no evidence of cross-reactivity between the two serogroups indicating antibody specificity to the different serogroups (Taylor *et al.* 1995).

Similar to the MSP-1 Block 2, antibody responses to MSP-2 are predominantly of the IgG3 followed by IgG1 subclass, although serogroup B responders tend to have approximately equal level of reactivity to both IgG3 and IgG1 (Taylor *et al.* 1995). This trend that is independent of age (Taylor *et al.* 1998). Again, similar to the comparison made between African and South American cohorts, the above trend is reversed in studies from South America (Tonhosolo *et al.* 2001). Several studies in which the full length MSP-2 antigens were expressed in *Escherichia coli* have shown that individuals with antibody response to these antigens have significantly reduced risk of clinical malaria episodes (Polley *et al.* 2006, Metzger *et al.* 2003, Taylor *et al.* 1998, al-Yaman *et al.* 1995).

1.7.5 General immuno-epidemiology trends to MSP antigens

General trends observed in immuno-epidemiology studies of antibody response to merozoite surface proteins (both MSP-1 and MSP-2) include higher prevalence responses in adults compared to children (Polley *et al.* 2006), higher prevalence in higher transmission areas and during peak transmission seasons than at the end of transmission seasons or during the long dry seasons when vector population is low and malaria is rare (John *et al.* 2005, Drakeley *et al.* 2005, John *et al.* 2004a, Braga *et al.* 2002), higher prevalence when blood samples are collected during the acute phase of malaria (Cavanagh *et al.* 1998), and higher prevalence in parasitaemic individuals compared to aparasitaemic cohorts (Osier *et al.* 2008, Osier *et al.* 2007, Polley *et al.* 2006, Polley *et al.* 2004, Tami *et al.* 2002, Tolle *et al.* 1993). These information on the response patterns based on transmission season and parasitaemic state of the patient were relevant in the selection of the study populations and samples used in this thesis, which are described in more details in Chapter 2.

1.8 Antibodies and immunity to malaria

Antibodies play a key role in humoral immunity to malaria. In this section I will briefly introduce the structure and functions of antibodies and their role in conferring immunity in malaria. One of the cardinal properties of antibodies which is of significant importance to the work in this thesis is their specificity to a target antigen or protein. Specificity of antibodies ensures that an immune response to a particular antigen or protein is targeted against that particular antigen or protein alone. This property of antibodies is the basis of the seroepidemiology work using *Plasmodium* MSP-1_{19} antigens described later in Chapter 3.

1.8.1 Characteristics of antibody

Antibodies are circulating proteins secreted by B-lymphocytes in vertebrates in response to exposure to antigens which the body considers foreign. An antibody is a large protein molecule that appears as a flexible Y-shape, and responsible for an organism's humoral immune responses (Abbas 2011). An antibody is a tetrameric molecule consisting of four polypeptide chains; two identical heavy chains (α, δ, ε, γ and μ) of about 50-70 kDa that pair with each other, as well as with two identical light chains (κ, λ) of about 25 kDa, covalently linked together by inter-chain disulfide bonds and by non-covalent interactions. The type of heavy chain present defines the class of antibody, and based on these heavy chains, there are five classes (also known as isotypes) of antibodies in humans and other vertebrates, namely, IgA, IgD, IgE, IgG and IgM (Abbas 2011, Paul 2008).

In humans, IgA and IgG can be subdivided into two and four subclasses respectively, namely, IgA1 and IgA2, and IgG1, IgG2, IgG3 and IgG4 (Abbas 2011, Paul 2008). The subclasses differ in the number of disulfide bonds and length of the hinge region. All these five classes of antibody molecules share the same basic structural characteristics although there is some variability among them with regards to the antigen binding sites. Variability within the antigen binding site accounts for the diversity with which the different antibodies recognise structurally diverse antigens (Abbas 2011).

Of the five immunoglobulin classes IgG is the most abundant immunoglobulin in blood and extracellular fluids, accounting for between 70 and 75% of the total serum immunoglobulin pool. It has a serum concentration of approximately 13.5 mg/mL and a half-life of between 12-25 days depending on the subclass. The secreted form of IgG exists mainly as monomers. IgG is responsible for the majority of humoral immunity against foreign antigens in human (Abbas 2011, Delves *et al.* 2011). It is the most well studied class of antibodies and due to the above characteristics has attracted most of the interest towards the development of antibody-based therapeutics for various diseases. The serological work in this thesis is also therefore mainly based on human IgG responses to *Plasmodium* merozoite proteins.

1.8.2 **Functions of antibodies**

Immunoglobulin G (IgG) in humans is involved in opsonisation, plays a role in complement activation, involved in antibody-dependent cell-mediated cytotoxicity, neonatal immunity, as well as providing feedback inhibition to the B-lymphocytes that produce them (Abbas 2011). It is the only antibody type capable of crossing the placenta. IgA is produced in large quantities for immune protection of mucosal surfaces, while IgE functions in antiparasite immunity and is also involved in immediate hypersensitivity reactions (Abbas 2011, Danilova and Amemiya 2009).

IgM appears in two forms, monomeric and pentameric forms. The monomeric form functions as a naïve B cell antigen receptor while the pentameric form is capable of activating complement. IgM is the first antibody to be produced in response to an antigenic challenge. Although δ expression follows that of μ in immature B cells it is subsequently shut down in activated B cells, and hence the function of IgD remains puzzling although it serves as a naïve B cell antigen receptor (Abbas 2011, Danilova and Amemiya 2009). Table 1.8.1 on the next page shows some of the characteristics of the different human immunoglobulin classes.

Table 1.8.1: Characteristics of human antibody classes.

Antibody class	Subclass (H chain)	Serum concentration (mg/mL)	Serum half-life (days)	Secreted form
IgA	IgA1, 2 (α1 or α2)	3.5	6	Mainly dimer; also monomer, trimers
IgD	None (δ)	Trace	3	Monomer
IgE	None (ε)	0.05	2	Monomer
IgG	IgG1-4 (γ1, γ2, γ3 or γ4)	13.5	23	Monomer
IgM	None (μ)	1.5	5	Pentamer

Abbreviations: H, heavy chain region. Table adapted and modified from (Abbas 2011).

1.8.3 Immune response to *Plasmodium* infection

Three levels of immune response to malaria parasites have been established in humans according to the parasite stages that are targeted; these are anti-infection, anti-parasite and anti-disease immune responses (Crompton *et al.* 2014, Doolan *et al.* 2009). Anti-infection immunity is targeted against the pre-erythrocytic sporozoites and infected hepatocytes (Roland *et al.* 2006, Pied *et al.* 2000). Antibodies may play a role by binding to and blocking sporozoite invasion of hepatocytes (John *et al.* 2008, John *et al.* 2005) and promote CD8+ cytotoxic T-cells killing of infected hepatocytes (McKenna *et al.* 2000, Pied *et al.* 2000). This may potentially lead to a reduction of blood-stage infection and thus manifestations of the disease. Additionally, cytokines induced by these cells, and CD4+ cells and other non-T cells are capable of killing infected hepatocytes or aiding hepatocytes to kill intracellular parasites (Roland *et al.* 2006, Pied *et al.* 2000).

Anti-parasite immunity is stage-specific, acquired slowly and over a long period of time, with the most important outcome being the decreased frequency and density of

blood parasitaemia (Doolan *et al.* 2009). Anti-disease immunity is also acquired rapidly and protects against severe malaria episodes. This level of immunity is normally observed readily in areas of intense transmission and protects children against death (Doolan *et al.* 2009). During the *Plasmodium* life cycle different parasite antigens are expressed at different stages and these elicit specific host responses. Immunity to malaria is therefore species, strain, and stage-specific (Holder 1999), and involves a concerted response from both the innate and adaptive arms of the human immune system.

1.8.3.1 Cellular immune response

Parasite antigens induce a specific immune response that causes the release of proinflammatory cytokines from mononuclear cells (Doolan *et al.* 1994), that in turn activate other host cellular machinery against both liver and blood stage antigens. Immune cells such as B and T cells, macrophages, dendritic cells and soluble pro-inflammatory cytokines such as interferon gamma (IFNγ), tumour necrosis factor (TNF) α, interleukin (IL)-12, IL-18 and anti-inflammatory cytokines IL-4, and IL-10 coordinate a regulated immune response against malaria infection (Doolan *et al.* 2009, Doolan *et al.* 1994).

Sporozoites antigens such as circumsporozoite protein and liver-specific antigens are the first to be recognized by the host and are rapidly processed on the surface of infected hepatocytes into peptides in association with the major histocompatibility complex class I (MHC-class I) and presented to cytotoxic T-cells (Weiss *et al.* 1990), leading to killing of infected hepatocytes. IFNγ produced by stimulated NK cells and CD4+ T-cells are able to trigger a cascade of immune response, leading to the death of the parasite (Wang *et al.* 1996, Weiss *et al.* 1990).

Merozoites from ruptured erythrocytes trigger TNFα response to merozoite proteins (Snounou *et al.* 2000). The effector functions of other proinflammatory cytokines such as IL-12 and IL-18 also help to propagate the immune response against *Plasmodium* parasites (Malaguarnera and Musumeci 2002). Surface antigens such as MSP-1 and MSP-2 are exposed to the host immune system and are therefore targets of host antibodies (Polley *et al.* 2006, Polley *et al.* 2004, Howard and Pasloske 1993). Host antibodies are capable of neutralizing parasite antigens, stimulating a fragment

crystallizing-mediated (Fc) killing by macrophages or activating complement pathways (Saul 1999). Naturally occurring antibodies are able to inhibit the secondary proteolytic processing of the MSP-1 protein, thereby preventing the dissociation of its C-terminus MSP-1_{19} from the entire protein complex (Nwuba *et al.* 2002).

1.8.3.2 Humoral immune responses

The serological work in this thesis is based on human IgG antibody responses to merozoite surface proteins. Humoral immune responses are important against blood-stage parasite antigens. Antibodies play a crucial role in malaria infection and have been implicated in protection against severe malaria. Acquired immunity to malaria is largely mediated by IgG (Hviid 2005), and there is evidence to suggest that passively acquired maternal IgG (Amaratunga *et al.* 2011) and secretary IgA from breast milk (Kassim *et al.* 2000) protect children under the age of six months from both severe disease and high parasitaemia (Doolan *et al.* 2009). These maternal antibodies, however, wane to undetectable levels as early as four months of life when infants begin to make their own antibodies (Duah *et al.* 2010). Maternal antibodies are acquired in the order of IgM, followed by IgG1 and IgA (Duah *et al.* 2010). IgG1 appears early in life and gradually switches towards IgG3 in older children as they become more exposed to malaria parasites (Duah *et al.* 2010). In primary infection, IgM predominate while in re-infection, IgG subclasses are the predominant isotypes seen (Garraud *et al.* 2003). Foetal haemoglobin also play a role in protection by inhibiting parasite growth and development (Snow *et al.* 1998).

Passively transferred sera from hyper-immune adults protects naïve individuals from severe malaria (Bouharoun-Tayoun *et al.* 1990). Moreover, sera from malaria endemic regions normally have antibodies to several malarial antigens (Cavanagh *et al.* 1998, Tolle *et al.* 1993), with some antigens correlating positively with protection from severe malaria in some regions (al-Yaman *et al.* 1996). These responses are however, short-lived in the absence of exposure to parasites and parasitaemia (Cavanagh *et al.* 2001), and are dependent on the longevity of the plasma cells that produce the antibodies (Duah *et al.* 2010, Akpogheneta *et al.* 2008).

Generally, anti-malarial immunity is acquired early in life, develops slowly with age, is enhanced by repeated exposure to the *Plasmodium* parasite and does not reach

'sterile immunity' (Tran *et al.* 2013, Marsh and Kinyanjui 2006, Smith *et al.* 1999), as shown by the persistent presence of gametocytes in asymptomatic adults (Doolan *et al.* 2009). The slow development of acquired immunity has been attributed to be partly due to suboptimal or defective induction of CD4+ T cells (Thompson *et al.* 2008) which are required for both antibody production and cytotoxic T cell responses (Weiss *et al.* 1993). The lack of sterile immunity is also partly explained by the extensive antigenic variation and polymorphism in several antigens of the *Plasmodium* parasite (Marsh and Kinyanjui 2006). It has been widely hypothesised that individuals may need to accumulate immune responses against various forms of the genetically distinct polymorphic antigens over a lifetime. In low endemicity, immunity in both children and adult develops slowly (Cavanagh *et al.* 1998). This is contrasted by protection against severe disease and high-density parasitaemia in people who are repeatedly exposed to the parasites, i.e. repeated exposure enhances anti-parasite immunity (Marsh and Kinyanjui 2006, Smith *et al.* 1999). Immunity against malaria wanes following a long absence of exposure to parasite and may render individuals susceptible to severe disease upon re-exposure to parasite (Doolan *et al.* 2009). A historic example was the malaria related loss of about 400,000 lives in Madagascar after malaria parasite re-emergence following intensive control measures which had reduced the exposure levels necessary for the development of acquired immunity (Romi *et al.* 2002, Mouchet *et al.* 1997, Fontenille and Rakotoarivony 1988).

Antigens with polymorphic and repetitive regions, such as MSP-2, MSP-3, MSP-7 and CSP, predominantly induce IgG3 responses, in contrast to IgG1 subclass responses that are induced by non-polymorphic and non-repetitive antigens, for example MSP-1_{19}, AMA-1, MSP-5 and MSP-6 (Tongren *et al.* 2006, Cavanagh *et al.* 2001). Within a single parasite protein, there are differential antibody responses to distinct regions (Cavanagh *et al.* 2001). For example while the MSP-1_{19} region induces an IgG1 response (Tongren *et al.* 2006, Cavanagh *et al.* 2001), responses to the polymorphic MSP-1 Block 2 is of the IgG3 subclass (Cavanagh *et al.* 2001). This may explain the short-lived responses to this unique region and requiring an individual to be continually exposed to malaria parasites to maintain protection against clinical episodes of the disease (Cavanagh *et al.* 2001). The frequency of IgG responses to MSP-2 antigens in malaria patients is very high, predominantly of the IgG3 subclass and correlate positively with age and previous malaria episodes (Taylor *et al.* 1995).

1.9 Malaria and the ABO blood group system

1.9.1 Biochemistry of ABO blood group antigens

Blood group antigens are surface markers present on the outside of the red blood cell membranes (Reid 2012). They are polymorphic traits genetically inherited from parents. There are currently 34 blood group systems recognized by the International Society for Blood Transfusion (Fung 2014, Reid 2012). The functions of many of the blood group antigens are unknown, and this is evident in individuals lacking certain antigens (such as blood group O individuals) not being seriously affected compared to those who possess it (Reid 2012). Among the blood group systems, the ABO is arguably the most immunogenic and the most important owing to its prime importance in blood group compatibility in transfusion medicine and haemolytic disease of the new born (Chung *et al.* 2005) and its associations with cancers and infectious disease (Rummel and Ellsworth 2016, Reid and Bird 1990).

The discovery of the ABO blood group systems dates back over a century ago (Larsen *et al.* 1990, Landsteiner 1900). The ABO blood group genes are located on chromosome 9q34.1-q34.2 (Yamamoto and Hakomori 1990) and are surface markers on the human erythrocytes that consist of proteins and carbohydrates attached to lipids or proteins. There are three major carbohydrate antigens expressed on glycosphingolipids and glycoproteins (A, B and H antigens) and four inheritable ABO blood types (types A, B, AB, and O) (Cooling 2015, Daniels 2002). The H antigen is the biosynthetic precursor and serves as the backbone to the A and B antigens which are the products of the ABO gene and are inherited as autosomal codominant (Cooling 2015). The group O phenotype is inherited as an autosomal-recessive and is due to a mutation that leads to premature termination of translation and, hence, no production of active glycosyltransferase (Cooling 2015, Berg *et al.* 2002).

The H antigen is synthesized by the *FUT1/H* gene, an α1,2-fucosyltransferase that adds a terminal fucose to lactosamine to form Fucα1-2Galβ1-4GlcNAc-R (Cooling 2015). The A, B and O blood groups have a common oligosaccharide structure called the O or H antigen (Berg *et al.* 2002) (see Figure 1.9.1 on page 51). For the A and B antigens a specific glycosyltransferase adds an extra monosaccharide to the basic O antigen through an α-1,3 linkage to a sub-terminal galactose moiety of the O antigen (Berg *et*

al. 2002). The type A transferase specifically adds N-acetylgalactosamine, whereas the type B transferase adds galactose (Figure 1.9.1) (Berg *et al.* 2002). In individuals with the Bombay (O_h) and para-Bombay phenotypes, the A, B, and H antigens are also absent as these individuals are unable to synthesize the basic H antigen due to mutations in *FUT1* (Cooling 2015).

In clinical practice, the ABO blood group of an individual is determined by a forward reaction for determining the presence or absence of A and B antigens on the surface of the red blood cells, and in a reverse typing for determining the presence or absence of antibody A or antibody B in the serum of an individual (Khan *et al.* 2013, Cooling 2008). Thus, the red blood cells of blood group A individuals possess antigen A with antibody B in their serum while blood group B individuals have antigen B on their red cells and antibody A in their serum. Blood type O individuals do not express antigen A nor B, but have both the A and B antibodies in their serum. Antibodies to ABO antigens are usually of the IgM class. The expression of ABO antigens is reduced on the surface of the red cells of new-borns, who also lack the ABO antibodies in the early stages of life until titres rise to adult levels between the ages of 5 to 10 years (Cooling 2010).

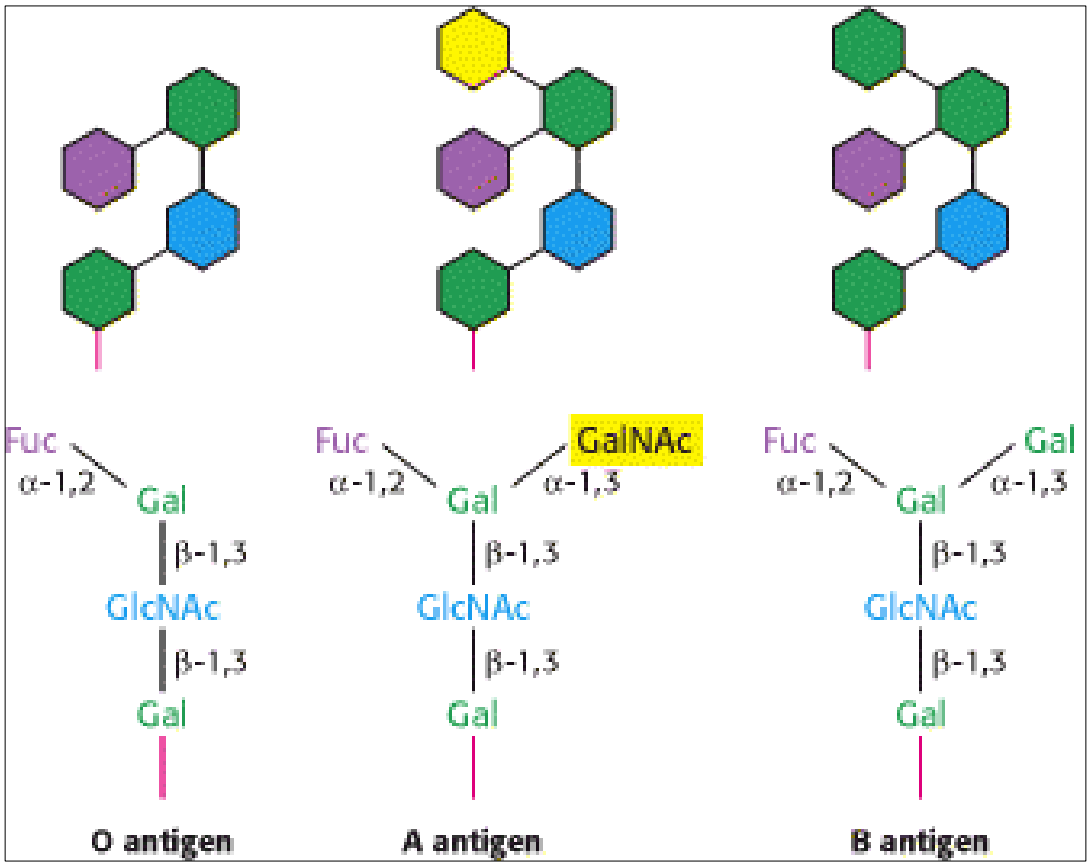


Figure 1.9.1: Structures of the O, A and B antigens. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine. Figure reproduced with permission from (Berg *et al.* 2002).

1.9.2 Malaria and the ABO blood group system

People with the most common ABO blood group type (blood group O) express neither the A nor B antigen on the surface of their red blood cells, but remain perfectly healthy, suggesting that there may be no association between the lack of expression of an ABO blood group antigens and disease (Dean 2005). However, several reports have associated a role of the ABO blood group type of the human host to increased susceptibility to certain diseases. For example, the ABO phenotype has been linked with gastric and duodenal ulcers which are more common in blood group O individuals (O'Donnell and Laffan 2001), while gastric cancer is commonly seen in blood group A individuals (Reid and Bird 1990). Another observation is that individuals with blood type O have lower plasma levels of the von Willebrand factor and clotting factor VII, two essential proteins involved in the blood clotting cascade (O'Donnell and Laffan 2001, Fuchs and Mayer 1995).

In malaria, several reports have proposed associations between the ABO blood group antigens and the development of severe or mild disease caused by *P. falciparum* (Pathirana *et al.* 2005, Beiguelman *et al.* 2003, Lell *et al.* 1999). The ABO blood group influences the susceptibility or resistance of an individual to developing severe malaria (Vigan-Womas *et al.* 2012, Fry *et al.* 2008, Rowe *et al.* 2007). Blood group O individuals have been reported to be significantly protected against the development of severe malaria through a mechanism of reduce rosetting compared with non-group O individuals (Rowe *et al.* 2007, Lowe *et al.* 1998). During rosetting, *Plasmodium*-infected erythrocytes spontaneously bind to uninfected erythrocytes (Rowe *et al.* 1995), leading to microvascular obstruction that results in severe malaria with its complications of hypoxia, acidosis, organ dysfunction and death (Kaul *et al.* 1991). This process is mediated by a sub-group of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) adhesins encoded by the large *var* gene family (Vigan-Womas *et al.* 2012, Rowe *et al.* 2009a).

ABO blood group has been identified as the main receptor for rosetting parasites, with a higher rosetting preference for blood group A erythrocytes (Vigan-Womas *et al.* 2012, Heddini *et al.* 2001, Barragan *et al.* 2000). This may possibly be due to the differential glycosylation on the surface of erythrocytes of the different blood group antigens (Fry *et al.* 2008). In *vitro* studies in support of the above showed that enzymatic removal of the A antigen on the erythrocytes and the introduction of soluble A antigen decreased *P. falciparum* rosette formation in blood group A erythrocytes (Barragan *et al.* 2000). Rosettes do form in blood group O erythrocytes but are smaller in size, easily disrupted compared to that formed in blood groups A and B, and involves receptors such as the complement receptor 1 (Rowe *et al.* 2009b) and increased phagocytosis of *P. falciparum*-infected type O erythrocytes (Wolofsky *et al.* 2012). Rosetting is more commonly seen in *P. vivax* than *P. falciparum* malaria (Lee *et al.* 2014) and is associated with anaemia (Resende *et al.* 2017, Marin-Menendez *et al.* 2013). Although *P. vivax* preferentially infects reticulocytes (Bruce and Day 2002), its rosetting complexes are commonly formed in mature erythrocytes (Lee *et al.* 2014). This has led to the suggestion that rosetting in *P. vivax* (Lee *et al.* 2014) or *P. falciparum* (Clough *et al.* 1998) may not be directly involved in merozoite invasion of host erythrocytes.

One of the host environmental challenges the schizont ruptured merozoite faces is the different red cell antigens when parasite infects individuals of different ABO blood groups. Whiles extensive research on the relationship between malaria and the ABO blood group have been done (as outlined above), there has been no study of the *Plasmodium* parasite gene expression changes when a single clone infects host of different blood groups. This knowledge gap is addressed in Chapter 6 of this thesis.

1.10 **Project aims and rationale**

Both parasite and host factors contribute to heterogeneity in the risk of developing malaria, clinical manifestation of the disease as well as the number of treatments required to clear parasites. This project endeavours to advance the knowledge and understanding behind host and parasite factors that drive heterogeneity in human malaria. Specifically, the project aims and rationale are as outlined below.

Aim 1: To determine the seroepidemiology of the different *Plasmodium* species in two mesoendemic African populations in Zimbabwe and Sudan (Chapters 3 and 4).

The above aim is achieved by developing an enzyme-linked immunosorbent assay (ELISA) based on antigens derived from *Plasmodium* merozoite surface proteins, also known as MSP-1_{19} from all four human *Plasmodium* species.

Rationale: The 'gold standard' blood film microscopy for the diagnosis of malaria has several limitations (requirement of a high level of skilled personnel, subtle difference in the morphology of *Plasmodium* species, lack of functional microscopes or electricity to run them, lack of or sub-standard reagents such as stains) (Obare *et al.* 2013, Payne 1988) that make the differentiation of the species of *Plasmodium* causing malaria very difficult, leading to under-reporting of mixed-species infections. There is therefore paucity of data on the epidemiology of the different *Plasmodium* species in many malaria endemic African populations, especially the non-falciparum species and their contribution to the overall malaria burden. This knowledge gap is the focus of using MSP-1_{19} antigens derived from four human *Plasmodium* species as a diagnostic tool to characterize the prevalence of the different *Plasmodium* species in Zimbabwean and Sudanese populations.

Aim 2: To determine if heterogeneity in clinical presentations of malaria (history of fever, body temperature and parasitaemia) and response to drug treatment is related to exposure to single *vs*. mixed-*Plasmodium* species infection (Chapter 4).

Rationale: Phenotypic differences in the biology of the four human *Plasmodium* species that cause malaria include the duration of the pre-erythrocytic and erythrocytic schizogony and the number of merozoites produced per cycle, host erythrocyte preference, and the ability to form dormant liver-stages or sequester infected

erythrocytes. These differences in the developmental cycles and the biological interaction between different *Plasmodium* species in mixed-species infections may partly influence the clinical and treatment outcomes of diseases in the host. However, the dynamics and interactions of these phenotypes when the host is infected with more than one *Plasmodium* species is not fully understood. In view of this the clinical presentation and response to drug treatment when exposed to single *vs*. mixed-*Plasmodium* species are not well documented, a knowledge gap this thesis hopes to address.

Aim 3: To determine the spatial and temporal dynamics of malaria prevalence and *Plasmodium* species distribution in a mesoendemic village in eastern Sudan (Chapter 5).

Rationale: Malaria risk can vary markedly between households within the same geographical area, but the determinants of variation in malaria risk remain poorly understood. Chemotherapy alone cannot reduce the burden of malaria even in a small geographical area. An understanding of the heterogeneity of malaria prevalence at a single village setting could have implications for designing small-area interventions targeted at eradicating disease at the local level.

Aim 4: To determine gene expression changes in 3D7 *P. falciparum* parasites as they infect erythrocytes of different ABO blood group donors (Chapter 6).

Rationale: The ABO blood group system is one of the red blood cell polymorphisms associated with a role in both infectious diseases and non-infectious diseases (Rummel and Ellsworth 2016, Reid and Bird 1990). The ABO blood group type of an infected host has an influence on the outcome of malaria, with the hosts of group O showing resistance to developing severe malaria, and the hosts of groups A and B being more susceptible to infection (Rowe *et al.* 2009b, Rowe *et al.* 2007). Moreover, malaria parasites have higher re-invasion rates in blood group A subtype A_1 than in blood group B and O individuals (Chung *et al.* 2005). Gene expression in clonal parasites when these are transmitted to hosts of different ABO blood groups have not been reported, a knowledge gap that I will be addressing.

1.11 **Thesis chapter outline**

The study conducted in this thesis explored the seroepidemiology of *Plasmodium* species infections in two mesoendemic African countries (Zimbabwe and Sudan) using antigens derived from merozoite surface proteins from four human *Plasmodium* species. The reasons for choosing these two countries are discussed in Chapter 2. The clinical presentations and treatment requirement as well as the spatio-temporal dynamics of malaria are also studied in the Sudanese cohort. Finally, the gene expression profile of *P. falciparum* infection in different blood group erythrocytes is also examined.

In Chapter 2 I provide an outline of the fieldwork carried out in Zimbabwe and Sudan that helped with sample collections and processing. I also outline the protein expression work done in the laboratory as well as the ELISA technique that was used for the seroepidemiological work described in this thesis. Statistical packages and tests used are also described.

In Chapter 3 I determined the seroepidemiology of the different *Plasmodium* species in Zimbabwe. I used competition ELISA to explore the specificity of IgG antibody responses to *Plasmodium* MSP antigens (MSP-1_{19}). Based on this I developed an ELISA-based technique using MSP-1_{19} from the four human *Plasmodium* species as a diagnostic tool for an immuno-epidemiological survey in three villages in Zimbabwe where *Plasmodium* transmission is described as mesoendemic (Hay *et al.* 2009, Mabaso *et al.* 2006).

In Chapter 4 I extend the seroepidemiology work of Chapter 3 to a village in eastern Sudan, Daraweesh, where malaria epidemiology has been studied over a period of eleven years (Creasey *et al.* 2004). The antibody responses to MSP-1_{19} antigens were categorised as single- or mixed-*Plasmodium* species and the clinical outcome (fever and parasitaemia) and the number of treatment required for parasite clearance compared between these two responder groups.

In Chapter 5 I explore the space-time analysis of malaria epidemiology at the household-level in Daraweesh to understand heterogeneity of malaria in a single village.

In Chapter 6 I investigate the gene expression changes when a single clone of *P. falciparum* 3D7 parasites infect erythrocytes of different ABO blood groups. Here I determined the growth rates of the parasite in the different blood group types as well as examining any differential expression of genes based on an individual's blood group type.

In Chapter 7 I discuss the major findings in this thesis against the body of scientific literature and summarise the key conclusions and the contribution of my work to knowledge in the field of human malaria.

Chapter 2. Study design, sample collection and materials and methods

2.1 Introduction

The overall objective of this project was to determine the host and parasite factors that drive heterogeneity in human malaria. Although several factors are important in driving heterogeneity in human malaria the two factors I have focussed on in this thesis are the epidemiology of the different *Plasmodium* species and gene expression changes when a single clone of *P. falciparum* infects erythrocytes of different blood groups.

Mixed-*Plasmodium* species infection can result in life-long chronic disease causing a wide range of morbidity such as nephropathy and chronic kidney diseases if undiagnosed and untreated (Badiane *et al.* 2014, Gilles and Hendrickse 1960). This has consequences on the health and general well-being of infected individuals. Although in malaria-endemic populations individuals may be infected with more than one species of *Plasmodium* the epidemiology of the non-*P. falciparum* species is poorly understood. This may be partly due to non-reporting of the type of infecting *Plasmodium* species diagnosed as the cause of malaria (Obare *et al.* 2013). Moreover, the conventional microscopy diagnosis of malaria has limitations (outlined previously in the general introduction) that do not allow the species to be properly reported, thereby attributing malaria to be solely caused by *P. falciparum*. Molecular methods such as PCR are not practicable in everyday diagnostic laboratories (Daniels *et al.* 2017, Nkumama *et al.* 2017), whilst the available rapid diagnostic tests (RDTs) on the market are limited to detecting only *P. falciparum* and *P. vivax* infections (Wilson 2012).

The *Plasmodium* parasite's developmental cycle in the human host involves a substantial amount of time in the host erythrocytes (Bannister and Mitchell 2003, Hawking *et al.* 1968). On the surfaces of the erythrocytes are blood group antigens, of which the ABO blood group is of much medical importance (Cooling 2015, Fung 2014). The ABO blood group has been described as an important phenotype which

influences susceptibility and resistance to severe malaria (Rowe *et al.* 2009b, Rowe *et al.* 2007). In this thesis, an aspect of this interaction I have focussed on is the gene expression changes, if any, when a single clone of *P. falciparum* parasites infect erythrocytes of different ABO blood group donors.

The study design outlined in this thesis uses quantitative analysis of epidemiological factors measured in the field associated with *Plasmodium* exposure and infection and compares the clinical outcomes (fever and parasitaemia) and treatment requirement of single- *versus* mixed-*Plasmodium* species anti-MSP-1_{19} responders in three villages in Zimbabwe, and Daraweesh, a village in eastern Sudan. It also uses bioinformatic analysis of gene expression changes in *Plasmodium falciparum* infecting different ABO blood groups. In this chapter, I describe the study design and statistical methods applied to address the specific research aims. The participants' selection criteria and epidemiological features of the study cohorts are also outlined.

2.2 Study sites and fieldwork methods

This project is made up of various studies that incorporate different populations for acquisition of data and analysis in addressing the specific aims outlined previously. Malaria parasite transmission i.e. endemicity is generally classified into four categories namely, holoendemic (transmission occurs all year long; disease affects a high proportion of the population at risk), hyperendemic (intense transmission, but with periods of no transmission during the dry season; disease affects a high proportion of the population at risk), mesoendemic (regular seasonal transmission that affects a moderate proportion of the population at risk) and hypoendemic (very intermittent transmission with disease affecting a small proportion of the population at risk) (Spencer 1963, Metselaar and Van Thiel 1959). These classifications are based on one of four indices, the entomological inoculation rate (EIR i.e. the number of infective bites per person per unit time), the spleen rate (number of palpable enlarged spleens per 100 individuals of similar ages, usually children aged 2-9 years), parasite rate (number of persons with parasitaemia per 100 individuals of similar ages) or annual parasite index (number of parasite infections in a well-defined geographical area; usually per 1,000 persons per year).

Table 2.2.1: Classification of malaria endemicity based on entomological inoculation rate, parasite rate and spleen rate.

Indices of malaria transmission	What is Measured	Holoendemic	Hyperendemic	Mesoendemic	Hypoendemic
Entomological Inoculation Rate (EIR)	The number of infectious mosquito bites per person per unit time.	>500	250 - 499	50 - 249	0 - 49
Parasite Rate (PR) (% of <5 years old children)	Proportion of the population found to carry asexual parasites in RBCs; can also assess gametocyte rates; by age group.	60-70	>50 <70	<20	0 - <10
Spleen Rate (SR) (% of <5 years old children)	The number of palpable enlarged spleens per 100 individuals of similar ages, usually children aged 2-9 years.	>75	51 - 75	>20 <50	0 - <10

Table adapted from (White 1996).

2.2.1 Criteria for selecting study sites

The blood samples for the seroepidemiology work described in Chapters 3 and 4 were collected from two African countries, Zimbabwe and Sudan, where malaria is endemic. The choice of these two countries as study sites for my research were based on the following reasons:

- malaria parasite transmission as determined by the EIR is described as mesoendemic in Zimbabwe (Mabaso *et al.* 2006, Taylor and Mutambu 1986), whilst parasite transmission in the Daraweesh village of Sudan where blood samples for the work described in Chapters 4 and 5 is described as holoendemic, with a very low EIR of 1-3 infective bites per person per year (Hamad *et al.* 2002, Hamad *et al.* 2000, Arnot 1998).
- although malaria epidemiology has been extensively studied in these two countries, there has been no focus on the epidemiology and disease burden of the non-*falciparum* species, as they are regarded as less frequent. The seroepidemiological data from this work highlights that the prevalence of the non-*falciparum* species is higher than has been reported in these two countries.
- malaria control programmes (against *P. falciparum*) are ongoing in these countries. Similar to what has been reported elsewhere (Oliveira-Ferreira *et al.* 2010), lack of knowledge of the epidemiology of the non-*falciparum* species has the potential to increase the prevalence and disease burdens of these minor species once *P. falciparum* infections decline.
- samples for the study were readily available for me to commence my work.

2.2.2 Zimbabwean samples

The plasma samples for the work described in Chapter 3 were from a cross-sectional study conducted in three villages (Burma Valley, Chiredzi and Mutoko) in Zimbabwe as part of a larger project investigating the immunoepidemiology of schistosomiasis in villages with *Plasmodium* coinfection. This work was a collaboration involving groups from the University of Zimbabwe, the National Institutes of Health Research in Zimbabwe (formerly the Blair Research Institute) and the University of Edinburgh. *Plasmodium* parasite transmission in Zimbabwe is generally described as mesoendemic (Hay *et al.* 2009, Mabaso *et al.* 2006, Mharakurwa *et al.* 2004, Taylor

and Mutambu 1986). However, malaria endemicity has been shown to be markedly influenced by altitude varying from hyperendemic in the low altitude areas to hypoendemic or absent on the central watershed (Taylor and Mutambu 1986). The seasonal peak of transmission occurs during the onset of the rainfall season from mid-February to May each year with very low transmission during the dry seasons of July to October (Taylor and Mutambu 1986).

The study sites were Burma Valley in the eastern Highlands, Manicaland Province (31°30'E; 17°45'S), where samples were collected in 1994 (Mutapi *et al.* 2000, Mutapi *et al.* 1997, Hagan *et al.* 1994), Chiredzi in the south-eastern Masvingo Province (31°90'E; 17°45'S) where samples were collected in 1999 (Mutapi *et al.* 2005, Mutapi *et al.* 2003) and Mutoko in the north eastern Mashonaland Province of Zimbabwe (32°13′E; 17°20′S) , where samples were collected in 2003 (Mutapi *et al.* 2006). All blood sampling in the three study sites were undertaken in early February prior to the annual malaria parasite transmission season, i.e. before the onset of the rainfall. The importance of this in relation to using MSP-1_{19} antigens as diagnostic tool (discussed in Chapter 3) is the demonstration that the levels of human IgG responses to MSP-1_{19} antigens are higher when blood samples are collected at the time of acute malaria and during malaria transmission seasons compared to lower levels observed when blood is taken from asymptomatic malaria patients outside of parasite transmission seasons (Braga *et al.* 2002, Cavanagh *et al.* 1998, Tolle *et al.* 1993)

P. falciparum is the predominant species of malaria parasite in Zimbabwe, with malaria transmission described largely as unstable in nature, low and sporadic (Hay *et al.* 2009, Mabaso *et al.* 2006, Mharakurwa *et al.* 2004). The study cohort consisted of 100 participants aged between five and 18 years (both males and females) in each study site (more details are given in Chapter 3). The study was school based, in which school children were recruited from the three villages. This is a common technique employed in epidemiological studies as it facilitates efficient recruitment and follow-up of school-aged individuals. IgG antibody responses to *Plasmodium* merozoite surface proteins were detected using ELISA.

2.2.3 **Sudanese samples**

Plasma samples for the work described in Chapters 4 and 5 are from an eleven year (1 January 1990 and 31 December 2000) longitudinal study of malaria epidemiology in Daraweesh, in eastern Sudan (14.02° N, 35.36° E), in the Gedaref district, 80km north of the Sudanese capital, Khartoum (Creasey *et al.* 2004). The Daraweesh area has been extensively described in previous publications (Creasey *et al.* 2004, Cavanagh *et al.* 1998, Elhassan *et al.* 1995). The principal activities in Daraweesh are farming of large scale sorghum and sesame. Daraweesh was founded by the Fulani grandfathers of many of the village elders (present at the time of the study) who migrated to Sudan from the Volta River region of present-day Burkina Faso in the 1880's (Creasey *et al.* 2004). The village is characterised by a long dry season (January to July) when malaria is rarely diagnosed, followed by a short rainfall season (August to October) after which the majority of malaria incidence is recorded (Creasey *et al.* 2004, Hamad *et al.* 2002). This seasonal pattern was observed to be the same over the 11 year study period. Vector-based measurements cannot absolutely establish the exact period of malaria parasite transmission in Daraweesh, (Arnot 1998), although malaria associated morbidities are always heavily concentrated between the months of September to November, with peak incidence in October (Hamad *et al.* 2000). Based on the EIR, malaria parasite transmission is described as holoendemic with an average infective bite per person per year estimated to be between 1 and 3 (Hamad *et al.* 2002, Hamad *et al.* 2000, Arnot 1998). The blood samples from Daraweesh for the work described in Chapters 4 and 5 were collected from individuals experiencing their first clinical malaria episode during the October to December transmission season years of 1991 to 2000.

Malaria was monitored by a village health team composed of doctors, malaria researchers and microscopists visiting from a permanent station in the nearby town of Gedaref. The team also included a health worker from Daraweesh who lived within a family compound in the village. The health team visited the village at least every second day during the malaria transmission season (September to November), and 2–3 times per week outside that period. The residents reported to the team or attended the village clinic if they had any medical complaint. Complaints or symptoms

suggestive of malaria warranted a blood smear to be made, and if found positive for malaria parasites, treatment was administered to the individual by the health team.

The first line of treatment for uncomplicated malaria was with chloroquine (given as 10-10.5 mg/kg/day), with sulfadoxine-pyrimethamine (Fansidar, given as 1.25 mg/kg body weight) treatment given in instances of chloroquine-resistant infections (A-Elbasit *et al.* 2006, Creasey *et al.* 2004). Quinine was used as a third line of treatment and also given to patients allergic to sulfadoxine-pyrimethamine (Andrews *et al.* 1990). Figure 2.4.1 on page 66 shows some photographs taken from the fieldwork in Daraweesh where the malaria experience of the residents was monitored.

2.2.4 Edinburgh samples

Samples used to generate data in Chapter 6 were collected from healthy adult volunteers from the University of Edinburgh King's Buildings. More details about these volunteers are given in Chapter 6.

2.3 Cohort selection

Both the cohorts from Zimbabwe and Daraweesh, Sudan met the following inclusion criteria:

1. Being lifelong residents of the study area
2. Having provided a blood sample for plasma extraction.

The volunteers who donated blood for the RNA work in Chapter 6 were all healthy Caucasian adults with no history of malaria and having undetectable plasma levels of IgG antibodies against MSP-1_{19} antigens of the four *Plasmodium* species tested.

2.4 Storage and transport of samples

Plasma samples collected from the study sites in Zimbabwe and Sudan were kept at -20°C and shipped to Edinburgh, UK on dry ice before placing at -80°C for long term storage until needed.

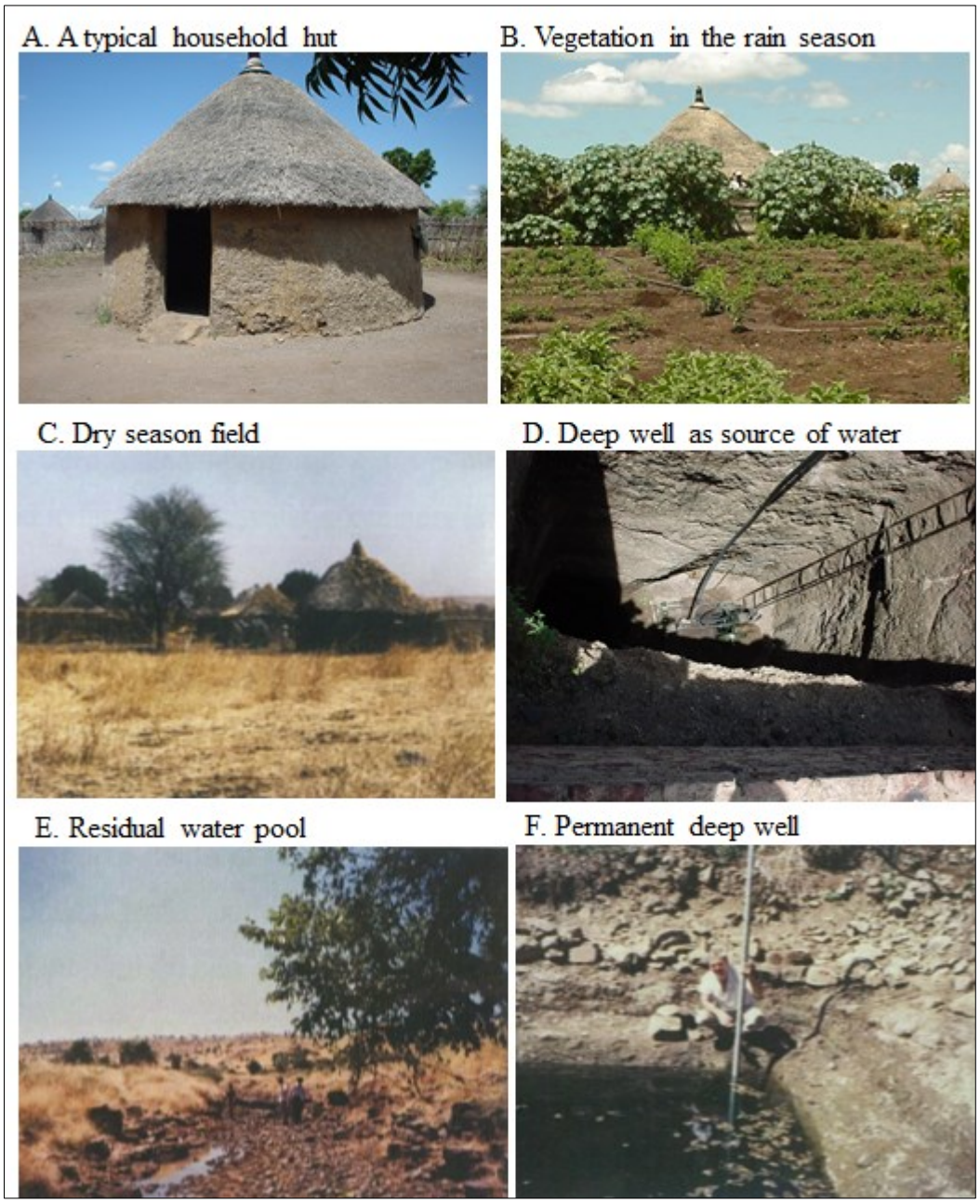


Figure 2.4.1: Photographs from the fieldwork in Daraweesh. A: a typical household hut, B: vegetation of Daraweesh in a rainy season; C: Daraweesh in the dry season; D: a well that served as a source of drinking water; E and F are respectively residual water pool and a well that served as breeding site for mosquitoes. These pools were located outside the village of Asar, 4 km south of Daraweesh. (Pictures courtesy of Prof. David Arnot, University of Edinburgh).

2.5 Ethical approval

The study in the three Zimbabwean study sites (Chapter 3) received ethical approval from the University of Zimbabwe's Ethics Review Board and the Medical Research Council of Zimbabwe. Permission to conduct the work in each of the three villages was obtained from the Provincial Medical Director, the District Educational Officer and Heads of schools in the study sites. Project aims and procedures were fully explained to the study participants and/or their guardian. Informed oral consent/assent in the local Shona language (if guardians could not read nor write) was obtained from parents/guardians, or participants if older than ten years, prior to enrolment of the participants into the study. The participants were recruited into the study on a voluntary basis and were free to withdraw with no further obligation.

The study in Daraweesh, Sudan received ethical approval from the Ethical Committee of the University of Khartoum and national clearance from the Sudan Ministry of Health. The study protocol and objectives were explained in Arabic at regular village assemblies. Informed oral consent/assent was obtained from parents/guardians, or participants if older than 10 years, prior to enrolment of the participants into the study. Participation in activities such as finger-pricking for blood sampling was entirely voluntary for all ages.

Finally, the study in healthy human volunteers in Edinburgh (Chapter 6) received ethical approval from the University of Edinburgh Ethics Committee.

2.6 Recombinant protein expression and purification

2.6.1 Overview of protein expression

Protein expression in bacteria is a widely used technique in biotechnology. It involves a DNA encoding a target protein being cloned downstream of a promoter in an expression vector (Structural Genomics *et al.* 2008). This vector is subsequently introduced into a host cell, usually a bacterium, and the cell's protein synthesis machinery produces the protein of interest. The cells are then lysed to extract the expressed protein for subsequent purification and quantification.

Escherichia coli is a Gram negative bacterium that is widely used in protein expression systems. The advantages of using this bacterium in protein expression include its

shorter culturing or replication time, its ability to grow on low cost medium, and the relative ease of genetically manipulating it (Sorensen and Mortensen 2005). Within the realm of *E. coli* expression, the T7 system is the most popular approach for producing proteins. In this system, an expression vector containing a gene of interest cloned downstream of the T7 promoter is introduced into a T7 expression host. T7 expression hosts such as DE3 strains or T7 Express strains carry a chromosomal copy of the phage T7 RNA polymerase gene (Structural Genomics *et al.* 2008, Sorensen and Mortensen 2005). When an inducer is added, T7 RNA polymerase is expressed and becomes dedicated to transcription of the protein of interest (Sorensen and Mortensen 2005).

2.6.2 **Expression of recombinant merozoite surface proteins**

The four MSP-1_{19} antigens (MSP-1_{19} Wellcome sequence) used in this thesis were expressed by myself in *E. coli* as recombinant proteins fused to the C terminus of *Schistosoma japonicum* glutathione S-transferase (GST) using pGEX vectors, as previously described (Cavanagh and McBride 1997, Smith and Johnson 1988). MSP-1_{19} antigens can also be expressed in *Saccharomyces cerevisiae*, with baculovirus/insect cell culture being another example of commonly used expression system (al-Yaman *et al.* 1996, Riley *et al.* 1992).

Plasmids of MSP-1 Block 2 antigens (3D7, MAD20, Wellcome, RO33), and MSP-2 serogroups A and B antigens were kindly donated by my co-supervisor (Dr. David Cavanagh, University of Edinburgh), and expressed in *E. coli* as GST-fusion proteins (Cavanagh and McBride 1997). The different expression systems have been cited as contributing to variations in the prevalence of antibody response to MSP-1_{19} antigen in immuno-epidemiological studies (Osier 2008). However, there is evidence from epidemiological studies showing that independent of the expression system used, strong correlations between human antibodies to the same MSP-l_{19} sequence are observed (Egan *et al.* 1995), although some correlations are seen to be weaker (John *et al.* 2004b).

All the procedures described below were carried out aseptically. *Plasmodium* MSP antigens were expressed using freshly transformed BL21 (DE3) *E. coli*. BL21 cells were thawed on ice and 1 µL of 100-200 ng plasmid DNA added to the cells and

mixed by flicking the tube. The cell-plasmid mixture was incubated on ice for 30 minutes, and the mixture heat-shocked at 42°C for 1 minute in a water bath. The mixture was transferred onto ice for 2 minutes. 500 µL of Luria broth (LB) was added and incubated at 37°C for 1 hour in a water bath. Aliquot of the mixture was pipetted and inoculated on LB agar containing 100 µg/ml of kanamycin. Plates were incubated at 37°C overnight.

After examination of bacteria growth, a single transformant colony was grown overnight at 37°C in 100 ml of LB supplemented with 100 µg/ml of kanamycin. 10 ml each of the overnight bacteria culture was dispensed into four 15 ml Cellstar tubes and centrifuged at 500*g* at 20°C for 10 minutes and resuspended in 10 ml of LB. Content of the resuspended culture was added to 1 L LB containing a final concentration of 100 µg/ml of kanamycin, and incubated with shaking at 37°C until an optical density (OD at 600nm) of the culture reached a log phase of OD 0.6. In order to monitor growth, aliquots were taken at an hourly interval.

Protein expression was induced with a final concentration of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and culture incubated in a shaking incubator at 30°C for a further 4 hours. Cells were harvested by centrifugation at 500*g* for 10 minutes at 4°C in a Sorvall SA3000 rotor. Cell paste was resuspended in 50 ml of 1X PBS (Sigma-Aldrich, P4417) and frozen down at -20°C until required. Cells were lysed by freeze-thawing and lysate treated with 1 µL/ml Benzonase (Novagen) and separated from cell debris by centrifugation at 1000*g* in GSA rotor at 4°C for 20 minutes. Supernatant was filtered through a 0.45 µm filters (Acrodisc (R) Syringe Filters, Pall Corporation, USA).

Protein purification was performed on the NGC-Chromatography machine using phosphate buffered saline (PBS) pre-equilibrated Glutathione Sepharose columns (GSTrap, GE Healthcare). After sample application, columns were washed with PBS and the GST fusion protein was then eluted with a buffer containing 1 M Tris pH8, 100 mM reduced glutathione buffer. Eluted proteins were collected and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions that contained purified product were investigated for purity by SDS-PAGE. Protein concentrations were estimated by the Bicinchoninic acid assay (BCA) (Thermo

Scientific Pierce, USA). The fractions were then pooled, dialysed against PBS and concentrated to the desired concentration by centrifugal ultrafiltration Amicon concentrator (Milipore, UK). Table 2.6.1 on the next page shows the recombinant *Plasmodium* merozoite surface proteins used for the work in this thesis.

Table 2.6.1: Recombinant *Plasmodium* merozoite surface proteins used for the study described.

Recombinant antigen	Amino acid position	Description	Reference
P. falciparum MSP-1_{19}	1584-1620	Wellcome isolate	(Burghaus and Holder 1994, Cavanagh *et al*. 1998
P. vivax MSP-1_{19}	1696-1728		Amanfo *et al*. 2016
P. malariae MSP-1_{19}	1700-1732		Amanfo *et al*. 2016
P. ovale MSP-1_{19}	1680-1711		Amanfo *et al*. 2016
P. falciparum MSP-1_B2_3D7	54-144	K1-like type	(Cavanagh and McBride 1997)
P. falciparum MSP-1_B2_MAD20	54-144	MAD20-like type	(Cavanagh and McBride 1997)
P. falciparum MSP-1_B2_RO33	54-144	RO33-like type	(Cavanagh and McBride 1997)
MSP-2_CH15019	1-184	Full length, allelic type A	(Taylor *et al*. 1995)
MSP-2_Dd2	22-247	Full length allelic type B	(Taylor *et al*. 1995)

Note: All the antigens were expressed in *E. coli*. *P. falciparum* MSP-1 Block 2 and MSP-2 antigens are designated as locus_*P. falciparum* strain.

2.6.3 **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein samples were resolved by polyacrylamide gel apparatus using the BioRad mini Protein II system. Glass plates (0.75 mm or 1.5 mm spacer plate and short plate) were cleaned with detergent and 70% ethanol, then sandwiched together and clamped in place in a casting frame and stand. SDS-PAGE was performed using gels consisting of 12% resolving gel (1.5 ml 40% acrylamide, 1.3 ml 1.5 M Tris pH8.8, 50 µl 10% SDS, 50 µl 10% APS, 2.1 ml dH_2O, 4 µl TEMED), and 4.5% stacking gel (560 µL 40% acrylamide, 0.63 ml 1M Tris pH 6.8, 50µl 10% SDS, 50µl 10% APS, 3.71 ml dH_2O, 5µl TEMED), (protocol provided by my co-supervisor).

The plates were filled to approximately 2 cm below the top of the short plate and the resolving gel was covered with isopropanol. After the gel was set the isopropanol was removed, the gel rinsed with distilled water and the stacking gel added. A 10 well comb was inserted and the gel allowed to set, after which it was rinsed with distilled water and the gel running apparatus assembled. The tank was filled with Tris-Glycine running buffer (7.55g Tris base, 47g glycine, 25 ml 10% SDS).

Protein samples were heated-up at 70^{o}C for 5 minutes and pipetted into wells (10 µL sample plus 5 µL sample buffer). Proteins were resolved alongside a protein size marker at a constant voltage of 200V for 45 minutes in the running buffer, until the bromophenol blue dye just ran off the bottom of the gel. Gels were then taken, rinsed with distilled water and stained with Coomassie blue (Life Technology) for 1 hour, followed by destaining with distilled water.

Protein concentration was measured by bicinchoninic acid (BCA) assay (Pierce, UK) and calculated against standard curve of bovine serum albumin (BSA, Sigma-Aldrich: A0336) diluted in the same buffer used for the samples.

2.6.4 **Protein quantification by Bicinchoninic acid assay**

Bicinchoninic acid assay (BCA) is a colorimetric assay for the detection and quantitation of the total protein concentration (Noble and Bailey 2009, Smith *et al.* 1985). Concentrations of the merozoite surface proteins from cell lysate were estimated by BCA (Thermo Scientific Pierce, USA) according to the manufacturer's

instruction. Duplicate test tubes containing 0.1 ml of protein standard, bovine serum albumin (BSA) or unknown samples were diluted in 2 ml of working reagent (50 part reagent A to 1 part reagent B) and incubated at 37^{o}C for 30 minutes. The tubes were cooled at room temperature for 10 minutes. Sample absorbances were measured at OD_{562nm} against blank. A standard curve of the average Blank-corrected OD_{562nm} measurement for each BSA standard *versus* its concentration in µg/mL was prepared, from which protein concentration of each unknown sample was determined.

2.7 Principles of Enzyme-Linked Immunosorbent Assay (ELISA)

The underlining principle of the ELISA technique extensively used in this thesis is the concept of a *Plasmodium*-specific antibody binding to its corresponding antigen coated on a solid phase, thereby allowing for the detection of very small quantities of these antibodies in the plasma samples of malaria patients (Hornbeck 2015, Gan and Patel 2013). There are three main types of ELISAs, namely; the direct, indirect, and sandwich ELISA all of which employ the basic steps illustrated in Appendix A.

Plasmodium antigens diluted in a suitable buffer were passively absorbed unto a 96-well polystyrene microtiter plate by hydrogen bonding and incubated for a specific time. These loose bonds allow for unbound reactants to be subsequently washed off the plate. The plates were washed and blocked with a non-specific protein (skimmed milk) to ensure the specific binding of the components added in the subsequent steps. Plasma samples diluted in the blocking buffer are added to the solid phase and incubated. An enzyme, horse radish peroxidase (HRP), for which hydrogen peroxide (H_2O_2) is a substrate, is covalently-linked to a secondary antibody that has a negligible effect on the binding properties of the primary antibody and acts as an amplifier of the signal is added (Hornbeck 2015). Unbound conjugates are washed and the action of the HRP enzyme on the H_2O_2 causes a colour change in the chromogen o-phenylenediamine dihydrochloride (OPD) (Hornbeck 2015, Hornbeck *et al.* 2001). The intensity of the colour formed is proportional to the amount of *Plasmodium*-specific antibody in the plasma. To ensure that the reaction does not proceed indefinitely, an additional step is performed by adding a stopping reagent H_2SO_4 to the

reaction. A spectrophotometer is used to quantify the amount of substance at a specific wavelength that is expressed in optical densities (OD).

2.7.1 ELISA of MSP-1 and MSP-2 antigens

In this thesis the ELISA technique used for the MSP antigens is as previously described (Cavanagh *et al.* 1998). Plasma samples were tested by ELISA for the presence of IgG antibodies able to recognize the recombinant merozoite surface proteins of the four *Plasmodium* species as an indication of recent or current exposure (Cavanagh *et al.* 1998) as well as *P. falciparum*-specific MSP-1 Block 2 and MSP-2 antigens.

96-well plates (Microlon, Greiner) were coated with 100 µL of 50 ng of each recombinant MSP-1_{19} antigens of the four *Plasmodium* species in carbonate bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) and incubated overnight at 4°C in a humidified atmosphere. Plates were washed four times in washing buffer [0.05% Tween-20 (Sigma-Aldrich: P9416) in PBS] using Skatron Skanwasher to remove unbound antigens and blotted on paper towels (Kimberley Clark 3-ply hand towels). Free binding sites in wells were blocked with 200 µL per well of blocking buffer (1% w/v skimmed milk powder in the PBS buffer) for 5 hrs at room temperature. The plates were further washed four times.

Human plasma diluted 1:500 in the blocking buffer (100 µL per well) was added in duplicate to the MSP antigens-coated wells and incubated overnight at 4°C, in accordance with published protocols (Cavanagh *et al.* 1998). After four washes, the wells were incubated for 3 hrs at room temperature with 100 µL per well of horseradish peroxidase-conjugated rabbit antihuman IgG (1:5000) (Dako Ltd, High Wycombe, UK).

Plates were washed four times to remove unbound secondary antibody before reaction development with 100 µL of substrate buffer [(0.04 mg/ml of *o*-phenylenediamine; Sigma, St Louis, MO, USA; 0.012% H_2O_2 in development buffer (24.5 mM citric acid monohydrate and 52 mM Na_2HPO4, pH 5.0)] for 10-15 min at room temperature. An unstopped positive control plate was read at an optical density (OD) 450 nm, with an OD 450 nm of 0.7-0.8 taken to be equivalent to OD 492 nm of 2.5-3.0. The reaction was stopped by the addition of 25 µL of 2 M H_2SO_4 per well, and the OD measured at 492 nm using Multiskan Ascent microtitre plate reader (Labsystems, UK).

GST protein, purified from *E. coli* transfected with pGEX-2T alone, was used as a control to determine the non-specific (background reactivity) binding of human IgG to the GST. A well containing only coating buffer also served as an additional internal control. Corrected OD values for each plasma sample were calculated by subtracting the mean OD value of wells containing control GST protein from the mean OD value obtained with each test sample MSP-1 antigen. Cut-off values at which binding of antibody from malaria-exposed individuals was regarded as significantly above background were calculated as corrected OD above the mean plus 3 standard deviation of OD readings obtained with plasma samples from eight Scottish blood donors with no history of exposure to malaria. The cut-off OD values for each MSP-1_{19} Ag were as follows: *P. falciparum* 0.217, *P. malariae* 0.114, *P. vivax* 0.110 and *P. ovale* 0.159.

Day to day and plate to plate variability in ELISA outputs are common owing the factors such as variations in room and incubating temperatures. To account for this possible outcome, the same malaria positive control, pooled plasma sample from Brefet, The Gambia, was run in duplicate at four plasma dilutions (1:500, 1:1000, 1:2000 and 1:4000), on each plate and on each day. Standard curves were plotted and the absorbances of the test samples calculated from it.

2.8 Data entry

Data such as a participant's fever history and body temperature measurements were double entered into Microsoft Office Excel (Microsoft, USA) by two independent researchers and verified for accuracy. Malaria blood film and parasitaemia count results were entered into a separate spreadsheet at the time of diagnosis and double entered in Excel. Records of blood samples that were collected and processed were recorded each day and at the end of the fieldwork transferred to Excel.

2.9 Statistical analysis

This section provides an overview of the main statistical methods used in the project to test different hypotheses, with specific details according to each chapter expanded on accordingly.

2.9.1 **Parametric tests**

In parametric statistics the data are assumed to come from a known underlying distribution (e.g. normal distribution) and hypothesis testing is based on the assumptions made (Rosnar 2000). The following parametric techniques were used for exploratory data analysis and to test for associations:

(i) Descriptive statistics was used to reveal the basic features of the data and provide summaries about the means, median and variability (standard deviations, SD and standard errors, SE) of the variables of interest.

(ii) The Pearson's correlation coefficient (r) was used to estimate the strength of association between pairs of continuous variables.

(iii) The logistic regression analysis was used to test relationships between variables while adjusting for the effects of other factors that can consist of discreet as well as continuous variables.

2.9.2 **Non-parametric tests**

When the assumptions of parametric test were not met, non-parametric tests were used instead. These tests work on the principle of ranking the data and thus the assumptions are less stringent than for parametric tests (Rosnar 2000). However, in making no assumptions about the data, the tests are sometimes considered less powerful. Non-parametric tests do not require normally distributed data and the data were therefore not normally transformed prior to conducting the test, however the test does require data that has the same distribution (Sprent and Smeeton 2001). The non-parametric statistics used included:

(i) The Chi-square (χ^2) test, used to test for associations between independent categorical variables.

(ii) The Spearman's rank correlation (p), used to measure the degree of association between two variables, appropriate for discrete data or when there were reasons to question the normality of the underlying data distribution.

(iii) The Fisher's exact test, used for to test associations between categorical variables.

Chapter 3. Seroepidemiology of *Plasmodium* species in a Zimbabwean population

Part of this work has been published I the *Malaria Journal* (Amanfo *et al.* 2016), and a copy of the publication is included in Appendix H.

3.1 Introduction

Malaria is a major public health problem in Sub-Saharan Africa, and is responsible for over half a million deaths annually, especially in children under the age of five years (WHO 2015, WHO 2013). Four major species of the protozoan parasite, *Plasmodium*, (*P. falciparum, P. vivax, P. malariae*, and *P. ovale*) cause human malaria in Sub-Saharan Africa. In malaria-endemic countries, there is an overlap in the geographic distribution of the different *Plasmodium* species and the *Anopheles* mosquito vectors that transmit these parasites. Individuals may be exposed to, and harbour multiple *Plasmodium* species (Zimmerman *et al.* 2004). Generally, the epidemiology of the non-*P. falciparum* species in malaria-endemic human populations is not well documented (Howes *et al.* 2015), with only a few specific studies on the epidemiology and clinical outcomes of *P. malariae* and *P. ovale* infections that are largely based on clinical case reports and other hospital-based studies (Sutherland 2016, Hase *et al.* 2013, Chadee *et al.* 2000). Diagnosis of malaria is important in enabling health practitioners to make informed decisions on treatment and management of malaria patients. Similarly, detection of mixed-species malaria infections helps to assess the prevalence and magnitude of the disease burden caused by these species in endemic populations.

In malaria-endemic clinical settings, disease diagnosis is predominantly by the 'gold standard' blood film microscopic examination, and rapid diagnostic tests (RDT), both of which lack sensitivity and specificity in differentiating the species of *Plasmodium* causing malaria (Wilson 2012). Microscopic examination of Giemsa stained peripheral blood smears has limitations such as the inability to detect low levels of parasitaemia, and the difficulty in species differentiation owing to subtle differences in the morphology of blood stage parasites (Mueller *et al.* 2007). This results in the

species of *Plasmodium* causing disease being rarely reported (Obare *et al.* 2013, Ohrt *et al.* 2002). In view of this almost all cases of malaria are attributed to *P. falciparum*, the species responsible for life-threatening disease (Howes *et al.* 2015). This has led to underestimates of the prevalence of both mixed-species and non-*P. falciparum* species infections (Snounou and White 2004). These non-*P. falciparum* species are of significant clinical importance; for example, *P. vivax* and *P. ovale* which form latent liver stage 'hypnozoites' are capable of causing disease several months or years after the primary infection (Coldren *et al.* 2007). Systemic diseases such as nephrotic syndrome caused by *P. malariae* several months or years after people have returned from malaria-endemic regions have been reported (Vinetz *et al.* 1998). In some cases, drug treatment failure attributable to the misdiagnosis of primary infections caused by the non-*P. falciparum* species or as co-infecting species with *P. falciparum* have been observed (Smith *et al.* 2011), while *P. malariae* has been diagnosed following antimalarial treatment of uncomplicated *P. falciparum* infections (Franken *et al.* 2012).

Even though the WHO recommends that all RDTs should be capable of detecting all four species of malaria parasite that infect humans, there is currently no available kit that is able to detect and differentiate among all four species, with most RDTs unable to differentiate the non-*P. falciparum* species (Howes *et al.* 2015). There is therefore an urgent need for additional sensitive diagnostic tools capable of rapid detection and differentiation of all four infecting *Plasmodium* species for effective treatment, management and control of malaria (Howes *et al.* 2015, Mueller *et al.* 2007, Zimmerman *et al.* 2004). This is because diagnostic tools that can detect exposure to all four parasite species, which are easy to use and readily available in settings where they are most needed will help with targeted treatment of the infecting species, increase our understanding of the epidemiology of the different species as well as their contribution to the overall malaria burden. Employing MSP-1_{19} antigens from the four human *Plasmodium* species may complement the existing tools for malaria diagnosis. In spite of the high sensitivity and specificity of PCR typing of infecting *Plasmodium* species the technique is not frequently available or applicable in many African clinical laboratory settings (Daniels *et al.* 2017), owing to its high cost and the requirement of qualified technical staff. These molecular surveys of parasite species have shown that mixed-infections comprising *P. falciparum* and/or *P. vivax* plus the low-density *P.*

malariae and/or *P. ovale* species are more common than previously reported (Daniels *et al.* 2017).

The surface of the invasive merozoite is coated in MSP-1 which constitutes 31% of the GPI-anchored proteins on *P. falciparum* merozoites (Gilson *et al.* 2006). MSP-1 is expressed by all four human *Plasmodium* species (Birkenmeyer *et al.* 2010, Pizarro *et al.* 2003). In *P. falciparum*, MSP-1 undergoes two proteolytic cleavages resulting in a C-terminal MSP-1_{19} fragment that is carried into the erythrocyte during merozoite invasion (Blackman *et al.* 1990, McBride and Heidrich 1987). Until recently, only the MSP-1 genes of *P. falciparum* and *P. vivax* had been characterized. In 2010, the sequences of the MSP-1_{19} gene fragments for *P. malariae* and *P. ovale* were determined, but there has been limited characterization of the responses to these parasite proteins in human populations (Birkenmeyer *et al.* 2010, Muerhoff *et al.* 2010). The authors found antibody detection assays of parasite exposure using recombinant MSP-1_{19} proteins to be highly sensitive, but did not determine the species specificity of the antibody responses in their assay (Muerhoff *et al.* 2010). Although the gene sequences of MSP-1_{19} antigens are unique to each of the four *Plasmodium* species, extensive homology can be found among them (Figure 3.1.1 on page 80).

For example, the number and relative positions of the cysteine residues within the C-terminal fragments of MSP-1_{19} are comparable in all four *Plasmodium* species (Birkenmeyer *et al.* 2010). In addition, there are about 32 amino acid sites within the MSP-1_{19} gene where all four parasite species share the same amino acid, and about 30 sites where the same amino acid is conserved in two or three *Plasmodium* species (Figure 3.1.1). Comparative analysis of the amino acids using the Clustal Omega programme (Sievers *et al.* 2011) shows that between any two species there is between 51 to 59.78% homology (Table 3.1.1 on page 81).

Figure 3.1.1: Sequence homology of *Plasmodium* MSP-1$_{19}$ antigens. Dots or semi-colons (. or :) indicate gene site where the same amino acid is shared between two or three *Plasmodium* species, while the asterisks (*) indicate conserved amino acid present in all four *Plasmodium* species. Pf: *P. falciparum*; Po: *P. ovale,* Pv: *P. vivax*; Pm: *P. malariae*; MSP: merozoite surface protein.

Table 3.1.1: Amino acid sequence comparison (%) of *Plasmodium* species MSP-1_{19}.

Plasmodium species	*P. falciparum*	*P. ovale*	*P. vivax*	*P. malariae*
P. falciparum	100			
P. ovale	51.65	100		
P. vivax	53.26	59.78	100	
P. malariae	51.09	54.34	56.99	100

The antigens for my work were chosen based on evidence that MSP-1_{19} of *P. falciparum* and *P. vivax* are highly recognised by more than 90% of malaria-exposed individuals in different geographical areas in Africa, Brazil and South Korea (Park *et al.* 2001, Cavanagh *et al.* 1998, Soares *et al.* 1997, Riley 1992), and that these MSP-1_{19} antigens of *P. falciparum* and *P. vivax* are frequently used either alone or in combination in many seroepidemiological studies. Moreover, the *P. falciparum* and *P. vivax* MSP-1_{19} antigens as well as *P. falciparum*-specific MSP-2 serogroups A and B antigens are components of Trinity Biotech's Captia^{TM} Malaria Total antibody EIA kit (formerly Newmarket Malaria EIA and marketed by Newmarket Laboratories Limited, UK) that is extensively used as a diagnostic kit for the screening of blood donors for malaria in many countries including the Scottish Blood Transfusion Services and other European and Australian Blood Banks (Seed *et al.* 2005a, Kitchen *et al.* 2004). Although this EIA kit's sensitivity (99.2-100%) has been shown to be comparable to that of the indirect fluorescence test, and its specificity sufficient to screen 'malaria-risk' donors, it still depends on antibody cross reactivity to identify recent *P. malariae* and *P. ovale* exposure (Seed *et al.* 2005a, Kitchen *et al.* 2004). Ideally, a pan-*Plasmodium* specific antibody test capable of detecting human exposure to all four major *Plasmodium* parasites is needed (Hemingway *et al.* 2016), and recombinant MSP-1_{19} antigens show promise in this regard (Amanfo *et al.* 2016, Muerhoff *et al.* 2010, Birkenmeyer *et al.* 2010).

In Zimbabwe over half of the population are exposed to malaria, with *P. falciparum* being the predominant species, accounting for almost all cases of the disease (Hay *et al.* 2009, Mabaso *et al.* 2006, Taylor and Mutambu 1986). There is little or no epidemiological data of exposure to the non-*P. falciparum* species and/or mixed-*Plasmodium* infections in Zimbabwe. To date, there is no commercial kit available in the market that incorporates MSP-1_{19} antigens of all four human *Plasmodium* species, and there has been no field study using all four recombinant MSP-1_{19} antigens to characterize the seroepidemiology of exposure to *Plasmodium* in any African population. This knowledge gap is addressed in this chapter with samples from Zimbabwe.

3.2 Study aim

To determine the seroepidemiology of the different *Plasmodium* species in a Zimbabwean population.

This was done by developing an ELISA based on antigens derived from merozoite surface proteins, also known as MSP-1_{19}, from four human *Plasmodium* species. This assay is capable of detecting exposure to all four human *Plasmodium* species, based on serum antibody responses to the C-terminal merozoite surface protein 1 (MSP-1_{19}). The assay is a relatively simple and inexpensive technique that could be of great applicability for epidemiological studies, the screening of blood donors and the serological diagnosis of the infecting *Plasmodium* species causing malaria if it is developed fully as a rapid diagnostic test.

3.3 Hypotheses

1. IgG antibody responses to *Plasmodium* MSP-1_{19} antigens will show species specificity.

2. People living in malaria-endemic areas in Zimbabwe are exposed to mixed-*Plasmodium* species

3.4 Materials and methods

3.4.1 Study populations

Plasma samples were collected in three Zimbabwean villages where malaria parasite transmission is described as mesoendemic (Taylor and Mutambu 1986), as part of a broader study investigating the immuno-epidemiology of schistosomiasis in villages with *Plasmodium* co-infection. The study sites were Burma Valley in the Eastern Highlands, Manicaland Province, where samples were collected in 1994 (Mutapi *et al.* 1997, Hagan *et al.* 1994), Chiredzi in the south eastern Masvingo Province, where samples were collected in 1999 (Mutapi *et al.* 2003), and Mutoko in the north eastern Mashonaland Province of Zimbabwe, where samples were collected in 2003 (Mutapi *et al.* 2006). The study cohort in each village consisted of 100 participants aged between five and 18 years (both males and females). Details of the age distribution of this cohort are shown in Table 3.4.1. Antibody responses to merozoite surface proteins from four human *Plasmodium* species were detected using enzyme-linked immunosorbent assays (ELISA).

Table 3.4.1: Age (in years) of study population from the three villages according to gender.

Study area	*Age range*	*Mean age*	*Median age*	*Male (%)*	*Female (%)*
Burma Valley	6-15	11.00	10	49	51
Mutoko	5-18	16.86	9.5	36	64
Chiredzi	7-16	15.06	11	52	48

3.4.2 Recombinant antigens

MSP-1_{19} antigens used in the ELISAs were expressed in *E. coli* transformed with pGEX-derived plasmid constructs (Polley *et al.* 2006, Cavanagh *et al.* 1998, Cavanagh and McBride 1997, Burghaus and Holder 1994) as recombinant proteins fused to glutathione S-transferase (GST), as described previously in Section 2.6.2. These were

purified by affinity chromatography using HiTrap glutathione Sepharose columns on an AKTAprime system and quantified by the Bicinchoninic acid assay.

3.4.3 ELISA procedure

The ELISA protocol has already been described in the materials and methods, in section 2.7.1.

3.4.4 Competition ELISA

Given that MSP-1_{19} antigens from the four human *Plasmodium* species share some but not all antibody epitopes, competition ELISA was performed to determine the contribution of shared or antigen-specific epitopes to antibody reactivity in plasma. Competition ELISA was performed on plasma samples from individuals with antibody reactivity (optical densities $\geq$1.0) to more than one MSP-1_{19} antigen, to assess whether human anti-MSP-1_{19} IgG antibodies specific for MSP-1_{19} were species-specific or cross-reacted with each other.

Each pre-diluted plasma sample was incubated with different concentrations of soluble MSP-1_{19} antigen, and then added to the wells of microtitre plates coated with either the homologous or heterologous MSP-1_{19} antigen. In this context, a homologous antigen is defined as identical to the competing antigen pre-incubated with the plasma sample, whereas a heterologous antigen is from a different *Plasmodium* species than the competitor antigen. The rationale of the competition ELISA is that shared epitopes between the competing and plate-bound antigens alleles will be blocked in the pre-incubation step and that appropriate antigen epitopes will react with their corresponding paratopes in the plasma, so that with increasing antigen concentration, all paratopes in the plasma react with the antigen, leaving none available to bind to antigen on the solid-phase microtitre plate (Gan and Patel 2013, Cavanagh and McBride 1997). In the case of antigens without corresponding paratopes in the plasma, there will be no prior reactivity between the antibodies and antigens, regardless of the antigen concentration, and binding to heterologous antigens will be unaffected.

The same ELISA protocol described in section 2.7.1 was followed with slight modification. Plates were coated with recombinant MSP-1_{19} Ag and incubated overnight at 4^{o}C. Plasma was diluted (1:500) and pre-incubated with increasing

concentrations (0 to 10 μg/ml) of soluble competing homologous or heterologous Ag, i.e., with up to 20-fold excess over the 0.50 μg/ml immobilized Ag to allow sera to bind to the antigen before reacting with the antigen bound on the plate, then tested on the plate-bound Ag overnight. This was followed by washing and incubation with a horseradish peroxidase-conjugated second Ab, as described previously in the indirect ELISA in section 2.7.1.

3.4.5 **Statistical analyses**

To determine if exposure prevalence derived from single species data differed from that based on mixed-species, Chi-square (χ^2) test was used. Data were analysed and graphs constructed with GraphPad Prism 7® software (San Diego, CA, USA). All differences were considered significant at $P<0.05$.

3.5 Results

3.5.1 Specificity of IgG antibodies reactivity to *Plasmodium* MSP-1_{19} antigens

300 plasma samples from the three study sites were screened by ELISA for antibody responses to MSP-1_{19} antigens. 140 of these had mixed-species responses of varying optical densities. Competition ELISA was performed on 30 plasma samples with antibody reactivity (of at least optical density ≥1.0) to more than one *Plasmodium* species MSP-1_{19} antigen. Competition ELISA showed that in plasma samples reactive against recombinant MSP-1_{19} antigens from more than one parasite species, anti-MSP-1_{19} IgG antibody responses were species-specific and did not cross-react. In positive control wells, for example, where *P. falciparum* MSP-1_{19} antigen was coated onto the microtitre plates (as capture antigen), plasma pre-incubated with *P. falciparum* MSP-1_{19} (competing homologous antigen), were inhibited from binding in a dose-dependent manner (e.g. Figure 3.5.1, red solid line). This inhibition occurred at competing homologous Ag concentration as low as 0.1 μg/ml with some plasma samples. Similar results were observed when the homologous competitor antigens were either *P. malariae* MSP-1_{19} (Figure 3.5.1., blue solid line), or *P. ovale,* or *P. vivax* (Appendix C).

To assess anti-MSP-1_{19} cross-reactivity, *P. falciparum* MSP-1_{19} antigen was coated onto microtitre plates and dual specificity sera were pre-incubated with increasing concentrations of the heterologous *P. malariae* or *P. ovale* or *P. vivax* MSP-1_{19} antigens. IgG binding to *P. falciparum* MSP-1_{19} antigen was not inhibited by soluble heterologous *P. malariae* or *P. ovale or P. vivax* MSP-1_{19}, even at concentration 20 times the capture antigen. This was also true when the coating and competing antigens were reversed in the assay (Figure 3.5.1 blue and red dashed lines, and Appendix C). The pattern of complete inhibition observed between homologous antigens and the lack of inhibition between heterologous antigens as shown in Figure 3.5.1 was typical in all the 30 plasma samples tested. The corrected absorbance readings for the 100 individuals screened in each of the three study sites are shown in Figure 3.5.2 on page 88.

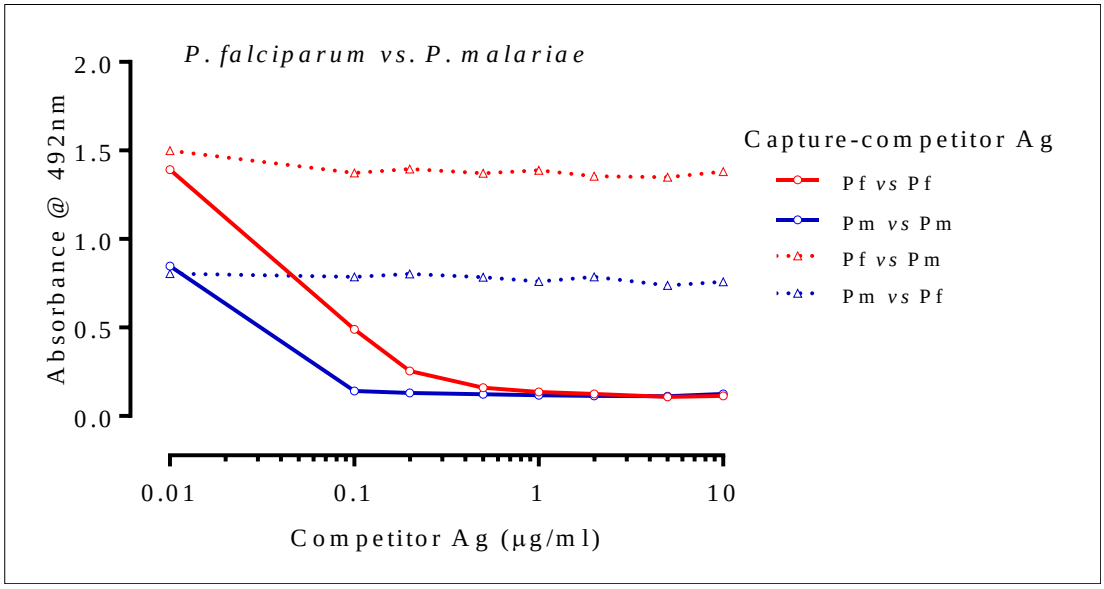


Figure 3.5.1: IgG antibodies to recombinant *Plasmodium* MSP-1$_{19}$ antigens are species specific. Plasma samples were tested at 1 in 500 dilution. Legends indicate the pairs of competing antigens used, with the well-bound capture antigen listed first and the competing homologous (control) or heterologous (test) antigen second. The capture antigens were coated at 50 ng/well. In each panel the red and blue solid lines with open circle symbols represent competition reactions involving homologous antigens, while the red and blue dashed lines with open triangle symbols represent reactions involving heterologous antigens. The y-axis indicates antibody absorbance measured at 492nm whiles the x-axis shows a titration with increasing concentrations of the competitor homologous or heterologous antigen added to the diluted plasma sample. The figure shows reactions between *P. falciparum* (Pf), and *P. malariae* (Pm). Ag is antigen. The reactions between other pairs of *Plasmodium* species are shown in Appendix C.

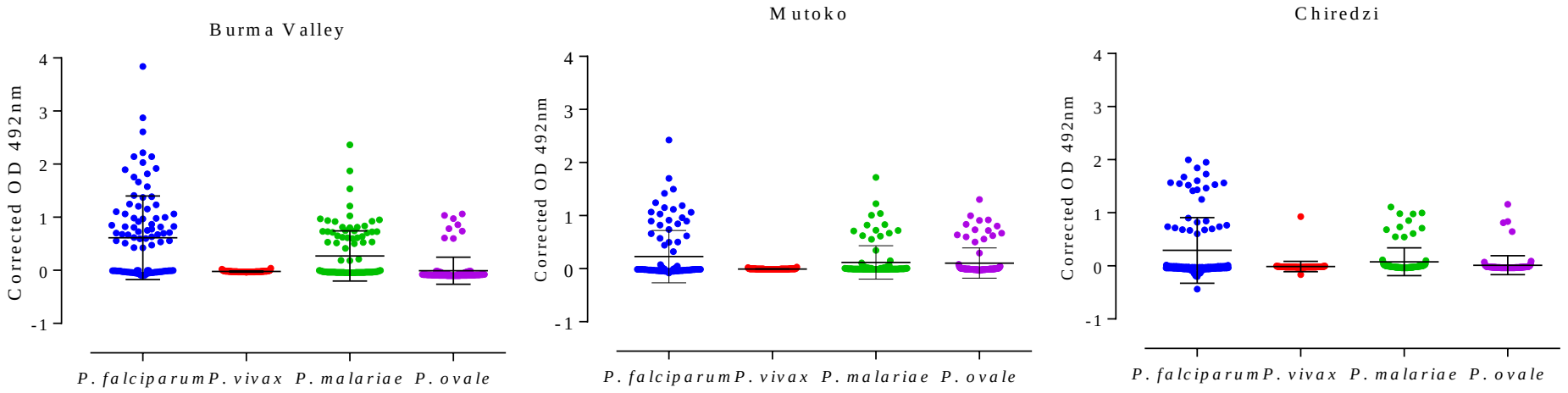


Figure 3.5.2: Scatter plots of the ELISA absorbances in each of the three study sites. Each dot is indicative of one individual.

3.5.2 **Different plasma dilutions do not affect antibody response**

My co-supervisor's laboratory (Dr. David Cavanagh) previously used checkerboard titrations to standardise its ELISA protocols and had determined an optimal serum or plasma dilution of 1:500 for immuno-epidemiological studies involving MSP-1_{19} antigens, and this has been used in previous epidemiological studies (Cavanagh *et al.* 2004, Cavanagh *et al.* 1998, Egan *et al.* 1995). This plasma dilution was chosen as optimal because it was the best compromise between sensitivity and specificity in the assay. The lack of standardized ELISA protocols with respect to serum or plasma dilution for determining antibody response to blood-stage antigens of *Plasmodium* parasites has been highlighted as a factor that accounts for variations observed in the reported prevalence of antibodies to MSP-1_{19} in different epidemiological studies (Osier 2008, John *et al.* 2005). For example, Osier highlighted in her PhD thesis two independent studies (Cavanagh *et al.* 2004, Dodoo *et al.* 1999) conducted on the same Ghanaian cohort measuring serum IgG response to the same *P. falciparum* MSP-1_{19} antigens. These studies reported different prevalence to the protein, partly due to the different serum dilutions used (Osier 2008). In these studies, while Dodoo *et al.* reported anti-MSP-1_{19} prevalence of approximately 30% at a serum dilution of 1:1000, Cavanagh *et al.* reported a higher prevalence of 56% at a serum dilution of 1:500. The higher serum dilutions used in the work by Dodoo *et al.* might partly account for the differences in the two reported prevalences, assuming the levels of antibodies present in the serum were low.

With the above differences in mind and to ensure that low concentrations of antibody responses to parasite antigens were not missed, or mixed-species responses were not misclassified as single response as a result of the plasma dilution of 1:500, individual plasma samples and a pooled plasma sample with high reactivity to single species MSP-1_{19} as well as samples with no reactivity to any parasite antigens (i.e. negative samples) were re-tested at two different dilutions, the original 1:500 and a lower dilution of 1:50 (high concentration, i.e. being only a 10^{th} of the original dilution).

As seen in Figure 3.5.3, in plasma samples with single species anti-MSP-1_{19} responses to either *P. falciparum* (panels A and B), *P. malariae* (panel C) or *P. ovale* (panel D) antigens, there was a marginal change in the absorbance at the two different plasma dilutions used. However, there was no significant change in

absorbance of the non-reacting species at the two different dilutions, indicating that mixed-species responses were not missed at the 1:500 plasma dilution. I did not use neat plasma, as this had the potential of giving a lot of background interference owing to high antibody reactivity, and also considering plasma volume of 100 µL required to perform the test. There is no plot for *P. vivax* as there was no individual with a response to this parasite antigen alone.

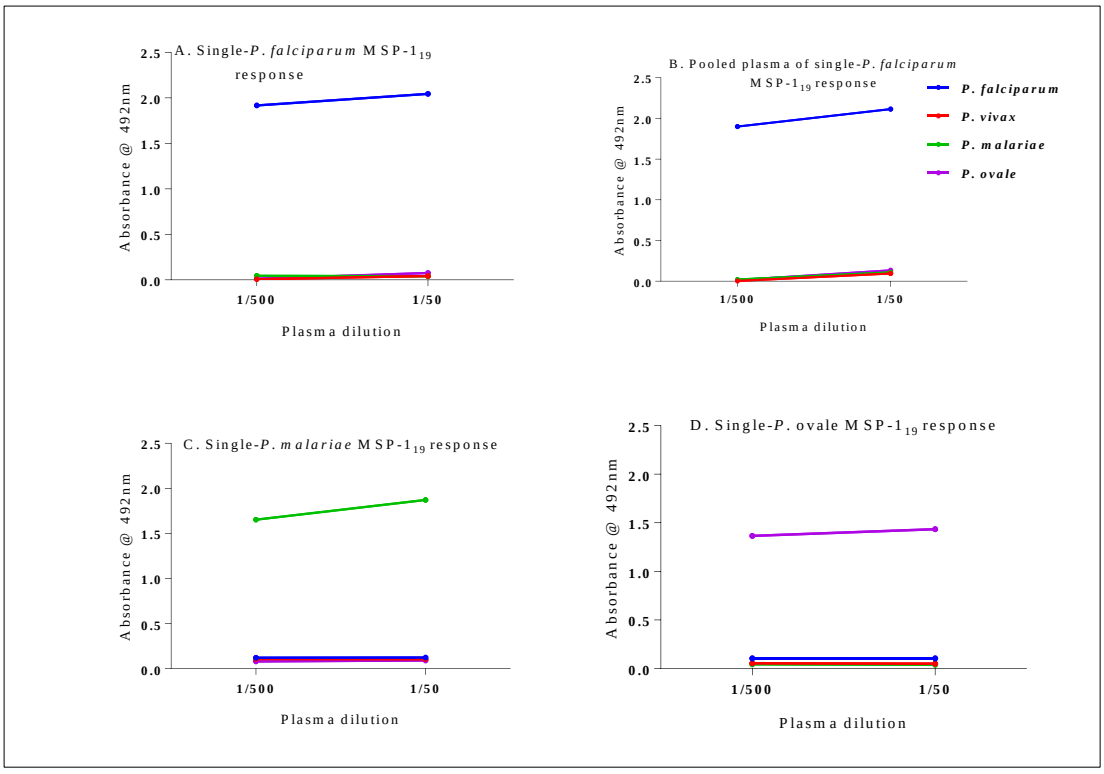


Figure 3.5.3: Plasma dilution has no effect on the specificity of IgG antibodies to MSP-1$_{19}$ antigens. Plasma samples with responses to single species were assayed by ELISA at two different dilutions (1:50 and 1:500) to determine whether the original 1 in 500 dilution did not underestimate mixed-species responses. The figure shows four independent plasma samples with single species responses to *P. falciparum* only (A), pooled samples of *P. falciparum* only (B), *P. malariae* only (C) and *P. ovale* only (D). The y-axis indicates antibody absorbance measured at 492nm whiles the x-axis shows the two dilutions of 50 and 500.

3.5.3 **Prevalence of human IgG antibodies to recombinant MSP-1_{19} from four *Plasmodium* species**

Antibody recognition of the panel of four *Plasmodium* recombinant MSP-1_{19} antigens was tested by ELISA against 100 plasma from each of the three study sites (Burma Valley, Mutoko and Chiredzi). Overall prevalence of IgG responses to MSP-1_{19} antigens in the three villages was 41.3% (95%CI: 31.6-50.9). The observed prevalence of IgG response to all recombinant MSP-1_{19} antigens was 61% (95%CI: 51.4-70.6), 31 (95%CI: 21.9-40.1) and 32% (95%CI: 22.9-41.1) in the Burma Valley, Mutoko and Chiredzi villages, respectively (Figure 3.5.4). There were no significant differences between the exposure prevalence between the villages (Burma Valley *vs* Mutoko: χ^2=0.002, df=1, *P*=0.97), (Burma Valley *vs* Chiredzi: χ^2=0.423, df=1, *P*=0.52) and (Mutoko *vs* Chiredzi: χ^2=0.001, df=1, *P*=0.97).

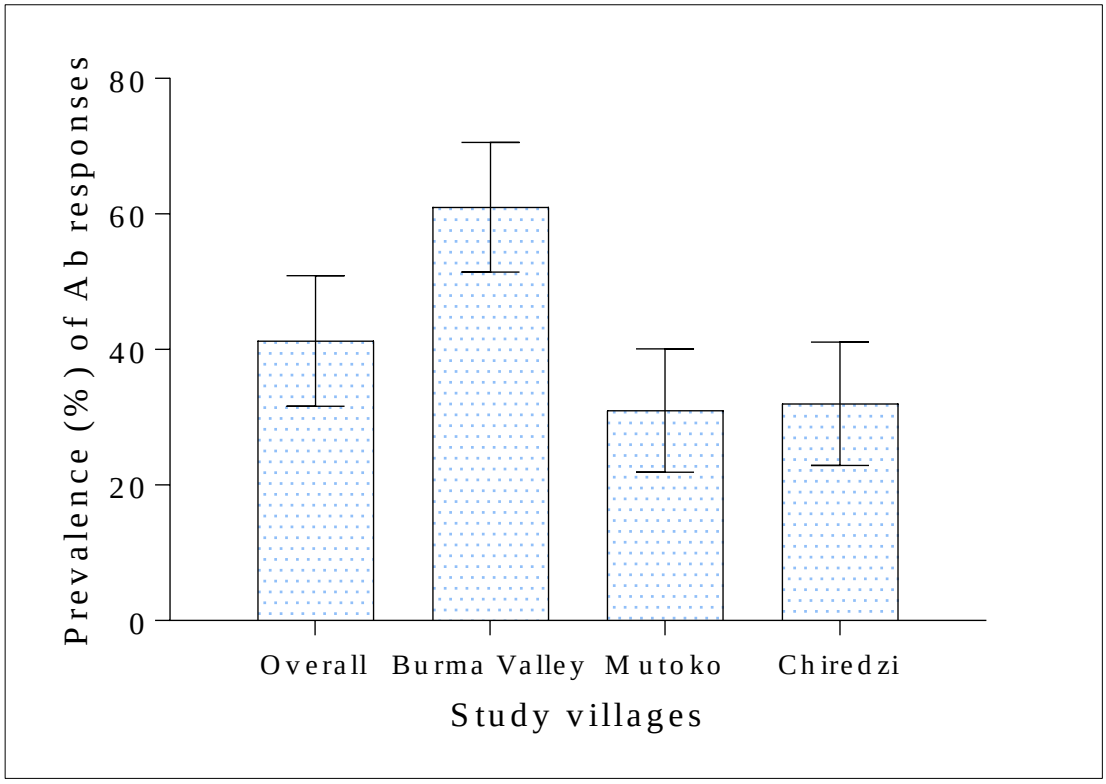


Figure 3.5.4: Prevalence of IgG response to MSP-1_{19} antigens in three Zimbabwean population. Comparisons by study village on a population of 100 participants in each study village. The error bars indicate 95% confidence intervals.

3.5.4 Single *vs*. mixed-*Plasmodium* species anti-MSP-1_{19} responses

Taking the three Zimbabwean villages as a single population, mixed-species IgG response to MSP-1_{19} accounted for nearly 50% of individuals in the study. Of the individuals with anti-MSP-1_{19} responses in all three study areas, Burma Valley (n=61), Mutoko (n=31) and Chiredzi (n=32), the prevalence of single- and mixed-species responses at a plasma dilution of 1:500 in Burma Valley and Mutoko were comparable, whiles in Chiredzi the proportion with single species responses were higher than mixed species responders (Table 3.5.1). In the single species response category, IgG antibodies were predominantly against *P. falciparum* MSP-1_{19} antigens (80, 68.8 and 78.3%) in Burma Valley, Mutoko and Chiredzi respectively. Antibody responses to *P. malariae* or *P. ovale* ranged between zero and 31.2% (Table 3.5.1). In responders with antibodies to mixed-*Plasmodium* species, antibody responses to the non-*P. falciparum* species were almost always accompanied by responses against *P. falciparum* MSP-1_{19} antigens in all three study areas (Table 3.5.1).

Mixed-*Plasmodium* species antibody responses were very common. IgG responses to *P. falciparum* and *P. malariae* MSP-1_{19} accounted for 78-87%, with responses between *P. falciparum* and *P. ovale* MSP-1_{19} accounting for ~50%. The high exposure prevalence of IgG responses to *P. malariae* MSP-1_{19} antigens suggests that infection with this parasite species is at a higher frequency in these populations than has been previously reported, and that co-infection, or co-exposure is predominantly associated with the main, usually stronger response to *P. falciparum* MSP-1_{19}.

While there were no single species responders to *P. malariae* MSP-1_{19} in Mutoko, a much higher proportion of this cohort (31.2%) responded to three parasite antigens (*P. malariae, P. falciparum,* and *P. ovale*) compared to the other two villages. Only one individual in Chiredzi had an antibody response to *P. vivax* antigen in addition to a response to *P. falciparum* (Table 3.5.1).

Table 3.5.1 Prevalence of antibody reactivity to single and mixed-*Plasmodium* species anti-MSP-1_{19}.

Study area	No Ab response	Single spp.	Mixed spp.	Single species anti-MSP-1_{19} responders			Mixed-species anti-MSP-1_{19} responders				
				P. falciparum	*P. malariae*	*P. ovale*	*P. falciparum*/ *P. malariae* only	*P. falciparum*/ *P. ovale* only	*P. falciparum*/ *P. vivax* only	*P. malariae*/ *P. ovale* only	*P. falciparum*/ *P. malariae* & *P. ovale*
Burma Valley	39	30	31	24 (80)	3 (10)	3 (10)	26 (83.9)	2 (6.5)	0	1 (3.1)	2 (6.5)
Mutoko	69	15	16	11 (68.8)	0	4 (31.2)	6 (37.5)	2 (12.5)	0	3 (18.8)	5 (31.2)
Chiredzi	68	23	9	18 (78.3)	3 (13)	2 (8.7)	6 (66.7)	0	1 (11.1)	0	2 (22.2)

Note: Values indicate the number of responders while those in parenthesis are expressed as percentage (%) of responders in the respective categories. One hundred individuals were screened in each study area.

When single species IgG responses to only *P. falciparum* were compared to mixed-species responses involving *P. falciparum* with *P. malariae* and/or *P. ovale*, no significant differences were observed in any of the three villages. When the cohort was divided into two age groups, based on the median age (i.e., those below ten years and those ten years and above), it was observed that the overall exposure prevalence in the two age groups was comparable in both Burma Valley (χ^2=3.5, df=1, *P*=0.06) and Mutoko (χ^2=0.05, df=1, *P*=0.83). However, in Chiredzi a significantly higher exposure prevalence was observed in responders aged ten years and above compared to those below ten years (χ^2=6.13, df=1, *P*=0.01) (Table 3.5.2). Differences between the two age groups in responders to single- and mixed-*Plasmodium* species in all three study areas were not compared because of the smaller sample sizes involved. An important observation was the consistent higher absolute ELISA absorbance of *P. falciparum* responses compared to the non-*falciparum* species in the context of co-infection, while single-species responses to the non-*falciparum* species always almost had a higher absorbance.

Table 3.5.2: Prevalence of antibody reactivity to single and mixed-*Plasmodium* species anti-MSP-1_{19} by age.

Study area	Age category, in years (n)	Overall prevalence (%)	Single spp.	Mixed spp.	Single species anti- MSP-1_{19} Ab responses			Mixed-species anti- MSP-1_{19} Ab responses			
					P. falciparum	*P. malariae*	*P. ovale*	*P. falciparum* /*P. malariae* only	*P. falciparum* /*P. ovale* only	*P. malariae* / *P. ovale* only	*P. falciparum*/ *P. malariae* / *P. ovale*
Burma Valley	<10 (45)	33 (73.3)	15 (45.5)	18 (54.5)	13 (86.7)	1 (6.6)	1 (6.6)	15 (83.2)	1 (5.6)	1 (5.6)	1 (5.6)
	≥10 (55)	28 (50.9)	15 (53.6)	13 (46.4)	11 (73.3)	2 (13.3)	2 (13.3)	11 (84.6)	1 (7.7)	0	1 (7.7)
Mutoko	<10 (50)	16 (32)	7 (43.8)	9 (56.2)	6 (85.7)	0	1 (14.3)	3 (33.3)	2 (22.2)	2 (22.2)	2 (22.2)
	≥10 (50)	15 (30)	8 (53.3)	7 (46.7)	5 (62.5)	0	3 (37.5)	3 (42.8)	0	1 (14.4)	3 (42.8)
Chiredzi	<10 (25)	3 (12)	2 (66.7)	1 (33.3)	2 (100)	0	0	1 (100)	0	0	0
	≥10 (75)	29 (38.7)	21 (72.4)	8 (27.6)	16 (76.2)	3 (14.3)	2 (9.5)	5 (62.5)	0	0	2 (25)

Note: Values in parenthesis are expressed as percentage (%) of responders in the respective categories. One hundred individuals were screened in each study area. Only one individual had a mixed- *P. falciparum* and *P. vivax* response in Chiredzi (data not shown on table).

3.5.5 **Discussion**

In this current study, IgG responses to recombinant MSP-1_{19} antigens (an indication of prior exposure to *Plasmodium* antigens) were determined by ELISA, from the four major human *Plasmodium* species in three Zimbabwean villages.

In the current study, the antibody response to individual *Plasmodium* recombinant MSP-1_{19} antigens in humans was seen to be highly species-specific. Furthermore, the study showed that naturally acquired human antibodies to individual species variants of MSP-1_{19} were not cross-reactive, despite the significant amino acid sequence similarities between the four *Plasmodium* MSP-1_{19} antigens. Specificity of antibody binding to its target antigen is a cardinal characteristic feature, often described as the lock and key system (Abbas 2011). This is because antibodies possess a highly complementary site structure to that of their target antigens. In experimental monkey and human studies utilizing all four *Plasmodium* species MSP-1_{19} antigens, a superior sensitivity was seen when compared to commercially available antibody assays (Muerhoff *et al.* 2010). The only commercially available kit (Malaria EIA) that incorporates MSP-1_{19} antigens is marketed by the Newmarket Laboratories, and mainly used for screening of blood donors (Seed *et al.* 2005a, Seed *et al.* 2005b). The assay is designed to detect antibodies in individuals infected with the four major *Plasmodium* species but incorporates only the MSP-1_{19} antigens of *P. falciparum* and *P. vivax* and depends on cross-reactivity in detecting the other two species (Kitchen *et al.* 2004). The package insert of this assay states that "the antigenic similarity between *Plasmodium* species means that antibodies to all species can be detected". Although the assay shows a high sensitivity and specificity against *P. falciparum* and *P. vivax*, these are lower in *P. malariae* and *P. ovale* infections (Kitchen *et al.* 2004, Chiodini *et al.* 1997), possibly due to its dependence on cross-reactivity as opposed to having the antigens of these two parasites incorporated in the assay.

As previously shown in this chapter (Figure 3.1.1 and Table 3.1.1), the sequence and phylogenetic analysis of the amino acid sequences of four *Plasmodium* MSP-1_{19} clearly shows that each parasite's sequence is unique and not simply a variant of each other, in spite of the similarities (Birkenmeyer *et al.* 2010). This suggests the need to

have a diagnostic tool that incorporates all four antigens to improve and increase the specificity and sensitivity of detecting the other two species. The specificity of IgG antibodies to these MSP-1_{19} antigens observed in this current study supports the evidence that these antigens could be used in pan-malaria diagnostic assay to enable the rapid detection of the type of *Plasmodium* species causing malaria (Muerhoff *et al.* 2010).

The overall antibody prevalence in the three villages was 41.3% ranging from 31 to 61% in the three study areas. In malaria endemic areas the prevalence of IgG antibody responses to MSP-1_{19} antigens are influenced by various factors and have been variedly reported to range from 4 to 96% (Dodoo *et al.* 1999, Hogh *et al.* 1995). Some of the factors implicated as contributing to increasing prevalence antibody response to MSP-1_{19} antigens are increasing age, (Cavanagh *et al.* 2004, Dodoo *et al.* 1999, Egan *et al.* 1995), the health status of the study population (Branch *et al.* 2000), when populations have documented clinical episodes of malaria (Dobano *et al.* 2008, Cavanagh *et al.* 1998) and when study populations are parasitaemic at the time of sampling (Osier *et al.* 2007, Polley *et al.* 2004, Tolle *et al.* 1993). The study population in Zimbabwe were of younger age group, and in 'good health' at the time of sampling, as none was hospitalised. The variation in the prevalence between Burma valley and the two villages might be accounted for by differences in the different transmission years when samples were taken in each of the villages, although all samples were collected just before the malaria transmission season.

In many malaria-endemic countries in Sub-Saharan Africa, *P. falciparum* is the predominant species that causes malaria, thus it was not surprising that the antibody response to *P. falciparum* MSP-1_{19} antigens was predominant in all three study sites. Since *P. falciparum* infections have higher parasitaemias than the other malaria parasite species (Oguike *et al.* 2011, Bruce *et al.* 2008), it is likely that individuals will have a stronger immune response to *P. falciparum* infection. One of the novel findings from this study was the indication that the exposure prevalence of *P. malariae* and *P. ovale* is higher than expected, as previous reports have attributed about 98% of all malaria cases in Zimbabwe to be caused by *P. falciparum* (Hay *et al.* 2009, Mabaso *et*

al. 2006). More importantly, the observed higher exposure prevalence of *P. malariae* in the Burma Valley district was striking, as reports suggest that this species only accounts for between 1 and 2.6% of all malaria cases diagnosed by light microscopy (Langford *et al.* 2015, Scopel *et al.* 2004, Al-Maktari and Bassiouny 2003). Microscopy has long been known to underestimate the prevalence of the non-*P. falciparum* species owing to difficulties in distinguishing the subtle differences in the morphology of the different species as well as the challenge posed in detecting minority species in a blood film with high density *P. falciparum* parasitaemia. It is therefore not surprising that these assays detected a higher sero-prevalence of these species, as this also reflects recent and concurrent parasite exposure. Studies employing nucleic acid based techniques for *Plasmodium* parasite detection and species identification in some African countries have reported prevalence of the non-*P. falciparum* species to be between 1 and 17% (Oguike *et al.* 2011, Bruce *et al.* 2008).

While antibody responses to single species *P. falciparum* antigens were common, single species responses to *P. malariae* and *P. ovale* antigens were infrequently detected. A significant proportion of individuals with IgG responses to *P. malariae* and/or *P. ovale* MSP-1_{19} almost always had responses to *P. falciparum* MSP-1_{19}. This results support the findings of a recent study in Ghana that reported frequent detection of *P. malariae* and *P. ovale* in individuals who were also PCR positive for *P. falciparum* (Dinko *et al.* 2013), and a previous findings in Ivory Coast in which *P. malariae* infections were always seen in mixed-infections with *P. falciparum* (Black *et al.* 1994). The plasma dilution (concentration of plasma IgG antibody) used did not affect the responses generated, with single species responders at both 1:50 and 1:500 plasma dilutions giving the same antibody response type. Existing kit uses undiluted plasma or serum (Kitchen *et al.* 2004), and would likely pick up much higher amounts of antibodies if adapted for the four antigens. My assay was not compared to any of the existing kits which could have added extra value to my claim of species specificity. However, the laboratory of my co-supervisor is undertaking a comparative study between the four *Plasmodium* MSP-1_{19} antigens and the Malaria EIA kit from the Newmarket Laboratory using the Zimbabwean and Daraweesh blood samples I used in my study, samples from two other African epidemiological settings (Gabon and The

Gambia), and malaria positive blood samples from the Scottish Blood Transfusion Services. The outcome of this comparative study would further demonstrate the sensitivity and specificity of these MSP-1_{19} antigens as potential diagnostic tools for accurate detection of the species of *Plasmodium* responsible for malaria.

The reasons for this co-occurrence of the non-*P. falciparum* species with *P. falciparum* may be both epidemiological and biological. Of the epidemiological reasons, it has been suggested that the same *Anopheles* mosquito circulating in a population might be responsible for the simultaneous or sequential inoculation of the different species (Snounou and White 2004), thereby increasing the likelihood of multiple species infections. Biological reasons may include selective advantages for these minor species when co-infecting with *P. falciparum*. For example, due to density-dependent regulation of immune responses directed against the majority species (*P. falciparum*), these non-*P. falciparum* species may be able to evade host immune responses and establish disease (Bruce and Day 2002, Bruce *et al.* 2000). There are also parallels in other infectious diseases, such as the obligate satellite virus hepatitis D, which is unable to establish disease independent of hepatitis B virus (Pascarella and Negro 2011). Hepatitis D virus co-infection in Hepatitis B-infected individuals worsens hepatic damage and inflammation, and is more likely to lead to hepatocellular carcinoma (Ho *et al.* 2013, Yurdaydin *et al.* 2010). The results show some single species *P. malariae* responses, indicating that this species is capable of establishing infection independent of other *Plasmodium* species. However, the significant proportion of individuals with co-occurrence of antibody responses to *P. falciparum* suggests a possible dependency on *P. falciparum* receptors or proteins for successful disease by *P. malariae*. These non-*P. falciparum* species, which usually exist as part of a complex mixed-infections with *P. falciparum* (Kasehagen *et al.* 2006, Zimmerman *et al.* 2004) may cause chronic, sub-clinical disease with potential health consequences, including treatment failure, disease relapse and long-term systemic consequences (Smith *et al.* 2011, Coldren *et al.* 2007, Vinetz *et al.* 1998). A recent study in Indonesia found *P. malariae* to be associated with a lower mean haemoglobin, nephrotic syndrome and death (Langford *et al.* 2015).

In mixed-species responders the absorbance of antibody responses to *P. falciparum* was always higher than the co-infecting species. This may be explained by the fact that malaria caused by *P. falciparum* is more common in malaria endemic areas and hence individuals may be more likely to be first exposed or infected with this species and subsequently with the non-*falciparum* species. Additionally, repeated exposure to this predominant *P. falciparum* species may result in boosting the antibody responses individuals make against this species compared to the non-*falciparum* species which are less frequently encountered.

Antibody responses to *P. vivax* MSP-1_{19} were rarely observed in this study population. *Plasmodium vivax* requires the presence of Duffy antigen on host erythrocytes to establish a successful infection (Miller *et al.* 1976). *P vivax* is predominantly endemic in Asian and Latin American countries where this Duffy antigen is prevalent on the surfaces of host erythrocytes. It has long been reported that the Duffy antigen is absent in most African populations (Miller *et al.* 1976) and this confers some protection against *P. vivax* malaria (Carter and Mendis 2002). It was therefore not surprising to observe a low frequency of responses to *P. vivax* MSP-1_{19}, which is consistent with a report obtained from nine African countries where no *P. vivax* was found despite extensive PCR screening (Culleton *et al.* 2008). In recent years however, there have been reports of *P. vivax* infections in both Duffy positive and negative individuals in Cameroon, suggesting that this species might have evolved and adapted to using other receptors to invade erythrocytes and establish disease (Fru-Cho *et al.* 2014).

Serological responses have generally been reported to increase with age (Perraut *et al.* 2005, Perraut *et al.* 2003, Egan *et al.* 1996, Egan *et al.* 1995). In this present study, age was not a confounding factor in Burma Valley and Mutoko, while in Chiredzi responders ten years old and above had higher overall frequencies of IgG antibodies to MSP-1_{19} antigens. Although all three villages are described as mesoendemic, the observed differences in age responses could be explained by the respective transmission dynamics of the different seasons in which sampling was done. In very low and unstable malaria transmission areas such as Daraweesh in eastern Sudan, reports suggest that the age dynamics associated with malaria and serological

responses are not apparent, as malaria affects all age groups (Giha *et al.* 1998, Roper *et al.* 1996).

This assay utilised IgG antibodies to detect both recent and long-term exposure (at least within 2-3 months after exposure) to *Plasmodium* parasite MSP-1_{19} antigens. Since antibodies to these antigens often wane in the absence of continuous exposure to parasite antigens (Cavanagh *et al.* 1998), its use in detecting exposure in individuals whose blood samples are taken long (more than six months) after they have cleared parasites by drug treatment may be of limited value. This however excludes blood samples taken at the acute phase of infection and stored appropriately until use, such as is the case of these Zimbabwean samples. The importance of this in malaria control is that when this assay is used in epidemiological surveys during the peak of malaria transmission seasons, there is the potential of detecting a higher prevalence of responses in a population compared to when it is used for surveys long after the transmission season when most individuals would have cleared their infections leading to reduced levels of antibody to parasite antigens. The assay can also be modified to detect IgM antibodies which are the first antibodies detectable following acute infections.

Enzyme labels in immunoassays have been significant and sensitive techniques since their introduction over four decades ago (Engvall and Perlmann 1971). The technique has become a standard procedure that is widely used in studying serological responses to parasitic infections and as diagnostic tool to demonstrate the appearance of antibody or to detect parasite antigens. One major advantage of the ELISA test is the ability to use this technique for large-scale batch testing in epidemiological studies (Rajasekariah *et al.* 1991). In spite of these, methodological issues with regards to ELISA standardisation are cited as contributing to variations observed in antibody prevalence in populations (Osier 2008, John *et al.* 2005).

The sensitivity and specificity of an ELISA test are influenced by almost every stage of the assay protocol. Being the major technique I used in this thesis, I ensured that internal quality controls were adhered to, to ensure the reliability and reproducibility of the results obtained. Antigen coating carried out at 4°C usually causes an ‘edge

effect' resulting in the cooler, outer wells of the microtitre plates binding less antigen than the inner wells (Venkatesan and Wakelin 1993, Oliver *et al.* 1981). To overcome this, I ensured that plates were incubated overnight in wrapped wet towels ensuring a humidified environment to minimise the 'edge effect'. It has been reported that, when microtitre plates are stacked, variations are usually observed between the lower and upper plates and the middle plates when incubation steps involve changes in temperature, such as from room temperature to 4°C or 37°C (Venkatesan and Wakelin 1993). Since my work involved working on many plates at a time, plates had to be stacked during incubation periods. To minimise any variations, a maximum of four plates were stacked at a time. Maximising specific antigen to antibody binding while minimising nonspecific binding are essential to improving the sensitivity and specificity of ELISA test. These were achieved through thorough washing steps using the detergent Tween 20 in phosphate-buffered saline (PBS) (Gardas and Lewartowska 1988), the use of skimmed milk as a blocking agent which is regarded as superior to others such as casein or bovine serum albumin (Venkatesan and Wakelin 1993), and diluting plasma samples in PBS containing skimmed milk. Protein bound to these antibody then reduces the nonspecific binding of the antibody.

The sensitivity of an ELISA is also affected by the plasma or serum dilution used (Venkatesan and Wakelin 1993). In my study, I used a plasma dilution of 1:500, as previously established by my co-supervisor's laboratory as the optimal dilution that gives the best compromise between sensitivity and specificity for MSP-1_{19} antigens (Cavanagh *et al.* 1998). To confirm this, plasma samples from single- and mixed-species responders were tested at two different dilutions (the standard 1:500, and 1:50 dilutions). It is reasonable to speculate that assays at 1:50 dilution should permit detection of a tenfold increase in antibody binding, compared to the original 1:500 dilution. However, the results showed no change in the outcomes of the response between the initial 1:500 plasma dilution and the increased antibody concentration at plasma dilution of 1:50. This confirms that the previously established plasma or serum dilution of 1:500 for serological work involving *P. falciparum* MSP-1_{19} antigens (Cavanagh *et al.* 1998) is applicable for these four antigens. It must be noted, however, that higher plasma concentrations may have potential inevitable consequence of

increasing background binding. While the sensitivity of the ELISA test may be affected by sample dilution, with a lower dilution favouring this (Venkatesan and Wakelin 1993), sample economy and nonspecific reactions may also influence the dilution taken to achieve the required endpoint (Venkatesan and Wakelin 1993). One of the main problem with ELISA is that of batch-to-batch, and within-batch variability owing to the type of plates used (Pruslin *et al.* 1986, Wreghitt and Nagington 1983). To overcome this I used high binding plates and included controls (positive malaria plasma samples from Brefet, The Gambia, and negative samples from the Scottish Blood Transfusion Services) in each batch to ensure that these variations were kept to a minimal, if any (Venkatesan and Wakelin 1993).

3.6 **Conclusions**

This study has shown for the first time, that IgG antibodies to recombinant MSP-1_{19} antigens of the four major human *Plasmodium* species are species-specific and that there is no cross reactivity to this antigen between the different *Plasmodium* species. This makes these antigens a good diagnostic tool for detecting the species of *Plasmodium* parasites a host has been exposed to. The study demonstrates that in Zimbabwean populations exposed to *Plasmodium* infections, the prevalence of the non-*P. falciparum* species responses is higher than previously reported in many other cross-sectional studies [reviewed by (Mueller *et al.* 2007)].

Finally, the study has demonstrated that a high proportion of individuals with antibody responses to non-*P. falciparum* MSP-1_{19} antigens also had antibodies to *P. falciparum* MSP-1_{19}. MSP-1_{19} antigens from all four major species of human malaria parasite offer a potential diagnostic tool for the rapid detection of exposure to the different *Plasmodium* species such as in blood transfusion screening services. It remains to be established if these exposure responses to mixed-*Plasmodium* species indicated by the serological survey correspond to concurrent or sequential exposure to previous or current infections.

In the next chapter I have extended this seroepidemiology work in Daraweesh, a village in eastern Sudan with a very low entomological inoculation rate of 1-3 infectious bites per person per year. I investigated some observable clinical outcomes (fever and parasitaemia) and the number of treatment requirement in individuals with anti-MSP-1_{19} response to single and mixed-*Plasmodium* species.

Chapter 4. Clinical and therapeutic impact of mixed-*Plasmodium* species infections in Daraweesh, Sudan

4.1 Introduction

In malaria endemic countries, there is heterogeneity in the clinical manifestation of malaria and the number of drug treatments needed to clear parasites. How infection with multiple *Plasmodium* species affects this heterogeneity remains poorly understood. Several phenotypic differences in the biology of the four human infecting *Plasmodium* species that commonly cause malaria in Sub-Saharan Africa (Bruce and Day 2002) may account for heterogeneity in malaria. These phenotypic differences include the duration of the pre-erythrocytic and erythrocytic schizogony (Garnham 1966) and the number of merozoites produced per cycle (Bruce and Day 2002, Garnham 1966), host erythrocyte preference (Garnham 1966), and the ability to form dormant liver-stages (Markus 2015, Markus 2011a, Markus 2011b) or sequester infected erythrocytes (Rowe *et al.* 2009a).

For example, *P. falciparum* has a shorter pre-erythrocytic cycle of 5.5-7 days (Garnham 1966) and produces more merozoites in the schizont (up to 30,000) compared to 6-16 days duration and the 10,000 to 15,000 merozoites produced by the non-*P. falciparum* species (Bruce and Day 2002, Garnham 1966, Fairley 1947). While *P. falciparum*, *P. vivax* and *P. ovale* have a 48-hour intra-erythrocytic developmental cycle resulting in tertian fever (fever occurring every two days), *P. malariae* completes its development in 72-hours and produces quartan fever (fever occurring every third day) (Collins and Jeffery 2007, Collins and Jeffery 2005, McKenzie and Bossert 1999). The host cell tropism in these species also differs; *P. falciparum* invades all ages of erythrocytes, *P. malariae* prefers older erythrocytes, while *P. vivax* and *P. ovale* establish infections in reticulocytes (Bruce and Day 2002, Garnham 1988b). *Plasmodium vivax* has long been thought to have a preferential restriction to Duffy-

positive erythrocytes (Miller *et al.* 1976), although several recent reports from some Sub-Saharan African countries revise this view and suggest that Duffy-negative individuals may in fact be equally susceptible to this species (Fru-Cho *et al.* 2014, Mbenda and Das 2014, Mendes *et al.* 2011, Wurtz *et al.* 2011, Menard *et al.* 2010, Ryan *et al.* 2006). Quiescent liver stage forms (hypnozoites) which serve as a chronic parasite reservoir that promotes disease relapse in *P. vivax* and *P. ovale* (Markus 2011b, Markus 2011a, Krotoski *et al.* 1980) are absent in *P. falciparum* and *P. malariae* (Garnham 1988a, Bray and Garnham 1982), although there is evidence of re-emergence of blood-stage infection by *P. malariae* after many years of blood-smear negativity (Garnham 1966, Kitchen 1939).

Morbidity and mortality associated with *P. falciparum* infections are usually higher than in *P. vivax, P. ovale* and *P. malariae* (Langford *et al.* 2015, Gilles 1993), although these non-*P. falciparum* species contribute significantly to morbidity (Langford *et al.* 2015). Severe anaemia is associated with both *P. falciparum* and *P. vivax*, but only *P. falciparum* causes cerebral malaria, and the metabolic and respiratory complications associated with malaria (Weatherall *et al.* 2002). *Plasmodium malariae* infections are characterised by prolonged chronicity that can persist for months or years after the primary infection (Franken *et al.* 2012, Vinetz *et al.* 1998).

The dynamics and interactions of these phenotypes when the host is infected with more than one *Plasmodium* species are not fully understood, especially in areas of very low and seasonal transmission, such as Daraweesh in Sudan. There are limited, and conflicting, reports on the clinical outcome of single- *versus* mixed-*Plasmodium* species infections. There are both reports of exacerbation and amelioration of disease severity in mixed-species infections (McKenzie *et al.* 2006, Black *et al.* 1994). Fever, although nonspecific, is a cardinal symptom associated with malaria, and in low-resource regions, it is often used as a surrogate indicator of presumptive malaria diagnosis (Singh and Sharma 2014). However, mixed species infections have been both negatively and positively associated with fever. In Thailand, higher body temperatures were reported in patients with mixed *P. vivax–P. falciparum* infections than those with single species infections (McKenzie *et al.* 2006), while an inverse

relationship between fever and mixed-species infections involving *P. falciparum* and *P. malariae* was found in a village in Ivory Coast (Black *et al.* 1994).

Malaria in the village of Daraweesh, in the Sub-Saharan savannah of eastern Sudan has been extensively studied in terms of parasitology, immunology and entomology of the disease (Creasey *et al.* 2004, Cavanagh *et al.* 1998, Roper *et al.* 1996, Elhassan *et al.* 1995). Malaria parasite transmission in this village is seasonal, following the rainfall that starts in June, peaks in August and ends by mid-late September. More than 95% of malaria cases occur between September and November, with the peak of clinical malaria cases in October each year (Hamad *et al.* 2000, Theander 1998). Entomological data reveal that there are perhaps 1-3 infectious bites per person per year, making this area a very low transmission zone by African standards (Hamad *et al.* 2002, Hamad *et al.* 2000, Arnot 1998). Some of the observed age-related dynamics of malaria in other high transmission areas are absent in Daraweesh (Roper *et al.* 1996).

To date, there has been no study in this village, or any African low malaria transmission area, comparing observable clinical outcomes (fever, body temperature, parasitaemia) and the drug treatment requirements associated with single- *vs*. mixed-*Plasmodium* species infections diagnosed using the four MSP-1_{19} antigens. In Chapter 3, I demonstrated that human antibodies to the recombinant MSP-1_{19} antigens from malaria-exposed donors show no cross-reactivity between the different *Plasmodium* species (Amanfo *et al.* 2016). Thus detection of anti-MSP-1_{19} antibodies is a robust diagnostic tool for detecting recent infection by each species of *Plasmodium* parasite. In this chapter I have extended the seroepidemiology work in the village of Daraweesh and compared the clinical manifestations (fever and parasitaemia) and treatment requirements between single and mixed-*Plasmodium* species anti-MSP-1_{19} responders.

4.2 Study aims

1. To determine the seroepidemiology of the different *Plasmodium* species in a mesoendemic Sudanese population.

2. To determine if heterogeneity in the clinical presentations of malaria (history of fever, body temperature and parasitaemia) and response to drug treatment is related to exposure to single *vs*. mixed-*Plasmodium* species infection. This was by comparing observable clinical manifestation (fever and parasitaemia) and the number of treatments required (single chloroquine or repeated chloroquine/sulfadoxine-pyrimethamine) between infections with single- *vs* mixed- *Plasmodium* species antibody response to MSP-1_{19} antigens in Daraweesh.

3. To determine whether the number of recognisable *P. falciparum* MSP-1 Block 2 and MSP-2 antigens differed in single and mixed-species responders and whether these influence the clinical outcome and the number of treatments required for effective parasite clearance.

4.3 Hypotheses

1. Clinical presentations of malaria (history of fever, body temperature and parasitaemia) would be differentially affected in individuals infected with *P. falciparum* alone *versus* those with mixed-*Plasmodium* species infections.

2. The number of drug treatments required for parasite clearance in malaria patients would be different in individuals infected with *P. falciparum* alone *versus* those with mixed-*Plasmodium* species infections.

4.4 Materials and methods

4.4.1 Study area and population

The malaria experience of the residents of Daraweesh village (14.0243° N, 35.3686° E) in eastern Sudan was monitored between 1 January 1990 and 31 December 2000. The study area has been extensively described in previous publications (Creasey *et al.* 2004, Cavanagh *et al.* 1998, Elhassan *et al.* 1995). Briefly, Daraweesh village is located 15-18 km from Gedaref town in Eastern Sudan (14.0243° N, 35.3686° E). Vector-based measurements cannot absolutely establish the exact period of malaria parasite transmission in Daraweesh (Arnot 1998), although, malaria associated morbidities are always heavily concentrated between September to November, with peak incidence in October (Cavanagh *et al.* 1998, Elhassan *et al.* 1995). Daraweesh is characterised by a long dry season (January to July) when malaria is rarely diagnosed, followed by a short raining season (August to October) after which the majority of malaria incidence is recorded (Creasey *et al.* 2004, Hamad *et al.* 2002). Malaria case incidence in Daraweesh in relation to rainfall pattern between 1990 and 2000 is shown in Figure 4.4.1 on page 110 (Creasey *et al.* 2004).

95% of malaria in Daraweesh has been reported to be caused by *P. falciparum* with *Anopheles arabiensis* being the only mosquito vector implicated in parasite transmission (Elhassan *et al.* 1993, Haridi 1972). Malaria was monitored by a village health team composed of doctors, malaria researchers and microscopists visiting from a permanent station in the nearby town of Gedaref. The team also included a health worker from Daraweesh who lived in his family compound in the village. The health team visited the village at least every second day during the malaria transmission season (September to November), and 2–3 times per week outside that period. The residents reported to the team or attended the village clinic if they had any medical complaint. Complaints or symptoms suggestive of malaria warranted making a finger-prick blood smear, and if found positive for malaria parasites, treatment was administered to the individual by the health team.

The Sudanese government's policy on malaria treatment at the time of the study, and during the entire study period was the administration of chloroquine as the first line of

treatment for uncomplicated malaria (El Sayed *et al.* 2000), with sulfadoxine-pyrimethamine (SP, commonly called Fansidar) treatment given in instances of chloroquine-resistant infections (Creasey *et al.* 2004). Quinine was used as third line of treatment and also given to patients allergic to sulfadoxine-pyrimethamine (Creasey *et al.* 2004).

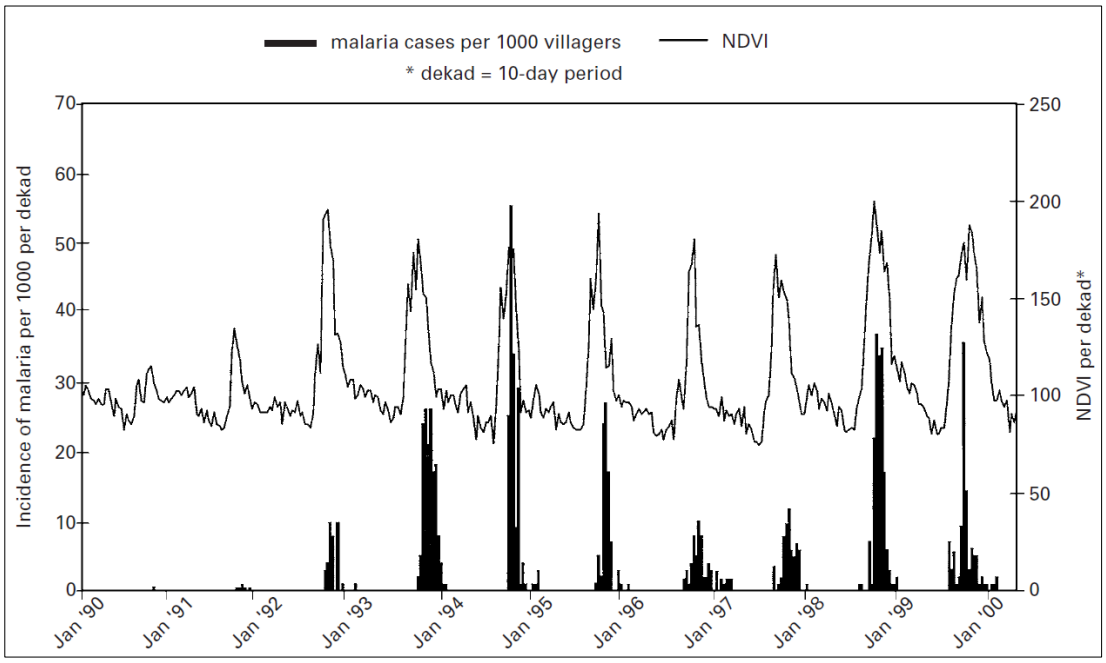


Figure 4.4.1: Malaria cases in relation to the Normalised Difference Vegetative Index (NDVI) in Daraweesh village between January 1990 and December 2000. Cases are expressed per 1000 inhabitants. The NDVI represents a measure of vegetative growth and is a reliable surrogate for rainfall. Both the NDVI and the number of cases per 1000 are expressed per dekad or 10-day period. Figure taken with permission from (Creasey *et al.* 2004)**.**

In the Daraweesh study an individual was categorised as requiring single treatment when there was no evidence of parasitaemia 7 days post standard chloroquine treatment, while requirement for repeated treatment was defined as the presence of parasites in blood 7 days post initial treatment and therefore requiring another dose of chloroquine and/or sulfadoxine-pyrimethamine treatment for effective parasite clearance. This latter group is usually considered as patients with late parasitological failure, defined as the presence of parasitaemia on any day between day 7 post initial treatment and last day of follow

up (usually 28 days) with axillary temperature < 37.5°C (WWARN 2012, Price *et al.* 2007).

The population of Daraweesh grew from 396 in the first quarter of 1990 to 560 at the end of 2000, with the birth of 164 children during the study period (Creasey *et al.* 2004). Over an eleven year period, 802 single clinical episodes of malaria were recorded in the village (Creasey *et al.* 2004). Individuals in the village rarely had more than one clinical malaria episode per year, however, the number of malaria episodes any single individual experienced varied between zero and eight throughout the eleven year study period (Creasey *et al.* 2004). A third of the village population did not experience any clinical malaria episodes, nor reported of signs and symptoms of malaria throughout the study period. The reasons underlying this striking finding are not fully understood (Creasey *et al.* 2004).

4.4.2 **Malaria case definition**

There was no age-specific criteria for defining clinical episodes of malaria with an individual defined as having malaria if both these two criteria were met; (a) febrile (body temperature measured with an oral probe at clinical examination was >37.5^{o}C), or reported fever in the preceding 48 hrs prior to attending the clinic, and (b) had slide detectable malaria parasites, evaluated by examining 200 fields under oil immersion (Giha *et al.* 2000, Roper *et al.* 1998, Roper *et al.* 1996). Parasites were counted per 300 leukocytes and standardized as parasites/µL (Bruce *et al.* 2008). Thick and thin blood smears were stained with Giemsa and examined for *Plasmodium* parasites under a magnification of ×1000 (Fleischer 2004, Giemsa 1904). Blood films were considered negative after the examination of 200 thick smear fields without detection of asexual parasites. Treatment for those with malaria was initiated immediately after diagnosis.

4.4.3 **Study cohort**

Three-hundred and thirty-three (333) individuals, aged 1 to 78 years (median age 11 years) with first clinical episode of malaria were recruited into this current study (see flowchart in Figure 4.4.2).

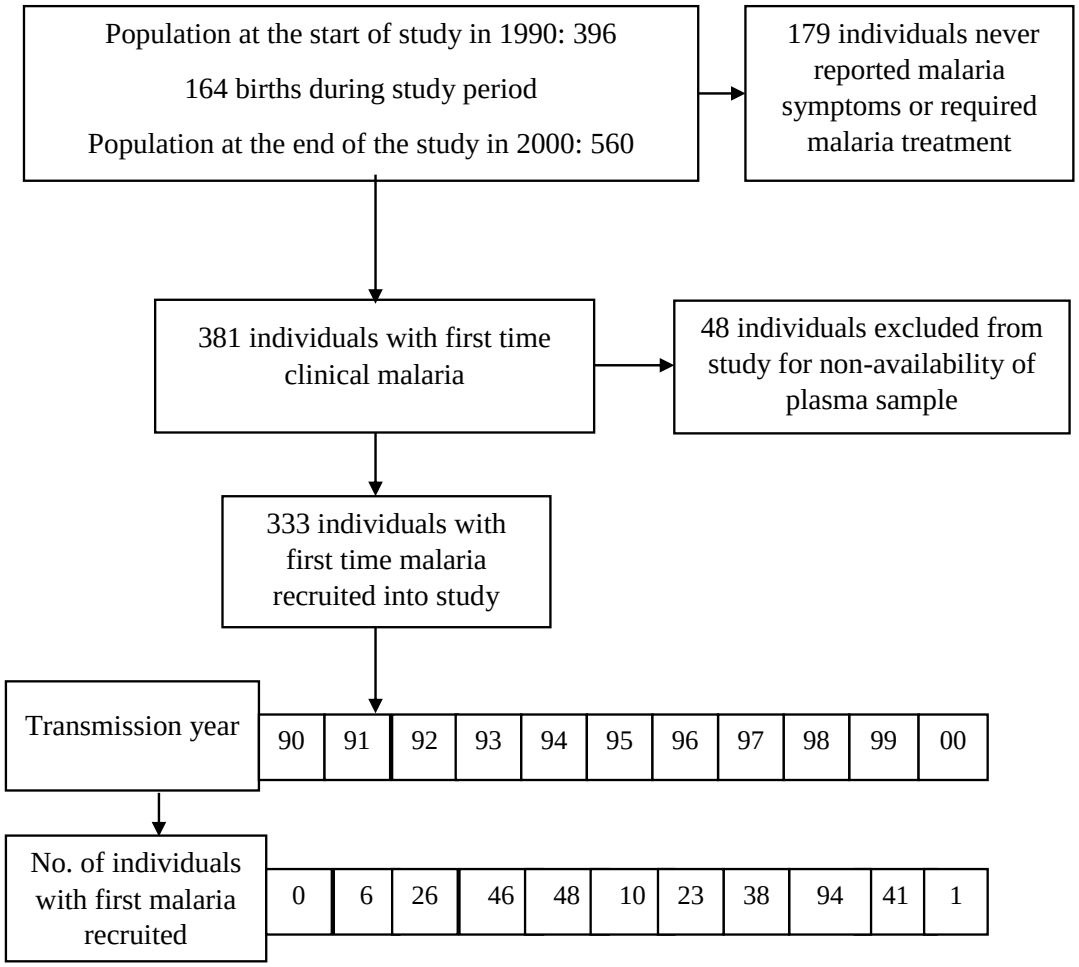


Figure 4.4.2: Flow chart of Daraweesh study participants' indicating the number of individuals recruited in each transmission year.

Between October 1989 and June 1990, there was severe drought in Daraweesh and the Gedaref plain (Roper *et al.* 1996), during which only one clinical malaria case was diagnosed in the 1990 transmission season (Creasey *et al.* 2004). This drought-clearance of patent parasitaemia and clinical malaria served as an informative baseline for patient anti-malarial antibody responses in the village (Cavanagh *et al.* 1998). All samples were from the first documented clinical malaria episode of individuals, confirmed by Giemsa-stained microscopy of asexual *Plasmodium* parasites, with complete information on history of fever, temperature, parasitaemia and treatment. These samples were collected during the malaria parasite transmission seasons of September to November (1991-2000) and occasionally post-transmission periods. The choice of the plasma of a first

malaria episode was more of a statistical consideration than biological, in order to avoid repeated measures on the same individual, and to exclude the temporary protective effects of chloroquine treatment and acquired immunity developed after malaria (van der Hoek *et al.* 1998). It has been hypothesised that individuals with previous clinical malaria episodes, may often carry parasites which have certain genotype(s) within which might be epitopes identical to a conserved antigen in a subsequent infection (Cavanagh *et al.* 1998). Thus using blood samples of the first malaria episode will help rule out possible memory responses to MSP-1_{19} antigens in previously infected individuals. Details of the characteristics of the study cohort are shown in Table 4.4.1.

Table 4.4.1: Study cohort and description (ages are in years).

Gender	Number of individuals	Mean age	Median age	Age range
Male	162 (48.6%)	18	12	1-78
Female	171 (51.4%)	19	16	4-68

4.4.4 Serological analysis

Plasma samples were screened by ELISA for IgG responses to MSP-1_{19} antigens from the four *Plasmodium* species and responders grouped as having antibody response to either single-*Plasmodium* or mixed-*Plasmodium* species antigens. Additionally, plasma samples were also screened for *P. falciparum* MSP-1 Block 2 antigens (the K-1 type, MAD20-type and RO33-type) as well as MSP-2 serogroups A and B antigens.

4.4.5 Blood sampling and blood film preparation

The malaria morbidity in Daraweesh was monitored throughout the malaria seasons (September-January) from September 1990 to December 2000 by a surveillance team that visited the village at least every second day. The malaria case detection was done by both passive and active case detection. In the passive case detection, the people of the village were instructed to report to the study team if they had complaints or symptoms suggestive of malaria. Actively, the village was visited by the study team every second day during malaria seasons. During the visit, health information was obtained and body

temperature measured. A blood smear was obtained from individuals with symptoms suggestive of malaria and/or a body temperature higher than 37.5°C. Three millilitres (3mL) of venous blood was obtained from participants into heparinized vacutainers (Becton Dickinson, Rutherford, NJ, USA). Plasma was separated from the blood by centrifugation and transferred into cryotubes, then stored at -20^{o}C in the study site. Aliquots of plasma samples were transported to Edinburgh and stored at -80 ^{o}C until used.

Thick blood smears were prepared at the time of blood collection with a drop of blood smeared within an area of 3 cm on a glass slide and allowed to air dry. Thin blood films were prepared by using approximately 1 µL of blood and spreading it thinly of a microscope slide with a cover glass or a glass spreader. This was allowed to air dry, and fixed with absolute methanol and air-dried. Both thick and thin slides were stained with 10% v/v Giemsa (TCS Biosciences, HS295) in Giemsa buffer made up of PBS pH 7.2. Thick blood films were examined for the presence of asexual *Plasmodium* parasites under a magnification of ×1000 as is routine (Fleischer 2004, Giemsa 1904). Blood films were considered negative after the examination of 200 smear fields without detection of asexual parasites. Thin films were used for species identification where practicable.

4.4.6 **Recombinant proteins and ELISA testing**

The MSP-1_{19} antigens from the four *Plasmodium* species and the *P. falciparum* MSP-1 Block 2 and MSP-2 serogroups A and B antigens are as shown previously in Table 2.6.1. The ELISA protocol for detection of IgG to MSP-1_{19} is as described in section 2.7.1 and is based on assays developed in earlier studies (Cavanagh *et al.* 1998). Briefly, plasma samples were tested at a dilution of 1 in 500 for the presence of IgG antibodies able to recognize recombinant MSP-1_{19} proteins. 96-well plates (Microlon, Greiner) were coated with 100 µL of 0.5 µg/mL of recombinant antigen in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4).

4.4.7 **Statistical Analyses**

Parasitaemia data were log-transformed in order to reduce the skewness of the data distribution. The Chi-square (χ^2) test, binary logistic regression and the Mann-Whitney *U* test and Spearman's rank correlation coefficient were used to determine if history of fever, body temperature, parasitaemia and the need for repeated drug treatment differed between single-*P. falciparum* species *versus* mixed-species responders. The *P* values given were considered significant at $< 0{\cdot}05$.

4.5 Results

4.5.1 Antibody response to MSP-1_{19} antigens in Daraweesh

IgG antibody recognition of the panel of four *Plasmodium* recombinant MSP-1_{19} antigens (*P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*) was tested by ELISA against three-hundred and thirty (333) plasma samples from individuals from Daraweesh who had been diagnosed as having malaria. Responders were grouped as either having antibodies to MSP-1_{19} from a single species, or with IgG responses to more than one MSP-1_{19} antigen defined as mixed-species responders. Serological responses correlated with microscopy, with all plasma samples tested showing reactivity to at least one recombinant MSP-1_{19} antigen. The mean level of IgG response (measured as OD at 492nm) to MSP-1_{19} of *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale* were 1.60, 0.34, 0.18 and 0.24 respectively.

Antibody responses to single-species MSP-1_{19} antigens accounted for 63.1% of all responses, and were almost exclusively against *P. falciparum* (Figure 4.5.1). Only three individuals had single species antibodies to *P. ovale* or *P. vivax* antigens. With the exception of four individuals, all mixed-species responses (36.9% of total) always involved responses to the *P. falciparum* MSP-1_{19} antigen. Dual responses against *P. falciparum* and *P. malariae* antigens (40.6%) were the predominant observation in the mixed-species category, although 22.8% of mixed-species responders had responses against all four *Plasmodium* species antigens. The median age of single and mixed-species responders was not significantly different (14 *vs*. 16 years, P=0.23, t-test).

Three individuals had single-species responses to *P. ovale* (2) and *P. vivax* (1). All three individuals were from different households (D, F and V; the detail of the Daraweesh household structure is described in Chapter 4). They were all males aged 7 years (individuals F13 and V14) and 12 years old (individual D9) at the time of their first clinical malaria episode and reported of a history of fever in the preceding 48hr before blood sampling. The *P. ovale* antibody responses of individuals D9 and V14 were observed in their first and only documented malaria episode in the malaria transmission seasons of 1995 and 1998 respectively. Individual F13 who was the only one to have single *P. vivax* MSP-1_{19} antibody response (in his first malaria episode in

the 1994 transmission season) subsequently had four additional clinical malaria episodes in the 1995, 1997, 1998 and 1999 transmission seasons. These subsequent infections showed antibody responses to more than one parasite antigen including *P. falciparum*. These individuals were excluded from further analysis involving antibody responses to single-species. Only the single-species *P. falciparum* responses were used for subsequent analysis comparing single- versus mixed-*Plasmodium* species responses.

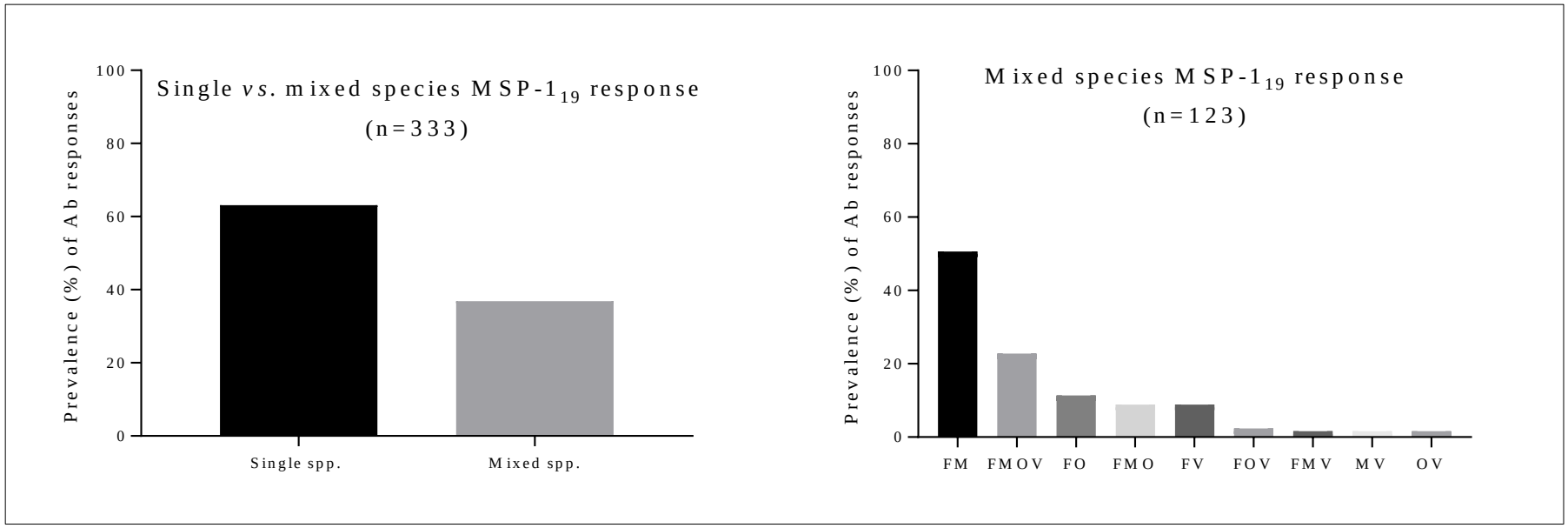


Figure 4.5.1: Single-species responses are almost exclusively against *P. falciparum* MSP-1$_{19}$ antigens. Note: With the exception of three individuals all single-species responses were against *P. falciparum* MSP-1$_{19}$ antigens. The predominant mixed-species responses were against *P. falciparum* and *P. malariae* MSP-1$_{19}$ antigens. F: *P. falciparum*, M: *P. malariae*, V: *P. vivax*, O: *P. ovale*. n is the total number of individuals represented in each panel.

4.5.2 Body temperature and reported fever in single-*P. falciparum versus* mixed-species responders

Body temperature data was normally distributed in the study population, and ranged between 35.0 and 41^{o}C. There was no significant difference (*P*=0.23, by Fisher's exact 2-tailed test.) in the median temperatures recorded for infections with *P. falciparum* only (37.9^{o}C, 95%CI 37.8-38.1) and mixed-species anti-MSP-1_{19} responders (37.8^{o}C 95%CI 37.5-38.5) (Figure 4.5.2A). Of the individuals with antibody response to *P. falciparum* antigens only, there was a significant difference (Pearson's Chi-square 4.7, DF=1, *P*=0.01) between the proportion with body temperature ≥37.5^{o}C and those below 37.5^{o}C (65.7% *vs*. 34.3%), however this was not so in mixed-species responders (*P*=0.62). When body temperature ≥37.5^{o}C was considered between the two antibody responder groups, there was a significant difference (*P*=0.03) between the proportion with responses to only *P. falciparum* (67.3%), who were approximately 1.7 times more likely to have body temperature ≥37.5^{o}C (OR: 1.65, 95%CI 1.04 – 2.62) than mixed-species responders (32.7%) (Figure 4.5.2B).

The proportion of individuals presenting with a history of fever (in the previous 48 hrs prior to attending clinic) was significantly higher (*P*=0.001) in both *P. falciparum* only and mixed-species responders (77.3 vs 22.7%, and 74.0 vs 26% respectively) compared to those who did not have a history of fever. Of responders with a history of fever, however, there was no significant difference between the proportion with *P. falciparum* only and mixed-species responders (*P*=0.50) (Figure 4.5.2C). Moreover, the relative risk of having a history of fever when an individual has an antibody response to *P. falciparum* only or mixed-*Plasmodium* species was the same (RR: 1.0, 95%CI (0.92 - 1.20).

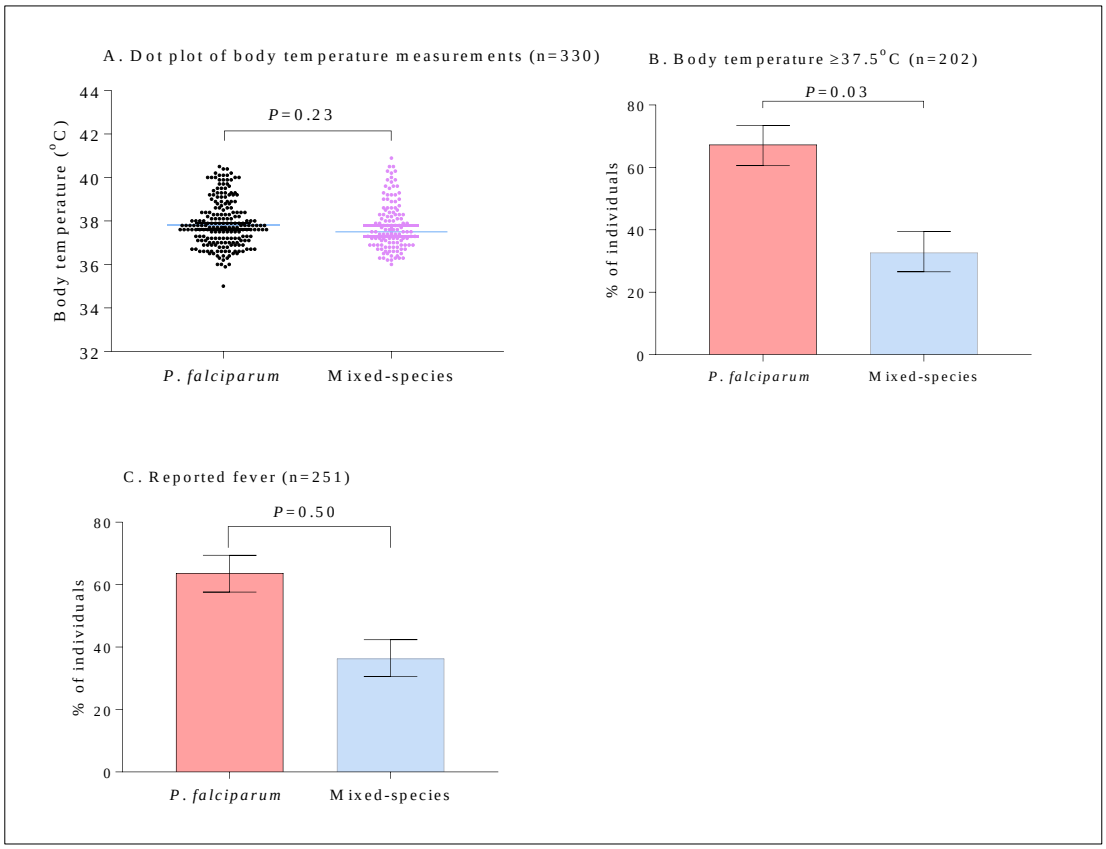


Figure 4.5.2: Median body temperature between *P. falciparum vs* mixed-species anti-MSP-1$_{19}$ responders is similar. Panel A: Dot plot of body temperature measurement showing no significant difference in the median body temperature, panel B: proportion of individuals with fever (body temperature ≥37.5ºC), panel C: proportion of individuals who reported a history of fever in the preceeding 48hr prior to blood sampling. n is the number of individuals represented in each panel. Error bars represent the 95% confidence intervals.

4.5.3 Parasitaemia in single-*P. falciparum versus* mixed-species anti-MSP-1_{19} responders

The World Health Organization defines hyperparasitaemia in low transmission areas such as Daraweesh as >2% of infected erythrocytes or >100,000 parasites/µL of blood (WHO 2011). In this study, the median parasitaemia was not significantly different (P=0.36, by the Mann-Whitney U test) between *P. falciparum* only and mixed-species anti-MSP-1_{19} responders (Figure 4.5.3A). The proportion of responders with low parasitaemia was significantly higher (P<0.001) compared to those with high parasitaemia in both the *P. falciparum* only (82.6%) and mixed-species (78.9%) groups. Among those with high parasitaemia, there was no significant difference between *P. falciparum* only and mixed-species responders (P=0.47) (Figure 4.5.3B). The odds of having a high parasitaemia in responders with *P. falciparum* only was only 1.3 times that of mixed-species responders (OR: 1.3, 95%CI 0.73 – 2.2).

A significant positive correlation (P<0.0001) was observed between parasitaemia and body temperature in the overall study population regardless of whether the patient has been exposed to single or mixed-*Plasmodium* species (Figure 4.5.4).

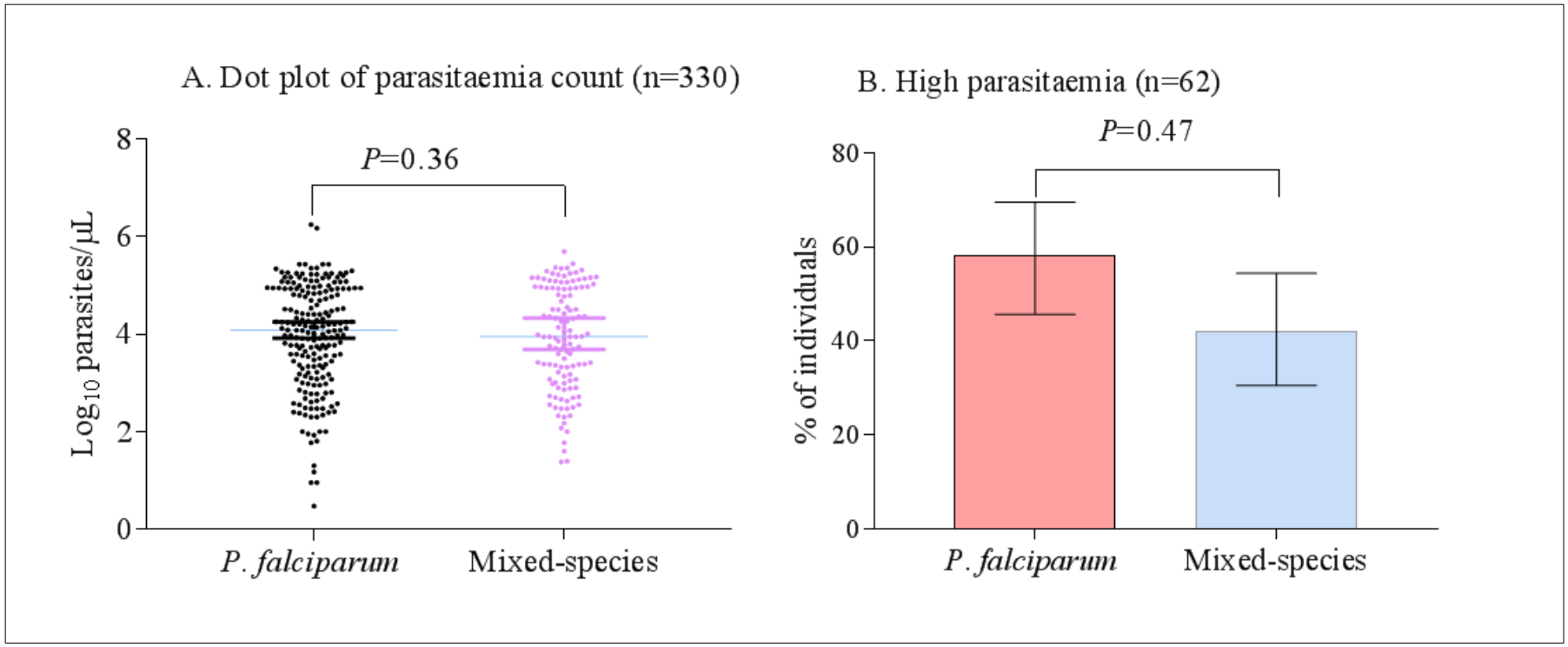


Figure 4.5.3: Median parasitaemia count in *P. falciparum vs* mixed-species anti-MSP-1_{19} responders is similar. Panel A: Dot plot of parasite count showing no significant difference in the median parasitaemia, panel B: proportion of individuals with high parasitaemia (>10^4 parasites/µL of blood). n is the number of individuals represented in each panel. Error bars represent the 95% confidence intervals.

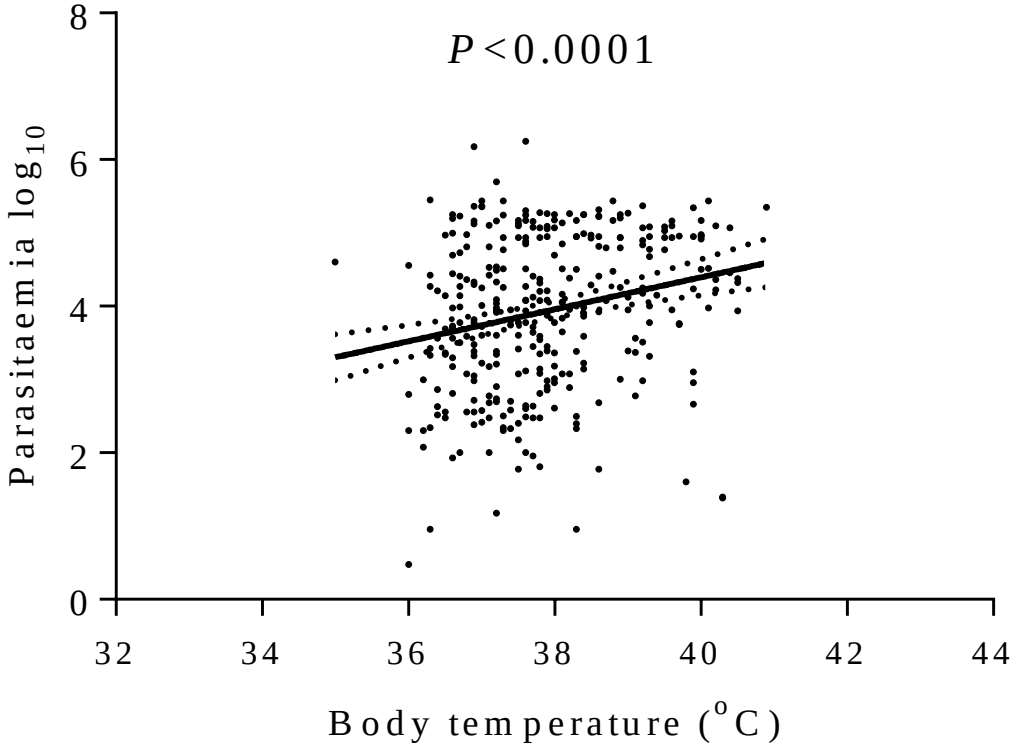


Figure 4.5.4: Parasitaemia correlates positively with body temperature. There is a positive association between body temperature and parasitaemia. Parasitaemia counts were log transformed.

4.5.4 Treatment requirement in single-*P. falciparum versus* mixed-species anti-MSP-1_{19} responders

In this study, 78% of those with antibodies to *P. falciparum* MSP-1_{19}, effectively cleared their infections with a single chloroquine treatment over the course of three days. By contrast, only 15% of those with antibodies to more than one species responded favourably to the same treatment. A significant proportion of individuals with antibodies to mixed-species (75%, *P*=0.001) required repeated treatment with chloroquine and/or sulfadoxine-pyrimethamine for effective clearance of parasites. In this study, repeated treatment is defined as the administration of chloroquine and/or sulfadoxine-pyrimethamine following the detection of asexual parasites in blood films three days after the initial chloroquine treatment of a malaria patient. There was no statistically significant difference in the mean age of those who required repeated treatment in either group (*P*=0.08). Of individuals requiring repeated treatment, the proportion of mixed-species responders was higher than those with antibodies to only *P. falciparum* MSP-1_{19} (66 *vs* 33.3%, *P*=0.001) (Figure 4.5.5). This was reflected in the odds of requiring repeated treatment in mixed-species responders, which was 10 times higher than the odds in *P. falciparum* only responders (OR: 10, 95%CI 6.2-18).

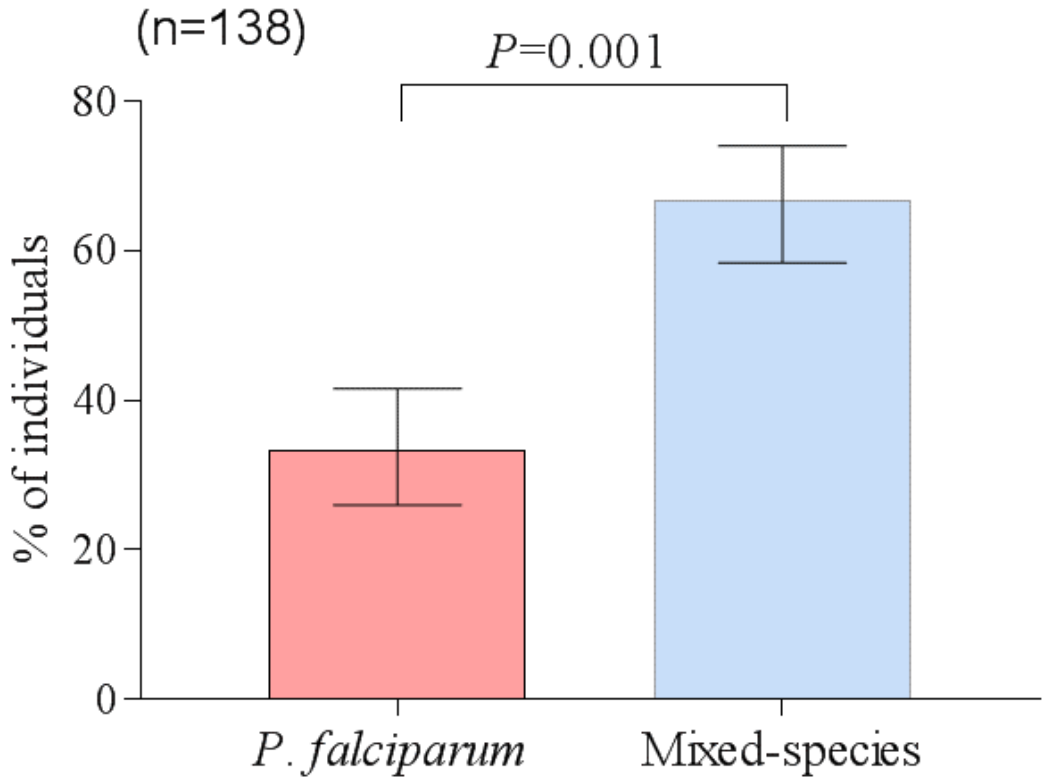


Figure 4.5.5: Significant proportion of mixed-species anti-MSP-1$_{19}$ responders require repeated treatment for parasite clearance. Repeated treatment is defined as the administration of chloroquine and/or sulfadoxine-pyrimethamine 3 days post-treatment owing to the presence of asexual parasites in blood smears. Error bars represent the 95% confidence intervals; n is the number of individuals.

4.5.5 Relationship between the number of recognized *P. falciparum* MSP-1 Block 2 antigens and clinical outcomes and treatment requirement

Humoral responses have been suggested to play a role in the efficacy of amodiaquine for the treatment of malaria (Mawili-Mboumba *et al.* 2003), with a significantly higher proportion of malaria patients with antibody response to more than one variant MSP-1 Block 2 antigen having a higher cure rate compared to those with responses to only one antigen (Mawili-Mboumba *et al.* 2003). With almost all mixed-species responses in this Daraweesh study involving a response to *P. falciparum*, I wanted to test if the

treatment outcomes observed in this population had any correlation with the number of *P. falciparum*–specific MSP-1 Block 2 and MSP-2 serogroup A and B antigens an individual recognises.

Of the 330 individuals with clinical malaria screened and included in the preceding analysis, 64.2% had detectable antibody response to at least one *P. falciparum* MSP-1 Block 2 antigen (Table 4.5.1). Of the MSP-1 Block 2 responders, 41.0 and 59% respectively had a response to either one, or more than one Block 2 antigen. Antibody response prevalence to the K1-type, MAD20-type and RO33-type were 78.3, 63.2 and 62.7% respectively. In individuals with antibody response to only *P. falciparum* MSP-1_{19} antigen (single species responders) the proportion who recognised only one Block 2 antigen and those with responses to more than one Block 2 antigens were similar (49.1 *vs*. 51.9%). However, in mixed-species anti-MSP-1_{19} responders the proportions of individuals who recognised more than one Block 2 antigen was significantly higher than those who recognised only one antigen (68.4 *vs*. 31.6%, χ^2=6.66, *P*=0.01) (Table 4.5.1 on page 127).

Of individuals with antibody response to only *P. falciparum* MSP-1_{19} antigen, independent of the type of response to Block 2 antigens, the proportions with a history of fever, body temperature above 37.5^{o}C, low parasitaemia and requiring single treatment with chloroquine were higher than those with no history of fever, body temperature below 37.5^{o}C, high parasitaemia and requiring repeated treatment for parasite clearance. In all the above parameters, the proportion of individuals with responses to one Block 2 and more than one Block 2 antigens were comparable (Table 4.5.2 on page 128).

Of mixed-species anti-MSP-1_{19} responders the proportion with a history of fever, low parasitaemia and requiring repeated treatment were higher than those with no history of fever, high parasitaemia and requiring single treatment, and was independent of Block 2 response an individual had. The proportions with body temperature above and below 37.5^{o}C were however similar.

Table 4.5.1: Prevalence of antibody response to *P. falciparum* MSP-1 Block 2 antigens.

	No response	Block 2 response	1 Block 2 Ag	>1 Block 2 Ag
All population (n=330)	118 (35.8%)	212 (64.2%)	87 (41.0%)	125 (59.0%)
***P. falciparum*-only responders (n=207)**	93 (44.9%)	114 (55.1%)	56 (49.1%)	58 (51.9%)
Mixed-species responders (n=123)	25 (20.3%)	98 (79.7%)	31 (31.6%)	67 (68.4%)

Note: Numbers represent absolute count while those in parenthesis represent the percentage prevalence.

Table 4.5.2: Block 2 response type and its relationship with clinical and treatment data in *P. falciparum*-only anti-MSP-1_{19} responders.

	***P. falciparum*-only anti-MSP-1_{19} responders (n=207)**								
MSP-1 Block 2 response	**Number of individuals**	**No history of fever**	**History of Fever**	**Temp <37.5ºC**	**Temp ≥37.5ºC**	**Low parasitaemia**	**High parasitaemia**	**Single treatment**	**Repeated treatment**
No response	93 (44.9)	22 (23.7)	71 (76.3)	34 (36.6)	59 (63.4)	77 (82.8)	16 (17.2)	76 (81.7)	17 (18.3)
1 Block 2 Ag	56 (27.1)	13 (23.2)	43 (76.8)	18 (32.1)	38 (67.9)	47 (83.9)	9 (16.1)	44 (78.6)	12 (21.4)
>1 Block 2 Ag	58 (28)	12 (20.7)	46 (79.3)	19 (32.8)	39 (67.2)	43 (74.1)	15 (25.9)	41 (70.7)	17 (29.3)

Note: Numbers represent absolute count while those in parenthesis represent the percentage prevalence.

Table 4.5.3: Block 2 response type and its relationship with clinical and treatment data in mixed-*Plasmodium* species anti-MSP-1_{19} responders.

	Mixed-species anti-MSP-1_{19} responders (n=123)								
MSP-1 Block 2 response		**No fever**	**Fever**	**Temp <37.5ºC**	**Temp ≥37.5ºC**	**Low parasitaemia**	**High parasitaemia**	**Single treatment**	**Repeated treatment**
No response	25 (20.3)	7 (28)	18 (72)	12 (48)	13 (52)	20 (80)	5 (20)	6 (24)	19 (76)
1 Block 2 Ag	31 (25.2)	7 (22.6)	24 (77.4)	13 (41.9)	18 (58.1)	26 (83.9)	5 (16.1)	9 (29)	22 (71)
>1 Block 2 Ag	67 (54.5)	18 (26.9)	49 (73.1)	32 (47.8)	35 (52.2)	52 (77.6)	15 (22.4)	16 (23.9)	51 (76.1)

Note: Numbers represent absolute count while those in parenthesis represent the percentage prevalence.

4.5.6 **Relationship between the number of recognized *P. falciparum* MSP-2 antigens and clinical outcomes and treatment requirement**

85.5% of the study population included in the analysis had an antibody response to MSP-2 antigens. Of these responders, a significant proportion (75.2%) had antibody response to both MSP-2A and MSP-2B serogroups compared to those with responses to only MSP-2A (11.3%) and MSP-2B (13.5%) antigens. When the population is divided into *P. falciparum*-only and mixed-*Plasmodium* species anti-MSP-1_{19} antibody responders, the pattern was the same with a high proportion recognising both MSP-2A and MSP-2B serogroups (70 and 83% respectively) (Table 4.5.4 on page 131). The proportion with antibody response to only MSP-2A or MSP-2B antigen was similar in both *P. falciparum*-only and mixed-*Plasmodium* species MSP-1_{19} response groups.

In *P. falciparum* anti-MSP-1_{19} responders, independent of an individual's IgG response to MSP-2 antigens, the patterns of fever history, body temperature, parasitaemia and treatment requirement were similar to that observed in Block 2 responders, with a higher proportion of individuals having a history of fever, body temperature above 37.5^{o}C, lower parasitaemia and requiring single treatment for parasite clearance (Table 4.5.5 on page 132). In mixed-*Plasmodium* species anti-MSP-1_{19} responders, the above pattern was the same except in treatment requirement in which all individuals with no antibody response to MSP-2 antigens as well as a higher proportion of individuals with responses to both MSP-2A and MSP-2B required repeated treatment (Table 4.5.6 on page 133). While in single-species responders a lack of antibody response to MSP-2 antigens is seen in a higher proportion of individuals clearing their infections with single treatment (Table 4.5.5), all mixed-species responders who had no antibody response to MSP-2 antigens required repeated treatment for parasite clearance (Table 4.5.6, on page 133).

Table 4.5.4: Prevalence of antibody response to *P. falciparum* MSP-2 antigens.

	No response	MSP-2 response	MSP-2A	MSP-2 B	MSP-2A and 2B
All population (n=330)	48 (14.5%)	282 (85.5%)	32 (11.3%)	38 (13.5%)	212 (75.2%)
***P. falciparum*-only responders (n=207)**	37 (17.9%)	170 (82.1%)	25 (14.7%)	26 (15.3%)	119 (70%)
Mixed-species responders (n=123)	11 (9.0%)	112 (91.0%)	7 (6.3%)	12 (10.7%)	93 (83%)

Note: Numbers represent absolute count while those in parenthesis represent the percentage prevalence.

Table 4.5.5: MSP-2 response type and its relationship with clinical and treatment data in responders to *P. falciparum*-only antigens.

	***P. falciparum*-only anti-MSP-1$_{19}$ responders (n=207)**								
MSP-2 response		**No fever**	**Fever**	**Temp <37.5ºC**	**Temp ≥37.5ºC**	**Low parasitaemia**	**High parasitaemia**	**Single treatment**	**Repeated treatment**
No response	37 (17.9)	10 (27.0)	27 (73.0)	13 (35.1)	24 (64.9)	28 (75.7)	9 (24.3)	34 (91.9)	3 (8.1)
MSP-2A only	25 (12.1)	9 (36.0)	16 (64.0)	11 (44.0)	14 (56.0)	21 (84.0)	4 (16.0)	21 (84.0)	4 (16.0)
MSP-2B only	26 (12.6)	6 (23.1)	20 (76.9)	4 (15.4)	22 (84.6)	21 (80.8)	5 (19.2)	17 (65.4)	9 (34.6)
MSP-2 A & B	119 (57.4)	22 (18.5)	97 (81.5)	43 (36.1)	76 (63.9)	99 (83.2)	20 (16.8)	89 (74.8)	30 (25.2)

Note: Numbers represent absolute count while those in parenthesis represent the percentage prevalence.

Table 4.5.6: MSP-2 response type and its relationship with clinical and treatment data in responders to mixed-*Plasmodium* species antigens.

	Mixed-species anti-MSP-1_{19} responders (n=123)								
MSP-2 response		**No fever**	**Fever**	**Temp <37.5ºC**	**Temp ≥37.5ºC**	**Low parasitaemia**	**High parasitaemia**	**Single treatment**	**Repeated treatment**
No response	11 (8.9)	5 (45.5)	6 (54.5)	5 (45.5)	6 (54.5)	6 (54.5)	5 (45.5)	0 (0.0)	11 (100.0)
MSP-2A only	7 (5.7)	1 (14.3)	6 (85.7)	3 (42.9)	4 (57.1)	6 (85.7)	1 (14.3)	2 (28.6)	5 (71.4)
MSP-2B only	12 (9.8)	3 (25.0)	9 (75.0)	6 (50.0)	6 (50.0)	10 (83.3)	2 (16.7)	5 (41.7)	7 (58.3)
MSP-2 A & B	93 (75.6)	23 (24.7)	70 (75.3)	43 (46.2)	50 (53.8)	72 (46.2)	21 (22.6)	24 (25.8)	69 (74.1)

Note: Numbers represent absolute count while those in parenthesis represent the percentage prevalence.

4.6 Discussion

While individuals living in malaria endemic regions are exposed to the different *Plasmodium* species, it is important to understand the drivers, if any, that exist of the clinical outcomes and treatment requirement of these individuals when infected with single or mixed-*Plasmodium* species. Clinical outcomes of single- and mixed-*Plasmodium* species infections have been variedly reported as being advantageous (Mohapatra *et al.* 2012, Lorenzetti *et al.* 2008, Black *et al.* 1994) and sometimes as detrimental (McKenzie *et al.* 2006) to the host. In the previous chapter I established the specificity of IgG antibodies to MSP-1_{19} antigens and used these to characterise the epidemiology of the four human *Plasmodium* species in three Zimbabwean villages (Amanfo *et al.* 2016). In this current chapter I extended the seroepidemiology work to characterise the full range of infecting *Plasmodium* species and strain variants of *P. falciparum* i.e. MSP-1 Block 2 and MSP2 in Daraweesh and asked if heterogeneity in clinical presentation and response to drug treatment was related to exposure to single vs. mixed-*Plasmodium* species. This was achieved by comparing fever and parasitaemia, and the number of treatments required for parasite clearance in malaria patients with *P. falciparum* only *versus* mixed-*Plasmodium* infections. The most significant findings were the higher response of mixed-species infections than have been previously reported by microscopy, and the associated drug treatment failure in a significant proportion of the population with mixed-species infections.

In this study all plasma samples tested (taken from first documented clinical episodes of malaria patients) had detectable antibody responses to MSP-1_{19} antigen of at least one *Plasmodium* species. This is consistent with observations by others that antibody responses to *Plasmodium* MSP-1 antigens are higher when plasma samples are taken at, or shortly after, the acute stage of malaria, or when blood films are positive for asexual-stage parasites and when patients are relatively highly parasitaemic (Osier *et al.* 2008, Osier *et al.* 2007, Polley *et al.* 2004, Tami *et al.* 2002, Cavanagh *et al.* 1998, Tolle *et al.* 1993). About 96% of all malaria cases in Daraweesh have been attributed, by optical microscopy of Giemsa-stained slides, to *P. falciparum* infections (Hamad *et al.* 2000). This is essentially confirmed here, as the great majority of plasma samples tested (all except 7 samples) had strong antibody reactivity against *P. falciparum* MSP-

1_{19} antigens, similar to the observations made in the Zimbabwean study described in Chapter 3. A previous study in this Daraweesh population showed the frequency of antibody recognition to MSP-1_{19} of *P. falciparum* of approximately 90% (Cavanagh *et al.* 1998). This is probably attributable to the close clinical monitoring and blood sampling in the Daraweesh study. As previously mentioned (in Section 4.4.1 of this chapter), in addition to the resident health worker in the village, the external health team visited the village at least every second day during malaria transmission seasons, and 2–3 times per week outside that period, with those found to be having malaria parasites, promptly treated.

One of two novel and striking observations made in this study is the higher than expected frequency of antibody responses to the non-*falciparum* species, especially responses against *P. malariae* in the context of mixed-species responses. This level of mixed species antibody responses (which in the context of malaria epidemiology in Daraweesh correlates with infection) was not detected by light microscopy (Hamad *et al.* 2000). This reveals the limitations of *Plasmodium* species detection and differentiation by microscopy alone (Barber *et al.* 2013, Mueller *et al.* 2007, McKenzie *et al.* 2003). With the exception of four individuals, all mixed-species antibody responses in Daraweesh always involved responses to *P. falciparum* antigens. This is similar to serological observations in Zimbabwe (Amanfo *et al.* 2016), and in Memni, a village in the Ivory Coast, where *P. malariae* infections were always found in association with *P. falciparum* by light microscopy (Black *et al.* 1994). *P. falciparum* is confirmed as indeed the heavily predominant human malaria parasite in Africa, but equally clearly, African malaria is far from being a monospecies problem. Using these new antigens as tools, has demonstrated that previous studies in this village have clearly underestimated the prevalence of *P. vivax*, *P. malariae* and *P. ovale* in this part of eastern Sudan.

Entomological surveys in Daraweesh and other northern Sudanese sites have found *Anopheles arabiensis* to be the sole vector responsible for *Plasmodium* transmission (Hamad *et al.* 2000). It is possible that the intrinsic vectorial capacity of this mosquito species might be higher for *P. falciparum* than the non-*falciparum* species, or that *P. falciparum's* capacity to produce infectious gametes outstrips its competitors. Since

mixed-species infections are caused by the simultaneous or sequential inoculation of parasites by the same or different mosquitoes (Snounou and White 2004, McKenzie and Bossert 1997), it seems highly likely that these mixed-species infections, are also being transmitted by the *An. arabiensis* vector. The preponderance of *P. falciparum* infections could be a function of the intrinsic vectorial capacity of the *Anopheles* mosquitoes being higher for *P. falciparum* than the non-*falciparum* species. Alternatively, *P. falciparum* may be out competing its rivals in capacity to produce infectious gametes. The very low EIR and the short transmission season of September to November in Daraweesh suggest these infections may have been initiated concurrently. This tentative assumption is supported by the observation that rising IgG responses to *P. falciparum* MSP-1_{19} antigens are only seen in Daraweesh when individuals are actually having clinical episodes of malaria (or, less commonly, have become PCR positive but remain asymptomatic) (Cavanagh *et al.* 1998). Antibodies to any species MSP1 antigen remain at undetectable levels in the absence of disease during the long dry seasons (December to August) when malaria is very rarely diagnosed in the population (Giha *et al.* 2000, Cavanagh *et al.* 1998).

Febrile episodes are the cardinal symptoms of malaria and usually coincide with the cyclical release of merozoites during schizont rupture of erythrocyte (Gazzinelli *et al.* 2014, Golgi 1889). Fever may alter the clinical course of the disease by influencing parasite replication, survival and virulence (reviewed by (Oakley *et al.* 2011)). In mixed-species infections a less defined 'non-classical' pattern of fever may be observed owing to differences in parasite synchronization, parasitaemia and the host immune response (reviewed by (Oakley *et al.* 2011)), as well as density dependent regulatory mechanisms (Bruce and Day 2002, Bruce *et al.* 2000). In this study, although a significantly high proportion of both monospecies-*P. falciparum* and mixed-species responders had a history of fever or with a body temperature above 37.5^{o}C, the median body temperature was not significantly different between the two groups. This observation is different to that reported in patients from western Thailand in which mixed *P. falciparum*-*P. vivax* infected patients presented with higher fevers than those with single-*P. falciparum* or single-*P. vivax* infections (McKenzie *et al.* 2006). This may reflect the more *P. vivax*-oriented nature of Southeast Asian malaria

and mixed-infection epidemiology. Fever involves a cascade of events involving parasite toxins and cytokine induction. The host immune response directed against one *Plasmodium* species has the potential to dampen the severity of malaria in the context of mixed-species infection (Chuangchaiya *et al.* 2010). Since these non-*P. falciparum* species are capable of establishing chronic, asymptomatic, low-level parasitaemia in the human host, antibody directed against parasite toxins in mixed-species infections may play a role in down-regulating the cytokine cascade and thereby dampening fever episodes (Black *et al.* 1994).

P. falciparum is capable of establishing infection in all stages of erythrocytes and generally causes higher parasitaemia than the non-*P. falciparum* species which have erythrocyte-stage restrictions (Bruce and Day 2003, Bruce and Day 2002). However, in the Daraweesh population a significantly higher proportion of both single- and mixed-species responders had lower parasitaemia levels. With a low EIR and the presence of accessible health care/team for the people to visit (i.e. close monitoring over the study period) it is possible that the compliance of the people in seeking accessible health care from the team as well as natural control of infection through immunity might have played a part in individuals having lower parasitaemia. Since almost every mixed-species response in Daraweesh involved *P. falciparum*, it is reasonable to assume that the contribution of the non-*P. falciparum* species to parasitaemia may be minimal, resulting in no significant difference in the parasitaemia of single-*P. falciparum versus* mixed-species responders. This assumption is supported by evidence that fever induced by the low density parasitaemic-*P. vivax* may protect against severe disease and limit *P. falciparum* parasitaemia (Smith *et al.* 2001, Luxemburger *et al.* 1997). Although co-infection with two rodent *Plasmodium* species have been reported to be highly virulent to the host (Ramiro *et al.* 2016), this was obviously not the case in this human population with respect to the parasitaemia data. In a recent mouse model study of malaria infection, the authors observed that when the rodent parasite species *P. chabaudi*, capable of infecting all stages of erythrocytes (similar to *P. falciparum* in humans) is inoculated first and establishes an infection, it facilitates the growth of *P. yoelii* species that have erythrocyte restriction (similar to the human *P. vivax* species) enabling it to reach significantly higher densities than in

either control infections or when both species were simultaneous inoculated in the mouse host, although the timing of infection is also critical (Ramiro *et al.* 2016).

Although in the Daraweesh study the certainty of which parasite species was first to infect cannot be deduced, what is certain is that the mixed-infections occurred within the tight transmission period of September to November of each years when samples were collected from patients with clinical episodes, as pre-transmission samples of these donors did not show detectable levels of antibody responses to the parasite antigens. Of the individuals with high parasitaemia in Daraweesh, there was no significant difference observed in the proportion of single- *versus* mixed-species responders. This finding is in agreement with a recent report that suggested that competition in mixed-strain *P. falciparum* infections does not increase the overall parasite density and that in the presence of competitors, densities of individual parasite strains are reduced (Bushman *et al.* 2016). Parasitaemia did not generally correlate with the level of antibody as measured by ELISA absorbance. It might be expected that the onset of an acute infection and the rising parasite density prior to treatment might correlate with the level of immune response against an infecting pathogen. The contrary observation in the Daraweesh population might be explained by the close monitoring of the population by the study team which included prompt treatment of all malaria cases diagnosed. In view of presence of a resident research team member in the village, the residents who presented with symptoms of malaria reported these to the team early and were diagnosed and treated before parasite densities could rise to levels often seen when disease is not diagnosed and left untreated for a longer duration.

In most Sub-Saharan African countries artemisinin-based combination therapy (ACTs) have currently been adopted as first-line treatment for uncomplicated malaria due to the widespread chloroquine resistance in most malaria endemic areas (Menard *et al.* 2016). Chloroquine was still an effective first line drug for treatment of uncomplicated malaria according to the Sudanese Ministry of Health guidelines, and was used extensively during the 1990s, with sulfadoxine-pyrimethamine treatment for chloroquine resistant cases (El Sayed *et al.* 2000). Self-medication in the Daraweesh population did not occur as the villagers had free health advice, drug treatment and care given by the project health team (Elhassan *et al.* 1995). Anti-malarial drug

treatment requirements were significantly different between those with single- and mixed-species antibody responses, with a significantly higher frequency of multiple drug administration in those with mixed-species infections. Most single *P. falciparum* infections were cleared by single course of the chloroquine treatment. By contrast, I observed that a significantly higher proportion of mixed-species infections required repeated treatment with sulfadoxine-pyrimethamine. This is in agreement with other studies, where co-infections with *P. falciparum*- and *P. malariae* (Smith *et al.* 2011) and *P. ovale* (Senn *et al.* 2014), resulted in artemether-lumefantrine, and oral atovaquone-proguanil treatment failures respectively, with patients requiring repeated treatment for full parasite clearance.

Furthermore, *P. malariae* infections have been reported to occur after antimalarial treatment in the absence of re-exposure of patient to the parasites (Franken *et al.* 2012, Muller-Stover *et al.* 2008, Hess *et al.* 1993). In a study of monoinfection of *P. knowlesi* (including microscopy result of *P. malariae*) in Malaysia, the authors reported a faster parasite clearance and fever resolution, and lower risk of anaemia within 28 days in patients treated with artesunate-mefloquine compared to those who received chloroquine alone (Grigg *et al.* 2016). However, what is not clear in the above study is the possible influence of the *P. malariae* species on the treatment outcome of the two groups.

Although there is widespread chloroquine resistance to *P. falciparum* in most malaria-endemic countries, *P. vivax* treatment in Ethiopia where this species is co-endemic with *P. falciparum* remained largely based on effective chloroquine monotherapy (Seifu *et al.* 2017). However, data is lacking on the susceptibility of *P. malariae* and *P. ovale* to chloroquine treatment. Some studies have reported recrudescent infection of these species, especially *P. malariae* infection months or years after *P. falciparum* treatment with chloroquine (Franken *et al.* 2012, Muller-Stover *et al.* 2008). In my study, the three individuals with single-species antibody response to *P. vivax* or *P. ovale* MSP-1_{19} antigens (who were excluded from further analysis) all cleared their infection with a three-day course of chloroquine treatment. Although this number is not of any statistical significance it probably shows the effectiveness of chloroquine against monospecies infections compared to mixed-species infections.

In the current study the proportion of individuals with antibody response to MSP-1 Block 2 antigens was 64.2%, with about 38% having a response to more than one variant antigen. These prevalences were higher than that observed in 77 post-transmission season samples collected from the same population between 1991 and 1995, in which 43% had a response to Block 2 antigens, with a lower prevalence of 24% responding to more than one Block 2 antigen (Cavanagh *et al.* 1998). This difference could be explained by the larger sample size and the fact that this current study sampling was until the year 2000, and included only those with documented evidence of clinical malaria as oppose to both symptomatic and asymptomatic patients screened by Cavanagh and colleagues. IgG response to MSP-1 Block 2 antigens in African population is of the IgG3 subclass, which are short-lived (half-life of 7 days) compared to response to the conserved MSP-1_{19} which is of the IgG1 subclass (half-life of 21 days) (Cavanagh *et al.* 2004, Cavanagh *et al.* 2001). This short half-life of IgG3 means that antibody responses might not be detectable in asymptomatic patients who have cleared their infections weeks or months prior to sampling. The Block 2 antigens are highly polymorphic and *P. falciparum* specific and therefore it is of interest to note that a higher proportion of mixed-*Plasmodium* species anti-MSP-1_{19} responders recognised more Block 2 antigens than those with responses to only *P. falciparum* antigens.

While the patterns of a history of fever, body temperature above 37.5^{o}C and having a lower parasitaemia was similar in *P. falciparum* only and mixed-*Plasmodium* species anti-MSP-1_{19} responders irrespective of the response type to, or the number of Block 2 antigens recognised, treatment requirement differed with a higher proportion of those with mixed-*Plasmodium* species responses requiring repeated treatment as against a higher proportion of *P. falciparum* only responders clearing their parasite with only single treatment. This suggests that the type or number of recognisable MSP-1 Block 2 antigens (i.e. a surrogate measure of the number of *P. falciparum* clones infecting an individual) is not as important in determining the treatment requirement as of the number of *Plasmodium* species an individual is exposed to. This is in contrast to what was observed in amodiaquine efficacy in Gabon, in which a significantly higher cure rate was observed in a higher proportion of malaria patients who recognised more than

one variant MSP-1 Block 2 antigen compared to those with responses to only one Block 2 antigen (Mawili-Mboumba *et al.* 2003). However, that study did not investigate the presence or exposure of individuals to the non-*P. falciparum* species, as is the case with my thesis.

Antibody responses to *P. falciparum*-specific MSP-2 antigens in the study population was higher in the overall population (85%), as well as in both single- and mixed-species responders. In both responders, the acquisition of antibodies to MSP-2 antigens correlated with lower parasitaemia and higher body temperature. While in single-species responders, anti-MSP-2 responses were associated with parasite clearance with single treatment, the opposite effect was seen in mixed-species responders who required repeated treatment independent of the number of MSP-2 antigens recognised. It has been shown that under natural exposure, the number of *Plasmodium* antigens an individual recognises (i.e. exposed to), and the level of antibody response (antibody titre) against these antigens confers a degree of protection, with children having high antibody levels to five or more antigens experiencing no clinical episode of malaria (Osier *et al.* 2008). In my study, recognition of Block-2 and MSP-2 antigens were associated with lower parasitaemia in a higher proportion of both single- and mixed-species responders. It would have been expected that an accumulation of Block 2 and MSP-2 antibodies would influence drug treatment, i.e. those recognising more of these antigens would be more likely to clear their infections with a single course of treatment. However, treatment outcomes seem to be more dependent on the *Plasmodium* species than the number of polymorphic MSP antigens an individual recognised. This study did not consider protection from malaria (as all individuals sampled had malaria), however, the dynamics of malaria epidemiology in Daraweesh may be unique, in that, although people had antibody responses to a diverse range of antigens, individuals experienced between zero and 8 malaria episodes throughout the study period (Creasey *et al.* 2004).

In the serological work of both Chapters 3 and 4, I could have further explored correlating the serological results obtained with PCR data from the samples I worked on. However, the limitation was that the plasma samples from the two study cohorts did not have corresponding whole blood or DNA to use for the molecular

characterisation of the *Plasmodium* species. However, in validating the use of these recombinant MSP-1_{19} antigens of the four *Plasmodium* species, work from others in my co-supervisor's laboratory has compared the plasma antibody responses to MSP-1_{19} antigens with immunofluorescence antibody test (IFAT) and PCR data of positive malaria blood samples collected from the Scottish Blood Transfusion Services. A clear correlation of the serology using MSP-1_{19} antigens with the IFAT and PCR results were obtained (D. Cavanagh, personal communication).

Anaemia is an important feature usually associated with malaria (Haldar and Mohandas 2009). Comparison of anaemia status in single- *versus* mixed-species infections would have added value to the current literature especially having a well characterised cohort like the Daraweesh population. Unfortunately, at the time of the Daraweesh study, measurement of haemoglobin was not included in the study design.

4.7 **Conclusion**

The study of malaria epidemiology in Daraweesh based on antibody response to *Plasmodium* MSP-1_{19} antigens has demonstrated for the first time that exposure to mixed-*Plasmodium* species are higher than previously reported in this village (Hamad *et al.* 2000). Similar to the observation in the Zimbabwean study (Chapter 3), this finding confirms the need for a standard and sensitive method of detecting mixed-infections to be implemented, as there are important clinical and therapeutic implications if minority species go undetected, or parasitaemia becomes sub-clinical without full cure. The requirement for multiple drug treatments was significantly higher in multi-species *Plasmodium* infections. Whilst a much higher proportion of *P. falciparum* infected individuals cleared their infections with a single chloroquine treatment course, two-thirds of mixed-species infections failed to clear their infection after one chloroquine course.

This significant finding of an associated drug treatment failure with mixed-species infections has potential health implications such as the development of drug-resistant strains. Treatment failure or partial parasite clearance has the potential to allow onward transmission, and to impact upon malaria morbidity and mortality. A better

understanding of the ecology and epidemiology of all the human *Plasmodium* species being transmitted in Africa would certainly contribute to designing more effective malaria treatment, control and elimination programmes in African populations.

I propose that this work should be replicated in other malaria epidemiological settings in light of the current shift to treating uncomplicated malaria with ACTs. This would confirm whether the observation in Daraweesh of the requirement of repeated treatment with chloroquine seen in mixed-species responders is similar or differs when ACTs are used to treat mixed-species infections.

Chapter 5. Household-level spatiotemporal dynamics of malaria prevalence in Daraweesh, Sudan

5.1 Introduction

The epidemiology of malaria is very complex, and involves infected mosquito vectors that spread *Plasmodium* parasites locally and typically over short distances to susceptible human hosts. In malaria, heterogeneity in parasite transmission within a defined geographical location is common (Bousema *et al.* 2012, Bousema *et al.* 2010a). This variation is also seen in parasitic diseases such as schistosomiasis (Manning *et al.* 1995) and other infectious diseases (Woolhouse *et al.* 1997), in what is described as the 20/80 rule (Woolhouse *et al.* 1997), in which about 20% of the population experience the heaviest burden of disease (about 80%), while the majority of the population carry only a few or no infections (Woolhouse *et al.* 1997). However, villages or towns with higher malaria incidence are usually targeted as a whole in malaria control programmes until such a time that individual episodes of malaria remain and become the focus of control (WHO 2007).

Variation in malaria risk are seen in both low to moderate transmission areas, where a considerable proportion of the population experience only little or no disease, while others experience multiple episodes of malaria over several years (Bousema *et al.* 2010a, Clark *et al.* 2008, Creasey *et al.* 2004, Woolhouse *et al.* 1997), and in high transmission intensity settings, where the majority of the population experience at least one malaria episode per year (Kreuels *et al.* 2008, Carter *et al.* 2000). This variation may be due to heterogeneity in contact rate between infected mosquitoes and the susceptible host (Acevedo *et al.* 2015, Smith *et al.* 1995b), which has the potential to alter the prevalence of malaria as well as the immunological response of the host to the parasite antigens (Smith *et al.* 1995b). In a single village there can be very marked variations in the risk of malaria between and within households (Bannister-Tyrrell *et*

al. 2017), but population-level analysis of malaria risk and epidemiology usually obscures these fine variations at the individual or household level. Factors underlying the variations in malaria risk may include the ecological characteristics facilitating breeding sites of mosquitoes (Reinbold-Wasson *et al.* 2012), household structural features and household size (Seyoum *et al.* 2017, Hulden *et al.* 2014), human behaviour such as outdoor sleeping at night (Clark *et al.* 2008) and economic activities that may increase exposure to infectious mosquito bites (Chuquiyauri *et al.* 2012).

Although individuals may be infected or challenged with the same strain of *Plasmodium* parasite, the host susceptibility to disease may vary owing to within and between host variations in immune responses. Malaria parasite transmission usually originates from a 'hot spot' within a geographical location (Bousema *et al.* 2012, Bousema *et al.* 2010a). A hot spot of malaria parasite transmission is defined as a "geographical part of a focus of malaria transmission where transmission intensity exceeds the average level" (Bousema *et al.* 2012). Hot spots are often more stable over time (Seyoum *et al.* 2017) and may serve as both sources of infection during transmission seasons as well as supporting continuing parasite transmission during the dry seasons (Bousema *et al.* 2012) when vector numbers are low. Identifying malaria hot spots within villages can help to predict areas of high risk of disease transmission and serve as a focal point of target in malaria control and elimination programmes (Bousema *et al.* 2012, Bousema *et al.* 2010a). It can also help focus health control measures and epidemiological surveillance in endemic areas where resources are inadequate (Bousema *et al.* 2010a).

The village of Daraweesh in eastern Sudan is considered a single homogeneous area, for which the malaria experience of its residents was closely monitored over time (Creasey *et al.* 2004). Although many studies in Daraweesh have looked at the malaria immunoepidemiology at the population level, there has been no study in this village looking at the household level dynamics of malaria epidemiology and mixed-*Plasmodium* species infections. The use of spatial-temporal tools to identify groups of households within this single village who are at increased risk of developing clinical episode of malaria has not been applied to analyse malaria transmission in this unique geographical location.

Knowledge of the biological, environmental and socioeconomic risk factors associated with malaria are important in implementing intervention programmes targeted at malaria control. A better understanding of malaria epidemiology at the household level, in relation to the population level, would provide important metrics for understanding the factors that contribute to variations in malaria incidence in a local area.

5.2 Study aim

To determine the spatial and temporal dynamics of malaria prevalence and *Plasmodium* species distribution in a mesoendemic village in eastern Sudan.

5.3 Hypothesis

The risk of having clinical malaria or being infected with a species of *Plasmodium* parasite is the same in all Daraweesh households and the same over time.

5.4 Materials and methods

5.4.1 The setting of the Daraweesh village

There were 52 families living in Daraweesh during the study period (1990-2000) with household sizes ranging from 2 to 26 occupants (mean household size 10.9 individuals). Families lived together in compounds comprising of a group of circular mud walled dwellings with thatched roofs (called tukul in the local Daraweesh community). Several families lived within compounds surrounded by woven sorghum-stalk fencing (designated in Figure 5.4.1 by solid lines on the next page). The yard spaces, locally called the *hawsh*, were used for outdoor sleeping except on rainy and cold nights in autumn and winter (Hamad *et al.* 2002). Traditional subsistence agriculture is centred around sorghum cultivation, which depends on the seasonal rains. There were also a number of market gardens irrigated by water pumps joined to deep-bore wells. No form of mosquito control was implemented during the study period, but during the early rainy season some local farmers used Malathion and other organophosphate insecticides for crop protection (Hamad *et al.* 2002).

There were either metal or concrete storage tanks of drinking water fed from a well, which were used throughout the year. Spillages, particularly from the larger tanks create small water pools and fill animal hoof prints that may provide breeding sites for mosquitoes (Hamad *et al.* 2002). The village lies on a flatter surface covered by black soil that allows rainwater to stay on the surface longer. These local environment characteristics may influence the presence of mosquito breeding sites.

For simplicity, families were designated with letters of the alphabet (e.g. families A to Z) or alphanumeric (e.g. family 2A, 2B etc.) for identification purposes. For this study, I labeled the compounds containing the family huts numerically. There was a mosque located between compounds 8 and 11, and a deep well closest to compound 10, that served as the source of water for the village (Figure 5.4.1 on page 149). A schematic outline of the village and the settings of the families or households and compounds at the time of the study is illustrated in Figure 5.4.1. It must be noted that the village has gone through a lot of remodelling and development in recent times compared to the layout of the village at the end of the study period in the year 2000.

Figure 5.4.1: Schematic layout of Daraweesh village indicating households and compounds. Families or households are designated by letters of the alphabet or alphanumeric combinations, with the numbers in red representing the different compounds. Solid lines indicate the woven sorghum-stalk fencing which separates compounds. Huts with no identification served as storage for agricultural materials and produce, as well as shelter for livestock. This schematic figure was provided by Prof. David E. Arnot (University of Edinburgh).

5.4.2 **Study participants**

The relationship between household and clinical malaria episodes included all 52 families and took into account the 802 single clinical malaria episodes diagnosed by microscopy in Daraweesh, which represented 1902600 person-days (i.e. an estimate of the actual time-at risk in days that all persons contributed to a study) over the eleven year study period (January 1990 to December 2000) (Creasey *et al.* 2004). Subsequent analysis of the spatiotemporal distribution of malaria included these 802 single episodes of clinical malaria as well as antibody response to *Plasmodium* MSP-1_{19}

antigens of the 333 individuals whose first clinical episode of malaria was discussed previously in Chapter 4. These individuals represented 49 of the 52 families in the village.

5.4.3 Antigens and ELISA

Antigens used for the ELISA included the MSP-1_{19} of the four *Plasmodium* species as well as *P. falciparum*–specific MSP-1 Block 2 antigens and the full-length and species-specific MSP-2A and MSP-2B serogroups (Table 2.6.1). ELISAs were performed as described in Section 2.7.1

5.4.4 Mapping of spatial data by QGIS

Maps showing year by year malaria prevalence and its distribution within the village were generated using QGISTM v.2.186 (QGIS developer team, Open Source Geospatial Foundation).

5.4.5 Statistical analysis

Space-time analysis of malaria prevalence and species of *Plasmodium* was done using a discrete Poisson model, with a generalised linear mixed model performed to identify differences in *Plasmodium* species between different households over time.

5.4.5.1 Discrete Poisson model

The discrete Poisson model was used as the number of cases in each household was known and the nature of the data were counts (Kulldorff 2015, Kulldorff 1997). Households with malaria were taken as cases, and the population as the combined number of person-years used to fit the Poisson model. Then, the Poisson data were analysed with the space-time scan statistics.

5.4.5.2 Cluster analysis

The scan statistics developed by Kulldorff and SaTScan™ software version 9.4 (Kulldorff 2015) were used to identify the presence of space-time malaria clusters. The scan statistics did scanning gradually across time and/or space to identify the number of observed and expected observations inside the window at each location. The

scanning window was an interval (in time), a circle (in space) or a cylinder with a circular base (in space-time) to which window sizes were determined, and the window with the maximum likelihood was the most likely cluster, and a *P*-value was assigned to this cluster.

5.5 Results

5.5.1 Trends in population and malaria prevalence in Daraweesh

Within the 11-year study of malaria epidemiology in Daraweesh, there were 802 single episodes of clinical malaria, with a third of the village population not experiencing a clinical malaria episode (Creasey *et al.* 2004). After the long dry season of 1988-1990, the first and only clinical episode of malaria case in the village was recorded in 1990. There was a steady rise in malaria incidence from 1992 peaking in 1994 following the rainfall in 1993 (Figure 5.5.1 on page 153). Following this there was a steady fall in malaria episodes until 1997. Malaria incidence peaked again in 1998 with the number of cases being about four times that observed in the previous year. The rainfall recorded in Daraweesh and across the entire Sudan was higher in 1998 compared to that of previous years. There was a decline in the number of malaria cases in both 1999 and 2000, with the cases recorded in 2000 being similar to levels observed in 1992 (Figure 5.5.1). It is obvious in Daraweesh that rainfall patterns influence the number of malaria cases in each transmission season, as it has a direct effect on the number of adult mosquitoes. In the dry seasons the annual rain-dependent expansion of the *Anopheles* population is not observed, reducing parasite transmission and malaria cases, as observed with only one documented clinical malaria case during the 1990 transmission season, when drought occurred.

The population of Daraweesh increased steadily throughout the study period. The mean malaria positive prevalence, which is the fraction of clinical malaria episodes to the population of the village, for the 11 year study period was 1.36 ± 0.068SD (range 0-3) and varied markedly between households. The number of households experiencing clinical malaria correlated positively with the number of cases in each transmission year, with more households experiencing clinical malaria in transmission seasons where higher numbers of malaria cases were recorded (Figure 5.5.1).

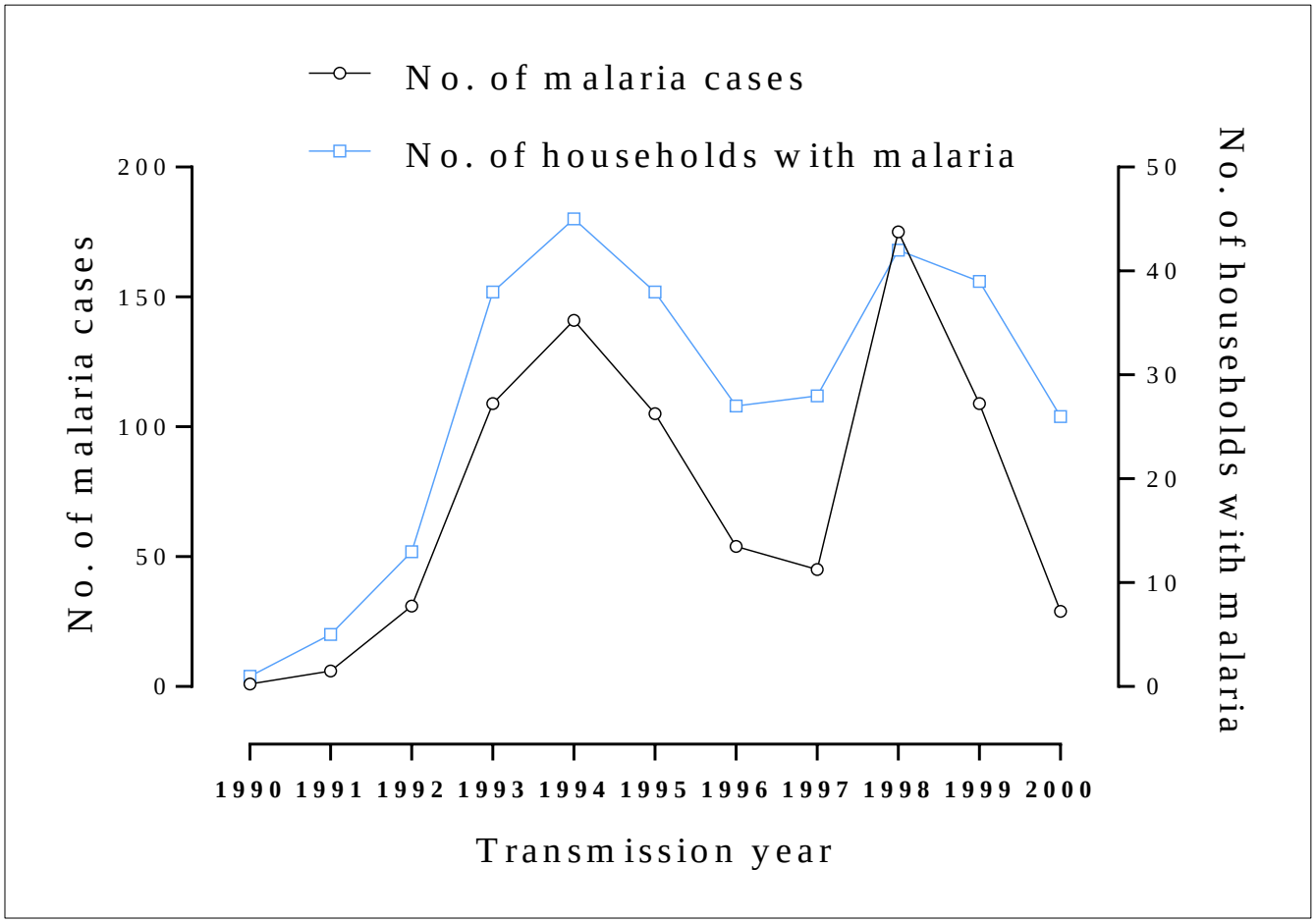


Figure 5.5.1: Incidence of malaria correlates positively with increasing household size in the Daraweesh village.

5.5.2 **Overview of clinical malaria cases in Daraweesh households**

The 802 individual clinical infections diagnosed in Daraweesh were treated over 63420 person-months, giving an overall 11-year average malaria case rate of 12.6 episodes/1000 person/months (Creasey *et al.* 2004). This average, however, conceals possible household to household variation in clinical disease incidence, which is addressed in this chapter. The single clinical malaria episode in 1990 (representing 0.2 malaria cases/1000 person months) was identified in an individual (aged 16 years) from family R in compound 7 (Figure 5.5.2 and Figure 5.5.3 on page 155 and 156 respectively). The location of the hut in which this individual resided is important in terms of the ecology of mosquitoes and parasite transmission. Family R is one of the families closest to the vegetation on the southeast part of the village.

In 1991, there were six new cases of clinical malaria. These were randomly distributed in the village within five households 2B, 2N, G, J, and V, residing in four different compounds (Figure 5.5.2). No member of family R experienced a malaria episode in that year, suggesting that the episodes seen in 1991 were not from any identifiable foci. This dispersed pattern may be indicative of the unusual biting behaviour of the mosquitoes. During the 1992 transmission season, there was a north and south divide of the incidence of malaria and this spread was both seen within and between households. The increased rainfall between 1993 and 1998 (Creasey *et al.* 2004) correlated positively with a corresponding rise in malaria episodes and this was uniformly distributed throughout the village, until 1997 when the north and south divide became similar to that observed in 1992.

Whiles some families (A, B, F, G, H, O, V, X, 2A, 2H, 2L, 2M, 2O and 2P) experienced higher incidence of malaria over the study period, there were two families (Y and 2U) with household sizes of 2 and 10 members respectively, who did not experience any clinical malaria episodes over the course of the eleven year study period (see previous Figure 5.5.2 and Figure 5.5.3). One, five and thirteen households experienced malaria in 1990, 1991 and 1992 respectively. Between 1993 and 2000, the mean number of households who experienced clinical malaria was 35.4 (range: 26-45 households).

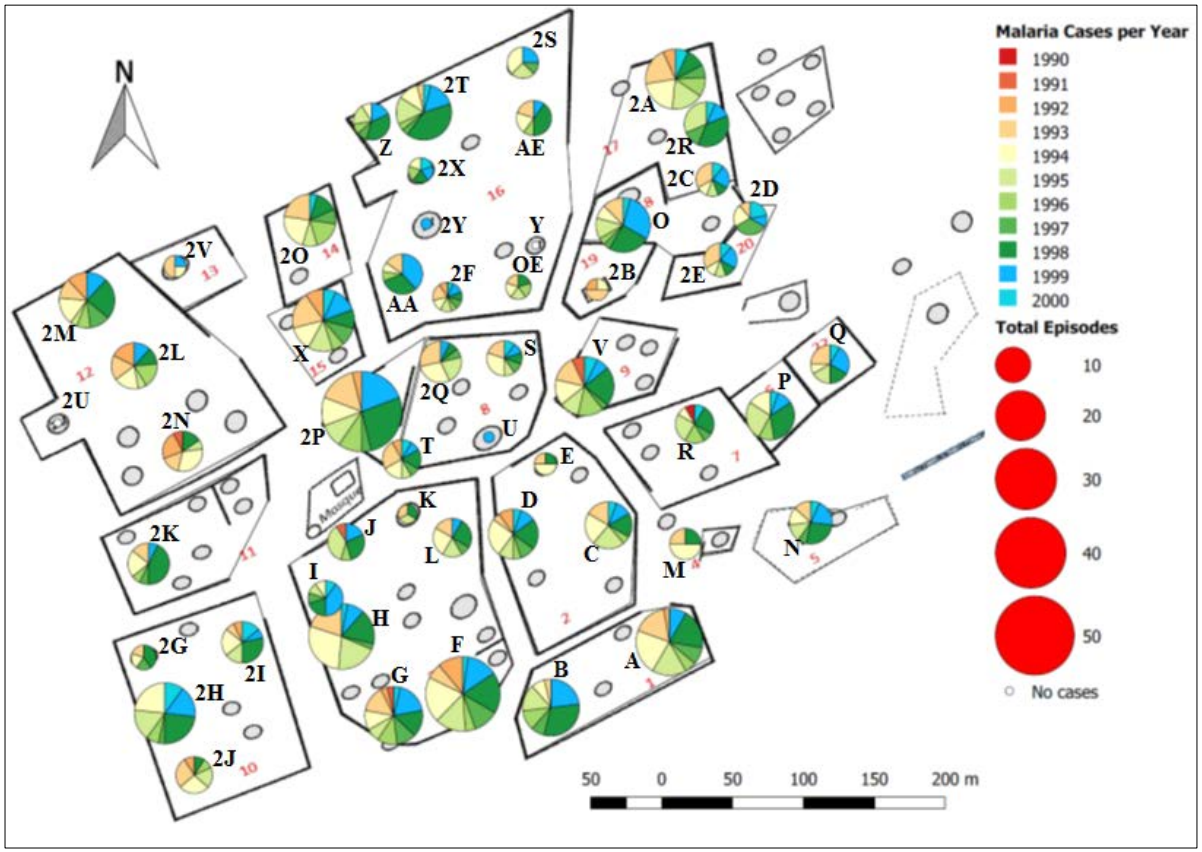


Figure 5.5.2: Malaria incidence is focal in Daraweesh households. The pie charts show the year by year episodes of malaria, with the sizes representative of the number of malaria cases. Households are defined by letters of the alphabet and alphanumeric combinations, while compounds are defined by the numbers in red. Huts with no identifications (white ovals) served as storage spaces for agricultural materials and produce as well as shelter for livestock.

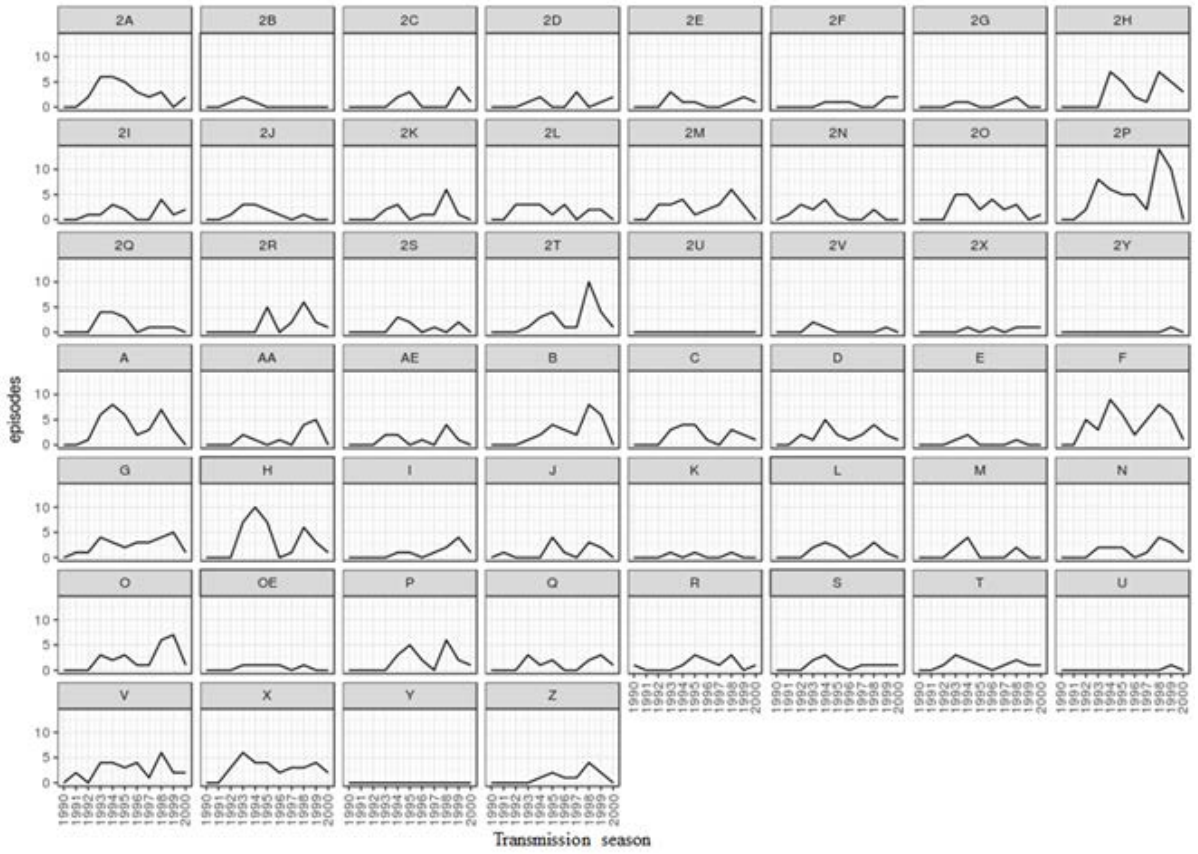


Figure 5.5.3: Malaria incidence occurs most frequently in certain households. Households are defined by letters of the alphabet and alphanumeric combinations. The y-axis represents the number of clinical malaria episodes, and the x-axis the transmission season.

5.5.3 **Malaria cases and household size**

Malaria transmission is generally within households and compounds and the propagation of the disease depends on the transmission that occurs between individuals and family members. A third of the Daraweesh population did not experience clinical malaria episode for the duration of the study, although some of these people were found to be PCR positive for malaria. The malaria experience ranged from 1 to 8 episodes, with two individuals experiencing 7 and 8 clinical malaria episodes over the course of 11 years. Family or household size has been reported to be proportional to the incidence and prevalence of malaria in communities where the disease is endemic (Hulden *et al.* 2014). As shown in Figure 5.5.4 on the next page, there was a direct relationship between household size and the incidence of malaria episodes in Daraweesh, with household level variations in the incidence of clinical malaria explained by variation in household size. Smaller family sizes (less than 5 individuals) experienced fewer episodes of disease (0-13, mean 4.75 malaria episodes) compared to families with more than 5 members in a household where between 0 and 52 clinical malaria episodes (mean 17.4 cases) were recorded over 11 years. The number of households experiencing clinical malaria in each transmission season correlated with the number of malaria cases diagnosed in that transmission season.

There was a general trend of a higher proportion of residents in households experiencing a clinical episode of malaria (Figure 5.5.5). Excluding the two households (families Y and 2U) where no clinical malaria was documented, the proportion of individuals experiencing clinical malaria per household ranged from 16.66 to 100% with a mean of 55.69%. The lower range was observed in family U where only one individual (out of a 6 household members) experienced one clinical malaria. Two households (family 2G, household size 2 and family 2A, household size 13) had all the residents experiencing at least one clinical malaria episode over the 11 year study period. The proportion of individuals per household who experienced a clinical malaria episode during the study period is shown in Figure 5.5.5.

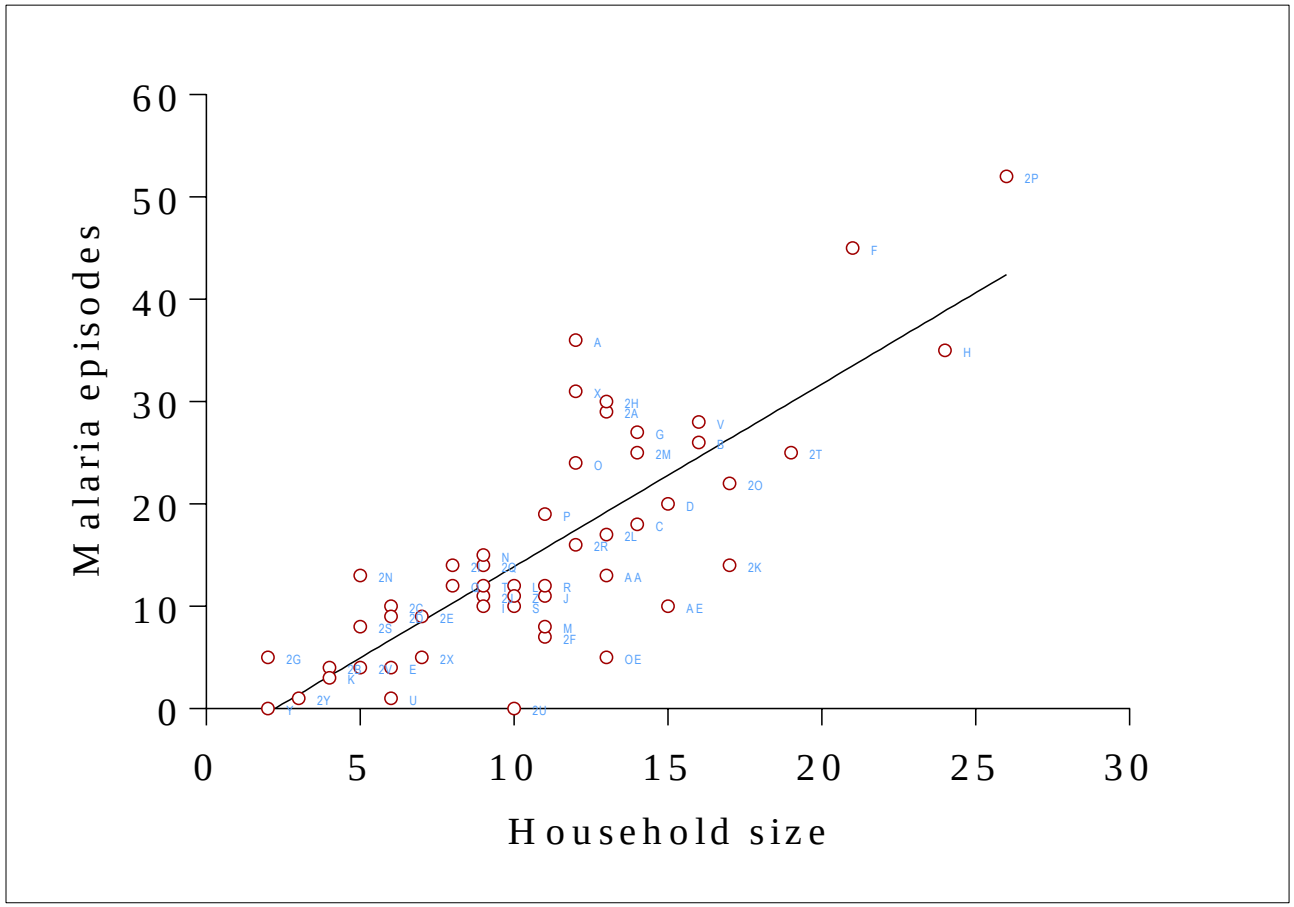


Figure 5.5.4: Household size correlates with the incidence of malaria in Daraweesh. Households are defined by letters of the alphabet and alphanumeric combinations and labelled in blue. Straight line indicates a regression line.

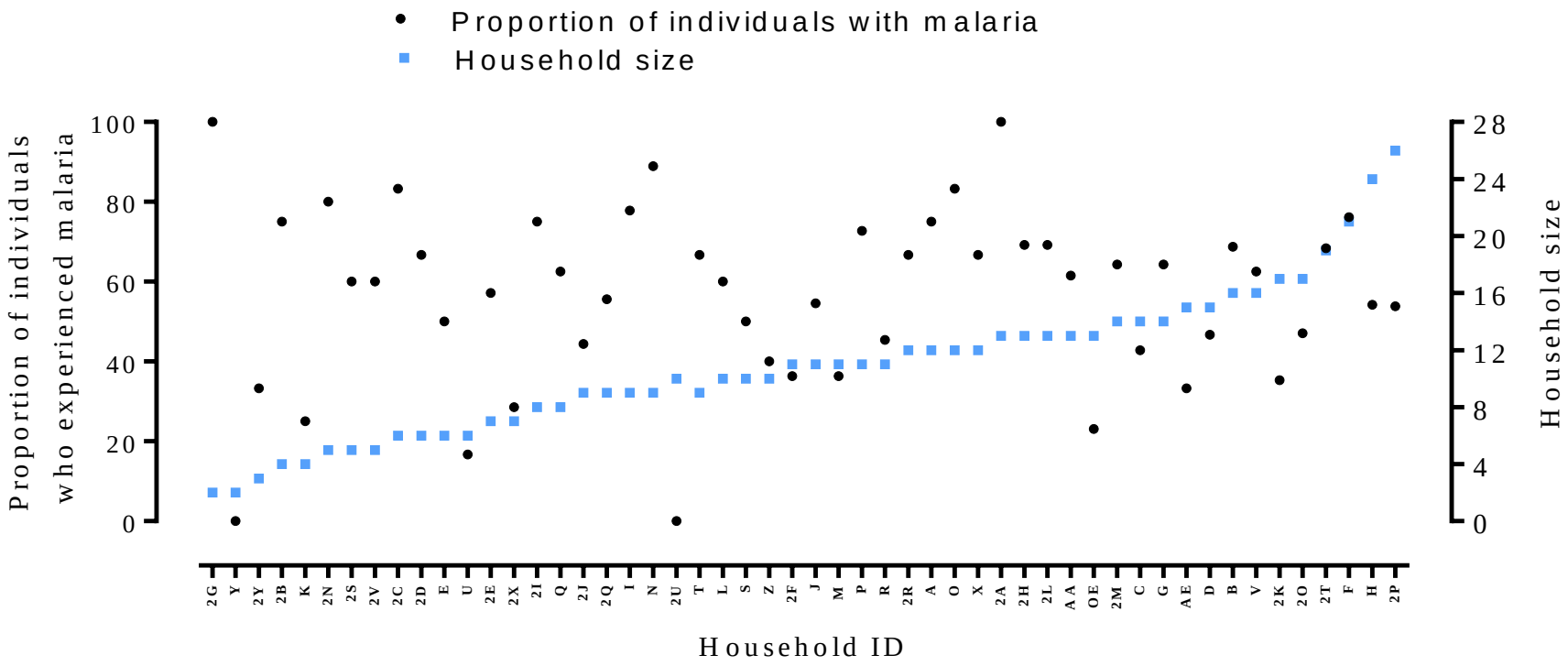


Figure 5.5.5: A higher proportion of residents of households experienced clinical malaria episodes. The proportion of individuals experiencing clinical malaria per household ranged from 16.66 to 100% with a mean of 55.69%. Two households Y and 2U had no documented malaria episode.

5.5.4 Space-time analysis of clinical malaria cases in Daraweesh households

Knowing the number of individuals and the number of clinical malaria cases recorded for each household in each transmission season, a retrospective space-time analysis was performed using the discrete Poisson model. This model confirmed that malaria episodes in Daraweesh were not randomly distributed within households. Two significant space-time clusters, and one temporal cluster were identified in the village. These clusters span different time periods and included different households in different geographical locations in the village. The first space-time cluster (cluster 1) was a 0.23 km radius area that spanned a longer time period between 1993 and 1999. It was largely concentrated in the southwest and central area of the village and included 23 households, with a population of 272 individuals experiencing 407 clinical malaria episodes over seven transmission seasons (Figure 5.5.6 on page 161). The observed number of malaria cases was higher than expected (1.66) (Figure 5.5.7 and Table 5.5.1; pages 162 and 163 respectively). Households and individuals within this cluster were 2.33 times more likely to be at risk of having a clinical malaria episode than households outside of this cluster (RR =2.33, $P < 10 \times 10^{-15}$) (Table 5.5.1).

The second cluster (cluster 2) occurred within a shorter time period between the 1998 and 1999 transmission seasons. It covered a 0.25 km radius area and included 22 households mainly in the northeast part of the village. Cluster 2 included 214 individuals experiencing 100 clinical malaria episodes in two years. Similar to cluster 1, the observed/expected ratio of malaria cases was higher (1.81). Households and individuals within cluster 2 were 1.93 times more likely at risk of having a clinical malaria episode than households outside this cluster (RR = 1.93, P=0.00032).

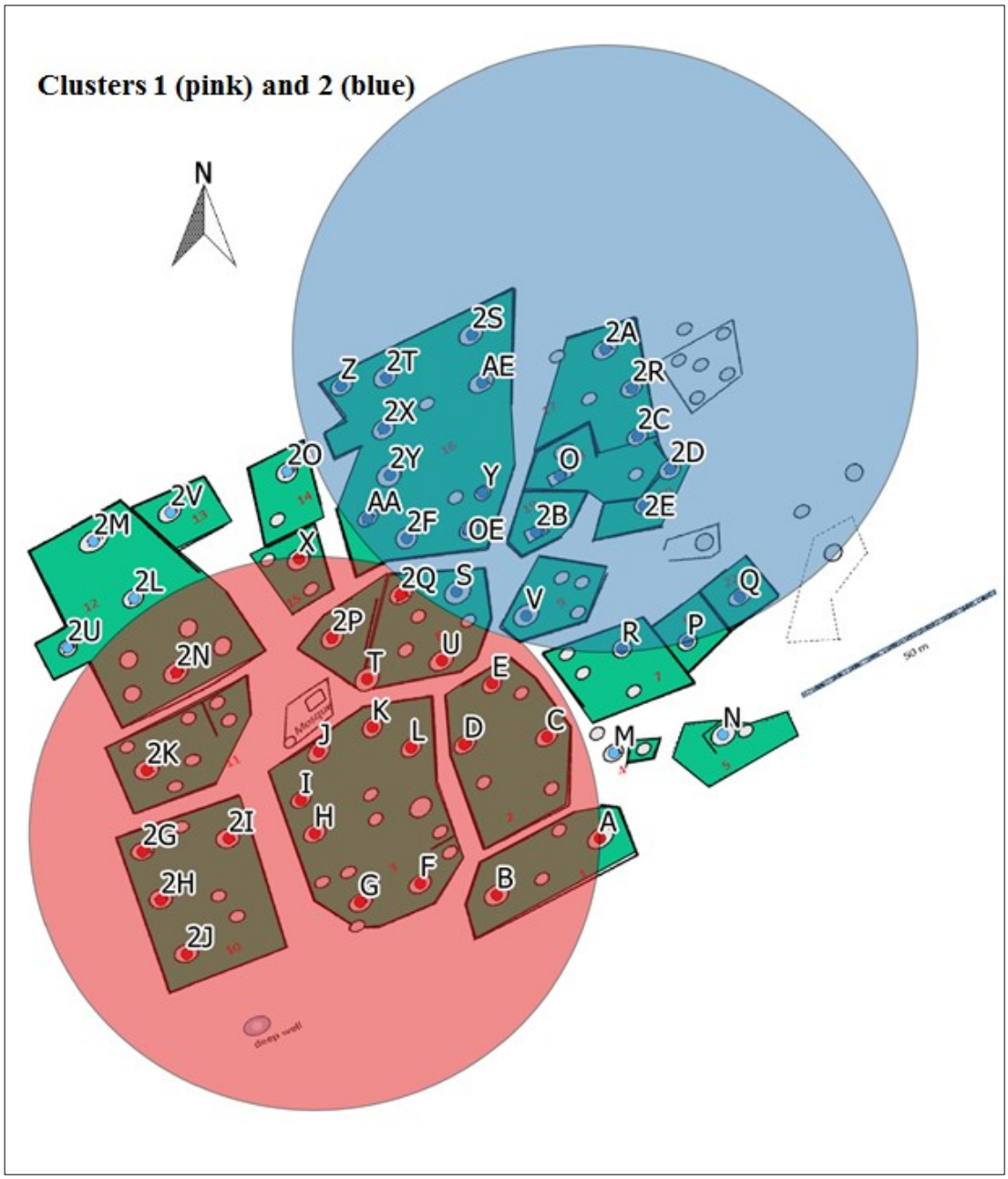


Figure 5.5.6: Two clusters of malaria incidence were identified in Daraweesh between 1991 and 2000. The red ovals in the large pink circle represents households in cluster 1, southwest and central area of the village (1993-1999), and the blue ovals in the large blue circle represent households in cluster 2, northeast part of the village (1998-1999). Households outside the pink and blue circles were outside the two clusters. Households are defined by letters of the alphabet and alphanumeric combinations.

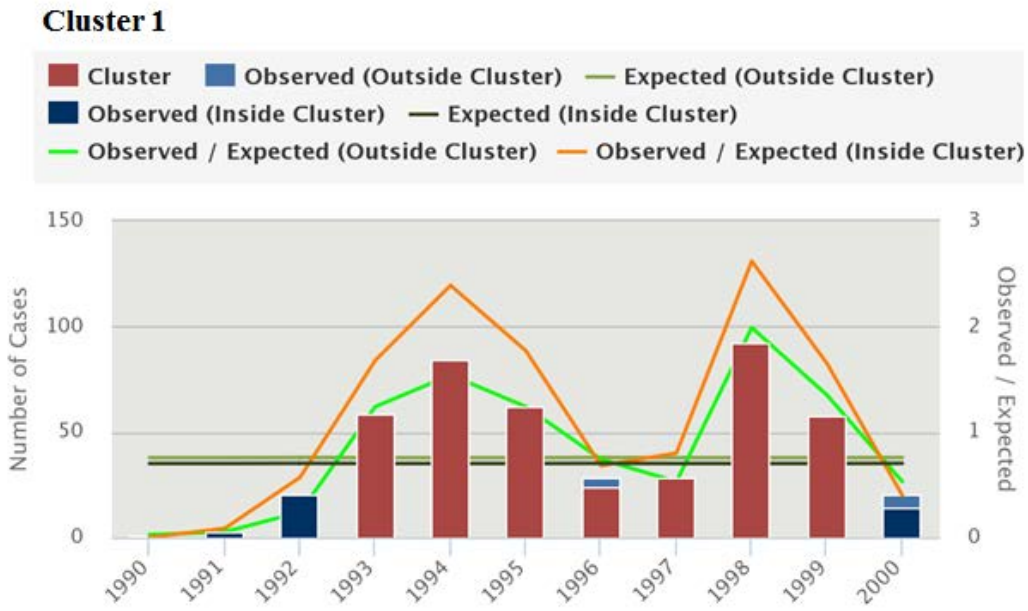


Figure 5.5.7: The first significant cluster of clinical malaria episodes covered seven transmission seasons (1993-1999). Cluster 1 covered the southwest and central area of the village and included 23 households, with a population of 272 individuals experiencing 407 clinical malaria episodes between 1993 and 1998. The observed number of malaria cases was higher than expected (1.66). Households and individuals within this cluster were 2.33 times more likely to be at risk of having a clinical malaria episode than households outside of this cluster.

Table 5.5.1: Retrospective space-time analysis of the most likely clinical malaria episodes.

Variables	Cluster 1	Cluster 2
Coordinates (N, E)	13.903490 N, 35.408406 E	13.907122 N, 35.410583 E
Radius (km)	0.23	0.25
Time frame	Jan 1993 to Dec 1999	Jan 1998 to Dec 1999
Number of households	23	22
Population	272	214
Clinical malaria cases	407	100
Expected cases	245.61	55.19
Observed/expected ratio	1.66	1.81
Annual cases/100,000	21381.9	23380.0
Relative risk	2.33	1.93
Log likelihood ratio	70.242380	16.001769
P-value	$10x10^{-15}$	3.2×10^{-4}
Household in cluster	(A, B, C, D, E, F, G, H, I, J, K, L, T, U, V, X, 2G, 2H, 2I, 2K, 2N, 2P and 2Q)	(P, Q, R, S, V, Y, Z, 2A, 2B, 2C, 2D, 2E, 2F, 2R, 2S, 2T, 2X, 2Y, AA, and AE)

Note: Households are defined by letters of the alphabet and alphanumeric combinations.

The start years of both cluster 1 (1993) and cluster 2 (1998) corresponded to the transmission seasons when heavy rainfalls were recorded in Daraweesh (Creasey *et al.* 2004). The two clusters were also found next to each other suggesting that for a while, and over a longer period of time the population in cluster 1 were continually being infected with *Plasmodium* parasites until an overlap in 1998 when the population in cluster 2 were also actively infected for a period of two years. The single temporal

cluster was observed from 1993 to 1999, with an overlap of the 1998 transmission season.

5.5.5 Space-time analysis of *Plasmodium* species in households

As previously discussed in Chapter 4, antibody response to MSP-1_{19} antigens of the four *Plasmodium* species were tested for 333 individuals who had experience a first malaria episode. Similar to the model used above for the total clinical malaria episodes over the course of eleven years, a retrospective space-time analysis was performed using the discrete Poisson model for all first time malaria diagnosed to understand if there were any significant clustering of the species in space and time.

This model confirmed that infection by the four *Plasmodium* species were not randomly distributed within the village. Multiple significant clusters were identified for the parasite species; three for *P. falciparum*, two for *P. vivax* and *P. malariae*, and one for *P. ovale* (Figure 5.5.8 on page 165). These clusters had overlapping time frames, with some of the species significantly infecting the same households.

For example, *P. falciparum* and *P. vivax* had two overlapping significant clusters spanning between 1992 to 1996 in the same household of 2A, with relative risk of 3.69 (P=4.4 x 10^{-5}) and 4.65 (P=1.5 x 10^{-2}) respectively, and between 1996 to 1999 in which these two species increasingly caused malaria in the same 9 households (RR=1.93, P=2.2x10^{-6} for *P. falciparum* and RR=3.94, P=1.7 x 10^{-15} for *P. vivax*) (Table 5.5.2 on page 166). The third significant cluster of malaria caused by *P. falciparum* was from 1995 to 1998 and was concentrated in six households located in the southeast of the village. Residents of these household were 1.7 times more likely to be infected with this species than households outside of this cluster (P=1.4 x 10^{-2}).

Similarly, between 1995 and 1999, residents of household 2P were significantly at risk of increasing malaria caused by *P. malariae* (RR=8.63, P=4.4 x 10^{-16}) and *P. ovale* (RR=7.74, P=1.7 x 10^{-15}) than other households (Table 5.5.2). The second cluster of malaria caused by *P. malariae* was seen during the 1996 to 1999 transmission seasons, with four households located in northeast of the village at significantly increased risk of exposure to this species (RR=4.23, P=1.7 x 10^{-2}) (Table 5.5.2).

Figure 5.5.8: Multiple significant clusters identified for the parasite species. Multiple significant clusters were identified for the parasite species; three for *P. falciparum*, two for *P. vivax* and *P. malariae*, and one for *P. ovale*.

Table 5.5.2: Retrospective space-time analysis of the most likely *Plasmodium* species infecting households.

	P. falciparum			*P. vivax*		*P. malariae*		*P. ovale*
Variables	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 1**	**Cluster 2**	**Cluster 1**	**Cluster 2**	**Cluster 1**
Coordinates (N, E)	13.907122, 35.410583	13.903029, 35.409770	13.904883, 35.406553	13.907122, 35.410583	13.904883, 35.406553	13.904956, 35.408536	13.906171, 35.410242	13.904956, 35.408536
Radius (km)	0.00	0.14	0.21	0.00	0.22	0.00	0.071	0.00
Time frame (year)	92- 95	95- 98	96- 99	92-96	96-99	95- 99	96- 99	95-99
Relative risk	3.68	1.70	1.93	4.65	3.94	8.63	4.23	7.74
LLR	16.9	10.4	20.2	11.1	44.1	48.2	10.6	45.8
P-value	4.4×10^{-5}	1.4×10^{-2}	2.2×10^{-6}	1.5×10^{-2}	1.7×10^{-15}	4.4×10^{-16}	1.7×10^{-2}	1.7×10^{-15}
Household in cluster	(2A)	(A, B, C, D, F, and G)	(X, 2G, 2I, 2K, 2L, 2M, 2N, 2P, 2U, and 2V)	(2A)	(J, 2G, 2I, 2K, 2L, 2M, 2N, 2P, 2U and 2V)	(2P)	(O, Y, 2B and 2C)	(2P)

Note: All time frames are from Jan to Dec. LLR: log likelihood ratio.

5.5.6 Variation in *Plasmodium* species within households and over time

Of the 333 individuals experiencing a first clinical episode of malaria (samples used in Chapter 4) the year by year proportion of the four different *Plasmodium* species was stable from 1991 to 1999 in the order of *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale* (Figure 5.5.9 on page 168). Of the 49 families in which blood samples were available and screened for IgG response to MSP-1_{19} antigens from the four *Plasmodium* species, mixed-*Plasmodium* infections-only were seen in three families (2K, 2X, 2Y) (Figure 5.5.10). Families 2X and 2Y lived in the same compound in the north of the village while family 2K lived alone in a compound in the western part of the village. Infections caused by single species only were seen in six families (families E, I, P, Q, R and U), all of whom lived in different compounds in the village. All other families experienced malaria comprising of both single and mixed-species infections (Figure 5.5.10 on page 169).

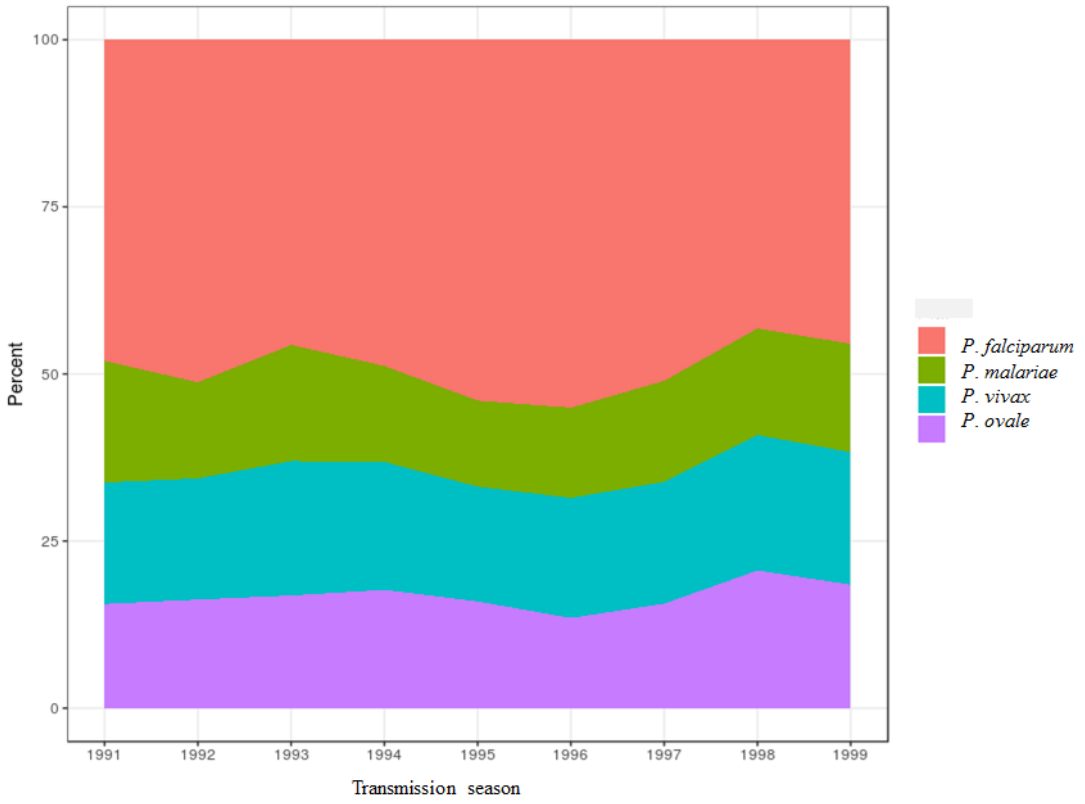


Figure 5.5.9: *Plasmodium* species are relatively stable over time. The proportions of the four *Plasmodium* species are relatively stable over the course of the study period.

Figure 5.5.10: Single- and mixed-*Plasmodium* infection prevalence in Daraweesh households. Households are defined by letters of the alphabet and alphanumeric combinations.

5.5.7 Heterogeneity in antibody responses to *Plasmodium* MSP antigens

There was no plasma sample available from the individual who contracted malaria in 1990 to determine the type of *Plasmodium* species or the genotype of *P. falciparum* antigens he carried. The first episodes of malaria with available plasma sample were from the 1991 transmission season. Plasma IgG antibody responses from these six individuals showed heterogeneous responses to MSP-1_{19} antigens of the four *Plasmodium* species as well as *P. falciparum*-specific MSP-1 Block 2 and MSP-2A and -2B antigens. *P. falciparum* MSP-1_{19} antigen was recognised by the plasma antibodies of all individuals, while two MSP-1 Block-2 antigen types, K-1 and MAD20 Block 2, were detectable by the plasma of five of these six individuals. RO33 Block 2 was detectable by only one individual, 2N, who also had antibody responses to all other antigens tested (Table 5.5.3, page 170). Two members of the same family (V4 and V5) had different responses. While both had a response to *P. falciparum* MSP-

1_{19} antigens, only individual V4 had additional responses to two MSP-1 Block-2 antigens. The antibody responses observed in these individuals in the 1991 transmission season illustrate no clear compound- or family-specific responses. This observation was also very common in subsequent transmission seasons.

Table 5.5.3: Antibody responses to *Plasmodium* MSP antigens are heterogeneous in Daraweesh (1991 transmission season).

	MSP-1_{19} antigen				*P. falciparum*-specific antigens				
ID	*P. falciparum*	*P. malariae*	*P. vivax*	*P. ovale*	K-1	MAD20	RO33	MSP-2A	MSP-2B
2B2	+	+	-	-	+	+	-	+	-
2N4	+	+	+	+	+	+	+	+	+
G7	+	-	+	-	+	+	-	+	+
J2	+	-	-	-	+	+	-	+	+
V4	+	-	-	-	+	+	-	-	-
V5	+	-	-	-	-	-	-	-	-

Note: The letters of the alphabet and alphanumeric combinations represent each individual's identity within a family. The + and - symbols represent detectable and undetectable antibody response respectively to the various *Plasmodium* antigens.

In the Daraweesh population, a previous study reported that a minority of the population often responded well to *P. falciparum* MSP-1_{19} antigen, but failed to response to the polymorphic MSP-1 Block 2 antigens (Cavanagh *et al.* 1998). In view of this I wanted to determine the patterns of antibody responses to *P. falciparum* MSP-1_{19} antigen and the polymorphic MSP-2 antigens in individuals who had more than one documented clinical malaria episode. A subset of the study population (136 individuals) who had more than one clinical malaria episode at different transmission

years had their plasma (from the second episode) tested to determine changes, if any, of antibody responses to *P. falciparum* MSP antigens between the first and subsequent malaria episodes. Selective non-responsiveness was observed in 11.03% of the population studied, with three different patterns of anti-MSP response to *P. falciparum*-specific antigens seen.

The first and the most common pattern (which was expected), consisted of individuals who had antibody responses to *P. falciparum* MSP-1_{19}, and at least one MSP-2 antigen in both the first and subsequent infections (121 of 136 individuals, Figure 5.5.11 panel A). The second pattern was observed in 11 individuals who made antibodies responses against *P. falciparum* MSP-1_{19} antigen, but not MSP-2 antigens, in the first infection, but responded to the other more polymorphic MSP-2 antigens in subsequent infection(s). An example of this pattern is illustrated in panel B of Figure 5.5.11. The final pattern is represented by a minority of individuals (4 of 136 cases) who responded well to both the conserved MSP-1_{19} and the polymorphic MSP-2 antigens at the first infection, but then failed to recognise MSP-2 antigens in subsequent infection(s). An example is illustrated in panel C of Figure 5.5.11. In this example, an individual having a malaria episode in October 1994 made an antibody response to both *P. falciparum* MSP-1_{19} and MSP-2A and 2B antigens, but then failed to make responses to the MSP-2 antigens during subsequent malaria episodes in August 1996 and October 1997, in spite of the strong response to *P. falciparum* MSP-1_{19}.

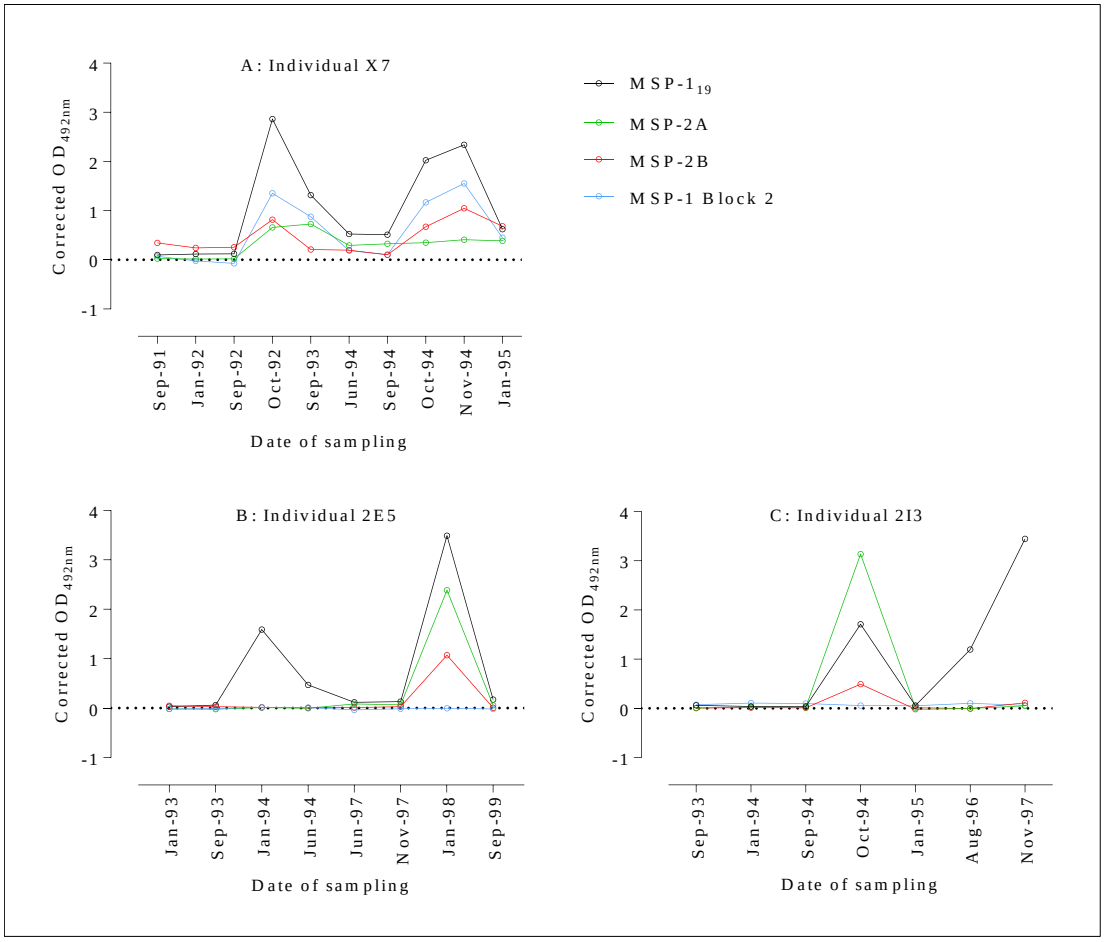


Figure 5.5.11: Selective non-responsiveness to *P. falciparum* polymorphic antigens is common in Daraweesh. A: individual X7 represents the majority of the population (121 individuals) who had antibody response to the polymorphic MSP-2 antigens at both the first and subsequent clinical malaria episode (October 1992 and 1994); B: individual 2E5 represents a smaller population (8 individuals) who only had a response to MSP-2 antigens at their second infection (January 1998 but not in the 1994 transmission season); C: individual 2I3 represents the minority group (4 individuals) who made a response to MSP-2 antigen at their first infection (October 1994), but failed to respond to these polymorphic antigens at subsequent clinical episodes of malaria (August 1996 and November 1997).

5.6 **Discussion**

In this chapter, a retrospective analysis of malaria incidence in Daraweesh allowed an in-depth characterisation of some fine scale details of malaria epidemiology in the village, including significant space-time clustering of malaria incidence, and identifying variations in malaria incidence between households and over time.

The epidemiology of malaria in the village showed a rainfall-dependent pattern in which higher number of clinical malaria cases were diagnosed in transmission seasons where there was heavy rainfall, and lower number of cases in the dry season periods. During the dry seasons the village continued to depend on the deep well as a source of water for domestic and vegetative purposes. This did not seem to play any role in creating breeding sites for adult mosquitoes, suggesting that drought has the capacity to break malaria parasite transmission in the village. The diagnosis of one case of malaria in 1990, however, suggests that blood feeding mosquitoes were not entirely absent in the village during the dry seasons, although the vector to human population ratio might have been very low. It has been shown that during the dry season, female *Anopheles* mosquitoes partially feed on human blood while undergoing an incomplete reproductive diapause (i.e. a period of suspended development in an insect) (Yaro *et al.* 2012, Lehmann *et al.* 2010). In this case malaria parasite transmission in the village could be described as endogenous, meaning the malaria cases originate within the village with a resident population of mosquitoes that are not being wind-dispersed over long distances into the village. This is supported by the fact that mosquitoes that breed around houses fly locally and do not have the capacity to fly longer distances (Biggs and Brown 2001, Gilles 1993).

Considering that the closest village to Daraweesh, Asar, was 4 km away it is possible the mosquitoes were inhabiting local vegetation during the time of drought, but had limited water source for breeding. It is worth noting that within this same period, low but significantly higher number of malaria cases were recorded in Asar, possibly due to there being more water sources and mosquito breeding sites in Asar relative to that in Daraweesh. (Hamad *et al.* 2002). The rainfall measurements in Daraweesh were based on satellite measurement of green colouration on the Gedaref plain, which was

indicative of levels of actively growing vegetation. This measure is a surrogate for rainfall in and around the plains within a 10 km square area, in which Daraweesh sits. Rainfall patterns can influence the ecology of the mosquito population and their breeding sites (Billingsley *et al.* 2005, Hamad *et al.* 2002). Mosquito population size and sporozoite rates have been shown to fluctuate between seasons and over years, increasing shortly after the onset of rainfall, and declining during long dry seasons as sources of water for breeding become scarce (Billingsley *et al.* 2005, Charlwood *et al.* 1995). This might have contributed to the fluctuating number of malaria cases seen during rainy and dry seasons.

In Daraweesh, household sizes correlated with malaria cases with larger family sizes experiencing higher numbers of malaria cases. In areas where malaria has been eradicated or prevalence has reduced over many years, it has been shown that declining household sizes play a key role in malaria case decline (Bannister-Tyrrell *et al.* 2017, Hulden *et al.* 2014, Hulden and Hulden 2009). Decreasing household size may result in fewer interactions between families, and accordingly decreasing contacts between the host and mosquito vectors (Hulden and Hulden 2009). However, in larger household in Daraweesh and other neighbouring villages, individuals are likely to sleep outdoors during the night (Hamad *et al.* 2002), thereby increasing the likelihood of being bitten by mosquitoes. The outdoor biting capacity of *An. arabiensis* has been shown to be significantly higher in Daraweesh than its indoor biting rate (Hamad *et al.* 2002), which may partly explain the higher number of malaria episodes observed in larger households where occupants are likely to sleep outdoors. Mosquito control complemented with other factors such as effective drug treatment and surveillance are important steps towards malaria eradication. The finding of my study of an apparent correlation between household size and decline in malaria cases is significant for both short and long term control of malaria in endemic regions. For example, malaria control agencies in Ministries of Health working in collaboration with other departments and stakeholders could ensure that land development, settlement planning and the planning of exploration of natural resources (Hulden *et al.* 2014) are taken into consideration when designing programmes for malaria eradication. Evidence of this is seen in the successful malaria control in Aneityum in Vanuatu since 1996, following

the provision of individual bed nets and effective antimalarial drug distribution and surveillance in smaller household size (average of 5.6 individuals) (Kaneko 2010, Kaneko *et al.* 2000). Such coordinated efforts through inter-ministerial collaboration would contribute significantly to reducing the burden of malaria.

The risk of having a clinical malaria episode in Daraweesh were significantly clustered in two geographical locations, herein referred to as 'hotspots'. These clusters spanned different transmission seasons and included different households in two geographical locations in the village, mainly the southwest and central, and the northeast of the village. These clusters were more stable at these locations and over time. In this study, the discrete Poisson model confirmed both the existence of clustering of households with clinical malaria episodes, and demonstrated that households with malaria episodes were more aggregated than those not experiencing clinical malaria, consistent with other observations of malaria clustering elsewhere (Alemu *et al.* 2013). The concentration of the heaviest burden of malaria in households which fell under cluster 1 over a long period of time (1993-1999), and cluster 2 for two years (1998-1999), both being within a radius of 0.25 km suggests a focal nature of *Plasmodium* parasite transmission in the village. Similar observations have been reported with Dengue fever, another mosquito-borne viral disease causes by Dengue virus, in which certain populations within a hotspot area are seen to be heavily infected over time and account for the transmission of the disease to other neighborhoods (Vincenti-Gonzalez *et al.* 2017).

The clustering of malaria incidence (cluster 1) suggests a higher exposure to infectious mosquitoes in the 23 households in the southwest and central areas of the village for a longer period of time (1993-1999), especially after the two heavy rainfalls in 1993 and 1998 (peak of cases in 1994 and 1998 according to the space-time analysis). It has been shown that mosquitoes have the tendency to feed and return to the first household they encounter (Smith *et al.* 1995b), with female mosquitoes visiting no more than 3 houses in their lifetime (Gubler 1998) and flying shorter distances of between 10 and 30 meters away from breeding or water sources (Getis *et al.* 2003). This may suggest that infectious mosquitoes were closer to these households than other parts of the village. Following heavy rainfalls, the mosquito population may increase, and pockets

of pools, as well as the greener vegetation may serve as potential breeding sites for the female mosquitoes (Seyoum *et al.* 2017). This may contribute to an increase in vector density as well as increased contact with the human host, leading to a greater number of clinical malaria episodes (Seyoum *et al.* 2016, Abate *et al.* 2013). Households which fell under cluster 1 had larger family sizes. As is the case in this village, members of larger households usually use the yard spaces in the compounds for outdoor sleeping (Hamad *et al.* 2002) which may explain their vulnerability to infectious bites. Although this study assumes that all malaria cases were occurring within households, occupation-related mobility and multiple residence system (Bannister-Tyrrell *et al.* 2017) such as household member(s) moving to other properties, or children staying overnight with relatives in other households could not be accounted for as these are challenging to assess.

The onset of the 1993 rainfall resulted in the cluster not randomly distributed over the entire village, but limited to only the 23 households in the southeast and central part of the village. This cannot be explained by some of the common factors associated with malaria clustering such as landscape, building structure or house construction (Bannister-Tyrrell *et al.* 2017). The characteristics of the local environment (e.g. the flat surface covered by black soil that allows rainwater to stay on the surface longer) which may influence the presence of mosquito breeding sites, was similar throughout the village (Hamad *et al.* 2002). Furthermore, the materials use for the construction of the household huts were the same for all the residents. What is known is that the deep well in the village was located at the southwest of the village, and may have contributed as a breeding site during both the rainy and dry seasons, culminating in households closest to it being more susceptible to infectious bites from mosquitoes. Genetic traits such as ABO blood group, and red blood cell haemoglobinopathies have also been suggested to influence clustering of malaria incidence in certain households within a population (Bannister-Tyrrell *et al.* 2017). These may not explain these differences in space-time analysis between the two geographical locations in Daraweesh where malaria cases clustered. This is because the people of the village are descendants from the Fulani tribe in Burkina Faso, and no genetic predisposition has

been found to contribute to increased susceptibility of malaria in some groups more than others (Creasey *et al.* 2004).

The northeast households in the village were a consistent and significant secondary cluster for malaria incidence (1998-1999), that corresponded to the start of heavy rainfall recorded in 1998. Although this cluster overlapped in time with cluster 1, the shift of malaria burden to another part of the village was dramatic and intense over a shorter period. Shift in malaria hotspots are common when control interventions are implemented in areas under intense transmission (Dhimal *et al.* 2014). However, apart from chemotherapy which was promptly administered to people who were diagnosed of having clinical malaria, no specific malaria control interventions were implemented in this village during the study period that could account for this shift (Creasey *et al.* 2004, Hamad *et al.* 2002). This shift may be explained in part, by some acquired immunity by households in cluster 1, who had been intensely exposed to parasites and infected over a longer period. Sterile immunity to malaria is rarely achieved (Langhorne *et al.* 2008, Marsh and Kinyanjui 2006), however, over many years of exposure, individuals develop a certain level of immunity against severe disease and the development of clinical malaria (Langhorne *et al.* 2008, Marsh and Kinyanjui 2006). It is possible that most individuals in households which fell under cluster 2 were 'non-immune' to disease owing to fewer exposure to parasite over a long periods, and therefore the new wave of disease in the 1998 and 1999 transmission seasons made them more susceptible to disease.

I also confirmed that infection by the four *Plasmodium* species were not randomly distributed within the village. Multiple significant clusters were identified for the parasite species; three for *P. falciparum*, two for *P. vivax* and *P. malariae*, and one for *P. ovale*. The significant observation was the overlap of clusters in time and space between the different *Plasmodium* species. *P. falciparum* and *P. vivax* infections overlapped in time and in the same households, with a similar trend observed with *P. malariae* and *P. ovale* infections. A biological reason for this interaction might be related to the erythrocyte preferences of each species (Bruce and Day 2002). Since *P. falciparum* can infect all stages of red cells (Bruce and Day 2002, Gilles 1993), the destruction of parasitised cells may generate new reticulocytes for *P. vivax* to establish

infections. The cluster of *P. vivax* infections in focal households may also suggest that residents of such households may either be Duffy positive (a measurement beyond the scope of this thesis) or may have unidentified erythrocyte receptors which this species binds to establish infection. Duffy antigens are relatively rare in the population of Sub-Saharan Africa, making them refractory to *P. vivax* infection (Miller *et al.* 1976). However, recent work has shown that *P. vivax* infections are increasingly being identified in Sub-Saharan African populations who are Duffy negative (Mbenda and Das 2014, Fru-Cho *et al.* 2014, Wurtz *et al.* 2011, Mendes *et al.* 2011), and perhaps this antigen is no longer a barrier to the ability of *P. vivax* to establish a successful infection. It is of interest for future research to explore the mechanism of these cluster interactions between the different species.

Following the long dry season in Daraweesh between 1988 and 1990, the first clinical episode of malaria was diagnosed in 1990. Unfortunately, the *Plasmodium* species and genotypes of the individual from family R who was infected in that year could not be identified owing to the non-availability of plasma sample for testing. Using the 1991 season as a case study, I observed heterogeneity in the parasite structure with reference to the type of antibody response to *Plasmodium* MSP antigens of the six individuals who were infected in that transmission season. Although the origin of these parasites is unknown, there is evidence from molecular studies showing that between 19-24% asymptomatic individuals in this village who carry some parasites during the long dry seasons when clinical malaria episodes are rare (Roper *et al.* 1996). This may also suggest the intrinsic capacity of the *Plasmodium* parasite to persist as chronic sub-clinical asymptomatic infections in some of the population over time. These individuals may serve as reservoirs for onward parasite transmission in subsequent seasons when environmental conditions are favourable for the mosquito vector (Bousema *et al.* 2012, Bousema *et al.* 2010a). In the preceding drought years where only one clinical malaria case was diagnosed in 1990 it was expected that the new infections of 1991 would arise from a limited number of sources and that antibody responses to these limited antigens in the population would reflect this. The heterogeneity in antibody response to MSP antigens in these six infections of 1991 may demonstrate the large parasite population size in Daraweesh even though

mosquito population may have been low. This assumption is supported by evidence that *P. falciparum* parasites isolated from clinical samples in both Daraweesh (Arnot *et al.* 1994) and its neighbouring villages (Babiker *et al.* 1991a, Babiker *et al.* 1991b) show extremely high genetic variability. This may further suggest that although the malaria episodes in Daraweesh may originate from identifiable foci, as seen in the clusters analysis, the parasite population is very diverse.

Selective non-responsiveness of individuals to parasite antigens between different seasons was observed in 11% of the subset of the Daraweesh population who had more than one malaria episode at different transmission seasons. It has been suggested that the phenomenon of "clonal imprinting," or "original antigenic sin" (Francis 1960) may be responsible for selective non-responsiveness of some individuals to certain parasite antigens during infection. This hypothesis suggests that an individual's B cell repertoire against parasite antigens might become fixed by his or her first or early exposure to a particular parasite antigenic variant, thereby preventing the recognition of other variant antigens in subsequent infections (Riley 1996, Taylor *et al.* 1996). This phenomenon is very much evident in viral diseases caused by the influenza virus (Kim *et al.* 2009) and dengue virus (Rothman *et al.* 2014, Halstead *et al.* 1983). However, the patterns of selective non-responsiveness to merozoite surface proteins in the Daraweesh population do not support this hypothesis as individuals do not show a fixation of their antibody response to any particular MSP antigen. Although a possible transitory antibody response to these polymorphic antigens could be argued (owing to the short half-life of IgG3 to these polymorphic antigens), this may not be the case as blood samples were collected at the acute phase of the disease, and not as might be the case with antibody responses to these antigens when blood sample is collected many months after illness (Cavanagh *et al.* 1998). A possible explanation to this observation in Daraweesh might be the limited number of variant antigens in the panel of polymorphic antigens used in this study that were unable to detect the presence of variant-specific antibodies that individuals might have been exposed to at different transmission seasons. There is the possibility of the full range of allelic variants in the MSP-2 antigens of the parasite population in Daraweesh being more than the recombinant antigens used in this study. Furthermore, the type of responses to

polymorphic MSP antigens might be as a result of the repertoire of parasite antigens an individual is exposed to at different transmission season, owing to the diversity of parasite population present at each season.

5.7 **Conclusions**

I have demonstrated in this chapter that malaria parasite transmission is more focal in Daraweesh and that hotspots of clinical malaria episodes were more stable at the geographical level and over time. Two space-time clusters of significantly increased malaria risk were identified (1993-1999, and 1998-1999) with marked variation between households, but little or no variation in the species of *Plasmodium* over time. This may suggest that even in a small geographic area malaria transmission shows heterogeneity, and that such data can provide useful information to guide malaria control efforts.

An important finding in this chapter is the correlation of household sizes with the incidence of malaria cases in Daraweesh. This finding provides new information that would be important in the long term planning and implementation of optimal control strategies targeting malaria in smaller geographical areas.

Chapter 6. Identifying gene expression signatures in *P. falciparum* infecting different ABO blood group donors

6.1 Introduction

A substantial part of the development of *P. falciparum* in humans takes place within the host erythrocyte where it replicates in a 48-hour cycle of intracellular growth, lysis and re-invasion (Bannister and Mitchell 2003, Hawking *et al.* 1968). This stage of the parasite's life cycle is responsible for the pathology of the disease, mediated by the parasite's rapid expansion in numbers (Simpson *et al.* 2002), the release of toxic by-products of erythrocyte lysis (White *et al.* 2013a), and the sequestration of infected erythrocytes within the microvasculature of the host's organs (Rowe *et al.* 2009a), leading to severe forms of malaria which have high rates of morbidity and mortality. Disease severity is related to parasite virulence factors, which interact with host parasite resistance factors, including the ABO blood groups.

It has been known for some years that the ABO blood group type of the infected host has an influence on the outcome of infectious diseases (Cooling 2015) and cancers (Rummel and Ellsworth 2016, Aird *et al.* 1953). The hosts of blood group O show resistance to severe malaria, possibly due to reduced parasite rosetting and sequestration than the non-O groups (Rowe *et al.* 2009b, Barragan *et al.* 2000). Group O is also far more common in areas of present day and historical malaria endemicity than elsewhere (Cserti and Dzik 2007). For example, in Sub-Saharan Africa and Central and Southern America where *P. falciparum* is endemic, a higher prevalence ratio of group O to group A is documented, whereas in the Western hemisphere and other temperate regions of the world with no malaria, a higher prevalence of blood group A individuals is observed (Fung 2014, Cserti and Dzik 2007). There is also evidence that malaria parasites have marginally higher re-

invasion rates in blood group A subtype A_1 compared to other ABO types (Chung *et al.* 2005).

What is not clear from any of these studies is the change, if any, in gene expression in clonal parasites when they are transmitted to hosts of different ABO blood groups. In bacteria, spontaneous switching between active and repressed states for a large number of genes generates transcriptional diversity within a clonal bacterial population before any environmental change is encountered, thus representing a risk-spreading strategy (Veening *et al.* 2008). Thus, upon a change in the environment, transcriptional heterogeneity provides the grounds for natural selection of pre-existing parasites, which have transcriptional patterns that confer maximum fitness under the new conditions. This form of epigenetic inheritance is likely to be important for malaria parasites encountering a new host, where environmental conditions will likely be different from those in the previous host.

I speculated that the transcriptional variability in *P. falciparum* might result not only in antigenic variation, but also in functional variation in the parasite's growth capabilities, which could be selected for or against by changes in the host environment that occur when parasites are transmitted between genetically different hosts. To date, no study of parasite gene expression profiling has been done to identify changes in parasite gene expression induced by changes in host ABO blood group. I hypothesised that there will be changes in the parasite's gene expression patterns when *P. falciparum* 3D7 clones infect erythrocytes of different ABO blood groups.

To test the above hypothesis, I cultured a single clonal isolate of *P. falciparum* (3D7, for which the complete genome sequence is known) in blood groups A, B and O erythrocytes, harvesting total mRNA at mid-trophozoite stage (Bunnik *et al.* 2013, Lacsina *et al.* 2011). The reason for harvesting at mid-trophozoite stage is because it is the stage where the parasite's metabolism is highest (Bunnik *et al.* 2013). I subsequently applied high-throughput transcriptome sequencing (RNA-seq) using previously described bioinformatic methods (Rovira-Graells *et al.* 2012, Vignali *et al.* 2011) to characterise the gene expression changes between different ABO blood groups.

6.2 Study aim

To determine gene expression changes in 3D7 *P. falciparum* parasites as they infect erythrocytes of different ABO blood group donors.

6.3 Hypothesis

P. falciparum infection will change the parasite's gene expression patterns in erythrocytes of different ABO blood group donors.

6.4 Materials and methods

6.4.1 Donor recruitment

15 adult Caucasians (5 each of known blood groups A, B and O) with no previous clinical history of malaria were recruited into this study. The blood collection procedure was carefully explained to them and each volunteer could leave the study at any time.

6.4.2 ABO blood group typing

To confirm the ABO blood group type of the donors finger prick blood was collected from each donor, and donor red blood cells (RBCs) evaluated for reactivity with commercial anti-A, and anti-B sera (Bio-Rad Medical Diagnostics, Germany), in what is described as the forward reaction (unknown donor ABO antigens against known anti-sera) (Khan *et al.* 2013, Cooling 2008). Two 50 µL aliquots of each donor's blood were placed on a white tile and a corresponding 50 µL of either anti-A or anti-B serum added to each drop of donor blood (Cooling 2008). Donors belonging to blood group A had the presence of the A antigens on their erythrocyte surface agglutinated by the anti-A serum, while the erythrocytes of blood group B individuals agglutinated upon the addition of anti-B serum. Blood group O individuals, having neither A nor B antigens on the RBCs showed no agglutination following the addition of anti-A or anti-B sera to their erythrocytes (Hosoi 2008).

6.4.3 **Blood collection and processing**

18 ml of whole blood from each donor was collected weekly for 2 weeks into Acid Citrate Dextrose tubes (Sigma-Aldrich, UK) (Carter *et al.* 1993). White cells/buffy coat was removed after centrifuging blood at 200*g* for 5 mins. Plasma and as much of the buffy coat layer on the surface of the red cells were removed as possible by aspiration. Erythrocytes were washed 3 times in Incomplete RPMI medium (RPMI 1640, 25 mM HEPES, 2 mM glutamine, 20% glucose, 25 μg/ml gentamicin, 1 M NaOH, 100 μM hypoxanthine, 20 mM $NaHCO_3$) (Thermo Fisher Scientific, UK), aspirating, re-suspending and mixing between each wash. An equal volume of the incomplete RPMI medium to the volume of cells was used for each round of wash. After the third wash, red cells were re-suspended at 50% haematocrit in complete medium (RPMI 1640, 25 mM HEPES, 2 mM glutamine, 20% glucose, 25 μg/ml gentamicin, 1 M NaOH, 100 μM hypoxanthine, 20 mM $NaHCO_3$, 10% of human AB negative serum) and stored at 4^{o}C for a week from the date of drawing.

6.4.4 **Thawing of deep-frozen 3D7 *P. falciparum***

All work involving 3D7 *P. falciparum* was done in a biosafety level 3 laboratory. Parasite culturing was done in a Class II biological safety cabinet under sterile conditions. All materials used for culturing were sterile and the solutions were warmed up to 37°C for use.

Three thawing solutions (A -12% NaCl, B - 1.6% NaCl, and C - 0.9% NaCl + 0.2% dextrose) were freshly prepared and sterilised by filtration through 0.22 micron filters (Carter *et al.* 1993). An ampoule of *P. falciparum* 3D7 parasites were removed from liquid nitrogen, placed in water bath at 37^{o}C for 2-3 minutes until fully thawed (Carter *et al.* 1993). The sample was transferred to a sterile tube and the volume of blood measured. Using 0.2 ml solution A for every 1 ml of thawed blood, solution A was added drop by drop, with constant mixing. The tube was allowed to stand for 3 minutes. Solution B was added drop by drop with constant mixing, using 10ml for each 1ml of original thawed blood. Finally, solution C was added drop by drop using 10ml per ml of original sample volume. The tube was centrifuged at 200*g* for 5

minutes. Supernatant was removed and cells re-suspended slowly in complete medium to a 5% haematocrit in a T-25cm^2 culture flask (Greiner Bio-One, UK).

6.4.5 Maintaining parasite cultures

The culture was then maintained at 37ºC overnight in a humidified tissue culture incubator at 5% CO_2. A 50µL drop of freshly washed, 50% haematocrit blood group O positive erythrocytes obtained from the Scottish National Blood Transfusion Service was added on the second day after thawing, when rings were preparing to burst and re-invade new erythrocytes. The culture was re-incubated at 37ºC overnight. On the third day, an aliquot of the culture was taken and a thin blood film prepared and examined for parasites by the Giemsa staining technique. A second preparation was made to estimate parasitaemia by staining parasite DNA with Hoechst 33342 and measuring by flow cytometry (Crowley *et al.* 2016). For RNAseq, these cultures were initially expanded to a parasitaemia of 1-10% in a 25 ml culture volume.

6.4.6 Parasitaemia count by Giemsa and Hoechst 33342 staining

Parasitaemia was monitored by both the Giemsa staining of thin blood films (Fleischer 2004, Giemsa 1904) and by the Hoechst staining technique for flow cytometry (Crowley *et al.* 2016, Chazotte 2011). For Giemsa staining, a 2 µL aliquot of the cultured erythrocytes was aspirated and smeared on a slide. The slide was allowed to air-dry, and submerged in methanol for 10 seconds, then allowed to air-dry. The slides were stained for 20 minutes in 10% Giemsa solution, allowed to air-dry, and observed by light microscopy under ×100 objective lens magnification for asexual *P. falciparum* parasites. Parasitaemia was measured as the percentage of infected erythrocytes among 1000 erythrocytes counted.

Hoechst 33342 is a specific fluorescent dye that labels DNA allowing for easy visualization of a cell's nucleus and other contents (Chazotte 2011). The dye binds within the minor groove of double-stranded adenine-thymine-rich regions of DNA, and can be used on both live and fixed cells owing to its membrane permeability (Chazotte 2011, Portugal and Waring 1988). The binding of Hoechst 33342 to DNA content results in increased fluorescence (Chazotte 2011). In addition to parasitaemia

count by Giemsa staining, Hoechst 33342 was used to label nuclear DNA of cells grown in culture. For Hoechst staining of parasite DNA, a 25 mg lyophilised powder of Hoechst 33342 (Cell Signalling Technology, USA) was dissolved at a 5 mg/ml in dimethylformamide and used at a final concentration of 1 µg/ml in phosphate buffered saline. 20 µL of parasite culture was resuspended in 300 µL of Hoesch solution and incubated at 37^{o}C for 45 minutes, according to the manufacturer's instructions. The tubes were protected from light and the flow cytometric analysis done with the BD LSR II flow cytometer with 355 and 488 nm argon lasers using the BD FACSDiva 6 software (BD Biosciences). For the analysis, an electronic acquisition gate was applied to the forward/side scatter plot around the red blood cell population, for the exclusion of debris from intact cells. 500,000 events were acquired in this gate. The blue fluorescence of Hoesch-stained parasite nuclei was measured through a 488 nm band pass filter. Data acquired was analysed using FlowJo software (Tree Star).

6.4.7 Transfer of stock culture into erythrocytes of different blood groups

Stock culture was diluted and transferred into 15 donor cultures (5 each of blood groups A, B and O) at a parasitaemia of 1% and maintained in the same pool of AB negative serum (TCS Biosciences, UK), with daily culture medium changes and monitoring of the parasitaemia by both Giemsa and Hoechst staining. The individual cultures were expanded to a volume of 50 ml in a T-75cm^2 flask. The parasites were maintained under standard growth conditions for a period of 2-weeks in each of the blood group erythrocyte, in all completing five growth cycles. Parasite RNA at mid-trophozoite stage was harvested for sequencing. The reason for the 2 weeks culture duration was to generate a preliminary data by investigating the short-term effect of *in vitro* parasite cultivation (Peters *et al.* 2007, Ringwald *et al.* 1999) on parasite gene expression in erythrocytes of different ABO blood group. Biologically it has been shown that the overall abundance of some parasite genes including *var* gene transcripts (one of the most abundant *P. falciparum* gene) reduces significantly during the first ~10 days of culturing 3D7 parasite (Peters *et al.* 2007).

6.4.8 **Sorbitol synchronization**

In order to produce a cohort of synchronous parasites 5% (w/v) D-sorbitol (Sigma, UK) was used to kill all the highly permeable stages of the *Plasmodium* parasite (i.e. trophozoites and schizonts); the ring forms which are less permeable are not affected by the action of the sorbitol (Radfar *et al.* 2009, Lambros and Vanderberg 1979). On the ninth day of parasite cultivation, cultures had high proportion of ring forms, and were transferred into 15 ml tubes and centrifuged at 200*g* and supernatant removed. The pellets were re-suspended to the original volume in 5% warm sorbitol (in sterile distilled water) and allowed to stand at room temperature for 5 minutes. The culture was centrifuged again at 200*g*, after which the sorbitol solution was removed and pellet re-suspended in Incomplete RPMI medium. The culture was washed twice by centrifuging in complete RPMI.

A smear was prepared using about 2 µL of pellet to assess ring enrichment and the absence of mature forms of the parasites. A new culture was set up by adding an aliquot of complete medium to the pellet obtained by gently resuspending the pellet by aspirating and releasing up and down using a plastic pipette and transferring into a new culture flask containing complete medium (Radfar *et al.* 2009). The smear of culture was examined after 24 hours and contained mainly large trophozoites. Parasitaemia more than 3% trophozoites were diluted with complete medium to 1%. Examination after 18 hours showed the appearance of early rings.

6.4.9 **Parasite harvesting**

Cultures were monitored daily with daily medium changes until they were at mid-trophozoite stage and at an approximate parasitaemia of 5% by Giemsa and Hoechst staining. For total RNA, erythrocytes of a 50 ml culture of each donor were pelleted by centrifugation at 500*g* at room temperature for 10 minutes, then washed twice with room temperature PBS. The volume of the pellet was estimated, and stored at -80ºC for RNA extraction.

6.4.10 **RNA isolation and purification**

Parasite total RNA samples were processed for RNA sequencing as described below in the flowchart (Figure 6.4.1).

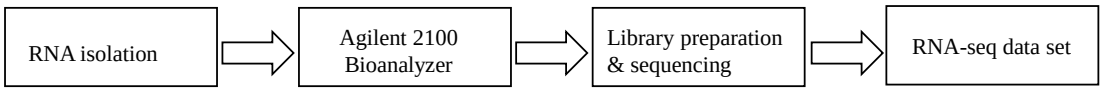


Figure 6.4.1: Schematic overview of the methodology and data processing used to analyse the RNA-seq.

Total RNA was extracted following the Direct-zol^{TM} RNA MiniPrep protocol (Zymo Research, USA). Three volumes of Trizol reagent was added to each erythrocyte pellet and mixed thoroughly for 5 minutes. To remove particulate debris, the mixture was centrifuged at 500×g for 5 minutes and supernatant transferred into an RNase-free tube. An equal volume of ethanol (95-100%) was added to each supernatant and thoroughly mixed. The mixture was transferred into a Zymo-Spin IIC Column in a collection tube and centrifuged at 10,000×g for 30 seconds (all centrifugation steps in this protocol were at this speed and time, unless otherwise stated). The flow-through and the collection tube were discarded, and the column transferred into a new collection tube. 400 µL of RNA wash buffer was added to the column and centrifuged. For DNase treatment, 5 µL of DNase I (6 U/µL) and 75 µL of DNA digestion buffer were mixed in an RNase-free tube and added directly to the column matrix. 400 µL of Direct-zol RNA prewash solution was added to the column, centrifuged and the flow-through discarded. This was followed by the addition of 700 µL of RNA wash buffer and centrifuged for 2 minutes to ensure complete removal of the wash buffer. The column was carefully transferred into an RNase-free tube. To elute highly concentrated RNA, 35 µL of DNase/RNase-free water was directly added to the column matrix and centrifuged.

6.4.11 **RNA quantification and quality**

The RNA obtained was quantified using Nanodrop microvolume spectrophotometer and stored at -70^{o}C. To determine the quality of RNA extracted, the samples were ran on the Agilent 2100 Bioanalyzer (Agilent Technology, Germany) at a concentration of 1 µg/ml. RNA integrity number (RIN) ranged between 8.7 and 9.9 while the 28S/18S ratio also ranged from 1.1 to 1.4 (Table 6.4.1). The Electropherogram summary of one of the samples as obtained on the Agilent 2100 bioanalyzer is shown in Figure 6.4.2. The RNA samples were sent to the Edinburgh Genomics, University of Edinburgh, for RNA sequencing, where all samples passed internal control assessment for quality, quantity and concentration.

Table 6.4.1: The mean RNA integrity number and 28S/18S ratios were similar in blood groups A, B and O donors.

Blood Group	RNA integrity number (mean ± SD)	28S/18S ratio (mean ± SD)
A	9.6 ± 0.20	1.3 ± 0.045
B	9.6 ± 0.21	1.2 ± 0.055
O	9.4 ± 0.53	1.3 ± 0.11

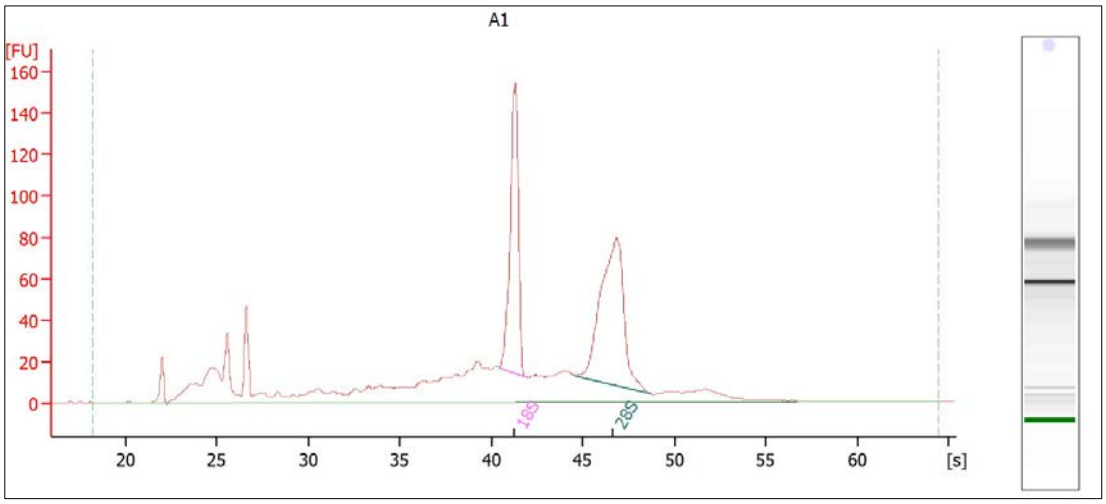


Figure 6.4.2: Electropherogram summary on 2100 expert Eukaryote Total RNA Pico for one sample (blood group A) showing the 18S and 28S. Overall results for one sample: RNA Area: 1,149.7, RNA Concentration: 3,190 pg/µl, rRNA Ratio [28s / 18s]: 1.3, RNA Integrity Number (RIN): 7.5.

6.4.12 **RNA sequencing**

RNA sequencing involves the conversion of a population of RNA to a library of complementary DNA (cDNA) fragments with adaptors attached to one or both ends (Wang *et al.* 2009), in order to determine the primary sequence and relative abundance of each RNA molecule in a biological sample.

The RNA sequencing was performed by the Edinburgh Genomics. Briefly, 5 µg of RNA for each sample was used to prepare cDNA libraries using a poly-A adaptor at the 3′ end. The libraries were sequenced using high throughput Illumina sequencing technology. Data from Edinburgh Genomics were downloaded as gzipped FASTQ files (with help from Dr. Al Ivens). The quality of the data was assessed using FastQC on each set of reads (i.e. 15 samples, 2 ends each) (Andrews 2010). A FastQC quantification of one of the samples is shown in Appendix D. Based on the output from FASTQC, the relevant primers were removed using Cutadapt v1.9.1 with parameters -O 12 -q 20 -m 25 -n 5 -e 0.1 (Martin 2011). The *P. falciparum* genome was obtained from ftp://ftp.sanger.ac.uk/pub/project/pathogens/*Plasmodium*/*falciparum*/3D7/3D7.latest_version/version3.1/2016/April_2016/ in EMBL format.

Chromosome, apicoplast and mitochondrial sequences were used in FASTA format to generate bowtie2 indices (Langmead and Salzberg 2012), (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) for alignment purposes. Chromosomes were viewed in Artemis (http://www.sanger.ac.uk/science/tools/artemis), and gene sets for each chromosome exported as nucleotide and protein. A BED file, containing the map locations for all 5541 *P. falciparum* genes and non-coding RNA genes, was generated from the annotation on the exported sequences.

Read alignment for each sample was performed using bowtie2 (parameters: -very-sensitive -p 16 --no-unal) and the resulting output sorted and indexed in BAM format using samtools v1.3 (http://www.htslib.org/). Alignment statistics were excellent (all >93%). Bedtools (v2.23.0, http://bedtools.readthedocs.org/en/latest/) was used to extract read-count numbers from the alignment files. Count data were explored in R statistical package. Counts within each sample were normalised by scaling to the sample with the lowest number of counts (Dillies *et al.* 2013, Bullard *et al.* 2010). The read counts before and after normalisations are shown in Table 6.4.2.

Table 6.4.2: Read count per sample before and after normalisation.

Donor ID	Before	After
A1	35559665	33356268
A2	34492897	33356228
A3	40726176	33356251
A4	36061260	33356208
A5	37123349	33356234
B1	33356241	33356241
B2	34046672	33356294
B3	38735410	33356289
B4	39322804	33356247
B5	38489232	33356246
O1	41135080	33356236
O2	40007266	33356288
O3	41406092	33356247
O4	37673569	33356248
O5	39271874	33356218

Note: The donor IDs A, B and O represent the three different ABO blood group types.

6.5 Results

6.5.1 Parasite growth in erythrocytes of different ABO blood groups

This study investigated the abilities of different blood group erythrocytes to support the growth of a single clone of 3D7 *P. falciparum* parasites. *P. falciparum* 3D7 strain was cultured in erythrocytes from three different blood groups (A, B, and O), using a common AB negative serum. The parasites were cultured for a period of 2-weeks in each of the blood group erythrocyte, thereby completing five growth cycles. Parasitaemia was monitored daily and calculated for each sample every 24 h as a percentage of infected erythrocytes per 1000 erythrocytes counted. After an initial steady growth, peak mean parasitaemia was observed on day six, following daily media changes (Group A: 8.2 ± 1.4, Group B: 8.6 ± 0.59, Group O: 8.8 ± 0.89) (Table 6.5.1).The cultures were diluted 1 in 4 with complete media on day 6 resulting in decreased parasitaemia on the 7^{th} day (Table 6.5.1 and Figure 6.5.1). Parasitaemia was monitored and media changed on days 7 and 8. On day 9 parasite growth reached that observed on day 6 and sorbitol treatment was used to obtain a cohort of synchronous parasites which can be seen as decreased parasitaemia 4 hours post-sorbitol treatment (Table 6.5.1 and Figure 6.5.1). Parasite cultures of all the blood group types were equally viable throughout the two-week period, as monitored by daily blood smears. The mean erythrocyte invasion (measured as percent parasitaemia) was similar in all blood types on each day and is summarized in Table 6.5.1.

The cultures were diluted 1 in 4 again on day 10, followed by media changes on days 11 and 12. Parasites at the trophozoite stage were harvested on day 13 and frozen at -80^{o}C until RNAs were extracted for sequencing.

Table 6.5.1: Daily mean parasitaemia of 3D7 *P. falciparum* in different ABO blood groups.

		Day of culture												
Blood group	Parasitaemia	1	2	3	4	5	6	7	8	9 (pre-sorbitol)	9 (4hr post sorbitol)	10	11	12	13
A	Mean	0.76	1.3	1.9	4.8	5.6	8.2	3	5.5	7.9	6.6	9.7	2.9	6.4	6.1
	SD	0.05	0.13	0.2	0.51	0.9	1.4	0.34	0.87	0.85	0.68	1.1	0.69	0.68	0.88
B	Mean	0.76	1.3	2	4.9	6	8.6	2.7	5.8	7.6	6.4	10	2.5	6	5.5
	SD	0.05	0.07	0.26	0.45	0.58	0.59	0.42	0.67	1.5	0.85	0.99	0.29	0.55	1.3
O	Mean	0.76	1.3	1.9	4.7	5.7	8.8	3.3	6	9	6.9	10	3	5.5	5.2
	SD	0.055	0.084	0.089	0.23	0.24	0.89	0.44	0.93	1.7	0.76	1.1	0.73	0.64	0.12

SD = standard deviation.

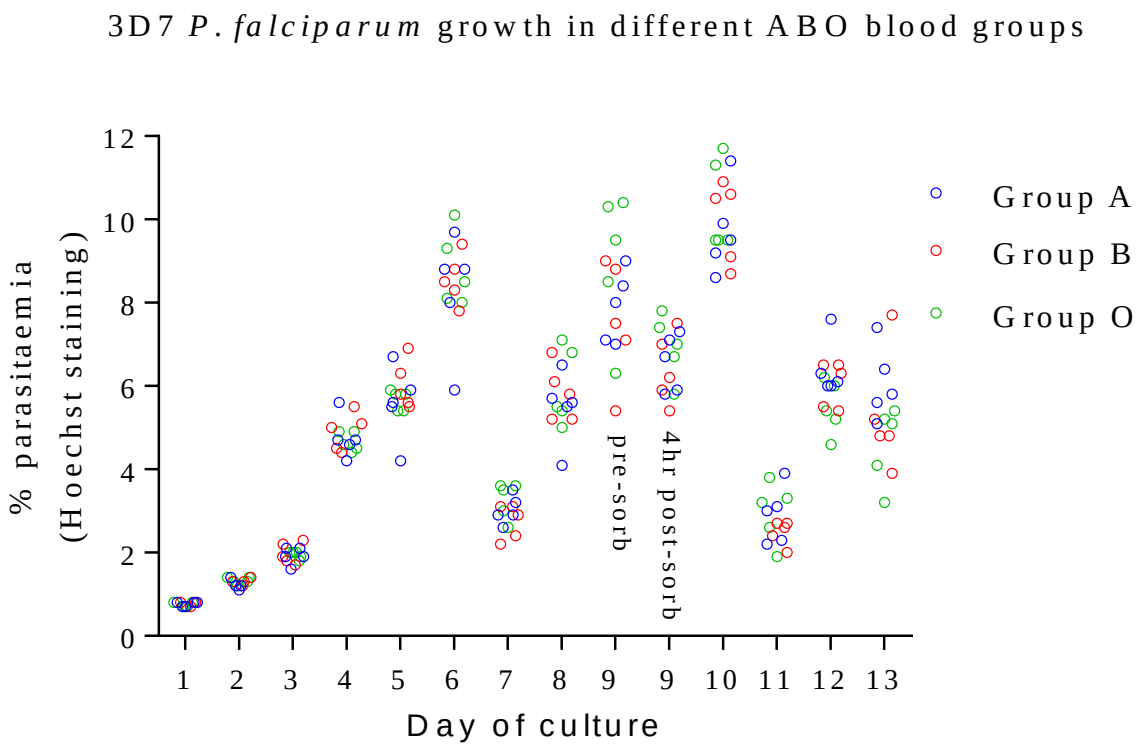


Figure 6.5.1: Parasite growth in erythrocytes of different ABO blood groups. Parasitaemia was monitored daily by Giemsa-stained thin blood films and Hoesch staining for flow cytometry. Each circle represent a donor. Culture medium was changed on days 1-5, 7-8, 11-12; cultures were diluted 1 in 4 on days 6 and 10; sorbitol synchronisation was performed on day 9 and parasitaemia assessed 4 hr post-sorbitol treatment; parasites at trophozoite stage were harvested on day 13.

6.5.2 Differential *P. falciparum* gene expression in different ABO blood groups

Gene expression levels for all 5541 *P. falciparum* genes and 102 non-coding RNA genes (ncRNA) of all classes were initially assessed using principal component analysis (PCA). PCA is a standard technique for reducing multivariate data down to its main independent features by extracting components according to the amount of variation in the data set (Sokal and Rohlf 1995). It was used to determine if gene expression levels clustered according to blood group types. PCA analysis of the gene expression levels showed no clear pattern of clustering of individuals of the same blood group type (Figure 6.5.2). Sample- to sample Pearson correlations (Appendix E) for the individual samples were very similar, and may probably explain the non-clustering of individuals of the same blood groups.

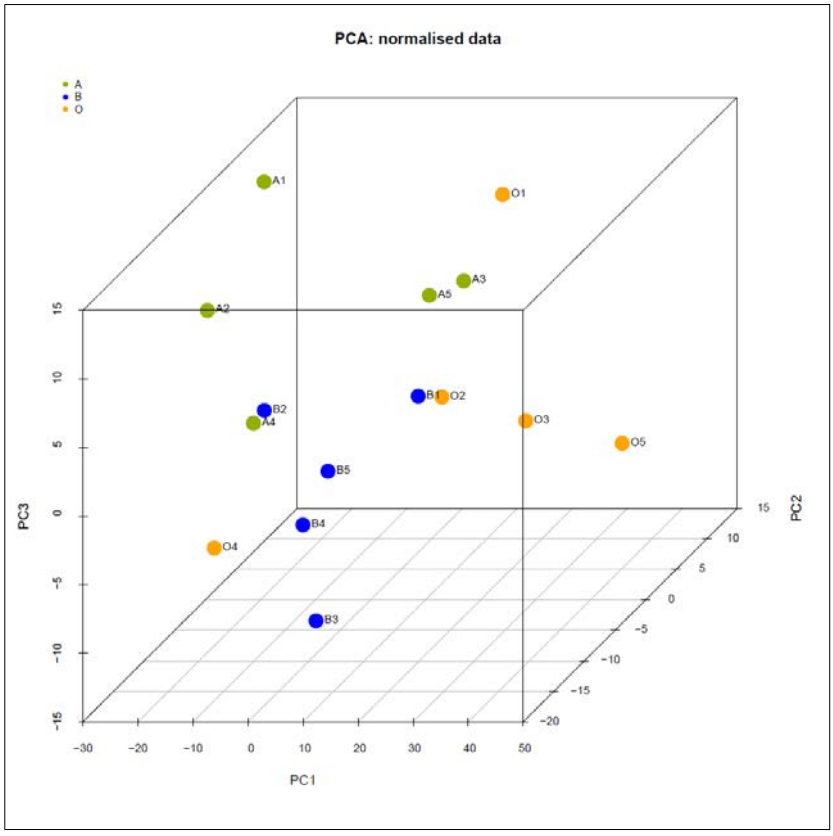


Figure 6.5.2: Principal component analysis of parasite genes showed no pattern of blood group clustering. The numbers assigned to the blood group types (A, B and O) indicate the donor identity.

Analysis of the normalised log2 expression data for the top 100 most variable expression levels (independent of sample blood groups) showed that donor samples do partition, but not according to blood group. Although statistically significant loci were obtained, there were no loci significant at the 5% level after p-value correction for multiple testing, and fold-differences were generally small. These loci included genes associated with *P. falciparum* merozoite surface such as the MSP and rhoptry-associated proteins; virulence genes such as *rifin* and *P. falciparum* erythrocyte membrane protein 1, and proteins of unknown functions but probably involved with exportation of cellular and host materials (Table 6.5.2 and shown as heat map on Figure 6.5.3). In Table 6.5.2, the first 20 of the 100 most variable genes are listed; the full list is found in Appendix G.

Table 6.5.2: First 20 of the 100 most variable genes in decreasing order of variance.

Chromosome	Gene name	Description	Variance
Pf3D7_02_v3	PF3D7_0221300	*Plasmodium* exported protein, unknown function, pseudogene	3.07
Pf3D7_01_v3	PF3D7_0102200	RESA ring-infected erythrocyte surface antigen	2.857
Pf3D7_10_v3	PF3D7_1002000	*Plasmodium* exported protein (hyp2), unknown function	2.628
Pf3D7_11_v3	PF3D7_1149200	Ring-infected erythrocyte surface antigen	2.013
Pf3D7_12_v3	PF3D7_1245700	ncRNA	2.01
Pf3D7_10_v3	PF3D7_1035200	S-antigen	1.99
Pf3D7_12_v3	PF3D7_1228600	MSP9 merozoite surface protein 9	1.889

Pf3D7_03_v3	PF3D7_0301100	*Plasmodium* exported protein (hyp13), unknown function	1.855
Pf3D7_09_v3	PF3D7_0905400	RhopH3 high molecular weight rhoptry protein 3	1.746
Pf3D7_12_v3	PF3D7_1255000	RIF rifin	1.733
Pf3D7_09_v3	PF3D7_0917300	Conserved *Plasmodium* membrane protein, unknown function	1.72
Pf3D7_10_v3	PF3D7_1029500	Conserved *Plasmodium* protein, unknown function	1.675
Pf3D7_13_v3	PF3D7_1352900	*Plasmodium* exported protein, unknown function	1.645
Pf3D7_05_v3	PF3D7_0501600	RAP2 rhoptry-associated protein 2	1.641
Pf3D7_14_v3	PF3D7_1449200	Conserved *Plasmodium* protein, unknown function	1.624
Pf3D7_02_v3	PF3D7_0201000	RIF rifin	1.615
Pf3D7_08_v3	PF3D7_0832800	RIF rifin	1.615
Pf3D7_02_v3	PF3D7_0207600	SERA5 serine repeat antigen 5	1.612
Pf3D7_14_v3	PF3D7_1410400	RAP1 rhoptry-associated protein 1	1.608
Pf3D7_03_v3	PF3D7_0301200	FIKK3 serine/threonine protein kinase, FIKK family	1.603

ncRNA: non-coding RNA.

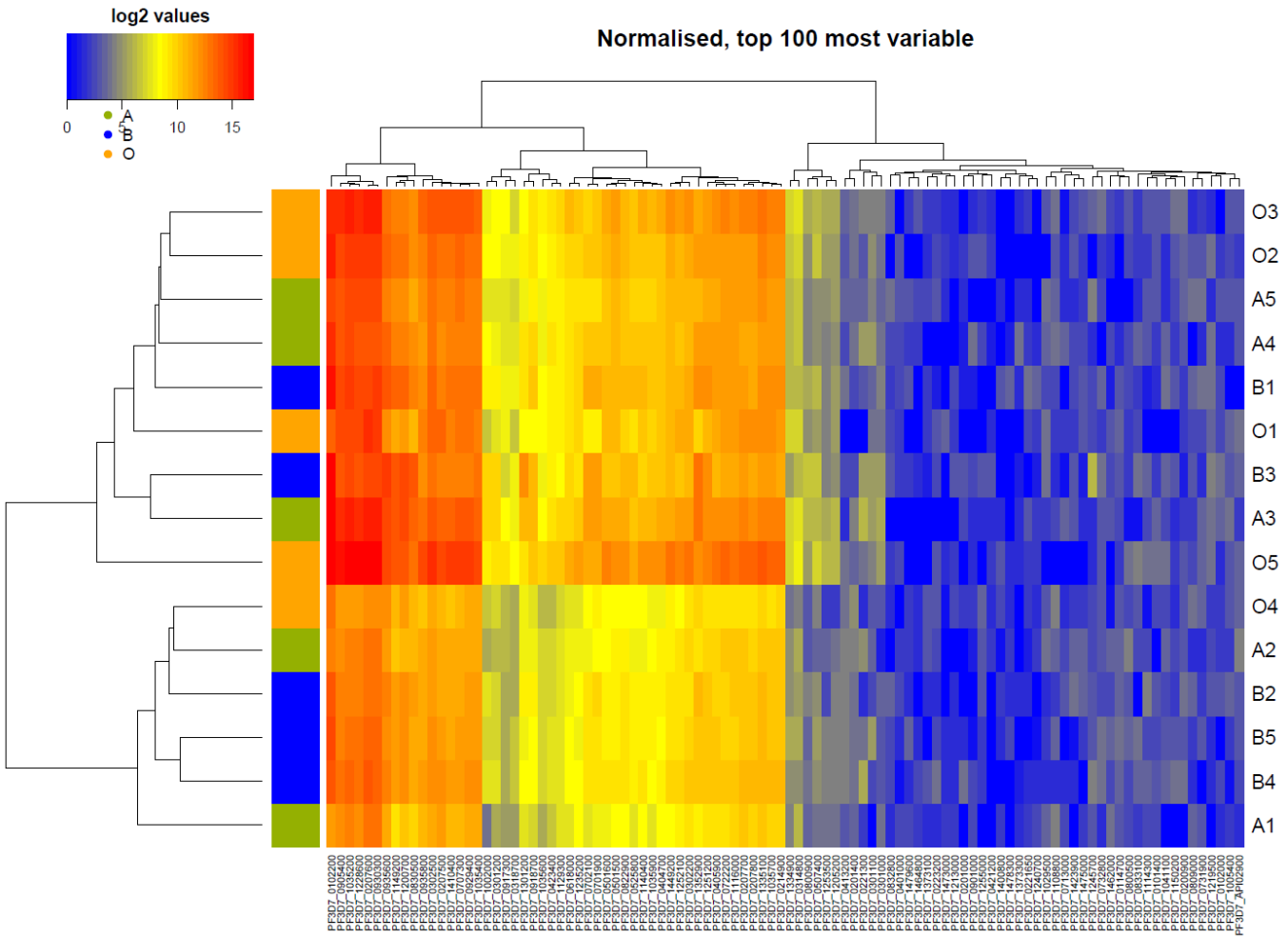


Figure 6.5.3: Normalised log2 expression data for the top 100 most variable expression levels (independent of sample blood groups).

Blood groups are shown as colours on the left hand side of the heat map. Data are partitioned by Euclidean distance, indicated by the dendrogram on the left.

Although there appeared to be no blood-group partitioning of the data, group-wise comparisons were nevertheless performed (using linear modelling) between the three blood groups, treating each sample as a biological replicate (i.e. 5 replicates per group). Gene level counts were subsequently normalised across the entire sample set by quantile normalisation after conversion to log2 with expression levels initially assessed using heat maps for the most variable genes within each blood group. These are shown in Appendix F for each blood group type.

6.5.3 Parasite gene expression levels between blood groups A and B donors

Gene expression profile between blood group types were compared. Taking unadjusted *p*-value of 0.05 as significant, there were 404 genes that were differentially expressed in blood group A relative to blood group B donors, 986 genes between blood group A relative to O donors, and 275 genes differentially expressed in blood group B relative to O donors. However, when these were adjusted for multiple testing using Benjamini and Hochberg method to control for 'false' discovery rate of all the genes analysed (5643 genes) (Reiner *et al.* 2003, Benjamini and Hochberg 1995), these significance were diminished. False discovery rate is defined as "the proportion of truly nondifferentially expressed genes that are declared differentially expressed" (Gusnanto *et al.* 2007), owing to the non-application of correction for multiple testing.

Considering the top ten significant loci in each comparison (see Table 6.5.3), six genes were differentially upregulated and four genes downregulated in blood group A compared to blood group B donors, although multiple correction diminished any observed statistical significance. Genes that were differentially upregulated included four conserved *Plasmodium* proteins of unknown functions, and two non-coding RNA genes (ncRNA) (PF3D7_1370800 and PF3D7_1148200) (Table 6.5.3). Genes that were differentially

downregulated in blood group A relative to blood group B donors included EPF4 exported protein family 4 (PF3D7_0114300), which alongside its other family members are known to encode proteins that are exported to the Maurer's clefts (Mbengue *et al.* 2013); CYP26 peptidyl-prolyl cis-trans isomerase (PF3D7_1202400), that accelerates the protein folding and catalyses the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (Marin-Menendez *et al.* 2012); TRAPPC5 trafficking protein particle complex subunit 5 (PF3D7_1437800), that has been suggested to play a role in vesicular transport from endoplasmic reticulum to Golgi (Gardner *et al.* 2002); and a conserved *Plasmodium* protein of unknown function (PF3D7_0312000) (Table 6.5.3).

Normalised data provide the input for statistical hypothesis testing, in which loci that are statistically significantly different between sample groups, (i.e. differences that are unlikely to be due to chance) can be identified (Bullard *et al.* 2010). The degree of difference, i.e. the fold-change is also of importance. In the outputs given in the tables below, the fold-changes (logFC) are given as log2 values, with a positive logFC representing upregulation, and a negative logFC indicating downregulation. For each comparison (e.g. A-B, which is the same as 'blood group A relative to 'blood group B'), the first group (A) is the numerator, while the second group (B) is the denominator. Thus, a positive logFC for the comparison 'A-B' indicates upregulation in A relative to B.

Table 6.5.3: Conserved and ncRNA genes are differentially expressed in blood group A relative to blood group B donors.

Gene name	Description	LogFC	Av. Expr	*P*-value ($x10^{-4}$)	Adj. *P*-value
PF3D7_1114600	Conserved *Plasmodium* protein, unknown function	0.594	7.90	16	0.091
PF3D7_1370800	ncRNA	0.94	10.53	1.7	0.367
PF3D7_1133000	Conserved *Plasmodium* protein, unknown function	0.652	7.14	2.3	0.367
PF3D7_0114300	EPF4 exported protein family 4, pseudogene	-1.004	5.66	2.6	0.367
PF3D7_1202400	CYP26 peptidyl-prolyl cis-trans isomerase	-0.388	10.33	5.2	0.392
PF3D7_1402800	Conserved *Plasmodium* protein, unknown function	0.416	11.10	5.6	0.392
PF3D7_1437800	TRAPPC5 trafficking protein particle complex subunit 5, putative	-0.428	10.31	6.4	0.392
PF3D7_0312000	Conserved *Plasmodium* protein, unknown function	-0.584	9.17	6.5	0.392
PF3D7_1145000	Conserved *Plasmodium* protein, unknown function	0.422	8.14	7.6	0.392
PF3D7_1148200	ncRNA	0.922	10.43	8.0	0.392

LogFC: Log base 2 fold change of the probeset; a positive value indicates upregulation, while a negative value indicates down regulation in group A relative to B; Av. Expr: Average log2 signal intensity of the probeset across all samples, ncRNA: non-coding RNA.

6.5.4 **Parasite gene expression levels between blood groups A and O donors**

Similar to the comparison between blood group A and B above, of the top ten significant loci, six genes were differentially upregulated in blood group A donors relative to blood group O with four genes downregulated in A relative to O donors (Table 6.5.4). Of the upregulated genes two are genes that code for conserved *P. falciparum* membrane protein or protein of unknown functions (PF3D7_1135300 and PF3D7_1217400) (Gardner *et al.* 2002), two are enzymes involved in the biosynthesis of haem: PBGS delta-aminolevulinic acid dehydratase (PF3D7_1440300), (Dhanasekaran *et al.* 2004, Sato *et al.* 2004) and SPP stromal-processing peptidase (PF3D7_1440200) (van Dooren *et al.* 2002, Hall *et al.* 2002). The others are Vacuolar transporter chaperone (PF3D7_0622300), which plays a role in vacuolar membrane fusion (Muller *et al.* 2003, Muller *et al.* 2002); and the ncRNA gene (PF3D7_1370800) (Table 6.5.4). The ncRNA gene PF3D7_1370800 (previously known as PF13TR009:ncRNA) is the only gene in this study that had a pre-adjusted significant locus and was relatively upregulated in blood group A individuals compared to both blood groups B (P=0.000174, adjusted P=0.367), and O (P=0.000135, adjusted P=0.077) (see Table 6.53 on the previous page, and Table 6.5.4 on the next page). Not much is known about this gene and its role in *Plasmodium* invasion or pathogenesis, except that it is found on chromosome 13 and downstream of the membrane-associated histidine-rich protein 1 (MAHRP1) and in between tRNA and rRNA genes. The MAHRP1 gene was however, downregulated in blood group A donors relative to group B donors, but marginally upregulated in blood groups A and B donors relative to blood group O donors.

The differentially expressed genes that were downregulated in blood group A donors relative to blood group O donors included chorismate synthase (PF3D7_0623000), which plays a role in the biosynthesis of aromatic fatty acids, chorismate and shikimate (Tapas *et al.* 2011, Fitzpatrick *et al.* 2001); CS domain protein (PF3D7_0813200), involved in protein folding; CDGSH iron-sulfur domain-containing protein (PF3D7_0302700) that is

involved with the biosynthesis of iron-sulfur (Paddock *et al.* 2007); as well as a conserved *Plasmodium* protein of unknown function (PF3D7_1303600) (Table 6.5.4).

Table 6.5.4: Parasite gene expression in blood group A relative to blood group O donors.

Gene name	Description	LogFC	Av. Expr	*P*-value ($x10^{-4}$)	Adj. *P*-value
PF3D7_1135300	Conserved *Plasmodium* membrane protein, unknown function	0.676	7.78	24	0.074
PF3D7_1440300	PBGS delta-aminolevulinic acid dehydratase	0.628	10.92	29	0.074
PF3D7_0623000	CS chorismate synthase	-0.682	11.32	6.0	0.074
PF3D7_0622300	Vacuolar transporter chaperone, putative	0.42	9.75	6.2	0.074
PF3D7_0813200	CS domain protein, putative	-0.476	11.60	7.9	0.074
PF3D7_0302700	CDGSH iron-sulfur domain-containing protein, putative	-0.534	9.24	7.9	0.074
PF3D7_1303600	Conserved *Plasmodium* protein, unknown function	-0.68	7.52	1.1	0.077
PF3D7_1370800	ncRNA	0.966	10.53	1.4	0.077
PF3D7_1217400	Conserved *Plasmodium* protein, unknown function	0.638	11.71	1.6	0.077
PF3D7_1440200	SPP stromal-processing peptidase, putative	0.468	11.41	1.7	0.077

LogFC: Log base 2 fold change of the probeset; a positive value indicates upregulation, while a negative value indicates down regulation in group A relative to O; Av. Expr: Average log2 signal intensity of the probeset across all samples, ncRNA: non-coding RNA.

6.5.5 **Parasite gene expression levels between blood groups B and O**

Of the ten most significant gene loci that were differentially expressed (before adjusting for multiple gene comparison), seven and three were respectfully upregulated and downregulated in blood group B individuals relative to group O donors (Table 6.5.5). Of the upregulated genes, one was a conserved protein of unknown function (PF3D7_1366100), with two being exported proteins of unknown functions (PF3D7_0115100 and PF3D7_1038700). The rest included Stevo, a pseudogene (PF3D7_0114600) that encodes the sub-telomeric variable open reading frame (stevo), which is clonally variant, and expressed on both merozoites and at the surface of infected erythrocytes, and involved in parasite immune evasion of the host (Niang *et al.* 2009, Blythe *et al.* 2008, Blythe *et al.* 2004); Dynein light chain type 2, Vacuolar transporter chaperone, which was also upregulated in blood group A donors relative to group O donors; and NOT5 CCR4-NOT transcription complex subunit 5 (PF3D7_1006100), which is involved in regulation of transcription (Shock *et al.* 2007).

The genes downregulated in blood group B donors relative to group O individuals included the SR1 serpentine receptor 1 that is involved in cell signalling (Madeira *et al.* 2008, Gardner *et al.* 2002); Haloacid dehalogenase-like hydrolase, (PF3D7_1118400), and a conserved *Plasmodium* membrane protein, unknown function (PF3D7_1005800).

Table 6.5.5: Parasite gene expression in blood group B relative to blood group O donors.

Gene name	Description	LogFC	Av. Expr	*P*-value ($x10^{-3}$)	Adj. *P*-value
PF3D7_0114600	Stevor, pseudogene	1.130	3.99	1.7	0.732
PF3D7_1131100	SR1 serpentine receptor, putative	-0.422	8.34	1.7	0.732
PF3D7_0115100	*Plasmodium* exported protein (PHISTa), unknown function	0.542	7.10	1.9	0.732
PF3D7_1114000	Dynein light chain type 2, putative	0.438	7.44	2.0	0.732
PF3D7_0622300	Vacuolar transporter chaperone, putative	0.274	9.75	2.5	0.732
PF3D7_1118400	Haloacid dehalogenase-like hydrolase, putative	-0.402	10.14	2.6	0.732
PF3D7_1366100	Conserved *Plasmodium* protein, unknown function	0.462	9.27	3.4	0.732
PF3D7_1005800	Conserved *Plasmodium* membrane protein, unknown function	-0.346	7.73	4.0	0.732
PF3D7_1038700	*Plasmodium* exported protein, unknown function	0.408	9.45	5.5	0.732
PF3D7_1006100	NOT5 CCR4-NOT transcription complex subunit 5, putative	0.392	9.74	5.5	0.732

LogFC: Log base 2 fold change of the probeset; a positive value indicates upregulation, while a negative value indicates down regulation in group A relative to B; Av. Expr: Average log2 signal intensity of the probeset across all samples, ncRNA: non-coding RNA.

6.5.6 Expression levels of *P. falciparum* MSP antigens

In Chapters 3 and 4 of this thesis I explored extensively the immunoepidemiology of *Plasmodium* using merozoite surface proteins 1 and 2 (MSP-1 and MSP-2). In view of that I wanted to find out whether these proteins are differentially expressed in relationship to parasite growth in the three donor blood groups. The data show that both MSP1 and MSP2 genes are very marginally up-regulated in blood group A individuals relative to blood group B (Table 6.5.6).

Table 6.5.6: Log2 fold change in selected *P. falciparum* genes of interest.

Chromosome	Gene name	Description	A relative to B	A relative to O	B relative to O
Pf3D7_09_v3	PF3D7_0930300	MSP-1	0.214	-0.774	-0.988
Pf3D7_02_v3	PF3D7_0206800	MSP-2	0.128	-0.742	-0.87

MSP: merozoite surface protein.

6.5.7 Barcoding of expression changes

I generated a file that contained a barcode representation of the log2 fold changes in the three blood group comparisons. The barcode represents only the direction of fold change, so no threshold was applied. In this case “Upregulation" means any log2 fold change greater than 0, and is represented as a "1", while "Down regulation" means any log2 fold change that is zero or negative, and is represented as a "0". As an example, a barcode of "111" means a particular gene was upregulated in all three comparisons, while a barcode

of 101 means upregulation in comparison 1 (blood group A vs B) and 3 (blood group B vs O), but down regulated in comparison 2 (blood group A vs O).

Although by permutation there are 8 possible barcodes that could be generated, only 6 were found in the analysis of this dataset, and are shown in Table 6.5.7 with their corresponding numbers of genes.

Table 6.5.7: Barcode representation of the log2 fold changes in the three blood group comparison.

Barcode	000	001	011	100	110	111
Number of genes	1182	767	935	755	516	1488

Note: Barcode 000 means the gene is down-regulated in all three blood groups comparison; barcode 101 means the gene is upregulation in blood group A relative to B and blood group B relative to O, but down regulated in comparison in blood group A relative O; barcode 111 indicates a gene that is upregulated in all three comparisons.

6.6 **Discussion**

The intra-erythrocytic niche provides a relatively stable environment for the survival of *P. falciparum* in the human host. In spite of this the parasite faces other environmental challenges in the human host which it must quickly adapt to in order to survive and establish its niche and cause disease. Some of the challenges encountered by the parasite when it infects different human hosts are immune and drug pressures, host genetics, and the presence of competing parasites (Rovira-Graells *et al.* 2012, Mackinnon and Marsh 2010). The surface structure of the erythrocyte and the different antigens present may also present challenges that the *Plasmodium* parasites must overcome to establish itself. Among these surface antigens, the ABO blood group antigens are of importance in malaria pathogenesis. In this chapter I have determined if there are any changes in parasite gene expression when *P. falciparum* infects erythrocytes of different ABO blood group individuals.

Through a mechanism of reduced rosetting (Rowe *et al.* 2007) and increased phagocytosis of infected erythrocytes (Wolofsky *et al.* 2012) individuals of blood group O have been reported to have a survival advantage with respect to life-threatening malaria compared to non-group O individuals. It stands to reason that parasitaemia would be expected to be lower in group O erythrocytes compared with erythrocytes of blood group A and B. In this study the mean daily parasitaemia was similar across all blood groups, and although there are differences in the surface structure and glycosylation of the different ABO blood groups, this did not influence the invasion and maturation of the *P. falciparum* parasites in the erythrocytes of the different donor blood groups. This is consistent with what has been shown in *P. falciparum* ITG, 3D7 and E8B clones, in which no statistical significant differences were observed in parasite invasion during two growth cycles in blood group A, B and O erythrocytes (Wolofsky *et al.* 2012). However, in an *in vitro* study using four clones of *P. falciparum* parasites (3D7, 7G8, Dd2 and RKL9), higher parasitaemia were observed in all clones in the erythrocytes of blood group O compared to group A and B (Pathak *et al.* 2016). The authors hypothesised that the 'O' group erythrocyte is not

‘resistant’ towards parasite entry and that the amount of H antigen, which is the blood group precursor, being abundant in group O than in group A and B might account for differences in parasite invasion of different erythrocytes (Pathak *et al.* 2016). In the current study, all the cultures were initiated at the same parasitaemia and the culture conditions and treatment was uniformly maintained throughout the experiment, with the only variable factor being the different erythrocytes of the different blood groups used.

Although significant differential gene expression were seen with respect to the different blood group types, this significance diminished after correction for multiple comparisons, possibly due to the sample size of five donors in each blood group. The ncRNA PF3D7_1370800 was significantly expressed (prior to multiple comparison) in blood group A erythrocytes relative to both blood groups B and O. Apart from the chromosome position (13) and the location of this gene, not much is known about it. However, the MAHRP1 gene where PF3D7_1370800 gene is located downstream is a transmembrane protein present throughout the erythrocyte life cycle and exported beyond the parasite's surface into parasite-derived structures known as Maurer's cleft (Spycher *et al.* 2006, Spycher *et al.* 2003). It has been proposed that this gene is essential for trafficking of *P. falciparum* erythrocyte membrane protein 1 to the surface of *P. falciparum*-infected red cells and provides stability to the Maurer's clefts (Spycher *et al.* 2008). While PF3D7_1370800 was upregulated in blood group A donors relative to both groups B and O donors, the MAHRP1 gene was marginally downregulated and upregulated in blood group A relative to B and O donors respectively. A possible suppression role of PF3D7_1370800 on MAHRP1 has yet to be determined. The marginal up and down regulations seen in this study owing to the smaller sample sizes and the diminishing significance after multiple gene comparison is however, not enough to make a tentative conclusion.

Although this experiment produced a negative result an alternative approach could have been to perform this experiment at different time points to identify significant changes, if any, that could be attributed to the length of time of parasite culture. For example, it has

been shown that rosette-forming *Plasmodium* parasites with a preference for blood group A and those with a preference for blood group B are capable of retaining their blood group preferences even after being cultivated for more than 100 cycles in blood group O erythrocytes (Barragan *et al.* 2000). This may suggest that *P. falciparum* parasites could have been maintained in the erythrocytes for a longer period of time. While my approach was to look at parasite gene expression changes in the host, an alternative approach would have been to investigate the possible host response on parasite in peripheral blood mononuclear cells.

In *P. falciparum* approximately 5,541 protein-coding genes are encoded in the 24 megabase genome, in addition to about 101 clusters of non-(protein)-coding RNA (ncRNAs) genes (Nagano and Fraser 2009). The ncRNAs are RNAs that are transcribed from DNA but are not translated into proteins. They are broadly divided into small (<200 nucleotides) and long ncRNAs (>200 nucleotides), based on the number of nucleotides (Bayer-Santos *et al.* 2017, Mercer *et al.* 2009), and play roles in the regulation of gene expression, RNA processing, and translation as well as protecting an organism's genome from foreign nucleic acids (Cech and Steitz 2014). Protozoan parasites of the apicomplexan group such as *Plasmodium*, *Toxoplasma*, *Leishmania*, and *Trypanosoma* are capable of reorganising host cell functions to allow their survival and replication (Plattner and Soldati-Favre 2008), including transferring their non-coding RNA molecules to the host cells to modulate their functions, via secreted extracellular vesicles (Bayer-Santos *et al.* 2017). The evasion of host immunity by *P. falciparum* involves a mechanism of antigenic variation largely mediated by a multifamily virulence *var* genes that encode variant surface antigens, *P. falciparum* erythrocyte membrane protein 1 (Baruch *et al.* 1995, Smith *et al.* 1995a). The chromatin surrounding these var genes have been shown to generate long sense and antisense ncRNAs that associate with it (Epp *et al.* 2009). The long antisense ncRNAs initiating from the *var* introns have been associated with the active *var* gene, and are capable of triggering the activation of a silent var gene in trans (Amit-Avraham *et al.* 2015), suggesting possible regulatory roles of ncRNA in antigenic variations. The human pathogen *Toxoplasma gondii* is one of the most widely distributed

protozoan parasites, infecting approximately one-third of the world's population. A non-coding RNA, known as Tg-ncRNA-1, plays a major role in cellular differentiation during the parasite's asexual replication in the human host from the rapidly growing tachyzoites to the latent bradyzoite, being upregulated over twenty times during this process (Patil *et al.* 2012, Matrajt 2010). A role for the PF3D7_1370800 gene and why it is differentially expressed in blood group A erythrocytes relative to blood groups B and O needs further investigation.

In this study I observed marginal upregulation of MSP-1 and MSP-2 genes in blood group A donors relative to blood groups B donors. Merozoite surface proteins play key roles in merozoite invasion of red blood cells (Das *et al.* 2015). Erythrocyte invasion of *P. falciparum* is a highly complex multi-step procedure that involves interaction between parasite ligands on the merozoite surfaces and receptors on the erythrocyte surface (Gaur *et al.* 2004). Immediately after schizont rupture, merozoites must find and invade a host erythrocyte. Blood group A phenotype has two subclasses, A_1 and A_2, of which the A_1 account for up to 80% (Dean 2005). Individuals of the A_1 subclass have higher number of oligosaccharide residues present on the surface of their red cell that enhances recognition and attachment of merozoite to their erythrocyte surface (Chung *et al.* 2005). This may enhance higher erythrocyte re-invasion rates by merozoites in blood group A_1 individuals (Chung *et al.* 2005). Although the subtypes of the blood group A donors were not confirmed in my study, it might be of interest to know whether the marginal upregulation of MSP-1 and MSP-2 genes observed in blood group A donors is related to an individual's blood group A subclass.

The results of the gene expression work in this chapter might have been influenced by some technical limitations and factors that were both within and beyond my ability to control. Variables in the study design that were within my ability to control, and implemented, included using the same AB serum and the same source of 3D7 parasite strain for the cultures, and performing the experiment on all the donors simultaneously to exclude any variability that may result from using different serum or parasites, or culturing

in different donor erythrocyte at different times. It is however clear that the sample size of five donors from each blood group type was probably inadequate to generate enough statistical power for analysis of the data, considering the need to account for corrections for multiple comparisons, which diminished the statistical significance of expression levels observed. It has been suggested that in RNA sequence experiment, 20-40% of significantly differential genes can be identified when three biological replicates are used, a proportion which increases to more than 85% when the replicates are increased to 20 (Schurch *et al.* 2016). Working on numbers more than the fifteen donors would certainly have been beyond my ability and would probably have needed the involvement of many hands, and over many days of parasite culture. From hindsight, perhaps increasing the number of donors in each blood group category would have led to a more robust significant differentiation of gene expression (Liu *et al.* 2014, Auer and Doerge 2010).

Although the design of the experiment was to harvest parasites at the mid-trophozoite stage, there were other asexual stages in the harvested culture, in spite of sorbitol synchronisation a few days prior to the harvest. This lack of synchronicity at the harvest stage might have had an effect on the RNA sequencing. Differences in parasite gene expression between different blood groups might be very subtle, and thus signal to background reactivity ratio obtained in this study may be very low and thereby obscure such small changes in gene expression. In *in vitro* studies, 3D7 parasites normally grow at between two- and six fold per cycle; however, this growth rate was not achieved in my experiment, possibly due to some culture conditions not being optimal. Parasite cultures are usually incubated in a mixture of 1% O_2, 3% CO_2 and 96% N gas (BOC Gases) (Carter *et al.* 1993). This step was not included in my protocol and could have accounted for the lower parasite growth rates obtained. Expanding parasite cultures to large scale volumes needed for sufficient RNA meant that the demand by parasites for glucose for metabolism was equally higher. 3D7 *P. falciparum* parasites use the glycolytic pathway to generate energy. From hindsight, this would probably have required more than the one daily medium change I followed, to ensure sufficient amount of energy source for improved parasite growth. Whole blood obtained from the Scottish National Blood Transfusion

Service for parasite cultures in my co-supervisor's laboratory usually have the white blood cells removed by passing the blood through a leucodepletion filter attached to the blood pack. My donors had their white blood cells removed manually by centrifugation and removing as much of the buffy coat as possible by aspiration. By this method I could not guarantee that all white blood cells were depleted and their effect on parasite growth and RNA sequencing could not be ruled out.

An alternative I could have chosen was to have performed this work on donors of one blood group at a time, completed the culture cycles, harvested parasites and processed for RNA extraction. Although this would have reduced the volume of work, it would have also introduced other variabilities such as different media preparations (although prepared using the same protocol, variations between solutions prepared weeks apart could not be ruled out) and the possible variability in incubation conditions. In spite of the above limitations, the study provides preliminary data on this ncRNA (PF3D7_1370800) gene and how it is differentially expressed in blood group A individuals relative to blood groups B and O individuals, for which further research might be needed.

6.7 **Conclusions**

In this chapter I sought to grow a single clone of 3D7 *P. falciparum* parasites in the erythrocytes of three different blood group donors. I observed that parasitaemia, which is a measure of parasite growth and viability, was similar in all blood groups. Although some *Plasmodium* genes appear to be differentially expressed in one blood group relative to the other, these differences were not very significant after correction for multiple gene comparisons. A gene that was prominently expressed in blood group A erythrocytes relative to group B and O donors is a non-coding RNA gene, ncRNA PF3D7_1370800, for which much is not known about its function, except its location, requiring further research to better understand its role, if any, in parasite growth, and pathogenesis of disease in different blood group erythrocytes. In the next chapter I discuss the major findings of this thesis and their implications with malaria research and global health.

Chapter 7. General discussions and conclusions

7.1 Introduction

In this thesis I focussed on malaria, an important parasitic disease that affects over half the world's population, most notably children under the age of five years from Sub-Saharan Africa (WHO 2015, WHO 2013). Two key aspects of my thesis were the epidemiology of mixed-*Plasmodium* species infections and gene expression changes when a single clone of parasite infects erythrocytes of different ABO blood groups. Diagnosis of malaria in endemic clinical settings is largely by microscopy, which usually reports the presence of *Plasmodium* parasites, but fails to report the species type or the presence of mixed-species infections (Obare *et al.* 2013). Currently available rapid diagnostic tools do not differentiate between all four major human *Plasmodium* species in Africa (Graves *et al.* 2015, Wilson 2012), while molecular techniques such as PCR are confined mainly to research environments (Daniels *et al.* 2017). Although the WHO recommends laboratory confirmation of malaria before treatment (WHO 2015, WHO 2014), most fevers in Sub-Saharan Africa are treated with antimalarial drugs (Singh and Sharma 2014, Kyabayinze *et al.* 2010) thereby possibly overestimating the burden of malaria (Bhatt *et al.* 2015). This may lead to over- or misuse of the limited range of anti-malarial drugs available, with the potential development of drug-resistant *Plasmodium* strains (Manguin *et al.* 2017, Chandler *et al.* 2008).

A phenotype associated with susceptibility or resistance to the development of severe malaria are the ABO blood group antigens (Cooling 2015, Cserti and Dzik 2007, Rowe *et al.* 2007, Reid and Bird 1990, Gupta and Chowdhuri 1980). The *Plasmodium* parasite, being an obligate intracellular parasite spends its entire blood-stage life-cycle in the human erythrocytes, except a brief period when merozoites burst from schizonts prior to

infecting new red blood cells (Bannister and Mitchell 2003). However, changes in parasite gene expression when a single clone of *P. falciparum* parasite infects different ABO blood group erythrocytes have not been investigated.

In this thesis I have determined the seroepidemiology of the different *Plasmodium* parasites in two African countries, Zimbabwe and Sudan (Chapters 3 and 4). I used recombinant merozoite surface antigens derived from merozoite surface protein 1, (also known as MSP-1_{19}) from all four major *Plasmodium* species, in an ELISA assay, to determine IgG antibody responses to these antigens in the two study populations. Furthermore, using some observable clinical manifestations (fever, parasitaemia) and treatment data from a well-characterised population from Daraweesh, in eastern Sudan, with longitudinal sampling spanning a period of eleven years (1990-2000), I compared these observable clinical manifestations and treatment requirements in individuals with documented clinical malaria who had antibody response to either *P. falciparum* alone or mixed-species MSP-1_{19} antigens (Chapter 4). Both the malaria parasitology and the antibody response data of the people of Daraweesh have been used to determine the spatial and temporal dynamics of malaria epidemiology in this village (Chapter 6). Finally, I determined the gene expression changes when a single clone of *P. falciparum* parasites infect erythrocytes of different blood groups (Chapter 6). This was done by culturing *Plasmodium* parasites in three erythrocytes from A, B and O blood group donors, harvesting total RNA and sequencing these to characterise gene expression changes.

In this present chapter, the major findings with regard to the research aims outlined previously in Chapter 1, (Section 1.10) are summarized. The main conclusions based on the findings of the studies set forth in the preceding chapters of this thesis are outlined. I will conclude the chapter with key suggestions for the diagnosis, disease treatment and clinical monitoring of malaria that may aid the design and monitoring of malaria control programmes targeting endemic areas where more than one parasite species may co-infect the population.

7.2 Malaria diagnosis: merozoite surface proteins as potential diagnostic tool

In accordance with the WHO guidelines, laboratory confirmation of suspected malaria cases or patients presenting with symptoms of malaria is a pre-requisite for malaria treatment and the development of intervention for the control of the disease (WHO 2015, WHO 2014). Accurate detection of the species of *Plasmodium* causing malaria is important to ensure timely and targeted treatment of the infecting parasite. The species of *Plasmodium*, if not confirmed and treated accordingly, has the potential consequences of drug treatment failure (Smith *et al.* 2011) and long term morbidity (Savargaonkar *et al.* 2014, Muller-Stover *et al.* 2008, Vinetz *et al.* 1998). Such untreated or partially cleared parasites can serve as potential source of parasite reservoirs in communities for onward transmission, and to impact upon malaria morbidity and mortality (Chang 2016, Kitchen *et al.* 2005). The confirmation of the presence or absence of malaria parasites in patients presenting with or suspected of having the disease is traditionally by microscopic examination of Giemsa-stained thick blood films for asexual parasites stages (Fleischer 2004, Giemsa 1904). Confirmation of the species of *Plasmodium* is usually by examining thin blood films for characteristic morphological features of the parasite species (Obare *et al.* 2013, Barber *et al.* 2013) (outlined previously in Section 1.1.1, Table 1.3.1). Although thick blood films are used routinely to detect *Plasmodium* parasites, thin blood films are seldom prepared and so the species of *Plasmodium* is seldom reported (Obare *et al.* 2013). In cases where thin blood films are examined, there are reports of misclassification of species and undiagnosed mixed-species infections even by expert microscopists (Obare *et al.* 2013, McKenzie *et al.* 2006).

Available rapid diagnostic tools also fail to detect *P. malaria* and *P. ovale* infections accurately, with most tests based on antigens from *P. falciparum* and *P. vivax* (Graves *et al.* 2015, Wilson 2012). Molecular techniques for malaria parasite speciation are confined to research laboratories. There is therefore the need for a diagnostic tool based on antigens

present in all four *Plasmodium* species that is sensitive and species-specific and capable of detecting the specific *Plasmodium* species causing malaria.

In Chapters 3 and 4, I focussed on using MSP-1_{19} antigens from all four major human *Plasmodium* species to determine the specificity of human IgG antibodies to these antigens. Using these antigens I determined the seroepidemiology of the different *Plasmodium* species in Zimbabwean and Sudanese populations. Several epidemiological studies use the MSP-1_{19} antigens of *P. falciparum* and/or *P. vivax* to determine exposure patterns of individuals to these antigens (Wang *et al.* 2016, Osier *et al.* 2008, Braga *et al.* 2002, Cavanagh *et al.* 1998). However, since the isolation and characterisation of the MSP-1_{19} genes for *P. malariae* and *P. ovale* (Birkenmeyer *et al.* 2010), there has been only one study that had used all four antigens in limited experimental human primate and human malaria studies (Muerhoff *et al.* 2010). The findings in this thesis show that MSP-1_{19} antigens are species-specific and that human IgG antibodies directed against these antigens do not cross-react with heterologous antigens (Amanfo *et al.* 2016).

This finding is important as it presents MSP-1_{19} antigens of the four *Plasmodium* species as the basis of a novel and sensitive assay for development into a diagnostic tool for species-specific detection of *Plasmodium*. To date, only one manufacturer incorporates MSP-1_{19} antigens of *P. falciparum* and *P. vivax* in their malaria EIA kit for malaria screening of blood donors and blood products, which are extensively used in blood transfusion services across major western countries (Seed *et al.* 2005a, Kitchen *et al.* 2004). Diagnostic tools currently in use supplement the efforts of the WHO in its quest to have all suspected malaria cases confirmed by laboratory test before treatment. Although the experimental design in this thesis was based on ELISA that usually takes three days to complete, the potential development of MSP-1_{19} antigens from all four *Plasmodium* species into a single assay format could complement microscopy in helping detect and report the types of *Plasmodium* species. Additionally, in peripheral health service areas where human resources and electricity are a limitation to using microscopy (Cibulskis *et*

al. 2011, Harvey *et al.* 2008), these antigens, formulated as RDTs, could be useful in reporting accurately the species of *Plasmodium* causing malaria.

The implication of using these antigens is that we will be able to assess the true prevalence and magnitude of the disease burden caused by the non-*falciparum* species in endemic populations. This will lead to targeted treatment with appropriate drugs, and enable governments and policy makers to develop effective control programmes that targets all *Plasmodium* species and not as is the current case in which control programmes are targeted only at *P. falciparum* elimination (Abeyasinghe *et al.* 2012, Oliveira-Ferreira *et al.* 2010).

The use of MSP-1_{19} antigens in this thesis has highlighted two important things about malaria epidemiology in the two African study sites. Firstly, the data in Chapters 3 and 4 have confirmed reports of previous studies indicating *P. falciparum* as being the predominant *Plasmodium* species in both Zimbabwe (Hay *et al.* 2009, Mabaso *et al.* 2006, Taylor and Mutambu 1986) and Sudan (Hamad *et al.* 2000, Theander 1998), and probably by extension the whole of Sub-Saharan Africa (Gemperli *et al.* 2006).

Secondly, my findings have confirmed that the frequency of mixed-species infections has been underestimated in African malaria epidemiology. In Zimbabwe I found mixed-species infections to account for almost half the cases of individuals with antibody responses to parasite antigens, while in the Sudanese cohort mixed-species was seen in more than a third of all individuals who experienced a first time clinical malaria episode.

What is of interest is that with the exception of a few individuals in both study countries, all mixed-species infections detected by antibody response to MSP-1_{19} antigens were in association with *P. falciparum* responses. What this may mean is that in the context of mixed-infections involving *P. falciparum* and one or more of the non-*falciparum* species, there is the tendency of higher parasitaemia associated with *P. falciparum* infections to obscure the presence of the other malaria parasite species (Collins and Jeffery 2007, Collins and Jeffery 2005) making it difficult to detect and report the presence of the non-

falciparum infections using microscopy (Obare *et al.* 2013). In this context, the majority of all infections are likely to be attributed to only *P. falciparum*. It has been shown that even expert microscopists are likely to miss mixed-species infections or misclassify them as *P. falciparum*-only infections (Obare *et al.* 2013, McKenzie *et al.* 2006). PCR data emerging from the continent of Africa support my findings that mixed-species infections are more frequent than previously reported (Fru-Cho *et al.* 2014, Dinko *et al.* 2013).

The consequence of this finding is that malaria morbidity and mortality will continuously be attributed to *P. falciparum* without a clear knowledge of the contributions of the non-*falciparum* species to the overall malaria burden (Mendis *et al.* 2001). Moreover, intervention programmes may only focus on *P. falciparum* and may fail to achieve maximum effectiveness in terms of prevention and control of disease, as these non-*falciparum* species may be potential sources of the next pandemic, as seen in areas where controls programmes against *P. falciparum* have seen some success (Oliveira-Ferreira *et al.* 2010).

In this thesis, I was able to demonstrate the importance of using a sensitive diagnostic tool based on antigens present in all four major human *Plasmodium* species to improve diagnostic accuracy of mixed-species infections as this has implications on the required treatment regimens for people with mixed-infections (Vinetz *et al.* 1998). Accurate diagnosis of the type of *Plasmodium* species infection provides a foundation for treatment and management of malaria.

7.3 Mixed-*Plasmodium* infections and associated drug treatment failure

Having established the sensitivity and specificity of MSP-1_{19} antigens as a diagnostic tool (Chapter 3) I assessed if heterogeneity in clinical presentation and response to drug treatment was related to exposure to single *vs*. mixed-*Plasmodium* species. The major finding in Chapter 4 of this thesis was the significant association of mixed-*Plasmodium* species infection with chloroquine treatment failure in the Daraweesh population. While

the clinical implications of mixed-infection dynamics remain controversial, this study provides one possible reason why initial therapy may fail to eradicate malaria parasites and/or alleviate clinical symptoms. Some studies have reported that the faster multiplying species in a co-infection may suppress the slower species (Mason and McKenzie 1999, Mason *et al.* 1999), perhaps in a density-dependent manner (Bruce and Day 2002). It has been reported that mixed-species infection can be a likely cause of treatment failure (Smith *et al.* 2011).

Drug resistant parasites are a major threat to disease control and eradication. Although chloroquine was the first line drug of use in Daraweesh at the time of the study, the lessons learnt from the results obtained could be applicable in current epidemiological settings where ACTs have become the first line treatment for uncomplicated malaria. For example, artemisinin derivatives in ACTs have been shown to be effective only against intra-erythrocytic parasites, but are unable to kill parasites in the hepatocytes (Chang 2016). This is advantageous to dormant liver stage hypnozoites of *P. vivax* and *P. ovale* that have the potential to recrudesce, and if undetected, or parasitaemia becomes sub-clinical without full cure may have important clinical and therapeutic implications (Kitchen *et al.* 2005, Vinetz *et al.* 1998). Although current ACTs have largely been reported to be highly effective against *Plasmodium* parasites, there are reports of the emergence of resistant strains to these drugs (Duffy and Sibley 2005). It is therefore important to extend such study of mixed-species *Plasmodium* infections into areas where resistances to ACTs are being reported to understand the contribution, if any, of the non-*falciparum* species to these developments.

The data on parasitaemia (Chapter 4) in the Daraweesh population supports the importance of close monitoring of communities where malaria is endemic. Higher proportions of individuals with malaria caused by both single- and mixed-*Plasmodium* species were diagnosed with low parasitaemia. Over the eleven year study period there were no malaria related deaths as no individual developed severe malaria with its associated complications. This suggests that closer surveillance, prompt diagnosis and

treatment of malaria, and adequate follow ups or management of disease can contribute significantly to reducing malaria related morbidities in malaria endemic communities.

7.4 Household dynamics of malaria in a Daraweesh

Having established in Chapters 3 and 4 that mixed-species infections are higher than previously reported and that these are associated with drug treatment failure, I assessed the fine details of malaria epidemiology, often described as mircroepidemiology (Bannister-Tyrrell *et al.* 2017) to understand heterogeneity associated with the risk of having disease within a single village. The epidemiology of malaria in Daraweesh showed a rain-dependent pattern (Creasey *et al.* 2004), similar to what is observed in other African settings (Bannister-Tyrrell *et al.* 2017, Seyoum *et al.* 2017, Alemu *et al.* 2013). The data in Chapter 5 showed that dry seasons temporarily break the transmission of parasites, resulting in fewer individuals being clinically infected. The onset of rainfall with its correlated increase in the number of clinical episodes is important information for greater planning and implementation of control programmes prior to rainy seasons. It is evident that closer monitoring, prompt diagnosis and treatment alone offered by the health team (Creasey *et al.* 2004) did not in any way reduce the burden of the disease or eradicate malaria in the village. It has been suggested that although mass drug administration (MDA) has the potential to reduce *Plasmodium* parasite transmission, it needs to be complemented with other vector control programmes (Brady *et al.* 2017). This calls for other intervention strategies to be implemented even in such a small village setting for effective elimination of disease. I also demonstrated that malaria parasite transmission in Daraweesh was focal. Two space-time clusters of significantly increased malaria risk were identified (1993-1999, and 1998-1999) with marked variation between households, but little or no variation in the species of *Plasmodium* over time. Why the households in the southwest and central part of the village experienced the heaviest burden of malaria over a longer period may be explained by local factors inherent to those households (Parizo *et al.* 2016). It is possible that the proximity of the village's deep well to these households and an increased density of vegetation during rainy seasons provide more hospitable

ecologic habitats for mosquito breeding, relative to other parts of the village. Proximity to local water bodies or wells has been shown to influence malaria incidence and clustering in rural areas (Peterson *et al.* 2009). The households identified in cluster 1 had larger family sizes and this could be a contributing factor in the increased number of malaria cases recorded. This finding is consistent with what has been found elsewhere in which households with larger family size experience more malaria (Hulden *et al.* 2014, Peterson *et al.* 2009, Hulden and Hulden 2009), while a decrease in household size to four individuals has been associated with reduced malaria incidence (Hulden *et al.* 2014, Hulden and Hulden 2009).

The data in this thesis suggests that even in a small geographic area malaria transmission shows heterogeneity, and that such data can provide useful information to guide malaria control efforts (Seyoum *et al.* 2017). Identification of a subset of a population who experience the heaviest burden of malaria at every point in time can provide useful information needed for implementing control programmes that can block the spread of malaria across an entire community. My present findings provide new information that would be important in the planning and implementation of optimal control strategies targeting malaria in smaller geographical settings. In addition, the possible utility of antibody screening based on recombinant *Plasmodium* MSP-1_{19} antigens will help to identify carriers of parasites in the later stages of malaria for drug treatment to eradicate asymptomatic infections.

7.5 Gene expression in different ABO blood groups

The different antigens expressed on the surface of erythrocytes might be a barrier to the invasion of the intra-erythrocytic merozoite. In chapter 6 of this thesis I explored the relationship between parasite growths in different ABO blood group erythrocytes and determined if parasite gene expression differed in a genetically identical parasite clone (3D7) grown in either A, B or O type erythrocytes. I observed that 3D7 *P. falciparum* parasite invasion of erythrocytes and their maturation as determined by parasitaemia count was similar in all blood groups. Since blood group O individuals are less susceptible to

developing severe malaria compared to non-group O individuals (Rowe *et al.* 2007, Barragan *et al.* 2000), it could be assumed that parasite invasion and maturation may differ between blood group O and the non-group O individuals. However, the data presented in this thesis showed no difference in *P. falciparum's* ability to invade the different blood groups erythrocytes, at least in terms of growth rates. A key finding in this thesis was the differential expression of the non-coding RNA gene, PF3D7_1370800, in blood group A donors relative to the other blood groups. A comprehensive search of the literature did not generate any information on this gene. It is therefore limiting to know the role this gene may play in blood group A individuals during malaria. Further studies need to be done to confirm the potential role, if any, of this gene.

7.6 Implications of my research findings for policy

Approximately 90% of malaria incidence and mortality occur in Sub-Saharan Africa with children under the age of five being more at risk (WHO 2015). Laboratory confirmation of malaria is important in the treatment and management of the disease. The findings in the thesis highlight the need to explore the development of antigens derived from merozoite surface proteins as diagnostic tools to complement existing microscopic and other diagnostic methods for accurate detection of the type of *Plasmodium* species in malaria endemic populations. This will enable accurate identification of the species type as well as mixed-species infections to enable appropriate and timely treatment strategies, aimed at improving the current and future health of populations where different species of *Plasmodium* coexist.

The finding in this thesis of the association of mixed-*Plasmodium* infections with treatment failures is of clinical significance. Health practitioners in clinical settings where species identification is possible should monitor closely the therapeutic effectiveness of patients diagnosed as being infected with mixed species. In settings where laboratory confirmation of the species of *Plasmodium* is not routine, practitioners should investigate further the possible contribution of mixed-species infections in patients who do not respond appropriately to drug treatment. In Daraweesh although the population was

closely monitored, diagnosed and treated for malaria, this did not suppress malaria incidence, confirming recent findings that mass drug administration alone, although effective for the short term, needs to be complemented with other vector control programmes for effective eradication of malaria (Brady *et al.* 2017). Spatial analysis of malaria epidemiology showed that malaria parasite transmission is very focal. The identification of clusters of significantly increased malaria risk suggests that even in a smaller geographic area malaria transmission shows heterogeneity. Such data can provide useful information to guide malaria control efforts. Such information shows that targeting communities at higher risk of infection may be better than targeting whole communities.

The role of the non-coding RNA gene PF3D7_1370800 identified in this study to be differentially expressed in blood group A donors relative to both group B and O individuals is unknown. However, this preliminary data on the differential expression of this gene presents an avenue to explore further research into the contribution of this gene to the dynamics of ABO blood group and malaria pathology.

The findings presented here will aid researchers and other stakeholders in making informed choices about intervention tools for malaria control programmes, as is the case of incorporating the important findings in this thesis into new Sudanese malaria research and malaria elimination planning. This work opens up an area of research to explore the use of MSP-1_{19} antigens in other malaria epidemiological settings and to better understand the contributions of all species of *Plasmodium* to the overall disease burden and drug treatment failures.

7.7 Future prospects and recommendations

In this thesis I have demonstrated that the shortcomings of malaria diagnosis by the gold standard microscopy method result in underestimating the prevalence of mixed-*Plasmodium* species infections in African populations. *Plasmodium* species identification can be improved by the use of sensitive serological methods based on merozoite surface proteins to complement microscopy and other diagnostic techniques currently in use in

both clinical and epidemiological settings (Chapters 3 and 4). These MSP-1_{19} antigens have shown that in both mesoendemic Zimbabwe and very low transmission village of Daraweesh, they can serve as a good reflection of exposure to *Plasmodium* parasites, and show promise to be developed as RDTs. Further epidemiological research in other malaria transmission settings using these antigens is proposed, as this will serve to contribute to validating these antigens and helping our understanding of the global epidemiology of the different *Plasmodium* species and their contribution to disease burden. I propose comparative studies of serological work based on MSP-1_{19} antigens and other molecular techniques such as PCR on the same study cohort in other epidemiological settings in Africa.

I have demonstrated that mixed-species infections are associated with chloroquine treatment failures in the Daraweesh population (Chapter 5). The Daraweesh study was conducted at a period when chloroquine-resistant *P. falciparum* in Sudan and other parts of Africa was becoming apparent (El Sayed *et al.* 2000). Nevertheless, my study demonstrated that a significant number of people with only *P. falciparum* infections, or single infections to *P. vivax* and *P. ovale* cleared their infections with a single course of chloroquine treatment. Since the year 2000, many African governments have introduced ACTs as their first line for treating uncomplicated malaria (WHO 2015). Thus, I propose further studies using these antigens in areas where ACTs are currently the first line of treatment, in order to compare the therapeutic effects in single- *versus* mixed-species infections detectable by antibody responses to MSP-1_{19} antigens. In view of recent reports of *Plasmodium*-resistant ACT strains in some parts of the world (Chang 2016, Dondorp *et al.* 2009, Duffy and Sibley 2005), it is important to identify at this early stage the contribution of the non-*falciparum* species to these reported resistances in settings of co-infections. From the historical information about antimalarial drugs I outlined in Table 1.5.1 in Chapter 1, it is clear that the development of antimalarial drugs takes many decades, and resistant parasite strains develop rapidly. Until an effective malaria vaccine becomes available, it is important to ensure that mixed-species infections are accurately

diagnosed and treatment effectively to prevent the development of drug-resistant strains to current ACTs.

The preliminary results obtained from the ABO and parasite studies should be scaled up and possibly, similar gene expression studies conducted in individuals naturally infected with malaria. On the basis of the data obtained from the sequencing, there might be no need to do RNAseq in subsequent validation experiments. I propose growing the parasites in the different blood group erythrocytes, and performing quantitative PCR on significantly differentially expressed genes, to determine whether there is an effect as this technique only amplifies one gene as oppose to the possibility of background readings associated with RNAseq and microarray techniques.

7.8 Final conclusions

My thesis has generated several results of research interest, which are of importance for health care practitioners, policy makers and stakeholders involved in the control of malaria. These findings are timely because of the current renewed global interest to address the contribution of the non-*falciparum* species to malaria burden.

- The study has confirmed that MSP-1_{19} antigens from the four major human *Plasmodium* parasites are species-specific and that human IgG antibodies directed against these antigens do not cross-react. These antigens could be developed into diagnostic tools to complement the use of microscopy in detecting malaria parasites and confirming the *Plasmodium* species individuals are infected with. This will ensure accurate epidemiology data on African malaria and help better our understanding of the contribution of the non-*falciparum* species to the overall malaria burden.

- Using these antigens, the study has confirmed that *P. falciparum* is indeed the predominant *Plasmodium* species in African malaria epidemiology, but equally and more importantly, mixed-species infections and the prevalence of the non-

falciparum species are higher than previously reported in the two study countries. This may suggest that microscopic detection of *Plasmodium* species often fails to accurately report on the epidemiology of mixed-species infections and the prevalence of the non-*falciparum* species. By using the more sensitive MSP-1_{19} IgG serological diagnostic method, I have been able to demonstrate that microscopy might misclassify and underestimate mixed-species infections in these two populations. These findings further reiterate the need for additional diagnostic tools capable of detecting the type of infecting *Plasmodium* in malaria endemic areas.

- Mixed-*Plasmodium* species infections are associated with drug treatment failures. This finding should prompt health care practitioners to evaluate closely the treatment regimen administered to individuals who are diagnosed with mixed-species infections. Since malaria drug development takes many years and resistances to drugs pose a major challenge in the elimination of the disease, it is important to administer the appropriate drug based on the infection an individual carries.

- Spatial analysis of malaria epidemiology showed that malaria parasite transmission in Daraweesh was focal. Two space-time clusters of significantly increased malaria risk were identified (1993-1999, and 1998-1999) with marked variation between households, but little or no variation in the species of *Plasmodium* over time. This may suggest that even in a small geographic area malaria transmission shows heterogeneity, and that such data can provide useful information to guide malaria control efforts.

- Although the ABO blood group systems plays a major role in the susceptibility or resistance to the development of severe malaria, *P. falciparum* growth was similar in all erythrocytes independent of blood groups. The preliminary data from this thesis has shown that ncRNA gene, PF3D7_1370800, is differentially expressed

in blood groups A individuals compared to the non-A blood groups, although this significance is diminished after correction for multiple comparisons.

References

A-Elbasit, I. E., Elbashir, M. I., Khalil, I. F., Alifrangis, M. & Giha, H. A. 2006. 'The efficacy of sulfadoxine-pyrimethamine alone and in combination with chloroquine for malaria treatment in rural Eastern Sudan: the interrelation between resistance, age and gametocytogenesis.' *Trop Med Int Health,* 11:5, 604-12.

Abate, A., Degarege, A. & Erko, B. 2013. 'Community knowledge, attitude and practice about malaria in a low endemic setting of Shewa Robit Town, northeastern Ethiopia.' *BMC Public Health,* 13, 312.

Abbas, A., Lichtman, A., Pillai, S. 2011. *Cellular and molecular immunology.* Elsevier Health Sciences,

Abeyasinghe, R. R., Galappaththy, G. N., Smith Gueye, C., Kahn, J. G. & Feachem, R. G. 2012. 'Malaria control and elimination in Sri Lanka: documenting progress and success factors in a conflict setting.' *PLoS One,* 7:8, e43162.

Acevedo, M. A., Prosper, O., Lopiano, K., Ruktanonchai, N., Caughlin, T. T., Martcheva, M., Osenberg, C. W. & Smith, D. L. 2015. 'Spatial Heterogeneity, Host Movement and Mosquito-Borne Disease Transmission.' *PLoS One,* 10:6.

Aird, I., Bentall, H. H. & Roberts, J. A. 1953. 'A relationship between cancer of stomach and the ABO blood groups.' *Br Med J,* 1:4814, 799-801.

Akpogheneta, O. J., Duah, N. O., Tetteh, K. K., Dunyo, S., Lanar, D. E., Pinder, M. & Conway, D. J. 2008. 'Duration of naturally acquired antibody responses to blood-stage *Plasmodium falciparum* is age dependent and antigen specific.' *Infect Immun,* 76:4, 1748-55.

Al-Maktari, M. T. & Bassiouny, H. K. 2003. 'Malaria status in Al-Hodeidah Governorate, Republic of Yemen. Part II: Human factors causing the persistence of chloroquine resistant *P. falciparum* local strain.' *J Egypt Soc Parasitol,* 33:3, 829-39.

Al-Maktari, M. T., Bassiouny, H. K., Al-Hamd, Z. S., Assabri, A. M., El-Massry, A. G. & Shatat, H. Z. 2003. 'Malaria status in Al-Hodeidah Governorate, Yemen: malariometric parasitic survey & chloroquine resistance *P. falciparum* local strain.' *J Egypt Soc Parasitol,* 33:2, 361-72.

al-Yaman, F., Genton, B., Anders, R., Taraika, J., Ginny, M., Mellor, S. & Alpers, M. P. 1995. 'Assessment of the role of the humoral response to *Plasmodium falciparum* MSP2 compared to RESA and SPf66 in protecting Papua New Guinean children from clinical malaria.' *Parasite Immunol,* 17:9, 493-501.

al-Yaman, F., Genton, B., Anders, R. F., Falk, M., Triglia, T., Lewis, D., Hii, J., Beck, H. P. & Alpers, M. P. 1994. 'Relationship between humoral response to *Plasmodium falciparum* merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea.' *Am J Trop Med Hyg,* 51:5, 593-602.

al-Yaman, F., Genton, B., Kramer, K. J., Chang, S. P., Hui, G. S., Baisor, M. & Alpers, M. P. 1996. 'Assessment of the role of naturally acquired antibody levels to

Plasmodium falciparum merozoite surface protein-1 in protecting Papua New Guinean children from malaria morbidity.' *Am J Trop Med Hyg*, 54:5, 443-8.

Alemu, K., Worku, A. & Berhane, Y. 2013. 'Malaria infection has spatial, temporal, and spatiotemporal heterogeneity in unstable malaria transmission areas in northwest Ethiopia.' *PLoS One*, 8:11, e79966.

Alker, A. P., Lim, P., Sem, R., Shah, N. K., Yi, P., Bouth, D. M., Tsuyuoka, R., Maguire, J. D., Fandeur, T., Ariey, F., Wongsrichanalai, C. & Meshnick, S. R. 2007. 'Pfmdr1 and in vivo resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border.' *Am J Trop Med Hyg*, 76:4, 641-7.

Amanfo, S. A., Mduluza, T., Midzi, N., Cavanagh, D. R. & Mutapi, F. 2016. 'Seroepidemiology of *Plasmodium* species infections in Zimbabwean population.' *Malar J*, 15:1, 267.

Amaratunga, C., Lopera-Mesa, T. M., Brittain, N. J., Cholera, R., Arie, T., Fujioka, H., Keefer, J. R. & Fairhurst, R. M. 2011. 'A role for fetal hemoglobin and maternal immune IgG in infant resistance to *Plasmodium falciparum* malaria.' *PLoS One*, 6:4, e14798.

Amit-Avraham, I., Pozner, G., Eshar, S., Fastman, Y., Kolevzon, N., Yavin, E. & Dzikowski, R. 2015. 'Antisense long noncoding RNAs regulate var gene activation in the malaria parasite *Plasmodium falciparum*.' *Proc Natl Acad Sci U S A*, 112:9, E982-91.

Anderson, T. J. & Roper, C. 2005. 'The origins and spread of antimalarial drug resistance: lessons for policy makers.' *Acta Trop*, 94:3, 269-80.

Andrews, B., Brown, G. W. & Creasey, L. 1990. 'Intergenerational links between psychiatric disorder in mothers and daughters: the role of parenting experiences.' *J Child Psychol Psychiatry*, 31:7, 1115-29.

Andrews, S. 2010. 'FastQC: a quality control tool for high throughput sequence data. Available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc.'

Angus, B. J., Thanikkul, K., Silamut, K., White, N. J. & Udomsangpetch, R. 1996. 'Short report: Rosette formation in *Plasmodium ovale* infection.' *Am J Trop Med Hyg*, 55:5, 560-1.

Ariey, F., Hommel, D., Le Scanf, C., Duchemin, J. B., Peneau, C., Hulin, A., Sarthou, J. L., Reynes, J. M., Fandeur, T. & Mercereau-Puijalon, O. 2001. 'Association of severe malaria with a specific *Plasmodium falciparum* genotype in French Guiana.' *J Infect Dis*, 184:2, 237-41.

Arnot, D. 1998. 'Unstable malaria in Sudan: the influence of the dry season. Clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission.' *Trans R Soc Trop Med Hyg*, 92:6, 580-5.

Arnot, D. E., Roper, C. & Sultan, A. A. 1994. 'MVR-PCR analysis of hypervariable DNA sequence variation.' *Parasitol Today*, 10:8, 324-7.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chuor, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T. T., Thuy-Nhien, N. T., Thanh, N. V., Phu, N. H., Htut, Y., Han, K. T., Aye, K. H., Mokuolu, O. A., Olaosebikan,

R. R., Folaranmi, O. O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P. N., Onyamboko, M. A., Fanello, C. I., Tshefu, A. K., Mishra, N., Valecha, N., Phyo, A. P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M. A., Ghose, A., Hossain, M. A., Samad, R., Rahman, M. R., Hasan, M. M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D. P., Bozdech, Z., Jeeyapant, A., Cheah, P. Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S. J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W. J., Yeung, S., Woodrow, C. J., Flegg, J. A., Das, D., Smith, J., Venkatesan, M., Plowe, C. V., Stepniewska, K., Guerin, P. J., Dondorp, A. M., Day, N. P., White, N. J. & Tracking Resistance to Artemisinin, C. 2014. 'Spread of artemisinin resistance in *Plasmodium falciparum* malaria.' *N Engl J Med,* 371:5, 411-23.

Atroosh, W. M., Al-Mekhlafi, H. M., Mahdy, M. A. K., Saif-Ali, R., Al-Mekhlafi, A. M. & Surin, J. 2011. 'Genetic diversity of *Plasmodium falciparum* isolates from Pahang, Malaysia based on MSP-1 and MSP-2 genes.' *Parasites & Vectors,* 4.

Auer, P. L. & Doerge, R. W. 2010. 'Statistical design and analysis of RNA sequencing data.' *Genetics,* 185:2, 405-16.

Babiker, H. A., Creasey, A. M., Bayoumi, R. A., Walliker, D. & Arnot, D. E. 1991a. 'Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the mdr1 genotype of recent isolates.' *Trans R Soc Trop Med Hyg,* 85:5, 578-83.

Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A., Arnot, D. E. & Walliker, D. 1991b. 'Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens.' *Trans R Soc Trop Med Hyg,* 85:5, 572-7.

Badiane, A. S., Diongue, K., Diallo, S., Ndongo, A. A., Diedhiou, C. K., Deme, A. B., Ma, D., Ndiaye, M., Seck, M. C., Dieng, T., Ndir, O., Mboup, S. & Ndiaye, D. 2014. 'Acute kidney injury associated with *Plasmodium malariae* infection.' *Malar J,* 13:1, 226.

Bain, B. J., Chiodini, P. L., England, J. M. & Bailey, J. W. 1997. 'The laboratory diagnosis of malaria.' *Clin Lab Haem,* 19, 165-70.

Baird, J. K. 2008. 'Real-world therapies and the problem of vivax malaria.' *N Engl J Med,* 359:24, 2601-3.

Baird, J. K. 2013. 'Evidence and implications of mortality associated with acute *Plasmodium vivax* malaria.' *Clin Microbiol Rev,* 26:1, 36-57.

Baird, J. K., Leksana, B., Masbar, S., Fryauff, D. J., Sutanihardja, M. A., Suradi, Wignall, F. S. & Hoffman, S. L. 1997. 'Diagnosis of resistance to chloroquine by *Plasmodium vivax*: timing of recurrence and whole blood chloroquine levels.' *Am J Trop Med Hyg,* 56:6, 621-6.

Bannister-Tyrrell, M., Verdonck, K., Hausmann-Muela, S., Gryseels, C., Ribera, J. M. & Grietens, K. P. 2017. 'Defining micro-epidemiology for malaria elimination: systematic review and meta-analysis.' *Malar J,* 16.

Bannister, L. & Mitchell, G. 2003. 'The ins, outs and roundabouts of malaria.' *Trends Parasitol,* 19:5, 209-13.

Bannister, L. H., Hopkins, J. M., Fowler, R. E., Krishna, S. & Mitchell, G. H. 2000. 'A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages.' *Parasitol Today,* 16:10, 427-33.

Bannister, L. H. & Mitchell, G. H. 2009. 'The malaria merozoite, forty years on.' *Parasitology,* 136:12, 1435-44.

Bannister, L. H., Mitchell, G. H., Butcher, G. A., Dennis, E. D. & Cohen, S. 1986. 'Structure and development of the surface coat of erythrocytic merozoites of *Plasmodium knowlesi*.' *Cell Tissue Res,* 245:2, 281-90.

Barber, B. E., William, T., Grigg, M. J., Yeo, T. W. & Anstey, N. M. 2013. 'Limitations of microscopy to differentiate *Plasmodium* species in a region co-endemic for *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium knowlesi*.' *Malar J,* 12, 8.

Barcus, M. J., Basri, H., Picarima, H., Manyakori, C., Sekartuti, Elyazar, I., Bangs, M. J., Maguire, J. D. & Baird, J. K. 2007. 'Demographic risk factors for severe and fatal vivax and falciparum malaria among hospital admissions in northeastern Indonesian Papua.' *Am J Trop Med Hyg,* 77:5, 984-91.

Barnes, K. I., Durrheim, D. N., Little, F., Jackson, A., Mehta, U., Allen, E., Dlamini, S. S., Tsoka, J., Bredenkamp, B., Mthembu, D. J., White, N. J. & Sharp, B. L. 2005. 'Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa.' *PLoS Med,* 2:11, e330.

Barragan, A., Kremsner, P. G., Wahlgren, M. & Carlson, J. 2000. 'Blood group A antigen is a coreceptor in *Plasmodium falciparum* rosetting.' *Infect Immun,* 68:5, 2971-5.

Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F. & Howard, R. J. 1995. 'Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes.' *Cell,* 82:1, 77-87.

Bayer-Santos, E., Marini, M. M. & da Silveira, J. F. 2017. 'Non-coding RNAs in Host-Pathogen Interactions: Subversion of Mammalian Cell Functions by Protozoan Parasites.' *Front Microbiol,* 8, 474.

Beeson, J. G., Drew, D. R., Boyle, M. J., Feng, G., Fowkes, F. J. & Richards, J. S. 2016. 'Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria.' *FEMS Microbiol Rev,* 40:3, 343-72.

Beiguelman, B., Alves, F. P., Moura, M. M., Engracia, V., Nunes, A. C., Heckmann, M. I., Ferreira, R. G., da Silva, L. H., Camargo, E. P. & Krieger, H. 2003. 'The association of genetic markers and malaria infection in the Brazilian Western Amazonian region.' *Mem Inst Oswaldo Cruz,* 98:4, 455-60.

Benjamini, Y. & Hochberg, Y. 1995. 'Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing.' *Journal of the Royal Statistical Society Series B-Methodological,* 57:1, 289-300.

Berg, J. M., Tymoczko, J. L. & Stryer, L. 2002. *Biochemistry 5th ed.* W.H. Freeman, New York,

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K. E., Moyes, C. L., Henry, A., Eckhoff, P. A., Wenger, E. A., Briet, O., Penny, M. A., Smith, T. A., Bennett, A., Yukich, J., Eisele, T. P., Griffin, J. T., Fergus, C. A.,

Lynch, M., Lindgren, F., Cohen, J. M., Murray, C. L., Smith, D. L., Hay, S. I., Cibulskis, R. E. & Gething, P. W. 2015. 'The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015.' *Nature,* 526:7572, 207-11.

Bhattarai, A., Ali, A. S., Kachur, S. P., Martensson, A., Abbas, A. K., Khatib, R., Al-Mafazy, A. W., Ramsan, M., Rotllant, G., Gerstenmaier, J. F., Molteni, F., Abdulla, S., Montgomery, S. M., Kaneko, A. & Bjorkman, A. 2007. 'Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar.' *PLoS Med,* 4:11, e309.

Biggs, B. A. & Brown, G. V. 2001. 'Malaria.' In Gillespie S & Pearson RD (Eds.) *Principles and Practice of Clinical Parasitology*: John Wiley & Sons Ltd, USA, 53-98.

Billingsley, P. F., Charlwood, J. D. & Knols, B. G. J. 2005. 'Rapid assessment of malaria risk using entomological techniques: taking an epidemiological snapshot.' *Environmental Change and Malaria Risk: Global and Local Implications,* 9, 51-68.

Birkenmeyer, L., Muerhoff, A. S., Dawson, G. J. & Desai, S. M. 2010. 'Isolation and characterization of the MSP1 genes from *Plasmodium malariae* and *Plasmodium ovale*.' *Am J Trop Med Hyg,* 82:6, 996-1003.

Bjorkman, A. & Phillips-Howard, P. A. 1990a. 'Drug-resistant malaria: mechanisms of development and inferences for malaria control.' *Trans R Soc Trop Med Hyg,* 84:3, 323-4.

Bjorkman, A. & Phillips-Howard, P. A. 1990b. 'The epidemiology of drug-resistant malaria.' *Trans R Soc Trop Med Hyg,* 84:2, 177-80.

Black, J., Hommel, M., Snounou, G. & Pinder, M. 1994. 'Mixed infections with *Plasmodium falciparum* and *P malariae* and fever in malaria.' *Lancet,* 343:8905, 1095.

Blackman, M. J., Heidrich, H. G., Donachie, S., McBride, J. S. & Holder, A. A. 1990. 'A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies.' *J Exp Med,* 172:1, 379-82.

Blythe, J. E., Surentheran, T. & Preiser, P. R. 2004. 'STEVOR--a multifunctional protein?' *Mol Biochem Parasitol,* 134:1, 11-5.

Blythe, J. E., Yam, X. Y., Kuss, C., Bozdech, Z., Holder, A. A., Marsh, K., Langhorne, J. & Preiser, P. R. 2008. '*Plasmodium falciparum* STEVOR proteins are highly expressed in patient isolates and located in the surface membranes of infected red blood cells and the apical tips of merozoites.' *Infect Immun,* 76:7, 3329-36.

Bosman, A. & Mendis, K. N. 2007. 'A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies.' *Am J Trop Med Hyg,* 77:6 Suppl, 193-7.

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T. & Druilhe, P. 1990. 'Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes.' *J Exp Med,* 172:6, 1633-41.

Bousema, T., Drakeley, C., Gesase, S., Hashim, R., Magesa, S., Mosha, F., Otieno, S., Carneiro, I., Cox, J., Msuya, E., Kleinschmidt, I., Maxwell, C., Greenwood, B., Riley, E., Sauerwein, R., Chandramohan, D. & Gosling, R. 2010a. 'Identification of Hot Spots of Malaria Transmission for Targeted Malaria Control.' *Journal of Infectious Diseases,* 201:11, 1764-74.

Bousema, T., Griffin, J. T., Sauerwein, R. W., Smith, D. L., Churcher, T. S., Takken, W., Ghani, A., Drakeley, C. & Gosling, R. 2012. 'Hitting Hotspots: Spatial Targeting of Malaria for Control and Elimination.' *PLoS Med,* 9:1.

Bousema, T., Okell, L., Shekalaghe, S., Griffin, J. T., Omar, S., Sawa, P., Sutherland, C., Sauerwein, R., Ghani, A. C. & Drakeley, C. 2010b. 'Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs.' *Malar J,* 9, 136.

Brady, O. J., Slater, H. C., Pemberton-Ross, P., Wenger, E., Maude, R. J., Ghani, A. C., Penny, M. A., Gerardin, J., White, L. J., Chitnis, N., Aguas, R., Hay, S. I., Smith, D. L., Stuckey, E. M., Okiro, E. A., Smith, T. A. & Okell, L. C. 2017. 'Role of mass drug administration in elimination of *Plasmodium falciparum* malaria: a consensus modelling study.' *Lancet Glob Health,* 5:7, e680-e87.

Braga, E. M., Barros, R. M., Reis, T. A., Fontes, C. J., Morais, C. G., Martins, M. S. & Krettli, A. U. 2002. 'Association of the IgG response to *Plasmodium falciparum* merozoite protein (C-terminal 19 kD) with clinical immunity to malaria in the Brazilian Amazon region.' *Am J Trop Med Hyg,* 66:5, 461-6.

Branch, O. H., Oloo, A. J., Nahlen, B. L., Kaslow, D. & Lal, A. A. 2000. 'Anti-merozoite surface protein-1 19-kDa IgG in mother-infant pairs naturally exposed to *Plasmodium falciparum*: subclass analysis with age, exposure to asexual parasitemia, and protection against malaria. V. The Asembo Bay Cohort Project.' *J Infect Dis,* 181:5, 1746-52.

Bray, R. S. & Garnham, P. C. 1982. 'The life-cycle of primate malaria parasites.' *Br Med Bull,* 38:2, 117-22.

Bronzan, R. N., McMorrow, M. L. & Kachur, S. P. 2008. 'Diagnosis of malaria: challenges for clinicians in endemic and non-endemic regions.' *Mol Diagn Ther,* 12:5, 299-306.

Bruce, M. C. & Day, K. P. 2002. 'Cross-species regulation of malaria parasitaemia in the human host.' *Curr Opin Microbiol,* 5:4, 431-7.

Bruce, M. C. & Day, K. P. 2003. 'Cross-species regulation of *Plasmodium* parasitemia in semi-immune children from Papua New Guinea.' *Trends Parasitol,* 19:6, 271-7.

Bruce, M. C., Donnelly, C. A., Alpers, M. P., Galinski, M. R., Barnwell, J. W., Walliker, D. & Day, K. P. 2000. 'Cross-species interactions between malaria parasites in humans.' *Science,* 287:5454, 845-8.

Bruce, M. C., Macheso, A., Kelly-Hope, L. A., Nkhoma, S., McConnachie, A. & Molyneux, M. E. 2008. 'Effect of transmission setting and mixed species infections on clinical measures of malaria in Malawi.' *PLoS One,* 3:7, e2775.

Buffet, P. A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G. D. & Mercereau-Puijalon, O. 2011. 'The pathogenesis of *Plasmodium*

falciparum malaria in humans: insights from splenic physiology.' *Blood,* 117:2, 381-92.

Bullard, J. H., Purdom, E., Hansen, K. D. & Dudoit, S. 2010. 'Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments.' *BMC Bioinformatics,* 11, 94.

Bunnik, E. M., Chung, D. W., Hamilton, M., Ponts, N., Saraf, A., Prudhomme, J., Florens, L. & Le Roch, K. G. 2013. 'Polysome profiling reveals translational control of gene expression in the human malaria parasite *Plasmodium falciparum*.' *Genome Biol,* 14:11, R128.

Burghaus, P. A. & Holder, A. A. 1994. 'Expression of the 19-Kilodalton Carboxy-Terminal Fragment of the *Plasmodium-Falciparum* Merozoite Surface Protein-1 in Escherichia-Coli as a Correctly Folded Protein (Vol 64, Pg 165, 1994).' *Mol Biochem Parasitol,* 67:2, 343-43.

Bushman, M., Morton, L., Duah, N., Quashie, N., Abuaku, B., Koram, K. A., Dimbu, P. R., Plucinski, M., Gutman, J., Lyaruu, P., Kachur, S. P., de Roode, J. C. & Udhayakumar, V. 2016. 'Within-host competition and drug resistance in the human malaria parasite *Plasmodium falciparum*.' *Proc Biol Sci,* 283:1826, 20153038.

Camus, D. & Hadley, T. J. 1985. 'A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites.' *Science,* 230:4725, 553-6.

Carrara, V. I., Sirilak, S., Thonglairuam, J., Rojanawatsirivet, C., Proux, S., Gilbos, V., Brockman, A., Ashley, E. A., McGready, R., Krudsood, S., Leemingsawat, S., Looareesuwan, S., Singhasivanon, P., White, N. & Nosten, F. 2006. 'Deployment of early diagnosis and mefloquine-artesunate treatment of falciparum malaria in Thailand: the Tak Malaria Initiative.' *PLoS Med,* 3:6, e183.

Carruthers, V. B. & Sibley, L. D. 1997. 'Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts.' *Eur J Cell Biol,* 73:2, 114-23.

Carter, R. & Mendis, K. N. 2002. 'Evolutionary and historical aspects of the burden of malaria.' *Clin Microbiol Rev,* 15:4, 564-94.

Carter, R., Mendis, K. N. & Roberts, D. 2000. 'Spatial targeting of interventions against malaria.' *Bull World Health Organ,* 78:12, 1401-11.

Carter, R., Ranford-Cartwright, L. & Alano, P. 1993. 'The culture and preparation of gametocytes of *Plasmodium falciparum* for immunochemical, molecular, and mosquito infectivity studies.' *Methods Mol Biol,* 21, 67-88.

Cavanagh, D. R., Dobano, C., Elhassan, I. M., Marsh, K., Elhassan, A., Hviid, L., Khalil, E. A., Theander, T. G., Arnot, D. E. & McBride, J. S. 2001. 'Differential patterns of human immunoglobulin G subclass responses to distinct regions of a single protein, the merozoite surface protein 1 of *Plasmodium falciparum*.' *Infect Immun,* 69:2, 1207-11.

Cavanagh, D. R., Dodoo, D., Hviid, L., Kurtzhals, J. A., Theander, T. G., Akanmori, B. D., Polley, S., Conway, D. J., Koram, K. & McBride, J. S. 2004. 'Antibodies to the N-terminal block 2 of *Plasmodium falciparum* merozoite surface protein 1 are

associated with protection against clinical malaria.' *Infect Immun,* 72:11, 6492-502.

Cavanagh, D. R., Elhassan, I. M., Roper, C., Robinson, V. J., Giha, H., Holder, A. A., Hviid, L., Theander, T. G., Arnot, D. E. & McBride, J. S. 1998. 'A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface protein-1 in an area of unstable malaria in Sudan.' *J Immunol,* 161:1, 347-59.

Cavanagh, D. R. & McBride, J. S. 1997. 'Antigenicity of recombinant proteins derived from *Plasmodium falciparum* merozoite surface protein 1.' *Mol Biochem Parasitol,* 85:2, 197-211.

Cech, T. R. & Steitz, J. A. 2014. 'The noncoding RNA revolution-trashing old rules to forge new ones.' *Cell,* 157:1, 77-94.

Certa, U., Rotmann, D., Matile, H. & Reber-Liske, R. 1987. 'A naturally occurring gene encoding the major surface antigen precursor p190 of *Plasmodium falciparum* lacks tripeptide repeats.' *EMBO J,* 6:13, 4137-42.

Chadee, D. D., Tilluckdharry, C. C., Maharaj, P. & Sinanan, C. 2000. 'Reactivation of *Plasmodium malariae* infection in a Trinidadian man after neurosurgery.' *N Engl J Med,* 342:25, 1924.

Chandler, C. I., Mwangi, R., Mbakilwa, H., Olomi, R., Whitty, C. J. & Reyburn, H. 2008. 'Malaria overdiagnosis: is patient pressure the problem?' *Health Policy Plan,* 23:3, 170-8.

Chang, Z. 2016. 'The discovery of Qinghaosu (artemisinin) as an effective anti-malaria drug: A unique China story.' *Sci China Life Sci,* 59:1, 81-8.

Charlwood, J. D., Kihonda, J., Sama, S., Billingsley, P. F., Hadji, H., Verhave, J. P., Lyimo, E., Luttikhuizen, P. C. & Smith, T. 1995. 'The Rise and Fall of Anopheles Arabiensis (Diptera, Culicidae) in a Tanzanian Village.' *Bulletin of Entomological Research,* 85:1, 37-44.

Chazotte, B. 2011. 'Labeling nuclear DNA with hoechst 33342.' *Cold Spring Harb Protoc,* 2011:1, pdb prot5557.

Chiodini, P. L., Hartley, S., Hewitt, P. E., Barbara, J. A., Lalloo, K., Bligh, J. & Voller, A. 1997. 'Evaluation of a malaria antibody ELISA and its value in reducing potential wastage of red cell donations from blood donors exposed to malaria, with a note on a case of transfusion-transmitted malaria.' *Vox Sang,* 73:3, 143-8.

Chotivanich, K., Silamut, K. & Day, N. P. J. 2007. 'Laboratory diagnosis of malaria infection – A short review of method.' *N Z J Med Lab Sci,* 61:1, 4-7.

Chuangchaiya, S., Jangpatarapongsa, K., Chootong, P., Sirichaisinthop, J., Sattabongkot, J., Pattanapanyasat, K., Chotivanich, K., Troye-Blomberg, M., Cui, L. & Udomsangpetch, R. 2010. 'Immune response to *Plasmodium vivax* has a potential to reduce malaria severity.' *Clin Exp Immunol,* 160:2, 233-9.

Chung, W. Y., Gardiner, D. L., Hyland, C., Gatton, M., Kemp, D. J. & Trenholme, K. R. 2005. 'Enhanced invasion of blood group A1 erythrocytes by *Plasmodium falciparum*.' *Mol Biochem Parasitol,* 144:1, 128-30.

Chuquiyauri, R., Paredes, M., Penataro, P., Torres, S., Marin, S., Tenorio, A., Brouwer, K. C., Abeles, S., Llanos-Cuentas, A., Gilman, R. H., Kosek, M. & Vinetz, J. M.

2012. 'Socio-demographics and the development of malaria elimination strategies in the low transmission setting.' *Acta Trop,* 121:3, 292-302.

Cibulskis, R. E., Aregawi, M., Williams, R., Otten, M. & Dye, C. 2011. 'Worldwide incidence of malaria in 2009: estimates, time trends, and a critique of methods.' *PLoS Med,* 8:12, e1001142.

Clark, I. A., Budd, A. C., Alleva, L. M. & Cowden, W. B. 2006. 'Human malarial disease: a consequence of inflammatory cytokine release.' *Malar J,* 5, 85.

Clark, T. D., Greenhouse, B., Njama-Meya, D., Nzarubara, B., Maiteki-Sebuguzi, C., Staedke, S. G., Seto, E., Kamya, M. R., Rosenthal, P. J. & Dorsey, G. 2008. 'Factors determining the heterogeneity of malaria incidence in children in Kampala, Uganda.' *Journal of Infectious Diseases,* 198:3, 393-400.

Clough, B., Atilola, F. A. & Pasvoi, G. 1998. 'The role of rosetting in the multiplication of *Plasmodium falciparum*: rosette formation neither enhances nor targets parasite invasion into uninfected red cells.' *Br J Haematol,* 100:1, 99-104.

Coatney, G., Collins, W., Warren, M. & Contacos, P. 1971. *The Primate Malarias.* National Institute of Allergy and Infectious Diseases, Bethesda, MD, 366.

Coatney, G. R. 1963. 'Pitfalls in a discovery: the chronicle of chloroquine.' *Am J Trop Med Hyg,* 12, 121-8.

Coldren, R. L., Jongsakul, K., Vayakornvichit, S., Noedl, H. & Fukudas, M. M. 2007. 'Apparent relapse of imported *Plasmodium ovale* malaria in a pregnant woman.' *Am J Trop Med Hyg,* 77:5, 992-4.

Collins, W. E. & Jeffery, G. M. 2005. '*Plasmodium ovale*: parasite and disease.' *Clin Microbiol Rev,* 18:3, 570-81.

Collins, W. E. & Jeffery, G. M. 2007. '*Plasmodium malariae*: parasite and disease.' *Clin Microbiol Rev,* 20:4, 579-92.

Conway, D. J., Cavanagh, D. R., Tanabe, K., Roper, C., Mikes, Z. S., Sakihama, N., Bojang, K. A., Oduola, A. M., Kremsner, P. G., Arnot, D. E., Greenwood, B. M. & McBride, J. S. 2000. 'A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses.' *Nat Med,* 6:6, 689-92.

Conway, D. J. & Polley, S. D. 2002. 'Measuring immune selection.' *Parasitology,* 125 Suppl, S3-16.

Cooling, L. 2008. 'ABO, H, and Lewis blood groups and structurally related antigens.' In Roback J, Combs MR, Grossmann B & Hillyer C (Eds.) *AABB Technical Manual:* Bethesda, MD: American Association of Blood Banks, 363–87.

Cooling, L. 2010. 'Polylactosamines, there's more than meets the "Ii": a review of the I system.' *Immunohematology,* 26:4, 133-55.

Cooling, L. 2015. 'Blood Groups in Infection and Host Susceptibility.' *Clin Microbiol Rev,* 28:3, 801-70.

Cowan, G. J., Creasey, A. M., Dhanasarnsombut, K., Thomas, A. W., Remarque, E. J. & Cavanagh, D. R. 2011. 'A malaria vaccine based on the polymorphic block 2 region of MSP-1 that elicits a broad serotype-spanning immune response.' *PLoS One,* 6:10, e26616.

Cowman, A. F., Berry, D. & Baum, J. 2012. 'The cellular and molecular basis for malaria parasite invasion of the human red blood cell.' *J Cell Biol,* 198:6, 961-71.

Cox-Singh, J., Davis, T. M., Lee, K. S., Shamsul, S. S., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J. & Singh, B. 2008. '*Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening.' *Clin Infect Dis,* 46:2, 165-71.

Cox-Singh, J. & Singh, B. 2008. 'Knowlesi malaria: newly emergent and of public health importance?' *Trends Parasitol,* 24:9, 406-10.

Creasey, A., Giha, H., Hamad, A. A., El Hassan, I. M., Theander, T. G. & Arnot, D. E. 2004. 'Eleven years of malaria surveillance in a Sudanese village highlights unexpected variation in individual disease susceptibility and outbreak severity.' *Parasitology,* 129:Pt 3, 263-71.

Crompton, P. D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L. S., Miller, L. H., Barillas-Mury, C. & Pierce, S. K. 2014. 'Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease.' *Annu Rev Immunol,* 32, 157-87.

Crowley, L. C., Marfell, B. J. & Waterhouse, N. J. 2016. 'Analyzing Cell Death by Nuclear Staining with Hoechst 33342.' *Cold Spring Harb Protoc,* 2016:9, pdb prot087205.

Cserti, C. M. & Dzik, W. H. 2007. 'The ABO blood group system and *Plasmodium falciparum* malaria.' *Blood,* 110:7, 2250-8.

Cui, L. & Su, X. Z. 2009. 'Discovery, mechanisms of action and combination therapy of artemisinin.' *Expert Rev Anti Infect Ther,* 7:8, 999-1013.

Culleton, R. L., Mita, T., Ndounga, M., Unger, H., Cravo, P. V., Paganotti, G. M., Takahashi, N., Kaneko, A., Eto, H., Tinto, H., Karema, C., D'Alessandro, U., do Rosario, V., Kobayakawa, T., Ntoumi, F., Carter, R. & Tanabe, K. 2008. 'Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing.' *Malar J,* 7, 174.

Da Silveira, L. A., Dorta, M. L., Kimura, E. A., Katzin, A. M., Kawamoto, F., Tanabe, K. & Ferreira, M. U. 1999. 'Allelic diversity and antibody recognition of *Plasmodium falciparum* merozoite surface protein 1 during hypoendemic malaria transmission in the Brazilian amazon region.' *Infect Immun,* 67:11, 5906-16.

Daniels, G. 2002. *Human blood groups, 2nd ed.*: Blackwell Science, Oxford, United Kingdom,

Daniels, R. F., Deme, A. B., Gomis, J. F., Dieye, B., Durfee, K., Thwing, J. I., Fall, F. B., Ba, M., Ndiop, M., Badiane, A. S., Ndiaye, Y. D., Wirth, D. F., Volkman, S. K. & Ndiaye, D. 2017. 'Evidence of non-*Plasmodium falciparum* malaria infection in Kedougou, Senegal.' *Malar J,* 16:1, 9.

Danilova, N. & Amemiya, C. T. 2009. 'Going adaptive: the saga of antibodies.' *Ann N Y Acad Sci,* 1168, 130-55.

Das, S., Hertrich, N., Perrin, A. J., Withers-Martinez, C., Collins, C. R., Jones, M. L., Watermeyer, J. M., Fobes, E. T., Martin, S. R., Saibil, H. R., Wright, G. J., Treeck, M., Epp, C. & Blackman, M. J. 2015. 'Processing of *Plasmodium falciparum* Merozoite Surface Protein MSP1 Activates a Spectrin-Binding Function Enabling Parasite Egress from RBCs.' *Cell Host Microbe,* 18:4, 433-44.

David, B. & Jacoby, R. M. Y. 2005. ' Encyclopedia of Family Health.'

de Roode, J. C., Culleton, R., Bell, A. S. & Read, A. F. 2004. 'Competitive release of drug resistance following drug treatment of mixed *Plasmodium* chabaudi infections.' *Malar J,* 3, 33.

Dean, L. 2005. *Blood Groups and Red Cell Antigens [Internet]*. Bethesda (MD): National Center for Biotechnology Information (US),

Delves, P. J., Martin, S. J., Burton, D. R. & Roitt, I. M. 2011. *Roitt's Essential Immunology*. Wiley-Blackwell,

Demissie, Y. & Ketema, T. 2016. 'Complicated malaria symptoms associated with *Plasmodium vivax* among patients visiting health facilities in Mendi town, Northwest Ethiopia.' *BMC Infect Dis,* 16:1, 436.

Dhanasekaran, S., Chandra, N. R., Chandrasekhar Sagar, B. K., Rangarajan, P. N. & Padmanaban, G. 2004. 'Delta-aminolevulinic acid dehydratase from *Plasmodium falciparum*: indigenous versus imported.' *J Biol Chem,* 279:8, 6934-42.

Dhimal, M., O'Hara, R. B., Karki, R., Thakur, G. D., Kuch, U. & Ahrens, B. 2014. 'Spatio-temporal distribution of malaria and its association with climatic factors and vector-control interventions in two high-risk districts of Nepal.' *Malar J,* 13, 457.

Dillies, M. A., Rau, A., Aubert, J., Hennequet-Antier, C., Jeanmougin, M., Servant, N., Keime, C., Marot, G., Castel, D., Estelle, J., Guernec, G., Jagla, B., Jouneau, L., Laloe, D., Le Gall, C., Schaeffer, B., Le Crom, S., Guedj, M., Jaffrezic, F. & French StatOmique, C. 2013. 'A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis.' *Brief Bioinform,* 14:6, 671-83.

Dinko, B., Oguike, M. C., Larbi, J. A., Bousema, T. & Sutherland, C. J. 2013. 'Persistent detection of *Plasmodium falciparum*, *P*. malariae, *P*. *ovale curtisi* and *P*. *ovale wallikeri* after ACT treatment of asymptomatic Ghanaian school-children.' *Int J Parasitol Drugs Drug Resist,* 3, 45-50.

Djimde, A., Doumbo, O. K., Cortese, J. F., Kayentao, K., Doumbo, S., Diourte, Y., Coulibaly, D., Dicko, A., Su, X. Z., Nomura, T., Fidock, D. A., Wellems, T. E. & Plowe, C. V. 2001. 'A molecular marker for chloroquine-resistant falciparum malaria.' *N Engl J Med,* 344:4, 257-63.

Dobano, C., Rogerson, S. J., Mackinnon, M. J., Cavanagh, D. R., Taylor, T. E., Molyneux, M. E. & McBride, J. S. 2008. 'Differential antibody responses to *Plasmodium falciparum* merozoite proteins in Malawian children with severe malaria.' *J Infect Dis,* 197:5, 766-74.

Dodoo, D., Theander, T. G., Kurtzhals, J. A., Koram, K., Riley, E., Akanmori, B. D., Nkrumah, F. K. & Hviid, L. 1999. 'Levels of antibody to conserved parts of *Plasmodium falciparum* merozoite surface protein 1 in Ghanaian children are not associated with protection from clinical malaria.' *Infect Immun,* 67:5, 2131-7.

Dondorp, A. M., Fanello, C. I., Hendriksen, I. C., Gomes, E., Seni, A., Chhaganlal, K. D., Bojang, K., Olaosebikan, R., Anunobi, N., Maitland, K., Kivaya, E., Agbenyega, T., Nguah, S. B., Evans, J., Gesase, S., Kahabuka, C., Mtove, G., Nadjm, B., Deen, J., Mwanga-Amumpaire, J., Nansumba, M., Karema, C., Umulisa, N., Uwimana, A., Mokuolu, O. A., Adedoyin, O. T., Johnson, W. B., Tshefu, A. K., Onyamboko,

M. A., Sakulthaew, T., Ngum, W. P., Silamut, K., Stepniewska, K., Woodrow, C. J., Bethell, D., Wills, B., Oneko, M., Peto, T. E., von Seidlein, L., Day, N. P., White, N. J. & group, A. 2010. 'Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial.' *Lancet*, 376:9753, 1647-57.

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W., Lee, S. J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S. S., Yeung, S., Singhasivanon, P., Day, N. P., Lindegardh, N., Socheat, D. & White, N. J. 2009. 'Artemisinin resistance in *Plasmodium falciparum* malaria.' *N Engl J Med*, 361:5, 455-67.

Doolan, D. L., Beck, H. P. & Good, M. F. 1994. 'Evidence for limited activation of distinct CD4+ T cell subsets in response to the *Plasmodium falciparum* circumsporozoite protein in Papua New Guinea.' *Parasite Immunol*, 16:3, 129-36.

Doolan, D. L., Dobano, C. & Baird, J. K. 2009. 'Acquired immunity to malaria.' *Clin Microbiol Rev*, 22:1, 13-36, Table of Contents.

Douglas, N. M., Nosten, F., Ashley, E. A., Phaiphun, L., van Vugt, M., Singhasivanon, P., White, N. J. & Price, R. N. 2011. '*Plasmodium vivax* recurrence following falciparum and mixed species malaria: risk factors and effect of antimalarial kinetics.' *Clin Infect Dis*, 52:5, 612-20.

Drakeley, C. J., Corran, P. H., Coleman, P. G., Tongren, J. E., McDonald, S. L., Carneiro, I., Malima, R., Lusingu, J., Manjurano, A., Nkya, W. M., Lemnge, M. M., Cox, J., Reyburn, H. & Riley, E. M. 2005. 'Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure.' *Proc Natl Acad Sci U S A*, 102:14, 5108-13.

Driss, A., Hibbert, J. M., Wilson, N. O., Iqbal, S. A., Adamkiewicz, T. V. & Stiles, J. K. 2011. 'Genetic polymorphisms linked to susceptibility to malaria.' *Malar J*, 10, 271.

Duah, N. O., Miles, D. J., Whittle, H. C. & Conway, D. J. 2010. 'Acquisition of antibody isotypes against *Plasmodium falciparum* blood stage antigens in a birth cohort.' *Parasite Immunol*, 32:2, 125-34.

Duffy, P. E. & Sibley, C. H. 2005. 'Are we losing artemisinin combination therapy already?' *Lancet*, 366:9501, 1908-9.

Egan, A. F., Chappel, J. A., Burghaus, P. A., Morris, J. S., McBride, J. S., Holder, A. A., Kaslow, D. C. & Riley, E. M. 1995. 'Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1(19), the carboxy-terminal fragment of the major merozoite surface protein of *Plasmodium falciparum*.' *Infect Immun*, 63:2, 456-66.

Egan, A. F., Morris, J., Barnish, G., Allen, S., Greenwood, B. M., Kaslow, D. C., Holder, A. A. & Riley, E. M. 1996. 'Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1.' *J Infect Dis*, 173:3, 765-9.

Ekue, J. M., Ulrich, A. M. & Njelesani, E. K. 1983. '*Plasmodium* malaria resistant to chloroquine in a Zambian living in Zambia.' *Br Med J (Clin Res Ed)*, 286:6374, 1315-6.

El Sayed, B. B., Arnot, D. E., Mukhtar, M. M., Baraka, O. Z., Dafalla, A. A., Elnaiem, D. E. & Nugud, A. H. 2000. 'A study of the urban malaria transmission problem in Khartoum.' *Acta Trop,* 75:2, 163-71.

Elhassan, I. M., Hviid, L., Jakobsen, P. H., Giha, H., Satti, G. M., Arnot, D. E., Jensen, J. B. & Theander, T. G. 1995. 'High proportion of subclinical *Plasmodium falciparum* infections in an area of seasonal and unstable malaria in Sudan.' *Am J Trop Med Hyg,* 53:1, 78-83.

Elhassan, I. M., Satti, G. H., Ali, A. E., Fadul, I., Elkhalifa, A. A., Abedelrahim, A. M., Ming, C. & Theander, T. G. 1993. 'The efficacy of artemether in the treatment of *Plasmodium falciparum* malaria in Sudan.' *Trans R Soc Trop Med Hyg,* 87:6, 685-6.

Engvall, E. & Perlmann, P. 1971. 'Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G.' *Immunochemistry,* 8:9, 871-4.

Epp, C., Li, F., Howitt, C. A., Chookajorn, T. & Deitsch, K. W. 2009. 'Chromatin associated sense and antisense noncoding RNAs are transcribed from the var gene family of virulence genes of the malaria parasite *Plasmodium falciparum*.' *RNA,* 15:1, 116-27.

Eyles, D. E., Hoo, C. C., Warren, M. & Sandosham, A. A. 1963. '*Plasmodium falciparum* Resistant to Chloroquine in Cambodia.' *Am J Trop Med Hyg,* 12, 840-3.

Fairley, N. H. 1947. 'Sidelights on Malaria in Man Obtained by Subinoculation Experiments.' *Trans R Soc Trop Med Hyg,* 40:5, 621-76.

Farrow, R. E., Green, J., Katsimitsoulia, Z., Taylor, W. R., Holder, A. A. & Molloy, J. E. 2011. 'The mechanism of erythrocyte invasion by the malarial parasite, *Plasmodium falciparum*.' *Semin Cell Dev Biol,* 22:9, 953-60.

Felger, I., Steiger, S., Hatz, C., Smith, T. & Beck, H. P. 2003. 'Antigenic cross-reactivity between different alleles of the *Plasmodium falciparum* merozoite surface protein 2.' *Parasite Immunol,* 25:11-12, 531-43.

Fenton, B., Clark, J. T., Khan, C. M., Robinson, J. V., Walliker, D., Ridley, R., Scaife, J. G. & McBride, J. S. 1991. 'Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*.' *Mol Cell Biol,* 11:2, 963-71.

Ferreira, M. U., Kimura, E. A., Katzin, A. M., Santos-Neto, L. L., Ferrari, J. O., Villalobos, J. M. & de Carvalho, M. E. 1998. 'The IgG-subclass distribution of naturally acquired antibodies to *Plasmodium falciparum*, in relation to malaria exposure and severity.' *Ann Trop Med Parasitol,* 92:3, 245-56.

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M., Sidhu, A. B., Naude, B., Deitsch, K. W., Su, X. Z., Wootton, J. C., Roepe, P. D. & Wellems, T. E. 2000. 'Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance.' *Mol Cell,* 6:4, 861-71.

Fitzpatrick, T., Ricken, S., Lanzer, M., Amrhein, N., Macheroux, P. & Kappes, B. 2001. 'Subcellular localization and characterization of chorismate synthase in the apicomplexan *Plasmodium falciparum*.' *Mol Microbiol,* 40:1, 65-75.

Fleischer, B. 2004. 'Editorial: 100 years ago: Giemsa's solution for staining of plasmodia.' *Trop Med Int Health,* 9:7, 755-6.

Fogh, S., Jepsen, S. & Effersoe, P. 1979. 'Chloroquine-resistant *Plasmodium falciparum* malaria in Kenya.' *Trans R Soc Trop Med Hyg,* 73:2, 228-9.

Fontenille, D. & Rakotoarivony, I. 1988. 'Reappearance of Anopheles funestus as a malaria vector in the Antananarivo region, Madagascar.' *Trans R Soc Trop Med Hyg,* 82:4, 644-5.

Fowkes, F. J., Richards, J. S., Simpson, J. A. & Beeson, J. G. 2010. 'The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: A systematic review and meta-analysis.' *PLoS Med,* 7:1, e1000218.

Francis, T. 1960. 'On the doctrine of original antigenic sin.' *Proc Am Philos Soc,* 104, 572–78.

Franken, G., Muller-Stover, I., Holtfreter, M. C., Walter, S., Mehlhorn, H., Labisch, A., Haussinger, D. & Richter, J. 2012. 'Why do *Plasmodium malariae* infections sometimes occur in spite of previous antimalarial medication?' *Parasitol Res,* 111:2, 943-6.

Fru-Cho, J., Bumah, V. V., Safeukui, I., Nkuo-Akenji, T., Titanji, V. P. & Haldar, K. 2014. 'Molecular typing reveals substantial *Plasmodium vivax* infection in asymptomatic adults in a rural area of Cameroon.' *Malar J,* 13, 170.

Fry, A. E., Griffiths, M. J., Auburn, S., Diakite, M., Forton, J. T., Green, A., Richardson, A., Wilson, J., Jallow, M., Sisay-Joof, F., Pinder, M., Peshu, N., Williams, T. N., Marsh, K., Molyneux, M. E., Taylor, T. E., Rockett, K. A. & Kwiatkowski, D. P. 2008. 'Common variation in the ABO glycosyltransferase is associated with susceptibility to severe *Plasmodium falciparum* malaria.' *Hum Mol Genet,* 17:4, 567-76.

Fuchs, C. S. & Mayer, R. J. 1995. 'Gastric carcinoma.' *N Engl J Med,* 333:1, 32-41.

Fung, M. K., Grossman, B.J., Hillyer, C.D., Westhoff, C.M. 2014. *ABO,H and Lewis blood groups and structurally related antigens.* AABB Press, Bethesda, MD., 291-315.

Gan, S. D. & Patel, K. R. 2013. 'Enzyme immunoassay and enzyme-linked immunosorbent assay.' *J Invest Dermatol,* 133:9, e12.

Garcia, C. R., de Azevedo, M. F., Wunderlich, G., Budu, A., Young, J. A. & Bannister, L. 2008. '*Plasmodium* in the postgenomic era: new insights into the molecular cell biology of malaria parasites.' *Int Rev Cell Mol Biol,* 266, 85-156.

Gardas, A. & Lewartowska, A. 1988. 'Coating of proteins to polystyrene ELISA plates in the presence of detergents.' *J Immunol Methods,* 106:2, 251-5.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M. & Barrell, B. 2002. 'Genome sequence

of the human malaria parasite *Plasmodium falciparum*.' *Nature,* 419:6906, 498-511.

Garnham, P. 1966. *Malaria Parasites and Other Haemosporidia.*: Blackwell Scientific Publications, 1132.

Garnham, P. C. 1988a. 'Swellengrebel lecture. Hypnozoites and 'relapses' in *Plasmodium vivax* and in vivax-like malaria.' *Trop Geogr Med,* 40:3, 187-95.

Garnham, P. C. C. 1988b. 'Malaria.' In Wernsdorfer WH & McGregor I (Eds.) *Principles and Practice of Malariology*: Churchill Livingstone, 781–814.

Garraud, O., Mahanty, S. & Perraut, R. 2003. 'Malaria-specific antibody subclasses in immune individuals: a key source of information for vaccine design.' *Trends Immunol,* 24:1, 30-5.

Gaur, D., Mayer, D. C. & Miller, L. H. 2004. 'Parasite ligand-host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites.' *Int J Parasitol,* 34:13-14, 1413-29.

Gazzinelli, R. T., Kalantari, P., Fitzgerald, K. A. & Golenbock, D. T. 2014. 'Innate sensing of malaria parasites.' *Nat Rev Immunol,* 14:11, 744-57.

Gemperli, A., Sogoba, N., Fondjo, E., Mabaso, M., Bagayoko, M., Briet, O. J., Anderegg, D., Liebe, J., Smith, T. & Vounatsou, P. 2006. 'Mapping malaria transmission in West and Central Africa.' *Trop Med Int Health,* 11:7, 1032-46.

Genton, B., Smith, T., Baea, K., Narara, A., al-Yaman, F., Beck, H. P., Hii, J. & Alpers, M. 1994. 'Malaria: how useful are clinical criteria for improving the diagnosis in a highly endemic area?' *Trans R Soc Trop Med Hyg,* 88:5, 537-41.

Gething, P. W., Casey, D. C., Weiss, D. J., Bisanzio, D., Bhatt, S., Cameron, E., Battle, K. E., Dalrymple, U., Rozier, J., Rao, P. C., Kutz, M. J., Barber, R. M., Huynh, C., Shackelford, K. A., Coates, M. M., Nguyen, G., Fraser, M. S., Kulikoff, R., Wang, H., Naghavi, M., Smith, D. L., Murray, C. J., Hay, S. I. & Lim, S. S. 2016. 'Mapping *Plasmodium falciparum* Mortality in Africa between 1990 and 2015.' *N Engl J Med,* 375:25, 2435-45.

Getis, A., Morrison, A. C., Gray, K. & Scott, T. W. 2003. 'Characteristics of the spatial pattern of the dengue vector, Aedes aegypti, in Iquitos, Peru.' *Am J Trop Med Hyg,* 69:5, 494-505.

Giemsa, G. 1904. 'Eine Vereinfachung und Vervollkommnung meiner Methylenblau-Eosin-Färbemethode zur Erzielung der Romanowsky-Nocht'schen Chromätinfarbung.' *Centralblatt fur Bakteriologie I Abteilung,* 32, 307–13.

Giha, H. A., Rosthoj, S., Dodoo, D., Hviid, L., Satti, G. M., Scheike, T., Arnot, D. E. & Theander, T. G. 2000. 'The epidemiology of febrile malaria episodes in an area of unstable and seasonal transmission.' *Trans R Soc Trop Med Hyg,* 94:6, 645-51.

Giha, H. A., Theander, T. G., Staalso, T., Roper, C., Elhassan, I. M., Babiker, H., Satti, G. M., Arnot, D. E. & Hviid, L. 1998. 'Seasonal variation in agglutination of *Plasmodium falciparum*-infected erythrocytes.' *Am J Trop Med Hyg,* 58:4, 399-405.

Gilles, H. M. 1993. 'Diagnostic methods in malaria.' In Gilles HM & Warrell DA (Eds.) *Bruce-Chwatt's Essential malariology 3rd edition*: Edward Arnold,London, United Kingdom, 78-95.

Gilles, H. M. & Hendrickse, R. G. 1960. 'Possible aetiological role of *Plasmodium malariae* in "nephrotic syndrome" in Nigerian children.' *Lancet,* 1:7128, 806-7.

Gilson, P. R., Nebl, T., Vukcevic, D., Moritz, R. L., Sargeant, T., Speed, T. P., Schofield, L. & Crabb, B. S. 2006. 'Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*.' *Mol Cell Proteomics,* 5:7, 1286-99.

Golgi, C. 1889. 'On the cycle of development of malarial parasites in tertian fever: differential diagnosis between the intracellular parasites of tertian and quartan fever.' *Archivio per le Scienza Mediche,* 13, , 173–96.

Goncalves, B. P., Fried, M. & Duffy, P. E. 2014. 'Parasite burden and severity of malaria in Tanzanian children.' *N Engl J Med,* 371:5, 482.

Graves, P. M., Gelband, H. & Garner, P. 2015. 'Primaquine or other 8-aminoquinoline for reducing *Plasmodium falciparum* transmission.' *Cochrane Database Syst Rev*:2, CD008152.

Greenberg, A. E., Ntumbanzondo, M., Ntula, N., Mawa, L., Howell, J. & Davachi, F. 1989. 'Hospital-based surveillance of malaria-related paediatric morbidity and mortality in Kinshasa, Zaire.' *Bull World Health Organ,* 67:2, 189-96.

Greenwood, B. M., Bojang, K., Whitty, C. J. & Targett, G. A. 2005. 'Malaria.' *Lancet,* 365:9469, 1487-98.

Grigg, M. J., William, T., Menon, J., Dhanaraj, P., Barber, B. E., Wilkes, C. S., von Seidlein, L., Rajahram, G. S., Pasay, C., McCarthy, J. S., Price, R. N., Anstey, N. M. & Yeo, T. W. 2016. 'Artesunate-mefloquine versus chloroquine for treatment of uncomplicated *Plasmodium knowlesi* malaria in Malaysia (ACT KNOW): an open-label, randomised controlled trial.' *Lancet Infect Dis,* 16:2, 180-8.

Gubler, D. J. 1998. 'Dengue and dengue hemorrhagic fever.' *Clin Microbiol Rev,* 11:3, 480-96.

Gupta, M. & Chowdhuri, A. N. 1980. 'Relationship between ABO blood groups and malaria.' *Bull World Health Organ,* 58:6, 913-5.

Gupta, S., Hill, A. V., Kwiatkowski, D., Greenwood, A. M., Greenwood, B. M. & Day, K. P. 1994. 'Parasite virulence and disease patterns in *Plasmodium falciparum* malaria.' *Proc Natl Acad Sci U S A,* 91:9, 3715-9.

Gusnanto, A., Calza, S. & Pawitan, Y. 2007. 'Identification of differentially expressed genes and false discovery rate in microarray studies.' *Curr Opin Lipidol,* 18:2, 187-93.

Hagan, P., Chandiwana, S., Ndhlovu, P., Woolhouse, M. & Dessein, A. 1994. 'The epidemiology, immunology and morbidity of *Schistosoma haematobium* infections in diverse communities in Zimbabwe. I. The study design.' *Trop Geogr Med,* 46:4 Spec No, 227-32.

Haldar, K. & Mohandas, N. 2009. 'Malaria, erythrocytic infection, and anemia.' *Hematology Am Soc Hematol Educ Program*, 87-93.

Hall, N., Pain, A., Berriman, M., Churcher, C., Harris, B., Harris, D., Mungall, K., Bowman, S., Atkin, R., Baker, S., Barron, A., Brooks, K., Buckee, C. O., Burrows, C., Cherevach, I., Chillingworth, C., Chillingworth, T., Christodoulou, Z., Clark, L., Clark, R., Corton, C., Cronin, A., Davies, R., Davis, P., Dear, P., Dearden, F.,

Doggett, J., Feltwell, T., Goble, A., Goodhead, I., Gwilliam, R., Hamlin, N., Hance, Z., Harper, D., Hauser, H., Hornsby, T., Holroyd, S., Horrocks, P., Humphray, S., Jagels, K., James, K. D., Johnson, D., Kerhornou, A., Knights, A., Konfortov, B., Kyes, S., Larke, N., Lawson, D., Lennard, N., Line, A., Maddison, M., McLean, J., Mooney, P., Moule, S., Murphy, L., Oliver, K., Ormond, D., Price, C., Quail, M. A., Rabbinowitsch, E., Rajandream, M. A., Rutter, S., Rutherford, K. M., Sanders, M., Simmonds, M., Seeger, K., Sharp, S., Smith, R., Squares, R., Squares, S., Stevens, K., Taylor, K., Tivey, A., Unwin, L., Whitehead, S., Woodward, J., Sulston, J. E., Craig, A., Newbold, C. & Barrell, B. G. 2002. 'Sequence of *Plasmodium falciparum* chromosomes 1, 3-9 and 13.' *Nature,* 419:6906, 527-31.

Hall, R., Hyde, J. E., Goman, M., Simmons, D. L., Hope, I. A., Mackay, M., Scaife, J., Merkli, B., Richle, R. & Stocker, J. 1984. 'Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria.' *Nature,* 311:5984, 379-82.

Halstead, S. B., Rojanasuphot, S. & Sangkawibha, N. 1983. 'Original antigenic sin in dengue.' *Am J Trop Med Hyg,* 32:1, 154-6.

Hamad, A. A., El Hassan, I. M., El Khalifa, A. A., Ahmed, G. I., Abdelrahim, S. A., Theander, T. G. & Arnot, D. E. 2000. 'Chronic *Plasmodium falciparum* infections in an area of low intensity malaria transmission in the Sudan.' *Parasitology,* 120 (Pt 5), 447-56.

Hamad, A. A., Nugud Ael, H., Arnot, D. E., Giha, H. A., Abdel-Muhsin, A. M., Satti, G. M., Theander, T. G., Creasey, A. M., Babiker, H. A. & Elnaiem, D. E. 2002. 'A marked seasonality of malaria transmission in two rural sites in eastern Sudan.' *Acta Trop,* 83:1, 71-82.

Haridi, A. M. 1972. 'Partial exophily of Anopheles gambiae species B in the Khashm Elgirba area in eastern Sudan.' *Bull World Health Organ,* 46:1, 39-46.

Harris, I., Sharrock, W. W., Bain, L. M., Gray, K. A., Bobogare, A., Boaz, L., Lilley, K., Krause, D., Vallely, A., Johnson, M. L., Gatton, M. L., Shanks, G. D. & Cheng, Q. 2010. 'A large proportion of asymptomatic *Plasmodium* infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting.' *Malar J,* 9, 254.

Harris, P. K., Yeoh, S., Dluzewski, A. R., O'Donnell, R. A., Withers-Martinez, C., Hackett, F., Bannister, L. H., Mitchell, G. H. & Blackman, M. J. 2005. 'Molecular identification of a malaria merozoite surface sheddase.' *PLoS Pathog,* 1:3, 241-51.

Harvey, S. A., Jennings, L., Chinyama, M., Masaninga, F., Mulholland, K. & Bell, D. R. 2008. 'Improving community health worker use of malaria rapid diagnostic tests in Zambia: package instructions, job aid and job aid-plus-training.' *Malar J,* 7, 160.

Hase, R., Uwamino, Y., Muranaka, K., Tochitani, K., Sogi, M., Kitazono, H. & Hosokawa, N. 2013. '[*Plasmodium malariae* malaria with more than a 4-month incubation period: difficult to distinguish from a relapse of *Plasmodium vivax* malaria].' *Kansenshogaku Zasshi,* 87:4, 446-50.

Hawking, F., Worms, M. J. & Gammage, K. 1968. '24- and 48-hour cycles of malaria parasites in the blood; their purpose, production and control.' *Trans R Soc Trop Med Hyg,* 62:6, 731-65.

Hay, S. I., Guerra, C. A., Gething, P. W., Patil, A. P., Tatem, A. J., Noor, A. M., Kabaria, C. W., Manh, B. H., Elyazar, I. R., Brooker, S., Smith, D. L., Moyeed, R. A. & Snow, R. W. 2009. 'A world malaria map: *Plasmodium falciparum* endemicity in 2007.' *PLoS Med,* 6:3, e1000048.

Hay, S. I., Guerra, C. A., Tatem, A. J., Noor, A. M. & Snow, R. W. 2004. 'The global distribution and population at risk of malaria: past, present, and future.' *Lancet Infect Dis,* 4:6, 327-36.

Heddini, A., Pettersson, F., Kai, O., Shafi, J., Obiero, J., Chen, Q. J., Barragan, A., Wahlgren, M. & Marsh, K. 2001. 'Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors.' *Infect Immun,* 69:9, 5849-56.

Hemingway, J., Shretta, R., Wells, T. N., Bell, D., Djimde, A. A., Achee, N. & Qi, G. 2016. 'Tools and Strategies for Malaria Control and Elimination: What Do We Need to Achieve a Grand Convergence in Malaria?' *PLoS Biol,* 14:3, e1002380.

Hess, F. J., Kilian, H. D., Nothdurft, H. D. & Loscher, T. 1993. 'Problems in the therapy of mixed malarial infections: a case of infection with *Plasmodium falciparum* and *P*. malariae treated with mefloquine and halofantrine.' *Trans R Soc Trop Med Hyg,* 87:6, 688.

Ho, E., Deltenre, P., Nkuize, M., Delwaide, J., Colle, I., Michielsen, P. & Belgian Association for the Study of the, L. 2013. 'Coinfection of hepatitis B and hepatitis delta virus in Belgium: a multicenter BASL study. Prospective epidemiology and comparison with HBV mono-infection.' *J Med Virol,* 85:9, 1513-7.

Hogh, B., Marbiah, N. T., Burghaus, P. A. & Andersen, P. K. 1995. 'Relationship between maternally derived anti-*Plasmodium falciparum* antibodies and risk of infection and disease in infants living in an area of Liberia, west Africa, in which malaria is highly endemic.' *Infect Immun,* 63:10, 4034-8.

Holder, A. A. 1988. 'The precursor to major merozoite surface antigens: structure and role in immunity.' *Prog Allergy,* 41, 72-97.

Holder, A. A. 1999. 'Malaria vaccines.' *Proc Natl Acad Sci U S A,* 96:4, 1167-9.

Holder, A. A., Blackman, M. J., Burghaus, P. A., Chappel, J. A., Ling, I. T., McCallum-Deighton, N. & Shai, S. 1992. 'A malaria merozoite surface protein (MSP1)-structure, processing and function.' *Mem Inst Oswaldo Cruz,* 87 Suppl 3, 37-42.

Holder, A. A. & Freeman, R. R. 1982. 'Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody.' *J Exp Med,* 156:5, 1528-38.

Holder, A. A., Guevara Patino, J. A., Uthaipibull, C., Syed, S. E., Ling, I. T., Scott-Finnigan, T. & Blackman, M. J. 1999. 'Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria.' *Parassitologia,* 41:1-3, 409-14.

Holder, A. A., Lockyer, M. J., Odink, K. G., Sandhu, J. S., Riveros-Moreno, V., Nicholls, S. C., Hillman, Y., Davey, L. S., Tizard, M. L., Schwarz, R. T. & et al. 1985.

'Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites.' *Nature,* 317:6034, 270-3.

Holder, A. A., Sandhu, J. S., Hillman, Y., Davey, L. S., Nicholls, S. C., Cooper, H. & Lockyer, M. J. 1987. 'Processing of the precursor to the major merozoite surface antigens of *Plasmodium falciparum*.' *Parasitology,* 94 (Pt 2), 199-208.

Homewood, C. A., Warhurst, D. C., Peters, W. & Baggaley, V. C. 1972. 'Lysosomes, pH and the anti-malarial action of chloroquine.' *Nature,* 235:5332, 50-2.

Hommel, B., Galloula, A., Simon, A. & Buffet, P. 2013. 'Hyposplenism revealed by *Plasmodium malariae* infection.' *Malar J,* 12:1, 271.

Hornbeck, P., Winston, S. E. & Fuller, S. A. 2001. 'Enzyme-linked immunosorbent assays (ELISA).' *Curr Protoc Mol Biol,* Chapter 11, Unit11 2.

Hornbeck, P. V. 2015. 'Enzyme-Linked Immunosorbent Assays.' *Curr Protoc Immunol,* 110, 2 1 1-23.

Hosoi, E. 2008. 'Biological and clinical aspects of ABO blood group system.' *J Med Invest,* 55:3-4, 174-82.

Howard, R. J. & Pasloske, B. L. 1993. 'Target antigens for asexual malaria vaccine development.' *Parasitol Today,* 9:10, 369-72.

Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., Wellems, T. E., Rener, J. & Taylor, D. W. 1986. 'Secretion of a malarial histidine-rich protein (Pf HRP II) from *Plasmodium falciparum*-infected erythrocytes.' *J Cell Biol,* 103:4, 1269-77.

Howes, R. E., Battle, K. E., Mendis, K. N., Smith, D. L., Cibulskis, R. E., Baird, J. K. & Hay, S. I. 2016. 'Global Epidemiology of *Plasmodium vivax*.' *Am J Trop Med Hyg,* 95:6 Suppl, 15-34.

Howes, R. E., Patil, A. P., Piel, F. B., Nyangiri, O. A., Kabaria, C. W., Gething, P. W., Zimmerman, P. A., Barnadas, C., Beall, C. M., Gebremedhin, A., Menard, D., Williams, T. N., Weatherall, D. J. & Hay, S. I. 2011. 'The global distribution of the Duffy blood group.' *Nat Commun,* 2, 266.

Howes, R. E., Reiner, R. C., Jr., Battle, K. E., Longbottom, J., Mappin, B., Ordanovich, D., Tatem, A. J., Drakeley, C., Gething, P. W., Zimmerman, P. A., Smith, D. L. & Hay, S. I. 2015. '*Plasmodium vivax* Transmission in Africa.' *PLoS Negl Trop Dis,* 9:11, e0004222.

Hulden, L. & Hulden, L. 2009. 'The decline of malaria in Finland--the impact of the vector and social variables.' *Malar J,* 8, 94.

Hulden, L., McKitrick, R. & Hulden, L. 2014. 'Average household size and the eradication of malaria.' *J. R. Statist. Soc. A,* 177:3, 725–42.

Hurwitz, E. S., Johnson, D. & Campbell, C. C. 1981. 'Resistance of *Plasmodium falciparum* malaria to sulfadoxine-pyrimethamine ('Fansidar') in a refugee camp in Thailand.' *Lancet,* 1:8229, 1068-70.

Hviid, L. 2005. 'Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa.' *Acta Trop,* 95:3, 270-5.

Jambulingam, P., Mohapatra, S. S., Das, L. K., Das, P. K. & Rajagopalan, P. K. 1989. 'Detection of *Plasmodium ovale* in Koraput district, Orissa state.' *Indian J Med Res,* 89, 115-6.

Jiang, G., Daubenberger, C., Huber, W., Matile, H., Tanner, M. & Pluschke, G. 2000. 'Sequence diversity of the merozoite surface protein 1 of *Plasmodium falciparum* in clinical isolates from the Kilombero District, Tanzania.' *Acta Trop,* 74:1, 51-61.

John, C. C., Moormann, A. M., Pregibon, D. C., Sumba, P. O., McHugh, M. M., Narum, D. L., Lanar, D. E., Schluchter, M. D. & Kazura, J. W. 2005. 'Correlation of high levels of antibodies to multiple pre-erythrocytic *Plasmodium falciparum* antigens and protection from infection.' *Am J Trop Med Hyg,* 73:1, 222-8.

John, C. C., Moormann, A. M., Sumba, P. O., Ofulla, A. V., Pregibon, D. C. & Kazura, J. W. 2004a. 'Gamma interferon responses to *Plasmodium falciparum* liver-stage antigen 1 and thrombospondin-related adhesive protein and their relationship to age, transmission intensity, and protection against malaria.' *Infect Immun,* 72:9, 5135-42.

John, C. C., O'Donnell, R. A., Sumba, P. O., Moormann, A. M., de Koning-Ward, T. F., King, C. L., Kazura, J. W. & Crabb, B. S. 2004b. 'Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage *Plasmodium falciparum* infection in individuals in a malaria endemic area of Africa.' *J Immunol,* 173:1, 666-72.

John, C. C., Tande, A. J., Moormann, A. M., Sumba, P. O., Lanar, D. E., Min, X. M. & Kazura, J. W. 2008. 'Antibodies to pre-erythrocytic *Plasmodium falciparum* antigens and risk of clinical malaria in Kenyan children.' *J Infect Dis,* 197:4, 519-26.

Johnson, D. J., Fidock, D. A., Mungthin, M., Lakshmanan, V., Sidhu, A. B., Bray, P. G. & Ward, S. A. 2004. 'Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents.' *Mol Cell,* 15:6, 867-77.

Josling, G. A. & Llinas, M. 2015. 'Sexual development in *Plasmodium* parasites: knowing when it's time to commit.' *Nat Rev Microbiol,* 13:9, 573-87.

Kadekoppala, M. & Holder, A. A. 2010. 'Merozoite surface proteins of the malaria parasite: the MSP1 complex and the MSP7 family.' *Int J Parasitol,* 40:10, 1155-61.

Kallander, K., Nsungwa-Sabiiti, J. & Peterson, S. 2004. 'Symptom overlap for malaria and pneumonia--policy implications for home management strategies.' *Acta Trop,* 90:2, 211-4.

Kaneko, A. 2010. 'A community-directed strategy for sustainable malaria elimination on islands: short-term MDA integrated with ITNs and robust surveillance.' *Acta Trop,* 114:3, 177-83.

Kaneko, A., Taleo, G., Kalkoa, M., Yamar, S., Kobayakawa, T. & Bjorkman, A. 2000. 'Malaria eradication on islands.' *Lancet,* 356:9241, 1560-4.

Kang, Y. & Long, C. A. 1995. 'Sequence heterogeneity of the C-terminal, Cys-rich region of the merozoite surface protein-1 (MSP-1) in field samples of *Plasmodium falciparum*.' *Mol Biochem Parasitol,* 73:1-2, 103-10.

Kasehagen, L. J., Mueller, I., McNamara, D. T., Bockarie, M. J., Kiniboro, B., Rare, L., Lorry, K., Kastens, W., Reeder, J. C., Kazura, J. W. & Zimmerman, P. A. 2006.

'Changing patterns of *Plasmodium* blood-stage infections in the Wosera region of Papua New Guinea monitored by light microscopy and high throughput PCR diagnosis.' *Am J Trop Med Hyg,* 75:4, 588-96.

Kassim, O. O., Ako-Anai, K. A., Torimiro, S. E., Hollowell, G. P., Okoye, V. C. & Martin, S. K. 2000. 'Inhibitory factors in breastmilk, maternal and infant sera against in vitro growth of *Plasmodium falciparum* malaria parasite.' *J Trop Pediatr,* 46:2, 92-6.

Kats, L. M., Black, C. G., Proellocks, N. I. & Coppel, R. L. 2006. '*Plasmodium* rhoptries: how things went pear-shaped.' *Trends Parasitol,* 22:6, 269-76.

Kaufman, T. S. & Ruveda, E. A. 2005. 'The quest for quinine: those who won the battles and those who won the war.' *Angew Chem Int Ed Engl,* 44:6, 854-85.

Kaul, D. K., Roth, E. F., Jr., Nagel, R. L., Howard, R. J. & Handunnetti, S. M. 1991. 'Rosetting of *Plasmodium falciparum*-infected red blood cells with uninfected red blood cells enhances microvascular obstruction under flow conditions.' *Blood,* 78:3, 812-9.

Khan, M. N., Khan, T. A. & Ahmed, Z. 2013. 'Discrepancy in ABO blood grouping.' *J Coll Physicians Surg Pak,* 23:8, 590-2.

Kim, J. H., Skountzou, I., Compans, R. & Jacob, J. 2009. 'Original antigenic sin responses to influenza viruses.' *J Immunol,* 183:5, 3294-301.

Kitchen, A., Mijovic, A. & Hewitt, P. 2005. 'Transfusion-transmitted malaria: current donor selection guidelines are not sufficient.' *Vox Sang,* 88:3, 200-1.

Kitchen, A. D., Lowe, P. H., Lalloo, K. & Chiodini, P. L. 2004. 'Evaluation of a malarial antibody assay for use in the screening of blood and tissue products for clinical use.' *Vox Sang,* 87:3, 150-5.

Kitchen, S. 1939. 'The infection of mature and immature erythrocytes by *Plasmodium falciparum* and *Plasmodium malariae*.' *Am J Trop Med Hyg,* 19:47–62.

Kreuels, B., Kobbe, R., Adjei, S., Kreuzberg, C., von Reden, C., Bater, K., Klug, S., Busch, W., Adjei, O. & May, J. 2008. 'Spatial variation of malaria incidence in young children from a geographically homogeneous area with high endemicity.' *Journal of Infectious Diseases,* 197:1, 85-93.

Krotoski, W. A., Krotoski, D. M., Garnham, P. C., Bray, R. S., Killick-Kendrick, R., Draper, C. C., Targett, G. A. & Guy, M. W. 1980. 'Relapses in primate malaria: discovery of two populations of exoerythrocytic stages. Preliminary note.' *Br Med J,* 280:6208, 153-4.

Kulldorff, M. 1997. 'A spatial scan statistic.' *Communications in Statistics-Theory and Methods,* 26:6, 1481-96.

Kulldorff, M. 2015. 'SaTScan™ User Guide for version 9.4. Available at http://www.satscan.org. Accessed 12 May 2017.'

Kwiatkowski, D. 1990. 'Tumour necrosis factor, fever and fatality in falciparum malaria.' *Immunol Lett,* 25:1-3, 213-6.

Kyabayinze, D. J., Asiimwe, C., Nakanjako, D., Nabakooza, J., Counihan, H. & Tibenderana, J. K. 2010. 'Use of RDTs to improve malaria diagnosis and fever case management at primary health care facilities in Uganda.' *Malar J,* 9, 200.

Lacsina, J. R., LaMonte, G., Nicchitta, C. V. & Chi, J. T. 2011. 'Polysome profiling of the malaria parasite *Plasmodium falciparum*.' *Mol Biochem Parasitol,* 179:1, 42-6.

Lalremruata, A., Magris, M., Vivas-Martinez, S., Koehler, M., Esen, M., Kempaiah, P., Jeyaraj, S., Perkins, D. J., Mordmuller, B. & Metzger, W. G. 2015. 'Natural infection of *Plasmodium* brasilianum in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon.' *EBioMedicine,* 2:9, 1186-92.

Lambrechts, T., Bryce, J. & Orinda, V. 1999. 'Integrated management of childhood illness: a summary of first experiences.' *Bull World Health Organ,* 77:7, 582-94.

Lambros, C. & Vanderberg, J. P. 1979. 'Synchronization of *Plasmodium falciparum* erythrocytic stages in culture.' *J Parasitol,* 65:3, 418-20.

Landsteiner, K. 1900. 'Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe.' *Zentralbl Bakteriol,* 27, 357-62.

Langford, S., Douglas, N. M., Lampah, D. A., Simpson, J. A., Kenangalem, E., Sugiarto, P., Anstey, N. M., Poespoprodjo, J. R. & Price, R. N. 2015. '*Plasmodium malariae* Infection Associated with a High Burden of Anemia: A Hospital-Based Surveillance Study.' *PLoS Negl Trop Dis,* 9:12, e0004195.

Langhorne, J., Ndungu, F. M., Sponaas, A. M. & Marsh, K. 2008. 'Immunity to malaria: more questions than answers.' *Nat Immunol,* 9:7, 725-32.

Langmead, B. & Salzberg, S. L. 2012. 'Fast gapped-read alignment with Bowtie 2.' *Nat Methods,* 9:4, 357-9.

Langreth, S. G., Jensen, J. B., Reese, R. T. & Trager, W. 1978. 'Fine structure of human malaria in vitro.' *J Protozool,* 25:4, 443-52.

Larsen, R. D., Ernst, L. K., Nair, R. P. & Lowe, J. B. 1990. 'Molecular cloning, sequence, and expression of a human GDP-L-fucose:beta-D-galactoside 2-alpha-L-fucosyltransferase cDNA that can form the H blood group antigen.' *Proc Natl Acad Sci U S A,* 87:17, 6674-8.

Lee, W. C., Malleret, B., Lau, Y. L., Mauduit, M., Fong, M. Y., Cho, J. S., Suwanarusk, R., Zhang, R., Albrecht, L., Costa, F. T., Preiser, P., McGready, R., Renia, L., Nosten, F. & Russell, B. 2014. 'Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes.' *Blood,* 123:18, e100-9.

Lehmann, T., Dao, A., Yaro, A. S., Adamou, A., Kassogue, Y., Diallo, M., Sekou, T. & Coscaron-Arias, C. 2010. 'Aestivation of the African malaria mosquito, Anopheles gambiae in the Sahel.' *Am J Trop Med Hyg,* 83:3, 601-6.

Lell, B., May, J., Schmidt-Ott, R. J., Lehman, L. G., Luckner, D., Greve, B., Matousek, P., Schmid, D., Herbich, K., Mockenhaupt, F. P., Meyer, C. G., Bienzle, U. & Kremsner, P. G. 1999. 'The role of red blood cell polymorphisms in resistance and susceptibility to malaria.' *Clin Infect Dis,* 28:4, 794-9.

Liu, Y., Zhou, J. & White, K. P. 2014. 'RNA-seq differential expression studies: more sequence or more replication?' *Bioinformatics,* 30:3, 301-4.

Lorenzetti, A., Fornazari, P. A., Bonini-Domingos, A. C., de Souza Rodrigues Penhalbel, R., Fugikaha, E., Bonini-Domingos, C. R., Fraga, V. D., Conceicao, L. M., Rossit, A. R., Cavasini, C. E., Couto, V. S. & Machado, R. L. 2008. 'Mixed *Plasmodium falciparum* infections and its clinical implications in four areas of the Brazilian Amazon region.' *Acta Trop,* 107:1, 8-12.

Lowe, B. S., Mosobo, M. & Bull, P. C. 1998. 'All four species of human malaria parasites form rosettes.' *Trans R Soc Trop Med Hyg,* 92:5, 526.

Lu, F., Culleton, R., Zhang, M., Ramaprasad, A., von Seidlein, L., Zhou, H., Zhu, G., Tang, J., Liu, Y., Wang, W., Cao, Y., Xu, S., Gu, Y., Li, J., Zhang, C., Gao, Q., Menard, D., Pain, A., Yang, H., Zhang, Q. & Cao, J. 2017. 'Emergence of Indigenous Artemisinin-Resistant *Plasmodium falciparum* in Africa.' *N Engl J Med,* 376:10, 991-3.

Luxemburger, C., Ricci, F., Nosten, F., Raimond, D., Bathet, S. & White, N. J. 1997. 'The epidemiology of severe malaria in an area of low transmission in Thailand.' *Trans R Soc Trop Med Hyg,* 91:3, 256-62.

Luzzatto, L. 1974. 'Genetic factors in malaria.' *Bull World Health Organ,* 50:3-4, 195-202.

Lysenko, A. J. & Beljaev, A. E. 1969. 'An analysis of the geographical distribution of *Plasmodium ovale*.' *Bull World Health Organ,* 40:3, 383-94.

Mabaso, M. L., Vounatsou, P., Midzi, S., Da Silva, J. & Smith, T. 2006. 'Spatio-temporal analysis of the role of climate in inter-annual variation of malaria incidence in Zimbabwe.' *Int J Health Geogr,* 5, 20.

Mackinnon, M. J. & Marsh, K. 2010. 'The selection landscape of malaria parasites.' *Science,* 328:5980, 866-71.

Mackintosh, C. L., Beeson, J. G. & Marsh, K. 2004. 'Clinical features and pathogenesis of severe malaria.' *Trends Parasitol,* 20:12, 597-603.

Madeira, L., Galante, P. A., Budu, A., Azevedo, M. F., Malnic, B. & Garcia, C. R. 2008. 'Genome-wide detection of serpentine receptor-like proteins in malaria parasites.' *PLoS One,* 3:3, e1889.

Makler, M. T. & Hinrichs, D. J. 1993. 'Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia.' *Am J Trop Med Hyg,* 48:2, 205-10.

Malaguarnera, L. & Musumeci, S. 2002. 'The immune response to *Plasmodium falciparum* malaria.' *Lancet Infect Dis,* 2:8, 472-8.

Manguin, S., Foumane, V., Besnard, P., Fortes, F. & Carnevale, P. 2017. 'Malaria overdiagnosis and subsequent overconsumption of antimalarial drugs in Angola: Consequences and effects on human health.' *Acta Trop,* 171, 58-63.

Manning, S. D., Woolhouse, M. E. J. & Ndamba, J. 1995. 'Geographic Compatibility of the Fresh-Water Snail Bulinus-Globosus and Schistosomes from the Zimbabwe Highveld.' *Int J Parasitol,* 25:1, 37-42.

Marin-Menendez, A., Bardaji, A., Martinez-Espinosa, F. E., Botto-Menezes, C., Lacerda, M. V., Ortiz, J., Cistero, P., Piqueras, M., Felger, I., Mueller, I., Ordi, J., del Portillo, H., Menendez, C., Wahlgren, M. & Mayor, A. 2013. 'Rosetting in *Plasmodium vivax*: a cytoadhesion phenotype associated with anaemia.' *PLoS Negl Trop Dis,* 7:4, e2155.

Marin-Menendez, A., Monaghan, P. & Bell, A. 2012. 'A family of cyclophilin-like molecular chaperones in *Plasmodium falciparum*.' *Mol Biochem Parasitol,* 184:1, 44-7.

Markus, M. B. 2011a. 'The hypnozoite concept, with particular reference to malaria.' *Parasitol Res*, 108:1, 247-52.
Markus, M. B. 2011b. 'Malaria: origin of the term "hypnozoite".' *J Hist Biol*, 44:4, 781-6.
Markus, M. B. 2015. 'Do hypnozoites cause relapse in malaria?' *Trends Parasitol*, 31:6, 239-45.
Marsh, K. & Kinyanjui, S. 2006. 'Immune effector mechanisms in malaria.' *Parasite Immunol*, 28:1-2, 51-60.
Martin, M. 2011. 'Cutadapt removes adapter sequences from high-throughput sequencing reads.' *2011*, 17:1.
Masinde, G. L., Krogstad, D. J., Gordon, D. M. & Duffy, P. E. 1998. 'Immunization with SPf66 and subsequent infection with homologous and heterologous *Plasmodium falciparum* parasites.' *Am J Trop Med Hyg*, 59:4, 600-5.
Mason, D. P. & McKenzie, F. E. 1999. 'Blood-stage dynamics and clinical implications of mixed *Plasmodium vivax-Plasmodium falciparum* infections.' *Am J Trop Med Hyg*, 61:3, 367-74.
Mason, D. P., McKenzie, F. E. & Bossert, W. H. 1999. 'The blood-stage dynamics of mixed *Plasmodium malariae-Plasmodium falciparum* infections.' *J Theor Biol*, 198:4, 549-66.
Matrajt, M. 2010. 'Non-coding RNA in apicomplexan parasites.' *Mol Biochem Parasitol*, 174:1, 1-7.
Mawili-Mboumba, D. P., Borrmann, S., Cavanagh, D. R., McBride, J. S., Matsiegui, P. B., Missinou, M. A., Kremsner, P. G. & Ntoumi, F. 2003. 'Antibody responses to *Plasmodium falciparum* merozoite surface protein-1 and efficacy of amodiaquine in Gabonese children with *P. falciparum* malaria.' *J Infect Dis*, 187:7, 1137-41.
Mayxay, M., Pukrittayakamee, S., Newton, P. N. & White, N. J. 2004. 'Mixed-species malaria infections in humans.' *Trends Parasitol*, 20:5, 233-40.
Mbenda, N. H. G. & Das, A. 2014. 'Molecular evidence of *Plasmodium vivax* mono and mixed malaria parasite infections in Duffy-negative native Cameroonians.' *PLoS One*, 9:8, e103262.
Mbengue, A., Audiger, N., Vialla, E., Dubremetz, J. F. & Braun-Breton, C. 2013. 'Novel *Plasmodium falciparum* Maurer's clefts protein families implicated in the release of infectious merozoites.' *Mol Microbiol*, 88:2, 425-42.
McBride, J. S. & Heidrich, H. G. 1987. 'Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex.' *Mol Biochem Parasitol*, 23:1, 71-84.
McGready, R., Lee, S. J., Wiladphaingern, J., Ashley, E. A., Rijken, M. J., Boel, M., Simpson, J. A., Paw, M. K., Pimanpanarak, M., Mu, O., Singhasivanon, P., White, N. J. & Nosten, F. H. 2012. 'Adverse effects of falciparum and vivax malaria and the safety of antimalarial treatment in early pregnancy: a population-based study.' *Lancet Infect Dis*, 12:5, 388-96.
McKenna, K. C., Tsuji, M., Sarzotti, M., Sacci, J. B., Jr., Witney, A. A. & Azad, A. F. 2000. 'gammadelta T cells are a component of early immunity against preerythrocytic malaria parasites.' *Infect Immun*, 68:4, 2224-30.

McKenzie, F. E. & Bossert, W. H. 1997. 'Mixed-species *Plasmodium* infections of Anopheles (Diptera:Culicidae).' *J Med Entomol*, 34:4, 417-25.

McKenzie, F. E. & Bossert, W. H. 1999. 'Multispecies *Plasmodium* infections of humans.' *J Parasitol*, 85:1, 12-8.

McKenzie, F. E., Sirichaisinthop, J., Miller, R. S., Gasser, R. A., Jr. & Wongsrichanalai, C. 2003. 'Dependence of malaria detection and species diagnosis by microscopy on parasite density.' *Am J Trop Med Hyg*, 69:4, 372-6.

McKenzie, F. E., Smith, D. L., O'Meara, W. P., Forney, J. R., Magill, A. J., Permpanich, B., Erhart, L. M., Sirichaisinthop, J., Wongsrichanalai, C. & Gasser, R. A., Jr. 2006. 'Fever in patients with mixed-species malaria.' *Clin Infect Dis*, 42:12, 1713-8.

McQueen, P. G. & McKenzie, F. E. 2006. 'Competition for red blood cells can enhance *Plasmodium vivax* parasitemia in mixed-species malaria infections.' *Am J Trop Med Hyg*, 75:1, 112-25.

Mehlotra, R. K., Lorry, K., Kastens, W., Miller, S. M., Alpers, M. P., Bockarie, M., Kazura, J. W. & Zimmerman, P. A. 2000. 'Random distribution of mixed species malaria infections in Papua New Guinea.' *Am J Trop Med Hyg*, 62:2, 225-31.

Meier, B., Dobeli, H. & Certa, U. 1992. 'Stage-specific expression of aldolase isoenzymes in the rodent malaria parasite *Plasmodium* berghei.' *Mol Biochem Parasitol*, 52:1, 15-27.

Menard, D., Barnadas, C., Bouchier, C., Henry-Halldin, C., Gray, L. R., Ratsimbasoa, A., Thonier, V., Carod, J. F., Domarle, O., Colin, Y., Bertrand, O., Picot, J., King, C. L., Grimberg, B. T., Mercereau-Puijalon, O. & Zimmerman, P. A. 2010. '*Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people.' *Proc Natl Acad Sci U S A*, 107:13, 5967-71.

Menard, D., Khim, N., Beghain, J., Adegnika, A. A., Shafiul-Alam, M., Amodu, O., Rahim-Awab, G., Barnadas, C., Berry, A., Boum, Y., Bustos, M. D., Cao, J., Chen, J. H., Collet, L., Cui, L., Thakur, G. D., Dieye, A., Djalle, D., Dorkenoo, M. A., Eboumbou-Moukoko, C. E., Espino, F. E., Fandeur, T., Ferreira-da-Cruz, M. F., Fola, A. A., Fuehrer, H. P., Hassan, A. M., Herrera, S., Hongvanthong, B., Houze, S., Ibrahim, M. L., Jahirul-Karim, M., Jiang, L., Kano, S., Ali-Khan, W., Khanthavong, M., Kremsner, P. G., Lacerda, M., Leang, R., Leelawong, M., Li, M., Lin, K., Mazarati, J. B., Menard, S., Morlais, I., Muhindo-Mavoko, H., Musset, L., Na-Bangchang, K., Nambozi, M., Niare, K., Noedl, H., Ouedraogo, J. B., Pillai, D. R., Pradines, B., Quang-Phuc, B., Ramharter, M., Randrianarivelojosia, M., Sattabongkot, J., Sheikh-Omar, A., Silue, K. D., Sirima, S. B., Sutherland, C., Syafruddin, D., Tahar, R., Tang, L. H., Toure, O. A., Tshibangu-wa-Tshibangu, P., Vigan-Womas, I., Warsame, M., Wini, L., Zakeri, S., Kim, S., Eam, R., Berne, L., Khean, C., Chy, S., Ken, M., Loch, K., Canier, L., Duru, V., Legrand, E., Barale, J. C., Stokes, B., Straimer, J., Witkowski, B., Fidock, D. A., Rogier, C., Ringwald, P., Ariey, F., Mercereau-Puijalon, O. & Consortium, K. 2016. 'A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms.' *N Engl J Med*, 374:25, 2453-64.

Mendes, C., Dias, F., Figueiredo, J., Mora, V. G., Cano, J., de Sousa, B., do Rosario, V. E., Benito, A., Berzosa, P. & Arez, A. P. 2011. 'Duffy negative antigen is no longer a barrier to *Plasmodium vivax*--molecular evidences from the African West Coast (Angola and Equatorial Guinea).' *PLoS Negl Trop Dis,* 5:6, e1192.

Mendis, K., Sina, B. J., Marchesini, P. & Carter, R. 2001. 'The neglected burden of *Plasmodium vivax* malaria.' *Am J Trop Med Hyg,* 64:1-2 Suppl, 97-106.

Mercer, T. R., Dinger, M. E. & Mattick, J. S. 2009. 'Long non-coding RNAs: insights into functions.' *Nat Rev Genet,* 10:3, 155-9.

Metselaar, D. & Van Thiel, P. H. 1959. 'Classification of Malaria.' *Trop Geogr Med,* 11:2, 157-61.

Metzger, W. G., Okenu, D. M., Cavanagh, D. R., Robinson, J. V., Bojang, K. A., Weiss, H. A., McBride, J. S., Greenwood, B. M. & Conway, D. J. 2003. 'Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria.' *Parasite Immunol,* 25:6, 307-12.

Mharakurwa, S., Mutambu, S. L., Mudyiradima, R., Chimbadzwa, T., Chandiwana, S. K. & Day, K. P. 2004. 'Association of house spraying with suppressed levels of drug resistance in Zimbabwe.' *Malar J,* 3, 35.

Miller, L. H., Ackerman, H. C., Su, X. Z. & Wellems, T. E. 2013. 'Malaria biology and disease pathogenesis: insights for new treatments.' *Nat Med,* 19:2, 156-67.

Miller, L. H., Baruch, D. I., Marsh, K. & Doumbo, O. K. 2002. 'The pathogenic basis of malaria.' *Nature,* 415:6872, 673-9.

Miller, L. H., Mason, S. J., Clyde, D. F. & McGinniss, M. H. 1976. 'The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy.' *N Engl J Med,* 295:6, 302-4.

Milner, D. A., Jr. 2017. 'Malaria Pathogenesis.' *Cold Spring Harb Perspect Med*.

Mohapatra, M. K., Dash, L. K., Barih, P. K. & Karua, P. C. 2012. 'Profile of mixed species (*Plasmodium vivax* and *falciparum*) malaria in adults.' *J Assoc Physicians India,* 60, 20-4.

Moody, A. H. & Chiodini, P. L. 2002. 'Non-microscopic method for malaria diagnosis using OptiMAL IT, a second-generation dipstick for malaria pLDH antigen detection.' *Br J Biomed Sci,* 59:4, 228-31.

Mouchet, J., Laventure, S., Blanchy, S., Fioramonti, R., Rakotonjanabelo, A., Rabarison, P., Sircoulon, J. & Roux, J. 1997. '[The reconquest of the Madagascar highlands by malaria].' *Bull Soc Pathol Exot,* 90:3, 162-8.

Mueller, I., Zimmerman, P. A. & Reeder, J. C. 2007. '*Plasmodium malariae* and *Plasmodium ovale*--the "bashful" malaria parasites.' *Trends Parasitol,* 23:6, 278-83.

Muerhoff, A. S., Birkenmeyer, L. G., Coffey, R., Dille, B. J., Barnwell, J. W., Collins, W. E., Sullivan, J. S., Dawson, G. J. & Desai, S. M. 2010. 'Detection of *Plasmodium falciparum*, *P*. vivax, *P*. ovale, and *P*. malariae merozoite surface protein 1-p19 antibodies in human malaria patients and experimentally infected nonhuman primates.' *Clin Vaccine Immunol,* 17:10, 1631-8.

Muller-Stover, I., Verweij, J. J., Hoppenheit, B., Gobels, K., Haussinger, D. & Richter, J. 2008. '*Plasmodium malariae* infection in spite of previous anti-malarial medication.' *Parasitol Res,* 102:3, 547-50.

Muller, O., Bayer, M. J., Peters, C., Andersen, J. S., Mann, M. & Mayer, A. 2002. 'The Vtc proteins in vacuole fusion: coupling NSF activity to V-0 trans-complex formation.' *Embo Journal,* 21:3, 259-69.

Muller, O., Neumann, H., Bayer, M. J. & Mayer, A. 2003. 'Role of the Vtc proteins in V-ATPase stability and membrane trafficking.' *Journal of Cell Science,* 116:6, 1107-15.

Murphy, G. S. & Oldfield, E. C., 3rd 1996. 'Falciparum malaria.' *Infect Dis Clin North Am,* 10:4, 747-75.

Murray, C. J. & Lopez, A. D. 1997. 'Mortality by cause for eight regions of the world: Global Burden of Disease Study.' *Lancet,* 349:9061, 1269-76.

Mutambu, S. L., Dallas, A. B. & Olweny, C. L. 1986. 'Chloroquine resistant *Plasmodium falciparum* malaria in Zimbabwe.' *Br Med J (Clin Res Ed),* 292:6519, 522.

Mutapi, F., Hagan, P., Woolhouse, M. E., Mduluza, T. & Ndhlovu, P. D. 2003. 'Chemotherapy-induced, age-related changes in antischistosome antibody responses.' *Parasite Immunol,* 25:2, 87-97.

Mutapi, F., Mduluza, T., Gomez-Escobar, N., Gregory, W. F., Fernandez, C., Midzi, N. & Maizels, R. M. 2006. 'Immuno-epidemiology of human *Schistosoma haematobium* infection: preferential IgG3 antibody responsiveness to a recombinant antigen dependent on age and parasite burden.' *BMC Infect Dis,* 6, 96.

Mutapi, F., Mduluza, T. & Roddam, A. W. 2005. 'Cluster analysis of schistosome-specific antibody responses partitions the population into distinct epidemiological groups.' *Immunol Lett,* 96:2, 231-40.

Mutapi, F., Ndhlovu, P. D., Hagan, P. & Woolhouse, M. E. 1997. 'A comparison of humoral responses to *Schistosoma haematobium* in areas with low and high levels of infection.' *Parasite Immunol,* 19:6, 255-63.

Mutapi, F., Ndhlovu, P. D., Hagan, P. & Woolhouse, M. E. 2000. 'Anti-schistosome antibody responses in children coinfected with malaria.' *Parasite Immunol,* 22:4, 207-9.

Mwingira, F., Nkwengulila, G., Schoepflin, S., Sumari, D., Beck, H. P., Snounou, G., Felger, I., Olliaro, P. & Mugittu, K. 2011. '*Plasmodium falciparum* msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa.' *Malar J,* 10, 79.

Nagano, T. & Fraser, P. 2009. 'Emerging similarities in epigenetic gene silencing by long noncoding RNAs.' *Mamm Genome,* 20:9-10, 557-62.

Newbold, C., Craig, A., Kyes, S., Rowe, A., Fernandez-Reyes, D. & Fagan, T. 1999. 'Cytoadherence, pathogenesis and the infected red cell surface in *Plasmodium falciparum*.' *Int J Parasitol,* 29:6, 927-37.

Newbold, C., Warn, P., Black, G., Berendt, A., Craig, A., Snow, B., Msobo, M., Peshu, N. & Marsh, K. 1997. 'Receptor-specific adhesion and clinical disease in *Plasmodium falciparum*.' *Am J Trop Med Hyg,* 57:4, 389-98.

Niang, M., Yan Yam, X. & Preiser, P. R. 2009. 'The *Plasmodium falciparum* STEVOR multigene family mediates antigenic variation of the infected erythrocyte.' *PLoS Pathog*, 5:2, e1000307.

Nkumama, I. N., O'Meara, W. P. & Osier, F. H. 2016. 'Changes in Malaria Epidemiology in Africa and New Challenges for Elimination.' *Trends Parasitol*.

Nkumama, I. N., O'Meara, W. P. & Osier, F. H. 2017. 'Changes in Malaria Epidemiology in Africa and New Challenges for Elimination.' *Trends Parasitol*, 33:2, 128-40.

Noble, J. E. & Bailey, M. J. 2009. 'Quantitation of protein.' *Methods Enzymol*, 463, 73-95.

Noedl, H., Se, Y., Schaecher, K., Smith, B. L., Socheat, D., Fukuda, M. M. & Artemisinin Resistance in Cambodia 1 Study, C. 2008. 'Evidence of artemisinin-resistant malaria in western Cambodia.' *N Engl J Med*, 359:24, 2619-20.

Nosten, F. & White, N. J. 2007. 'Artemisinin-based combination treatment of falciparum malaria.' *Am J Trop Med Hyg*, 77:6 Suppl, 181-92.

Nwuba, R. I., Sodeinde, O., Anumudu, C. I., Omosun, Y. O., Odaibo, A. B., Holder, A. A. & Nwagwu, M. 2002. 'The human immune response to *Plasmodium falciparum* includes both antibodies that inhibit merozoite surface protein 1 secondary processing and blocking antibodies.' *Infect Immun*, 70:9, 5328-31.

O'Dempsey, T. J., McArdle, T. F., Laurence, B. E., Lamont, A. C., Todd, J. E. & Greenwood, B. M. 1993. 'Overlap in the clinical features of pneumonia and malaria in African children.' *Trans R Soc Trop Med Hyg*, 87:6, 662-5.

O'Donnell, J. & Laffan, M. A. 2001. 'The relationship between ABO histo-blood group, factor VIII and von Willebrand factor.' *Transfus Med*, 11:4, 343-51.

O'Grady, N. P., Barie, P. S., Bartlett, J. G., Bleck, T., Carroll, K., Kalil, A. C., Linden, P., Maki, D. G., Nierman, D., Pasculle, W., Masur, H., American College of Critical Care, M. & Infectious Diseases Society of, A. 2008. 'Guidelines for evaluation of new fever in critically ill adult patients: 2008 update from the American College of Critical Care Medicine and the Infectious Diseases Society of America.' *Crit Care Med*, 36:4, 1330-49.

Oakley, M. S., Gerald, N., McCutchan, T. F., Aravind, L. & Kumar, S. 2011. 'Clinical and molecular aspects of malaria fever.' *Trends Parasitol*, 27:10, 442-9.

Obare, P., Ogutu, B., Adams, M., Odera, J. S., Lilley, K., Dosoo, D., Adhiambo, C., Owusu-Agyei, S., Binka, F., Wanja, E. & Johnson, J. 2013. 'Misclassification of *Plasmodium* infections by conventional microscopy and the impact of remedial training on the proficiency of laboratory technicians in species identification.' *Malar J*, 12, 113.

Oguike, M. C., Betson, M., Burke, M., Nolder, D., Stothard, J. R., Kleinschmidt, I., Proietti, C., Bousema, T., Ndounga, M., Tanabe, K., Ntege, E., Culleton, R. & Sutherland, C. J. 2011. '*Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities.' *Int J Parasitol*, 41:6, 677-83.

Ohrt, C., Purnomo, Sutamihardja, M. A., Tang, D. & Kain, K. C. 2002. 'Impact of microscopy error on estimates of protective efficacy in malaria-prevention trials.' *J Infect Dis*, 186:4, 540-6.

Oliveira-Ferreira, J., Lacerda, M. V., Brasil, P., Ladislau, J. L., Tauil, P. L. & Daniel-Ribeiro, C. T. 2010. 'Malaria in Brazil: an overview.' *Malar J,* 9, 115.

Oliver, D. G., Sanders, A. H., Hogg, R. D. & Hellman, J. W. 1981. 'Thermal gradients in microtitration plates. Effects on enzyme-linked immunoassay.' *J Immunol Methods,* 42:2, 195-201.

Onchiri, F. M., Pavlinac, P., Singa, B. O., Mulongo, J. N., Farquhar, C., John-Stewart, G. C. & Walson, J. 2014. 'Frequency and correlates of malaria overdiagnosis and treatment in western Kenya.' *The Lancet Global Health,* 2, S45.

Osier, F. H. 2008. 'Immune responses to polymorphic antigens and protection against severe malaria in Kenyan children (Doctoral dissertation).' Open University. Retrieved from the British Library ethesis online service.

Osier, F. H., Fegan, G., Polley, S. D., Murungi, L., Verra, F., Tetteh, K. K., Lowe, B., Mwangi, T., Bull, P. C., Thomas, A. W., Cavanagh, D. R., McBride, J. S., Lanar, D. E., Mackinnon, M. J., Conway, D. J. & Marsh, K. 2008. 'Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria.' *Infect Immun,* 76:5, 2240-8.

Osier, F. H., Polley, S. D., Mwangi, T., Lowe, B., Conway, D. J. & Marsh, K. 2007. 'Naturally acquired antibodies to polymorphic and conserved epitopes of *Plasmodium falciparum* merozoite surface protein 3.' *Parasite Immunol,* 29:8, 387-94.

Pachebat, J. A., Kadekoppala, M., Grainger, M., Dluzewski, A. R., Gunaratne, R. S., Scott-Finnigan, T. J., Ogun, S. A., Ling, I. T., Bannister, L. H., Taylor, H. M., Mitchell, G. H. & Holder, A. A. 2007. 'Extensive proteolytic processing of the malaria parasite merozoite surface protein 7 during biosynthesis and parasite release from erythrocytes.' *Mol Biochem Parasitol,* 151:1, 59-69.

Packard, R. M. 2014. 'The origins of antimalarial-drug resistance.' *N Engl J Med,* 371:5, 397-9.

Paddock, M. L., Wiley, S. E., Axelrod, H. L., Cohen, A. E., Roy, M., Abresch, E. C., Capraro, D., Murphy, A. N., Nechushtai, R., Dixon, J. E. & Jennings, P. A. 2007. 'MitoNEET is a uniquely folded 2Fe 2S outer mitochondrial membrane protein stabilized by pioglitazone.' *Proc Natl Acad Sci U S A,* 104:36, 14342-7.

Palmer, C. J., Lindo, J. F., Klaskala, W. I., Quesada, J. A., Kaminsky, R., Baum, M. K. & Ager, A. L. 1998. 'Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria.' *J Clin Microbiol,* 36:1, 203-6.

Parizo, J., Sturrock, H. J., Dhiman, R. C. & Greenhouse, B. 2016. 'Spatiotemporal Analysis of Malaria in Urban Ahmedabad (Gujarat), India: Identification of Hot Spots and Risk Factors for Targeted Intervention.' *Am J Trop Med Hyg,* 95:3, 595-603.

Park, J. W., Moon, S. H., Yeom, J. S., Lim, K. J., Sohn, M. J., Jung, W. C., Cho, Y. J., Jeon, K. W., Ju, W., Ki, C. S., Oh, M. D. & Choe, K. 2001. 'Naturally acquired antibody responses to the C-terminal region of merozoite surface protein 1 of *Plasmodium vivax* in Korea.' *Clin Diagn Lab Immunol,* 8:1, 14-20.

Parra, M., Hui, G., Johnson, A. H., Berzofsky, J. A., Roberts, T., Quakyi, I. A. & Taylor, D. W. 2000. 'Characterization of conserved T- and B-cell epitopes in *Plasmodium falciparum* major merozoite surface protein 1.' *Infect Immun,* 68:5, 2685-91.

Pascarella, S. & Negro, F. 2011. 'Hepatitis D virus: an update.' *Liver Int,* 31:1, 7-21.

PATH 2011. 'Staying the Course? Malaria Research and Development in a Time of Economic Uncertainty.' Seattle: PATH.

Pathak, V., Colah, R. & Ghosh, K. 2016. 'Correlation between 'H' blood group antigen and *Plasmodium falciparum* invasion.' *Ann Hematol,* 95:7, 1067-75.

Pathirana, S. L., Alles, H. K., Bandara, S., Phone-Kyaw, M., Perera, M. K., Wickremasinghe, A. R., Mendis, K. N. & Handunnetti, S. M. 2005. 'ABO-blood-group types and protection against severe, *Plasmodium falciparum* malaria.' *Ann Trop Med Parasitol,* 99:2, 119-24.

Patil, V., Lescault, P. J., Lirussi, D., Thompson, A. B. & Matrajt, M. 2012. 'Disruption of the expression of a non-coding RNA significantly impairs cellular differentiation in *Toxoplasma gondii*.' *Int J Mol Sci,* 14:1, 611-24.

Paul, W. E. 2008. *Fundamental immunology.* Philadelphia: Lippincott Williams & Wilkins,

Payne, D. 1987. 'Spread of chloroquine resistance in *Plasmodium falciparum*.' *Parasitol Today,* 3:8, 241-6.

Payne, D. 1988. 'Use and limitations of light microscopy for diagnosing malaria at the primary health care level.' *Bull World Health Organ,* 66:5, 621-6.

Perraut, R., Diatta, B., Marrama, L., Garraud, O., Jambou, R., Longacre, S., Krishnegowda, G., Dieye, A. & Gowda, D. C. 2005. 'Differential antibody responses to *Plasmodium falciparum* glycosylphosphatidylinositol anchors in patients with cerebral and mild malaria.' *Microbes Infect,* 7:4, 682-7.

Perraut, R., Marrama, L., Diouf, B., Fontenille, D., Tall, A., Sokhna, C., Trape, J. F., Garraud, O. & Mercereau-Puijalon, O. 2003. 'Distinct surrogate markers for protection against *Plasmodium falciparum* infection and clinical malaria identified in a Senegalese community after radical drug cure.' *J Infect Dis,* 188:12, 1940-50.

Peters, J. M., Fowler, E. V., Krause, D. R., Cheng, Q. & Gatton, M. L. 2007. 'Differential changes in *Plasmodium falciparum* var transcription during adaptation to culture.' *J Infect Dis,* 195:5, 748-55.

Peters, W. 1970. *Chemotherapy and Drug Resistance in Malaria.* Academic Press, London,

Peters, W. 1982. 'Antimalarial drug resistance: an increasing problem.' *Br Med Bull,* 38:2, 187-92.

Peters, W. 1987a. 'Resistance in human malaria IV: 4-aminoquinolines and multiple resistance.' *Chemotherapy and drug resistance in malaria*: Academic Press, London, 659–786.

Peters, W. 1987b. 'Resistance in human malaria. III. Dihydrofolate reductase inhibitors.' *Chemotherapy and drug resistance in malaria*: Academic Press, London, 593–657.

Peters, W. 1990. '*Plasmodium*: resistance to antimalarial drugs.' *Ann Parasitol Hum Comp,* 65 Suppl 1, 103-6.

Peterson, I., Borrell, L. N., El-Sadr, W. & Teklehaimanot, A. 2009. 'A temporal-spatial analysis of malaria transmission in Adama, Ethiopia.' *Am J Trop Med Hyg,* 81:6, 944-9.

Peterson, M. G., Marshall, V. M., Smythe, J. A., Crewther, P. E., Lew, A., Silva, A., Anders, R. F. & Kemp, D. J. 1989. 'Integral membrane protein located in the apical complex of *Plasmodium falciparum*.' *Mol Cell Biol,* 9:7, 3151-4.

Pied, S., Roland, J., Louise, A., Voegtle, D., Soulard, V., Mazier, D. & Cazenave, P. A. 2000. 'Liver CD4-CD8- NK1.1+ TCR alpha beta intermediate cells increase during experimental malaria infection and are able to exhibit inhibitory activity against the parasite liver stage in vitro.' *J Immunol,* 164:3, 1463-9.

Pizarro, J. C., Chitarra, V., Verger, D., Holm, I., Petres, S., Dartevelle, S., Nato, F., Longacre, S. & Bentley, G. A. 2003. 'Crystal structure of a Fab complex formed with PfMSP1-19, the C-terminal fragment of merozoite surface protein 1 from *Plasmodium falciparum*: a malaria vaccine candidate.' *J Mol Biol,* 328:5, 1091-103.

Plattner, F. & Soldati-Favre, D. 2008. 'Hijacking of host cellular functions by the Apicomplexa.' *Annu Rev Microbiol,* 62, 471-87.

Poespoprodjo, J. R., Fobia, W., Kenangalem, E., Lampah, D. A., Hasanuddin, A., Warikar, N., Sugiarto, P., Tjitra, E., Anstey, N. M. & Price, R. N. 2009. 'Vivax malaria: a major cause of morbidity in early infancy.' *Clin Infect Dis,* 48:12, 1704-12.

Polley, S. D., Conway, D. J., Cavanagh, D. R., McBride, J. S., Lowe, B. S., Williams, T. N., Mwangi, T. W. & Marsh, K. 2006. 'High levels of serum antibodies to merozoite surface protein 2 of *Plasmodium falciparum* are associated with reduced risk of clinical malaria in coastal Kenya.' *Vaccine,* 24:19, 4233-46.

Polley, S. D., Mwangi, T., Kocken, C. H., Thomas, A. W., Dutta, S., Lanar, D. E., Remarque, E., Ross, A., Williams, T. N., Mwambingu, G., Lowe, B., Conway, D. J. & Marsh, K. 2004. 'Human antibodies to recombinant protein constructs of *Plasmodium falciparum* Apical Membrane Antigen 1 (AMA1) and their associations with protection from malaria.' *Vaccine,* 23:5, 718-28.

Portugal, J. & Waring, M. J. 1988. 'Assignment of DNA binding sites for 4',6-diamidine-2-phenylindole and bisbenzimide (Hoechst 33258). A comparative footprinting study.' *Biochim Biophys Acta,* 949:2, 158-68.

Price, R. N., Dorsey, G., Ashley, E. A., Barnes, K. I., Baird, J. K., d'Alessandro, U., Guerin, P. J., Laufer, M. K., Naidoo, I., Nosten, F., Olliaro, P., Plowe, C. V., Ringwald, P., Sibley, C. H., Stepniewska, K. & White, N. J. 2007. 'World Antimalarial Resistance Network I: clinical efficacy of antimalarial drugs.' *Malar J,* 6, 119.

Price, R. N., Uhlemann, A. C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N. J., Nosten, F. & Krishna, S. 2004. 'Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number.' *Lancet,* 364:9432, 438-47.

Price, R. N., von Seidlein, L., Valecha, N., Nosten, F., Baird, J. K. & White, N. J. 2014. 'Global extent of chloroquine-resistant *Plasmodium vivax*: a systematic review and meta-analysis.' *Lancet Infect Dis,* 14:10, 982-91.

Prudencio, M., Rodriguez, A. & Mota, M. M. 2006. 'The silent path to thousands of merozoites: the *Plasmodium* liver stage.' *Nat Rev Microbiol,* 4:11, 849-56.

Pruslin, F. H., Rodman, T. C., Jones, J. & Winston, R. 1986. 'An ELISA for IgM titer of human serum.' *J Immunol Methods,* 94:1-2, 99-103.

Pukrittayakamee, S., Supanaranond, W., Looareesuwan, S., Vanijanonta, S. & White, N. J. 1994. 'Quinine in severe falciparum malaria: evidence of declining efficacy in Thailand.' *Trans R Soc Trop Med Hyg,* 88:3, 324-7.

Quakyi, I. A., Currier, J., Fell, A., Taylor, D. W., Roberts, T., Houghten, R. A., England, R. D., Berzofsky, J. A., Miller, L. H. & Good, M. F. 1994. 'Analysis of human T cell clones specific for conserved peptide sequences within malaria proteins. Paucity of clones responsive to intact parasites.' *J Immunol,* 153:5, 2082-92.

Radfar, A., Mendez, D., Moneriz, C., Linares, M., Marin-Garcia, P., Puyet, A., Diez, A. & Bautista, J. M. 2009. 'Synchronous culture of *Plasmodium falciparum* at high parasitemia levels.' *Nat Protoc,* 4:12, 1899-915.

Rajasekariah, G. R., Parab, P. B. & Subrahmanyam, D. 1991. 'Detection of Wuchereria bancrofti specific antigens in the serum of endemic residents.' *Trop Med Parasitol,* 42:2, 103-5.

Ramasamy, R. 1998. 'Molecular basis for evasion of host immunity and pathogenesis in malaria.' *Biochim Biophys Acta,* 1406:1, 10-27.

Ramiro, R. S., Pollitt, L. C., Mideo, N. & Reece, S. E. 2016. 'Facilitation through altered resource availability in a mixed-species rodent malaria infection.' *Ecol Lett.*

Rayner, J. C. 2015. '*Plasmodium malariae* Malaria: From Monkey to Man?' *EBioMedicine,* 2:9, 1023-4.

Reid, M. E. & Bird, G. W. 1990. 'Associations between human red cell blood group antigens and disease.' *Transfus Med Rev,* 4:1, 47-55.

Reid, M. E., Lomas-Francis, C., Olsson, M.L. 2012. *Blood group antigen FactsBook, 3rd ed.*: Academic Press, Waltham, MA.,

Reinbold-Wasson, D. D., Sardelis, M. R., Jones, J. W., Watts, D. M., Fernandez, R., Carbajal, F., Pecor, J. E., Calampa, C., Klein, T. A. & Turell, M. J. 2012. 'Determinants of Anopheles Seasonal Distribution Patterns Across a Forest to Periurban Gradient near Iquitos, Peru.' *American Journal of Tropical Medicine and Hygiene,* 86:3, 459-63.

Reiner, A., Yekutieli, D. & Benjamini, Y. 2003. 'Identifying differentially expressed genes using false discovery rate controlling procedures.' *Bioinformatics,* 19:3, 368-75.

Resende, S. S., Milagres, V. G., Chaves, D. G., Fontes, C. J., Carvalho, L. H., Sousa, T. N. & Brito, C. F. 2017. 'Increased susceptibility of blood type O individuals to develop anemia in *Plasmodium vivax* infection.' *Infect Genet Evol,* 50, 87-92.

Riley, E. M. 1996. 'The role of MHC- and non-MHC-associated genes in determining the human immune response to malaria antigens.' *Parasitology,* 112 Suppl, S39-51.

Riley, E. M., Allen, S. J., Wheeler, J. G., Blackman, M. J., Bennett, S., Takacs, B., Schonfeld, H. J., Holder, A. A. & Greenwood, B. M. 1992. 'Naturally acquired

cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity.' *Parasite Immunol,* 14:3, 321-37.

Riley, R. S. 1992. 'Cellular proliferation markers in the evaluation of human cancer.' *Clin Lab Med,* 12:2, 163-99.

Ringwald, P., Meche, F. S., Bickii, J. & Basco, L. K. 1999. 'In vitro culture and drug sensitivity assay of *Plasmodium falciparum* with nonserum substitute and acute-phase sera.' *J Clin Microbiol,* 37:3, 700-5.

Rock, E. P., Marsh, K., Saul, A. J., Wellems, T. E., Taylor, D. W., Maloy, W. L. & Howard, R. J. 1987. 'Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP-I, HRP-II and HRP-III in malaria parasites of diverse origin.' *Parasitology,* 95 (Pt 2), 209-27.

Rodriguez-Morales, A. J., Benitez, J. A. & Arria, M. 2008. 'Malaria mortality in Venezuela: focus on deaths due to *Plasmodium vivax* in children.' *J Trop Pediatr,* 54:2, 94-101.

Roland, J., Soulard, V., Sellier, C., Drapier, A. M., Di Santo, J. P., Cazenave, P. A. & Pied, S. 2006. 'NK cell responses to *Plasmodium* infection and control of intrahepatic parasite development.' *J Immunol,* 177:2, 1229-39.

Romi, R., Razaiarimanga, M. C., Raharimanga, R., Rakotondraibe, E. M., Ranaivo, L. H., Pietra, V., Raveloson, A. & Majori, G. 2002. 'Impact of the malaria control campaign (1993-1998) in the highlands of Madagascar: parasitological and entomological data.' *Am J Trop Med Hyg,* 66:1, 2-6.

Roper, C., Elhassan, I. M., Hviid, L., Giha, H., Richardson, W., Babiker, H., Satti, G. M., Theander, T. G. & Arnot, D. E. 1996. 'Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan.' *Am J Trop Med Hyg,* 54:4, 325-31.

Roper, C., Richardson, W., Elhassan, I. M., Giha, H., Hviid, L., Satti, G. M., Theander, T. G. & Arnot, D. E. 1998. 'Seasonal changes in the *Plasmodium falciparum* population in individuals and their relationship to clinical malaria: a longitudinal study in a Sudanese village.' *Parasitology,* 116 (Pt 6), 501-10.

Rosnar, B. 2000. *Fundamentals of Biostatistics.* Brooks/Cole, Boston,

Rothman, A. L., Medin, C. L., Friberg, H. & Currier, J. R. 2014. 'Immunopathogenesis Versus Protection in Dengue Virus Infections.' *Curr Trop Med Rep,* 1:1, 13-20.

Roucher, C., Rogier, C., Dieye-Ba, F., Sokhna, C., Tall, A. & Trape, J. F. 2012. 'Changing malaria epidemiology and diagnostic criteria for *Plasmodium falciparum* clinical malaria.' *PLoS One,* 7:9, e46188.

Rovira-Graells, N., Gupta, A. P., Planet, E., Crowley, V. M., Mok, S., Ribas de Pouplana, L., Preiser, P. R., Bozdech, Z. & Cortes, A. 2012. 'Transcriptional variation in the malaria parasite *Plasmodium falciparum*.' *Genome Res,* 22:5, 925-38.

Rowe, A., Obeiro, J., Newbold, C. I. & Marsh, K. 1995. '*Plasmodium falciparum* rosetting is associated with malaria severity in Kenya.' *Infect Immun,* 63:6, 2323-6.

Rowe, J. A., Claessens, A., Corrigan, R. A. & Arman, M. 2009a. 'Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications.' *Expert Rev Mol Med,* 11, e16.

Rowe, J. A., Handel, I. G., Thera, M. A., Deans, A. M., Lyke, K. E., Kone, A., Diallo, D. A., Raza, A., Kai, O., Marsh, K., Plowe, C. V., Doumbo, O. K. & Moulds, J. M. 2007. 'Blood group O protects against severe *Plasmodium falciparum* malaria through the mechanism of reduced rosetting.' *Proc Natl Acad Sci U S A,* 104:44, 17471-6.

Rowe, J. A., Opi, D. H. & Williams, T. N. 2009b. 'Blood groups and malaria: fresh insights into pathogenesis and identification of targets for intervention.' *Curr Opin Hematol,* 16:6, 480-7.

Rummel, S. K. & Ellsworth, R. E. 2016. 'The role of the histoblood ABO group in cancer.' *Future Sci OA,* 2:2, FSO107.

Ryan, J. R., Stoute, J. A., Amon, J., Dunton, R. F., Mtalib, R., Koros, J., Owour, B., Luckhart, S., Wirtz, R. A., Barnwell, J. W. & Rosenberg, R. 2006. 'Evidence for transmission of *Plasmodium vivax* among a duffy antigen negative population in Western Kenya.' *Am J Trop Med Hyg,* 75:4, 575-81.

Salem, M. S. O. A., Ndiaye, M., OuldAbdallahi, M., Lekweiry, K. M., Bogreau, H., Konate, L., Faye, B., Gaye, O., Faye, O. & Boukhary, A. O. M. S. O. 2014. 'Polymorphism of the merozoite surface protein-1 block 2 region in *Plasmodium falciparum* isolates from Mauritania.' *Malar J,* 13.

Saliba, K. J., Folb, P. I. & Smith, P. J. 1998. 'Role for the *Plasmodium falciparum* digestive vacuole in chloroquine resistance.' *Biochem Pharmacol,* 56:3, 313-20.

Sato, S., Clough, B., Coates, L. & Wilson, R. J. 2004. 'Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite *Plasmodium falciparum*.' *Protist,* 155:1, 117-25.

Saul, A. 1999. 'The role of variant surface antigens on malaria-infected red blood cells.' *Parasitol Today,* 15:11, 455-7.

Savargaonkar, D., Shah, N., Das, M. K., Srivastava, B. & Valecha, N. 2014. '*Plasmodium malariae* infection: a case of missed diagnosis.' *J Vector Borne Dis,* 51:2, 149-51.

Schellenberg, D., Menendez, C., Kahigwa, E., Font, F., Galindo, C., Acosta, C., Schellenberg, J. A., Aponte, J. J., Kimario, J., Urassa, H., Mshinda, H., Tanner, M. & Alonso, P. 1999. 'African children with malaria in an area of intense *Plasmodium falciparum* transmission: features on admission to the hospital and risk factors for death.' *Am J Trop Med Hyg,* 61:3, 431-8.

Schofield, L. & Grau, G. E. 2005. 'Immunological processes in malaria pathogenesis.' *Nat Rev Immunol,* 5:9, 722-35.

Schurch, N. J., Schofield, P., Gierlinski, M., Cole, C., Sherstnev, A., Singh, V., Wrobel, N., Gharbi, K., Simpson, G. G., Owen-Hughes, T., Blaxter, M. & Barton, G. J. 2016. 'How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use?' *RNA,* 22:6, 839-51.

Scopel, K. K., Fontes, C. J., Ferreira, M. U. & Braga, E. M. 2005. '*Plasmodium falciparum*: IgG subclass antibody response to merozoite surface protein-1 among

Amazonian gold miners, in relation to infection status and disease expression.' *Exp Parasitol,* 109:2, 124-34.

Scopel, K. K., Fontes, C. J., Ferreira, M. U. & Braga, E. M. 2006. 'Factors associated with immunoglobulin G subclass polarization in naturally acquired antibodies to *Plasmodium falciparum* merozoite surface proteins: a cross-sectional survey in Brazilian Amazonia.' *Clin Vaccine Immunol,* 13:7, 810-3.

Scopel, K. K., Fontes, C. J., Nunes, A. C., Horta, M. F. & Braga, E. M. 2004. 'Low sensitivity of nested PCR using *Plasmodium* DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region.' *Malar J,* 3, 8.

Seed, C. R., Cheng, A., Davis, T. M., Bolton, W. V., Keller, A. J., Kitchen, A. & Cobain, T. J. 2005a. 'The efficacy of a malarial antibody enzyme immunoassay for establishing the reinstatement status of blood donors potentially exposed to malaria.' *Vox Sang,* 88:2, 98-106.

Seed, C. R., Kitchen, A. & Davis, T. M. 2005b. 'The current status and potential role of laboratory testing to prevent transfusion-transmitted malaria.' *Transfus Med Rev,* 19:3, 229-40.

Seifu, S., Zeynudin, A., Zemene, E., Suleman, S. & Biruksew, A. 2017. 'Therapeutic efficacy of chloroquine for the treatment of *Plasmodium vivax* malaria among outpatients at Shawa Robit Health Care Centre, North-East Ethiopia.' *Acta Trop,* 171, 44-51.

Senn, H., Alattas, N., Boggild, A. K. & Morris, S. K. 2014. 'Mixed-species *Plasmodium falciparum* and *Plasmodium ovale* malaria in a paediatric returned traveller.' *Malar J,* 13, 78.

Seyoum, D., Kifle, Y. G., Rondeau, V., Yewhalaw, D., Duchateau, L., Rosas-Aguirre, A. & Speybroeck, N. 2016. 'Identification of different malaria patterns due to *Plasmodium falciparum* and *Plasmodium vivax* in Ethiopian children: a prospective cohort study.' *Malar J,* 15, 208.

Seyoum, D., Yewhalaw, D., Duchateau, L., Brandt, P., Rosas-Aguirre, A. & Speybroeck, N. 2017. 'Household level spatio-temporal analysis of *Plasmodium falciparum* and *Plasmodium vivax* malaria in Ethiopia.' *Parasit Vectors,* 10:1, 196.

She, R. C., Rawlins, M. L., Mohl, R., Perkins, S. L., Hill, H. R. & Litwin, C. M. 2007. 'Comparison of immunofluorescence antibody testing and two enzyme immunoassays in the serologic diagnosis of malaria.' *J Travel Med,* 14:2, 105-11.

Shi, Y. P., Sayed, U., Qari, S. H., Roberts, J. M., Udhayakumar, V., Oloo, A. J., Hawley, W. A., Kaslow, D. C., Nahlen, B. L. & Lal, A. A. 1996. 'Natural immune response to the C-terminal 19-kilodalton domain of *Plasmodium falciparum* merozoite surface protein 1.' *Infect Immun,* 64:7, 2716-23.

Shiff, C. J., Minjas, J. & Premji, Z. 1994. 'The ParaSight-F test: a simple rapid manual dipstick test to detect *Plasmodium falciparum* infection.' *Parasitol Today,* 10:12, 494-5.

Shiff, C. J., Premji, Z. & Minjas, J. N. 1993. 'The rapid manual ParaSight-F test. A new diagnostic tool for *Plasmodium falciparum* infection.' *Trans R Soc Trop Med Hyg,* 87:6, 646-8.

Shock, J. L., Fischer, K. F. & DeRisi, J. L. 2007. 'Whole-genome analysis of mRNA decay in *Plasmodium falciparum* reveals a global lengthening of mRNA half-life during the intra-erythrocytic development cycle.' *Genome Biol,* 8:7, R134.

Shute, G. T. 1988. 'Malaria ' In Wernsdorfer WH & McGregor I (Eds.) *Principles and Practice of Malariology*: Churchill Livingstone, 781-814.

Sibley, C. H., Hyde, J. E., Sims, P. F., Plowe, C. V., Kublin, J. G., Mberu, E. K., Cowman, A. F., Winstanley, P. A., Watkins, W. M. & Nzila, A. M. 2001. 'Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next?' *Trends Parasitol,* 17:12, 582-8.

Siddiqui, W. A., Tam, L. Q., Kramer, K. J., Hui, G. S., Case, S. E., Yamaga, K. M., Chang, S. P., Chan, E. B. & Kan, S. C. 1987. 'Merozoite surface coat precursor protein completely protects Aotus monkeys against *Plasmodium falciparum* malaria.' *Proc Natl Acad Sci U S A,* 84:9, 3014-8.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D. & Higgins, D. G. 2011. 'Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega.' *Mol Syst Biol,* 7, 539.

Simpson, J. A., Aarons, L., Collins, W. E., Jeffery, G. M. & White, N. J. 2002. 'Population dynamics of untreated *Plasmodium falciparum* malaria within the adult human host during the expansion phase of the infection.' *Parasitology,* 124:Pt 3, 247-63.

Singh, N. & Sharma, R. K. 2014. 'Improving diagnosis and treatment of uncomplicated malaria.' *Lancet Glob Health,* 2:6, e304-5.

Singh, S., Plassmeyer, M., Gaur, D. & Miller, L. H. 2007. 'Mononeme: a new secretory organelle in *Plasmodium falciparum* merozoites identified by localization of rhomboid-1 protease.' *Proc Natl Acad Sci U S A,* 104:50, 20043-8.

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., Kabaria, C. W., Okara, R. M., Van Boeckel, T., Godfray, H. C., Harbach, R. E. & Hay, S. I. 2010. 'The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis.' *Parasit Vectors,* 3, 117.

Skinner, T. S., Manning, L. S., Johnston, W. A. & Davis, T. M. 1996. 'In vitro stage-specific sensitivity of *Plasmodium falciparum* to quinine and artemisinin drugs.' *Int J Parasitol,* 26:5, 519-25.

Smith, A., Denholm, J., Shortt, J. & Spelman, D. 2011. '*Plasmodium* species co-infection as a cause of treatment failure.' *Travel Med Infect Dis,* 9:6, 306-9.

Smith, D. B. & Johnson, K. S. 1988. 'Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase.' *Gene,* 67:1, 31-40.

Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I. & Miller, L. H. 1995a. 'Switches in expression of *Plasmodium falciparum* var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes.' *Cell,* 82:1, 101-10.

Smith, J. D., Rowe, J. A., Higgins, M. K. & Lavstsen, T. 2013. 'Malaria's deadly grip: cytoadhesion of *Plasmodium falciparum*-infected erythrocytes.' *Cell Microbiol,* 15:12, 1976-83.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. 1985. 'Measurement of protein using bicinchoninic acid.' *Anal Biochem,* 150:1, 76-85.

Smith, T., Charlwood, J. D., Takken, W., Tanner, M. & Spiegelhalter, D. J. 1995b. 'Mapping the Densities of Malaria Vectors within a Single Village.' *Acta Trop,* 59:1, 1-18.

Smith, T., Felger, I., Tanner, M. & Beck, H. P. 1999. 'Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections.' *Trans R Soc Trop Med Hyg,* 93 Suppl 1, 59-64.

Smith, T., Genton, B., Baea, K., Gibson, N., Narara, A. & Alpers, M. P. 2001. 'Prospective risk of morbidity in relation to malaria infection in an area of high endemicity of multiple species of *Plasmodium*.' *Am J Trop Med Hyg,* 64:5-6, 262-7.

Smythe, J. A., Coppel, R. L., Brown, G. V., Ramasamy, R., Kemp, D. J. & Anders, R. F. 1988. 'Identification of two integral membrane proteins of *Plasmodium falciparum*.' *Proc Natl Acad Sci U S A,* 85:14, 5195-9.

Smythe, J. A., Coppel, R. L., Day, K. P., Martin, R. K., Oduola, A. M., Kemp, D. J. & Anders, R. F. 1991. 'Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2.' *Proc Natl Acad Sci U S A,* 88:5, 1751-5.

Snounou, G., Jarra, W. & Preiser, P. R. 2000. 'Malaria multigene families: the price of chronicity.' *Parasitol Today,* 16:1, 28-30.

Snounou, G., Viriyakosol, S., Zhu, X. P., Jarra, W., Pinheiro, L., do Rosario, V. E., Thaithong, S. & Brown, K. N. 1993. 'High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction.' *Mol Biochem Parasitol,* 61:2, 315-20.

Snounou, G. & White, N. J. 2004. 'The co-existence of *Plasmodium*: sidelights from falciparum and vivax malaria in Thailand.' *Trends Parasitol,* 20:7, 333-9.

Snow, R. W., Nahlen, B., Palmer, A., Donnelly, C. A., Gupta, S. & Marsh, K. 1998. 'Risk of severe malaria among African infants: direct evidence of clinical protection during early infancy.' *J Infect Dis,* 177:3, 819-22.

Soares, I. S., Levitus, G., Souza, J. M., Del Portillo, H. A. & Rodrigues, M. M. 1997. 'Acquired immune responses to the N- and C-terminal regions of *Plasmodium vivax* merozoite surface protein 1 in individuals exposed to malaria.' *Infect Immun,* 65:5, 1606-14.

Sokal, R. R. & Rohlf, J. 1995. *Biometry: the principles and practice of statistics in biological research.* Freeman and Company, New York,

Sorensen, H. P. & Mortensen, K. K. 2005. 'Advanced genetic strategies for recombinant protein expression in Escherichia coli.' *J Biotechnol,* 115:2, 113-28.

Spencer, T. E. T. 1963. *The classification of malaria endemicity*. G. W. H. Organization,

Sprent, P. & Smeeton, N. C. 2001. *Applied Nonparametric Statistical Methods.* Chapman & Hall/CRC, London,

Spycher, C., Klonis, N., Spielmann, T., Kump, E., Steiger, S., Tilley, L. & Beck, H. P. 2003. 'MAHRP-1, a novel *Plasmodium falciparum* histidine-rich protein, binds ferriprotoporphyrin IX and localizes to the Maurer's clefts.' *J Biol Chem,* 278:37, 35373-83.

Spycher, C., Rug, M., Klonis, N., Ferguson, D. J., Cowman, A. F., Beck, H. P. & Tilley, L. 2006. 'Genesis of and trafficking to the Maurer's clefts of *Plasmodium falciparum*-infected erythrocytes.' *Mol Cell Biol,* 26:11, 4074-85.

Spycher, C., Rug, M., Pachlatko, E., Hanssen, E., Ferguson, D., Cowman, A. F., Tilley, L. & Beck, H. P. 2008. 'The Maurer's cleft protein MAHRP1 is essential for trafficking of PfEMP1 to the surface of *Plasmodium falciparum*-infected erythrocytes.' *Mol Microbiol,* 68:5, 1300-14.

Structural Genomics, C., China Structural Genomics, C., Northeast Structural Genomics, C., Graslund, S., Nordlund, P., Weigelt, J., Hallberg, B. M., Bray, J., Gileadi, O., Knapp, S., Oppermann, U., Arrowsmith, C., Hui, R., Ming, J., dhe-Paganon, S., Park, H. W., Savchenko, A., Yee, A., Edwards, A., Vincentelli, R., Cambillau, C., Kim, R., Kim, S. H., Rao, Z., Shi, Y., Terwilliger, T. C., Kim, C. Y., Hung, L. W., Waldo, G. S., Peleg, Y., Albeck, S., Unger, T., Dym, O., Prilusky, J., Sussman, J. L., Stevens, R. C., Lesley, S. A., Wilson, I. A., Joachimiak, A., Collart, F., Dementieva, I., Donnelly, M. I., Eschenfeldt, W. H., Kim, Y., Stols, L., Wu, R., Zhou, M., Burley, S. K., Emtage, J. S., Sauder, J. M., Thompson, D., Bain, K., Luz, J., Gheyi, T., Zhang, F., Atwell, S., Almo, S. C., Bonanno, J. B., Fiser, A., Swaminathan, S., Studier, F. W., Chance, M. R., Sali, A., Acton, T. B., Xiao, R., Zhao, L., Ma, L. C., Hunt, J. F., Tong, L., Cunningham, K., Inouye, M., Anderson, S., Janjua, H., Shastry, R., Ho, C. K., Wang, D., Wang, H., Jiang, M., Montelione, G. T., Stuart, D. I., Owens, R. J., Daenke, S., Schutz, A., Heinemann, U., Yokoyama, S., Bussow, K. & Gunsalus, K. C. 2008. 'Protein production and purification.' *Nat Methods,* 5:2, 135-46.

Suhrbier, A., Holder, A. A., Wiser, M. F., Nicholas, J. & Sinden, R. E. 1989. 'Expression of the precursor of the major merozoite surface antigens during the hepatic stage of malaria.' *Am J Trop Med Hyg,* 40:4, 351-5.

Sutherland, C. J. 2016. 'Persistent Parasitism: The Adaptive Biology of Malariae and Ovale Malaria.' *Trends Parasitol,* 32:10, 808-19.

Tami, A., Grundmann, H., Sutherland, C., McBride, J. S., Cavanagh, D. R., Campos, E., Snounou, G., Barnabe, C., Tibayrenc, M. & Warhurst, D. C. 2002. 'Restricted genetic and antigenic diversity of *Plasmodium falciparum* under mesoendemic transmission in the Venezuelan Amazon.' *Parasitology,* 124:Pt 6, 569-81.

Tanabe, K., Mackay, M., Goman, M. & Scaife, J. G. 1987. 'Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*.' *J Mol Biol,* 195:2, 273-87.

Tanabe, K., Murakami, K. & Doi, S. 1989. '*Plasmodium falciparum*: dimorphism of the p190 alleles.' *Exp Parasitol,* 68:4, 470-3.

Tangpukdee, N., Duangdee, C., Wilairatana, P. & Krudsood, S. 2009. 'Malaria diagnosis: a brief review.' *Korean J Parasitol,* 47:2, 93-102.

Tapas, S., Kumar, A., Dhindwal, S., Preeti & Kumar, P. 2011. 'Structural analysis of chorismate synthase from *Plasmodium falciparum*: a novel target for antimalaria drug discovery.' *Int J Biol Macromol,* 49:4, 767-77.

Taylor, P. & Mutambu, S. L. 1986. 'A review of the malaria situation in Zimbabwe with special reference to the period 1972-1981.' *Trans R Soc Trop Med Hyg,* 80:1, 12-9.

Taylor, R. R., Allen, S. J., Greenwood, B. M. & Riley, E. M. 1998. 'IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria.' *Am J Trop Med Hyg,* 58:4, 406-13.

Taylor, R. R., Egan, A., McGuinness, D., Jepson, A., Adair, R., Drakely, C. & Riley, E. 1996. 'Selective recognition of malaria antigens by human serum antibodies is not genetically determined but demonstrates some features of clonal imprinting.' *Int Immunol,* 8:6, 905-15.

Taylor, R. R., Smith, D. B., Robinson, V. J., McBride, J. S. & Riley, E. M. 1995. 'Human antibody response to *Plasmodium falciparum* merozoite surface protein 2 is serogroup specific and predominantly of the immunoglobulin G3 subclass.' *Infect Immun,* 63:11, 4382-8.

ter Kuile, F., White, N. J., Holloway, P., Pasvol, G. & Krishna, S. 1993. '*Plasmodium falciparum*: in vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria.' *Exp Parasitol,* 76:1, 85-95.

Tetteh, K. K., Cavanagh, D. R., Corran, P., Musonda, R., McBride, J. S. & Conway, D. J. 2005. 'Extensive antigenic polymorphism within the repeat sequence of the *Plasmodium falciparum* merozoite surface protein 1 block 2 is incorporated in a minimal polyvalent immunogen.' *Infect Immun,* 73:9, 5928-35.

Theander, T. G. 1998. 'Unstable malaria in Sudan: the influence of the dry season. Malaria in areas of unstable and seasonal transmission. Lessons from Daraweesh.' *Trans R Soc Trop Med Hyg,* 92:6, 589-92.

Thera, M. A., Doumbo, O. K., Coulibaly, D., Laurens, M. B., Ouattara, A., Kone, A. K., Guindo, A. B., Traore, K., Traore, I., Kouriba, B., Diallo, D. A., Diarra, I., Daou, M., Dolo, A., Tolo, Y., Sissoko, M. S., Niangaly, A., Sissoko, M., Takala-Harrison, S., Lyke, K. E., Wu, Y., Blackwelder, W. C., Godeaux, O., Vekemans, J., Dubois, M. C., Ballou, W. R., Cohen, J., Thompson, D., Dube, T., Soisson, L., Diggs, C. L., House, B., Lanar, D. E., Dutta, S., Heppner, D. G., Jr. & Plowe, C. V. 2011. 'A field trial to assess a blood-stage malaria vaccine.' *N Engl J Med,* 365:11, 1004-13.

Thompson, J., Millington, O. R., Garside, P. & Brewer, J. M. 2008. 'What can transgenic parasites tell us about the development of *Plasmodium*-specific immune responses?' *Parasite Immunol,* 30:4, 223-33.

Tjitra, E., Anstey, N. M., Sugiarto, P., Warikar, N., Kenangalem, E., Karyana, M., Lampah, D. A. & Price, R. N. 2008. 'Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia.' *PLoS Med,* 5:6, e128.

Tolle, R., Fruh, K., Doumbo, O., Koita, O., N'Diaye, M., Fischer, A., Dietz, K. & Bujard, H. 1993. 'A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections.' *Infect Immun,* 61:1, 40-7.

Tomiyama, C., Watanabe, M., Honma, T., Inada, A., Hayakawa, T., Ryufuku, M. & Abo, T. 2015. 'The effect of repetitive mild hyperthermia on body temperature, the autonomic nervous system, and innate and adaptive immunity.' *Biomed Res,* 36:2, 135-42.

Tongren, J. E., Drakeley, C. J., McDonald, S. L., Reyburn, H. G., Manjurano, A., Nkya, W. M., Lemnge, M. M., Gowda, C. D., Todd, J. E., Corran, P. H. & Riley, E. M. 2006. 'Target antigen, age, and duration of antigen exposure independently regulate immunoglobulin G subclass switching in malaria.' *Infect Immun,* 74:1, 257-64.

Tonhosolo, R., Wunderlich, G. & Ferreira, M. U. 2001. 'Differential antibody recognition of four allelic variants of the merozoite surface protein-2 (MSP-2) of *Plasmodium falciparum*.' *J Eukaryot Microbiol,* 48:5, 556-64.

Tournier, J. N., Hellmann, A. Q., Lesca, G., Jouan, A., Drouet, E. & Mathieu, J. 2003. 'Fever-like thermal conditions regulate the activation of maturing dendritic cells.' *J Leukoc Biol,* 73:4, 493-501.

Tran, T. M., Li, S., Doumbo, S., Doumtabe, D., Huang, C. Y., Dia, S., Bathily, A., Sangala, J., Kone, Y., Traore, A., Niangaly, M., Dara, C., Kayentao, K., Ongoiba, A., Doumbo, O. K., Traore, B. & Crompton, P. D. 2013. 'An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to *Plasmodium falciparum* infection.' *Clin Infect Dis,* 57:1, 40-7.

Trape, J. F., Pison, G., Preziosi, M. P., Enel, C., Desgrees du Lou, A., Delaunay, V., Samb, B., Lagarde, E., Molez, J. F. & Simondon, F. 1998. 'Impact of chloroquine resistance on malaria mortality.' *C R Acad Sci III,* 321:8, 689-97.

Trucco, C., Fernandez-Reyes, D., Howell, S., Stafford, W. H., Scott-Finnigan, T. J., Grainger, M., Ogun, S. A., Taylor, W. R. & Holder, A. A. 2001. 'The merozoite surface protein 6 gene codes for a 36 kDa protein associated with the *Plasmodium falciparum* merozoite surface protein-1 complex.' *Mol Biochem Parasitol,* 112:1, 91-101.

Udomsanpetch, R., Thanikkul, K., Pukrittayakamee, S. & White, N. J. 1995. 'Rosette formation by *Plasmodium vivax*.' *Trans R Soc Trop Med Hyg,* 89:6, 635-7.

van der Hoek, W., Konradsen, F., Dijkstra, D. S., Amerasinghe, P. H. & Amerasinghe, F. P. 1998. 'Risk factors for malaria: a microepidemiological study in a village in Sri Lanka.' *Trans R Soc Trop Med Hyg,* 92:3, 265-9.

van Dooren, G. G., Su, V., D'Ombrain, M. C. & McFadden, G. I. 2002. 'Processing of an apicoplast leader sequence in *Plasmodium falciparum* and the identification of a putative leader cleavage enzyme.' *J Biol Chem,* 277:26, 23612-9.

Veening, J. W., Stewart, E. J., Berngruber, T. W., Taddei, F., Kuipers, O. P. & Hamoen, L. W. 2008. 'Bet-hedging and epigenetic inheritance in bacterial cell development.' *Proc Natl Acad Sci U S A,* 105:11, 4393-8.

Venkatesan, P. & Wakelin, D. 1993. 'ELISAs for parasitologists: or lies, damned lies and ELISAs.' *Parasitol Today,* 9:6, 228-32.

Verdrager, J. 1986. 'Epidemiology of the emergence and spread of drug-resistant falciparum malaria in South-East Asia and Australasia.' *J Trop Med Hyg,* 89:6, 277-89.

Verdrager, J. 1995. 'Localized permanent epidemics: the genesis of chloroquine resistance in *Plasmodium falciparum*.' *Southeast Asian J Trop Med Public Health,* 26:1, 23-8.

Vigan-Womas, I., Guillotte, M., Juillerat, A., Hessel, A., Raynal, B., England, P., Cohen, J. H., Bertrand, O., Peyrard, T., Bentley, G. A., Lewit-Bentley, A. & Mercereau-Puijalon, O. 2012. 'Structural basis for the ABO blood-group dependence of *Plasmodium falciparum* rosetting.' *PLoS Pathog,* 8:7, e1002781.

Vignali, M., Armour, C. D., Chen, J., Morrison, R., Castle, J. C., Biery, M. C., Bouzek, H., Moon, W., Babak, T., Fried, M., Raymond, C. K. & Duffy, P. E. 2011. 'NSR-seq transcriptional profiling enables identification of a gene signature of *Plasmodium falciparum* parasites infecting children.' *J Clin Invest,* 121:3, 1119-29.

Vincenti-Gonzalez, M. F., Grillet, M. E., Velasco-Salas, Z. I., Lizarazo, E. F., Amarista, M. A., Sierra, G. M., Comach, G. & Tami, A. 2017. 'Spatial Analysis of Dengue Seroprevalence and Modeling of Transmission Risk Factors in a Dengue Hyperendemic City of Venezuela.' *PLoS Negl Trop Dis,* 11:1, e0005317.

Vinetz, J. M., Li, J., McCutchan, T. F. & Kaslow, D. C. 1998. '*Plasmodium malariae* infection in an asymptomatic 74-year-old Greek woman with splenomegaly.' *N Engl J Med,* 338:6, 367-71.

Walter, E. J., Hanna-Jumma, S., Carraretto, M. & Forni, L. 2016. 'The pathophysiological basis and consequences of fever.' *Crit Care,* 20:1, 200.

Wang, Q., Zhao, Z., Zhang, X., Li, X., Zhu, M., Li, P., Yang, Z., Wang, Y., Yan, G., Shang, H., Cao, Y., Fan, Q. & Cui, L. 2016. 'Naturally Acquired Antibody Responses to *Plasmodium vivax* and *Plasmodium falciparum* Merozoite Surface Protein 1 (MSP1) C-Terminal 19 kDa Domains in an Area of Unstable Malaria Transmission in Southeast Asia.' *PLoS One,* 11:3, e0151900.

Wang, R., Charoenvit, Y., Corradin, G., De La Vega, P., Franke, E. D. & Hoffman, S. L. 1996. 'Protection against malaria by *Plasmodium* yoelii sporozoite surface protein 2 linear peptide induction of CD4+ T cell- and IFN-gamma-dependent elimination of infected hepatocytes.' *J Immunol,* 157:9, 4061-7.

Wang, Z., Gerstein, M. & Snyder, M. 2009. 'RNA-Seq: a revolutionary tool for transcriptomics.' *Nat Rev Genet,* 10:1, 57-63.

Weatherall, D. J., Miller, L. H., Baruch, D. I., Marsh, K., Doumbo, O. K., Casals-Pascual, C. & Roberts, D. J. 2002. 'Malaria and the red cell.' *Hematology Am Soc Hematol Educ Program,* 35-57.

Weiss, W. R., Mellouk, S., Houghten, R. A., Sedegah, M., Kumar, S., Good, M. F., Berzofsky, J. A., Miller, L. H. & Hoffman, S. L. 1990. 'Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes.' *J Exp Med,* 171:3, 763-73.

Weiss, W. R., Sedegah, M., Berzofsky, J. A. & Hoffman, S. L. 1993. 'The role of CD4+ T cells in immunity to malaria sporozoites.' *J Immunol,* 151:5, 2690-8.

Wellems, T. E. 2002. '*Plasmodium* chloroquine resistance and the search for a replacement antimalarial drug.' *Science,* 298:5591, 124-6.

Wellems, T. E. 2004. 'Transporter of a malaria catastrophe.' *Nat Med,* 10:11, 1169-71.

Wellems, T. E., Walker-Jonah, A. & Panton, L. J. 1991. 'Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7.' *Proc Natl Acad Sci U S A*, 88:8, 3382-6.

Wernsdorfer, W. H., Landgraf, B., Wiedermann, G. & Kollaritsch, H. 1995. 'Chloroquine resistance of *Plasmodium falciparum*: a biological advantage?' *Trans R Soc Trop Med Hyg*, 89:1, 90-1.

Whitby, M. 1997. 'Drug resistant *Plasmodium vivax* malaria.' *J Antimicrob Chemother*, 40:6, 749-52.

Whitby, M., Wood, G., Veenendaal, J. R. & Rieckmann, K. 1989. 'Chloroquine-resistant *Plasmodium vivax*.' *Lancet*, 2:8676, 1395.

White, N. 1999a. 'Antimalarial drug resistance and combination chemotherapy.' *Philos Trans R Soc Lond B Biol Sci*, 354:1384, 739-49.

White, N. J. 1996. 'Malaria.' In Cook GC (Ed.) *Manson's Tropical Disease*: W. B. Saunders: London, 1087–164.

White, N. J. 1999b. 'Delaying antimalarial drug resistance with combination chemotherapy.' *Parassitologia*, 41:1-3, 301-8.

White, N. J. 2004. 'Antimalarial drug resistance.' *J Clin Invest*, 113:8, 1084-92.

White, N. J. 2008. 'Qinghaosu (artemisinin): the price of success.' *Science*, 320:5874, 330-4.

White, N. J. 2011. 'Determinants of relapse periodicity in *Plasmodium vivax* malaria.' *Malar J*, 10, 297.

White, N. J., Nosten, F., Looareesuwan, S., Watkins, W. M., Marsh, K., Snow, R. W., Kokwaro, G., Ouma, J., Hien, T. T., Molyneux, M. E., Taylor, T. E., Newbold, C. I., Ruebush, T. K., 2nd, Danis, M., Greenwood, B. M., Anderson, R. M. & Olliaro, P. 1999. 'Averting a malaria disaster.' *Lancet*, 353:9168, 1965-7.

White, N. J., Pukrittayakamee, S., Hien, T. T., Faiz, M. A., Mokuolu, O. A. & Dondorp, A. M. 2013a. 'Malaria.' *Lancet*.

White, N. J., Turner, G. D., Day, N. P. & Dondorp, A. M. 2013b. 'Lethal malaria: Marchiafava and Bignami were right.' *J Infect Dis*, 208:2, 192-8.

WHO 1990. 'Severe and complicated malaria.' *Trans R Soc Trop Med Hyg*, 84, 1-65.

WHO 2006. *WHO guidelines for the treatment of malaria*. G. WHO Press, Switzerland, 43–46.

WHO 2007. *Malaria elimination: a field manual for low and moderate endemic countries*. Geneva,

WHO 2011. *World Malaria Report 2011*. S. W. H. O. Geneva,

WHO 2013. *World Malaria Report 2013*. S. W. H. O. Geneva, 1-255.

WHO 2014. 'Severe malaria.' *Tropical Medicine and International Health*, 19(Suppl. I), 7-131.

WHO 2015. *World Malaria Report 2015*. S. W. H. O. Geneva, 1-188.

WHO, R. B. M., United States Agency for International Development 2000. *New perspectives : malaria diagnosis : report of a joint WHO/USAID informal consultation, 25-27 October 1999*. G. W. H. Organization,

Wilson, M. L. 2012. 'Malaria rapid diagnostic tests.' *Clin Infect Dis*, 54:11, 1637-41.

Wolofsky, K. T., Ayi, K., Branch, D. R., Hult, A. K., Olsson, M. L., Liles, W. C., Cserti-Gazdewich, C. M. & Kain, K. C. 2012. 'ABO blood groups influence macrophage-mediated phagocytosis of *Plasmodium falciparum*-infected erythrocytes.' *PLoS Pathog,* 8:10, e1002942.

Wongsrichanalai, C. & Meshnick, S. R. 2008. 'Declining artesunate-mefloquine efficacy against falciparum malaria on the Cambodia-Thailand border.' *Emerg Infect Dis,* 14:5, 716-9.

Woolhouse, M. E. J., Dye, C., Etard, J. F., Smith, T., Charlwood, J. D., Garnett, G. P., Hagan, P., Hii, J. L. K., Ndhlovu, P. D., Quinnell, R. J., Watts, C. H., Chandiwana, S. K. & Anderson, R. M. 1997. 'Heterogeneities in the transmission of infectious agents: Implications for the design of control programs.' *Proc Natl Acad Sci U S A,* 94:1, 338-42.

Wreghitt, T. G. & Nagington, J. 1983. 'Variability in EIA plates used in ELISA tests for the detection of antibodies to Legionella pneumophila.' *J Clin Pathol,* 36:2, 238-9.

Wright, G. J. & Rayner, J. C. 2014. '*Plasmodium falciparum* erythrocyte invasion: combining function with immune evasion.' *PLoS Pathog,* 10:3, e1003943.

Wu, Y., Kirkman, L. A. & Wellems, T. E. 1996. 'Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine.' *Proc Natl Acad Sci U S A,* 93:3, 1130-4.

Wurtz, N., Mint Lekweiry, K., Bogreau, H., Pradines, B., Rogier, C., Ould Mohamed Salem Boukhary, A., Hafid, J. E., Ould Ahmedou Salem, M. S., Trape, J. F., Basco, L. K. & Briolant, S. 2011. 'Vivax malaria in Mauritania includes infection of a Duffy-negative individual.' *Malar J,* 10, 336.

WWARN 2012. *Clinical Module: Data Management and Statistical Analysis Plan, Version 1.2*.

Yamamoto, F. & Hakomori, S. 1990. 'Sugar-nucleotide donor specificity of histo-blood group A and B transferases is based on amino acid substitutions.' *J Biol Chem,* 265:31, 19257-62.

Yaro, A. S., Traore, A. I., Huestis, D. L., Adamou, A., Timbine, S., Kassogue, Y., Diallo, M., Dao, A., Traore, S. F. & Lehmann, T. 2012. 'Dry season reproductive depression of Anopheles gambiae in the Sahel.' *Journal of Insect Physiology,* 58:8, 1050-59.

Yayon, A., Cabantchik, Z. I. & Ginsburg, H. 1984. 'Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine.' *EMBO J,* 3:11, 2695-700.

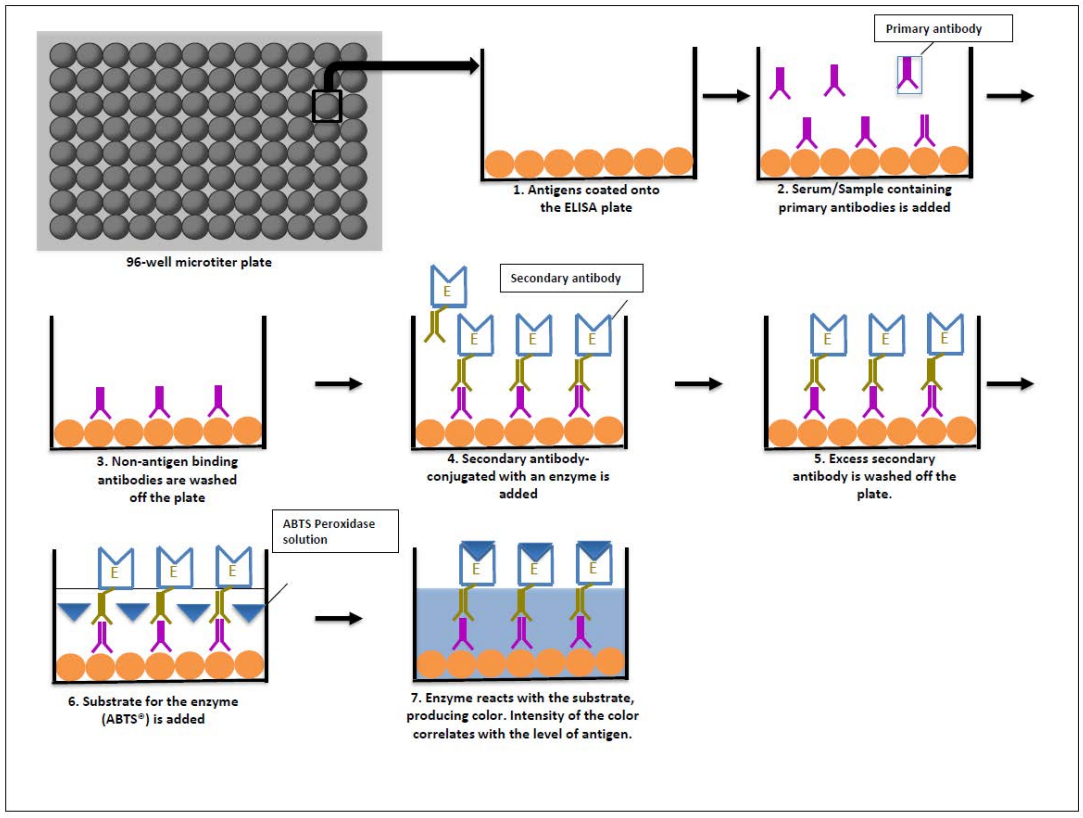
Yurdaydin, C., Idilman, R., Bozkaya, H. & Bozdayi, A. M. 2010. 'Natural history and treatment of chronic delta hepatitis.' *J Viral Hepat,* 17:11, 749-56.

Zhang, X., Perugini, M. A., Yao, S., Adda, C. G., Murphy, V. J., Low, A., Anders, R. F. & Norton, R. S. 2008. 'Solution conformation, backbone dynamics and lipid interactions of the intrinsically unstructured malaria surface protein MSP2.' *J Mol Biol,* 379:1, 105-21.

Zimmerman, P. A., Mehlotra, R. K., Kasehagen, L. J. & Kazura, J. W. 2004. 'Why do we need to know more about mixed *Plasmodium* species infections in humans?' *Trends Parasitol*, 20:9, 440-7.

Appendices

Appendix A: ELISA outline



Enzyme-linked immunosorbent assay (ELISA) technique for the detection of *Plasmodium* antibody in serum samples.

The MSP-1_{19} antigens are added to the wells, where it passively binds to the walls. Primary IgG antibody (anti-MSP-1_{19}) in the test serum binds specifically to the MSP-1_{19} antigen. An enzyme-linked secondary antibody (HRP) is added that reacts with a chromogen, producing a colour that is proportional to the amount of antibody present in the serum. Reproduced from (Gan and Patel 2013) with permission from the licensed content publisher.

Appendix B: Solutions Used In Enzyme-Linked Immunosorbent Assay

1. Phosphate buffer (PBS)

   1.9mNaH_2PO_4
   8.1mM Na_2HPO_4
   150mM NaCl
   pH to 7.2-7.4

2. Coating Buffer

   1.59g Na_2CO_3 [anhydrous] (15mM)
   2.93g $NaHCO_3$ [anhydrous] (35mM)
   Made up to 1 litre with distilled water. pH adjusted to 9.4-9.6
   Stored in 4^oC fridge.

3. Washing Buffer

   PBS plus 0.05% Tween 20

4. Blocking Buffer

   1% Marvel Skimmed Milk Powder in Washing Buffer

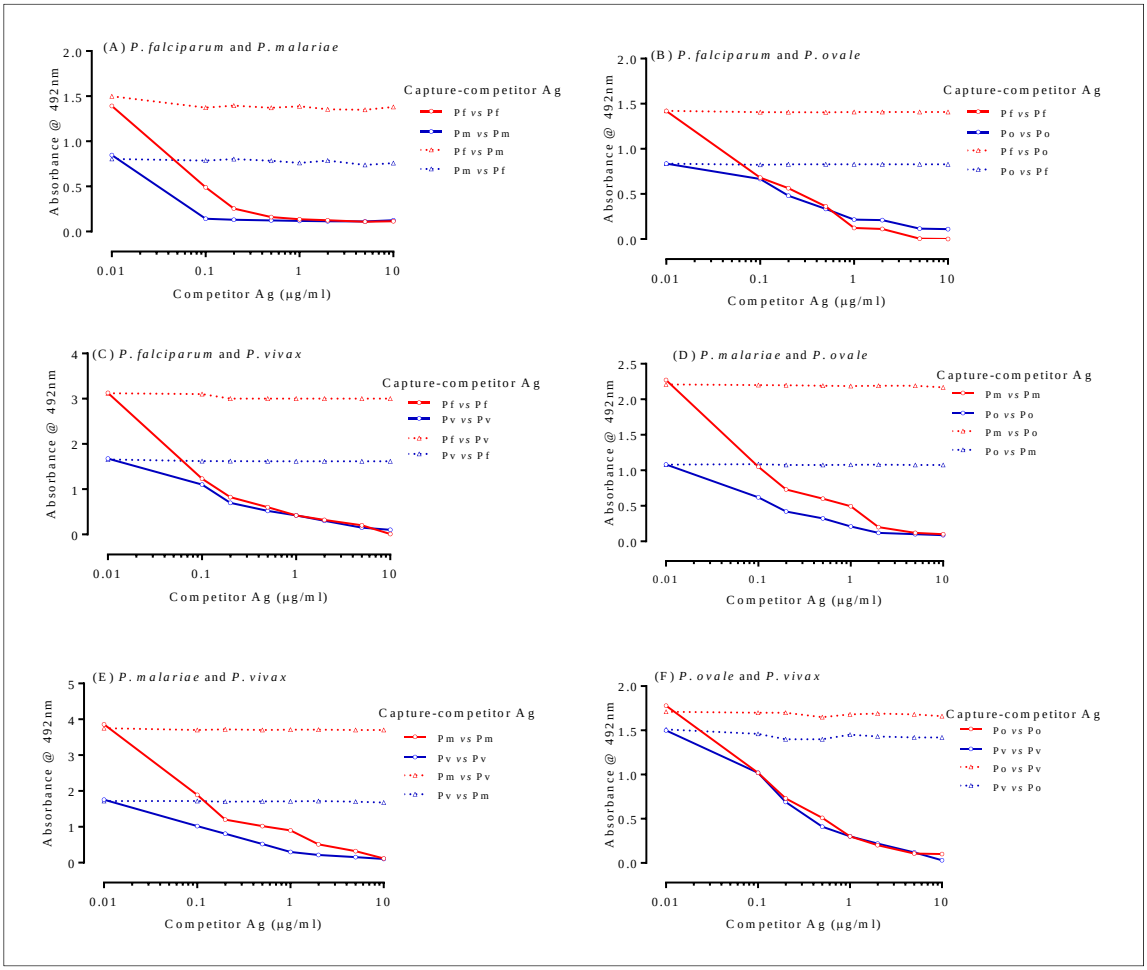
5. Substrate Buffer for 25 ml

   6ml 0.1M citric acid (stored at 4^oC)
   6.4ml 0.2M Na_2HPO_4
   12.5ml double distilled H_2O
   10 µL H_2O_2
   10 mg 0-Phenylenediamine Dihydrochloride (OPD) (Sigma)

6. Stopping Buffer

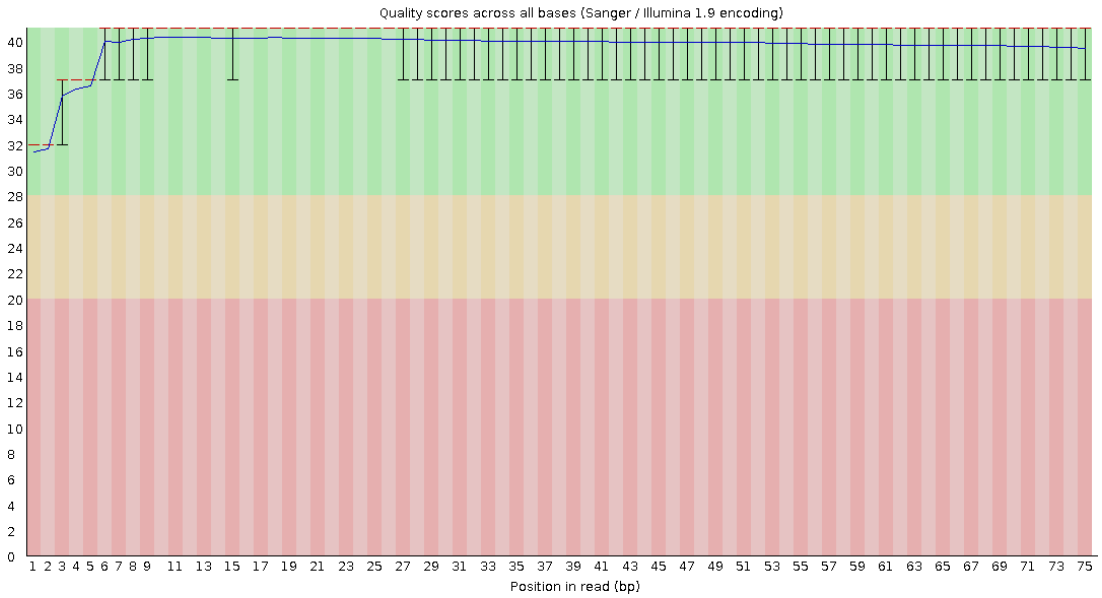
   2M H_2SO_4

Appendix C: Competition ELISA showing species specificity of IgG antibodies to recombinant *Plasmodium* MSP-1_{19} antigens



Plasma samples were tested at 1 in 500 dilution. Legends indicate the pairs of competing antigens used, with the well-bound capture antigen listed first and the competing homologous (control) or heterologous (test) antigen second. The capture antigens were coated at 50 ng/well. In each panel the red and blue solid lines with open circle symbols represent competition reactions involving homologous antigens, while the red and blue dashed lines with open triangle symbols represent reactions involving heterologous antigens. The y-axis indicates antibody absorbance measured at 492nm whiles the x-axis shows a titration with increasing concentrations of the competitor homologous or heterologous antigen added to the diluted serum sample. Panels A, B and C show reactions between *P. falciparum* (Pf), and *P. malariae* (Pm), *P. ovale* (Po) and *P. vivax* (Pv) respectively; Panels D and E involve *P. malariae* with *P. ovale* and *P. vivax*, while panel F shows *P. ovale* and *P. vivax*. Ag is antigen.

Appendix D: An example of FastQC quantification for one sample

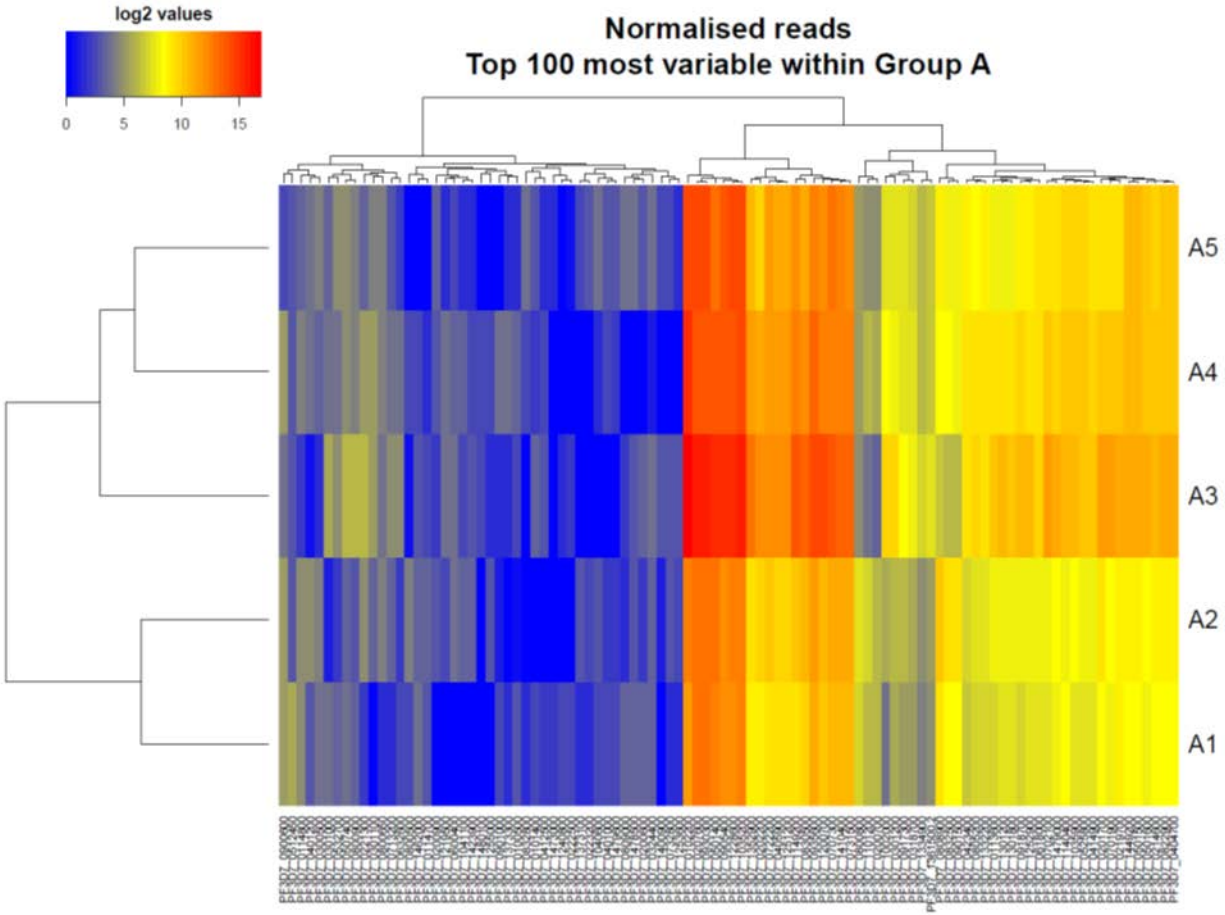


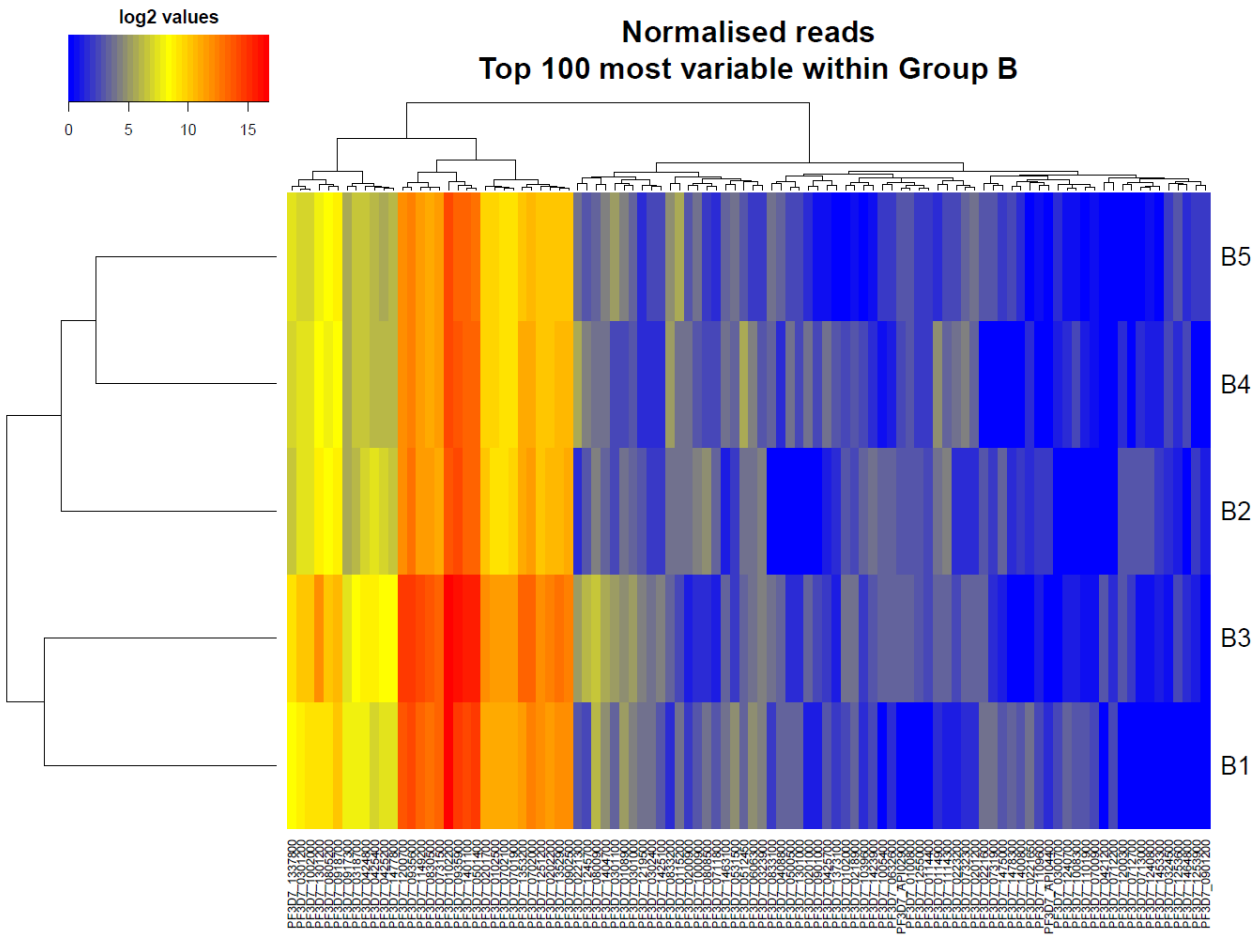
Appendix E: Sample- to Sample Pearson correlation of gene expression levels in different blood group donors.

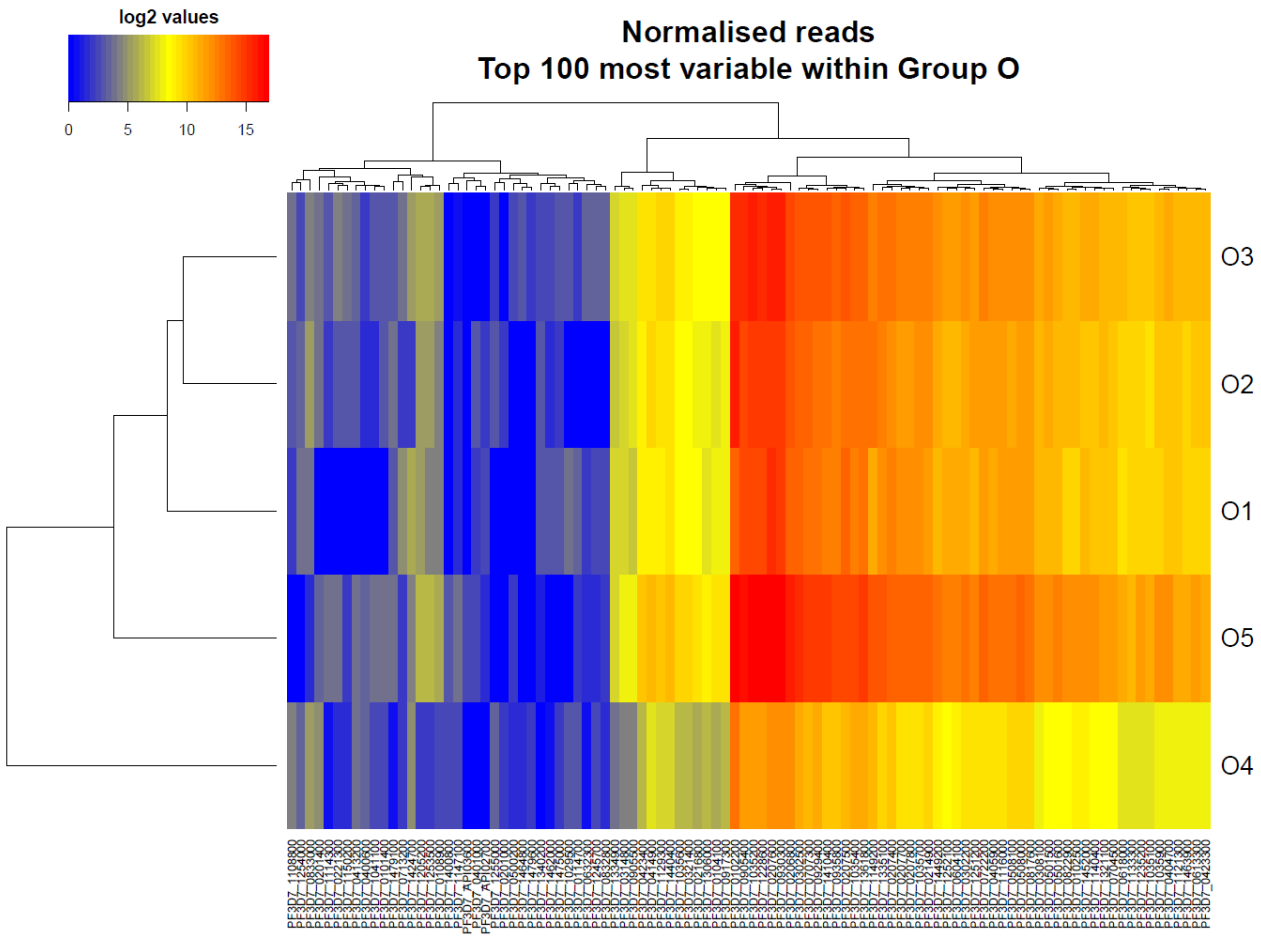
Donor ID	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	O1	O2	O3	O4	O5
A1	1	0.99262	0.96732	0.98354	0.98651	0.97916	0.99061	0.97476	0.98873	0.98965	0.98222	0.98154	0.97382	0.98992	0.94967
A2	0.99262	1	0.96463	0.9852	0.98454	0.97648	0.98946	0.97529	0.98971	0.99016	0.97732	0.98172	0.9728	0.99019	0.94622
A3	0.96732	0.96463	1	0.98259	0.98512	0.9883	0.97318	0.98841	0.97501	0.97396	0.98653	0.98739	0.98813	0.95958	0.98664
A4	0.98354	0.9852	0.98259	1	0.98896	0.98819	0.98776	0.98882	0.98842	0.98565	0.98426	0.98727	0.98349	0.98213	0.97111
A5	0.98651	0.98454	0.98512	0.98896	1	0.99246	0.98934	0.98682	0.99083	0.99149	0.99336	0.99379	0.9916	0.98288	0.97738
B1	0.97916	0.97648	0.9883	0.98819	0.99246	1	0.98642	0.99022	0.98689	0.98597	0.99168	0.99126	0.98942	0.9778	0.98259
B2	0.99061	0.98946	0.97318	0.98776	0.98934	0.98642	1	0.98332	0.99165	0.9912	0.98493	0.98608	0.97913	0.99191	0.96043
B3	0.97476	0.97529	0.98841	0.98882	0.98682	0.99022	0.98332	1	0.98624	0.98442	0.98424	0.98904	0.98735	0.97688	0.97892
B4	0.98873	0.98971	0.97501	0.98842	0.99083	0.98689	0.99165	0.98624	1	0.99411	0.98552	0.989	0.98468	0.99102	0.96608
B5	0.98965	0.99016	0.97396	0.98565	0.99149	0.98597	0.9912	0.98442	0.99411	1	0.98583	0.99034	0.98483	0.99143	0.96267
O1	0.98222	0.97732	0.98653	0.98426	0.99336	0.99168	0.98493	0.98424	0.98552	0.98583	1	0.99151	0.99083	0.9767	0.98115
O2	0.98154	0.98172	0.98739	0.98727	0.99379	0.99126	0.98608	0.98904	0.989	0.99034	0.99151	1	0.99332	0.98073	0.98018
O3	0.97382	0.9728	0.98813	0.98349	0.9916	0.98942	0.97913	0.98735	0.98468	0.98483	0.99083	0.99332	1	0.97231	0.98792
O4	0.98992	0.99019	0.95958	0.98213	0.98288	0.9778	0.99191	0.97688	0.99102	0.99143	0.9767	0.98073	0.97231	1	0.94705
O5	0.94967	0.94622	0.98664	0.97111	0.97738	0.98259	0.96043	0.97892	0.96608	0.96267	0.98115	0.98018	0.98792	0.94705	1

The five donors in each blood group are assigned a 1 to 5 identification numbers.

Appendix F: Normalised log2 expression data for the top 100 most variable expression levels




log2 values
0
5
10
15
Normalised reads
Top 100 most variable within Group B
B5
B4
B2
B3
B1


log2 values
0
5
10
15
Normalised reads
Top 100 most variable within Group O
O3
O2
O1
O5
O4

Appendix G: The 100 most variable genes in decreasing order of variance independent of blood group type.

Chromosome	GeneName	Description	Variance
Pf3D7_02_v3	PF3D7_0221300	PF3D7_0221300 *Plasmodium* exported protein, unknown function, pseudogene	3.07
Pf3D7_01_v3	PF3D7_0102200	RESA ring-infected erythrocyte surface antigen	2.857
Pf3D7_10_v3	PF3D7_1002000	PF3D7_1002000 *Plasmodium* exported protein (hyp2), unknown function	2.628
Pf3D7_11_v3	PF3D7_1149200	PF3D7_1149200 ring-infected erythrocyte surface antigen	2.013
Pf3D7_12_v3	PF3D7_1245700	ncRNA	2.01
Pf3D7_10_v3	PF3D7_1035200	PF3D7_1035200 S-antigen	1.99
Pf3D7_12_v3	PF3D7_1228600	MSP9 merozoite surface protein 9	1.889
Pf3D7_03_v3	PF3D7_0301100	PF3D7_0301100 *Plasmodium* exported protein (hyp13), unknown function	1.855
Pf3D7_09_v3	PF3D7_0905400	RhopH3 high molecular weight rhoptry protein 3	1.746
Pf3D7_12_v3	PF3D7_1255000	RIF rifin	1.733
Pf3D7_09_v3	PF3D7_0917300	PF3D7_0917300 conserved *Plasmodium* membrane protein, unknown function	1.72
Pf3D7_10_v3	PF3D7_1029500	PF3D7_1029500 conserved *Plasmodium* protein, unknown function	1.675
Pf3D7_13_v3	PF3D7_1352900	PF3D7_1352900 *Plasmodium* exported protein, unknown function	1.645
Pf3D7_05_v3	PF3D7_0501600	RAP2 rhoptry-associated protein 2	1.641
Pf3D7_14_v3	PF3D7_1449200	PF3D7_1449200 conserved *Plasmodium* protein, unknown function	1.624
Pf3D7_02_v3	PF3D7_0201000	RIF rifin	1.615
Pf3D7_08_v3	PF3D7_0832800	RIF rifin	1.615
Pf3D7_02_v3	PF3D7_0207600	SERA5 serine repeat antigen 5	1.612
Pf3D7_14_v3	PF3D7_1410400	RAP1 rhoptry-associated protein 1	1.608
Pf3D7_03_v3	PF3D7_0301200	FIKK 3 serine/threonine protein kinase, FIKK family	1.603
Pf3D7_07_v3	PF3D7_0702100	PF3D7_0702100 *Plasmodium* exported protein (PHISTb), unknown function, pseudogene	1.587
Pf3D7_12_v3	PF3D7_1200700	ACS7 acyl-CoA synthetase	1.555
Pf3D7_14_v3	PF3D7_1462000	TLP1 thioredoxin-like protein	1.554
Pf3D7_07_v3	PF3D7_0707300	RAMA rhoptry-associated membrane antigen	1.548
Pf3D7_12_v3	PF3D7_1219500	VAR erythrocyte membrane protein 1 (PfEMP1), exon 2, pseudogene	1.546
Pf3D7_07_v3	PF3D7_0713000	RIF rifin	1.526
Pf3D7_10_v3	PF3D7_1035900	M566 probable protein, unknown function	1.523
Pf3D7_10_v3	PF3D7_1041100	RIF rifin	1.481
Pf3D7_07_v3	PF3D7_0722200	RALP1 rhoptry-associated leucine zipper-like protein 1	1.468
Pf3D7_01_v3	PF3D7_0114700	RIF rifin	1.449
Pf3D7_05_v3	PF3D7_0501500	RAP3 rhoptry-associated protein 3	1.44
Pf3D7_04_v3	PF3D7_0423400	AARP asparagine-rich protein	1.422
Pf3D7_13_v3	PF3D7_1301200	GBPH2 glycophorin binding protein	1.422
Pf3D7_14_v3	PF3D7_1479600	RIF rifin	1.413
Pf3D7_14_v3	PF3D7_1400800	RIF rifin	1.409
Pf3D7_09_v3	PF3D7_0930300	MSP1 merozoite surface protein 1	1.409
Pf3D7_04_v3	PF3D7_0413200	RIF rifin	1.398
Pf3D7_02_v3	PF3D7_0201400	PF3D7_0201400 *Plasmodium* exported protein (hyp10), unknown function	1.397
Pf3D7_11_v3	PF3D7_1108800	PF3D7_1108800 conserved *Plasmodium* protein, unknown function	1.395
Pf3D7_11_v3	PF3D7_1140400	PF3D7_1140400 conserved *Plasmodium* protein, unknown function	1.383
Pf3D7_08_v3	PF3D7_0833100	RIF rifin	1.359
Pf3D7_13_v3	PF3D7_1335100	MSP7 merozoite surface protein 7	1.35
Pf3D7_10_v3	PF3D7_1035700	DBLMSP duffy binding-like merozoite surface protein	1.35
Pf3D7_11_v3	PF3D7_1114300	ncRNA	1.35
Pf3D7_07_v3	PF3D7_0701900	PF3D7_0701900 *Plasmodium* exported protein, unknown function	1.348
Pf3D7_11_v3	PF3D7_1150200	RIF rifin	1.347
Pf3D7_09_v3	PF3D7_0901000	RIF rifin	1.339

Chromosome	GeneName	Description	Variance
Pf3D7_09_v3	PF3D7_0935800	CLAG9 cytoadherence linked asexual protein 9	1.318
Pf3D7_04_v3	PF3D7_0404700	DPAP3 dipeptidyl aminopeptidase 3	1.311
Pf3D7_13_v3	PF3D7_1373100	RIF rifin	1.302
Pf3D7_04_v3	PF3D7_0405900	ASP apical sushi protein	1.3
Pf3D7_12_v3	PF3D7_1252100	RON3 rhoptry neck protein 3	1.296
Pf3D7_09_v3	PF3D7_0929400	RhopH2 high molecular weight rhoptry protein 2	1.288
Pf3D7_03_v3	PF3D7_0302500	CLAG3.1 cytoadherence linked asexual protein 3.1	1.285
Pf3D7_13_v3	PF3D7_1334900	PF3D7_1334900 MSP7-like protein, fragment, pseudogene	1.27
Pf3D7_08_v3	PF3D7_0800900	PF3D7_0800900 *Plasmodium* exported protein (hyp7), unknown function, pseudogene	1.265
Pf3D7_14_v3	PF3D7_1473000	PF3D7_1473000 conserved *Plasmodium* protein, unknown function	1.261
Pf3D7_05_v3	PF3D7_0507400	PF3D7_0507400 conserved *Plasmodium* protein, unknown function	1.259
Pf3D7_06_v3	PF3D7_0618000	PF3D7_0618000 conserved *Plasmodium* membrane protein, unknown function	1.256
Pf3D7_12_v3	PF3D7_1235200	VP2 V-type K+-independent H+-translocating inorganic pyrophosphatase	1.256
Pf3D7_12_v3	PF3D7_1253500	PF3D7_1253500 *Plasmodium* exported protein, unknown function	1.251
Pf3D7_02_v3	PF3D7_0207700	SERA4 serine repeat antigen 4	1.247
Pf3D7_09_v3	PF3D7_0935600	GIG gametocytogenesis-implicated protein	1.246
Pf3D7_09_v3	PF3D7_0918700	PF3D7_0918700 conserved *Plasmodium* protein, unknown function	1.238
Pf3D7_08_v3	PF3D7_0830500	TryThrA sporozoite and liver stage tryptophan-rich protein, putative	1.236
Pf3D7_14_v3	PF3D7_1478300	PF3D7_1478300 *Plasmodium* exported protein, unknown function, pseudogene	1.233
Pf3D7_04_v3	PF3D7_0401000	RIF rifin	1.226
Pf3D7_14_v3	PF3D7_1423900	ncRNA	1.226
Pf3D7_02_v3	PF3D7_0223200	RIF rifin	1.223
Pf3D7_03_v3	PF3D7_0301000	ACS2 acyl-CoA synthetase	1.219
Pf3D7_01_v3	PF3D7_0101400	EPF1 exported protein family 1, pseudogene	1.207
Pf3D7_08_v3	PF3D7_0809000	ncRNA	1.207
Pf3D7_10_v3	PF3D7_1035600	H101 merozoite surface protein	1.196
Pf3D7_01_v3	PF3D7_0102000	PF3D7_0102000 *Plasmodium* exported protein (PHISTa), unknown function, pseudogene	1.192
Pf3D7_14_v3	PF3D7_1475000	MED31 mediator of RNA polymerase II transcription subunit 31, putative	1.186
Pf3D7_08_v3	PF3D7_0800500	RIF rifin	1.185
Pf3D7_14_v3	PF3D7_1464800	PF14_615a:mRNA:1 conserved *Plasmodium* protein, unknown function	1.176
Pf3D7_10_v3	PF3D7_1035400	MSP3 merozoite surface protein 3	1.167
Pf3D7_02_v3	PF3D7_0200900	PF3D7_0200900 stevor, pseudogene	1.165
Pf3D7_11_v3	PF3D7_1116000	RON4 rhoptry neck protein 4	1.164
Pf3D7_02_v3	PF3D7_0221650	PF3D7_0221650 *Plasmodium* exported protein, unknown function, pseudogene	1.155
Pf3D7_07_v3	PF3D7_0732800	PF3D7_0732800 erythrocyte membrane protein 1 (PfEMP1), exon 2	1.155
Pf3D7_12_v3	PF3D7_1240700	RIF rifin, pseudogene	1.145
Pf3D7_11_v3	PF3D7_1129300	PF3D7_1129300 conserved *Plasmodium* protein, unknown function	1.144
Pf3D7_02_v3	PF3D7_0207800	SERA3 serine repeat antigen 3	1.144
Pf3D7_02_v3	PF3D7_0207500	SERA6 serine repeat antigen 6	1.137
Pf3D7_03_v3	PF3D7_0314800	PF3D7_0314800 conserved *Plasmodium* protein, unknown function	1.113
Pf3D7_07_v3	PF3D7_0731900	RIF rifin, pseudogene	1.112
Pf3D7_04_v3	PF3D7_0421200	RIF rifin	1.109
Pf3D7_02_v3	PF3D7_0214900	RON6 rhoptry neck protein 6	1.106
Pf3D7_12_v3	PF3D7_1213000	ncRNA	1.103
Pf3D7_13_v3	PF3D7_1373300	RIF rifin	1.095
Pf3D7_05_v3	PF3D7_0525800	IMC1g inner membrane complex protein 1g, putative	1.094
Pf3D7_12_v3	PF3D7_1251200	PF3D7_1251200 coronin	1.093
Pf3D7_03_v3	PF3D7_0318700	PF3D7_0318700 trophozoite stage antigen	1.089
Pf3D7_10_v3	PF3D7_1005400	PF3D7_1005400 conserved *Plasmodium* protein, unknown function	1.089
Pf3D7_08_v3	PF3D7_0822900	PF3D7_0822900 conserved *Plasmodium* protein, unknown function	1.084
Pf3D7_API_v3	PF3D7_API02900	RIFA elongation factor Tu, putative	1.083
Pf3D7_12_v3	PF3D7_1205200	HADO HAD domain ookinete protein, putative	1.075
Pf3D7_03_v3	PF3D7_0302200	CLAG3.2 cytoadherence linked asexual protein 3.2	1.059

Appendix H: Publication of work related to the thesis

Seroepidemiology of *Plasmodium* species infections in Zimbabwean population. In *Malaria Journal*, 2016.

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Malaria Journal

RESEARCH Open Access

Seroepidemiology of *Plasmodium* species infections in Zimbabwean population

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Abstract

Background: Individuals living in malaria-endemic regions may be exposed to more than one *Plasmodium* species; there is paucity of data on the distribution of the different species of *Plasmodium* in affected populations, in part due to the diagnostic method of microscopy, which cannot easily differentiate between the species. Sero-epidemiological data can overcome some of the shortcomings of microscopy.

Methods: The specificity of IgG antibodies to recombinant merozoite surface protein 1 (MSP-1_{19}) derived from four human *Plasmodium* species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*) was investigated using competition enzyme-linked immunosorbent assay. Subsequently, these antigens were used to determine the exposure prevalence to the different *Plasmodium* species in serum samples of participants. One-hundred individuals, aged five-18 years, from each of the three *Plasmodium* meso-endemic Zimbabwean villages (Burma Valley, Mutoko, Chiredzi) were recruited in the study.

Results: The study demonstrated that the host serum reactivity to MSP-1_{19} antigens was species-specific and that no cross-reactivity occurred. The overall prevalence of antibody response to MSP-1_{19} antigens was 61 % in Burma Valley, 31 % in Mutoko and 32 % in Chiredzi. Single species IgG responses to MSP-1_{19} were most frequent against *P. falciparum,* followed by *P. malariae* and *P. ovale*, with responses to *P. vivax* being the least prevalent. Interestingly, 78–87 and 50 % of sera with IgG responses to *P. malariae* and *P. ovale* MSP-1_{19}, respectively, also had IgG specific response for *P. falciparum* MSP-1_{19} antigens, indicating that exposure to these species is a common occurrence in these populations. Single species IgG responses to the non-falciparum species were at a very low frequency, ranging between 0 and 13 % for *P. malariae*.

Conclusions: There is evidence of a higher exposure to the non-falciparum parasite species than previously reported in Zimbabwe. The recombinant MSP-1_{19} antigens could be used as additional diagnostic tools in antibody assays for the detection of exposure to the different *Plasmodium* species. The results also introduce an interesting concept of the co-infection of non-falciparum *Plasmodium* almost always with *P. falciparum*, which requires further validation and mechanistic studies.

Keywords: Antibody, Merozoite surface protein 1 (MSP-1_{19}), *Plasmodium*, Microscopy

Background

Malaria is a major public health problem in sub-Saharan Africa, and is responsible for over half a million deaths annually, especially in children under the age of 5 years [1]. Four major species of the protozoan parasite, *Plasmodium*, (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*) cause human malaria in sub-Saharan Africa. In malaria-endemic countries, there is an overlap in the geographic distribution of the different *Plasmodium* species and the *Anopheles* mosquito vectors that transmit these parasites, and that individuals may be exposed to, and harbour multiple *Plasmodium* species [2]. However, the epidemiology of the different *Plasmodium* species in endemic human

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populations is not well documented [3]. Diagnosis of malaria in endemic clinical settings is predominantly by the 'gold standard' blood film microscopic examination, and rapid diagnostic tests (RDT), both of which lack sensitivity in differentiating the species of *Plasmodium* causing malaria. Microscopic examination has several limitations such as the inability to detect low levels of parasitaemia, and the difficulty in species differentiation owing to subtle differences in the morphology of blood stage parasites [4]. This results in the species of *Plasmodium* causing disease being rarely reported, and almost all cases of malaria are therefore attributed to *P. falciparum*, the species causing the most serious form of malaria [3]. This has led to underestimates of the prevalence of both mixed-species and non-falciparum species infections [5]. These non-falciparum species are of significant clinical importance; for example, *P. vivax* and *P. ovale* which form latent liver stage 'hypnozoites' are capable of causing disease several months or years after the primary infection [6]. Incidences of the diagnosis of systemic diseases caused by *P. malariae* several months or years after people have returned from malaria-endemic regions have been reported [7]. In some cases, drug treatment failure attributable to the misdiagnosis of primary infections caused by the non-falciparum species or as co-infecting species with *P. falciparum* have been observed [8]. While PCR typing of infecting *Plasmodium* species is not frequently available or applicable in many African field settings, most RDTs may not differentiate non-falciparum species [3]. There is an urgent need for additional diagnostic tools [2–4] capable of rapid detection of all four infecting *Plasmodium* species for effective treatment and control of malaria.

In this study, a new assay has been developed that detects exposure to all four human *Plasmodium* species based on serum antibody responses to merozoite surface protein 1 (MSP-1). The surface of the invasive merozoite is coated in MSP-1 that constitutes 31 % of the GPI-anchored proteins on *P. falciparum* merozoites [9]. MSP-1 is expressed by all four human *Plasmodium* species. In *P. falciparum*, MSP-1 undergoes two proteolytic cleavages resulting in a C-terminal MSP-1_{19} fragment that is carried into the erythrocyte during merozoite invasion [10, 11]. Until recently, only the MSP-1 genes of *P. falciparum* and *P. vivax* had been characterized. Recently, the sequences of the MSP-1_{19} gene fragments for *P. malariae* and *P. ovale* have been determined with limited characterization of the responses to these parasite proteins [12]. Although the gene sequences of MSP-1_{19} antigens are unique to each of these four *Plasmodium* species, extensive homology can be found among them. The number and relative positions of cysteine residues within the C-terminus fragments of MSP-1_{19} are comparable in all four *Plasmodium* species [12]. For example, there are about 32 amino acid sites within the MSP-1_{19} gene where all four parasite species share the same amino acid, and about 30 sites where the same amino acid is conserved in two or three species (Additional file 1: Figure S1). To date, there has been no field study using MSP-1_{19} antigens from all four malaria parasite species to characterize the epidemiology of exposure to *Plasmodium* in any African population.

In Zimbabwe over half of the population are exposed to malaria, with *P. falciparum* being the predominant species, accounting for almost all cases of the disease [13]. There is little epidemiological data of exposure to non-falciparum species and/or mixed *Plasmodium* infections in Zimbabwe. The aim of this study is to determine the species specificity of IgG antibody responses to recombinant *Plasmodium* MSP-1_{19} antigens in three meso-endemic villages of Zimbabwe: Burma Valley, Mutoko and Chiredzi. Using these antigens as diagnostic tools, this study describes the sero-epidemiology of multiple *Plasmodium* species infections in these study sites.

Methods

Study sites and population

Serum samples were collected in three Zimbabwean villages where malaria parasite transmission is described as meso-endemic [13], as part of studies investigating the immuno-epidemiology of schistosomiasis in villages with *Plasmodium* co-infection. The study sites were Burma Valley in the northeast, where samples were collected in 1994, Chiredzi in the southeast where samples were collected in 1999 and Mutoko in central Zimbabwe, where samples were collected in 2003. The study cohort consisted of 100 participants aged between 5 and 18 years (both males and females) in each study site (Table 1). Antibody responses to merozoite surface proteins were detected using enzyme-linked immunosorbent assays (ELISA).

Ethical approval and consent

The studies in the different study sites received ethical approval from the Medical Research Council of Zimbabwe. Permission to conduct the work in each of the three

Table 1 Summary of study population

Study area	Age range (years)	Median age (years)	Sex	
			Male (%)	Female (%)
Burma valley	6–15	10	49	51
Mutoko	5–18	9.5	36	64
Chiredzi	7–16	11	52	48

100 individuals from each village were recruited into the study

villages was obtained from the Provincial Medical Director, the District Educational Officer and Heads of schools in the study sites. Project aims and procedures were fully explained to the study participants and/or their guardian. Informed oral consent/assent was obtained from parents/guardians, or participants if older than 10 years, prior to enrolment of the participants into the study. The participants were recruited into the study on a voluntary basis and were free to withdraw with no further obligation.

Recombinant antigens

MSP-1 antigens used in the ELISAs were expressed in *Escherichia coli* transformed with pGEX-derived plasmid constructs [14–17] as recombinant proteins fused to glutathione S-transferase (GST). These were purified by affinity chromatography using HiTrap glutathione Sepharose columns on an AKTAprime system and quantified by the Bradford protein assay.

Serology

Sera were tested by ELISA for the presence of IgG antibodies able to recognize the recombinant merozoite surface proteins as an indication of recent or current exposure; 96-well plates (Immulon4 HBX; Dynex, Greiner Microlon) were coated with 100 µL of 0.5 µg/mL of recombinant antigen in carbonate bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) and incubated overnight at 4 °C in a humidified atmosphere. Plates were washed four times in washing buffer (0.05 % Tween-20 in PBS) using Skatron Skanwasher to remove unbound antigens and blotted on paper towels (Kimberley Clark 3-ply hand towels Cat No. 6771). Free binding sites in wells were blocked with 200 µL per well of blocking buffer (1 % (w/v) skimmed milk powder in the PBS buffer) for 5 h at room temperature and then plates further washed four times. Human serum diluted 1:500 in the blocking buffer (100 µL per well) was added in duplicate to the Ag-coated wells and incubated overnight at 4 °C. After four washes, the wells were incubated for 3 h at room temperature with 100 µL per well of horseradish peroxidase-conjugated rabbit antihuman IgG (1:5000) (Dako Ltd, High Wycombe, UK). Plates were washed four times to remove unbound secondary antibody before reaction development with 100 µL of substrate buffer [(0.04 mg/mL of *o*-phenylenediamine; Sigma, St Louis, MO, USA; 0.012 % H_2O_2 in development buffer (24.5 mM citric acid monohydrate and 52 mM Na_2HPO4, pH 5.0)] for 10–15 min at room temperature. An unstopped positive control plate was read at an optical density (OD) 450 nm, with an OD 450 nm of 0.7–0.8 taken to be equivalent to OD 492 nm of 2.5–3.0. The reaction was stopped by the addition of 25 µL of 2 M H_2SO_4 per well, and OD was measured at 492 nm (Labsystems Multiskan Ascent microtitre plate reader). GST protein, purified from *Escherichia coli* transfected with pGEX-2T alone, was used as a control to determine the non-specific (background) binding of human IgG to the GST. Corrected OD values for each plasma sample were calculated by subtracting the mean OD value of wells containing control GST protein from the mean OD value obtained with each test MSP-1 antigen. Cut-off values at which binding of Ab from malaria-exposed individuals was regarded as significantly above background were calculated as corrected OD above the mean plus 4 standard deviation of OD readings obtained with sera from eight Scottish blood donors with no history of exposure to malaria. The same positive controls (pooled sera from Brefet, The Gambia) were run in duplicate, on each plate, to allow for standardization of plate-to-plate variations.

Competition ELISA

Competition ELISA was performed for individuals with substantial antibody reactivity to more than one MSP-1_{19} antigen, to assess whether human anti-MSP-1_{19} IgG antibodies specific for MSP-1_{19} were species-specific or cross-reacted with each other. Serum was pre-incubated with different concentrations of MSP-1_{19} antigen, and then added to the wells of microtitre plates coated with either the homologous or heterologous MSP-1_{19} antigen. The rationale of the competition ELISA is that appropriate antigen epitopes will react with their corresponding paratopes in the sera, so that with increasing antigen concentration, all paratopes in the sera react with the antigen, leaving none available to bind to antigen on the plate [18]. In the case of antigens without corresponding paratopes in the sera, there will be no prior reactivity between the serum and antigens, regardless of the antigen concentration. The same ELISA protocol above was followed with slight modification. Plates were coated with recombinant MSP-1_{19} Ag and incubated overnight at 4 °C. Serum was diluted (1:500) and pre-incubated with increasing concentrations (0–10 µg/mL) of soluble competing homologous or heterologous Ag, i.e., with up to 20-fold excess over the 0.50 µg/mL immobilized Ag to allow sera to bind to the antigen before reacting with the antigen bound on the plate, then tested on the plate-bound Ag overnight. This was followed by washing and incubation with a horseradish peroxidase-conjugated second Ab, as described above.

Statistical analyses

To determine if exposure prevalence derived from single species data differed from that based on multiple species, Chi square (χ^2) tests were used.

Results

Specificity of *Plasmodium* MSP-1_{19} antigens

Competition ELISA showed that in sera reactive against recombinant MSP-1_{19} antigens from more than one parasite species, anti-MSP-1_{19} IgG antibody responses were species-specific and did not cross-react. As a positive control, when *P. falciparum* MSP-1_{19} antigen was coated onto microtitre plates (as capture Ag), sera pre-incubated with *P. falciparum* MSP-1_{19} (competing homologous Ag), were inhibited from binding in a dose-dependent manner (Fig. 1). This inhibition occurred at competing homologous Ag concentration as low as 0.1 μg/mL. Similar results were observed when the homologous competitor Ags were either *P. malariae* or *P. ovale* MSP-1_{19}. To assess anti-MSP-1_{19} cross-reactivity, *P. falciparum* MSP-1_{19} antigen was coated onto microtitre plates and dual specificity sera were pre-incubated with increasing concentrations of the heterologous *P. malariae* or *P. ovale* MSP-1_{19} antigens. IgG binding to *P. falciparum* MSP-1_{19} antigen was not inhibited by soluble heterologous *P. malariae* or *P. ovale* MSP-1_{19}, even at concentration 20 times the capture antigen. This was also true when the coating and competing antigens were reversed in the assay (Fig. 1).

Prevalence of human IgG antibodies to recombinant MSP-1_{19} antigens from four *Plasmodium* species

Antibody recognition of the panel of four *Plasmodium* recombinant MSP-1_{19} antigens was tested by ELISA against 100 sera from each of the three study sites (Burma Valley, Mutoko, Chiredzi). The observed overall prevalence of IgG response to all recombinant MSP-1_{19} antigens was 61, 31 and 32 % in the Burma Valley, Mutoko and Chiredzi villages, respectively (Table 2). There were no significant differences between the exposure prevalence between the villages (Burma Valley vs Mutoko: $\chi^2 = 0.002$, df = 1, $P = 0.97$), (Burma Valley vs Chiredzi: $\chi^2 = 0.423$, df = 1, $P = 0.52$) and (Mutoko vs Chiredzi: $\chi^2 = 0.001$, df = 1, $P = 0.97$).

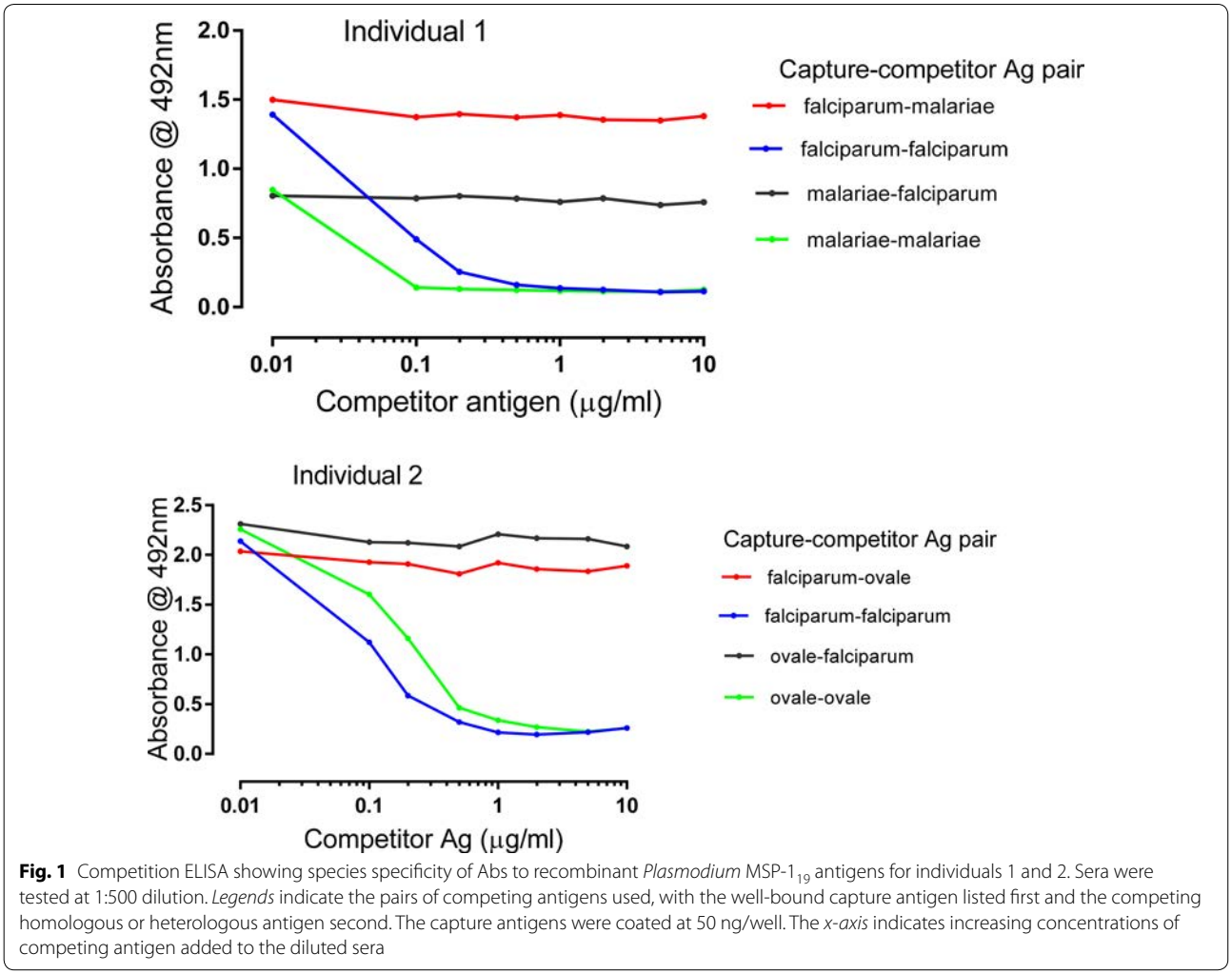

Fig. 1 Competition ELISA showing species specificity of Abs to recombinant *Plasmodium* MSP-1_{19} antigens for individuals 1 and 2. Sera were tested at 1:500 dilution. *Legends* indicate the pairs of competing antigens used, with the well-bound capture antigen listed first and the competing homologous or heterologous antigen second. The capture antigens were coated at 50 ng/well. The *x-axis* indicates increasing concentrations of competing antigen added to the diluted sera

Table 2 Prevalence of antibody reactivity to single and multiple *Plasmodium* spp. anti-MSP-1$_{19}$

Study area	Overall prevalence (%)	Single *Plasmodium* spp. response	Multiple *Plasmodium* spp. response	Single spp. anti-MSP-1$_{19}$ Ab responses			Multiple spp. anti- MSP-1$_{19}$ Ab responses				
				Falciparum	Malariae	Ovale	Falciparum/ malariae only	Falciparum/ ovale only	Falciparum/ vivax only	Malariae/ ovale only	Falciparum/ malariae and ovale
Burma valley (n = 100)	61	30 (49.2)	31 (50.8)	24 (80)	3 (10)	3 (10)	26 (83.9)	2 (6.5)	0	1 (3.1)	2 (6.5)
Mutoko (n = 100)	31	15 (48.4)	16 (51.6)	11 (68.8)	0	4 (31.2)	6 (37.5)	2 (12.5)	0	3 (18.8)	5 (31.2)
Chiredzi (n = 100)	32	23 (71.9)	9 (28.1)	18 (78.3)	3 (13)	2 (8.7)	6 (66.7)	0	1 (11.1)	0	2 (22.2)

Values indicate the number of responders while those in parenthesis are expressed as % of responders in the respective categories

n number of individuals

Single *vs* multiple *Plasmodium* species anti-MSP-1_{19} responses

Of the individuals with anti-MSP-1_{19} responses in all three study areas, Burma Valley (n = 61), Mutoko (n = 31) and Chiredzi (n = 32), single species responses were the most common occurrence, and these were predominantly directed against *P. falciparum* MSP-1_{19} antigens (80, 68.8 and 78.3 %, respectively), with responses to *P. malariae* or *P. ovale* ranging from 0 to 31.2 % (Table 2). The proportion of individuals with single *versus* multiple species anti-MSP-1_{19} responses in all three study villages were comparable.

In responders with antibodies to multiple *Plasmodium* species, antibody responses to the non-falciparum species were almost always accompanied by responses against *P. falciparum* MSP-1_{19} antigens in all three study areas. Multiple responses to *P. falciparum*- and *P. malariae* MSP-1_{19} were the most common, with about 78–87 and 50 % of all sera with IgG responses to *P. malariae* and *P. ovale* MSP-1_{19}, respectively, also having IgG-specific response for *P. falciparum* MSP-1_{19} antigens. The high exposure prevalence of IgG responses to *P. malariae* MSP-1_{19} antigens suggests that infection with this parasite species is at a higher frequency in these populations than has been previously reported, and are predominantly co-responses to the main response to *P. falciparum* MSP-1_{19} antigen.

While there were no single species responders to *P. malariae* MSP-1_{19} in Mutoko, a much higher proportion (31.2 %) responded to three parasite antigens (*P. malariae*, *P. falciparum, P. ovale*) compared to the other two villages. Only one individual in Chiredzi had an antibody response to *P. vivax* antigen in addition to a response to *P. falciparum* (Table 2). When single species responses involving only *P. falciparum* was compared to multiple species responses involving *P. falciparum* with *P. malariae* and/or *P. ovale*, no significant difference was observed in all three villages (Fig. 2).

When the cohort was divided into two age groups, based on the median ages (i.e., those below 10 years and those 10 years and above), it was observed that the overall exposure prevalence in the two age groups were comparable in both Burma Valley ($\chi^2 = 3.5$, df = 1, $P = 0.06$) and Mutoko ($\chi^2 = 0.05$, df = 1, $P = 0.83$). However, in Chiredzi a significantly higher exposure prevalence was observed in responders aged 10 years and above compared to those below 10 years ($\chi^2 = 6.13$, df = 1, $P = 0.01$) (Table 3). Differences between the two age groups in responders to single and multiple species in all three study areas were not compared because of the smaller sample sizes involved.

Discussion

In this current study, IgG responses to recombinant MSP-1_{19} antigens (an indication of prior exposure to *Plasmodium* antigens) from the four major human *Plasmodium* species were evaluated in three Zimbabwean villages with meso-endemic malaria transmission dynamics. Individuals living in malaria-endemic regions may harbour multiple *Plasmodium* species owing to the

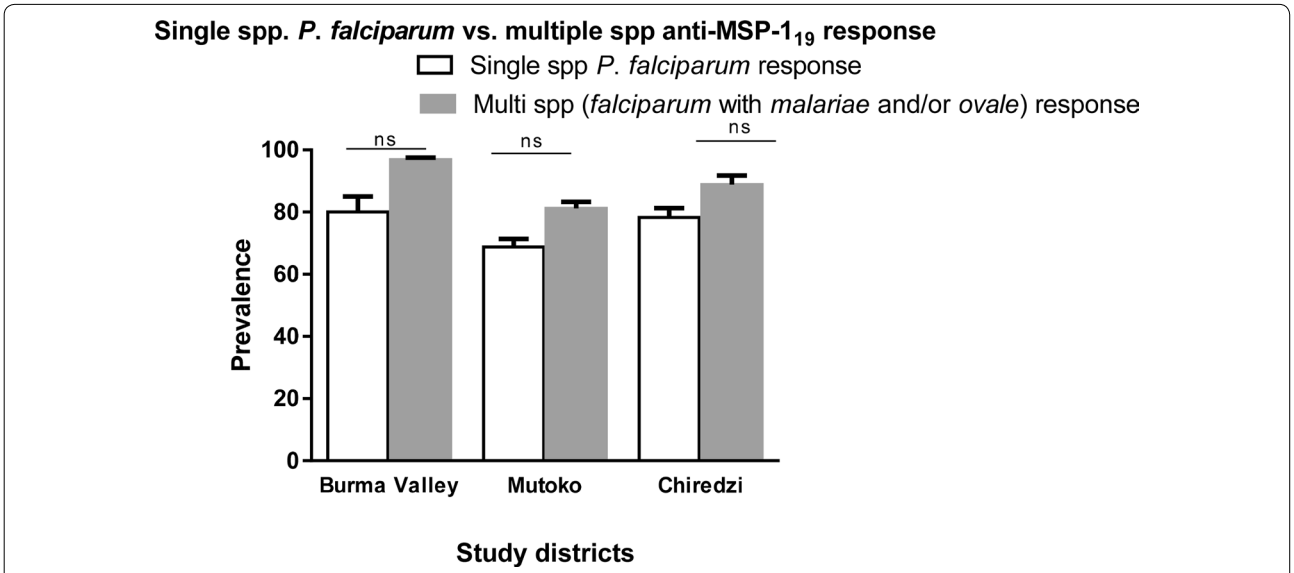

Fig. 2 Prevalence of single *Plasmodium falciparum* vs multiple species anti-MSP-1_{19} responses. Observed prevalence of single spp. *P. falciparum* anti-MSP-1_{19} responses were compared with multiple responses involving *P. falciparum* with *P. malariae* and/or *P. ovale* in all three study sites

Table 3 Prevalence of antibody reactivity to single and multiple *Plasmodium* spp. anti-MSP-1_{19} by age

Study area	Age range years (n)	Overall prevalence (%)	Single *Plasmodium* spp. response	Multiple *Plasmodium* spp. response	Single spp. anti-MSP-1_{19} Ab responses			Multiple spp. anti- MSP-1_{19} Ab responses				
					Falciparum	Malariae	Ovale	Falciparum/ malariae only	Falciparum/ ovale only	Falciparum/ vivax only	Malariae/ ovale only	Falciparum/ malariae/ ovale
Burma valley (n = 100)	6–9 (45)	33 (73.3)	15 (45.5)	18 (54.5)	13 (86.7)	1 (6.6)	1 (6.6)	15 (83.2)	1 (5.6)	0	1 (5.6)	1 (5.6)
	10–15 (55)	28 (50.9)	15 (53.6)	13 (46.4)	11 (73.3)	2 (13.3)	2 (13.3)	11 (84.6)	1 (7.7)	0	0	1 (7.7)
Mutoko (n = 100)	5–9 (50)	16 (32)	7 (43.8)	9 (56.2)	6 (85.7)	0	1 (14.3)	3 (33.3)	2 (22.2)	0	2 (22.2)	2 (22.2)
	10–18 (50)	15 (30)	8 (53.3)	7 (46.7)	5 (62.5)	0	3 (37.5)	3 (42.8)	0	0	1 (14.4)	3 (42.8)
Chiredzi (n = 100)	7–9 (25)	3 (12)	2 (66.7)	1 (33.3)	2 (100)	0	0	1 (100)	0	0	0	0
	10–16 (75)	29 (38.7)	21 (72.4)	8 (27.6)	16 (76.2)	3 (14.3)	2 (9.5)	5 (62.5)	0	1 (12.5)	0	2 (25)

Values indicate the number of responders while those in parenthesis are expressed as % of responders in the respective categories

n number of individuals

geographical overlap of the four major human *Plasmodium* species [4, 19, 20]. Malaria diagnosis in most African field settings is largely by microscopy of blood films, which reports the presence or absence of *Plasmodium* parasites without cognisance to the species causing disease. Low-level parasitaemia of the non-falciparum species in mixed infection with *P. falciparum* accounts for the misdiagnosis of these species. There have been case study reports of treatment failures [21] and acute renal injury [22] attributable to undiagnosed *P. malariae* infection or co-infection. Knowledge of the type of infecting species is therefore essential for effective treatment as well as the implementation of control programmes. IgG responses to *P. falciparum* MSP-1_{19} antigens have been shown to rise following clinical episodes of malaria and decline in the absence of the disease [15]. In the current study, the antibody response to *Plasmodium* species recombinant MSP-1_{19} antigens in humans was seen to be highly species-specific. Furthermore, the study showed that the responses were not cross-reactive, despite the amino acid sequence similarities between the four *Plasmodium* MSP-1_{19} antigens. In experimental monkey and human studies utilizing all four *Plasmodium* species MSP-1_{19} antigens, a superior sensitivity was seen when compared to commercially available antibody assays which only utilize MSP-1_{19} antigens from *P. falciparum* and *P. vivax* and depend on cross-reactivity in detecting the other two species [23]. The specificity of antibodies to these antigens supports the evidence that these antigens could be used in pan-malaria diagnostic assay to enable the rapid detection of the type of *Plasmodium* species causing malaria [23].

In many malaria-endemic countries in sub-Saharan Africa, *P. falciparum* is the predominant species that causes malaria, thus it was not surprising that the antibody response to *P. falciparum* MSP-1_{19} antigens was predominant in all three study sites. Since *P. falciparum* infections have higher parasitaemias than the other malaria parasite species [20, 24], it is likely that individuals will have a stronger immune response to *P. falciparum* infection. The novel results from this study were the indication that the exposure prevalence of *P. malariae* and *P. ovale* is higher, as previous reports have attributed about 98 % of malaria in Zimbabwe to be caused by *P. falciparum*. More importantly, the observed higher exposure prevalence of *P. malariae* in the Burma Valley district was striking, as reports suggest that this species only accounts for between 1 and 2.6 % of all malaria cases by light microscopy [25–27].

Microscopy has long been known to underestimate the prevalence of the non-falciparum species owing to difficulties in distinguishing the subtle differences in the morphology of the different species as well as the challenge posed in detecting minority species in a blood film with high density *P. falciparum* parasitaemia. It is therefore not surprising that these assays detected a higher sero-prevalence of these species, as this also reflects recent and concurrent parasite exposure. Studies employing nucleic acid based techniques for *Plasmodium* parasite detection and species identification in some African countries have reported prevalence of the non-falciparum species to be between 1 and 17 % [20, 24].

While antibody responses to single species *P. falciparum* antigens were common, single species responses to *P. malariae* and *P. ovale* antigens were infrequently detected. A significant proportion of individuals with IgG responses to *P. malariae* and/or *P. ovale* MSP-1_{19} almost always had responses to *P. falciparum* MSP-1_{19}. This results support the findings of a recent study in Ghana, which reported frequent detection of *P. malariae* and *P. ovale* in individuals who are also PCR positive for *P. falciparum* [28]. The reasons for this co-occurrence of the non-falciparum species with *P. falciparum* may be both epidemiological and biological. Of the epidemiological reasons, it has been suggested that the same *Anopheles* mosquito circulating in a population might be responsible for the simultaneous or sequential inoculation of the different species [4], thereby increasing the likelihood of multiple species infections. Biological reasons may include selective advantages for these minor species when co-infecting with *P. falciparum*. For example, due to density-dependent regulation of immune responses directed against the majority species (*P. falciparum*), these non-falciparum species may be able to evade host immune responses and establish disease [29, 30]. There are parallels in other infectious diseases, such as the obligate satellite virus hepatitis D, which is unable to establish disease independent of hepatitis B virus [31]. Hepatitis D virus co-infection in Hepatitis B-infected individuals worsens hepatic damage and inflammation, and is more likely to lead to hepatocellular carcinoma [32, 33]. The results show some single species *P. malariae* responses, indicating that this species is capable of establishing infection independent of other *Plasmodium*. However, the significant proportion of individuals with co-occurrence of antibody responses to *P. falciparum* suggests a possible dependency on *P. falciparum* receptors or proteins for successful disease by *P. malariae*. These non-falciparum species, which usually exist as part of a complex mixed-infections with *P. falciparum* [2, 34] may cause chronic, sub-clinical disease with potential health consequences, including treatment failure, disease relapse and long-term systemic consequences [5–7]. A recent

study in Indonesia found *P. malariae* to be associated with a lower mean haemoglobin, nephrotic syndrome and death [27].

Antibody responses to *P. vivax* MSP-1_{19} were rarely observed in this study. *Plasmodium vivax* requires the Duffy antigen to establish a successful infection [35], and is predominantly endemic in Asian and Latin American countries. It has long been known that the Duffy antigen is absent in most African populations [35]; it was therefore not surprising to observe a low frequency of responses to *P. vivax* MSP-1_{19}. In recent years however, there have been reports of *P. vivax* infections in both Duffy positive and negative individuals in Cameroon [36] suggesting that this species might have evolved and adapted to using other receptors to invade erythrocytes and establish disease.

Serological responses generally increase with age. In this present study, age was not a confounding factor in Burma Valley and Mutoko, while is Chiredzi responders 10 years old and above had a higher overall exposure prevalence to parasite antigens. Although all three villages are described as meso-endemic, the observed differences in age responses could be due to the respective transmission dynamics of the different seasons in which sampling was done. In very low and unstable malaria transmission areas such as Daraweesh in eastern Sudan, reports suggest that the age dynamics associated with malaria and serological responses are not apparent, as malaria affects all age groups [37, 38].

Conclusions

This study has shown for the first time that IgG antibodies to recombinant MSP-1_{19} antigens of the four major human *Plasmodium* species are species-specific. The study also demonstrates that in Zimbabwean populations exposed to *Plasmodium* infections, the prevalence of the non-falciparum species responses is higher than previously reported in many other cross-sectional studies (reviewed in [3]). Finally, the study has demonstrated that a high proportion of individuals with antibody responses to non-falciparum MSP-1_{19} antigens also had antibodies to *P. falciparum* MSP-1_{19}. MSP-1_{19} antigens from all four major species of human malaria parasite offer a potential diagnostic tool for the rapid detection of exposure to multiple *Plasmodium* species such as in blood transfusion screening services. It remains to be established if these exposure responses to multiple *Plasmodium* species indicated by the serological survey correspond to concurrent or sequential exposure to previous or current infections.

Additional file

Additional file 1: Figure S1. Sequence homology of *Plasmodium* MSP-1_{19} antigens. Dots or semi-colons (. or :) indicate gene site where the same amino acid is shared between two or three *Plasmodium* species, while the stars (*) indicate conserved amino acid present in all four *Plasmodium* species.

Abbreviations

MSP: merozoite surface protein; RDT: rapid diagnostic test; PCR: polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; GST: glutathione S-transferase; OD: optical density.

Authors' contributions

FM, DRC and SAA conceived, designed and performed the experiments: FM, NM and TM participated in the fieldwork: FM, DRC, SAA, NM, and TM contributed to draft manuscript editing/reviewing. All authors contributed to the revisions. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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References

1. WHO. World Malaria Report 2013. Geneva: World Health Organization; 2013.
2. Zimmerman PA, Mehlotra RK, Kasehagen LJ, Kazura JW. Why do we need to know more about mixed *Plasmodium* species infections in humans? Trends Parasitol. 2004;20:440–7.
3. Howes RE, Reiner RC Jr, Battle KE, Longbottom J, Mappin B, Ordanovich D, et al. *Plasmodium vivax* transmission in Africa. PLoS Neglect Trop Dis. 2015;9:e0004222.
4. Mueller I, Zimmerman PA, Reeder JC. *Plasmodium malariae* and *Plasmodium ovale*—the "bashful" malaria parasites. Trends Parasitol. 2007;23:278–83.

5. Snounou G, White NJ. The co-existence of *Plasmodium*: sidelights from falciparum and vivax malaria in Thailand. Trends Parasitol. 2004;20:333–9.
6. Coldren RL, Jongsakul K, Vayakornvichit S, Noedl H, Fukudas MM. Apparent relapse of imported *Plasmodium ovale* malaria in a pregnant woman. Am J Trop Med Hyg. 2007;77:992–4.
7. Vinetz JM, Li J, McCutchan TF, Kaslow DC. *Plasmodium malariae* infection in an asymptomatic 74-year-old Greek woman with splenomegaly. N Engl J Med. 1998;338:367–71.
8. Smith A, Denholm J, Shortt J, Spelman D. *Plasmodium* species co-infection as a cause of treatment failure. Travel Med Infect Dis. 2011;9:306–9.
9. Gilson PR, Nebl T, Vukcevic D, Moritz RL, Sargeant T, Speed TP, et al. Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*. Mol Cell Proteom. 2006;5:1286–99.
10. McBride JS, Heidrich HG. Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex. Mol Biochem Parasitol. 1987;23:71–84.
11. Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J Exp Med. 1990;172:379–82.
12. Birkenmeyer L, Muerhoff AS, Dawson GJ, Desai SM. Isolation and characterization of the MSP1 genes from *Plasmodium malariae* and *Plasmodium ovale*. Am J Trop Med Hyg. 2010;82:996–1003.
13. Taylor P, Mutambu SL. A review of the malaria situation in Zimbabwe with special reference to the period 1972–1981. Trans R Soc Trop Med Hyg. 1986;80:12–9.
14. Cavanagh DR, McBride JS. Antigenicity of recombinant proteins derived from *Plasmodium falciparum* merozoite surface protein 1. Mol Biochem Parasitol. 1997;85:197–211.
15. Cavanagh DR, Elhassan IM, Roper C, Robinson VJ, Giha H, Holder AA, et al. A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface protein-1 in an area of unstable malaria in Sudan. J Immunol. 1998;161:347–59.
16. Burghaus PA, Holder AA. Expression of the 19-Kilodalton carboxy-terminal fragment of the *Plasmodium falciparum* merozoite surface protein-1 in *Escherichia coli* as a correctly folded protein. Mol Biochem Parasitol. 1994;64:165–9.
17. Polley SD, Conway DJ, Cavanagh DR, McBride JS, Lowe BS, Williams TN, et al. High levels of serum antibodies to merozoite surface protein 2 of *Plasmodium falciparum* are associated with reduced risk of clinical malaria in coastal Kenya. Vaccine. 2006;24:4233–46.
18. Gan SD, Patel KR. Enzyme immunoassay and enzyme-linked immunosorbent assay. J Invest Dermatol. 2013;133:e12.
19. Senn H, Alattas N, Boggild AK, Morris SK. Mixed-species *Plasmodium falciparum* and *Plasmodium ovale* malaria in a paediatric returned traveller. Malar J. 2014;13:78.
20. Bruce MC, Macheso A, Kelly-Hope LA, Nkhoma S, McConnachie A, Molyneux ME. Effect of transmission setting and mixed species infections on clinical measures of malaria in Malawi. PLoS ONE. 2008;3:e2775.
21. Savargaonkar D, Shah N, Das MK, Srivastava B, Valecha N. *Plasmodium malariae* infection: a case of missed diagnosis. J Vector Borne Dis. 2014;51:149–51.
22. Badiane AS, Diongue K, Diallo S, Ndongo AA, Diedhiou CK, Deme AB, et al. Acute kidney injury associated with *Plasmodium malariae* infection. Malar J. 2014;13:226.
23. Muerhoff AS, Birkenmeyer LG, Coffey R, Dille BJ, Barnwell JW, Collins WE, et al. Detection of *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* merozoite surface protein 1-p19 antibodies in human malaria patients and experimentally infected nonhuman primates. Clin Vaccine Immunol. 2010;17:1631–8.
24. Oguike MC, Betson M, Burke M, Nolder D, Stothard JR, Kleinschmidt I, et al. *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities. Int J Parasitol. 2011;41:677–83.
25. Scopel KK, Fontes CJ, Nunes AC, Horta MF, Braga EM. Low sensitivity of nested PCR using *Plasmodium* DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region. Malar J. 2004;3:8.
26. Al-Maktari MT, Bassiouny HK. Malaria status in Al-Hodeidah Governorate, Republic of Yemen. Part II: human factors causing the persistence of chloroquine resistant *P. falciparum* local strain. J Egypt Soc Parasitol. 2003;33:829–39.
27. Langford S, Douglas NM, Lampah DA, Simpson JA, Kenangalem E, Sugiarto P, et al. *Plasmodium malariae* infection associated with a high burden of anemia: a hospital-based surveillance study. PLoS Negl Trop Dis. 2015;9:e0004195.
28. Dinko B, Oguike MC, Larbi JA, Bousema T, Sutherland CJ. Persistent detection of *Plasmodium falciparum*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri* after ACT treatment of asymptomatic Ghanaian school-children. Int J Parasitol Drugs Drug Resist. 2013;3:45–50.
29. Bruce MC, Donnelly CA, Alpers MP, Galinski MR, Barnwell JW, Walliker D, et al. Cross-species interactions between malaria parasites in humans. Science. 2000;287:845–8.
30. Bruce MC, Day KP. Cross-species regulation of malaria parasitaemia in the human host. Curr Opin Microbiol. 2002;5:431–7.
31. Pascarella S, Negro F. Hepatitis D virus: an update. Liver Int. 2011;31:7–21.
32. Ho E, Deltenre P, Nkuize M, Delwaide J, Colle I, Michielsen P. Belgian Association for the study of the liver. Coinfection of hepatitis B and hepatitis delta virus in Belgium: a multicenter BASL study. Prospective epidemiology and comparison with HBV mono-infection. J Med Virol. 2013;85:1513–7.
33. Yurdaydin C, Idilman R, Bozkaya H, Bozdayi AM. Natural history and treatment of chronic delta hepatitis. J Viral Hepat. 2010;17:749–56.
34. Kasehagen LJ, Mueller I, McNamara DT, Bockarie MJ, Kiniboro B, Rare L, et al. Changing patterns of *Plasmodium* blood-stage infections in the Wosera region of Papua New Guinea monitored by light microscopy and high throughput PCR diagnosis. Am J Trop Med Hyg. 2006;75:588–96.
35. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med. 1976;295:302–4.
36. Fru-Cho J, Bumah VV, Safeukui I, Nkuo-Akenji T, Titanji VP, Haldar K. Molecular typing reveals substantial *Plasmodium vivax* infection in asymptomatic adults in a rural area of Cameroon. Malar J. 2014;13:170.
37. Giha HA, Theander TG, Staalso T, Roper C, Elhassan IM, Babiker H, et al. Seasonal variation in agglutination of *Plasmodium falciparum*-infected erythrocytes. Am J Trop Med Hyg. 1998;58:399–405.
38. Roper C, Elhassan IM, Hviid L, Giha H, Richardson W, Babiker H, et al. Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. Am J Trop Med Hyg. 1996;54:325–31.