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**Title: Serological and molecular tools for the evaluation of
malaria transmission blocking vaccines**

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**Thesis submitted in accordance with the requirements for the
degree of Doctor of Philosophy (PhD) of the University of
London**

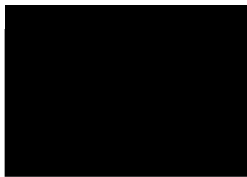
September 2014

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Funded by: The European FP7 Project (#242079)

Declaration of own work

I, Sophie Jones, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



Signed:

(SOPHIE JONES)

September 2014

Acknowledgements

I am extremely grateful to my supervisors Teun Bousema and Chris Drakeley for providing me with the opportunity to do this project and for all their guidance, support, training and encouragement over the years, all of which were supplied with humour! I am thankful to them for arranging funding and am very grateful to The European FP7 Project for funding this research.

I would like to express thanks to the team at Gennova Biopharmaceuticals Limited for supplying me with the vaccine antigens derived from Pfs48/45, and to Fraunhofer Center for Molecular Biotechnology for sharing their vaccine candidate 230CMB. I wish to thank Will Roeffen and Spencer Polley for being my advisory committee and providing valuable training and support over the years. Special thanks to Patrick Corran for always being so willing to help, and to Kevin Tetteh for ongoing advice and mentoring. I am very grateful to Carolynne Stanley for help with countless complicated orders and shipping over the years.

The work described in this thesis would not have happened without the help of many people. Special acknowledgements go to all the study participants, without them this research would not be possible. I am grateful to staff at Noguchi Memorial Institute of Medical Research in Ghana, for sample collection, slide reading and allowing me to host a training course at their institute. I would also like to thank the staff at Kilimanjaro Clinical Research Institute in Tanzania for sample collection and slide reading. Special thanks to staff at Centre National de Recherche et de Formation sur le Paludisme in Burkina Faso, not only for hosting me during my field work but for performing mosquito husbandry, slide reading, and oocyst counting. I am extremely grateful for all your hard work and kind hospitality. I am also thankful to the team at Mulago Hospital in Uganda for accommodating me for my stay in the lab.

I am also appreciative to Professor Robert Sauerwein for repeatedly hosting me in his department, and to my friends in Nijmegen, Amrish, Will, Evelinn, Simon and Anja, who have always made me feel so welcome.

I wish to express my gratitude to David, Will, Bronner and Spencer for kindly reading parts of my thesis, and to Michael, Bronner (again!) and Gillian for help with statistics over the years.

Special thanks to David, Michael, Sarah, Bekah, Ali, Laura, Christian, Kathrin, Bronner and Lynn. Your friendship and moral support has kept me going.

I wish to thank my parents and Ellen for all your encouragement and for being such a solid support system over the years. Finally, I am especially grateful to Trevor, not only for relocating to the UK so I could do this project, but for your affection and care, and for always believing in me.

Abstract

Malaria transmission blocking vaccines (TBV) have been prioritized as an intervention to facilitate malaria elimination, but tools are required to support roll-out and evaluation. This thesis presents research that aids development of pre-fertilization TBV candidate 10C (amino acids 159-428 of Pfs48/45).

Gametocytes must be detected to identify the infectious reservoir and support mosquito infectivity studies. I proposed filter papers as a novel, cost effective, practical approach for collection and detection of mRNA in low density gametocytes. Comparing 3 filter papers, 2 RNA extraction methods and 2 molecular detection techniques, I concluded Whatman 903 and Whatman 3MM filter papers, combined with guanidine based nucleic acid extraction and detection using QT-NASBA, were operationally most appealing and most sensitive.

To identify natural recognition to 10C and 230CMB (a vaccine candidate including amino acids 444-730 of Pfs230), cross sectional surveys were performed sampling school children (n=510) in 3 countries. I demonstrated naturally exposed individuals had antibodies against 10C and 230CMB which displayed age dependent acquisition ($p \leq 0.03$). Supportive datasets demonstrated 10C and 230CMB antibodies are significantly associated with $\geq 90\%$ transmission reducing activity (TRA) ($p \leq 0.003$).

To assess the TRA of 10C-immunized rats against genetically diverse parasites, I sampled venous blood from naturally infected participants in Burkina Faso (n=53), and performed direct membrane feeding assay combined with serum replacement using European control serum spiked with IgG from 10C vaccinated rats. I demonstrated 10C vaccine induced IgG significantly reduced transmission in 4/5 participants who were infectious and infected >2 mosquitoes. This resulted in 80.9-100% reduction in oocyst prevalence ($p \leq 0.042$), and 85.2-100% reduction in oocyst density ($p \leq 0.023$).

My research identified an attractive combination of tools for detecting low density gametocytes to facilitate sampling in remote field settings. I advanced progress of

10C vaccine candidate by indicating antibodies are acquired following natural malaria exposure and are associated with functional TRA.

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My publications

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Abbreviations

°C	degrees Celsius
µg	micrograms
µL	microlitre
µM	micromolar
ACT	artemisinin combination therapy
AL	artemether-lumefantrine
AMA-1	apical membrane antigen -1
AMV-RT	avian myeloblastosis virus reverse transcriptase
AU	arbitrary units
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CMB	Fraunhofer Center for Molecular Biotechnology
CNRFP	Centre National de Recherche et de Formation sur le Paludisme
CSP	circumsporozoite protein
DMFA	direct membrane feeding assay
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EBA	erythrocyte binding antigen
ECL	electrochemiluminescence
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetraacetic acid
EIR	entomological inoculation rate
ELISA	enzyme linked immunosorbent assay
EXP	exported protein

g	gram
<i>g</i>	g force
GLURP	glutamate rich protein
GPI	glycosylphosphatidylinositol
GPS	global positioning system
GuSCN	guanidine thiocyanate
g/μL	gametocytes per microlitre
H ₂ SO ₄	sulphuric acid
H ₂ O	water
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HNB	hydroxy naphthol blue
HRP	horseradish peroxidase
HRP2	histidine rich protein 2
Ig	immunoglobulin
IQR	interquartile range
IRS	indoor residual spraying
ITN	insecticide treated nets
kb	kilobase
LSA	liver stage antigen
LSHTM	London School of Hygiene and Tropical Medicine
MBP	maltose binding protein
MDA	mass drug administration
MDM	magnetic deposition microscopy
mg	milligram
mL	millilitre
mM	millimolar
mRNA	messenger RNA
MSP	merozoite surface protein

MTBV	malaria transmission blocking vaccine
MVI	Malaria Vaccine Initiative
NAI	naturally acquired immunity
NASBA	nucleic acid sequence based amplification
NB	nota bene
ng	nanogram
nPCR	nested PCR
NPV	negative predictive value
NS	not statistically significant
OD	optical density
OPD	O-phenylene diamine
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PfEMP-1	<i>Plasmodium falciparum</i> erythrocyte membrane protein-1
PKU	Phenylketonuria
pLDH	<i>Plasmodium</i> lactate dehydrogenase
PPV	positive predictive value
PVC	polyvinyl chloride
QBC	quantitative buffy coat
QT-NASBA	quantitative nucleic acid sequence based amplification
RDT	rapid diagnostic test
RESA	ring-infected erythrocyte surface antigen
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
RT	room temperature
RT-LAMP	reverse transcription loop mediated isothermal amplification

RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMFA	standard membrane feeding assay
SNP	single nucleotide polymorphism
SOP	standard operating procedures
SP	sulfadoxine-pyrimethamine
SP166	synthetic peptide 166
TBE	Tris Borate
TBV	transmission blocking vaccine
TMB	tetramethylbenzidine substrate
TNF- α	tumour necrosis factor alpha
TPP	target product profile
TR	transmission reducing
TRA	transmission reducing activity
TRAP	thrombospondin-related adhesion protein
TTP	time to positivity
UK	United Kingdom
USA	United States of America
USD	United States dollars
UV	ultra violet
VIMT	vaccine that interrupts malaria transmission
VLP	virus like particle
WBC	white blood cell
WHO	World Health Organization

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1.1 Parasite biology

1.11 Malaria pathology and parasite life cycle

Malaria continues to be a serious global health problem with approximately half the world population at risk (World Health Organization 2011). In 2010, there were an estimated 225 million clinical malaria episodes, not accounting for multiple episodes in the same individual, resulting in around 660,000 deaths and a financial cost of approximately 12 billion USD (World Health Organization 2011). Distributed across 106 countries worldwide, malaria is mostly found in tropical climates with favourable conditions that support the life cycle of both the insect vector responsible for transmission, and the *Plasmodium* parasites (Karl, David et al. 2008; Hay, Okiro et al. 2010; World Health Organization 2010; World Health Organization 2011). In 2010, approximately 91% of all malaria related deaths occurred within the Africa continent, with the majority of the disease burden located in Sub-Saharan Africa where fatalities are predominantly among pregnant women and children under 5 years of age (Hay, Okiro et al. 2010; World Health Organization 2011). Often, malaria endemic regions are in developing countries which struggle to finance the healthcare infrastructure required to effectively control such a pathogenic and infectious disease (Williams, Hering et al. 2013). The healthcare cost to these countries severely undermines economic stability, thereby hindering further development (Doolan, Dobano et al. 2009).

Malaria is a vector borne protozoan parasitic disease with 6 main species capable of infecting humans, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri* and *Plasmodium knowlesi* (Sutherland, Tanomsing et al. 2010; Lau, Fong et al. 2011; Lee, Divis et al. 2011). *P. ovale* was recently recognized as having two distinct sub-species that may exhibit varying symptom severity (Sutherland, Tanomsing et al. 2010). *P. knowlesi* was more

recently identified as an important human pathogen, but long tailed macaques are the natural reservoir (Singh, Kim Sung et al. 2004). Plasmodia are protozoa belonging to the family Apicomplexa and have morphologically distinct species which differ in disease severity (CDC 2010). While *P. vivax* is more widely distributed, *P. falciparum* is responsible for the most morbidity and over 90% of malaria related deaths, and is the species this thesis focuses on (Bousema and Drakeley 2011; WHO 2013).

The symptoms caused by *P. falciparum* are the consequence of the asexual proliferation cycle which occurs within the human host's erythrocytes (Wipasa, Elliott et al. 2002). Symptoms appear approximately 7-28 days post sporozoite inoculation and typically begin with fever, chills, headache, vomiting and general malaise (CDC 2010). If not treated within 24 hours, parasites can rapidly proliferate and disease can quickly progress to complicated infection resulting in severe anaemia, renal failure, cerebral malaria, acute respiratory distress, coma, and death (Beeson and Brown 2002; CDC 2010). Complicated infection can manifest as a result of delayed drug treatment (or due to treatment failure), but is also common in 'at risk' groups who have limited immunity such as children under the age of 5, immunocompromised individuals, or pregnant women who are at risk of developing placental malaria (Meerman, Ord et al. 2005). Plasmodia have a complex life cycle within the human host, and insect vector responsible for disease transmission (Figure 1.0.).

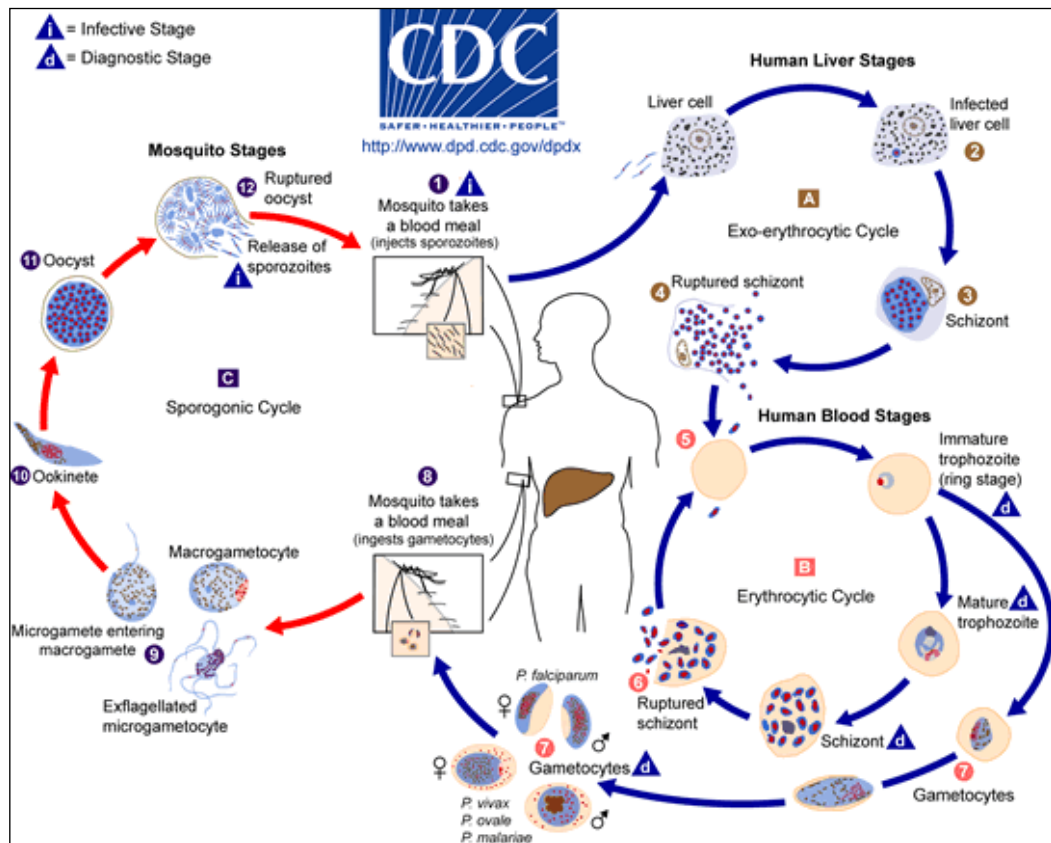


Figure 1.0. The life cycle of *Plasmodium* species. (Figure taken directly from the referenced source (CDC 2012))

The asexual cycle takes place within the human host and generates parasites with a haploid genome, whereas the sexual reproduction cycle, which occurs in the insect vector, results in parasites with a diploid genome (Gonzales, Patel et al. 2008). Malaria is transmitted when an infected female Anopheline mosquito takes a blood meal from a human. When feeding, a parasitized mosquito will inject up to several hundreds of sporozoites from its salivary glands (which harbour the infectious parasites) into the human dermis where they may reside for up to 6 hours (CDC 2012). After this period, the sporozoites migrate and enter the blood stream or lymph system (Amino, Thiberge et al. 2006; Yamauchi, Coppi et al. 2007; Doolan, Dobano et al. 2009). Pre-erythrocytic parasite stages, sporozoites and liver stage parasites express surface antigens including circumsporozoite protein (CSP), thrombospondin-related adhesion protein (TRAP), liver stage antigen 1 and 3 (LSA1,

LSA3) and exported protein 1 (EXP1) (Winzeler 2006; Kafsack, Rovira-Graells et al. 2014).

Once inside a human host, sporozoites migrate to hepatocytes, where they may traverse several cells before finally infecting one to undertake the exo-erythrocytic cycle (CDC 2010; Offeddu, Thathy et al. 2012). Inside hepatocytes, over approximately 1 week, sporozoites develop into mature schizonts, which harbour merozoites. The schizonts burst to release up to 30,000 merozoites which enter the blood stream where they invade erythrocytes, the site of the asexual proliferation cycle (CDC 2012). During the asexual cycle, the parasite expresses antigens such as apical membrane antigen -1 (AMA-1), *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1), merozoite surface protein-1-5 (MSP-1-5), ring-infected erythrocyte surface antigen precursor (RESA) and glutamate rich protein (GLURP) (Wipasa, Elliott et al. 2002; Hill 2011).

Within the erythrocytes, merozoites enter a cycle beginning with maturation into trophozoites, which then develop over days to form into schizonts (CDC 2010). Mature schizonts erupt to produce more merozoites, ensuring the continuation of the asexual cycle in the human host. A small proportion of newly released merozoites leave the asexual cycle to develop into male and female gametocytes; a process which may be regulated by DNA-binding protein PfAP2-G (Kafsack, Rovira-Graells et al. 2014). Gametocytes are the sexual stage of the parasite that are not associated with clinical symptoms but are solely responsible for transmission to the insect vector (Bousema and Drakeley 2011). Gametocytes are produced approximately 7-15 days following the first asexual reproduction cycle, and appear in five morphologically different developmental stages (Talman, Domarle et al. 2004; Drakeley, Sutherland et al. 2006). Developing inside the erythrocyte, gametocytes gradually increase in size until occupying the entire cell (Baker 2010). Due to the presence of a microtubular cytoskeleton and double membrane, gametocyte infected erythrocytes can be identified by their distinctive curved shape (Meszoely, Erbe et al. 1987). Early stage gametocytes (I-IV) are not found in human peripheral blood, but instead sequester within cells in the bone marrow, and possibly the spleen

(Sinden and Smalley 1979; Bousema and Drakeley 2011; Peatey, Watson et al. 2013). Sequestration is thought to be a tactic that enables gametocyte maturation to occur undisturbed in regions with low blood flow where they will not be targeted and cleared by host immunity (Wang, Mwakalinga et al. 2010; Bousema and Drakeley 2011; Farfour, Charlotte et al. 2012; Peatey, Watson et al. 2013; Aguilar, Magallon-Tejada et al. 2014). From stage V onwards, gametocyte sex can be determined and females identified by a smaller nucleus and more concentrated pigment and, depending on the staining procedure, a different coloured cytoplasm (Bousema and Drakeley 2011). During the first 6 days of their development, gametocytes appear to be metabolically inactive, with nucleic acid synthesis restricted to just ribonucleic acid (RNA) synthesis. Here, mRNA is synthesized for proteins such as P25 and P28 that will be translated once inside the mosquito midgut (Baker 2010; Bousema and Drakeley 2011).

Following maturation, stage V gametocytes emerge from sequestration and can be found in sub dermal capillaries, the optimal site for ingestion by a female mosquito when it takes a blood meal (Lensen, Bril et al. 1999; Bousema and Drakeley 2011). Once released into the blood stream, a lag period of several days must pass until gametocytes become infectious to mosquitoes (Bousema, Okell et al. 2010; Bousema and Drakeley 2011). When released into the blood stream, gametocytes circulate for an average of 4-6 days (Bousema, Okell et al. 2010).

Mosquitoes can become infected if they passively ingest infectious mature gametocytes when taking a blood meal (CDC 2012). Once inside the mosquito, gametocytes become spherical gametes, and discard the membrane of the erythrocyte they developed in (Bousema and Drakeley 2011). Male gametes then undergo 3 rounds of deoxyribonucleic acid (DNA) replication to produce up to 8 male microgametes, whereas females produce 1 macrogamete (Paul, Brey et al. 2002; Aly, Vaughan et al. 2009). Gametocyte activation to become gametes is thought to be stimulated by the different environment in the mosquito, specifically, a lower temperature, the presence of xanthurenic acid or a more alkaline surrounding (Bousema and Drakeley 2011; Guttery, Holder et al. 2012). The sexual replication

cycle begins with gametes proceeding to the mosquito midgut where they fuse to generate a diploid zygote, which undergoes meiosis to form a motile ookinete, which then penetrates the midgut wall (CDC 2010). Inside the midgut wall, ookinetes develop into oocysts which enlarge until they eventually rupture to release sporozoites which migrate to mosquito salivary glands, ready to be inoculated into another human host during the next blood meal (CDC 2010).

Plasmodium produces an array of antigens to ensure development and survival in the mosquito midgut. Within these, known gametocyte antigens can be split into pre and post fertilization antigens, depending on whether they are present before or after fertilization of female macrogametes by exflagellated male microgametes in the mosquito midgut (Bousema and Drakeley 2011). Pre-fertilization antigens include Pfs48/45 and Pfs230 and post fertilization antigens include Pfs25 and Pfs28, which are discussed in more detail in section 1.55.(Drakeley, Sutherland et al. 2006; CDC 2010).

1.2 Malaria epidemiology

1.21 Malaria transmission and the human infectious reservoir

While asexual parasites are responsible for the symptoms associated with malaria, gametocytes are responsible for transmission. Infectious gametocyte carriers form the infectious reservoir that maintains and seeds transmission (Bousema and Drakeley 2011). While gametocytes do not cause symptoms, gametocyte carriers can be both symptomatic or asymptomatic malaria-infected individuals, the symptoms being attributed to concurrent asexual parasite carriage (Bousema and Drakeley 2011). Gametocytes were once thought to be uncommon in malaria infections, but the development of sensitive molecular detection methods revealed they are present in most infections, but often circulating at low (submicroscopic) densities, where they escape detection by microscopy, the routine method for asexual parasite

detection (Carter 1980; Ouedraogo, Bousema et al. 2009; Ouedraogo, Bousema et al. 2010). Gametocytes only comprise a small proportion of all parasites that are present in infections with approximately 1 produced for every 156 asexual parasites (Eichner, Diebner et al. 2001; Bousema and Drakeley 2011). To some extent, gametocyte carriage mirrors asexual parasite dynamics. For example, high density gametocyte prevalence (detectable by microscopy) mimics patterns of asexual parasite prevalence, peaking during the high transmission season and following waves of asexual parasites (Boudin, Lyannaz et al. 1991). In contrast, submicroscopic gametocytes are often circulating at low levels for a much longer duration (Shekalaghe, Bousema et al. 2007).

Submicroscopic gametocyte carriers may still have a significant impact on transmission with extremely low densities of 0.1-10/ μ L capable of infecting mosquitoes (Carter 1980; Pradel 2007; Schneider, Bousema et al. 2007; Ouedraogo, Bousema et al. 2009). It is not currently well understood who within the population is the most important contributor to the human infectious reservoir. While all gametocyte positive individuals should be considered capable of transmission, and submicroscopic infections certainly play a role, higher density infections (those microscopically detected) are more likely to infect mosquitoes and could therefore comprise a larger proportion of the human infectious reservoir (Ouedraogo, Bousema et al. 2009).

Gametocyte presence is influenced by malaria transmission intensity, season, age, duration and type of symptoms, anti-malarial drugs and other host and external influences (Jones, McElroy et al. 1997; Hogh, Gamage-Mendis et al. 1998; Price, Nosten et al. 1999; Robert, Awono-Ambene et al. 2000; Sokhna, Trape et al. 2001; Targett, Drakeley et al. 2001; Tjitra, Suprianto et al. 2002; Bousema and Drakeley 2011). Malaria infections are complex but can be broadly categorized according to symptoms, symptomatic or asymptomatic, and their parasite densities, referred to as detected by microscopy, or too low to be detected by microscopy (submicroscopic) (Lin, Saunders et al. 2014). The consensus opinion is that gametocytes are more likely to be seen in symptomatic infection where rounds of asexual proliferation, typical of

symptomatic disease, are followed by an increase in gametocyte prevalence and density (Drakeley, Akim et al. 2000; Bousema, Gouagna et al. 2004; Dunyo, Milligan et al. 2006; McKenzie, Jeffery et al. 2007; Ouedraogo, Schneider et al. 2007).

However, research conducted in The Gambia and Nigeria found there to be a positive association between gametocyte carriage and a lack of fever (von Seidlein, Drakeley et al. 2001; Sowunmi, Fateye et al. 2004; Bousema and Drakeley 2011).

Studies have also shown that recrudescence infections can result in higher gametocyte prevalence (Price, Nosten et al. 1999; Robert, Awono-Ambene et al. 2000; Sokhna, Trape et al. 2001; Sowunmi, Fateye et al. 2004). Similarly, haemoglobinopathies may impact on gametocyte carriage with genetic differences in the B-globin of haemoglobin resulting in HbAS, HbAC, HbCC genotypes which are associated with increased gametocytaemia (Robert, Tchuinkam et al. 1996; Gouagna, Banccone et al. 2010; Bousema and Drakeley 2011). Differences in gametocyte densities have also been observed between ethnic tribes in Burkina Faso, with individuals belonging to the Fulani tribe generally carrying lower densities than individuals in the sympatric Mossi tribe, a phenomenon thought to be attributed to a stronger immunoglobulin G (IgG) response (Paganotti, Palladino et al. 2006; Bousema and Drakeley 2011; Tiono, Sirima et al. 2013).

Gametocytes are a product of asexual stage parasites. Following exposure to parasites, individuals acquire asexual stage immunity which can reduce asexual parasite density and therefore also affect gametocyte presence (Bousema, Gouagna et al. 2004). Figure 1.1. presents the prevalence of microscopically detectable gametocytes by age group in different regions of endemicity. In high endemic settings, younger children have yet to acquire asexual parasite immunity and therefore have higher asexual parasite densities, which results in the generation of more gametocytes (Molineaux and Gramiccia 1980; Drakeley, Bousema et al. 2006; Bousema and Drakeley 2011). For this reason, children under the age of 10 tend to harbour more microscopically detectable gametocyte infections and have been suggested to form the main human infectious reservoir (Boudin, Lyannaz et al. 1991;

Githeko, Brandling-Bennett et al. 1992; Toure, Doumbo et al. 1998; Drakeley, Akim et al. 2000; Mabunda, Casimiro et al. 2008; Ouedraogo, Bousema et al. 2010).

Since immunity to the asexual parasite stages is acquired with cumulative exposure which is inherently linked with age, this results in an age dependent decrease of gametocyte prevalence (Drakeley, Sutherland et al. 2006). It has been suggested this reduction in gametocyte carriage with age could also be attributed to sexual stage immunity that is associated with clearance of gametocytes from the blood stream although this requires confirmation, and identification of the responsible gametocyte antigens (Bousema, Gouagna et al. 2004; Saeed, Roeffen et al. 2008).

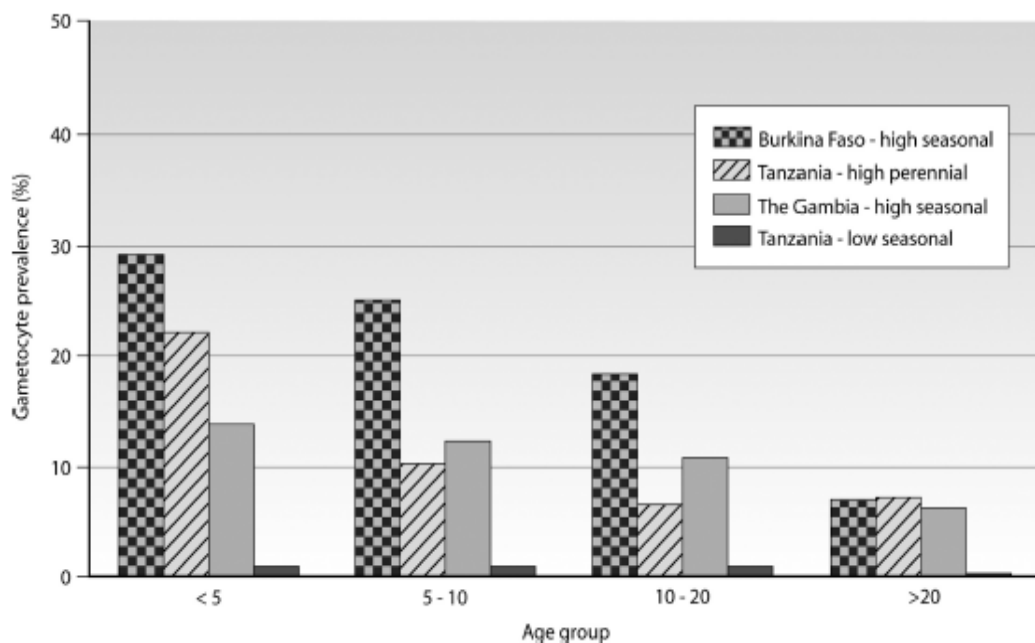


Figure 1.1. Gametocyte prevalence by age group in populations sampled from different endemic settings in Burkina Faso, Tanzania and The Gambia. Gametocyte prevalence was determined by microscopy. Figure taken directly from the referenced publication. (Bousema and Drakeley 2011).

In low transmission or low seasonal transmission regions asexual stage immunity may wane between seasons and the result is no distinct relationship between age and gametocyte carriage, and similar proportions of each age group are

gametocytaemic (Figure 1.1.) (Bousema and Drakeley 2011). In areas with marked seasonality, gametocyte prevalence during the dry season may be low, and chronic low density parasite carriage may be responsible for maintaining the infectious reservoir to the following season (Babiker, Abdel-Muhsin et al. 1998; Abdel-Wahab, Abdel-Muhsin et al. 2002; Nassir, Abdel-Muhsin et al. 2005; Bousema and Drakeley 2011).

1.22 The flexibility of gametocyte production

Despite the low prevalence of gametocytes in the dry season, within these sparse infections the proportion of all parasites that are gametocytes may be relatively high (Bousema and Drakeley 2011). This suggests a relative shift to gametocyte production, which may be a strategy to ensure transmission still occurs during the dry season (Boudin, Lyannaz et al. 1991; Drakeley, Sutherland et al. 2006; Mair, Lasonder et al. 2010).

While the highest gametocyte densities are found in younger age groups, the proportion of total parasites that are gametocytes is lowest within this age group, with approximately only 2% of their total parasite load being gametocytes (Ouedraogo, Bousema et al. 2010). In contrast, adults have lower total parasite numbers, but a higher proportion of both microscopic and submicroscopic gametocytes amounting to 15% of their overall parasite burden (Drakeley, Sutherland et al. 2006; Ouedraogo, Bousema et al. 2010; Bousema and Drakeley 2011). This complex dynamic (Figure 1.2.) may be considered a strategy to ensure transmission is ongoing even when parasite densities are low (Roper, Elhassan et al. 1996). However, the data revealing this pattern was derived from a high transmission setting and this finding requires confirmation in other levels of endemicity and preferably should be supported by detection of asexual parasites that are committed to the gametocyte pathway (Bousema and Drakeley 2011).

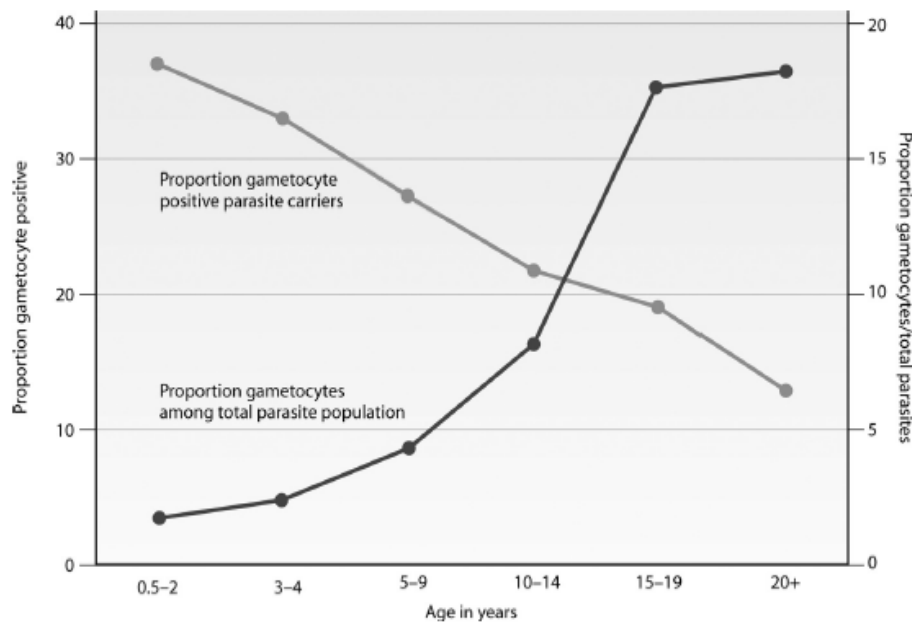


Figure 1.2. The relationship between age and gametocyte carriage in Burkina Faso (Ouedraogo, Bousema et al. 2010). Gametocyte prevalence was determined by microscopy, Figure taken directly from the referenced publication.

1.23 Who is infectious to mosquitoes?

The human infectious reservoir (the proportion of the human population that is capable of infecting mosquitoes) is determined by gametocyte carriage, infectivity, mosquito biting patterns and the relative contribution of different age groups to the total human population (Bousema and Drakeley 2011). Generally, there is an increasing relationship between gametocyte density and prevalence of infection in mosquitoes; however, submicroscopic gametocyte densities, including those undetectable using molecular methods, have been demonstrated to result in infection, both in the field, and under laboratory conditions (Jeffery and Eyles 1955; Bonnet, Gouagna et al. 2000; Schneider, Bousema et al. 2007). A study performing membrane feeding assays in Kenya demonstrated 4.1% of mosquitoes were infected by submicroscopic infection compared to 8.3% infected by microscopically detected gametocytes (Schneider, Bousema et al. 2007). The authors suggested because submicroscopic carriers are more prevalent, their contribution to the infectious

reservoir is in fact comparable to that of microscopically detectable parasites (Schneider, Bousema et al. 2007).

Body size, human behaviour and foot odour are among the variables indicated to influence mosquito biting behaviour (Bousema and Drakeley 2011; Omolo, Njiru et al. 2013). Despite having lower gametocyte densities, since adults have more body mass this increases the likelihood of mosquito contact and therefore feeding, so their influence on transmission must not be underestimated (Drakeley, Sutherland et al. 2006; Bousema, Drakeley et al. 2007; Ouedraogo, Bousema et al. 2010).

Furthermore, as adults in high endemic settings are likely to be asymptomatic carriers, they may not seek treatment and will continue to be potential transmitters of parasites (Ganguly, Saha et al. 2013). Consequently, as asymptomatic infections are often long lasting, this increases the likelihood of gametocyte presence and increases the likelihood of human-mosquito contact (Bousema and Drakeley 2011). Human behavioural patterns also influence who is likely to be bitten. For example, studies have revealed if households have a bed net, it will not necessarily be used for children; since they are likely to be sleeping at dusk when mosquitoes start feeding, this makes them especially vulnerable to being bitten (Korenromp, Miller et al. 2003). In a similar vein, adults are likely to be awake (at least for part of the mosquito feeding time) and therefore also unprotected by a bed net (Korenromp, Miller et al. 2003).

Attempts have been made to identify which age groups are most infectious to mosquitoes. With this knowledge, targeted control interventions could be adopted (WHO 2012; Sturrock, Hsiang et al. 2013). Studies looking at populations in Burkina Faso, The Gambia and Tanzania suggested different groups were contributing most to the infectious reservoir. The study in Burkina Faso concluded all age groups contributed equally, whereas data from The Gambia suggested those over the age of 20 were most infectious, and the Tanzanian research concluded 1-4 year olds and those over the age of 20 were most infectious (Boudin, Olivier et al. 1993; Drakeley, Akim et al. 2000). Since these studies were performed, the advent of sensitive molecular gametocyte detection methods has enabled investigation of the impact of

submicroscopic carriers, by age, to gain a more complete understanding of infectiousness.

1.3 Natural infections and acquisition of immunity

1.31 Acquired immunity against asexual stages of malaria

As touched on briefly, antibodies may develop and immunity gained against asexual parasite stages following natural infection. The concept of naturally acquired immunity (NAI) was first introduced by Robert Koch in 1900 when he examined prevalence and density of parasites in blood films from regions of high and low malaria endemicity, and deduced that immunity against malaria was acquired following uninterrupted and heavy exposure to parasites (Koch 1900; Koch 1900; Koch 1900; Koch 1900; Doolan, Dobano et al. 2009). By 1920, it had been recognized that NAI was gained in relation to the intensity of the exposure, lost when exposure is removed, and was specific to the species and parasite stage (Sergente 1910; Schuffner 1919).

Since immunity is acquired following exposure, which accumulates over time, the outcome is an age dependent antibody acquisition pattern, which results in an age dependent decrease in vulnerability to severe disease (Doolan, Dobano et al. 2009). As malaria exposure varies with season, and transmission intensity, both of these impact on the dynamics of antibody acquisition (Drakeley, Corran et al. 2005; Doolan, Dobano et al. 2009). Age dependent immunity develops throughout life, first manifesting as clinical immunity, which decreases parasite pathogenicity and associated symptoms, then eventually leading to anti-parasitic immunity, where asexual parasites are actively controlled (Baird 1995; Good 2001; Doolan, Dobano et al. 2009). The relationship between antibodies and derived protection is complex and the presence of antibodies or a high antibody titre does not necessarily equate to protection, or a high level of protection, respectively (Struik and Riley 2004).

1.32 Immunity in high transmission regions

Immunity is acquired in different stages. Newborn babies are generally protected for approximately their first 6 months by passively acquired maternally derived IgG, obtained in the final trimester of pregnancy (Riley, Wagner et al. 2000). During the first 18 weeks post birth, this immunity protects from high parasitaemia with infections appearing as asymptomatic, and generally resolving quickly (Franks, Koram et al. 2001; Doolan, Dobano et al. 2009). After 18 weeks, maternal antibodies begin to wane and there is a significant increase in risk of severe infection as active immunity has yet to develop (Wagner, Koram et al. 1998; Riley, Wagner et al. 2000). This leaves young children extremely vulnerable to severe disease and death and this age group carries the highest parasitaemias, but is also susceptible to symptomatic infection from very low densities (McGuinness, Koram et al. 1998). However, after surviving infections, infants acquire their own immunity that becomes more protective following subsequent exposures (Doolan, Dobano et al. 2009).

Anti disease immunity develops next which protects against clinical disease, reducing the extent of morbidity (Marsh and Kinyanjui 2006). The functionality and memory of immunity seems variable, with no clear association between the number or duration of parasite exposures and the level of protection induced; in some individuals a single malaria infection is sufficient to protect against death and clinical disease, whereas for others, multiple infections will only induce limited protection (Struik and Riley 2004). Generally, in a high transmission setting, the risk of severe malaria is highest in the first years of life, which reduces the risk of clinical disease at approximately 2-5 years of age (Doolan, Dobano et al. 2009).

In older children and adults, anti-parasite immunity develops which controls parasite densities and protects from severe disease (Baird, Owusu Agyei et al. 2002). While mild clinical infections may still be common, infections are often asymptomatic (Doolan, Dobano et al. 2009). This is thought to be due to lower parasite density,

although recent evidence challenges an exact relationship between parasite density and severity of symptoms (Goncalves, Huang et al. 2014). As immunity builds, parasite densities in subsequent infections become progressively lower and lower (Langhorne, Ndungu et al. 2008).

Recent studies have reiterated that while high levels of clinical immunity to blood stage parasites is common among adults in high endemic settings, there is no evidence of naturally acquired sterile immunity, even though it can be experimentally induced in human and mouse models (Nussenzweig, Vanderberg et al. 1967; Hoffman, Goh et al. 2002; Roestenberg, McCall et al. 2009; Tran, Li et al. 2013). While possible that partial immunity is acquired against sporozoites or liver stage parasites, the theory that sterile immunity is not gained is supported by the repeated re-infections commonly seen (when using molecular methods to determine parasite prevalence) in high endemic settings among all age groups (Tran, Li et al. 2013). Though there is speculation about the role of genetic polymorphisms or low density sporozoite inoculation in preventing the natural development of sterile immunity, it largely remains a mystery why such immunity can be induced experimentally, and not through natural infection (Barry, Schultz et al. 2009; Offeddu, Thathy et al. 2012). It is certainly of interest to vaccine developers to gain an understanding of the underlying mechanisms leading to sterile immunity (Tran, Li et al. 2013).

1.33 Immunity in low transmission regions

In regions with low or unstable transmission of malaria, the entomological inoculation rate (EIR) is often between 1-5 per year, which slows the acquisition of protective immunity ((WHO) 2010). Consequently, acute clinical cases are prevalent in all age groups, including older children and adults (Carneiro, Roca-Feltrer et al. 2010; Griffin, Ferguson et al. 2014). Furthermore, the risk of cases progressing to

severe malaria (if untreated) is high, and the populations living in these settings are at risk from epidemics((WHO) 2010).

While it is expected that individuals in low transmission settings would not benefit from the same degree of immunity experienced in high transmission areas, and that asymptomatic infections including those harbouring submicroscopic parasites would be less common, recent studies challenge these ideas (Okell, Ghani et al. 2009; Okell, Bousema et al. 2012). A study by Baliraine *et al*, discovered a high prevalence of asymptomatic infections, including those detected by microscopy in a region of hypo-endemicity in Kenya (Baliraine, Afrane et al. 2009). Similarly, findings from Okell and colleagues showed submicroscopic parasite carriage is not only more common among adults in low transmission settings but accountable for 20-50% of mosquito infections (Okell, Bousema et al. 2012). The authors hypothesized that low density asymptomatic carriage was due to older and monoclonal infection; they suggested when individuals become infected with a second clone, this results in a higher parasite density and clinical disease (Okell, Bousema et al. 2012). Leading from this they speculated that super infections, typical of high endemic areas, are the cause of higher parasite densities, and that acquired immunity may only have partial cross protection against different clones (Okell, Bousema et al. 2012).

1.34 Serological markers of exposure

Along with protecting from the clinical consequences of malaria infection, antibodies are also valuable markers of malaria exposure. While malaria transmission can be measured using parasite prevalence data, EIR or clinical data, these indicators can be subject to seasonal and geographical variation (Cook 2010; Cook, Reid et al. 2010). Monitoring of serological responses is cheaper, simpler to sample (since filter papers samples can be used), and is more stable due to antibody longevity (Cook, Reid et al. 2010). Asexual serological markers such as AMA-1, GLURP and MSP-1 are all routinely measured for this purpose using enzyme linked immunosorbent assay

(ELISA) (Corran, Cook et al. 2008; Drakeley and Cook 2009; Cook, Reid et al. 2010; Proietti, Pettinato et al. 2011; Stone, Bousema et al. 2012; Proietti, Verra et al. 2013).

The rate of antibody acquisition (seroconversion) to these asexual stage antigens can be modelled to demonstrate the force of infection as indicator of transmission intensity (Drakeley, Corran et al. 2005). In high transmission regions seroconversion occurs faster, and consequently younger age groups are more likely to be antibody positive, whereas in low transmission regions, the rate of antibody acquisition is slower, and the age of seroconversion may therefore be older (Drakeley, Corran et al. 2005). These differences across transmission settings also reflect the time between infections, which is longer in low transmission settings (therefore removing the stimulus for boosting), whereas individuals in high transmission regions may suffer from chronic low parasite density carriage which acts to maintain their antibody levels (Drakeley, Corran et al. 2005). The rate of seroconversion varies by plasmodial antigen and it has been hypothesized that individuals seroconvert against AMA-1 after just one or two infections and that antibodies may circulate for a longer time (compared to those derived against other antigens) in absence of boosting (Drakeley, Corran et al. 2005). MSP-1 and GLURP antibody responses are generally lower than that for AMA-1 (Drakeley, Corran et al. 2005; Stone, Bousema et al. 2012). Since antibody levels decline in absence of parasite exposure, antibody prevalence and density can be used as a proxy to measure the impact of parasite reducing interventions (Cook, Reid et al. 2010). In this thesis serological markers of malaria exposure are measured to provide information about parasite exposure.

1.35 Evidence for naturally acquired sexual stage immunity from the field

Most gametocytes are not ingested by mosquitoes, but are instead removed by the human immune system which breaks down infected erythrocytes to expose gametocytes (and their surface proteins) which can result in the production of anti-gametocyte immune responses (Bousema and Drakeley 2011). Anti-gametocyte

immunity has been suggested to function in several ways, by clearing gametocytes from the site of sequestration, or from blood circulation or by blocking transmission to the mosquito (Bousema and Drakeley 2011).

Immunity hypothesized to clear gametocytes circulating within the human host can be split into gametocyte specific or non specific. It has been suggested gametocyte specific antibodies recognize molecules on the gametocyte infected erythrocyte, and serum reacting to this (demonstrated using immunofluorescence) has been linked with reduced gametocyte prevalence (Saeed, Roeffen et al. 2008; Sutherland 2009; Bousema and Drakeley 2011). Overall there is limited evidence for this theory. For non specific immunity, it has been suggested that immunity derived against molecules presented on the surface of the infected erythrocytes or trophozoites, such as PfEMP-1, could cross react with young gametocytes which may have identical adhesion molecules (Day, Hayward et al. 1998; Cham, Turner et al. 2009; Bousema and Drakeley 2011). This has been suggested to affect gametocyte maturation during sequestration (Day, Hayward et al. 1998; Cham, Turner et al. 2009; Bousema and Drakeley 2011). Once more, there is limited evidence to validate this hypothesis.

Whilst innate and cellular immune responses do play a role in immunity that effects the parasite's sexual stage (such as release of tumour necrosis factor (TNF)-alpha, which may reduce gametocyte infectiousness) antibody responses play the largest protective role (Naotunne, Karunaweera et al. 1991; Karunaweera, Carter et al. 1992; Bousema, Drakeley et al. 2006; Bousema and Drakeley 2011). As antibodies are produced against many of the surface sexual stage antigens, they have a larger impact, compared to cell-mediated immune responses (Bousema and Drakeley 2011).

Individuals in endemic settings may have antibodies against gametocyte proteins and for a subset of this population, these antibodies have been demonstrated to have functional transmission reducing activity (TRA) (Carter, Graves et al. 1989; Drakeley, Mulder et al. 1998; Healer, McGuinness et al. 1999; Struik and Riley 2004; Bousema and Drakeley 2011). This has been evidenced by a reduced oocyst density in the mosquito, which is demonstrated using membrane feeding assays, discussed further

in section 1.47.(Bousema and Drakeley 2011). This observation of naturally acquired TRA initiated interest in using gametocyte antigens as vaccine candidates that aim to reduce the parasite reservoir in the mosquito vector (Sauerwein 2007).

Pfs48/45 and Pfs230 are 2 gametocyte proteins that transmission blocking immunity develops against (Bousema, Roeffen et al. 2010). Pfs48/45 is concerned with gametocyte attachment during fertilization, and Pfs230 has a suggested role in arrangement of exflagellation centres, (function is further discussed in section 1.55.) (Vermeulen, Ponnudurai et al. 1985; Kocken, Jansen et al. 1993; Williamson, Criscio et al. 1993; van Dijk, Janse et al. 2001; Eksi, Czesny et al. 2006; van Schaijk, van Dijk et al. 2006; Bousema and Drakeley 2011). Membrane feeding assays, specifically the standard membrane feeding assay (SMFA) and direct membrane feeding assay (DMFA) have confirmed the TRA of high levels of antibodies against Pfs230 and Pfs48/45 in endemic sera from Africa and Australasia (Graves, Carter et al. 1988; Roeffen, Mulder et al. 1996; Healer, McGuinness et al. 1999; Mulder, Lensen et al. 1999; Drakeley, Eling et al. 2004; Bousema, Roeffen et al. 2006; Drakeley, Bousema et al. 2006; van der Kolk, de Vlas et al. 2006; Bousema, Drakeley et al. 2007; Bousema, Roeffen et al. 2010; Bousema and Drakeley 2011).

These results are promising, but not all studies found a relationship between antibody presence and TRA, with some indicating individuals with no antibodies against Pfs48/45 or Pfs230 still demonstrated TRA (Graves, Carter et al. 1988; Premawansa, Gamage-Mendis et al. 1994; Drakeley, Bousema et al. 2006; Bousema, Drakeley et al. 2007; Bousema, Roeffen et al. 2010). Results from a study performed in a low transmission region in Sri Lanka indicated there was no relationship between TRA and Pfs48/45 or Pfs230 antibodies (Premawansa, Gamage-Mendis et al. 1994). This suggests transmission reducing (TR) functionality of these antibodies may be dependent on repeated exposure, typical of higher endemicity, or alternatively, TRA is caused by antibodies derived against other antigens not measured in the study (Premawansa, Gamage-Mendis et al. 1994; Bousema and Drakeley 2011).

The degree of transmission reduction also varies. A study in Tanzania showed gradual deterioration of TRA (at the $\geq 50\%$ level) with age which was hypothesized to be attributed to lower gametocyte densities (and therefore lower antibody levels), or possibly reduced antibody avidity (Drakeley, Bousema et al. 2006). The same study found superior transmission blockers, i.e. individuals with $\geq 90\%$ transmission reduction, were associated with higher antibody titre, and these individuals were from each age category (Drakeley, Bousema et al. 2006). For this reason, it was suggested that submicroscopic gametocyte densities may be sufficient to elicit functional TRA at the $\geq 90\%$ level (Drakeley, Bousema et al. 2006). While high antibody titres may be more effective at transmission reduction, low antibody levels may actually enhance transmission, which has been reported for *P. vivax* (Ponnudurai, Van Gemert et al. 1987; Peiris, Premawansa et al. 1988; Naotunne, Rathnayake et al. 1990; Gamage-Mendis, Rajakaruna et al. 1992; Healer, McGuinness et al. 1999; van der Kolk, de Vlas et al. 2006; Bousema and Drakeley 2011).

1.36 Evidence for age dependent acquisition of Pfs48/45 and Pfs230 antibodies

The rate and pattern of naturally acquired antibodies to Pfs48/45 and Pfs230 with age is not clear. It has been suggested sexual stage antibodies (derived against Pfs48/45 and Pfs230) develop quickly, are reliant on recent rather than cumulative exposure, and are short lived (Riley, Bennett et al. 1994; Drakeley, Eling et al. 2004; Drakeley, Bousema et al. 2006; Bousema, Roeffen et al. 2010; Bousema and Drakeley 2011). Since gametocyte exposure is highest in children, the majority of studies have reported that Pfs48/45 and Pfs230 antibodies are more commonly found in children, and decrease with age, as gametocyte load and therefore stimulus for antibody production also decreases (Bousema, Sutherland et al. 2011). Continuing with this idea, it has therefore also been suggested that Pfs48/45 and Pfs230 antibodies will be less prevalent in the older age groups in high transmission areas (Bousema, Drakeley et al. 2007).

However, this age dependent decrease is not always evident, with studies both supporting and contradicting this theory. For Pfs230, evidence from a study in The Gambia showed no relationship with age and antibody prevalence or titre (Riley, Bennett et al. 1994). Alternatively, a study in Papua New Guinea found an increase of antibody prevalence and density for Pfs48/45 and Pfs230 with age (Graves, Doubrovsky et al. 1991), whereas evidence from Tanzania showed an age dependent decrease (Drakeley, Sutherland et al. 2006; Bousema, Drakeley et al. 2007; Bousema and Drakeley 2011). The dynamics of sexual stage exposure and antibody prevalence, titre and functionality still remain unclear and need further investigation, especially in the context of transmission blocking vaccine (TBV) candidate development, to ensure host immune-responsiveness is not a limitation.

1.4 Measuring the human infectious reservoir

1.41 Gametocyte detection

Detection of malaria infection routinely relies on the use of microscopy or rapid diagnostic tests (RDTs), depending on available resources (Bell 2002). Both assays are sufficiently sensitive to detect asexual parasite densities that are clinically relevant in the majority of malaria endemic settings (WHO 2009). Whilst it is sufficient to detect asexual parasites for the sake of routine clinical care, there are circumstances where it is required to specifically detect gametocytes. Gametocyte detection is especially valuable in epidemiological studies to understand transmission intensity and detect the human infectious reservoir, for infectivity studies to understand gametocyte prevalence and densities relevant for mosquito infection, and for malaria control purposes, such as determining efficacy of transmission blocking interventions or gametocidal drugs (Bousema, Drakeley et al. 2006; Schneider, Bousema et al. 2007; Wampfler, Mwingira et al. 2013).

1.42 Rapid diagnostic test (RDT) and microscopy based gametocyte detection

Microscopy and RDTs have limited sensitivity to detect all malaria infections since up to 80% of infections may be present at submicroscopic parasite densities (Okell, Bousema et al. 2012). Moreover, RDTs are not parasite stage-specific so have no role in specifically detecting gametocytes (Moody 2002). While microscopy can detect as few as 16 gametocytes/ μL (Churcher, Bousema et al. 2013; Karl, Laman et al. 2014), often densities in the range of 20-50/ μL are not seen (when screening 100 views) (Dowling and Shute 1966; Moody 2002; Karl, Davis et al. 2009; Bousema and Drakeley 2011). This is insufficient for accurate measurement since gametocytes often circulate at much lower densities (Ouedraogo, Bousema et al. 2009), and evidence has indicated microscopy negative blood samples are capable of infecting mosquitoes, which spurred the development of more sensitive detection tools (Muirhead-Thomson 1954; Jeffery and Eyles 1955; Muirhead-Thomson 1998; Bousema and Drakeley 2011).

The sensitivity of gametocyte detection can be improved in several ways, by reading more microscopy fields, by increasing the blood volume screened using concentration methods, or by using molecular detection techniques (Bousema and Drakeley 2011). In one study, increasing the number of fields that were viewed from 100 to 1000 (on a thick blood film) reportedly increased gametocyte prevalence by 300% (Colbourne 1956). An alternative approach is an enhanced microscopy method, magnetic deposition microscopy (MDM) which concentrates asexual stage parasites due to the magnetic properties of their hemozoin crystals (Karl, David et al. 2008). Whilst hemozoin crystals are not gametocyte specific, this method enables specific reading of parasite infected blood, which is more time efficient; a study assessing symptomatic individuals reported an increased gametocyte prevalence of 7.3% to 45% when using MDM compared to reading a normal blood smear (Drakeley, Sutherland et al. 2006; Karl, David et al. 2008; Bousema and Drakeley 2011).

Another technique is the quantitative buffy coat (QBC) method which separates and concentrates parasites from platelets, monocytes, granulocytes and lymphocytes in

a whole blood sample (Spielman, Perrone et al. 1988; Bousema and Drakeley 2011). The blood is drawn in an acridine orange capillary tube which is then centrifuged to separate the parasite infected erythrocytes from other blood cell components (Pinto, Rodrigues et al. 2001). When illuminated under ultra violet (UV) light, acridine orange stained parasites fluoresce, thereby enabling rapid and specific parasite counting under the microscope (Pinto, Rodrigues et al. 2001). A transmission study conducted in Cameroon by Mulder *et al.* reported an increased gametocyte estimate from 4.6% (determined by conventional microscopy) to 19.1% when using QBC (Mulder, van der et al. 1998; Bousema and Drakeley 2011). Even with the enhanced sensitivity offered by these adapted methods, microscopy remains labour intensive, requires skilled technicians (and repeated quality checks to ensure skills are maintained) (Fernando, Karunaweera et al. 2004), is insufficient for low gametocyte densities (Bousema and Drakeley 2011), and not appropriate for remote field settings.

1.43 Molecular detection of gametocytes

Individuals with microscopy negative gametocytes have been reported to be infectious to mosquitoes (Boudin, Lyannaz et al. 1991; Bousema, Dinglasan et al. 2012), indicating the need for improved detection methods. To enable sensitive detection of submicroscopic gametocytes, molecular techniques targeting gametocyte specific mRNA transcripts have been developed (Bousema and Drakeley 2011). Whilst DNA is present in both asexual and sexual stage parasites, RNA transcription of specific genes is up-regulated in just gametocytes, thereby providing a gametocyte specific target (Talman, Domarle et al. 2004; Karl, David et al. 2008). The mRNA of approximately 300 genes is exclusively produced in gametocytes, providing the opportunity for detection, quantification or genotyping, depending on the gene assessed (Silvestrini, Bozdech et al. 2005; Young, Fivelman et al. 2005; Bousema and Drakeley 2011). Since many *Plasmodium* genes have no introns, DNA and RNA appears identical, and methods seeking to specifically detect gametocytes

must remove genomic DNA by digest prior to RNA detection or use alternative approaches to avoid the amplification of double stranded DNA (Babiker and Schneider 2008).

Pfs25mRNA is abundantly expressed in mature gametocytes, zygotes and ookinetes (Jones, Chichester et al. 2013). This transcript has so far been the most commonly used target for detection of mature gametocytes (Wampfler, Mwingira et al. 2013). Although translationally suppressed until after fertilization in the mosquito, Pfs25mRNA transcripts can be detected in stage V gametocytes circulating in the human host (Bousema and Drakeley 2011). Currently, three molecular methods: reverse transcriptase polymerase chain reaction (RT-PCR), reverse transcription loop mediated isothermal amplification (RT-LAMP) and quantitative nucleic acid sequence based amplification (QT-NASBA) have been adapted for gametocyte detection targeting Pfs25mRNA (Trager and Jensen 1976; Ponnudurai, Meuwissen et al. 1982; Boom, Sol et al. 1990; Babiker, Abdel-Wahab et al. 1999; Schneider, Schoone et al. 2004; Bousema, Schneider et al. 2006; Mens, Schoone et al. 2006; Mens, Sawa et al. 2008; Bousema and Drakeley 2011). Reportedly, QT-NASBA detects gametocyte densities as low as 0.02-0.1 gametocytes/ μ L, while both RT-PCR and RT-LAMP have detection thresholds of approximately 1-2 gametocytes/ μ L (Babiker, Abdel-Wahab et al. 1999; Notomi, Okayama et al. 2000; Schneider, Schoone et al. 2004; Mlambo, Vasquez et al. 2008). As well as varying sensitivity, these techniques differ in terms of assay cost, ease of use, time to result, equipment needed and if read-out is qualitative or quantitative. The use of molecular techniques has vastly improved the accuracy of estimations of the human infectious reservoir, with studies consistently showing a 3-10 fold increase in gametocyte prevalence when using molecular tools, compared to microscopy (Ali, Mackinnon et al. 2006; Bousema, Schneider et al. 2006; Schneider, Bousema et al. 2006; Shekalaghe, Drakeley et al. 2007; Mens, Sawa et al. 2008; Bousema and Drakeley 2011).

1.44 Reverse transcription polymerase chain reaction (RT-PCR)

RT PCR is a qualitative PCR method informing only of presence or absence of gametocytes (Mlambo, Vasquez et al. 2008). Due to the lack of introns in Pfs25mRNA, parasite RNA and DNA is identical in coding sequence, therefore residual DNA from asexual stage parasites will also produce positive results (Babiker and Schneider 2008; Joice, Narasimhan et al. 2013). To avoid this, test samples must be subjected to a DNA digestion step prior to use. Amplification occurs as follows; starting with an RNA sample, cDNA copies are generated using reverse transcriptase, then nested PCR is performed from the cDNA template (Babiker and Schneider 2008). To confirm specificity of positive samples (i.e. to ensure asexual DNA has been digested), DNA controls (where the reverse transcriptase is heat inactivated, and substituted for a DNA polymerase) must be conducted for every positive sample (Babiker and Schneider 2008). While RT-PCR is convenient as it can be performed on a conventional thermocycler, and the methodology is straight forward to conduct and interpret, reagent cost is increased since 2 reactions must be included for each positive result. Additionally, it has been speculated the DNA digest may degrade a fraction of the RNA copies and result in reduced sensitivity. A quantitative version of RT- PCR (qRT-PCR) has more recently been developed which uses a titration curve of cultured gametocytes to enable quantification. The reported sensitivity is 1 copy number / μL (Wampfler, Mwingira et al. 2013).

1.45 Quantitative nucleic acid sequence based amplification (QT-NASBA)

QT-NASBA is a quantitative molecular detection method that amplifies RNA under the action of three enzymes, AMV-RT (avian myeloblastosis virus reverse transcriptase), RNase H and T7 RNA polymerase, and two target specific primers (Schneider, Schoone et al. 2004). Amplification occurs as follows, a primer containing a T7 promoter hybridizes to single stranded RNA in the sample. AMV-RT then

extends the sequence and RNA is hydrolyzed from the cDNA:RNA complex by RNase H, leaving the cDNA from which double stranded DNA is amplified with the second primer (Schneider, Schoone et al. 2004). The newly generated double stranded DNA contains a T7 promoter which allows continual generation of new RNA under the action of T7 RNA polymerase. When primers have been exhausted, amplification occurs solely due to transcription. Here, fluorescent dye binds to the new DNA and electrochemiluminescence (ECL) is measured as the output. As there is a linear relationship between the newly transcribed material and the number of gametocytes in the reaction, sample quantification is possible (Schneider, Schoone et al. 2004; Schneider, Wolters et al. 2005).

QT- NASBA is advantageous since RNA is amplified isothermally at 41°C, a thermocycler is theoretically not required and because the reaction temperature is too low to denature DNA this ensures primers only anneal to single stranded RNA therefore resulting in RNA specific amplification (Schneider, Schoone et al. 2004; Babiker and Schneider 2008). QT-NASBA is rapid with an incubation time of 90 minutes, compared to hours of thermocycling needed for conventional PCR, and has a higher level of sensitivity as previously mentioned. Whilst the initial investment in quantitative equipment is high, the reduction in person hours to result means it is more cost effective and less labour intensive (Schneider, Schoone et al. 2004; Schneider, Wolters et al. 2005).

1.46 Reverse transcription loop mediated isothermal amplification (RT-LAMP)

RT-LAMP is an alternative isothermal amplification method which has also been optimized for gametocyte detection targeting Pfs25mRNA (Buates, Bantuchai et al. 2010). The methodology functions as follows, under the action of AMV and Bst enzymes, 8 target regions are amplified using 6 primer sets . RNA is firstly reverse transcribed to cDNA which contains complementary sequences at both ends that self anneal. Auto-cycling strand displacement takes place which results in a hairpin

looped product from which new DNA is generated (Notomi, Okayama et al. 2000). A fluorophore calcein is included in the reaction which is initially quenched by manganese; when the reaction starts, pyrophosphate is generated as a by-product and it binds to the calcein displacing it from the manganese which allows it to fluoresce (Tomita, Mori et al. 2008). Along with fluorescence, a positive result also manifests as visible turbidity in the reaction mixture and this can be measured either by using a turbidimeter or by visualizing fluorescence using a UV light source (Tomita, Mori et al. 2008). RT-LAMP is advantageous as it occurs within a closed tube system, which reduces contamination risk (Chang, Chen et al. 2012), and it also does not require a thermocycler which makes it more potentially more amenable for use in the field. Whilst it has been suggested to be more sensitive than RT-PCR, it is currently unknown how it performs compared to QT-NASBA.

1.47 Membrane feeding assays

While gametocyte presence is suggestive of transmission potential, mosquito feeding assays are the only assays that can provide direct evidence of infectiousness to mosquitoes (Lin, Saunders et al. 2014). There are 3 main mosquito feeding assays that will be discussed, which are used for 2 purposes in this thesis, to determine the human infectious reservoir and to quantify the impact of naturally acquired sexual stage antibodies on transmission efficiency (Bousema and Drakeley 2011).

Gaining an understanding of who forms the infectious reservoir could be used to target interventions towards infectious hotspots, for a more specific approach to malaria control (Bonnet, Gouagna et al. 2003; Ouédraogo, Sattabongkot et al. 2013; Sturrock, Hsiang et al. 2013). Two methods are used to determine the human infectious reservoir by assessing the infectiousness of gametocytes to mosquitoes, direct skin feeding and membrane feeding using whole blood samples (Muirhead-Thomson 1957; Ponnudurai, Lensen et al. 1989; Bousema and Drakeley 2011) (see Figure 1.3.).

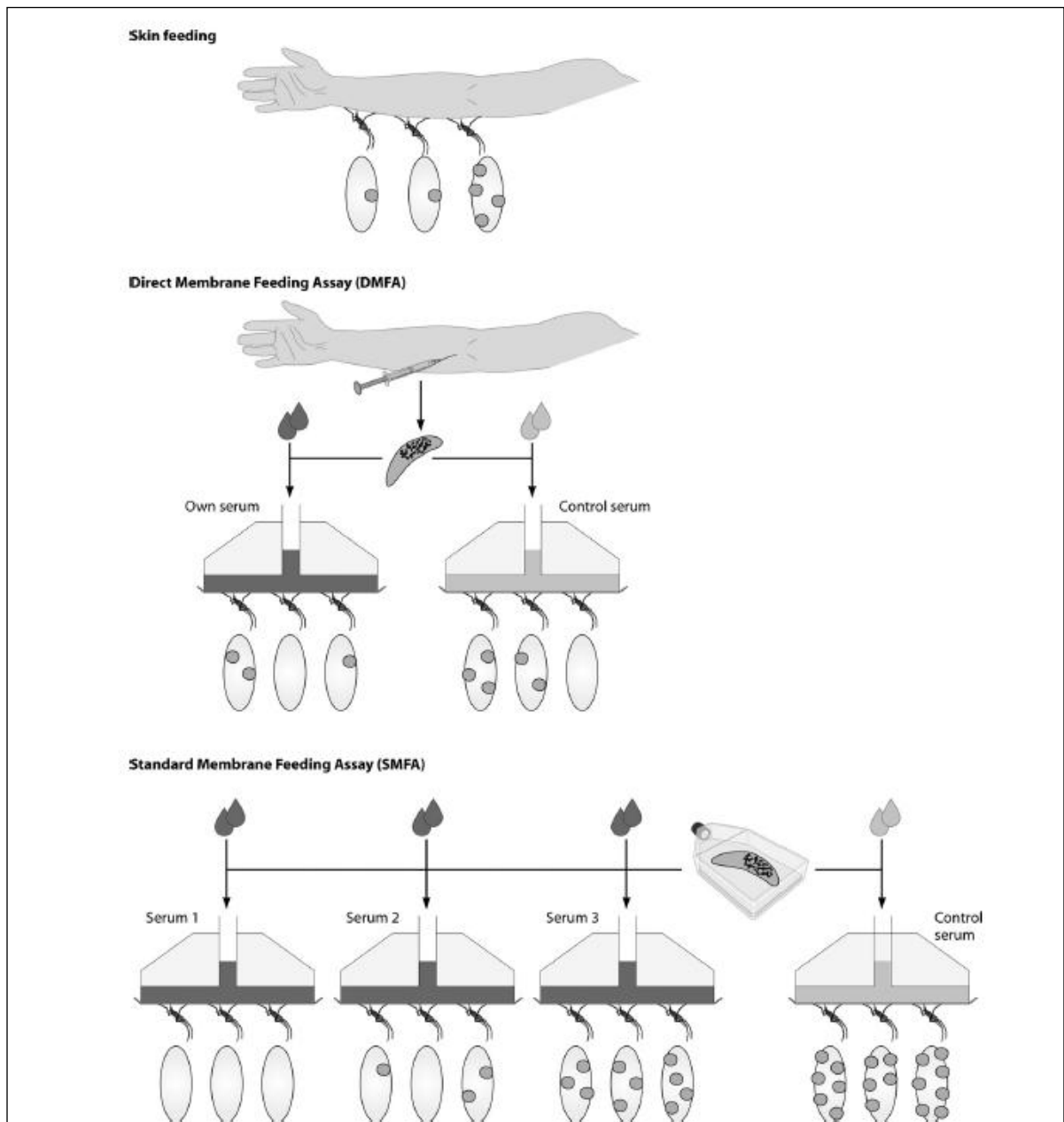


Figure 1.3. Membrane feeding assays for assessing malaria transmission to mosquitoes (Bousema and Drakeley 2011). Methodologies for skin feeding, direct membrane feeding assay (DMFA) and standard membrane feeding assay (SMFA) are indicated. Figure taken directly from the referenced publication.

For direct skin feeding, colony reared uninfected mosquitoes are fed directly on infected individuals, in an endemic setting (Bousema and Drakeley 2011). While this is most representative of a natural infection, it raises ethical concerns about transmission of other mosquito borne pathogens (particularly when feeding

mosquitoes on children) (Bousema, Dinglasan et al. 2012). This method is currently only accepted and used in a limited number of countries (Bousema and Drakeley 2011). DMFA using a whole blood sample offers an alternative method to circumvent ethical concerns. DMFA is performed in the field where mosquito colonies derived from local mosquitoes are offered whole blood from naturally infected gametocyte carriers through a membrane covered water jacketed feeder (Bousema and Drakeley 2011). To emulate natural infection as much as possible, blood is drawn from the participant and fed to mosquitoes immediately (Bousema and Drakeley 2011).

To determine the TRA of naturally acquired sexual stage antibodies, two methods can be used, the DMFA or the SMFA (Figure 1.3.). DMFA is performed in combination with serum replacement to determine the TRA of naturally occurring antibodies (André Lin Ouédraogo, Jetsumon Sattabongkot et al. 2013). To investigate this, whole blood samples are drawn from participants, the volume is split in half and both aliquots centrifuged. While one volume is resuspended in autologous plasma then offered to mosquitoes, the autologous plasma is removed from the second aliquot and replaced with naive plasma from an unexposed individual, before being fed to mosquitoes (Tchuinkam, Mulder et al. 1993; Bousema, Roeffen et al. 2006; Bousema and Drakeley 2011; Bousema, Sutherland et al. 2011). These paired conditions allow comparison of infectiousness to mosquitoes and calculation of transmission reduction, which may be attributed to the presence of sexual stage antibodies (André Lin Ouédraogo, Jetsumon Sattabongkot et al. 2013). This is discussed in more detail in Chapter 6.0.

SMFA is usually performed in a non endemic country and uses uninfected laboratory reared mosquitoes that are offered blood meals of cultured gametocytes combined with uninfected blood and either autologous endemic sera or control unexposed (naive) serum, which once more enables comparative assessment of TRA of antibodies in endemic serum (Bousema and Drakeley 2011). After the feeding process, methodologies for DMFA and SMFA are the same. Approximately 1 week after feeding, (when oocysts have had sufficient time to develop), mosquito midguts are dissected and if infection is successful, oocysts are visible (Ponnudurai, Van

Gemert et al. 1987; Lensen, van Druten et al. 1996; Mulder, Lensen et al. 1999). Oocyst prevalence or density can be compared as an outcome measure between control and endemic serum fed mosquitoes (Bousema and Drakeley 2011). Higher oocyst prevalence and/or density would be expected in mosquito midguts offered naive serum, compared to autologous serum, if the autologous serum contained transmission blocking antibodies that could control or reduce oocyst development (Diallo, Toure et al. 2008).

While these methods have improved our understanding of the infectious reservoir and the TRA of naturally acquired antibody responses, there are advantages and disadvantages (Ponnudurai, Lensen et al. 1989; Bonnet, Gouagna et al. 2000; Sattabongkot, Maneechai et al. 2003; Bousema, Sutherland et al. 2011). Generally, infection prevalence for skin feeding is higher than that for DMFA. For example, a study from Burkina Faso resulted in 78.6% of infected mosquitoes using skin feeding, compared to 41.4% using DMFA (Bousema, Dinglasan et al. 2012; Gouagna, Yao et al. 2013). Similarly, a study in Mali suggested infectivity was three fold higher for mosquitoes fed on skin feeding compared to mosquitoes fed using DMFA (Diallo, Toure et al. 2008). Despite this, results are strongly related and DMFA provides a more ethical measure to determine infectiousness (Diallo, Toure et al. 2008).

Compared to SMFA, DMFA is advantageous since it is more representative of natural infection, uses local mosquitoes and more genetically diverse endemic gametocytes occurring at natural densities, however, it is more difficult to standardize (Mulder, Lensen et al. 1999; Drakeley, Eling et al. 2004; Bousema and Drakeley 2011). Since large gametocyte cultures can be grown, SMFA has the advantage of exploring a greater number of test conditions at the same time while allowing more control to be exerted over variables such as gametocyte density, for this reason it is the current gold standard technique for determining TRA (van der Kolk, De Vlas et al. 2004; Bousema and Drakeley 2011). Studies performed in The Gambia and Cameroon have indicated there is an overall relationship between infectiousness determined using DMFA and SMFA, but differences do exist (Mulder, Lensen et al. 1999; Drakeley, Eling et al. 2004).

While these methods are labour intensive and there is notable inter lab protocol variation which complicates comparability, they are extremely valuable for understanding transmission and efforts are continuing to develop protocols that represent natural infection as accurately as possible (Roeffen, Mulder et al. 1996).

1.5 Future interventions for malaria elimination

1.51 Recent successes and the move to elimination

Malaria has been successfully reduced or eliminated from some endemic areas where intense control programme(s) have been well managed and maintained (Barat 2006). These successful control efforts are thought to be greatly facilitated by a combination of good infrastructure, political stability, effective tools, good technical support and the financial means to roll out the interventions (Barat 2006). In these regions scaling up existing control measures, mostly involving vector control such as the roll out of ITNs and IRS, was found to be effective (Targett and Greenwood 2008). Parasite prevalence has also been reduced through use of improved diagnostics (namely RDTs) and use of artemisinin combination therapy (ACTs) which also benefits from gametocidal activity (Sutherland, Ord et al. 2005; Kleinschmidt, Schwabe et al. 2009). As a result of wide-scale implementation of vector control and improved diagnosis and treatment, a number of low transmission countries have successfully reduced malaria related deaths and disease including South Africa, The Gambia, Zanzibar and Kenya, among others (Barat 2006). In South Africa, following a household spraying campaign and use of artemether-lumefantrine (AL) the number of cases over three years decreased by 99% (Duffy and Mutabingwa 2005). Similarly, parasite prevalence dropped from 35-40% prevalence to 2% in Zanzibar over a period of 15 years (Bhattarai, Ali et al. 2007). This has been suggested to be due to a high level of coverage of preventative measures with 96% of surveyed individuals having

at least one preventative method used in their house (Anon 2009). The Gambia achieved success in reducing their parasite prevalence by 50-85% across 4 sites over a period of 4 years by rolling out of chemoprophylaxis and insecticide treated bed nets (Ceesay, Casals-Pascual et al. 2008). Similarly, the same interventions were used in Kenya between from 1999-2007, where a decline of 28-63% was found in paediatric malaria cases (Okiro, Hay et al. 2007).

Stimulated by these successes, 32 out of the 99 countries with endemic malaria were preparing an elimination strategy in 2010 (Feachem, Phillips et al. 2010). Moving from malaria control to elimination requires a change in tactics from focusing on prompt diagnosis and treatment of symptomatic infection, to treating all infections (Mendis, Rietveld et al. 2009). Elimination is most realistic when transmission intensity is already low and it has been suggested there will need to be a shift from targeting specific groups like young children or pregnant women, to adopting a community based approach to treat every infection, including those that are asymptomatic (Targett and Greenwood 2008). With this objective in mind, the high prevalence of low density parasitaemias and asymptomatic infections complicates efforts, since detection of these infections requires a different set of more sensitive tools (Okell, Bousema et al. 2012). In line with this, mathematical modelling has suggested that malaria elimination is unlikely to be achieved in the majority of African settings by further scaling-up of currently available tools (Griffin, Hollingsworth et al. 2010).

Despite the promising advances in malaria control so far, a combination of drug and insecticide resistance, the emergence of counterfeit drugs, and an anticipated reduction in funding due to the economic crises all threaten to jeopardize the progress made, and reiterates the need for new approaches to malaria control (World Health Organization 2011). The current theory is that eradication of malaria is not possible with the tools that are currently available (malERA 2011), and an emphasis has been placed on developing gametocidal or transmission blocking tools (Nunes, Woods et al. 2014). Whilst chloroquine, quinine and ACTs can be effective against young gametocytes, and useful for reducing gametocyte carriage, they are

not completely effective against mature gametocytes and therefore do not prevent transmission shortly after treatment (Adjalley, Johnston et al. 2011). In addition to transmission-blocking drugs, malaria vaccines that interrupt malaria transmission are also high on the priority list for malaria elimination (Alonso, Brown et al. 2011).

1.52 Vaccines

Throughout the duration of the *Plasmodium* life cycle, the parasite undergoes numerous morphological changes, producing antigenically distinct stages (Ferreira, da Silva Nunes et al. 2004). Antigens presented on the parasite cell surface are exposed to a host's blood system presenting an opportunity for vaccination strategies. Clyde and colleagues had the first described success in vaccine development when they inoculated volunteers with irradiated sporozoites, using mosquitoes, which upon challenge effectively prevented blood stage infection (Nussenzweig, Vanderberg et al. 1967; Clyde, Most et al. 1973). In addition to 'whole parasite approaches' to vaccination, there has been considerable investment in vaccine development focused on specific antigens targets from the different parasite life stages (see Figure 1.4.).

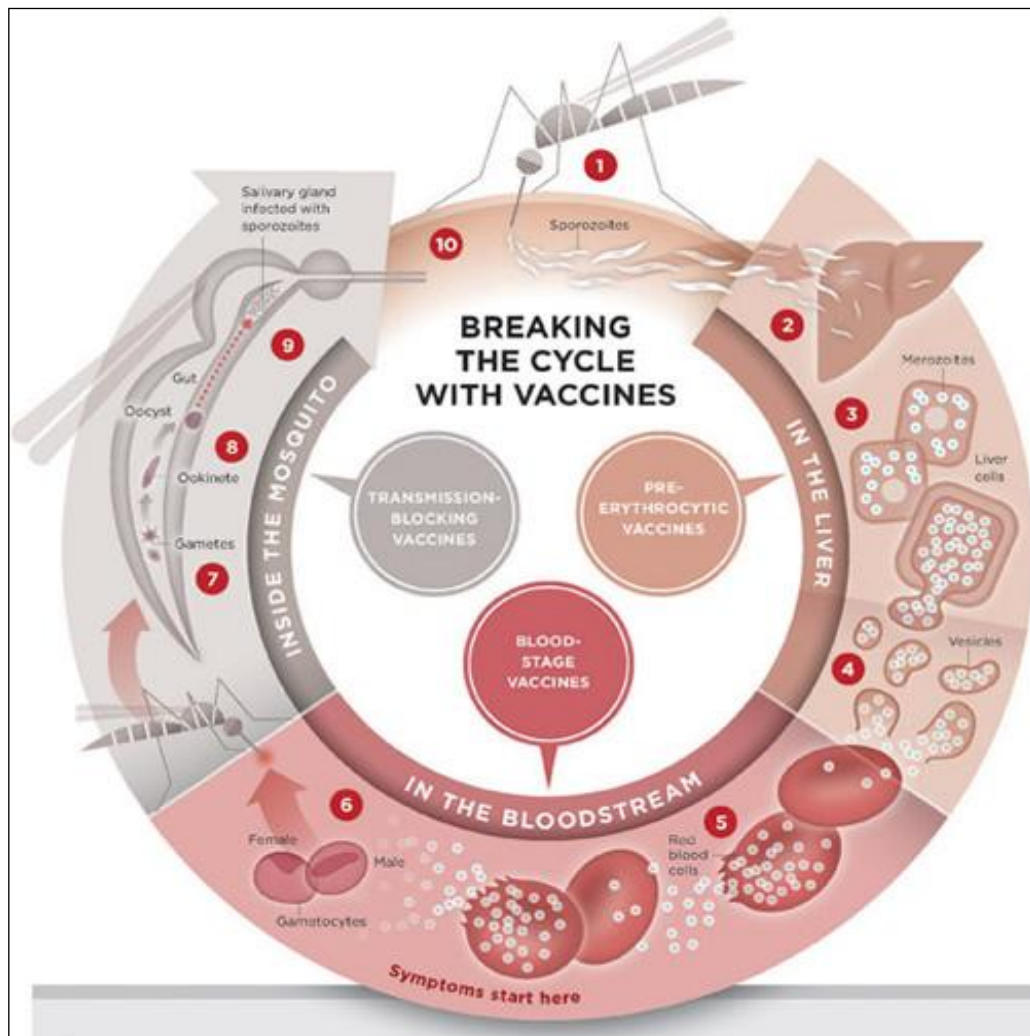


Figure 1.4. Approaches to malaria vaccines based on different stages of the parasite life cycle (MVI.PATH 2011). Figure taken directly from the referenced source.

1.53 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines are regarded as highly appealing as, if fully effective, they would prevent invasion by evoking immunity against sporozoites and liver stage parasites before they entered the blood stream (Targett, Moorthy et al. 2013). As they would prevent infection, clinical malaria and subsequent transmission potential (Hill 2011), a successful pre-erythrocytic vaccine has the greatest appeal. If fully effective, this would also qualify as a vaccine that interrupts malaria transmission

(VIMT) (MalERA 2011). The most advanced vaccine to date is recombinant pre-erythrocytic vaccine candidate RTS,S /AS01 which is comprised of the 19 NANP epitope repeats of CSP fused with hepatitis B surface antigen (Agnandji, Lell et al. 2012). CSP is a promising vaccine candidate as it covers the entire surface of the sporozoite and is essential for several important functions including salivary gland infection, and the targeting and invading of hepatocytes (Aly, Vaughan et al. 2009). Development of RTS,S /AS01 began in 1987 and phase III trials are still ongoing, but initial results indicate the vaccine confers approximately 32.1% protection against severe malaria and clinical episodes in children and infants, which dropped to 16.8% protection after 4 years (The RTS 2011; Offeddu, Thathy et al. 2012; Olotu, Fegan et al. 2013). While an important achievement resulting from decades of research, due to the relatively low protection induced, it is unlikely to lead to the eradication of malaria, but will provide another useful control or tool for decreasing malaria incidence (Targett, Moorthy et al. 2013). Alternate pre-erythrocytic candidates include thrombospondin-related anonymous protein (TRAP) and LSA-1, which were being developed in combination with 3 other antigens as a multistage DNA vaccine; the initial results indicated limited efficacy (Wang, Richie et al. 2005), but both antigens continue to be utilized in several vaccine candidates and feature highly in the WHO Rainbow Table of candidates undergoing clinical projects, including 1 that combines RTS,S and TRAP (Schwartz, Brown et al. 2012; (WHO) 2013).

1.54 Blood stage vaccines

Vaccines targeting the asexual stage do not aim to prevent infection, but instead have the objective of preventing or lessening the symptoms associated with malaria by reducing the asexual proliferation cycle (Hill 2011). Blood stage targets are mostly molecules found on the surface of the merozoite that are believed to be involved in invasion of the erythrocytes (Hu, Chen et al. 2008; Targett and Greenwood 2008). These antigens include AMA-1, erythrocyte binding antigen (EBA-175), MSP-1,2, 3, erythrocyte membrane protein (PfEMP-DBL3), and synthetic peptide 166 (SP166)

(Patarroyo, Amador et al. 1988). AMA-1 and MSP-1, 83kDa and ~200kDa respectively, are two of the leading asexual stage candidates with antibodies found in naturally exposed populations that are linked with clinical immunity (Hu, Chen et al. 2008). Currently, a chimeric vaccine comprised of sequences from both antigens is under development and has so far proven to be safe, immunogenic and induces inhibitory antibodies in monkeys and rabbits (Hu, Chen et al. 2008). The polymorphic nature of these antigens can be problematic, for example AMA-1 has more than 300 unique haplotypes and there is concern that vaccine induced immunity may not protect against all strains of parasites (Takala, Coulibaly et al. 2009).

GLURP is a 220-kDa protein present on pre-erythrocytic and erythrocytic parasites that has 3 main regions, the N terminal non repetitive region called R0, R2 which is the C-terminal repetitive region, and R1 which is the central repeated region (Babiker, Ranford-Cartwright et al. 1999; Agyeman-Budu, Brown et al. 2013). Antibodies in naturally exposed populations have been found against R0 and R2, but not R1 which therefore has not been of interest as a vaccine candidate (Theisen, Dodoo et al. 2001). Candidates named GMZ1 and GMZ2 have both been developed using GLURP, although GMZ1 (Imoukhuede, Ventura et al. 2010), was dropped in favour of developing candidate GMZ2, a recombinant fusion of two antigens, R0 of GLURP and a conserved section of the C terminus of MSP-3 (Wampfler, Mwingira et al. 2013). This candidate is currently undergoing phase I trials in Germany and Gabon, and so far seems to be immunogenic and safe (Mouchet 1994; Roberts, Manguin et al. 2000; Baumann, Magris et al. 2012; Agyeman-Budu, Brown et al. 2013).

While asexual vaccines ideally would provide protection against disease, it is accepted they may only result in a reduction in parasite density, rather than a reduction in parasite prevalence, which was the outcome for a vaccine comprised of MSP-1 and MSP-2 (Genton, Betuela et al. 2002; Targett and Greenwood 2008).

1.55 Transmission blocking vaccines

Since malaria eradication has been a long term goal for many funding bodies, the funds available for developing sexual stage vaccines is expected to increase (Schwartz, Brown et al. 2012). Although efficacious pre-erythrocytic vaccines may also prevent transmission by preventing or reducing malaria infection rates, the term malaria transmission blocking vaccine (MTBV) is traditionally used for vaccines that specifically target sexual stage malaria proteins. Different from the other two classes of malaria vaccines, MTBV do not provide direct protection to the vaccinated individual, but instead work on a population level, which is somewhat different to many conventional vaccines (Sauerwein 2007).

Transmission blocking vaccinated individuals will generate an antibody response that acts inside the mosquito after antibodies are passively ingested when the mosquito takes a blood meal. Inside the mosquito, antibodies may prevent midgut invasion, the development of the zygote or further sporogonic development, thereby preventing generation of sporozoites and blocking onwards transmission (Carter 2001; van Dijk, Janse et al. 2001; Baton and Ranford-Cartwright 2005; Sauerwein 2007). Parasite densities are at their lowest in the mosquito (Figure 1.5.) which makes it an appealing stage to introduce control since lower densities could be easier to overcome (Sinden 2010).

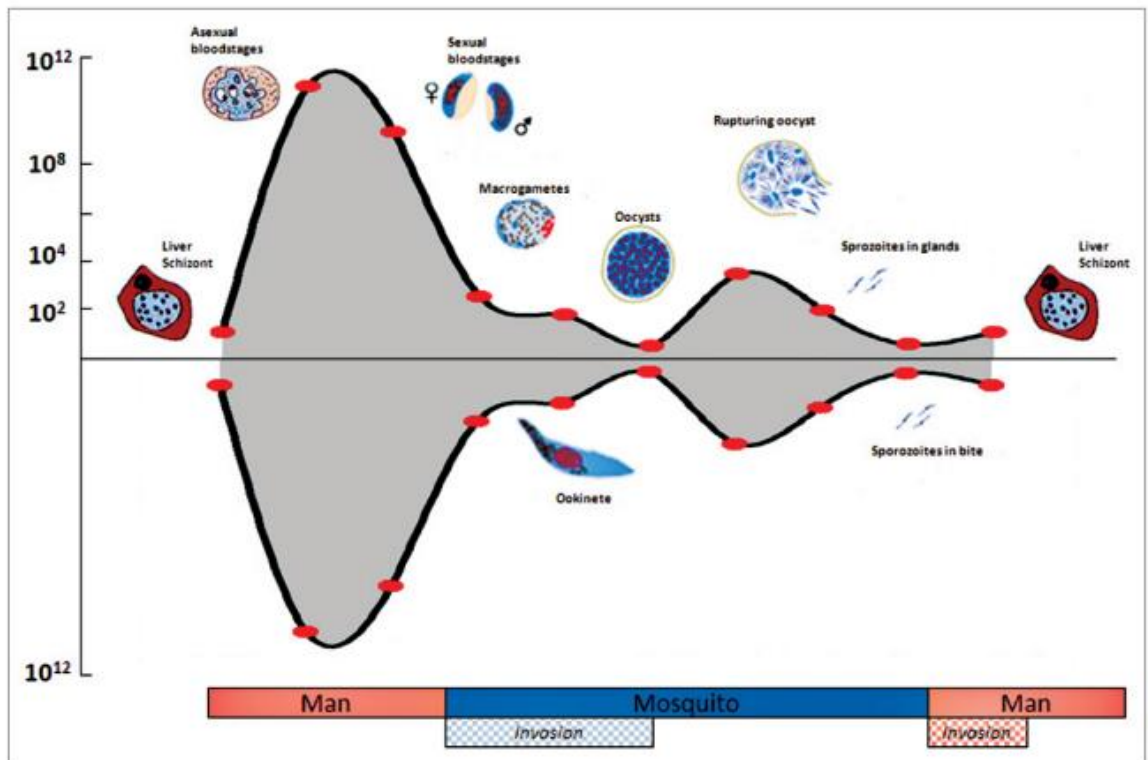


Figure 1.5. Approximate parasite load at each stage of the parasite life cycle (Sinden 2010). Figure taken directly from the referenced publication.

The concept of sexual stage vaccines was first developed approximately 35 years ago where transmission blocking antigens on gametes and ookinetes were identified by use of surface labelling experiments and monoclonal antibodies (Reiner, Graves et al. 1983; Carter, Miller et al. 1984). Early transmission blocking successes were achieved by immunizing chickens with formalin treated erythrocytes infected with *P. gallinaceum* (Carter and Chen 1976; Gwadz 1976). This was later replicated immunizing mice with formalin fixed *P. yoelii* gametes and rhesus monkeys with *P. knowlesi* gametes (Gwadz, Carter et al. 1979; Mendis and Targett 1979).

Since then, transmission blocking vaccines have predominantly focused on individual antigen targets which are sexual stage proteins that can be divided into pre and post-fertilization candidates according to when they are expressed (Bousema and Drakeley 2011). Pre-fertilization proteins are expressed before fertilization, on stage V gametocytes in the human host, whereas post fertilization proteins are expressed after ookinete development in the mosquito midgut (Carter 2001; Targett and

Greenwood 2008; Bousema, Sutherland et al. 2011). Pre-fertilization candidates include Pfs48/45, Pfs230, HAP2 and Pfs47 (Bousema and Drakeley 2011). HAP2 is required for the fusion of gamete membranes and knock-out parasites are incapable of gamete fusion and therefore fertilization (Blagborough and Sinden 2009). Development of this candidate has not advanced as far as others, but recently, it has been confirmed for the first time that antibodies against HAP2 are capable of transmission blocking activity (Miura, Takashima et al. 2013).

Pfs48/45, Pfs47 and Pfs230 all play a role in fertilization and belong to the 6-Cys protein group which is defined by six conserved cysteine residues (Outchkourov, Vermunt et al. 2007; Outchkourov, Roeffen et al. 2008; Bousema and Drakeley 2011). While functions remain unclear for some proteins, as many members of this group have glycosylphosphatidylinositol (GPI) anchors (except Pfs230), it has been suggested they are located on the parasite surface and therefore exposed to host immunity, which has sparked interest in the potential for natural antibody boosting of these vaccine candidates (van Schaijk, van Dijk et al. 2006). Pfs47 is a female fertility protein (Bousema and Drakeley 2011), located on the surface of macrogametes (van Schaijk, van Dijk et al. 2006). Knock-out experiments indicated *pfs47* deletion does not impact on fertility, but does reduce oocyst survival (van Schaijk, van Dijk et al. 2006; Molina-Cruz, Garver et al. 2013).

Pfs48/45 is located on the surface membrane of gametocytes (male and female), gametes (macrogametes only) and zygotes, where it plays a role in male fertility, specifically, in the attachment of microgametes (males) to macrogametes (females) during fertilization (van Dijk, van Schaijk et al. 2010; Bousema and Drakeley 2011). Experiments using knock-out parasites indicated male gametes cannot attach to and fertilize the females (which remain fertile) (van Dijk, Janse et al. 2001; van Dijk, van Schaijk et al. 2010). Antibodies derived against both Pfs48/45 and Pfs230 are thought to block transmission by inhibiting zygote formation, resulting in reduced oocyst formation (Vermeulen, Ponnudurai et al. 1985; Carter, Graves et al. 1990; Roeffen, Geeraedts et al. 1995; Williamson, Keister et al. 1995; Roeffen, Mulder et al. 1996;

Healer, McGuinness et al. 1997; Outchkourov, Roeffen et al. 2008; van Dijk, van Schaijk et al. 2010).

Pfs48/45 has 5 B cell epitopes, and antibodies against epitopes 1 and 5 have been demonstrated to be most effective at blocking transmission, but antibodies produced against epitopes 2b and 3 can block transmission in combination (Targett 1988; Carter, Graves et al. 1990; Targett, Harte et al. 1990; Roeffen, Mulder et al. 1996; Roeffen, Teelen et al. 2001; Outchkourov, Vermunt et al. 2007; Outchkourov, Roeffen et al. 2008). Pfs48/45 is being developed as a vaccine candidate by several groups, and has been shown to induce strong humoral antibody titres capable of inducing transmission blocking in 90% of mice and rats (Outchkourov, Roeffen et al. 2008; Bousema and Drakeley 2011; Theisen, Roeffen et al. 2014). Due to the large number of cysteine residues, development has been hindered as it is difficult to produce a properly folded product capable of inducing high levels of transmission blocking immunity (Outchkourov, Roeffen et al. 2008). The overexpression vector used impacts on correct folding and attempts have been made to express part of Pfs48/45 in *Escherichia coli*, which resulted in a yield of just 10-20% of correctly folded protein (Outchkourov, Vermunt et al. 2007). Another approach has been to use *Lactococcus lactis* where successful folding is dependent on the fermentation conditions, rather than co expression with bacterial folding catalysts (Theisen, Roeffen et al. 2014).

Pfs230 has a similar role to Pfs48/45 and is also concerned with gamete recognition and fertilization (van Dijk, van Schaijk et al. 2010). Knock-out parasites have been indicated to be unable to interact with red blood cells or form exflagellation centres, resulting in reduced oocyst production (Eksi, Czesny et al. 2006; Bousema and Drakeley 2011). Pfs230 has 5 epitopes and has proven to be complicated to produce as a vaccine due to its 363kDa size and 70 cysteine residues (Farrance, Rhee et al. 2011). Whilst full length Pfs230 is too large to produce by expression vectors, amino acids 444-730 in the N terminal region have been successfully overexpressed in *Agrobacterium tumefaciens* using a plant based system (Farrance, Rhee et al. 2011). Serum from rabbits vaccinated against this section has been shown to result in

approximately 99% reduction in oocyst prevalence (in the presence of complement) compared to control serum using SMFA (Farrance, Rhee et al. 2011). Another research group expressed the C fragment of Pfs230 in *E.coli* which demonstrated a reduction in oocyst prevalence of 70-90% (Williamson, Keister et al. 1995).

Whilst antibodies derived against Pfs48/45 are capable of transmission blocking on their own, transmission reducing immunity derived against Pfs230 is complement mediated (Read, Lensen et al. 1994; Roeffen, Geeraedts et al. 1995; Bousema and Drakeley 2011). It has been suggested pre-fertilization antigens Pfs48/45 and Pfs230 may have the advantage of boosting vaccine induced immunity, during natural infection (Bousema and Drakeley 2011; Bousema, Sutherland et al. 2011). Although, this natural exposure may act as a double-edged sword by forming a stimulus for antigenic variation and, in the case of vaccination, escape mutants (Nunes, Woods et al. 2014).

Pfs25 and Pfs28 are post fertilization candidates expressed on the surface of gametes, zygotes and ookinetes (Sharma 2008). Studies exploring homologues in *P. berghei* suggest their function is concerned with ookinete survival, traversal of the ookinete into the midgut epithelium and oocyst production (Tomas, Margos et al. 2001; Baton and Ranford-Cartwright 2005; Bousema and Drakeley 2011). Post fertilization proteins are only exposed within the mosquito and since they are not exposed to human immunity there is no human selection pressure, and antibodies against Pfs25 and Pfs28 are not found in the serum of people living in endemic areas (van Dijk, Janse et al. 2001). While advantageous since a lack of selection pressure may mean less genetic polymorphism (Bousema and Drakeley 2011), as there is no possibility of natural immune boosting, vaccines directed against these targets will need to induce a long lasting effective antibody response.

Due to the high number of cysteine residues and specific tertiary structure, Pfs25 has also been complicated to produce in the correct conformation (Kaslow, Quakyi et al. 1988). Groups have also struggled to produce immunogenic and safe candidates, and adverse side effects were encountered with Pfs25 combined with emulsion Montanide ISA 51 (Wu, Ellis et al. 2008; Bousema and Drakeley 2011). Other groups

have suffered problems with insufficient immunogenicity (Vogel 2010), and have produced Pfs25 as a chemically conjugated nanoparticle in an attempt to improve this, which so far looks promising (Shimp, Rowe et al. 2013). More recent advancements have been achieved by expressing Pfs25 in a plant based system fused to Alfalfa mosaic virus coat protein which produces as a virus like particle (VLP) (Jones, Chichester et al. 2013). The highly immunogenic nature of VLPs makes this technique appealing and early results are encouraging with complete transmission blocking achieved in mice (following 1 or 2 vaccinations) which remains even following a period of 6 months (Jones, Chichester et al. 2013). Other researchers expressed codon harmonized Pfs25 in *E. coli* and recently published results stating vaccine induced antibodies in mice gave 100% transmission blocking (Kumar, Angov et al. 2014).

1.56 The rationale for deployment of transmission blocking vaccines

TBV generally function as a community control method and current thinking suggests their specific use would be guided by transmission intensity (Sauerwein 2007). In regions of high or moderate transmission intensity, TBV (when available) could have more impact combined with an asexual stage vaccine with the overall aim of contributing towards a reduction in morbidity, mortality, and transmission (Smith, Chitnis et al. 2011). In regions of seasonal transmission, the vaccine could be used to protect against epidemics, or reduce the survival of drug or vaccine resistant parasites (Sauerwein 2007; Bousema and Drakeley 2011). It is currently thought TBV would most likely be used in regions of low transmission where the vaccine could even be used alone, or in combination with other control methods to move towards elimination of the infectious reservoir to stop malaria transmission (Carter, Mendis et al. 2000; Sauerwein 2007; Bousema and Drakeley 2011). An alternative approach would be to use TBV as a quarantine tool to prevent reintroduction of malaria from potentially infectious migrants into regions that have eliminated, but could support malaria reintroduction (Sauerwein 2007).

In order to impact on transmission reduction, a high level of vaccine coverage would be needed and it has been suggested that 80% population coverage could result in an overall 3.5 fold reduction in transmission (Saul 2007). Natural boosting of a vaccine induced immune response would be potentially advantageous (Carter, Mendis et al. 2000; Chowdhury, Angov et al. 2009). Not only would this boost the antibody titre of poor responders but could eliminate or reduce the need for follow up booster vaccinations which would make roll out of the vaccine logistically easier.

The research performed in this PhD helps to support development of a TBV, by investigating the natural recognition, diversity and functionality of a candidate named 10C, which contains the 10 C terminal cysteine residues of Pfs48/45.

Chapter 2: Aims of the thesis

1. To compare methodologies for collection and detection of low densities of *P. falciparum* gametocytes from filter papers, and conclude on the optimal combination (Chapter 4).
2. To determine the sensitivity and specificity of different diagnostic methods to identify individuals who are infectious to mosquitoes during the dry season in Burkina Faso (Chapter 6).
3. To identify if there are naturally acquired antibody responses to two leading transmission blocking vaccine candidates, 10C (Pfs48/45) and 230CMB (Pfs230), in school aged children in 3 different endemic settings in Africa (Chapter 5).
4. To identify genetic polymorphisms in *pfs48/45* (containing vaccine candidate 10C) in 3 endemic settings in Africa (Chapter 5).
5. To assess the ability of R0-10C (Pfs48/45) vaccine induced IgG in rats to reduce transmission of *P. falciparum* gametocytes from naturally infected gametocyte carriers in Burkina Faso (Chapter 6).
6. To determine the association between naturally acquired antibody responses to 10C (Pfs48/45) and 230CMB (Pfs230) and naturally acquired transmission reducing immune responses (Chapters 5 and 6)

Detailed chapter specific objectives are embedded within each chapter, in sections 4.2., 5.2., and 6.2. While research performed in this thesis investigated antibody responses (and their functionality) to 230CMB antigen, 10C was the primary focus since this was the vaccine candidate produced by collaborators.

3.1 Serological methods

3.11 Antigens selected for antibody measurement using enzyme linked immunosorbent assay (ELISA)

Antibodies that develop against asexual antigens are commonly used as a proxy to indicate malaria exposure. Because of the longevity of antibody responses, they provide markers of exposure that are suitable indicators of stable transmission intensity (Cook, Reid et al. 2010). In this thesis, *P. falciparum* antigens were used to measure antibodies produced against asexual antigens AMA-1, MSP-1₁₉ and GLURP (protein functions are covered in section 1.54.), along with recombinant transmission blocking vaccine candidates maltose binding protein (MBP)-10C (Pfs48/45), R0-10C (Pfs48/45) and 230CMB (Pfs230) (provided by Fraunhofer Center for Molecular Biotechnology).

The AMA-1 antigen used in these experiments encompassed amino acids 22-545 from the 3D7 strain, which is a conserved portion with a high level of cross reactivity with other strains (Hodder, Crewther et al. 2001). MSP-1₁₉ is a 19kDa sub fragment derived from the C-terminal domain of MSP-1₄₂. While largely conserved, 4 main polymorphic amino acids have been previously described (Miller, Roberts et al. 1993; Kang and Long 1995; Hui and Hashimoto 2007). The antigen used in this study represented amino acids 1631-1726 from the K1/Wellcome allele of MSP-1₁₉ which is a commonly found allele across Africa (Drakeley, Corran et al. 2005). GLURP is predominantly found on mature schizont infected erythrocytes, but is also expressed in liver stage parasites (Pratt-Riccio, Bianco et al. 2011). Studies investigating geographically diverse samples have revealed low levels of polymorphisms (Theisen, Soe et al. 1998). For this thesis unless otherwise stated, antibodies were quantified against GLURP-R2.

Pfs48/45 is a gametocyte surface protein, which was discussed in section 1.55. Both MBP-10C and R0-10C are transmission blocking vaccine candidates produced by collaborators (at Radboud University Medical Centre, Nijmegen, The Netherlands and Genova Biopharmaceuticals, Ltd. Pune, India). MBP-10C consists of amino acids 159-428 found in the 10 C-terminal cysteine residues of Pfs48/45. This includes epitopes 1, 2b and 3, (Figure 3.0.). 10C was fused to MBP which was expressed in *E. coli* as described elsewhere (Outchkourov, Roeffen et al. 2008). A factor Xa cleavage site is situated between MBP and 10C.

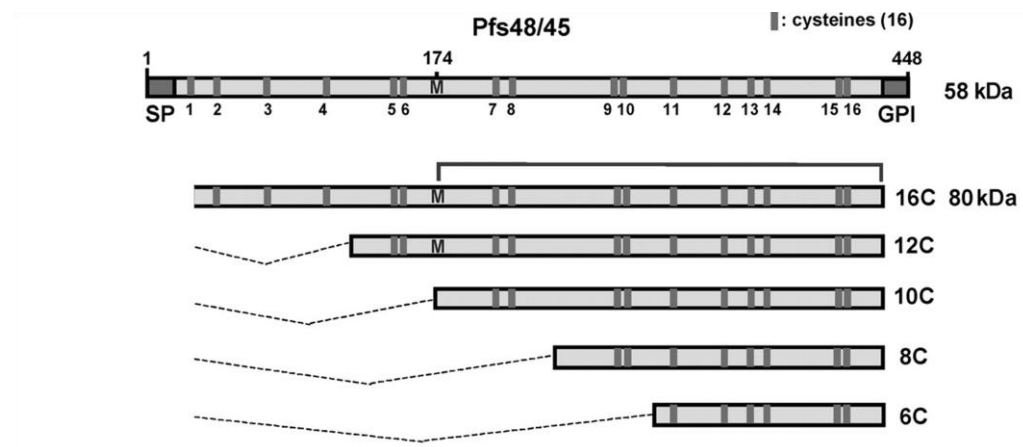


Figure 3.0. The structure of Pfs48/45 protein and the location of 10C vaccine candidate (Outchkourov, Roeffen et al. 2008). Figure adapted from the referenced publication.

R0-10C is a chimeric blood/liver stage and transmission blocking vaccine candidate that is comprised of an asexual stage antigen and a sexual stage antigen. The 10C terminal cysteine residues of Pfs48/45 were fused to R0, the N-terminal non repetitive region (residues 24-489) of asexual stage antigen GLURP, Figure 3.1. (Will Roeffen, personal communication). A FXa site was positioned between R0 and 10C to enable cleavage of 10C for specific sexual stage antibody detection, and a HIS-tag was included at the C terminal end.

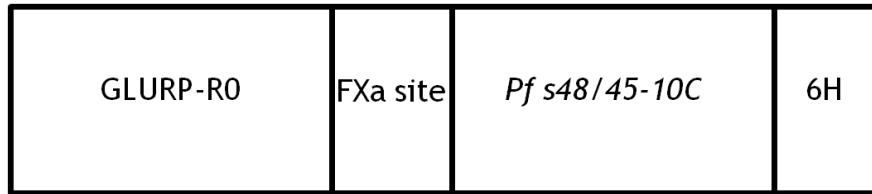


Figure 3.1. Schematic representation of the components of R0-10C vaccine (provided by Will Roeffen, Radboud UMC, Nijmegen, The Netherlands).

Antibodies were measured against another TBV candidate termed 230CMB which corresponds to amino acids 444-730 from the C domain of Pfs230 (Figure 3.2.), (Farrance, Rhee et al. 2011). This candidate was produced by Fraunhofer CMB, but antibodies were quantified against it since it occupies a similar functional niche to our vaccine candidate. The main focus of this thesis was development of our candidate, MBP-10C which was later replaced with R0-10C.

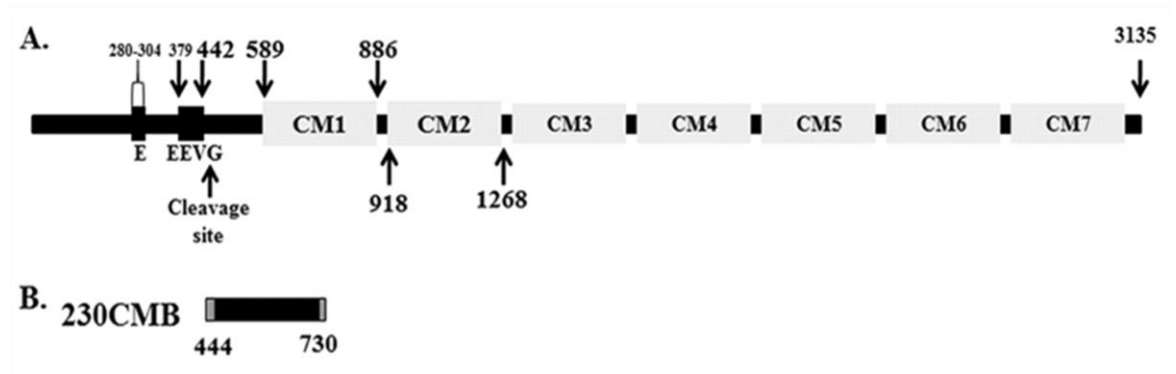


Figure 3.2. Structure of Pfs230. (a) Cysteine motifs in Pfs230, and location of 230CMB vaccine candidate. (b) Representation of the amino acids included in vaccine candidate 230CMB (Farrance, Rhee et al. 2011). The numbers refer to the amino acid locations. CM= cysteine motifs. Figure taken directly from the referenced publication.

3.12 ELISA to detect asexual antibodies

Total IgG binding to AMA-1, MSP-1₁₉ and GLURP R2 antigens was quantified using the same ELISA protocol which was performed as follows. Fifty μL of antigen per well was coated onto high binding Immulon 4 microtitre 96 well plates (VWR, Bedfordshire, UK) diluted in coating buffer (15mM Na_2CO_3 , 35mM NaHCO_3 , pH 9.5) to an end concentration of 1.25 $\mu\text{g}/\text{mL}$ for AMA-1 and 0.5 $\mu\text{g}/\text{mL}$ for MSP-1₁₉ and GLURP. Plates were incubated overnight at 4°C, then the following day they were washed 3 times with phosphate buffered saline (PBS) 0.05% v/v Tween 20 wash buffer. Plates were then blocked with 150 μL per well of 1% w/v milk powder (Marvel, Lincolnshire, UK) in PBS Tween 20 (0.05% v/v) for 3 hours at room temperature. Plates were washed 3 times as before, then test samples added to the plate in duplicate, diluting samples in blocking buffer (1% w/v milk powder (Marvel, Lincolnshire, UK) in PBS Tween 20 (0.05% v/v) to the following serum dilutions, 1/2000 for AMA-1 and 1/1000 for MSP-1₁₉ and GLURP. A positive control standard curve was generated from hyper immune serum to enable quantification of the antibody titre, and standardization of optical densities (ODs) to correct for plate to plate variation. A 7 point standard curve was titrated in 4 fold dilutions starting at 1/50 for GLURP and MSP-1₁₉ and 1/100 for AMA-1, then added to the plate in duplicate. Two blank wells were left per plate, where no sample was added, to allow correction for reagent background reactivity. Plates were incubated overnight once more, then washed 5 times in PBS Tween 20 (0.05% v/v) wash solution. Next, 50 μL of horseradish peroxidase (HRP) conjugated rabbit anti-human IgG –HRP (Dako, Cambridgeshire, UK) diluted 1/5000 in PBS Tween 20 (0.05% v/v) was added per well, which was incubated at room temperature for 3 hours, then plates were washed 5 more times as before. One hundred μL of SigmaFast O-phenylene diamine (OPD),

(Sigma-Aldrich, Dorset, UK) substrate was added per well and left to develop in the dark for 14 minutes for AMA-1 and 15 minutes for GLURP and MSP-1₁₉. Reactions were stopped with 25µL of 2M H₂SO₄ per well, then ODs determined using an ELISA microplate reader (DynexTechnologies, Virginia, USA) at 492nm.

3.13 ELISA to detect sexual stage antibodies

Antibodies against recombinant sexual stage antigens 10C and 230CMB were quantified using a different protocol to keep results comparable with a collaborating laboratory. This was done as follows, 96 well Maxisorp Nunc plates (Nunc, Stockholm, Sweden) were coated overnight at 4°C with 100µL per well of 0.1µg/mL of 10C or 230CMB diluted in PBS (Gibco, Life Technologies, Bleiswijk, The Netherlands). The following day, plates were blocked for 30 minutes using 150µL of 5% w/v milk powder (Marvel, Lincolnshire, UK) in PBS. Plates were then washed 3 times with PBS, and 100µL of test serum was diluted to 1/500 in PBS (with 1% w/v milk powder and 0.05% v/v Tween 20), which was incubated for 4 hours at room temperature. As before, samples were tested in duplicate, and hyper immune serum was used as a positive control which was titrated from 1/200 in doubling dilutions 7 times to produce a standard curve. Plates were washed 3 times, then incubated with 100µL per well of human-IgG-HRP (Pierce Biotechnology, Illinois, USA) diluted to 1/40,000 in PBS (with 0.05% v/v Tween 20), for 2 hours at room temperature. Plates were washed 4 times, then 100µL of tetramethylbenzidine substrate (TMB) solution was added per well and incubated for 20 minutes to allow development of the substrate. Reactions were stopped using 50µL per well of 0.2M H₂SO₄ and ODs were measured at 450nm (BioRad iMark Microplate Reader, Hertfordshire, UK).

3.14 Analyzing ELISA results

Different positive controls were used for the asexual and sexual stage ELISA. For the asexual stage ELISA, serum taken from 3 hyper-immune adults who lived in a hyperendemic region in Tanzania was used. For the sexual stage ELISA, serum was used from an expatriate with high levels of antibodies against native sexual stage proteins, and functional transmission reducing activity (van der Kolk, De Vlas et al. 2004; Saeed, Roeffen et al. 2008). Antibodies in European naive control serum was quantified to enable identification of non specific ELISA assay reactivity. Serum from European volunteers (recruited from the London School of Hygiene and Tropical Medicine (LSHTM)), who had not travelled to a malaria endemic region was used for this negative control population.

Results were analyzed using a macro enabled Excel spreadsheet (Microsoft, Washington, USA) that had been optimized to average, correct and normalize OD values, then convert them to titre. All test samples were assayed in duplicate, and repeated if duplicates differed by 50% or more. OD values from good pairs were averaged and the blank well OD was subtracted, to correct for background noise from assay reagents. The standard curve was plotted and a line of best fit was fitted to the curve. ODs were normalized against the fitted value corresponding to the mid-point dilution on the standard curve. Normalization aims to correct for plate to plate variation, which could occur due to substrate development times varying slightly from assaying plates in high throughput.

To determine antibody prevalence in test samples, positive and negative samples were firstly identified using the mixture model which was supplied as algorithms written by Jamie Griffin (Imperial College London), in STATA (Statacorp, Texas, USA) (Corran, Cook et al. 2008). The mixture model splits the normalized OD values into two Gaussian distributions, a narrow curve (typically) for the sero-negatives representing the lower ODs and a broader one for the sero-positives representing the higher ODs, using maximum likelihood methods. The mean OD of the sero-negative population plus 3 standard deviations was used to define the cut off of

positive and negative test samples (Corran, Cook et al. 2008; Bousema, Roeffen et al. 2010).

To calculate antibody density (titre), a titration curve was fitted to the control serum data by least squares minimisation using a three parameter sigmoid model and a 'solver add in' in a macro enabled Excel spreadsheet (Microsoft, Washington, USA), using an arbitrary value of 1000 units/mL of antibody in the control sample. Using the standard curve, averaged ODs were converted to units/mL and antibody titre was calculated using the following equation:

$$\text{Titre} = \text{dilution} / [\text{maximum OD} / (\text{OD test serum} - \text{minimum OD}) - 1]$$

(Drakeley, Corran et al. 2005; Proietti, Verra et al. 2013).

3.2 Molecular parasite detection

3.21 Nested PCR for parasite detection

Parasite prevalence was determined using nested PCR (nPCR) developed by Snounou *et al.*, which detects DNA copies of 18S rRNA (Snounou, Viriyakosol et al. 1993). This was performed as follows. Reactions were assembled in 0.2mL PCR tubes (BioRad, Veenendaal, The Netherlands) as outlined in Table 3.0. using the following genus specific primers for the primary PCR reaction: forward primer 5'-TTAAAATTGTTGCAGTTAAAACG-3' and reverse primer: 5'-CYTGTTGTTGCCTTAACTTC-3', and adding the italicized reagents indicated in the table to the reaction tubes.

Table 3.0. Reagents and volumes for nest 1 nPCR reaction for qualitative detection of all parasite stages.

reagent	Final Concentration	volume per reaction (μL)
Nuclease free H ₂ O	-	8.4
10 X NH ₄ buffer	1X	2.0
50 mM MgCl ₂	2 mM	0.8
10 mM dNTPs (2.5 mM each)	(200 μM each)	1.6
primer mix 1 (2.5 μM each primer)	250 nM (each primer)	2.0
Bioline Taq polymerase	1.0 unit	0.2
<i>mastermix total</i>		<i>15.0</i>
<i>template(added separately)</i>		<i>5.0</i>

The following thermocycling conditions were used for amplification: 95°C for 5 minutes, (58°C for 2 minutes, 68°C for 2 minutes and 94°for 1 minute) for 25 cycles, then 58°C for 2 minutes, 68°C for 5 minutes, then held at 4°C until use (Snounou, Viriyakosol et al. 1993).

Nested PCR (referred to as nest 2) was performed in 0.2mL PCR tubes using the following *P. falciparum* specific primers, forward primer 5'–TTAAACTGGTTTGGGAAAACCAAATATATT–3' and reverse primer 5'–ACACAATAGACTCAATCATGACTACCCGTC–3'. Reactions were otherwise assembled as outlined in Table 3.1., (adding the italicized reagents to the reaction tubes) and using the same thermocycling conditions as previous, but performing 30 cycles, instead of 25.

Table 3.1. Reagents and volumes for nest 2 nPCR reaction for qualitative detection of all parasite stages.

reagent	Final Concentration	volume per reaction (μL)
Nuclease free H_2O	-	12.4
10 X NH_4 buffer	1X	2.0
50 mM MgCl_2	2 mM	0.8
10 mM dNTPs (2.5 mM each dNTP (each)	(200 μM each)	1.6
primer mix 2 (2.5 μM each primer)	250 nM (each primer)	2.0
Bioline Taq	1.0 unit	0.2
<i>mastermix total</i>		19.0
<i>nest 1 product (added separately)</i>		1.0

To visualize amplified product, a 2.0% TBE (Tris Borate) agarose gel was prepared as follows. Two g of agarose (Sigma-Aldrich, Dorset, UK) was mixed with 100mls of 1x TBE (BioRad, Hertfordshire, UK). This was heated by microwave until dissolved, then when cool, 5 μL of ethidium bromide (Sigma-Aldrich, Dorset, UK) was added. The mixture was gently swirled to combine, then poured into a gel cast (BioRad, Hertfordshire, UK) with combs and allowed to set. Five μL of the nest 2 PCR product was mixed with 3 μL of loading dye (Thermo Fisher Scientific, Hertfordshire, UK), then added to the gel. Five μL of Hyperladder I (Bioline, London, UK) was included to allow estimation of the product size. PCR products were resolved by electrophoresis (60 minutes, 80 volts) and visualized using a transilluminator (BioRad, Hertfordshire, UK). Positive results were identified as a band of 205 base pairs in size.

3.3 Chapter specific methodologies

3.31 Preparation of work space and equipment for RNA extraction and molecular detection

See section 4.41.

3.32 Collection of cultured gametocytes on filter paper

See section 4.42.

3.33 RNA extraction from filter papers

See sections 4.43 for excision of filter paper blood spots, 4.44 for guanidine based RNA extraction and section 4.45 for RNA extraction using the Qiagen mRNA kit.

3.34 DNA digest performed on extracted nucleic acid

See sections 4.51 and 4.54.

3.35 Gametocyte detection using RT-LAMP

See sections 4.52 and 4.56.

3.36 Gametocyte detection using QT-NASBA

See section 4.61.

3.37 Gametocyte detection using RT-PCR

See section 4.64.

3.38 MSP-2 genotyping of natural parasite infections

See section 5.71.

3.39 DNA extraction from filter papers

See section 5.73.

3.40 Sequencing *pfs48/45* in endemic parasites

See sections 5.74-5.81.

3.41 Purification and validation of functionality of IgG from R0-10C vaccinated rats

See sections 6.31-6.35.

3.42 Field sampling for collection of endemic parasites for membrane feeding assays in Burkina Faso

See sections 6.41-6.46.

3.43 Mosquito husbandry, direct membrane feeding assay (DMFA) (with serum replacement), and oocyst counting

See sections 6.48-6.51.

3.44 QT-NASBA for detection of 18S rRNA

See section 6.52.

Chapter 4: Collection and detection of low density gametocytes

4.1 Introduction to publication

4.11 The importance and operational challenges of detecting low gametocyte densities

The infectious reservoir consists of all individuals in a population with infectious gametocytes in their peripheral blood. The presence of gametocytes thereby serves as a valuable indicator of malaria transmission potential (Bousema and Drakeley 2011). To fully understand local transmission dynamics, a detailed understanding of who within the population is carrying gametocytes is essential. As described in section 1.2., gametocytes often circulate at low densities below the detection of microscopy (Ouedraogo, Bousema et al. 2009). Decreasing malaria prevalence with accompanying lower parasite densities in many regions in Africa reinforces the urgency for improved techniques able to consistently detect sub-microscopic gametocyte densities (Okell, Bousema et al. 2012; Baidjoe, Stone et al. 2013).

4.12 Challenges with sampling gametocytes

While molecular detection of 18S rRNA enables identification of infection, detection of Pfs25mRNA allows the specific identification of submicroscopic gametocytes (Schneider, Schoone et al. 2004). Detecting mRNA is a challenge, due to its labile nature it can rapidly degrade outside of cells. Consequently, samples need to be handled and stored carefully to maintain mRNA integrity and facilitate accurate detection (Kast, Berens-Riha et al. 2013). Historically, RNA sampling has been

performed by diluting whole blood in RNA preserving buffer which inactivates RNases and permeates cells and tissues to protect and stabilize intercellular RNA (Kast, Berens-Riha et al. 2013). While useful, RNA diluted in buffer still needs to be stored at -80°C until use. This is unproblematic for well equipped labs, but less attractive for resource poor environments where accessibility to freezers and continuous power supply can be a constraint.

To circumvent this problem, finger prick sampling and filter paper sample collection provides the most operationally attractive combination of methods that enable large scale sampling with minimal discomfort (Toledo, Januario et al. 2005; Kast, Berens-Riha et al. 2013). For this purpose, small blood volume finger prick samples can be directly transferred to filter papers which are then no longer categorized as hazardous, and can be sent between laboratories without specialist shipping (Pritsch, Wieser et al. 2012). Furthermore, they can be shipped at room temperature which avoids the need for a cold chain, contributing to a cheaper cost. While the use of filter papers does not mean results can be obtained in the field, unless equipment is available, they do provide an inexpensive tool for collection and transport of samples to an equipped laboratory and thereby facilitate the incorporation of molecular gametocyte detection in epidemiological surveys of malaria endemic regions (Pritsch, Wieser et al. 2012).

Whilst filter papers are generally a user friendly collection method, due to the susceptibility of RNA to degradation it is expected that correct filter paper drying and storage will significantly impact on detection success. Currently, there are no clear guidelines for filter paper handling, or indicators of RNA integrity when sampling is performed on the different filter paper types. For antibody collection, a study demonstrated filter paper and glass fibre papers offered similar stability, but both resulted in antibody decay (compared to paired serum samples), when incubated at room temperature with ambient humidity for more than a week (Corran, Cook et al. 2008). This study emphasized the importance of storing blood spots with desiccant and in frozen conditions (Corran, Cook et al. 2008). However, for collection of DNA, storage temperature appears less relevant, whereas desiccating is essential and

allows storage of nucleic acid on filter papers for several years (Hwang, Jaroensuk et al. 2012).

4.13 Different filter paper types for sample collection

A range of filter papers are commercially available, some of which have been tailored for specific purposes. The widespread use of filter papers was initiated in the 1960s with the Guthrie card which was produced by Robert Guthrie for the collection of neonatal blood to diagnose phenylketonuria (PKU). Since then, their use has expanded to diagnostics, drug resistance screening, evaluating drug pharmacokinetics, and disease surveillance (Stevens, Pass et al. 1992; Toledo, Januario et al. 2005; Steegen, Luchters et al. 2007; Ashley, Stepniewska et al. 2010; Cook, Reid et al. 2010). Filter paper material spans from the commonly used cellulose paper, to glass, quartz, nylon and polyvinyl chloride (PVC). Cellulose papers are either plain or treated with chemicals, with the plain papers more commonly used for serological and nucleic acid collection (Baidjoe, Stone et al. 2013). As well as treatment with chemicals, filter papers vary in thickness and pore size. While untreated cellulose papers can be adapted for collection of a range of samples, including antibody and nucleic acid collection, the treated cellulose papers are only appropriate for nucleic acid collection due to impregnation with lysis buffers and/or nucleic acid stabilizing reagents.

4.14 Previous research investigating gametocyte collection on filter papers

A previous study evaluated the 903 filter paper (Schleicher and Schuell, New Hampshire, USA) and the FT2 filter paper (GE Healthcare, Buckinghamshire, UK) as tools for collection and storage of gametocytes which were then detected using RT-PCR (Mlambo, Vasquez et al. 2008). Filter papers were incubated at 25°C or at 37°C (with or without pre treatment with *RNAlater* (Qiagen, Surrey, UK). Non-filter paper

controls of whole blood diluted in RNA later were included as controls and were stored at -80°C , or 37°C overnight followed by -80°C . RNA from gametocytes stored on the 903 paper was successfully extracted and detected from all incubation conditions, whereas those stored on the FT2 paper were not well detected (Mlambo, Vasquez et al. 2008).

While a useful starting point, the study did not examine low densities of gametocytes that comprise the bulk of the infectious reservoir in many endemic settings and may pose additional challenges for successful gametocyte detection. Furthermore, it was not clear if the same results could be found with a larger range of incubation conditions, or when using the commonly available Whatman 3MM paper for sampling, or QT-NASBA as the detection method. In this chapter, I therefore performed a more rigorous study, testing the detectability of gametocytes at densities of 0.1-10 gametocytes/ μL , which are commonly observed in the field (Drakeley, Sutherland et al. 2006).

4.15 RNA extraction from samples collected on filter paper

RNA extraction methodologies commonly follow the same set of steps. The guanidine based extraction method extracts total nucleic acid. The process begins by adding buffer to the filter paper in a microcentrifuge tube which is then shaken. The buffer contains chaotropic agent, guanidine thiocyanate (GuSCN) which lyses red blood cells to release the nucleic acid, and exposes DNases and RNases which are concurrently inactivated (Boom, Sol et al. 1990). In the next step, supernatant is mixed with silica which binds to the nucleic acids due to the high concentration of GuSCN. A series of wash steps with ethanol and acetone follow to remove PCR inhibitors and contaminants and reduce the concentration of chaotropic GuSCN in preparation for nucleic acid elution. The silica is then dried and nucleic acid is eluted into nuclease free buffer, ready for use. As there is genomic DNA present in extracted material, it must be digested prior to performing detection with techniques

that could result in DNA amplification, such as RT-PCR (Boom, Sol et al. 1990; Casanova 2007). The Qiagen RNeasy kit (Qiagen, Surrey, UK) has the same principle but uses silica embedded centrifugable columns for purification. Although more costly, it has the benefit of being a more convenient method using spin columns with pre-prepared reagents.

4.2 Study design and objective

This chapter was devised to identify the optimal filter paper sampling method, RNA extraction technique and nucleic amplification method for detection of low density gametocytes. While this was performed in this chapter in a standardized laboratory setting, this information was needed for field sample collection carried out in subsequent chapters in this thesis.

Three filter papers were evaluated for preservation of low density gametocytes, when stored under a range of temperature conditions for up to 3 months. The 903 Protein Saver Card (GE Healthcare, Buckinghamshire, UK) was selected due to previous validation for gametocyte collection and the FTA Classic Card chosen since Whatman recommend this card specifically for RNA sampling. The Whatman 3MM filter paper was evaluated under a subset of conditions. Despite its extensive use for antibody and DNA collection, at the time of devising this study it had not been validated for RNA collection, and was included as a secondary objective. Three gametocyte densities (10, 1.0 and 0.1/ μL) spanning the previously reported microscopic detection threshold were selected to test the sensitivity of different approaches (Karl, Davis et al. 2009; Bousema and Drakeley 2011).

Two RNA extraction methods, guanidine based extraction (Boom, Sol et al. 1990) and the Qiagen RNeasy kit (Qiagen, Surrey, UK) were compared. Guanidine based RNA extraction was chosen due to its previous successful combination with molecular detection technique QT-NASBA, and potential for scaling up to high throughput which is useful in large epidemiological studies (Schneider, Schoone et al. 2004;

Schneider, Wolters et al. 2005). Qiagen extraction was chosen due to previous validation with the 903 Protein Saver Card and because it is one of the more widely used commercial extraction kits (Mlambo, Vasquez et al. 2008).

The outcome measure for this study was successful gametocyte detection by QT-NASBA, RT-PCR (and RT-LAMP was initially intended to be included). QT-NASBA was included due to the reported high level of sensitivity for low density detection and extensive previous use in field studies, including detection from filter paper (Schneider, Schoone et al. 2004). RT-PCR was assessed since it has previously been validated for detection of gametocytes from filter paper (Mlambo, Vasquez et al. 2008). While just one study had been published using RT-LAMP, and this was performed from whole blood samples, RT-LAMP was included due to its portability and use of a heat block, rather than thermocycler, which increases its appeal for field use (Buates, Bantuchai et al. 2010).

Specific objectives

1. Test the ability of filter papers to preserve mRNA when stored under a range of incubation conditions, compared to whole blood diluted in RNA stabilizing buffer and stored at -80°C.
2. Identify the RNA extraction method that is operationally most attractive and results in the highest or equivalent sensitivity of gametocyte detection.
3. Identify the molecular detection method that results in the highest sensitivity of gametocyte detection.
4. Conclude on the optimal combination of filter paper, RNA extraction and molecular detection method for identification of low density gametocytes from filter papers.

4.3 Publication: Filter paper collection of *Plasmodium falciparum* mRNA for detecting low-density gametocytes

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **Malaria Journal**
 - 1.2. When was the work published? **2012**
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion
not applicable
 - 1.3. Was the work subject to academic peer review? **Yes**
 - 1.4. Have you retained the copyright for the work? **Yes**

If yes, attach evidence of retention

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2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? not applicable
 - 2.2. List the paper's authors in the intended authorship order
 - 2.3. Stage of publication – Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

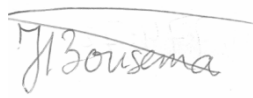
I designed and conducted all experiments, performed the statistical analysis and wrote the article. Parasites were cultured by colleagues.

Candidate's signature



A handwritten signature in cursive script, appearing to read "J. Bouwma". The signature is enclosed within a faint, hand-drawn rectangular border.

Supervisor or senior author's signature to confirm role as stated in (3)



A handwritten signature in cursive script, appearing to read "J. Bouwma". The signature is enclosed within a faint, hand-drawn rectangular border.

Filter paper collection of *Plasmodium falciparum* mRNA for detecting low-density gametocytes

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Abstract

Background

Accurate sampling of sub-microscopic gametocytes is necessary for epidemiological studies to identify the infectious reservoir of *Plasmodium falciparum*. Detection of gametocyte mRNA achieves sensitive detection, but requires careful handling of samples. Filter papers can be used for collecting RNA samples, but rigorous testing of their capacity to withstand adverse storage conditions has not been fully explored.

Methods

Three gametocyte dilutions: 10/ μ L, 1.0/ μ L and 0.1/ μ L were spotted onto Whatman™ 903 Protein Saver Cards, FTA Classic Cards and 3MM filter papers that were stored under frozen, cold chain or tropical conditions for up to 13 weeks. RNA was extracted, then detected by quantitative nucleic acid sequence-based amplification (QT-NASBA) and reverse-transcriptase PCR (RT-PCR).

Results

Successful gametocyte detection was more frequently observed from the Whatman 903 Protein Saver Card compared to the Whatman FTA Classic Card, by both techniques ($p < 0.001$). When papers were stored at higher temperatures, a loss in sensitivity was experienced for the FTA Classic Card but not the 903 Protein Saver Card, or Whatman 3MM filter paper. The sensitivity of gametocyte detection decreased when papers were stored at high humidity.

Conclusions

This study indicates the Whatman 903 Protein Saver Card is better for Pfs25 mRNA sampling compared to the Whatman FTA Classic Card, and that the Whatman 3MM filter paper may prove to be a satisfactory cheaper option for Pfs25 mRNA sampling. When appropriately dried, filter papers provide a useful approach to Pfs25 mRNA sampling, especially in settings where storage in RNA-protecting buffer is not possible.

Keywords: Reverse-transcription polymerase chain reaction (RT-PCR), Quantitative nucleic acid sequence-based amplification (QT-NASBA), Sub-microscopic, Gametocyte, Detection, Elimination, Transmission, Ribonucleic acid (RNA), Filter paper.

Background

The recent decline in the burden of malaria has placed malaria elimination and eradication back on the agenda of the international research community and health policy makers (World Health 2007; O'Meara, Mangeni et al. 2010; MalERA 2011; malERA 2011). As a consequence, vaccines and anti-malarial drugs that specifically target the transmission stages of malaria parasites, gametocytes, are a high priority (MalERA 2011; malERA 2011). These interventions aim to reduce the infectious reservoir of malaria: the number of individuals capable of infecting mosquitoes. Infectiousness of humans to mosquitoes depends on the presence of mature gametocytes in the peripheral blood, and sensitive detection of gametocytes is therefore paramount. In the last decade, it has become evident that detection of gametocytes by microscopy is insufficiently sensitive to assess potential infectivity. Gametocyte densities below the microscopic threshold for gametocyte detection (~ 5 gametocytes/ μL) frequently result in mosquito infection (Carter 1980; Pradel 2007; Schneider, Bousema et al. 2007; Ouedraogo, Bousema et al. 2009). Molecular detection of low levels of gametocyte specific mRNA enables identification of submicroscopic gametocyte carriers, and has revealed gametocyte prevalence to be four to ten times higher than estimated by microscopy (Abdel-Wahab, Abdel-Muhsin et al. 2002; Sutherland, Allouche et al. 2002; Nassir, Abdel-Muhsin et al. 2005; Bousema, Schneider et al. 2006; Drakeley, Sutherland et al. 2006; Paganotti, Palladino et al. 2006; Ouedraogo, Schneider et al. 2007; Sauerwein 2007; Schneider, Bousema et al. 2007; Shekalaghe, Drakeley et al. 2007; Karl, David et al. 2008; Nwakanma, Kheir et al. 2008). However, whilst mRNA-based gametocyte detection has aided epidemiological research concerned with low density *Plasmodium falciparum* gametocytes, the labile

nature of RNA and the ubiquitous presence of RNAses pose challenges to sampling under field conditions, where high heat and humidity may lead to mRNA degradation (Bauer, Polzin et al. 2003). Optimal handling of RNA samples, e.g., stabilizing in buffer, shipping on dry ice, and storing at -80°C, allows maintenance of integrity of nucleic acids but restricts sampling to well-equipped laboratories. To facilitate RNA sampling and storage in settings with limited resources a filter paper-based approach has been proposed (Mlambo, Vasquez et al. 2008). Filter papers have been routinely used for reliable collection and storage of whole blood samples for both antibody and DNA recovery (Corran, Cook et al. 2008; Taylor, Martin et al. 2011). The value of filter-paper matrices for malaria mRNA collection remains to be confirmed, in particular for detecting low-density infections under field conditions. In this study, the suitability of three different filter papers that are recommended for DNA or RNA storage has been determined for the mRNA-based detection of low-density gametocyte concentrations. Samples were stored on two filter papers under different conditions, ranging from those available in well-equipped laboratories to humid and hot tropical conditions, whereas the third paper was included in a smaller subset of conditions. Two commonly used RNA extraction protocols were compared and gametocytes were detected by both reverse-transcriptase PCR (RT-PCR) and quantitative nucleic acid sequence based amplification (QT-NASBA).

Methods

Figure 4.0 depicts an overview of the methodology.

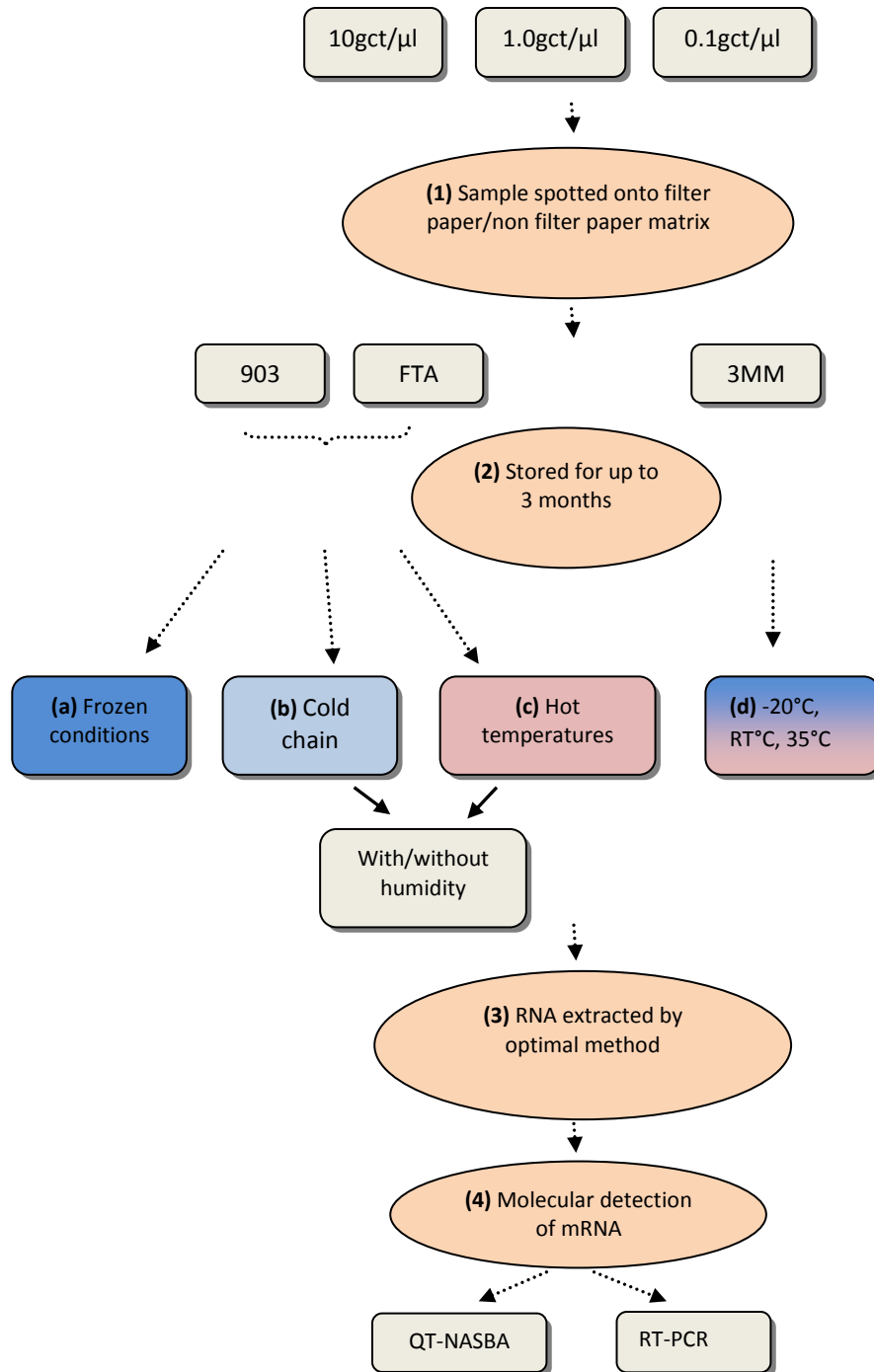


Figure 4.0. Overview of experimental procedure.

Plasmodium falciparum gametocytes were cultured as previously described (Ponnudurai, Lensen et al. 1982; Ponnudurai, Lensen et al. 1986), quantified in

counting chambers by two independent microscopists and diluted to densities of 10, 1.0 and 0.1 gametocytes per μL in parasite negative European whole blood. These concentrations were chosen because they span the microscopic threshold for gametocyte detection (Bousema and Drakeley 2011) but may still be detectable by molecular methods (Babiker, Schneider et al. 2008). Large blood spots of 50 μL of the different dilutions were aliquoted in five replicates per filter paper, incubation condition and time point, and allowed to dry overnight before being sealed into plastic bags with a silica desiccant sachet. To evaluate the impact of sub-optimal drying on mRNA recovery, after aliquoting the sample some papers were immediately sealed into bags without air drying overnight, and were stored without desiccant in containers with $\sim 80\%$ relative humidity. Two sets of papers were handled this way and are referred to as the conditions 'with humidity' in Figure 4.0. Three filter paper types were evaluated: the Whatman 903 Protein Saver Card, Whatman FTA Classic Card and the Whatman 3MM filter paper (GE Healthcare Ltd., New Jersey, USA). The FTA Classic Card was included as it is impregnated with chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases, oxidative and UV damage. The 903 Protein Saver Card, has no stabilizing properties but has previously been shown to be suitable for RNA collection (Mens, Schoone et al. 2006; Mlambo, Vasquez et al. 2008). The 3MM plain filter paper has no nucleic acid stabilizing properties, was not expected to perform well and was therefore evaluated under a smaller subset of conditions (Whatman 2009). Storage conditions were considered to replicate likely conditions that may be encountered at field sites for sampling and during transportation back to the laboratory. Frozen and cold chain incubation temperatures were: -80°C , -20°C and 4°C . To represent laboratories without access to any cold storage, ambient/room temperature (an average of 22°C , but with a range from $20-24^{\circ}\text{C}$) and hot tropical temperatures (35°C , in an incubator) conditions were included. To mimic sampling under humid conditions, papers were also stored at 22°C and at 35°C in a wet box with a hygrometer, reaching $\sim 80\%$ relative humidity. Filter papers were incubated for one week under a 'transportation/shipping' temperature, then moved to a

second 'storage' temperature, where replicates were kept for an additional four weeks or twelve weeks before RNA extraction.

As non- filter paper controls, 50 μ L aliquots of the three gametocyte densities and parasite negative diluent blood were stored in RNA stabilizing guanidine isothiocyanate buffer (Boom, Sol et al. 1990), at -80°C. A total of twenty replicates of gametocyte negative human blood was blotted onto 903 Protein Saver Cards; twenty onto the FTA Classic Card and ten onto the 3MM papers. These negative controls were stored at -20°C or 35°C with silica gel desiccant before extraction and analysis.

RNA extraction

To determine optimum extraction methods, a small-scale comparison extracting total RNA with the RNeasy Mini Blood kit (QIAGEN Ltd, Crawley, UK) and a guanidine based extraction protocol (Boom, Sol et al. 1990; Mlambo, Vasquez et al. 2008) was conducted. Replicates of all three gametocyte densities were spotted onto the 903 Protein Saver Card (n=21) and the FTA Classic Card (n=21) which were air dried overnight then stored at -80°C or -20°C for twenty-four hours. RNA was extracted by both methods and sensitivity was determined by QT-NASBA, and measured in terms of the number of positive results. RNeasy extraction from filter papers was performed as described elsewhere, following an initial homogenization step where entire 50 μ L blood spots were excised from filter papers then rocked horizontally in individual microcentrifuge tubes at 150 rpm for one hour with 500 μ L of RNeasy Lysis Buffer (QIAGEN Ltd, Crawley, UK) plus β -mercaptoethanol (0.14M final concentration). Following this, samples were centrifuged through QIAshredder columns (QIAGEN Ltd, Crawley, UK) as described elsewhere (Mlambo, Vasquez et al. 2008).

For guanidine-based extraction, filter papers were similarly homogenized in 700 μ L of guanidine isothiocyanate L6 buffer (8.3M GuSCN, 82mM Tris-HCL pH 6.4, 36mM EDTA pH 8 and 2% v/v Triton-X-100) and rocked for 1.5 hours at 150 rpm. After this, the supernatant was kept and an additional 700 μ L of guanidine isothiocyanate L6 buffer was added to the filter paper that was rocked for thirty more minutes at 150

rpm. Then the supernatants were pooled and RNA extraction performed as described (Boom, Sol et al. 1990). Non filter paper controls were gametocyte concentrations of 10, 1.0 and 0.1 gametocytes per μL in parasite negative European whole blood that was stored immediately in L6 buffer, and were extracted according to protocol from this media (Boom, Sol et al. 1990).

Gametocyte detection

Gametocyte specific Pfs25 mRNA transcripts were detected in extracted material using quantitative nucleic acid sequence based amplification (QT-NASBA) and reverse transcription polymerase chain reaction (RT-PCR). QT-NASBA was conducted as previously described using the NucliSENS Easy Q Basic Kit, Version 2 (BioMérieux Benelux B.V, Boxtel, The Netherlands), see Table S1 in the additional files for primers and molecular beacon (Schneider, Schoone et al. 2004). Prior to RT-PCR, potentially contaminating DNA was digested with DNA-freeTM (Applied Biosystems, Warrington, UK). Twenty μL reactions were conducted according to the manufacturer's protocol with the following amendments: RNA sample was added to 2 μL of rDNase I, 2 μL of 10X DNase I buffer, and inactivated with 4 μL of DNase inactivation reagent. Nested RT-PCR was conducted using a previously published protocol with novel primers, described in Table S1 in the additional files and amended cycling conditions (Mlambo, Vasquez et al. 2008; Stresman, Kamanga et al. 2010). Reactions were set up using lyophilized Illustra Ready to Go RT-PCR beads (Illustra, GE Healthcare UK Ltd, Buckinghamshire, UK) and PCR cycling was conducted as described below. To generate cDNA, reactions were held at 42°C for 30 minutes, followed by 95°C for five minutes. Primary PCR conditions were 94°C for two minutes, then 45 cycles of: 94°C for thirty seconds, 52°C for one minute and 68°C for 2.5 minutes. Nested PCR was performed using 2 μL of primary PCR product and the same conditions as before, but with thirty cycles instead. The PCR product was analysed on a 1.5% agarose gel and produced a band of ~124 base pairs.

Data analysis

Statistical comparisons of the filter paper types and detection methods were performed using statistical software STATA Version 11 (Statacorp, Texas, USA). Comparisons of filter paper or extraction performance were done by Pearson's χ^2 or trend test. Where appropriate, multivariate logistic regression models were used to allow for the effect of gametocyte concentrations when comparing the effect of storage temperature by filter paper type and to allow for the effects of gametocyte concentrations and storage temperature when comparing the performance of filter papers. GraphPadPrism Version 5 (GraphPad Software Inc., La Jolla, USA) was used for graphical presentation.

Results

Selection of an RNA extraction method

In a pilot experiment RNA extraction from bloodspots on desiccated 903 Protein Saver Cards stored at -80°C or -20°C was found to be similar using guanidine-based extraction (18/21 successful amplifications) or RNeasy based extraction (19/21 successful amplifications), with no significant difference found between the two ($p=0.635$), Table 4.0.

Table 4.0. RNeasy Mini Blood kit (QIAGEN Ltd, Crawley, UK) and Boom RNA extraction technique comparison, determined by QT-NASBA.

	-80°C			-20°C			-80°C			-20°C		
	903 Protein Saver						FTA™ Classic Card					
extraction type	10	1	0.1	10	1	0.1	10	1	0.1	10	1	0.1
Boom	(5/5)	(5/5)	(4/5)	(2/2)	(1/2)	(1/2)	(5/5)	(5/5)	(1/5)	(2/2)	(2/2)	(2/2)
RNeasy	(5/5)	(5/5)	(4/5)	(2/2)	(2/2)	(1/2)	(5/5)	(3/5)	(1/5)	(2/2)	(1/2)	(0/2)

RNA extraction from the FTA Classic Card was less efficient by RNeasy (12/21 successful amplifications) compared to guanidine-based extraction (17/21 successful amplifications), although this difference was not significant ($p=0.095$), Table 4.0. Therefore, all subsequent experiments were continued with guanidine-based extraction, which is less laborious and less expensive.

Positive and negative controls

For non-filter paper controls when gametocyte dilutions were stored at -80°C in protective guanidine buffer, all of the samples with 10 gametocytes / μL or 1 gametocyte / μL were detected by QT-NASBA, but six out of eight were detected for both dilutions by RT-PCR. For the 0.1 gametocytes/ μL samples five of eight were successfully detected by QT-NASBA, and three of eight for RT-PCR (Table 4.1.). None of the negative European donor blood showed RNA amplification by either QT-NASBA or RT-PCR (0/23).

Table 4.1. Detection comparison for QT-NASBA and RT-PCR for whole blood stored directly in guanidine RNA preservation buffer at -80°C.

	Gametocyte density/ μL			
	10	1	0.1	negative
QT NASBA	100 (8/8)	100 (8/8)	62.5 (5/8)	0 (0/4)
RT-PCR	75 (6/8)	75 (6/8)	37.5 (3/8)	0 (0/4)

Storage duration

There was no evidence for a difference in the proportion of successful amplifications between samples stored in their second condition for 4 weeks compared to those stored for 12 weeks by either QT-NASBA (regression analysis adjusting for gametocyte concentration and storage condition, $p=0.89$) or RT-PCR (regression analysis adjusting for gametocyte concentration and storage condition, $p=0.49$) (Tables S2 and S3 in the additional files). Therefore, data from the two time-points was combined for the comparison of storage conditions for subsequent analysis.

Detection of gametocytes from filter papers

Detection of gametocytes from filter papers stored at -80°C or -20°C

All gametocyte concentrations that were blotted onto the 903 Protein Saver Cards and stored with desiccant at -80°C were successfully detected by QT-NASBA and RT-PCR (Figure 4.1. (a), Tables S2 and S3 in additional files). All gametocyte concentrations that were blotted onto the FTA Classic Cards and stored with desiccant at -80°C were successfully detected by QT-NASBA but not by RT-PCR (Figure 4.1. (b), $p=0.007$).

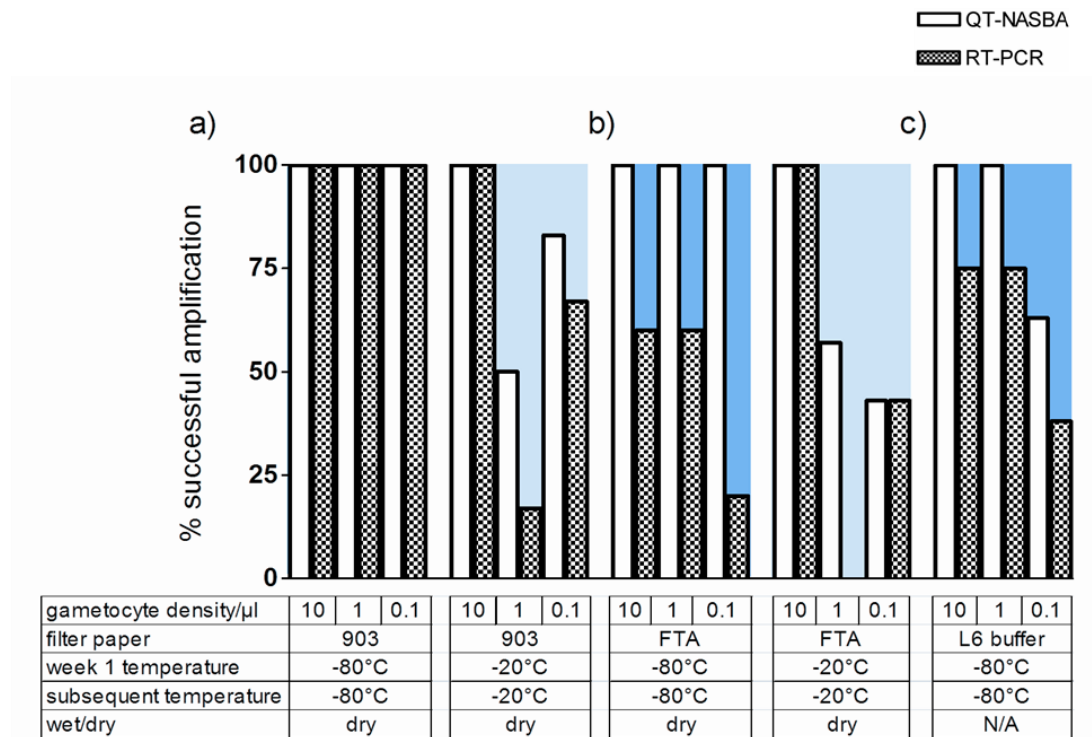


Figure 4.1. Effect of frozen storage on gametocyte mRNA preserved on filter papers. Percentage of mRNA amplification in 10, 1.0 and 0.1 gametocyte/ μ l dilutions, determined by QT-NASBA (open bars) and RT-PCR (hatched bars), when stored on filter paper. **(a)** shows the Whatman 903 Protein Saver filter paper, **(b)** the Whatman FTA Classic Card, and **(c)** sample stored in guanidine isothiocyanate buffer, when stored under frozen temperatures for up to thirteen weeks. Raw data are shown in Tables S2 and S3, additional files.

Detection of gametocytes from filter papers stored at increasing temperatures

The proportion of cards that gave successful amplification for all gametocyte dilutions was unaffected by increasing storage temperature if using the 903 Protein Saver Card (Figures 4.2. and 4.3., Tables S2 and S3 in the additional files; (test for trend adjusting for gametocyte concentration, $p=0.18$).

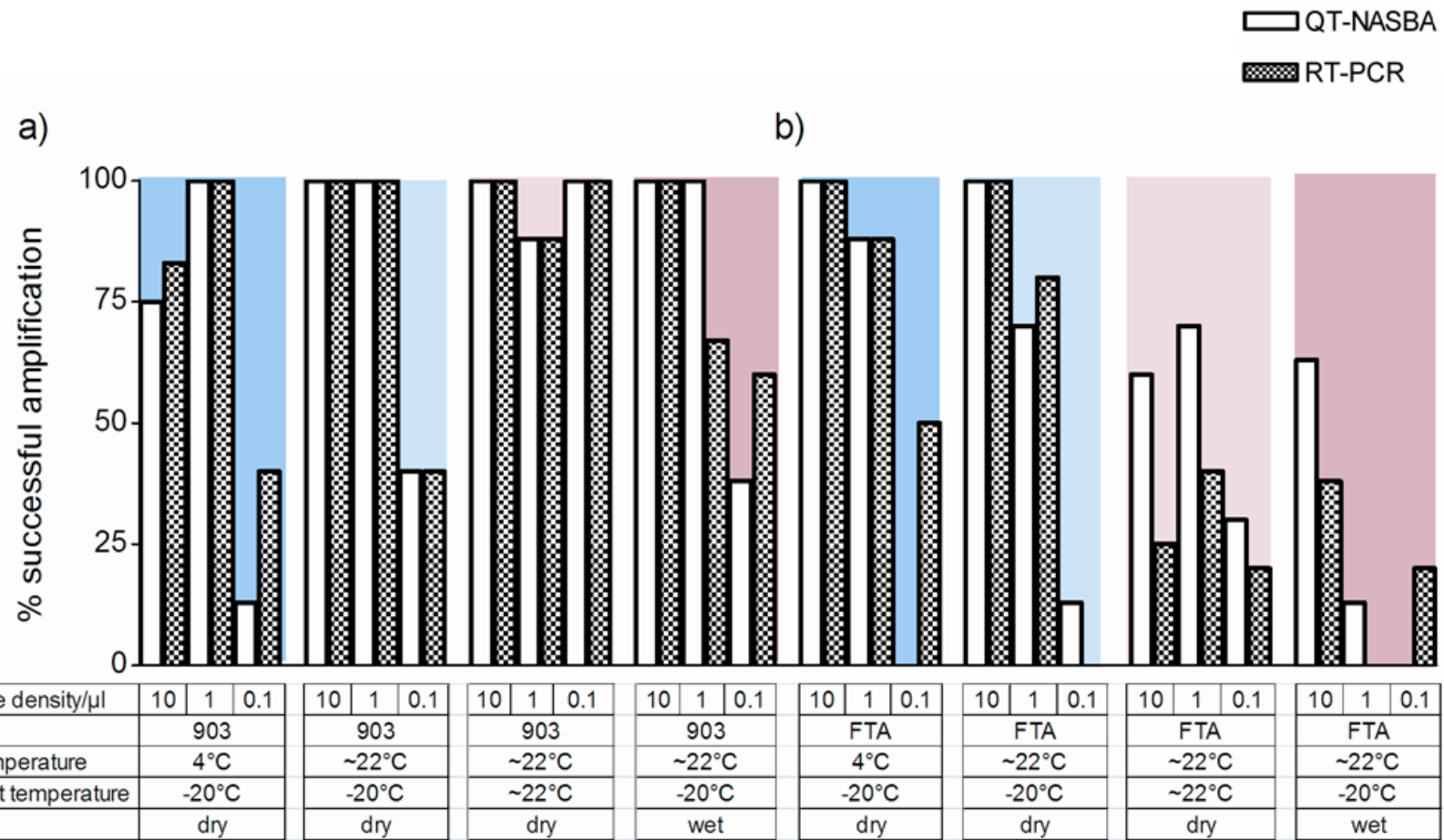


Figure 4.2. Effect of cold chain storage on gametocyte mRNA preserved on filter papers. Percentage of mRNA amplification in 10, 1.0 and 0.1 gametocyte/ μ l dilutions, determined by QT-NASBA (open bars) and RT-PCR (hatched bars), when stored on **(a)** the Whatman 903 Protein Saver filter paper and **(b)** the Whatman FTA Classic Card, under cold chain conditions with variable humidity for up to thirteen weeks.

In contrast, there was evident loss in sensitivity when samples on FTA Classic Cards were stored at a higher temperature. Successful detection by QT-NASBA of gametocytes blotted on FTA Classic Cards was achieved for 100% (9/9), 63.2% (12/19), 53.3% (16/30) and 31.6% (6/19) of the samples that were continuously stored with desiccant at -80°C, -20°C, 22°C and 35°C, respectively (test for trend, $p=0.002$). Similarly, successful detection by RT-PCR of gametocytes blotted on FTA Classic Cards was achieved for 46.7% (7/15), 50.0% (5/10), 27.8% (5/18) and 10.5% (2/19) of the samples that were continuously stored with desiccant at -80°C, -20°C, 22°C and 35°C, respectively (test for trend adjusting for gametocyte concentration, $p=0.02$). The use of RT-PCR for gametocyte detection led to similar conclusions about the effect of increasing storage temperature on the detectability of gametocytes from 903 Protein Saver Cards (test for trend adjusting for gametocyte concentration, $p=0.83$) or FTA Classic Cards (test for trend adjusting for gametocyte concentration, $p=0.01$).

Detection of gametocytes from filter papers stored at changing temperatures

Compared to 903 Protein Saver Cards that were continuously stored at -20°C, the chance of detecting gametocytes by QT-NASBA from 903 Protein Saver Cards that were first stored at higher temperatures (4°C, 22°C or 35°C) was not reduced (test for trend, $p=0.41$). In contrast, FTA Classic Cards that were first stored at higher temperatures (4°C, 22°C or 35°C) were less frequently successfully amplified by QT-NASBA (test for trend adjusting for gametocyte concentration, $p=0.09$). The use of RT-PCR for gametocyte detection resulted in similar conclusions about changing storage temperature and a loss in the detectability of gametocytes from 903 Protein Saver Cards (test for trend adjusting for gametocyte concentration, $p=1.00$) and FTA Classic Cards (test for trend adjusting for gametocyte concentration, $p=0.09$).

Detection of gametocytes from filter papers stored under humid conditions

A subset of filter papers were examined in humid and dry conditions; these samples were stored at 22°C for one week and subsequently shifted to -20°C (Figure 4.2.) or were stored at 35°C for an additional 12 weeks (Figure 4.3.).

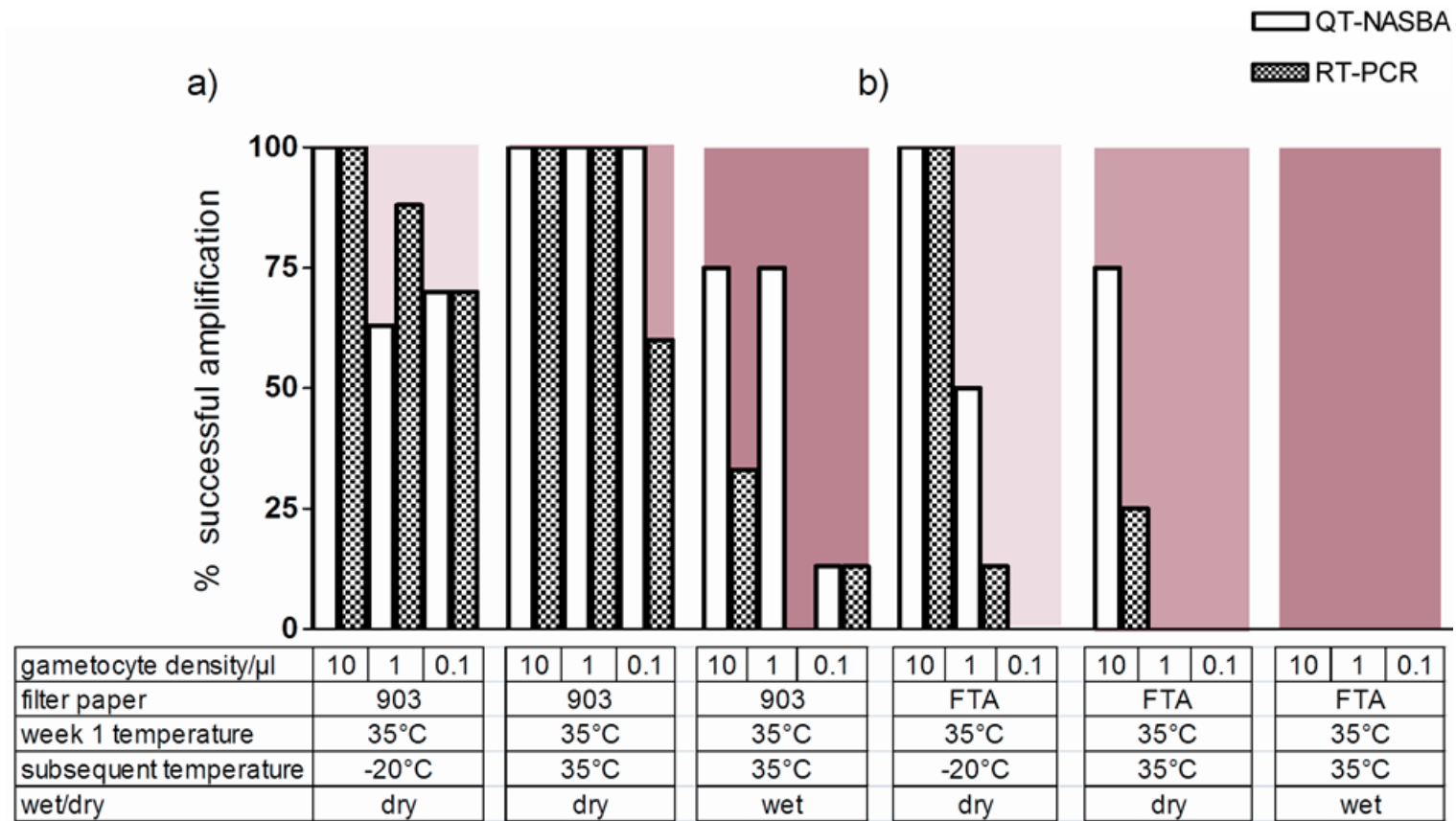


Figure 4.3. Effect of tropical condition storage on gametocyte mRNA preserved on filter papers. Percentage of mRNA amplification in 10, 1.0 and 0.1 gametocyte/ μ l dilutions, determined by QT-NASBA (open bars) and RT-PCR (hatched bars), when stored on **(a)** the Whatman 903 Protein Saver filter paper and **(b)** the Whatman FTA Classic Card, under tropical conditions with variable humidity for up to thirteen weeks.

For the first condition, 22°C for one week followed by -20°C for the next 12 weeks, there was no reduction in the proportion of successfully amplified samples from 903 Protein Saver Card by QT-NASBA (regression analysis adjusting for gametocyte concentration, $p=0.87$). However, there was a clear reduction in the proportion of successfully amplified samples from FTA Classic Cards by QT-NASBA (regression analysis adjusting for gametocyte concentration, $p=0.049$). Comparisons between humid and dry conditions for these storage temperatures gave similar results when RT-PCR was used for gametocyte detection from 903 Protein Saver Cards (regression analysis adjusting for gametocyte concentration, $p=1.00$) or FTA Classic Cards (regression analysis adjusting for gametocyte concentration, $p=0.009$). When stored at 35°C, there was a clear reduction in the proportion of successfully amplified samples from 903 Protein Saver Card by QT-NASBA (regression analysis adjusting for gametocyte concentration, $p<0.001$). For FTA Classic Cards, too few samples gave successful amplification for statistical analysis; none of the samples stored under wet conditions at 35°C were successfully detected. Comparisons between humid and dry conditions for these storage temperatures gave similar results when RT-PCR was used for gametocyte detection.

Detection of gametocytes from Whatman 3MM filter papers

Whatman 3MM papers were not expected to be a suitable media for mRNA storage, so were incubated under fewer conditions (Figure 4.4., Tables S2 and S3 additional files). These samples were tested under three conditions: at -20°C for thirteen weeks, at 22°C for thirteen weeks and at 35°C for one week followed by -20°C storage for the next twelve weeks.

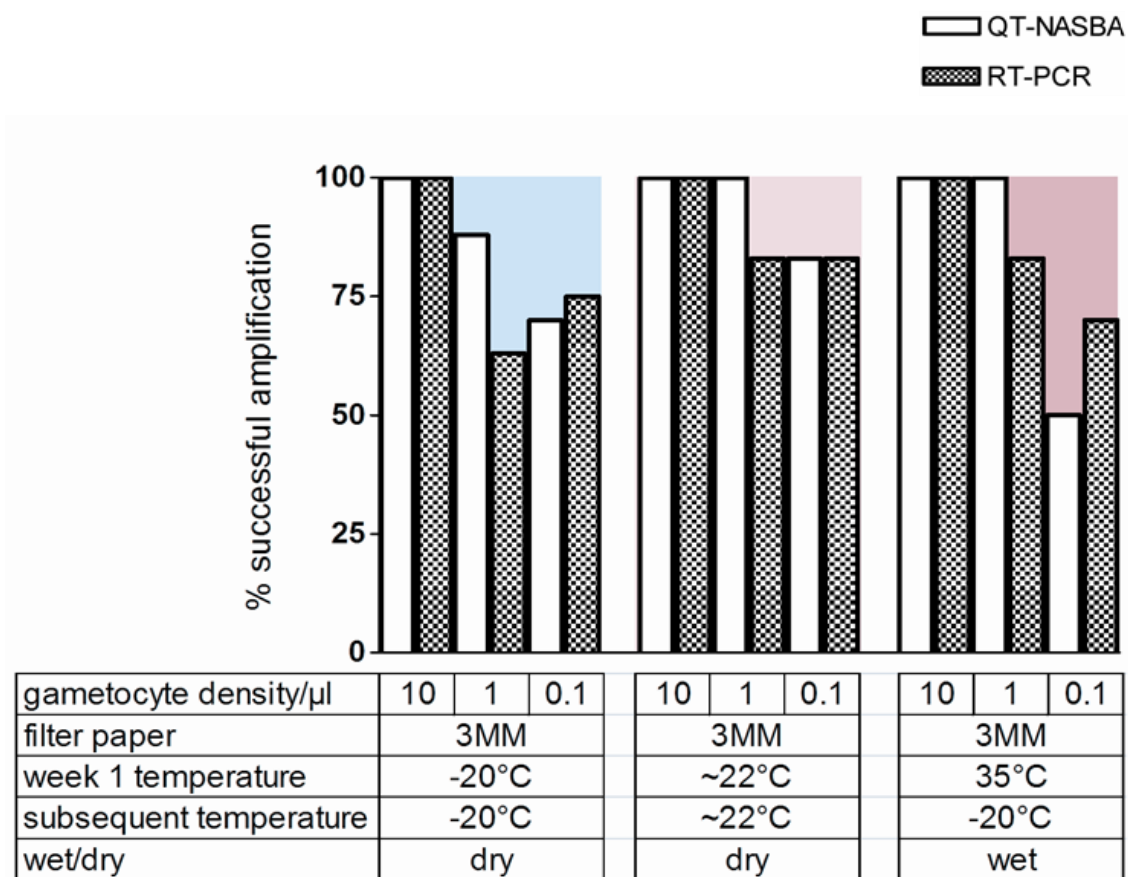


Figure 4.4. Performance of Whatman 3MM as a storage media for gametocyte mRNA. Percentage of mRNA amplification in 10, 1.0 and 0.1 gametocyte/ μ l dilutions, determined by QT-NASBA (open bars) and RT-PCR (hatched bars), when stored on the Whatman 3MM filter paper, incubated under different conditions for up to thirteen weeks.

When continuously stored at -20°C , there was no difference between the proportion of successfully detected samples for all gametocyte dilutions by QT-NASBA between 3MM and 903 Protein Saver Cards (regression analysis adjusting for gametocyte concentration= 0.34) or FTA Classic Cards (regression analysis adjusting for gametocyte concentration, $p=0.10$). When stored continuously at 22°C , 3MM filter papers performed as well as 903 Protein Saver Cards (regression analysis adjusting for gametocyte concentration, $p=0.95$) but better than FTA Classic Cards (regression analysis adjusting for gametocyte concentration, $p=0.01$). When stored at 35°C for one week followed by -20°C for the next twelve weeks, 3MM filter papers performed as well as 903 Protein Saver Cards (regression analysis adjusting for gametocyte concentration, $p=0.75$) but better than FTA Classic Cards (regression analysis adjusting

for gametocyte concentration, $p=0.01$). When RT-PCR was used, similar results were obtained.

Discussion

This study was designed to investigate optimal collection and storage conditions to enable reliable detection of *P. falciparum* gametocyte densities found in natural infections. Filter papers were found to be a suitable media for Pfs25 mRNA detection from low gametocyte concentrations after storage for up to thirteen weeks.

Furthermore, it was found that gametocyte Pfs25 mRNA stored on 903 Protein Saver Cards is remarkably stable at temperatures higher than -80°C .

Unlike the general detection of malaria parasites, which is based on amplification of *Plasmodium* nuclear or mitochondrial DNA, the specific detection of gametocytes depends on mRNA. Reliable sampling for gametocytes currently depends on RNA extraction in the field (Sutherland, Allouche et al. 2002; Schneider, Bousema et al. 2007) or storage of samples in stabilizing buffer at -80°C in order to protect the integrity of mRNA, which is much more labile than DNA. The main aims of this study were to determine which filter papers were most suitable for storage of gametocyte mRNA in dried whole blood samples, and to explore flexibility of storage conditions and whether less stringent conditions are permissible without affecting estimates of gametocyte prevalence. This study builds upon similar research evaluating RNA detection from filter paper (Mlambo, Vasquez et al. 2008) but provides more rigorous testing with a larger number of replicates in diverse incubation conditions including both high and low humidity, which is of particular relevance for sampling in the tropics. QT-NASBA reliably detected gametocyte densities of ten or one gametocyte/ μL when immediately stored in RNA-stabilizing buffer at -80°C and was found to be more sensitive than RT-PCR for detecting low gametocyte concentrations. The higher sensitivity of QT-NASBA is unlikely to be explained by amplification of ssDNA (Pritsch, Wieser et al. 2012); but we do consider it plausible that the DNase digestion step may have negatively influenced the sensitivity of RT-PCR. It is also important to note that we compared QT-NASBA with RT-PCR as it was previously used for Pfs25 mRNA

detection (Mlambo, Vasquez et al. 2008; Stresman, Kamanga et al. 2010); more sensitive quantitative RT-PCR assays may become available that make this assay comparable in sensitivity to QT-NASBA. Under optimal conditions, storage in RNA-stabilizing buffer at -80°C, it was observed that not all samples with 0.1 gametocyte/μL were detected by QT-NASBA. This may reflect variability in extraction efficiency or RNA amplification (Churcher *et al* in preparation) or an inevitable consequence of very low pathogen concentrations and sample volumes (an average of five gametocytes per 50μL blood sample blotted on the paper). The use of an extraction control could address uncertainties on extraction efficiency between samples but was not used in this study or in other studies using Pfs25 mRNA RT-PCR or NASBA (Schneider, Schoone et al. 2004; Schneider, Wolters et al. 2005; Mens, Schoone et al. 2006; Schneider, Bousema et al. 2006; Mens, Spieker et al. 2007; Ouedraogo, Schneider et al. 2007; Schneider, Bousema et al. 2007; Mens, Sawa et al. 2008; Ouedraogo, Bousema et al. 2009).

Detection of gametocytes from filter papers stored at -80°C succeeded with a similar, but slightly higher frequency compared to detection of gametocytes from samples stored directly in RNA stabilizing buffer. The unexpected improved detection from filter paper samples has been previously acknowledged in similar studies (Pritsch, Wieser et al. 2012) where it has been suggested the drying step enhances blood cell lysis, leading to a higher probability of detection. It is currently unclear whether this apparent superiority of filter papers over RNA stabilizing buffer is specific to Pfs25 mRNA and if other gametocyte mRNA targets are less well stored or recovered using filter papers.

The successful detection of gametocytes from filter papers stored at -80°C formed a useful starting point for analyses that aimed to determine the suitability of filter papers for storage under less favourable conditions. No evidence was found that increasing storage temperature resulted in a lower success rates for gametocyte detection from 903 Protein Saver Cards. Contrary to this, gametocyte detection from the FTA Classic Cards was most efficient when stored at -80°C, lower when stored at -20°C or 22°C and lowest when stored at 35°C. FTA Classic Cards were also more susceptible to changes in storage temperature with short-term storage at

temperatures higher than -20°C resulting in a loss in detectability of gametocytes from these cards. Qualitative gametocyte detection from 903 Protein Saver Cards was not affected by different storage temperatures.

Incomplete drying of filter papers resulted in a loss in efficiency of gametocyte detection. This effect was most apparent at the highest temperature tested (35°C) and FTA Classic Cards appeared most susceptible to this detrimental effect of moisture on gametocyte detection. This loss in sensitivity could be a result of RNA degradation and/or incomplete sample elution from insufficiently dried filter papers. It has previously been acknowledged that insufficient drying impacts on the sample elution which is likely to contribute to the loss in detection seen here. On visual inspection following blood spot elution, filter papers stored under humid conditions remained red, suggesting incomplete recovery of the blood-product (Mei, Alexander et al. 2001). Exposure to heat and moisture has been suggested to cause damage or denaturation of RNA not only by the formation of nicks in the nucleic acid strands, or by strand hydrolysis, but also by amplifying degradation caused by UV light exposure (Rogers and Burgoyne 2000; Moscoso, Raybon et al. 2005; Kansagara AG 2008). Removing moisture by overnight drying and inclusion of desiccant not only prevents physical destruction of the paper, but will also slow RNA degradation.

The general-purpose 3MM paper permitted detectable RNA recovery as often as the 903 Protein Saver Card and more often than the FTA Classic Card under all conditions. As this filter paper has no specific sample preservation properties and is generally used for DNA and serological sample collection, it was not expected to perform as well as it did. Previous success has been reported for viral RNA storage on the 3MM filter paper, with transcripts being detected after twelve weeks of storage at 32°C, without a loss in sensitivity (Michaud, Gil et al. 2007). Studies conducting RT-PCR from measles virus RNA stored on the 3MM filter paper have shown successful amplification following twenty-five weeks of storage at room temperature, but a loss in sensitivity with increasing temperature, and detection for just one week post storage for papers incubated at 37°C in humid conditions, due to fungal growth (De Swart, Nur et al.

2001). These results are promising, and use of 3MM is particularly appealing due to its low cost and common use as a substrate for DNA collection (Corran, Cook et al. 2008; Cook, Reid et al. 2010). A recent publication provided evidence that this paper is indeed suitable for Pfs25 mRNA storage (Pritsch, Wieser et al. 2012). When this publication was in preparation, Pritsch *et al* published similar research showing the suitability of 3MM for Pfs25 mRNA storage for low density gametocytes (Pritsch, Wieser et al. 2012). Results are in broad agreement: 3MM paper is suitable for Pfs25 mRNA storage, higher storage temperatures may lead to a lower sensitivity of gametocyte detection, and filter paper Pfs25 mRNA collection may lead to higher RNA yield than RNA extraction from whole blood samples. The manuscript by Pritsch provides additional details on the stability and quantification of Pfs25 mRNA; the current manuscript has limitations in the sense that RNA yield from filter papers was not determined. The analyses were restricted to gametocyte prevalence and some RNA degradation may therefore have remained undetected. However, the current manuscript extends previous reports on the use of filter paper for RNA storage by the concurrent examination of the sensitivity of RT-PCR, by showing a side-by-side comparison of three filter papers and by examining the effects of a large range of storage conditions including a combination of high temperature and high humidity, which is particularly relevant for field sample collection.

In addition to Pfs25 mRNA stability and recovery, the cost and operational ease are important considerations when choosing a methodology. The filter papers used in this study differ substantially in cost from USD \$2 for 100 papers for the 3MM, \$133 for 100 903 Protein Saver Cards, to approximately \$400 for 100 of the FTA classic cards, in 2011. Whilst the extraction methods performed equally well in the small comparison conducted, the guanidine-based extraction is more affordable at \$1.2 per reaction and is more amenable to large-scale extraction. The Qiagen protocol, complete with QiaShredder homogenization and DNA digest, is more expensive at \$4.5 per extraction. The individual expense of molecular detection is \$7 per reaction for QT-NASBA, but amounts to ~\$14 for RT-PCR as it requires a DNA digest and a DNA control per sample, to exclude the possibility of DNA contamination. The combination of 903 Protein Saver

Cards (which allows a minimum of five spots per card), guanidine extraction and QT-NASBA detection gives a total cost of \$8.5 per sample. The cheapest combination of methods is the 3MM paper, guanidine-based extraction and QT-NASBA for detection, which would amount to approximately \$8.22 per sample.

Conclusions

In summary, these findings indicate that filter paper cards can be used for collection and storage of Psf25 mRNA for detecting low gametocyte concentrations. The 903 Protein Saver Card and 3MM filter papers are both recommended as they appear particularly promising in this respect and appear to be robust storage media under temperatures higher than those conventionally used for RNA storage. Thorough drying of papers was more important for successful gametocyte detection than the temperature under which samples were stored.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SJ, TB, CD, CH and RH designed the experiments, RS contributed reagents for the study, MVB cultured the parasites and SJ, TA, KT and CH set up the study. SJ extracted the RNA, performed the RT-PCR and QT-NASBA and CS provided RT-PCR primers and contributed to RT-PCR protocol design. SJ, TB and CD interpreted the data, SJ, TB and PC performed the statistical analysis and SJ and TB wrote the manuscript. CD and CS revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Filter papers were kindly provided by GE Healthcare Bio-Sciences Corp. This study was funded by the European FP7 project REDMAL (#242079). The authors thank Dr Godfree Mlambo at the Parasite Core Facility at Johns Hopkins School of Public Health, USA, Dr Tamaki Kobayash from the Department of Epidemiology at Johns Hopkins School of Public Health, USA, Professor Srinivasan Villuppanoor of Indian Immunologicals Limited, Hyderabad, India, and Gillian Stresman in the Department of Immunology and Infection at the London School of Hygiene and Tropical Medicine, UK for sharing their RNA extraction and RT-PCR protocols and providing advice. The authors would also like to thank Amrish Baidjoe from the Department of Medical Microbiology at Radboud University Nijmegen Medical Centre, The Netherlands, for help with QT-NASBA.

4.4 Linking methods and results:

The publication discusses the main findings of this chapter. This section details optimization of protocols, including RT-LAMP (which had initially been intended to be included in the comparative study) and additional work that supports the results in the publication. To improve the clarity, the methods and results follow each other in some instances, and headings are labelled with either 'methods' or 'results'.

This section begins with general methods detailing preparation of this study, then it is split into the following overall headings:

- Gametocyte detection using reverse transcriptase loop mediated isothermal amplification (RT-LAMP) from RNA treated with a DNA digest.
- Molecular detection using quantitative nucleic acid sequence based amplification (QT-NASBA) and reverse transcription polymerase chain reaction (RT-PCR).
- Detection of Pfs25mRNA from filter paper blood spots collected in an endemic setting.

An extended discussion follows at the end.

4.41 Methods: Preparation of work space and equipment for RNA extraction and molecular detection

There were 2 designated cabinets used for:

- 1) Excision of blood spots and nucleic acid extraction.
- 2) Preparation of molecular detection reaction mastermixes.

Before use, all bench space and cabinets were sterilized as described next. Bench surfaces, pipettes and lab equipment (pipette tip boxes, tube racks etc) were sterilized

thoroughly using 100% ethanol followed by RNase Zap (Sigma-Aldrich Ltd, Dorset, UK). Specific pipettes and filter tips were designated for RNA extraction protocols, with a separate set used for molecular protocols. All microcentrifuge tubes used were nuclease free (Life Technologies, Lincolnshire, UK). Cabinets were sterilized as follows, UV light exposure for 30 minutes in a closed hood with a UV light and timer, followed by cleaning with 100% ethanol, then cleaning with RNase Zap. For molecular protocols, addition of test sample nucleic acid (or amplified product for a nest 2 reaction) was performed in a separate clean area, and was added with a specific pipette.

4.42 Methods: Collection of cultured gametocytes on filter paper

To provide low density gametocytes for spotting onto filter papers for the comparative study, 3D7 gametocytes were cultured and supplied as a kind gift from Marga van der Vegte-Bolmer (Radboud UMC, Nijmegen, The Netherlands). Gametocytes were diluted in malaria negative European Blood provided from individuals with no travel history to a malaria endemic country (supplied by Radboud UMC, Hospital Blood Bank, Nijmegen, The Netherlands) to 10, 1.0 and 0.1 gametocytes/ μL . Fifty μL of gametocyte spiked blood was spotted five times onto each filter paper per incubation condition. The 903 Protein Saver Card has 5 stamped circles printed on it to aid with sample collection. Since the FTA card had 4 large circles, the 5th blood spot was spotted in the centre of the others. The 3MM paper is provided on a continuous roll with a fixed width of 0.78 inches. Sections were cut that were 5 inches in length, which were stapled into cardboard covers, then the 5 samples were spotted evenly spaced onto the paper. Since the 3MM paper is thin, sample was pipetted slowly to allow absorption and avoid sample run off. Table 4.2. indicates the total number of sample replicates stored per gametocyte density, incubation condition and filter paper type.

Table 4.2. The number of filter paper replicates incubated for each filter paper type, gametocyte density and incubation condition. RT = room temperature, AD= filter papers were air dried overnight before storage (average temperature: 22°C, relative humidity ~40%), NT=condition not tested, WK=week. Filter papers were stored at the temperature indicated for ‘temp wk 1’ for the first week, then were moved to the temperature indicated in ‘temp wk 2’, where they remained until extraction after 1 or 3 months (half the number of filter paper replicates indicated in this table were extracted at each time point).

Filter paper:				Whatman 903 Protein Saver Card				Whatman FTA Classic Card				Whatman 3MM							
Incubation condition				gametocyte density/ μ L															
drying	silica gel	temp wk 1	temp wk 2	10	1	0.1	0	10	1	0.1	0	10	1	0.1	0				
AD	Yes	-80 °C	-80 °C	5	5	5	NT	5	5	5	NT	NT	NT	NT	NT				
AD	Yes	-20 °C	-20 °C	10	10	10	10	10	10	10	10	10	10	10	10				
AD	Yes	35 °C	35 °C	10	10	10	10	10	10	10	10	NT	NT	NT	NT				
AD	Yes	35 °C	-20 °C	10	10	10	NT	10	10	10	NT	10	10	10	NT				
AD	Yes	RT	RT	10	10	10	NT	10	10	10	NT	10	10	10	NT				
AD	Yes	RT	-20 °C	10	10	10	NT	10	10	10	NT	NT	NT	NT	NT				
AD	Yes	4 °C	-20 °C	10	10	10	NT	10	10	10	NT	NT	NT	NT	NT				
wet	No	35 °C	35 °C	10	10	10	NT	10	10	10	NT	NT	NT	NT	NT				
wet	No	RT	-20 °C	10	10	10	NT	10	10	10	NT	NT	NT	NT	NT				

Twenty additional replicate blood spots per filter paper and gametocyte dilution were prepared to enable protocol optimization before extraction of test papers. Following sample aliquoting, filter papers were air dried overnight at ambient temperature then sealed into individual air tight plastic bags with a 0.5g sachet of colour changing desiccant (GeeJay, Bedfordshire, UK), before incubated under test conditions. Filter papers evaluated in humid conditions were sealed into plastic bags immediately following sample aliquoting and were incubated without silica gel desiccant.

Incubation conditions were chosen that span temperatures typical for the field, a well equipped laboratory, or transportation between the 2. A selection of conditions had incubation at one temperature for the first week, followed by movement to a second temperature for the rest of the duration, which was devised to mimic storage during transport of filter papers from the field back to the laboratory. Specifically, standard storage temperatures were included such as 4°C (fridge temperature), -20°C and -80°C

(freezer temperatures) along with room temperature storage (~22°C) and storage at a tropical temperature, 35°C. Due to high humidity commonly experienced in the tropics, paired incubation conditions with and without humidity were included for two temperatures: room temperature for the first week, followed by -20°C for the second week, and for papers incubated at 35°C throughout. A benchtop incubator was used for 35°C incubation, and humidity maintained by incubating filter papers (sealed in plastic bags) inside Tupperware[®] plastic boxes with a small pad of water soaked tissue that was changed every day 2/3 days (as needed) to maintain an average relative humidity of 82% (range 44-98%). Humidity was monitored daily with a hygrometer (Screwfix, Somerset, UK). Half the filter papers (5/10) were stored for 1 month and the other half were stored for 3 months to assess if longer term storage led to a measurable loss in mRNA detection. Following incubation, filter papers stored for 1 month were kept at -80°C until 3 month filter papers had finished incubating, then RNA extraction and mRNA detection was performed.

4.43 Methods: Excision of filter paper blood spots

In preparation for extraction, entire 50µL blood spots were excised from filter papers in a laminar flow cabinet that had been sterilized as outlined previously. All equipment used for excision, including microcentrifuge tube racks, scissors and tweezers, were sterilized in the same manner. To excise the blood spots, scissors were flame sterilized using a Bunsen burner, allowed to cool, then blood spots were cut allowing a ~2mm margin to avoid contact of the scissors with the sample. Using sterilize tweezers, the blood spot was removed into a microcentrifuge tube ready for extraction.

4.44 Methods: Guanidine based RNA extraction

To compare guanidine based extraction and Qiagen RNA extraction methods, gametocytes (10, 1.0 and 0.1/ µL) were spotted onto Whatman 903 Protein Saver

(n=42, total) and FTA Classic Card filter papers (n=42), which were stored at -20°C or -80°C overnight before extraction. Half were extracted using guanidine based extraction and the other half were extracted using Qiagen extraction, as outlined in the publication.

Guanidine based extraction was performed as follows from excised blood spots, 700µL of L6 lysis buffer (8.3M GuSCN, 82mM Tris-HCl (hydrochloric acid) [pH 6.4], 36 mM ethylenediaminetetraacetic acid (EDTA) and 2% v/v Triton X-100), see appendix 1 for preparation, was added per microcentrifuge tube which was horizontally shaken at 150 rpm for 1.5 hours. Supernatant was removed to a new microcentrifuge tube and another 700µL added to the filter paper which was shaken as before for 30 more minutes. The supernatant was removed to the same microcentrifuge tube as previous, and 30µL of silica suspension was added (see appendix 1 for preparation) and mixed thoroughly by vortex. Tubes were shaken horizontally for 10 minutes at 150 rpm as before, vortexed for 5 seconds, then centrifuged for 15 seconds at 12,000g. Supernatant was removed, then 1mL of L2 wash buffer (5.25 M GuSCN, 50 mM Tris HCl [pH 6.4],) see appendix 1 for preparation, was added and microcentrifuge tubes were vortexed to resuspend the pellet. Tubes were centrifuged at 12,000g and supernatant was removed by aspiration. This was repeated once more with 1mL of L2 buffer, twice more with 1mL of 70% ethanol, then once with 1mL of acetone. Following this, microcentrifuge tubes were incubated in a hot block at 56°C for 10 minutes with the lids off to dry the pellets. Next 50µL of RNase free water (Ambion, Renfrewshire, UK) was added to the pellet which was vortexed thoroughly to mix. Samples were incubated at 56°C with the lids on for 10 minutes, then centrifuged for 2 minutes at 12,000g. The top 35µL of supernatant containing the eluted nucleic acid was transferred to a new microcentrifuge tube and stored at -80°C until use.

4.45 Methods: RNA extraction using Qiagen RNA mini kit

To complete the RNA extraction comparison, the second half of the filter papers (discussed in 4.44) were extracted using the Animal Cells protocol (RNA Mini Kit, Qiagen, Surrey, UK) as follows. Entire 50µL blood spots were aseptically excised (as described before) into 1.5mL microcentrifuge tubes containing 500µL of RTL buffer (Qiagen) plus β-mercaptoethanol (0.14 M end concentration). This was horizontally shaken at 150 rpm for 1 hour on a rotary shaker. Then, the filter paper and buffer were transferred to a QiaShredder Column (Qiagen, Surrey, UK) inside a collection tube, and centrifuged for 2 minutes at 14,000 rpm. Supernatant was removed to a new microcentrifuge tube, then 500µL of 70% ethanol was added and this was mixed by pipetting. Half this volume was added to a spin column in a collection tube which was centrifuged for 2 minutes at 13,000 rpm, then flow through was discarded and the procedure repeated with the remaining volume. The column was washed with 700µL of RW1 buffer (Qiagen) which was centrifuged for 2 minutes at 13,000 rpm. The flow through was discarded and another wash performed with 500µL of RPE buffer (Qiagen) that had been pre-diluted with 4 volumes of 100% ethanol. This was centrifuged for 2 minutes at 13,000 rpm, the flow through discarded again, then the wash step repeated and spin columns transferred to fresh collection tubes and centrifuged for 1 minute at 13,000 rpm to dry the column membrane. Columns were moved to new microcentrifuge tubes and 50µL of RNase free water (Ambion, Renfrewshire, UK) was added directly to the membrane, then columns were centrifuged at 10,000 rpm for 1 minute to elute the RNA. Samples were stored at -80°C until use.

4.5 Linking methods and results: Gametocyte detection using reverse transcriptase loop mediated isothermal amplification (RT-LAMP) from RNA treated with a DNA digest

4.51 Methods: DNA digestion of the extracted nucleic acid using DNase I (RNase-free)

Guanidine based extraction extracts total nucleic acid. To eliminate DNA that may amplify during the RT-PCR reaction, a DNA digest had to be performed. Similarly, while the Qiagen kit should result in extraction of RNA, the manufacturers recommend following with a DNA digest, if performing an amplification technique that is sensitive to small amounts of DNA.

Initially, DNA was digested using DNase I (RNase-free) (Ambion, Renfrewshire, UK). Digest reactions were set up according to the supplied protocol as follows. In a microcentrifuge tube, the following were combined, 5µL of 10x DNase I buffer, 1µL (2 units) of DNase I, 9µL of RNase free water (Ambion, Renfrewshire, UK) and 10µL of 25mM of EDTA. Twenty five µL of the previously extracted nucleic acid was added and reactions incubated at 37°C for 30 minutes on a thermocycler (C1000 Thermal Cycler, BioRad, Hertfordshire, UK), followed by 75°C for 5 minutes to inactivate the enzyme. RNA was stored at -80°C until use.

4.52 Methods: Reverse transcription loop mediated isothermal amplification (RT-LAMP) performed on DNase I (RNase-free) treated RNA

Using the RNA that had been treated with the DNase I (RNase-free) digest, gametocyte detection with RT-LAMP was performed using the protocol published by Buates and colleagues and the RT-LAMP Loopamp kit, produced by Eiken (Tokyo, Japan) (Buates, Bantuchai et al. 2010).

First, a primer mix was prepared as follows, 40pmol of FIP and BIP was combined with 20pmol of LOOP-F and LOOP-B and 5pmol of F3 and B3 (see Table 4.3. for primer sequences).

Table 4.3. Primer sequences for detection of Pfs25mRNA using RT-LAMP.

Primer name	Sequence (5' to 3' orientation)
F3	TTTAATTCAGATGAGTGGTCAT
B3	CTTACATTCATTTGGTATACAAACA
FIP	GTCTTTTCGTCACATTTCAGAACTTATGTAAATGTGAAAATGATTTGGTG
BIP	ACCATGTGGAGATTTTCCAAATGTCAAGATTACATTTACAAGCGTATG
LOOP-F	CTTCACATGTTTCTTCATTTACTAA
LOOP-B	ATTAAAATAGATGGAAATCC

Maintaining reagents on ice, the mastermix was prepared as outlined in Table 4.4. and italicized reagents were added to the reaction tubes.

European malaria negative blood was used as a negative control and a high density cultured sample (1000 gametocytes/ μL (g/ μL)) was used as a positive control. Both were included with each assay. Positive control sample was provided by Marga van der Vegte-Bolmer, and the negative control blood came from Radboud Hospital Blood Bank (Radboud UMC, Nijmegen, The Netherlands).

Table 4.4. RT-LAMP mastermix reagents and volumes using Loopamp RNA amplification kit (Eiken, Tokyo, Japan)

reagent	Volume for 1 reaction (μL)
2 x reaction mix	12.5
primer mix	1.3
Enzyme mix	1.0
Water	7.2
calcein	1.0
<i>Mastermix total</i>	<i>23.0</i>
<i>Template RNA (added separately)</i>	<i>2.0</i>

Amplification reactions were performed in a thermocycler (C1000 Thermal Cycler, BioRad, Hertfordshire, UK), using the following conditions: 65°C for 90 minutes,

followed by 80°C for 5 minutes. Positive samples were determined by fluorescence when using a hand held UV lamp (Mini Fluorescent UV Lamp, Fujitsu, London, UK) and as turbidity when detected by eye. Results were read by 3 lab personnel and test samples were compared to controls, to aid with interpretation of results (Buates, Bantuchai et al. 2010).

4.53 Results: Gametocyte detection using reverse transcription loop mediated isothermal amplification (RT-LAMP) performed on DNase I (RNase free) treated nucleic acid

To assess if the protocol was optimized, the following samples were amplified by RT-LAMP: the positive control sample (1000g/μL) (already extracted), 4 positive samples extracted using Qiagen, 4 x positive samples extracted using guanidine based extraction, and 2 negative control samples (all of which were treated with the DNase I digest). The 4 positive samples were 2 x 100 g/μL and 2x 10g/μL. One sample of each density (and the negative controls) had been spotted onto both the Whatman 903 Protein Saver Card and the FTA Classic Card. All 11 samples produced positive results (determined by fluorescence/eye, data not shown) including the negative control blood, which was from individuals with no travel history to a malaria endemic country. The false positive results were thought to be attributed to the presence of EDTA which was added to the digest reaction to prevent RNA from undergoing scission during heat inactivation of the enzyme. EDTA has been recognized as resulting in false positive results, when using detection by fluorescence (Francois, Tangomo et al. 2011). Figure 4.5.demonstrates the suspected reason for these false positive results. In the reaction, the fluorophore calcein is initially quenched by Mn^{2+} , since EDTA is a chelator of 2^{+} ions, it is likely EDTA chelated Mn^{2+} from the calcein which resulted in fluorescence and a false positive result, for all reactions (Ambion 2007; Francois, Tangomo et al. 2011).

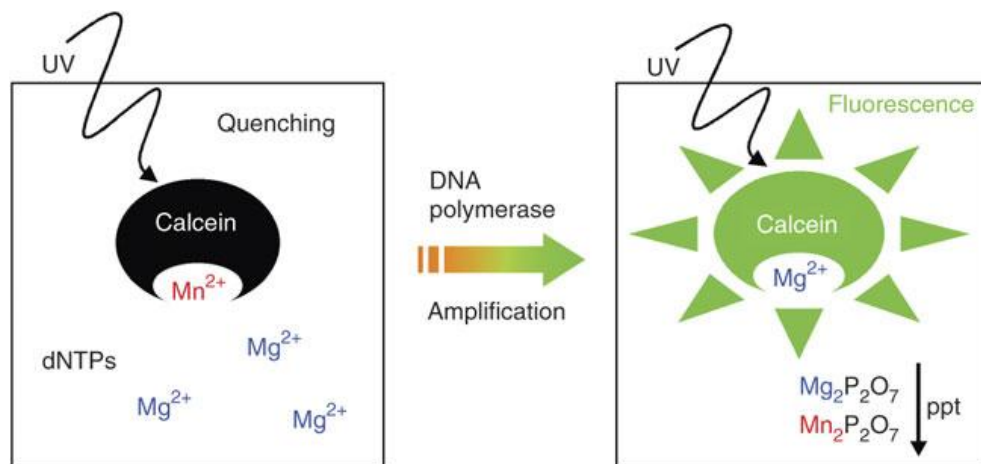


Figure 4.5. Principle of the LAMP reaction using calcein as a fluorophore (Tomita, Mori et al. 2008). Figure taken directly from the referenced publication.

To avoid using EDTA in the DNA digest reaction, the DNA Free Kit (Ambion) was used instead.

4.54 Methods: DNA digestion of the extracted nucleic acid using the DNA Free Kit

A second DNA digest was tried using the DNA Free kit (Ambion, Renfrewshire, UK), as follows. Sixteen μL of extracted nucleic acid was mixed with 2 μL of 10x DNase I buffer and 2 μL of rDNase I in a microcentrifuge tube. This was gently mixed by pipetting, and then was incubated at 37°C for 30 minutes on a thermocycler (C1000 Thermal Cycler, BioRad, Hertfordshire, UK). Four μL of DNase inactivation reagent was then added to the reaction, which was incubated at room temperature for 2 minutes, during which it was mixed 3 times by pipetting to disperse the reagent. Tubes were then centrifuged at 10,000g for 1.5 minutes and $\sim 18\mu\text{L}$ of supernatant was removed to a new microcentrifuge tube, which was stored at -80°C until use.

4.55 Results: Gametocyte detection using reverse transcription loop mediated isothermal amplification (RT-LAMP) performed on nucleic acid treated with the DNA Free Kit

RT-LAMP was attempted once more (as before) using the same extracted nucleic acid for the test samples, but performing the new DNA Free digest on the extracted samples first. For guanidine based extraction, digest reaction volume was halved since there was less extracted material remaining. Results were determined by fluorescence/ turbidity as before. Positive and negative results were difficult to distinguish. This resulted in 5/11 positive for reader 1, 8/11 for reader 2 and 7/11 for reader 3, with poor inter reader agreement including for positive and negative controls.

4.56 Methods: Reverse transcription loop mediated isothermal amplification (RT-LAMP) using hydroxy naphthol blue to indicate positive results

To try to improve the sensitivity of RT-LAMP with the aim of achieving better discrimination of positive and negative results (and therefore improving inter reader agreement), the fluorophore calcein was replaced with hydroxy naphthol blue (HNB), a metal indicator that changes from purple to blue if by-product magnesium pyrophosphate is produced and the pH changes (Goto, Honda et al. 2009; Wastling, Picozzi et al. 2010). Reactions were assembled as before (in Table 4.4.) substituting calcein with 0.15 μ L of HNB per reaction and adjusting the final volume of water to 8.05 μ L. Along with a colour change from purple to blue, positive results also cause a change in turbidity which can be measured on an ELISA microplate reader at an absorbance of 650nm (Goto, Honda et al. 2009). To distinguish positive and negative results using the microplate reader, the OD value of the negative controls plus 2 standard deviations was used as a cut off value. To determine if filter paper sampling was interfering with reagents in the RT-LAMP reaction, whole blood samples (10, 1.0

and 0.1 gametocytes/ μL) preserved in L6 buffer were extracted and gametocytes detected by RT-LAMP.

4.57 Results: Performance of RT-LAMP

Despite varying the DNA digest protocol and positive result indicator, it remained difficult to identify positive results with 3 readers having poor agreement. There was no improvement when fluorophore calcein was replaced with HNB, and use of the microplate reader did not result in positive samples. There was also no improvement when using gametocytes preserved in L6 buffer as the starting material. Rather than investing in a technique that was not performing well in controlled conditions in my hands, and therefore likely to be problematic in less well equipped field conditions, further work using RT-LAMP was suspended and RT-LAMP was not included for comparison in the publication.

4.6 Linking methods and results: Molecular detection using quantitative nucleic acid sequence based amplification (QT-NASBA) and reverse transcription polymerase chain reaction (RT-PCR)

4.61 Methods: Quantitative nucleic acid sequence based amplification (QT-NASBA)

QT-NASBA reactions were prepared in an RNA cabinet that had been decontaminated as previously outlined. A cultured gametocyte sample (3D7) was used to generate a trend line which was titrated 10 fold from 10^6 , 10^5 , 10^4 , 10^3 , 10^2 to 10 gametocytes/mL, to serve as an internal control to identify positive results, enable calculation of gametocyte load and indicate efficiency of amplification. Pfs25mRNA was amplified using the NucliSens EasyQ Basic Kit 2 (BioMérieux, Benelux B.V, Boxtel, The Netherlands) which was kept at 4°C until use. The enzyme and mastermix preparations are provided as lyophilized pellets and once reconstituted the volume is

enough for 16 reactions, consequently, all experiments were assembled in multiples of 16. For each experiment, 3 water controls were included along with the 6 trend line samples. The mastermix was prepared as follows. The enzyme pellet comprised of AMV-RT, RNase H and T7 RNA polymerase in bovine serum albumin (BSA), was reconstituted in 42µL of NASBA enzyme diluent which was left to dissolve at room temperature without mixing. Next, 53.3µL of NASBA buffer was added to the mastermix pellet and then vortexed. The remaining reagents, outlined in Table 4.5., were added to this tube which was mixed by vortex.

Table 4.5. QT-NASBA mastermix reagents and volumes

Reagent	Volume for 16 reactions (µL)
Mastermix pellet (Reconstituted first in 53.3µL of H ₂ O)	53
Molecular Beacon (Texas Red Pfs25 beacon (20µM)	2.4
NASBA water (BioMérieux)	7.0
Pfs25 primer (forward) (10µM)	3.3
Pfs25 primer (reverse) (10µM)	3.3
KCl (640mM)	10.7
<i>Mastermix total</i>	<i>5.0</i>
<i>Enzyme (added separately)</i>	<i>2.5</i>
<i>Template nucleic acid (added separately)</i>	<i>2.5</i>

The primer and molecular beacon sequences are indicated in Table 4.6.

Table 4.6. QT-NASBA primer and molecular beacon sequences

Primer name	Sequence (5' to 3' orientation)
Pfs25 forward	GACTGTAAATAAACCATGTGGAGA
Pfs25 Reverse	T7-CATTTACCGTTACCACAAGTTA
Molecular beacon Texas Red Pfs25 beacon	TexasRed-CGATCGCCCGTTTCATACGCTTGTAACGATCG-DABSYL

Five µL of reaction mastermix was aliquoted into 0.2mL Low Profile PCR Tube Strips (BioRad, Veenendaal, The Netherlands) then tubes were centrifuged at 10,000 rpm for 10 seconds. Then the italicised reagents (Table 4.5.) were added separately as follows.

2.5µL of template nucleic acid, water control or positive control trend line was added per tube which was centrifuged as before. Tubes were inserted into the Nuclisens EasyQ heat block (BioMérieux, Benelux B.V, Boxtel, The Netherlands) without the lids on, then held at 65°C for 2 minutes, followed by 41°C for 2 minutes. Next, the enzyme aliquot was centrifuged, then 2.5µL of enzyme added into the tube lids (Optical Flat 8-cap strips, BioRad, Veenendaal, The Netherlands) which were securely added to the tubes, then centrifuged once more. This ensures reactions begin at the same time to improve comparability of results. Samples were amplified for 90 minutes at 41°C in a BioRad CFX96 Real Time System (BioRad, Veenendaal, The Netherlands).

Results were analyzed using BioRad CFX Manager (BioRad, Veenendaal, The Netherlands) which provides a time to positivity value (TTP) referring to the cycle number when sample amplification crosses the background noise threshold. A positive sample is identified when the TTP value exceeds the mean fluorescence of the 3 negative water controls +20 standard deviations (Schneider, Wolters et al. 2005). TTP can be converted to log parasitaemia from the equation off the slope of the trend line values, using regression. Higher gametocyte density samples produce a lower (faster) TTP due to the larger quantity of mRNA which amplifies more quickly. Low density samples take longer to amplify and cross the threshold, and often have larger TTP values.

4.62 Results: Comparison of RNA extraction methods assessed using QT-NASBA

Gametocyte detection was performed using QT-NASBA on RNA extracted with Qiagen or guanidine based extraction from extra replicate Whatman 903 Protein Saver and FTA Classic Card filter papers which had been stored at -20°C or -80°C (which is covered in the attached publication). The objective was to conclude on an optimal extraction method (determined by higher detection using QT-NASBA), prior to extraction of the test papers. There was no statistically significant difference between the 2 extraction methods, but since guanidine based extraction was more cost

effective and amenable to high throughput, it was used to extract all filter papers outlined in the attached publication.

4.63 Results: Performance of QT-NASBA

Variation had been observed in the quantitative results, when comparing the RNA extraction techniques. Further extractions were performed to explore the variation seen, using low density replicate samples detected by QT-NASBA. For this purpose 5 replicate blood spots of 0.1 gametocytes/ μL were spotted onto both the 903 Protein Saver Card, and the FTA Classic Card, which were stored at -80°C overnight, then guanidine based extraction was performed followed by detection using QT-NASBA. The data in Table 4.7. demonstrates the variation between TTP for filter paper replicates with results ranging from a rapid TTP of 33.22 to negative, for the Whatman 903 Protein Saver Card and 38.24 to negative for the FTA Classic Card within the same replicate group.

Table 4.7. Time to positivity (TTP) values for amplification of Pfs25 mRNA using QT-NASBA detecting 0.1 gametocyte/ μL spotted onto Whatman 903 Protein Saver Card and FTA Classic Card. Nucleic acid was extracted using guanidine based extraction.

RNA extraction method:	Filter paper type	
	Whatman 903 Protein Saver Card	FTA Classic Card
Guanidine extraction	47.64	56.8
	34.97	negative
	negative	negative
	33.8	negative
	33.22	38.24
Number positive	4/5	2/5

To attempt to gain an understanding of the variation seen with these extractions, TTP values for the same trend line samples included across 17 QT-NASBA experiments from this study were compared, as shown in Figure 4.6. The more concentrated gametocyte

densities, 10^6 and 10^5 were detected 100% (17/17), which decreased to 94.1% (16/17) for 10^4 and 10^3 , and furthermore, 76.5% for 10^2 (13/17), and 58.8% for 10^1 (10/17).

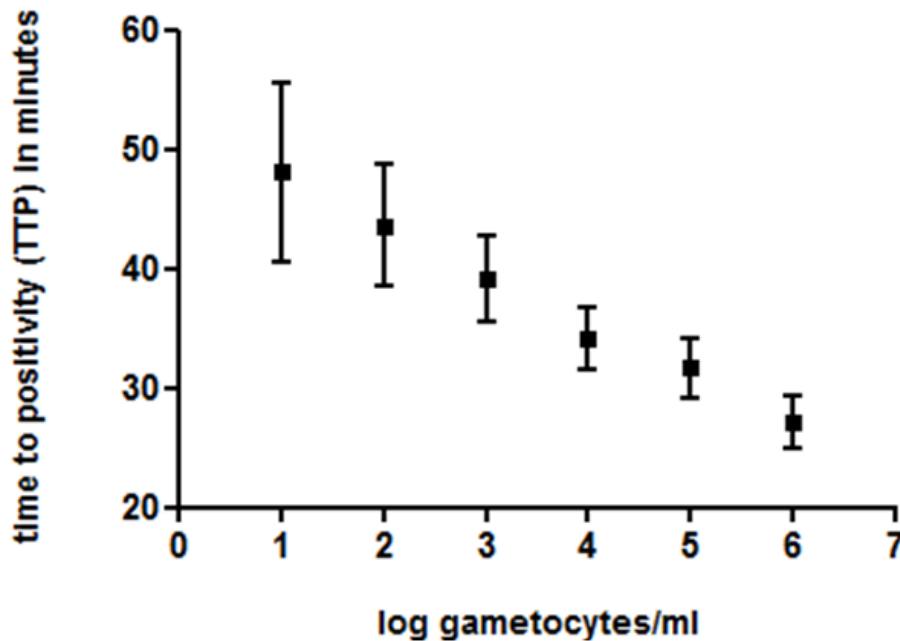


Figure 4.6. Time to positivity (TTP) values for titrated gametocyte trend line samples performed across multiple experiments. Trend line samples were titrated from 10^6 - 10^1 gametocytes/mL. Time is expressed as minutes. This data was sourced from 17 QT-NASBA experiments performed for the publication within this chapter, samples were extracted using guanidine based extraction. Mean TTP values are shown (indicated by the central dots) with bars indicating the 95% confidence interval.

The confidence intervals indicate high variation in detection time for the lower gametocyte densities. This is likely to reflect normal stochastic variation seen when detecting very low density material. Therefore, not all replicates with low density gametocytes would be expected to be positive. Additionally, the use of small volumes of extracted nucleic acid (2.5 μ L) further amplifies the random element of a QT-NASBA reaction actually containing gametocytes, and producing a positive result. Due to this wide variation observed at low gametocyte densities, it was decided to display

qualitative results (gametocyte prevalence) instead of quantitative results (gametocyte density) for the publication in this chapter.

Performance of QT-NASBA for detection of low density gametocytes spotted onto filter papers stored under different temperature conditions is discussed in the attached publication. For detection using QT-NASBA, RNA was not treated with a DNA digest.

4.64 Methods: Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was conducted from RNA (extracted using guanidine based extraction) that had been treated with the DNA digest (DNA Free). RT-PCR was performed using Illustra Ready to go RT-PCR beads (GE Healthcare, Buckinghamshire, UK) and primers designed by Professor Colin Sutherland. Asexual controls were performed for each sample to ensure contaminating DNA was not response for amplification. Reactions were set up as follows. Reaction tubes containing mastermix beads were maintained on ice while the following was added per reaction, 2.5µL of primer 2F (10µM- TAATGCGAAAGTTACCGTGG), 2.5µL of primer 1R (10µM- TCCATCAACAGCTTTACAGG) and 37.5µL of nuclease free water. This was incubated for 5 minutes at room temperature to allow the lyophilized bead to dissolve. At this point, the asexual control reactions (only) were incubated at 95°C for 10 minutes to inactivate the reverse transcriptase, then aliquots were cooled on ice and the protocol was continued. For all samples, 7.5µL of RNA template was added, then reactions were inserted into the thermocycler (C1000, Thermal Cycler, BioRad, Hertfordshire, UK), and the following was performed to synthesise cDNA from the RNA: 42°C for 30 minutes, then 95°C for 5 minutes to inactivate the enzyme. Without removing reactions from the thermocycler, the primary PCR reaction was performed using the following conditions: 94°C for 2 minutes, then 45 cycles of (94°C for 30 seconds, 52°C for 1 minute, then 68°C for 2.5 minutes). The nested PCR reaction was assembled as shown in Table 4.8. using the following primers, 6F (AACCATGTGGAGATTTTCC) and 7R (ACATTCTTACATTCATTTGG). The italicized reagents were added to the reaction tubes, then the following

amplification conditions were performed: 94°C for 2 minutes, then 30 cycles of (94°C for 30 seconds, 52°C for 1 minute and 68°C for 2.5 minutes).

Table 4.8. Pfs25mRNA RT-PCR mastermix reagents and volumes.

Reagent	Volume for 1 reaction (μL)
Nuclease free water (Ambion)	8.5
GoTaq Greenmastermix (Promega)	12.5
6F primer (10μM)	1.0
7R primer (10μM)	1.0
<i>Mastermix total</i>	<i>23.0</i>
<i>Primary PCR product (added separately)</i>	<i>2.0</i>

Positive reactions produced a product of ~125 base pairs in size which was visualized on a 2% TBE gel (Figure 4.7.) using Hyper ladder I 100 base pair molecular ladder (Biolone, London, UK) as a size marker. In the instance of a positive result for the DNA control, the DNA digest was repeated increasing the volume of rDNase I to 4μL to eliminate all remaining contaminant DNA.

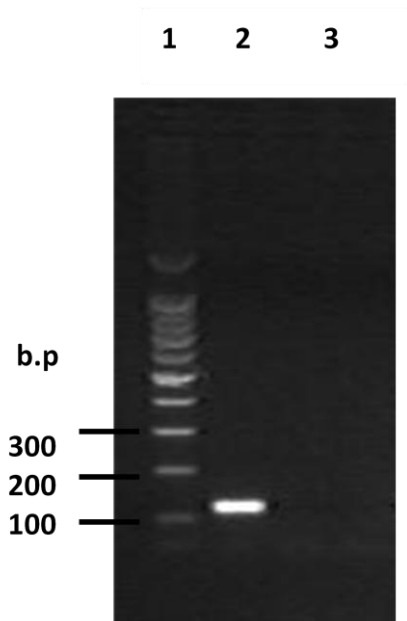


Figure 4.7. RT-PCR positive and negative control results using novel primers. Lane 1: 5 μ L of Molecular ladder Hyperladder I (100 base pairs (b.p.), Bioline, Lane 2: 2 μ L of amplified positive control (~125 base pairs), lane 3: 2 μ L of amplified negative control. Visualized on a 2% TBE gel.

4.65 Results: Performance of RT-PCR

Performance of RT-PCR for detection of low density gametocytes spotted onto filter papers stored under different temperature conditions is discussed in the attached publication.

4.66 Results: Comparative sensitivity of RT-PCR and QT-NASBA

This study was devised to compare extraction and detection of gametocytes from filter papers stored under a temperature range rather than conclude on a limit of detection for the molecular detection methods, hence why lower gametocyte densities were not included. However, to give an indication of the relative sensitivity of the methods,

detection was compared for samples on Whatman 903 Protein Saver Cards and FTA Classic cards stored at -20°C or -80°C, for 3 months which were extracted by guanidine based extraction, Table 4.9.

Table 4.9. Comparison of detection of low density gametocyte samples stored frozen on filter papers using RT-PCR and QT-NASBA. Nucleic acid was extracted from 50µL blood spots using guanidine based extraction. Eight replicate samples were stored on Whatman 903 Protein Saver and 8 on the FTA Classic Card per gametocyte density (data are consolidated). Half the filter papers were stored at -80°C and the other half at -20°C. n/N of detected replicates is shown in the table with percent detection in brackets, and the corresponding p-value comparing detection with RT-PCR and QT-NASBA.

	Gametocytes/µL		
	10	1.0	0.1
QT-NASBA	16/16 (100%)	12/16 (75.0%)	12/16 (75.0%)
RT-PCR	15/16 (93.8%)	4/16 (25.0%)	9/16 (56.3)
p-value	0.310	0.005	0.264

While QT-NASBA does not consistently show a statistically significant higher level of detection compared to RT-PCR, when comparing combined gametocyte density results QT-NASBA is significantly more sensitive when considering both the results in this table ($p=0.007$) and for the experiment outlined in the publication ($p=0.042$). Possibly, the lower sensitivity for RT-PCR could be partly attributed to loss of RNA during the DNA digestion step.

4.7 Linking methods and results: Detection of Pfs25mRNA from filter paper blood spots collected in an endemic setting

4.71 Methods: Detection of Pfs25mRNA from filter paper blood spots collected on Whatman 3MM in an endemic setting

To extend the research shown in the publication using cultured gametocytes handled in a laboratory environment, to field isolates collected in an endemic setting, RNA was

extracted from 48 Whatman 3MM filter paper blood spots collected in Kanungu, by staff from Mulago Hospital, Kampala, Uganda. Whole blood was dabbed from finger prick onto filter papers which were air dried overnight. Filter papers were then stored in individual sealable plastic bags along with desiccant and were kept at room temperature for several months before being transferred to -20°C. Nucleic acid was then extracted as before using guanidine based extraction followed by Pfs25mRNA detection using QT-NASBA.

4.72 Results: Detection of Pfs25mRNA from field collected samples stored on Whatman 3MM filter paper

Pfs25mRNA was successfully detected in nucleic acid extracted from blood spots stored on Whatman 3MM collected in an endemic setting. While accurate parasite counts and demographic information are currently not available for these samples, 33.3% (16/48) of individuals were positive for gametocytes, indicating it seems possible to detect field collected gametocytes from the Whatman 3MM filter paper.

4.8 Extended discussion

As discussed in the publication, this chapter aimed to identify the optimal combination of methods for collecting, extracting and detecting low density gametocytes. The range of gametocyte densities and incubation conditions selected in this study demonstrated decay of mRNA and subsequent loss of detection sensitivity in suboptimal sampling and storage conditions, which enabled discrimination of better performing filter papers, the 903 Protein Saver and the Whatman 3MM, the most user friendly extraction RNA method, guanidine-based extraction, and the more sensitive detection method, QT-NASBA.

4.81 The suitability of filter papers for RNA storage and extraction

The most surprising findings from this chapter was the poor detection of gametocytes from the FTA classic card and the good detection from the Whatman 3MM. The FTA classic card preserved RNA best when stored at -80°C, then detection sensitivity decreased with warmer incubation temperatures. Incomplete filter paper drying also impacted most on detection from the FTA classic card, with no RNA detected from any filter papers stored wet. These results question the applicability of use of the FTA card in the tropics where exposure to high temperature and moisture is expected.

This study importantly revealed that Whatman 3MM filter paper is suitable for RNA collection. These results are new and promising since this filter paper is commonly used in epidemiological studies for DNA and antibody collection. The duration of filter paper storage was fairly short (3 months, plus 1 week) but it now seems plausible that gametocytes could be detected from retrospectively collected filter papers. However, since little is known about long term degradation of mRNA from historically collected filter papers, this idea must be approached with caution, since it could be difficult to interpret if negative results were truly negative or the result of degraded undetectable mRNA. A safe approach to testing this could be to evaluate batches of samples that

have been collected and stored under the same conditions for the same amount of time.

Furthermore, in this chapter I provided evidence that detection from Whatman 3MM extends from laboratory prepared samples by indicating gametocytes can also be detected from blood samples collected on Whatman 3MM in an endemic setting. Gametocyte detection from Whatman 3MM was unexpected as this filter paper is made from standard cellulose, is not endorsed for RNA collection and is the cheapest of the 3 evaluated papers at \$2 for 100 papers. Using the Whatman 3MM, collection of gametocytes is now more affordable compared to the filter papers specifically marketed, or previously used for RNA collection, such as the FTA Classic Card (\$400 for 100), or the 903 Protein Saver Card (\$133 for 100).

4.82 My findings in context of recent research

At the time of submitting this manuscript, another study was published that also demonstrated RNA could be preserved and detected from Whatman 3MM filter paper. My study supports their findings but builds upon it by providing a more comprehensive comparison of several filter papers and using a wider range of incubation conditions, while comparing 2 molecular detection techniques.

In contrast to the Whatman 3MM and 903 Protein Saver Cards, the FTA Classic Card is specifically marketed for nucleic acid collection. According to Whatman, the filter paper contains reagents that stabilize nucleic acids and protect them from damage by oxidation, enzymatic activity and UV light (Whatman 2013). In my study, detection of sample eluted from the FTA Classic Card was found to be less sensitive, compared to the other filter papers. In contrast, the study by Pritsch *et al*, found no difference for detection from the FTA Classic Card, compared to the 903 Protein Saver Card and the 3MM, but papers were stored for just 1 day at room temperature (Pritsch, Wieser *et al*. 2012). Another study published more recently by Wampfler *et al* found poor detection from the FTA Classic Card, which is in line with my findings, and the authors

judged the elution step to be the major stumbling block for RNA retrieval from this paper Felger, personal communication, (Wampfler, Mwingira et al. 2013).

It was assumed using filter paper samples instead of whole blood would result in a trade off with reduced sample detection for a more user friendly collection method. In this study, there was similar or better detection from filter paper collected RNA compared to whole blood in L6 RNA stabilizing buffer. This was unexpected as it is assumed some RNA would be lost during sample elution. While previous studies have shown RNA can degrade over a period of 2 weeks when stored at -20°C in L6 lysis buffer, it should be more stable when stored at -80°C (Damen, Sillekens et al. 1998; Schneider 2006). Interestingly, Pritsch *et al.* had the same finding that samples were better detected from filter papers compared to whole blood. In their study, they suggested this could be due to the presence of single stranded DNA, not RNA. To test this, they divided samples into two groups and subjected them to either a DNase or RNase digest. Samples digested with RNase all produced negative results, whereas those digested with DNase remained positive, confirming it was RNA that was detected. Similarly, interaction between fresh blood and extraction kit reagents were ruled out, since the same detection sensitivity was obtained for filter paper and gametocyte spiked medium (Pritsch, Wieser et al. 2012). The higher detection seen from filter papers has been suggested to be the result of improved red blood cell lysis, which may occur during sample drying (Pritsch, Wieser et al. 2012).

4.83 Potential contributors to good and poor gametocyte detection from filter paper

While all 3 filter papers are made of cellulose, the key differences are filter paper thickness and whether the paper has undergone pre-treatment with nucleic acid preserving chemicals. Detection was best from the Whatman 3MM or the 903 Protein Saver Card, and worst from the FTA Classic Card. The Whatman 3MM is thinnest at 335µM, the FTA card is between 480-580µM and the 903 Protein Saver is thickest at 520µM (Personal communication, Whatman representative). It seems logical the

thickness of the card is not negatively impacting sample elution as detection is comparable for the 3MM and the 903 Protein Saver Card. Therefore it seems plausible that the remaining difference, impregnation of the FTA card with RNA stabilizing buffers is contributing to poor extraction and/or detection. According to Whatman, the FTA Card has been treated with sodium dodecyl sulphate (SDS) detergent, a buffering agent, a metal ion chelator and a compound that neutralizes free-radicals (Whatman 2013). Following sample drying the nucleic acids are supposedly immobilized within the fibres of the card (Personal communication, Whatman representative). Possibly, the GuSCN based lysis buffer is not sufficient to mobilize nucleic acids out of the paper matrix, which could be resulting in incomplete elution. Whilst Whatman do provide a specific purification reagent buffer for the FTA Card, it was not used due to the significant cost which prevented it being useful for large epidemiological surveys. This reagent has been tested in other studies where its use was discontinued due to unsatisfactory RNA yield, and high cost (Wampfler, Mwingira et al. 2013).

The 3MM paper is primarily used for blotting and chromatography and is designed to have rapid wicking (Whatman 2013). As a consequence, this paper is thinnest which is evident from the wider diameter a 50 μ L sample produces, compared to the other papers. Possibly, the good detection from this paper could be partly attributed to the larger surface area a sample occupies which is resulting in better contact with elution buffer, and therefore more complete elution and better downstream detection.

Filter papers that were not properly dried were very difficult to detect mRNA from. The loss in detection could be attributed to mRNA degradation or incomplete sample elution. As addressed in the publication, exposure to heat and moisture may cause damage by allowing nicks to form in the RNA strand (Dobbs, Madigan et al. 2002; Moscoso, Raybon et al. 2005). Successful elution can be confirmed by visual inspection of the filter paper, which should be white following this step. Many of the FTA cards evaluated under wet conditions remained red following elution, which implies at minimum, there is still haemoglobin bound to the filter paper matrix, and possibly also mRNA.

4.84 The RNA extraction methods

Two RNA extraction methods were initially compared with the objective of identifying one as being more sensitive, cost effective and amenable to high throughput testing. While it could be assumed samples processed with the Qiagen kit might result in better detection as reagents are supplied, therefore reducing operator error, it is possible use of columns can result in reduced yield. It has previously been indicated that small RNA fragments may remain in the spin column matrix following extraction. Use of larger weight carrier RNA has been suggested as a solution that improves binding of the target RNA, while protecting it from degradation, therefore resulting in improved yield (Qiagen 2010). While carrier RNA was not used in this study, it may have improved the Qiagen extraction protocol, although since Pfs25 is larger in size (654 nucleotides) this target may not suffer this problem. Detection from guanidine and Qiagen extracted gametocytes were found to be comparable, but guanidine based extraction was used for the study due to cheaper cost.

4.85 Gametocyte molecular detection methods

RT-LAMP was initially included as a 3rd molecular detection method for comparison. Whilst unknown at the time, the problems experienced in this study with EDTA causing false positive results, can be avoided by using heparin as an anticoagulant in the blood collection tubes (Francois, Tangomo et al. 2011). However, this would have deemed the material less suitable for RT-PCR (unless treated with heparinise), since heparin interferes with reverse transcriptase (Bai, Fischer et al. 2000). While poor inter reader agreement lead to discontinued use of RT-LAMP, results were determined by eye, and microplate reader (which has not been extensively validated as a method). Possibly if a turbidimeter had been available this technique may have been adequate.

QT-NASBA has clear advantages over RT-PCR (other than being more sensitive, overall), such as fewer steps and no need for the DNA digest, during both of which RNA

may be lost. Due to the necessity to include a DNA digest and non- reverse transcriptase control to confirm specificity of positive results, RT-PCR is not only twice as expensive (using the Ready to Go kit) but takes approximately 1 day to process 48 samples, compared to 2.5 hours for QT-NASBA.

Despite these disadvantages, the sensitivity of RT-PCR was improved using new primers, which often detected 0.1 gametocytes/ μL , compared to the detection limit of 1-2 gametocytes/ μL for the previously published primers (Mlambo, Vasquez et al. 2008). Although the cost of the QT-NASBA Real Time equipment is high, which does restrict use to more affluent laboratories, it was found to be both faster and cheaper per sample. Despite the high level of sensitivity of molecular techniques, studies have still reported infected mosquitoes from gametocyte negative (by microscopy and QT-NASBA) samples, but this is likely to be the result of RNA degradation in samples, or just very low gametocyte densities (Schneider, Bousema et al. 2006). It is important to note, the sensitivity of molecular detection is limited by the volume of blood that is processed and by increasing this volume, the sensitivity may also increase.

4.86 QT-NASBA gametocyte detection versus quantification

In this study, gametocyte prevalence was used as the QT-NASBA outcome to compare molecular detection methods, rather than gametocyte density. While I felt confident about the accuracy of gametocyte detection, there was variation in quantitative readings between sample replicates of the same density. The use of QT-NASBA as a qualitative method was questioned by a peer reviewer but it was considered justified for our purposes. Firstly, the majority of epidemiological surveys focus on the prevalence of gametocyte infections, not the densities (which fluctuate daily) and filter paper collection is the most convenient sampling medium for large surveys in remote settings. While it is important for some studies to have accurate gametocyte density counts, such as clinical trials looking at effects of gametocidal drugs, these studies are more likely to have the facilities for storing RNA in stabilizing buffer at -80°C and

would therefore not rely on filter papers for sample collection. Secondly, from this study, and since confirmed by others, it came to light there are concerns with reliable quantification of mRNA using TTP values generated by QT-NASBA and a high degree of variation has been seen at low gametocyte concentrations (Churcher, Bousema et al. 2013; Dinko 2013). It is important to note NASBA was originally developed as a qualitative method that has since been adapted to quantitative measuring and still needs some refining (Compton 1991). Furthermore, since Pfs25mRNA is thought to be a female specific marker or at minimum more abundantly expressed in female gametocytes, this also contributes to the uncertainty around these density estimations, and what precisely is being measured, and how representative it is of total gametocyte load (Schneider, Bousema et al. 2006).

Previous studies have reported uncertainty around gametocyte density estimates when using QT-NASBA and it has been highlighted that confidence intervals and standard errors are rarely revealed (Churcher, Bousema et al. 2013). I have shown the confidence intervals for trend line values, which have the largest number of replicates at the same density, to demonstrate variation. It has been speculated this intra assay measurement error could be attributed to varying enzyme efficiency, or inaccuracies from pipetting small volumes. But the most likely reason can be explained by the 'Monte Carlo effect', which suggests that the presence or absence of template in a low density sample is random (Wampfler, Mwingira et al. 2013). In this experiment, the gametocyte densities used are very low, and it is likely the variation simply reflects that there may or may not be a gametocyte in the reaction. Furthermore, small volumes of template are used for molecular detection, which further decreases the likelihood of there being a gametocyte in the reaction to detect. Due to these concerns, gametocyte prevalence was presented, rather than gametocyte density as this was the most robust version of the results.

4.87 Future perspectives

While performed under controlled conditions, this study was devised, to answer specific sampling questions to plan for gametocyte collection in the field. The incubation time of up to 3 months was selected for logistical reasons but may not necessarily represent the longest time filter papers would be left before being processed. Detection was compared from papers incubated for 1 week and 1 or 3 months and no statistically significant difference was found between the 2 time points. Whilst useful in the scope of shorter epidemiological studies, this time frame is too short for surveys that may wish to bank filter paper samples for years before processing them. While a study by Wampfler *et al.* incubated filter papers for up to 2 years, they firstly excised blood spots and diluted them in Trizol, therefore good detection could be attributed to stabilization of the buffer and not the filter paper, so this study also does not answer questions about long term storage using just filter papers.

To build upon research in this chapter, it would be valuable to repeat the study, focusing on the Whatman 3MM paper using longer incubation periods. It would be useful to look at the effect of freeze thawing the papers which would likely occur if for example, if papers are stored at -20°C in the field, shipped at room temperature, frozen on arrival, then finally thawed before processing, as the effect of condensation is likely to contribute to mRNA degradation.

4.88 Conclusions

This study indicates filter papers are appropriate tools for detection of low density gametocytes, which importantly highlights that routinely, possibly retrospectively collected filter papers can be used for gametocyte detection. In my hands the FTA classic Card performed poorly, unless stored under optimal conditions, but the 903 Protein Saver Card was found to be appropriate for use. Detection from the Whatman

3MM performed equally well compared to the 903 Protein Saver Card, but needs to be evaluated under the full range of conditions. Whilst the Qiagen Kit and guanidine based extraction were comparable in terms of detection, the guanidine extraction method is cheaper and more amenable to high throughput. With the new primers, RT-PCR was more sensitive than the previously reported primers (Mlambo, Vasquez et al. 2008), but QT-NASBA was more sensitive overall. The cheaper cost per reaction and faster turn-around time made QT-NASBA more amenable to high throughput.

What is evident from this study is that careful handling of filter papers is imperative to successful sample elution, extraction and detection. Filter papers must be handled with gloves at all times. Thorough drying of filter papers in the most sterile environment possible and storing them in sealable plastic bags with desiccant is recommended for all filter papers. When implementing filter paper sampling for RNA in the field, it is important to ensure health care workers have been properly trained in correct collection, drying and storage of papers prior to beginning a study.

Chapter 5: Assessing naturally acquired antibody responses to 10C and 230CMB transmission blocking vaccine candidates in endemic settings

5.1 Introduction to the manuscript

Previous studies assessing recognition of native sexual stage proteins, Pfs48/45 and Pfs230 have confirmed antibody presence in malaria exposed populations in endemic settings, but provided conflicting evidence regarding patterns of antibody acquisition following exposure to gametocytes, as discussed in section 1.36. (Bousema, Roeffen et al. 2006). Gaining an understanding of how these antibodies are acquired, and their functionality may provide insight into how a TBV based on the same antigens may function in an endemic setting. Cumulative antibody exposure (over time) would be reflected by an increase in antibody prevalence with age (Bousema, Roeffen et al. 2010). In contrast, a short lived antibody response would be reflected by no obvious age pattern, or an age dependent decrease in antibody response that is associated with immune control of asexual, and therefore sexual stage parasites densities, resulting in lower antigen exposure (Drakeley, Bousema et al. 2006). While an age dependent antibody pattern remains uncertain, previous studies indicated antibodies to native proteins Pfs48/45 and Pfs230 are acquired following exposure and are associated with functional transmission reducing activity that results in a reduction of oocyst prevalence or density in mosquitoes (Bousema, Roeffen et al. 2006). This information is highly relevant with regard to vaccines targeting pre-fertilization antigens (expressed on gametocytes) that may benefit from boosting of immunity following natural exposure (Chowdhury, Angov et al. 2009).

While antibody prevalence has been explored for native proteins, the laboriousness of the production of native antigen for these assays has hindered large-scale epidemiological surveys. This chapter focuses on the assessing the recognition and functionality of two new recombinant transmission blocking vaccines that are under development and were briefly described in section 3.11. Both Pfs48/45 and Pfs230 are well established vaccine candidates due to their essential role in parasite fertilization.

However the development of recombinant antigens has been complicated by the large number of disulphide bonds in both proteins (van Dijk, Janse et al. 2001; Eksi, Czesny et al. 2006). Since the majority of sexual stage B cell epitopes depend on tertiary structures, correct protein folding is critical to the induction of functional antibodies (Theisen, Roeffen et al. 2014).

5.12 The recombinant vaccine candidates

MBP-10C was the primary vaccine candidate produced under the REDMAL collaborative project that this thesis supports. The C-terminal 10C fragment was selected as the subunit for this vaccine candidate since it induced the highest titres of functional transmission blocking antibodies and was the most stable product when stored following production (Outchkourov, Roeffen et al. 2008). MBP-10C was over expressed in *E. coli* along with 4 folding catalysts (chaperone proteins) which ensured that folding occurred within the periplasmic space which has the optimal redox environment for correct formation of disulphide bonds (Outchkourov, Roeffen et al. 2008). Due to complications with this candidate, which are discussed in this chapter, MBP-10C was replaced with polyvalent candidate R0-10C (see Figure 3.1. for candidate schematic) (Theisen, Roeffen et al. 2014).

R0-10C vaccine candidate contains an asexual and transmission blocking component and therefore may have the benefit of inducing immunity that both protects the vaccinated individual and provides community level protection (Theisen, Roeffen et al. 2014). Furthermore, the transmission blocking component may help protect the asexual component by reducing the likelihood of vaccine escape mutants (Theisen, Roeffen et al. 2014). In addition to altering the fusion partner, R0-10C was produced in *Lactococcus lactis*, which was selected due to previous successful use for good manufacturing practice (GMP) production of GMZ2 and LSA3 among other antigens (Esen, Kremsner et al. 2009). Since *L. lactis* has no chaperones, correct protein folding

is entirely dependent on the fermentation process. To enable quantification of 10C antibodies in this chapter, R0 was cleaved first.

The second candidate 230CMB, introduced in section 3.11., was produced by Fraunhofer, USA (Farrance, Rhee et al. 2011). Functionality was demonstrated in vaccinated rabbits which produced high titres of antibodies that resulted in >99% reduction in oocyst density in the presence of complement (Farrance, Rhee et al. 2011). While 10C was the focus of the REDMAL project and this thesis, since Pfs48/45 and Pfs230 occupy similar functional niches and 230CMB recombinant antigen was kindly supplied, natural antibody responses were explored for both.

5.13 Genetic polymorphisms

Since 10C and 230CMB are exposed to host immunity during clearance of infected erythrocytes, they may also be subject to selection pressure and therefore be antigenically diverse (Takala and Plowe 2009). Vaccines are often produced using just one allele variant, therefore it is extremely important to assess genetic polymorphisms of the target antigen in the population prior to selecting an allele/alleles for vaccine development (Barry, Schultz et al. 2009; Takala and Plowe 2009). Previously, genetic polymorphisms have led to vaccine failure, for example the strain of MSP-2 used in the Combination B malaria vaccine only protected against that strain and enabled escape of other variants which ultimately led to vaccine failure (Klenerman and Zinkernagel 1998; Genton, Al-Yaman et al. 2003; Dutta, Lee et al. 2007). While it has been suggested sexual stage antigens (especially post fertilization targets, such as Pfs25) are not as immunogenic or polymorphic as asexual stage antigens (such as AMA-1, or MSP-1 and 2), polymorphisms have still been identified in 23 out of 449 amino acids in Pfs48/45 (Escalante, Grebert et al. 2002; Struik and Riley 2004; Takala and Plowe 2009). The implications of these polymorphisms are unknown but previous studies assessing transmission reducing immunity in The Gambia and Cameroon speculated

immunity of variable function could be due to sequence polymorphisms in *pfs48/45* (Drakeley, Mulder et al. 1998).

5.2 Objectives

This chapter has five main objectives.

1. Optimize and validate an ELISA assay to enable quantification of antibody responses against 10C and 230CMB recombinant antigens.
2. Assess if there are naturally acquired antibodies to 10C and 230CMB vaccine candidates in school aged children in 3 malaria endemic settings in Africa.
3. Determine whether the acquisition of antibody responses to 10C and 230CMB is dependent on age, season, transmission intensity and concurrent parasite carriage in 3 malaria endemic settings in Africa.
4. Explore the relationship between transmission reducing activity and 10C and 230CMB antibody prevalence and density, using supportive datasets with mosquito membrane feeding data.
5. Assess the presence (or absence) of polymorphisms in the *pfs48/45* gene, with special consideration to the 10C (subunit of Pfs48/45) vaccine region, in samples from 3 malaria endemic settings.

5.3 Manuscript: Naturally acquired antibody responses to recombinant Pfs230 and Pfs48/45 transmission blocking vaccine candidates

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published

1.1 Where was the work published?

1.2 When was the work published?

1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

1.3. Was the work subject to academic peer review?

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2. For a 'research paper' prepared for publication but not yet published

2.1 Where is the work intended to be published? **Journal of Infection**

2.2 List the paper's authors in the intended authorship order

Sophie Jones, Lynn Grignard, Issa Nebie, Jaffu Chilongola, Daniel Dodoo, Robert Sauerwein, Michael Theisen, Will Roeffen, Shrawan Kumar Singh, Rajesh Kumar Singh, Sanjay Singh, Eric Kyei-Baafour, Kevin Tetteh, Chris Drakeley & Teun Bousema

2.3 Stage of publication **Submitted**

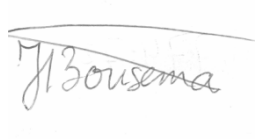
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed and conducted all experiments, omitting: the membrane feeding which was performed by others (as referenced in the manuscript), MSP2 genotyping which was performed by Dr. Lynn Grignard (LSHTM), and field collection of samples and microscope slide reading which was performed by staff at each of the field sites. I performed the statistical analysis and wrote the article.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



**Naturally acquired antibody responses to recombinant Pfs230 and Pfs48/45
transmission blocking vaccine candidates.**

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Abstract.

Pfs48/45 and Pfs230 are *P. falciparum* sexual stage proteins and promising malaria transmission-blocking vaccine candidates. These proteins are expressed by gametocytes while in the human bloodstream and, consequently, antibody responses may be naturally acquired and target antigens may be under selective pressure. This has consequences for the future evaluation of vaccine immunogenicity and efficacy in populations naturally exposed to malaria. We determined naturally acquired antibody responses to the recombinant proteins Pfs48/45 -10C and Pfs230-230CMB in children from three malaria endemic settings in Ghana, Tanzania and Burkina Faso. We also examined genetic polymorphisms in the *P. falciparum* gene *pfs48/45*. Antibody prevalence was 1.1-18.2% for 10C and 6.7-18.9% for 230CMB. Antibody responses were markedly lower for these sexual stage antigens compared to responses against asexual stage malaria antigens. In Burkina Faso we observed evidence of an age-dependent acquisition pattern for both 10C ($p < 0.001$) and 230CMB ($p = 0.031$). Membrane feeding assays confirmed transmission reducing activity of antibodies for both 10C ($p = 0.017$) and 230CMB ($p = 0.049$). 17 single nucleotide polymorphisms were found in *pfs48/45* (from 126 samples), with 5 non-synonymous SNPs in the Pfs48/45 10C region. We conclude there are naturally acquired antibody responses to both vaccine candidates which have functional relevance by reducing the transmissibility of infected individuals. We identified genetic polymorphisms, in *pfs48/45* which exhibited geographical specificity.

Keywords:

Plasmodium falciparum, gametocyte, gamete, transmission, immunity, polymorphism

Abbreviations:

SNP (single nucleotide polymorphism), MTBV (Malaria transmission blocking vaccines), TRA (transmission reducing activity), RDT (rapid diagnostic test), GLURP (glutamate rich protein), SMFA (standard membrane feeding assay), MSP-2 (merozoite surface protein-2), MOI (multiplicity of infection).

INTRODUCTION

The recent decline in the burden of malaria, particularly in sub-Saharan Africa has re-emphasized elimination as an attainable goal for many malaria endemic countries (Ceesay, Casals-Pascual et al. 2008; Barnes, Chanda et al. 2009; World Health Organization 2012). Novel malaria control strategies that specifically aim to reduce malaria transmission may be required to move from malaria control to elimination (Okell, Griffin et al. 2011). Malaria transmission blocking vaccines (MTBV) are high on the priority list for malaria elimination and eradication strategies (Sauerwein 2007; Targett and Greenwood 2008; Griffin, Hollingsworth et al. 2010). The transmission of malaria from man to mosquito depends on the presence of mature sexual stage parasites, gametocytes, in the human peripheral blood. Once ingested by blood feeding mosquitoes, male and female gametocytes activate to become gametes that fuse to form zygotes that penetrate the mosquito midgut wall as ookinetes to form oocysts. These oocysts enlarge over time to release sporozoites that migrate to the mosquito salivary glands and render the mosquito infectious to human beings upon their next feeding. MTBV aim to elicit antibodies that are passively ingested when a mosquito takes a blood meal which reduce or arrest parasite development, thereby blocking transmission to the next host (Kaslow 1993). Transmission-blocking antigens can be categorized as those that play a role before zygote formation (pre-fertilization) and those that affect the subsequent development of mosquito stages (post-fertilization). Pre-fertilization proteins Pfs48/45 and Pfs230 are both found on the surface of gametocytes and become exposed in humans (Vermeulen, Ponnudurai et al. 1985; Kaslow 1993). This exposure allows the acquisition of immune response during natural malaria infections. Antibody responses to both proteins have been detected in naturally exposed populations and have been associated with functional transmission reducing activity (TRA) (Bousema, Roeffen et al. 2006; Drakeley, Bousema et al. 2006; Bousema, Roeffen et al. 2010). Immune recognition may also result in selective pressure that gives rise to genetic polymorphisms associated with reduced susceptibility of parasites to natural or vaccine-induced immune responses. For

pfs48/45, 5 main non synonymous genetic polymorphisms have been described previously with clear geographical clustering (Conway, Machado et al. 2001). Both the presence of naturally acquired antibody responses and genetic polymorphisms in vaccine protein regions are of great importance for the planning and evaluation of vaccine trials in naturally exposed populations.

Here we determine for the first time, naturally acquired antibody responses to MTBV candidates Pfs48/45-10C and Pfs230-230CMB, assess their functionality and describe genetic polymorphisms in local isolates of *pfs48/45*.

MATERIALS AND METHODS

Study areas and populations

Three study sites were selected to reflect different levels of transmission intensity: a site of hyper endemicity in Ouahigouya, Burkina Faso, meso endemicity in Bondo, Tanzania and hypo endemicity in Asutsuare in Ghana. One hundred and eight children were randomly sampled from 1 school in Ghana, which was 200 children sampled in total across 2 schools in Burkina Faso and 202 children sampled in total across 2 schools in Tanzania using strategies described by Brooker *et al* (Brooker, Kolaczinski et al. 2009). Two cross sectional surveys were conducted during the peak transmission season and at the end of the dry season in 2011 and 2012 at each study site (Brooker, Kolaczinski et al. 2009). Ethical permission was granted from LSHTM (approval number 5946) and from local and national ethics committees in Burkina Faso, Tanzania and Ghana. Written consent was gained from participant's guardians prior to sampling. At each survey, finger prick samples of approximately 300µL were taken in BD microtainers (Becton Dickinson, Oxford, UK) for microscopy, plasma collection and filter paper storage (Whatman 3MM, Maidstone, UK). Plasma was diluted to 1/20 in 0.05% sodium azide in phosphate buffered saline (PBS), which was

stored at -20°C until use. Blood spot filter papers were air dried overnight, then sealed into individual plastic bags with silica desiccant, and stored at -20°C until use. Parasite detection was done by microscopy and Rapid Diagnostic Test (RDT, Premier Medical First Response, India). Clinical decision making was based on the RDT result and all RDT positive individuals with reported fever in the past 24-48 hours were treated for malaria according to national guidelines. Parasite counts were obtained by microscopy and slides were read by two independent microscopists, both examining 100 fields. Parasite densities were determined after reading against 1000 leukocytes. Questionnaires were administered to participants to gain details about malaria symptoms, bed net use and socio economic status.

Antigens

Pfs48/45-10C was obtained from the chimeric R0-10C vaccine protein produced at the cGMP-facility of Gennova, India, using the standard proposed medium composition as described by Theisen *et al.* (Theisen, Roeffen *et al.* 2014). The R0-10C vaccine protein includes 10 cysteine residues spanning epitopes I-III from the C terminal domain of Pfs48/45, fused to GLURP-R0. R0 was firstly cleaved from Pfs48/45-10C and successful removal of GLURP-R0 was confirmed by testing plasma samples from GLURP-vaccinated volunteers (Hermsen, Verhage *et al.* 2007) (see supporting information). Pfs230-230CMB was obtained as a transmission blocking candidate that was developed by Fraunhofer USA Center for Molecular Biotechnology. The recombinant protein encompasses a conserved region including the pro-domain and part of the first cysteine residue of Pfs230, corresponding to amino acids 444-730 (Farrance, Rhee *et al.* 2011). Additional asexual stage antigens were included to contrast sexual stage antibody responses to asexual stage antibody responses. These recombinant asexual proteins were, apical membrane antigen (AMA-1 3D7, Biomedical Primate Research Centre, Rijswijk, the Netherlands), merozoite surface protein 1₁₉ (MSP-1₁₉ Wellcome allele, provided by Patrick Corran, London School of Hygiene & Tropical Medicine with

permission of Tony Holder) and R2 region of GLURP, provided by Michael Theisen, Statens Serum Institut, Copenhagen.

Enzyme linked immunosorbent assays

10C and 230CMB antibodies were quantified as follows; 96 well Maxisorp NUNC plates (Nalge Nunc International Corp., Naperville, IL, USA) were coated overnight at 4°C with 100µl per well of 0.1µg/ml of antigen diluted in PBS. Plates were blocked for 30 minutes with 150µl of 5% non-fat skimmed milk (Marvel, Premier International Foods Ltd., Spalding, UK) in PBS. Following this, plates were washed 3 times with PBS, and 100µl of test serum was diluted to 1/500 in PBS (with 1% milk and 0.05% Tween 20), and incubated on the plates for 4 hours at room temperature. Plates were then washed 3 times as before and incubated with 100µl per well of human-IgG-HRP (Pierce Biotechnology Inc., Rockford, IL, USA) diluted to 1/40,000 in PBS with 0.05% Tween 20, for 2 hours at room temperature. Next plates were washed 4 times, then 100µl of tetramethylbenzidine substrate (TMB) solution was added per well and incubated for 20 minutes. Reactions were stopped using 50µl per well of 0.2M sulphuric acid and optical densities were measured at 450nm (Bio-Rad iMark Microplate Reader, Hertfordshire, UK). Serum from an expatriate with established high levels of sexual stage immune responses and functional transmission reducing activity (TRA) was used as a positive control (Saeed, Roeffen et al. 2008) and titrated from 1/200 in doubling dilutions 7 times to produce a standard curve. Antibody responses were quantified against asexual antigens MSP-1₁₉, AMA-1 and, GLURP as described elsewhere (Proietti, Verra et al. 2013). For all assays, averaged sample ODs were normalized (using the midpoint dilution as reference), against a titration curve fitted to the positive control sample by least squares minimisation using a three variable sigmoid model and assigning an arbitrary value of 1000u/mL to calculate titre (Drakeley, Corran et al. 2005; Proietti, Verra et al. 2013). The mixture model was used to distinguish positive and negative samples by fitting test sample ODs to two Gaussian distributions using

maximum likelihood methods in STATA (Version 11, Statacorp, Texas, USA). The mean OD of the seronegative (the test samples with low ODs) population plus 3 standard deviations was used as the cut off value. This resulted in a cut off OD value above which a sample was considered antibody positive of; 0.17 for AMA-1, 0.23 for MSP-1₁₉, 0.2 for GLURP, 0.35 for 10C and 0.44 for 230CMB (Corran, Cook et al. 2008; Bousema, Roeffen et al. 2010).

Analysis of functional transmission-blocking immunity

While it was not possible to perform membrane feeding assays in this study where small-volume finger prick blood samples were obtained, we performed 10C and 230CMB ELISA on a sample set (n=58) with a known functional transmission-blocking phenotype in the standard membrane feeding assay (SMFA) (Bousema, Roeffen et al. 2010). The methodology for SMFA is described in detail elsewhere (Bousema, Roeffen et al. 2010). Briefly, 3-5 day old *Anopheles stephensi* mosquitoes were offered a blood meal containing NF54 *P. falciparum* gametocytes with purified control serum or test sample at a final dilution of 1:3 in the feeder. Twenty blood-fed, surviving mosquitoes were dissected 7 days later and oocyst counts were made. Transmission reducing activity (TRA) was determined as the percentage reduction in arithmetic mean oocyst number in test samples as compared to paired controls. The test samples were taken from gametocyte positive individuals recruited from a health centre in a region of hypo endemicity in Lower Moshi, Tanzania (Bousema, Roeffen et al. 2010) of whom 32.8% blocked transmission of NF54 parasites by $\geq 50\%$ and 15.5% by $\geq 90\%$.

Sequencing of the pfs48/45 gene

Samples were selected at random for sequencing of the *pfs48/45* gene. In Burkina Faso and Tanzania, this selection was made from microscopy-positive parasite carriers. In

Ghana, where microscopy data was not available at the time of sequencing, we selected samples regardless of asexual parasite presence but first confirmed parasite prevalence by nested PCR, which was performed according to standard methods (Baidjoe, Stone et al. 2013). In total we obtained 126 readable sequences for samples from Burkina Faso (n=39), Ghana (n=39) and Tanzania (n=48). Sequencing was performed as described elsewhere (Anthony, Polley et al. 2007). Briefly, DNA was extracted from filter paper blood spots using the QIAamp DNA Micro Kit (Qiagen™ Hilden, Germany) and the dried blood spot protocol according to manufacturer's instructions. *pfs48/45* was sequenced between nucleotides 44 and 1303 in 2 segments (Anthony, Polley et al. 2007). Firstly nested PCR was performed, then the secondary PCR product was purified using the QIAquick PCR purification kit (Qiagen™ Hilden, Germany), and sequencing PCR was performed using the BigDye V3.1 sequencing kit (Applied Biosystems, San Francisco, CA, USA). Products were ethanol precipitated then sequenced on an ABI3730 sequencer (Applied Biosystems, San Francisco, CA, USA), and chromatograms analyzed using CLC Sequence Viewer 6 (Cambridge, MA, USA). To gauge the number of clones per sample, MSP-2 genotyping of asexual parasites was performed using capillary electrophoresis as described elsewhere (Liljander, Wiklund et al. 2009; Schoepflin, Valsangiacomo et al. 2009). GeneMapper version 4.0 (Applied Biosystems, Paisley, UK) and FSTAT version 293 (Jérôme Goudet, Lausanne, Switzerland) software was used to analyze results and calculate the expected heterozygosity.

Statistical analysis

All statistical analyses were performed using STATA Version 11 (Statacorp, Texas, USA) and graphical presentation of data was done using GraphPadPrism Version 5 (GraphPad Software Inc., La Jolla, USA). Study participants were categorized into 3 age categories; 3-8, 9-11 and >11 to determine age dependent antibody acquisition patterns. Pearson's Chi Square and Spearman's Rank were used to determine the

association between categorical and continuous variables; odds ratio's with 95% confidence intervals (95% CI) and correlation coefficients were used to quantify effect sizes. Generalized estimating equations (GEE) were used to determine associations between antibody prevalence and age, season, site and parasite status, adjusting for multiple observations per individual.

RESULTS

A total of 208 samples were collected from Ghana (108 individuals), 521 from Tanzania (202 individuals) and 389 from Burkina Faso (200 individuals), from participants sampled during the peak transmission season and at the end of the dry season for Tanzania and Ghana, and during the peak transmission season and in the middle of the dry season for Burkina Faso (Table 5.0.).

Table 5.0. Characteristics of survey participants. IQR, interquartile range based on 25th and 75th percentiles, GM, geometric mean, CI, 95% confidence interval. n=number positive, N=total number in category. This table contains data for participants where we know their age (38 people are missing from this table).

Age group	Burkina Faso			Tanzania			Ghana		
	≤8.0	9-11	>11	≤8.0	9-11	>11	≤8.0	9-11	>11
Number	195	163	22	40	175	305	55	58	67
Age, median (IQR)	7 (6-8)	10(9-11)	12 (12-13)	8 (7-8)	10 (10-11)	14(12-15)	7 (6-8)	10 (9-10)	12 (13-14)
Gender, % female (n/N)	47.2 (92/195)	53.7 (87/162)	33.3 (7/21)	75.7 (28/37)	58.9 (103/175)	47.5 (145/305)	45.5 (25/55)	37.9 (22/58)	34.9 (22/63)
Asexual prevalence, % (n/N)	38.0 (74/195)	40.7 (66/162)	38.1 (8/21)	22.5 (9/40)	11.4 (20/175)	11.5 (35/304)	3.6 (2/55)	10.3 (6/58)	4.5 (3/66)
Asexual density, GM (IQR)	970 (245-2796)	462 (120-1196)	818 (397-2304)	1204 (1120-2707)	501 (128-1960)	384 (112-996)	119 (32-440)	321 (128-480)	142 (64-400)
Gametocyte prevalence, % (n/N)	9.7 (19/195)	10.5 (17/162)	9.5 (2/21)	2.5 (1/40)	2.3 (4/175)	1.3 (4/305)	0 (0/55)	0 (0/58)	0 (0/67)
Gametocyte density, GM (IQR)	18.3 (8-38)	15.9 (8-20)	11.0 (8-15)	8.0 (8.0-8.0)	16.0 (8-40)	33.8 (24-56)	n/a	n/a	n/a

The majority of children were sampled in both surveys, with 5.5-7.9% of children sampled just once. Overall asexual parasite prevalence by microscopy was 39.2% (148/378) in Burkina Faso, 12.3% (64/519) in Tanzania and 6.1% (11/180) in Ghana; gametocyte prevalence was 10.1% (38/378) in Burkina Faso, 1.7% (9/520) in Tanzania and 0% (0/180) for Ghana. The prevalence of both asexual parasites (OR= 1.01, 95% CI 0.94-1.09; p=0.70) and gametocytes (OR= 1.00, 95% CI, 0.88-1.15; p=0.94) did not vary significantly with age in this school-age population, after adjusting for season and study setting. Asexual parasite density in parasite positive individuals declined with increasing age ($\beta=-0.079$, $se=0.022$; $p<0.001$); and gametocyte density in gametocyte carriers was not associated with age ($\beta=-0.013$, $se=0.022$; $p=0.55$), after adjusting for season and study setting. Asexual parasite prevalence was significantly higher in the transmission season compared to the dry season (OR= 2.00, 95% CI 1.50-2.68; $p<0.001$), but this seasonality was not observed for patent gametocyte prevalence (OR= 1.18, 95% CI 0.65 - 2.14 $p=0.59$), after adjusting for age and study setting.

The complexity of infection was determined by MSP2 genotyping for a subset of the samples that were sequenced for *pfs48/45*, Ghana (n=27), Tanzania (n= 37), and Burkina Faso (n=34). Multiplicity of infection (MOI) followed the same between-country pattern as parasite prevalence: Ghana had the lowest MOI, with an average of 1.96, followed by Tanzania with 3.35, and Burkina Faso with 3.88 (Table 5.1.).

Table 5.1. Multiplicity of infection (MOI) and *pfs48/45* sequencing results by study location. *A sub population of the sequenced samples were assessed to determine multiplicity of infection.

	Burkina Faso	Tanzania	Ghana
Number of analyzed samples (MOI)	34*	37*	27*
Average number of clones	3.88	3.35	1.96
% single clone infections	17.6	10.8	44.0
Allelic richness	46.9	38.8	14.0
Expected heterozygosity	0.973	0.970	0.903
Number of analyzed samples (sequencing)	39	48	39
Number of <i>pfs48/45</i> haplotypes	15	21	11
<i>pfs48/45</i> haplotypes, % 3D7	17.9	50.0	69.2
<i>pfs48/45</i> haplotypes, % mixed clone	56.4	43.8	15.4

Allelic richness, a measure of genetic diversity, was lowest in Ghana (14.0), followed by Tanzania (38.8) and Burkina Faso (46.9). The heterozygosity index showed a similar pattern with 0.903 for Ghana, 0.970 for Tanzania and 0.973 for Burkina Faso (Table 5.1.).

Antibody responses in relation to age, parasite carriage and study setting

Antibody profiles for asexual stage antigens AMA-1, MSP-1₁₉ and GLURP, and transmission blocking vaccine candidates 10C and 230CMB are shown in Table 5.2.

	Burkina Faso			Tanzania			Ghana		
Age group	≤8.0	9-11	>11	≤8.0	9-11	>11	≤8.0	9-11	>11
AMA-1, prevalence (n/N)	61.5 (118/192)	67.7 (107/158)	90.9 (20/22)	92.1 (35/38)	79.8 (126/158)	79.9 (226/283)	14.3 (7/49)	24.6 (14/57)	44.3 (27/61)
Titre, GM (95% CI)	909 (728-1136)	1129 (896-1422)	2364 (1198-4667)	1194 (822-1738)	827 (678-1008)	1080 (928-1259)	854 (290-2520)	700 (395-1240)	517 (346-773)
MSP-1, prevalence (n/N)	24.2 (47/194)	26.5 (43/162)	15 (3/20)	32.5 (13/40)	40.8 (71/174)	44.6 (136/305)	18.9 (10/53)	0 (0/58)	19.7 (13/66)
Titre, GM (95% CI)	1179 (844-1646)	931 (648-1338)	548 (187-1610)	1106 (656-1867)	1300 (1029-1643)	1085 (926-1271)	594 (389-905)	0 (0-0)	1240 (821-1874)
GLURP, prevalence (n/N)	32.4 (56/173)	61.9 (91/147)	63.2 (12/19)	77.5 (31/40)	60.1 (104/173)	66.8 (197/295)	7.6 (4/53)	12.3 (7/57)	24.2 (16/66)
Titre, GM (95% CI)	968 (728-1288)	1433 (1126-1824)	1317 (528-3285)	871 (605-1255)	879 (739-1045)	1195 (1028-1389)	1116 (156-7990)	841 (489-1446)	539 (382-761)
10C, prevalence (n/N)	8.33 (16/192)	28.1 (45/160)	31.8 (7/22)	18.8 (6/32)	11.8 (17/144)	16.5 (43/261)	1.82 (1/55)	0 (0/57)	1.5 (1/66)
Titre, GM (95% CI)	81 (60-110)	80 (70-91)	70 (49-99)	77 (45-131)	107 (75-151)	86 (71-104)	50	0	53
230CMB, prevalence (n/N)	6.8 (13/192)	16.8 (27/161)	22.7 (5/22)	12.5 (4/32)	13.2 (19/144)	22.9 (60/262)	5.5 (3/55)	1.8 (1/57)	12.1 (8/66)

	Burkina Faso			Tanzania			Ghana		
Age group	≤8.0	9-11	>11	≤8.0	9-11	>11	≤8.0	9-11	>11
230CMB Titre, GM (CI)	384 (204-722)	265 (235-299)	598 (139-2574)	273 (78-957)	299 (202-442)	307 (256-367)	623 (192-2021)	202	284 (190-426)

Table 5.2. Antibody prevalence and density for asexual stage and sexual stage antigens. Titres refer to antibody positive individuals only. GM, geometric mean, CI, 95% confidence interval.

Combining all age categories, the proportion of people antibody positive for any asexual antigen was 77.1% (300/389) for Burkina Faso, 85.0% (443/521) for Tanzania and 31.1% (64/206) for Ghana (Figure 5.0).

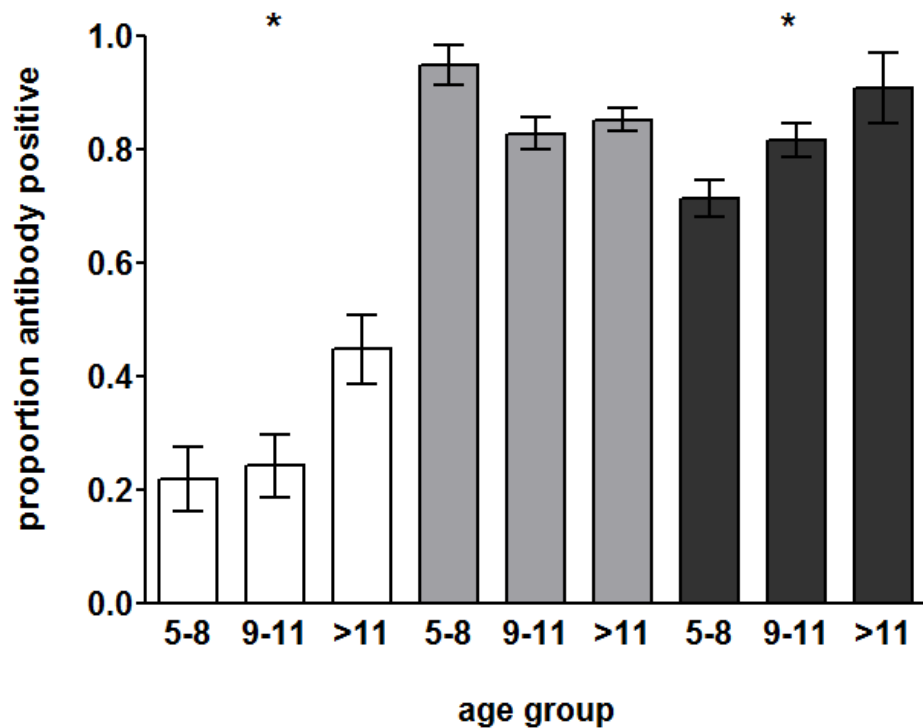


Figure 5.0. Antibody prevalence for asexual antigens in relation to age group and country of origin. Individuals counted as asexual positive have antibodies to any or multiple of the following antigens; AMA-1, MSP-1₁₉ or GLURP. The bars show the antibody prevalence in the age groups, shaded by country; white for Ghana, light grey for Tanzania and dark grey, Burkina Faso. The error bars indicate the 95% confidence interval, and the asterisks indicates a statistically significant positive increasing trend at the following levels: 0.05 – 0.01 (*), 0.01- 0.001 (**), and <0.001 (***). Results did not change significance boundary when using age as either a categorical or continuous variable.

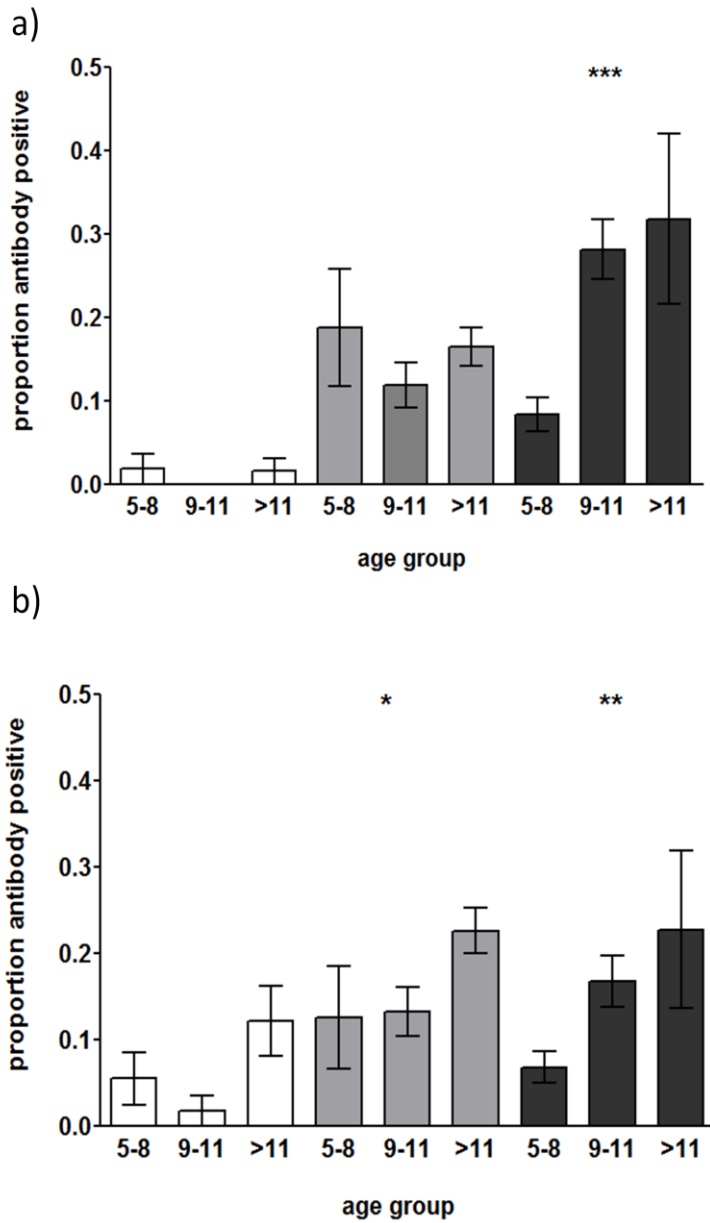


Figure 5.1. Antibody prevalence for sexual stage antigens in relation to age group and country of origin. (a) Responses to 10C, (b) Responses to 230CMB. The bars show the antibody prevalence in the age groups, shaded by country; white for Ghana, light grey for Tanzania and dark grey, Burkina Faso. The error bars indicate the 95% confidence interval, and the asterisks indicates a statistically significant positive increasing trend at the following levels: 0.05 – 0.01 (*), 0.01- 0.001 (**), and <0.001 (***). Results did not change significance boundary when using age as either a categorical or continuous variable. An interaction between parasite prevalence and age was assessed for antibody prevalence trends depicted in this graph. An association was only found in Burkina Faso.

The overall proportion positive for 10C antibodies was considerably lower with 18.0% (69/383) antibody prevalence in Burkina Faso, 15.1% (66/438) in Tanzania and 1.9% (4/206) in Ghana; and antibody titre followed the same pattern (Table 5.2., Figure 5.1 (a)). 230CMB antibody prevalence was 12.2% (47/384) in Burkina Faso, 18.9% (83/439) in Tanzania and 6.3% (13/205) in Ghana (5.1.(b)). The prevalence and density of antibodies against all antigens were all correlated ($p < 0.001$ for all comparisons; Table 5.3.).

Table 5.3. Associations between antibody titres for the different antigens. The spearman correlation coefficient is presented with the related p-value.

	AMA-1	MSP-1	GLURP	10C	230CMB
AMA-1					
MSP-1	r=0.39, p<0.001				
GLURP	r=0.55, p<0.001	r=0.64, p<0.001			
10C	r=0.38, p<0.001	r=0.30, p<0.001	r=0.44, p<0.001		
230CMB	r=0.32, p<0.001	r=0.33, p<0.001	r=0.42, p<0.001	r=0.53, p<0.001	

Overall, the prevalence of antibody responses to any of the asexual stage antigens increased significantly with age in years (OR= 1.12, 95% CI, 1.05 -1.21; $p=0.001$), after adjusting for concurrent parasite carriage, season and study setting. When this age-dependency of asexual antibody prevalence trend was examined per study setting, there was a significant positive association in Burkina Faso ($p=0.022$) and Ghana ($p=0.012$) but not Tanzania ($p=0.41$). Antibody prevalence to 10C increased with age (Figure 5.1. (a)), but this trend was only statistically significant in Burkina Faso (OR= 1.39, 95% CI 1.18- 1.65; $p < 0.001$). Similarly, antibody responses to 230CMB increased with age, but only in Burkina Faso (OR= 1.21, 95% CI 1.02 -1.44; $p=0.031$) and Tanzania (OR= 1.22, 95% CI, 1.07 -1.38, $p=0.003$), with no clear pattern for Ghana ($p=0.32$). The concurrent presence of asexual parasites was significantly associated with the prevalence of antibodies against any asexual stage antigen (OR 3.55, 95% CI 2.32-5.42, $p < 0.001$) and against the individual asexual antigens; AMA-1, MSP-1₁₉ and GLURP ($p \leq 0.012$) and antibody titre for AMA-1 ($p < 0.001$), GLURP ($p < 0.001$) but not MSP-1₁₉

($p=0.64$). The concurrent presence of asexual parasites was also associated with a higher prevalence of antibodies against 10C (OR 2.01, 95% CI 1.30-3.11, $p=0.002$) and 230CMB (OR 1.65, 95% CI 1.06-2.57; $p=0.027$) and a higher antibody titre for 10C (\log_{10} titre: $\beta=0.35$, $se=0.081$, $p<0.001$) and 230CMB (\log_{10} titre: $\beta=0.37$, $se=0.076$, $p<0.001$), after adjustment for age, season and study setting. There was no association between microscopically detectable gametocytes and antibody prevalence for 10C ($p=0.456$) or 230CMB ($p=0.281$).

Functional transmission reducing activity

A total of 58 samples with SMFA results from a previous study were successfully tested for 10C and 230CMB antibodies, of which 19/58 reduced transmission by $\geq 50\%$ and 9/58 reduced transmission by $\geq 90\%$. Both 10C and 230CMB antibody prevalence was significantly associated with TRA at both the 50% and 90% levels (Table 5.4.).

Table 5.4. Relationship between sexual stage antibody responses and functional transmission reducing activity (TRA). Transmission reducing activity was categorized at $\geq 50\%$ and $\geq 90\%$. Odds ratios are presented with 95% confidence intervals, and the associated p-values.

Antibody prevalence	TRA $\geq 50\%$		TRA $\geq 90\%$	
	OR (95% CI)	p-value	OR (95% CI)	p-value
10C	5.54 (1.21-25.43)	0.028	7.04(1.41-35.13)	0.017
230CMB	8.48(1.91-37.64)	0.005	4.69(1.00-21.85)	0.049

pfs48/45 sequencing

We sequenced *pfs48/45* in a subset of samples from Tanzania ($n=48$), Ghana ($n=39$) and Burkina Faso ($n=39$). All variation discussed here is compared to the 3D7 reference

sequence (PlasmoDB 2012). 17 single nucleotide polymorphisms (SNPs) were found, 10 of which were synonymous and 7 non-synonymous. 12 of the SNPs (5 of the non-synonymous SNPs) are within the vaccine candidate region (Table 5.5.). Two new non-synonymous substitutions were found at nucleotide locations 396 (amino acid position 132) and 468 (amino acid position 156), that appeared once in both Ghana and Tanzania, and are outside of the 10C recombinant protein region. For Ghana, the majority of samples (69.2%) were identical to 3D7 for *pfs48/45*; for Tanzania and Burkina Faso this proportion was 50.0% and 17.9%, respectively (Table 5.1.). Ghana had the lowest number of haplotypes (n=11), (defined as a specific collective of SNPs), Burkina Faso had 15 and Tanzania, 21, resulting in 44 unique haplotypes overall. Ghana also had the lowest number of infections with mixed *pfs48/45* haplotypes (15.4%), followed by Tanzania (43.8%) and Burkina Faso (56.4%). Five SNPs were found most frequently in terms of appearing in different haplotypes and also appearing in the largest numbers of samples. These are located at nucleotide positions 757, 762, 911, 940 and 965 and are all non-synonymous amino acid changes (Table 5.5.).

Table 5.5. Single nucleotide polymorphisms (SNPs) in *pfs48/45* per study location. The shading defines the SNPs found within the different epitope regions

location and type of nucleotide change			SNP frequency by country			
Pfs48/45 epitope location	nucleotide location (corresponding amino acid if changed)	amino acid change	Ghana	Burkina Faso	Tanzania	Total
epitope V	328		1	0	0	1
	336		0	1	0	1
	396 (132)	I=T	1	0	0	1
	419		0	0	1	1
	468 (156)	F=V	0	0	1	1
epitope II & III	492		1	0	0	1
	502		0	0	1	1
	556		1	0	1	2
	753		1	0	0	1
	757 (253)	K=E	2	5	13	20
	762 (254)	N=K	9	12	21	42
epitope I	911 (304)	V=D	7	3	8	18
	940 (314)	L=I	2	29	6	37
	965 (322)	S=N	7	4	6	17
	989		1	0	0	1
	1023		2	0	1	3
	1047		0	0	1	1

DISCUSSION

The present study is the first to determine natural recognition of MTBV candidates Pfs48/45-10C and Pfs230-230CMB in three malaria endemic settings and extends our appreciation of genetic polymorphisms in *pfs48/45*. These observations need to be taken into consideration in the planning of vaccine trials that have to allow for the presence of protein recognition prior to vaccination and potential immune boosting following natural antigen exposure.

Pre-fertilization and post-fertilization antigens for MBTV differ not only in their mechanisms of action but also in their potential for immune-boosting following natural infections. Whilst the possible immune boosting of Pfs230 and Pfs48/45 antibodies has been commonly mentioned (Bousema and Drakeley 2011), it is only with the availability of recombinant proteins that the extent of natural immune recognition can be fully explored. The availability of recombinant proteins also allows addressing immuno-epidemiological questions about the rate of antibody acquisition and its relationship with age and transmission intensity (Kaslow 1997). All studies up to date have relied on assays using natural antigen from cultured gametocyte extract, limiting throughput and sensitivity because of high background reactivity (Roeffen, Mulder et al. 1996; Bousema, Drakeley et al. 2006; Bousema, Roeffen et al. 2006; Drakeley, Bousema et al. 2006; Bousema, Drakeley et al. 2007; Bousema, Roeffen et al. 2010; Ouedraogo, Roeffen et al. 2011). We examined antibody responses to *P. falciparum* transmission blocking vaccine candidates and asexual stage vaccine candidates in three endemic settings in Africa. Multiplicity of infection (MOI), allelic richness, asexual parasite prevalence and serological markers of malaria exposure were used to characterize sites in Ghana, Tanzania and Burkina Faso (Arnot 1998; Babiker, Ranford-Cartwright et al. 1999; Schoepflin, Valsangiacomo et al. 2009; Agyeman-Budu, Brown et al. 2013). All indices used, indicate low transmission intensity in our site in Ghana and intense transmission intensity in our site in Burkina

Faso. Tanzania was selected as a site of meso endemicity. A reduction in malaria burden of 65.4% was reported between 2003-2008, which was attributed to declining numbers of mosquitoes (Mmbando, Vestergaard et al. 2010; Ishengoma, Francis et al. 2011; Meyrowitsch, Pedersen et al. 2011). However, in our survey higher antibody prevalence combined with higher parasite prevalence within our youngest age group suggests transmission may once more be on the rise.

Naturally acquired antibody responses recognizing transmission blocking vaccine candidates 10C and 230CMB broadly followed the pattern of asexual stage antibody responses with lowest antibody responses in Ghana, the area of low transmission intensity. 10C and 230CMB antibody responses were also associated with concurrent asexual parasite prevalence, indicative of higher malaria exposure (Bousema, Kreuels et al. 2011), and a general trend of higher antibody prevalence in older children. The interaction between parasite prevalence and age in Burkina Faso suggests there is higher probability of being 10C or 230CMB antibody positive when both the parasite is present and when children are older.

The age-dependency of sexual stage malaria immunity has not been firmly established (Drakeley, Bousema et al. 2006; Bousema, Roeffen et al. 2010; Ouedraogo, Roeffen et al. 2011) and, although more detailed studies across a larger age-range are needed, our findings indicate that the recombinant vaccine proteins are suitable tools for such studies. Importantly, we observed a highly significant association between naturally acquired antibody responses recognizing 10C and 230CMB and functional TRA, as was previously reported for the native proteins Pfs48/45 and Pfs230 (Graves, Carter et al. 1988; Bouharoun-Tayoun, Oeuvery et al. 1995; Healer, McGuinness et al. 1999; Drakeley, Eling et al. 2004; Bousema, Roeffen et al. 2006; Drakeley, Bousema et al. 2006; Bousema, Roeffen et al. 2010). While TRA in the supportive dataset was significantly associated with antibodies against 10C and 230CMB, there may be antibodies to other candidate transmission-blocking antigens that we have not ruled out as contributors. In general, our assays fall short of providing definitive evidence for the transmission-blocking activity of naturally acquired 230CMB and 10C antibodies. Whilst, antibodies generated by immunization

of rodents with 230CMB or 10C and monoclonal antibodies against Pfs230 and Pfs48/45 are causally associated with transmission blocking activity, our epidemiological findings do not provide the same level of evidence (Roeffen, Beckers et al. 1995; Chowdhury, Angov et al. 2009; Farrance, Rhee et al. 2011; Theisen, Roeffen et al. 2014). To formally conclude that naturally acquired 10C and 230CMB antibodies in humans are responsible for the observed transmission-blocking activity, future studies may affinity purify antibodies against these two antigens and test these purified antibodies using SMFA.

Taken together, our findings of natural recognition of 10C and 230CMB antibody responses and their association with commonly used proxy-markers of malaria exposure (age, concurrent parasite prevalence, serological markers of cumulative exposure to asexual stage antigens) suggest that natural parasite exposure may boost vaccine induced immunity (Farrance, Rhee et al. 2011; Theisen, Roeffen et al. 2014), and that field trials with these vaccine candidates have to take into account naturally acquired immune responses to the vaccine proteins.

For the 10C vaccine candidate, we provided relevant information on genetic variation in the *pfs48/45* gene in our three field sites. We identified 5 previously documented non-synonymous substitutions (Conway, Machado et al. 2001) and discovered 2 new non-synonymous substitutions that were both detected once and are present outside the part of the *pfs48/45* gene that forms the basis of the 10C vaccine. The genetic sequence of 10C in the R0-10C vaccine was based on cultured isolate 3D7/NF54. In a recent study, R0-10C induced immunity showed statistically significant TRA ($\geq 90\%$) against cultured isolates 3D7/NF54, and NF135 which have polymorphisms in amino acids 254, 304 and 322 (Theisen, Roeffen et al. 2014), indicating that these polymorphisms do not necessarily translate in lower vaccine efficacy. While a substantial proportion of field isolates were different from 3D7/NF54 (30.8% in Ghana, 50.0% in Tanzania and 82% in Burkina Faso), it is currently unknown whether this has consequences for 10C vaccine efficacy. Polymorphisms on positions 757 and 940 may be of particular interest in this respect, since these were commonly detected in field isolates but not in NF135, a cultured

strain for which efficient transmission blocking activity of anti-10C antibodies was confirmed (Theisen, Roeffen et al. 2014). Previous research highlighted that the 5 non synonymous substitutions resulted in 9 haplotypes that showed geographical clustering across Africa, Asia and South America (Conway, Machado et al. 2001; Escalante, Grebert et al. 2002; Anthony, Polley et al. 2007). These findings are in line with our study which found these substitutions to be common, but resulted in a larger number of haplotypes, with many being mixed infections. While we only analysed samples from African settings, we also found clear geographical clustering of haplotypes. A high number of mixed clone infections were seen in our samples. As it was not possible to determine which SNPs belonged to different clones, mixed sequencing results were designated new haplotype identities. It is possible that dominant clones may shroud lower density clones resulting in an underestimation in the MOI and *pfs48/45* sequencing data (Agyeman-Budu, Brown et al. 2013). Genetic polymorphisms in vaccine candidates can threaten success by enabling immune escape mutants (Cooreman, Leroux-Roels et al. 2001). The majority of non synonymous substitutions found in these samples, and in previous studies are within the vaccine candidate region. It is currently unclear, but of utmost importance, to determine if vaccine induced immunity is likely to be efficacious against strains with a different genetic composition on the functional epitopes.

In summary, we have found that antibodies against two new recombinant vaccine candidates are naturally acquired, found in serum from 3 endemic regions across both East and West Africa, and show age dependency in Burkina Faso. The functional importance of these antibodies has been confirmed using samples with SMFA data which showed a significant correlation between antibody prevalence and TRA. Our samples show genetic variation within the vaccine candidate that exhibit geographical clustering, the relevance of which is currently not known.

ACKNOWLEDGMENTS

We are grateful to the volunteers for their participation in the study, and to the teams from Centre National de Recherche et de Formation sur le Paludisme (CNRFP), Burkina Faso, Noguchi Memorial Institute for Medical Research, Ghana and Kilimanjaro Clinical Research Institute, Tanzania, for fieldwork and microscope slide reading.

We would like to thank Dr. Jessica Chichester at Fraunhofer USA Center for Molecular Biotechnology for sharing their recombinant 230 CMB protein.

This work was completed as part of the REDMAL project and was funded by the European FP7 project (#242079).

Conflict of interest

We declare there are no competing interests.

Supplementary methods and results

A factor Xa site was included between R0 and 10C to enable cleavage and allow quantification of sexual stage antibodies. To cleave R0, the following mastermix was prepared; 200µL of 100% properly folded R0-10C (300µg) was incubated with 20µl of 100mM of CaCl₂, 20µl of 1% DOC in Tris-buffer and 4µl of undiluted Factor Xa Protease (New England BioLabs Inc, Leiden, The Netherlands) overnight at room temperature.

To confirm complete cleavage, product was visualized using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and a 4-12% NuPAGE^(R) gel (Invitrogen Life Technologies, Bleiswijk, The Netherlands). 15µl of cleaved product was incubated with 15µl of Tris-Glycine SDS, for 20 minutes at 80°C, then 15µl of product was visualized on the gel (Figure 5.2.).

Protein reactivity against epitope 45.2b of Pfs48/45, was confirmed using Western Blotting. Protein bands were transferred from a second SDS gel to Hybond-C-extra paper (Amersham), which was blocked with 5% milk in PBS for 30 minutes on a shaking incubator. The gel was incubated in 1.5µg/ml of HRP-mAb85RF45.2b (epitope 45.2b of Pfs48/45) in PBS with 0.05% Tween 20 for 2 hours on a shaking incubator. The gel was then incubated with peroxidise substrate Kit Vector SG (SK4700, Vector Laboratories), which enabled identification of protein bands that reacted against epitope 45.2b.

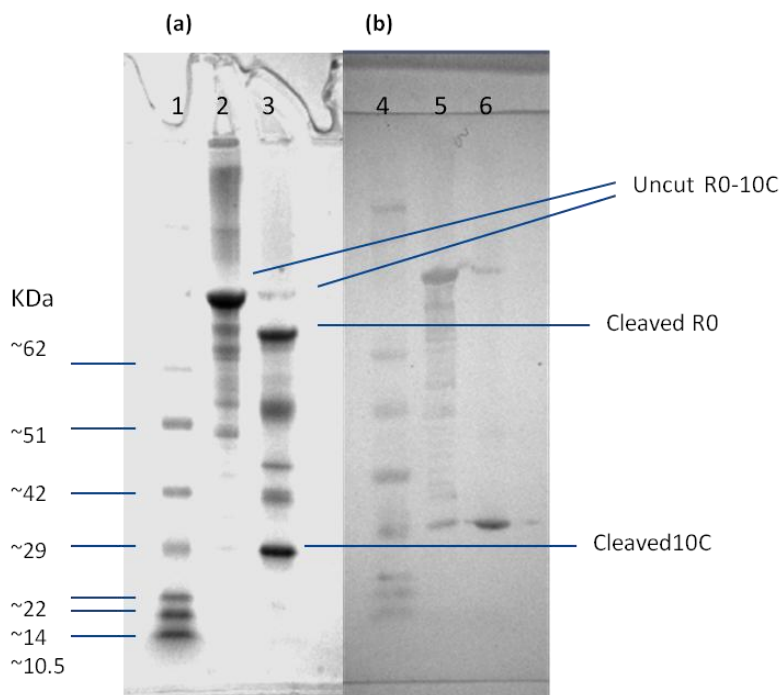


Figure 5.2. Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (a) and, Western blotting analysis of cut and uncleaved protein (b). Lanes 1 and 4 contain molecular weight markers (SeeBlue Pre-stained standard (Invitrogen LC5625) lanes 2 and 5 have 100% uncleaved protein and the cleaved product is in lanes 3 and 6.

Figure 5.2. shows the product of the cleavage reaction (a), and the reactivity of the products to epitope 2b (b) in a Western blot. Uncleaved R0-10C is 89.8kDa in size, which following cleavage divides into R0, 58.3kDa in size, and 10C-6His which is 31.5kDa. Figure 5.2 (b) shows there is reactivity against epitope 2b Pfs48/45 to two bands in the cleaved product, which are at the correct size for cleaved 10C and a minor band for uncleaved protein, which confirms that there is some uncleaved R0-10C in the 'cleaved' product.

Reactivity of 10C was checked with a two site ELISA using anti-Pfs48.45 mAbs recognizing epitopes 1 and 2B, which was otherwise performed as before. Samples were assayed from known gametocyte carriers who had antibodies against Pfs48/45 and had been shown to demonstrate transmission reducing activity using membrane feeding assays. To confirm specificity, a control sample was used from an individual who had been vaccinated with GLURP. High reactivity against gametocyte positive individuals and low reactivity against the GLURP vaccinated individual indicated the cleaved product was just 10C.

10C was then purified by gel filtration on a Sephacryl S-200 column (GE Healthcare, The Netherlands) size exclusion column to remove the uncleaved protein. 150 μ L of 10C was mixed with 1000 μ L of buffer (EDTA 250mM, Tris 1M pH 8.8, NaCl 100mM, 0.8% sodium deoxycholate in ddH₂O), then loaded onto the column and run for 3 hours to enable separation. Fractions corresponding to 10C were pooled and concentrated according to their expected molecular weight using VivaSpin columns (GE Healthcare, The Netherlands) which were centrifuged at 4000 rpm for 25 minutes. Following this, supernatant was checked on SDS and Western blot once more, concentration estimated using Nanodrop and BCA and reactivity confirmed once more using ELISA. Product was concluded to be free of R0.

5.4 Linking methods and results: MBP-10C

The linking information in this chapter is comprised of protocol optimization and building upon results covered in the manuscript through use of supportive sample sets. To improve clarity, this section has been split into sections addressing:

- MBP-10C, R0-10C and the cross sectional survey sample sets.
- 10C and 230CMB antibody responses and their relationship with TRA (using supportive sample sets).
- Genetic analysis of the cross sectional survey samples.

MBP-10C was the primary version of the vaccine candidate (different to the one discussed in the attached manuscript). The first section below outlines development using this candidate, prior to its replacement. Within each section the methods are followed by the results, to improve the clarity. A final extended discussion follows at the end.

5.41 Methods: MBP-10C ELISA assay

An ELISA assay was optimized to enable quantification of MBP-10C antibodies. Total IgG was measured against recombinant MBP-10C (which was produced and supplied by W.Roeffen (Radboud UMC)), using ELISA as follows. Nunc plates (Nunc, Stockholm, Sweden) were coated with 100 μ L per well of 2 μ g/mL of MBP-10C diluted in PBS (Invitrogen, Life Technologies, Bleiswijk, The Netherlands), then incubated overnight at 4°C. Plates were washed 3 times with PBS then blocked with 150 μ L per well of PBS with 5% w/v milk powder (Sigma-Aldrich, Zwijndrecht, The Netherlands), for 15 minutes at room temperature. Plates were washed 3 more times with PBS, then incubated at room temperature for 3 hours with 100 μ L of test serum, diluted 1/200 in PBS (with 0.05% v/v Tween 20 (Sigma-Aldrich) and 1% w/v milk powder).

Plates were washed 3 times, then incubated for 2 hours with 100µL per well of anti-human-IgG-HRP (Dako, Cambridgeshire, UK) diluted 1/50,000 in PBS Tween 20 (0.05% v/v). Plates were washed 4 times then incubated with 100µL of TMB (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 20 minutes at room temperature. The reaction was stopped with 50µL per well of 0.2M H₂SO₄ (Sigma-Aldrich, Zwijndrecht, The Netherlands) and OD was read at 450nm, using an iMark Microplate reader (BioRad, Veenendaal, The Netherlands). Samples were tested in duplicate and two blank wells were included per plate to adjust for background reactivity of reagents.

To identify reactivity to the fusion protein MBP, IgG was measured using ELISA as outlined previously, but plates were coated with MBP at a concentration of 1µg/mL. Results were analyzed as discussed in the general methods in section 3.14.

5.42 Methods: Samples used for validation of MBP-10C ELISA

Serum samples from previous studies performed in The Gambia (n=200) and Tanzania (n=360) were used to validate the ELISA assay. Tanzanian samples were collected from the Tanzanian Plantation Company in 2003-2004 from a village of low endemicity in Lower Moshi, with an estimated EIR of 3.4 per year (Bousema, Roeffen et al. 2010). This sample set was used for validation since antibody responses against native proteins Pfs48/45 and Pfs230 had previously been measured and it was hypothesized antibody responses against recombinant antigens would be comparable (Oesterholt, Bousema et al. 2006; Bousema, Roeffen et al. 2010). The Gambian samples were collected in 1990-1991 from the North bank of the Farafenni river, in a region of intense seasonal transmission where there was an asexual parasite prevalence of 5% during the dry season, and 35% during the wet season, which was determined by microscopy (Drakeley, Secka et al. 1999; Drakeley, Akim et al. 2000; Lulat 2003). MBP-10C antibodies were also quantified in the European negative controls (n=23), (which were discussed in section 3.14.).

5.43 Results: MBP-10C antibodies quantified in samples from malaria exposed individuals from the Tanzania Plantation Company (Lower Moshi, Tanzania) and The Gambia

Results for the Tanzanian sample set are discussed first. In the graph below (Figure 5.3.), all data are from the previous study, excluding MBP-10C and GLURP antibodies which were quantified in this chapter. MBP-10C antibody positivity was 2.5% for <5 years, 11.3 % for 5-14 years and 8.7% for ≥ 15 years (Figure 5.3.). Antibody prevalence for native (Pfs48/45) and recombinant (MBP-10C) protein was not expected to be identical, but these patterns appeared similar enough for confidence to be gained in antibody measurement using MBP-10C.

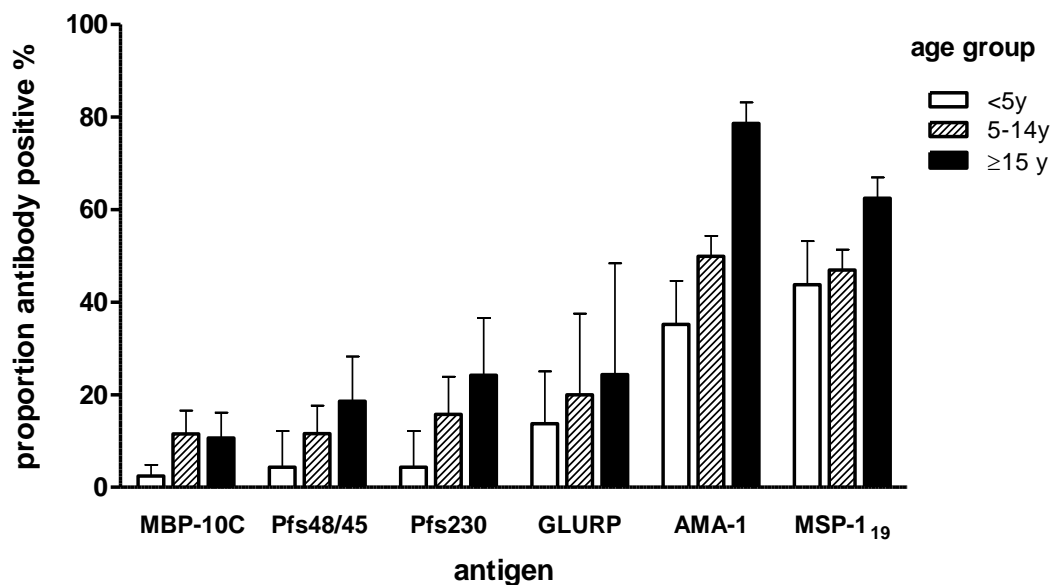


Figure 5.3. Tanzania Plantation Company: Proportion of individuals antibody positive for MBP-10C, Pfs48/45, Pfs230, GLURP, AMA-1 and MSP-1₁₉ by age group. Error bars indicate the 95% confidence interval. Age groups are represented by different bar shading. NB: This data has been re-drawn with permission, excluding results for MBP-10C and GLURP, which were determined in this chapter and plotted here to allow comparison with previous data (Bousema, Roeffen et al. 2010).

To further increase confidence in results, the ELISA assay was validated against a larger set of samples from The Gambia (n=178). MBP-10C antibody prevalence overall was 42.1% in these samples which is higher than commonly reported for sexual stage antigens (typically 10-40%) (Bousema, Roeffen et al. 2006).

5.44 Results: MBP-10C antibody quantification in malaria unexposed European controls using ELISA

Following unexpectedly high MBP-10C antibody prevalence in The Gambian dataset, MBP-10C antibodies were measured in malaria unexposed European individuals (n=23) to assess if there was non-specific reactivity. Negative controls typically produce low OD values not much higher than that obtained for a blank well, which on average is 0.02. In an ELISA assay where there is little non-specific response, negative controls would be expected to have an OD within the range of approximately 0.02-0.2. Figure 5.4. indicates the normalized ODs for European negative control samples which ranged from 0.13-0.85. This is high and indicates antibodies that bind to MBP-10C may not all be specific to 10C.

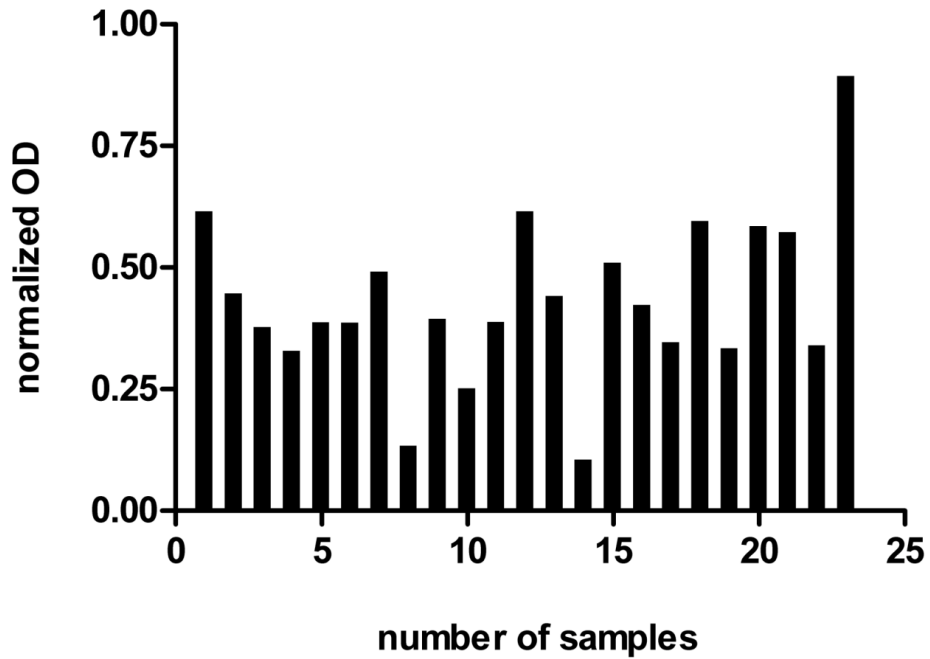


Figure 5.4. MBP-10C antibody responses measured in European negative controls. Antibody responses are expressed as normalized OD, and were measured in 23 European negative controls.

5.45 Results: Assessing reactivity to maltose binding protein (MBP) in malaria exposed and unexposed populations

It was suspected some of the measured antibodies were specific to MBP, not 10C. To confirm this, IgG was measured as before coating plates with MBP. Figure 5.5. demonstrates there is a positive correlation ($R^2=0.561$) between sample ODs for MBP-10C and MBP coated plates, suggesting reactivity is partly antibodies that bind to MBP.

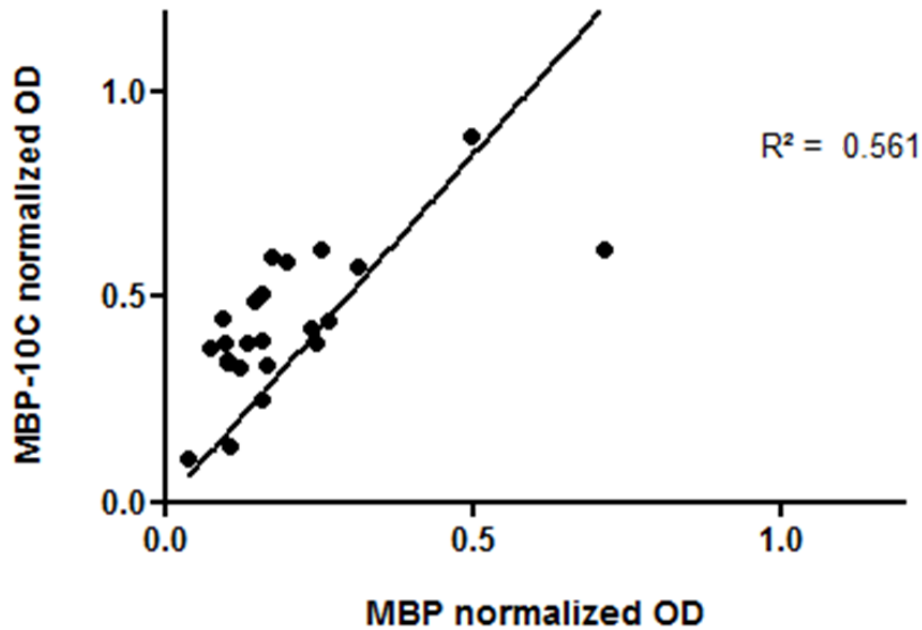


Figure 5.5. The relationship between MBP-10C and MBP antibody responses measured in European negative controls. Antibody responses are expressed as normalized OD and were measured in 22 European negative control samples. The relationship was assessed using regression, $R^2=0.561$.

MBP antibodies were then measured in endemic serum. Figure 5.6. (a). indicates there is a also a relationship between MBP-10C and MBP antibody responses in serum from Tanzania and The Gambia, suggesting these populations also react against fusion protein MBP. To attempt to isolate 10C antibody responses, OD values for MBP were subtracted from ODs to MBP-10C, (referred to as 10C adjusted ODs). Figure 5.6. (b) demonstrates there was no discernible pattern between the adjusted results (10C) and MBP-10C ODs. For example, some samples with high MBP-10C ODs had zero or negative 10C-specific reactivity after subtraction of MBP. While MBP-10C had initially showed promising reactivity with antibodies in the Tanzanian sera samples, responses were found to be strongly related to MBP (Figure 5.6.(b)).

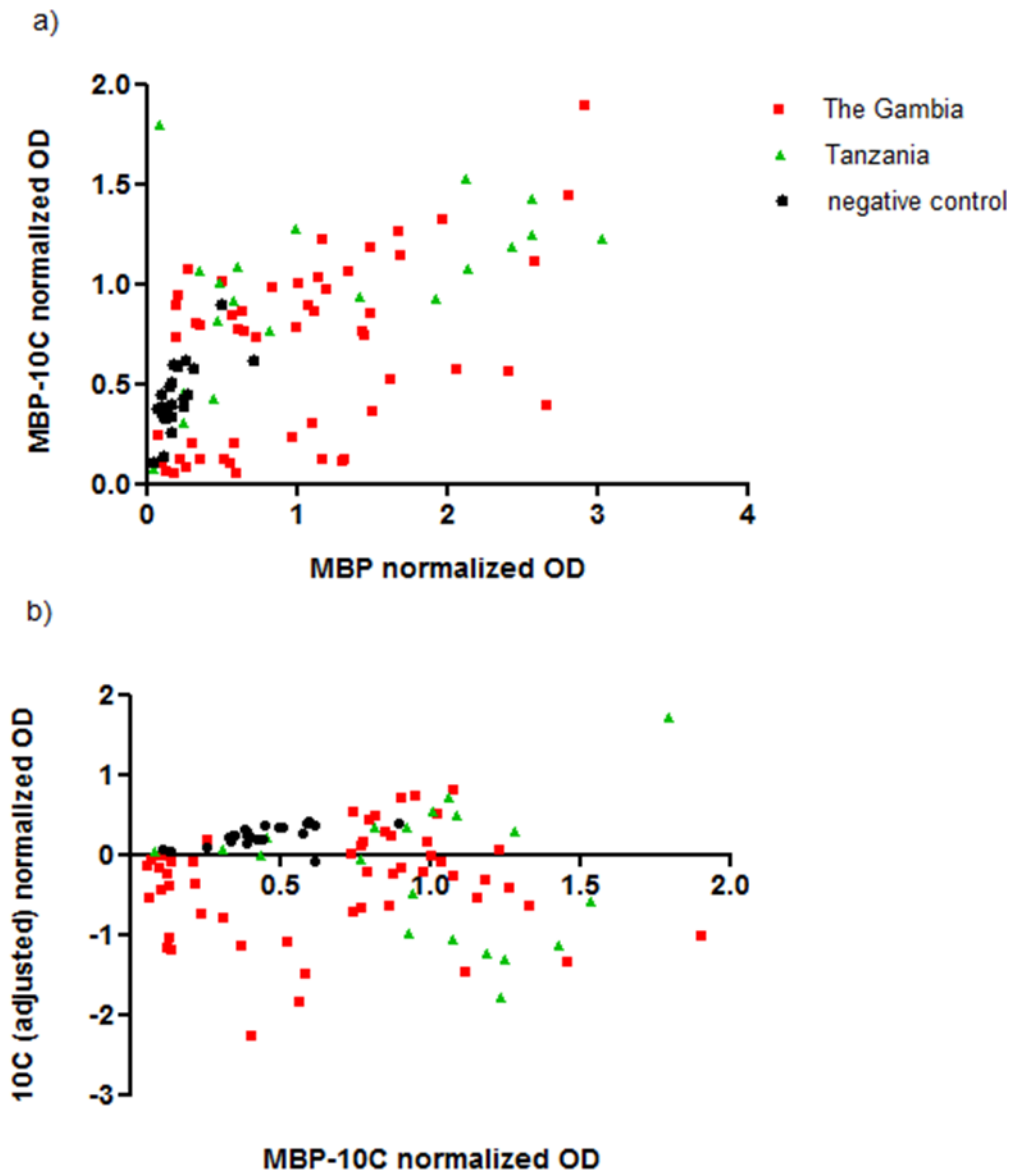


Figure 5.6. The relationship between MBP-10C and MBP antibody responses in endemic and European control serum. (a) The relationship between antibody responses measured against MBP-10C and MBP. (b) The relationship between adjusted OD responses for 10C and MBP-10C. Dots represent European negative controls, squares represent serum from The Gambia and triangles represent Tanzanian serum.

While the presence of a factor Xa cleavage site between MBP and 10C would have enabled cleavage to assess reactivity to 10C, problems with production of MBP-10C

as a vaccine candidate, which are described next, simultaneously occurred and led to a revised vaccine candidate being used.

The REDMAL project vaccine development objectives were to produce $\geq 95\%$ properly folded clinical grade material, a yield of 10mg/L, protein stability for at least 9 months, and induction of high titres of functional transmission blocking antibodies in rodents (REDMAL 2011). MBP-10C overexpression was performed by Will Roeffen at Genova Biopharmaceuticals, Pune, India. The majority of MBP-10C that was overexpressed was multimeric protein which induced high titres of antibodies, with no transmission reducing activity, whereas monomeric protein induced functional TR antibodies (Will Roeffen, personal communication). While variations of the fermentation protocol were attempted to try to increase the proportion that was monomeric, protein yields remained too low and all experiments with MBP-10C were discontinued and the revised candidate, R0-10C was developed instead.

5.5 Linking methods and results: 10C ELISA and cross sectional surveys

To allow assessment of antibodies specific to 10C, R0 was cleaved, then 10C was purified by Will Roeffen.

5.51 Methods: Optimization of 10C ELISA

To determine optimal ELISA reagent dilutions a checkerboard titration was performed, titrating coating antigen (purified 10C) and conjugate down and across the plate respectively. Antigen was titrated from 4 μ g/mL to 0.03 μ g/mL and the conjugate from 1/10,000-1/320,000. The objective was to select concentrations that achieved low reactivity for a Radboud Hospital blood bank negative control sample that previously produced higher reactivity in malaria ELISA assays (Will Roeffen, personal communication), and high reactivity for the positive control sample. ELISA

was otherwise performed as outlined in 3.13. , but samples were diluted to 1/200 in PBS and added to half the plate each. Optimal concentrations were determined to be 1.0µg/mL of 10C coating antigen and 1/80,000 dilution for the conjugate (see Table 5.6.). These concentrations were optimized against 10C but were also found to be suitable for 230CMB (data not shown).

Table 5.6. Titration of 10C coating antigen and conjugate using ELISA. Optical densities (ODs) are shown for a positive and negative control sample. Optimal concentrations were 1µg/mL of coating antigen and 1/80,000 conjugate dilution (highlighted in boxes). Rational for selection is explained in the text.

10C Conc. (µg/ml)	Positive control 1/200 dilution						blood bank negative 5 (high responder) 1/200 dilution					
	Conjugate dilution						Conjugate dilution					
	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/320,000	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/320,000
4	3.50	3.50	3.50	3.50	2.03	1.04	1.99	0.87	0.45	0.22	0.10	0.09
2	3.50	3.50	3.50	2.59	1.70	0.81	1.29	0.60	0.34	0.17	0.09	0.07
1	3.50	3.50	3.50	2.68	1.33	0.70	1.10	0.50	0.28	0.16	0.09	0.05
0.5	3.50	3.50	3.50	2.11	1.15	0.55	0.99	0.53	0.27	0.14	0.08	0.05
0.25	3.50	3.50	2.71	1.58	0.79	0.38	0.81	0.46	0.31	0.13	0.07	0.04
0.125	3.50	3.50	1.87	0.98	0.48	0.25	0.80	0.43	0.32	0.11	0.06	0.04
0.063	3.50	2.33	1.14	0.58	0.27	0.15	0.80	0.42	0.25	0.09	0.05	0.04
0.030	2.62	1.74	0.71	0.32	0.19	0.09	0.78	0.39	0.18	0.09	0.05	0.03

Following this, a larger batch of antigen was produced and purified by Will Roeffen, to provide sufficient material for evaluating all sample sets assessed in this thesis. Due to slight variation in antigen production between batches, a new coating concentration of 0.1µg/mL, a conjugate dilution of 1/40,000, and test sample dilution of 1/500, were used for both 10C and 230CMB ELISA. These were determined in the same manner as previously described. The final ELISA protocol is in section 3.13.

5.52 Methods: GLURP controls

A concern with serological assessment using R0-10C vaccine candidate was that incomplete cleavage of R0 would result in measurement of asexual stage antibodies in addition to 10C responses. Since GLURP-R0 is widely found in naturally exposed individuals, R0 reactivity has the potential to mask 10C reactivity (Dodoo, Aikins et al. 2008; Pratt-Riccio, Bianco et al. 2011). To ensure R0 had been cleaved from 10C, serum from individuals who had been vaccinated with AMA-1 or GLURP (and produced high antibody titres), was included on ELISA plates coated with 10C or 230CMB antigens, Table 5.7. (Hermsen, Verhage et al. 2007; Esen, Kremsner et al. 2009; Mordmuller, Szywon et al. 2010).

Table 5.7. indicates OD values for GLURP are low and comparable to asexual antigen AMA-1, which demonstrates there is not additional reactivity to GLURP. Since OD values measured on 10C plates are comparable to 230CMB responses, which did not have a GLURP fusion partner, this further validates removal of R0 from 10C.

Table 5.7. Reactivity of GLURP and AMA-1 positive serum to 10C and 230CMB antigens using ELISA. Reactivity is expressed as normalized optical density (OD).

Control serum	normalized OD response	
	10C	230CMB
GLURP	0.10	0.11
AMA-1	0.15	0.20

5.53 Methods: The cross sectional survey field sites, and my role in the surveys

Immuno-epidemiological cross sectional surveys were performed to provide snapshot information about antibody prevalence for the two vaccine candidates, as outlined in the attached manuscript. My role in cross sectional survey sample collection included developing and supplying standard operating procedures (SOPs), and shipping reagents to the field sites. In each country, team leaders were designated who co-ordinated trips to the schools and ensured SOPs were understood and sampling performed as required. Following sample collection, I taught a 1 week training course at Noguchi Memorial Institute for Medical Research in Accra, Ghana for participants from each of the field sites. While the primary objective was capacity building to teach participants how to perform ELISA and analyze results, some results contributed to the manuscript in this chapter. Following the training session, filter paper samples and plasma were transferred to London (at ambient temperature, and -20°C respectively) for DNA extraction, sequencing and sexual stage antibody quantification using ELISA.



Figure 5.7. Map of Ghana indicating the location of the field site in Asutsuare. Asutsuare is marked by 'A'. The inset map in the top left hand corner shows the location of Ghana within the African continent. Sourced from Google Maps (www.google.co.uk/maps), August 2014.

Three field sites were selected to represent different levels of endemism. In Ghana, samples were collected from Asutsuare which is in the Dangme district, approximately 120km northeast of Accra (Figure 5.7.). This is a rural area, where the land is characterized by a network of irrigation canals and the majority of the

population is subsistence farmers. Asutsuare has a long rainy season from March to August and a shorter rainy period from November to December, with dry seasons in between. Malaria transmission is low and seasonal and parasite prevalence was reported as 2.7% (by microscopy) in 2009 (Kusi, Bosomprah et al. 2014).



Figure 5.8. Map of Tanzania indicating the location of the field site in Bondo. Bondo is marked by 'A'. The inset map in the bottom right hand corner shows the location of Tanzania within the African continent. Sourced from Google Maps (www.google.co.uk/maps), August 2014.

Bondo was selected as the field site location in Tanzania. It is a village within the Handeni district in the coastal region called Tanga located in northeast Tanzania, where the population is predominantly subsistence farmers (Figure 5.8.). Malaria transmission is perennial and there are 2 rainy seasons: a long season from March-

June and a shorter season from October to November (personal communication, Dr. Jaffu Chilogola). Parasite prevalence was 23% in 2011, which was determined by PCR (unpublished results, Dr. Jaffu Chilogola).

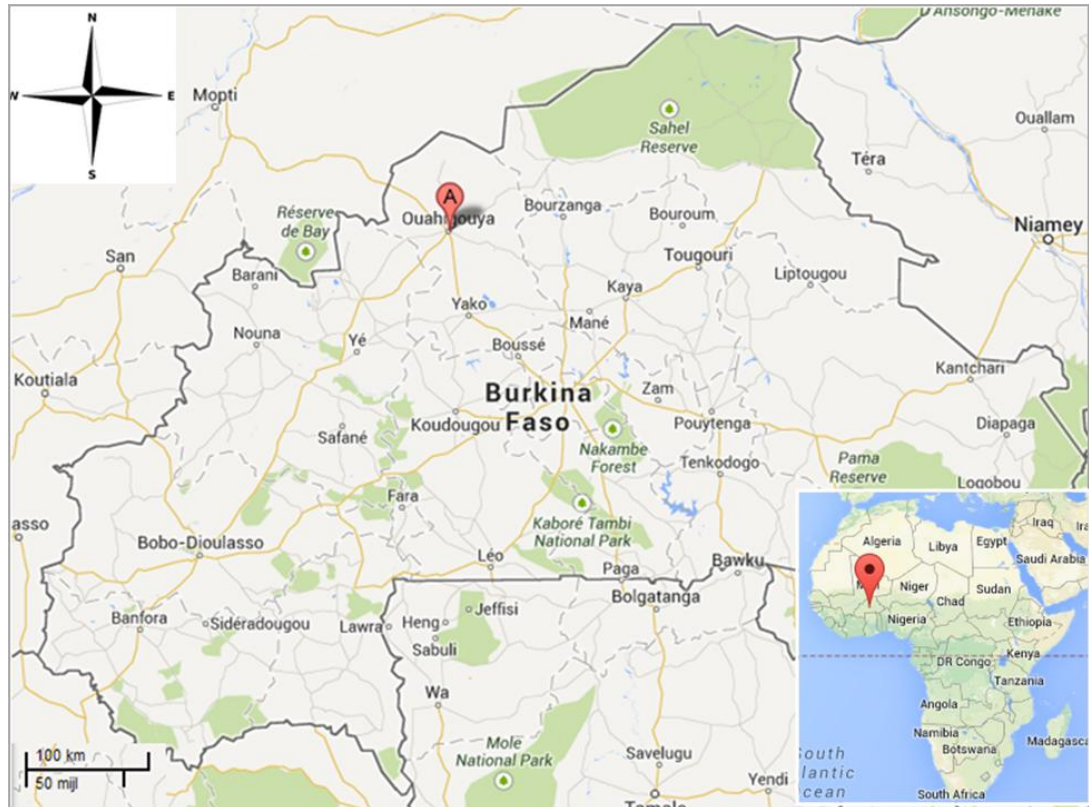


Figure 5.9. Map of Burkina Faso indicating the location of the field site in Ouahigouya. Ouahigouya is marked by 'A'. The inset map in the bottom right hand corner shows the location of Burkina Faso within the African continent. Sourced from Google Maps (www.google.co.uk/maps), August 2014.

Ouahigouya was chosen as the sample collection site in Burkina Faso (Figure 5.9.). The terrain in the northern part of the country is desert, the central region is Sudanese savannah and the southern part is characterized by forest. Malaria transmission is stable and seasonal with 300-500 infectious bites per person per year (Ouedraogo, De Vlas et al. 2008). The dry season runs from November to May and the rainy season spans June to October (Ouedraogo, De Vlas et al. 2008).

5.54 Methods: Sample size calculation to determine the number of children to recruit for the surveys

Without previous information on 10C and 230CMB reactivity in naturally exposed populations, sample size was based on school surveys to demonstrate parasite prevalence rates with adequate precision.

Parasite rate estimations taken from school surveys performed in a lower endemic site in Ghana and a higher endemic site in Tanzania indicated a difference in parasite rate of 55-88% between sites for school aged children (5-14) (Brooker, Kolaczinski et al. 2009). Using this data, it was estimated sampling 200 children would enable parasite detection by microscopy of 20.2% (95% CI; 15.1-26.5%) in a high endemic setting and 10.1% (95% CI; 7.1-14.2%) slide prevalence in a low endemic setting.

5.55 Methods: Ethical permission and field sampling

Ethical permission was obtained from LSHTM (approval number 5946) and local and national ethics committees in each country. Sampling was performed as outlined in the attached manuscript. Information sheets, consent forms, questionnaires and protocols for taking the blood sample and preparing the RNA sample, filter paper, microscopy blood slide, and plasma and cell pellet, are included in appendix 2-10. RDT was performed according to manufacturer's instructions.

5.56 Methods: Membrane feeding assays

In addition to the membrane feeding referenced in the attached manuscript, SMFA was performed by colleagues on a wider range of samples, using protocols described elsewhere (Bousema, Sutherland et al. 2011; Boissiere, Gimonneau et al. 2013; Tchioffo, Boissiere et al. 2013). 10C and 230CMB antibodies were quantified in these

samples to enable assessment of the relationship between naturally acquired antibodies and transmission reduction (a reduction in oocyst density).

5.57 Main results: Cross sectional surveys

Please refer to the manuscript for the main results from the cross sectional surveys.

5.58 Results: The sampling population

School children were sampled in this study since they provide an easily accessible population that is highly informative to gain knowledge about local epidemiology (Stevenson, Stresman et al. 2013). They are likely to reveal patterns of antibody acquisition since they may have some level of asexual stage immunity but still carry high parasite burdens, and are therefore more exposed to microscopically detectable gametocyte densities, compared to adults (Smith, Dushoff et al. 2005; Brooker, Kolaczinski et al. 2009).

Although all field sites used the same sampling protocols, there were variations in the way field workers visited different classes which resulted in a different age distribution for each of the field sites, see Table 5.8. Consequently, the data was divided into the following age categories ≤ 8.0 , 9-11 and >11 , to minimize the effect of different age skewing per country. Table 5.0. in the attached manuscript indicates the median age per each country within these age groups and demonstrates they are similar.

Table 5.8. Age distribution of study participants for each field site. NB: age data was not available for all individuals.

age	Tanzania	Ghana	Burkina Faso
Median	12	10	8
IQR	10-14	8-13	7-10
Range	5-17	3-15	5-16
Total number	520	180	380

5.59 Results: Effect of season on sexual stage antibody prevalence in cross-sectional populations in Burkina Faso, Ghana and Tanzania

To try to understand what influences antibody prevalence, the relationship with season was explored. Table 5.9. indicates the prevalence of 230CMB antibodies is higher during the wet season for all countries when compared to the dry season, but this difference is only statistically significant in Burkina Faso, or when analysing the dataset as one population.

Table 5.9. 10C and 230CMB antibody prevalence (%) in school aged children according to sampling season in Ghana, Tanzania and Burkina Faso. p-values were generated using logistic regression after adjusting for age.

	Antibody prevalence according to season					
	10C dry	10C wet	p-value	230CMB dry	230CMB wet	p-value
Ghana % (n/N)	3.0% (3/100)	0.9% (1/106)	0.285	4.0% (4/100)	8.5% (9/106)	0.114
Tanzania % (n/N)	13.0% (33/254)	17.9% (33/184)	0.150	16.6% (42/254)	22.2% (41/185)	0.167
Burkina Faso % (n/N)	20.7% (39/188)	15.4% (30/195)	0.049	7.9% (15/189)	16.4% (32/195)	0.002
Overall p-value			0.785			0.009

10C antibody prevalence is higher during the dry season for Ghana and Burkina Faso, which is only a statistically significant difference for Burkina Faso. When considering the difference in antibody titre by season, there was no statistically significant difference for 230CMB ($p \geq 0.206$), excluding Burkina Faso where titre was significantly higher during peak transmission season ($p = 0.005$). For 10C, while there was no statistically significant difference with titre and season in Tanzania ($p = 0.771$) titre was lower during the peak transmission season in Burkina Faso and Ghana ($p < 0.001$). To explore how exposure changes with season, the relationship between gametocyte prevalence (microscopically detected) and season was explored but there was no statistically significant difference in gametocyte carriage between seasons for Burkina Faso or Tanzania ($p \geq 0.347$), (Ghana had no gametocytes). In contrast, asexual parasite prevalence was significantly higher during the peak transmission season for Tanzania and Burkina Faso ($p \leq 0.006$), but not for Ghana ($p = 0.343$), where parasite prevalence (determined by microscopy) was very low overall.

5.6 Linking methods and results: Supportive data sets and further exploration of antibody responses and transmission reducing activity

5.61 Methods: Explanation of the supportive sample sets

To expand on the results seen with the cross sectional survey samples (from Burkina Faso, Tanzania and Ghana) collected in this chapter, 10C and 230CMB antibodies were quantified in 2 additional sample sets. These supportive sample sets were:

- 1) The Ugandan cross sectional study: This study sampled individuals from all ages, and was used in this chapter to explore antibody response patterns into adulthood since the cross sectional surveys focused on school children.
- 2) A compilation of samples with known transmission-reducing activity: These samples were predominantly taken from gametocyte carriers, from several African

countries. TRA was assessed using SMFA (by colleagues at Radboud UMC), and 10C and 230CMB antibodies were quantified in this chapter to explore their association with TRA.

To clarify which sample sets are discussed in the following sections they are referred to as: the cross sectional survey samples, the Ugandan cross sectional study and the SMFA compilation sample set.

5.62 Results: Acquisition of 10C and 230CMB antibodies with age in samples from a cross sectional study performed in Uganda

Upon detection of an age dependent antibody acquisition pattern in school aged children in a high endemic setting (in Burkina Faso), and meso endemic site in Tanzania (for 230CMB only), the next objective was to investigate if this pattern continued into adulthood. To answer this question, 10C and 230CMB antibody responses were quantified in samples collected in all age groups (6 months to 72 years) from Kanungu, Uganda. This is a region of low to moderate malaria transmission with an estimated EIR of 26.6, which was determined in 2011-2012 (Kilama, Smith et al. 2014). Overall, 17.4% (308/1769) of individuals were positive for 10C antibodies and 13.2% (233/1766) positive for 230CMB antibodies.

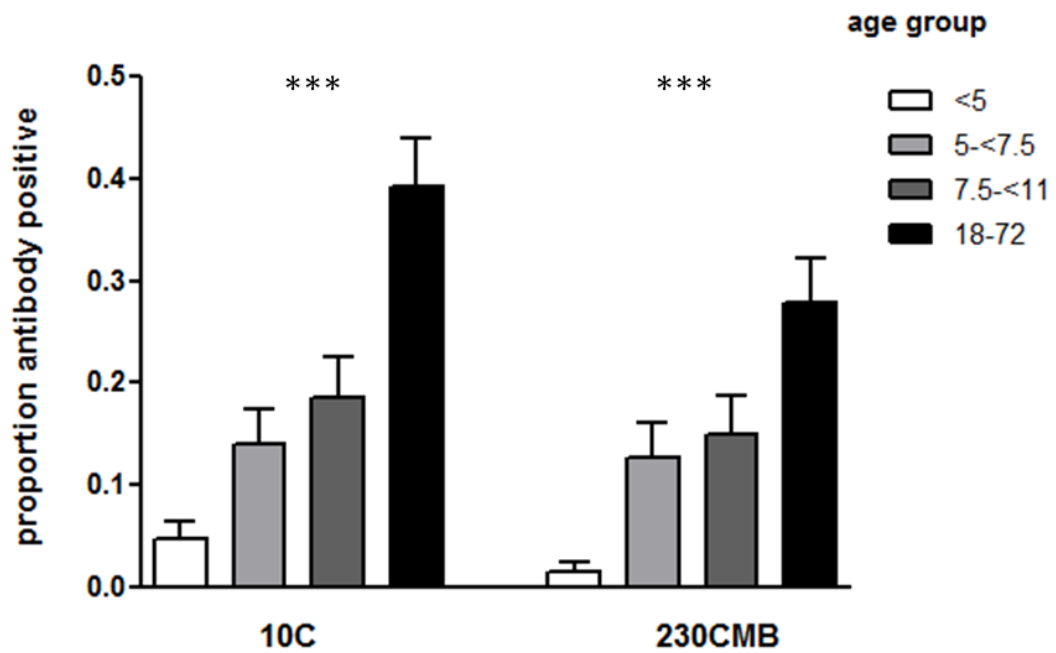


Figure 5.10. Ugandan cross sectional survey samples: Prevalence of 10C and 230CMB antibodies according to study participant age group. The bars indicate the proportion antibody positive according to age group (shaded in different colours), and the error bars demonstrate the 95% confidence interval. The asterisks indicates a statistically significant positive increasing trend at the <0.001 level (***), which was assessed using logistic regression. Results did not change significance boundary when using age as either a categorical or continuous variable.

Figure 5.10. demonstrates a statistically significant age dependent 10C and 230CMB antibody increase in prevalence, and confirms that the pattern previously seen in children extends into adults who have the highest antibody prevalence in this population.

5.63 Results: SMFA compilation sample set. The association between transmission reducing activity and 10C and 230CMB antibodies

Data from a previous study performed in Tanzania was used to confirm the association between TRA and 10C and 230CMB antibody responses in the attached

manuscript. Since then, antibodies have been quantified in additional samples from a SMFA compilation sample set with TRA data. Samples (n=375) were obtained from gametocyte carriers (median age 7 years, quartiles 4 and 10 years) from Burkina Faso, The Gambia, Cameroon, Gabon and from Dutch Missionaries who had lived in malaria endemic regions and were repeatedly infected with malaria.

To further elucidate the relationship between more effective TRA and antibody prevalence, TRA was categorized at $\geq 50\%$ (representing 50-89%) and $\geq 90\%$ levels (representing 90-100%). The % TRA reflects the % reduction of oocyst density when comparing control mosquitoes which were fed gametocytes with non-immune sera, to test mosquitoes which were fed gametocytes with endemic serum.

The association between antibody prevalence and TRA was explored using logistic regression. Table 5.10. indicates there is a statistically significant association with 10C and 230CMB antibody prevalence and TRA at the $\geq 90\%$ level.

Table 5.10. SMFA compilation sample set: Association between transmission reducing activity at the $\geq 50\%$ and $\geq 90\%$ levels and 10C and 230CMB antibody prevalence. Transmission reducing activity was categorized at $\geq 50\%$ (representing 50-89%) and $\geq 90\%$ (representing 90-100%) levels. p-values are presented along with odds ratios with 95% confidence intervals (CI). # indicates there were no 10C positive individuals who exhibited $\geq 50\%$ TRA.

Antibody	TRA $\geq 50\%$ (n=31)		TRA $\geq 90\%$ (n=16)	
	OR (95%CI)	p-value	OR (95%CI)	p-value
10C	#	#	5.37 (1.8-16.0)	0.003
230CMB	0.98(0.35-2.68)	0.962	7.74 (2.44-24.55)	0.001

There were 16 participants who exhibited $\geq 90\%$ TRA, 15 of whom had antibody data for 10C, and 13 had antibody data for 230CMB. Six individuals (40.0%) were positive for 10C, 8 (61.5%) positive for 230CMB, and 5 of these individuals were positive for both antibodies. Five other individuals had $\geq 90\%$ TRA, but no detectable antibodies against either transmission blocking vaccine candidate, indicating other antibody responses may be responsible for TRA. There was no statistically significant relationship between TRA at the $\geq 50\%$ level and 230CMB antibodies, and none of these participants had 10C antibodies, so no calculation was performed, Table 5.10.

5.64 Results: SMFA compilation sample set. Antibody acquisition patterns with age

There is age data for 199 participants from the SMFA compilation sample pool. Age was once again divided into categories <5 (n=25), 5-14 (n=123) and ≥ 15 years (n=51). Figure 5.11.(a) indicates the SMFA compilation samples also demonstrate statistically significant increasing acquisition of 10C and 230CMB antibodies with age. Figure 5.11. (b) demonstrates 230CMB titre significantly increases with age, and while 10C titre also increases, this was not a statistically significant finding.

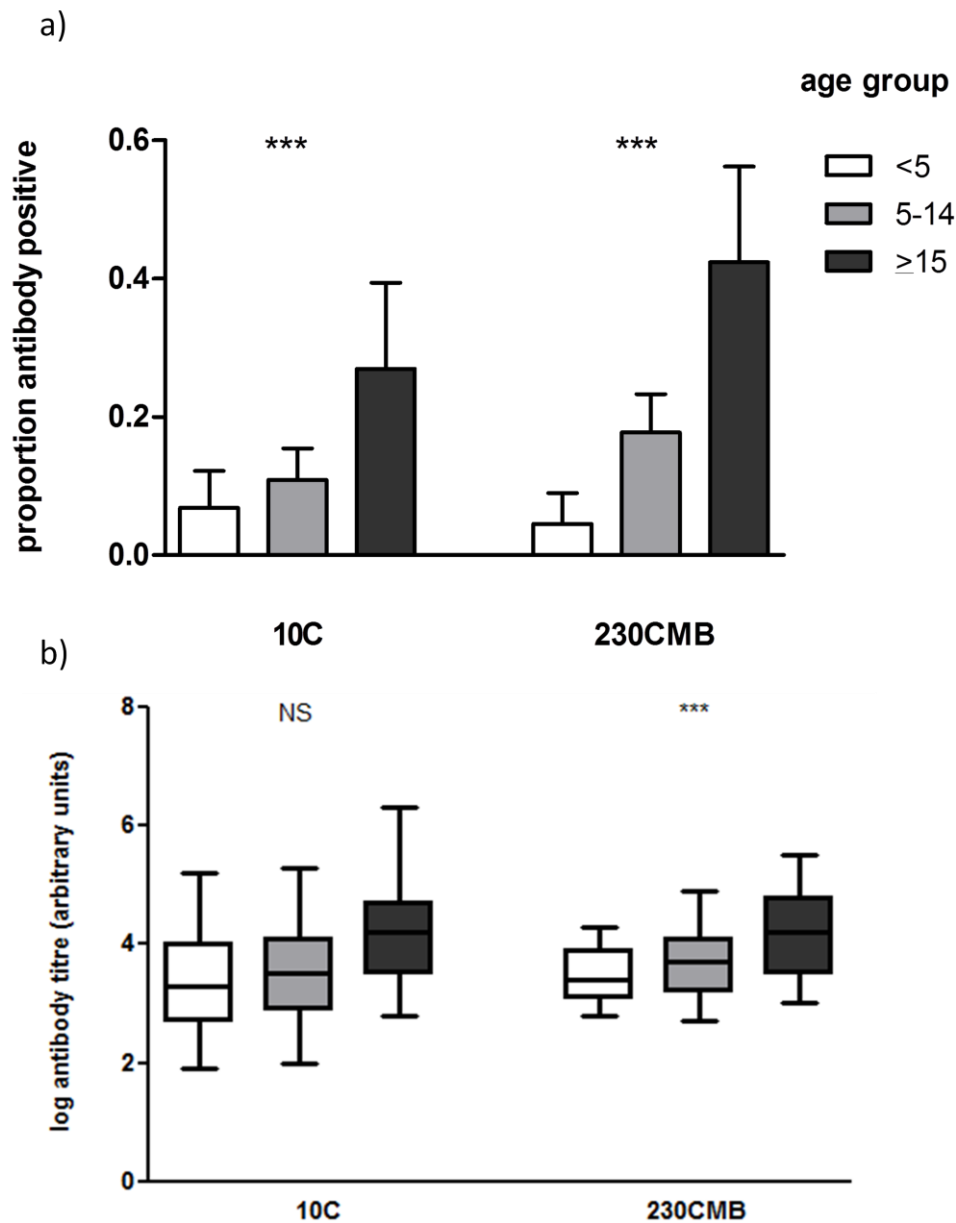


Figure 5.11. SMFA compilation sample set: Prevalence and density (log antibody titre) of 10C and 230CMB antibodies according to study participant age group. (a) The proportion of individuals positive for 10C and 230CMB antibodies according to age group. The error bars indicate the 95% confidence interval. The asterisk indicates a statistically significant positive increasing trend at the <0.001 level (***), determined by logistic regression (adjusting for country). Results did not change significance boundary when using age as either a categorical or continuous variable. (b) Log antibody titre for 10C and 230CMB antibodies according to participant age group. Box plots indicate the median (horizontal line), 25th and 75th percentiles (lower and upper box limits) and whiskers indicate the 5th and 95th percentiles. The asterisk indicates a statistically significant difference at the following levels: >0.05 (not statistically significant= NS) and <0.001 (***), which was assessed using linear regression. Different colour shading represents the different age categories.

5.65 Results: SMFA compilation sample set. The relationship between transmission reducing activity and age

When considering functional TRA (categorized at the higher levels) and age (Figure 5.12.), there was no statistically significant association between age and transmission reduction at the $\geq 50\%$ level ($n=31$) (OR 0.98; CI: 0.92-1.03, $p=0.437$) or the $\geq 90\%$ level ($n=16$) (OR 1.02; CI: 0.96-1.10, $p=0.423$), indicating similar proportions of more functional transmission reducers are found within each age category. This suggests that while antibody prevalence and titre (for 230CMB) may increase with age, antibody functionality does not.

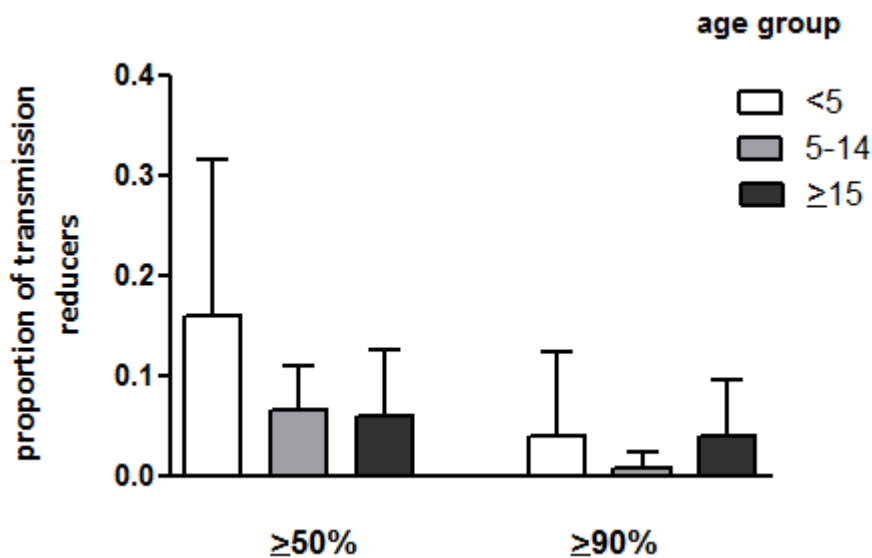


Figure 5.12. SMFA compilation sample set: The proportion of individuals in each age group who demonstrate TRA at the $>50\%$ and $>90\%$ level. $\geq 50\%$ TRA represents 50-89% ($n=31$) and $\geq 90\%$ TRA represents 90-100% ($n=16$). The error bars indicate the 95% confidence interval. The number of participants per age group is: <5 years ($n=25$), 5-14 years ($n=123$) and >15 years ($n=51$). Age groups are represented by different colour shading. Age trends were assessed using logistic regression (adjusting for country) and were not significant when using age as either a categorical or continuous variable (see text).

5.66 Results: SMFA compilation sample set. The relationship between transmission reducing activity and antibody titre

TRA was categorized according to the following groups 0 (n=208), >0-<50 (n=90), 50-<90 (n=31) and >90 (n=16) then the relationship was assessed with mean log antibody titre between the groups, using ANOVA. Pairwise t-tests were then performed between TRA groups to determine which differences were statistically significant. Figure 5.13. demonstrates the log antibody titre according to the % TRA. Compared to 0% TRA, there was no statistically significant difference between mean titre for the different TRA categories and 10C antibodies ($p \geq 0.122$), apart from 50-<90% which had a significantly lower titre than 0% ($p=0.003$). For 230CMB titre, there was no statistically significant difference between the TRA groups ($p \geq 0.272$).

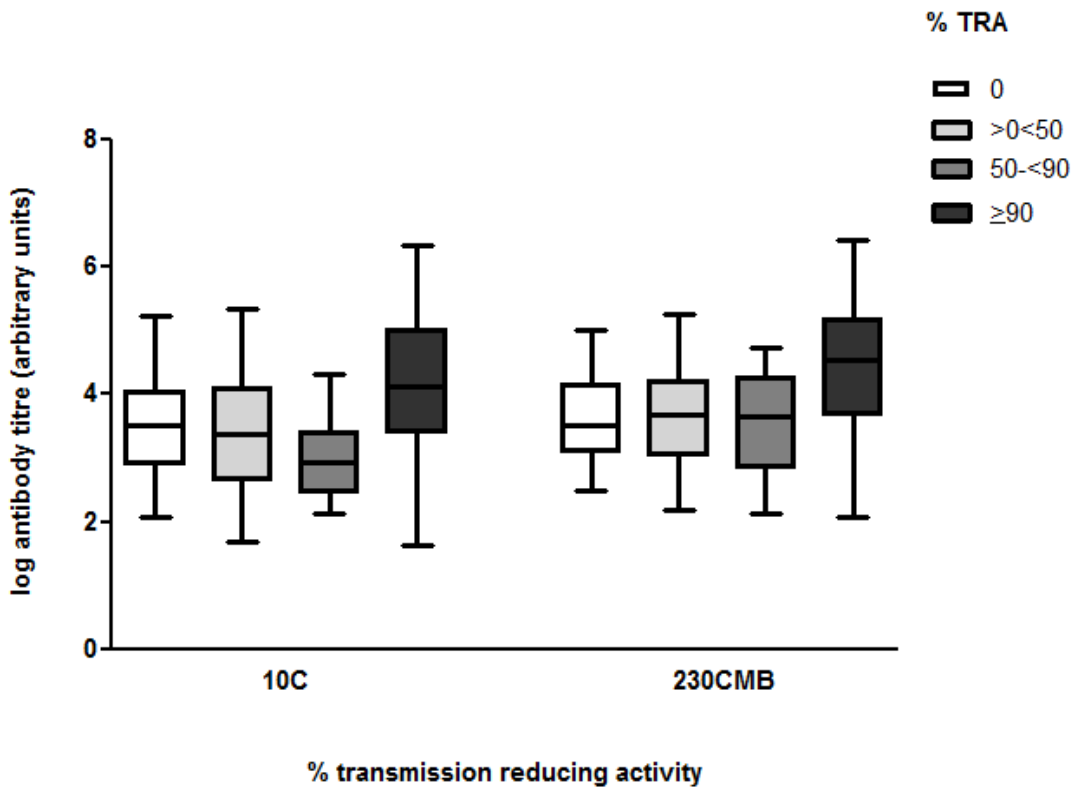


Figure 5.13. SMFA compilation sample set: Log titre for 10C and 230CMB antibodies according to the level of transmission reducing activity (TRA). Log antibody titre is represented as arbitrary units. Box plots indicate the median (horizontal line), 25th and 75th percentiles (lower and upper box limits). Statistically significant differences between groups are discussed in the text above. TRA categories are indicated by

different colour shaded boxes. The numbers of individuals in each TRA group is as follows: TRA 0 (n=208), >0-<50 (n=90), 50-<90 (n=31) and >90 (n=16).

5.7 Linking methods and results: Genetic analysis of cross sectional survey field samples from Ghana, Tanzania and Burkina Faso

5.71 Methods: MSP2 genotyping to assess clonal diversity of field sites

MSP2 genotyping by capillary electrophoresis was performed by Dr. Lynn Grignard at LSHTM to characterize the field sites in terms of clonal diversity. This was performed as referenced in the manuscript.

5.72 Methods: BLAST search to explore previously reported polymorphisms in *pfs48/45*

To investigate the genetic diversity of our vaccine candidate 10C, the *pfs48/45* gene was sequenced in the cross sectional survey field samples. First, a BLAST search was performed to identify which regions are polymorphic; 55 sequences were found predominantly from a study by Escalante *et al*, which compared *pfs48/45* diversity in samples from Kenya, India, Thailand and Venezuela (Table 5.11.) (Escalante, Grebert *et al*. 2002). The authors identified 42 single nucleotide polymorphisms (SNPs) which spanned 39 codon locations, 24 of which resulted in non synonymous changes.

Table 5.11. Location and frequency of SNPs and resulting amino acid changes in *pfs48/45*. Evaluated in samples uploaded to BLAST by other researchers (www.ncbi.nlm.nih.gov/BLAST/) visited November 2012). * the location is from the beginning of *pfs48/45*.

Nucleotide Location* of SNP	Codon location	Amino acid change	SNP frequency (in 55 samples)
42	14	synonymous	1
60	20	synonymous	1
69	23	synonymous	1
95	32	C=Y	2
99	33	K=N	1
106	36	S=A	1
132	44	synonymous	1
159	53	synonymous	2
172	58	N=D	1
215	72	T=N	1
276	92	synonymous	5
297	99	synonymous	1
424	142	I=L	1
448	150	K=E	1
454	152	I=L	1
531	177	synonymous	1
651	217	synonymous	1
757 and 759	253	E=K	8 and 4
762	254	K=N	7
777	259	synonymous	1
827	276	K=R	1
911	304	D=V	25
937	313	S=G	1
940	314	I=L	3
944 and 945	315	D=G	1
964 and 965	322	S=N	1 and 28
996	332	synonymous	1
998	333	E=G	1
1045	349	I=V	2
1056	352	synonymous	2
1064	355	Q=L	1
1067	356	V=A	3
1074	358	synonymous	2
1185	395	synonymous	1
1211	404	K=R	1
1240	414	K=E	1
1265	422	T=I	1
1272	424	synonymous	1
1315	439	F=L	1

High frequency SNP 'hotspots' were observed in nucleotide locations 911 and 965 where variation was seen in 25 and 28 samples (n=55) respectively (Table 5.11.). Twenty-seven SNPs appeared just once and could be singletons since this study used cloned isolates. SNPs that were observed more than once, and resulted in non synonymous amino acid changes were of most interest. Since non synonymous changes were found throughout, the entire gene was sequenced in the field samples collected in the cross sectional surveys.

5.73 Methods: DNA extraction from filter papers

DNA was extracted from filter paper samples for *pfs48/45* sequencing and MSP2 genotyping. For Burkina Faso and Tanzania, microscopy positive samples were selected at random for sequencing. As the result of a delay before microscopy results were available for Ghana, Ghanaian samples were selected at random, then screened for parasite presence using nested PCR (described in section 3.21.) (Snounou, Viriyakosol et al. 1993; Snounou, Viriyakosol et al. 1993). Genomic DNA was extracted from Whatman 3MM filter papers using the QIAamp DNA Mini kit (Qiagen™) following the "isolation of genomic DNA from dried blood spots" protocol as described next. For each sample, three 3mm circles were aseptically punched from the centre of the filter blood spot into a sterile 1.5mL microcentrifuge tube using a 3mm hole punch (Staples, High Wycombe, UK). Forceps and punch were ethanol sprayed and flame sterilized between cuts for the different samples, allowing them to cool before use. One hundred and eighty µL of buffer ATL (Qiagen™) was added per tube, which was incubated at 85°C for 10 minutes. Samples were pulse centrifuged to 14,000 rpm in a benchtop microcentrifuge to gather liquid adhering to the microcentrifuge tube lid, then 20µL of Proteinase K (Qiagen™) was added and tubes mixed by vortex. Samples were incubated at 56°C for 1 hour then centrifuged briefly. Next, 200µL of Buffer AL was added, tubes mixed by vortex, then incubated

at 70°C for 10 minutes. Samples were centrifuged at 14,000 rpm in a benchtop microcentrifuge, then 200µL of 100% ethanol was added, mixed by vortex, then centrifuged once more. Sample and buffer was added to a QIAamp Mini Spin Column inside a 2mL collection tube, then centrifuged at 8000 rpm for 1 minute. The spin column was inserted into a new collection tube, 500µL of Buffer AW1 was added, then tubes were centrifuged at 8000 rpm for 1 minute. This was repeated with Buffer AW2, but centrifugation performed at 14,000 rpm for 3 minutes. The spin column was inserted into a new collection tube which was centrifuged at 14,000 rpm for 1 minute. Then the column was placed in a microcentrifuge tube and 150µL of Buffer AE was added, which was incubated at room temperature for 1 minute, then centrifuged at 8000 rpm for 1 minute to elute DNA off the membrane. Extracted DNA was stored in microcentrifuge tubes at -20°C until use.

5.74 Methods: PCR amplification of *pfs48/45*

Figure 5.14. (a) indicates the *pfs48/45* gene sequence and location of SNPs found in samples uploaded to BLAST. *pfs48/45* is a large 1347 base pair single copy gene without introns (PlasmODB 2012). Due to the large size, the primary PCR was performed using two primer sets that amplify *pfs48/45* in two overlapping segments (Kocken, Jansen et al. 1993), using protocols developed elsewhere (Anthony, Polley et al. 2007). Figure 5.14.(b) shows the location and coupling of the primer pairs which are referred to as set 1 and 2, for the first and second half of the gene, respectively, for the nest 1 reaction. Set 3 and 4 refer to the first and second half of the gene (respectively) for the nest 2 reaction.

(a)

ATGATGTTATATATTTCTGCGAAAAAGGCTCAAGTTGCTTTTATCTTATATATAGTATTAGTA
TTAAGATAATAAGTGGAAACAATGATTTTTGTAAAGCCTAGCTCTTTGAATAGTGAAATATC
TGGATTATAGGATATAAGTGTAAATTTTCAAATGAAGGTGTTTCATATTAAAGCCAGATA
TGCCTGAACGTAGGTCTATTTTTGCAATCCATTCGATTTTATATATGATAAGATAAGAT
TAATAATACCTAAAAAAGTTCGTCTCCTGAGTTTAAAATATTACCAAGAAAAATGTTTTCAA
AAGTATATACTGATTATGAGAATAGAGTTGAACTGATATATCGGAATTAGGTTAATTGAA
TATGAAATAGAAGAAAATGATACAAACCCTAATTATAATGAAAGGACAATAACTATATCTCC
ATTTAGTCCAAGACATTGAATTTTTTTGTTTTGTGATAAATACTGAAAAGGTTATATCAAG
TATAGAAGGGAGAAAGTGCTATGGTACATGTAGTGTATTTAAATATCCACATAATTTTTAT
TTACTAATTTAACAATGATCTTTTTACATATTTGCCGAAAACATATAATGAATCTAATTTTGT
AAGTAATGTATTAGAAGTAGAATTGAATGATGGAGAATTATTTGTTTTAGCTTGTGAACATA
TTAATAAAAAATGTTTTCAAGAAGGAAAAGAAAAAGCCTTATATAAAAGTAATAAAATAATT
TATCATAAAACCTTAACTATCTTTAAAGCTCCATTTTATGTTACATCAAAGATGTTAATACA
GAATGTACATGCAATTTAAAAATAATAATTATAAAATAGTTTTAAACCAAATATGAAAA
AAAAGTCATACACGGATGTAACCTCTCTTCAAATGTTAGTTCTAAACATACTTTTACAGATAG
TTTAGATTTCTTTAGTTGATGATAGTGCACATATTTTATGTAACGTACATTTGTCTGAACC
AAAATATAATCATTGGTAGGTTTAAATTGCCTGGTATATTATACCAGATTGCTTTTTTCA
AGTATATCAACCTGAATCAGAAGAACTTGAACCATCCAACATTGTTATTTAGATTACAAAT
AAATATAGGAGATATTGAATATTATGAAGATGCTGAAGGAGATGATAAAATTAATTATTG
GTATAGTTGGAAGTATACCAAAAACGACATCTTTACTTGTATATGTAAGAAGGATAAAAAA
AGTGCTTATATGACAGTTACTATAGATTCAGCATATTATGGATTTTTGGCTAAAACATTTATA
TTCCTAATTGATGCAATATTATTATATTTAG

(b)

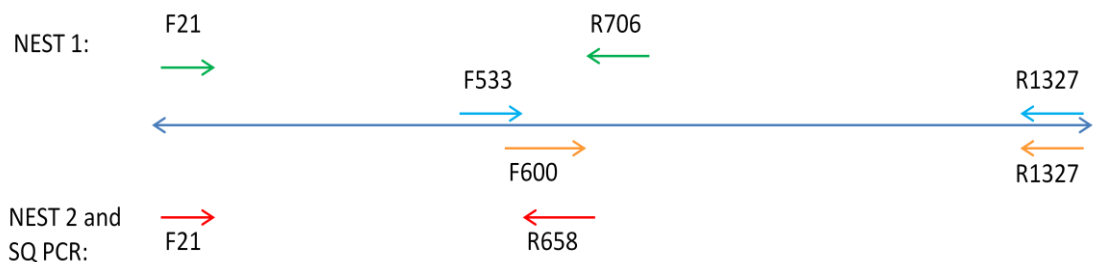


Figure 5.14. Sequence of *pfs48/45* and PCR primer pairing. (a) Sequence of the *pfs48/45* gene and location of SNPs and 10C vaccine candidate. The underlined portion indicates the location of vaccine candidate 10C. The nucleotides highlighted in red are SNPs that appear more than once, whereas blue SNPs appear in just in 1/55 samples. Primer set 1 consists of the turquoise (F21) and pink (R706) primers and set 2 is light (F533) and dark green (R1327) primers. Primer set 3 is turquoise (F21) and grey (R658) combined, and set 4 is yellow (F600) and dark green (R1327). (b) Location of primers used for amplification and sequencing of the *pfs48/45* gene.

Primers of the same colour represent forward and reverse pairs. Sequencing PCR was performing using the same primers as the nest 2 PCR reaction.

The nested PCR reaction used the same external primers, but different overlapping internal primers, as indicated in Table 5.12.

Table 5.12. Primer sequences for amplification and sequencing of the *pfs48/45* gene. NB: the nest 2 primers were also used for the sequencing PCR reactions. b.p. = base pairs.

reaction	primer pair	primer name	sequence 5'-3'	product size (b.p.) for each primer set	
Nest 1	set 1	F21	ATACATGATGTTATATATTTCTGCG	730	
		R706	CAAGAAGGAAAAGAAAAGCC		
	set 2	F533	AGTGCTATGGTACATGTACG		840
		R1327	GCAATATTATTATATATTTAGCTCATGA		
Nest 2	set 3	F21	ATACATGATGTTATATATTTCTGCG	687	
		R658	GAGAATTATTTGTTTAGCTTGTGA		
	set 4	F600	CAAATGATCTTTTACATATTTGCC		780
		R1327	GCAATATTATTATATATTTAGCTCATGA		

DNA extracted from cultured parasites (NF54, NF166 and NF135), was used to validate PCR and sequencing protocols, and support another study where the transmission-blocking properties of 10C vaccine induced antibodies was determined against these gametocyte-producing strains *in vitro* (Theisen, Roeffen et al. 2014). PCR reactions were set up as follows: 1µL of extracted DNA was pipetted into a VWR 96 well skirted plate (VWR, Bedfordshire, UK), which was kept on ice. Expand High Fidelity enzyme mix (Roche, Hertfordshire, UK) was used for amplification with the aim of producing high quality sequence traces. Due to the exonuclease activity of the enzyme, two separate mastermixes were prepared to prevent primer degradation. For each sample, 5.25µL of mastermix 1 (Table 5.13.) was combined with 6.25µL of mastermix 2 (Table 5.14.), then added to the 96 well plate, which was sealed with StarsSeal film (Starlab, Buckinghamshire, UK).

Table 5.13. Mastermix 1 reagents and volumes for primary and nested PCR reactions.

Reagent	Final concentration	Volume per reaction (µL)
Nuclease free water (Ambion)	N/A	3.75
dNTP mix (10mM each) (Bioline)	200µM of each	0.25
PCR primer mix (3µM each) (Sigma-Aldrich)	300nM of each primer	1.25
<i>Mastermix total</i>		5.25

Table 5.14. Mastermix 2 reagents and volumes for primary and nested PCR reactions.

Reagent	Final concentration	Volume per reaction (µL)
Nuclease free water (Ambion)	N/A	3.56
Expand high fidelity buffer (Roche)	1x	1.25
Mg ²⁺ (25mM stock) (Roche)	2.5mM	1.25
Expand High Fidelity enzyme mix (Roche)	2.6units/reaction	0.19
<i>Mastermix total</i>		6.25

Cycling conditions for nest 1 and 2 PCR reactions were as follows: 94°C for 4 minutes, then 45 cycles of (94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute), followed by a final extension step of 72°C for 10 minutes. The nested reaction used the same reagents and volumes, but used 1µL of nest 1 product as the template. To confirm product amplification, a 0.7% agarose gel was prepared as follows, 0.7g of agarose (Sigma-Aldrich, Dorset, UK), was combined with 100mls of 1x TBE (BioRad, Hertfordshire, UK), which was heated by microwave until dissolved. When sufficiently cool, 5µL of ethidium bromide (Sigma-Aldrich, Dorset, UK), was added, the mixture gently swirled to combine, then poured into a gel cast (BioRad, Hertfordshire, UK) with combs and allowed to set.

One µL of PCR product was combined with 2µL of loading dye (Thermo Fisher Scientific, Hertfordshire, UK) which was resolved by agarose gel electrophoresis (45 minutes, 80 volts) and visualized using a transilluminator (BioRad, Hertfordshire, UK).

Positive amplification resulted in a band size of 687 base pairs for the first half of the gene, and a band size of 780 nucleotides for the second half. Five μL of Hyperladder I (Bioline, London, UK) was included on the gel to enable product size estimation.

5.75 Methods: PCR product clean up

PCR products from the next 2 reactions were cleaned using the QIAquick Spin kit (Qiagen), according to the manufacturer instructions, as follows. Forty μL of buffer PB was combined with 8 μL of PCR product, which was mixed by vortex. Ten μL of 3M NaOAc (pH 5.0) was added and mixed by pipetting until a uniform yellow colour was observed. Next, sample was added to a spin column inside a 2mL collection tube which was centrifuged for 45 seconds at 14,000 rpm to bind the product to the membrane. Flow through was discarded and 750 μL of Buffer PE was added to the column and centrifuged at 14,000 rpm for 45 seconds. Flow through was discarded again and the spin column centrifuged once more for 1 minute. Next the column was inserted into a new 1.5mL microcentrifuge tube and 50 μL of nuclease free water added to the membrane and incubated for 1 minute at room temperature. This was centrifuged at 14,000 rpm for 1 minute to elute the cleaned product.

5.76 Methods: Estimation of PCR product concentration

For sequencing PCR, the optimal PCR product concentration for amplification of a 500-1000 base pair fragment is 5-20ng per sequencing reaction. Concentration was estimated by analyzing 5 μL of cleaned PCR product on a 1.0 % agarose gel alongside 5 μL of Hyperladder I (Bioline, London, UK). The objective was to compare the density of the band (demonstrated in Figure 5.15.) to estimate the volume of product needed for the sequencing PCR reaction, and if dilution was required. In instances where the product was too concentrated, it was diluted in Nuclease-free water (Ambion, Renfrewshire, UK).

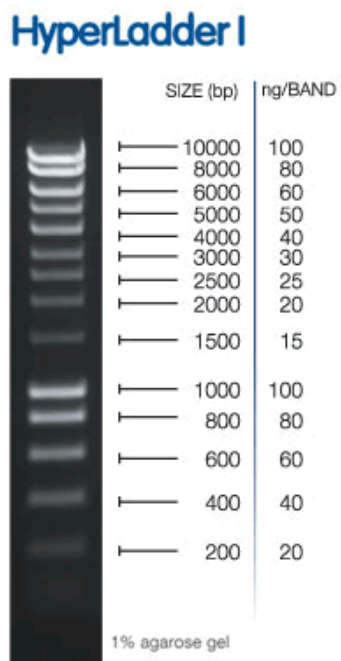


Figure 5.15. Hyperladder I size marker and corresponding estimated protein concentration. Hyperladder I was sourced from Bioline, London, UK. Protein concentration (ng/band) indicated in this picture is estimated according to the strength of the band. Picture was sourced from (www.bioline.com/us/hyperladder-1kb).

5.77 Methods: Sequencing PCR reactions

Sequencing reactions were set up on ice as indicated in Table 5.15. using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Renfrewshire, UK).

Table 5.15. Mastermix reagents and volumes for sequencing PCR reaction.

reagent	volume (μL) per reaction
BigDye (Applied Biosystems)	1.0
Buffer (Applied Biosystems)	1.5
Primer (10pmole/ μL) (F21,F600, R658 or R1327)	3.2
Nuclease free water (Ambion)	2.3
<i>Mastermix total</i>	<i>8.0</i>
<i>Purified PCR product (added separately)</i>	<i>2.0</i>

Italicized reagents (Table 5.15.) were added to the PCR plates as follows. Two μL of purified PCR product (previously diluted, if needed) was transferred into barcoded 96 well PCR plates (Thermo Fisher Scientific, Hertfordshire, UK) on ice, then $8\mu\text{L}$ of mastermix was added per reaction. Since each primer amplifies product separately, 4 reactions were performed per sample. F21 and R658 primers were combined with the purified nest 1 product, and F600 and R1327 were combined with the purified nest 2 product. The following thermocycling conditions were used: 25 cycles of (94°C for 1 minute, 55°C for 1 minute, 65°C for 1 minute), followed by 65°C for 5 minutes for final extension, which was performed using a thermocycler (C1000 Thermal Cycler, BioRad, Hertfordshire, UK).

5.78 Methods: Ethanol precipitation of sequencing product

PCR products were ethanol precipitated which was performed as described next. The following was added per well, $3.0\mu\text{L}$ of 3M NaOAc (pH 4.6), $62.5\mu\text{L}$ of 100% BioUltra Ethanol (Sigma-Aldrich, Dorset, UK), and $24.5\mu\text{L}$ of MQ- H_2O . Plates were covered with PCR Foil (Thermo Fisher Scientific, Hertfordshire, UK), pulse vortexed, then kept on ice for 20 minutes. Plates were centrifuged at $3000g$ for 30 minutes at 4°C , the foil

was removed, and plates were inverted onto blue roll to drain. Next, plates were centrifuged inverted at 50g for 1 minute, then 150µL of ice cold 70% ethanol was added per well. Plates were sealed with PCR foil, inverted 5 times to mix then centrifuged for 10 minutes at 3000g at 4°C. The foil was removed and contents drained once more. Plates were centrifuged inverted at 50g for 1 minute, then 10.5µL of HiDi formamide (Applied Biosystems, Renfrewshire, UK) was added to each well, and samples sequenced using a ABI3730 sequencer (Applied Biosystems, Renfrewshire UK).

5.79 Methods: Sequence trace analysis

Sequence chromatograms were analyzed using Chromas LITE, Version 2.1 (Technelysium Pty Ltd, Queensland, Australia) and aligned within CLC Sequence Viewer 6.7.1. (Massachusetts, USA). To detect SNPs, sample sequences were compared to cultured isolate 3D7, gene ID PF13_0247, accessed from PlasmoDB (www.plasmodb.org), November 2012. It was not possible to consistently obtain nucleotide sequences for the first and last 44 nucleotides. Since these stretches did not contain the vaccine candidate, they were excluded from analysis.

5.8 Methods and results: PCR and sequencing protocol optimization

The protocols detailed above are the final protocols used but prior to concluding on these, the following PCR and sequencing optimization was performed. Initially, sequence traces were short with approximately only 290/687 readable base pairs for the first half of the gene and 200/780 for the second half of the gene. Sequences were poor quality with undefined sample peaks and high background (identified as multiple smaller peaks). To improve sequence quality, the magnesium concentration, PCR annealing temperature and primer concentrations were titrated, one at a time. The following final magnesium concentrations were attempted in the PCR reaction,

1.5mM, 2.0mM, 2.5mM, 3.0mM, 3.5mM and 4.0mM, and the volume of water was adjusted accordingly. Since the final concentration of 2.5mM produced slightly longer readable traces, this concentration was used. To try to further improve sequence trace quality, a gradient was tried for the PCR annealing temperature, 44.9°C, 45.4°C, 46.5°C, 48°C, 50°C, 51.6°C, 52.5°C, and 52.9°C. Fifty °C was the temperature used in the published protocol, which was also found to deliver optimal sequence traces. The primer concentration was titrated from 8-64pmol per reaction, but no observable difference in sequence trace quality was found so the recommended concentration of 32pmol/reaction was used for subsequent reactions. To try to conserve costly reagents, a range of PCR reaction end volumes were attempted, 6.25µL, 12.5µL, 25µL and 50µL, and 12.5µL was selected since it produced sufficiently strong bands and readable sequences for both primer sets.

The volume of PCR product in the sequencing PCR reaction was titrated from 4.0-0.5µL, and 2.0µL was found to be optimal. Similarly, the volume of BigDye used per reaction was titrated from 0.5-1.5µL and 1µL per reaction was found to result in longer traces.

5.81 Methods and results: Design of new primers for amplification of *pfs48/45*

It was not possible to amplify the second half of the gene (base pairs 488-1347) for 20/146 samples (Tanzania=7, Ghana=4 and Burkina Faso=9). Following nested PCR there was no visible product on an agarose gel, and while sequencing was attempted, these samples did not have readable peaks on the sequencing chromatogram. Since the first half of the gene amplified well, poor quality DNA was doubted as the cause. While the BLAST search did not reveal previously identified SNPs in the primer sequence regions, the majority of uploaded sequences were from studies conducted over 10 years ago (Escalante, Grebert et al. 2002; Anthony, Polley et al. 2007). It was speculated the presence of a novel SNP(s), or an indel could be preventing amplification. PCR was attempted using a gradient across the annealing

temperature with the aim of decreasing binding specificity to hope to accommodate potential SNPs that could be impacting on melting temperature. No product was amplified.

Different primer sets were paired to identify which part of the gene was resulting in amplification failure. To assess if there was a mutation in the original forward primer (F600), primers F533 and R1327 were combined but produced no amplicon. Next, F600 and R706 were combined to exclude SNPs in the forward primer, and this produced amplified product, suggesting the problem was in the later part of the sequence. Following this, four new primers were designed in the second half of the gene (Table 5.16.). Figure 5.16. demonstrates the location of the original, and new primer pairs (shaded in orange).



Figure 5.16. Sequence of *pfs48/45* (nucleotides 488-1347) and location of SNPs and primers. The nucleotides highlighted in red are SNPs that appear more than once, and SNPs in blue appear in just in 1/55 samples, (from samples uploaded to BLAST). SNPs highlighted in pink were identified in endemic samples, from this study. Novel primers are shaded in orange, and other colour primers are as previous, light green (F533), yellow (F600), grey (R658), pink (R706), and dark green (R1327).

Table 5.16. Sequence of new primers for amplification of fragments within nucleotides 488-1347 of *pfs48/45*.

name	primer sequence 5'-3'
SJF1	GAATCTAATTTTGTAAGTAATG
KTrev2	CTGATTCAGGTTGATATAC
SJR1	TTAGGAATATAAATGTTTTAGCC
SJR2	TTAATTTTATCATCTCCTTCAGCA

The following primer pairs were then combined, 1) SJF1 and SJR1 and 2) SJF1 and KTrev2, attempting an 8 point annealing temperature gradient of 40-55°C. The new primers were tested in PCR against a small number of samples (n=4), since gradient PCR consumes a large volume of DNA. Both primer sets (SJF1 and KTrev2) and (SJF1 and SJR1) produced amplification bands for these samples, when using 52.4°C as the annealing temperature. Due to time constraints, the remaining samples were not tested, but the results imply there could be SNP/s or indels in the later region of the *pfs48/45* gene, but this remains to be confirmed.

5.9 Extended discussion

The main results and discussion are outlined in the attached manuscript. Additional findings discussed here are predominantly concerned with assay optimization, and further validation of results from the cross sectional surveys, using supportive sample sets.

5.91 MBP-10C vaccine candidate

The primary version of the REDMAL vaccine candidate, MBP-10C suffered 2 developmental setbacks. Firstly, both endemic and European naive serum demonstrated reactivity to fusion partner MBP, and secondly, the level of properly folded clinical grade protein (produced by collaborators), was lower than the funding body requirement of $\geq 95\%$ properly folded product (Will Roeffen, personal communication).

MBP is a protein expression tag that improves yield of overexpressed proteins, and enhances their solubility (Sun, Tropea et al. 2011). MBP was not cleaved from 10C, since removal can result in protein aggregation, and reactivity of test serum to MBP was not anticipated (Fox, Routzahn et al. 2003). There is no obvious explanation for the apparent background reactivity to MBP in European controls and endemic sera. A BLAST search (www.ncbi.nlm.nih.gov/BLAST) was performed and all close matches found to be sugar transporters found among Enterobacteriaceae. Since MBP is a bacterial protein, it could be speculated that individuals carry antibodies as a result of exposure during immune clearance of natural bacterial infection. However, MBP is commonly used as an overexpression fusion partner and there is no evidence in the literature to support this hypothesis. Possibly, *E.coli* contaminants from production of 10C may have remained and resulted in ELISA reactivity. The source of the high reactivity to MBP remains unconfirmed at present. Since the thesis objectives are related to measuring specific antibodies to the sexual stage vaccine candidate and

because the product changed to reach higher concentrations of conformational protein, the remainder of this work focused on 10C from the R0-10C product.

5.92 Acquisition of 10C and 230CMB antibodies with age

Gaining a more thorough understanding about how antibodies are acquired following natural exposure could provide insight into how long vaccine induced antibody responses may last for and what is required to boost them. Ideally, from a TBV viewpoint, it is desirable for a functional antibody response to be evoked following as few exposures (vaccination, or natural) as possible, last a long time and be boosted from low parasite densities. Attempts have been made to determine how sexual stage antibodies are acquired and what results in TRA. There has been conflicting evidence so far regarding an age dependent pattern of sexual stage antibody acquisition. As discussed in section 1.36., previous research indicated sexual stage antibody prevalence decreased with age, which implies higher density gametocytes (typically found in children) are needed to elicit antibody production. Furthermore, previous research suggests sexual stage antibodies are reliant on recent gametocyte exposure and are short lived rather than forming the cumulative immunological memory response, which is commonly seen for asexual stage antigens (Bousema, Drakeley et al. 2007; Bousema, Sutherland et al. 2011; Ouedraogo, Roeffen et al. 2011).

The field sites described in this chapter were selected for sample collection to explore differences in antibody prevalence in varying levels of malaria exposure, and because these sites are under consideration for future vaccine trials. The schools sampled in Burkina Faso were located in a region of hyper endemicity, whereas Tanzanian schools were in a site of meso-endemicity, and Ghana in a site of hypo endemicity. As indicated in the attached manuscript, participants from Burkina Faso demonstrated a statistically significant age dependent increase in antibody prevalence for both 10C and 230CMB. In Tanzania there was a significant increasing

relationship with age only for 230CMB antibodies. This finding raises questions regarding antibody boosting and longevity and contrasts with previous results. However, it must be noted that prior studies measured antibodies against native proteins Pfs48/45 and Pfs230. Therefore they are not directly comparable since previous research has indicated there can be variation in immunogenicity between protein epitopes, and 10C contains 3 epitopes out of the 5 found in native protein, (Anders, Coppel et al. 1988; Taylor, Egan et al. 1996). Similarly, 230CMB represents a portion of 1 of the 7 paired domains of Pfs230 (Farrance, Rhee et al. 2011). Consequently, there could be a different pattern of recognition between native and recombinant protein. This is discussed more in section 7.2.

Generally in high endemic settings, the majority of infections with high density, microscopically detectable gametocytes are found in children, and the gametocyte burden in adults is predominantly submicroscopic gametocytes (Ouedraogo, Bousema et al. 2010). Since an age dependent antibody acquisition pattern was observed with sexual stage antibodies, and adults have the highest antibody prevalence, this implies that sexual stage antibody responses may accumulate on exposure and be boosted from submicroscopic gametocytes (Ouedraogo, Bousema et al. 2010), although this requires confirmation using molecular gametocyte detection. The discrepancies between sites in the age-dependent acquisition of 10C and 230CMB antibodies could be explained by infrequent transient infections in Ghana which result in limited exposure, insufficient to sustain antibody responses, whereas in Burkina Faso, there may be more frequent (or continuous) exposure to infections, with accompanying (low densities) of gametocytes. One potential modulating factor may be a longer antibody half life in adults compared to children, which could contribute to the cumulative response (Bousema, Roeffen et al. 2010).

There was no consistent pattern with antibody prevalence and season for the countries explored. While anticipated antibody prevalence would be higher in the transmission season due to more prolonged exposure to potentially higher density gametocytes, this was not consistently (or statistically significantly) the case. Once more, this implies submicroscopic gametocytes play an important role in eliciting a

sexual stage antibody response. Furthermore, there was no statistically significant relationship with microscopically detectable gametocytes and antibody prevalence (which is in line with previous research) which again suggests submicroscopic densities are sufficient to boost antibody responses (Riley, Bennett et al. 1994; Bousema, Roeffen et al. 2006; Drakeley, Bousema et al. 2006; Bousema, Drakeley et al. 2007).

A relationship between parasite carriage and concurrent antibody responses is difficult to interpret since there is a time lag between antigen exposure and antibody production. For this reason, current antibody responses may reflect long term differences in parasite exposure (Ouedraogo, Roeffen et al. 2011). A longitudinal study repeatedly sampling participants over time and performing molecular gametocyte detection would help to gain a more accurate understanding of this relationship.

To explore antibody prevalence in adults, 10C and 230CMB antibodies were quantified in samples from participants aged 6 months to 72 years from Kanungu, Uganda. This revealed the previously indicated age acquisition pattern seen in school aged children extended into adults who had the highest 10C and 230CMB antibody prevalence in the population, once more supporting the hypothesis of a cumulative sexual stage antibody response.

In terms of the vaccine, this data demonstrates all age groups are capable of producing antibodies against both vaccine candidates which may translate to boosting of a vaccine induced response. However, the functionality of these antibodies remains in question since it has been indicated only a relatively small of proportion of antibodies demonstrate TRA (Drakeley, Bousema et al. 2006; Bousema, Roeffen et al. 2010).

5.93 Transmission reducing activity and the relationship with 10C and 230CMB antibodies

10C and 230CMB antibodies were quantified in the compilation of samples with SMFA data from Burkina Faso, The Gambia, Cameroon, Gabon and missionaries from The Netherlands. While these individuals represent different populations in terms of previous malaria exposure (and acquired immunity), genetic status and ethnicity, data was combined to increase the sample size and strength of the investigated associations. All individuals, with the exception of the missionaries, were microscopically confirmed gametocyte carriers. Since there was no age data for the missionaries and they were first exposed to malaria at an adult age, they were excluded from age associated analysis.

It has previously been revealed estimates of TRA on a continuous scale should be interpreted with caution and only TRA $\geq 90\%$ can be reproducibly measured (van der Kolk, de Vlas et al. 2004). Therefore, TRA was analyzed at the $\geq 50\%$ (including 50-89% TRA) and $\geq 90\%$ (including 90-100% TRA) level. It is important to note within our sample population, 31 individuals exhibited TRA at the 50% level and 16 people at the 90% level. Since these are low numbers, these results should be interpreted with caution. There was no statistically significant relationship between 230CMB antibodies and TRA at the $\geq 50\%$ level, and none of these individuals had antibodies to 10C. When categorized at the $\geq 90\%$ level, TRA was significantly associated with both 10C and 230CMB antibodies. Forty % of the individuals who demonstrated $\geq 90\%$ TRA had 10C antibodies (most of whom were also positive for 230CMB), 61.5% had 230CMB antibodies, and 5 people had no measurable antibody responses for either antigen. It has previously been indicated Pfs230 is more immunogenic compared to Pfs48/45, which has been suggested to be a consequence of the larger size of Pfs230 and presence of more epitopes (Williamson 2003). The higher immunogenicity may be the reason for the larger proportion positive for 230CMB antibodies within the $\geq 90\%$ TRA category (Ouedraogo, Roeffen et al. 2011).

The SMFA compilation sample set also demonstrated a statistically significant age dependent increase in antibody prevalence for both vaccine candidates. Since there was no evidence that TRA increases with age. This suggests the functionality of antibody responses in this sample set is not well reflected by the ELISA assays. It is unclear what contributes towards functional TRA.

As discussed in section 1.35. previous studies have provided conflicting evidence regarding TRA and native protein, Pfs48/45 and Pfs230, antibody prevalence and density. Studies have confirmed a relationship between the 2 (Drakeley, Bousema et al. 2006; Ouedraogo, Roeffen et al. 2011), found no relationship (Premawansa, Gamage-Mendis et al. 1994), or indicated high antibody titre samples demonstrate TRA (Drakeley, Mulder et al. 1998). A study performed in Tanzania suggested the proportion of individuals exhibiting $\geq 50\%$ TRA decreased with age, and just a small proportion of individuals from all age categories demonstrated $\geq 90\%$ TRA (Drakeley, Bousema et al. 2006). While I confirmed there is a relationship with 10C and 230CMB antibodies and TRA, no increasing association was found with titre. My findings were in broad agreement with the Tanzanian study and showed the proportion of individuals demonstrating $\geq 50\%$ TRA decreased with age, but this was not a significant trend. Furthermore, my findings also agreed that a small proportion of each age group exhibited $\geq 90\%$ TRA. Potential contributions to functional TRA are further discussed in section 7.2.

5.94 *pfs48/45* gene sequencing

Mutations were found in field sample *pfs48/45* gene sequences. It has previously been indicated mutations induced in the cysteine residues resulted in a loss of epitope function (Outchkourov, Vermunt et al. 2007). Since none of the non synonymous substitutions affected cysteine residues, it seems possible that epitopes will remain properly folded in samples with these polymorphisms. Intriguingly, it was not possible to amplify the second half of the gene for 20 samples, which could be

caused by the presence of an indel or SNP/s in the region of the reverse primer thereby preventing primer binding. It would certainly be of relevance to identify the source of the problem, since transmission blocking epitopes 1-3 are all within this region and a deletion, particularly within epitope 1 which is correlated with higher TRA, could reduce vaccine efficacy (Roeffen, Teelen et al. 2001; Outchkourov, Vermunt et al. 2007).

5.95 Conclusions

While previous studies indicated malaria endemic populations are antibody positive to native proteins Pfs48/45 and Pfs230, it was uncertain if these findings would extend to the recombinant proteins which represent just a portion of the native proteins. Furthermore, due to the large number of disulphide bonds, overexpressing recombinant proteins in the correct conformation in order to achieve good functionality is an obstacle. The research discussed within this chapter represents the next step in development of 2 novel vaccine candidates.

The presence of antibodies was demonstrated in participants of all ages from regions of hyper, meso and hypo endemicity, and higher antibody prevalence was found in the high endemic setting. What appears to be a cumulative antibody memory response was identified in the site with high endemicity, and also within the additional supportive sample sets. This pattern could reflect antibody boosting from submicroscopic gametocyte densities, a longer antibody half life, or improved antibody affinity in adults. The observations from this chapter suggest vaccine induced antibodies against these specific candidates could be boosted (in all age groups) within different endemic settings, which may improve the TRA of vaccine induced immunity, although this has not been tested.

While a statistically significant relationship was demonstrated between 10C and 230CMB antibodies and TRA, it must be noted what is described falls short of formal evidence for a causal association. In order to directly prove this association, 10C and

230CMB antibodies in test samples would need to be affinity purified against the recombinant antigens then assessed using SMFA. Within the SMFA compilation sample set, there is evidence that while 10C and 230CMB antibody prevalence increases with age (and titre, for 230CMB), TRA does not, which implies more than just antibody prevalence and density is needed to result in functional TRA.

While antibody responses against 2 transmission blocking candidates were investigated in this chapter, it is important to note there are other antigens for which antibodies have not been measured, that could be responsible for TRA. HAP2 is an example of another known pre-fertilization transmission blocking antigen being developed as a vaccine candidate (Miura, Takashima et al. 2013). Beyond the known antigens, proteomics revealed there are approximately 300 genes specific to sexual stages, and among these could be other pre-fertilization transmission blocking antigens that may contribute to naturally acquired TRA (Florens, Washburn et al. 2002; Gardner, Hall et al. 2002; Lasonder, Ishihama et al. 2002; Le Roch, Zhou et al. 2003; Bousema and Drakeley 2011).

Vaccine research has predominantly focused on pre-erythrocytic and blood stage antigens, and there are currently 28 vaccine candidates under development that are featured in the clinical trials listed in the WHO Rainbow Table (WHO 2013). Comparatively less research has focused on TBV development and as a result, just a handful of candidates have been considered for development (Bousema and Drakeley 2011). Only 2 are currently undergoing clinical trials, both of which are based on Pfs25 (WHO 2013), which has previously been reported to be of low immunogenicity (Wu, Ellis et al. 2008).

The biggest hurdle for the most advanced transmission blocking candidates (Pfs48/45, Pfs230 and Pfs25) has been the large number of disulphide bonds which has complicated the production of recombinant proteins in the correct conformation (Outchkourov, Roeffen et al. 2008; Farrance, Rhee et al. 2011; Gregory, Li et al. 2012). These challenges highlight the importance of developing new transmission blocking antigens. Exploring some of the uncharacterized sexual stage specific genes would form a good starting point in order to identify new candidates.

Chapter 6: Determining the transmission reducing activity of IgG purified from vaccinated rats against endemic parasites, and assessing the suitability of parasite detection methods to detect infectious individuals

6.1 Introduction

Following confirmation of the presence and functionality of naturally acquired 10C antibodies in different endemic settings, the next goal was to confirm the ability of anti R0-10C IgG from vaccinated rats to reduce transmission of field isolates. Previously, plasma from rats vaccinated with R0-10C was confirmed to demonstrate functional TRA against a panel of genetically diverse *P. falciparum* cultured strains (NF54, NF166 and NF135) from different geographical locations, using SMFA (Theisen, Roeffen et al. 2014). While results using cultured parasites were encouraging, there are differences with natural infection such as parasite genetic complexity and varying parasite densities that could affect the success of transmission reducing antibodies. To rigorously test the efficacy of any malaria vaccine, it needs to be challenged against endemic parasites in a setting in which it is intended for use (Bousema, Dinglasan et al. 2012). While touched on briefly in section 5.13., a major concern with development of any vaccine is that it may only elicit protection against strains that are identical or closely related to the vaccine genotype, which could result in vaccine -induced escape mutants. AMA-1 is an example of an asexual stage polymorphic candidate where development has been hindered as a consequence of the genetic diversity of natural isolates (Dutta, Dlugosz et al. 2013). A clinical trial performed in Mali assessed response to a monovalent AMA-1 vaccine candidate and revealed protection was only induced against identical or similar strains to the vaccine sequence (Thera, Doumbo et al. 2011). Similarly, other researchers determined the genetic variation of MSP-1₁₉ in a vaccine field site in Mali, and discovered the majority of circulating strains were different haplotypes to the vaccine candidate. This reiterates the importance of examining endemic parasite genetic diversity when developing a vaccine, and exploring the degree of cross allele protection that will determine the occurrence of escape mutants (Takala, Coulibaly et al. 2007).

In this chapter, this was explored by performing membrane feeding combined with serum replacement to determine the ability of vaccine derived IgG to reduce transmission of endemic parasites. As discussed in section 1.47., there are variations of membrane feeding protocols, such as DMFA and SMFA that have been devised to address these questions. The techniques have different strengths and while SMFA allows control over variables such as parasite density, it has the disadvantage of using a small number of parasite isolates (commonly 3D7) for experiments. While DMFA performed in the field lacks some control, it has the advantage of using naturally infected parasite carriers as the gametocyte source, who are often carrying more genetically diverse gametocytes (Bousema and Drakeley 2011). Serum replacement is a valuable adaptation of the protocol that can be coupled with DMFA or SMFA to enable determination of transmission blocking immunity in naturally exposed, or vaccinated populations, compared to naive serum.

Results from the previous chapter indicated *pfs48/45* is genetically diverse, especially in parasites from Burkina Faso. For this reason, and due to availability of good membrane feeding facilities, the study described in this chapter was performed in Burkina Faso.

6.2. Study design and objectives

My study was nested within a larger study which randomly selected individuals for mosquito feeding experiments in order to assess the human infectious reservoir. The objective of my study was to determine the ability of IgG purified from serum from rats that had been vaccinated with R0-10C to reduce or block transmission of endemic parasites, using DMFA combined with serum replacement. Six test membrane feeding conditions were used in total, whole blood, autologous plasma, control plasma, naive rat IgG (0.6mg/mL), positive rat IgG (0.6mg/mL) and positive rat IgG (0.2mg/mL). Whole blood was included since it is the most widely used approach to determine infectiousness of participants, which was the main outcome

of the overarching study. Autologous plasma was included as a quality control condition, where blood samples were pelleted then the red blood cell pellet was once more mixed with autologous plasma. This sample should give a similar infection outcome as the whole blood sample, with the only difference being sample handling. This enables identification of a loss in infectiousness as a result of centrifugation, which has previously been reported (Bousema, Dinglasan et al. 2012). The European naive control plasma was included to allow comparative determination of the TRA of naturally acquired antibodies in the participant plasma. For example, if the participant serum contained antibodies with functional TRA, the expected outcome would be higher prevalence/density of infection in mosquitoes fed with control serum, compared to mosquitoes fed with autologous plasma (Bousema and Drakeley 2011). Similarly, naive rat IgG (Abcam, Cambridgeshire, UK) was used as a control to assess the TRA of the vaccinated rat IgG. The vaccinated rat IgG was diluted to 2 concentrations, 0.6mg/mL and 0.2mg/mL to assess if a dose dependent effect would be reflected with TRA.

Specific chapter objectives were as follows:

1. To purify IgG from rats vaccinated with R0-10C vaccine candidate and confirm the ability of the IgG to prevent the transmission of cultured *P. falciparum* gametocytes, using standard membrane feeding assay.
2. Determine the sensitivity and specificity of parasite detection techniques to identify individuals who infected mosquitoes, which was assessed using direct membrane feeding assay.
3. To test the ability of vaccine-induced IgG in rats to reduce transmission of *P. falciparum* gametocytes from naturally infected gametocyte carriers in Burkina Faso using serum-replacement membrane feeding experiments.
4. Assess the transmission reducing activity of endemic serum using serum replacement membrane feeding experiments, and the relationship between transmission reducing activity and 10C and 230CMB antibodies.

6.3. Materials and Methods

Work outlined in sections 6.31 and 6.32 was performed by colleagues at Radboud UMC.

6.31 Vaccination of rats with R0-10C

Recombinant R0-10C was purified using a Ni-NTA Purification System (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) followed by a Sephacryl affinity column (GE Healthcare, Eindhoven, The Netherlands), a Size Exclusion Column (GE Healthcare, Eindhoven, The Netherlands), followed by a HiTrap column (GE Healthcare, Eindhoven, The Netherlands), containing epitope 45.1. This was performed by colleagues at Statens Serum Institut, Denmark as described in more detail elsewhere (Theisen, Roeffen et al. 2014). Following this, purified R0-10C was sent to Radboud UMC, The Netherlands, where purity and reactivity was confirmed by SDS PAGE, Western blot and 2 site ELISA, by colleagues. Next rat vaccination was performed using the purified product by Will Roeffen as follows. Three groups of 5 Wistar Hanover rats were vaccinated with R0-10C combined with 1 of the following adjuvants, SE/GLA, ALUM or AbiSCO. Rats were administered 3 subcutaneous vaccinations with 200 μ L of 20 μ g of 100% properly folded R0-10C over an 8 week period. Fourteen days following the final vaccination, rats were bled (~2mL per rat) and serum separated from the blood pellet by centrifugation at 3000 rpm for 3 minutes, in preparation for use in SMFA.

6.32 Assessing the transmission reducing activity of vaccinated rat serum using standard membrane feeding assay (SMFA)

To assess TRA using SMFA, 30µL of rat serum was diluted to an end dilution of 1/9 with cultured (3D7) gametocytes (150µL) combined with 120µL of naïve human AB serum. This was then offered to 20 starved *Anopheles stephensi* mosquitoes per each vaccine/adjuvant combination. Serum taken from rats prior to vaccination was used for the control mosquito feeding arm, but was otherwise handled in the same manner as described above. Following feeding, fully and partially unfed mosquitoes were removed and fed mosquitoes were maintained on glucose soaked pads for seven days. Mosquitoes were then dissected and oocyst density was compared between test and control serum fed mosquitoes, (Table 6.0.). This was performed by colleagues at Radboud UMC.

Table 6.0. Transmission reducing activity of vaccinated rat serum assessed using SMFA. Data provided by Will Roeffen, p-values were determined comparing arithmetic means in oocyst density, as routinely done at Radboud UMC (van der Kolk, de Vlas et al. 2004).

Adjuvant Group	Rat #	number infected mosquitoes (n=20)	% TRA	p-value
Alum	1	1	99	<0.001
	2	0	100	<0.001
	3	0	100	<0.001
	4	1	99	<0.001
	5	0	100	<0.001
SE/GLA	11	2	99	<0.001
	12	2	99	<0.001
	13	1	99	<0.001
	14	1	99	<0.001
	15	0	100	<0.001
AbISCO	16	0	100	<0.001
	17	0	100	<0.001
	18	1	100	<0.001
	19	2	99	<0.001

6.33 Purification of IgG from vaccinated rat serum

The vaccinated serum demonstrated 99-100% TRA for all rats, Table 6.0. Since TRA was similar within each of the 3 adjuvant groups, serum from individual rats was pooled for each adjuvant group, then purified by affinity chromatography using a HiTrap MabSelect Xtra column (GE Healthcare, Eindhoven, The Netherlands). Prior to purification, 200 μ L of serum from each adjuvant pool was reserved to enable pre and post purification comparison of protein concentration and functionality. Serum pools were prepared as follows 1.5mL of serum from each rat was pipetted into a 50mL falcon tube, then 25mls of 10x PBS was added and tubes centrifuged for 15 minutes at 4000 rpm. IgG purification was performed according to manufacturer instructions (Healthcare 2013). To provide a negative control for membrane feeding experiments, naïve rat IgG (Abcam, Cambridgeshire, UK) was purified in the same manner as the 3 test samples. Following fractionation, protein peaks indicated IgG remained in fractions 5-9. These fractions (2mls each) were pooled, neutralized to pH 7.5 by adding 500 μ L of 1M Tris pH 8.0, then this volume was adjusted to 20mls with 1x PBS and added to a Vivaspin 20 column (Sartorius Stedim Biotech GmbH, Goettingen, Germany). This centrifugable column concentrated IgG by removing lower weight proteins, and removed residual salt from the column purification which could interfere with the membrane feeding assay. The spin column was centrifuged at 4000 rpm for 35 minutes to allow low molecular weight material to pass through the column, while retaining higher molecular weight proteins in the spin column matrix. Flow through was retained until it was later confirmed to be free of IgG. The concentrated material (~500 μ L) within the matrix of the spin column was then resuspended in 20mls of 1x PBS, and centrifuged as before, to ensure removal of all salt. Following this, the purified protein in the column matrix was resuspended in 1.5mL of 1x PBS and IgG concentration was determined. IgG concentration was assessed in both the concentrated product and flow through using a Nanodrop, Table 6.1.

Table 6.1. Concentration of IgG purified from vaccinated rats from different adjuvant groups. Results are shown for the concentrated sample and the column flow through.

Adjuvant group	Sample type	Concentration mg/mL
ALUM	Concentrated sample	1.36
	Flow through	0
AbiSCO	Concentrated sample	1.35
	Flow through	0
SE/GLA	Concentrated sample	1.33
	Flow through	0

6.34 Reactivity of vaccinated rat IgG against 10C using ELISA

While Table 6.1. indicates there is no IgG in the column flow through material, before discarding samples, total IgG was measured reacting to 10C using ELISA for 3 samples per each adjuvant group, a) starting material before column purification, b) purified IgG and, c) column flow through. The 10C ELISA was performed as previously outlined in methods section 3.13., but the human IgG conjugate was substituted for a rabbit-anti rat IgG-HRP (Dako, Glostrup, Denmark). Each IgG sample was titrated down the plate from row A-H in tripling dilutions from 1/100- 1/218,700.

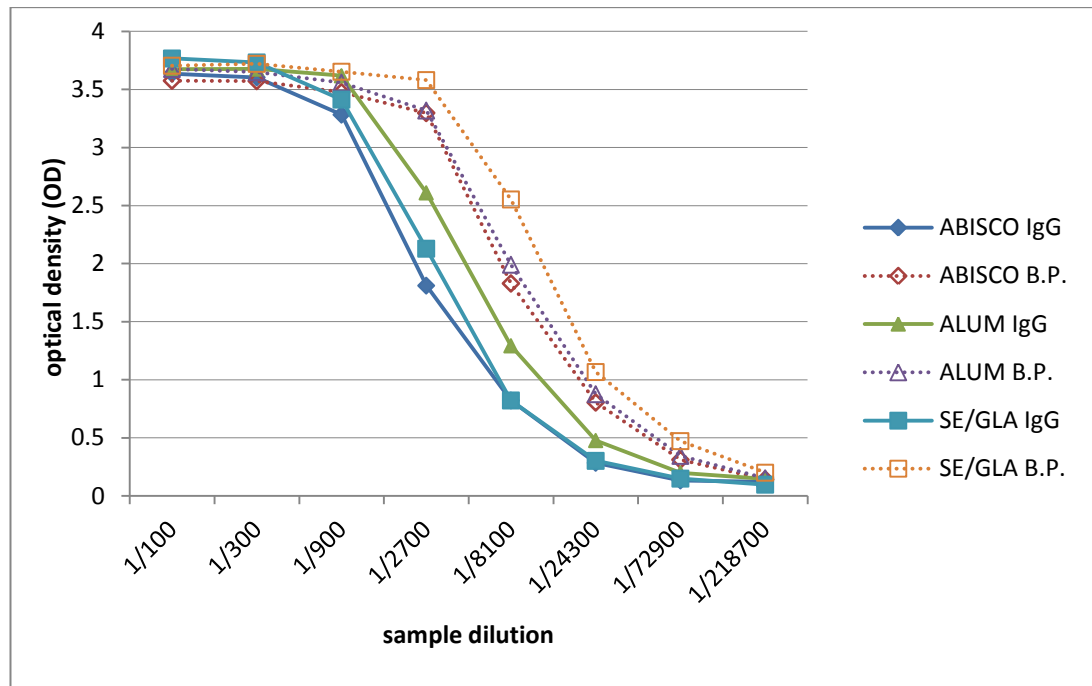


Figure 6.0. Reactivity of titrated rat serum before purification (B.P) and after purification (IgG samples) assessed against 10C antigen using ELISA, for each of the 3 adjuvant groups. Reactivity in ELISA is indicated by optical density. Dotted lines and un-filled shapes represent un-purified material and solid shapes and solid lines represent purified IgG. Diamonds represent the AbiSCO group, triangles represent the alum adjuvant group, and squares represent the SE/GLA group.

IgG purified from the 3 adjuvant pools shows comparable reactivity on an ELISA plate coated with 10C, as shown in Figure 6.0., and demonstrates IgG is specific to 10C. To increase the volume of material available for membrane feeding experiments in the field, IgG was pooled from the 3 adjuvant groups to produce 1 test sample.

6.35 Standard membrane feeding assay (SMFA) and dilution of test IgG dilution

To determine an appropriate IgG test concentration, 2 dilutions were made (0.3mg/mL and 0.1mg/mL) which were based on previous titration experiments. This was tested using SMFA which was performed by colleagues at Radboud UMC. IgG was diluted in European AB serum (Blood Bank, Radboud UMC), which was taken

from donors with no travel history to malarious regions. When compared to the control group, mosquitoes fed with 0.3mg/mL of IgG from vaccinated rats demonstrated a reduction in oocyst density by 99.2% (CI: 97.8-100, $p < 0.001$) which was 84.8% (CI: 75.3-91.6, $p < 0.001$) for mosquitoes fed with 0.1mg/mL of IgG (determined by one sided Mann Whitney, by Will Roeffen). Maintaining an approximate ratio of 1:1 of IgG to diluent, purified IgG was diluted in European naïve AB serum to 0.6mg/mL and 0.2mg/mL, which was aliquoted into volumes of 300 μ L in the field, ready for serum replacement experiments.

6.4 Fieldwork performed in Burkina Faso to collect endemic parasites

Fieldwork was planned and performed with Dr. Bronner Goncalves, LSHTM. Samples were collected together, but used for different purposes. Household selection and participant enrolment was performed with a team of demographers, translators and drivers.

6.4.1 Sampling population

Participants were recruited from a village called Laye located 33km North-West from Ouagadougou in Burkina Faso. This is a rural region comprised of 3 districts and is defined by Sudanese Savannah, where the population are mainly farmers. Malaria transmission is high and seasonal, and the transmission season spans July to October (Ouedraogo, De Vlas et al. 2008). The EIR has been estimated at 300-500 infectious bites per person per year (Cuzin-Ouattara, Van den Broek et al. 1999; Ouedraogo, De Vlas et al. 2008). A survey performed in 2002-2003, demonstrated asexual parasite carriage was 60-90% in children under 15 years, and 20-50% in adults (Ouedraogo, De Vlas et al. 2008; Ouedraogo, Bousema et al. 2009). The study in this chapter was performed at the end of the dry season to complement the main study that was

assessing the human infectious reservoir. The target population was healthy individuals over the age of 2, with no upper limit.

The experiment assessing TRA of vaccine induced rat IgG was exploratory in nature (no formal sample size calculation was performed) and the first 53 RDT positive individuals who were recruited for the larger study were sampled. The larger study was descriptive and aimed to obtain estimates of infection prevalence during the dry season. Since the insectary was able to provide mosquitoes to allow processing of 6 individuals per day (each with potentially 6 membrane feeding conditions), 200 participants were enrolled for the month before the beginning of the dry season, which enabled enough time to complete sampling before commencement of the rainy season. One hundred children were sampled from age 5-15 were since they form the most informative part of the population with the highest parasite carriage (Goncalves, in preparation).

6.42 Ethical approval

Ethical approval was acquired from the London School of Hygiene and Tropical Medicine (#6271) and the Ethical Review Committee of the Ministry of Health of Burkina Faso.

6.43 Participant sensitization and household enrolment

Sensitization was typically performed 1-5 days before participants were brought to the centre for sampling. First, houses were selected using the random walk method. This involved spinning a pen to select a direction to walk in, then walking from the centre of the 1st district in Laye to the outer perimeter of the village counting the houses on the way (UNICEF 2010). On the return trip every 'n'th house was selected for enrolment, where 'n' is a randomly selected number that results in 10-15 houses

being chosen per day. For example, when there were 24 houses, every second house ($n=2$) was selected (12 total), this ensured that while the direction selected was random, there was fairly even sampling from the centre outwards. In each week, sampling was performed in a different part of the village, so by the end of the study all neighbourhoods were represented.

For each selected house, the sensitization team who spoke the local dialect explained the project objectives and procedures, including the purpose of the samples that would be taken. From each house 4 individuals: 1 child aged 2-4, 2 children aged 5-14 and 1 adult ≥ 15 years, were randomly selected by drawing numbers from a box. The highest numbers were then asked if they would participate in the study. Consent forms were read and signed by participants and/or guardians and care was taken to ensure participants understood what had been said to them, then questions about the study were answered, and household global positioning system (GPS) coordinates collected.

6.44 Screening day

Screening was performed in the field several days before sampling with the objective of determining if participants were symptomatic before transporting them to Centre National de Recherche et de Formation sur le Paludisme (CNRFP). The study nurse asked participants about medical history and current symptoms, and then obtained axillary temperature. If febrile ($> 37.5^{\circ}\text{C}$) or suffering from other acute symptomatic disease, individuals were treated, referred to their local health facility and excluded from the study.

6.45 Participant sampling

On the sampling day, participants were brought to the insectary at CNRFP, sampling one family at a time where possible and ensuring children were accompanied by an adult. At the centre the study nurse asked about medical history once more, including development of new symptoms. A physical examination was performed and study questionnaire completed on a case report form.

Finger prick and venous blood samples were taken for RDT, microscopy, serology, filter paper collection, parasite detection by PCR and QT-NASBA, gametocyte detection by QT-NASBA and membrane feeding assays. A hemocue test (Angelholm, Sweden) was performed immediately (according to manufacturer instructions) to test and treat for anaemia. Participants were excluded if their haemoglobin level was <8g/dL, otherwise they were issued a study ID number and the rest of the sampling was performed as follows. Approximately 300µL was taken for the finger prick blood sample in a BD Microtainer (Becton Dickinson, Oxford, UK), according to the manufacturer guidelines. To store samples for RNA based gametocyte detection, 100µL of whole blood was immediately transferred into a microcentrifuge tube containing 500µL of RNA protect (Qiagen, Surrey, UK) using filter tips. Microcentrifuge tubes were inverted 5 times to mix, then samples were maintained on ice and transferred to -80°C as soon as possible. On return to the laboratory, thick and thin microscopy slides were prepared for each participant along with 3x 20µL filter paper blood spots which were taken on Whatman 3MM filter paper.

6.46 RDT scoring

The intention was to use gametocyte positive blood for the serum replacement experiments with rat IgG. While it would have been preferable to confirm gametocyte presence before performing serum replacement, it was not possible to hold participants while waiting for microscope slides to be read. Furthermore, many

studies have indicated gametocyte carriers often harbour gametocytes at submicroscopic densities which are still capable of infecting mosquitoes (Schneider, Bousema et al. 2007). Therefore, RDT positivity was used as a proxy for gametocyte presence since RDTs offer rapid parasite detection. While RDTs are not gametocyte specific, individuals positive for asexual parasites at a density sufficiently high to be detected by RDT (sensitivity ~ 100 parasites/ μL) were expected to also harbour infectious gametocytes (Bell 2002; Bell, Wongsrichanalai et al. 2006). Since studies have indicated an increasing relationship between RDT test line colour density and parasitaemia (Rodulfo, De Donato et al. 2007), results were scored against the colour chart that was provided by the WHO for the RDT product testing project (WHO 2009). Negative results were scored 0, while positive results were scored from 1-4, with 1 corresponding to a faint line, and 4 corresponding to a dark line (see appendix 11 for the colour chart). RDT results were initially determined as positive or negative much sooner than the manufacturer recommended time in order to minimise the amount of time between blood draw and feeding mosquitoes. The colour intensity scores that were analyzed in this chapter were determined at the manufacturer recommended reading time.

While symptomatic individuals were excluded, in some instances symptoms developed after arriving at CNRFP. In this situation, an RDT was performed, and following a positive result, the participants were excluded from the study and treated with AL (CoArtem, Novartis, Basel, Switzerland).

6.47 Membrane feeding conditions

Six membrane feeding conditions were included for RDT positive individuals: whole blood, autologous plasma, control plasma, naive rat IgG (0.6mg/mL), positive rat serum (0.6mg/mL) and positive rat serum (0.2mg/mL), using the venous blood sample, (see Table 6.2.).

Table 6.2. Membrane feeding conditions and sample handling according to participant RDT results.

Condition	Purpose	RDT results	Sample handling
Whole blood	Assess who is infectious Allow determination of effects of centrifugation on infectiousness	All	<ul style="list-style-type: none"> • Directly into the feeder
Autologous plasma	Determine effects of centrifugation on infectiousness Assess who is infectious	All	<ul style="list-style-type: none"> • Centrifuged, then resuspended
European naive human serum	Control to assess TRA of endemic antibodies	All	<ul style="list-style-type: none"> • Centrifuged, serum removed and replaced with same volume of European naive serum
Naive rat IgG (0.6mg/mL)	Control for rat IgG conditions	Positive only	<ul style="list-style-type: none"> • Centrifuged, serum removed and replaced with same volume of naive rat IgG
Vaccinated rat IgG (0.6mg/mL)	Determine TRA of vaccinated rat IgG	Positive only	<ul style="list-style-type: none"> • Centrifuged, serum removed and replaced with same volume of vaccinated rat IgG (0.6mg/mL)
Vaccinated rat IgG (0.2mg/mL)	Identify possible dose dependent effect of TRA	Positive only	<ul style="list-style-type: none"> • Centrifuged, serum removed and replaced with same volume of vaccinated rat IgG (0.2mg/mL)

6.48 Mosquito husbandry

Mosquito husbandry and dissection was performed by staff at CNRFP. A brief overview of processes is described below. Membrane feeding experiments were

performed using mosquitoes that had been acquired locally but adapted to colony 9 years previously (Ouedraogo, Bousema et al. 2009). Four-five day old female *Anopheles gambiae sensu stricto* (s-form) mosquitoes were used for feeding experiments. Mosquitoes were starved for a minimum of 5 hours before experiments, then shortly before sampling, batches of 60 mosquitoes were aspirated into children's cardboard ice cream cups (500mls) which were covered with mesh and secured with an elastic band.

6.49 Equipment preparation

Mini water jacketed glass membrane feeders (Coelen Glastechniek, Weldaad, The Netherlands) were connected to each other using plastic tubing attached to an Isotemp Immersion Circulator water bath (Thermo Fisher Scientific, Hertfordshire, UK) that maintained water at 37-38°C. Water was circulated through the feeders for 10 minutes before use to acclimatize instruments to temperature. Following this, the glass feeders were sealed with 2x2cm pieces of Parafilm (Camlab, Cambridgeshire, UK), then were ready for use. To prevent gametocytes from exflagellating due to a drop in temperature, plastic wear and serum was also heated to human body temperature (37-38°C). An Eppendorf ThermoStat Plus dry incubator (Eppendorf, Hertfordshire, UK) was used to heat the following equipment: blunt needles (0.7mm x38.1mm, Kendall Monoject, Massachusetts, USA), microcentrifuge tubes, pipette tips, and all serum aliquots (European naive control, naive rat IgG, and 2 vaccinated rat IgG conditions). A water bath was used to heat: 10 mL syringes (BD Plastipak, Becton Dickinson, Oxford, UK), BD heparin vacutainers (Becton Dickinson, Oxford, UK) for taking blood, and additional syringes for transferring blood into the glass feeders. All centrifugation steps required for serum replacement were performed in a heated Eppendorf centrifuge 5702 RH (Eppendorf, Hertfordshire, UK) which was also kept at 37°C. Attempts were made to estimate the number of serum aliquots that would be needed per session to avoid freeze thawing, and effort was taken to ensure serum was heated for just 15 minutes before use.

6.50 Membrane feeding and serum replacement sampling

Blood was taken by a trained CNRFP staff member. The volume of venous blood taken varied according to the age of the participant, 2 x 2.5mls of blood was taken for children <5 years and 2 x 4mls of blood for participants >5 years. Blood was collected in a warmed heparin vacutainer (appendix 12) using warmed syringes, and was then gently inverted 6 times to mix the blood and heparin.

The first venous blood tube was maintained in a cold box (ensuring no direct contact with the ice packs) and was processed for RNA detection, serology and DNA pellet (appendix 10 and 13). The second tube was used for membrane feeding and the following steps were performed as quickly as possible to ensure no longer than 10 minutes passed between taking the blood and feeding it to the mosquitoes. Keeping the blood tube warm by holding it, an RDT (First Response Premier Medical, India) was performed according to the manufacturer's instructions. While waiting for the RDT to develop, 400 μ L of blood was pipetted into 6 heated microcentrifuge tubes (1 for each condition) using a heated pipette tip. The whole blood sample was processed first and the entire volume of the first aliquot (~400 μ L) was pipetted directly into a membrane feeding device using a heated syringe and heated blunt needle, ensuring no air bubbles were introduced. A labelled mosquito cup was placed below the corresponding membrane feeder and was raised to touch the Parafilm (covering the bottom of the membrane feeder), by inserting folded paper below the cup. A sheet of dark plastic tarpaulin was used to cover the top of the feeding chamber to allow the mosquitoes to feed in the dark for 20 minutes. The remaining 5 blood aliquots were then centrifuged at 3000 rpm for 3 minutes at 37°C, and the next two aliquots were prepared, placing the other 3 in the dry heated incubator. The autologous plasma aliquot was processed next, which was inverted 10 times to mix, then the entire volume was transferred into the membrane feeder using a heated blunt needle, and the mosquito cup was positioned below the feeder as before. For the European control serum aliquot, all autologous serum was removed from the centrifuged blood pellet, noting the volume taken which was on

average 200µL (from 400µL of whole blood). Autologous serum was replaced with the same volume of heated European control serum, the blood pellet was mixed with the replaced serum by inverting 10 times, then the entire volume was loaded into the membrane feeder and mosquitoes fed as before.

RDT results were now decided and if positive, serum replacement was performed using the 3 rat IgG conditions: naive rat IgG (0.6mg/mL), vaccinated rat IgG (0.6mg/mL) and vaccinated rat IgG (0.2mg/mL), processing the samples one at a time as described for the European control serum. Individual timers were set for the 6 conditions to ensure uniform feeding duration.

After 20 minutes, the mosquito husbandry team processed the mosquitoes as follows. Mosquito cups were placed into holding cages (25x 35x25cm), the mesh cover was removed and cups gently tapped to expel the mosquitoes. After a settling period of 10 minutes, unfed and partially unfed mosquitoes (identified by the smaller size and lighter abdominal colour) were removed by aspiration. Fully fed mosquitoes were maintained at 26-28°C with 80% humidity and care was taken not to disturb them for 24 hours post feeding. Mosquitoes were fed with glucose (10%) soaked pads that were soaked daily and changed 3 times per week.

6.51 Mosquito dissection and oocyst counting

Mosquito dissection and oocyst counting was performed by staff at CNRFP. Mosquitoes were dissected on average 8 days after membrane feeding, then midguts were examined for the presence of oocysts as follows. Mosquitoes were removed from their holding cage by aspiration into paper cups, then transferred to a Petri dish which was stored at -20°C for 10 minutes to sedate mosquitoes in preparation for staining and dissection. Using forceps, a mosquito was placed onto the un-frosted side of a microscope slide and a droplet of 1.0% mercurochrome was added. Moving the slide under a dissection microscope, the midgut was then removed into the mercurochrome droplet which was covered with a cover slip. The slide was

incubated in a wet chamber for 5 minutes, then examined for the presence of oocysts under a microscope. Each slide was read by two individuals, and all positive midguts confirmed by a third reader. The number of oocysts was recorded for each mosquito dissected.

6.52 Microscopy, molecular and serological laboratory protocols

Microscopy was performed by colleagues at CNRFP to determine presence and density of asexual parasites and gametocytes. I performed sexual and asexual antibody quantification using ELISA as described in section 3.12 and 3.13., for 10C and AMA-1, and GLURP and 230CMB ELISA was performed by Nicolas Ouedraogo, from CNRPF. Nucleic acid extraction and molecular detection of parasites was performed by colleagues at Radboud UMC, as follows. The nucleic acid was extracted from 100µL of whole blood diluted in 500µL of RNA protect (Qiagen, Surrey, UK) using an automated nucleic acid extractor (MagNAPure, Roche, Almere, The Netherlands). QT-NASBA for Pfs25mRNA detection was performed as previously described (section 4.61.), and QT-NASBA for detection of all parasite stages (targeting 18S rRNA) was performed using the same methodology, but different primers outlined in Table 6.3. (Mens, Schoone et al. 2006).

Table 6.3. QT-NASBA primer and molecular beacon sequences for detection of 18S rRNA.

Primer	Sequence (5'-3' orientation)
Forward	GTCATCTTTCGACGTGACTT
Reverse	AATTCTAATACGACTCACTATAGGGAGAAGGAACTTCTCGCTTGCGCGAA
Molecular beacon	6-carboxyfluorescein CGATCGGAGAAATCAAAGTCTTTGGGCGATCG-dimethylaminoazosulfonic acid

Along with QT-NASBA detecting 18S rRNA, parasite prevalence was also determined by colleagues at Radboud, UMC, using nested PCR developed by Snounou *et al.*, which detects DNA for the same target. This was performed as described in section 3.22.

6.53 Statistical analysis for calculating the reduction of oocyst prevalence and density determined using membrane feeding assays

Often transmission reduction is either expressed as a change in oocyst prevalence or oocyst density. In this chapter, both are shown. While reduction in oocyst prevalence is more important in terms of a vaccine that aims to completely block all infection (Stone, Eldering et al. 2013), examining the reduction in density allows identification of an effect in absence of complete transmission blocking and is more commonly used in systems with artificially high mosquito infection intensities such as SMFA (van der Kolk, de Vlas et al. 2004).

When evaluating natural TRA, the control arm was mosquitoes fed with European naive serum and the test arm was mosquitoes fed with autologous plasma. When assessing TRA of rat IgG, the control arm was mosquitoes fed with naive rat IgG and the test arm was mosquitoes fed with vaccinated rat IgG (0.6mg/mL or 0.2mg/mL). For analysis of a reduction in oocyst prevalence or density, a cut off of >2 infected mosquitoes was imposed, so conclusions were not derived from very small sample numbers.

Fishers Exact Test was used to determine if differences between oocyst prevalence in control and test arms were statistically significant. A one sided Mann-Whitney test was used to determine if reduction in oocyst density was statistically significant between test and control arms. This was performed in R (version 3.0.2) using code supplied by Dr. Michael Bretscher. This test was used since oocyst density is not normally distributed. The percentage of transmission reduction (and confidence intervals) was determined by bootstrapping.

6.6 Results

6.61 Participant population and data collected

Two hundred participants aged 2-74 were recruited from 59 households in Laye, at the end of the dry season in May-July 2013 for the overarching study that assessed the human infectious reservoir for malaria. The first 53 RDT positive individuals were selected for serum replacement with rat IgG and were from the following age groups <5 (n=7), 5-14 (n=40) and ≥ 15 (n=6). Along with undergoing medical screening and completing the medical questionnaire, the following data was collected for each participant: RDT result, gametocyte and asexual parasite prevalence and density determined by microscopy, parasite prevalence by nested PCR (nPCR), parasite (all stages) and gametocyte prevalence by QT-NASBA, antibody prevalence and titre for 10C, 230CMB, GLURP and AMA-1, and mosquito oocyst prevalence and density for each feeding assay.

6.62 Parasite carriage by participant age group

Table 6.4. illustrates participant information and parasite carriage according to age group. To assess the differences by age, 3 age categories were adopted <5, 5-14 and ≥ 15 years. These reflect different populations according to the degree of acquired immunity, which impacts on parasite carriage as discussed in 1.31. and 1.32.

Table 6.4. Characteristics of study participants according to age group. p-values compare all age groups and were generated using chi square or regression. IQR= interquartile range, GM= geometric mean.

Age group:	<5	5-14	≥15	p-value
characteristics				
Number of participants enrolled	50	100	50	
Median age (IQR)	3(3-4)	9(7-11)	39(27-47)	
Female % (n/N)	57.1(28/49)	56.1(55/98)	30.0(15/50)	
RDT positivity % (n/N)	38.8(19/49)	63.0(63/100)	20.0(10/50)	<0.001
Asexual parasite prevalence by microscopy % (n/N)	27.1(13/48)	37.4(37/99)	2.0(1/50)	<0.001
Asexual stage density by microscopy GM (IQR)	666(456-1259)	908(409-1727)	39	0.102
Asexual prevalence by nPCR% (n/N)	44.4(20/45)	62.9(61/97)	32(16/50)	0.001
Asexual parasite prevalence by QT-NASBA % (n/N)	44.4(20/45)	65.0(63/97)	42.0(21/50)	0.010
Proportion (%) (n/N) of asexual infections that are submicroscopic	40.9(9/22)	43.9(29/66)	95.7(22/23)	<0.001
Gametocyte prevalence by microscopy % (n/N)	4.2(2/48)	8.1(8/99)	2.0(1/50)	0.276
Gametocyte density by microscopy GM (IQR)	25(20-32)	41(15.5-68)	68 (NA)	0.988
Gametocyte prevalence by QT-NASBA % (n/N)	39.6(19/48)	62.2(61/98)	32.0(16/50)	0.001
Proportion (%) (n/N) of gametocytes that are submicroscopic	89.5(17/19)	86.9(53/61)	93.8(15/16)	0.724

In total 25.9% (51 /197) of individuals were positive for asexual parasites detected by microscopy, which was 46.2% (92/199) detected by RDT, 50.5% (97/192) detected by PCR and 54.2% (104/192) detected by QT-NASBA. Gametocyte prevalence was 5.6% (11/197) detected by microscopy and 49.0% (96/196) detected by QT-NASBA.

The age groups were compared to assess if differences in parasite carriage were statistically significant (see Table 6.4. for p-values). Children under the age of 15 had the highest prevalence of microscopically detectible parasites. The highest

submicroscopic asexual parasite prevalence (detected by nPCR or QT-NASBA) was seen in children age 5-14 (Table 6.4.). No difference was seen with parasite density (determined by microscopy) and age groups.

While there was no difference in the prevalence of microscopically detectable gametocytes between the age groups, submicroscopic gametocyte prevalence (determined by QT-NASBA) was significantly higher for age group 5-14. Gametocyte density in gametocyte carriers did not vary with age, (Table 6.4.).

6.63 Proportion of submicroscopic and microscopic parasite infections

Along with parasite burden, the proportion of infections harbouring submicroscopic and microscopic parasites varies with age. The proportion of asexual stage infections that are submicroscopic is similar for <5 and 5-14 years but significantly higher for adults, where the majority of infections (95.7%) are submicroscopic, Table 6.4. In contrast, the proportion of infections with submicroscopic gametocytes is similarly high (86.9-93.8%) for all groups, with no statistically significant difference between age groups.

6.64 Parasite detection method comparison

Several detection methods were used to determine parasite prevalence and density, and characterize participants according to their infection type, i.e. microscopic or submicroscopic (as discussed above) in order to explore which densities are relevant for infection in mosquitoes. Here, methodologies are compared in order to determine their sensitivity and specificity. RDT antigen detection lines were assessed separately since they do not detect exactly the same parasite stages.

Figure 6.1. graphically represents some of the data shown in Table 6.4, and indicates the proportion of individuals parasite positive according to the detection method used.

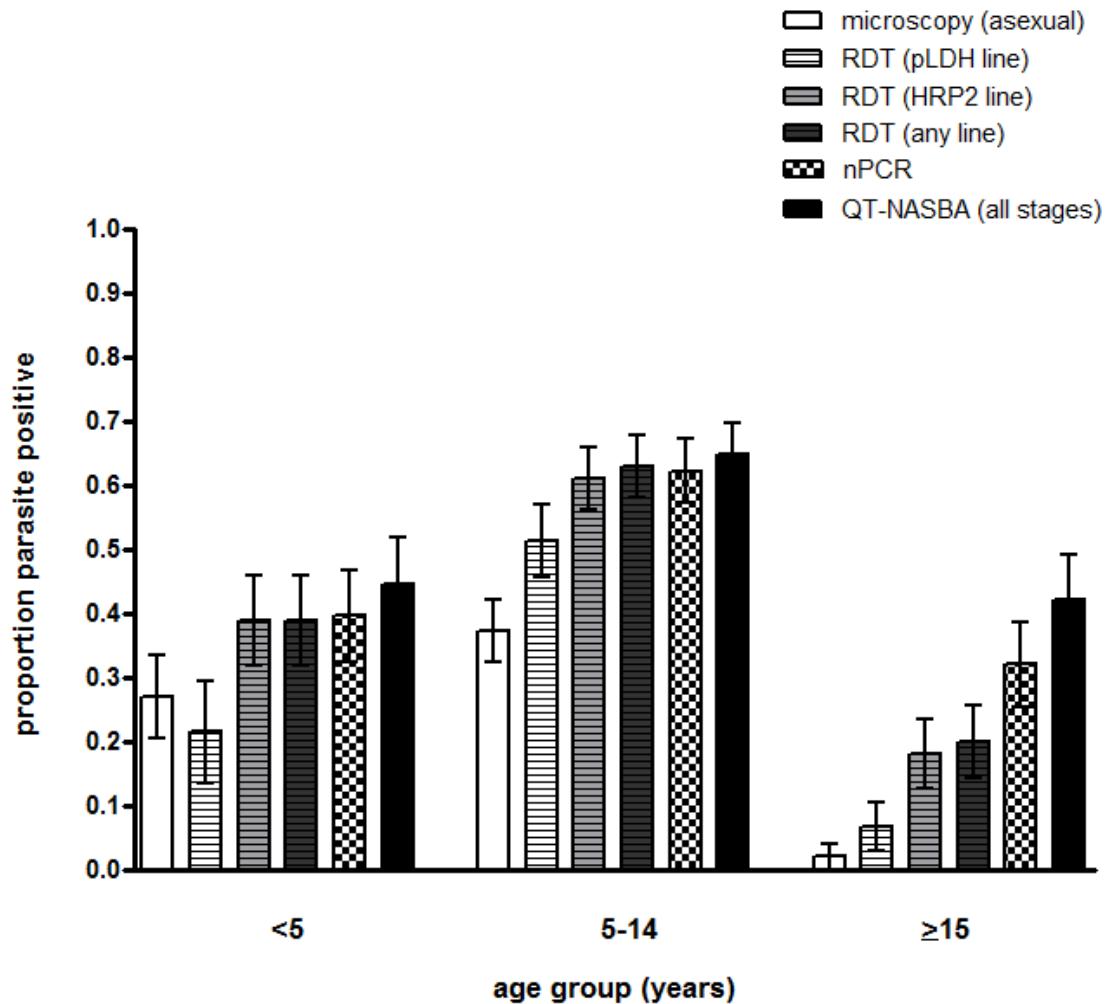


Figure 6.1. The proportion of parasite positive individuals according to the detection method by age group. Parasite detection methods are represented with different bar shading. The error bars indicate the 95% confidence interval.

The pattern seen is in line with previous reports (Bell 2002; Mens, Spieker et al. 2007), and indicates the following general pattern. Microscopy is least sensitive (except in <5 years), (determined by comparatively lower parasite prevalence), followed by RDT, with the most sensitive being molecular detection methods. From

Figure 6.1., as discussed previously, it can be seen more microscopically detectable parasites are found in the younger ages, with a higher proportion of submicroscopic infections (detected by nPCR and QT-NASBA but not microscopy) in the older age groups. Intriguingly, what can be observed from Figure 6.1. is that RDTs are comparatively less sensitive (although not a statistically significant difference) for participants 15 years or over, which again is likely to reflect the shift towards submicroscopic parasite carriage, which may result in less diagnostic antigen being available for detection. For individuals <15 years, parasite prevalence by RDT (any line, or HRP2) gave comparable prevalence to that determined by nPCR.

Using QT-NASBA (18S rRNA detection) as reference, the performance of each diagnostic test was expressed in terms of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) (Table 6.5., shows how this was calculated, and results are shown in Table 6.6.).

Table 6.5. Calculation of parasite detection method sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Calculations were performed as follows: sensitivity ($a/a+c$), specificity ($d/b+d$), positive predictive value ($a/a+b$) and negative predictive value (NPV) ($d/c+d$).

Test result	Disease is truly present	Disease is truly absent
+	True positive (a)	False positive (b)
-	False negative (c)	True negative (d)

In this setting with very high parasite prevalence by QT-NASBA, a strikingly lower detection sensitivity of microscopy and RDT can be seen. nPCR had the highest sensitivity (detected the largest proportion of QT-NASBA confirmed parasite carriers), followed by RDT any line, HRP2 line, pLDH line and microscopy. While microscopy has the lowest sensitivity, it has the highest specificity and PPV, with all other methods being lower, but comparable to each other. NPV broadly followed the same pattern as sensitivity, but pLDH RDT line had the lowest NPV.

Table 6.6. Sensitivity, specificity, positive predictive value (PPV) and negative predictive (NPV) value of parasite detection methods compared to detection by 18S rRNA QT-NASBA. 95% confidence intervals (CI) are indicated in brackets.

Detection method	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Microscopy	45.6(35.8-55.7)	97.7(91.9-99.7)	95.9(86.0-99.4)	60.3(51.7-68.4)
RDT (pLDH line)	52.3(41.3-63.2)	93.8(84.8-98.2)	91.8(80.4-97.7)	59.4(49.2-69.1)
RDT (HRP2 line)	77.9(68.7-85.4)	93.1(85.6-97.4)	93.1(85.6-97.4)	77.9(68.7-85.4)
RDT (any line)	79.8(70.8-87.0)	92.0(84.1-96.7)	92.2(84.6-96.8)	79.2(70.0-86.6)
nPCR	88.5(80.7-93.9)	94.3(87.2-98.1)	94.8(88.4-98.3)	87.4(79.0-93.3)

6.65 Mosquito feeding and mortality

For 198 participants, the following membrane feeding conditions were assessed, whole blood feeding, autologous plasma and European naive control plasma. For 53 people 3 additional experimental conditions were included, naive rat IgG (0.6mg/mL), vaccinated rat IgG (0.6mg/mL) and vaccinated rat IgG (0.2mg/mL). An average of 42 mosquitoes was dissected per cup and feeding condition. Initially, the proportion of mosquitoes that fed was low at 82.2% (6708/8160) in the first week which improved to 90.2% (7086/7860) in the second week, after the number of people in the mosquito feeding room was reduced. Similarly mortality was initially high at 25.8% (2108/8160) for the first week which decreased to 14.3% (1127/7860) in the second week after mosquitoes had been moved from cardboard cups into more spacious mesh cages.

6.66 Using parasite detection methods to detect/predict infection in mosquitoes

Twenty four/198 people (12.1%) infected a mosquito in one or more of the feeding conditions. Children under the age of 15 were significantly more infectious than adults ($p=0.011$). Twenty-five % (6/24) of infectious people were gametocytaemic by microscopy, 62.5% (15/24) positive for asexual parasites by microscopy (which is not

directly related to infection in mosquitoes but may be related to higher gametocyte densities), 87.5% (21/24) positive for 18S rRNA QT-NASBA, 95.8% (23/24) positive by RDT (any line) and 95.8% (23/24) positive for Pfs25mRNA QT-NASBA. While there was a statistically significant relationship between gametocyte density and infectious participants after adjusting for age ($p < 0.001$), the majority, 70.8% (17/24) of mosquito infections were from submicroscopic gametocyte carriers i.e. negative for gametocytes by microscopy. The 1 infectious individual that was negative by Pfs25mRNA QT-NASBA, was carrying *P. malariae* parasites, and the 1 infectious individual that was negative by RDT was positive by both QT-NASBA methods.

Since RDTs give the most rapidly available results, they were used to identify infected, and therefore potentially infectious, individuals for the serum replacement membrane feeding assays. Table 6.7. assesses the discriminative value of different diagnostic methods to identify individuals who infected at least one mosquito. While the techniques used are diagnostic/detection tools, here they are referred to in terms of their ability to 'predict infection' in mosquitoes. Sensitivity, specificity, PPV and NPV were calculated as before, comparing each method to the outcome, which is infection prevalence in mosquitoes.

Table 6.7. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of detection methods to predict/detect infection in mosquitoes. 95% confidence intervals (CI) are indicated in brackets.

Detection method	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Microscopy (gametocytes)	25.0(9.8-46.7)	97.1(93.4-99.0)	54.6(23.5-83.1)	90.3(85.1-94.2)
Microscopy (asexual)	62.5(40.6-81.2)	79.2(72.4-85.0)	29.4(17.5-43.8)	93.8(88.6-97.1)
RDT (pLDH line)	81.8(59.7-94.7)	76.0(67.7-83.1)	36.7(23.4-51.7)	96.1(90.3-98.9)
RDT (HRP2 line)	87.5(67.6-97.2)	60.9(53.3-68.2)	23.6(15.2-33.8)	97.3(92.2-99.4)
RDT (any line)	95.8(78.8-99.3)	60.3(52.7-67.7)	25.0(16.6-35.1)	99.1(94.8-99.8)
nPCR	87.5(67.6-97.2)	54.5(46.6-62.2)	21.7(13.9-31.2)	96.8(90.9-99.3)
QT-NASBA 18S rRNA	87.5 (67.6-97.2)	50.3(42.5-58.1)	12.6(8.2-18.1)	20.2(13.0-29.2)
QT-NASBA Pfs25mRNA	95.8(78.8-99.3)	57.6(49.8-65.1)	24.0(15.8-33.8)	99.0(94.5-99.8)

The data shows RDTs (any line) and QT-NASBA targeting Pfs25mRNA have the highest sensitivity for detecting infection that results in infected mosquitoes. In terms of specificity, microscopy detecting gametocytes is the most specific predictor of infection, followed by microscopy detecting asexual parasites, then RDT detecting the *Plasmodium* lactate dehydrogenase (pLDH) line. Gametocyte detection using microscopy and RDTs detecting the pLDH line have the highest PPV indicating if these results are positive, they more likely to translate to infection in mosquitoes. NPVs are highest for QT-NASBA (detecting Pfs25mRNA) and RDT (any line). In summary, while QT-NASBA (detecting Pfs25mRNA) and RDT (any line) are most sensitive, the specificity and PPV indicate they may not necessarily predict infection. Since none of the PPVs were very high, this indicates none of these methods can reliably predict mosquito infection.

To further investigate the relationship between RDT positivity and mosquito infection, the density of the colour of the RDT lines (scored against the WHO colour chart) was examined in relation to mosquito infection. The strength of the association is indicated between the density of the positive test line for histidine rich

protein 2 (HRP2) (Table 6.8.) and pLDH (Table 6.9.) and infection in mosquitoes. While overall, both diagnostic antigens provide similar associations with mosquito infection, the effect estimates are larger for pLDH, indicating a stronger association compared to HRP2. Use of the pLDH line prevalence therefore seems a slightly more reliable predictor of infection in mosquitoes compared to the HRP2 line.

Table 6.8. The association of HRP2 line colour density with infection in mosquitoes.

This was performed using logistic regression, after adjusting for age, using RDT score 0 as the reference category. RDT colour scores 3&4 were combined due to low numbers of observations for colour intensity 3 for both the HRP2 and pLDH lines. Odds ratios (OR) are presented with the 95% confidence interval (CI), along with the associated p-value. Overall trend was assessed using logistic regression, colour score 0 was compared to colour scores 1-4 (combined).

RDT score	% infectious (n/N)	OR (95% CI)	p-value
0	3.7(3/82)	1.00	
1	25.0(2/8)	11.08(1.29-95.31)	0.029
2	9.09(1/11)	2.26(0.20-25.63)	0.511
3&4	32.0(16/50)	10.09(2.58-39.44)	0.001
Overall trend		1.98(1.32-3.00)	0.001

Table 6.9. The association of pLDH line colour density with infection in mosquitoes.

This was performed using logistic regression, after adjusting for age, using RDT score 0 as the reference category. RDT colour scores 3&4 were combined due to low numbers of observations for colour intensity 3 for both the HRP2 and pLDH lines. Odds ratios (OR) are presented with the 95% confidence interval (CI), along with the associated p-value. Overall trend was assessed using logistic regression, colour score 0 was compared to colour scores 1-4 (combined).

RDT score	% infectious (n/N)	OR(95% CI)	p-value
0	3.9(4/102)	1.00	
1	26.7(4/15)	12.44(2.19-70.81)	0.004
2	42.3(11/26)	14.65(3.79-56.73)	<0.001
3&4	37.5(3/8)	11.47(1.87-70.49)	0.008
Overall trend		2.59(1.61-4.16)	<0.001

When only assessing the association between mosquito infection and RDT scores 1-4, neither HRP2 (p=0.671), or pLDH (p=0.770) demonstrated a statistically significant increasing trend. This indicates that the presence of a positive band, not the band intensity, discriminates infectious and non infectious individuals.

6.67 Infection in mosquitoes fed with autologous plasma compared to whole blood

Infection in mosquitoes fed with autologous plasma and whole blood was compared to determine if centrifugation (performed for serum replacement) negatively impacted upon gametocyte infectiousness, as suggested by previous research (Bousema, Dinglasan et al. 2012). While there was slightly higher infection prevalence in mosquitoes fed with autologous plasma, compared to whole blood, this was not a statistically significant difference ($p=0.084$).

6.68 Naturally acquired transmission reducing activity affecting mosquito infection prevalence and density

To determine if naturally acquired antibodies demonstrated TRA, oocyst prevalence and density was compared in mosquitoes fed with autologous plasma and European control serum spiked blood. This was explored in individuals who infected >2 mosquitoes ($n=6$), Table 6.10. One participant demonstrated a statistically significant reduction in oocyst prevalence ($p=0.013$), and 5 demonstrated a statistically significant reduction in oocyst density ($p\leq 0.048$).

Table 6.10. Percent reduction in oocyst prevalence and density comparing mosquitoes fed with European control serum spiked blood to mosquitoes fed with autologous plasma. The reduction in oocyst prevalence is shown in the left side of the table and the reduction of oocyst density is in the right hand side. 95% confidence interval (CI) is shown in brackets, NB: individuals infecting >2 mosquitoes were included in comparisons.

Gametocyte carrier ID	Number of infected/no of dissected mosquitoes				Oocyst density arithmetic mean (95% CI)			
	European control	Autologous plasma	% reduction in prevalence	p-value	European control	Autologous plasma	% reduction in density	p-value
AF1023	13/39	10/35	16.7(-68.5-62.1)	0.802	1.00(0.44-1.56)	0.94(0.37-1.52)	8.3(-112.2-62.4)	0.426
AF1028	19/25	12/30	47.4(16.7-70.8)	0.013	6.08(2.51-9.65)	0.90(0.41-1.39)	85.2(68.4-93.3)	<0.001
AF1054	11/47	3/36	64.4(-4.4-100)	0.083	1.49(0.49-2.50)	0.17(-0.04-0.37)	88.8(60.8-100.0)	<0.001
AF1071	21/42	14/43	34.9(-9.2-63.4)	0.125	4.07(1.83-6.31)	1.58(0.36-2.81)	61.2(6.6-86.7)	0.048
AF1135	15/55	5/36	49.1(-14.6-88.2)	0.195	0.91(0.36-1.46)	0.22(0.01-0.44)	75.6(33.6-95.1)	0.003
AF1152	14/43	11/44	23.2(-53.6-64.0)	0.484	1.63(0.65-2.61)	0.48(0.16-0.80)	70.7(27.2-88.7)	0.006

There was no statistically significant association between age and naturally acquired TRA ($p=0.251$). The relationship between TRA and gametocyte density was assessed, but no association was observed ($p=0.338$).

6.69 Vaccine-induced transmission reducing activity affecting mosquito infection prevalence

Next, TRA of IgG purified from immunized rats was assessed by comparing oocyst prevalence and density between the two test conditions (0.6mg/mL and 0.2mg/mL), and the naive rat IgG condition. Table 6.11. indicates the percent reduction in oocyst prevalence. In total, there were 14 individuals who infected at least one mosquito in these membrane feeding conditions, 5 of which infected >2 mosquitoes and were included in the analysis. Two participants showed a statistically significant reduction in oocyst prevalence for mosquitoes fed with 0.2mg/mL of IgG ($p\leq 0.029$), and 4 participants showed a significant reduction for 0.6mg/mL of IgG ($p\leq 0.015$), Table 6.11.

Table 6.11. Percent reduction in oocyst prevalence comparing mosquitoes fed with naive rat IgG to mosquitoes fed with vaccinated rat IgG (0.2mg/mL and 0.6mg/mL). 95% confidence interval (CI) is shown in brackets, $-\infty$ refers to minus infinity, for the lower limit of the confidence interval, ND= not determined. NB: individuals infecting >2 mosquitoes were included in comparisons.

Gametocyte carrier ID	No. Of infected/no of dissected mosquitoes:		% reduction in prevalence 0.2mg/mL (CI)	p-value (0.2mg/mL)	% reduction in prevalence 0.6mg/mL(CI)	p-value (0.6mg/mL)
	Naive IgG	vaccinated IgG				
AF1016	3/33	1/32	65.6($-\infty$ -100)	0.317	60.7($-\infty$ -100)	0.385
AF1023	14/40	1/37	85.0(54.9-100)	<0.001	85.0(56.1-100)	0.001
AF1063	5/38	1/45	83.1(15.6-100)	0.055	100(ND)	0.021
AF1071	17/40	2/43	89.1(69.0-100)	<0.001	80.9(52.0-100)	0.001
AF1089	4/36	1/44	79.5.0($-\infty$ -100)	0.104	100(ND)	0.042

6.70 Vaccine-induced transmission reducing activity affecting mosquito infection intensity

Three individuals showed a statistically significant reduction in oocyst density for the 0.2mg/mL condition ($p \leq 0.027$), and 4 individuals for the 0.6mg/mL condition ($p \leq 0.023$), Table 6.12.

Table 6.12. Percent reduction in oocyst density comparing mosquitoes fed with naive rat IgG to mosquitoes fed with vaccinated rat IgG (0.2mg/mL and 0.6mg/mL). 95% confidence interval (CI) is shown in brackets, $-\infty$ refers to minus infinity, for the lower limit of the confidence interval, ND= not determined. NB: individuals infecting >2 mosquitoes were included in comparisons.

Oocyst density arithmetic mean (CI)							
Gametocyte carrier ID	Naive rat IgG	vaccinated IgG		% reduction	p-value	% reduction	p-value
		0.2mg/mL	0.6mg/mL	0.2mg/mL (CI)	0.2mg/mL	0.6mg/mL (CI)	0.6mg/mL
AF1016	0.09(-0.01-0.19)	0.03(-0.03-0.09)	0.07(-0.08-0.22)	65.6($-\infty$ -100)	0.164	21.4($-\infty$ -100)	0.214
AF1023	1.56(0.51-2.61)	0.03(-0.03-0.08)	0.24(-0.15-0.64)	85.2(41.9-100)	<0.001	85.2(43.7-100)	<0.001
AF1063	0.29(0.03-0.55)	0.02(-0.02-0.07)	0	92.3(51.7-100)	0.027	100(ND)	0.011
AF1071	3.63(1.62-5.63)	0.07(-0.03-0.17)	0.41(-0.06-0.87)	98.1(93.7-100)	<0.001	88.8(68.3-100)	<0.001
AF1089	0.14(-0.0-0.28)	0.02(-0.02-0.07)	0	83.6($-\infty$ -100)	0.053	100(ND)	0.023

No difference was observed with oocyst prevalence ($p=0.954$) or density ($p=0.450$) between 0.6mg/mL and 0.2mg/mL conditions.

6.71 Antibody responses to sexual and asexual antigens

Antibody responses were quantified against asexual stage antigens AMA-1 and GLURP and sexual stage antigens 10C and 230CMB. Figure 6.2. demonstrates antibody profiles and titre by age group.

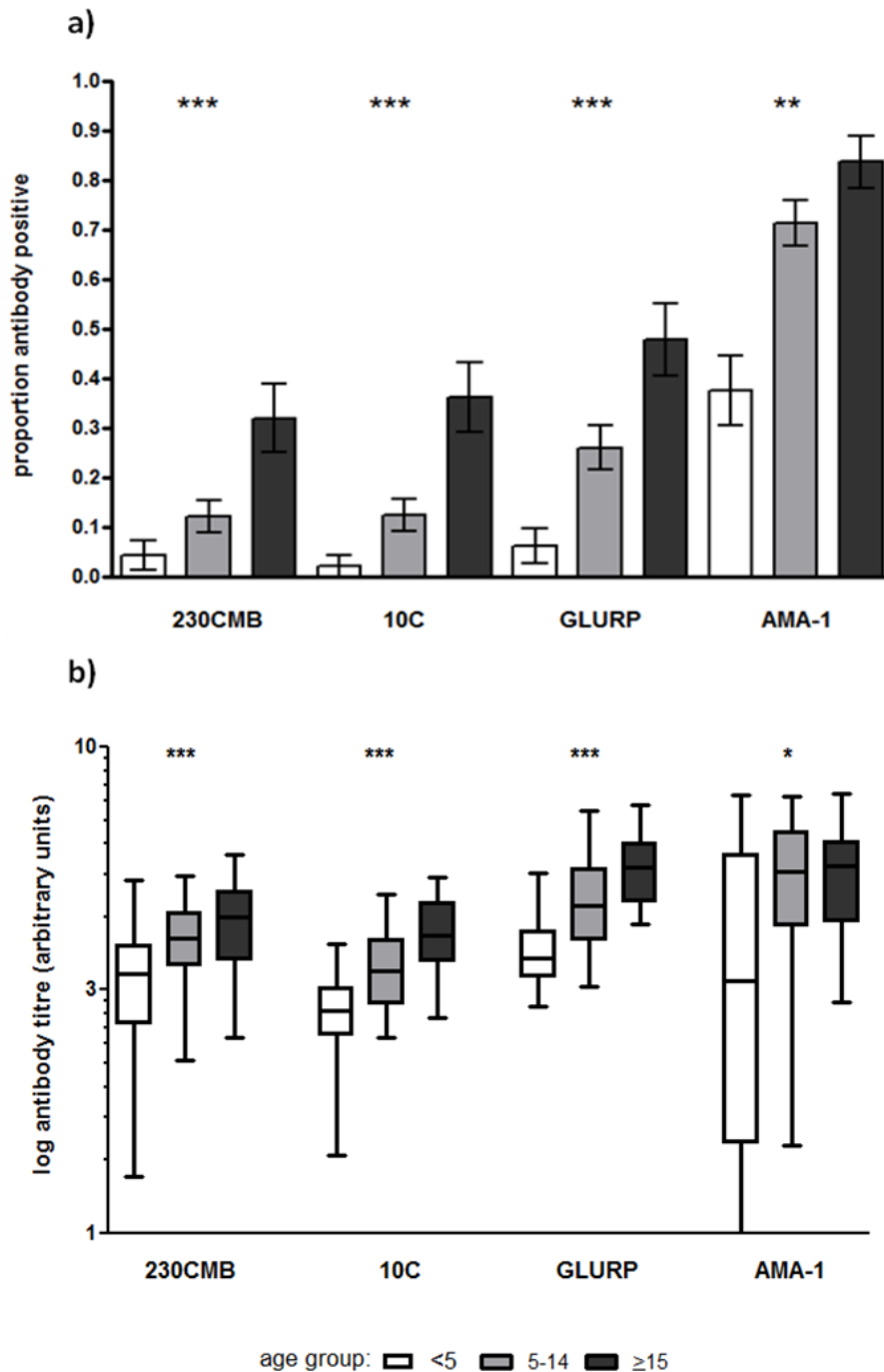


Figure 6.2. Antibody profiles for 230CMB, 10C, GLURP and AMA-1 in relation to age group. (a) The proportion of individuals antibody positive by age group. The error bars indicate the 95% confidence interval, (b) Log antibody titre (arbitrary units) by age group. Box plots indicate the median (horizontal line), 25th and 75th percentiles (lower and upper box limits) and whiskers indicate the 5th and 95th percentiles. The asterisk indicates a statistically significant positive increasing trend with age at the following levels: 0.05 – 0.01 (*), 0.01- 0.001 (**), and <0.001 (***), after adjusting for parasite carriage and density. Age groups are shaded in different colours.

Combining all age categories, the proportion of people positive for GLURP antibodies was 26.5% (52/196) and 66.2% (129/195) for AMA-1. Responses to sexual stage antigens were lower, with 14.7% (29/197) positive for 230CMB and 15.3% (30/196) positive for 10C. Both antibody prevalence and titre significantly increased with age for all antigens, after adjusting for parasite prevalence and density, Figure 6.2. (a) and (b).

6.72 Relationship between antibody prevalence to 10C and 230 and naturally acquired TRA and infectiousness to mosquitoes

Having confirmed the presence of naturally acquired 10C and 230CMB antibodies, the next objective was to assess if the reduction in oocyst prevalence or density demonstrated in autologous plasma (in section 6.68.) was associated with 10C or 230CMB antibodies.

TRA was classified as a binary variable to distinguish individuals with or without statistically significant TRA who had infected >2 mosquitoes (those shown in Table 6.10.). The relationship with antibody prevalence and density was assessed using logistic regression, after adjusting for age. Out of the infectious individuals (n=6) listed in Table 6.10., just 1 (age 3) had sexual stage antibodies, but demonstrated no statistically significant relationship with TRA and, 10C or 230CMB antibody prevalence ($p=0.923$, for both) or titre ($p\geq 0.394$).

Next, the relationship between the presence of 10C or 230CMB antibodies and mosquito infection was explored for gametocyte positive individuals for the autologous plasma feeding conditions. The hypothesis was that gametocyaemic individuals with 10C or 230CMB antibodies were less likely to infect mosquitoes compared to gametocyte carriers without 10C or 230CMB antibodies. There was no statistically significant relationship between the prevalence of mosquito infection and antibody prevalence for 10C antibodies ($p=0.370$) or 230CMB antibodies ($p=0.409$), after adjusting for gametocyte density and age, for gametocyte positive individuals, using logistic regression.

Neither was there a statistically significant association between the prevalence of mosquito infection and antibody titre for 10C ($p \leq 0.376$) and 230CMB ($p \leq 0.992$) in individuals gametocyte positive by QT-NASBA, or just by microscopy (Figure 6.3.). There also was no statistically significant relationship with oocyst density and antibody titre for 10C ($p = 0.640$) and 230CMB ($p = 0.110$).

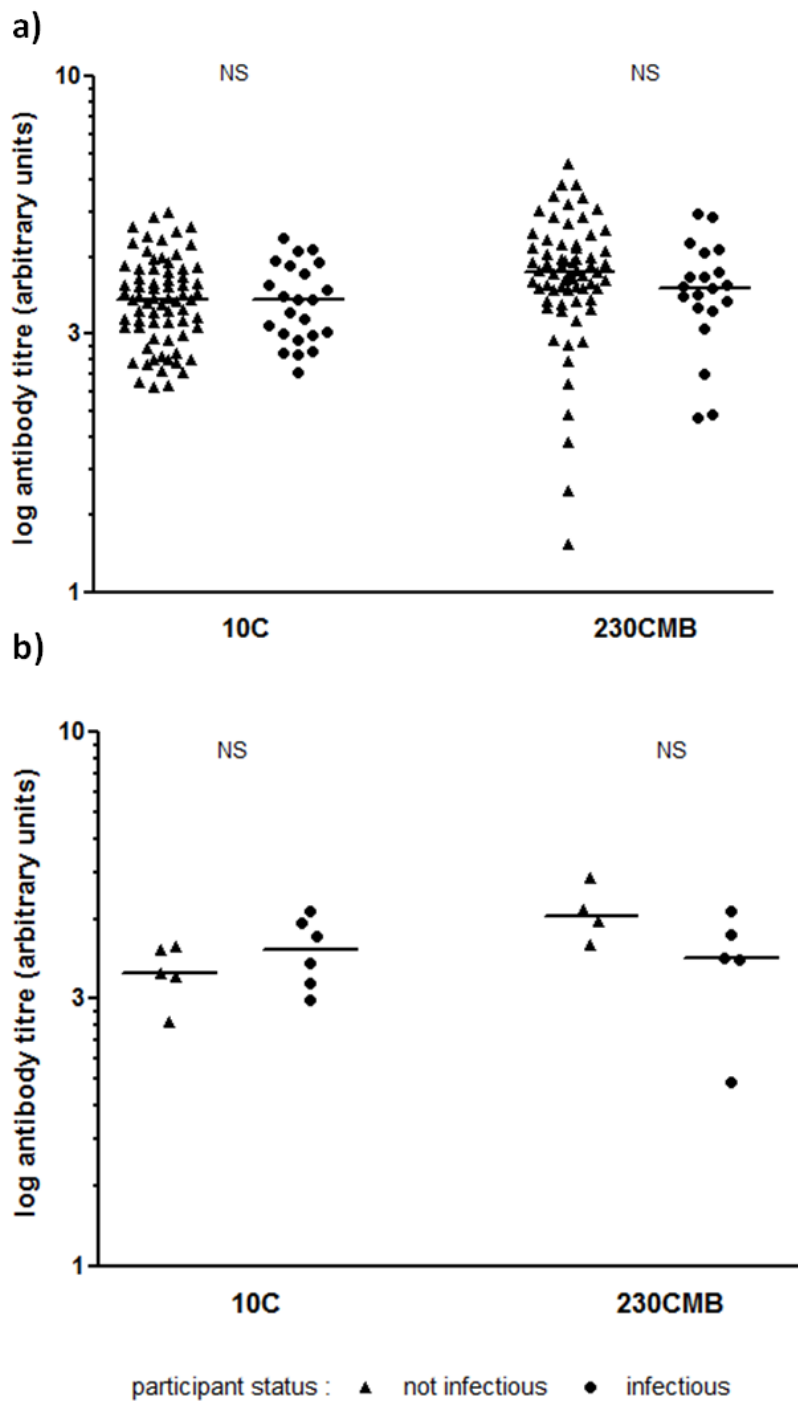


Figure 6.3. 10C and 230CMB log antibody titre in relation to participant infection status. (a) Log antibody titre (arbitrary units) for infectious and non infectious individuals for all gametocyte carriers. (b) Log antibody titre for infectious and non infectious individuals with microscopically detectable gametocytes only. Infectious participants are represented with triangles and not infectious with dots. The median titre is demonstrated by the horizontal line. NS= no statistically significant difference.

6.8 Discussion

6.8.1 General findings

The objective of the overarching project was to identify who within the population was the largest contributor to the infectious reservoir. These questions are being addressed by a colleague and my activities focused on exploring different diagnostic methods for detecting malaria infections, and infectious individuals, and assessing the transmission reducing activity of vaccine-induced or naturally acquired sexual stage antibodies.

In the study area, during the dry season, children <15 years harboured the highest asexual parasite prevalence determined by microscopy, with 5-14 year olds carrying significantly more submicroscopic asexual infections, compared to the other ages. Children aged 5-14 formed the majority of the gametocytaemic reservoir, harbouring the highest overall gametocyte burden (microscopically and submicroscopically detected combined), and also the highest prevalence of submicroscopic gametocytes. This finding is in agreement with other studies that also concluded gametocyte carriage was highest in children, which is likely a consequence of the higher asexual parasite burden found in this age group, leading to the generation of more gametocytes (Bousema, Gouagna et al. 2004; Ouedraogo, Bousema et al. 2010). This information is highly relevant for application of control measures and targeting children 5-14 years old could be an appropriate approach to reducing the infectious gametocytaemic reservoir, within the dry season.

While microscopy suggested low gametocyte prevalence, QT-NASBA revealed many infections harboured gametocytes, therefore indicating the gametocytaemic reservoir, was mostly comprised of submicroscopic gametocytes. This emphasises the need for sensitive gametocyte detection methods, when determining the infectious reservoir.

6.82 Sensitivity of different diagnostic methods to detect the parasitaemic reservoir

Several parasite detection tools were used in this study and were compared to assess their relative sensitivity. Compared to RDTs and molecular detection, microscopy is least sensitive for asexual parasite detection (excluding those <5 years), especially in older age groups where the majority of infections are submicroscopic. The lower sensitivity of microscopy has been extensively reported, and while it is in part attributed the labour intensiveness of preparing and reading slides, sequestered parasites will also go undetected. This gives some techniques, such as RDTs an advantage since they detect antigen that may be present in absence of detectable parasites in the circulation (Kattenberg, Tahita et al. 2012).

The sensitivity of the RDT HRP2 line is consistently higher than the pLDH line ((WHO) 2012), which is likely to be because more HRP2 is produced by parasites, compared to pLDH (Cunningham, in preparation). In addition, HRP2 lingers post clearance (Makler and Piper 2009), and allows infections that have already been cleared from the circulation to be detected, resulting in a higher proportion of HRP2 positive individuals. The relative sensitivity of RDTs is highest in age group 5-14 (who have highest density infections), and lowest in adults who mostly have submicroscopic infections and therefore less antigen.

Molecular detection methods QT-NASBA and nPCR were the most sensitive parasite detection tools. While both methods detect 18S rRNA, the targets for amplification are different. QT-NASBA amplifies transcripts of 18S rRNA which are found in high abundance in all parasite stages (hundreds of copies per parasite) whereas nPCR amplifies DNA copies of 18S rRNA which are present in lower abundance (~5 per parasite) (Wampfler, Mwingira et al. 2013). QT-NASBA therefore offers more sensitive detection, when compared to nPCR (and all other techniques), an effect which is especially pronounced within the older age groups. While RDTs and microscopy fall short for detection of all malaria infections across the different age groups, molecular methods offer more sensitive detection.

6.83 Sensitivity of different diagnostic techniques to detect the infectious reservoir

Serum replacement with rat IgG was performed in this study and the idea was to conduct this for confirmed gametocyte positive individuals only. While screening participants for microscopically detectable gametocytes would have been preferable, it was not logistically feasible to ask participants to wait until microscopy slides were read, or visit CNRFP twice, which is a considerable distance from Laye, to be pre-screened. At the time of performing the field work, RDT positivity (HRP2 line) was used as a proxy to detect the infectious reservoir since it is reasonable to assume if asexual parasites are present, gametocytes may also be present. The ability of RDTs and other diagnostic methods to detect the infectious reservoir was considered.

There was a statistically significant increasing relationship between the presence of microscopically detectable gametocytes and mosquito infection (oocyst prevalence), which is in line with findings from other studies (Boudin, van der Kolk et al. 2004; Schneider, Bousema et al. 2007; Ouedraogo, Bousema et al. 2009). Despite this finding, the majority (70.8%) of mosquito infections were from submicroscopic gametocyte carriers, which reiterates their importance and reemphasises the need for sensitive methods to accurately detect the infectious reservoir. Detection methods were compared to assess their ability to detect/predict infection in mosquitoes. QT-NASBA (Pfs25mRNA) and RDT (any line) had the highest sensitivity, but microscopy and the pLDH line had the highest specificity and PPV.

RDTs were assessed in further detail looking at the association between mosquito infection and colour density of the RDT test lines, to reveal if this provides additional information about participant infectiousness. While colour intensity for both HRP2 and pLDH (compared to 0) were significantly associated with infection in mosquitoes, the strength of the association was slightly stronger for pLDH. However, when assessing infectiousness and the relationship between the degree of positivity (line scores 1-4) there was no statistically significant increasing relationship, indicating while prevalence of RDT positivity is related to infectiousness, the colour density is not.

Premier Medical First Response Combo RDT was used in this study. For this RDT, pLDH seems a slightly better choice for detection of gametocytes and detection/prediction

of infection, but does suffer from lower sensitivity compared to the HRP2 line. It is possible there may be another pLDH detecting RDT on the market that offers higher sensitivity and specificity, and could form a useful tool for gametocyte detection. It is important to note, in this project RDTs were scored against the WHO colour chart using procedures developed by the Centers for Disease Control and Prevention (CDC), but colour scores were recorded for 1 person only. This was performed within this study in an exploratory manner, but to make this protocol more robust, ideally results would be recorded for two readers (to allow inter person validation) who had undergone training to ensure streamlining of interpretation of results.

RDT lines were assessed separately since the targets differ in several ways. Firstly HRP2 is a residual parasite metabolite and can linger for several weeks post clearance of parasites, and therefore could suggest recent, rather than active parasite infection (Kattenberg, Tahita et al. 2012). Secondly, HRP2 is produced by young gametocytes, but not mature gametocytes (which have the potential to infect mosquitoes), whereas pLDH is produced by mature gametocytes (Bell, Wongsrichanalai et al. 2006; Nyunt, Kyaw et al. 2013). A previous study monitoring the gametocidal effects of drug compounds, found gametocytaemia determined by microscopy to correlate well with pLDH and the authors suggested pLDH as a suitable proxy for gametocytaemia. Furthermore, they reported low levels of pLDH to correlate with lower densities of oocysts in the mosquito (D'Alessandro, Silvestrini et al. 2013). While this supports the findings linking pLDH prevalence and infectiousness in my study, it must be noted the study described above was performed using higher density gametocytes than seen in my participants.

Pre-screening with QT-NASBA (Pfs25mRNA detection) or microscopy would theoretically be the favourable approach to detect potentially infectious gametocyte carriers in this study. However, QT-NASBA is time consuming and has so far not been widely adapted for use in the field and since gametocytes often circulate at low densities and may dip below microscopic detection from a day to day basis, microscopy may misrepresent the gametocytaemic reservoir. Therefore, screening against a sensitive pLDH RDT could offer a more stable indicator for mature

gametocyte presence and infection potential, although an HRP2 RDT was also found suitable in this study.

6.84 The occurrence of naturally acquired transmission reducing activity

Previously performed meta-analysis revealed centrifugation of blood conducted during serum replacement can result in lower gametocyte infectiousness compared to whole blood samples (Bousema, Dinglasan et al. 2012). There was slightly higher mosquito infection from autologous plasma feeds, but this was not a statistically significant difference. Since infection was not lower in mosquitoes fed with autologous plasma, I feel confident that methods used for serum replacement did not negatively affect infectiousness of gametocytes.

To assess if there was naturally occurring TRA, oocyst prevalence and density was compared between mosquitoes fed with European control serum and autologous plasma samples. This revealed that 16.7% (1/6) of infectious participants infecting >2 mosquitoes exhibited a statistically significant reduction in oocyst prevalence, and 83.3% (5/6) participants showed a significant reduction in oocyst density in the autologous plasma fed mosquitoes. This finding (although derived from few data points) is in general agreement with data from a publication that compared studies performed across 8 studies in 3 countries. Here, the authors demonstrated in 68.2% of experiments there was a significantly lower proportion of infected mosquitoes fed with autologous plasma compared to control serum (Bousema, Sutherland et al. 2011).

6.85 Transmission reduction of vaccine-induced IgG

Generally there was low mosquito infection in this experiment which restricted the number of robust comparisons. Regardless, the vaccinated rat IgG (0.6m/mL) demonstrated a statistically significant reduction in oocyst prevalence of 80.9-100%, which was an 85.2-100% reduction in oocyst density, for 4/5 people who infected >2

mosquitoes. For the 0.2mg/mL condition, there was a statistically significant reduction in oocyst prevalence of 85.0 and 89.1% (in 2/5 people) which was a reduction in oocyst density of 85.2-98.1% in 3/5 people. Within this study, 2 IgG concentrations were tested but no dose dependent effect was seen.

This study builds upon our previous research where TRA of vaccinated rat IgG was assessed against 3 cultured isolates (Theisen, Roeffen et al. 2014). While *pfs48/45* has not been sequenced in the endemic samples collected in this chapter, it is very encouraging I now have evidence that vaccine induced IgG blocks transmission within an endemic setting. While it is certainly relevant to test TBV efficacy against all infection levels including submicroscopic infections, which represent a large portion of the gametocyaemic reservoir (Schneider, Bousema et al. 2007), higher oocyst prevalence and density would provide a larger number of comparisons to derive robust conclusions about the TRA of vaccine induced rat IgG.

There have been few other studies that tested TRA of vaccine induced serum against endemic parasites. One study assessed the TRA of serum from humans vaccinated with Pfs25 in a different region of Burkina Faso and achieved an average reduction in oocyst density of 80.3% and a reduction in oocyst prevalence of 31.0% (Da, Dixit et al. 2013). There are differences between the study methodologies, firstly, they screened participants using microscopy to ensure gametocytes were present which resulted in a higher mean oocyst density (Da, Dixit et al. 2013). Secondly, the authors fed larger numbers of mosquitoes (150) across 3 feeders for a longer time of 30 minutes. Increasing the number of mosquitoes fed, and using multiple feeders helps to eliminate 'feeder effect' and also increases the sample size and power of the study. Another study confirmed the TRA of serum from mice vaccinated with Pfs25, but once more they firstly selected for gametocyte positive individuals and also fed mosquitoes for a longer duration of 30 minutes (Arakawa, Komesu et al. 2005).

6.86 The functional importance of naturally acquired antibody responses to 10C and 230CMB

10C and 230CMB antibodies were quantified to confirm recognition to the vaccine candidates in this population, and enable assessment of TRA attributed to sexual stage antibody prevalence. 10C and 230CMB antibody prevalence demonstrated a statistically significant increasing acquisition pattern with age, which is not the commonly seen pattern for sexual stage antigens (covered in section 1.36.), but is in line with findings from Chapter 5.

After confirming the presence of 10C and 230CMB antibodies, next TRA was assessed by comparing oocyst prevalence and density in the autologous plasma and European control plasma conditions. One person (age 11) demonstrated a statistically significant reduction in oocyst prevalence of 47.4%, and 5 people (aged 4-15) showed a significant reduction in oocyst density of 61.2-88.8%. Only 1 of these individuals had antibodies for 10C and 230CMB and there was no statistically significant association with TRA and antibody prevalence or titre. To determine if 10C or 230CMB antibodies were protecting from infection in mosquitoes (irrespective of the % of TRA), the association was assessed between infection prevalence and density in mosquitoes and antibody prevalence and density (for 10C or 230CMB), but no statistically significant relationship was found.

In this study it was not possible to link together 10C or 230CMB antibody prevalence or density with a reduction of infection in mosquitoes. Since antibody prevalence and density was measured for just two sexual stage targets, 10C and 230CMB, it must be noted there may be other pre-fertilization antigens such as HAP2 that may result in TRA but have not been measured (Miura, Takashima et al. 2013).

There are varying ideas regarding what induces functional TRA. A previous study assessing TRA in The Gambia, Kenya and Cameroon reported higher levels of TRA were associated with recent gametocyte carriage the week before membrane feeding (Bousema, Sutherland et al. 2011). Data from another study in Cameroon suggested that intensity of TRA was associated with current gametocyte density. The authors reported a high level of functional TRA in individuals harbouring gametocyte densities

over 150/ μ L (Boudin, van der Kolk et al. 2004). While the relationship between TRA and gametocyte density was assessed in my samples, there was no association. Since sampling was performed in the dry season, gametocyte densities were low and just 1 participant harboured a density over 150/ μ L, and they did not exhibit statistically significant TRA. It has been suggested sampling later in the transmission season has been associated with higher TRA which implies recent gametocyte exposure may play a role in functionality (Bousema, Sutherland et al. 2011). This hypothesis supports the low levels of TRA found in our samples that were taken during the dry season.

Previous studies in Tanzania have indicated TRA is more common in children and decreases with age. This decrease has been hypothesized to a consequence of asexual immunity controlling asexual parasite density and subsequent gametocyte production (Drakeley, Bousema et al. 2006). My data indicates sexual stage antibodies accumulate with age, which suggests sufficient antibody boosting still occurs in adults, or 10C and 230CMB antibodies have substantial longevity. Since adults are predominantly carrying submicroscopic gametocytes, it is tempting to conclude this low density is sufficient to boost antibody responses. However, longitudinal studies are needed to confirm this and determine whether antigen exposure from submicroscopic gametocytes is sufficient to induce or sustain functional TRA.

While results in Chapter 5 indicated a statistically significant association between 10C and 230CMB antibodies and TRA using SMFA, it was not possible to extend that finding to this chapter using DMFA. It is important to note, SMFA performed in Chapter 5 used purified IgG, while membrane feeding performed in this chapter used endemic whole blood. SMFA is easier to standardize and the ability to grow high gametocyte densities in culture makes it easier to reach statistical power when comparing oocysts from mosquitoes fed with control and test blood (Bousema and Drakeley 2011). DMFA suffers more individual variation e.g. participants have variable and generally lower gametocyte densities and could have residual antimalarial drugs in their blood stream, all of which make assessment of TRA more difficult. Regardless, the data in this chapter suggests naturally acquired antibodies to 10C and 230CMB may not have a key role in influencing the human infectious reservoir during the dry season in Burkina Faso. This needs to be explored in other settings and during the peak transmission season.

The main limitation of this study is that low mosquito infection resulted in few robust analyses to compare the TRA of vaccine induced IgG. To increase chance of infection, the study could be repeated during the peak transmission season, when gametocyte prevalence and density have been indicated to be significantly higher, and more people will be infectious (Ouedraogo, De Vlas et al. 2008). To screen for potential gametocyte carriers, a sensitive pLDH RDT could be used with the hope of more specific gametocyte detection. To further boost the power of the study a larger number of mosquitoes could be fed (~100 per feeder), and to account for the membrane feeder effect, multiple membrane feeders could be used per condition. If the study were to be repeated, it would certainly also be of value to sequence *pfs48/45* in the individuals who exhibited TRA to explore which strains vaccine derived immunity protects against.

6.87 Conclusions

This study indicates children comprise the majority of the gametocyte reservoir and are responsible for the most mosquito infections, during the dry season in Laye in Burkina Faso. This information could be used for targeted control to reduce the infectious reservoir within the dry season and in particular, this group could be ideal to vaccinate with a TBV to prevent seeding of transmission to the surrounding population. Since the majority of mosquito infections were from submicroscopic gametocyte carriers, this reiterates the need for more sensitive and rapid detection tools to enable detection of all relevant gametocyte densities. Until such tools are available, guided by data from this study I speculate use of a sensitive pLDH RDT could provide slightly more specific gametocyte detection, compared to an HRP2 RDT.

An age dependent antibody acquisition pattern for 10C and 230CMB antibodies was observed in this sample population which reinforces findings from Chapter 5. While infection prevalence and density was low in samples from this chapter, some participants had high enough infectivity to enable demonstration that vaccinated rat IgG significantly reduced or blocked transmission. Since transmission reduction was not 100% in all participants evaluated this indicates a higher concentration than

0.6mg/mL of IgG is needed to achieve more complete transmission blocking. These encouraging findings provide a valuable starting point towards bridging the gap between transmission reduction confirmed using SMFA and transmission reduction in an endemic setting, against more genetically diverse parasite strains.

7.1 Diagnostics for malaria elimination and malaria transmission trials

Following recognition of the value of transmission blocking vaccines, new challenges have arisen, such as identifying tools to facilitate their development (Vogel 2010). One aspect of this, is accurately detecting gametocytes to understand who is capable of transmitting their infection to mosquitoes. The first chapter of this thesis explored methods for mRNA collection for detection of low density gametocytes stored on filter papers kept at different temperatures and humidity conditions. This chapter demonstrated Pfs25mRNA can be detected from both the FTA Classic Card and Whatman 903 Protein Saver Card, but RNA was detected more often from the 903 Protein Saver Card when stored under more challenging warmer temperatures, or humid conditions. What may be of more importance is that this chapter revealed the cheapest and most commonly used filter paper, Whatman 3MM, appears to be appropriate for gametocyte mRNA collection and storage. These findings have to be interpreted with caution since Whatman 3MM was evaluated under just 3 conditions not including humidity which was revealed to have the largest impact on diminishing RNA stability. Nevertheless, my findings demonstrated that the most widely used filter paper may allow the detection of submicroscopic gametocyte densities. An important part in sensitive parasite detection is optimal sample collection, storage and extraction. Hopefully the results in the publication in Chapter 4 could help towards forming an SOP to recommend optimal sampling methods for detection of gametocytes and move towards standardizing methodology to aid inter-lab comparability of results.

The information revealed by Chapter 4 is valuable since use of filter papers reduces the cost of field sampling in several ways. Firstly, filter papers themselves are cheap compared to nuclease-free microcentrifuge tubes that are commonly used for whole blood sampling, secondly the study performed in my thesis (along with others) (Jones, Sutherland et al. 2012; Pritsch, Wieser et al. 2012; Kast, Berens-Riha et al. 2013) have indicated costly freezers may not be necessary for filter paper sample storage

(although this may depend on the storage duration), and finally since filter paper samples are not categorized as biohazardous they can be transported at room temperature without specialist shipping permits or dry ice. This expands the feasibility of sampling gametocytes to remote field sites in resource poor settings, and to projects with smaller budgets. Furthermore, filter paper sampling is advantageous since only small blood volumes are needed, finger prick samples can be used which obviates the need for performing venipuncture (which requires more safety precautions, specialised personnel and results in more discomfort to the participant) (Kast, Berens-Riha et al. 2013).

An important next step to advance this research would be to confirm if RNA can be detected from routinely collected finger prick samples directly applied to filter paper, in contrast to finger prick samples collected in a microtainer then pipetted onto filter paper. While I made a start at addressing this, by indicating gametocytes were detected from routinely collected filter paper samples in Uganda, this was using a small number of samples. Ideally, this finding should be confirmed with larger sample sets that preferably have gametocyte density data (determined by microscopy) to aid comparison, at least for the higher density samples. Additionally, it would then be useful to assess the effect of long term filter paper storage on mRNA sample stability and detection success.

If RNA filter paper sampling was to be introduced into a field study, staff would need specific training on sample collection and handling, since RNA is less robust compared to DNA or antibodies, which are routinely collected on filter paper. Sample collection precautions include wearing gloves for filter paper preparation and handling, ensuring filter papers are dried in a dust free sterile environment out of direct sunlight and only storing papers once thoroughly dry. Furthermore, filter papers must be stored in sealable plastic bags along with colour changing desiccant. Although these precautions should be commonly exercised for DNA and antibody collection on filter papers, they may be more important for RNA collection to prevent introduction of ubiquitous RNases and consequent degradation. Humidity indicator Cards (Adsorbents & Desiccants Corporation of America, California, USA) may prove a useful tool to monitor the presence of moisture.

There is considerable debate about the circumstances in which it is important to detect gametocytes. In many situations, the specific detection of gametocytes may not be essential. Previous research indicated the majority of malaria infections carry gametocytes, and even if not gametocytaemic at one time point, they are likely to become gametocytaemic (Bousema and Drakeley 2011). Therefore detecting infections (any parasite stages) is sufficient for most purposes since every infection is plausibly contributing to the gametocytaemic reservoir, and to transmission potential. The development of sensitive molecular parasite detection tools has led to an unveiling of unexpected parasite carriage patterns. For example, recent research revealed the majority of infections (70-80%) in regions of low transmission intensity are submicroscopic and asymptomatic. It was previously thought that limited exposure (and therefore limited immunity) would result in higher density infections in low transmission settings (Bousema, in press). It is currently unknown how parasite densities are seemingly controlled so efficiently despite limited cumulative exposure to malaria (Bousema, in press). This finding is in agreement with a previous study (Okell, Bousema et al. 2012), that also indicated the high prevalence of submicroscopic infections in low endemic areas. This has implications for epidemiological studies and malaria control and elimination programmes (WHO 2014), and emphasises that rapid field friendly malaria detection methods are needed that detect submicroscopic parasites.

In general, these findings suggest that the specific detection of gametocytes may be less important for routine surveillance, and certainly for public health purposes, the sensitive detection of all infections (with or without concurrent gametocytes) is most relevant. However, there are several circumstances where gametocyte detection is desirable. One example is for population studies to assess the infectious reservoir to gain an understanding of who is responsible for malaria transmission (Wampfler, Mwingira et al. 2013). With this information, it could be possible to perform targeted interventions toward responsible groups, which the aim of a more efficient cost effective approach to control. For example, in the village sampled in Burkina Faso in Chapter 6, it was revealed that children aged 5-14 comprised the majority of the infectious reservoir within the dry season. It could be speculated targeting this age group with transmission reducing interventions e.g. bednet coverage (or use of a TBV

when available), could have a larger impact on overall reduction of parasite prevalence.

Another circumstance where gametocytes need to be detected is to evaluate gametocidal drugs e.g. primaquine. For this purpose, mean gametocyte circulation time post treatment is commonly used to determine drug efficacy (Eziefula, Bousema et al. 2014). Alternatively, gametocyte detection is required when performing mosquito infectivity studies to gain an understanding of which gametocyte densities are relevant for mosquito infection (André Lin Ouédraogo, Jetsumon Sattabongkot et al. 2013).

7.2 Naturally acquired and vaccine induced transmission reducing immune responses

An anticipated advantage of a TBV is that natural gametocyte exposure may boost a vaccine derived response which perhaps could improve antibody functionality by resulting in an enhanced antibody response with better antibody avidity. In Chapter 5 (and 6) evidence indicated exposed populations have antibodies to 10C and 230CMB vaccine candidates. Furthermore, these antibodies appeared to accumulate with repeated exposure, as reflected by the age-dependent antibody acquisition pattern and the higher antibody prevalence in the high endemic sites. In the sample sets assessed, 10C and 230CMB antibody presence (but not increased titre) was demonstrated to be associated with TRA at the $\geq 90\%$ level. Since antibody titre was not found to impact upon TRA, it could be speculated functional antibodies (with $\geq 90\%$ TRA) are the result of improved antibody affinity. Possibly, this could occur when B cells are stimulated by an antigen for a second time therefore resulting in the production of new daughter B cells. During this process, single point mutations may occur within both the heavy and light immunoglobulin chains, which have the potential to result in affinity maturation e.g. the production of antibodies with improved cytophilic activity. It has been theorized that over time, mature antibodies would out compete others (Honjo and Habu 1985; Or-Guil, Wittenbrink et al. 2007).

While it could be hypothesized that TRA should increase with age since 10C and 230CMB antibody prevalence increases with age, no significant age relationship was found. Just a small proportion of individuals from all age groups exhibited $\geq 90\%$ TRA, and this proportion displayed no age dependent pattern. This indicates antibody presence alone does not accurately predict TRA.

Alternatively, the discordance between antibody presence and TRA could be attributed to sequence polymorphisms in *pfs48/45*. In Chapter 5 I indicated *pfs48/45* has 5 non-synonymous amino acid substitutions in the functional epitopes (I-III). I also demonstrated the proportion of isolates identical to 3D7 varies by country, with 17.9% in Burkina Faso ranging to 69.2% in Ghana. The compilation sample sets assessed for TRA in Chapter 5 are from a range of countries, Burkina Faso, The Gambia, Cameroon, Gabon (and Dutch missionaries) and were assessed for their ability to block or reduce transmission of 3D7 using SMFA. Possibly, individuals exhibiting $\geq 90\%$ TRA are those who have been exposed the most to 3D7 and mounted antibodies predominately against this allele. The majority of individuals demonstrating $\geq 90\%$ TRA are from Cameroon, for which I have no information regarding *pfs48/45* allele types. In a separate study, we provided evidence that R0-10C vaccine induced immunity blocks transmission against 3D7, NF135 and NF165 (Theisen, Roeffen et al. 2014). Since the vaccine allele is 3D7, this indicates a degree of cross allele protection, but there are non-synonymous amino acid substitutions not represented by these cultured isolates, that appear in endemic parasites and their relevance needs to be confirmed.

Asides from this, there are several possible explanations for the lack of an age-dependent acquisition of TRA, despite the age dependent increase in 10C and 230CMB antibody prevalence. These include 1) differential acquisition of antibodies to the different epitopes (which induce antibodies of varying functionality), 2) variation in antibody avidity, or 3) production of different antibody isotypes/subclasses, which may also exhibit different functionality (Tongren, Drakeley et al. 2006).

Epitopes I, II and III of Pfs48/45 are present in vaccine candidate 10C. It has previously been indicated epitopes II and III stimulate production of transmission reducing antibodies but epitope I induces the most effective antibodies (Outchkourov, Roeffen et al. 2008). However, epitope III is immunodominant and it could be speculated the

increasing age dependent antibody acquisition pattern predominantly reflects antibodies produced against epitope III. A previous study performed in Cameroon supports this hypothesis. Here, endemic antibodies reacting to native protein Pfs48/45 were found to be predominantly specific to epitope III (Mulder, Lensen et al. 1999).

This could explain the lack of increasing relationship with individuals exhibiting $\geq 90\%$ TRA and age (which is linked to cumulative antibody acquisition) (Roeffen, Raats et al. 2001; Outchkourov, Vermunt et al. 2007). If this is the case, this suggests detection of antibodies directed against epitope I, while only reflecting 1/3 of the vaccine candidate functional epitopes, might reveal a stronger correlation with TRA.

Recognition to malaria antigens has been demonstrated to be selective with some individuals producing antibodies to some epitopes, but not other epitopes in the same protein, which has been suggested to be attributed to clonal imprinting (Taylor, Egan et al. 1996). Since epitope III of 10C may be immunodominant, it could be speculated the primary immune response is more commonly mounted against this epitope. If this is the case, subsequent immune responses may continue to be produced against epitope III (as a result of clonal imprinting). To investigate this, the reactivity to different 10C epitopes could be determined for sera with a known TRA phenotype.

There are several other features that could impact on antibody-antigen binding (and therefore affect TRA), such as antibody avidity and the functionality of the antibody isotype (e.g. IgG, IgA, IgM, IgD, IgE) or subclass (e.g. IgG1-4) against the different epitopes. Since previous research indicated TRA below 90% is not reliably replicated between experiments, it would be logical to assess avidity and identify isotypes or subclasses only in serum demonstrating $\geq 90\%$ TRA (van der Kolk, de Vlas et al. 2004). Antibody avidity could be explored using dissociation ELISA, or BiaCore technology to test the strength of the antibody-antigen binding. While antibody avidity could be hypothesized to mature and improve upon increasing cumulative exposure (and therefore age), this has not been explored for 10C and the different epitopes.

Possibly, specific antibody isotypes or subclasses could be responsible for the most effective TRA (which again could be related to their avidity). Generally, for antibodies produced against asexual/pre erythrocytic malaria antigens, IgG has been suggested to be the most important (most cytophilic) antibody isotype for malaria protection, which

was demonstrated using passive transfer experiments (Cohen, Mc et al. 1961; Sabchareon, Burnouf et al. 1991; Dobano, Quelhas et al. 2012). Furthermore, subclasses IgG1 and IgG3 have been suggested to be most important for clearance of parasites, and are more commonly produced against malaria antigens (Tongren, Drakeley et al. 2006; Eisenhut 2007; Mewono, Matondo Maya et al. 2008; Stanistic, Richards et al. 2009; McCarra, Ayodo et al. 2011; Dobano, Quelhas et al. 2012).

Antibody subclass switching has been observed for MSP (Tongren, Drakeley et al. 2006). In this thesis, total IgG was measured for all ELISA assays; if the functional antibody isotype or subclass was not dominant in the serum samples, it is possible these responses could be shrouded and not sufficiently captured by a total IgG measurement. To explore antibody isotypes in the serum (and their relationship with TRA), ELISA could be performed but detecting with different secondary antibodies specific to the range of isotypes or subclasses (e.g. using IgG1-HRP, IgG3-HRP etc). With this knowledge, if an isotype/subclass was found to be particularly important for functional TRA, efforts could be made for the vaccine to specifically elicit a response to that isotype/subclass. For example, in the instance of MSP2, IgG3 was suggested to be the most functional subclass for reducing parasite densities. It was therefore proposed that future vaccines based on MSP2 could include a T-cell epitope that specifically induces IgG3 production (Tongren, Drakeley et al. 2006).

Research performed in Papua New Guinea assessed antibody subclass produced against RESA and discovered only IgG3 demonstrated an age dependent pattern (Beck, Felger et al. 1995). This could form another potential hypothesis for the difference in age dependent patterns observed with native Pfs48/45 and Pfs230 (some studies did not show an age dependent acquisition pattern (Ouedraogo, Roeffen et al. 2011)) and the recombinant proteins 10C and 230CMB (which did display an age dependent increase).

Antibodies can develop against (and bind to) a wide array of *Plasmodium* antigens; for this reason they are most important for transmission reduction (Bousema and Drakeley 2011). However, it has been suggested that white blood cells (WBCs) and cytokines may have a small effect on transmission reduction. For example, leucocytes have been suggested to phagocytose gametes that have been opsonised (which is

dependent on antibody presence) (Lensen, Bolmer-Van de Vegte et al. 1997; Lensen, Mulder et al. 1998; Bousema and Drakeley 2011). Alternatively, cytokines (e.g. tumour necrosis factor alpha (TNF- α)) in combination with WBCs have been suggested to play a role in transmission reduction (Naotunne, Karunaweera et al. 1993; Bousema and Drakeley 2011). Neither of these ideas have been explored in this project, which was concerned with vaccine candidate antibody responses and functionality, but, peripheral blood mononuclear cells (PBMCs) were collected in Burkina Faso which will be assessed for their activity in another project.

While results from Chapter 5 indicated a strong association with 10C and 230CMB antibodies and TRA using SMFA, this association was not observed in the field in Chapter 6 where DMFA was performed. In Chapter 6, naturally acquired antibodies to 10C and 230CMB did not appear to play an important role in defining the human infectious reservoir or predicting which gametocyte carriers showed evidence of TRA. However, it appears antibodies to both candidates do block transmission when using SMFA. It must be noted the lack of an association (with TRA and antibodies) in Chapter 6 was based on data from a low number of infectious individuals, during the dry season. Low gametocyte density infections (while typical of natural infection), introduce complications when aiming to use membrane feeding in the field and reach adequate statistical power. This is something that needs further development and standardization before membrane feeding can be considered a robust tool for use in clinical trials. Repeating DMFA in larger studies, during the peak transmission season, and within different endemic settings, would be a useful next step to inform how naturally acquired TRA varies when exposure to gametocytes may be more prolonged and gametocyte densities may be higher. Asides from this, it must be reiterated there are methodological differences between SMFA and DMFA. SMFA is much more standardized and forms the current gold standard method for assessing TRA (Bousema and Drakeley 2011).

In Chapter 6, I confirmed the functional TRA of vaccine induced antibody responses in rats. This represents an important step in development of the vaccine candidate by extending the statistically significant TRA demonstrated using SMFA and cultured isolates (Theisen, Roeffen et al. 2014) to the field where the vaccine will be deployed.

My finding that the highest tested concentration of pooled rat IgG did not completely prevent transmission of all isolates may indicate the assay needs to be optimised with a higher starting IgG concentration to achieve more complete blocking. While performing this experiment during the dry season where gametocyte densities were low was relevant and reflected the low densities seen in the majority of infections (Bousema and Drakeley 2011), to improve the statistical power, the study should be repeated with patent gametocyte carriers who may infect larger numbers of mosquitoes and form a more sensitive starting point to measure TRA (Ouedraogo, Bousema et al. 2009; Bousema, Dinglasan et al. 2012).

However, recent experiments using an animal model system suggested a transmission blocking intervention that resulted in transmission reduction of 32% was sufficient to eliminate transmission in a low endemic site (Blagborough, Churcher et al. 2013). As part of the target product profile for a TBV, it was stated the vaccine should reduce transmission by $\geq 85\%$ (Penny, Maire et al. 2008). The TRA seen in the field with our candidate (in a small number of participants) is close to this goal of $\geq 85\%$, but needs confirming in a larger population. In light of the data derived from modelling, perhaps improvements may not be necessary, depending on the transmission intensity of the intended site of vaccine roll out. The $\geq 85\%$ transmission reduction benchmark could be excluding potentially useful vaccine candidates and perhaps the target product profiles should be re-evaluated according to transmission intensity in the location of use.

7.3 A toolbox for community trials with transmission blocking vaccines

Research on TBV has been prioritized in light of global malaria eradication initiatives. A recent publication that summarized the Malaria Vaccine Technology Roadmap strategy (Nunes, Woods et al. 2014) reflects these changes by including TBV in the goals. The authors highlighted important gaps in current knowledge that need to be filled to enable evaluation of TBV (Nunes, Woods et al. 2014). Along with tools to assess the human infectious reservoir, the need for standardized membrane feeding assays was discussed. Another specific potential problem that was mentioned was that pre-

fertilization TBV candidates may suffer immune escape mutants since vaccine candidates are exposed to host immunity (Nunes, Woods et al. 2014).

In my thesis I have made progress towards addressing several of the concerns expressed by the Malaria Vaccine Initiative (MVI) (Nunes, Woods et al. 2014). I have demonstrated transmission stage parasites can be detected from filter papers, and proposed a robust combination of sampling methods which play a role in identifying the human infectious reservoir. I have shown naturally acquired antibodies to 10C and 230CMB vaccine candidates are detectable in all age groups in several sample sets from different endemic settings. This has implications for future transmission blocking vaccine trials which need to consider malaria endemic populations may have antibodies prior to vaccination. Furthermore, within these populations, it is possible the vaccine derived immune response could elicit an enhanced reaction since immune memory cells may already be present from natural exposure (Siegrist 2014).

I provided evidence of functionality of naturally acquired antibodies and showed that antibodies produced against our vaccine candidate (10C) significantly blocked transmission of endemic parasites. I have demonstrated *pfs48/45* contains polymorphisms with strong geographical variation, which forms a starting point to determine their relevance for 10C vaccination. These findings have all contributed towards filling in these knowledge gaps to aid further TBV development. A hurdle for TBV development that was highlighted by MVI, was the variability in how membrane feeding assays are performed and assessed (Nunes, Woods et al. 2014). The serum replacement method I performed, along with other membrane feeding protocols performed by colleagues were published in a methodological paper with the aim of encouraging standardization of protocols to aid inter laboratory comparability (André Lin Ouédraogo, Jetsumon Sattabongkot et al. 2013).

Our vaccine candidate is still in the early stages of development and the next step is toxicity studies of a batch of GMP produced R0-10C. An important consideration for all groups developing TBV candidates is trying to reduce the cost per vaccination. Since it is likely the vaccine will be deployed to all age groups, this significantly increases the cost and there are concerns the vaccine may exceed malaria elimination budgets (Nunes, Woods et al. 2014). Furthermore, attempts need to be made to improve

acceptance of a community based vaccine that provides delayed benefit on an individual level. Efforts have been made by MVI, WHO and the Gates Foundation to encourage collaboration between research groups, and there is optimism that progress may now advance more rapidly (Nunes, Woods et al. 2014).

8.0 References

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Appendix

Please note: These protocols were all supplied by colleagues, as detailed below.

Appendix 1: Preparation of L6 buffer, L2 buffer, and silica, for guanidine based nucleic acid extraction

Protocol provided by Medical Microbiology Department, Radboud University Medical Centre, Nijmegen, The Netherlands.

Reagents and catalogue numbers:

SiO ₂ , Sigma 5631 (80% 1-5 micron)	EDTA, Tritriplex, Merck, 8418
Tris, Trizma base Sigma T6066 (T12), Mw 121.1	NaOH, Merck 6498
GuSCN, Fluka 50990, Mw 118.16 (Sigma –Aldrich)	Triton-X-100, Packard 6008084

The Tris-HCl is a component of L2 and L6 buffers, make this first as shown below.

0.1M Tris-HCl, pH 6.4 (1 litre)

- Dissolve 12.1g Tris in 800 ml of ddH₂O
- In a fume cupboard, slowly add 8.1ml of HCl (37%)
- Adjust the pH to 6.4
- Then top up to 1 litre with ddH₂O
-

L2 buffer

- Dissolve 120g GuSCN in 100 ml of 0.1M Tris-HCl, pH 6.4
- Gently shake in a 60°C water bath until dissolved, then aliquot into 50ml falcon tubes.

0.2M EDTA pH8 (500mls):

- Dissolve 37.2g of EDTA and 4.4g NaOH in 400 ml of autoclaved MQH₂O, pH and top up to 500mls

L6 buffer (keep dark)

- Gently dissolve 1200g of GuSCN in 1 litre of pre-made 0.1M Tris-HCl, pH 6.4, at 60°C in a water bath
- Add 220 ml of 0.2M EDTA pH8
- Add 26ml of Triton-X-100
- Mix and aliquot into 50ml falcon tubes.

Silica

In a cylinder (diameter 5cm, height 27.5cm), do the following:

- Suspend 60 g of silica in 500 ml of ddH₂O
- Leave to sediment for 24 hours at room temperature
- Remove 430 ml of supernatant by aspiration
- Add ddH₂O up to 500 ml and resuspend by shaking
- Leave to sediment for 5 hours
- Remove 440 ml of supernatant
- Add 600 µl of HCl 37% and resuspend by vortex
- Aliquot into small bottles (1 mL) and autoclave wet for 20 min at 121°C, in tightly closed bottles

Appendix 2: Cross sectional survey information sheets

Supplied by Teun Bousema

Overview

A cross-sectional school survey to determine the recognition of antibody responses to Pfs48/45- 10C malaria transmission blocking vaccine candidate

Introduction

Dr. [Chilongola/Dodoo/Nebie] of [Kilimanjaro Christian Medical Centre/Noguchi Memorial Institute for Medical Research/ Centre National de Recherche et de Formation sur le Paludisme] is leading a team that will be conducting studies on immunity against malaria in the area of [Bondo/Asutsuare/Ouahigouya]. In the current study, we want to determine how common immune responses are that influence the spread of malaria. This information is very valuable for the future testing of a malaria vaccine. In the current study, we will not give anybody a vaccine. We will determine natural immunity to malaria.

Why is this study being done?

Malaria is one of the most important health problems in [Tanzania/Ghana/Burkina Faso]. The vaccine that we are trying to develop could play an important role in fighting malaria. For this, we need to study natural immune responses to malaria. We also want to determine how many children are infected with malaria parasites and how this is related to mosquito exposure.

What will happen if I agree to let my child take part in this study?

If you agree to let your child take part in this study, we will ask them some questions about age, residence and other general information. In addition, we will ask him/her to provide a small blood sample by finger prick. This will be used to check for malaria and to determine their level of immunity.

We may also contact you later to catch mosquitoes in your house.

How long will my child be in the study?

Your child will be sampled twice: once in the rainy season and once in the dry season. Sampling will not normally take more than 15 minutes.

What risks can I expect if my child takes part in the study?

The risks of drawing blood from a finger prick include temporary discomfort and bruising. The amount of blood removed per time-point is less than a quarter of a teaspoon so it is too small to affect your child's health.

Are there benefits if my child takes part in the study?

Your child will receive free clinical care from the study staff medical officers and nurses if a clinical malaria attack is detected. In addition, the knowledge gained from this study will help the country of [Tanzania/Ghana/Burkina Faso] in determining the best way to treat and prevent malaria in children.

What other choices do I have if I do not allow my child to participate in this study?

Whether you allow your child to participate or not is completely your choice. If you decide not to take part in this study, there will be no penalty to you.

Will information about my child be kept private?

The study personnel will treat all information gathered as private, and records will be kept securely in locked filing cabinets and offices. No personal identification information such as names will be used in any reports arising out of this research. The findings of this study will be presented in a village meeting but individual findings will not be reported.

Who pays for this study?

The European Union has funded this study.

What are the costs of taking part in this study?

There are no costs to you for taking part in this study.

Will I be paid for taking part in this study?

You will not be paid for taking part in this study.

What are my rights if my child takes part in this study?

Taking part in this study is your choice. You may choose for your child to either take part or not take part. If you decide your child will participate in this study, you may change your mind at any time. No matter what decision you take, there will be no penalty to you in any way.

Who can answer my questions about the study?

You can talk to the researchers at the study clinic or on telephone number [...] about any questions or concerns you have. You may also contact [Chilongola/Dodoo/Nebie] or other members of the team on telephone number [...]. If you have any questions, comments or concerns about taking part in this study, please talk to the researchers first. If for any reason you do not wish to do so, or you still have concerns, you may contact the chairman of the ethical committee on [...].

Appendix 3: Cross-sectional survey consent form

Supplied by Teun Bousema

A cross-sectional study to determine antibody responses to the Pfs48/45-10C malaria transmission blocking vaccine candidate

INSTITUTIONS: Kilimanjaro Christian Medical Centre (Moshi, Tanzania), Noguchi Memorial Institute for Medical Research (Kintampo, Ghana), Centre National de Recherche et de Formation sur le Paludisme (Ouagadougou, Burkina Faso), AMANET (Dar es Salaam, Tanzania), London School of Hygiene & Tropical Medicine (London, UK), Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands)

PRINCIPAL INVESTIGATOR: Chilongola/Dodoo/Nebie

PARTICIPATION INFORMATION: We would like your child to participate in a medical study. It is very important that you understand the following general principles, which apply to all participants within our studies

- i) Participation is entirely voluntary.
- ii) Persons may withdraw from participation in this study or any part of the study at any time.
- iii) After you read the explanation, please feel free to ask any questions that will allow you to clearly understand the nature of the study.

INTRODUCTION:

Malaria is common in [Tanzania/Ghana/Burkina Faso]. A vaccine would be a very valuable tool to protect people from malaria. One of the possible vaccines is a vaccine that blocks the spread of malaria. The current study wants to determine this type of immunity in people who are naturally exposed to malaria.

PROCEDURES TO BE FOLLOWED:

A single 0.25-0.35mL blood sample, approximately a quarter of a teaspoon, will be taken from the finger. This blood will be used to determine parasite carriage and detect antibodies against malaria parasites. It will be stored in the laboratory in [Moshi, Accra, Ouagadougou] and will only be used for the mentioned purposes. Some

of the samples may be transported to Europe for additional analyses. A short questionnaire will be administered.

RISKS AND DISCOMFORTS:

Mild discomfort and bruising is possible at the site where the finger prick blood sample is obtained.

The volume of blood is approximately a quarter of a teaspoon and is too small to affect your child's health.

DURATION OF VOLUNTEER'S PARTICIPATION: 1 day, to be repeated twice during the year

NUMBER OF VOLUNTEERS IN THE STUDY: 100 per school, a total of five schools are included in three countries.

ASSURANCE OF CONFIDENTIALITY OF VOLUNTEER'S IDENTITY: Records relating to your/your child's participation in the research will remain confidential. Identifying information will not be used in any report resulting from this study. You will receive a copy of this consent form.

BENEFITS/MEDICAL CARE FOR ILLNESS: You/your child will be entitled to free malaria treatment if this is considered beneficial by the study physician.

FOR INFORMATION OR ANSWERS TO QUESTIONS CONCERNING YOUR RIGHTS AS A RESEARCH SUBJECT YOU MAY CONTACT: Dr. [Chilongola/Dodoo/Nebie]; Mobile phone: [...]

IF THERE IS ANY PORTION OF THIS CONSENT EXPLANATION SHEET THAT YOU DO NOT UNDERSTAND, ASK THE INVESTIGATOR BEFORE SIGNING.

I have read the information sheet concerning this study [or have understood the verbal explanation] and I understand what will be required of me and what will happen to me if I take part in it. Questions have been answered by Dr. [Chilongola/Dodoo/Nebie]. I

agree to take part in this study and understand that at any time I may withdraw from this study without giving a reason and without affecting my normal care and management.

I **approve/disapprove** (delete one) that part of my sample may be shipped abroad for analysis

Name

Child's name (if applicable)

Signature / finger print:

Date_____

Signature/fingerprint child (if applicable)

Date_____

Witness's Signature:

Date_____

Appendix 4: Child Questionnaire

Supplied by Teun Bousema.

Parasitological survey – 2011

Stool	Urine	Blood	exam
-------	-------	-------	------

Pupil Study ID no: |__|__|__|__|

School: _____

Date survey: |__|__| / |__| / 2011

Class: |__|

Age: |__|__|

Sex: Male Female

Complete the following with each child recruited into the survey

A.Residence	Response	Codes
A1. Since Primary one, where have you lived?/ umeishi wapi tangu uanze shule ya msingi?	Within District/ Katika wilaya Outside district/ Nje ya wilaya	[]
A2. What is the name of the village you reside in now? / Kijiji chako chaitwaje?	_____	[]

A3. In minutes, how long does it take you to walk to schools? / Unachukua dakika ngapi kutembea hadi shuleni?	[] []	
B.Socio-economic status	Response	Codes
B1. In your house, are there any of the following? / Kwa nyumba yenu kuna vitu vifuatavyo? READ OUT & FILL IN WITH Y/N <i>(More than one option maybe selected)</i>	Electricity/ Stima/umeme Solar power/ Umeme kutoka kwa jua Flush toilet/ Choo cha maji Owns a pit latrine/ Choo chenu binafsi cha shimo Uses someone elses pit latrine/ Mnatumia choo cha shimo na wengine Telephone; landline/ Simu ya meza Telephone; mobile phone/ Simu ya mkononi	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
B2.What type of wall does your house have? / Je kuta za nyumba yenu ni za aina gani?	1=Stone or bricks or cement/ Mawe, Matofali au simiti 2=Clay/mud/ Udongo	

<p>READ OUT OPTIONS (Select only one answer)</p>	<p>3=Wood/ Mbao</p> <p>4=Other (specify)/ Zinginezo (eleza)</p> <p>[_____]</p>	<p>[_____]</p>
<p>B3. What type of flooring does your house have?/ Je sakafu ya nyumba yenu ni ya aina gani?</p> <p>READ OUT OPTIONS (Select only one answer)</p>	<p>1=Cement or tiles or linoleum / Simiti au vigae</p> <p>2=Wooden planks /Sakafu ya mbao</p> <p>3=Earth or sand/ Udongo au mchanga</p> <p>4=Other (specify)/ Zinginezo (eleza)</p> <p>[_____]</p>	<p>[_____]</p>
<p>B4. What type of roof does your house have? / Je paa la Nyumba lenu ni la aina gani?</p> <p>READ OUT OPTIONS (Select only one answer)</p>	<p>1=Tiles/ Vigae</p> <p>2=Iron sheets/ Mabati</p> <p>3=Grass or thatch/ Nyasi</p> <p>4=Makuti/ Makuti</p> <p>5=Other (specify)/ Zinginezo (eleza)</p>	

	[_____]	[]
<p>B5. What is the <u>main</u> type of fuel used by your family for cooking?/<i>Kwa kawaida, mnatumia nini kwa kupika?</i></p> <p>READ OUT OPTIONS (Select only one answer)</p>	<p>1=Electricity or gas/ <i>Stima au gesi?</i> 2=Kerosene/ <i>Mafuta ya taa</i> 3=Charcoal/ <i>Makaa</i> 4=Firewood/ <i>Kuni</i> 5=Dung/ <i>Samadi</i> 6=Others (specify)/ <i>Zinginezo (eleza)</i></p> <p>[_____]</p>	[]
<p>B6.What is the <u>main</u> source of water for drinking or cooking in your home? / <i>Kwa Kawaida, maji ya kunywa au kupika yatoka wapi?</i></p> <p>READ OUT OPTIONS (Select only one answer)</p>	<p>1=Piped/tap water/ <i>Maji ya bomba</i> 2=Borehole or well/ <i>Kisimani/chemichemi</i> 3=Rain water/ <i>Maji ya mvua</i> 4=Stream or river/ <i>Maji ya mtoni</i> 5=Bought/ <i>Mnanunua</i> 6=Bottled water/ <i>Maji ya chupa</i></p>	[]

	7=Others (<i>specify</i>)/ Zinginezo (eleza) [_____]	[]
C.Bed net use	Response	Code
C1. Do you normally sleep under a bednet?/ Je kwa kawaida unalala ndani ya neti?	Yes/ ndio No/ La Don't know/ Sijui	[]
C2. Did you sleep under a bed net last night?/ Je ulilala ndani ya neti jana usiku?	Yes/ ndio No/ La Don't know/ Sijui	[]
C3. What is the colour of your bed net?/ Neti yako ni ya rangi gani?	[_____]	[]
C4. When do you receive your bed net? / Ulipata neti yako lini?	This school term/ Muhula huu	

	Last school term/ Muhula uliopita Last school year/ Mwaka jana	
D: Animals	Response	Code
What animals are found in your compound? / Je mna wanyama wagani kwa boma yenu? D1.Cattle / Ng’ombe D2. Sheep/ kondoo D3. Goats/ Mbuzi READ OUT OPTIONS	CIRCLE CORRECT RESPONSE Yes/No Ndio/La Yes/No Ndio/La Yes/No Ndio/La	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
F.Fever	Response	Code
F1. Do you have fever or hot body at the moment ? Je, una joto jingi au mwili moto hivi sasa?	Yes/No/Don’t know Ndio/La/Sijui	<input type="checkbox"/>

<p>F2. In the last 2 weeks have you had fever or hot body? / Wiki mbili zilizopita ulikuwa na joto jingi au mwili moto?</p> <p>F3. In the last 2 weeks have you had blood in urine/ Wiki mbili zilizopita ulikuwa na damu au kichocho katika mkojo wako?</p>	<p>Yes/No/Don't know Ndio/La/Sijui</p> <p>Yes/No/Don't know Ndio/La/Sijui</p>	<p><input type="checkbox"/></p> <p><input type="checkbox"/></p>
<p>G. Family and siblings</p>	<p>Response</p>	<p>Code</p>
<p>G1. How many people live in your household? / watu wangapi wanaishi kwa nyumba yenu?</p> <p>G2. How many siblings do you have? / Je watoto wa mama yako ni wangapi?</p>	<p><input type="text"/></p> <p><input type="text"/></p>	<p><input type="text"/></p> <p><input type="text"/></p>

	Name of sibling (Jina la ndungu)	Sex Kike/ Mume	Age Miaka	In same school? /Anasoma hapa?) (N/Y)	Name of school if 'N' (Kama la, anasoma shule gani?)	Class number (Darasa)	Does he/she normally sleep under a bednet? / Je hula ndani ya chandarua?	Did he/she sleep under a bed net last night? / Alilala ndani ya chandarua jana usiku?	What is the colour of their bed net? / Chandarua yenu ni rangi gain?
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									

Appendix 5: School Questionnaire

Supplied by Teun Bousema

SCHOOL SURVEY QUESTIONNAIRE: _____ District

Date: ____/____/2011

School name:

Year school established:

Person interviewed:

Head-teacher (yes ,no) if No, describe:

Longitude: [] [] . [] [] [] [] [] Latitude: - [] . [] [] [] [] []

A. General demographics	Response	Code
A1. Total number of children in school	[] []	
A2 Total number of teachers in school	[] []	
A3.Village nearest to school	_____]	[] []
A4.Village furthest from school	[] [] [] [] []	[] []
B. Health programmes	Response	Code
B1.Does the school have a feeding program?	Y/N	[] []
B2.Did pupils receive free bednets in school campaigns?	Y/N	[] []
B3.Is the school involved in a deworming programme?	Y/N	[] []

Appendix 6: Blood sampling procedure

Protocol supplied by Teun Bousema

Definition

This SOP describes how to collect blood for thick and thin film, DNA sampling, RNA sampling, plasma, RDT and filter paper.

Staff

All trained study staff

Materials

- Dry cotton wool
- Gauze
- Cotton wool swabs soaked in 70% Ethanol (provided with Premier Medical First Response RDT).
- Swab containers
- Disposable sterile puncture device (provided with Premier Medical First Response RDT).
- EDTA microtainer
- Screw cap tubes for plasma
- Glass microscope slides
- Slide box
- Pre-labelled stickers
- Permanent marker pen or pencil
- Sharps bin
- Clean plastic disposable gloves
- Whatman 3 filter papers, previously arranged into their protective card jackets
- RDTs (Premier Medical First Response)
- L6 buffer (guanidine isothiocyanate buffer) (Severn Biotech) pre-measured in eppendorfs
- Extraction Matrix (Severn Biotech)
- Silica gel
- Sealable bags
- Barrier tips for 40 μ L, 50 μ L and 900 μ L volumes (i.e. 200 and 1000 μ L tips)
- Pipettes to measure above mentioned volumes
- Cryoboxes for sample storage
- Cool boxes
- Ice
- Blue roll
- 70% ethanol
-

Procedure for collecting blood slide from finger/heel prick:

1. Before going out to the field, please confirm that the following are correctly labelled according to the pre-agreed labelling protocol (see India Meeting minutes), slides, EDTA microtainers, filter papers, L6 eppendorfs, and screw cap tubes for plasma. Take the following tubes out to the field with you, EDTA microtainers, filter papers L6 eppendorfs and slides. The plasma samples can be dealt with on return to the lab as described in protocol 5.
2. When in the field, prepare the equipment and consumables making sure sharps are safely placed out of reach of children.
3. Explain to the participant/parent/carer the procedure you are about to do and the reason why.
4. Wear a clean pair of disposable gloves
5. Squeeze the finger from the base towards the top, stimulating the blood flow.
6. Make sure the finger is pointing downwards, to aid the flow of blood
7. Make sure the site of pricking is clean
8. Disinfect finger with a swab moistened with methylated spirit or 70% ethanol (or use the swab provided with the RDT).
9. Allow the finger to air-dry
10. Apply a thin layer of Vaseline on the place where you want to prick
11. Using a sterile blood lancet, prick the finger deeply on the side of the tip of the finger.
12. Discard used lancets directly into the sharps disposal container
13. Wipe away the first droplet of blood with gauze
14. Hold the microtainer tube in an angle of $\sim 45^\circ$ angle from surface of finger.
15. Squeeze gently from the base of the finger towards the tip, allowing the blood to flow. The blood should flow easily and without interruption, only the first droplets take some effort. If the squeezing ('milking') is done with too much force, haemolysis can occur which can affect the results.
16. Take approximately 300 μ L of blood in the microtainer. Lines are indicated, make sure to take not more than 500 μ L, not less than 200 μ L.
17. Gently invert the tube 10 times. This is done to mix the blood with the EDTA which will prevent the blood from clotting.
18. Remove 50 μ L of blood for the RNA sample. This is to be immediately put into an eppendorf with 900 μ L of previously aliquoted L6 buffer. Vortex the L6 aliquot straight away if possible, or invert 10 times. If tubes can be processed within 1 hour, leave at room temperature. If it will take >1 (up to 5 hours, not longer), store in cool box or fridge.
19. The remaining volume of blood in the microtainer tube will be used for plasma and DNA extraction. For this, the microtainer should be kept in a cool box or fridge until further processing. It should not be frozen before it has been processed.
20. Collect three drops of blood on the Whatman 3 filter paper (see appendix 8 for further details). Make sure that the drops wet the full thickness of the paper (i.e. the blood is also visible at the back of the paper)
21. Perform RDT (First Response Combo, Premier Medical, India) according to the manufacturer's instructions.
22. Take the slide as described in appendix 9.
23. On return to the lab, prepare the plasma sample, as outlined in appendix 10.

Appendix 7: Procedure for RNA collection in L6 buffer

Protocol supplied by Teun Bousema

Definition

This protocol is for taking the RNA sample from the EDTA microtainer tube. This sample will later be used to determine gametocytaemia. As the target is RNA, these samples need to be handled as carefully and as aseptically as possible to avoid introduction of RNAses.

Material

- L6 buffer (Severn Biotech)
 - Extraction matrix (Severn Biotech)
 - gloves
 - Eppendorfs
 - 70% ethanol
 - RNA sample log book
 - Cryoboxes
 - Barrier tips for 900 μ L
 - Pipette that measures 900 μ L
 - Barrier tips for 40 μ L
 - Pipette that measures 40 μ L
 - Vortex
 - centrifuge
-
1. When handling all RNA equipment, ensure that all surfaces, pipettes, tube racks and any other equipment is wiped down first with 70% ethanol prior to use to avoid introduction of RNases into the eppendorfs.
 2. Label the eppendorfs according to the naming protocol.
 3. Within the lab in the most sterile environment possible, aliquot out 900 μ l of L6 buffer into the eppendorf using a barrier tip, but before aliquotting the buffer, change your gloves to reduce the possibility of introduction of RNAses.
 4. Next transport the eppendorfs out to the field site.
 5. These tubes should be kept in the dark at room temperature and should not be prepared >7 days before the actual sample collection to prevent evaporation.
 6. When taking the microtainer EDTA sample in the field, after inverting the blood in the EDTA tube (10 times), using a barrier tip, pipette off 50 μ L and aliquot this into the 900 μ L of the L6 buffer.
 7. If possible, mix immediately by vortex.
 8. If not, immediately invert the tube 10 times.

9. Samples can be stored at room temperature for 1 hour or in a cool box or fridge for 5 hours.
10. Following return to the lab, vortex the sample thoroughly. In the most aseptic environment possible, do the following. Change your gloves, then add 40 μ L of extraction matrix to the eppendorf.
11. Vortex this for 5 seconds then shake gently at room temperature for 10 minutes.
12. Then vortex again for 5 seconds, spin for 25 seconds at full speed in a microfuge. Change your gloves, then open the tube and remove the supernatant. The remaining pellet is now ready to be shipped for RNA extraction. It can be shipped with icepacks that have been stored overnight at -80°C after consultation of the LSHTM coordinator.

Appendix 8: Whole blood collection on filter paper

Protocol supplied by Teun Bousema

Definition

This SOP is to be used when processing DNA blood spots collected on filter paper. For best results the blood spot should be dried as soon as possible. This usually occurs in the field and at the health facility. The drying process is **crucial** and takes place in two steps. This sample will be used for species determination via PCR, and for ELISA.

Staff

Laboratory technicians and fieldworkers

Material

- Whatman 3MM filter paper
- manila or cardboard paper
- clean forceps
- gloves
- plastic medicine dispensing bags
- colour indicating silica either as individual grains or as small bags
- Filter paper log book
- Filter paper storage container

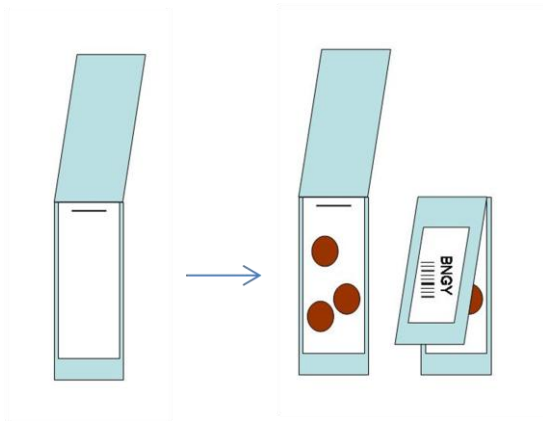
Prepare filter paper:

1. Wear gloves during all steps.
2. Cut Whatman number 3MM filter paper in pieces of ~5x2cm.
3. Cut manila/cardboard paper or paper card in pieces of ~14x2.5cm. Each piece of card should make 11 protective jackets.
4. Place a strip of filter paper and a strip of cardboard end-to-end and overlapping by 0.5 cm and staple together.
5. Fold down the stapled part so that the filter strip lies down the cardboard strip.
6. Fold up the bottom of the cardboard strip over the filter paper to form a protective cover.
7. The filter paper is now ready for use.

Filter paper blood collection and processing:

1. Wear clean gloves during all steps
2. Do not directly touch the filter paper, only touch the protective cover
3. Label the filter paper with correct sticker label

4. Collect three large drops of blood on the filter paper (approximately 20 μ L each) and make sure the droplets are in the middle of the filter paper (see figure below).



5. Make sure that the drops wet the full thickness of the paper (i.e. the blood is also visible at the back of the paper)
6. Place the filter papers in a drying container to allow it to dry but protect it from the wind and sand
7. After a minimum of one hour, fold the filter paper and place the filter paper in a plastic bag, combining up to 5 filter papers per plastic bag. Do not close the bag. Realize that this is only the first step of the drying process. After collection of all samples (i.e. samples from all individuals) upon returning in the laboratory/clinic or project house, commence with step 8.
8. Remove the filter papers from the plastic bags with clean forceps and gloves and place them on top of the plastic bags
9. Ensure that the filter papers are on a surface that cannot be disturbed by the wind, insects, animals or individuals.
10. Allow them to dry overnight at room temperature
11. Put five filter papers into each plastic bag
12. Add one bag of silica into each plastic bag with the filter papers.
13. Push the air out of the bags and close the bags tightly ensuring the seal has completely closed.
14. Combine plastic bags in a larger sealable plastic bag with another quantity of silica gel inside.
15. Place the plastic bag in an airtight container.
16. Place at -20°C and check silica gel every two weeks for changes in colour.
17. If the colour changes from orange to green, replace the silica gel sachet
18. Keep at -20°C until processed for PCR or ELISA. When samples are taken from the freezer, allow them to come to room temperature for at least 2 hours before you open the bag to prevent condensation.

Appendix 9: Preparation of a thick and thin blood slide

Protocol supplied by Teun Bousema

Definition

This protocol describes how to collect a thick and thin film. The follows the protocol where it is described how blood is collected from a finger prick sample.

Staff

All trained study staff

Materials required:

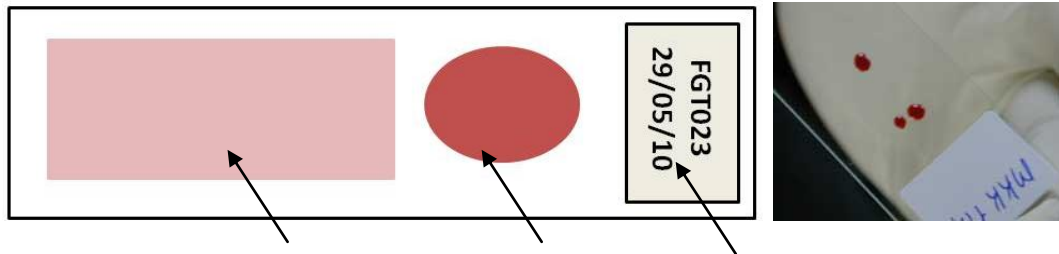
- Glass slides that are clean and grease-free (this may require cleaning with ethanol prior to going to the field).
- Slide box
- Permanent marker pen
- Sticker label
- Alcohol pads
- Lancets
- Sharps bin

Procedure for preparing a blood slide from finger/heel prick sample (following from protocol describing how to take a finger prick):

1. Wipe away the first droplet of blood.
2. Squeeze gently to obtain drop of blood.
3. Discard used lancets directly into the sharps disposal container
4. Using two completely clean *grease-free* microscope slides collect blood directly onto labelled slides: a small drop of blood to the centre of the slide for thin smear and a large drop about 1-2 cm away from the frosted end of microscope slide.

Making thick and thin blood films

Thick and thin blood films should be made on the same slide as shown below. The picture on the right indicates the required quantity of blood.



Thin smear

Thick smear

Sticker

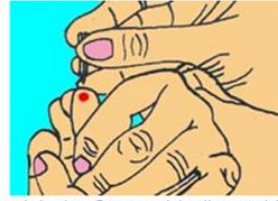
5. Immediately spread the thin film using a smooth edged slide spreader (Clean slide) inclined at 45° to horizontal line (a well made blood film should have a smooth tail end not ragged and be free of vertical lines and holes (a poorly spread film is extremely difficult to report because the red cells, parasites, and white cells will appear distorted))
6. Allow the blood film to air-dry with the slide in a horizontal position and placed in a slide boxes or any other safe place (where there is no risk of the blood coming into contact with dust or flies) for transport and staining.
7. Use the hairdryer to speed up the process of drying but keep it at least 30 cm away from the slide.
8. Be aware of flies and insect that might disturb the slide when drying in the open air. Try to find a place without flies or if not possible keep disturbing them.
9. Do not stain the slide until back in the laboratory.

Step 1



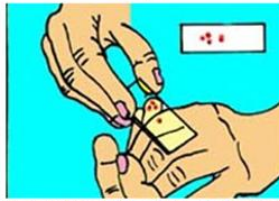
Hold the third finger of the left hand and wipe its tip with spirit/Savlon swab; allow to dry

Step 2



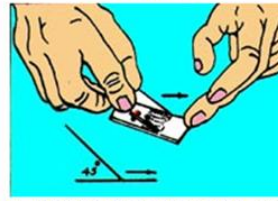
Prick the finger with disposable needle/lancet; allow the blood to ooze out

Step 3



Take a clean glass slide. Take 3 drops of blood 1 cm from the edge of the slide, take another drop of blood one cm from the first drop of blood

Step 4



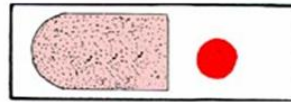
Take another clean slide with smooth edges and use it as a spreader...

Step 5



...and make thick and thin smears. Allow it to dry

Step 6



Prepared smear.
(Slide number can be marked on the thin smear with a lead pencil.)

Appendix 10: Plasma and cell pellet separation procedure

Protocol supplied by Teun Bousema

Definition

This protocol is for the separation of plasma from the finger prick samples that are collected in the cross-sectional surveys. This plasma will later be used in the ELISA, the cell pellet can be used for DNA extraction and parasite molecular detection.

Material

- Previously taken EDTA blood samples
- Screw cap tubes
- Centrifuge
- gloves
- Ice
- Pipette that measures 100µL
- Tips for 100µL
- Plasma sample log book

Procedure: to be done from previously taken blood samples in EDTA tubes.

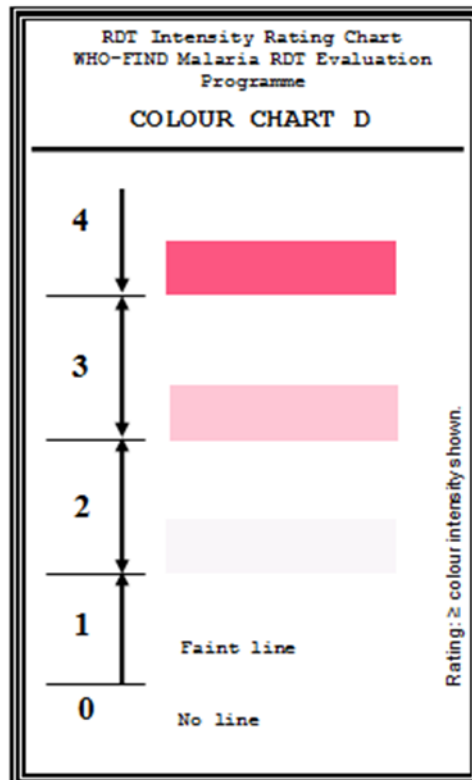
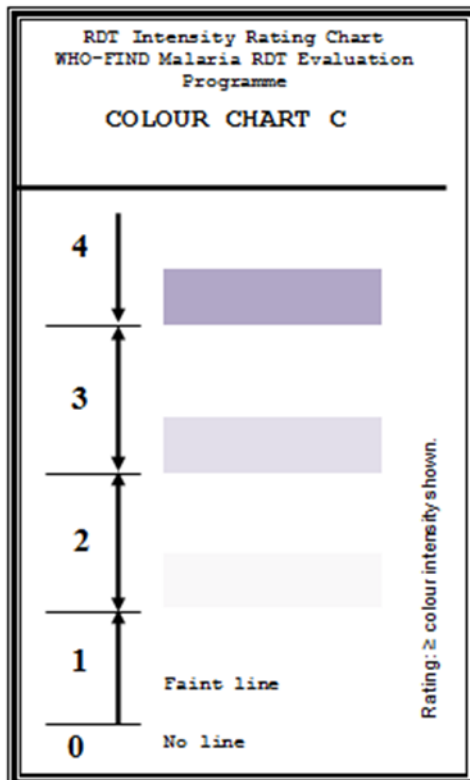
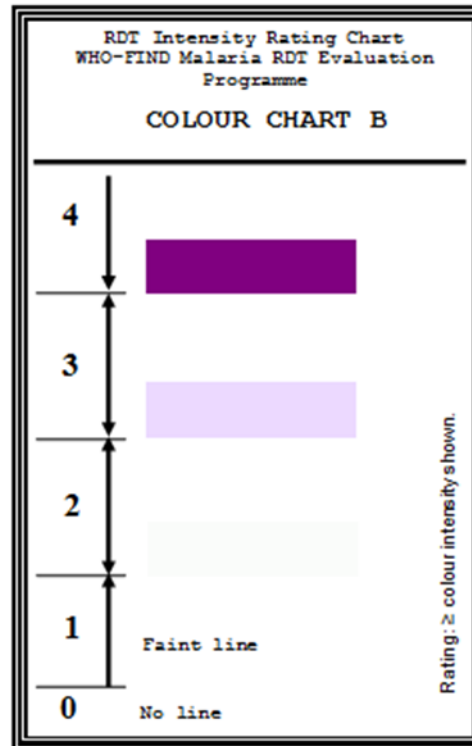
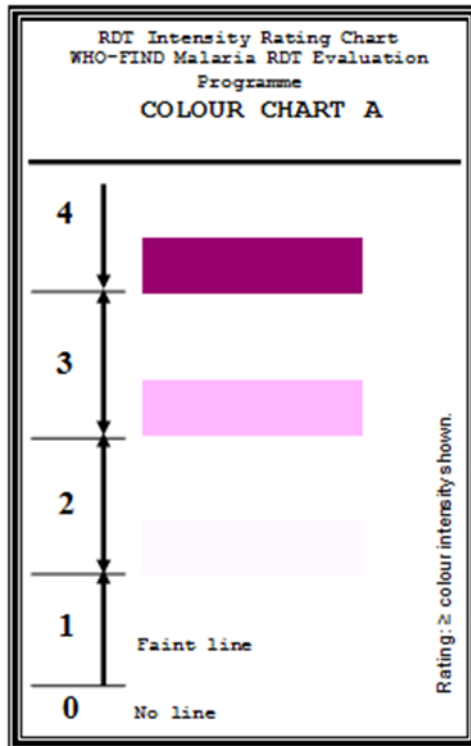
1. Label the screw cap tubes with the patient ID.
2. Blood should be transported to the lab in the original EDTA microtainer tubes in which the sample was collected, packed in cool boxes pre-packed with ice packs or ice cubes.
3. Avoid direct contact of the microtainer tubes with ice packs as this can cause haemolysis of the red blood cells. Also make sure that tubes are not frozen.
4. Remove tubes from cool boxes and put them into a tube rack.
5. If you are unable to process the samples on same day store blood tubes in the fridge (+4°C, not in the freezer) overnight.
6. Use the centrifuge to spin the blood down at a low speed for 3 minutes. The exact speed depends on the centrifuge; for a centrifuge with a maximum speed of 13000 rpm, the speed used is 3000 rpm. In general, the lowest speed at which there is a clear plasma separation in 3 minutes should be used; higher speeds can cause haemolysis.
7. Remove tubes carefully from centrifuge and put in tube rack in an upright position. Do this slowly to make sure that the plasma and blood cells do not mix. (If you make a mistake and the cells mix, just centrifuge again)

8. Label one empty clean tube with screw cap. You need to pre print stickers with the labelled patient ID for this purpose.
9. Transfer all of the plasma to the screw cap tube
10. Keep the cell pellet in the original tube. Close this tube and place it in -20°C (or -80°C) for future DNA extraction.
11. Carefully close the lid of the tubes.
12. Place the plasma tubes in -20°C (or -80°C)
13. Write in PLASMA log book that samples have been separated with the date and sign that you have stored them.

Appendix 11: WHO RDT scoring colour chart

(Provided by Dr. Jane Cunningham, WHO).

RDT Intensity Rating Charts for Stability Assessment



Appendix 12: Vacutainer blood collection

Protocol supplied by Teun Bousema

Collecting a venous bleed sample.

NOTE: Two venous bleed samples will be taken per person. 2x 4mls for individuals over the age of 5 and 2x 2.5ml for children under the age of 5. The first venous bleed is for the membrane feeding and serum replacement experiments. It is essential that the membrane feeders are prepared and ready before the venous blood samples are taken- please check!

MATERIALS

- Clean plastic disposable gloves
- Dry and wet swabs soaked in methylated spirit (70% ethanol)
- Swab containers
- Disposable venipuncture sampling set
- Sharps disposal container
- Heparinized tubes for venous blood collection
- Labels for Venous bleed 1 and 2.

VENOUS BLOOD SAMPLING

1. Write the unique patient number (3 digits) on the venous bleed sample labels (one label called venous1, the second called venous2) after 'AF1', along with the date of sampling. Attach the labels to the heparin tubes.
2. Support the forearm from which blood will be taken by a pillow or arm of chair.
3. Apply the tourniquet, place a finger behind the tourniquet clip to prevent the skin or hairs being pinched.
4. Place the tourniquet approximately 5-15 cm above planned puncture site.
5. Assess subject for a suitable vein.
6. Assemble equipment. Holding the coloured end of the needle sheath/luer adaptor in one hand, twist and remove the clear translucent end. Thread rubber covered needle into holder. For Luer adaptor attach winged infusion set.
7. Clean the skin with an alcohol swab and allow to dry.
8. Position the needle and holder in the direction of the vein. Do not connect the tube before the needle has pierced the skin.
9. Inform the subject that he/she may feel a sharp sting when the skin is pierced.
10. Pierce the skin directly over the vein and enter with a smooth and quick entry. The needle should enter the vein at a 10-15° angle.
11. Use the Heparin tube to collect blood.

12. With one hand secure the needle and holder. With the other hand press the tube onto the needle with the thumb. Once the blood is running, give a sign to the person in the insectary that he/she can prepare the feeders.
13. Remove tube when filled with the required amount of blood and mix by inverting 5-6 times gently.
14. Make sure 2 venous samples are taken per person (as mentioned in the notes).
15. Withdraw needle from vein in a quick movement. (Always remove the tube from holder before withdrawing the needle from the vein).
16. Discard needle and holder immediately into an approved sharps container. Needles should never be recapped.
17. Place cotton balls over the puncture site immediately after the needle has been withdrawn and apply direct pressure to the site.
18. The donor may apply pressure to the puncture site.
19. Remove gloves.

Appendix 13: Procedure for RNA collection in RNAProtect

Protocol supplied by Teun Bousema

Collecting, processing and storing RNA samples from venipuncture.

MATERIALS

- Clean plastic disposable gloves
- Disposal container for pipette tips
- 200 μ l pipette
- Disposable 200 μ L filter/barrier tips
- Eppendorf tubes with 500 μ L of RNAProtect (Qiagen) stabilizing buffer
- Labels for venous bleed RNAProtect samples
- Boxes for sample storage

RNA SAMPLE PROCESSING AND STORAGE

1. As soon as possible after blood has been taken in the heparin tube, do the following steps in a sterile environment.
2. Write the unique patient number (3 digits) on the venous bleed RNAProtect sample labels after 'AF1', along with the date of sampling. Attach the labels to the 3 RNAProtect aliquots.
3. Transfer 100 μ L of blood with a filter/barrier tip in the first pre-aliquoted and labeled tube with 500 μ L of RNAProtect stabilizing buffer, mix well by inverting the tube 5 times.
4. Repeat this for the second and third RNA samples.
5. Store all three at -80C in separate boxes for different locations.