TOWARDS THE DEVELOPMENT AND EVALUATION OF A TRANSMISSION-BLOCKING VACCINE FOR *PLASMODIUM FALCIPARUM* MALARIA

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Towards the Development and Evaluation of a Transmission-Blocking Vaccine for *Plasmodium falciparum* malaria

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ABSTRACT

Plasmodium falciparum malaria continues to evade control efforts in part through the complexity of its life cycle. While asexual parasites cause clinical illness in the human host, the highly specialized sexual stages transmit infection by uptake as intra-erythrocytic gametocytes from peripheral blood into a mosquito vector. Evidence from endemic settings suggests that naturally acquired immunity to sexual stage parasites may disrupt this cycle. Indeed in a vaccination model, antibodies to sexual stage antigens, ingested in the mosquito blood meal, can inhibit parasite growth in the insect midgut as judged by functional *ex vivo* experiments such as the standard membrane feeding assay (SMFA). To date, only a fraction of sexual stage antigens have been screened by this methodology; the list of proteins capable of eliciting the robust inhibitory responses required of a transmission-blocking vaccine (TBV) has changed little over the past 30 years. This thesis aims to strengthen the pre-clinical TBV pipeline by improving the characterization of known and novel candidate antigens. This study first establishes an independent and unbiased performance ranking of historical TBV antigens, and then uses this ranking as a benchmark to evaluate a diverse set of sexual stage proteins by heterologous expression and functional prioritization in SMFA. Finally, both known and novel proteins with exposure to human immunity are evaluated for seroreactivity in malaria-endemic Burkina Faso and Mali to better assess longitudinal antibody responses to potential TBVs. Proteins of little interest to vaccine development are considered as possible markers of gametocyte exposure in a Malian cohort with defined gametocyte positivity.

Preface

The following papers include work presented in this thesis.

A version of Chapter 1 has been published.

Nikolaeva, D, Draper SJ, Biswas S. *Toward the development of effective transmission-blocking vaccines for malaria*. Expert Rev Vaccines. 2015 May;14(5):653-80.

Adult Pfs230C, HAP2, and Pfs25 seroprevalence data has been published.

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Chapters

Chapter 1 Introduction

Chapter 2 Materials and Methods

Results: Part I

Chapter 3 Functional Comparison of Leading TBV Antigens

Chapter 4 Selection and Expression of a Panel of *P. falciparum* Sexual Stage Proteins

Chapter 5 Functional Evaluation of a Panel of *P. falciparum* Sexual Stage Proteins

Results: Part II

Chapter 6 Naturally Acquired Immune Responses to TBV Antigens

Chapter 7 Screening of a Panel of Sexual Stage Antigens for Markers of Gametocyte Exposure

Chapter 8 Conclusion

Table of Contents

Abstract.....i
Preface.....ii
Chapters.....iii
Table of Contents.....iv
List of Tables.....viii
List of Figures.....x
List of Abbreviations.....xiii
Acknowledgements.....xv
Chapter 1: Introduction.....1
1.1 Malaria.....1
1.1.1 Etiology.....2
1.1.2 Life cycle.....2
1.1.3 Pathophysiology.....4
1.2 Malaria Control Strategies.....5
1.2.1 Targeting the parasite with vaccines.....6
1.2.2 Pre-erythrocytic vaccines.....6
1.2.3 Blood stage vaccines.....7
1.2.4 Transmission-blocking vaccines.....7
1.3 Natural immune responses to sexual stage malaria.....8
1.3.1 Immune response and gametocyte density.....9
1.3.2 Immune response and transmission.....10
1.4 Vaccine-mediated immune responses to sexual stage malaria.....12
1.4.1 Experimental precedent for transmission-blocking vaccines.....12
1.5 The leading transmission-blocking vaccine candidate antigens.....13
1.5.1 Considerations for the ideal candidate.....13
1.5.1.1 Pre-fertilization antigens.....14
1.5.1.2 Post-fertilization antigens.....14
1.5.2 Leading pre-fertilization targets Pfs230 and Pfs48/45.....15
1.5.2.1 Pfs230 development.....16
1.5.2.2 Pfs48/45 development.....19
1.5.3 Leading zygote/ ookinete (post-fertilization) target Pfs25.....21
1.6 Additional transmission-blocking vaccine targets.....26
1.6.1 Pre-fertilization targets.....27
1.6.1.1 Pfs47.....28
1.6.1.2 HAP2.....28
1.6.2 Post-fertilization targets.....29
1.6.2.1 Crystalloid-derived proteins.....30
1.6.2.2 Micronemal proteins.....30
1.6.2.3 Parasite enolase and chitinase.....32
1.6.2.4 Plasmodium perforin-like proteins.....33
1.6.3 Mosquito midgut targets.....34
1.7 Improving antigen immunogenicity.....37
1.7.1 Antigen conjugation and viral-like particles (VLPs).....37
1.7.2 Viral vectors.....39
1.8 Thesis Overview.....40
Chapter 2: Material and Methods.....42

2.1 Materials 42
2.1.1 Reagents....42
2.1.2 Solutions, buffers, and cell culture media....44
2.1.3 Adjuvants 47
2.2 Methods 47
2.2.1 Molecular biology 47
2.2.1.1 Antigen inserts 47
2.2.1.2 Plasmids....52
2.2.1.3 Viral vectors 53
2.2.1.4 Agarose gel electrophoresis....54
2.2.1.5 Polymerase Chain Reaction (PCR)....54
2.2.1.6 Restriction endonuclease digests....55
2.2.1.7 Ligation reactions 56
2.2.1.8 Bacterial transformation 56
2.2.1.9 Plasmid DNA preparations 57
2.2.2 Protein production 58
2.2.2.1 Maintenance of HEK293E cell line 58
2.2.2.2 Transformation 58
2.2.2.3 SDS-PAGE....59
2.2.2.4 Western blotting 60
2.2.3 Immunology 60
2.2.3.1 Animals 60
2.2.3.2 Immunizations 61
2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA) 62
2.2.5 Indirect Fluorescence Assay (IFA) 63
2.2.6 IgG purification 64
2.2.7 Parasitology 65
2.2.7.1 *P. falciparum* sexual stage culture 65
2.2.7.2 Standard Membrane Feeding Assay (SMFA)....65
2.2.8 Epidemiology 66
2.2.9 Statistics....68
Chapter 3: Functional Comparison of Leading TBV Antigens 69
3.1 Introduction 69
3.2 Results 77
3.2.1 Generation of TBV candidate ChAd63 and MVA viral vectors .77
3.2.2 Expression of TBV antigens in HEK293E cells 78
3.2.3 Immunogenicity of protein-in-adjuvant and viral-vectored vaccination 85
3.2.4 Inhibitory activity of vaccine-induced purified IgG by SMFA....90
3.3 Discussion....94
Chapter 4: Selection and Expression of a Panel of *P. falciparum* Sexual Stage Proteins....98
4.1 Introduction 98
4.2 Results 102
4.2.1 Selection of Candidates 102
4.2.2 Generation of a panel of sexual stage *P. falciparum* proteins 107
4.2.3 Recombinant *P. falciparum* proteins generate high titer IgG as vaccine antigens....114

4.2.4 Recombinant P. falciparum proteins recreate native parasite epitopes 120
4.3 Discussion....125
Chapter 5: Functional Evaluation of a Panel of *P. falciparum* Sexual Stage Proteins....128
5.1 Introduction....128
5.2 Results....135
5.2.1 Screening for transmission-blocking epitopes by SMFA....135
5.2.2 Modeling inhibition of oocyst intensity from multiple feeding experiments....145
5.2.3 Evaluation of additive effects between anti-enolase and anti-Pfs25 IgG....150
5.2.4 Recombinant *P. falciparum* Proteins are immunoreactive....159
5.3 Discussion....163
Chapter 6: Naturally Acquired Immune Responses to TBV Antigens....166
6.1 Introduction....166
6.1.1 Host immunity and gametocytemia....167
6.1.2 Transmission-reducing immunity....168
6.2 Results....170
6.2.1 Malian adult serology....170
6.2.1.1 Malian adult antibody response to erythrocytic stage antigens 172
6.2.1.2 Malian adult antibody response to sexual stage antigens..173
6.2.1.3 Effect of age on Malian adult antibody response....175
6.2.2 Malian adult serology relative to Malian children....178
6.2.3 Longitudinal changes in Pfs230C-specific IgG response in Malian children....183
6.2.3.1 Effect of season on Pfs230C-specific IgG response in Malian children 184
6.2.3.2 Correlation of antibody responses between antigens by season in Malian children....187
6.2.3.3 Effect of age on Pfs230C-specific IgG response in Malian children 189
6.2.4 Functional activity of plasma from Malian children....191
6.2.5 Entomology....195
6.3 Discussion....196
Chapter 7: Screening a Panel of Sexual Stage Antigens for markers of Gametocyte Exposure....199
7.1 Introduction....199
7.2 Results....203
7.2.1 Study design and sample collection....203
7.2.2 IgG responses to sexual stage antigens in Malian individuals 204
7.2.3 Optimization of ELISA....206
7.2.4 Down-selections of antigens for broader serological screening 211
7.2.5 Positive IgG titer correlates to gametocyte positivity in a subset of antigens....212
7.3 Discussion....218

Chapter 8: Conclusions and Future Directions 220
8.1 Summary.....220
8.2 Conclusions and future directions 222
8.2.1 TBV antigens demonstrate a rank order of inhibitory IgG 222
8.2.2 Qualified SMFA with purified IgG provides a powerful platform for antigen testing.....224
8.2.3 HEK293 system enables moderate-throughput approach to rationally screen *P. falciparum* proteins.....225
8.2.4 Rational screen identifies novel components of the *Plasmodium* transmission pathway.....227
8.2.5 TBV targets are also the subject of naturally acquired immune response 228
8.2.6 Protein panel enables screen for markers of parasite exposure 230
8.3 Final remarks 231
Bibliography 232
Appendix: Standard Operating Procedures.....251

List of Tables

Table 2.1 Commercially available reagents used in this thesis 42

Table 2.2 Commercially available antibodies used in this thesis.....44

Table 2.3 Components of Phusion® High-Fidelity PCR mixture 55

Table 2.4 Standard reaction settings for Phusion® High-Fidelity PCR 55

Table 2.5 Oligonucleotide primers used in this study.....55

Table 2.6 Restriction endonuclease digest reaction.....56

Table 2.7 DNA ligation reaction 56

Table 3.1 Antigen sequences used for generation of ChAd63/MVA and protein TBV constructs.....80

Table 4.1 Defining features of a panel of *P. falciparum* sexual stage proteins 106

Table 5.1 SMFA A: Percent inhibition of mean oocyst intensity at 0.75 mg/ml 141

Table 5.2 SMFA B: Percent inhibition of mean oocyst intensity at 0.75 mg/ml 141

Table 5.3 Percent inhibition of mean oocyst intensity at 1.5 mg/ml.....145

Table 5.4 Percent inhibition of mean oocyst intensity with serial concentrations of anti-Pfs25 IgG 151

Table 5.5 Mix A: Percent inhibition of mean oocyst intensity of anti-Pfs25 and anti-enolase IgG.....153

Table 5.6 Mix B: Percent inhibition of mean oocyst intensity of anti-Pfs25 and anti-enolase IgG.....156

Table 6.1 Demographic Data: Malian Adult Cohort (2009) 171

Table 6.2 Demographic Data: Malian Children Cohorts.....179

Table 7.1 Prevalence of positive IgG response among Malian cohort (n=16, paired).....206

Table 7.2 Antigen expression by IFA 211

Table 7.3 *P. falciparum* stage-specific expression 216

List of Figures

Figure 1.1 The *P. falciparum* life cycle 4

Figure 3.1 Dose-dependent inhibition of oocyst intensity by anti-TBV IgG in SMFA 73

Figure 3.2 Effect of mouse IgG induced by ChAd63-MVA immunization on P. *falciparum* NF54 parasite infectivity in *A. stephensi* mosquitoes. 75

Figure 3.3 Expression construct design 79

Figure 3.4 Heterologous expression of TBV antigens 82

Figure 3.5 Pfs48/45$_{+NGln}$ expression and purification 83

Figure 3.6 Pfs230C expression and purification.....84

Figure 3.7 Vaccination schedule 86

Figure 3.8 Immunogenicity of protein-in-adjuvant and viral-vectored regimes ..89

Figure 3.9 Standard membrane feeding assay 91

Figure 3.10 Multi-feed prediction model 93

Figure 4.1 Selection of a panel of sexual stage antigens for expression in HEK293E cells 105

Figure 4.2 HEK293E expression construct design 109

Figure 4.3 Heterologous expression of surface and secreted *P. falciparum* sexual stage proteins 111

Figure 4.4 PNGase F treatment of recombinant proteins reveals N-glycosylation 113

Figure 4.5 Endpoint titer ELISA reveals high titer IgG responses to vaccine antigens 115

Figure 4.6 IgG responses generated to the protein tag can be depleted 116

Figure 4.7 Pfs25 titers to clinical construct, TBV25H, parallel recognition of the vaccine antigen 119
Figure 4.8 Recombinant *P. falciparum* sexual stage antigens recreate native parasite epitopes.....124
Figure 5.1 Metrics of malaria transmission.....134
Figure 5.2 Several *P. falciparum* sexual stage proteins demonstrate transmission-blocking epitopes by SMFA 140
Figure 5.3 Test IgG are evaluated by SMFA at 1.5 mg/ml 144
Figure 5.4 Two-feed prediction model estimates oocyst inhibition by SMFA ..147
Figure 5.5 Multiple-feed prediction model estimates oocyst inhibition by SMFA 149
Figure 5.6 Percent inhibition of mean oocyst intensity with serial concentrations of anti-Pfs25 IgG 152
Figure 5.7 Evaluation of anti-Pfs25 and anti-enolase IgG in one membrane feeder by SFMA 154
Figure 5.8 Repeat evaluation of anti-Pfs25 and anti-enolase IgG in one membrane feeder by SMFA.....157
Figure 5.9 Overlay of two SMFA studies evaluating interaction of anti-Pfs25 and anti-enolase IgG.....158
Figure 5.10 *P. falciparum* proteins demonstrate seroreactivity in two independent cohorts from malaria-endemic regions. 160
Figure 5.11 Protein seroreactivity correlates between independent cohorts from Burkina Faso and Mali 162
Figure 6.1 Malian adult IgG antibody responses against erythrocytic and sexual stage antigens.....174

Figure 6.2 Malian adult IgG antibody responses against erythrocytic and sexual stage antigens by age and season 177
Figure 6.3 Comparison of IgG antibody responses to Pfs230C and HAP2 in Malian child and Malian adult cohorts 181
Figure 6.4 Comparison of IgG responses to Pfs230C in Malian children and adults by age and season 182
Figure 6.5 Effect of season on longitudinal Pfs230C antibody responses in Malian children 186
Figure 6.6 Correlation of IgG levels by antigen 188
Figure 6.7 Effect of age on longitudinal Pfs230 IgG response in Malian children 190
Figure 6.8 Functional SMFA of purified IgG from cohort of Malian children 193
Figure 6.9 Functional GIA of purified IgG from a cohort of Malian children 194
Figure 7.1 Development time-course of sexual stage antigen ELISA 208
Figure 7.2 Time-course depicting ratio of median IgG response to antigen panel 210
Figure 7.3 Serological response relative to gametocyte positivity 213
Figure 7.4 Serological response relative to gametocyte positivity by season..215
Figure 7.5 Protein expression in asexual stage schizonts by IFA 217

List of Abbreviations

ACT	Artemisinin-based combination therapy
ARDS	Acute respiratory distress syndrome
AMA1	Apical membrane antigen 1
CI	Confidence interval
ChAd63	Adenovirus chimpanzee serotype 63
CHO	Chinese hamster ovary
CSP	Circumsporozoite protein
CTRP	Circumsporozoite and TRAP related protein
CtxB	Cholera toxin subunit B
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
EPA	*Pseudomonas aeuruginosa* exoprotein A
ER	Endoplasmic reticulum
GLA-SE	Glucopyranosyl lipid adjuvant in a stable emulsion
GLURP	Glutamate-rich protein
GPI	Glycosylphosphatidylinositol
GST	Glutathione-S-transferase
HEK	Human embryonic kidney
HuAd5	Human adenovirus serotype 5
im	Intramuscular
IFNγ	Interferon gamma
ITN	Insecticide-treated bed net
LCCL	Limulus clotting factor C. Coch-5b2, Lgl 1 domain
mAb	Monoclonal antibody
MACPF	Membrane attack complex/perforin
MAOP	Membrane attack ookinete protein
MFA	Membrane feeding assay
mRNA	Messenger ribonucleic acid
MSP1	Merozite surface protein 1

MVA	Modified vaccinia virus Ankara
NF54	*P. falciparum* parasite isolate
OMPC	Outer membrane protein complex
PbLAP	*P. berghei* LCCL-lectin adhesive-like proteins
PfCCP	*P. falciparum* Limulus coagulation factor C domain proteins
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PNGase	Peptide N-glycosidase
PSOP	Putative secreted ookinete protein
RBM	Roll Back Malaria Partnership
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMFA	Standard membrane feeding assay
SOAP	Secreted ookinete adhesive protein
TBI	Transmission-blocking immunity
TBV	Transmission-blocking vaccine
TNFα	Tumor necrosis factor alpha
VLP	Viral-like particle
WARP	von Willebrand factor A domain-related protein
WGCFS	Wheat germ cell-free system
WHO	World Health Organization

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Chapter 1: Introduction

1.1 Malaria

The continued burden of malaria profoundly impacts public health, overwhelming treatment centers and limiting economic stability in the worst affected regions. Its effect on global morbidity and mortality ranks malaria with tuberculosis and HIV, which together comprise the so-called 'Big Three' infectious diseases – intertwined epidemics responsible for 10% of deaths worldwide [1]. In 2014, 97 countries and territories had documented malaria transmission, with an estimated 3.3 billion people at risk of infection. By the most recent reports, in 2013 there were an estimated 584,000 deaths out of an estimated 198 million cases, with the greatest burden of clinical infection (80% of global cases) and death (90% of global cases) in sub-Saharan Africa, among children under the age of five and women with a first pregnancy [2]. Globally, the disease was responsible for 435,000 deaths in children under five in 2013 alone. The widespread toll of malaria infection can also be felt through loss of workforce productivity and reduced population growth, which when coupled with the massive costs of control and preventative measures, translates into a direct negative correlation with long-term economic output [3].

1.1.1 Etiology

Clinical malaria is the result of infection with protozoan parasites from the genus *Plasmodium*, transmitted through the bite of an infected female *Anopheles* mosquito. Six species have been shown to infect humans, of which *P. falciparum* confers the greatest mortality, with significant contributions in morbidity from *P. vivax* and additional species (human malaria parasites *P. malariae* and *P. ovale spp.*, and the occasionally zoonotic *P. knowlesi* [4] and *P. cynomolgi* [5]) to a lesser degree. Unless otherwise specified, subsequent reference to malaria will denote the *P. falciparum* parasite.

1.1.2 Life cycle

The *P. falciparum* life cycle is divided between the human host, giving rise to pre-erythrocytic and blood stages, and the mosquito vector, supporting parasite sporogonic development. Importantly the sexual stages span the human and mosquito hosts (Figure 1.1).

During pre-erythrocytic infection, several hundred sporozoites are injected into the skin during probing by an infected *Anopheles* mosquito. These sporozoites target, invade, and proliferate in human liver hepatocytes before rupturing the host cell to initiate blood stage infection. Notably, a proportion will remain in the skin or drain to lymph nodes, where they are susceptible to immune recognition [6, 7]. Blood stage infection begins when merozoites released from infected hepatocytes invade human erythrocytes. Inside the erythrocyte, the merozoite undergoes an asexual replication process wherein it passes through several

developmental forms including the ring-stage trophozoite, mature trophozoite, and schizont. Schizonts rupture their host erythrocyte to release merozoites, which continue the cycle of blood stage infection by re-invading new erythrocytes.

During blood stage asexual replication, a small subset of parasites undertakes the sexual pathway [8, 9] as gametocytes. Gametocytes undergo four developmental forms (stages I-IV) while sequestered from the peripheral circulation [10] before appearing as mature stage V intra-erythrocytic male and female gametocytes in the peripheral blood. Stage V parasites are taken up in the blood meal of a female *Anopheles* mosquito, where due to environmental triggers, parasites exit the erythrocyte as gametes to undergo fertilization in the insect midgut. Within 24 hours of mosquito infection, zygotes develop into motile ookinetes that by 48 hours will traverse the midgut epithelium. At the basal lamina, ookinetes differentiate into oocysts that after approximately 2 weeks and multiple rounds of division, rupture to release sporozoites that then travel to the mosquito salivary glands to subsequently re-initiate infection of a human host.

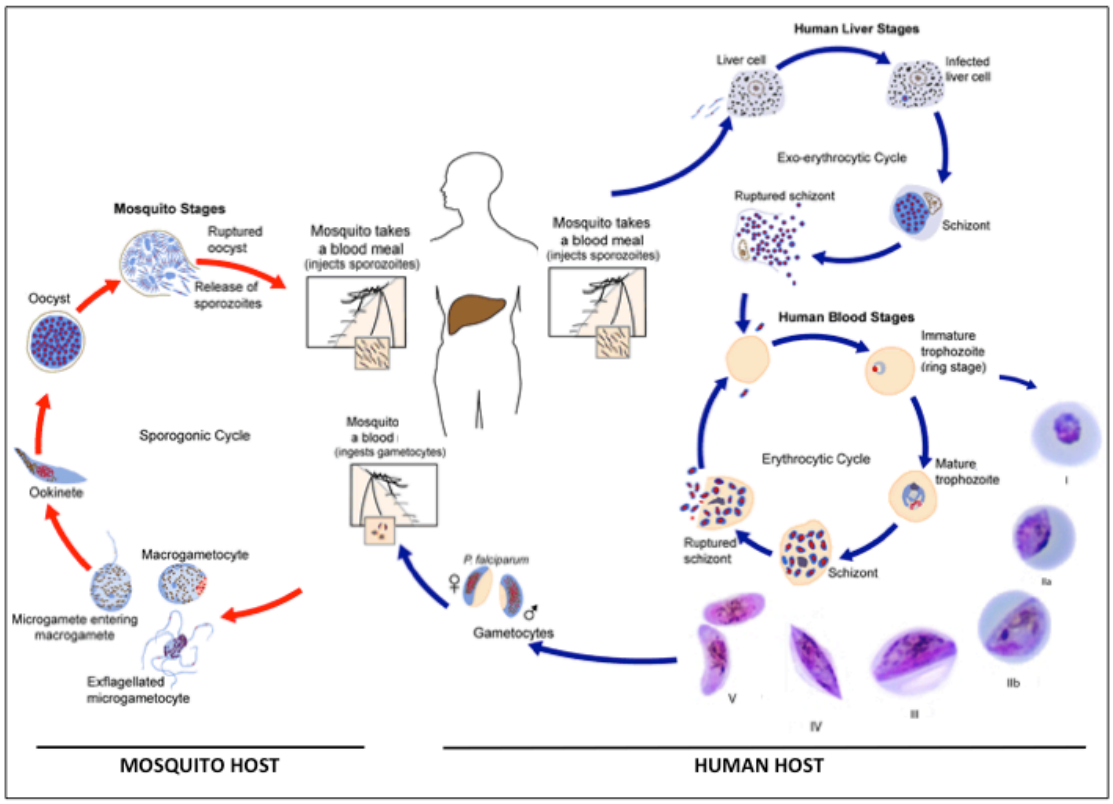


Figure 1.1 The *P. falciparum* life cycle

The life cycle of *Plasmodium* parasites is comprised of multiple stages, divided between the human host (blue arrows) and mosquito vector (red arrows). Adapted from [11, 12].

1.1.3 Pathophysiology

Malaria infection has a spectrum of presentations ranging from asymptomatic or mild – classified broadly as uncomplicated – to severe or complicated disease. Complicated malaria typically undertakes one of three possible overlapping clinical pictures, which include cerebral malaria, severe malarial anemia, or acute respiratory distress syndrome (ARDS). *P. falciparum* is most often associated with a severe clinical presentation. Clinical symptoms of malaria result entirely from the asexual-blood stage cycle of erythrocyte rupture and re-invasion. Parasite by-products such as hemozoin and

glycosylphosphotidylinositol (GPI) released during erythrocytic lysis stimulate production of pro-inflammatory cytokines. These factors, including TNFα and IFNγ, promote the classic cyclic malarial fever as well as severe pathophysiology defined by blood and metabolic abnormalities and end-organ damage. So too, PfEMP1-mediated adherence of infected erythrocytes, corresponding to the mature trophozoite parasite stage, to host endothelium, may act in vascular occlusion and end-organ dysfunction as seen in cerebral malaria [13].

1.2 Malaria Control Strategies

Efforts to limit the spread of infection and reduce the burden of disease have targeted the parasite as well as the vector, but these two branches of control experienced dramatic setbacks towards the end of the last century [14]. An unexpected surge in the resistance of parasites to synthetic antimalarial drugs, most notably chloroquine, as well as a reluctance to promote the use of environmentally-damaging insecticides in the face of growing mosquito resistance – all compounded by the increasing pressure of operational costs – made the call to eradication increasingly untenable [14]. As a result, over the last decade, the global community has made a remarkable, renewed commitment to malaria control. Interventions including improved antimalarial regimens such as artemisinin-based combination therapies (ACTs), insecticide-treated bed nets (ITNs), and indoor insecticide residual spraying campaigns, led to reductions of 17% in malaria case incidence and 26% in malaria-specific mortality between 2000 and 2010 [15, 16].

These gains were substantial enough that at the World Malaria Forum in October 2007, partners such as the Bill and Melinda Gates Foundation, the World Health Organization (WHO), and the Roll Back Malaria Partnership (RBM) once again emphasized eradication as the ultimate goal of the international malaria research and control agenda [17, 18]. In line with the research strategy born out of the 2007 call to action, the current burden of malarial disease emphasizes that target objectives will not be achieved without new approaches and innovative technological breakthroughs, such as vaccines [19]. Most notably, the updated Malaria Vaccine Technology Roadmap calls for vaccines to eliminate malaria in multiple settings by 2030, and specifies vaccines to prevent parasite transmission as a critical component technology to support this aim [20, 21].

1.2.1 Targeting the parasite with vaccines

The evasive nature of the malaria parasite in spite of multi-faceted control strategies is due, in part, to its diverse life cycle stages. Vaccines are currently in development against multiple life cycle targets, and can be divided broadly into pre-erythrocytic, asexual blood stage, and sexual stage candidates.

1.2.2 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines act after a mosquito first transmits sporozoites into the human circulation with an infectious bite. Vaccine candidates aim to generate an antibody-mediated response against parasites travelling towards the liver, as

well as a cytotoxic $CD8^+$ T cell response to infected hepatocytes. Pre-erythrocytic vaccines intend to prevent blood stage infection, the cause of clinical malaria, and include the most clinically advanced vaccine candidate RTS,S – a subunit vaccine based on the parasite circumsporozoite protein (CSP) [17]. In theory, a highly effective pre-erythrocytic vaccine would have the added benefit of indirectly arresting transmission by stopping parasite development at or before the liver. At this time there is no evidence to suggest that antibodies specific for CSP directly inhibit oocyst or sporozoite formation in the mosquito [22] to elicit so-called classical transmission blockade (discussed in Section 1.2.4).

1.2.3 Blood stage vaccines

Blood stage vaccines are primarily meant to minimize clinical symptoms of disease after infection has occurred, but could, if highly effective, aid in parasite clearance and block transmission. Most vaccine candidates target various checkpoints during the process of erythrocyte reinvasion. Protection by blood stage vaccines is thought to be primarily antibody-mediated, although there is evidence to suggest a role for T cell effector mechanisms in generating the response [14, 23].

1.2.4 Transmission-blocking vaccines

Unique in their strategy, vaccines against the sexual stage do not directly prevent infection or clinical symptoms within the human host, but rather impact upon the parasite life cycle in the mosquito vector. Human immune factors

taken up with gametocytes in the mosquito blood meal remain active in the mosquito midgut and are able to interfere with parasite survival, primarily though the action of antibodies, but in some cases via activation of the complement lysis cascade [24, 25].

In effect, arrest of the life cycle at this stage acts to reduce transmission; vaccine candidates aimed at antigens with roles in sexual development and survival in the mosquito midgut are termed classical transmission-blocking vaccines (TBV). A distinction can be made between TBVs and vaccines that interrupt malaria transmission (VIMT); VIMTs encompass vaccines acting at pre-erythrocytic and blood stages that can also result in reduced parasite transmission.

1.3 Natural immune responses to sexual stage malaria

As delineated by Bousema and Drakeley [11], natural immunity to the malaria sexual stage can theoretically impact parasite development and survival in three ways.

1. Immune responses interfering with sequestration mechanisms may prevent gametocyte maturation by promoting premature clearance, thus reducing the gametocyte infectious reservoir in the human host.
2. Active recognition of circulating gametocytes may increase immune-mediated clearance and reduce gametocyte density.

3. Immune recognition of gametocyte antigens, which persist and act during sporogonic development in the mosquito midgut, may interrupt transmission.

While the basis for the first two means is primarily theoretical, evidence for the third, while controversial, is in concert with the rationale for transmission-blocking vaccine development.

1.3.1 Immune response and gametocyte density

Currently, there is no strong evidence to suggest that natural immune responses to the gametocyte stage, whether targeting sequestration mechanisms or by direct clearance, significantly affect circulating gametocyte density during infection.

While sequestration is a critical step in gametocyte maturation, presumably allowing early developmental forms to avoid immune clearance by the spleen, the differential contributions of cytoadherence by receptor-ligand mediated interaction versus biophysical properties, such as rigidity of the infected erythrocyte, have yet to be fully elucidated. One line of research suggests that deformability of immature intra-erythrocytic gametocytes *in vitro* progressively decreases and is rapidly restored in mature stage V forms, potentially allowing their release into the peripheral blood [26]. *In vitro* data on receptor-ligand mediated adhesion, a mechanism more vulnerable to immune-mediated blockade, shows that similarly to trophozoites, early stage gametocytes (stages I and IIa) use CD36 as the main host ligand for adherence [27]; consequently,

asexual stage immunity may also exert an effect on early gametocytes though this has not been directly reported. A parallel line of inquiry into PfEMP1, the parasite-specific CD36 binding partner, supports the possibility that asexual cross-reactivity may affect early gametocyte sequestration [28]. PfEMP1 recognition has also been reported to be a direct target of antibody-dependent cell-mediated cytotoxicity, resulting in decreased density of gametocytes and trophozoites [29].

Stage-specific, parasite proteins on the surface of circulating erythrocytes infected with mature, stage V gametocytes offer another potential target of host immunity [30], but little evidence for such protein targets exists. One study, using the plasma of children from the Gambia, reported recognition of erythrocytes infected with stage V gametocytes by flow cytometry, and noted that recognition of surface antigens was associated with decreased gametocyte densities four weeks after anti-malarial therapy [31]. An earlier field study – one of the only to suggest a suppression of gametocyte density independent of asexual immunity – described diminished incidence of gametocytemia in individuals with seroreactivity to gametocytes by indirect fluorescence assay [32]. While parasite proteins on the surface of the gametocyte-infected erythrocyte could present interesting vaccine targets, this avenue has been left largely unexplored.

1.3.2 Immune response and transmission

After gametocytes are taken up in the mosquito blood meal, the ensuing

parasite stages are especially vulnerable to immune recognition. In addition to mosquito immune factors, the blood meal components include human antibodies, leukocytes, and complement. Transmission-blocking immunity, therefore, may include both antibody-mediated and cell-mediated effector mechanisms [33]. An early study demonstrated the ability of human leukocytes to survive for several hours in the mosquito midgut, but questioned whether mosquito midgut conditions would support normal functional activity [34]. Still, the presence of viable leukocytes may support the action of antibodies in transmission reduction [35, 36]. One study demonstrated gamete opsonization and phagocytosis by chemiluminescence assay using serum from donors living in malaria-endemic Cameroon and Tanzania. Parallel mosquito feeding experiments, using the blood of a gametocyte carrier, confirmed that sera showed transmission reduction in the presence of viable leukocytes [35]. Finally, outside of antibody- and cell-mediated effects there is evidence for nonspecific immune factors influencing gametocyte transmission. TNFα has been shown to inactivate gametocytes in the presence of leukocytes [218,312] and may decrease the infectivity of gametocytes during paroxysm [37-39], defined as the periodic chills, fever, and sweats occurring between relatively symptom-free periods.

Membrane feeding assays (MFA) have been further used to assess plasma from individuals living in malaria-endemic areas for naturally acquired antibody-mediated transmission-blocking activity (TBA). Briefly, mosquitoes are fed on *P. falciparum* gametocytes from *in vitro* culture (standard membrane feeding assay, SMFA) or from naturally infected individuals (direct membrane feeding

assay, DMFA) in the presence or absence of test plasma. Studies based in the Gambia, Cameroon, and Kenya report a reduction in both oocyst intensity and the prevalence of infected mosquitoes in the presence of test plasma relative to control serum from malaria naïve individuals [40, 41]. The significance of these immune responses remains controversial although functional TBA does associate with host antibody responses against known gametocyte/gamete surface antigens Pfs230 and Pfs48/45 in most, but not all studies [42-44].

1.4 Vaccine-mediated immune responses to sexual stage malaria

With recent reports of parasite resistance to first-line artemisinin [45, 46] and a plateau in the commitment of international funds [16], the traditionally understudied sexual stages (defined here to include developmental forms from the gametocyte through the oocyst) have re-entered the spotlight as increasingly interesting targets for vaccine strategies. The main aim for these approaches is to induce antibodies against parasite surface antigens, which when taken up in a mosquito blood meal, prevent successful parasite development in the midgut.

1.4.1 Experimental precedent for transmission-blocking vaccines

The scientific rationale for TBVs is not a new concept. It was first reported in 1958 in turkey and chicken models [14, 47]. This observation was revisited in 1976 with an immunization regime of formalin-fixed *P. gallinaceum* gametocytes in chickens, reaffirming that it is experimentally possible to achieve transmission-blocking immunity (TBI) [14, 48]. A further study immunizing

chickens with X-irradiated exo-erythrocytic gametes elicited more robust TBI and further suggested that the effectors of the response were serum-based factors acting on parasites in the mosquito vector rather than the avian host [14, 49]. Analogous reports soon demonstrated a similar induction of TBI in mice with *P. yoelii* [50] and in primates with *P. knowlesi* [51]. Specialized improvements in the *in vitro* culture of *P. falciparum* for the sexual stage [52] enabled the first production of transmission-blocking monoclonal antibodies (mAbs) in 1983. Generated via immunization of BALB/c mice with *P. falciparum* gametes, several mAbs were identified which could arrest parasite development in the mosquito midgut [53].

Collectively, these studies emphasized the role of antibodies against surface antigens of the malaria parasite [53-55] as the critical mediator of TBV efficacy, and surface radio-isotope labeling of gametes followed by precipitation with transmission-blocking mAbs identified the first list of TBI targets [53]. These included Pfs25, Pfs48/45, and Pfs230, named for their size migration by SDS-PAGE. In *ex vivo* models such as SMFA, vaccine-induced antibodies generated against these individual candidate antigens have been shown to reduce the transmission capacity of the parasite.

1.5 The leading transmission-blocking vaccine candidate antigens

1.5.1 Considerations for the ideal candidate

Existing parasite-specific TBV targets can be divided broadly into candidates that have expression in the human host, i.e. pre-fertilization targets such as

Pfs48/45 and Pfs230, and candidates expressed at later stages exclusively in the mosquito vector, i.e. post-fertilization targets such as Pfs25 and Pfs28. This theoretical divide is relevant from the perspective of TBV candidate prioritization.

1.5.1.1 Pre-fertilization antigens

The induction of sustained, high-titer antibody responses with transmission-blocking activity is a necessary criterion for antigens to move forward into human clinical vaccine studies. Exposure to pre-fertilization targets in the human host offers the possibility of boosting antibody responses through the course of natural infection. Sero-epidemiology studies of anti-Pfs48/45 and anti-Pfs230 IgG responses in malaria-exposed individuals suggest that specific antibodies do occur in endemic settings. Furthermore, as previously mentioned, immune serum with specificity for Pfs230, and occasionally Pfs48/45, has been shown to correlate to transmission-blocking activity of that serum [42-44].

1.5.1.2 Post-fertilization antigens

Post-fertilization targets, by contrast, do not benefit from natural boosting responses, but also do not experience immune-selective pressure in the human host; such candidates on the whole have a lower incidence of antigenic polymorphism as a result. There is remarkably little polymorphism in the Pfs25 gene between field isolates of *P. falciparum* [56]. A recent study evaluated transmission-blocking activity of anti-Pfs25 plasma from the Pfs25/ISA51 Phase Ia trial [57] against *P. falciparum* parasites from patients living in either Burkina

Faso or Thailand. The plasma had a significant transmission-blocking effect against parasite isolates from both field sites relative to malaria-naïve controls, and microsatellite sequencing revealed only one non-synonymous nucleotide polymorphism in 1 of 41 patients from both sites [58]. But of note, both Pfs230 and Pfs48/45 also display lower levels of sequence variation than blood stage antigens, such as AMA1, [59, 60]. This is possibly the result of gametocyte sequestration [26] or modifications to the host cell surface of these intraerythrocytic parasites, both suggested to play a role in immune evasion.

1.5.2 Leading pre-fertilization targets Pfs230 and Pfs48/45

Despite the attention given to two of the earliest identified TBV candidates – Pfs230 and Pfs48/45, their conformational expression as recombinant antigens is still in progress several decades later. While these proteins remain two of the best-characterized targets of vaccine-induced transmission-blocking immunity, both have yet to be evaluated in human clinical trials. Pfs48/45 and Pfs230 are co-expressed mid-way through gametocyte development, on the female macrogamete as well as the male microgamete, and remain until the completion of fertilization [53, 61-63]. Functional knockout studies suggest an essential role for Pfs48/45 (as well as the *P. berghei* ortholog, Pbs48/45) exclusively in male fertility [64]. So too, disruption of Pfs230 demonstrates an inability of microgametes to form foci of fertilization with adjacent red blood cells, although the reduction in fertilization is incomplete, as demonstrated by a 92-96% inhibition of oocyst formation, suggesting an important but non-essential role [65, 66].

Recreating the native conformation of Pfs48/45 and Pfs230 transmission-blocking epitopes as vaccine immunogens has proven difficult. Both candidates are members of a protein family defined by a structural domain resulting from the disulfide bonding pattern of six conserved cysteine residues [67]. Pfs230 contains 14 sets of the 6-cysteine motif (resulting in 7 double domains of two structurally similar components), while Pfs48/45 contains 3 copies (forming 1.5 double domains) – a complication for both in the generation of correctly folded antigen [67]. Production of full-length Pfs230 antigen has been particularly challenging, with pre-clinical development lagging behind its partner Pfs48/45 – the greater focus of vaccine-development due to its more manageable size. Prior to gametocyte emergence, Pfs230 exists as a roughly 360-kDa precursor which then undergoes proteolytic cleavage to yield either 300- and 35-kDa or 307- and 47-kDa forms [68]. The larger 300- and 307-kDa fragments do not contain a glycosylphosphatidylinositol (GPI) anchor, but are membrane associated as a complex with the GPI-anchored Pfs48/45 on the gamete surface [69, 70].

1.5.2.1 Pfs230 development

The early characterized mAbs raised against Pfs230 – generated by intra-peritoneal vaccination of BALB/c mice with fresh NF54 strain *P. falciparum* macrogametes in the presence of lyophilized *Bordetella pertussis* – failed to block transmission and nearly resulted in the antigen being dismissed [62], until the observation that the functional activity was complement-mediated [71]. In

the latter study, CAF_1 mice were immunized with 3D7 clone *P. falciparum* macrogametes and zygotes in Freund's adjuvant by intra-peritoneal injection. Two isotype IgG2a mAbs, 1B3 and 2B4, were isolated and reported to give a 95-100% inhibition of oocyst intensity when used at concentrations of at least 100 µg/mL. In attempts to broadly identify the functional regions of interest, six sections (A-F) of the full-length Pfs230 protein were expressed as a series of recombinant antigens fused to maltose-binding protein (MBP) in an *Escherichia coli* system, purified on amylose resin and used to vaccinate mice [72]. The transmission-blocking epitopes were localized to the 300/307-kDa proteins. In particular, anti-serum generated against region C – an N-terminal 76-kDa fragment (amino acids 443 to 1132) – significantly reduced oocyst development, with inhibition of 71.2-89.8%, in the mosquito midgut as determined by SMFA. This was the first report of antisera to a recombinant gametocyte-stage protein, with demonstrated immunogenicity in natural human malaria infection [73], blocking transmission in an *ex vivo* assay [72]. However, unlike transmission-blocking mAbs 1B3 and 2B4, the antiserum generated to recombinant Pfs230C was unable to block transmission completely. Nevertheless, Pfs230C became the focus for subsequent vaccine development (see [66]).

An important breakthrough came in 2005 with sequence-based, fold prediction analysis of Pfs230 suggesting a structural similarity to another apicomplexan parasite protein, *Toxoplasma gondii* SAG1 [74]. Since this structural insight, there have been several encouraging reports of heterologous protein expression with improved folding. In the wheat germ cell-free system (WGCFS),

recombinant Pfs230C, along with a series of truncated sub-regions, were used to immunize rabbits in an attempt to identify the minimal region sufficient to induce a transmission-blocking response. Resulting antisera recognized the 360-kDa unprocessed Pfs230 precursor and induced a complement-mediated transmission-blocking activity against NF54 strain *P. falciparum* parasites. Moreover, unexpectedly all recombinant truncated forms partially reduced oocyst intensity in the presence and absence of active complement. In particular, the study draws attention to the Pfs230C0 cysteine-free pro-domain (amino acids 443-588), as the region of interest [75]. In addition to this study, more recently, a plant-based production system was used to generate soluble 'Pfs230CMB' (amino acids 444-730), which is highly immunogenic in rabbits and induces complement-mediated inhibition of oocyst intensity by SMFA (estimated at 99% when whole rabbit antiserum is tested at a final dilution of 1:3 in the membrane feeding apparatus) [76]. Perhaps the most advanced in the pre-clinical testing pipeline, Pfs230 subdomain 1 (D1), which contains the 6 cysteine-rich motif (amino acids not published), was generated using a modified *Pichia pastoris* system and has been tested in an *Aotus* model. As a formulation with synthetic Toll-like receptor 4 agonist GLA-SE (Glucopyranosyl Lipid Adjuvant in a Stable Emulsion) antibodies generated to Pfs230D1 block transmission by SMFA when used alone or in the presence of antibodies to Pfs25, generated in the same regime. Nanoparticles containing chemically conjugated Pfs25 and Pfs230D1 have been suggested as a promising future direction [77]. A Phase I trial with Pfs25 and Pfs230D1, dosed separately and in combination, in which each antigen is conjugated to *Pseudomonas aeruginosa*

ExoProtein A (EPA) and adjuvanted with Alhydrogel®, is ongoing (ClinicalTrials.gov NCT02334462).

1.5.2.2 Pfs48/45 development

Still, Pfs48/45 is arguably the more developed of the partner proteins in the pre-clinical pipeline [78], though its trajectory as a vaccine antigen has been similar to Pfs230. The full-length candidate was first cloned and expressed in 1993 in the insect baculovirus system in a conformation that was recognized by transmission-blocking mAbs [79], with no reported SMFA data. Two attempts were made in *E. coli* in 1998, with and without a glutathione *S*-transferase (GST) fusion, but neither induced a transmission-blocking immune response in either mice or rabbits [80]. An attempt by the same group in the same year incorporated the gene into a recombinant vaccinia virus, and an N-glycosylated product was expressed *in situ* in mammalian cells; immunization of mice and rabbits elicited Pfs48/45-specific antibodies, but the antisera failed to block transmission by SMFA [81]. Induction of expression in *S. cerevisiae* resulted in prematurely terminated transcripts, and despite assembly of an entirely synthetic gene with higher GC content to allow for completion of transcription, no recombinant protein could be detected [82]. Low yields were obtained in *P. pastoris* of a disulfide bridge constrained variant, which was not recognized by transmission-blocking mAbs and did not induce transmission-blocking serum in mice [82]. In 2007, expression of a two-domain fragment denoted 10C (amino acids 173-427) – comprising the middle four-cysteine and the C-terminal six-cysteine modules – in *E. coli* followed by a refolding protocol resulted in a

product estimated to have 10-20% conformational integrity. Of five mice vaccinated with the 10C construct in Freund's adjuvant, one was reported to have substantial transmission-blocking immunity [83].

Significant progress came a year later, with production of C-terminal Pfs48/45, (amino acids 159-428) as a fusion with MBP, with an estimated 90% conformationally native product in *E. coli*. As mentioned, while *E. coli* conveniently does not produce N-glycosylation variants, it also lacks the advanced ER machinery necessary for disulfide bond formation. Hence, disulfide bonds are formed in the periplasmic space catalyzed by a set of periplasmic oxidoreductases. These enzymes were upregulated in addition to a peptidyl-prolyl cis/trans-isomerase and a chaperone protein to aid in protein folding, with a resulting significant improvement in appropriate disulfide bond formation efficiency. In immunological assessment in BALB/c mice, 12/12 immunized mice produced high titers of antibody, and 11/12 of the anti-sera generated had transmission-blocking activity between 88 and 100% by SMFA (performed with a 1:10 dilution of whole mouse anti-serum in the membrane feeding apparatus) [84]. In 2009, full-length Pfs48/45 protein (amino acids 28-427) was successfully expressed in *E. coli* after codon-harmonization, a process whereby codon usage is manipulated to replicate the speed of translation of the native species in the non-native expression platform. The protein was thought to be of the correct conformation, as assessed by mAbs recognizing reduction-sensitive epitopes. Transmission-blocking antibodies were elicited in mice and non-human primates (*Papio anubis*) [85].

In addition to the above, most recently, a C-terminal version was produced in green algae *Chlamydomonas reinhardtii* [86], and a non-glycosylated form was produced in *Nicotiana benthaniana* by co-expression with bacterial PNGase F [87]. These have yet to be functionally evaluated. Most recently, the REDMAL project funded through the European Commission has supported the development of a chimeric antigen which fuses a "correctly folded" (as evaluated by recognition by conformation-dependent mAbs to domains I, IIb, and III) region of Pfs48/45 (Pfs48/45.10C) to a portion of asexual stage candidate, Glutamate-Rich Protein (GLURP.R0). GLURP is part of the GMZ2 vaccine, an asexual hybrid protein currently being tested in a Phase IIb multicenter field trial in Africa. The chimera denoted R0-PF10C is produced in *Lactococcus lactis*, and slated for clinical grade production and testing in the field [88].

1.5.3 Leading zygote/ ookinete (post-fertilization) target Pfs25

The most developed candidate in the clinical pipeline is Pfs25 – the only TBV target with published human clinical trials data. Of note, the *P. vivax* ortholog, Pvs25 has also been evaluated in human vaccination studies, and it is a unique facet of the transmission-blocking field relative to other life cycle stages that *P. vivax* vaccine research has kept pace with the benchmarks set by *P. falciparum*. In contrast to Pfs48/45 and Pfs230, Pfs25 expression begins from the point of gamete emergence through the ookinete-stage in the mosquito midgut, with evidence for continued expression in the oocyst. A closely related paralog, Pfs28 – with an adjacent position in the genome, the likely result of a gene

duplication event [89] – is expressed slightly later in development, predominantly in the ookinete [90]. The female gametocyte contains abundant Pfs25 and Pfs28 transcripts but the translation of these proteins is repressed prior to egress from the red blood cell [91].

Similar to Pfs48/45 and Pfs230, Pfs25 was identified as the target of very effective transmission-blocking mAbs [53]. Pfs28, was described first as Pbs21 in *P. berghei*, and has since been identified in other *Plasmodium* species. Due to a redundancy of function, Pfs28 is being considered as an adjunct candidate to Pfs25 in a multi-antigen TBV. Antibodies against either antigen inhibit oocyst development in *ex vivo* assays [92], but debate still exists as to whether the effect of combined antibody response to both targets is synergistic [92, 93]. While both Pfs25- and Pfs28-specific IgG have been shown to prevent the ookinete to oocyst developmental transition in the mosquito midgut, the mechanism of action remains to be determined and could be due to impaired ookinete survival [94] or ineffective traversal of the midgut epithelium [95], possibly as a result of impaired epithelial recognition [96]. Studies of gene disruption in *P. berghei* demonstrate that deletion of either Pbs25 or Pbs21 results in a low level of inhibition of oocyst development relative to wild-type parasites. A double knockout meanwhile results in a nearly complete block of oocyst intensity, but this effect is still not complete [94, 97].

Structural analyses were first finished for Pvs25 and sequence comparison suggests that these findings can be extrapolated to other orthologs and paralogs [98]. The molecule is triangular and flat, which allows it to form a

protective interlocking sheet on the surface of the ookinete. Indeed, imaging studies confirm this pattern for both Pfs25 and Pfs28, demonstrating the two proteins to be expressed extensively on the ookinete surface [99, 100], compatible with a role in ookinete protection. Pfs25 and Pfs28 each contain a C-terminal GPI-anchor, as well as four epidermal growth factor (EGF)-like domains; these domains are found primarily in eukaryotic extracellular proteins with roles in adhesion and signaling. EGF domains are characterized by a disulfide-bonding pattern [101]; Pfs25 has 22 cysteines with 11 disulfide bonds, compared with 20 and 10 respectively in Pfs28 [102, 103].

EGF-like domain 2 has been shown to induce the highest transmission-blocking response in a study [104] which immunized mice with each of the four EGF-like domains produced in *S. cerevisiae* and boosted the responses with the analogous full-length Pfs25 (TBV25H: amino acids 22-193 [105]). An EGF2/TBV25H vaccination regime induced antiserum able to block oocyst development by SMFA, and so too rabbit antiserum to TBV25H depleted of anti-EGF2 IgG lost its transmission-blocking response. The same study mapped domain 3 as the target of known Pfs25 transmission-blocking mAbs, including conformation-independent 4B7 and conformation-dependent 1D2 by enzyme-linked immunosorbent assay (ELISA) [104].

While recreating these epitopes as recombinant protein has proven more feasible than for previously described candidates [106], human clinical trials suggest that higher antibody titers will be required to block transmission in the mosquito midgut relative to levels sufficient in pre-clinical work. Quantitative

evidence indicating antigen-specific antibody concentrations to achieve these effects is extremely limited, although such analysis will inevitably be vital to any future comparisons of transmission-blocking targets or characterizations of antibody response quality. The only published report of functional inhibition of oocyst intensity given as antigen-specific IgG suggests that significantly different concentrations of Pfs25-specific IgG antibody are needed to inhibit oocyst intensity in the SMFA by 50% (IC_{50}) between species; the IC_{50} value for mouse antigen-specific antibodies was 15.9 μg/mL while, rabbit, monkey and human, were 4.2, 41.2, and 85.6 μg/mL, respectively [107]. Antibody titers against Pfs25 in excess of 100 μg/mL are therefore likely to be required in humans to achieve high-level TBI, and such titers require highly immunogenic delivery platforms and chemical adjuvants, as evidenced by the long period of iterative vaccine development that led to the final RTS,S/AS01 formulation [108]. Given the need to also maintain such titers over transmission seasons, producing sufficiently immunogenic recombinant antigens with a long-lived high titer response remains almost certainly the greatest challenge for the field [109, 110].

In keeping with this observation, in animal models, vaccination with a *S. cerevisiae*-generated Pfs25 or Pvs25 in aluminum hydroxide adjuvant results in a transmission-blocking antibody response [111]. But *S. cerevisiae*-formulated Pvs25 with the same adjuvant did not produce sufficiently high antibody titers in a Phase Ia clinical trial, confirming that more potent adjuvants are likely to be necessary for clinical development. In the study, the five volunteers with the highest vaccine-induced antibody titers only showed a modest transmission-

blocking activity by SMFA with, on average, 20-30% reduction in mean oocyst intensity [112]. By contrast, a Phase Ia clinical trial with *P. pastoris*-produced Pfs25 and *S. cerevisiae*-produced Pvs25 in Montanide ISA51 was stopped due to high reactogenicity that was later attributed to probable effects of the adjuvant. Of the data available, five volunteers were given two doses of the protein-in-adjuvant formulation. Serum from the best responders to Pfs25 had a greater than 90% reduction in oocyst intensity by SMFA; three of the remaining volunteers also produced specific antibodies, with 18-47% transmission-blocking activity [57]. Despite the interruption in scheduled dosing, it remains to be determined why the antibody titers were insufficient for complete transmission-blocking response.

In accordance with the version of Pfs25 and Pvs25 taken forward into human clinical studies, the best-characterized production remains in a yeast-based platform. Of note, it has been previously shown that Pfs25 produced in yeast is a heterogeneous mixture of two isoforms, which differ in the quality of the induced antibody response. Production in *P. pastoris* rather than *S. cerevisiae* improves the yield of the appropriate isoform but does not exclude the unwanted conformational variant [106]. Alternate vaccine expression platforms have been applied in efforts to improve conformational production, and successful expression has been demonstrated in alternate systems. Despite a failed early attempt [111], the relative cost-effectiveness and ease of recombinant protein production in *E. coli* resulted in a second effort with a codon-harmonized version in the appropriate monomeric conformation which elicits a strong transmission-blocking activity by SMFA [113]. Given that the

wheat germ cell free system (WGCFS) has been used for the generation of a broad selection of *Plasmodium* proteins [114], Pfs25 expression in this system has enabled comparison of antigens as transmission-blocking targets within the same expression system in pre-clinical work [115]. Plant-based *N. benthamiana* [116] and the algal chloroplast of *C. reinhardtii* [117] were explored with the downstream aim of generating fusion variants in the same platforms for improved immunogenicity (Section 1.7).

1.6 Additional transmission-blocking vaccine targets

The sequencing of the *P. falciparum* genome in 2002 [118], followed by the *P. vivax* genome in 2008 [119], was a critical turning point for TBV research. The former identified over 5000 genes, a number of which are specifically up-regulated at the mRNA level in the sexual stages of parasite development [120, 121]. Closer characterization of the *P. falciparum* proteome highlights over 900 proteins expressed in gametocytes, around 300 of which are specific to the gametocyte [11, 122]. Furthermore, a variety of transcriptomic [123, 124] and proteomic [124-127] datasets predict proteins with sex-specific expression in gametes and surface expression in the ookinete. Alternatively, possible targets expressed by the mosquito vector have been identified in a similar manner [128, 129], with a number of proteins shown to be on the surface of the midgut luminal microvilli and possibly relevant to ookinete traversal processes. Any of the above could comprise interesting vaccine targets, and their comparison to, and combination with, current leading TBV candidates as components of a

subunit vaccine is critical to the progress of vaccine research, as well as for an improved understanding of parasite biology and host interactions.

1.6.1 Pre-fertilization targets

The leading pre-fertilization targets Pfs48/45 and Pfs230 are members of a protein family characterized by 6-cysteine domains. Following screening of the *P. falciparum* transcriptome, a total of 10 members have been identified [74, 130, 131]. All 6-cysteine proteins have a predicted signal peptide, and most also contain a putative GPI anchor sequence, suggesting that these proteins localize to the surface of the parasite membrane, making the remaining family members possible vaccine targets. Genes of this family are arranged in pairs or tandem arrays, and include Pfs230 and Pfs230p on chromosome 2; Pf36, Pf36p, and Pfs41 on chromosome 4; Pf38 alone on chromosome 5; Pf12 and Pf12p on chromosome 6; and Pfs48/45 and Pfs47 on chromosome 13 [66, 132]. 6-cysteine family proteins are expressed across multiple life cycle stages. Pb36 and Pb36p play roles in the liver-stage, with possible functions in invasion or intra-hepatocytic development [65, 133, 134]. Pf12, Pf38, and Pf41 are merozoite surface proteins and are proposed to act in erythrocyte recognition and invasion or in host-immune interactions [135-137]. Six family members also have predicted sexual stage expression (Pfs230, Pfs230p, Pfs48/45, Pfs47, Pf36, and Pf38) [74].

1.6.1.1 Pfs47

Pfs47, the paralog of Pfs48/45 has received the most attention for its role in the biology of sexual development. Pfs47 is expressed only by female gametocytes, and is located on the macrogamete after emergence from the red blood cell. Gene disruption suggests that it is not essential for female fertility, with females producing normal oocyst numbers. Rat mAbs generated by gametocyte extract vaccinations were not able to inhibit transmission by SMFA nearly dismissing the target as interesting to transmission biology [138]. However, further characterization suggests that the protein plays an interesting role in allowing for parasite immune evasion in the *Anopheles gambiae* vector by suppressing midgut nitration – a process necessary for activation of the mosquito complement system. Disruption of this pathway may be an effective transmission-reducing strategy in the *A. gambiae* mosquito, although not necessarily through a vaccine-based platform [139].

1.6.1.2 HAP2

Of surface targets with a demonstrated role in sexual stage biology, HAP2/GCS1, a homolog of a sterility gene in both *Arabidopsis* and green alga *Chlamydomonas*, localizes to the surface of male gametocytes and microgametes. Suggestive of a conserved function, gene disruption in *P. berghei* is consistent with an essential role in the membrane fusion event between male and female gametes during fertilization; knockout parasites do not form oocysts [140]. Immunization of rabbits with heterologously expressed *P. berghei* HAP2, generated in *E. coli*, resulted in an antibody response which

reduced *in vitro* ookinete formation by 81%, inhibited oocyst intensity by 81.1%, and lowered the prevalence of mosquito infection by up to 34% in a *P. berghei* SMFA. This inhibition was found to be dose-dependent [141]. More recently, using a WGCFS-produced HAP2, vaccination of CD-1 mice demonstrated a dose-dependent 97-100% transmission-blocking activity in *P. falciparum* SMFA, with a lower inhibition than Pfs25 by dose titration, but no statistical difference with that of Pfs230C [115]. Very few studies comparatively assessing vaccine targets exist in the literature and, as more candidates are evaluated [142], this will be an important direction for future work.

1.6.2 Post-fertilization targets

The ookinete is the largest motile cell of the parasite life cycle, with apical machinery that secretes proteins during mosquito-midgut recognition and traversal. Unlike the invasive pre-erythrocytic sporozoite and blood stage merozoite, which contain a set of secretory organelles including micronemes, rhoptries, and dense granules, the ookinete apex contains only numerous, small micronemes [24, 127]. These organelles house the majority of invasins and adhesins necessary for the traversal process. In addition, crystalloid – a cytoplasmic organelle unique to ookinetes and young oocysts – is believed to contain a store of macrogametocye-secreted proteins, that later aid in sporozoite development and transmission.

1.6.2.1 Crystalloid-derived proteins

LCCL proteins (named for the Limulus clotting factor C. Coch-5b2, Lgl 1 domain), localize to the crystalloid, in addition to earlier gametocyte-stage expression, and have been considered as TBV targets [24, 143, 144]. Six family members have been identified to date due to predicted expression of a number of adhesive domains, with five containing the LCCL. These proteins have been studied by several groups independently and are called the PfCCPs (Limulus coagulation factor C domain-containing proteins) in *P. falciparum* and the PbLAPs (LCCL-lectin adhesive-like proteins) in *P. berghei* (reviewed in [132]). Consistent with the most recent localization reports, gene disruption studies available for four of the six family members demonstrate a failure at the oocyst to sporozoite transition [145].

1.6.2.2 Micronemal proteins

A number of ookinete micronemal components have been considered as vaccine targets. These include circumsporozoite and TRAP related protein (**CTRP**); von Willebrand factor A domain-related protein (**WARP**); secreted ookinete adhesive protein (**SOAP**); membrane attack ookinete protein (**MAOP**, also known as *Plasmodium* perforin-like protein 3); putative secreted ookinete proteins 2 and 7 (**PSOP2 and PSOP7**); cell-traversal protein for ookinetes and sporozoites (**CelTOS**); and parasite **chitinase**. While initially internal to the micronemal compartment, these proteins either translocate to the apical ookinete surface or are secreted. CTRP is one of the better-studied proteins of the ookinete, identified first in *P. falciparum* [146], and now known to have

expression in other *Plasmodium* species [147-149], including *P. vivax* [150]. Together with WARP [151] and thrombospondin type-1 related proteins, the extracellular portion has a series of adhesive domains and has been shown to bind to midgut basement membrane glycosaminoglycans [151]. Gene disruption in both *P. berghei* and *P. falciparum* shows CTRP to have an essential role in ookinete motility; knockout parasites are unable to invade the mosquito midgut [147-149]. The protein is not essential for the transition of ookinetes to oocysts, suggesting a distinct role in gliding motility mediated by the A domains of the protein [152]. This candidate has not been heterologously expressed or evaluated as a TBV candidate, although the knockout phenotype suggests that it may be an interesting target.

A number of micronemal proteins act directly in ookinete recognition of the midgut epithelium, which is necessary for colonization and the initiation of traversal. WARP is presumed to play a role in adhesion between the ookinete surface and the midgut on the ookinete's path towards the basal lamina [153]. SOAP is a cysteine-rich, soluble protein able to form complexes at a high molecular weight via disulfide bonds. SOAP associates with the parasite surface, with a reported affinity for mosquito laminin. Knockout parasites are unable to invade the mosquito midgut, suggesting a role in the initial recognition of the epithelium [154]. The putative secreted ookinete proteins or PSOPs are not grouped by a functional or structural relationship. Characterized by gene disruption in a recent functional knockout screen of *P. berghei*, PSOP2 knockouts produce reduced ookinete numbers, while PSOP7 knockouts are unable to invade the midgut [155].

1.6.2.3 Parasite enolase and chitinase

Enolase is also suggested to have a role in midgut recognition and traversal. The protein, while expressed at multiple life cycle stages, is secreted onto the ookinete surface, and then thought to have a dual function as a ligand for a hypothetical ookinete midgut receptor as well as a source for co-opting mammalian plasminogen, which when converted to active plasmin, supports the midgut traversal process. Unlike the aforementioned candidates, enolase has been heterologously expressed in *E. coli* and assessed as a possible TBV by immunizing rabbits followed by whole serum SMFA. It was shown to induce a 70% inhibition of oocyst intensity [156]. There is some data to suggest that enolase is also expressed on the merozoite surface and that antibodies generated to enolase are able to inhibit blood stage parasites growth as determined by *in vitro* studies such as the growth inhibitory assay (GIA) [157]. One study has reported high-titer anti-enolase antibodies in a serology screen of malaria patients [158]. These reports suggest that enolase could serve as both a blood stage and transmission-blocking vaccine target with the possibility for natural boosting of vaccine-generated immunity.

There has been some interest in the development of parasite-secreted chitinases as possible immunogens for a TBV. These enzymes serve a key biological function in the passage of the parasite through the mosquito, which is an attractive target for antibody-mediated disruption. Upon feeding, the female *Anopheles* mosquito forms a chitin-rich membrane around the ingested blood meal, effectively walling in any potentially invasive pathogens. In order to

traverse the midgut epithelium, the ookinete needs to penetrate this physical barrier. The parasite secreted chitinases, PCHT1 and PCHT2, serve this function and also display a high level of inter-species conservation [159]. A single chain monoclonal antibody raised against PfCHT1 can substantially reduce mean oocyst intensity by 84-97% in SMFA not only with *P. falciparum* but also *P. gallinaceum* parasites [160]. This study adds to previous reports in which PCHT gene knockout and chitinase enzyme inhibition were demonstrated to significantly impair *P. berghei* and *P. falciparum* infectivity. [161-163].

1.6.2.4 Plasmodium perforin-like proteins

The biology of traversal is itself not well understood. The *Plasmodium* perforin-like proteins (PPLPs) play a role in this process, and are defined by the presence of a membrane attack complex/perforin superfamily (MACPF) domain. MACPF domains are known to play a role in transmembrane pore formation in lipid bilayers [164]. Ookinetes express three PPLPs in total, two of which, PPLP3 or MAOP [164-166] and PPLP5 [167] have been shown to be essential in midgut invasion [166, 167]. The third PPLP (PPLP2) is not fully characterized [155], but recent reports suggest that it may have a role in macrogametocyte egress from the red blood cell [168]. Further, CelTOS is suspected to play an important role in ookinete traversal. Gene disruption results in parasites that are unable to complete the path to the basal lamina, and specifically become arrested in the cytoplasm of midgut epithelial cells. Interestingly, while CelTOS knockout parasites dramatically reduce infectivity of the liver, motility and hepatocyte invasion are not affected [169]. At this point there is insufficient

characterization of the above antigens to indicate whether they could rival existing targets in candidate vaccines.

1.6.3 Mosquito midgut targets

The only successful malaria control strategies to date have included a vector control component [170-172]. As such, there is significant interest in exploring mosquito antigens, such as proteins essential for parasite colonization of the midgut epithelium, for vaccine development. In parallel to the demonstration of transmission-blocking immune responses directed against parasite antigens, mice immunized with mosquito midgut preparations significantly reduced the infectivity of *P. berghei* [173]. In addition to the predicted effect on the ookinete to oocyst transition, anti-midgut antibodies partially inhibited sporozoite development, acting in particular on the translocation of sporozoites to the salivary gland [174]. Similar studies using anti-midgut polyclonal antibodies were repeated for *P. falciparum* [175] and *P. vivax* [176], although as in previous reports, the transmission-blocking effect was never complete. Several mAbs generated from mouse immunization with *A. gambiae* lysate, on the other hand, were able to block transmission, with a dual effect in limiting vector survival [175]. Further characterization of these mAbs suggested that they were able to disrupt ookinete to oocyst development in both *P. falciparum* and *P. berghei* in five different *Anopheles* species. Moreover, they were not directed exclusively against proteins, but also thought to recognize carbohydrate moieties [177, 178].

Several mosquito digestive enzymes, activated with ingestion of the blood meal, have been identified as possible TBV targets [179]. *cpbag1* and homolog *cpbag2* were identified from a screen of genes with modified expression after *P. falciparum* infection [180-182], and the former protein product has been evaluated as a TBV candidate. Mice immunized with baculovirus-expressed CPBAg1 (carboxypeptidase B) demonstrated a 51-63% inhibition of oocyst intensity following challenge with *P. berghei* and a direct mosquito feed. Meanwhile, rabbit antiserum to recombinant CPBAg1 used in a *P. falciparum* SMFA resulted in a reproductive fitness cost to *A. gambiae* mosquitoes [182]. Compatible with this observation, the primary function of digestive enzymes in the female *Anopheles* mosquito is to break down the blood meal into amino acid components, which then support egg maturation. While the precise function of mosquito CPB in parasite survival has not been fully elucidated, one theory is that it supplements parasite lysine and arginine needs [179].

Microvillar glycoconjugates have been broadly implicated in parasite attachment to the midgut. In support of this view, free glycans such as N-acetylglucosamine, and lectins, such as jacalin, reduce ookinete attachment, presumably by blocking midgut epithelial glycan ligands. Further to this, a mAb (MG96) generated against midgut microvillar glycans fully inhibits the ookinete to oocyst transition in a dose-dependent manner [178]. Jacalin-affinity chromatography and tandem mass spectrometry was used to identify the O-glycosylated midgut microvillar protein, *Anopheline* midgut alanyl aminopeptidase 1 (AgAPN1), coincidentally also recognized by MG96. It is abundantly expressed on the midgut lumen and thought to play a role as an

attachment ligand during parasite midgut colonization. Rabbit polyclonal antibodies against N-terminal AgAPN1 passively transferred into *P. berghei* infected mice inhibited transmission in both *A. stephensi* and *A. gambiae*, and demonstrated a 70-85% reduction in *P. falciparum* NF54 oocyst intensity [183]. Meanwhile following vaccination of CD-1 mice with WGCFS generated soluble, immunogenic antigen (amino acids not specified), no inhibition was observed [115]. The most recent report argues that conformational reproduction of a single, conserved epitope is necessary for the transmission-blocking effect [184].

The most promising aspect of targeting the mosquito midgut is the potential it provides for blocking the transmission of multiple malarial species with one vaccine formulation. Still, midgut invasion is a complex process and may exhibit partial redundancy of yet to be defined receptor/ligand pathways. Thus, future attempts to produce more effective transmission-blocking immunity using mosquito antigens as targets may require a multi-subunit approach. However, for this field to expand, biological studies with the goal of delineating the molecular pathways underlying *Plasmodium* invasion of the midgut epithelium are most urgently needed. So too is a clearer understanding of mosquito immune mechanisms, which could provide a further avenue for TBV development. It is presently observed in the natural setting that mosquito innate immunity clears the majority of *Plasmodium* parasites, a defense reaction primarily acting at the stage of ookinete invasion. Indeed a TBV strategy which targets *A. gambiae* immune regulatory molecule, serpin-2, a serine protease of the mosquito midgut, inhibits *P. berghei* development in two *Anopheles* species

[185]. But so too, targeting mosquito innate defenses can be achieved by transmission-blocking technologies beyond TBVs, and a number of elegant studies of modulation of mosquito immunity [186-188] and theoretical analyses of release of genetically modified mosquitoes are discussed in detail elsewhere [189].

1.7 Improving antigen immunogenicity

Novel antigen modifications and delivery strategies may circumvent the need for understudied or controversial adjuvants to improve antibody titer or longevity of response. Examples are described below for the most developed TBV antigen – Pfs25.

1.7.1 Antigen conjugation and viral-like particles (VLPs)

An effective TBV must generate high antibody titers and sustain the response for, at minimum, the duration of a transmission season. In particular, candidates such as Pfs25 and Pfs28 do not have protein expression in the human host, and vaccine-induced immunity would not be boosted by natural infection. The majority of recent efforts to overcome this problem rely on conjugation of Pfs25 to carrier proteins or to itself in attempts to increase vaccine efficacy, with reports of significant increases in antibody titer and duration in animal models. Given reactogenicity concerns with more potent, experimental adjuvants [57], this will be an important direction for future research. Covalent conjugation of yeast-produced Pfs25 with the outer membrane protein complex (OMPC) of *Neisseria meningitides*, the carrier protein used in the *Haemophilus influenzae*

type b (Hib) vaccine, formulated with aluminum hydrophosphate, produced antibody responses that were reported to persist for over 18 months in rhesus macaques [109]. The improved immunogenicity of protein conjugates was confirmed in a subsequent study comparing conjugation strategies – drawing attention to the finding that self-conjugation of Pfs25 alone improves the immunogenicity and longevity of response [190]. Recently, Pfs25 conjugated with cholera toxin subunit B (CtxB), a potent mucosal adjuvant, was expressed in *C. reinhardtii* for use as a whole-cell, algal-based, freeze-dried oral vaccine [191]. The principle has been previously demonstrated with cholera toxin delivered in the presence of *P. pastoris*-produced Pfs25 in mice by the intranasal route [192].

Several such approaches to improve immunogenicity have recently progressed to human clinical studies. One methodology employs conjugation of Pfs25 to EPA [193], with a Pfs28-EPA construct in development as well [194]. Safety testing has already been initiated in US malaria-naïve volunteers, and a second trial is ongoing in Bancoumana, Mali (ClinicalTrials.gov NCT01867463). Also currently in a safety clinical trial, a VLP consisting of Pfs25 fused to Alfalfa mosaic virus coat protein (CP) was generated in *N. benthamiana* using a Tobacco mosaic virus-based 'launch' vector. Even with an Alhydrogel® adjuvant, mice receiving one or two doses generated a transmission-blocking immunity which was sustained over a 6 month period [195]. The Phase Ia dose-escalation study to evaluate this plant-derived Pfs25 VLP is ongoing (ClinicalTrials.gov NCT02013687).

1.7.2 Viral vectors

In addition to alternate expression systems and antigen conjugation strategies to improve the immunogenicity of the vaccine product, delivery platforms other than recombinant-protein-based formulations have been explored for improved antibody response. An early human trial in 1996 primed volunteers with a highly attenuated vaccinia strain viral vector expressing seven genes from *P. falciparum* (denoted NYVAC-Pf7), including Pfs25, followed by a boost of Pfs25 recombinant protein. In the study, volunteers developed low level Pfs25 antibodies with incomplete transmission-blocking activity [196]. A subsequent pre-clinical study in mice, evaluated an alternate viral-vectored platform in delivering Pfs25 without a multi-antigen prime. A heterologous prime-boost regime, consisting of an adenovirus (chimpanzee ChAd63 or human AdHu5) delivery vector followed by a poxviral modified vaccinia Ankara (MVA) boost after a 10 week interval, elicited Pfs25-specific antibodies capable of blocking transmission by membrane feeding (96% reduction in oocyst intensity and 78% reduction in mosquito infection prevalence at a 1 in 5 serum dilution) [197]. This result was replicated in a subsequent study comparing leading vaccine candidates Pfs25, Pfs230C, Pfs48/45, AgAPN1, and HAP2 in the Chad63/MVA prime-boost regime in SMFA using purified IgG at a concentration of 0.75mg/mL [198]. When the same regime is given with Pfs25 expressed in the presence of an added heptamerization domain (IMX313, PMID: 18474650), antibody titers are improved by a log with more robust transmission-blocking activity [199]. Human clinical trials will begin in 2015 under EU FP7 MultiMalVax.

1.8 Thesis Overview

Broadly, this thesis aims to improve the functional characterization of known and novel TBV candidate antigens by two complementary approaches.

Part I relies on recent advances in heterologous expression of *Plasmodium* proteins to generate a panel of sexual stage antigens for functional characterization by SMFA. Chapter 3 describes expression of a panel of leading TBV candidate antigens in HEK293 cells (Pfs25, Pfs28, Pfs48/45, Pfs230C), which are compared and ranked by functional transmission-blocking activity of generated anti-serum. A viral-vectored delivery platform comprised of an adenovector prime and poxviral modified vaccinia Ankara boost has previously been generated for these antigens. This chapter compares the ability of the viral-vectored platform to elicit transmission-blocking IgG relative to a protein-in-adjuvant delivery platform. Chapter 4 describes the expression of 12 previously uncharacterized sexual stage antigens in the HEK293E cell system and Chapter 5 delineates the functional evaluation of these proteins relative to current leading candidate Pfs25 in their ability to induce transmission-blocking antibodies.

Part II assesses natural humoral immunity to better characterize its antigenic targets. In accordance with this aim, Chapter 6 describes the testing of archived serum samples from children living in a malaria-endemic region of Mali for recognition of a small subset of sexual stage antigens (Pfs230C, HAP2) known to elicit transmission-blocking responses, which are expressed as recombinant protein in the WGCFS. Given that the archived samples were not collected for

studies of transmission, there was no accompanying data on gametocyte carriage at the time of sample collection. Consequently Chapter 7 describes the development and completion of sample collection for a new clinical protocol aimed to (1) track asexual parasite and gametocyte carriage at bimonthly time points in a cohort of 500 individuals (6 months to 65 years of age) for one calendar year and (2) provide serum samples for analysis of naturally acquired transmission-blocking immunity. Chapter 7 contains seroreactivity data to the panel of sexual stage proteins at the peak of the wet season relative to the peak of the dry season and correlates this reactivity to gametocyte density in aims to identify markers of gametocyte exposure.

Chapter 2: Material and Methods

Comprehensive materials and methods pertaining to this thesis are listed below. Where relevant, reference is made to standard operating procedures at the Jenner Institute at the University of Oxford (J-#) and the Laboratory of Malaria and Vector Research at the National Institutes of Health (IM-#), included in the Appendix. Plasmids are denoted by the Jenner Institute plasmid stock number (p-#).

2.1 Materials

2.1.1 Reagents

Details of all commercially available reagents used in this thesis are provided in Table 2.1. Commercially available antibodies used in this thesis are provided in Table 2.2. Oligonucleotide primers used in this thesis are listed in Table 2.5 in section 2.2.1.5.

Table 2.1 Commercially available reagents used in this thesis

Material	Supplier	Catalog Number
0.2µm syringe filter	Sartorius	16534
15mL centrifuge tubes	Fisher Scientific	FB55950
18-guage catheter 1.3x45mm	VWR International	TERUSR-DM1845PX
2-β Mercaptoethanol [50 µM]	Gibco	31350-010
2ml syringes	Nu-Care Products	M0185
50mL centrifuge tubes	Greiner	227261
50mL syringes	Nu-Care Products	M0865

5ml polystyrene tubes	VWR International	734-0001
5ml syringes	Nu-Care Products	M2187
96-well flat bottom plates	Fisher Scientific	TKT-180-070U
Agarose	Sigma Aldrich	A9539
Ampicillin	Sigma Aldrich	A5354
Bovine Serum Albumin	PAA Laboratories	K41-001
Cryogenic vials	Star Lab	E3110-6122
DAPI (4'-6'-diamidino-2-phenylindole)	Sigma Aldrich	101101353
DH5α maximum efficiency cells	Invitrogen	12297-016
Diethanolamine buffer	Pierce	34064
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650
Endotoxin-free water	Sigma Aldrich	W3500
Ethanol	Sigma Aldrich	32221
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E7889
Fetal Calf Serum (FCS)	Sigma Aldrich	F0127
Gentamicin solution	Sigma Aldrich	G1397
Glycerol	Sigma Aldrich	G7893
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma Aldrich	A3562
HEK293E cell line	Oxford Protein Production Facility	N/A
Heparin	Sigma Aldrich	H4784
HEPES	Sigma Aldrich	H3375
Hydrochloric acid (HCl) [1M]	Sigma Aldrich	S7653
Hydrochloric acid (HCl) [2M]	Fisher Scientific	J/4315/15
Immersion oil	Sigma Aldrich	10890
In-Fusion® HD Enzyme Premix	Clontech	638911
Isofluorane	Oxford University Veterinary Services	N/A
Kanamycin	Sigma Aldrich	K0254
KetasetTM (ketamine)	Oxford University Veterinary Services	N/A
LB agar tablet	Sigma Aldrich	L7025-500TAB
LB broth tablet	Sigma Aldrich	L7275-500TAB
L-glutamine [4mM]	Sigma Aldrich	G7513
LS Columns	Miltenyi Biotec	130-042-401
Magnesium chloride ($MgCl_2$)	Sigma Aldrich	M2393
Methanol	Fisher Scientific	M/3900/17
Microcentrifuge tubes	Fisher Scientific	FB74031
Micro-fine Insulin Syringe with needle 0.5ml 29g x 12.7mm	Nu-Care Products	M4892
Micro-fine Insulin Syringe with needle 1ml 29g x 12.7mm	Nu-Care Products	M4891
Microscope slides	Fisher Scientific	MNJ-200-010H
MinElute Gel Extraction Kit	Qiagen	28606
Mr. Frosty cryocontainer	Fisher Scientific	CRY-120-010T
Neutral buffered formalin	Sigma Aldrich	HT501128
NUNC Maxisorp 96-well plates	Fisher Scientific	DIS-971-030J
PBS	Sigma Aldrich	A9878
PCR Primers	Sigma Aldrich	N/A
Penicillin/Streptomycin [100 pen/ 100 µg	Sigma Aldrich	P0781

strep]		
Percoll	Sigma Aldrich	P1644
Phenylhydrazine	Sigma Aldrich	P26252
Phusion® High-fidelity DNA Polymerase	Fisher Scientific	FZF-531L
pNPP (20mg tablets)	Sigma Aldrich	N2765
Protein A/G Ultralink Resin	Fisher Scientific	PN53132
Proteinase K	Qiagen	19131
Protein G agarose	Pierce	22852
Qiagen MinElute DNA purification Kit	Qiagen	28604
QIAprep Spin MiniPrep Kit	Qiagen	27106
Qiagen Plasmid Midi Kit	Qiagen	12143
Qiagen Plasmid Endofree Maxi Kit	Qiagen	12362
Restriction and Ligation Enzymes	New England Biolabs	N/A
RPMI-1640	Sigma Aldrich	R0883
Scalpel (surgical blade)	Swann Morton	0302
SmartLadder DNA Marker	EurogenTec	MW-1700-02
SOC medium	Invitrogen	15544-034
Sodium azide (NaN_3)	Fluka Analytical	08591
Sodium bicarbonate ($NaHCO_3$)	Sigma Aldrich	S8761
Sodium chloride (NaCl)	Sigma Aldrich	S5886
Sodium dodecyl sulfate (SDS)	Sigma Aldrich	L4390
SYBR Safe	Invitrogen	S33102
Transfer pipettes	Fisher Scientific	FB55348
Tris-acetate-EDTA (TAE)	Fisher Scientific	FB55348
Triton™ X-100	Sigma Aldrich	X100
Trizma® HCl buffered solution (pH8)	Sigma Aldrich	T2694
Trypan Blue	Sigma Aldrich	T8154
Tubing	Fisher Scientific	PYH-700-020L
Xanthurenic acid	Sigma Aldrich	D102804

Table 2.2 Commercially available antibodies used in this thesis

Antibody	Supplier	Catalog number
Alexa Fluor®488 conjugated goat anti-mouse IgG	Life Technologies	A11008
Alexa Fluor® 488 conjugated goat anti-human IgG	Life Technologies	A11013

2.1.2 Solutions, buffers, and cell culture media

Complete *P. falciparum* culture medium: 45 mL of incomplete culture medium was mixed with 5 mL of pooled heat-inactivated filter-sterilized human serum and 50 µL of 10 mg/mL gentamicin.

Diethanolamine buffer: 5X stock was diluted with H_20 before use according to manufacturer instructions.

Exflagellation medium: RPMI-1640 was supplemented with 25 mM HEPES, 20% FCS, 10 mM sodium bicarbonate and 50 µm xanthurenic acid. pH was adjusted to 7.6.

Giemsa: 5% Giemsa in H_2O.

HisTrap protein binding buffer: 20 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.4.

HisTrap protein elution buffer: 20 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 7.4.

IgG purification binding buffer: 0.144 M Na_2HPO_4, 0.056 M NaH_2PO_4, pH 7.2 in H_20.

IgG purification elution buffer: 7.5 mg glycine was dissolved in 800 mL H_20 and the pH was adjusted to 2.7 with concentrated HCl. The solution was made up to 1L with H_20 to yield a final concentration 0.1 M glycine, pH 2.7.

Incomplete *P. falciparum* culture medium: 2.97 g HEPES, 0.025 g hypoxanthine, and 5 mL 100X L-glutamine solution were added to 500 mL RPMI-1640 and mixed with a magnetic stirrer for at least an hour.

LB agar/broth: Tablets were dissolved in H_2O (1 tablet per 50 ml). Antibiotics were added at working concentrations of 100 µg/ml for ampicillin and 25 µg/ml for kanamycin.

Mowiol: 6 g glycerol and 2.4 g polyvinyl alcohol 4-88 were dissolved in 6 ml H_2O for two hours at 50°C with agitation. 12 ml Tris pH 8.5 (0.2 M) added and the solution dissolved for a further three hours at 50°C with agitation. The solution was centrifuged at 5000 x *g* for five minutes to remove any undissolved solids. DAPI was then added at a final concentration of 0.1 µg/ml.

PBS (0.1 M): 0.138 M NaCl, 0.0027 M KCl, pH 7.4; made by dissolving tablets in H_2O according to the manufacturer instructions.

PBS/Tween (PBS/T) (0.1 M): 0.138 M NaCl, 0.0027 M KCl, Tween 0.05%, pH 7.4; made by dissolving sachets in H_2O.

PBS/BSA: 15 g BSA (3%) and 250 µl sodium azide (0.05%) were added to 500 ml PBS.

***Plasmodium falciparum* freezing medium:** 11 ml FCS, 4.2 ml 5% $NaHCO_3$ and 5.5 mg neomycin were added to 96 ml RPMI-1640.

TAE Buffer: Made from 50x concentrate diluted in H_2O.

2.1.3 Adjuvants

Addavax, pre-clinical grade: Nano-emulsion (160 nm) of sorbitan trioleate (0.5 w/v) in squalene oil (5% v/v) and Tween-80 (0.5% w/v) in sodium citrate buffer (10mM, pH 6.5) (InvivoGen, distributed by Source BioScience, UK).

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Antigen inserts

P. falciparum proteins were expressed as recombinant antigens and delivered as either protein-in-adjuvant (Chapter 3 & 4) or viral-vectored vaccines (Chapter 3).

2.2.1.1.1 Antigen inserts for viral vectors

The rationale for the selection and expression of *P. falciparum* TBV targets in a viral-vectored system is included in Chapter 3. The viral vectors for these targets were generated previously by the Vector Core Facility of the University of Oxford (HAP2, Pfs28, Pfs230 region C, Pfs48/45) and Dr. Anna Goodman (Pfs25 [197]). Antigen constructs for HAP2, Pfs230C, and Pfs48/45 were

designed by Dr. Melissa Kapulu [198]. The antigen construct for Pfs28 was designed by Kerry Jewell (Clinical Master's Thesis). These antigen inserts are described in detail below:

- **HAP2:** The 3D7 strain of the *P. falciparum* HAP2 gene (NCBI PF3D7_1014200, amino acids 31-684) was obtained from PlasmoDB (http:/plasmodb.org/plasmo/) and used as the basis for the expression construct. Predicted N-glycosylation sites – defined as Asn-Xaa-Ser/Thr – were identified at amino acids 78, 145, 155, 268, 297, 449, 481, 518, 535, 541, 593, and 612 using the NetNGlyc 1.0 Server (Center for Biological Sequence Analysis). Each site was modified to a glutamine residue (Gln-Xaa-Ser/Thr).
- **Pfs25:** The Pfs25 sequence based on the NF54 isolate of *P. falciparum* (NCBI PF3D7_1031000), between the predicted signal peptide and first transmembrane domain (amino acids 22-192) using SignalP and THMHH Servers (Center for Biological Sequence Analysis). Predicted N-glycosylation sites – defined as Asn-Xaa-Ser/Thr – were identified at amino acids 112, 165, 187, and 202 using the NetNGlyc 1.0 Server (Center for Biological Sequence Analysis). Each site was modified to a glutamine residue (Gln-Xaa-Ser/Thr).
- **Pfs28:** The 3D7 strain of the *P. falciparum* Pfs28 sequence (NCBI PF3D7_1030900, amino acids 23-218) was obtained from PlasmoDB (http:/plasmodb.org/plasmo/) and used as the basis for the expression construct. No N-glycosylation sites were predicted by the NetNGlyc 1.0 Server (Center for Biological Sequence Analysis).

- **Pfs230C:** The 3D7 strain of the *P. falciparum* Pfs230 gene region C (NCBI PF3D7_0209000, amino acids 443-1132) was obtained from PlasmoDB (http:/plasmodb.org/plasmo/) and used as the basis for the expression construct. Predicted N-glycosylation sites – recognized as Asn-Xaa-Ser/Thr – at amino acids 585, 821, 829, 889, 961, 1029, 1079, and 1089 were identified at amino acids 78, 145, 155, 268, 297, 449, 481, 518, 535, 541, 593, and 612 using the NetNGlyc 1.0 Server (Center for Biological Sequence Analysis). Each site was modified to a glutamine residue (Gln-Xaa-Ser/Thr).
- **Pfs48/45:** The 3D7 strain of the *P. falciparum* Pfs48/45 gene (NCBI PF3D7_1346700, amino acids 28-427) was obtained from PlasmoDB (http:/plasmodb.org/plasmo/) and used as the basis for the expression construct. Predicted N-glycosylation sites were left intact. In a previous study by Kapulu *et al*, two different constructs were designed; one with all seven N-glycosylation sites substituted with a glutamine residue ($Pfs48/45_{-NGln}$) and the other with the native amino acid sequence ($Pfs48/45_{+NGln}$). Of these, anti-$Pfs48/45_{+NGln}$ IgG outperformed anti-$Pfs48/45_{-NGln}$ IgG by SMFA [198], and as a result the $Pfs48/45_{+NGln}$ construct was used in this study.

For all antigens, the gene sequence was codon optimized for expression in *Homo sapiens*. A human tissue plasminogen activator (tPA) leader sequence (GenBank Accession K03021) [200] was added at the N-terminus in lieu of the endogenous leader sequence, and two stop codons were added at the C-terminus. The resulting insert sequence was assembled from synthetic oligonucleotides and/or PCR products, and cloned into the pMA vector

(ampicillin resistance) by GeneArt® (Life Technologies, Germany). The inserts were then PCR cloned into the pENTR4™LPTOS plasmid (p1990) with flanking Acc65I and NotI cloning sites (GeneArt® Life Technologies, Germany) [201]. The synthetic genes were then introduced into the chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) viral vectors from the pENTR4™LPTOS shuttle plasmid by previously published methods [200, 202] described in section 2.2.1.3.

2.2.1.1.2 Antigen inserts for mammalian expression vector

For the purpose of comparing leading TBV candidate performance in both a viral-vectored and protein-in-adjuvant platform (Chapter 3), antigen inserts corresponding to HAP2, Pfs25, Pfs28, Pfs230C, and Pfs48/45 were cloned into a modified pENTR4™LPTOS vector (p3223), which differs from the viral vector shuttle plasmid (p1990) primarily in the inclusion of epitope tags to aid in protein purification and downstream applications. Modifications to the plasmid were made by Dr. Joseph Illingworth (DPhil Thesis) and are documented in Section 2.2.1.2. Notably, p3223 includes the human tPA leader sequence in the backbone of the vector, and unique restriction enzyme sites (KpnI and BamHI) enable cloning of genes between the tPA leader sequence and the C-terminal epitope tag (hexahistidine). Gene sequences for HAP2, Pfs230C, and Pfs48/45 were therefore amplified by PCR from the corresponding pENTR4™LPTOS-[antigen] entry plasmid in Section 2.2.1.1.1 with primers encoding an N-terminal KpnI and C-terminal BamHI restriction site (Table 2.5). Inserts were then cloned into the p3223 plasmid by restriction enzyme digestion and ligation. Pfs25 and

Pfs28 had been previously cloned into pENTR4™LPTOS vector p3129, which is identical to p3223 with the exception of a StrepII tag (WSHPQFEK) in place of the hexahistidine sequence, by Kerry Jewell (Master's Thesis). As a result, the Pfs25 and Pfs28 genes were directly transferred from p3129 to p3223 by restriction enzyme digestion and ligation.

For the purpose of antigen screening using the HEK293E platform (Chapter 4), inserts for 22 sexual stage antigens (including the native-sequence Pfs25, amino acids 22-192) were based on the *P. falciparum* 3D7 strain gene sequences obtained from PlasmoDB (http:/plasmodb.org/plasmo/). Unless otherwise specified, the sequence corresponding to the full-length ectodomain was determined by software predicting both the signal peptide sequence (SignalP 3.0) [203] and any membrane-tethering sequences consistent with either a transmembrane domain or GPI-anchor (TMHMM Servers from the Center for Biological Sequence Analysis) [204, 205]. Following codon optimization for expression in *Homo sapiens*, synthetic genes were generated by GeneArt® (Life Technologies, Germany). Predicted N-linked glycosylation sites were left intact to avoid potentially disruptive modifications to the native sequence. The coding region, flanked by unique KpnI and BamHI restriction enzyme sites, was subcloned into plasmid p3223 (GeneArt® Life Technologies, Germany).

2.2.1.2 Plasmids

The pENTR4™LPTOS plasmid (p1990) contains an *E. coli* origin of replication, a long hCMV promoter (LP), the tetracycline operator in proximity to the TATA box at the SacI site (TOS), a tPA leader sequence, a bovine growth hormone polyA signal [206], and a kanamycin resistance gene. The plasmid is notable for elements which enable DNA shuttling into the adenovirus genome by Gateway® LR clonase™ homologous recombination (Invitrogen, UK). The long hCMV promoter is described in detail elsewhere [206, 207] and, in addition to the short hCMV promoter sequence, contains downstream elements and enhancers, notably intron A [207].

Following the observation that pENTR4™LPTOS (p1990) performs competitively as a mammalian expression vector, several modifications were made to the plasmid by Dr. Joseph Illingworth (DPhil Thesis) to promote use of the plasmid for the generation of recombinant proteins (p3223). Unlike p1990, p3223 contains the human tPA leader sequence within the vector backbone, as well as a C-terminal antigen tag consisting of the 15 amino acid biotin acceptor peptide (GLNDIFEAQKIEWHE), which serves as a substrate mimic for biotin ligase enzyme BirA, followed by a hexahistidine tag consisting of six consecutive histidine residues. Unique KpnI and BamHI restriction enzyme sites enable cloning of multiple genes of interest between these elements.

2.2.1.3 Viral vectors

Standard methods were used to clone antigen-specific ChAd63 and MVA viral vectors. As described in 2.2.1.1.1, for each antigen of interest, the predicted endogenous signal peptide was replaced with a human tissue plasminogen activator (tPA) signal peptide (GenBank Accession K03021), which has been demonstrated to promote efficient antigen expression. Antigen sequences were optimized for human codon usage bias and predicted N-glycosylation sites were modified to glutamine residues for all antigens except for $Pfs48/45_{+NGln}$. A Kozac sequence was placed upstream to maximize mammalian expression. The hCMV long promoter (containing intron A) [207] was selected to drive expression in ChAd63, while the vaccinia P7.5 promoter was used to drive expression in the MVA vector. Antigen inserts, flanked by Acc65I and NotI restriction enzyme sites, were first each cloned into the pENTR4™LPTOS entry vector.

Recombinant ChAd63 vaccines were constructed using a novel Gateway® cloning approach established by Dr. Matthew Cottingham at the Jenner Institute, University of Oxford. For the ChAd63 vector, the Gateway® LR Clonase™ II reaction (Invitrogen, UK) was used to recombine the entry vector pENTR4™LPTOS with the destination vector, ChAd63-DEST. Herein, site-specific recombination occurs between attachment (attL) sites within the pENTR4™LPTOS entry vector (containing the gene of interest) and attachment R (attR) sites within ChAd63-DEST. Recombinant MVA vaccines were constructed by cloning antigen inserts into an MVA-GFP-TD shuttle vector at

the thymidine kinase (TD) locus (between the TKL and TKR sites) by restriction enzyme digestion and ligation between Acc65I and XhoI. This clone was then linearized and the antigen inserted into the MVA genome (parental virus, MVA-RFP) by homologous recombination. The resulting recombinant viruses were cultured and purified by previously published methods [6,7]. Control ChAd63 and MVA vectors expressing GFP have been described previously [197].

2.2.1.4 Agarose gel electrophoresis

DNA samples were resolved by agarose gel electrophoresis. Gels were prepared as 1% agarose in 1x Tris-acetate-EDTA (TAE) buffer with SYBR Safe Stain (1:10,000). DNA samples were diluted in loading buffer (1:6) prior to loading and separated by electrophoresis (100 V, 60 minutes). Sample visualization was performed using a transilluminator, and band size was determined against SmartLadder (Eurogentec, Belgium) or GeneRuler 1kb (Thermo Scientific, USA) DNA ladders. For sequencing and cloning applications, DNA bands were excised as needed and purified using the QIAquick® Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's published protocol.

2.2.1.5 Polymerase Chain Reaction (PCR)

Unless stated otherwise, PCR reactions in this study were performed using the Phusion® High-Fidelity PCR Master-Mix (New England Biolabs, UK). The standard PCR reaction mixture is summarized below (Table 2.3) along with reaction conditions (Table 2.4).

Table 2.3 Components of Phusion® High-Fidelity PCR mixture

Components	25 µL Reaction	Final Concentration
10 µM forward primer	1.25 µL	0.5 µM
10 µM reverse primer	1.25 µL	0.5 µM
2X Phusion® Master Mix	12.5 µL	1X
Template DNA	variable	<250 ng
Nuclease-free H_20	To 25 µL	N/A

Table 2.4 Standard reaction settings for Phusion® High-Fidelity PCR

Step	Temperature	Time
Initial denaturation	98°C	30 sec
30 cycles	98°C	10 sec
	Variable (55°C-72°C)	30 sec
	72°C	30 sec/kb
Final extension	72°C	10 min
Hold	4°C	

Table 2.5 Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')
F-HAP2	TCGGTACCCTGAACGACTACATCCGGACCAAGTAC
R-HAP2	ATCGGATCCGGTCACGAAGCGGCCCAGG
F-4845	TCGGTACCGGCAACAACGACTTCTGCAAGC
R-4845	ATCGGATCCGCTGTCGATGGTCACGGTCA
F-230C	TCGGTACCGAGTACGTGGACGAGAAAGAGCGGC
R-230C	ATCGGATCCCTTGGAGATCAGCAGTTCCACGATTC

2.2.1.6 Restriction endonuclease digests

Restriction endonuclease digestion reactions were carried out as 2 hour incubations at 37°C using endonucleases from New England Biolabs (NEB). According to the manufacturer specifications, appropriate NEB buffers were

selected with or without bovine serum albumin (BSA). The components of a standard 20 µL reaction are outlined below (Table 2.6).

Table 2.6 Restriction endonuclease digest reaction

Components	20 µL Reaction
NEB restriction enzyme A (10,000 U/mL)	1 µL
NEB restriction enzyme B (10,000 U/mL)	1 µL
NEB buffer (10X)	2 µL
BSA (10X) (as needed)	2 µL
DNA	0.5-1 µL
Nuclease-free H_20	To 20 µL

2.2.1.7 Ligation reactions

DNA fragments were ligated as necessary following excision from a 1% agarose gel and purification by Qiagen® MinElute Gel Extraction Kit (Qiagen, UK) per the manufacturer's instructions. Ligation reactions were incubated at room temperature (RT) for 15 minutes. Components of a standard reaction mixture are as listed below (Table 2.7).

Table 2.7 DNA ligation reaction

Components	10 µL Reaction
Insert	6 µL
Plasmid backbone	2 µL
T4 DNA ligase	1 µL
10x T4 ligase buffer	1 µL

2.2.1.8 Bacterial transformation

Ligation reactions were transformed into chemically competent *E. coli* as follows. High efficiency DH5α competent cells (New England Biolabs, UK) were

thawed on ice for 10 minutes (20 µL per reaction). Cells were mixed with 1-5 µL plasmid DNA (maximum 100 ng), and the mixture was chilled on ice for 30 minutes. The mixture was heat-shocked at 42°C for 45 seconds and then placed back on ice for 5 minutes. 950 µL pre-warmed S.O.C medium (New England Biolabs, UK) was added to the mixture to support *E. coli* growth at 37°C for 1 hour. The bacterial growth mixture was plated onto LB agar plates prepared with the appropriate selection antibiotic for the plasmid. Plates were incubated at 37°C for overnight to promote colony growth. If needed, plates were stored at 4°C for up to 2 weeks.

2.2.1.9 Plasmid DNA preparations

Plasmid DNA was produced by growing individual colonies from agar-plated *E. coli* or archived bacterial glycerol stocks in LB medium under antibiotic selection (1:1000 v/v ampicillin or 1:2000 v/v kanamycin) at 37°C in a shaking incubator (225 rpm) for 16-24 hrs. Bacteria were pelleted by centrifugation at 6000 x *g* for 10 minutes, and plasmids were purified using the QIAGEN® Mini-, Midi-, Maxi-, or Endofree Maxi- Plasmid Kit (Qiagen, UK) as needed. DNA was eluted into ddH_20, and the concentration was determined by the absorbance at 260 nm by NanoDrop™ (Thermo Scientific, UK). Final expression constructs were confirmed by sequencing (Sanger Sequencing Services, UK) before archiving at −20°C for downstream applications. For long-term storage, 1 mL bacterial culture from the original LB stock was mixed with 70% glycerol and stored at −80°C.

2.2.2 Protein production

2.2.2.1 Maintenance of HEK293E cell line

Suspension HEK293E cells were cultured in Exp293 media (Life Technologies, UK) as described in protocol J238 (Appendix A). The HEK293E cell stock was initially obtained from Dr. Ray Owens in the Oxford Protein Production facility at the University of Oxford.

2.2.2.2 Transformation

Transfection of non-adherent HEK293E cells [208] for heterologous protein expression is detailed in protocol J238 (Appendix A). In brief, 24 hours prior to transfection, HEK293E cells were passaged to a density of 2 x 10^6. On the day of transfection, for every 100 mL of culture to be transfected, 100 µg of expression plasmid DNA was added to 4 mL of Freestyle 293 medium (without FCS). After mixing to ensure a homogenous distribution, 100 µg of linear 25 kDa PEI (Alfa Aesar, UK) [35] was added. Plasmid DNA contained 10% plasmid encoding non-secreted GFP. The mixture was vortexed for 10 seconds, and then incubated at 37°C for 10 minutes to support DNA/PEI complex formation. The mixture was applied drop-wise to the HEK293E cell culture. Transfection efficiency was determined after 24 hours by measuring the proportion of cells with green fluorescence. At 4 days of expression, cells were spun at 1800 x *g* to harvest whole supernatant. Soluble recombinant proteins with a hexahistidine tag were affinity-purified from HEK293E supernatant (adjusted to 300 mM NaCl, 20 mM imidazole) using a HisTrap Excel Ni-Sepharose Column (GE

Healthcare, UK) and subsequently eluted with protein elution buffer containing 300 mM imidazole. If necessary, proteins were concentrated using a spin filter device (Millipore, USA). Recombinant proteins were resolved on 4-20% BioRad Mini-PROTEAN® TGX™ precast gels (Pierce, UK) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2.2.2.3 under reducing conditions and visualized by Coomassie Brilliant Blue (Bio-Rad, USA). If visualized as a uniform band of appropriate molecular weight, proteins were quantified by a NanoDrop™ spectrophotometer (Thermo Scientific, UK) by absorbance at 280 nm. Sequences were confirmed to have a sufficient percentage of aromatic residues prior to spectrophotometric quantification. The final concentration was corrected by the extinction coefficient as calculated by the ExPASy ProtParam Server [72] (SIB Bioinformatics Resource Portal, www.expasy.org). Until use, protein samples were stored in aliquots at −80°C.

2.2.2.3 SDS-PAGE

SDS-PAGE was used to detect protein nonspecifically as described in protocol J119 (Appendix A). Samples were run on 4-20% BioRad Mini-PROTEAN® TGX™ precast gels (Pierce, UK) for 1 hour at 100 V along either ColorPlus Protein Ladder (New England Biolabs, USA) or PrecisionPlus Unstain Standards (Bio-Rad, USA) to estimate molecular weight. Gels were washed 3 times in H_20 before staining with Coomassie Brilliant Blue (Bio-Rad, USA) for 1 hour. Gels were then washed with H_20 for 4 x 15 min before overnight de-staining in H_20.

2.2.2.4 Western blotting

Standard methods were used to detect proteins from supernatants and purified protein fractions by Western blot [209], described in protocol J119 (Appendix A). After resolution on a 4-20% BioRad Mini-PROTEAN® TGX™ precast gel (Pierce, UK) by SDS-PAGE and transfer to a nitrocellulose membrane using the Turbo-Blot system (Bio-Rad, USA), blots were blocked in 3% BSA/PBS overnight and incubated for 1 hour at room temperature with antibody. Tag-specific detection of the hexahistidine sequence was achieved by anti-pentahistidine monoclonal antibody (Sigma-Aldrich, UK). Antigen-specific detection was performed either with pooled day 70 sera (n=5 BALB/c mice) or 4B7 Pfs25-specific monoclonal antibody [104] (provided by the NIH Reference Center, USA). Blots were washed with PBS-Tween 20 (PBS/T) for 30 minutes before incubation with alkaline-phosphatase conjugated goat anti-mouse antibody for 1 hour. Blots were then washed for 30 minutes with PBS/T, rinsed with deionized water and incubated with SIGMAFAST™ BCIP®/NBT substrate (Sigma-Aldrich, UK) for direct band visualization. If needed, PNGase F treatment (New England Biolabs, USA) was carried out in accordance with manufacturer instructions.

2.2.3 Immunology

2.2.3.1 Animals

All animal experiments and procedures were performed in accordance with the UK Animals (Scientific Procedures) Act Project License (PPL 30/2414 and

30/2889) and approved by the University of Oxford Animal Care and Local Ethical Review Committee. Mice used in this study were housed in a specific-pathogen free (SPF) environment in the Wellcome Trust Centre for Human Genetics animal facility.

2.2.3.2 Immunizations

Age-matched 6-8 week old female BALB/c mice (Harlan, UK) were vaccinated using a viral-vectored or protein-in-adjuvant prime-boost regime. Mice were anesthetized before immunization with inhaled isofluorane (3.5%) and oxygen (2 L/min). All vaccine doses were administered by the intramuscular (im) route (equal volume into the left and right *musculus tibialis*) with a 26G needle.

2.2.3.2.1 Viral-vectored vaccines

Viral-vectored vaccines were stored in sterile, endotoxin-free PBS at −80°C until use and delivered in a prime-boost regimen consisting of a priming dose of $1x10^8$ infectious units (IU) ChAd63-[antigen] followed by a boosting dose of $1x10^7$ plaque forming units (PFU) MVA-[antigen] at an 8 week interval.

2.2.3.2.2 Protein-in-adjuvant vaccines

Proteins, diluted in sterile, endotoxin-free PBS, were formulated with the adjuvant prior to vaccination by mixing a 1:1 volume ratio with 50 µL of Addavax™ (Sigma-Aldrich, UK) per dose. Doses were delivered on days 0, 28, and 56. Vaccine-induced antibody responses were evaluated by small volume tail-bleed on days 27 and 55 and terminal cardiac bleed on day 70.

2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

A standard methodology for performing ELISA has been described previously [210] and is detailed in protocol IM-6024-00 (Appendix B). Briefly, NUNC-ImmunoMaxisorp plates (Thermo Scientific, UK) were coated overnight with recombinant protein (0.1 µg per well). After washing with PBS/T and blocking for 2 hours with 5% skimmed milk in PBS, plates were incubated with diluted test sera for 2 hours at room temperature. Plates were then washed with PBS/T and incubated with 1:5000 goat anti-mouse total IgG conjugated to alkaline phosphatase diluted in PBS (Jackson ImmunoResearch Laboratories, USA) for 1 hour at room temperature. Signal was detected by addition of visualization substrate p-nitrophenylphosphate (Sigma-Aldrich, UK) diluted in diethanolamine buffer (Thermal Scientific, UK). Optical density (OD) was read at 405nm using an ELx800 absorbance microplate reader (Biotek, UK). Specific deviations from this protocol are addressed in the relevant results chapters.

2.2.4.1.1 Whole IgG endpoint titer ELISA

For endpoint titer quantification of IgG generated to the vaccine antigen panel, day 70 sera (n=5 BALB/c mice) were evaluated in duplicate test samples and serially diluted 3-fold down the plate. The endpoint titer was defined as the *x*-axis intercept of the dilution curve at an absorbance value three standard deviations greater than the mean OD of pooled sera generated to HEK293E-derived OVA protein or ChAd63/MVA-GFP (n=5 BALB/c mice per group).

2.2.4.1.2 Screening ELISA

For antigen immunoreactivity screening, test human sera were diluted by 1:100 for sexual stage antigens and 1:500 for asexual stage antigens (Chapter 6). IgG levels were recorded as raw OD. Positive signal was defined as a test sample absorbance at least 0.01 greater than 3 standard deviations above the mean OD of malaria-naïve adults (USA or UK, n=10) run against the same antigen on the same 96-well NUNC-ImmunoMaxisorp plates (Thermo Scientific, UK). Adjusted OD was calculated as raw OD minus the cut-off of 3 standard deviations above the mean OD of malaria-naïve adults.

2.2.4.1.3 Standardized ELISA

Standardized ELISA to quantify and compare vaccine-induced IgG generated to Pfs25 was performed by a previously reported method [210]. The optical density value of individual test samples was converted into antibody units (AU) using a standard curve generated by serially diluting reference serum of known antibody titer. The reference serum was established by using pooled high-titer day 70 sera derived from BALB/c mouse vaccination with TBV25H [211]. TBV25H protein was generated in *Pichia pastoris* by the Jenner Institute (Oxford, UK).

2.2.5 Indirect Fluorescence Assay (IFA)

Protein localization studies were performed with pooled day 70 sera (n=5 BALB/c mice) by indirect fluorescence assay. Briefly *P. falciparum* NF54 strain gametocytes and gametes were air dried onto glass slides, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 as previously

described [198]. Briefly, slides were blocked for 1 hour in blocking buffer (3% BSA/PBS) followed by incubation with test sera (1:100) or control monoclonal antibody 3E12 (1:500) in 3% BSA/PBS at 37°C in a wet chamber for 1 hour. Slides were washed three times in PBS and then incubated with AlexaFluor 488-conjugated goat anti-mouse IgG (1:2000) for 1 hour. Slides were washed five times and mounted with DAPI Vecta-shield (Vector Laboratories, USA) and analyzed by fluorescence microscopy on a DMI3000B microscope (Leica Microsystems, Germany).

2.2.6 IgG purification

Total IgG was purified from pooled BALB/c mouse sera as previously described [107]. In brief, day 70 mouse sera (n=5 BALB/c mice per test group) were pooled in equal volume (300µL serum per mouse) from all mice irrespective of individual antibody titer following protein-in-adjuvant and viral-vectored vaccinations. Protein G columns (Pierce, USA) were equilibrated with Immunopure IgG binding buffer (Pierce, USA). Pooled mouse sera were diluted in binding buffer (1:1 volume ratio) and applied to the column through gravity flow. Columns were washed once with binding buffer, and purified IgG were eluted with Immunopure IgG elution buffer (Pierce, USA). Eluted fractions were collected in 1 M Tris-HCl (pH 9.0, Teknova, USA) and transferred for buffer exchange to Amicon centrifugal filters (Millipore, USA) using PBS. The eluent was concentrated in PBS (Invitrogen, UK) and filtered using a 0.22 µm Millipore Ultrafree sterile centrifugal unit. Total protein was quantified using a NanoDrop spectrophotometer at 280 nm and adjusted to 3.5 µg/mL.

2.2.7 Parasitology

2.2.7.1 *P. falciparum* sexual stage culture

P. falciparum NF54 strain parasites were maintained in *in vitro* blood culture to generate stage V gametocytes (day 16) by routine methods. For gamete preparation, day 16 gametocytes were activated by incubation at room temperature for 30 minutes to promote differentiation to the gamete stage. For ookinete preparation, *P. falciparum* NF54 strain gametocyte culture (16-18 days old) was adjusted to 0.15-0.2 % stage V gametocytemia at 50% hematocrit. Sixty µL purified human IgG at 0.75mg/mL in PBS was mixed with 200 µL of the gametocyte mixture, and the final mixture was immediately fed to 20 of 3-6 day old female *Anopheles stephensi* (Nijmegen strain) mosquitoes through a membrane feeding apparatus. Mosquitos midguts were dissected to harvest the blood meal after 24hrs. Pooled blood meals (n=20) containing ookinetes were washed three times in RPMI before use.

2.2.7.2 Standard Membrane Feeding Assay (SMFA)

A qualified methodology for performing SMFA has been previously described [212] and is detailed in protocol IM-6008-02 (Appendix B). All SMFA studies were performed by the NIH SMFA Reference Center (Rockville, MD, USA). Briefly, gametocyte culture (16-18 days old) of *P. falciparum* NF54 strain (initially provided by Steve Hoffman, Sanaria, USA) was adjusted at 0.15-0.2 % stage V gametocytemia at 50% hematocrit. 60 µL of a test sample (purified IgG at 0.75mg/mL in PBS) was mixed with 200 µL of the gametocyte mixture, and

the final mixture was immediately fed to ~50 of 3-6 day old female *Anopheles stephensi* (Nijmegen strain) mosquitoes through a membrane feeding apparatus. Purified IgG generated to ovalbumin (OVA) was used as a negative control for protein vaccinations and purified IgG generated to ChAd63/MVA-[GFP] was used as a negative control for viral-vectored vaccinations. Mosquitoes were kept for 7 days and dissected (n=20 per sample) to enumerate the oocysts in the midgut. Only midguts from mosquitoes with any eggs at the time of dissection were analyzed to avoid counting mosquitoes that had not fed on the blood meal. Read-out of the assay is percent inhibition of oocyst development in test samples as a percentage of the relevant purified IgG negative control sample fed at the same concentration.

2.2.8 Epidemiology

In Chapter 6, serological screening was performed using archived plasma samples from Malian child and adult volunteers. Plasma samples tested were from a 4-year cohort study of malaria incidence in Kenieroba, a village 75 km southwest of Bamako, where malaria transmission is intense and highly seasonal. The original study followed approximately 1350 children aged 6 months to 17 years and diagnosed nearly 4200 episodes of malaria in 2008-2011 [213, 214]. Up to 50 adults were sampled for plasma to serve as controls in immunological assays during each year of the study. Gametocyte prevalence was not measured in this study population during this study and had never been measured previously. All samples obtained in the serological survey (ClinicalTrials.gov, NCT00669084) were collected in accordance with the Ethics

Committee of the Faculty of Medicine, Pharmacy and Odontostomatology at the University of Bamako and the Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Diseases (NIAID). Individual written informed consent was obtained from all participants.

Given the lack of adequate gametocyte prevalence data in the study site for detailed assessments of naturally acquired sexual stage immunity, as part of this thesis work, a new clinical protocol was developed in 2012 (ClinicalTrials.gov NCT01829737). Sample collection was completed in 2014, and the materials were used, in part, for the seroreactivity screening in Chapter 7. In design, a cohort of 500 individuals, aged 6 months to 65 years, were enrolled in proportion to the age distribution of Kenieroba, Mali. When possible, entire families living in single compounds (i.e., housing units) were consented for participation in the study. All participants were encouraged to present to the Kenieroba Health Center for treatment in the event of symptoms of malaria. The study received approval from the Ethics Committee of the Faculty of Medicine, Pharmacy and Odontostomatology at the University of Bamako and the Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Diseases (NIAID). Individual written informed consent was obtained from all participants.

Sera from Burkina Faso adults (n=32) were collected with approval from the Centre MURAZ ethical review committee (003-2009/CE-CM). Individual written informed consent was obtained from all participants.

Sera donated by adults from the USA were purchased from Interstate Blood Bank, Inc. (Memphis, TN). Sera donated by adults from the UK were obtained from the Jenner Institute Clinical Trials Division.

2.2.9 Statistics

All statistical analysis was performed using Prism 5 (Graphpad Software Inc., La Jolla, CA, USA). Probability values less than 0.05 were considered significant.

ELISA endpoint titers between vaccination time points were compared by the Mann-Whitney test.

Percent inhibition of mean oocyst intensity (PIm) was calculated by the following formula: 100 × [1 − (mean number of oocysts in the test group)/(mean number of oocysts in the OVA group)], where the 95% confidence interval of the percent inhibition is estimated as previously described [115]. True inhibition and 95% confidence interval was estimated from multiple feeds by the NIH SMFA Reference Center (Rockville, MD, USA) based on a published multi-feed prediction negative binomial, zero-inflation model [212].

All statistical tests were performed in R (version 2.15.2) or Prism 5 (GraphPad Software Inc., La Jolla, CA). P values of <0.05 were considered significant.

Chapter 3: Functional Comparison of Leading TBV Antigens

3.1 Introduction

The successful licensing and field-deployment of a *P. falciparum* TBV will depend on pre-clinical studies that not only evaluate but also prioritize vaccine antigens for subsequent clinical trials. At present, development efforts have focused largely on optimizing the recombinant expression, vaccine formulation, and delivery schedules of known transmission-blocking targets. Numerous published reports have described improvements in the titer and quality of antibodies generated to select antigens in animal models. While studies have been limited to a small number of leading candidates, exceedingly few analyses have ranked well-characterized proteins relative to one another by a standardized functional assay.

SMFA is widely accepted as the gold standard for determining functional transmission-blocking activity of vaccine-induced antibodies. In the assay, *Plasmodium* gametocytes generated through *in vitro* culture are mixed with test antibodies and fed to *Anopheles* mosquitos through a warmed, membrane-covered container. Approximately one week after feeding, mosquitos are dissected and oocysts are enumerated in each midgut. Testing antibody recognition of native proteins expressed by live parasites is a distinct advantage

of SMFA, but the inherent variability of the assay prevents easy comparison of the inhibitory activity of antibodies run in independent assays, particularly at low levels of inhibition. Further to this, despite the general reliance on SMFA to evaluate pre-clinical vaccine antigens, the assay is often not performed by a uniform methodology and results are not reported in a consistent format. The multitude of antigen formulations and delivery schedules during pre-clinical evaluation of leading TBVs further precludes easy comparison of the performance of different antigens relative to one another in independent studies. Chapter 5 will introduce the relative advantages and limitations of SMFA in greater detail.

SMFA was instrumental to the identification of the first panel of TBV candidates. The assay was used to comparatively assess the inhibitory effects of monoclonal antibodies generated to sexual stage parasite extracts. Several targets from these early studies of transmission biology – pre-fertilization proteins Pfs230 and Pfs48/45, and post-fertilization protein Pfs25 – remain the best-developed TBV candidates. Pfs230 and Pfs48/45 contain multiple 6-cysteine structural domains – the result of a pattern of disulfide bonds between 6 conserved cysteine residues – which has complicated heterologous expression and slowed the progress of both proteins through pre-clinical development. Nevertheless, Pfs230 and Pfs48/45 remain leading candidates in part due to their favorable expression pattern from the standpoint of vaccine development. Co-expression of these antigenic targets begins mid-way though the gametocyte stage and is detectable on the surface of the female macrogamete and male microgamete, persisting until the fertilization event.

Human antibody responses resulting from natural infection have been observed to both proteins, opening the possibility that vaccine-generated antibody responses may be boosted.

Pfs25, as a post-fertilization target, does not have exposure to human immune responses over the course of natural infection. Expression begins on newly emerged gametes in the mosquito midgut and continues through the ookinete stage. A potential advantage of late sexual stage expression lies in the opportunity to evade immune-selective pressure resulting in polymorphism, and indeed there is remarkably limited polymorphism in the Pfs25 gene between independent field isolates [56]. To date, of the leading TBVs, only Pfs25 and the *P. vivax* homolog Pvs25 have been tested in clinical trials. Pvs25 was tested in 2005 in a formulation with an alum adjuvant [112], followed by a second Phase I study in 2008 in which Pfs25 and Pvs25 were delivered with experimental adjuvant ISA51 [57]. The former trial did not elicit sufficient antibody titers in volunteers, but the latter had to be stopped prematurely due to reactogenicity concerns that were presumed to be associated with the adjuvant rather than the antigen.

More recently, limited pre-clinical studies of additional sexual stage candidates have shown evidence of TBA. At present, the candidates receiving the most attention for their pre-clinical functional activity are *Plasmodium* gamete surface antigen HAP2 and *Anopheles* midgut protein AgAPN1. HAP2, reported to play an essential role in the membrane fusion of the male and female gamete, has demonstrated TBA with a heterologously expressed *P. falciparum* homolog.

AgAPN1 has abundant expression in the mosquito midgut lumen and may act as an attachment ligand during ookinete recognition and traversal. It too has been evaluated by SMFA in a recombinant *P. falciparum* version. While not a primary focus of this thesis, the targeting of mosquito midgut proteins for TBV development is theoretically appealing; a single vaccine formulation could interrupt the life cycle of multiple malaria species. Additional research into possible redundancy of mosquito midgut recognition and traversal pathways will be critical to promoting this type of technology. A more complete list of candidates of interest in varying stages of pre-clinical development is reviewed in detail in Chapter 1.

The time and tremendous resources associated with vaccine regulatory approvals reinforce the need to critically compare leading candidates in pre-clinical stages and to establish a priority ranking for testing leading formulations in clinical trials. At present few head-to-head studies exist. One report tested recombinant Pfs25, Pfs48/45, Pfs230 (region C), HAP2, and AgAPN1 produced in the WGCFS for functional transmission-blocking epitopes by SMFA. Anti-Pfs25, -Pfs230C, and -HAP2 purified IgG all demonstrated significant inhibition with anti-Pfs25 IgG exhibiting the strongest head-to-head inhibitory effect (Figure 3.1). While IgG generated to WGCFS-produced Pfs25 and Pfs230C had previously been reported [75, 114], this was the first study to show TBA of anti-HAP2 IgG against *P. falciparum* [115].

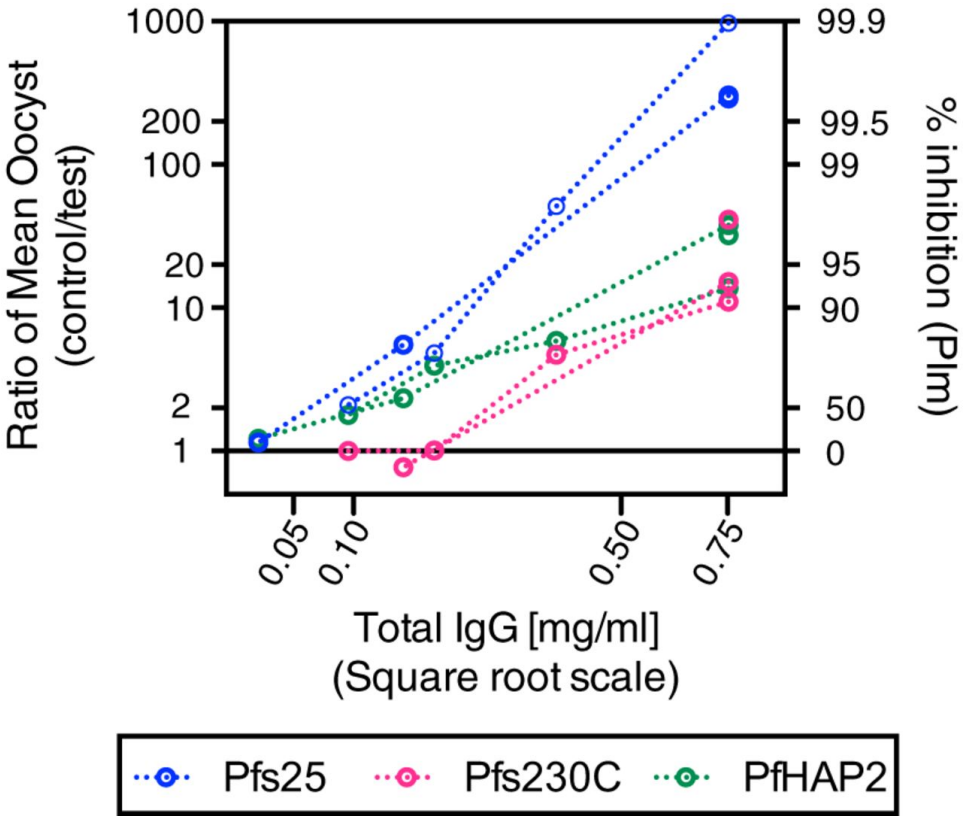
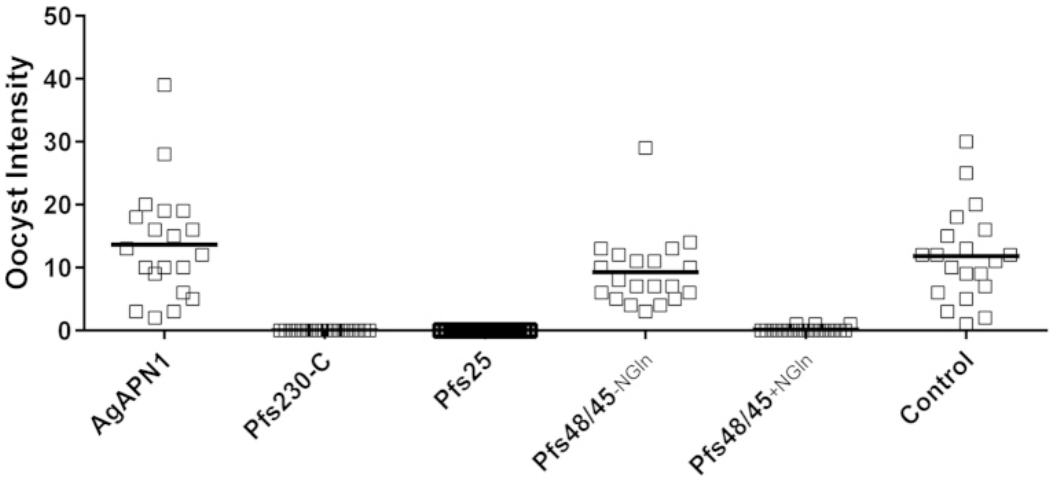


Figure 3.1 Dose-dependent inhibition of oocyst intensity by anti-TBV IgG in SMFA

Purified anti-Pfs25, anti-Pfs230C, and anti-HAP2 IgG were tested at 0.75 mg/ml in the first SMFA, at 0.75, 0.15, and 0.03 mg/ml in the second SMFA, and 0.75, 0.38, 0.19, and 0.09 mg/ml in the third SMFA. Mean oocyst numbers of anti-HisGST IgG tested at 0.75 mg/ml (14.5, 15.2, and 48.6 oocysts per mosquito in the first, second, and third SMFAs, respectively) were used as controls. The square root of total IgG level is shown on the x-axis. The ratios of mean oocyst counts in control and test samples are plotted on a log scale along the left y-axis, and the associated percent inhibition of mean oocyst intensity is plotted along the right y-axis. Data points from the same SMFA are connected by dotted lines for the second and third SMFAs. Figure and legend adapted from [115].

More recently, leading TBV candidates Pfs25, Pfs48/45, Pfs230C, and AgAPN1 were each delivered head-to-head in a viral-vectored regime consisting of a ChAd63 adenovirus prime followed by a modified vaccinia Ankara (MVA) boost [198]. Purified IgG resulting from the vaccinations demonstrated a hierarchy of inhibition by SMFA. Anti-Pfs230C and anti-Pfs25 IgG conferred complete transmission block. These experiments used antigen sequences based on the 3D7 strain of *P. falciparum*, consistent with the report by Miura *et al*. So too SMFA in both publications was performed by the NIH SMFA Reference Center using *A. stephensi* mosquitoes and purified IgG. Notably, the observed rank order demonstrated with the Chad63/MVA prime-boost regime was replicated against field isolates of *P. falciparum* from Burkina Faso in *A. gambiae* mosquitos by direct membrane feeding assay (DMFA), providing the first head-to-head comparison in a single study with functional data from two parasite species and two vector sources (Figure 3.2).



	AgAPN1	Pfs230-C	Pfs25	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	20	20	20	20	20	20
Mean	13.7	0	0	9.3	0.2	11.8
Inhibition of Infection (%) (lower, upper 95% CI)	-16 (-73, 23)	100 (100, 100)	100 (100, 100)	22 (-13, 46)	99 (96, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	0 (0, 1)	100 (0.8, 1)	100 (0.8, 1)	0 (0, 0)	85 (0, 1)	-

Figure 3.2 Effect of mouse IgG induced by ChAd63-MVA immunization on P. *falciparum* NF54 parasite infectivity in *A. stephensi* mosquitoes.

Pooled day 70 serum ($n = 5$) was used to purify total IgG against each of the antigens. 655 µg/mL of each of the total IgG was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* mosquitoes ($n = 20$) in SMFA. Midguts were dissected 7 days post-feeding. Data points represent the number of oocysts in individual mosquitoes and the lines show the arithmetic mean (from one experiment). The table shows the number of mosquitoes dissected (N) per antigen, mean number of oocysts, percent inhibition of infection intensity and prevalence calculated relative to the IgG from mice immunized with vectors expressing GFP. Figure and legend adapted from [198].

While historically, viral vectors have been valuable tools for the induction of antigen specific T-cells, it is important to note that adenovirus vectors such as HuAd5 and ChAd63, when delivered in prime-boost with MVA, have been reported to induce functional antibodies in pre-clinical studies and Phase I/IIa clinical trials [200, 215-217]. Clinical translation of adenoviruses with human tropism such as HuAd5 has been complicated by the presence of neutralizing antibodies from previous infections. In turn, chimpanzee adenoviruses such as ChAd63, a human-compatible delivery platform reported to elicit lower levels of anti-vector neutralizing antibodies, have offered an alternative to HuAd5 for vaccine testing. A relative advantage of viral-vectored platforms in general is that the delivery of each antigen is not subject to the inherent variability of recombinant protein purification *ex vivo*. Nevertheless, given the system's dependence on protein expression *in vivo*, easy assessment of the amount of protein needed for a given immune response is not possible.

A criticism of both the Miura *et al* and Kapulu *et al* studies is that each derived conclusions about head-to-head efficacy based on TBV antigens generated in only one expression system. The aim of Chapter 3 is to determine whether the leading TBV antigen performance ranking changes in different expression platforms. A panel of leading candidate TBV antigens was delivered to BALB/c mice in both a ChAd63/MVA viral-vectored regime and a protein-in-adjuvant formulation. Purified IgG was evaluated for functional inhibition of oocyst intensity by SMFA. Chapter 3 reports a consistent head-to-head rank order between both the protein-in-adjuvant and viral-vectored delivery platforms; anti-Pfs25 and anti-Pfs230C IgG exhibit the strongest inhibitory effect.

3.2 Results

A panel of leading *P. falciparum* TBV candidate antigens was selected for characterization by SMFA. In an effort to expand upon the two previously published head-to-head comparisons of TBV antigen efficacy, recombinant Pfs25, Pfs28, Pfs48/45, Pfs230C and HAP2 were selected for assessment in BALB/c mice by either (1) viral-vectored heterologous ChAd63 prime and MVA boost or (2) protein-in-adjuvant vaccination.

3.2.1 Generation of TBV candidate ChAd63 and MVA viral vectors

Recombinant ChAd63 and MVA vectors for Pfs25 [197], Pfs48/45, and Pfs230C [198] have been previously published. Additional paired ChAd63 and MVA vectors were developed in the present study for HAP2 and Pfs28, a paralog of Pfs25 with reported inhibition of oocyst intensity by SFMA. Parasite antigen sequences were based on the *P. falciparum* 3D7 strain. Predicted N-glycosylation cites, recognized as Asn-Xaa-Ser/Thr, were mutated to Gln-Xaa-Ser/Thr for all antigens except Pfs48/45. Of note, Kapulu *et al* created two versions of Pfs48/45 corresponding to both the native amino acid sequence ($Pfs48/45_{+NGln}$) and a modified construct with 7 predicted N-glycosylation cites removed ($Pfs48/45_{-NGln}$). Of the two, anti-$Pfs48/45_{+NGln}$ purified IgG demonstrated superior inhibition of oocyst intensity by SMFA. Concern that mutation of N-glycosylation sites impacted Pfs48/45 transmission-blocking epitope conformation led to the use of only $Pfs48/45_{+NGln}$ in the current analysis.

Vector constructs were based on full-length ectodomains for all antigens except Pfs230, for which region C (amino acids 443-1132) has been reported as the domain of interest for vaccine design. All antigen sequences were codon optimized for expression in *Homo sapiens*. Predicted endogenous signal peptides for each antigen were replaced with the human tissue plasminogen activator (tPA) leader sequence.

3.2.2 Expression of TBV antigens in HEK293E cells

For protein-in-adjuvant vaccination, recombinant proteins were expressed in the HEK293E cell system [218]. Identical antigen sequences to those used in the recombinant ChAd63 and MVA vectors were subcloned into a modified pENTR4-LPTOS™ [201] expression construct (p3223, Section 2.2.1.2) encoding an exogenous N-terminal human tissue plasminogen activator (tPA) signal peptide and C-terminal biotin-acceptor and hexahistidine affinity tag (Figure 3.3). Expression plasmids were complexed with PEI for HEK293E transfection followed by transient protein expression. Soluble recombinant proteins were affinity purified from whole supernatant by Ni-Sepharose column and subsequently eluted with imidazole.

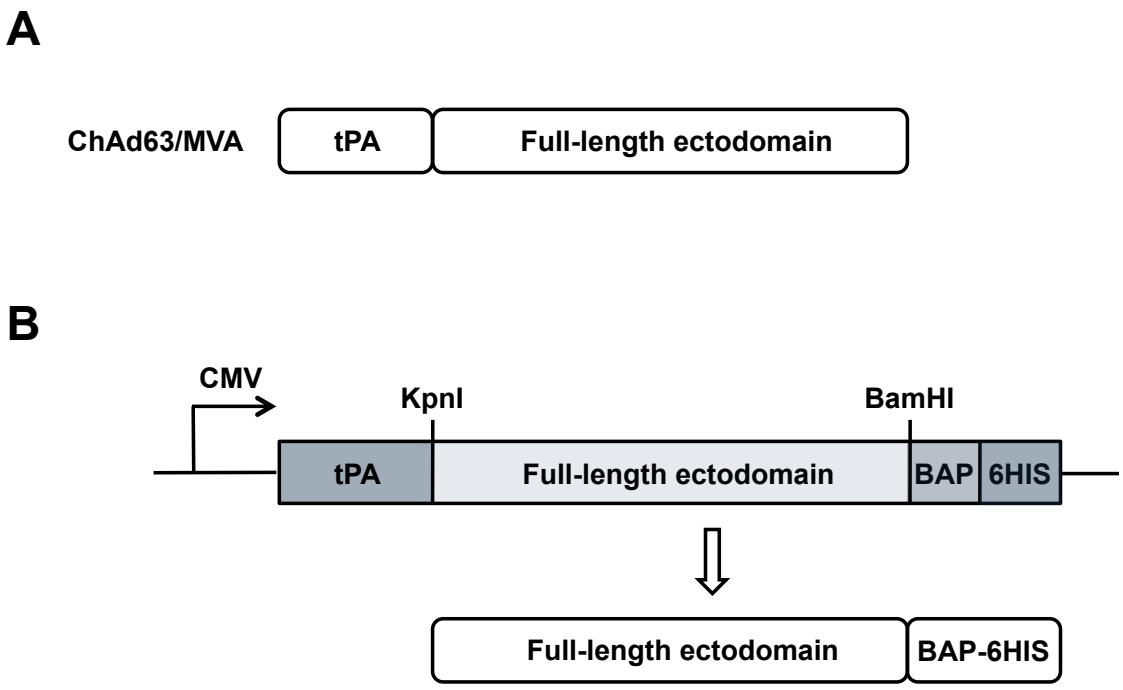


Figure 3.3 Expression construct design

Constructs encoding TBV antigens (Pfs25, Pfs28, Pfs48/45$_{+NGln}$, Pfs230C, HAP2) were generated to enable (**A**) viral-vectored delivery and (**B**) heterologous protein expression and purification. The same gene sequence was used in the expression construct for both platforms. Synthetic genes were based on the *P. falciparum* 3D7 strain. Endogenous signal peptides were replaced with the human tissue plasminogen activator (tPA) leader sequence. A biotinylatable (BAP) and hexahistidine (6HIS) sequence was included in the construct designed for affinity purification of soluble protein products from HEK293E cell supernatant.

Table 3.1 Antigen sequences used for generation of ChAd63/MVA and protein TBV constructs

Antigen	Accession #	Amino Acids	N-glycosylation
Pfs25	PF3D7_1031000	22-192	0*
Pfs28	PF3D7_1030900	23-218	0*
Pfs230C	PF3D7_0209000	443-1132	0*
Pfs48/45$_{+NGln}$	PF3D7_1346700	28-427	7
HAP2	Pf3D7_1014200	31-684	0*

*Represents N-glycosylation sites in the expression construct, not predicted N-glycosylation sites in the native antigen sequence.

The yield and solubility of Pfs25, Pfs28, Pfs48/45$_{+NGln}$, and Pfs230C were sufficient to pursue a BALB/c mouse vaccination study. Ni-Sepharose column eluates resolved by SDS-PAGE and visualized by Coomassie stain revealed bands within the predicted range for these antigens (Figure 3.4 A). Specificity was confirmed by Western blot detection with anti-pentahistidine mAb (Figure 3.4 B). HAP2 expression was not detectable by anti-pentahistidine Western blot and as a result this antigen was removed from further consideration within the study. While affinity purification resolved Pfs25 and Pfs28 into single bands, Pfs48/45$_{+NGln}$ and Pfs230C preparations contained additional bands outside of the predicted sizes for both antigens (Figure 3.5 A and Figure 3.6 A). These bands were not detectable in Western blots performed using whole anti-serum specific for either Pfs48/45$_{+NGln}$ or Pfs230C generated by BALB/c mouse vaccination with ChAd63/MVA viral vectors [198], suggesting that they may be the result of co-purification from whole HEK293E supernatant rather than degradation of proteins of interest (data not shown).

In an effort to improve the protein preparations for Pfs48/45$_{+NGln}$ and Pfs230C, first the affinity-based elution protocol was modified from a one-step elution with 300mM imidazole to a gradient ranging from 70 mM to 300 mM imidazole. This

process was not successful in identifying a fraction with improved protein purity as assessed by AKTA™ chromatogram and Coomassie stain (data not shown). Similarly, use of size exclusion chromatography was unable to effectively separate either Pfs48/45$_{+NGln}$ or Pfs230C from lower and higher molecular weight contaminants (Figure 3.5 B and Figure 3.6 B). Quantitative assessment of the proportion of antigen of interest relative to total protein – as visualized by Coomassie stain – was attempted in the interest of normalizing protein vaccine dosage, but due to the high incidence of background banding, this approach was ultimately abandoned in favor of calculating vaccine doses based on total protein eluted from the Ni-Sepharose column.

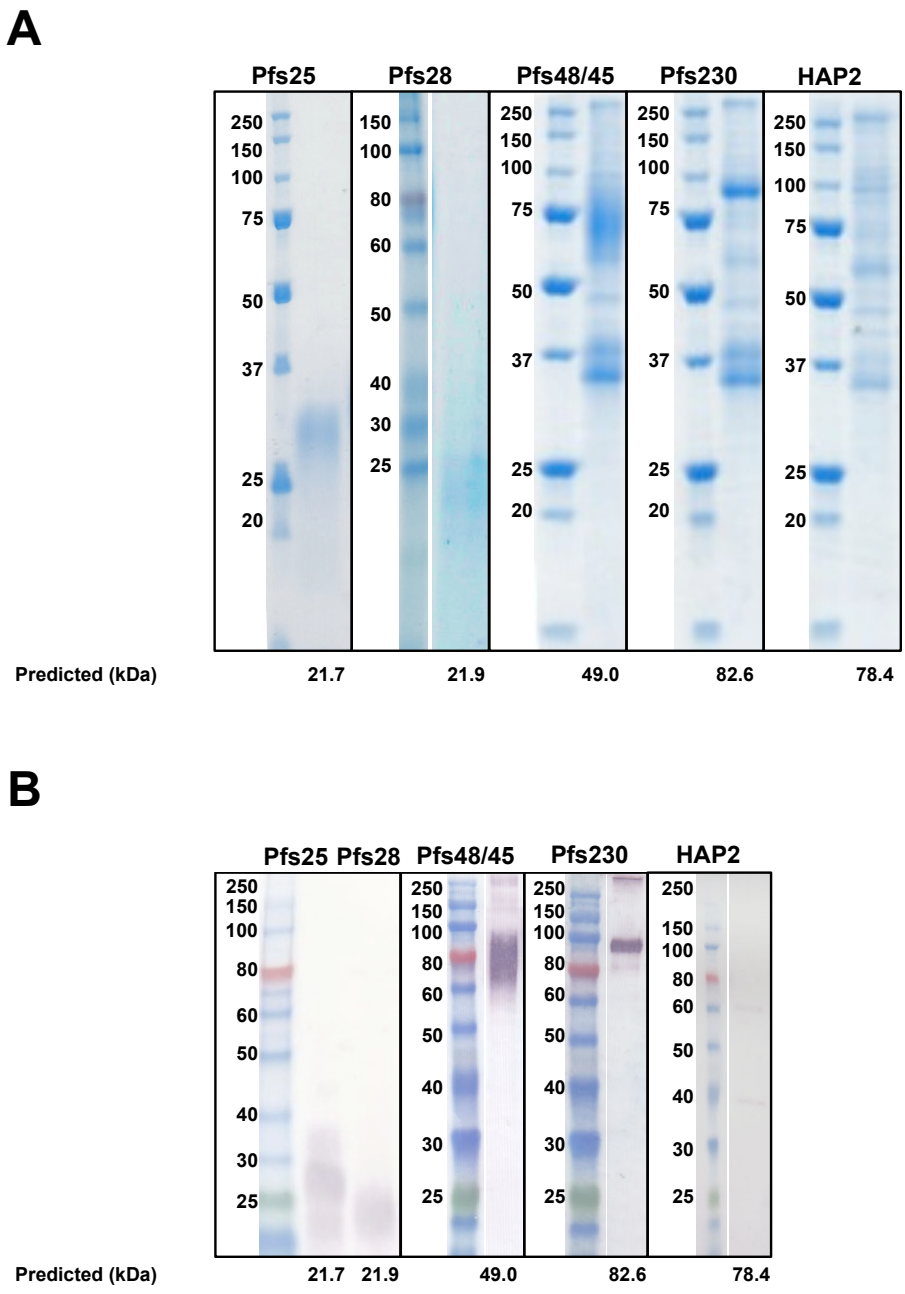


Figure 3.4 Heterologous expression of TBV antigens

A. Coomassie brilliant blue R-250 staining of recombinant proteins resolved by reducing SDS-PAGE. Each protein has a C-terminal biotin-acceptor peptide and hexahistidine tag (2.95 kDa) and was purified from HEK293E cell supernatant by Ni-Sepharose column prior to visualization. **B.** Western blot detection of affinity-purified TBV antigens by anti-pentahistidine monoclonal antibody after SDS-Page under reducing conditions and nitrocellulose transfer.

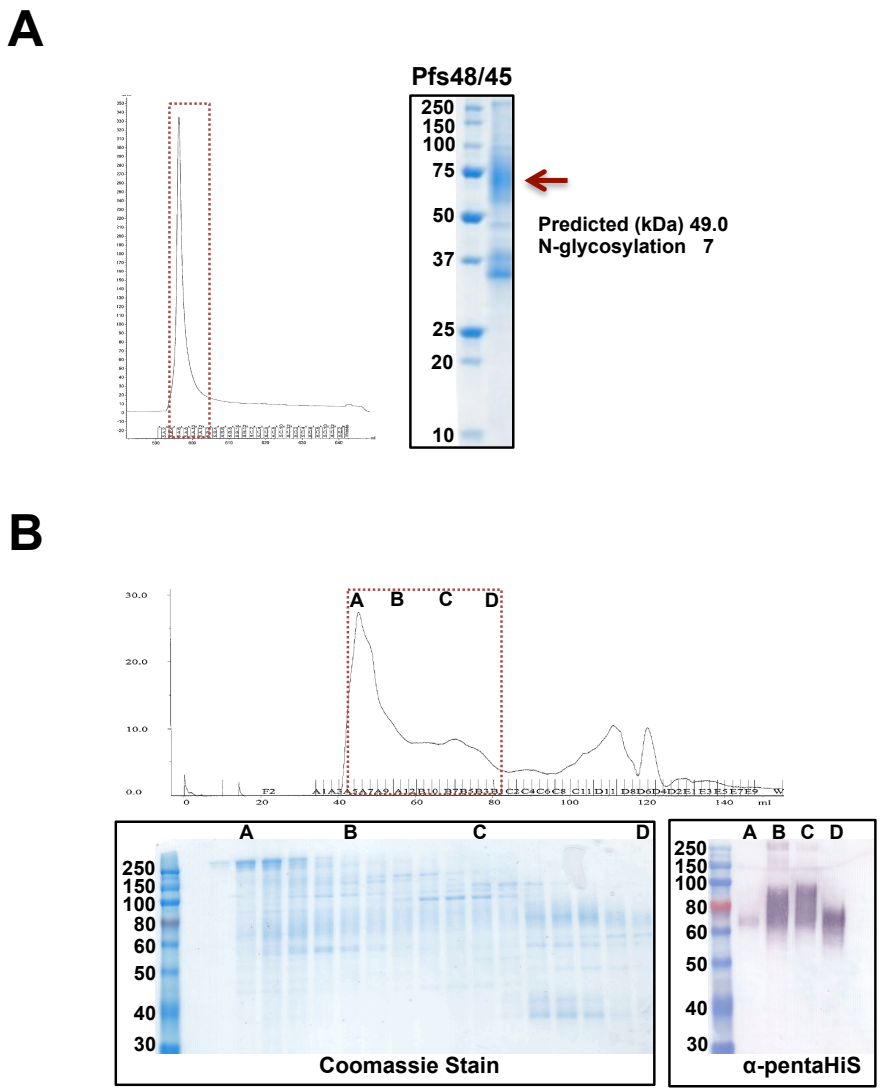


Figure 3.5 Pfs48/45$_{+NGln}$ expression and purification

A. AKTA™ chromatogram tracing the elution step during affinity purification of Pfs48/45$_{+NGln}$. Soluble protein derived from HEK293E supernatant was purified with a Ni-Sepharose column and eluted at 300 mM imidazole. Pooled fractions from the chromatogram peak were resolved by reducing SDS-PAGE and stained with Coomassie brilliant blue R-250. **B.** AKTA™ chromatogram tracing the elution step of size-exclusion chromatography (SEC) purification. Pooled, purified Pfs48/45$_{+NGln}$ fractions from a Ni-Sepharose column were loaded onto a Superdex 200 pg column. Individual fractions from the chromatogram peak were resolved by reducing SDS-PAGE and stained with Coomassie brilliant blue R-250. Select fractions (labeled A, B, C, D) were visualized by Western blot with anti-pentahistidine monoclonal antibody following reducing SDS-PAGE.

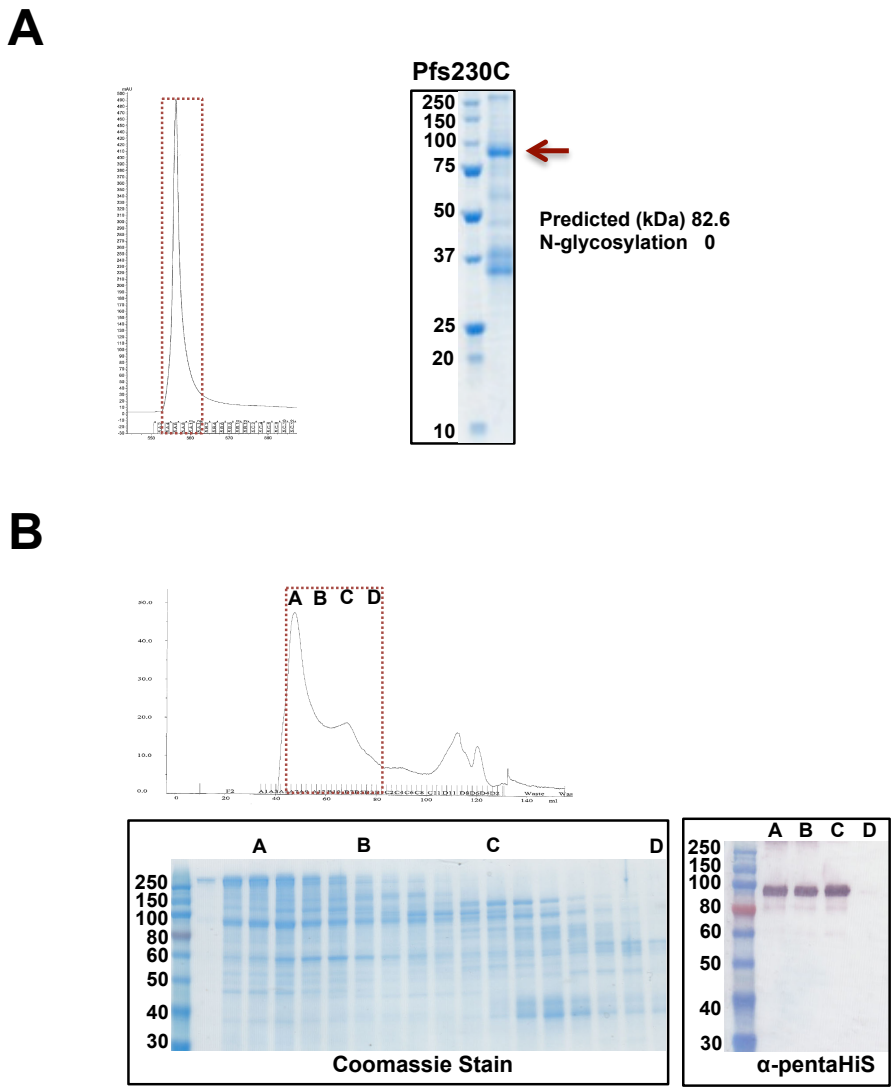


Figure 3.6 Pfs230C expression and purification

A. AKTA™ chromatogram tracing the elution step during affinity purification of Pfs230C. Soluble protein derived from HEK293E supernatant was purified with a Ni-Sepharose column and eluted at 300 mM imidazole. Pooled fractions from the chromatogram peak were resolved by reducing SDS-PAGE and stained with Coomassie brilliant blue R-250. **B.** AKTA™ chromatogram tracing the elution step of size-exclusion chromatography (SEC) purification. Pooled, purified Pfs230C fractions from a Ni-Sepharose column were loaded onto a Superdex 200 pg column. Individual fractions from the chromatogram peak were resolved by reducing SDS-PAGE and stained with Coomassie brilliant blue R-250. Select fractions (labeled A, B, C, D) were visualized by Western blot with anti-pentahistidine monoclonal antibody following reducing SDS-PAGE.

3.2.3 Immunogenicity of protein-in-adjuvant and viral-vectored vaccination

To assess immunogenicity and generate purified IgG for functional studies, age-matched BALB/c mice (n=5) received either protein-in-adjuvant or viral-vectored delivery of each TBV antigen (Figure 3.7). For each candidate vaccine, animals were vaccinated with a ChAd63 priming dose of $1x10^8$ IU (day 0) followed by a MVA boosting dose of $1x10^7$ PFU (day 56). Control vaccinations were delivered with ChAd63 and MVA expressing GFP. In a parallel protein-in-adjuvant study arm, animals were vaccinated with 20 μg of affinity-purified protein in Addavax™ adjuvant in three intramuscular doses delivered four weeks apart (days 0, 28, 56). OVA protein in the same formulation and regime was used as a vaccination control. Over the course of the study, sera were collected from all groups at days 27, 55 and 70. All animal experiments and procedures were performed in accordance with the UK Animals (Scientific Procedures) Act Project License (PPL 30/2414 and 30/2889) and approved by the Oxford University Local Ethical Review Committee.

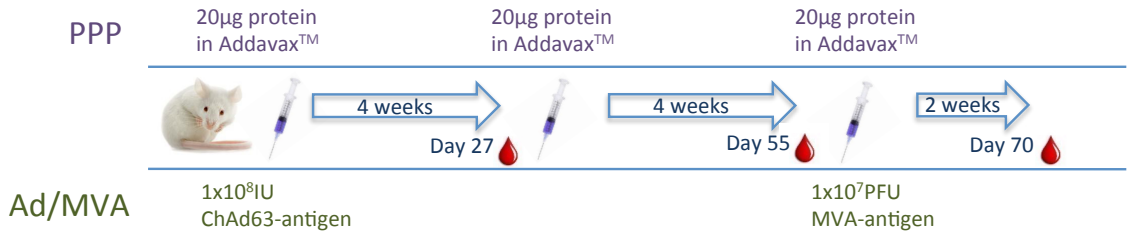


Figure 3.7 Vaccination schedule

TBV antigens were delivered to BALB/c mice (n=5 per group) in parallel protein-in-adjuvant (PPP) and viral-vectored (Ad/MVA) regimes. Protein was given in a 20 µg dose in Addavax™ adjuvant by the intramuscular route (days 0, 28, 56). Viral-vectors encoding recombinant antigens were given in a ChAd63 priming dose ($1x10^8$ IU, day 0) followed by a MVA boosting dose ($1x10^7$ PFU, day 56) by the intramuscular route. Sera were collected at days 27, 55, and 70 for immunological studies.

Immunogenicity was assessed using day 0, 27, and 55 sera samples by endpoint titer ELISA to HEK293E-derived protein. Within the viral-vectored regime, MVA delivery significantly boosted IgG responses between day 27 and day 70 for all antigens as assessed by Mann-Whitney (Pfs25: p=0.0317; Pfs28: p=0.0159; Pfs230C: p=0.0079; Pfs48/45$_{+NGln}$: p=0.0397) (Figure 3.8). Notably, Kapulu *et al* did not observe a statistically significant boosting with MVA corresponding to Pfs25 – a result in contrast to previously reported significant boosting effect following HuAd5 priming. The present findings suggest that this may have indeed been due to variability in antibody responses of animals within the Kapulu *et al* study rather than a true lack of effect following ChAd63 priming.

Similarly in the protein-in-adjuvant study arm, two boosting doses significantly increased IgG endpoint titer between day 27 and day 70 for all antigens (Pfs25: p=0.0159; Pfs28: p=0.0079; Pfs230C: p=0.0238; Pfs48/45$_{+NGln}$: p=0.0079). The first boost of protein translated into a significant boost in antibody titer between day 27 and 55 for Pfs25 (p=0.0159), Pfs230C (p=0.0079), and Pfs4845$_{+NGln}$ (p=0.0079), but not Pfs28 (p=0.3095). By contrast, the second boost resulted in a significant increase in titer between day 55 and 70 for Pfs28 (p=0.0159) and Pfs48/45$_{+NGln}$ (p=0.0397), but not Pfs25 (p=0.6571) and Pfs230 (p=0.1984) (Figure 3.8). The variation in this study between the effect of the first and second boost for the panel of antigens may be due to insufficient data points to see a significant effect rather than inherent differences in vaccination kinetics.

Importantly endpoint titer was not significantly different between viral-vectored and protein-in-adjuvant vaccination for Pfs25 (p=0.4127), Pfs28 (p=0.3968), or

Pfs230C (p=0.6667) by Mann-Whitney. Endpoint titers were significantly different between Pfs48/45$_{+NGln}$ delivered as a protein-in-adjuvant versus viral vector (p=0.0079). Again, it is possible an effect would be seen with more observations in individual test groups.

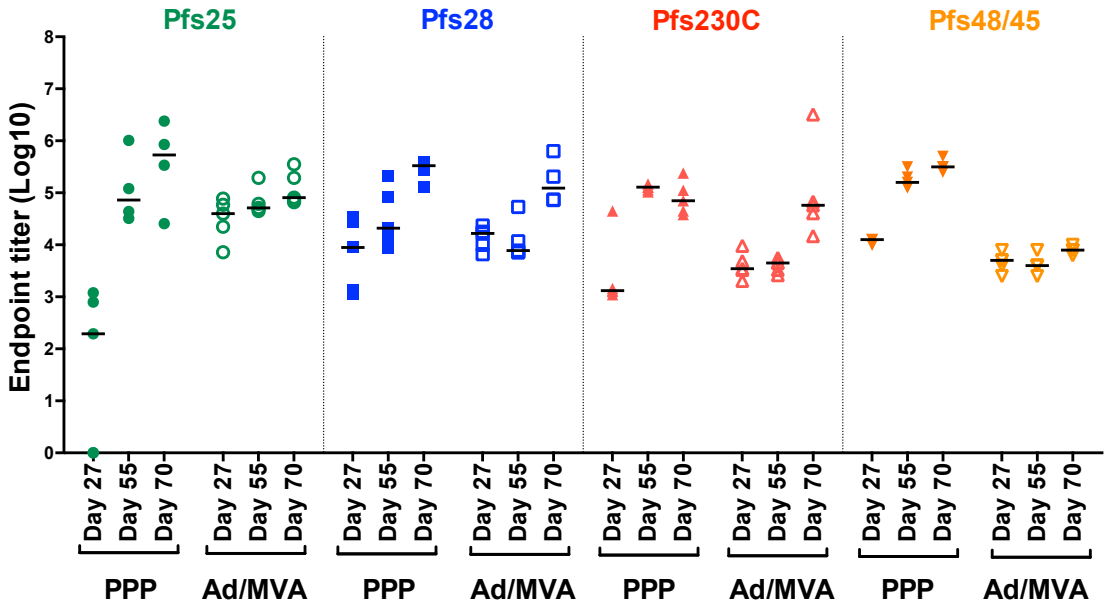


Figure 3.8 Immunogenicity of protein-in-adjuvant and viral-vectored regimes

IgG responses generated to each TBV antigen in both protein-in-adjuvant (PPP) and viral-vectored (Ad/MVA) vaccination regimes were assessed by endpoint titer ELISA using day 70 sera. All PPP sera were depleted of anti-tag responses, achieved by co-incubation of test-sera with peptide representing the biotin acceptor peptide and hexahistidine sequence (method described in Section 4.2.3). Responses of both individual BALB/c mice and the group arithmetic mean are shown.

3.2.4 Inhibitory activity of vaccine-induced purified IgG by SMFA

Purified IgG was generated from pooled day 70 BALB/c mouse sera (n=5) for assessment by SMFA for inhibitory effects on oocyst intensity in the *A. stephensi* midgut. The aim of the analysis was to determine whether the head-to-head rank order of antigen performance differs between viral-vectored and protein-in-adjuvant delivery. All feeds were performed at 0.75 mg/ml. Anti-Pfs25 IgG and Pfs230C demonstrated the highest level of inhibition in both the viral-vectored and protein-in-adjuvant systems. Pfs28 showed intermediate performance. Pfs48/45$_{+NGln}$ delivered by viral vectors demonstrated the lowest inhibition of oocyst intensity corresponding to the lowest transmission-blocking effect (Figure 3.9).

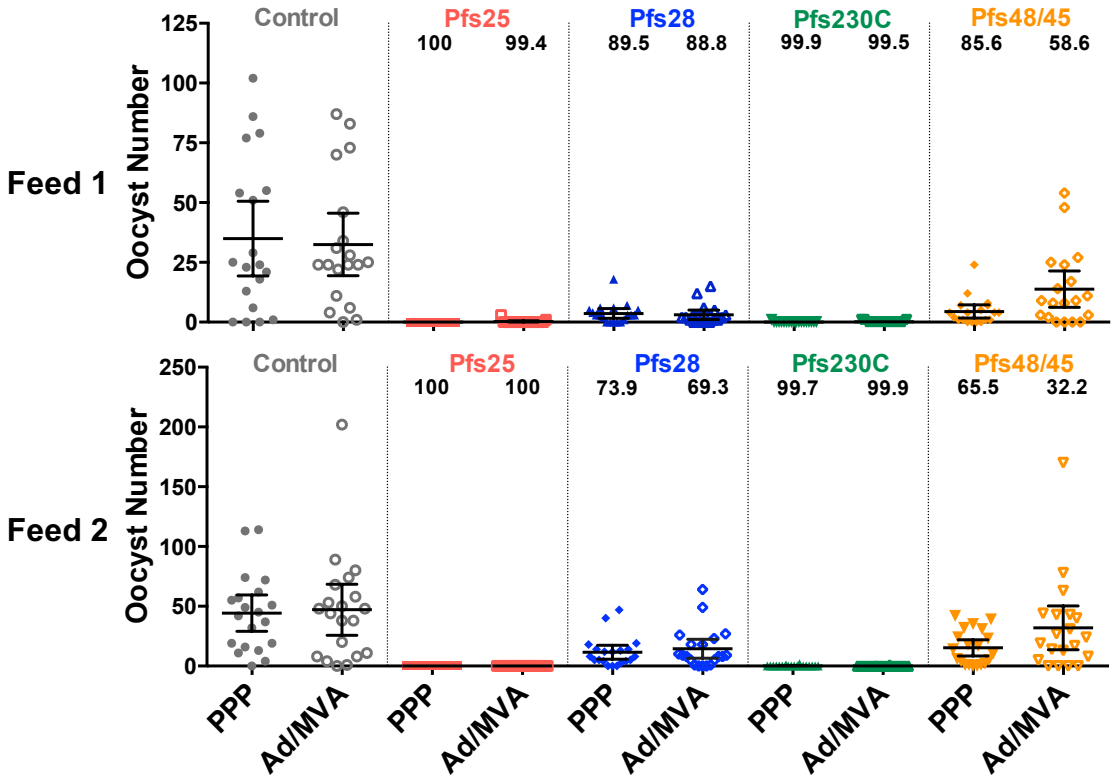


Figure 3.9 Standard membrane feeding assay

Purified IgG from pooled anti-serum (day 70) generated to recombinant TBV antigens in either a protein-in-adjuvant (PPP) or viral-vectored (Ad/MVA) regime was tested by SMFA in the presence of human complement at a final concentration of 0.75 mg/mL in the mosquito blood feeder. Two independent feeds are shown in **A** and **B** for all candidates and regimes evaluated. Individual oocyst counts per mosquito and mean oocyst intensity and standard deviation of the test group are shown. The value of the inhibition of oocyst intensity is calculated relative to the appropriate experimental control IgG at a final concentration 0.75 mg/ml (PPP: OVA protein, Ad/MVA: ChAd63/MVA-GFP) and listed above each group.

Based on a previously published multi-feed prediction model [212], the data from two independent feeds was compiled to estimate the true percent inhibition and 95% confidence interval to establish a rank-order of performance for both viral-vectored and protein-in-adjuvant delivery (Figure 3.10). In both the protein-in-adjuvant and viral-vectored delivery platforms, IgG generated to Pfs25 and Pfs230C had the highest inhibitory effect, followed by Pfs28. This is the first study to evaluate Pfs28 in a head-to-head ranking. While the antigen is well described, redundancy of function with Pfs25 prevented its careful consideration as a TBV antigen. The activity of Pfs48/45$_{+NGln}$ in a protein-in-adjuvant delivery system was indistinguishable from Pfs28 given as protein or viral vector, but IgG generated to ChAd63/MVA-Pfs48/45$_{+NGln}$ demonstrated the lowest inhibitory effect. Notably, the antigen rank order did not change with delivery platform and was in keeping with previous reports [115, 198].

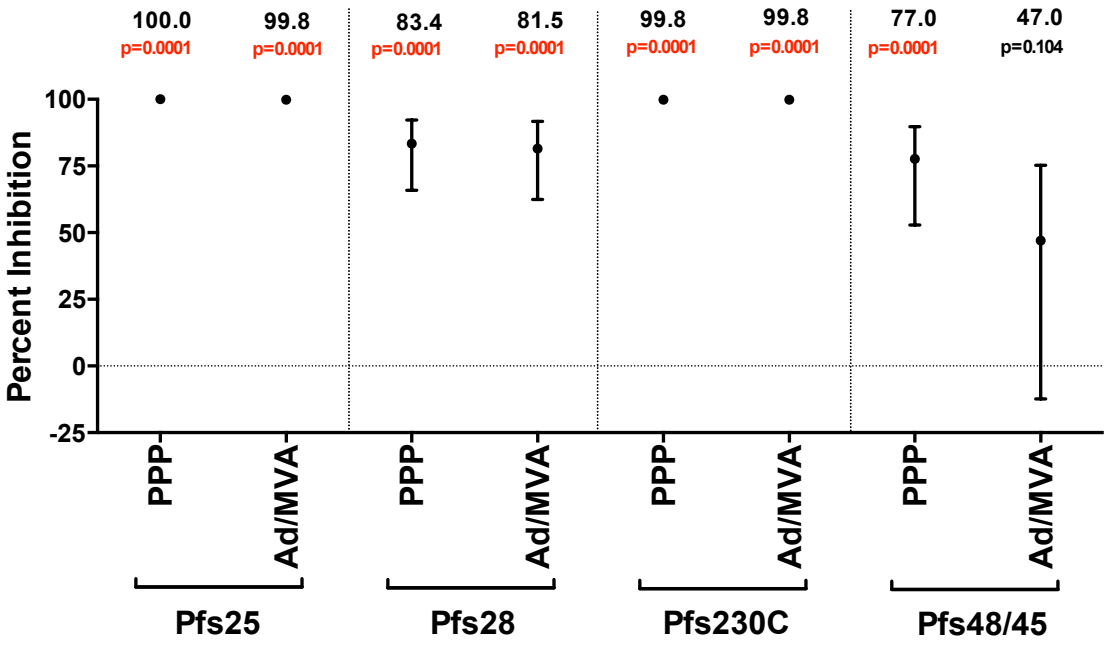


Figure 3.10 Multi-feed prediction model

To estimate the percent inhibition of oocyst intensity and 95% confidence interval (CI) based on two independent feeding assays conducted using 0.75 mg/ml test IgG, an R script model was implemented which incorporates data from multiple independent feeding experiments to account for assay variability due to feed-to-feed and mosquito test group differences [212].

3.3 Discussion

With expansion of the research agenda to prioritize TBV development, pre-clinical work increasingly employs a variety of expression systems and vaccine delivery platforms. At present, it is difficult to unify SFMA findings from these reports into a pre-clinical priority ranking of historical and novel TBV candidates. For one, purified IgG induced by vaccination may elicit variable TBA against *P. falciparum* relative to the expression system or vaccine delivery platform used. The analysis in this chapter is the first to establish a rank order of TBV antigen performance in two independent vaccination approaches within the same study. Notably, the rank order of inhibitory activity was replicated in both a protein-in-adjuvant and viral-vectored system. Pfs25 and Pfs230C outperformed all TBVs tested, followed by Pfs28 and Pfs48/45$_{+NGln}$.

Pfs25 has demonstrated consistency as a vaccine antigen in pre-clinical work (Section 1.5.3). By contrast, challenges surrounding heterologous expression of recombinant Pfs230 and Pfs48/45 have been significant enough to slow progression of these candidates into clinical trials. Multiple efforts to optimize construct design are documented in the literature (Section 1.5.2). Following interest in Pfs230 region C (amino acids 443-1132) [72], versions that elicit inhibitory IgG have been published using *E. coli* (amino acids 443-1132) [72], the WGCFS (amino acids 443-1132) [75, 115], *N. benthamiana* (amino acids 444-730) [76], modified *P. pastoris* (amino acids not available) [77][1], and a

[1] Pfs230 subdomain 1 (Pfs230D1), fused with EPA, is currently in a Phase I clinical trial (ClinicalTrials.gov NCT02334462).

ChAd63/MVA viral-vectored expression platform (amino acids 443-1132) [198]. In this chapter, Pfs230C (amino acids 443-1132) generated in the HEK293E and viral-vectored system both elicited 99.8% (p=0.0001) inhibition of oocyst intensity in a qualified SMFA performed by the NIH SMFA Reference Center using purified IgG. The strong inhibitory activity of IgG generated to HEK293E-derived protein was unexpected in the setting of concerns about protein purity and integrity based on SDS-PAGE visualization. Accepting that the hexahistidine tag is subject to a higher level of impurity relative to alternate tag-based purification systems, affinity tags such as the StrepII or C-tag may improve the protein purity for future work.

Native Pfs48/45 epitopes have proven particularly challenging to recreate (Section 1.5.2). Two constructs expressed in *E. coli* have been reported to elicit transmission-blocking antibodies in the literature. In one study, a C-terminal Pfs48/45 fragment (amino acids 159-428) was expressed in an *E. coli* strain modified to up-regulate enzymes that promote disulfide bond formation and protein folding [84]. A second study expressed the full-length Pfs48/45 sequence in *E. coli* following codon harmonization [85]. More recently, regions denoted 10C (amino acids 159-428) [219][2] and the single epitope 6Cc (amino acids 291-428) [220] were generated in *L. lactis* and reported to induce strong TBI. In this chapter, the HEK293E-derived full-length Pfs48/45$_{+NGln}$ exhibited an inhibition of 77.0% (p=0.0001) in the two-feed prediction model. By contrast, the

[2] The 10C antigen will enter clinical trials as a chimera, fused to asexual stage candidate GLURP, as part of the REDMAL project funded by the European Commission.

ChAd63/MVA-Pfs48/45$_{+NGln}$ vaccine inhibited oocyst intensity by 47.0% (p=1.04). This is less than reported by Kapulu *et al*, wherein Pfs48/45$_{+NGln}$ administered by viral-vectored delivery ranged between 89-99% inhibition in SMFA with *A. stephensi* mosquitoes. The same construct demonstrated an inhibition of 46% in SMFA with *A. gambiae* mosquitoes. The results of Chapter 3, using an identical ChAd63/MVA-Pfs48/45$_{+NGln}$ vaccine and immunization schedule, suggest that some of this variation may be a byproduct of the vaccine construct itself. The version with mutated glycans (ChAd63/MVA-Pfs48/45$_{-NGln}$) was not included in our study. In the Kapulu *et al* analysis, this version elicited 22% and 46% inhibition in *A. stephensi* and *A. gambiae* mosquitoes respectively.

Due to a redundancy of function with paralog Pfs25, Pfs28 has been primarily studied as a multi-subunit TBV component (Section 1.5.3). Of available reports on the functionality of the antigen without conjugation, expression of Pfs28 in *S. cerevisiae* yielded inhibition of 92-100% (amino acids 24-179) using whole serum [93] and 69% (amino acids 24-196) in a 1:3 whole serum dilution [92] in SMFA. The results of Chapter 3 are the first to evaluate Pfs28 in a qualified SMFA using purified IgG. Performance was similar between both vaccine delivery platforms with an inhibition of 83.4% (p=0.0001) by HEK293E-derived protein and 81.5% (p=0.0001) by ChAd63/MVA-Pfs28. The unexpectedly strong activity of anti-Pfs28 IgG supports the consideration of this protein in a multi-antigen TBV.

Outside of the well-characterized, historical TBV candidates, HAP2 is perhaps the only parasite protein to have demonstrated encouraging inhibitory activity against *P. falciparum*. Protein expressed in the WGCFS (amino acids 195-684) elicited 97% inhibition in a qualified SMFA using purified IgG [115]. Unfortunately our study was not able to test HAP2 (amino acids 31-684) due to challenges expressing the antigen in HEK293E cells. Optimized expression of the shorter fragment (amino acids 195-684) in HEK293E cells is ongoing. In the study by Miura *et al*, which evaluated the WGCFS-derived protein, it was noted that low yield of HAP2 protein precluded two-step purification by both affinity and size exclusion chromatography; low-molecular-mass proteins were visible in the preparation by SDS-PAGE and Coomassie staining [115].

Both the protein-in-adjuvant and viral-vectored vaccine delivery platforms were versatile in their ability to express complex vaccine antigens and induce strong, inhibitory antibody responses. While the methodology for antigen expression and delivery was standardized, for each antigen included in this study, there is a body of literature dedicated to improving the expression platform and construct specifications. As recombinant vaccines are optimized for epitope-recognition and immunogenicity, it is hoped that antigens will continue to be evaluated by head-to-head qualified SFMA using purified IgG. At this stage, the analysis relied on standard test concentrations of total IgG and did not establish a rank order of inhibition based on metrics such as antigen-specific IC_{50}, or the concentration of antigen-specific IgG necessary to inhibit oocyst intensity by 50%. This is an important area for expanding the priority ranking.

Chapter 4: Selection and Expression of a Panel of *P. falciparum* Sexual Stage Proteins

4.1 Introduction

Identification of new antigenic targets will be critical to developing a robust pre-clinical TBV pipeline. As delineated in Chapter 3, few antigens have been functionally evaluated for the ability to induce transmission-blocking antibodies. The majority of these were identified over 30 years ago – broadly grouped into pre-fertilization antigens (gametocyte, gamete) with potential exposure to human immunity, such as Pfs230 and Pfs48/45, and post-fertilization antigens (zygote, ookinete) expressed exclusively in the mosquito vector, such as Pfs25 [221]. The sequencing of the *P. falciparum* genome in 2002 identified all possible targets across the sexual stages [118] and has been followed by a myriad of global studies of protein expression. Just one analysis of the *P. falciparum* proteome identified 931 pre-fertilization proteins expressed during the gametocyte stage, of which 315 are gametocyte-specific [122]. A sex-specific evaluation of the mouse malaria *P. berghei* proteome found the male and female gametocyte to share only 69 proteins [126]. Proteome analysis of *Plasmodium* stages in the mosquito predicted 65 proteins on the surface of the male gamete, 6 on the surface of the female gamete and 100 on the surface of the post-fertilization ookinete [222]. Still, the field has yet to capitalize on the wealth of knowledge accrued over the past decade. There has been no rational

selection or comparative functional assessment of potential TBV targets by a standard methodology.

For the characterization and comparative assessment of a growing list of interesting targets, a major technological hurdle lies in successful, high-throughput synthesis of recombinant protein. For vaccination studies, the challenge is two-fold with a need for heterologous production of conformational antigens as well as easily scalable expression [223]. High-throughput production of recombinant malaria proteins has been notoriously difficult and has hindered the breadth of antigen selection. The *Plasmodium* genome is A/T rich with abundant repeat regions [118], which complicates the ability of heterologous expression platforms to decode and express conformational recombinant antigen. Moreover *Plasmodium* proteins do not lend themselves easily to an obvious expression platform; *Plasmodium* parasites are one of few eukaryotes without N-linked glycosylation machinery accessible to surface proteins [224]. Hence, recombinant protein expression in a eukaryotic platform can result in aberrant glycosylation variants. But the complex tertiary structure and multiple structural domains, also characteristic of malaria proteins, can preclude heterologous prokaryotic expression due to improper folding and poor solubility [223]. For example, leading TBV candidate antigens such as Pfs48/45 and Pfs230 are known to be cysteine-rich, with multiple domains that require correct disulfide bridge pairing. Prokaryotic systems, such as *E. coli*, may have the advantage of also lacking N-glycosylation, but so too lack the advanced endoplasmic reticulum machinery critical for correct disulfide bond formation [84].

Several large-scale attempts have been made to express proteins from the *Plasmodium* genome (see [223]). In brief, in a prokaryotic *E. coli* platform, only 39 out of 292 genes attempted as GST fusions were successfully expressed [225]. Another attempt at expressing 1000 genes encoding small, cytosolic proteins, resulted in detectable expression of 337 proteins, only 63 of which were soluble [226]. This success rate can be improved with the use of genetically engineered *E. coli* that express tRNAs able to decode A/T-rich genes in both cell-based [227] and cell-free platforms [228, 229], although the same concerns about conformational integrity remain. Eukaryotic systems – including cell-based and cell-free iterations of yeast, wheat germ, insect cells, rabbit reticulocytes, Chinese hamster ovary (CHO) cells, and tumor cells – meanwhile have traditionally suffered from low throughput or the production of non-conformational proteins [230]. The WGCFS has the advantage of also lacking glycosylation machinery, and among eukaryotic expression platforms has been most widely applied for high-throughput screening in the malaria field; out of 567 genes from the liver-stage sporozoite, blood stage merozoite, and gametocyte stages, 478 give soluble protein product [223]. Protein antigens, made in this manner, have progressed to comparative vaccine studies [115]. More recently, following improvement in mammalian expression constructs and cell line performance [208, 231, 232], a cell-based human embryonic kidney (HEK293) platform has enabled a moderate throughput, heterologous synthesis of difficult malaria protein targets [218]. This approach yields sufficient quantities of protein for vaccination and is gaining more widespread use in immuno-epidemiological and receptor-ligand interaction studies [233, 234]. Still,

while heterologous expression and screening has been implemented in both pre-erythrocytic [235] and erythrocytic vaccine development [201, 218] for the rational identification of new targets, this approach has never been reported for TBVs.

The robust nature of a reverse vaccinology strategy would allow for evaluation of candidates from a broader selection of life cycle stages in the aim of blocking transmission. So too, the sequencing of the *P. falciparum* genome enables the selection of proteins with putative surface localization or predicted adhesive motifs. Despite the wealth of new datasets, a minority of sexual stage proteins have been expressed in heterologous systems and characterized to show firm evidence for induction of transmission-blocking antibodies. The list of proteins suggested to have an important role in sexual stage biology, as well as features of a potential vaccine candidate, is longer, but there has not been a comprehensive effort to express and compare these targets using functional assays. In addition to current strategies to improve the immunogenicity of the known leading candidates (Section 1.7), the field urgently needs a robust pre-clinical pipeline of additional target antigens in order to progress [16].

In determining whether additional proteins may generate transmission-blocking antibodies, Chapter 4 and 5 describe the heterologous expression of a panel of *P. falciparum* sexual stage proteins and their evaluation as TBV antigens by SFMA. Chapter 4 reports the selection of 21 candidates from across the *P. falciparum* sexual stages for heterologous expression and functional characterization. In brief, HEK293E cell-based expression of native sequence,

full-length ectodomains successfully yielded a panel of 12 proteins, which recapitulate parasite-derived epitopes by indirect fluorescence assay. Chapter 5 reports the functional characterization of this panel of sexual stage antigens. Purified IgG generated to three proteins – one previously reported, one unreported, and one hypothetical – demonstrated a moderate inhibition of parasite development in the mosquito midgut. So too, a proportion exhibited seroreactivity in cohorts from malaria-endemic Burkina Faso and Mali.

4.2 Results

4.2.1 Selection of Candidates

In assembling a panel of recombinant *P. falciparum* sexual stage proteins for immunological and functional studies, we included surface-associated candidates previously described to play a relevant biological role (n=12), as well as an unbiased selection from the proteome (n=9) with an emphasis on those proteins which have not been systematically evaluated as TBV candidates (Figure 4.1).

Literature-based pre-fertilization targets were limited to Pfs48/45 paralog, Pfs47 [138], a female gametocyte protein reported to act in parasite evasion of the *A. gambiae* complement system [139]. Post-fertilization targets included those of functional interest as well as five proteins demonstrating a transmission-blocking phenotype in a systematic knockout screen [155]. Of the former we selected two ookinete micronemal proteins, SOAP (secreted ookinete adhesive protein) and WARP (von Willebrand factor A domain-related protein), implicated

respectively in the ookinete's initial recognition of [154] and adhesion to the mosquito midgut epithelium during traversal [153]. Two proteins, PPLP3[3] [164] [134] [166] and PPLP5 [167], were chosen from a class of Plasmodium perforin-like proteins – implicated in midgut traversal by the ookinete and defined by the presence of a membrane attack complex/perforin superfamily (MACPF) domain. Two additional proteins, enolase and chitinase, have been previously reported to partially block transmission by SMFA; enolase is secreted onto the ookinete surface with a role in midgut recognition and traversal [156], whereas parasite chitinase disrupts the *Anopheles*-derived chitin-rich membrane encompassing the mosquito blood meal [160]. The remaining antigens were selected from study by Ecker *et al*, which characterized 20 putatively secreted ookinete proteins by gene disruption. The five proteins shown to be essential to early *P. berghei* development in the mosquito (PSOP7, PSOP9, PSOP13, CCP5, ASP), can be divided into the PSOPs (putative secreted ookinete proteins) a category not defined by structure or function, CCPs[4] (Limulus coagulation factor C domain-containing proteins) which localize to crystalloid, a cytoplasmic organelle present only in ookinetes and young oocysts, and ASP, an aspartyl protease[5] (Figure 4.1, Table 4.1).

Given the anticipated bias towards post-fertilization candidates in the literature-based screen, we emphasized pre-fertilization stages in the proteome-based selection. In brief, upon commitment to the sexual pathway, gametocytes store

[3] PPLP3 is also referred to as MAOP, or membrane attack ookinete protein.
[4] Referred to as CCPs in the *P. falciparum* literature, *P. berghei* homologs are called LAPs (LLCL-lectin adhesive-like proteins).
[5] ASP is also known as plasmepsin IV.

mRNA transcripts, repressing translation until the appropriate environmental cues signal the parasite to proceed to the next life cycle stage. Consequently, in lieu of transcriptomic data, we relied on a comprehensive analysis of the *P. falciparum* proteome, which identified 1289 unique proteins by high accuracy mass spectroscopy between the asexual blood stages, gametocytes and gametes [122]. Of these 511 are expressed in both the gametocyte and gamete stage. 41 of these are predicted to have a signal peptide. Excluding proteins of a size that may preclude successful heterologous expression (>100kDa) leaves 31 candidates, including well-characterized stage-specific proteins Pfs16 and Pfs48/45. Of this list, we chose 5 named and 4 hypothetical targets (Figure 4.1). HSP70-2, PDI-8, and PDI-11 are predicted by Wass *et al* to localize to the *P. berghei* ookinete surface suggesting a biological function in multiple stages [222]. The primary study objective was to develop a sexual stage protein panel to characterize biological targets of transmission-blocking antibodies. As such, we did not exclude proteins, which for a variety of reasons may not ultimately be competitive vaccine antigens, for example electing to express enolase and HSP70-2 for biological interest despite speculative surface expression and the existence of human homologs (Figure 4.1, Table 4.1).

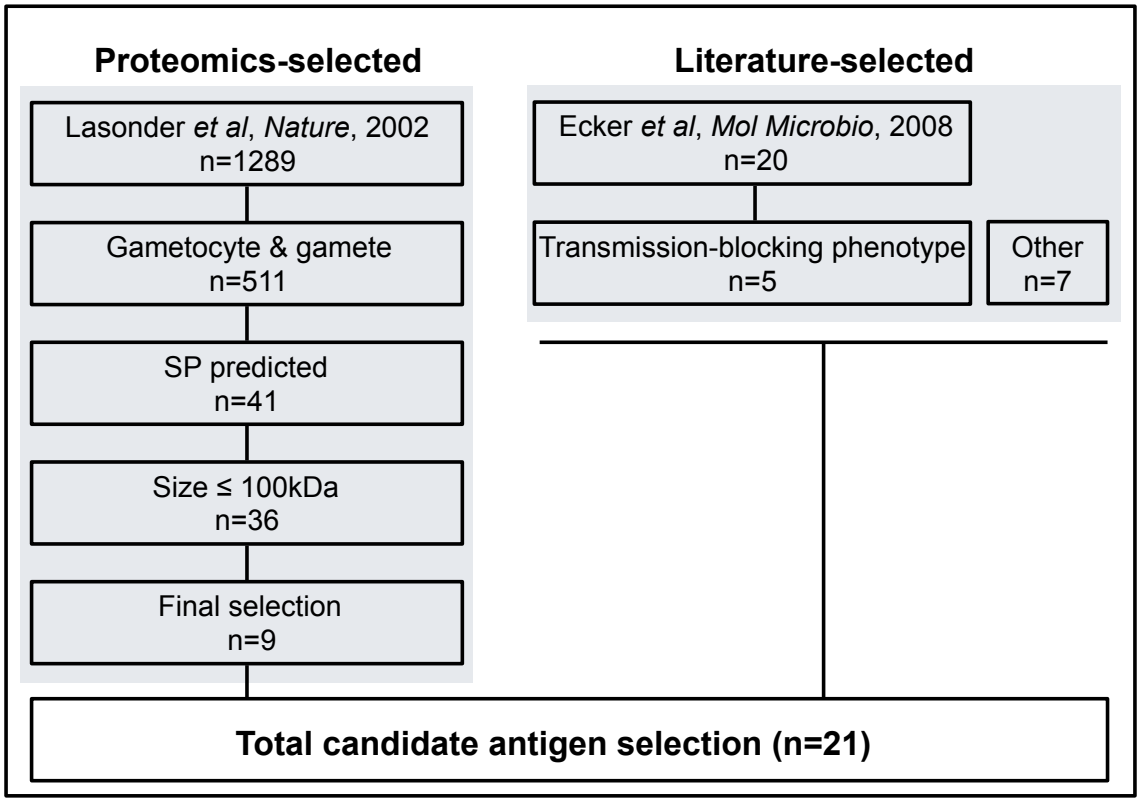


Figure 4.1 Selection of a panel of sexual stage antigens for expression in HEK293E cells

Candidate antigens (n=21) were selected from both an unbiased screen of the *P. falciparum* sexual stage proteome (n=9) and proteins described to be important to sexual stage biology (n=12).

Table 4.1 Defining features of a panel of *P. falciparum* sexual stage proteins

			Protein characteristics					
Antigen	**Literature-based: protein class**	**Pf Accession #**	**Size kDa**	**SP**	**TMD**	**N-Glyc**	**Exp**	**Ref**
PSOP7	Putative secreted ookinete protein	PF3D7_1340000	72.2	+	1	7	-	[155]
PSOP9	Putative secreted ookinete protein	PF08_0008	85.3	+	2	5	+	[155]
PSOP13	Putative secreted ookinete protein	PF3D7_0518800	23.5	+	0	1	+	[155]
ASP	Aspartyl protease	PF3D7_0311700	49.4	+	1	1	-	[155]
PPLP3	*Plasmodium* perforin-like protein	PF3D7_0923300	93.3	+	1	8	-	[155, 164, 166]
PPLP5	Plasmodium perforin-like protein	PF3D7_0819200	79.4	+	1	4	-	[155, 164]
Pfs47	6-cysteine domain	PF3D7_1346800	50.8	+	2	2	+	[138, 236]
CCP5	Lectin adhesive protein	PF3D7_1451600	100	+	0	8	-	[145, 155]
Chitinase		PF3D7_1252200	42.8	+	0	3	Low	[159, 237]
SOAP	Secreted ookinete adhesive protein	PF3D7_1404300	22.7	+	0	6	+	[154]
WARP	von Willebrand factor A-domain related protein	PF3D7_0801300	32	+	0	1	+	[151, 153, 238]
Enolase		PF3D7_1015900	48.7	-	0	4	+	[156]
Antigen	**Proteomics-based: protein class**	**Pf Accession #**	**Size kDa**	**SP**	**TMD**	**N-Glyc**	**Exp**	**Ref**
HSP70-2	Heat shock protein	PF3D7_0917900	72.4	+	0	2	+	
PDI-8	Putative disulfide isomerase precursor	PF3D7_0827900	55.5	+	1	0	+	
GEST	Gamete egress and sporozoite traversal	PF3D7_1449000	28.8	+	0	0	Low	[239]
PDI-11	Putative disulfide isomerase precursor	PF3D7_1134100	49.2	+	0	4	+	
ICP	Inhibitor of cysteine proteases	PF3D7_0911900	47	+	0	8	+	[240]
HYP1	Hypothetical	PF3D7_0925900	24.7	+	0	3	+	
HYP2	Hypothetical	PF3D7_0303900	23.2	+	0	2	+	
HYP3	Hypothetical	PF3D7_0912400	52.7	+	1	1	-	
HYP4	Hypothetical	PF3D7_0417000	32.9	-	1	3	-	

Abbreviations Pf: *P. falciparum*, kDa: kiloDaltons, SP: presence or absence of signal peptide, TMD: number of predicted transmembrane domains, N-Glyc: number of N-glycosylation sites, Exp: expression, Ref: references

4.2.2 Generation of a panel of sexual stage *P. falciparum* proteins

Synthetic genes representing 22 proteins (including Pfs25 as a positive control for the expression construct and platform) were generated based on the *P. falciparum* 3D7 strain sequence. Prediction software was used to identify the coding region after the parasite signal peptide [203] up to the first transmembrane domain or GPI-anchor [204, 205], if present, to ensure solubility of the recombinant product (Table 4.1). The native sequence was codon-optimized for expression in *Homo sapiens* and predicted N-glycosylation sites were left intact. The full-length ectodomains – flanked by unique KpnI and NotI restriction enzyme sites – were then subcloned into a modified pENTR4-LPTOS™ [201] expression construct encoding an exogenous N-terminal human tissue plasminogen activator (tPA) signal peptide and C-terminal biotin-acceptor and hexahistidine affinity tag (Figure 4.2).

Crosnier *et al* recently evaluated the relative contributions of codon-optimization, an exogenous signal peptide, and mutation of N-glycosylation sites on expression of asexual blood stage protein PfRH5 in HEK293E cells, reporting the relative increases in protein expression to be equivocal, ~3.5-fold, and ~5.5-fold respectively compared with the native sequence [218]. And while mutated N-glycosylation sites allowed for a marginal increase of functional PfRH5 protein product, reports for other antigens are variable. As discussed in Chapter 3, a recent study using a viral-vectored heterologous prime-boost vaccination regime to deliver Pfs48/45, a protein known to have a complex 6-

cysteine domain fold, suggested a disruption of transmission-blocking epitopes following mutation of N-glycosylation sequences relative to the native construct [198]. Given that our candidate list includes proteins that are not well characterized structurally or functionally, we chose not to alter native sequences.

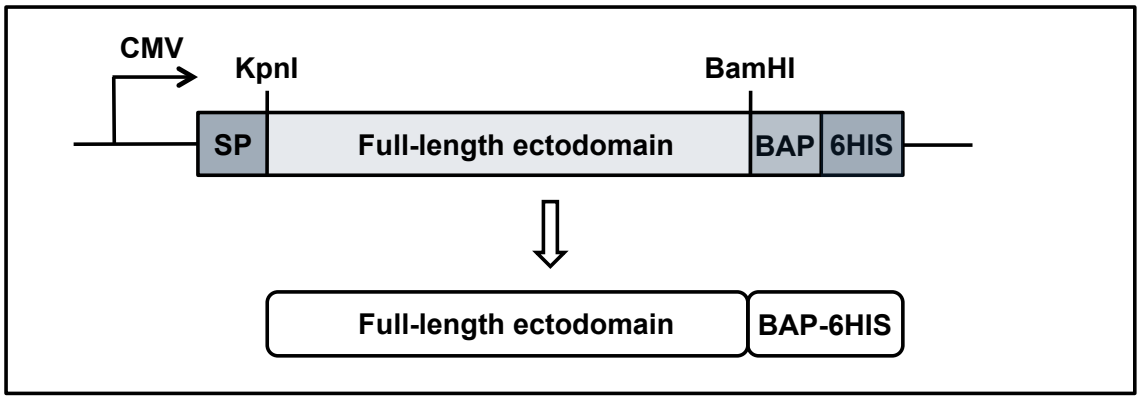


Figure 4.2 HEK293E expression construct design

Synthetic genes of full-length ectodomains based on the *P. falciparum* 3D7 sequence were generated and subcloned into an expression plasmid encoding an exogenous human tPA signal peptide (SP) and tag containing both a biotinylatable (BAP) and hexahistidine (6HIS) sequence for affinity purification of soluble protein product from HEK293E cell supernatant.

All 22 plasmids corresponding to our candidate antigens were successfully generated. Expression plasmids were complexed with PEI, and proteins were transiently expressed in non-adherent HEK293E cells. Soluble recombinant proteins were affinity purified from whole supernatant by Ni-Sepharose column and subsequently eluted by imidazole. 15 out of 22 proteins (68%), including Pfs25, showed detectable expression by Western blot using an anti-pentahistidine antibody, which is in concert with previous reports for P. falciparum protein expression in HEK293E cells. 13 out of 22 proteins (59%), including Pfs25, were purified in sufficient quantities for a vaccination study in BALB/c mice (n=5 per group), i.e. equal to or exceeding 300µg. Insufficient expression was noted for Chitinase and GEST and no detectable expression was observed for PSOP7, PPLP3, PPLP5, CCP5, ASP, HYP3 (PF3D7_0912400), and HYP4 (PF3D7_0417000) (Table 4.1). Successful expression did not correlate with molecular weight, predicted N-glycosylation sites, or other protein parameters[6]. Affinity purified, proteins were visualized by Coomassie staining following reducing SDS-PAGE, with bands at sizes at or above the predicted molecular weight, with the exception of Pfs47, which contained possible co-purification products (Figure 4.3, Table 4.1). Notably the Pfs25 protein product was detectable by known transmission-blocking monoclonal antibody 4B7 [104], suggesting that this epitope is intact (Figure 4.3).

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[6] The total number of negatively charged residues (Asp+Glu), total number of positively charged residues (Arg+Lys), instability index (II), aliphatic index, and grand average of hydrophobicity (GRAVY) were determined using the ExPASy ProtParam Server, a tool provided by the SIB Bioinformatics Resource Portal (www.expasy.org).

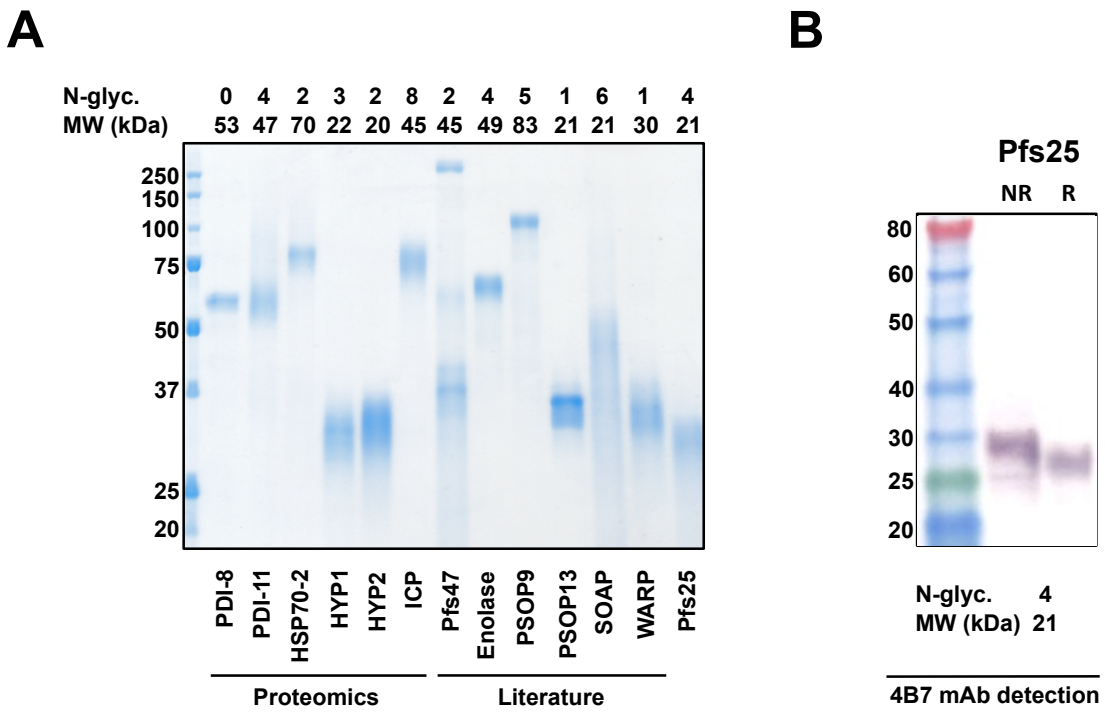


Figure 4.3 Heterologous expression of surface and secreted *P. falciparum* sexual stage proteins

A. Coomassie brilliant blue R-250 staining of recombinant proteins resolved by reducing SDS-PAGE. Each protein has a C-terminal biotin-acceptor peptide and hexahistidine tag (2.95 kDa) and was purified from HEK293E cell supernatant by Ni-Sepharose column prior to visualization. **B.** Western blot detection of affinity-purified Pfs25 by 4B7 monoclonal antibody after SDS-Page under reducing and non-reducing conditions and nitrocellulose transfer.

To evaluate whether N-glycosylation variants could explain the diffuse banding and increased size, proteins in the sexual stage panel were treated with PNGaseF and reduced (Figure 4.4). Specific anti-pentahistidine detection by Western blot following reducing SDS-PAGE indeed showed a decreased molecular weight and more uniform banding for all proteins except PDI-8 and a diminished effect for PSOP13 and WARP; indeed PDI-8 does not contain any predicted N-glycosylation sites and PSOP13 and WARP only have one predicted site each (Table 4.1).

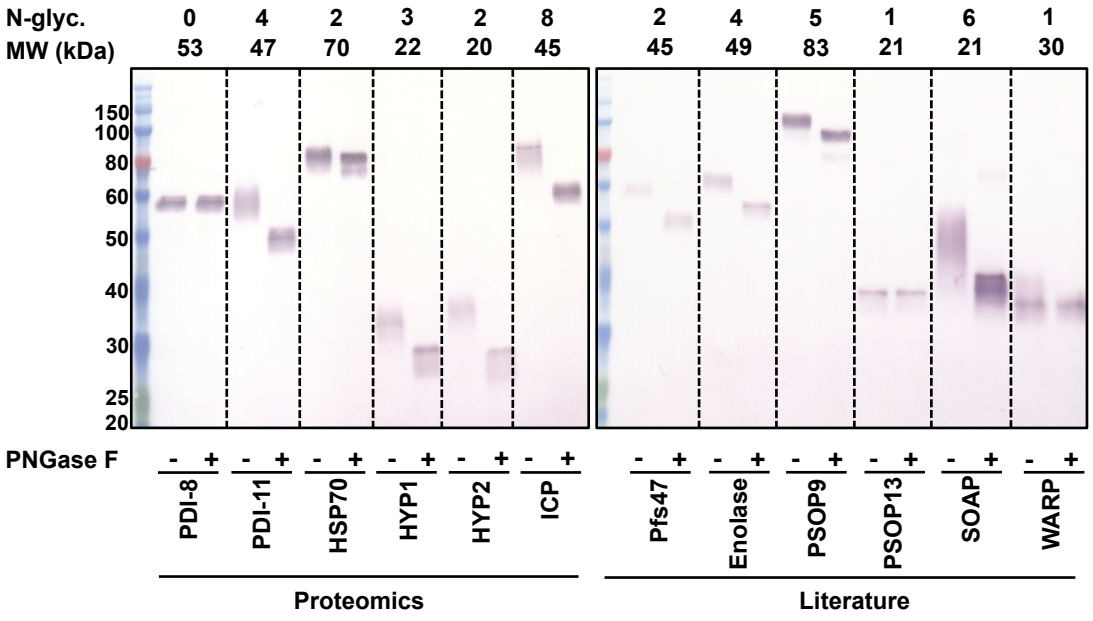


Figure 4.4 PNGase F treatment of recombinant proteins reveals N-glycosylation

Western blot detection of hexahistidine tag affinity-purified proteins, which were resolved by reducing SDS-page, blotted, and probed with monoclonal anti-pentahistidine antibody. Each protein is shown with and without treatment by PNGaseF, an amidase that cleaves N-linked glycan residues.

4.2.3 Recombinant *P. falciparum* proteins generate high titer IgG as vaccine antigens

To generate anti-sera to our panel of proteins, age-matched BALB/c mice (n=5) were vaccinated with 20μg of affinity-purified protein in Addavax™ adjuvant in three intramuscular doses delivered four weeks apart. Sera were collected at days 27, 55 and 70 to measure antibody titers generated by the priming dose, first boost, and second boost respectively. All animal experiments and procedures were performed in accordance with the UK Animals (Scientific Procedures) Act Project License (PPL 30/2414 and 30/2889) and approved by the Oxford University Local Ethical Review Committee.

Animals seroconverted with the priming dose for all test antigens except the Pfs25 control. Endpoint titer ELISA confirmed a statistically significant rise in IgG titers with the first boost for all antigens by Mann-Whitney; the majority reached a plateau with the second dose (Figure 4.5). The vaccination regime generated IgG titers of a sufficient magnitude at day 70 for all antigens to proceed with immunological and functional studies including indirect fluorescence assay detection of native parasite epitopes by whole serum and evaluation of transmission-blocking activity of purified IgG. Of note, IgG responses to the biotin acceptor sequence and hexahistidine tag (2.95 kDa) were detected by ELISA, and these responses were boosted over the course of the vaccination regime. Whole sera were depleted of anti-tag antibodies prior to quantifying vaccine-induced antigen-specific IgG titers by ELISA (Figure 4.6).

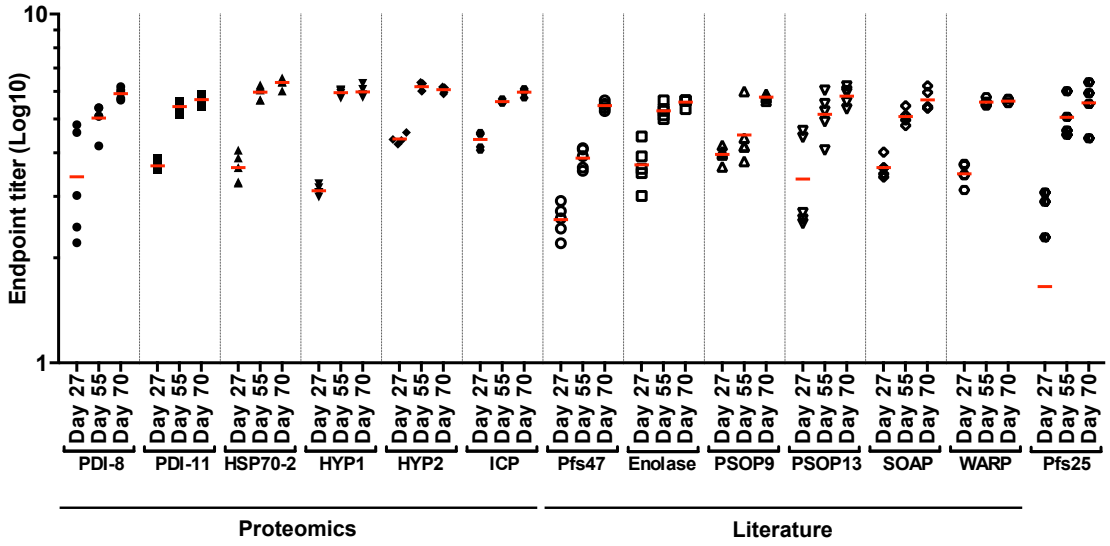


Figure 4.5 Endpoint titer ELISA reveals high titer IgG responses to vaccine antigens

BALB/c mice were vaccinated intramuscularly with 20 µg of affinity-purified protein in Addavax™ adjuvant on days 0, 28 and 56. Sera were collected at days 27, 55 and 70. For all quantitative immunogenicity studies, sera were depleted of antibodies specific to the biotin acceptor peptide and hexahistidine tag. IgG responses were quantified by endpoint titer ELISA. Responses of both individual BALB/c mice and the group arithmetic mean are shown.

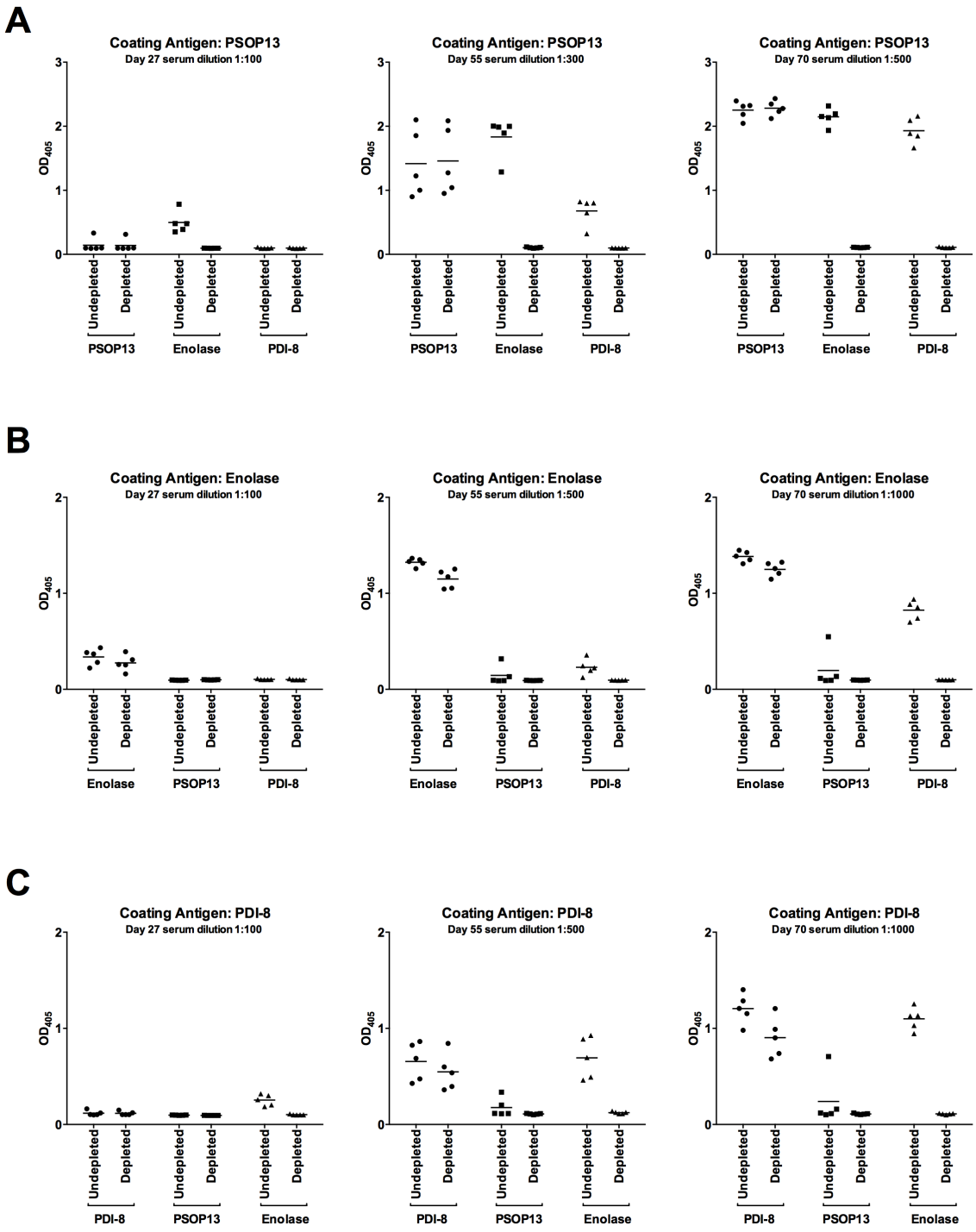


Figure 4.6 IgG responses generated to the protein tag can be depleted

Representative panels show ELISA OD_{405} values generated against coating antigens: PDI-8 (**A**), PSOP13 (**B**), and enolase (**C**). Test sera from at days 27, 55, and 70 are denoted as being either undepleted or depleted of anti-tag responses. Depletion of anti-tag responses was achieved by co-incubation of test-sera with peptide representing the biotin acceptor peptide and

hexahistidine sequence. In each panel (**A, B, C**) test sera generated by vaccination against the coating antigen was evaluated in addition to two irrelevant test sera, as specified below the *x*-axis.

Notably, by standardized ELISA, antisera generated to Pfs25 exhibited comparably high titer responses to *P. pastoris-derived* TBV25H, the previously described clinical construct [105], as to the vaccination antigen, i.e. the HEK293E-derived protein. This suggests that the construct developed for expression in the HEK293E system, which importantly differs from TBV25H in that predicted N-glycan sequences have been left intact, nonetheless exhibits similar epitopes to the clinical construct. This data, in addition to recognition of HEK293E-derived Pfs25 by monoclonal antibody 4B7 (Figure 4.3 B), serve to validate design of the mammalian expression construct.

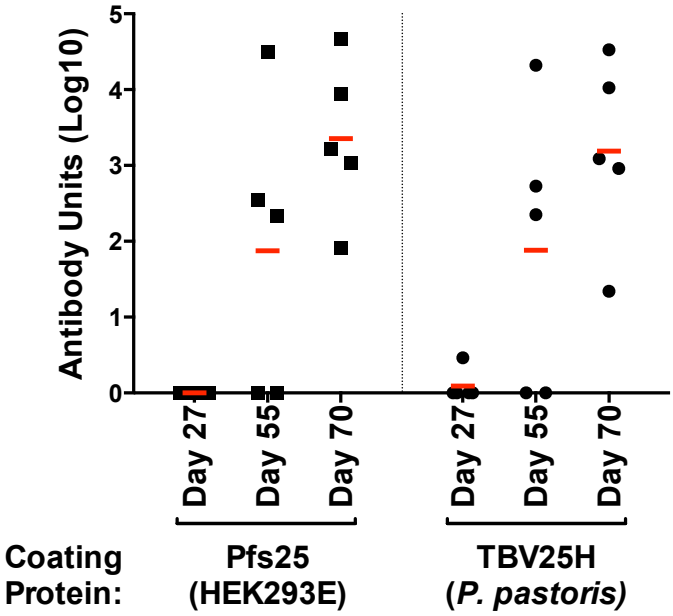
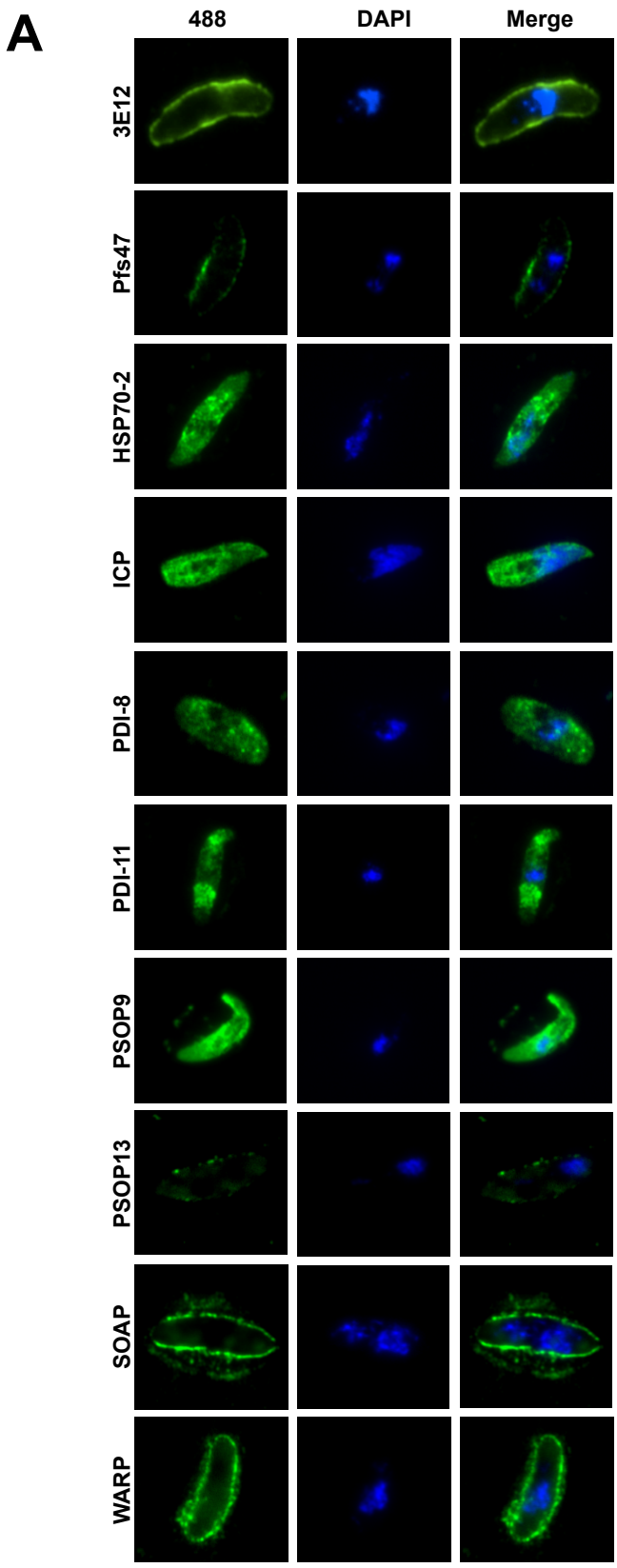


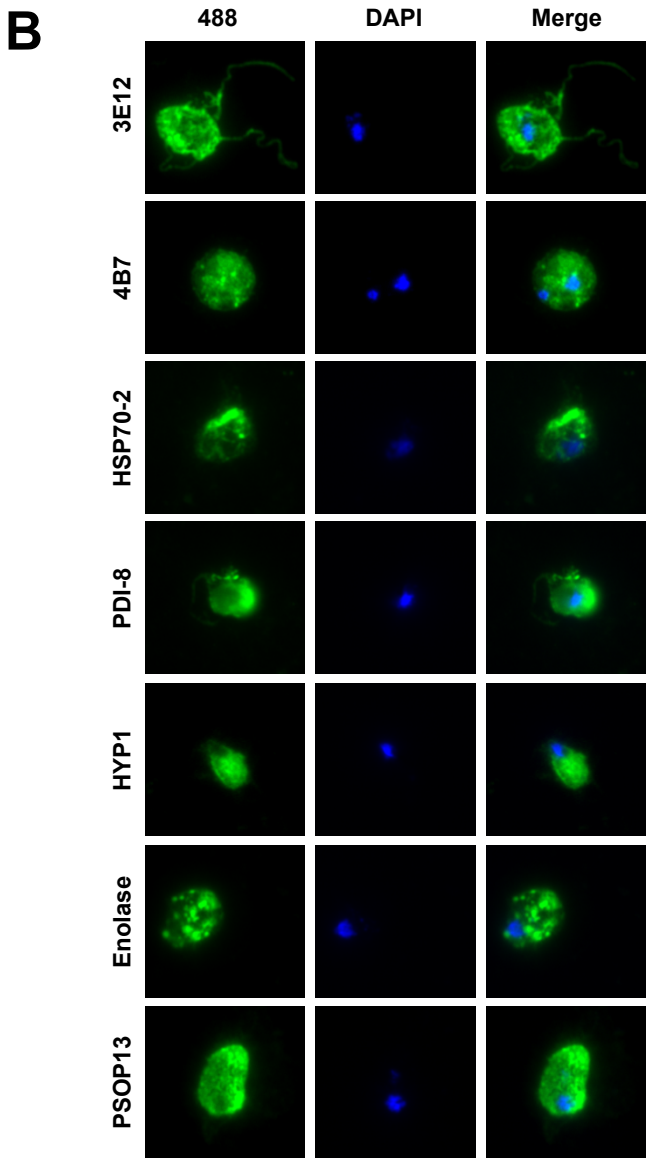
Figure 4.7 Pfs25 titers to clinical construct, TBV25H, parallel recognition of the vaccine antigen

Standardized ELISA was used to quantitate IgG responses following HEK293E-derived Pfs25 vaccination and evaluate whether the magnitude of response was similar to both the HEK293-derived vaccine antigen, and TBV25H, the yeast-derived clinical-grade construct.

4.2.4 Recombinant P. falciparum proteins recreate native parasite epitopes

To indirectly assess if HEK293E-derived proteins in the sexual stage panel contain native parasite epitopes, pooled day 70 sera (n=5 BALB/c mice per antigen) specific to each antigen were used to visualize *P. falciparum* NF54 strain parasites. Indirect fluorescence assay (IFA) was performed to evaluate serum recognition of gametocytes and gametes generated *in vitro* and ookinetes generated *in vivo* through mosquito blood feeding and dissection (Figure 4.8). 9 of 12 sera stained Triton X-100 permeabilized intraerythrocytic gametocyte stages (Figure 4.8 A). Five of 12 anti-sera stained unpermeabilized exo-erythrocytic gamete stages (Figure 4.8 B). Despite predicted expression in the gametocyte and gamete stage, anti-sera generated to hypothetical protein HYP2 (PF3D7_0303900) failed to recognize these life cycle stages by IFA; it is possible that it has recognition of the ookinete stage, which was not assessed.


A
488
DAPI
Merge
3E12
Pfs47
HSP70-2
ICP
PDI-8
PDI-11
PSOP9
PSOP13
SOAP
WARP


B
488
DAPI
Merge
3E12
4B7
HSP70-2
PDI-8
HYP1
Enolase
PSOP13

C

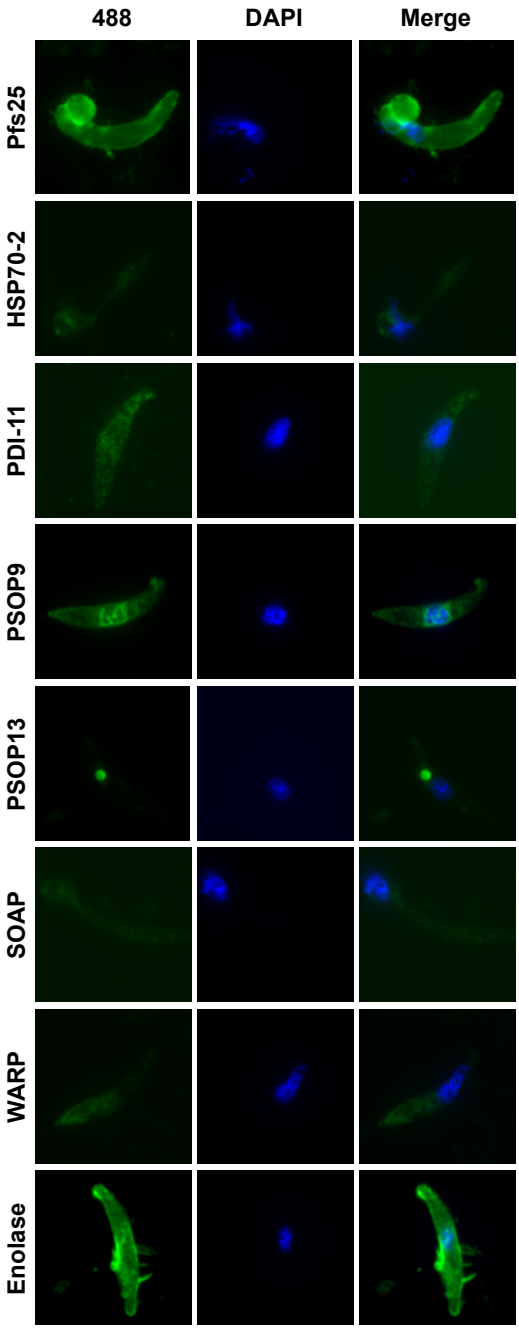

488
DAPI
Merge
Pfs25
HSP70-2
PDI-11
PSOP9
PSOP13
SOAP
WARP
Enolase

Figure 4.8 Recombinant *P. falciparum* sexual stage antigens recreate native parasite epitopes

Pooled day 70 anti-sera (n=5 BALB/c mice) generated to the panel of sexual stage antigens were used to probe parasite epitopes by indirect fluorescence assay. *P. falciparum* NF54 strain stage V gametocytes (**A**) and gametes (**B**) were generated *in vitro*. NF54 strain ookinetes (**C**) were generated in vivo and dissected from infected *A. stephensi* mosquito midguts. Parasites were air-dried onto glass slides and fixed with 4% paraformaldehyde. Intraerythrocytic gametocytes were permeabilized with 0.01% TritonX-100 prior to immunostaining.

4.3 Discussion

Chapter 4 reports the use of a mammalian platform for the expression of a panel of sexual stage proteins of sufficient yield for use in a rodent vaccination study. Vaccine antigens generated in the HEK293E system are able to recapitulate native parasite epitopes by immunological assays such as IFA.

The majority of *P. falciparum* malaria-endemic geography is currently defined by low transmission (entomologic inoculation rate < 5) [241] marking a pressing need for innovative technologies such as VIMTs, and specifically TBVs, to target the parasite reservoir in regions working to achieve elimination. Yet, vaccine development has been significantly impeded by difficulties in expressing full-length, high-quality recombinant *P. falciparum* proteins. Indeed, defined TBV targets, such as cysteine-rich protein Pfs48/45, have not progressed through clinical development in part due to difficulties in recapitulating conformation-dependent target epitopes in pre-clinical studies [84, 242]. Moreover in establishing a robust pre-clinical pipeline, moderate to high-throughput heterologous expression platforms are critical for allowing the rational selection and unbiased, comparative screening of potential vaccine targets in functional assays.

Historically, large-scale efforts to express proteins from the *Plasmodium* genome have been limited to bacterial and cell-free systems. *E. coli*, with the advantage of no N-glycosylation machinery, has arguably been the most widely employed heterologous expression system, but with fewer than 20% of

attempted genes carried through to soluble protein. Genetically engineered *E. coli*, expressing tRNAs better suited for decoding A/T-rich genes, is an improvement, but cumbersome refolding procedures preclude high-throughput expression. Likewise, the wheat-germ cell free system (WGCFS) also has the advantage of lacking N-glycosylation machinery, and among eukaryotic expression platforms has been the most extensively utilized in large scale expression efforts, generating soluble protein products from the liver-stage sporozoite and blood stage merozoite and gametocyte stages for over 80% of targets (478 out of 567) [223]. While the WGCFS benefits from throughput, limited data exist on the conformational integrity of the protein products; complex immunogen Pfs48/45 does not yield functional product [115].

Consequently this screen employed transiently transfected, non-adherent HEK293 cells for protein expression. As recently reported by other groups, recent improvements in expression plasmid design and cell lines has enabled a scale-up in terms of speed of production and cost for generating recombinant malaria proteins. With the HEK293 cells system, screening larger numbers of candidates is faster, cheaper, and higher throughput with the benefit of more controlled dosing, relative to viral vectored platforms, for antigen comparisons. While we cannot rule out the negative impact of not mutating N-glycosylation sites, Pfs25 generated in this platform was recognized by transmission-blocking monoclonal antibody 4B7 and elicited IgG which was also reactive to clinical construct TBV25H. So too, the recombinant proteins generated in this regime recreated native parasite epitopes by IFA.

In summary, this chapter describes the heterologous expression of 12 proteins from across the *P. falciparum* sexual stages. These proteins were generated as full-length ectodomains in a mammalian HEK293E cell system. Immunogenicity was evaluated in a vaccination study of BALB/c mice. Anti-sera generated by immunization were assessed for the ability to recognize native parasite epitopes. Chapter 5 will report seroreactivity to these proteins in cohorts from malaria-endemic Mali and Burkina Faso. Data will also be presented on the ability of purified IgG generated to the candidates to inhibit oocyst intensity by SMFA.

Chapter 5: Functional Evaluation of a Panel of *P. falciparum* Sexual Stage Proteins

5.1 Introduction

Assays that evaluate and prioritize vaccine candidates are essential to the development of a robust, pre-clinical pipeline. So too, standard protocols are particularly important for interpreting data from an increasingly global network of vaccine-development laboratories. At present, SMFA is considered the gold standard for assessing transmission-blocking vaccines through both the pre-clinical and clinical stages. SMFA tests the functional activity of antibodies using naturally expressed protein targets on the surface of live parasites, and as such represents the closest available measure of true physiological efficacy *ex vivo*. Yet for the same reasons, the assay is limited by a dependence on reliable gametocyte cultures and a healthy mosquito colony. As a result, SMFA can be labor-intensive, time-consuming, and subject to poor reproducibility, especially for antibody samples with low inhibitory activity.

Early observations of parasite uptake by the mosquito vector were instrumental to defining antibody-mediated TBI [47]. As discussed in Chapter 1, in 1958 direct feeding studies showed that gametocytes from *Plasmodium*-infected birds, which had been previously immunized with killed parasites, were less infectious to mosquitos than gametocytes from unvaccinated controls. This

observation was investigated further in 1976 through a series of experiments in chickens immunized with gametocyte and gamete extracts prior to challenge with *P. gallinaceum*. Following a direct feed, mosquito infection was assessed by dissection for both the presence and number of oocysts. Mosquitos fed on the vaccinated chickens had diminished oocyst counts relative to controls [48, 49].

Improvements to routine *in vitro* sexual stage parasite culture [52] were necessary before *P. falciparum* parasitized blood samples could be fed to mosquitos through a membrane. Membrane feeding allowed for the characterization of the first panel of transmission-blocking monoclonal antibodies (mAbs) in 1983 [53, 243]. In these studies, test mAbs were mixed with laboratory-generated *P. falciparum* gametocytes and fed to *A. freeborni* mosquitos with and without complement through nylon, monofilament mesh over a water-jacketed, warmed feeder. Mean oocyst intensity per midgut was reported [53, 243]. As *P. falciparum* proteins corresponding to transmission-blocking mAbs were expressed in heterologous systems, SMFA became the standard for evaluating them as vaccine antigens.

Despite the assay's widespread use, pre-clinical laboratories often do not adhere to standard protocols and results are not reported in a consistent format, precluding easy exchange of data between research groups. One sample tested in independent assays may not return a predictable efficacy – most often reported either as the inhibition of mean oocyst intensity, the number of oocysts per infected mosquito, or a reduction in prevalence, the number of infected

mosquitos [244, 245]. Rigorous consistency in *in vitro* gametocyte maintenance (human serum batch, starting parasitemia, hematocrit, culture volume, and temperature), mosquito husbandry, and feeding method improves concordance, but the accuracy of SMFA is still sensitive to variables outside of experimental control.

It is well accepted that mean oocyst intensity of control mosquitos is the greatest source of variability. (For clarity, hereafter a group of mosquitos corresponding to a single test sample will be referred to as a pint.[7]) An early study of SMFA reproducibility established that the control pint should have a mean oocyst intensity of greater than or equal to 35 for significant efficacy to be observed in a test sample [244]. This is not possible to achieve in all cases. In a single study, which followed a strict gametocyte culture and mosquito maintenance protocol, mean NF54 strain *P. falciparum* oocyst intensity in the control pint varied from 5.6 to 67 in 9 feeding experiments [212]. Moreover, the range of experimental oocyst intensities in most studies is dramatically different from natural mosquito infections in the field. In both direct feeding assays (DFA) on malaria patients [246] or dissection of field-caught mosquitos [247], infection typically consists of fewer that 5-6 oocysts per midgut.

Present methods do not allow for the regulation of oocyst intensity within a narrow interval, and taking this into consideration, several attempts have been made to formally evaluate the assay's accuracy and dynamic range. Churcher

[7] This is the volume of the carton container which houses a test mosquito group fed on the same mixture of *Plasmodium*-infected blood and test IgG or serum.

et al compiled SFMA data from 536 feeds on 132 different samples – performed with *P. falciparum* and *P. berghei* parasites in *A. gambiae* or *A. stephensi* mosquitos. Their findings determined that the number of mosquitos dissected in both the test and control pints as well as the number of replicate feeds have a dramatic effect on assay accuracy [248]. This was in parallel with an earlier study by Medley *et al* which maintained that using fewer than 50-100 mosquitos per test pint would result in a diminished reliability of efficacy estimate [249].

Increasing mosquito number and replicate number adds not only to labor-intensiveness of dissection, but also to cost; these limitations have largely prevented standardization between research groups. In weighing the relative importance of mosquito number per pint against replicate number, Miura *et al* compiled SMFA data from 9 feeding experiments to develop an R-script model to predict the impact of assay modifications [212]. In keeping with previous findings, by the model, increasing the number of mosquitos per pint from 20 to 60 resulted in a significant reduction in variability. Yet for a set total number of dissected mosquitos for a unique test sample, it was determined that it is preferable to dissect multiple groups of mosquitos in smaller numbers (i.e. 20 mosquitos each in 3 independent pints) than one group of a larger number (i.e. 60 mosquitoes in one pint). This finding suggests that inter-assay variability supersedes intra-assay variability, particularly for samples with low inhibition, and makes an argument for increasing replicate number [212].

Taking into account the impact of oocyst intensity in the control, a major finding of the validation studies from both Churcher *et al* and Miura *et al* maintained

that inhibition of oocyst intensity is more consistent for test samples with high inhibitory effect; samples with weak inhibitory activity were more impacted by error of the assay. In determining the dynamic range of SMFA as defined by validation criteria provided by International Conference on Harmonization (ICH) Harmonized Tripartite Guideline Q2 (R1), Miura *et al* tested the transmission-blocking monoclonal antibody 4B7 [250] at four concentrations (1, 6, 23, 94 µg/ml) in three feeding experiments. Smaller inter-feed variation was observed for 4B7 concentrations over 23 µg/ml, corresponding to percent inhibition of over 80%, suggesting that for samples demonstrating over 80% inhibition it may be possible to estimate the percent inhibition of mean oocyst intensity from only one feeding experiment. The study acknowledged that establishing a definitive cutoff of 80% is in effect arbitrary, given that the ICH Q2 (R1) defines the dynamic range to fall between two test concentrations that yield "suitable" precision, accuracy, and linearity. Inter-feed variation correlated predictably with test IgG concentration. It is thus possible, that the optimal range could be expanded to include lower levels of inhibition based on the assay's intent and usage [212]. The range is informally set at 60-100% for studies at the NIH SMFA Reference Center.

Defining the complex effects of inhibitory antibodies on oocyst intensity and prevalence within the SMFA experimental model is a small part of transmission dynamics in the field, for which we have experimental tools to measure some but certainly not all components (Figure 5.1). The relative constraints and advantages of DMFA, which relies on natural human infection as a gametocyte source, are not discussed in this thesis although SMFA is an equally critical tool

for determining the effects of vaccine mediated and naturally derived transmission-blocking immunity. As vaccine candidates move forward to field trials, assay readouts need to be evaluated for their relative impact on population transmission. This will require innovative trial design and provide an opportunity to validate pre-clinical assays.

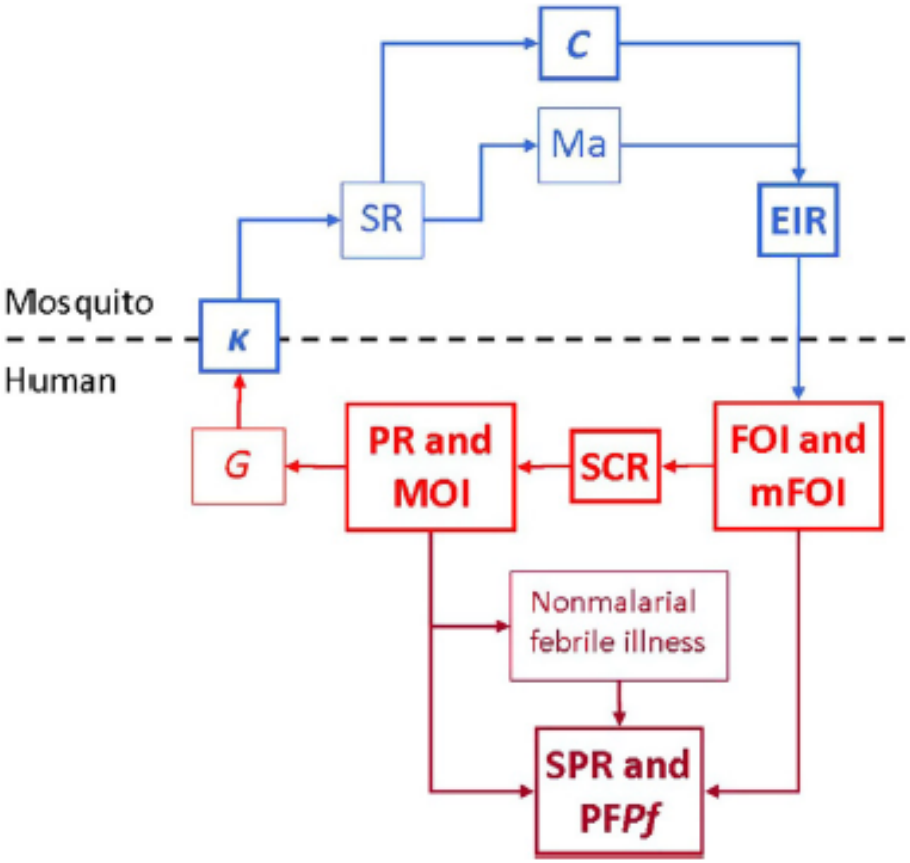


Figure 5.1 Metrics of malaria transmission

Blue indicates entomological metrics; red indicates clinical metrics; dark red indicates asymptomatic and symptomatic infections identified in health facilities. *κ*: net infectiousness of humans; SR: sporozoite rate; *C*: vectorial capacity; *Ma*: human biting rate; EIR: entomological inoculation rate; FOI: force of infection; mFOI: molecular force of infection; MOI: multiplicity of infection; SCR: seroconversion rate; PR: parasite rate; *G*: gametocyte rate; SPR: slide positivity rate; PF*Pf*: proportion of fevers parasitaemic. Parasite rate (PR) is the proportion of the proportion of people who are infected with parasites and the gametocyte prevalence is the proportion of people carrying gametocytes in their blood. The human biting rate is the number of bites by vector mosquitoes received per human per day, denoted *Ma*, and some portion of mosquitoes biting infectious humans become infected. Figure and legend sourced from [251].

Chapter 4 described the heterologous expression of a panel of *P. falciparum* sexual stage proteins. This chapter employs SMFA, as performed by the NIH SMFA Reference Center, with the primary aim of evaluating the panel of proteins for transmission-blocking epitopes. So too the sexual stage panel is screened for seroreactivity in cohorts from malaria-endemic Burkina Faso and Mali to determine the presence of native *P. falciparum* epitopes to complement SMFA data.

5.2 Results

5.2.1 Screening for transmission-blocking epitopes by SMFA

The panel of recombinant sexual stage proteins expressed in Chapter 4 was evaluated for transmission-blocking epitopes. The generation of anti-sera to these proteins was previously described (Section 4.2.3). In brief, BALB/c mice were vaccinated with 20µg of protein in Addavax™ adjuvant in three doses delivered four weeks apart by the intramuscular route. Serum harvested at day 70 was pooled from 5 BALB/c mice for each vaccine antigen. To assess antibody-mediated inhibition of oocyst intensity in the mosquito midgut, total IgG was purified from pooled anti-sera and tested by SMFA. In a mosquito membrane feeder, a blood meal consisting of *P. falciparum* NF54 strain gametocytes was mixed with purified IgG (final concentration 0.75 mg/ml) in the presence of human complement. *A. stephensi* mosquitoes were allowed to feed on the blood mixture; oocyst intensity in the midgut was quantified by dissection 7 days later.

Publications differ in reporting oocyst prevalence (the proportion of infected mosquitos) and oocyst intensity (the oocyst number per mosquito) [249]. Intensity and prevalence have been show in several publications to be related metrics [247, 248]. The analysis by Churcher *et al*, which compiled SMFA from 536 feeds on 132 different samples, identified a non-linear relationship between oocyst intensity and prevalence [248], which is present in both control and experimental groups, suggesting that if an IgG sample reduces intensity by a certain amount, the reduction in prevalence can be predicted. Given that the primary aim of chapter 5 is to evaluate the presence of transmission-blocking epitopes in a panel of sexual stage proteins, inhibition of mean oocyst intensity was selected as the more descriptive metric. Of note, previous studies by van der Kolk *et al* and Miura *et al* suggest that using the arithmetic mean to calculate the percent inhibition of oocyst intensity results in a lower variance as compared to calculations based on the geometric mean [245] or median [212] of a test group. Subsequent reference to transmission blocking refers to the percent inhibition of mean oocyst intensity.

Test IgG samples were evaluated at a concentration of 0.75 mg/ml in two independent feeding assays denoted A and B (Figure 5.2, Table 5.1, Table 5.2). Given the well-documented variability of membrane feeding studies, both a positive 4B7 monoclonal antibody assay control (final concentration 0.094 mg/ml) and negative human serum pool assay control were included with each feed. A feed was defined as a failure if (1) the negative control yielded a mean oocyst intensity less than or equal to 4 or (2) the positive control inhibited oocyst intensity by less than 80% relative to the negative control. Purified IgG samples

specific to HEK293E-derived proteins Pfs25 and OVA – both containing a biotin-acceptor peptide and hexahistidine affinity tag – were generated in the same vaccination regime as the test samples and served as positive and negative experimental controls respectively (final concentration of 0.75 mg/ml in the membrane feeder). SMFA data is reported as both oocyst number per mosquito (Figure 5.2) and percent inhibition of mean oocyst intensity relative to anti-OVA IgG (Table 5.1 and Table 5.2).

Purified IgG for PSOP13, enolase, PDI-8, HYP1, and HYP2 became available earlier during the study and were tested first. Consequently, the first feeding experiment was conducted in two parts on separate days (Figure 5.2 A). The mean oocyst intensity in the negative assay control (human serum pool) between the two days was 42.3 and 43.3 respectively, whereas the negative experimental control (anti-OVA IgG) had a mean oocyst intensity of 38.5 and 29.5 respectively. Similarly in the second feeding experiment wherein all IgG samples were tested on the same day (Figure 5.2 B), the negative assay control had higher mean oocyst intensity (51.1) than the negative experimental control (39.9). This trend is consistent with a nonspecific inhibition of oocyst intensity by purified mouse IgG, which has been documented previously [212]. At a concentration of 0.75 mg/ml, this inhibition is not statistically significant (feed A: $p=0.644$, $p=0.103$; feed B: $p=0.311$ by Mann-Whitney), but highlights the importance of evaluating percent inhibition relative to an appropriate experimental control rather than to human serum pool alone.

In independent feeding experiments, anti-Pfs25 IgG demonstrated a statistically significant inhibition of mean oocyst intensity; inhibition was 100% and 99.8% in feed A (95% CI 99.7-100, p= 0.001; 95% CI 98.7-100, p=0.001 respectively) and 99.9% in feed B (95% CI 99.1-100, p=0.001) (Table 5.1 and Table 5.2). This range of inhibitions is in keeping with previously published reports using IgG specific to TBV25H, the original clinical construct generated in *P. pastoris*, at a final concentration of 0.75 mg/ml [250]. Of note, the Pfs25 expression construct was generated by the same parameters as the sexual stage protein panel. Predicted N-glycosylation sites in the native sequence were left intact, and in the instance of Pfs25, such antigen design did not result in obstruction of transmission-blocking epitopes by glycan residues.

Of the sexual stage protein panel, in feed A, enolase and HYP1 demonstrated a moderate inhibition of mean oocyst intensity of 46.6% (95% CI −50.8-81.3, p=0.225) and 45.7% (−56.4-81.4, p=0.259) respectively. This effect was similar in feed B, with enolase inhibiting mean occyst intensity by 52.9 (95% CI −40.7-84.2, p=0.179) and HYP1 by 33.8% (95% CI −101.6-77.1, p=0.448). So too, in feed B, HSP70-2 elicited a statistically significant inhibition of 98.2% (95% CI 94.5-99.7, p=0.001) (Table 5.2). Of the remaining antigens in the panel, the activity of Pfs47 has been previously shown to be vector-specific. Pfs47 promotes parasite immune evasion in the *A. gambiae* mosquito – the main vector for *P. falciparum* in large regions of Africa – through suppression of midgut nitration, a process necessary for activation of the mosquito complement system [139]. By contrast, *P. falciparum* development in *A. stephensi* is not Pfs47-dependent; *A. stephensi* mosquitos successfully support the

development of Pfs47 knockout parasites of the *P. falciparum* NF54 strain (median of 60 oocysts/midgut) [139]. Taking vector specificity into account, SMFA was repeated for Pfs47 with *A. gambiae* mosquitos. 0.75 mg/ml anti-Pfs47 IgG did not inhibit transmission relative to anti-OVA IgG (data not shown).

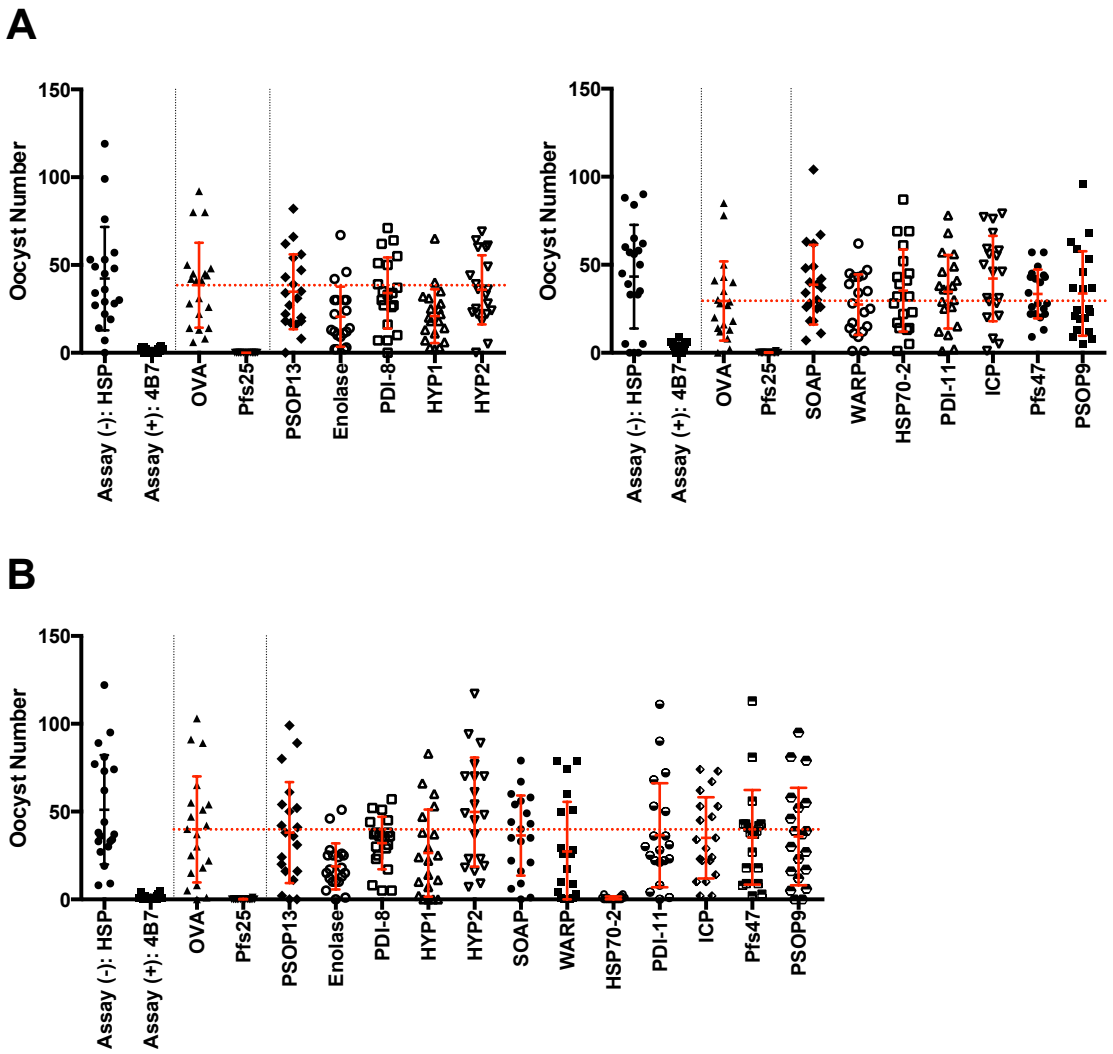


Figure 5.2 Several *P. falciparum* sexual stage proteins demonstrate transmission-blocking epitopes by SMFA

Purified IgG from pooled anti-serum generated to recombinant sexual stage proteins was tested by SMFA in the presence of human complement at a final concentration of 0.75 mg/mL in the mosquito blood feeder. Mean oocyst intensity and standard deviation is shown in red. Two independent feeds are shown in **A** and **B** for all candidates evaluated. The first independent feed was conducted over two separate days. The second independent feed was conducted on the same day. In each feed, human serum pool (HSP) served as the positive assay control and Pfs25 monoclonal antibody 4B7 (final concentration 0.094 mg/ml) served as the negative assay control. The mean oocyst intensity of the experimental control (purified anti-OVA IgG, final concentration 0.75 mg/ml) is denoted by a red dotted line.

Table 5.1 SMFA A: Percent inhibition of mean oocyst intensity at 0.75 mg/ml

Sample	IgG (mg/ml)	Mean Oocyst	% Oocyst Inhibition	95% CI Low	95% CI High	*p*-value
HSP	0.750	42.3				
4B7	0.094	1.3	96.9	91.0	99.0	0.001
OVA	0.750	38.5				
Pfs25	0.750	0.0	100.0	99.7	100.0	0.001
PSOP13	0.750	34.8	9.7	-160.7	68.4	0.910
Enolase	0.750	20.6	46.6	-50.8	81.3	0.225
PDI-8	0.750	34.1	11.6	-164.7	69.9	0.816
HYP1	0.750	20.9	45.7	-56.4	81.9	0.259
HYP2	0.750	35.9	6.8	-164.9	70.2	0.927

HSP	0.750	43.3				
4B7	0.094	3.0	93.2	81.0	97.7	0.001
OVA	0.750	29.5				
Pfs25	0.750	0.1	99.8	98.7	100.0	0.001
SOAP	0.750	38.7	-31.4	-283.1	53.5	0.580
WARP	0.750	27.4	7.0	-170.6	68.6	0.907
HSP70-2	0.750	35.4	-20.0	-227.8	58.8	0.750
PDI-11	0.750	34.7	-17.7	-254.4	59.2	0.762
ICP	0.750	42.2	-43.3	-321.3	47.6	0.499
Pfs47	0.750	33.5	-13.6	-228.1	62.0	0.797
PSOP9	0.750	33.7	-14.4	-232.8	60.9	0.781

Table 5.2 SMFA B: Percent inhibition of mean oocyst intensity at 0.75 mg/ml

Sample	IgG (mg/ml)	Mean Oocyst	% Oocyst Inhibition	95% CI Low	95% CI High	*p*-value
HSP	0.750	51.1				
4B7	0.094	1.2	97.7	93.4	99.3	0.001
OVA	0.750	39.9				
Pfs25	0.750	0.1	99.9	99.1	100.0	0.001
PSOP13	0.750	38.1	4.5	-174.7	66.6	0.953
Enolase	0.750	18.8	52.9	-40.7	84.2	0.179
PDI-8	0.750	32.1	19.5	-137.8	70.5	0.727
HYP1	0.750	26.4	33.8	-101.6	77.1	0.448
HYP2	0.750	49.8	-24.8	-271.7	55.9	0.698
SOAP	0.750	36.4	8.8	-165.2	68.9	0.863
WARP	0.750	27.4	31.5	-109.4	76.9	0.481
HSP70-2	0.750	0.7	98.2	94.5	99.7	0.001
PDI-11	0.750	36.6	8.4	-171.2	69.6	0.880
ICP	0.750	35.1	12.2	-152.8	69.3	0.784
Pfs47	0.750	35.4	11.4	-153.4	68.9	0.811
PSOP9	0.750	35.9	10.2	-157.4	69.8	0.855

Inherent feed-to-feed and pint-to-pint variability contributes to assay error, and consequently, SMFA does not effectively distinguish moderate oocyst inhibition – defined to be 60% or below based on published qualification of the assay [212] – from 0% inhibition. As a result, SMFA was repeated to determine whether a higher concentration of test IgG (final concentration 1.5 mg/ml) would bring the mean inhibition of candidates that performed below 60% inhibition into an interpretable range (Figure 5.3, Table 5.3). In considering nonspecific effects of normal mouse IgG (purified from pooled whole sera of unimmunized mice) on oocyst inhibition, test IgG concentrations of up to 3 mg/ml were evaluated in previous studies by the NIH reference center (unpublished). In brief, to determine the optimal concentration of test IgG for use in SMFA, normal mouse IgG at 0.5-3 mg/ml was tested in 5 separate feeds. There was an observed increase in percent inhibition at IgG concentrations over 2.25 mg/ml, though too few feeds were performed to establish a statistically significant difference between inhibitions at 2.25 mg/ml and lower IgG concentrations. This is in part due to the inability of SMFA to resolve small differences in oocyst inhibition. While these studies did not conclusively disprove statistically significant inhibition by normal mouse IgG below 2.25 mg/ml, they suggest it may be possible to gain useful information from the assay at these IgG concentrations when calculating percent inhibition relative to a relevant experimental control such as anti-OVA IgG.

Upon repeating SMFA at a sample concentration of 1.5 mg/ml – double the previous test IgG concentration – the negative experimental control, anti-OVA IgG, had a mean oocyst intensity of 25.5, representing a percent inhibition of

mean oocyst intensity of 57.1% (p=0.003 by Mann-Whitney) relative to the negative assay control, human serum pool (mean oocyst intensity 59.5). This was a greater inhibitory effect than seen with 0.75 mg/ml anti-OVA IgG. As before, percent inhibition values for test samples were calculated relative to anti-OVA IgG (Table 5.3). In keeping with previous experiments, anti-Pfs25 IgG had a significant effect on oocyst inhibition of 99.9% (95% CI 99.3-100, p=0.001). Enolase IgG continued to demonstrate an inhibition in the moderate range of 40.3% (95% CI −75.3-79.4, p=0.348), but HYP1 and HSP70-2 did not inhibit oocyst intensity (Figure 5.3).

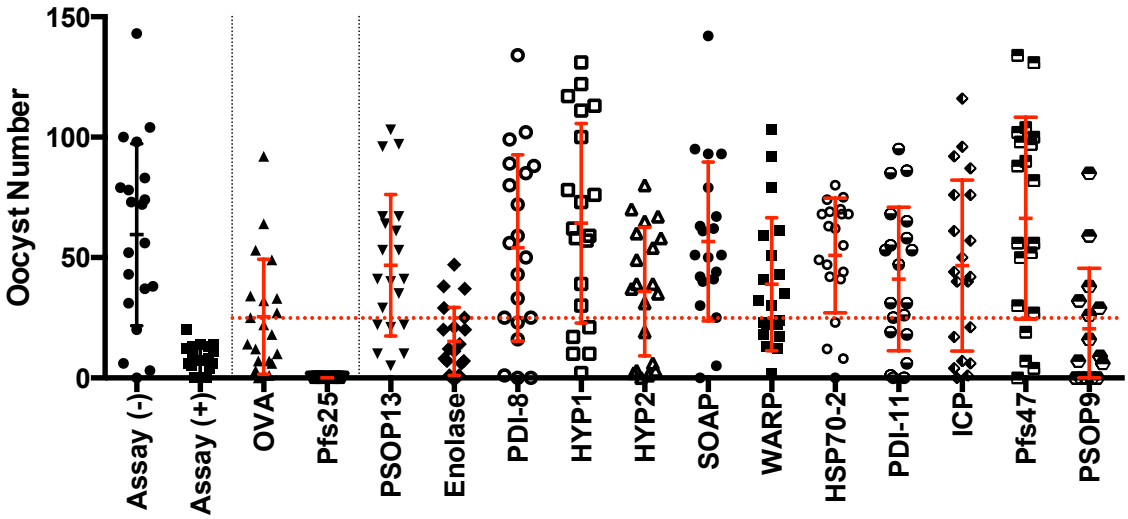


Figure 5.3 Test IgG are evaluated by SMFA at 1.5 mg/ml

Purified IgG from pooled anti-sera generated to recombinant sexual stage proteins was tested by SMFA in the presence of human complement at a final concentration of 1.5 mg/ml in the mosquito blood feeder. Mean oocyst intensity and standard deviation is shown in red. Human serum pool (HSP) served as the negative assay control and Pfs25 monoclonal antibody 4B7 (0.094 mg/ml) was included as a positive assay control. The mean oocyst intensity of the experimental control (purified anti-OVA IgG, final concentration 1.5 mg/ml) is denoted by a red dotted line.

Table 5.3 Percent inhibition of mean oocyst intensity at 1.5 mg/ml

Sample	IgG (mg/ml)	Mean Oocyst	% Oocyst Inhibition	95% CI Low	95% CI High	*p*-value
HSP	1.500	59.5				
4B7	0.094	7.5	87.4	63.2	95.6	0.001
OVA	1.500	25.5				
Pfs25*	1.500	0.0	99.9	99.3	100.0	0.001
PSOP13	1.500	46.9	-84.1	-438.3	37.5	0.256
Enolase	1.500	15.2	40.3	-75.3	79.4	0.348
PDI-8	1.500	54.0	-112.2	-523.8	26.7	0.170
HYP1	1.500	64.3	-152.7	-651.4	14.8	0.097
HYP2	1.500	35.9	-41.1	-321.0	51.1	0.543
SOAP	1.500	56.7	-122.8	-556.6	20.9	0.124
WARP	1.500	39.0	-53.0	-352.9	46.0	0.415
HSP70-2	1.500	50.9	-100.0	-469.4	31.7	0.225
PDI-11	1.500	41.1	-61.5	-360.7	42.1	0.354
ICP	1.500	46.7	-83.3	-421.1	35.5	0.252
Pfs47	1.500	66.4	-160.7	-715.7	8.3	0.071
PSOP9*	1.500	20.5	19.6	-128.9	73.6	0.683

* Mosquito death in the pint resulted in 18 test mosquitos and 15 test mosquitos in the Pfs25 and PSOP9 pints respectively.

5.2.2 Modeling inhibition of oocyst intensity from multiple feeding experiments

To determine which proteins merit further consideration as possible components of the parasite transmission pathway, the aforementioned SMFA data was compiled to establish a rank order. Multiple sources of assay error, attributable to the differences between feeds (inter-assay variations) and between pints (intra-assay variation), complicate the interpretation of data from multiple experiments. The previously described R script model based on data from multiple independent feeding studies, accounts for the differential contributions of feed-to-feed versus pint-to-pint variation [212] and was used to estimate the percent inhibition of oocyst intensity and 95% confidence intervals based on two independent SMFA results. Given the high inhibitory effect of anti-

OVA IgG at 1.5 mg/ml relative to human serum pool, analysis was based on the two SFMA results with sample IgG concentrations of 0.75 mg/ml. In the two-feed prediction model, anti-Pfs25 inhibition was estimated at 99.8% (95%CI 99.3-100.0, p=0.001). IgG generated to enolase and HYP1 was estimated to elicit moderate inhibitions of 48.9 and 38.6 respectively (95%CI −47.1-82.6, p=0.211; 95% CI −72.3-78.0, p=0.343 respectively). Inhibition for HSP70-2 was statistically significant at 85.5% (95% CI 70.3-93.5, p=0.001), but was the composite of one statistically significant inhibition (SMFA B) and one insignificant inhibition (SMFA A) (Figure 5.4).

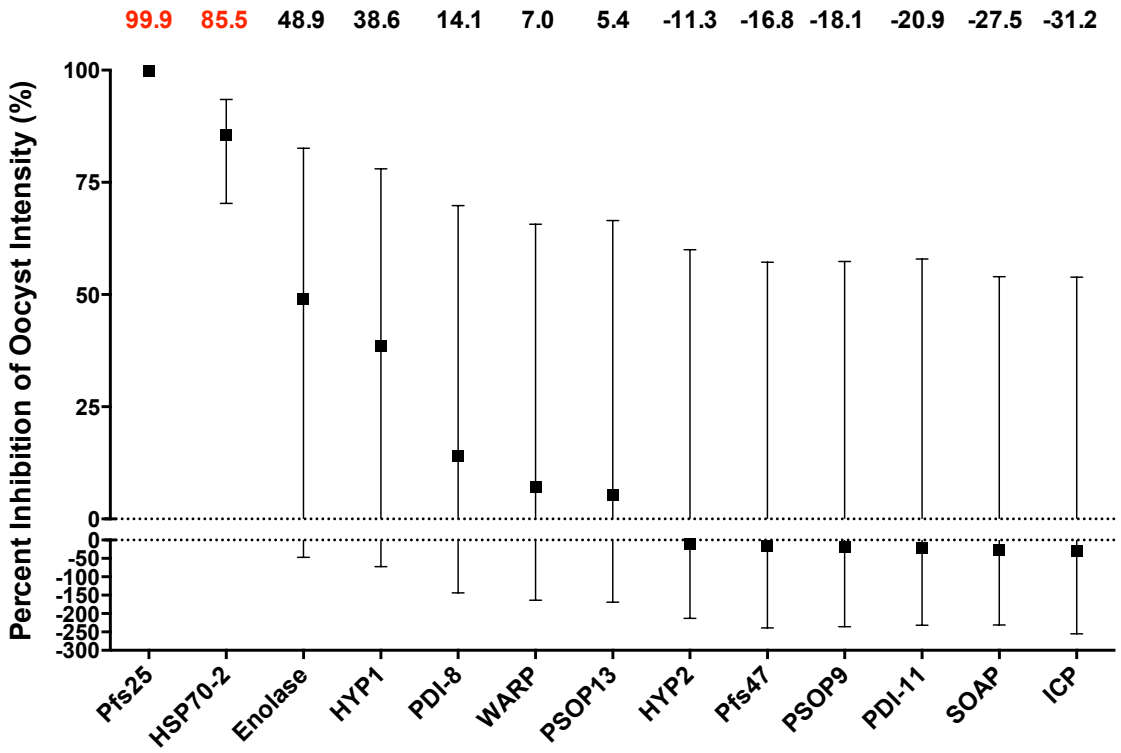


Figure 5.4 Two-feed prediction model estimates oocyst inhibition by SMFA

To estimate the percent inhibition of oocyst intensity and 95%CI based on two independent feeding assays conducted using 0.75 mg/ml test IgG, an R script model was implemented which incorporates data from multiple independent feeding experiments to account for assay variability due to feed-to-feed and pint-to-pint differences [212]. Percent inhibition of mean oocyst intensity and 95% confidence interval (95%CI) was predicted.

While enolase and HYP1, are estimated to show moderate inhibition of oocyst intensity, the wide 95% confidence intervals for each candidate do not exclude 0% inhibition. As previously discussed, inhibition values below 60% were considered effectively outside of the dynamic range of the assay. Per the R script model, assay error is largely attributable to variation in oocyst intensity between mosquitos fed on *P. falciparum* infected blood by a standard methodology. IgG samples which inhibit oocyst intensity to a moderate degree are more susceptible to this variability. Consequently SMFA cannot effectively distinguish inhibition below 60% from 0%, and it is difficult to know if moderate inhibition by SMFA represents a true biological effect.

In evaluating whether the findings for enolase, HSP70-2, and HYP1 are of biological relevance, two additional independent feeding assays were performed with anti-enolase, anti-HSP70-2, and anti-HYP1 IgG. Despite the limitations of the assay, within a multiple feed prediction model, it is possible for reproducibility of inhibition to outweigh assay error. Applying the R script model, in both a 3-feed and 4-feed prediction, anti-enolase IgG demonstrated a statistically significant inhibition of 49.7% (95% CI 8.92-72.6, p= 0.025) and 56.9% (95% CI 27.0-73.3, p=0.006), respectively (Figure 5.5). Inhibition by anti-HSP70-2 and anti-HYP1 was not statistically significant.

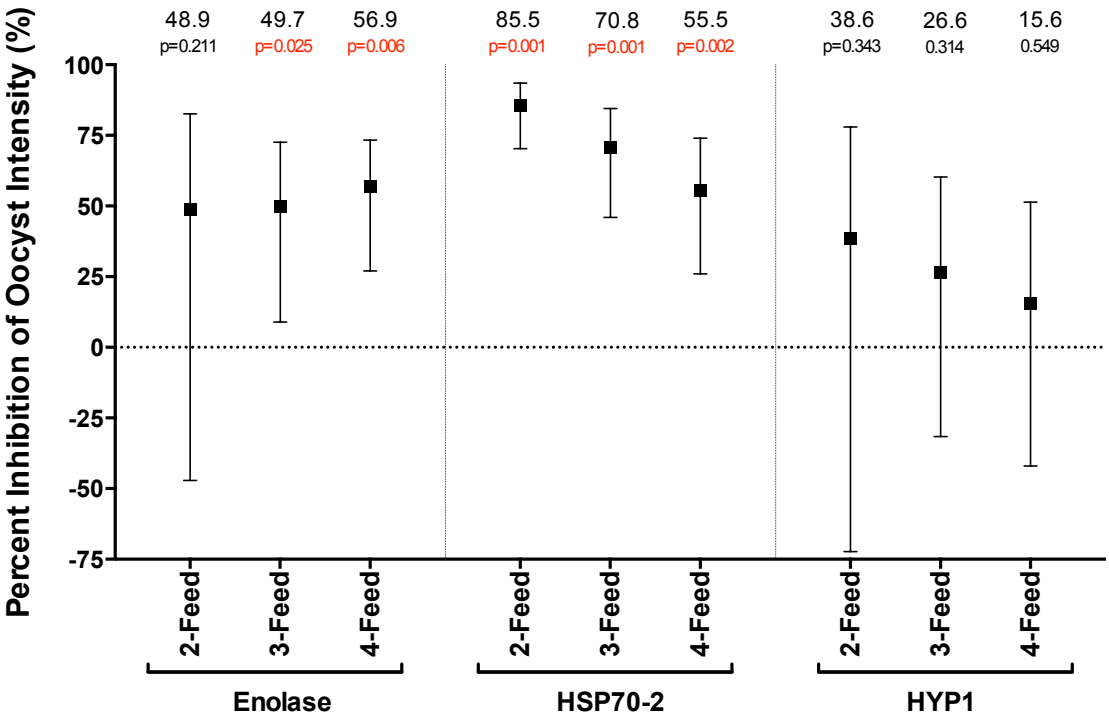


Figure 5.5 Multiple-feed prediction model estimates oocyst inhibition by SMFA

To estimate the percent inhibition of oocyst intensity and 95%CI based on 3 and 4 independent feeding assays conducted using 0.75 mg/ml test IgG, an R script model was implemented which incorporates data from multiple independent feeding experiments to account for assay variability due to feed-to-feed and pint-to-pint differences [212]. Percent inhibition of mean oocyst intensity and 95% confidence interval (95%CI) was predicted.

5.2.3 Evaluation of additive effects between anti-enolase and anti-Pfs25 IgG

While the primary objective of chapter 5 was to characterize a panel of sexual stage proteins for transmission-blocking epitopes, a secondary aim was to consider these proteins as potential vaccine antigens. The previous data support a strong inhibition of oocyst intensity by anti-Pfs25 IgG, and within the scope of this thesis, IgG generated to Pfs25 outperformed other HEK293E-expressed candidates in a protein-in-adjuvant formulation (Chapter 3). This is in keeping with published literature [115, 198], and at present, Pfs25 remains the best-characterized TBV antigen. Given the moderate inhibition displayed by the three highest ranked candidates in the two-feed prediction model, to be of value for TBV development, these antigens should enhance functional antibody response generated to Pfs25. Of the three, inhibition by anti-enolase IgG was most consistent. Therefore IgG generated to enolase was evaluated for additive and synergistic interactions in a mixing study with anti-Pfs25 IgG by SMFA.

In the two-feed prediction model, anti-Pfs25 IgG at 0.75 mg/ml was estimated to inhibit oocyst intensity by 99.8% (95%CI 99.3-100.0, p=0.001), representing the maximum limit of the assay's read-out. At this concentration it is not possible to quantify added functional improvement. To determine the minimum IgG concentration resulting in a functional plateau, SMFA was performed with two-fold serial concentrations of test anti-Pfs25 IgG from 0.023 mg/ml to 0.75 mg/ml. Percent inhibition of mean oocyst intensity with 95% confidence intervals was calculated relative to anti-OVA IgG at 0.75 mg/ml (Table 5.4). Percent inhibition

did not reach an upper plateau within the dilution series; maximum oocyst inhibition was 98.2% (95% CI 94.3-99.5, p=0.001) at 0.75 mg/ml IgG.

The ratio of mean oocyst intensity (control sample/ test sample) was then plotted on a log scale against the test anti-Pfs25 IgG concentration in mg/ml on a square root scale (Figure 5.6). Concentrations corresponding to the linear portion of the curve (0.094, 0.188, 0.375, 0.750 mg/ml) were selected as test anti-Pfs25 IgG concentrations for the mixing experiment. Antibodies generated to enolase were established to inhibit oocyst intensity by less than 60% in previous sections; mean inhibition is 56.9% (95% CI 27.0-73.3, p=0.006) at 0.75 mg/ml by the 4-feed prediction model. Therefore a parallel anti-enolase IgG titration experiment was not performed.

Table 5.4 Percent inhibition of mean oocyst intensity with serial concentrations of anti-Pfs25 IgG

Sample	IgG (mg/ml)	Mean Oocyst	% Oocyst Inhibition	95% CI Low	95% CI High	*p*-value
OVA	0.750	21.8				
Pfs25	0.750	0.4	98.2	94.3	99.5	0.001
Pfs25	0.375	2.8	87.4	60.5	96.2	0.001
Pfs25	0.188	8.0	63.4	-7.9	87.5	0.068
Pfs25	0.094	19.7	9.7	-152.8	69.7	0.827
Pfs25	0.047	21.8	0.0	-188.4	65.8	0.984
Pfs25	0.023	24.2	-11.3	-205.8	62.1	0.881

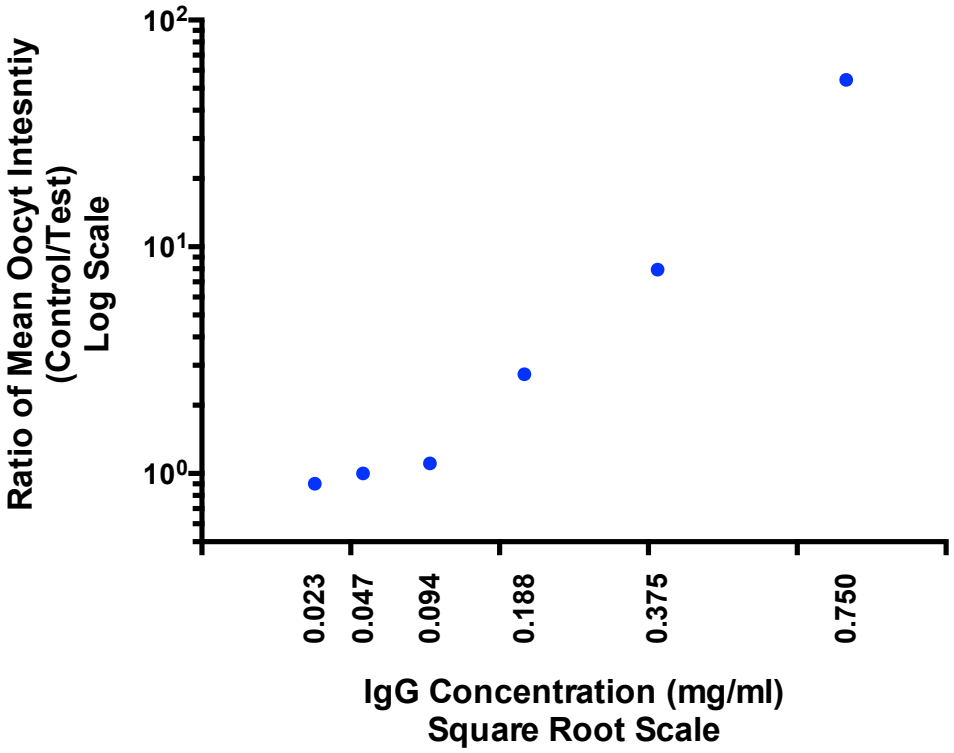


Figure 5.6 Percent inhibition of mean oocyst intensity with serial concentrations of anti-Pfs25 IgG

To determine concentration resulting in a functional plateau of inhibition of mean oocyst intensity, SMFA was performed with two-fold serial concentrations of test anti-Pfs25 IgG from 0.023 mg/ml to 0.75 mg/ml. The ratio of mean oocyst intensity (control sample/ test sample) was then plotted on a log scale against the test anti-Pfs25 IgG concentration in mg/ml on a square root scale to determine the linear range of the assay for anti-Pfs25 IgG. Concentrations corresponding to the linear portion of the curve (0.094, 0.188, 0.375, 0.750 mg/ml) were selected as test anti-Pfs25 IgG concentrations for subsequent mixing experiments with anti-enolase IgG.

To assess additive or synergistic effects between antibody responses generated to Pfs25 and enolase, anti-Pfs25 IgG samples (0.094, 0.188, 0.375, and 0.75 mg/ml) were tested with and without anti-enolase IgG (0.75 mg/ml) by SMFA. Per the previous analysis with anti-Pfs25 IgG alone, this range of test concentrations should allow for functional inhibition of mixed samples to fall within the dynamic range of the assay, i.e. 60-100% (Figure 5.7). Mixed IgG samples were evaluated for a statistically significant increase in percent inhibition of oocyst intensity relative to anti-Pfs25 IgG alone. In the first mixing study (Table 5.5, Figure 5.7), anti-enolase IgG at 0.75 mg/ml inhibited oocyst intensity by 72.8% (95% CI 19.4-91.2, p=0.023). Inhibition by anti-Pfs25 IgG was also higher that anticipated with sample concentrations of 0.094-0.75 mg/ml corresponding to 67.4-99.5% mean inhibition. Given the unexpectedly high inhibitory effect of anti-Pfs25 IgG, when mixed with enolase at 0.75mg/ml, inhibition was statistically significant at all anti-Pfs25 test IgG concentrations but reached a plateau at 0.375 mg/ml anti-Pfs25 IgG mixed with 0.75 mg/ml enolase.

Table 5.5 Mix A: Percent inhibition of mean oocyst intensity of anti-Pfs25 and anti-enolase IgG

Sample	IgG (mg/ml)	Mean Oocyst	% Oocyst Inhibition	95% CI Low	95% CI High	p-value
OVA	0.750	9.2				
Enolase	0.750	2.5	72.8	19.4	91.2	0.023
Pfs25	0.750	0.1	99.5	96.2	100.0	0.001
Pfs25	0.375	0.2	98.4	89.5	100.0	0.001
Pfs25	0.188	1.3	85.9	52.5	96.4	0.002
Pfs25	0.094	3.0	67.4	-1.9	90.5	0.051
Pfs25+Enolase	0.075+0.750	0.0	100.0	98.4	100.0	0.001
Pfs25+Enolase	0.375+0.750	0.0	100.0	98.2	100.0	0.001
Pfs25+Enolase	0.188+0.750	0.9	90.8	71.4	97.2	0.001
Pfs25+Enolase	0.094+0.750	0.5	95.1	83.5	99.0	0.001

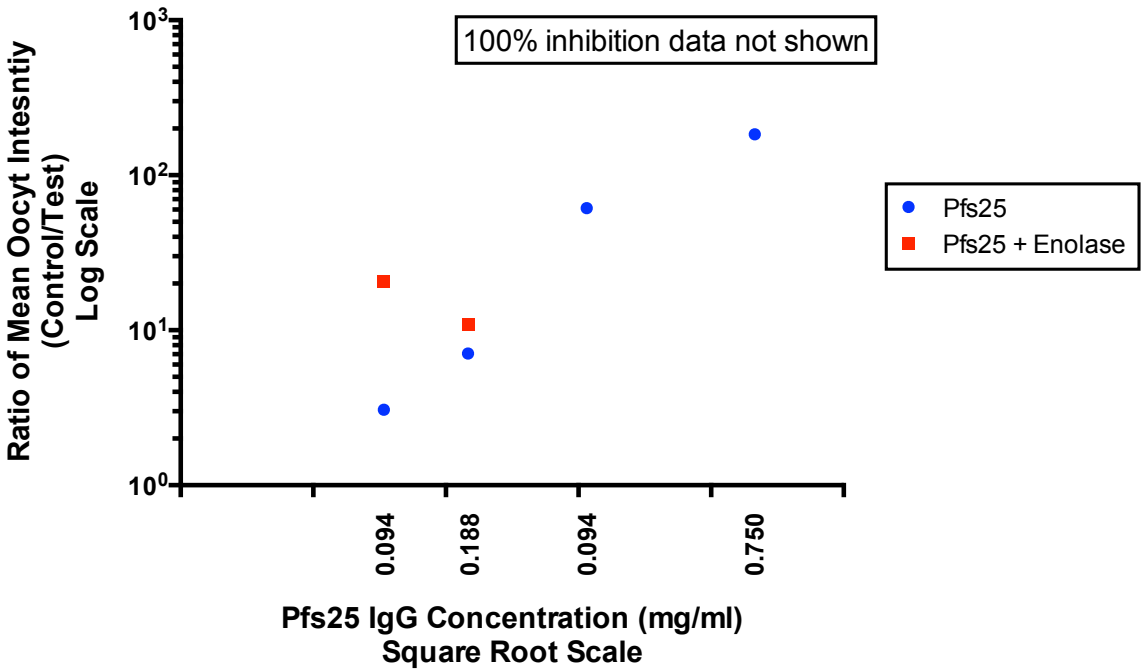


Figure 5.7 Evaluation of anti-Pfs25 and anti-enolase IgG in one membrane feeder by SFMA

Serial concentrations of anti-Pfs25 IgG (0.094, 0.188, 0.375, 0.750 mg/ml) were mixed with anti-enolase IgG at 0.75 mg/ml and evaluated by SMFA. The ratio of mean oocyst intensity (control sample/ test sample) was then plotted on a log scale against the test anti-Pfs25 IgG concentration in mg/ml on a square root scale to linearize percent inhibition with respect to IgG.

The mixing study was repeated as previously. In the second study, given the known inhibitory effect of purified normal mouse IgG, serial concentrations of anti-Pfs25 IgG (0.094, 0.188, 0.375, and 0.75 mg/ml) were also tested in the presence of 0.75 mg/ml normal mouse IgG. In contrast to the previous study, mean oocyst intensity in the anti-OVA IgG control was significantly higher 68.1. Anti-enolase IgG at 0.75 mg/ml performed at an anticipated 41.0% inhibition (95% CI −69.0-79.8, p=0.342). And while again, anti-Pfs25 IgG mixed with anti-enolase IgG demonstrated a statistically significant inhibition for all test concentrations, anti-Pfs25 IgG mixed with normal mouse IgG demonstrated the same effect suggesting that the presence of anti-enolase IgG may be nonspecific (Table 5.6, Figure 5.8). An overlay of both mixing studies suggests that this experimental design is not able to prove an additive effect of anti-Pfs25 IgG with anti-enolase IgG, in part because the moderate inhibition of anti-enolase IgG alone necessitates use of 0.75 mg/ml IgG which may result in nonspecific effects within a mixed sample (Figure 5.9).

Table 5.6 Mix B: Percent inhibition of mean oocyst intensity of anti-Pfs25 and anti-enolase IgG

Sample	IgG (mg/ml)	Mean Oocyst	% Oocyst Inhibition	95% CI Low	95% CI High	*p*-value
OVA	0.750	68.1				
Enolase	0.750	40.2	41.0	-69.0	79.8	0.342
Pfs25	0.750	0.2	99.8	99.0	100.0	0.001
Pfs25	0.375	4.3	93.7	80.5	98.0	0.001
Pfs25	0.188	10.9	84.1	55.9	94.8	0.001
Pfs25	0.094	31.3	54.1	-31.6	83.9	0.166
Pfs25+Enolase	0.075+0.750	0.1	99.9	99.7	100.0	0.001
Pfs25+Enolase	0.375+0.750	0.5	99.3	97.7	99.8	0.001
Pfs25+Enolase	0.188+0.750	4.8	92.9	79.5	97.7	0.001
Pfs25+Enolase	0.094+0.750	11.3	83.5	52.4	94.0	0.002
Pfs25+NMAb	0.075+0.750	0.2	99.8	99.2	100.0	0.001
Pfs25+NMAb	0.375+0.750	0.9	98.8	96.0	99.7	0.001
Pfs25+NMAb	0.188+0.750	4.7	93.2	81.1	97.6	0.001
Pfs25+NMAb	0.094+0.750	8.6	87.4	62.5	95.8	0.001

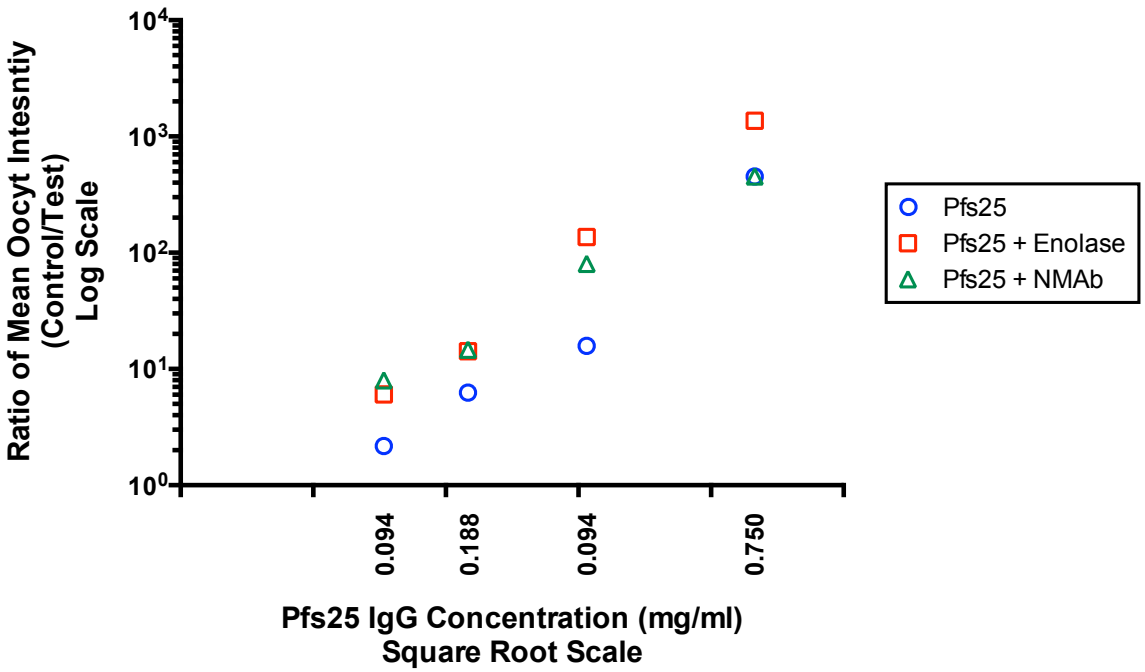
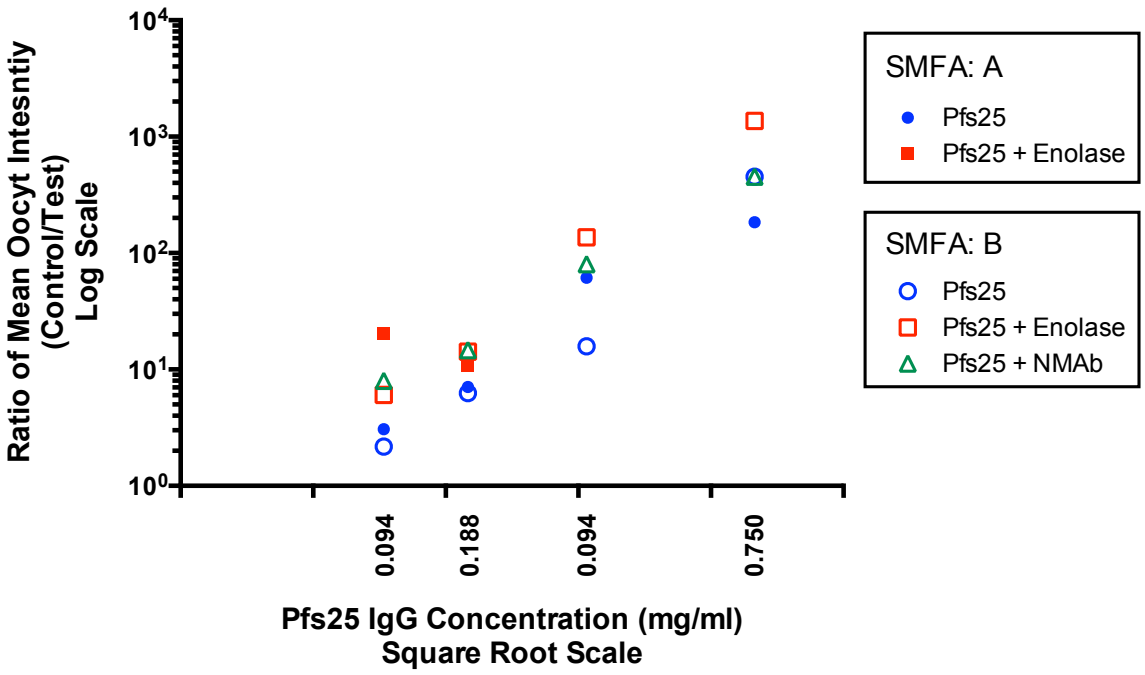


Figure 5.8 Repeat evaluation of anti-Pfs25 and anti-enolase IgG in one membrane feeder by SMFA

Serial concentrations of anti-Pfs25 IgG (0.094, 0.188, 0.375, 0.750 mg/ml) were mixed with anti-enolase IgG at 0.75 mg/ml and evaluated by SMFA. The ratio of mean oocyst intensity (control sample/ test sample) was then plotted on a log scale against the test anti-Pfs25 IgG concentration in mg/ml on a square root scale to linearize percent inhibition with respect to IgG. Serial concentrations of anti-Pfs25 IgG (0.094, 0.188, 0.375, 0.750 mg/ml) mixed with normal mouse IgG at 0.75 mg/ml are plotted in green.



100% inhibition data from assay A not shown

Figure 5.9 Overlay of two SMFA studies evaluating interaction of anti-Pfs25 and anti-enolase IgG

Serial concentrations of anti-Pfs25 IgG (0.094, 0.188, 0.375, 0.750 mg/ml) were mixed with anti-enolase IgG at 0.75 mg/ml and evaluated by SMFA. The ratio of mean oocyst intensity (control sample/ test sample) was then plotted on a log scale against the test anti-Pfs25 IgG concentration in mg/ml on a square root scale to linearize percent inhibition with respect to IgG. Serial concentrations of anti-Pfs25 IgG (0.094, 0.188, 0.375, 0.750 mg/ml) mixed with normal mouse IgG at 0.75 mg/ml are plotted in green. Results from two independent mixing studies are shown.

5.2.4 Recombinant *P. falciparum* Proteins are immunoreactive

To further determine whether the recombinant sexual stage antigens display native epitopes, we tested immunoreactivity using serum from two cohorts of adults living in malaria-endemic Burkina Faso (n=32) and Mali (n=36) – regions defined by intense and highly seasonal *P. falciparum* transmission [2] – relative to US adults naïve to malaria infection (n=10). Individuals from Burkina Faso demonstrated positive responses to 7 out of 12 antigens as defined by a cutoff of 3 standard deviations above the mean optical density of US naïve response by ELISA (Figure 5.10 A). In rank order, the highest level of response as defined by median raw OD was to PSOP9, PSOP13, ICP, PDI-8 and HSP70-2, all of which recognized gametocyte stage parasites by IFA. Immunoreactivity in a cohort of adults in malaria-endemic Mali demonstrated an identical rank order in an independent field site, suggesting that these recombinant antigens indeed display epitopes seen in native parasite proteins (Figure 5.10 B).

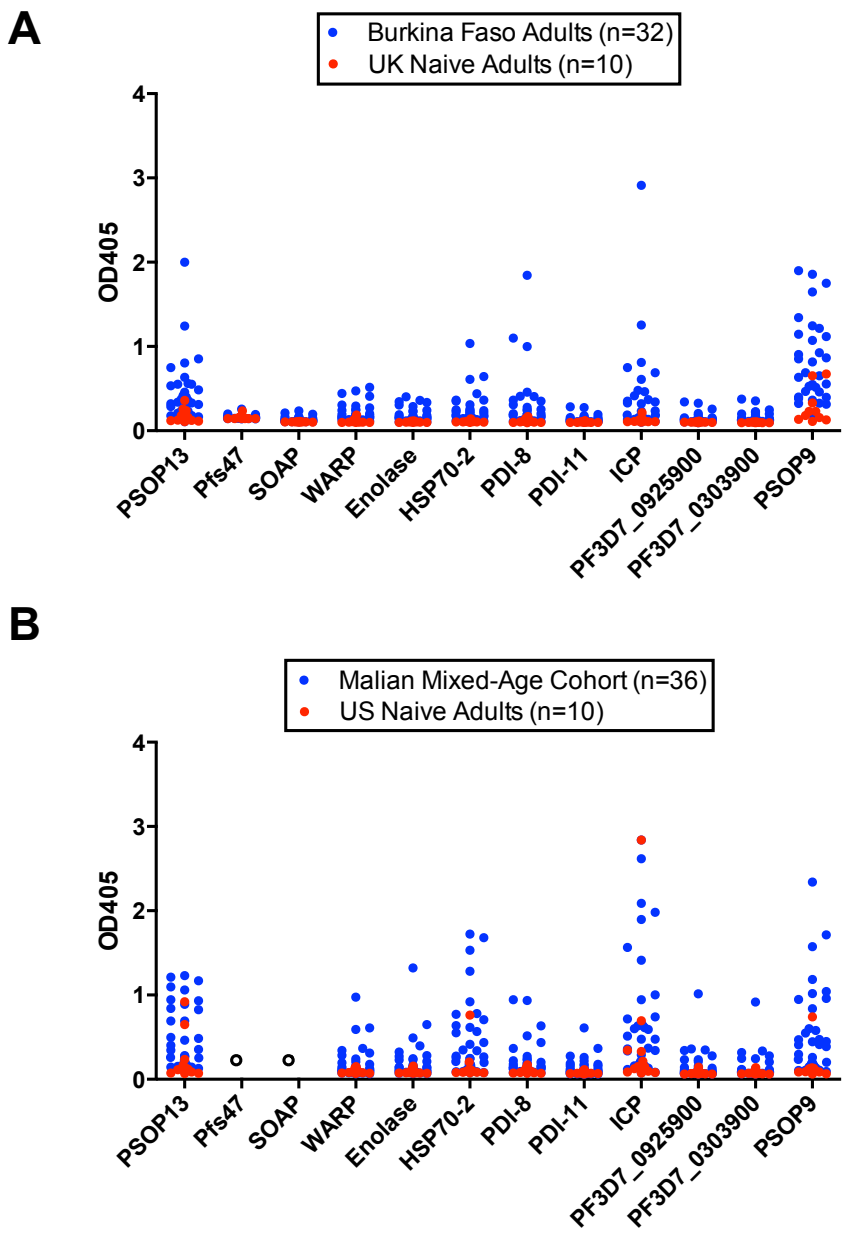


Figure 5.10 *P. falciparum* proteins demonstrate seroreactivity in two independent cohorts from malaria-endemic regions.

ELISA was used to determine the immunoreactivity of heterologously expressed proteins in a cohort from malaria-endemic Burkina Faso (n=36) (**A.**) and Mali (n=32) (**B.**) by measuring IgG responses, reported as optical density at an absorbance of 405nm. Raw OD_{405} values for Burkina Faso, Mali, and US malaria-naïve volunteers are shown. The antigens with lowest median raw OD_{405} in the Burkina Faso cohort – Pfs47 and SOAP – were not tested in the Malian cohort.

In rank order, the highest level of response as defined by median raw OD was to PSOP9, PSOP13, ICP, PDI-8 and HSP70-2, all of which recognized gametocyte stage parasites by IFA. Immunoreactivity in a cohort of adults in malaria-endemic Mali demonstrated an identical rank order in an independent field site, suggesting that these recombinant antigens indeed display epitopes seen in native parasite proteins (Figure 5.11).

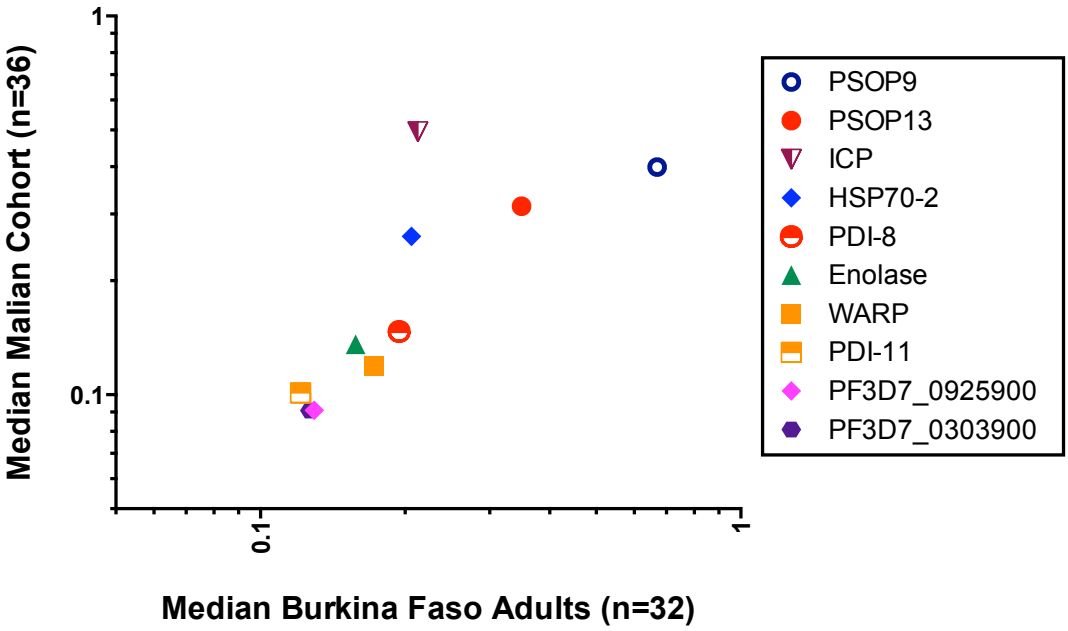


Figure 5.11 Protein seroreactivity correlates between independent cohorts from Burkina Faso and Mali

ELISA was used to determine the immunoreactivity of heterologously expressed proteins in a cohort of adults from malaria-endemic Burkina Faso (n=36). The median IgG response to the panel of sexual stage correlated between the Burkina Faso and Malian cohort.

5.3 Discussion

Chapter 5 reports the screening of a panel of sexual stage antigens for transmission-blocking epitopes by SMFA and the testing of these proteins for seroreactivity in two independent cohorts from malaria-endemic Burkina Faso and Mali.

In a two-feed prediction model based on independent feeding experiments performed with 0.75 mg/ml IgG, three antigens demonstrated a moderate predicted inhibition of oocyst intensity by SMFA. IgG generated to enolase, HSP70-2, and hypothetical protein HYP1 inhibited oocyst intensity by 48.9%, 38.8%, and 38.6% respectively (95%CI −47.1-82.6, p=0.211; 95% CI −163.7-65.7, p=0.938; 95% CI −72.3-78.0, p=0.343 respectively). By contrast, in a four-feed prediction model, while enolase demonstrated a statistically significant inhibition of oocyst intensity, HSP70-2 and HYP1 did not inhibit oocyst intensity. In the case of HSP70-2, one feed which demonstrated an inhibition of 98.2% (95% CI 94.5-99.7, p=0.001) appeared to be an outlier, possibly due to experimental error; the inhibitory activity of anti-HSP70-2 IgG was not statistically significant in three additional, independent feeds.

Notably purified IgG samples tested in this study were of whole IgG. Due to the limited quantity of IgG derived from BALB/c mouse vaccination, it was not possible to evaluate antigen-specific IgG responses by SMFA. While all anti-sera demonstrated high endpoint titers to the respective vaccine immunogen by ELISA, this result does not discount variable levels of antigen-specific IgG

between candidates. It would be interesting in further studies to rank vaccine immunogens by SFMA relative to antigen specific-antibody titer in prioritizing candidates for clinical studies.

The findings for anti-enolase are consistent with a previous publication by Ghosh *et al*, which identified enolase as a target of transmission-blocking antibodies. While enolase does not have a predicted signal peptide, it is secreted onto the ookinete surface. The antigen was suggested to have a role in midgut recognition and traversal by acting as a hypothetical ligand for a mosquito midgut receptor and means for co-opting mammalian plasminogen, which when converted to active plasmin, aids in midgut traversal. In the Ghosh *et al* study, enolase was heterologously expressed in *E. coli*. Anti-sera were generated to the recombinant antigen by rabbit immunization. Whole sera showed 70% inhibition of *P. falciparum* oocyst intensity by SMFA. The use of whole sera, which is known to exert nonspecific inhibitory effects, may have contributed to the higher inhibition compared with the present analysis.

While anti-enolase IgG performed consistently and demonstrated a statistically significant inhibition of oocyst intensity in parallel with previous reports, the moderate range of inhibition suggests that to be considered as a vaccine immunogen, enolase would need to be used in a multi-subunit TBV. Establishing additivity by SMFA is not straightforward. SMFA does not effectively resolve small differences in inhibition, and the dynamic range of the assay requires that the inhibition of pure and mixed samples fall between 60 and 100%. The inability to test anti-enolase in a mixing experiment at a

concentration below 0.75 mg/ml – given the already moderate inhibition – coupled with nonspecific inhibitory effects on normal mouse IgG at concentrations above 0.75 mg/ml made it difficult to gain useful data from the SMFA mixing study. In subsequent analysis, availability of antigen-specific IgG may circumvent this problem.

Finally, seroreactivity in selected proteins from the sexual stage panel confirms the presence of native *P. falciparum* epitopes as suggested by IFA studies in Chapter 4. Chapter 7 will evaluate seroreactive proteins as potential markers of gametocyte exposure.

Chapter 6: Naturally Acquired Immune Responses to TBV Antigens

6.1 Introduction

While immune recognition of *P. falciparum* – acquired through malaria infection – may impact TBV immunogenicity and functionality, naturally acquired immune responses to TBV candidate antigens remain understudied. Chapter 6 and 7 will shift focus from vaccine-induced immunity to consider naturally acquired recognition of the *P. falciparum* sexual stage. In theory, naturally acquired human immune response may disrupt parasite sexual stage development by (1) decreasing gametocyte density in the human host or (2) targeting parasite antigens exposed within the mosquito midgut. Currently, apart from naturally acquired immunity to the asexual stages, which indirectly reduces gametocyte numbers, the evidence for the former is limited. While the latter is the theoretical foundation for TBVs, naturally acquired TBI remains controversial.

Chapter 6 describes the profiling of antibody responses to known TBV antigens, Pfs230C and HAP2, in an area of intense and highly seasonal transmission in Mali, the screening of a subset of individuals in the same cohort for TRI, and assessment of whether antibody titers may correlate to diminished gametocyte transmissibility. Chapter 7, by contrast, profiles antibody responses to the 12 sexual stage antigens previously characterized by SMFA.

6.1.1 Host immunity and gametocytemia

Among epidemiologic studies which evaluate the relative effects of seasonality [252-254], transmission intensity [255] and host factors [256, 257] on gametocyte dynamics, relatively few have been designed to assess the effect of host immunity. Early and foundational epidemiologic analyses borne out of the Garki Project proposed that immunity might develop more rapidly to gametocytes than to asexual stage parasites [258], but this observation has had little follow-up. One report based on a cohort from the Papua province (formerly Irian Jaya), Indonesia, where malaria is seasonally intense, identified an association between antibody specificity for sexual stage antigens and reduced gametocyte prevalence. The study cohort represented two distinct subpopulations including life-long residents and recent migrants. Recent migrants were defined as having arrived less than 20 months before the study's surveillance by bi-weekly blood collection was initiated. An analysis of parasite densities by microscopy suggested that the rate of *P. falciparum* positivity was similar in both groups, whereas the rate of gametocytemia was higher among recent migrants, an effect unrelated to potential confounders such as number of clinical episodes, severity of disease, or recent antimalarial use [32].

Moreover, in one village of the Papua province study, it was observed that gametocyte prevalence fell from 83% to 25% between the 11^{th} and 25^{th} month of residence for recent migrants while asexual parasite prevalence remained the same. Antibody titer to acetone-fixed gametocytes correlated to a lower incidence of gametocytemia in recent-migrants but not life-long residents

suggesting a functional antibody response specific to gametocytes and independent of asexual stage immunity [32]. Circulating gametocytes in the human host are intra-erythrocytic, so direct recognition is presumably mediated by parasite-specific proteins on the erythrocyte surface; no such proteins unique to the gametocyte stage have been identified to date. As described in Section 1.3.1, early stage gametocytes (I and IIa) mediate their sequestration phenotype in part by PfEMP1, a known erythrocyte surface antibody target in asexual stage parasites [38]. And while immune sera have been shown to specifically recognize erythrocytes infected with the stage V gametocytes by flow cytometry, no specific targets have been defined as of yet [31].

6.1.2 Transmission-reducing immunity

As gametocytes activate and emerge from human erythrocytes in the mosquito midgut, stage-specific surface antigens critical to fertilization and sporogony become exposed to human immune factors in the blood meal. It has been proposed that those gametocytes not sampled by feeding *Anopheles* mosquitos, die within the human host allowing immune recognition of previously intra-erythrocytic sexual stage antigens. The concept of TBI resulting from malaria infection remains controversial. Field studies have evaluated individuals living in malaria-endemic areas for transmission-reducing antibody activity by mosquito feeding studies such as the SMFA and DMFA, though the lack of standard methodology and need for more intensive longitudinal study design has limited conclusions about rate of TBI development and functional persistence [40].

Pfs48/45 and Pfs230 are abundant gametocyte and gamete surface proteins as demonstrated by their identification via monoclonal antibodies to parasite sexual stage extracts [53, 62, 63, 71]. While the true targets of natural TBI are not known, antibody responses against Pfs230 and Pfs48/45 correlate to transmission-reducing potential of sera in most, but not all studies. Pfs230 antibody titers are arguably more predictive of TBI with strong correlation reported in sera from Gambian [42, 259, 260], Tanzanian [261, 262], and Papua New Guinean [43, 263] cohorts; in Cameroon [245, 264] and Sri Lanka [265] similar analyses did not yield statistical significance. Outside of one recent study using recombinant proteins Pfs48/45-10C and Pfs230-230CMB produced in *L. lactis* [88] and *N. benthamiana* [76] respectively [266], most serological analyses have relied on capture of antigens from lab strain parasite-extracts by monoclonal antibodies due to historical difficulties in the conformational expression of Pfs48/45 and Pfs230; limited sequence variation in Pfs48/45 and Pfs230 [266-268] has supported use of lab strains in such studies. Interestingly, several reports have identified transmission-reducing activity in sera with no detectable Pfs48/45 or Pfs230 antibody titers [259, 261, 262] suggesting that additional antigenic targets of TBI may exist in the field.

Chapter 6 aims to better characterize seroreactivity to known TBV candidates HAP2 and Pfs230 (region C) in an area of highly endemic and seasonal malaria, as well as to better define incidence of TBI in the same field site by a standard methodology used to evaluate pre-clinical vaccine candidates – SMFA with purified IgG.

6.2 Results

6.2.1 Malian adult serology

In 2009, adult plasma samples were collected in two independent cross-sectional surveys of Kenieroba – a village 75 km southwest of Bamako, Mali. The study site is characterized by a cycle of intense and highly seasonal *P. falciparum* transmission [214], and consequently the sample collections were timed in accordance with the peak of the wet season and the peak of the dry season. In a preliminary study aimed to explore naturally occurring immunity to TBV antigens among adult participants, a random subset of donors were screened for serological reactivity to a panel of TBV antigens by ELISA.

Recombinant Pfs48/45, Pfs230C, HAP2 (predicted to localize to the male gametocyte and microgamete surface [140]), and Pfs25 proteins – expressed in the WGCFS [115] – were provided as a generous gift by Dr. Takafumi Tsuboi.[8] Recombinant Pfs48/45, HAP2, and Pfs25 proteins were expressed as full-length ectodomains; all protein expression was based on the 3D7 parasite strain sequence [115]. Notably, the final ELISA screen was limited to Pfs230C, HAP2, and Pfs25 due to concerns about the conformational integrity of Pfs48/45; as previously discussed, in a rabbit immunization study, this antigen failed to elicit antibodies capable of functional transmission reduction by SMFA [115]. Based on previous reports, HAP2 and Pfs25 expression predominates in

[8] This analysis pre-dated expression of TBV antigens in the HEK293E system (Chapter 3). Due to concerns regarding purity of the hexahistidine tagged HEK293E-derived Pfs230C and Pfs48/45, the serology studies described in Chapter 6 have not been repeated with HEK293-derived proteins.

the gamete and ookinete stages respectively. While neither antigen is predicted to have expression in the human host, these proteins were included with the aim of confirming this expression pattern and validating the sero-epidemiological analysis using WGCFS-derived recombinant protein.

As summarized in Table 6.1, Pfs25 and Pfs230C antibody responses were measured in 52 adults with a median age of 34.5 (IQR 25-47). Anti-HAP2 antibody responses were assessed in a smaller subset of this cohort (n=41, median age=34.5, IQR 24-46). Titers to AMA1 (3D7) and $MSP1_{42}$ (3D7), erythrocytic stage antigens, also expressed in the WGCFS, were available from an earlier analysis by Ababacar Diouf for the majority of the 52 individuals (n=42, median age=34.5, IQR 25-47), and serve as markers of malaria exposure at the study site. All plasma was diluted 1:100 for sexual stage antigen ELISA and 1:500 for erythrocytic stage antigen ELISA.

Table 6.1 Demographic Data: Malian Adult Cohort (2009)

Serological Study	Cohort size, n	Age, median (range, IQR)	Gender, % female	Ethnicity %		
				Dogon	Fulani	Malinke
Pfs25, Pfs230C	52	34.5 (18-60, 25-47)[b]	54.8 (23/42)[b]	11.9[b]	42.9[b]	45.2[b]
mid-dry	23	25 (18-50, 21-32)	34.8 (18/23)	21.7	78.3	0
mid-wet	29	45 (28-60, 40-58)[b]	78.9 (15/19)[b]	0[b]	0[b]	100[b]
HAP2[a]	41	34.5 (18-60, 24-46)[b]	58.8 (20/34)[b]	0[b]	52.9[b]	47.1[b]
mid-dry	18	24.5 (18-50, 21-31)	44.4 (8/18)	0	100	0
mid-wet	23	43.5 (28-60, 38-52.5)[b]	75.0 (12/16)[b]	0[b]	0[b]	100[b]
AMA1/MSP1[a]	42	34.5 (18-60, 25-47)	54.8 (23/42)	11.9	42.9	45.2
mid-dry	23	25 (18-50, 21-32)	34.8 (8/23)	21.7	78.3	0
mid-wet	19	45 (28-60, 40-58)	78.9 (15/19)	0	0	100

a. HAP2, AMA1, and $MSP1_{42}$ serology was performed on two different subsets of the 52 samples with available Pfs25 and Pfs230C data due to limitations in available plasma. As a result a total of 34 samples have serological data for all five antigens.
b. A minority of samples lacked corresponding demographic data in the wet season.

The initial screen of Malian adult plasma aimed to:

1. Confirm a pattern of intense and highly seasonal transmission via IgG antibody responses to AMA1 and $MSP1_{42}$.
2. Identify antibody responses to Pfs230C, with a predicted absence of response to HAP2 and Pfs25 antigens, not thought to be expressed in the human host.
3. Determine Pfs230C IgG prevalence and antibody level relative to age.
4. Develop hypotheses for pursuing a similar analysis in a larger and better-characterized cohort of Malian children.

6.2.1.1 Malian adult antibody response to erythrocytic stage antigens

Despite the lack of parasite quantification from peripheral blood at the time of plasma collection, a positive serology for asexual stage antigens in the majority of adult samples is in accordance with intense and highly seasonal transmission at the field site. In this analysis, to determine positivity, raw optical density (OD) values were normalized according to a cut-off and expressed as “adjusted OD” according to the method of Yildiz Zeyrek *et al* [269]. Briefly, cut-off values were set at three standard deviations (SD) above the mean OD of US naïve sera diluted 1:100 (n=10) run on the same ELISA plate. After background subtracting the cut-off, adjusted OD measurements greater than 0.01 were defined as positive. 97.6% (41/42) of adults had a positive IgG antibody titer to AMA1; 83.3% (35/42) of adults had a positive response to $MSP1_{42}$. Without corresponding clinical records and parasitology, it is not possible to conclude whether these responses are reflective of recent infection or immunological

memory, but these results confirm a widespread immune recognition of malarial antigens in the study population.

6.2.1.2 Malian adult antibody response to sexual stage antigens

The prevalence of IgG antibodies, i.e. the proportion of individuals with a positive adjusted OD, to Pfs230C was 50% (26/52). Surprisingly, IgG antibody response to HAP2 and Pfs25 was present in a minority of samples: 19.5% (19/41) to HAP2 and 13.5% (7/52) to Pfs25 (Figure 6.1 A). Notably the majority of individuals denoted as positive responders to Pfs230C, HAP2 or Pfs25, had antigen-specific IgG levels very near the set threshold for positive response; for context, the proportion of adults with an adjusted OD above 0.1 was 11.5% (6/52) to Pfs230C, 9.8% (4/41) to HAP2, and 1.9% (1/52) for Pfs25. Consequently it becomes difficult to distinguish background variability in OD from true positive serology. When considering the plasma levels of antigen-specific IgG, reported as adjusted OD, asexual stage antigens elicit a higher level of IgG response across the population as a whole (Figure 6.1 B).

There were no significant correlations between the levels of IgG specific to either of the erythrocytic stage antigens (AMA1, $MSP1_{42}$) and any of the sexual stage antigens (Pfs230C, HAP2, Pfs25) in the adult cohort. Such statistical inferences are limited by the relatively small sample size, which was intended to serve only as a preliminary screen of sexual stage antibody responses in Kenieroba.

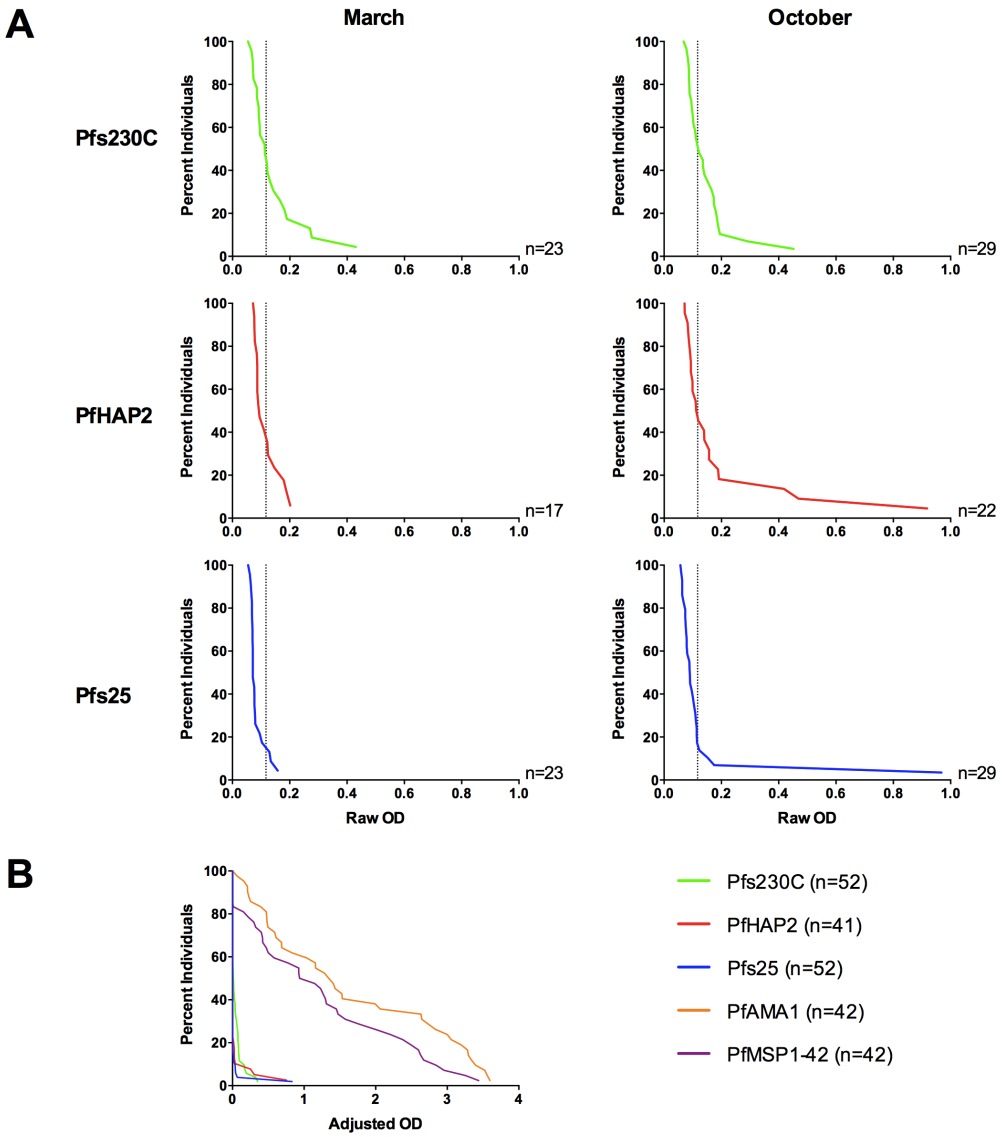


Figure 6.1 Malian adult IgG antibody responses against erythrocytic and sexual stage antigens

Population-based representation of IgG antibody levels, as measured by ELISA optical density (OD) to erythrocytic stage antigens AMA1 and $MSP1_{42}$ and sexual stage antigens Pfs230C, HAP2, and Pfs25 by season (**A**) and in aggregate (**B**). Responses are shown for the sexual stage antigens (Pfs230C, HAP2, and Pfs25) as a raw OD with the plotted cut-off for positive response **A.** Adjusted ODs for all adults tested relative to antigen are also plotted on the same axes for comparison **B.** Briefly, to determine positivity, raw OD values were normalized according to a cut-off and expressed as "adjusted OD". Cut-off values were set at three standard deviations above the mean OD of US naïve serum run on the same ELISA plate. Adjusted OD measurements above 0.01 were defined as positive.

6.2.1.3 Effect of age on Malian adult antibody response

Plasma antibody responses were assessed with regards to age, first among all adults tested (Figure 6.2 A) and second, with stratification of responses by mid-dry season and mid-wet season (Figure 6.2 B). Given that the adult plasma samples are from independent cross-sectional collections and thus un-paired, serological comparisons with respect to season alone were not performed. Of note, as outlined in Table 6.1, the wet season samples are female biased with a higher mean age. This is reflective of working-age men leaving their places of residence during the rainy, agricultural months – a trend captured in the sampling scheme.

As expected, a positive response to asexual stage parasite antigens AMA1 and $MSP1_{42}$ was present in the majority of samples across all age categories. Relative to the erythrocytic stage antigens, the prevalence of Pfs230C-specific IgG response appeared to increase definitively with age (Figure 6.2 A). Yet when comparing the 18-40 year olds to 41-60 year olds within the same cross-sectional cohort (i.e. within the mid-dry season and mid-wet season sample collections respectively), the trend was no longer consistent (Figure 6.2 B). Given that the randomized cohort from the wet season had a mean age of 45 (IQR 40-58) and the cohort from the dry season had a mean age of 25 (IQR 21-32), this is likely the result of the inherent age bias and diminished sample size after stratification. By contrast, prevalence of responses to HAP2 and Pfs25 show no obvious trends with regards to age (Figure 6.2 A), with some evidence

that 41-60 year olds may have a higher prevalence of HAP2 and Pfs25-specific IgG after stratifying by season (Figure 6.2 B).

Assessing the levels of antigen-specific IgG relative to age categories reiterates that sexual stage antigens elicit a much lower titer response than erythrocytic stage antigens. In this analysis, antibody levels to $MSP1_{42}$ correlated significantly with age (Spearman r=0.594, 95% CI 0.346-0.765, p<0.0001), whereas antibody levels to AMA1 did not (Spearman r=0.302, 95% CI −0.0119-0.561, p=0.0523). There is a subtle increase in Pfs230C-specific IgG levels with increasing age (Figure 6.2 A), but there is no significant correlation in the raw data (Spearman r=0.0271, 95% CI −0.0449-0.538, p=0.0823). The levels of HAP2- and Pfs25-specific IgG responses do not trend with age (HAP2: Spearman r=0.0250, 95% CI −0.336-0.380, p=0.0.892; Pfs25: Spearman r=0.0145, 95% CI −0.299-0.325, p=0.927).

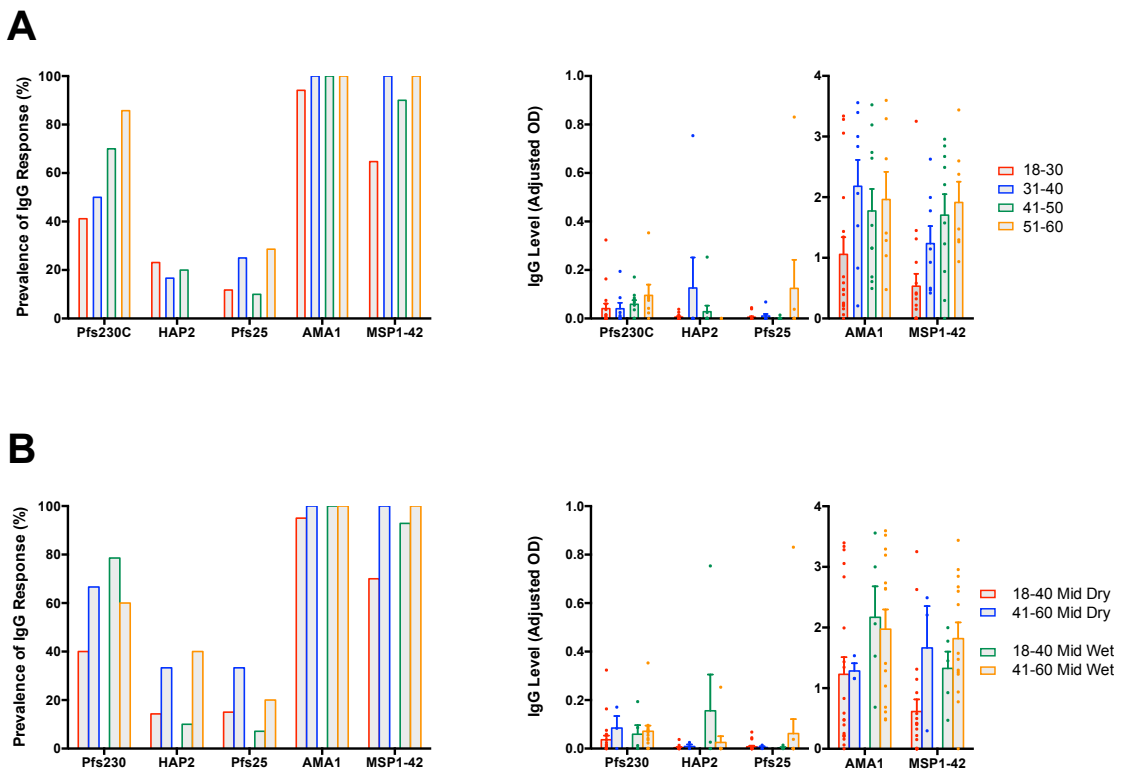


Figure 6.2 Malian adult IgG antibody responses against erythrocytic and sexual stage antigens by age and season

A. Prevalence of positive responders and IgG antibody levels by age group. Raw OD has been converted to adjusted OD, and serves as a measure of IgG level. The number of plasma samples tested per age group (18-30, 31-40, 41-50, 51-60) are 17, 8, 10, 7 for Pfs230C; 13, 6, 10, 3 for HAP2; and 17, 8, 10, 7 for Pfs25, AMA1 and $MSP1_{42}$. **B.** Proportion of positive responders and IgG antibody levels by age group relative to season. Error bars are shown as standard error of the mean (SEM). The number of plasma samples tested per group (18-40 mid-dry season, 41-60 mid-dry season, 18-40 mid-wet season, 41-60 mid-wet season) was 14, 3, 5, 10 for HAP2; and 20, 3, 5, 14 for Pfs230C, Pfs25, AMA1, and $MSP1_{42}$.

6.2.2 Malian adult serology relative to Malian children

The adult plasma samples used above were collected as controls in conjunction with a 4-year study (2008-2011) of malaria incidence in children living in Kenieroba, Mali. The aim for the original sample collection was to enroll all children at the field site into a longitudinal cohort and to correlate clinical episodes of malaria to hemoglobin phenotype and α-thalassemia and G6PD deficiency genotypes [214]. Plasma for immune assays was collected from a sub-cohort (n=394) at the start and at the end of each wet season, May and December respectively. Archived plasma from this cohort study was used for the analysis of immune responses to WGCFS-derived TBV antigens.

To explore whether antibody responses to sexual stage antigens differ between children and adults at the study site, a preliminary set of children's plasma samples was selected. These samples were collected at the end of the 2009 wet season (n=27, median age=6, IQR=4-7) and screened for IgG specific to Pfs230C, HAP2, AMA1 and $MSP1_{42}$. Given that Pfs25 had the lowest population prevalence and lowest average level of IgG response in adults; this antigen was not characterized further. In addition, as it was not possible to compare the effect of season on immune response to TBV antigens in the adult cohort, a paired set of children's plasma was tested from the start and the end of the 2010 wet season (n=24, median age=7, IQR=4.5-8) (Table 6.2).

Table 6.2 Demographic Data: Malian Children Cohorts

Cohort	Sampling		Cohort size, n	Age, median (range, IQR)	Gender, % female	Ethnicity %	
	Year	Season				Bambara	Malinke
Screening	2009	end-wet	27	6 (3-11, 4-7)	37.0	0.04	99.96
Screening	2010	paired	24	7 (3-11, 4.5-8)[a]	54.2	0.08	99.92
Longitudinal	2009,10,11	paired	123	6 (3-11, 4-8) [a]	51.2	1.02	98.98
Functional	2009	end-wet	73	7 (3-11, 4-10)	38.4	0.06	99.94

a. Age taken at start of sampling.

Children had a lower prevalence of Pfs230C-specific IgG response than adults. The prevalence of response to Pfs230C in children at the end of the 2009 wet season was about half that of adults at the middle of the 2009 wet season, 33% (9/27) and 55.2% (16/29) respectively (Figure 6.3). When comparing IgG levels, children and adults have the same adjusted OD in Pfs230C ELISA: 0.039 (SEM=0.016) and 0.042 (SEM=0.014) for children and adults respectively (Figure 6.3). By contrast, the prevalence of HAP2 response in children at the end of the wet season in 2009 was comparable to that of adults at mid-wet season 2009, 18.2% (4/22) and 20.8% (5/24), but the levels of IgG responses in children were effectively negligible (Figure 6.3). This observation reiterates the concern that background variability in the assay may be greater than our set threshold for positive titer.

The effect of season (Figure 6.4 A) and age (Figure 6.4 B) on Pfs230C-specific IgG responses in children were evaluated. There appeared to be an increase in prevalence and antibody level at the end of the 2010 wet season relative to the start (Figure 6.4 A). The presence of anti-Pfs230C IgG appeared to increase with age. By contrast, Pfs230C-specific IgG levels were similar across all age groups, with potentially negligible titers in children aged 1-4 as compared to

older children and adults. Previous serological studies are inconsistent concerning age [259, 261, 270] and season [271, 272] as significant mediators of Pfs230 antibody responses. It was therefore interesting to see whether the trends from our preliminary analysis would be valid in a comprehensive study of longitudinal plasma samples collected from children in Kenieroba.

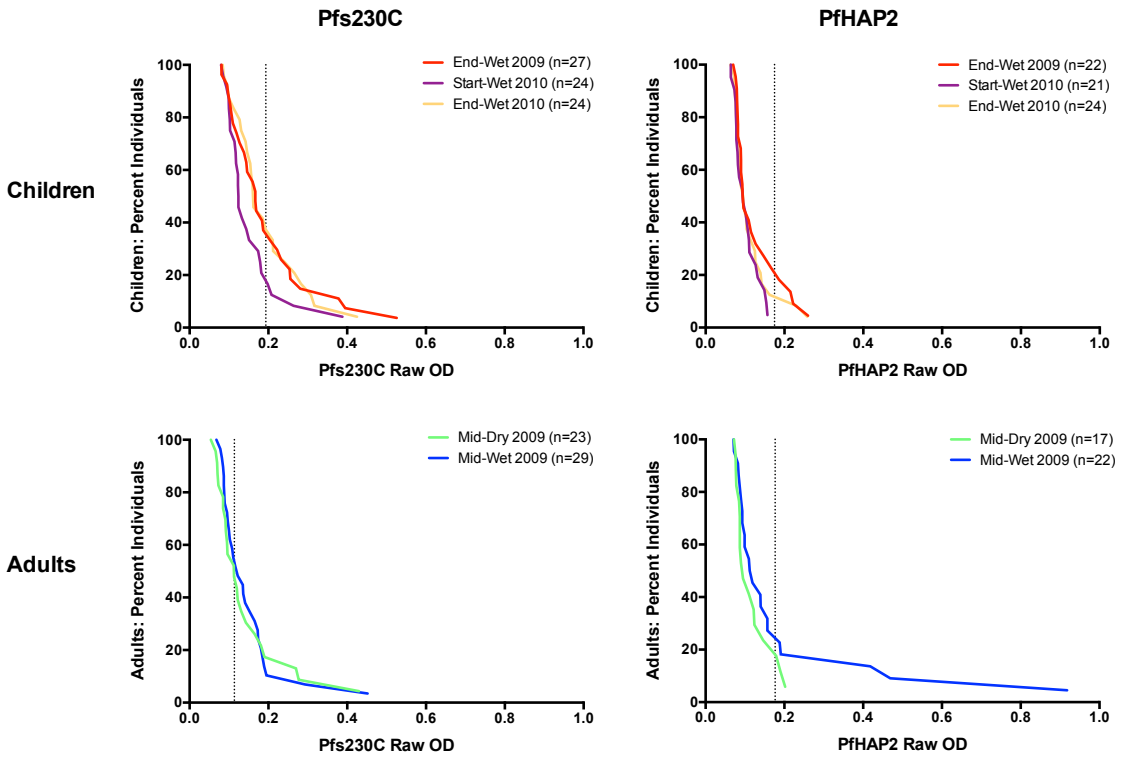


Figure 6.3 Comparison of IgG antibody responses to Pfs230C and HAP2 in Malian child and Malian adult cohorts

Population based schematic of IgG antibody responses to Pfs230C and HAP2, as measured by raw ELISA OD with the plotted cut-off for positive response.

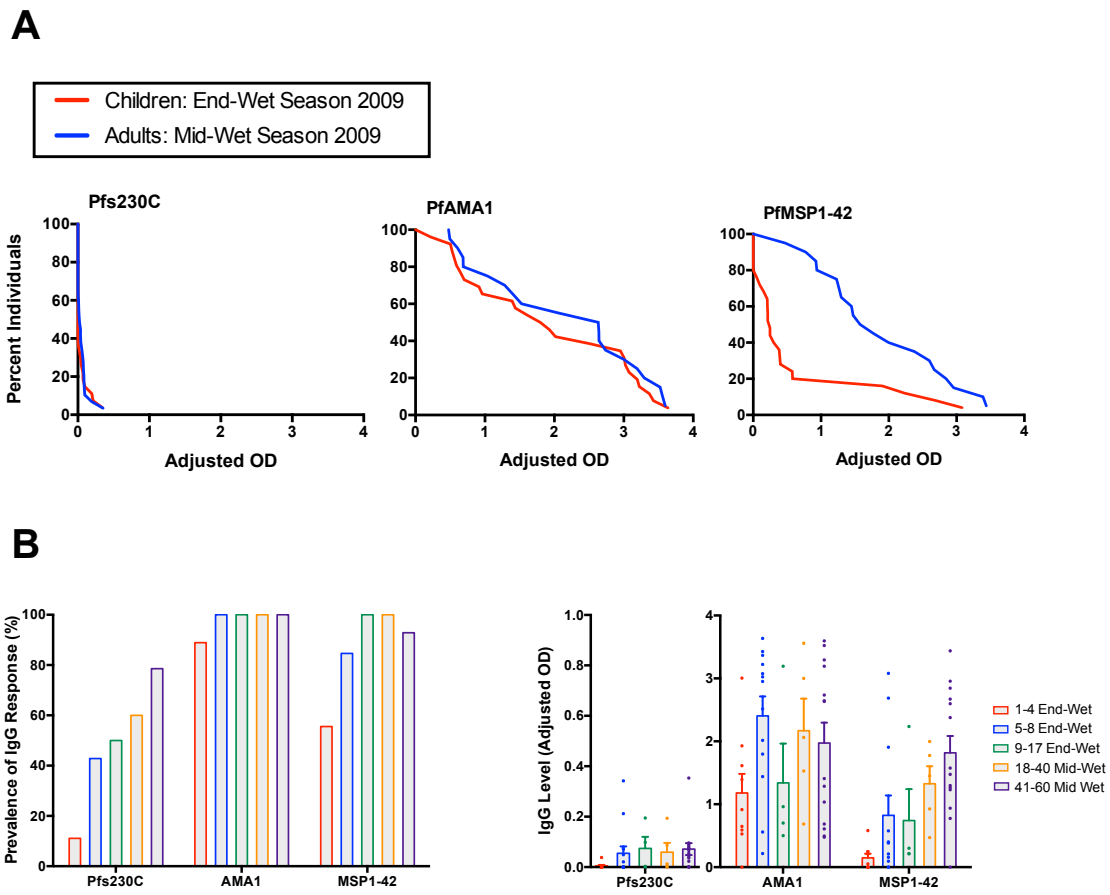


Figure 6.4 Comparison of IgG responses to Pfs230C in Malian children and adults by age and season

A. A population-based comparison of IgG antibody responses, as measured by adjusted ELISA OD, to Pfs230C, AMA1, and MSP1$_{42}$ between children and adults in the 2009 wet season. The number of plasma samples tested (child end-wet 2009, adult mid-wet 2009) are 27, 29 for Pfs230C; 26, 20 for AMA1; and 25, 20 for MSP1$_{42}$. **B.** Prevalence of positive responders and IgG levels by age group during the 2009 wet season. The number of plasma samples tested per group (1-4, 5-8, 9-17, 18-40, 41-60) was 9, 14, 4, 19, 13 for Pfs230C; and 9, 13, 4, 25, 17 for AMA1 and MSP1$_{42}$.

6.2.3 Longitudinal changes in Pfs230C-specific IgG response in Malian children

Given that age-dependent increase in antibody titer is used as a surrogate for cumulative immune memory, the inconsistency of age as a predictor of Pfs230-specific antibodies in previous publications across multiple endemic settings has called into question the longevity of these immune responses. Longitudinal sample sets to assess this phenomenon are limited. From the Kenieroba study described above, samples were available from a longitudinal sub-cohort of 123 children, with stored plasma from the start and end of the wet season for three consecutive years (2009, 2010, and 2011). Pfs230C, AMA1 and $MSP1_{42}$ responses were assessed in this sample set by ELISA. At the start of the 2009 wet season, median age in the cohort was 6 (IQR 3-11) (Table 6.2 Demographic Data: Malian Children Cohorts). AMA1 and $MSP1_{42}$ titers were available from an earlier analysis for 118 out of the 123 children at all six time-points. These samples do not overlap with the preliminary set tested in the previous section.

Given the antibody responses of Malian adults and the small cohort of children from the previous analysis, the comprehensive survey of a longitudinal cohort of children aimed to:

1. Determine the effect of season and age as mediators of Pfs230C IgG response in children, hypothesizing that:

a. Responses will be stronger at the end of each wet season due to malaria exposure.
b. Level of Pfs230C IgG response will correlate to the level of AMA1 and $MSP1_{42}$ IgG.
c. Responses will increase in prevalence and intensity with age.

2. Form a justification and hypotheses for assessing TBA in children at the study site.

6.2.3.1 Effect of season on Pfs230C-specific IgG response in Malian children

In accordance with the few existing reports from comparable transmission settings, no obvious trend was observed in prevalence of the Pfs230C response in children with regards to season. In 2009 and 2010, average overall prevalence was 24.4% (24.4% start wet season, 24.4% end wet season) and 24.0% (25.2% start wet season, 22.8% end wet season) respectively. By contrast, in 2011 overall prevalence was 29.2%, but varied more between the start and the end of the wet season, 17.9% and 40.7% prevalence respectively (Figure 6.5 A). When comparing the level of Pfs230C-specific IgG between seasons it appears that levels increased slightly at the end of the wet season relative to the start for all three years. Resting levels at the start of the wet season were comparable across all three years: an adjusted OD of 0.025 (SEM=0.0064) in 2009, 0.030 (SEM=0.010) in 2010, and 0.037 (SEM=0.0095) in 2011. In parallel to prevalence, the greatest difference in IgG levels occurred between the start and the end of the wet season in 2011.

This result could be interpreted in one of two ways. Since the cohort is longitudinal, in 2011, median age is 8 (IQR 5-13). Potentially there is an effect of increasing immunity in older children, and while responses appear to return to a resting baseline after the dry season, the cohort is better able to boost an immune response upon repeat exposure to Pfs230C. Alternatively, given that we do not have data on the entomological inoculation rate (EIR) – a commonly used measure of transmission intensity, to accompany each sampling time point, it is possible that there were underlying environmental changes in 2011 to explain the differences in prevalence and level of antibody. Of note, the rate of clinical infection (i.e. children presenting to our healthcare center for symptomatic malaria) remained consistent from 2009-2011 (data not shown). When testing for responses to erythrocytic stage antigens AMA1 and $MSP1_{42}$, there was no dramatic annual variation in prevalence of response or specific IgG levels (Figure 6.5 B).

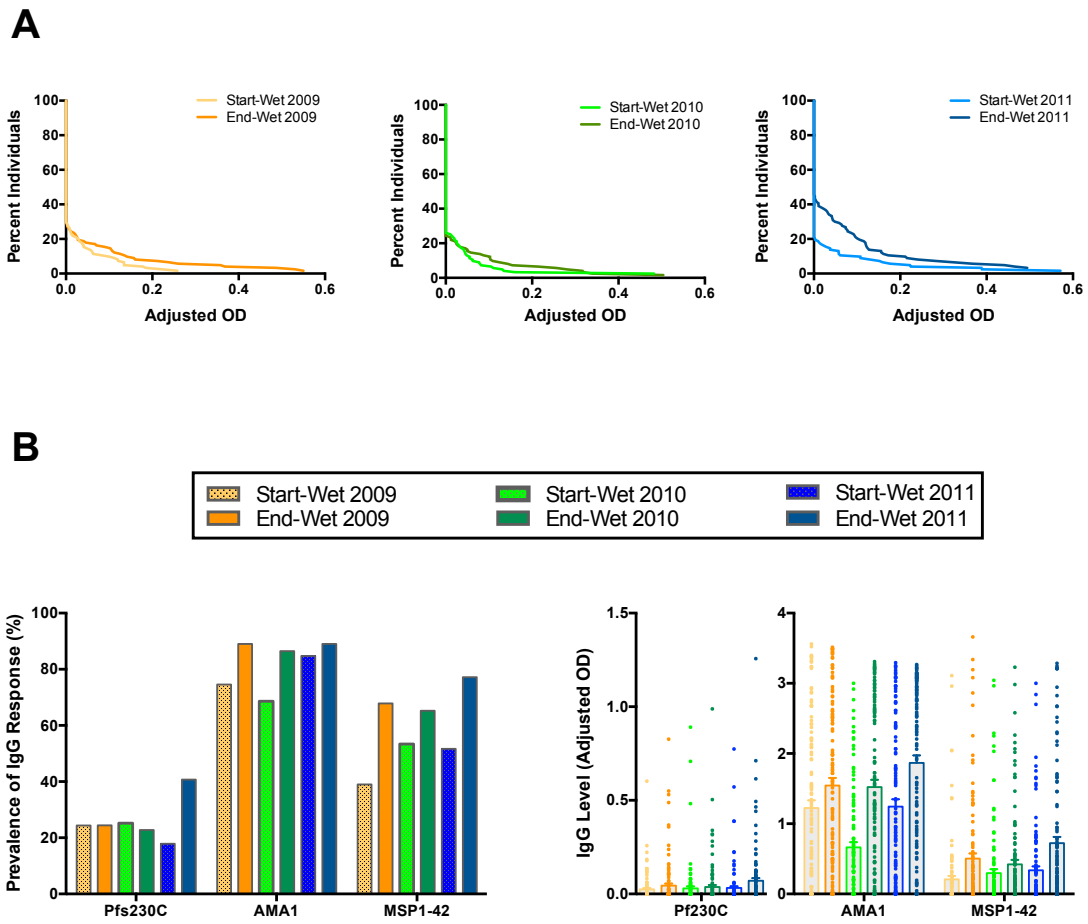


Figure 6.5 Effect of season on longitudinal Pfs230C antibody responses in Malian children

A. Population based representation of IgG antibody responses to Pfs230C, as measured by adjusted ELISA OD, with respect to season. A cohort of 123 Malian children was followed at 6 time points over three years. **B.** IgG antibody prevalence and level specific to Pfs230C, AMA1, and $MSP1_{42}$ for comparison. 118 plasma samples are tested per time-point for AMA1 and $MSP1_{42}$.

6.2.3.2 Correlation of antibody responses between antigens by season in Malian children

Unlike the adult cohort, significant season-specific correlations were observed between the levels of Pfs230C IgG and levels of IgG to erythrocytic stage antigens AMA1 and $MSP1_{42}$. Pfs230C antibody levels correlated with AMA1 levels in three out of our six sample collection time-points, and with $MSP1_{42}$ in four out of our six time-points. Notably there was no positive correlation between Pfs230C-specific IgG levels and either of the erythrocytic stage antigens in 2011 (Figure 6.6).

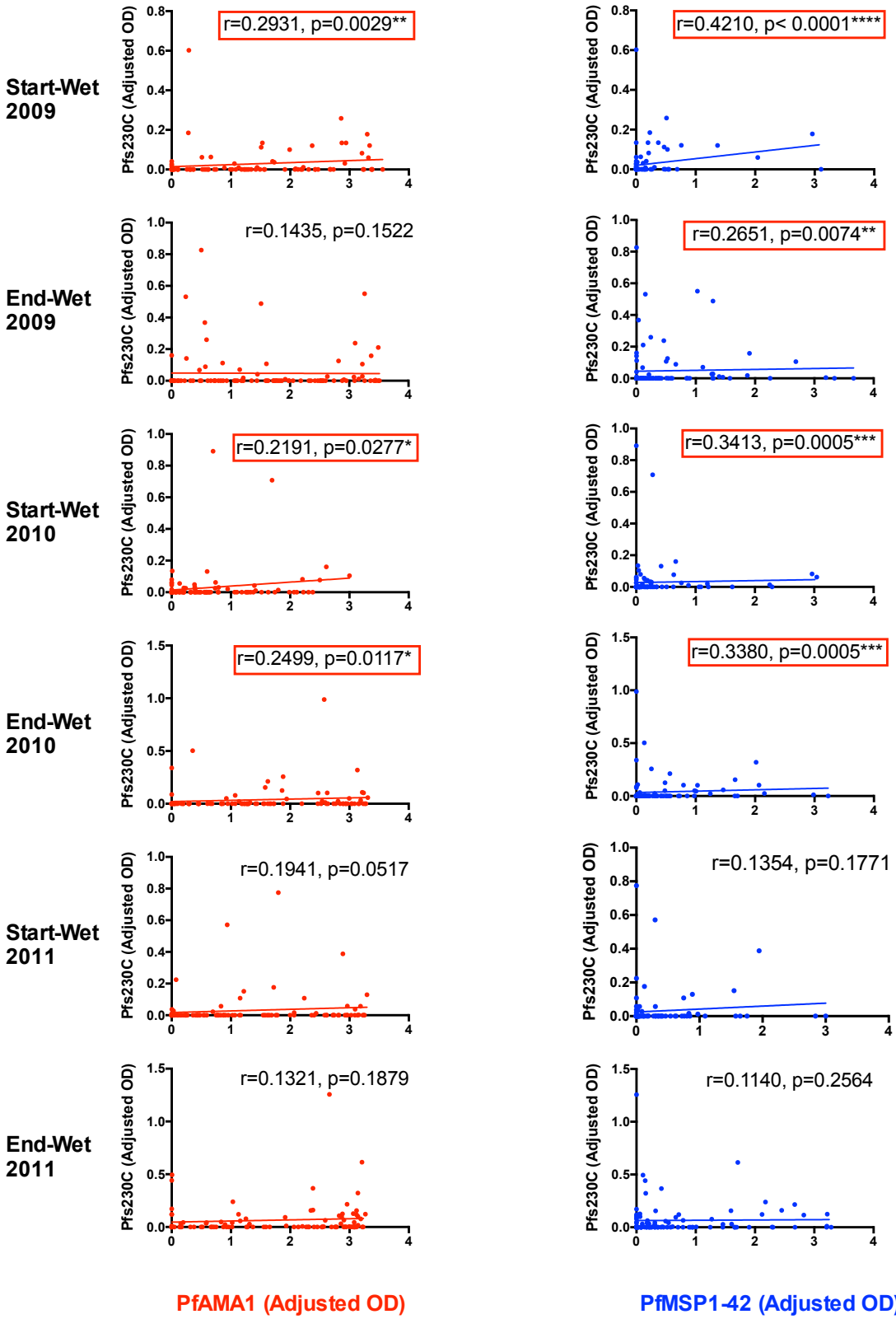


Figure 6.6 Correlation of IgG levels by antigen

Correlation of IgG levels (given as adjusted ELISA OD) to Pfs230C relative to asexual stage antigens (n=118 for each association). Spearman rank coefficient (r) and p value shown.

6.2.3.3 Effect of age on Pfs230C-specific IgG response in Malian children

When considering the total set of children's plasma tested (n=738), in contrast with previously published reports [259, 261, 270], a significant correlation between age and Pfs230C-specific IgG level was also observed (Spearman r=0.253, 95% CI 0.182-0.322, p<0.0001). Less surprisingly, a significant correlation between age and specific IgG level was noted for AMA1 (Spearman r=0.0.258, 95% CI 0.186-0.328, p<0.0001) and $MSP1_{42}$ (Spearman r=0.289, 95% CI 0.218-0.357, p<0.0001). When these responses are grouped by season, there is a significant positive correlation between Pfs230C-specific IgG level and age at the start and end of the 2009 wet season, and at the start of the 2011 wet season only (Figure 6.7). Similarly there is a significant correlation in four out of six sample collection time-points for AMA1 and in two out of six time-points for $MSP1_{42}$ (data not shown). It is unclear whether this is the result of true season-specific variation or the result of underlying variability in the read-out of the assay.

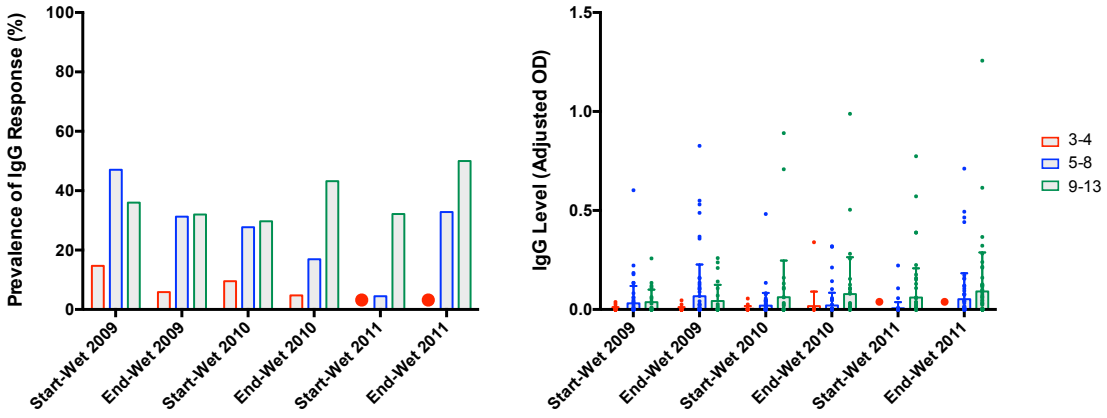


Figure 6.7 Effect of age on longitudinal Pfs230 IgG response in Malian children

Pfs230C IgG antibody prevalence and levels by age group relative to season. The number of plasma samples tested (age 3-4, 5-8, 9-13) are 34, 64, 25 for both time-points in 2009; 21, 65, 37 for 2010; and 0, 67, 56 for 2011. Red icons indicate that n=0 for the particular grouping.

6.2.4 Functional activity of plasma from Malian children

Previous studies have implicated antibody titer to Pfs230 as a correlate of functional transmission-blocking activity in human plasma (The Gambia [259, 260, 273], Tanzania [261, 262], Papua New Guinea [43, 263]). The volume of plasma from the above analyses was insufficient to perform IgG purification for the purpose of functional studies. A subset of children's plasma with sufficient volume was selected from the end of the wet season in 2009 (n=36, median age=6, IQR 4-8). All samples were tested for reactivity to Pfs230C by ELISA. Titers towards AMA1 and $MSP1_{42}$ were available from a previous analysis for the majority of the test population (n=34). All samples were tested for inhibition of the proliferation of erythrocytic stage malaria by growth inhibition assay (GIA) in the reference center within our lab and screened by SMFA for functional TBA.

First, it was confirmed that the test samples for functional analysis have immune responses broadly in accordance to those already seen in the previous analyses (Figure 6.8 A). 8.3% (3/36) plasma samples demonstrated a significant level of inhibition (positive threshold is 60% inhibition relative to control serum based on previous statistical modeling [212]). Given the small sample size, it is difficult to draw conclusions about the effect of age on functional TBA (Figure 6.8 B). Given the limitations of available material, by chance the cohort selected had a lower median age, and perhaps as a consequence, lower anti-Pfs230 IgG. Unsurprisingly, no correlation is observed between titer to Pfs230C and functional TBA of plasma by SMFA (Spearman r=0.007, p=0.969) in this analysis (Figure 6.8 C).

32.5% (25/73) of plasma samples had a positive GIA (positive threshold 40%). A positive trend was observed in the prevalence of positive GIA with age, although the average inhibition is comparable across all age groups (

Figure 6.9). There is a positive correlation between AMA1 (Spearman r=0.416, p=0.0006) and $MSP1_{42}$ (Spearman r=0.319, p=0.010) IgG titer and GIA in this analysis.

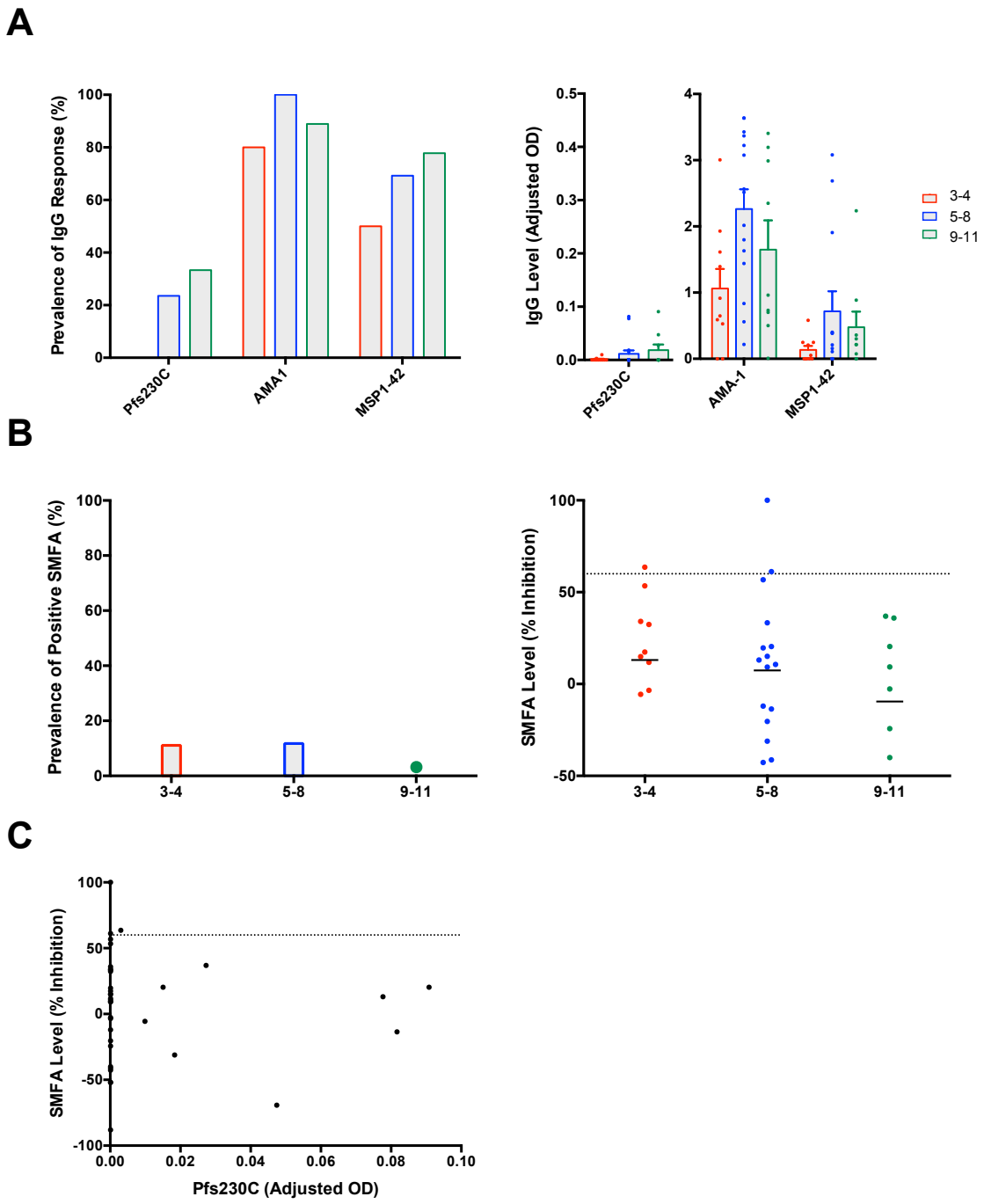


Figure 6.8 Functional SMFA of purified IgG from cohort of Malian children

A. Prevalence of Pfs230C, AMA1 and MSP1$_{42}$–specific IgG response and antibody levels by age. The number of plasma samples tested (age 3-4, 5-8, 9-11) was 10, 17, 9 for Pfs230C and 10, 15, 9 for AMA1 and MSP1$_{42}$. **B.** SMFA responses relative to age by prevalence of positive TBA and % inhibition (total n=36). Cut-off for positive TBA set at 60%. **(C)** Correlation between Pfs230C IgG level and SMFA response (total n=27).

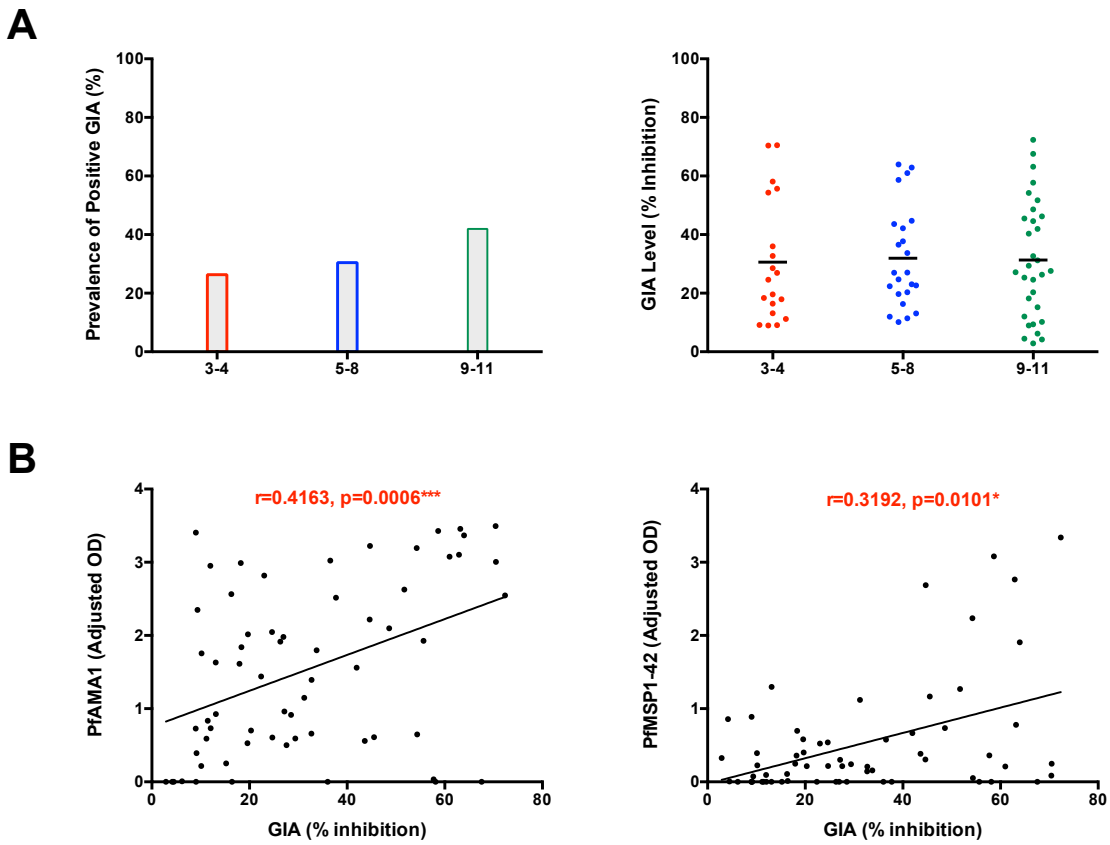


Figure 6.9 Functional GIA of purified IgG from a cohort of Malian children

A. GIA responses relative to age by prevalence of positive growth inhibitory activity and % inhibition (total n=73). **(B)** Correlation between AMA1 and $MSP1_{42}$ IgG level and GIA response (total n=64). Spearman rank coefficient (r) and p-value are shown.

6.2.5 Entomology

Tracking a proxy measure of *P. falciparum* transmission over time is important to establishing whether changing environmental conditions are confounding serological measurements in a study population. Unfortunately, careful analysis was not available for the study site at the sample collection time-points described above. An earlier analysis carried out by Dr. Nafomon Sogoba at the University of Bamako, approximated exposure to *P. falciparum* infected mosquitoes at the field site in Kenieroba, Mali (personal communication) between 2007 and 2009. The study captured mosquitoes from the same set of homes on a monthly basis. The baseline level of mosquito exposure and mosquito infection remained broadly consistent over the three years of this study. On average, each home had a mean density of 1.7 mosquitoes (SEM=0.55) in the dry season (January-June) and 6.1 mosquitoes (SEM=0.20) in the wet season (May-December). Harvesting of the head-thorax portion of the insects with subsequent ELISA-based analysis to assess the prevalence of circumsporozoite protein (CSP) established that 2.3% of mosquitoes (SEM=0.40) were positive for CSP in the dry seasons and 2.4% of mosquitoes (SEM=0.73) were positive in the wet seasons.

6.3 Discussion

Plasma samples collected in Kenieroba, Mali were screened for (1) the acquisition of immune responses to known *P. falciparum* TBV candidate antigens and (2) the functional capacity to block transmission by SMFA. Pfs230C responses were detected in both children and adults in the samples tested and subsequently examined more thoroughly relative to season and age in a longitudinal cohort of Malian children. The findings suggest a low prevalence of antibodies with TBA in children, and TBI did not associate with Pfs230C-specific antibodies in the current sample set, possibly due to the limited number of observations.

There are few existing studies on seasonality, an indicator of recent malaria exposure, as a mediator of immune response to sexual stage antigens [271, 272]. In one report from Burkina Faso, antibody prevalence to Pfs230 is highest at the peak of malaria transmission during the wet season, relative to the start and end of the rains when prevalence is comparable [272]. This finding may indicate that recent exposure to parasite antigens in the short-term has a greater effect on sexual stage-specific immune response than cumulative immune memory from repeated infections. The observation that migrants demonstrate a rapid immune recognition of Pfs48/45 and Pfs230 shortly after relocating to an area where malaria is endemic has been used to lend merit to this claim and poses concerns about how rapidly these responses may decline [263]. Yet these associations have not been fully characterized in longitudinal studies; in the longitudinal cohort assessed here, there wasn't a strong effect on Pfs230C IgG prevalence or antibody level with regards to season. Notably, it

was not possible to relate these responses to recent gametocyte exposure and level of gametocytemia, as these data are not available for the cohort described.

Age-dependent increase of antigen-specific immune responses is used as a correlate of cumulative immune memory, likely the result of repeated infections. The lack of a positive correlation between age and sexual stage specific antibodies in previous studies [259, 261, 270] has also implied that the responses may be short-lived or require frequent boosting. However, age may be a relevant factor to acquiring or maintaining sexual stage specific immunity; in this study, despite similar overall antibody levels, a difference in prevalence of Pfs230C-specific IgG was observed between children and adults, 24.4% at the end of the wet season 2009 and 55.2% at the middle of the wet season 2009 respectively. Given the seasonal findings from Burkina Faso [272], the differences in prevalence may be exaggerated by having sampled adults at the mid-wet season. However within the larger sample size of our full cohort of children, when analyzing all samples tested irrespective of season, there is a strong association between age and level of Pfs230C antibodies. Consequently, it may be relevant to assess age as a modulator of immune response to the gametocyte, in particular relative to recent exposure, in future analyses.

The majority of studies on naturally acquired immune responses to sexual stage antigens are structured with a cross-sectional study design [42-44, 259, 260, 264, 274-276] making it difficult to assess factors that may influence longevity. Very few published studies have quantified sexual stage immune responses in

a longitudinal manner. These reports suggest that sexual stage antibody responses and functional antibodies that have TBA can be acquired rapidly [263] and may be more prevalent in children than adults [259, 261, 270]. In the analyses reported here, it appears that very few children in Kenieroba have TBI, and these responses do not associate with Pfs230C antibody levels for our sample set. (Of note, the Pfs230C-specific IgG levels seen in this study are broadly in accordance with previously published data [262]). Given the small sample size, we are unable to correlate these responses with additional factors such as age.

A shortcoming of this analysis is the inability to relate sexual stage immune responses to recent gametocyte exposure, and thus interpret the findings relative to the level of transmission at the study site, reported as EIR. This makes it difficult to adequately assess the effect of age and seasonality on antigen-specific responses and functional capacity. Chapter 7 complements the current observations with a 1-year cohort study in Kenieroba, Mali that addressed these points directly; the study enrolled 500 individuals, including children and adults, and longitudinally quantified both asexual parasite and gametocyte densities. Serum collected in the middle of the 2013 wet season and the 2014 dry season was characterized for IgG responses to the panel of sexual stage antigens described in Chapter 4 and 5 with the aim of identifying markers of gametocyte infection.

Chapter 7: Screening a Panel of Sexual Stage Antigens for markers of Gametocyte Exposure

7.1 Introduction

About 2.4 billion people are estimated to be at risk of *P. falciparum* transmission [277], but it remains unclear which individuals constitute the primary gametocyte reservoir in a given human population. Existing gametocyte prevalence data rely heavily on microscopic analysis of blood smears wherein gametocytes are often present at levels below the limit of detection [278-280]. Indeed, individuals found to be gametocyte-negative by microscopy can nonetheless infect mosquitoes in functional transmission assays [281]. Within the last decade, more sensitive molecular methods [280, 282, 283] have identified individuals with sub-microscopic gametocytemia [284-286] and suggested that these carriers contribute significantly to overall transmission [287-289]. Further studies applying these methods to field settings are urgently needed to accurately measure gametocyte prevalence.

During blood stage infection of humans, a small fraction of asexual parasites commit to gametocytogenesis. Gametocytes typically represent <5% of detectable parasites in peripheral blood smears [279] and are identified by microscopy in <50% of infections regardless of endemic setting [41]. To the contrary, molecular methods have spurred speculation that most infections will

produce gametocytes but at very low levels [41]. The correlation of asexual parasitemia to gametocytemia in humans remains to be fully defined [290]. In part, this interplay depends on host factors; for example, immune recognition of circulating gametocytes may be limited by their low density in the peripheral blood [31, 279, 291]. On the other hand, the prevalence and density of asexual parasites decline with age – a surrogate for naturally acquired immunity in high transmission settings. In low transmission settings, the prevalence of asexual parasitemia is more evenly distributed among age groups [292]. The prevalence of gametocytemia largely follows that of asexual parasitemia by microscopy [285, 293] and molecular methods [286, 294]; however, this trend may be influenced by more than immunity to asexual parasites alone [252]. As delineated in Section 6.1.1, epidemiological data suggest that immunity may develop faster to gametocytes than to asexual parasites [295], and one serological study reported that gametocyte-specific antigens on the surface of infected erythrocytes may be targeted by human immune responses [31].

In addition to immunity, host genetic factors may also influence the dynamics of parasitemia. Sickle hemoglobin S (HbS), for example, has been associated with increased gametocyte prevalence [256]. Another report suggests that HbS and HbC enhance transmission of gametocytes from humans to mosquitoes [257]. Determining how host factors relate to gametocyte carriage is critical to illuminating which hosts drive transmission. While in many settings children bear the greatest burden of malaria with the highest asexual parasite densities, they may not be the majority contributor to the human infectious reservoir. One study suggests that adults are responsible for 28-38% of mosquito infections

even when accounting for their greater body size and larger area of exposure to mosquitoes [293].

Along with host factors, the seasonality and intensity of transmission impact gametocyte prevalence. At present, the majority of existing *P. falciparum* epidemiology is based on the study of asexual parasites [41]. Another barrier to defining the human transmission reservoir comes from relatively limited data on gametocyte dynamics in "healthy" individuals; i.e., the majority of epidemiological data follows people who had a malaria episode [296, 297]. Consequently, the true effects of transmission seasonality and intensity on gametocyte prevalence remain to be fully defined. In high transmission settings, most infections are asymptomatic. Specifically in regions with seasonal endemicity, malaria episodes are very uncommon during the dry months as mosquitoes become less abundant, but asymptomatic infections can persist [298]. Asymptomatic parasite carriers are known to produce gametocytes [285, 293, 294].

While the duration of gametocytemia remains controversial, presumably such infections are in part responsible for re-initiation of transmission after the long dry season. Despite the number of gametocyte carriers being lower in the dry season, cross-sectional surveys suggest that the relatively few persisting asexual parasite infections may have a higher likelihood of producing gametocytes at a higher density [252-254]. A longitudinal, microscopy-based study in Burkina Faso examined gametocyte density and prevalence relative to two divergent environmental transmission patterns. In both settings, the

analysis showed increased gametocyte prevalence when asexual prevalence became low, as well as a stable rate of gametocyte production in the dry season [255]. These associations remain to be confirmed with longitudinal studies based on more sensitive molecular methods.

Recent technical advances in molecular detection of gametocytes have set the stage for a better understanding of gametocyte epidemiology and biology in humans. These methods rely on detecting gametocyte-specific mRNA, such as quantitative real-time polymerase chain reaction (qRT-PCR) of Pfs25, a late gametocyte-specific transcript [280, 299]. Other methods of quantification, such as nucleic acid sequence-based amplification with electrochemical luminescence (QT-NASBA) [283] and reverse transcription loop-mediated isothermal amplification (RT-LAMP) [282] are designed for convenience in field-based assays [300]. While QT-NASBA primers exist for Pfs16 mRNA, which is the earliest known transcript specific for immature gametocytes, the assay has been used in studies on gametocyte development but has never been applied in a field setting [283]. Notably, there has not been a study to longitudinally examine the relative contributions of parasite stages (asexual, immature gametocyte, mature gametocyte) to the circulating parasite pool within the same host using current molecular approaches. In this context it is important to note that immature gametocytes do not circulate in the peripheral blood, as determined by microscopy. However, detailed studies of molecular markers to detect such cells have not been done.

As part of this thesis work, a new, year-long epidemiology protocol was developed to take place in Mali beginning in the wet-season of 2013 and concluding in the dry-season of 2014. The primary aim was to explore longitudinal variation in gametocyte prevalence, taking into account age, asexual parasite prevalence, season, and red blood cell (RBC) polymorphisms. Serum samples from this cohort analysis, collected in the middle of the 2013 dry season and the 2014 wet season, were used to assess IgG responses to the panel of sexual stage antigens expressed as recombinant proteins in the HEK293E system in Chapter 4. The aim of Chapter 7 is to determine whether IgG responses to any of the antigens generated are associated with gametocyte-positive infections.

7.2 Results

7.2.1 Study design and sample collection

As in Chapter 6, sample collection was conducted in Mali, approximately 75 km southwest of Bamako, where malaria transmission is highly endemic and seasonal [214]. Gametocyte prevalence has never been measured in the population prior to the initiation of this study. To track gametocyte prevalence in a longitudinal, mixed-age cohort of Kenieroba residents, 500 individuals, aged 6 months to 65 years, were enrolled in proportion to the age distribution of the overall village. When possible, entire families living in single housing units were consented for participation in the study.

Over 12 months, a finger-prick blood sample was obtained from each participant to make a filter paper blood spot for DNA isolation and store 100 µL in RNAProtect™ (Qiagen, USA) for RNA preparation. Patient DNA and RNA samples were tested after completion of the study. To detect parasitemia, total DNA was extracted from filter paper blood spots and tested for the presence of parasite DNA by 18S PCR. In parallel, a subset of cross-sectional time-points was selected for detection of gametocyte stage-specific RNA by qRT-PCR based on the presence of Pfs25 mRNA. Dr. Yaw Adomako-Ankomah and Matthew Chenoweth (NIH, USA) are performing parasite quantification; quantification for all time-points is ongoing.

In addition, for the purpose of serological studies, a venipuncture of up to 10 mL of blood (up to 4 mL of blood if aged 6 months to 2 years) was collected from all participants with Hb level ≥8.5 g/dl in April 2013 (dry season), October 2013 (wet season), and March 2014 (dry season). The April 2013 blood sample, corresponding to enrollment, was used to determine Hb type (by HPLC), ABO/Rh blood group (by agglutination assay), and G6PD/α-globin genotypes (by PCR amplification of DNA extracted from filter paper blood spots), which are not relevant for the analysis below.

7.2.2 IgG responses to sexual stage antigens in Malian individuals

Chapter 4 described the expression and purification of a panel of sexual stage antigens in the HEK293E cell system for functional evaluation by SMFA. Of 12 proteins in the panel, 10 were selected for seroreactivity studies in Malian volunteers. Pfs47 and SOAP were excluded, but may be considered in future

analyses pending optimization of expression of these candidates. Notably, in a serological screen of 32 adults from Burkina Faso, median IgG response to Pfs47 and SOAP ranked lowest out of the 12 sexual stage antigens tested (Figure 5.10 A).

Initially 16 volunteers were selected at random from the cohort to evaluate antigen seroreactivity; paired sera from the October 2013 mid-wet season and March 2014 mid-dry season time points were tested alongside sera from 10 adult US volunteers naïve to malaria infection. All sera were diluted by 1:100 for ELISA screening; antigen-specific human IgG was detected with goat anti-human antibody conjugated to alkaline phosphatase enzyme. Pfs25, a post-fertilization antigen expressed primarily during infection of the mosquito vector, was included as a negative control; Pfs25 was also expressed in HEK293E cells (Chapter 4).

For each antigen, raw optical density at an absorbance wavelength of 405 (OD_{405}) was recorded for Malian and US volunteer sera. After a development time of 20 minutes, HSP70-2, PSOP9, PDI-8, enolase, and PSOP13 were the five antigens with highest prevalence of positive IgG responses (71.9, 62.5 50.0, 43.8, and 40.6%, respectively) relative to a cut-off defined as at least 0.01 above three standard deviations plus the mean OD_{405} of the US naïve serum (n=10) (Table 7.1, Figure 7.1). IgG responses to Pfs25 had the lowest prevalence of positivity (18.8%). All samples demonstrating positive detection of Pfs25 also recognized at least 8 of the remaining 10 antigens suggesting that these responses may be due to cross-reactivity with a common epitope sequence (such as the biotin acceptor peptide and hexahistidine tag present in

all proteins in the panel) or a co-purified component in the HEK293E preparations.

Table 7.1 Prevalence of positive IgG response among Malian cohort (n=16, paired)

Antigen	Prevalence of Positive IgG Response (%)*
PfHSP70-2	71.9 (23 of 32)
PSOP9	62.5 (20 of 32)
PDI-8	50.0 (16 of 32)
Enolase	43.8 (14 of 32)
PSOP13	40.6 (13 of 32)
WARP	37.5 (12 of 32)
ICP	34.4 (11 of 32)
HYP2	28.1 (9 of 32)
HYP1	25.0 (8 of 32)
PDI-11	21.9 (7 of 32)
Pfs25	18.8 (6 of 32)

*Development time 20 minutes

7.2.3 Optimization of ELISA

In Chapter 6, IgG specific to sexual stage antigens were observed at low levels in Malian cohort studies also based in Kenieroba. As a result, in the present work, OD_{405} was measured in a development time-course at 20, 30, 40, 50, and 60 minutes to identify the point of maximal separation between OD_{405} corresponding to Malian and US sera (Figure 7.1). While using a background cut-off of three standard deviations above the mean of malaria naïve IgG response is a common convention for the analysis of serology data [269], arithmetic mean is a more helpful metric for data with a normal distribution. Moreover, the arithmetic mean is more sensitive to outlier data points. For

example, one US naïve volunteer with uncharacteristically high response to PSOP13 is responsible for a high background cut-off value for this antigen (Figure 7.1).

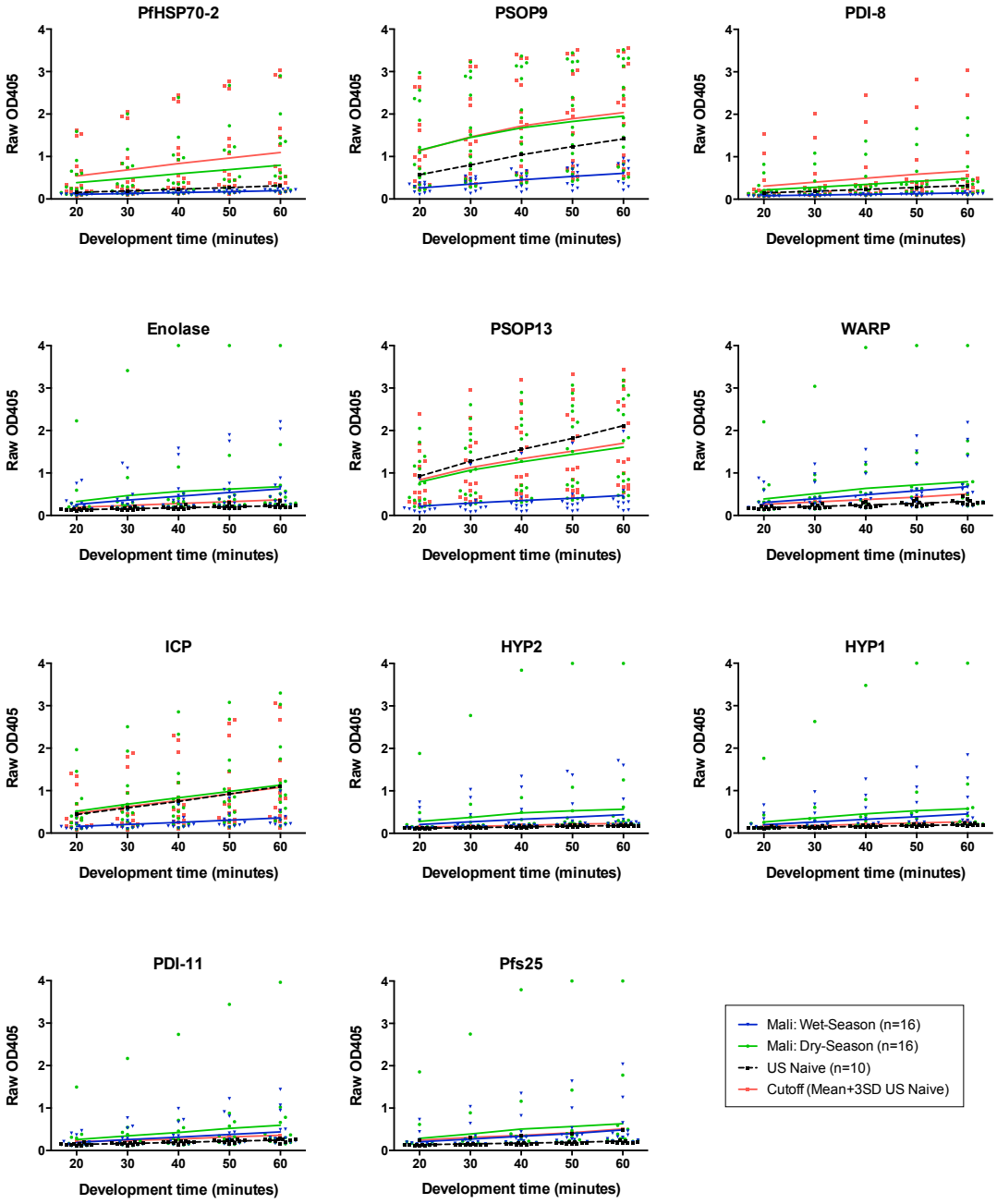


Figure 7.1 Development time-course of sexual stage antigen ELISA

IgG antibody levels, as measured by ELISA OD_{405}, to a panel of sexual stage antigens in an alkaline-phosphatase development time-course of 20, 30, 40, 50, and 60 minutes. Paired sera collected in the wet and dry season from Malian volunteers (n=16) were tested alongside US malaria-naïve adults (n=10). Cut-off values were set at three standard deviations above the mean OD of US naïve serum (n=10) run on the same ELISA plate for each antigen.

To consider the data another way, the cut-off was redefined as three standard deviations above the median IgG response of US volunteers. The median IgG level of Malian sera (as measured by OD_{405}) was calculated as a ratio of this cut-off and plotted for each time point (20, 30, 40, 50, and 60 minutes) with the aim of determining the optimal development time for the assay. Longer development times did not correspond to improved separation between Malian and US OD_{405} values, so an intermediate time-point of 40 minutes was selected for subsequent experiments.

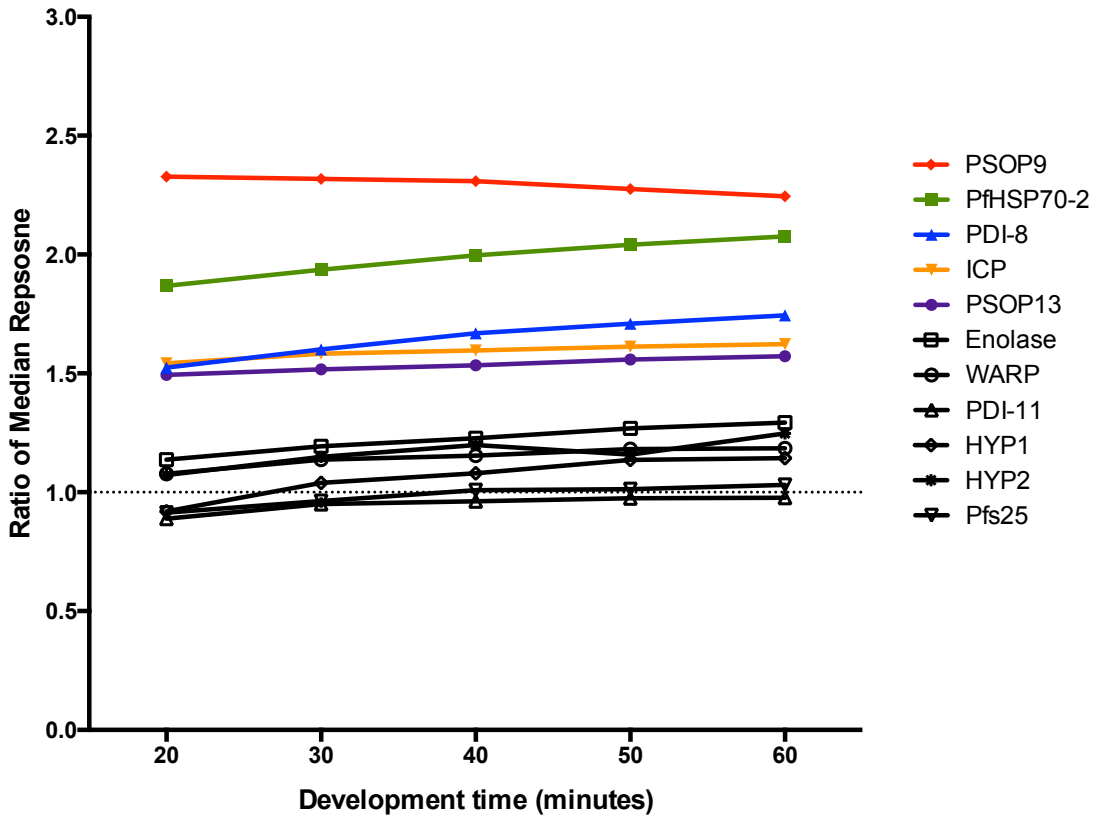


Figure 7.2 Time-course depicting ratio of median IgG response to antigen panel

IgG levels (OD_{405}) specific for a panel of sexual stage *P. falciparum* antigens were measured in paired sera collected in the wet and dry season from Malian volunteers (n=16) and US malaria-naïve adults (n=10). A cut-off value was calculated as three standard deviations plus the median response of US adults to each antigen in the panel. The ratio of median response to this cut-off value was calculated for each antigen at a development time of 20, 30, 40, 50, and 60 minutes (raw data portrayed in Figure 7.1).

7.2.4 Down-selections of antigens for broader serological screening

The primary aim of the study is to identify markers of gametocyte carriage. Based on the findings of Chapter 4, pooled anti-serum (n=5 BALB/c mice) generated to a proportion of proteins in the sexual stage panel recognized fixed and permeabilized *P. falciparum* NF54 strain gametocytes by indirect fluorescence assay (IFA) (Table 7.2). Anti-enolase, -HYP1, and -HYP2 sera did not recognize gametocytes by IFA and consequently are not candidates to pursue under the main aim of identifying potential markers of gametocyte positivity. Favoring antigens with moderate overall seroreactivity as defined by the ratio of median IgG responses relative to the cut-off, PSOP9, HSP70-2, PDI-8, ICP, and PSOP13 were selected for broader serological screening (Figure 7.2).

Table 7.2 Antigen expression by IFA

Antigen	Gametocyte IFA	Gamete IFA
PSOP9	+	
PfHSP70-2	+	+
ICP	+	
PDI-8	+	+
PSOP13	+	+
Enolase		+
WARP	+	
HYP2		
HYP1		+
Pfs25		
PDI-11	+	

7.2.5 Positive IgG titer correlates to gametocyte positivity in a subset of antigens

In an effort to identify gametocyte serological markers, IgG responses to the sexual stage antigen panel were compared between individuals with gametocyte positivity and those without at the time of sample collection (Figure 7.3). Malian individuals (n=126, median age=10, IQR 6-17) were screened for IgG specific to PSOP13, HSP70-2, PDI-8, ICP, and PSOP9. ELISA was used to measure the IgG level (OD_{405}) in paired sera corresponding to the 2013 mid-wet season and 2014 mid-dry season time-points. All samples were also screened for IgG responses to a peptide encoding the biotin acceptor sequence and hexahistidine tag – coated at the same concentration as test proteins (1µg/mL). Anti-peptide OD_{405} readings were treated as background and subtracted from antigen-specific OD_{405} prior to data analysis.

Pfs25 qRT-PCR was used to determine gametocyte positivity in archived RNA corresponding to the 2013 mid-wet season and 2014 mid-dry season serum samples. Initially, paired samples were analyzed in aggregate, without consideration for whether collection was performed in the wet or dry season. IgG levels were significantly higher in gametocyte positive samples than gametocyte negative samples for PSOP13 ($p< 0.0001$), PfHSP70-2 ($p=0.0006$), and ICP ($p=0.0004$) by Mann-Whitney test; the difference in IgG level for PDI-8 ($p=0.2024$) and PSOP9 ($p=0.5785$) was not significant.

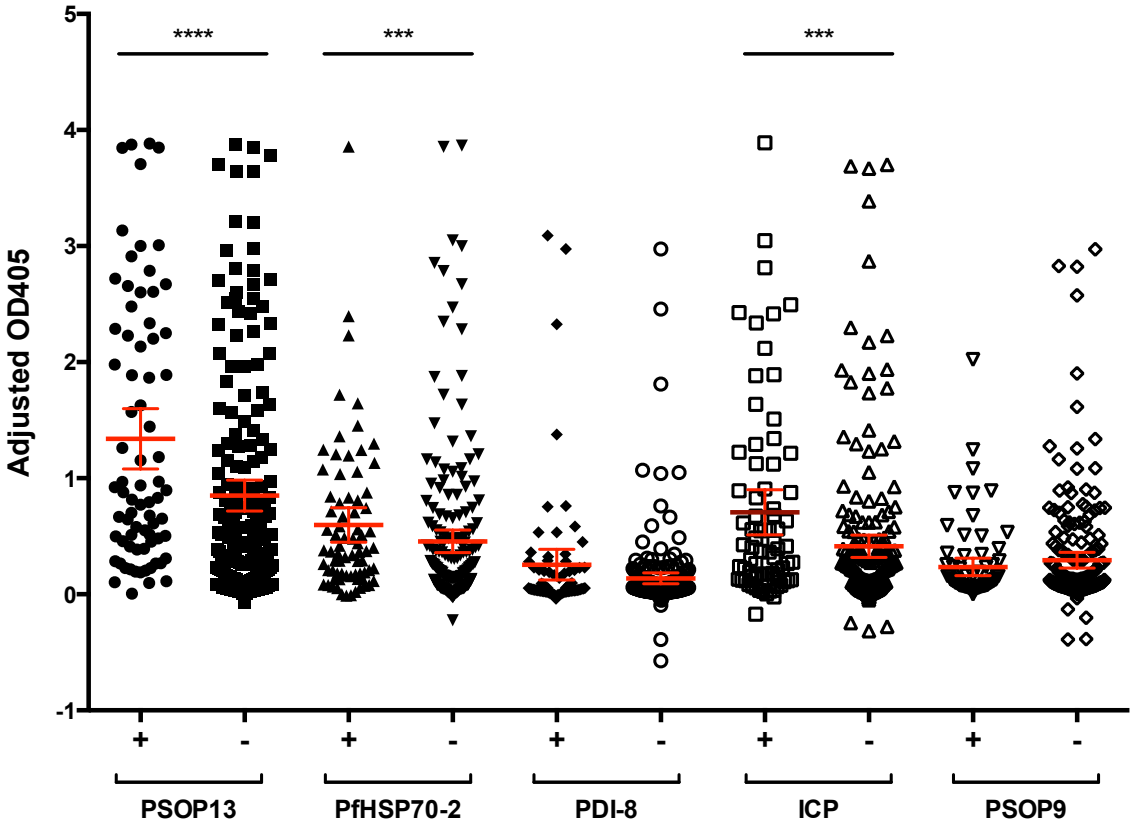


Figure 7.3 Serological response relative to gametocyte positivity

IgG levels specific for a panel of sexual stage *P. falciparum* antigens were measured in paired sera collected in the wet and dry season from Malian volunteers (n=126) – corresponding to 252 total samples per antigen. Adjusted OD_{405} was calculated by subtracting background responses to a peptide encoding the biotin acceptor sequence and hexahistidine tag. Mean response with 95% CI is depicted in red for samples separated by gametocyte positivity (+) and gametocyte negativity (-) at the time of serum collection. Groups were compared by Mann-Whitney.

Similarly, wet season samples divided into gametocyte positive and gametocyte negative groups demonstrated a statistically significant difference in IgG levels specific for PSOP13 (p=0.0002), HSP70-2 (p=0.0002), and ICP (p=0.0005) by Mann-Whitney (Figure 7.4). Again, PDI-8 (p=0.1889) and PSOP9 (p=0.3703) IgG levels were not significantly different relative to gametocyte positivity as with all samples analyzed without consideration for season. By contrast, among dry season samples, the differences relative to gametocyte carriage were less pronounced; still, anti-PSOP13 (p=0.0196) and anti-ICP (p=0.0366) IgG levels were significantly higher among gametocyte positive samples. HSP70-2 (p=0.3120), PDI-8 (p=0.9962), and PSOP9 (p=0.3485) IgG responses were comparable between gametocyte positive and gametocyte negative groups.

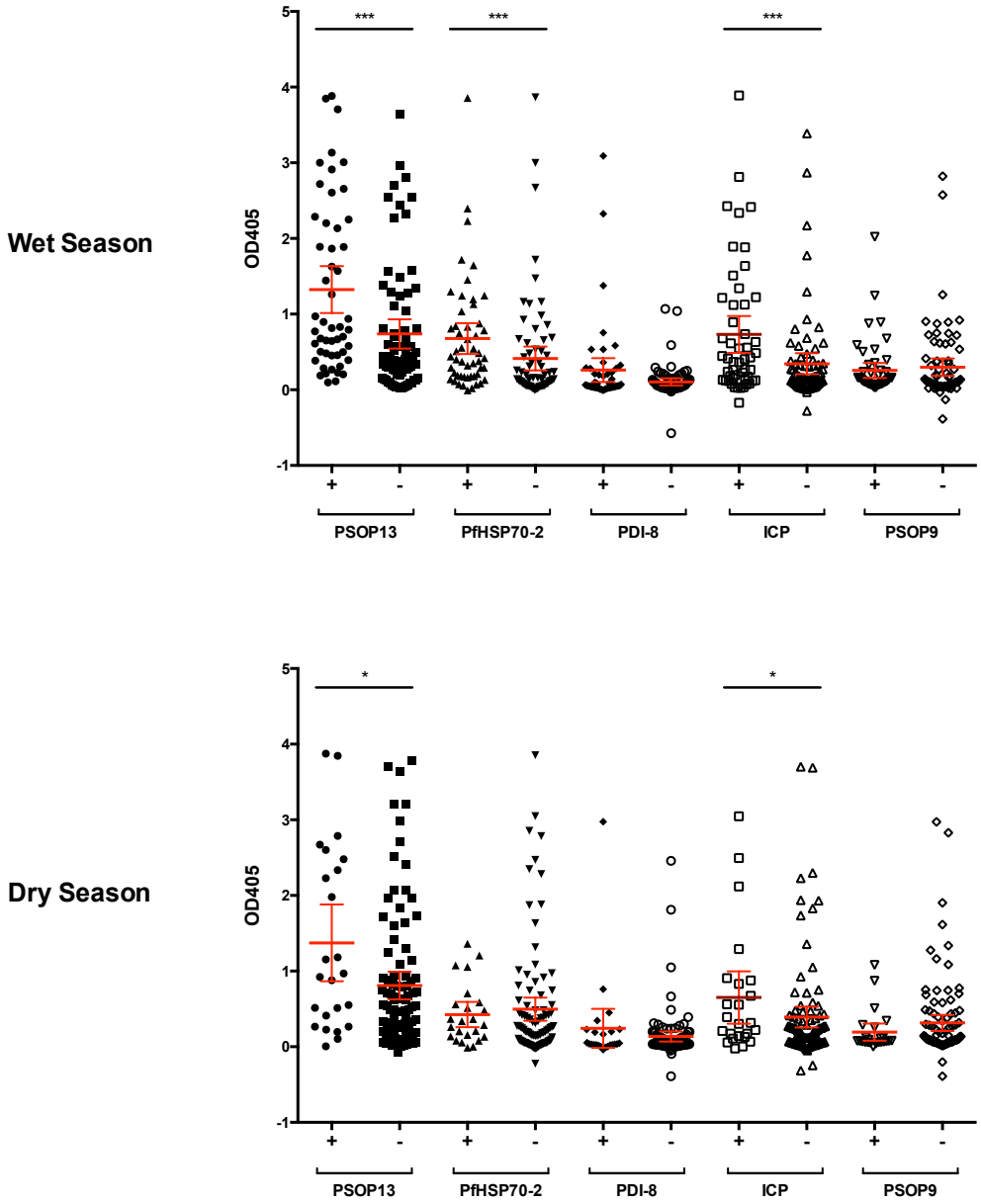


Figure 7.4 Serological response relative to gametocyte positivity by season

IgG levels specific for a panel of sexual stage *P. falciparum* antigens were measured in paired sera collected in the wet and dry season from Malian volunteers (n=126) – corresponding to 252 total samples per antigen. Adjusted OD_{405} was calculated by subtracting background responses to a peptide encoding the biotin acceptor sequence and hexahistidine tag. Mean response with 95% CI is depicted in red for samples separated by gametocyte positivity (+) and gametocyte negativity (-) at the time of serum collection. Groups were compared by Mann-Whitney.

While the antigens of interest – PSOP13, HSP70-2, and ICP – are expressed in the gametocyte stage based on IFA data (Figure 4.8), it is important to consider whether these proteins have also have expression in the asexual stage. Asexual parasites and gametocytes are typically present at the same time, and therefore asexual stage expression could also explain the differential effect. A *P. falciparum* proteomics analysis reports peptides specific to HSP70-2 and PDI-8 in preparations of asexual blood stage trophozoites and schizonts (Table 7.3) [122]. Further to this, a literature review suggests that ICP has expression in the asexual blood stage [240]. We confirm these findings, in part by, IFA to asexual blood stage parasites. HSP70-2, PDI-8, and PSOP9 (GAMA) are weakly positive.

Table 7.3 *P. falciparum* stage-specific expression

			Number of Peptides			
Name	**Gene ID**	**Mr**	**Troph/ Schiz.**	**Gametocytes**	**Gametes**	**Total**
PSOP13	PF3D7_0518800			No Data		
HSP70-2	PF3D7_0917900	72457	151	122	135	408
PDI-8	PF3D7_0827900	55808	76	55	80	211
ICP	PF3D7_0911900	47214		1	2	3
PSOP9	PF3D7_0828800	85710		2	1	3

Data obtained from [122].

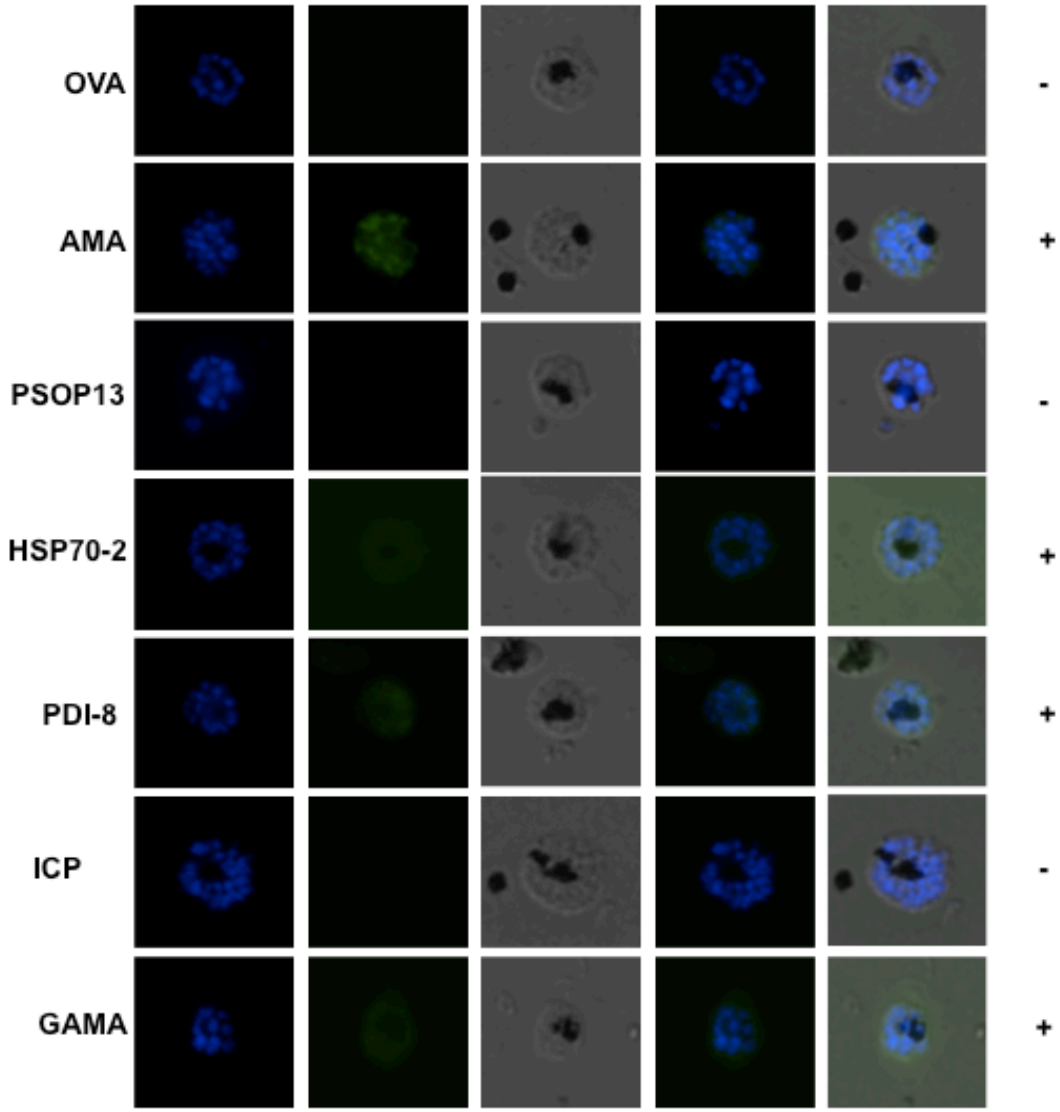


Figure 7.5 Protein expression in asexual stage schizonts by IFA

Pooled day 70 anti-sera (n=5 BALB/c mice) generated to the panel of sexual stage antigens were used to probe parasite epitopes by indirect fluorescence assay. *P. falciparum* NF54 strain asexual stage parasites were generated *in vitro*. Parasites were air-dried onto glass slides and fixed with 4% paraformaldehyde and permeabilized with 0.01% TritonX-100 prior to immunostaining.

7.3 Discussion

Chapter 7 reports the development and execution of a seroepidemiology study to assess gametocyte carriage in a longitudinal cohort of Malian children and adults. While it is hoped that this effort will contribute to more extensive inquiry into gametocyte dynamics and sexual stage immunity, the results presented aim solely to identify serological markers of gametocyte exposure. The study identifies three antigens – PSOP13, HSP70, and ICP – to have increased seroreactivity in individuals that are gametocyte positive at the time of sample collection.

A major limitation of the assessment lies in the possible expression of the antigens presented in the asexual stage. The serological responses to the five antigens selected for evaluation at two cross-sectional time points are on average higher than those seen towards TBV candidate antigens in Chapter 6. There may be many reasons for this, but it nonetheless proposes that the response may be due to asexual expression. Optimization of HEK293E expression of Pfs230C, Pfs48/45, and other TBVs will be required before testing these antigens in parallel with PSOP13, HSP70-2, PDI-8, ICP, and PSOP9 for a direct comparison. IFA to asexual stage NF54 strain *P. falciparum* parasites indeed shows weakly positive staining for anti-sera to HSP70-2, PDI-8, and PSOP9. From reviews of available literature and proteomics data, ICP may have asexual stage expression as well. While PSOP13 may be specific to the sexual stage, this remains to be confirmed with more careful assessment. While 18S PCR data is available for all samples collected, this metric is not

exclusive to asexual stage parasites. Moreover it is less sensitive than Pfs25 qRT-PCR, and as such it is not possible to identify a cohort of individuals who are negative for asexual parasites with existing data. One imperfect approach to aid in interpretation of the results in Chapter 7 may be to screen all serum samples against *P. falciparum* asexual stage extract by ELISA, to normalize responses to our panel based on this information, and to repeat the analysis.

Another limitation is that in the analysis, antibody response and gametocyte carriage were quantified at the same time point. Gametocyte carriage by microscopy is often short-lived, and there is delay between gametocyte exposure and antibody detection to known sexual stage antigens Pfs230C and Pfs48/45. As such, antibody responses may be more indicative of previous, rather than active gametocytemia. Given the longitudinal nature of this study, as additional time points are quantified for gametocyte carriage, it is hoped that this issue may be explored.

With a renewed focus on elimination strategies in the malaria control agenda, it is of great importance to better define the human infectious reservoir in endemic regions. Serological markers of exposure could present a powerful tool, but due to limitations in heterologous production of *Plasmodium* proteins, it has been estimated that serological responses have been evaluated to less than 0.5% of the proteome [266]. Very little is known about naturally acquired immunity to *P. falciparum* sexual stage antigens, and the analysis in Chapter 7 serves foremost as an illustration of screening-based approaches which are generating attention [266].

Chapter 8: Conclusions and Future Directions

8.1 Summary

Vaccine-induced transmission-blocking immunity was demonstrated over half a century ago in an animal model. Transitioning these observations into a safe and effective vaccine strategy has yet to be realized, and three decades after the identification of the first transmission-blocking target antigens, these candidates still represent the majority of development efforts. In part, progress was delayed by a historical de-prioritization of inquiry into *Plasmodium* sexual biology; sexual forms do not directly contribute to clinical symptoms of disease. As elimination and eradication re-enter the control agenda, sexual stage vaccine targets have become an increasingly important focus. Phase Ia studies with Pfs25 suggest that existing formulations with non-controversial adjuvants will not elicit sufficient antibody titer or longevity to achieve functional TBI. Strong and sustained responses may require more potent adjuvants and new strategies such as protein conjugation or novel delivery platforms, which are an active area of study. New vaccine targets may circumvent these issues, but at present there is little effort to expand the pool of TBV candidates paired with exceedingly few head-to-head comparisons to prioritize existing antigens for clinical trials. Since the sequencing of the *P. falciparum* genome, both transcriptomic and proteomic studies have identified new proteins of interest, which remain to be assessed relative to existing antigens. In the setting of a

rapidly growing understanding of sexual stage biology, there is no excuse for the absence of an active pre-clinical pipeline in TBV development.

The work in this thesis prioritizes existing TBV antigens for future study and characterizes a panel of sexual stage proteins for the presence of transmission-blocking epitopes in an effort to expand the priority ranking. In Chapter 3, known TBV targets in varying stages of development (Pfs25, Pfs28, Pfs48/45, Pfs230C) were expressed in two independent vaccine-delivery platforms to approximate a rank order of performance. The performance ranking was not affected by delivery of antigens through a protein-in-adjuvant or a viral-vectored route and was consistent with previous publications in which Pfs25 and Pfs230C demonstrated a nearly complete inhibition of oocyst intensity by SMFA. With the aim of increasing the number of TBV candidates under active development, *P. falciparum* proteins known to play an important role in sexual stage biology, as well as proteins of unknown function profiled through global studies of the transcriptome and proteome, were heterologously expressed and evaluated for transmission-blocking epitopes by SMFA. Two out of 12 antigens, one previously described and one hypothetical, demonstrated a partial inhibition of oocyst intensity suggesting that additional epitopes exist which may benefit multi-subunit and so-called rational vaccine design. Finally, deployment of TBV strategies into the field setting will necessitate a better understanding of natural humoral immunity to sexual stage antigens. In addition to characterizing longitudinal responses to current TBV candidates, Part II of this thesis evaluated IgG responses to a panel of sexual stage antigens in a cohort from malaria-endemic Mali in an effort to identify markers of gametocyte exposure.

8.2 Conclusions and future directions

8.2.1 TBV antigens demonstrate a rank order of inhibitory IgG

Malaria vaccine research has been biased in its focus on a handful of historical antigens, a trend that extends to TBV development. While efforts to improve heterologous expression and antigen immunogenicity are well documented in the literature, it is surprising that few studies have compared leading TBV antigen performance in functional assays during pre-clinical development. To-date, two studies have established a pre-clinical priority ranking of TBV antigens as determined by a qualified SMFA using purified IgG [115, 198]. While the findings of these studies were complementary, reliance on one antigen expression platform to establish a priority ranking was a limitation of both. The first by Miura *et al* expressed recombinant Pfs25, Pfs230C, Pfs48/45, HAP2, and AgAPN1 in the WGCFS, evaluating vaccine antigens in a CD-1 mouse model [115]. A subsequent report by Kapulu *et al* evaluated Pfs25, Pfs230C, Pfs48/45$_{+Ngln}$, and AgAPN1 head-to-head using a ChAd63/MVA heterologous prime-boost viral-vectored regime in BALB/c mice [198]. Anti-Pfs25 and anti-Pfs230C IgG demonstrated a statistically significant and superior inhibition of over 98% in both studies at a test IgG concentration of 0.75 mg/mL; Miura *et al* did not observe a significant difference between Pfs230C and HAP2 performance.

Indeed, in Chapter 3, when evaluating TBV antigens Pfs25, Pfs28, Pfs48/45$_{+Ngln}$, and Pfs230C expressed in a mammalian HEK293E system and the viral-vectored ChAd63/MVA heterologous prime boost regime in parallel

vaccination schedules in BALB/c mice, Pfs25 and Pfs230C outperformed Pfs28 and Pfs48/45$_{+Ngln}$. While not a central aim of this thesis, the work in Chapter 3 reinforces the utility of viral-vectored antigen delivery, particularly adenovector prime followed by MVA boost after an 8 week interval [200], in the induction of functional antibodies for antigen screening [201]. When a two-feed prediction model [115] was used to estimate antigen inhibition in both platforms, there was no difference in inhibitory activity between protein-in-adjuvant and viral-vectored delivery for all antigens, except Pfs48/45$_{+Ngln}$. The protein-in-adjuvant Pfs48/45$_{+Ngln}$ outperformed the viral vectored formulation with an activity indistinguishable from Pfs28 given as protein or viral vector. This is the first study to evaluate Pfs28 head-to-head by SMFA. Redundancy of function with Pfs25 has limited its consideration as a solitary TBV antigen, but its ease of production and strong performance in this analysis warrant further exploration of potential additivity or synergy with other TBV candidates, which at present remain controversial [92, 93].

Research into additivity or synergy between responses to competitive vaccine antigens – whether through IgG mixing studies, co-vaccination, or chimeric proteins – promises to be a productive area for future research. Based on the priority ranking, the interaction of Pfs25 with Pfs230C is of particular interest; a Phase I trial with Pfs25 and Pfs230D1, dosed separately and in combination, in which each antigen is conjugated to EPA and adjuvanted with Alhydrogel®, is ongoing (ClinicalTrials.gov NCT02334462). Nanoparticles containing chemically conjugated Pfs25 and Pfs230D1 have been suggested as a promising future direction [77]. While technically challenging due to the dynamic range of SMFA,

identifying antigen specific IC_{50} – the antigen-specific antibody concentration at which 50% of oocyst development is inhibited in the mosquito – or alternatively IC_{80}, will aid in further prioritizing antigens, both alone and in combination.

8.2.2 Qualified SMFA with purified IgG provides a powerful platform for antigen testing

This work relied heavily on one of the few available tests of TBV efficacy – SMFA. Undeniably a key tool, the assay has been implemented widely in both the pre-clinical and clinical setting. Since 2010, key strides have been made in qualifying the assay for precision, linearity, range, and specificity [212], as well as evaluating its conclusions relative to the more biologically relevant DMFA [248]. Yet, SMFA is subject to low sensitivity and significant intra- and inter-assay variability particularly for samples with moderate or weak inhibitory activity. The limited dynamic range requires multiple feeds at multiple test IgG concentrations to yield interpretable results. This is most evident in efforts to study additivity and synergy in IgG mixing studies. A model for such experiments was presented in Chapter 5 with anti-Pfs25 and anti-enolase IgG; unfortunately, eliciting moderate transmission with anti-Pfs25 IgG proved inconsistent, even following antibody titration trials. As an extension of this, testing already strong targets in combination as protein fusions may present similar challenges; it is by definition impossible to achieve over 100% inhibition and within the context of the assay, interpreting small differences between titrations of inhibitory activity remains difficult.

At present, transmission-blocking activity in SMFA has not been proven to predict TBV function in a field setting, whether at the individual or population level. A major limitation is lack of clinical correlates for SMFA. No TBV candidate has entered field efficacy studies in clinical testing, but hopefully in light of recent attention to elimination campaigns, this will soon change. At present, several candidates with extensive support in pre-clinical work are slated to enter the clinical testing pipeline in preliminary safety and immunogenicity studies. In addition to the trial of Pfs25 and Pfs230D1 mentioned above, the REDMAL project, funded through the European Commission, will evaluate Pfs48/45 (subunit PF10C-MBP) in Phase Ia studies and the Malaria Vaccine Initiative is planning to conduct Phase Ia trials for mosquito midgut antigen APN1. So too there will be clinical testing of improved delivery platforms for Pfs25 such as a VLP formulation developed by the Fraunhofer Institute and an adenoviral prime, modified vaccinia virus Ankara boost vaccination regime to be evaluated by the Jenner Institute (University of Oxford, MultiMalVax EU FP7).

8.2.3 HEK293 system enables moderate-throughput approach to rationally screen *P. falciparum* proteins

The mammalian HEK293 system is versatile in enabling moderate-throughput expression of a *P. falciparum* sexual stage protein panel. The system was able to recreate native *P. falciparum* epitopes as assessed by IFA, including the transmission-blocking epitopes in known TBV candidates by SMFA. Following transient transfection, out of 27 known and novel antigens, 18 (67%) were detectable from HEK293E supernatant by anti-pentahistidine Western blot. 16

(40%) were expressed in sufficient quantity for a BALB/c immunization (>300 µg, n=5 mice). Comparative assessments and rational screens of the *P. falciparum* sexual stage proteome have been limited by the ability to express full-length, conformational proteins in heterologous systems. This technical limitation has impeded vaccine development efforts across all life cycle stages. The *Plasmodium* genome is A/T biased (~80%). Long regions of highly repetitive amino acid sequence predispose to unique challenges in identifying defined structural domains by standard prediction software [118]. Furthermore, unlike typical eukaryotes, secreted and surface-associated proteins are not modified by N-linked glycans due to a lack of appropriate enzymatic machinery [224].

As such, *Plasmodium* proteins are not easily amenable to popular expression platforms; high-throughput efforts in *E. coli* have been plagued by production of insoluble or non-functional protein. Moreover, bacterial and cell-free systems typically produce protein under reducing conditions, affecting correct disulfide bridge pairing and requiring complex refolding. Case by case mammalian expression of *Plasmodium* proteins has been reported previously but has only recently become more widely adopted following expression plasmids and cell line optimization [208, 231]. Recently, the HEK293 cell system has been used to develop protein libraries for the *P. falciparum* [218] and *P. vivax* [234] merozoite and is gaining more widespread use in immuno-epidemiological and receptor-ligand interaction studies [233, 234]. The former reports that 39 of 51 proteins (76%), and the latter 37 of 39 (95%) proteins, were detectable from HEK293 culture supernatant by ELISA. This is higher than the combined

success rate of Chapters 3 and 4 (67%), which included both named and hypothetical proteins. The use of high-expressing, stably transfected cell lines would bypass the lower transfection efficiency of our transient transfection protocol, and could be used to improve yield of low-expressing proteins in the future.

8.2.4 Rational screen identifies novel components of the *Plasmodium* transmission pathway

Rational screens of the *Plasmodium* proteome promise to strengthen understanding of transmission biology and expand the list of potential TBVs. Of 12 sexual stage proteins expressed in sufficient quantities for a vaccination study in BALB/c mice, two antigens – one previously reported and one hypothetical – reproducibly elicited inhibitory IgG as assessed by qualified SMFA performed by the NIH SMFA Reference Center. In a multi-feed prediction model incorporating four independent feeding experiments, enolase demonstrated statistically significant inhibition of oocyst intensity. While the inhibitory effect of anti-HYP1 IgG did not reach statistical significance, the level of inhibition was repeatable between four feeds, suggesting that there may be biological relevance to the transmission pathway. In the case of HSP70-2, one feed which demonstrated an inhibition of 98.2% (95% CI 94.5-99.7, p=0.001) appeared to be an outlier; the inhibitory activity of anti-HSP70-2 IgG was not statistically significant in three additional, independent feeds.

Ghosh *et al* initially characterized enolase as a target of transmission-blocking antibodies [156]. Expressed in multiple life cycle stages, the protein appears to

be secreted to the ookinete surface in the mosquito midgut despite the absence of an identifiable signal peptide. The Ghosh *et al* study suggests that the antigen may function in midgut recognition and traversal by acting as a hypothetical ligand for a mosquito midgut receptor and means for co-opting mammalian plasminogen, which when converted to active plasmin, aids in midgut traversal. The study heterologously expressed *P. falciparum* enolase in *E. coli* and generated anti-sera in a rabbit vaccination. The antigen inhibited *P. falciparum* oocyst intensity by 70% in SMFA using whole sera. While the inhibition observed in SMFA with purified IgG was lower, it was reproducible suggesting that the role of enolase in *Plasmodium* transmission warrants further study.

8.2.5 TBV targets are also the subject of naturally acquired immune response

TBVs can be broadly divided into pre-fertilization targets, expressed in the human host, and post-fertilization targets, expressed exclusively in the mosquito vector. Notwithstanding the strong and consistent inhibitory activity of anti-Pfs25 IgG – a post-fertilization target – in pre-clinical studies, existing formulations have failed to induce the sustained, high-titer antibody responses necessary for functional TBI in clinical trials. Antigens of the pre-fertilization class offer a possible advantage in that antibody responses may be boosted through the course of natural infection. Pfs230 and Pfs48/45 have been the subject of extensive study, with a multitude of reports observing Pfs230- and Pfs48/45-specific antibodies among individuals living in malaria endemic settings. At present, the durability of these responses is not well described, nor whether

they translate into functional TBI. Analyses to date have used antigen-specific mAbs to capture pre-fertilization proteins from gametocyte extract. These techniques limit the throughput of screening and impact assay sensitivity due to high background (Section 6.1). Improved production of recombinant protein promises to promote studies of natural immune recognition of TBV targets, the kinetics of antibody acquisition, and the impact of age and transmission intensity on these responses. The work in Chapter 6 is one of few analyses of Pfs230C serological responses using recombinant protein [266]. In a longitudinal cohort of Malian children (n=123) followed over three consecutive transmission seasons, antibody acquisition approximated the trend for IgG prevalence and level seen with asexual stage antigens AMA1 and $MSP1_{42}$ (Figure 6.5). The levels of anti-Pfs230C IgG were lower in magnitude relative to responses to the asexual stage, but antibody level was greater with increasing age. The association of Pfs230C responses with AMA1 and $MSP1_{42}$ IgG level suggest that cumulative parasite exposure may in fact promote sexual stage immune responses. The incidence of TBI in the same field-site was low in Malian children (n=36) when evaluating purified IgG in a qualified SMFA, and barring further study, it is unclear whether the inhibition seen in SMFA is compatible with transmission reduction in the field. The findings of Chapter 6 broadly support the notion that vaccine-induced immune responses to pre-fertilization antigens may be boosted in malaria-endemic settings, but these observations merit careful consideration in well-characterized cohort studies with a longitudinal study design.

8.2.6 Protein panel enables screen for markers of parasite exposure

In deploying public health interventions such as TBV strategies, it will be of paramount importance to identify reservoirs of infection. As an extension of the use of recombinant proteins to define serological responses to known TBV targets, conformational protein libraries enable more comprehensive assessments of serological responses to the sexual stage. The panel of antigens expressed in this thesis was limited in scope, with a bias towards proteins previously described in the literature as being of biological interest. Nevertheless, several antigens show strong seroreactivity in a cohort from Burkina Faso and Mali. Exploration in a larger study, which was well characterized for gametocyte carriage, suggests that differences in antibody titer to PSOP13, PfHSP70-2, and ICP exist between individuals who are positive for gametocytes by qRT-PCR and those who are negative. In this study, we are not able to comment conclusively on serological markers of gametocyte exposure due to cross-reactivity of antigens demonstrating positive titer with expression in the asexual stage. With optimization of *Plasmodium* protein production, microarrays will offer an unbiased platform for evaluating the seroprevalence and kinetics of gametocyte-specific IgG responses. A recent study probed plasma from Malian children against a protein microarray containing 1,204 *P. falciparum* proteins (expressed in *E. coli*) to compare antibody profiles between proteins exclusive to gametocytes and those not expressed in the gametocyte stage. The study reports several proteins reliably present in nearly all gametocyte carriers [301].

8.3 Final remarks

As more candidates enter the clinical pipeline in the coming years, the field will be increasingly able to link pre-clinical assays to field efficacy. Importantly, the pre-clinical evaluation of vaccine candidates across research institutes will need to become increasingly standardized for these findings to be effectively translated into a pre-clinical pipeline, which feeds into human trials. Recombinant protein expression systems will need to be optimized for high-throughput expression of vaccine targets. So too the generation of monoclonal libraries to a more diverse set of sexual life cycle stages will enable the identification and prioritization of protein candidates by another methodology. Ultimately an active TBV development pipeline paired with an improved understanding of natural immunity to TBV antigens in the field will be necessary for the development and licensure of a single or multi-subunit TBV as stated in the Malaria Vaccine Technology Roadmap.

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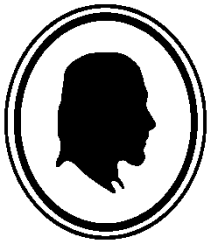
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Appendix: Standard Operating Procedures

Appendix A Standard Operating Procedures of the Jenner Institute

A.1 J238 Production of recombinant proteins in transiently transfected suspension 293 cells



This protocol uses GMOs. All users MUST be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.
If you are not currently registered with the Occupational Health Service for work with GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

Other Documents
MSDS: refer to MSDS for the relevant safety information on the individual reagents

See S:\PROTOCOLS\Protein expression\Transient 293:

a. "Transient 293 protocols Bushell Durocher and CSHL.docx"
    i. Summary of transient 293 production protocols from the following sources
        1. Gavin Wright's publications from the Sanger Centre
        2. Yves Durocher's publications from Canadian NRC
        3. Cold Spring Harbour Laboratory protocols, 2008
b. "Invitrogen Freestyle 293 system manual.pdf"

1. Definitions

293F cells - 293 cells available from Invitrogen, which are adapted to suspension growth in serum-free Freestyle medium

293T cells - 293 cells expressing SV40 large T antigen. This allows for episomal replication of transfected plasmids containing the SV40 origin of replication. This allows for amplification of transfected plasmids and extended temporal expression of the desired gene products. Adherent 293T cells originally obtained by Sandy Douglas from Strubi/ OPPF, and were adapted to suspension culture in serum-free medium by Sandy. Can be grown in the presence of G418, but not necessary as the SV40 large T

antigen gene is integrated into the genome along with the neomycin resistance cassette.

293E cells - 293 cells expressing EBV EBNA1 antigen. This allows for episomal replication of transfected plasmids containing the EBNA1 origin of replication. Adherent 293T cells originally obtained by Sandy Douglas from Strubi/ OPPF, and were adapted to suspension culture in serum-free medium by Sandy. As for 293Ts, it is not clear whether G418 selection is required during stock culture maintenance.

PEI - Polyethyleneimine.

2. Objective

Expression of soluble secreted recombinant proteins by transfection of suspension 293 cells.

Note: Associated methods (eg Western blotting to detect protein, ELISA to quantify protein, NiNTA or Streptactin purification of protein) may be discussed here, but detailed methods are elsewhere.

3. Reagents

Invitrogen Freestyle 293 expression medium or Invitrogen Expi293 expression medium (cat # 2082258)

G418 antibiotic solution at 50 mg/mL for cell culture (optional, for use with 293E cells, add to medium at 1:1000 ratio)

Foetal calf serum

Trypan blue

25KDa linear PEI from Alfa Aesar at 1 mg/mL in dH_2O. This needs to be prepared overnight in the molecular biology lab. Add an appropriate volume of ddH_2O to the PEI. PEI is insoluble at room temperature and so must be mixed at high temperature. Use the heated mixer on the molecular biology side, set the heater to 90°C. Loosen the lid to allow steam to escape and **clearly label the bottle and heater** so people are aware it is hot. Use an appropriate sized magnetic stirring rod. Initially the mixture will be cloudy but eventually it will become transparent. After at least 12 hours, the mixture can be put through a 0.22 um filter to remove insoluble clumps of PEI. Take an aliquot and check the pH, which should be pH 8.0. Aliquot and store at room temperature – **low temperatures will cause the PEI to come out of solution.**

Cells: 293F, 293T and 293E cells are banked in the ORCRB 40K LN2 tank, tower D6, box 7, at various stages of suspension and serum-free adaptation. It is critical that the records of remaining vials in the LN2

freezer spreadsheet are accurately maintained. *Do not take vials from the LN2 without updating the spreadsheet and consulting Joe Illingworth: S:\Reagent Storage Info\LN2, cells and serum storage\ORCRB LN2 Storage\40K ORCRB tank*

Useful Plasmids:
-pTT BirA #2938 – secreted biotin ligase for biotinylating proteins with a biotin acceptor peptide
-nonsecreted eGFP #1733 – useful for measuring transfection efficiency

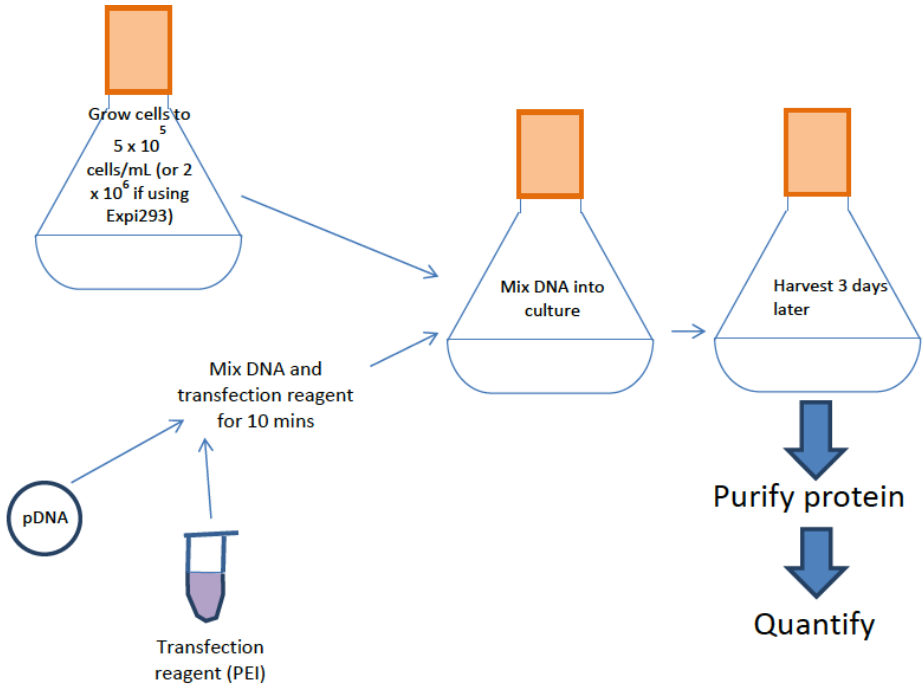
4. Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
Disposable haemocytometer	Immune Systems, cat# BVS100	

Trypan blue stain
V well plate (no need to be sterile)
Erlenmeyer culture flasks (vented or unvented are both fine, e.g. Corning 430421)
15 ml sterile tubes
5, 10 and 25 ml sterile pipettes
Sterile transfer pipette

5. Method

Overview: all performed in a sterile hood:



Central to the success of this procedure is the health of the cell culture. This can be fairly easily assessed microscopically: healthy cells tend to double approx every 20 hours and grow either as a single-cell suspension or small clumps of 2, 3 or 4 cells. If more than 20% of cells in a culture are in clumps of 5 or more cells, this is a sign of poor culture health and will likely result in low transfection efficiency.

5.1. Thawing and Establishing Cells

5.1.1. Store frozen cells in liquid nitrogen until ready to use. To thaw and establish cells:
5.1.2. Remove the cryovial of cells from the liquid nitrogen and thaw quickly in a 37°C water bath.
5.1.3. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol. Triturate and transfer the entire contents of the cryovial into a 125 ml polycarbonate, disposable, sterile Erlenmeyer shaker flask containing 30 ml of **pre-warmed** FreeStyle™ 293 Expression Medium. Incubate cells in a 37°C incubator containing a humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 125 rpm. Loosen the cap of the flask a quarter turn and tape into position to allow oxygenation/aeration.
5.1.4. Once the culture has reached greater than 1 x 10_6 viable cells/ml (typically 3 to 5 days), transfer the cell suspension aseptically into a centrifuge tube and vortex for 10 seconds.
5.1.5. Determine viable and total cell counts.

Important Note: Subculture cells for a minimum of 6 days (e.g. thaw on Friday for use the following Thursday) before use in transfection experiments to allow opportunity for recovery from thawing.

5.2. Counting cell density

5.2.1. Prepare a sterile 2 mL stripette and pipetteboy, open up and wipe down a class II safety cabinet.
5.2.2. Take the culture flask from the incubator.
5.2.3. Take a small (<100 uL) sample from the flask using the 2 mL stripette and put into a single well of a v-well plate.
5.2.4. Return the flask to the incubator.
5.2.5. Add 20 uL of Trypan blue to a well in the V-well plate adjacent to where the cell culture was deposited.
5.2.6. Take 20 uL of the cell culture and mix with the 20 uL of trypan blue by pipetting up and down.
5.2.7. Put 20 uL of 50:50 mixed trypan blue:cell culture into a disposable cell counting chamber.
5.2.8. Count all of the cells found in one of the large blocks of 4x4 smaller squares.
5.2.9. To obtain the cells/mL value, take the number of cells counted in the 4x4 square box, and multiply by $2x10^4$.

5.3. Subculturing cells

Splitting should be done on a Monday and a Friday. For routine maintenance, cells should be seeded into a new flask each time they are split. Seed cells at a density such that they will not reach a density of >$5x10^5$ cells/mL (or $4x10^6$ cells/mL if using Expi293) before they are next split (except before transfections). Don't split the culture so low that after one week the cell density will still be <$5x10^5$ (this is because Freestyle293 medium is light-sensitive and so should be replenished after not more than one week). It is crucial that the culture is kept at 37°C and experiences as few temperature perturbations as possible – otherwise the cells will clump. This can cause them slow down their growth-rate and become refractory to transfection.

5.3.1. Obtain a cell count.
5.3.2. For routine maintenance, split the cells so that they achieve a density of not more than $5x10^5$ cells/mL (or $2x10^6$ cells/mL is using Expi293) by the next splitting date. On a Friday, this means splitting the culture to ~$3.2x10^4$ cells/mL (or ≈$5x10^6$ cells/mL if using Expi293), if they'll next be looked at on Monday (assuming divisions every approx 20h). On Monday, split the culture to ~$1.6x10^4$ cells/mL (or $1.5x10^5$ cells/mL if using Expi293).
5.3.3. At least half an hour before splitting the cells, decide on how large a culture you want to set up. As a rule of thumb, try to avoid filling a cell culture flask more than one-third of its stated volume (e.g. for a 125 mL flask, ~40mL is the max volume).
5.3.4. Take a new flask and fill it with an appropriate volume of Freestyle 293 medium (or Expi293), and add G418 at a 1:1000 if culturing 293E cells.
5.3.5. Place in the shaking incubator for 30 minutes to allow equilibration to 37 degrees.
5.3.6. At the time of seeding, the fresh medium should be the same temperature as the cell culture will be split from. If it's perceptibly cooler, it is not warm enough and you should delay the splitting.
5.3.7. Once the medium is up to temperature, turn on and wipe down a microbiological safety cabinet.
5.3.8. Take the two flasks (one containing medium, one containing culture) to the hood, and seed an appropriate volume of culture into the fresh medium.
5.3.9. Return the newly inoculated culture to the incubator immediately.

5.4. Transfecting cells

This procedure needs to be planned in advance as it can take a while to grow up a culture of suitable size. The smallest transfection volume that should be performed in flasks is 30mL.Transfection in sterile 96 deep-well tissue culture plates has been described but never performed at the Jenner Institute. This protocol assumes that the transfection *per se* will be

performed on a Friday, but it could easily be shifted (e.g. make Friday day 1).

DNA guidelines

Every transfected DNA mix should contain 5% by mass of plasmid #1733. This plasmid expresses GFP and is useful for estimating transfection efficiency. To obtain sufficient DNA, at least a Maxiprep's worth of DNA is necessary, and occasionally a megaprep may be required. EndoFree prepped DNA is not essential to achieve good transfection efficiency, but it will improve cell health and can be considered as part of troubleshooting.

On Monday (day 1)

5.4.1. On Monday, whilst performing the routine culture splitting, seed a culture that will reach $1x10^6$ cells/mL (or $4x10^6$ cells/mL if using Expi293) on Thursday. Seeding the new culture at $1.2x10^5$ cells/mL (or $0.5x10^6$cells/mL is using Expi293) should achieve this. The culture seeded on Monday should be at least 25% of volume of the planned transfection culture.

On Thursday (day 4)

5.4.2. On Thursday, approx 24hrs before the transfection, get another cell count of the culture seeded on Monday. Calculate the dilution factor needed to seed a new culture at $2.5x10^5$ cells/mL ($1x10^6$ cells/mL is using Expi293).

5.4.3. Calculate the volume of medium required to start the transfection culture. E.g. if the stock is at $1x10^6$ cells/mL, and the total planned transfection culture volume is 500 mL, prepare 375 mL of medium.

5.4.4. If using Freestyle293, prepare the transfection medium by taking Freestyle 293 medium, adding G418 solution if using 293E cells at 1:1000 and adding FCS to 1% of the final culture volume (e.g. if the final culture volume will be 500 mL, take 375 mL medium, and add 375 uL G418 solution and 5 mL FCS). If using Expi293, no addition of FCS is necessary, simply add G-418 at 1:1000.

5.4.5. Place the flask containing the medium into the incubator and allow to equilibrate to 37 degrees for at least 30 minutes, preferably an hour. This is crucial as the culture will not have time to recover from any temperature perturbations before the transfections.

5.4.6. Once the medium is up to temperature, seed it with the culture at $2.5x10^5$ cells/mL (or $1x10^6$ cells/mL if using Expi293).

5.4.7. Place the culture in the incubator. It should not be removed again until the transfection.

On Friday (day 5) – stagger this process if more than one culture is being transfected

5.4.8. For every 100 mL of culture to be transfection, warm up 4 mL of Freestyle 293 medium to room temperature. Do not add 1% FCS as this can interfere with the interaction of PEI and DNA.

5.4.9. For every 100 mL of culture to be transfected, add 66 ug of DNA to the room temperature Freestyle 293 medium (or 100ug of DNA

per 100mL of culture if using Expi293). Vortex vigorously for 5 seconds. The DNA mixture should contain plasmid of interest and 5% by mass #1733. If the protein is to be biotinylated and contains a biotin acceptor peptide, also include 5% by mass plasmid #2938.

5.4.10. For every 100 mL of culture to be transfected at 100 uL of PEI to the room temperature medium/DNA mix (if using Expi293, use 200uL of PEI per 100mL of culture). Vortex vigorously for 5 seconds, and leave at room temperature for 10 minutes to allow PEI/DNA complexes to form.

5.4.11. After 10 minutes, remove the transfection culture from the incubator.

5.4.12. Add the DNA/PEI/medium mixture drop-wise to the culture, whilst swirling. Immediately return the culture to the incubator.

Next Monday (day 7)

5.4.13. Turn on the fluorescence laser attached to the microscope in the cell-line tissue culture room.

5.4.14. Perform a standard cell count as in 8.2. Cell density will by now be $>1x10^6$ ($>5x10^6$ if using Expi293) and the will likely be some cell clumping. This is normal at this density, especially as PEI is toxic. Importantly, cell viability should still be >95%.

5.4.15. Dim the lights in the room, turn the stage illuminator all the way down to nothing and switch to the blue filter. If the transfection has been successful, green circles representing GFP-transfected cells will be visible.

5.4.16. Calculate the ratio of GFP+ve vs GFP-ve cells to determine the transfection efficiency. Typically 40% of cells will be GFP+ve. Less than 30% indicates a less successful transfection. >80% GFP+ve cells is a bumper transfection.

5.4.17. Next Tuesday or Wednesday (day 8)

5.4.18. When cell viability falls below 80% the culture should be harvested. Spin at 750g for 10 minutes to pellet the cells and decant off the supernatant (cell pellet can be discarded, or stored to test for protein retention in the cells).

5.4.19. If intending to store for more than a few hours, add a protease inhibitor cocktail and refrigerate. If intending to store for more than a few days, freeze at -20 degrees

5.5. Freezing Cells

Introduction: You may freeze FreeStyle™ 293-F cells directly in FreeStyle™ 293 Expression Medium. When freezing the FreeStyle™ 293-F cell line, we recommend the following:

• Freeze cells at a density of 5-8 x 106 viable cells/ml.

• Use a freezing medium composed of 90% fresh FreeStyle™ 293 Expression
Medium and 10% DMSO.

Guidelines to prepare freezing medium and to freeze cells are provided in this section.

5.5.1. Preparing Freezing Medium

Prepare freezing medium immediately before use.

5.5.1.1. In a sterile, conical centrifuge tube, mix together the following reagents for every 1 ml of freezing medium needed:
- FreeStyle™ 293 Expression Medium 0.9 ml
- DMSO 0.1 ml

5.5.1.2. Filter-sterilize the freezing medium and place the tube on ice until use.

5.5.1.3. Discard any remaining freezing medium after use.

5.5.2. Freezing Cells

Before starting, label cryovials and prepare freezing medium. Keep the freezing medium on ice.

5.5.2.1. Grow the desired quantity of FreeStyle™ 293-F cells in shaker flasks, harvesting when the cell density reaches 0.5 to 1 x 106 viable cells/ml.

5.5.2.2. Transfer cells to a sterile, conical centrifuge tube.

5.5.2.3. Determine the viable and total cell counts (see protocol on page 4) and calculate the volume of freezing medium required to yield a final cell density of 5-8 x 106 viable cells/ml.

5.5.2.4. Centrifuge cells at 100 x *g* for 5 minutes at room temperature and carefully aspirate the medium.

5.5.2.5. Resuspend the cells in the pre-determined volume of chilled freezing medium.

5.5.2.6. Place cryovials in a microcentrifuge rack and aliquot 1 ml of the cell suspension into each cryovial.

5.5.2.7. Freeze cells in an automated or manual, controlled-rate freezing apparatus following standard procedures. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute.

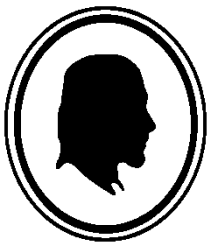
5.5.2.8. Transfer frozen vials to liquid nitrogen for long-term storage.

Note: You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen by following the procedure outlined in Thawing and Establishing Cells.

5.6. Scaling Up Transfections

Can be performed with linear increases of each ingredient. Largest volume attempted here is 500 mL.

A.2 J019 Detection of epitope-tagged proteins by western blotting.



Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:

\\lmsnw3_jenner_server\jenner\hill_group\Safety\COSHH assessments\Manufacturers material safety data sheets

C036 Protein gel electrophoresis

1. Definitions

PPE	Personal protective equipment
DNA	Deoxyribonucleic acid
SDS	sodium dodecyl sulphate
PAGE	Poly-acrylamide gel electrophoresis
BCIP/NBT	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt / Nitro-Blue Tetrazolium Chloride
ECL	chemiluminescence

2. Objective

To test for protein expression by SDS-PAGE followed by western blotting. The primary antibody may target an epitope tag engineered into the protein sequence. This protocol describes the detection by primary antibody, signal amplification by secondary antibody and then visualisation by either BCIP/NBT (colourimetric) reagent or ECL (chemiluminescence).

3. Reagents

Anti-penta-His monoclonal antibody	Qiagen	34660
Sheep Anti-mouse-HRP	Amersham	NA931V
Donkey anti-mouse-AP	Jackson labs	715-055-150
Colorplus Prestained Protein Ladder, Broad Range (10-230 kDa)	NEB	P7711S
10% SDS	Gibco	15553-035
Elix H_2O		
1 x PBS (in Elix water) pre-chilled	Oxoid	
Tween 20	Sigma	P2287
Laemmli sample buffer	Made in-house	
β-mercaptoethanol	Sigma	M3148
Methanol	Sigma	34860
Precast minigels	Pierce	

Fibre Pads		
Filter Paper	Whatman	
Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, 7 x 8.5 cm, 50 pack	Bio-Rad	162-0215
2 ml screw caps tubes	App Woods	AZ037
microcentrifuge	Hettich	
Hot Block	Techne	
Miniprotean 3 cell kit	Biorad	
Power pack argo	Anachen	
BCIP/NBT	Sigma	B5655
Ponceau S Staining Solution	Sigma	P7170
Digital camera		
10% sodium azide solution		

Reducing Laemmli buffer (For 20 ml):

125 mM Tris-HCl pH6.3	2.5 ml
20% v/v glycerol	4 ml
4% w/v SDS	0.8 g
20 mg/ml Brilliant Blue	400 mg
10% v/v β–mercaptoethanol	2 ml
Make up to 20 ml with 18Ωohm water	

Filter (0.45 µm) to remove any Brilliant Blue crystals.
(for non-reducing buffer, omit the β-mercaptoethanol).

Anti-penta-His antibody

Dilute lyophilised antibody with 500 µl dH_2O (0.2 mg/ml)
Store in 25 µl aliquots at -20°C
Working concentration = 1:1000 in 3% BSA in PBS.
For long-term storage add sodium azide to a final concentration o f 0.02% w/v

Tris-HEPES-SDS Running Buffer (10X)

Tris Base (MW = 121)	121 g
HEPES (free acid MW = 238)	238 g
SDS (MW = 288)	10 g
Add ultrapure water to 1 L	

• Before use dilute 10-fold with water. pH of the 1X buffer should be ~8.0; do not adjust pH.
• Final composition of the 1X buffer is 100 mM Tris, 100 mM HEPES, 1% (~3 mM) SDS, pH 8.0.

Transfer Buffer (1L)

Tris base	6 g
Glycine	30 g
Methanol	200 ml
Elix H_2O	up to 1 litre

Blocking buffer (50 ml)

BSA	1.5 g
PBS	up to 50ml

Washing buffer (1 L)

Tween 20	1 ml
PBS	up to 1 L

4. Equipment

Electrophoresis tank
Genie blotter or BioRad Trans-Blot Turbo
Power pack for electrophoresis tank
Large polystyrene box to contain blotting equipment and ice
Pipettes and tips
Orbital shaking platform

5. Method

5.1. Sample preparation

5.1.1. Harvest supernatant from S2 cells 3 days after induction of expression and transfer to a suitable container.
5.1.2. Pellet cells (1000 rpm, 5 min, RT)
5.1.3. Transfer supernatant to fresh tube
5.1.4. Add 4x reducing laemmli buffer to samples.
5.1.5. Heat to 95°C for 5 min.
5.1.6. Allow to cool.
5.1.7. Store at -20°C until ready to run gel.

For each gel, either use 10 µl of positope and heat this in the same manner as the samples, or use a previously qualified protein sample.

5.2. Running SDS-PAGE

5.2.1. Fill the electrophoresis tank up to the relevant fill line with Tris-HEPES-SDS running buffer.
5.2.2. Place the Pierce pre-cast gel/s in the gel holder, using a blanking plate if only one gel is being run.
5.2.3. Fill the central reservoir with 1 x Tris-HEPES-SDS running buffer.
5.2.4. Use a p200 pipette to rinse the wells with running buffer.
5.2.5. Load samples and molecular weight markers.
5.2.6. Close tank.
5.2.7. Run at 70 V until the samples have entered the gel, then increase the voltage to 100 V. Run time should be approximately 45 min.

5.3. Transfer method 1 (Genie Blotter)

5.3.1. Prepare 1L of transfer buffer and pre-chill
5.3.2. Pre-wet nitrocellulose membrane (just before use) in transfer buffer (use 1000 ml tip box).
5.3.3. Pre-soak the gel in transfer buffer for approx 5 min.

5.3.4. Assemble the sandwich, ensuring all components remain wet, as follows:
- 5.3.4.1. Two plastic grid spacers, one ridge down, 2nd one ridge up
- 5.3.4.2. Base electrode
- 5.3.4.3. Plastic grid spacer, ridges down.
- 5.3.4.4. Two sponge pads
- 5.3.4.5. Two blotting paper (once at this layer, top up cassette with buffer)
- 5.3.4.6. Membrane
- 5.3.4.7. Gel
- 5.3.4.8. Two blotting paper
- 5.3.4.9. Two pads
- 5.3.4.10. Plastic grid spacer, ridges upwards
- 5.3.4.11. Top electrode
- 5.3.4.12. Plastic top

5.3.5. Close the cassette and top up with transfer buffer
5.3.6. Keeping the tray level, insert cassette in the apparatus and gently raise it into the upright position.
5.3.7. Place entire apparatus in a large, polystyrene box.
5.3.8. Fill the box with ice.
5.3.9. Run for ~1h at 12V.

5.4. Transfer method 2 (BioRad Trans-Blot Turbo System)

5.4.1. Use pre-soaked Nitrocellulose transfer packs as described in the User Manual. Copy stored here: S:\PROTOCOLS\Protein expression\Trans-Blot Turbo System manuals (Read before use!)
5.4.2. Assemble transfer pack with SDS gel as described. Load into cassette. NOTE: Midi transfer packs can run 2 mini gels side by side and are more cost effective than running 2 mini transfer packs. Ensure no buffer is leaking.
5.4.3. Load cassette(s) into Trans-Blot Turbo unit. Switch on and select appropriate running programme (15 or 30 minute standard transfer is suitable for most gels. Only use 7 minute turbo programme if using BioRad TGX pre cast gels)
5.4.4. When run is finished, carefully disassemble cassette and transfer pack (NOTE: if running a fast programme, this can get quite hot). Remove membrane (use forceps) to a clean tip box lid for the next stage.
5.4.5. Without exception, carefully clean the cassette and electrode surfaces with distilled water. Dry thoroughly before re-assembling. Check the cassette contacts and visually inspect the electrode contacts within Turbo Blot unit for any buffer residue build up. If noted, clean as described in manual and Notification letter (S:\PROTOCOLS\Protein expression\Trans-Blot Turbo System manuals).

5.5. Ponceau S staining

5.5.1. After transferring proteins onto membrane, immerse membrane in sufficient Ponceau S staining solution, satin for 5 mins (use the lid of a 200 ul tips box, and 15 ml of ponceau solution).

5.5.2. Rinse membrane with Elix water three times, until background is clear

5.5.3. Take a picture of the membrane using the digital camera

5.5.4. Immerse membrane in aqueous solution of 0.1 M NaOH, protein bands will start to disappear.

5.5.5. Rinse membrane with water for 5 times

5.6. Blocking and antibodies

5.6.1. Wash the membrane twice for 10 min each in PBS.

5.6.2. Block the membrane for 1hr at R/T in 3% BSA/PBS, on the shaking platform.

5.6.3. Wash membrane 2 x 10 min in PBS-T.

5.6.4. Wash membrane for 10 min in PBS.

5.6.5. Incubate in 20 ml 1° antibody for 1 hour RT (anti-His Ab 1:1000 in blocking buffer).

5.6.6. Wash 2 x 10 min in PBS-T.

5.6.7. Wash 1 x 10 min in PBS.

5.6.8. Incubate with secondary antibody solution for 1 hour at RT. (Donkey anti-mouse-AP, diluted 1:3000 in blocking buffer - 6.7 μl in 20 ml)

5.6.9. Wash 4 x 10 min with PBS-T.

5.7. Development

5.7.1. Dilute 1 SIGMAFAST BCIP/NBT tablet in 10 ml water (vortex to dissolve)

5.7.2. Lay the nitrocellulose blot on some Saran wrap and pipette the BCIP/NBT solution carefully on top, ensuring it is fully covered.

5.7.3. Develop for 5 – 10 min.

5.7.4. Rinse with water.

5.7.5. Photograph or scan.

5.7.6. Dry and store away from light.

6. Appendix

Primary antibodies

Anti-5xHis 1:1000 in 3% BSA in PBS.
Anti-V5 1:1000 in PBS-T

Secondary antibodies

Sheep Anti-mouse-HRP diluted 1:5000 in 10% milk in PBS (4 μl in 20 ml)
Donkey anti-mouse-AP diluted 1:3000 in PBS-T (6.7 μl in 20 ml)

Notes:
BCIP/NBT colorimetric system of western blot detection

An ideal system for blotting or staining applications is the combination of BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) and NBT (Nitro-Blue Tetrazolium Chloride). Together they yield an intense, insoluble black-purple precipitate when reacted with Alkaline Phosphatase. The NBT/BCIP reaction is illustrated in the figure below. This reaction proceeds at a steady rate, allowing accurate control of the relative sensitivity and control of the development of the reaction.

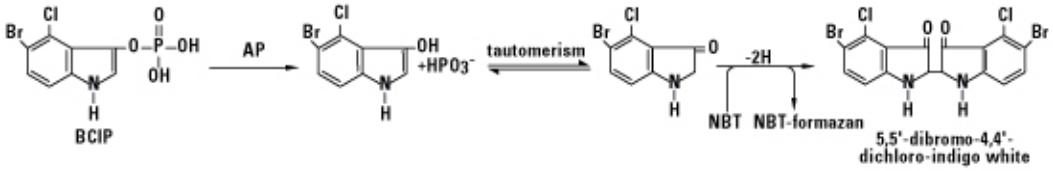
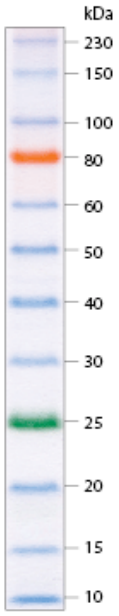
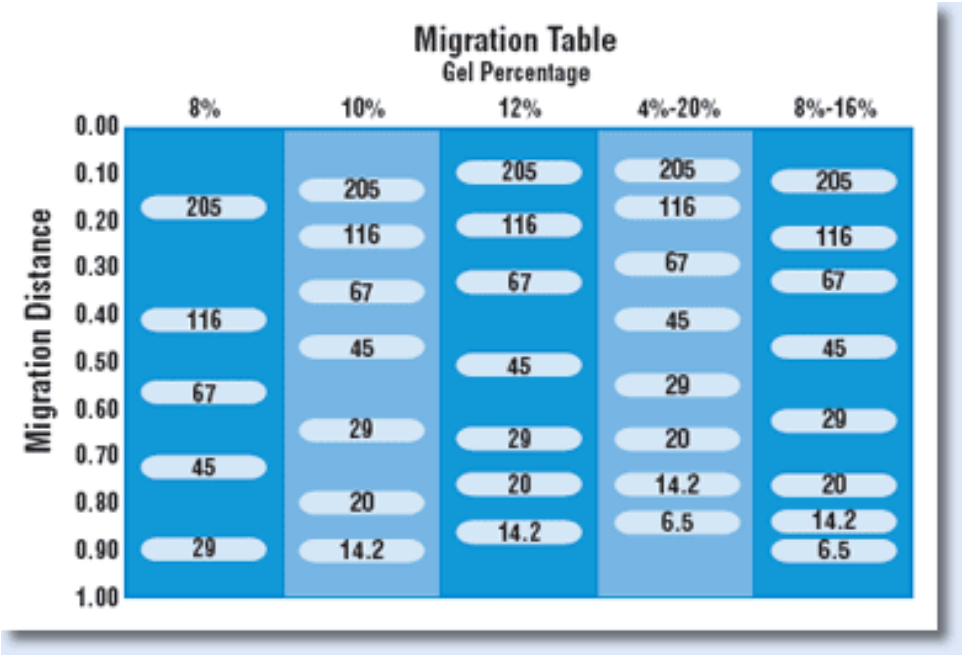


Figure 1. NBT/BCIP reaction scheme.
BCIP is hydrolyzed by alkaline phosphatase to form an intermediate that undergoes dimerization to produce an indigo dye. The NBT is reduced to the NBT-formazan by the two reducing equivalents generated by the dimerization.

Colorplus Prestained Protein Ladder, Broad Range (10-230 kDa) is a mixture of highly purified recombinant proteins covalently coupled to a remazol blue dye that resolves into 12 sharp bands when electrophoresed. The protein concentrations are carefully balanced for even intensity. Both the orange 80 kDa and the green 25 kDa bands serve as the reference indicators. The covalent coupling of the dye to the proteins affects their electrophoretic behavior on SDS-PAGE gels relative to unstained proteins (3). The apparent molecular weights of the ColorPlus Prestained Protein Ladder bands were determined on Invitrogen Novex 10–20% Tris-glycine SDS-PAGE gels (1,2) by comparison to the Protein Ladder (NEB #P7703). The ColorPlus Prestained Protein Ladder is ideally suited for use in SDS-PAGE and western blotting applications. It allows continuous monitoring of protein separations during electrophoresis and also provides a quick and easy way to assess blotting (3).



Pierce protein migration table



Appendix: Use of Ludwig Institute ChemiDoc MP system

Note: This equipment belongs to the Ludwig, not the Jenner. Any Jenner user wishing to use the ChemiDoc MUST have received clearance from both the Ludwig lab manager (Neil Carveth, at time of writing) AND Julie Furze AND have received training in use of the equipment. This appendix is intended as a memory aid, not a substitute for training.

IT IS ESSENTIAL THAT THE CHEMIDOC IS LEFT CLEAN AND IS NOT DAMAGED BY IMPROPERLY TRAINED USERS. DO NOT ENDANGER OTHER JENNER USERS' ACCESS TO THIS EQUIPMENT.

To use this protocol, you will need to have used an HRP-conjugated secondary antibody and have access to an ECL substrate (eg Pierce CN 32106, which is prepared for use by mixing equal parts of the two solutions).

Your membrane should be in a clean document wallet, with a minimal quantity of substrate added on the membrane up to a few minutes before imaging. You should not have large quantities of liquid flowing around on the membrane and risking contaminating the ChemiDoc.

1. Turn on the ChemiDoc (switch at bottom right, beneath door)
2. Log in to PC using your usual Novell network username and open ImageLab software
3. From the ImageLab start window, select 'New single channel' protocol.
4. Select an 'Application'- use Custom → Epiwhite to get white epi-illumination (which will allow you to take a picture of your MW markers).
5. Select 'Position Gel'. The membrane position can be altered by hand and the zoom adjusted in the software to get the desired view. It should **not** be necessary to adjust the focus on the ChemiDoc- **do not do so without seeking advice from an experienced Ludwig user.**
6. Click 'Run protocol' to take the epi-illuminated image. It can be saved to your H drive in ImageLab .scn format by clicking save on the ImageLab tool icon bar, or exported in TIFF or JPG format by going to File → Export → Export for Publication
7. Now return to the 'protocol' window. If it's not visible, you can return to it either using the 'Window' menu, or by reducing the image you've just taken from full-size window to partial-size (using the 'minimise' etc icons at the top right).
8. Change the 'Application' to 'Blots'→ 'Chemi' or 'Chemi Hi Sensitivity' or 'Chemi Hi Resolution'.
9. Under Image Exposure Mode, select 'Signal Accumulation', click on Setup and enter your desired settings (for example, first image at 1s, last image at 60s, take total of 10 images- this will give you a series of images with a variety of exposures).
7. The gel positioning/ zoom settings should remain the same as before, and don't need to be altered. Click 'Run Protocol'.
8. Assuming you want to save all the images for future use, right click on the resulting series of images at the bottom of the 'Protocol' window, click 'Save all', select the folder on your H drive you want the images to go to. They will be saved in .scn format.
9. Select the single image which looks most promising, click the yellow button saying 'Select Image and Continue'. You can now access the ImageLab analysis tools (beyond the scope of this appendix) or export the image as a TIFF or JPG. Unless you have better skills in ImageLab than the author, it will be necessary to use Photoshop or similar software to merge your epiwhite and ECL images so you can identify molecular weights on the ECL image.

NOW REMOVE YOUR BLOTS, CLEAN THE CHEMIDOC, TURN IT OFF, AND LOG OUT OF THE PC#

Appendix B Standard Operating Procedures of LMVR, NIAID, NIH

B.1 IM-6024-00 General Procedure of ELISA with Triplicates in One Plate

1. Purpose

This SOP describes the procedure for the Enzyme-Linked ImmunoSorbent Assay (ELISA). The purpose of the ELISA in this instance is to detect and evaluate the presence of antibodies to malarial proteins in human or animal samples.

2. Definitions

None

3. References

Instruction Manual for the Biomedical safety cabinet
Instruction Manual for the Microplate reader

4. Materials, Instruments and Equipment

Note: General materials and equipments (e.g., Distilled water, 12 channel pipette, disposable pipette tips, centrifuges, and personal protective equipment, etc) are not listed here.

4.1. Coating Antigen minimum of 10 □g per plate. Where available working aliquots of reference antigen batches will be used
4.2. ELISA Standard. Where available working aliquots of standard will be used
4.3. Sodium carbonate - Mallinckrodt AR Cat. No. 7521
4.4. Sodium bicarbonate - Mallinckrodt AR Cat. No. 7412
4.5. Difco Skim Milk, BD, 232100 or equivalent
4.6. TRIS Buffered Saline (TBS) 10x, Biofluids, Cat# 616NS-000
4.7. BSA, Sigma; Cat. # A-7906
4.8. Phosphatase substrate tablets: Sigma cat No. S0942-200tab, or equivalent
4.9. Tween-20 (Polyoxyethylenesorbitan Monolaurate), Sigma, Cat #: P-1379
4.10. Immulon 4 HBX Flat Bottom Microtiter Plates, Dynex Technologies, Inc,
4.11. Cat# 3855
4.12. Deep well ELISA dilution plates, Nalgene Nunc International, Cat #270140 or equivalent
4.13. 96-well ELISA dilution plate, Nunc, Cat #279944, or equivalent
4.14. 96-well caps, Nalgene Nunc International, Cat# 276002 or equivalent

4.15. Multichannel Solution Basin, Labcor Products, Inc. Cat. No 730-004 or equivalent
4.16. Alkaline Phosphatase labeled secondary antibody. Antibody used depends on the species of sample to be tested and will be chosen from the following list:
4.17. Alkaline Phosphatase Labeled Goat anti-Human IgG (H+L); Kirkegaard & Perry Labs, Inc. 1.0 mg Cat. #: 075-1006, or equivalent
4.18. Alkaline Phosphatase Labeled Goat anti-Mouse IgG (H+L); Kirkegaard & Perry Labs, Inc. 1.0 mg Cat. #: 075-1806, or equivalent
4.19. Alkaline Phosphatase Labeled Goat anti-Rabbit IgG (H+L); Kirkegaard & Perry Labs, Inc. 1.0 mg Cat. #: 075-1516, or equivalent
4.20. Alkaline Phosphatase Labeled Goat anti-Rat IgG (H+L); Kirkegaard & Perry Labs, Inc. 1.0 mg Cat. #: 15-16-06, or equivalent
4.21. ALP Rabbit X Monkey IgG, Accurate chemical & scientific corp., Cat # BYA44831, or equivalent
4.22. APStabilizer, KPL, Cat #55-15-00
4.23. ELISA Ultrawash Plus, Dynex Technologies
4.24. Microplate reader, Spectramax 190 Molecular Devices

5. Safety and Precautions

5.1. Blood derived specimens

All work with blood derived specimens should be performed in accordance with established NIH Biological Safety Regulations. Vinyl or latex gloves must be worn at all times. Procedures involving human blood derived specimens should be performed in a biological hood or in the laboratory with lab coats, masks, and goggles. Areas and equipment must be decontaminated after use.

5.2. ELISA plates

Avoid touching the bottom of the ELISA plate. Fingerprints, drops, etc., will interfere with the spectrophotometric reading of the absorbance.

5.3. Frozen samples

If samples have been frozen, use a vortex and/or invert to mix before use.

6. Procedures

6.1. Buffers and secondary antibody preparation

10x Coating Buffer: pH=9.6, 0.15M Sodium Carbonate, 0.35M Sodium Bicarbonate

To prepare 1000 ml of 10x Coating Buffer, dissolve 15.74 g of Sodium Carbonate $(Na)_2CO_3$ and 29.4 g of Sodium Bicarbonate ($NaHCO_3$) in 1000 ml of Distilled water.

Label the bottle as “10x Coating Buffer” and date prepared. Store at room temperature for up to one year.

To prepare 1x solution from a 10x solution, mix 100 ml of 10x solution and 900 ml of Distilled water. Label the bottle as “1x Coating Buffer” and date prepared. Store at room temperature for up to one month.

10x BSA Diluting Buffer: 1%BSA, 0.5%Tween-TBS

To make 1000 ml of 10x BSA Diluting Buffer, mix 10 g of BSA, 5 ml of Tween-20 and 1000 ml of 10x TBS.

Mix on a magnetic stirrer until BSA and Tween-20 dissolve (at least 20 min at room temperature). Sterilize by filtering the solution through a 0.2μm filter. Label the bottle as “10x BSA Diluting Buffer” and date prepared. Store at 4°C for up to one year.

To prepare 1x solution from a 10x solution, mix 100 ml of 10x solution and 900 ml Distilled water. Label the bottle as “1x BSA Diluting Buffer” and date prepared. Store at 4°C for up to two weeks.

10x Wash Buffer: 1%Tween-10xTBS

To make 1000 ml of 10x Wash Buffer, mix 10 ml of Tween-20 and 1000 ml of 10x TBS.

Mix on a magnetic stirrer until Tween-20 dissolves (at least 20 min at room temperature). Label the bottle as “10x Wash Buffer” and date prepared. Store at room temperature for up to one year.

To prepare 1x solution from a 10x solution, mix 100 ml of 10x solution and 900 ml of Distilled water. Label the bottle as “1x Wash Buffer” and date prepared. Store at room temperature for up to one year.

5% Skim Milk Diluent/Blocking Buffer: 5% Milk – 1X TBS

To make 300 ml of a 5% Skim Milk Buffer, mix 15 g of Skim Milk Powder, 30 ml of 10x TBS and 270 ml of Distilled water.

Mix on a magnetic stirrer until Milk dissolves (at least 20 min at room temperature). Label the bottle as “5% Milk Buffer” and date prepared. Store at 4°C for up to two weeks.

Optional: make 50 ml aliquots of prepared 5% Milk Buffer and store at -20°C for up to one year.

Secondary antibody

Add 1 ml of KPL AP Stabilizer to each vial of secondary antibody and mix until completely rehydrated. Label the vial with date rehydrated and store at 4°C for up to one year.

Optional: To minimize vial-to-vial difference, make a pool of secondary antibody using multiple vials, then aliquot the antibody and store the stock aliquot(s) at -80°C for up to two years.

6.2. Coating plates with antigen

6.2.1. Note: Coated plates must be stored at 4°C and must be used within one week

6.2.2. Label each plate with the antigen name and coating date with a permanent, waterproof marker on the side of the plate.

6.2.3. Each plate requires 10 ml of coating solution. Determine the total volume of coating solution needed to coat the number of plates in the assay.

6.2.4. Dilute the antigen in 1x Coating Buffer to a concentration of 1 µg/ml in a 50 ml tube (or a 250 ml Erlenmeyer flask).

6.2.5. Mix the solution by inverting the tube (or flask) and then pour the solution into a multichannel solution basin.

6.2.6. Using a multichannel pipette, fill each well with 100 µl of the coating solution.

6.2.7. Carefully stack the plates using an empty plate as the top plate, and wrap them in plastic wrap. Leave the plates for at least one night in the refrigerator at 4°C.

6.3. General precautions for primary antibody and ELISA standard dilutions

6.3.1. Dilution factor and final volume of dilutions will be determined based on the design of ELISA (e.g., how many antigens need to be tested, etc.). To make the dilutions, it is not recommended to handle less than 5 □L of liquid in any step.

6.3.2. One plate can be used for up to 24 different primary antibodies in triplicate (row A to F).

6.3.3. Serial 2 fold dilutions in duplicate of standard are included on each ELISA plate (rows G and H).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
B												
C												
D	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
E												
F												
G	Standard dilution										Diluent only	
H												

6.3.4. Diluent containing milk should be used for all human samples and diluent containing BSA should be used for animal samples.

6.3.5. If primary antibody or standard have been frozen, vortex and/or invert to mix, then centrifuge the tube/plate at 300-500xg for 30 seconds before use.

6.3.6. If there is not a specific reason for doing otherwise, all primary antibodies should be tested by ELISA at 1:500 dilution first, and then subsequently tested at higher dilution if required.

6.3.7. Primary antibody/standard dilutions can be made the day before ELISA is performed (or earlier), but the dilutions should be used before the buffer expires. If the dilutions will be kept less than 2 hours, keep the plate(s) at room temperature in a humid chamber or with a cover. If the dilutions will be kept for more than 2 hours, cover the dilution plate(s) and store at 2-8 °C. The primary antibody plate(s) can be stored at -80 °C or as required by a supervisor (do not freeze/thaw multiple times).

6.3.8. The treatment of the primary antibody (i.e., which sample is tested which plate/which well at what dilution) and the way of making dilution(s) for primary antibody/standard (e.g., 1:50 dilution was made by mixing 5 □l of original serum with 245 □l of diluent. Then 1:500 dilution was made by mixing 15 □l of 1:50 dilution with 135 □l of diluent, etc) must be recorded.

6.3.9. To achieve the same shape of the Standard Curve, first standard dilution should have 10-50 ELISA units (usually 20 ELISA units). The number of ELISA units in a Standard should be determined in advance using IM-6025-XX.

6.3.10. To minimize the variation in the standard, it is highly recommended to make enough volume of the serial dilutions for a day as a single lot (i.e., it is not recommended to make a series of dilutions for each row of ELISA plate).

6.3.11. In some cases, ELISA will be performed without an ELISA standard (e.g., O.D. will be reported as a final outcome, instead of ELISA units). Ask a supervisor for details.

6.4. Primary antibody preparation

6.4.1. Obtain the sufficient number of 96-well dilution plate(s) for sample dilution. Label each dilution plate.

6.4.2. Using the appropriate size multichannel pipette, put 1x diluent in desired rows for all dilution plate(s). For the initial dilution plate(s), it is optional to put tape over rows D and E to write sample numbers or other comments (put samples rows A-C or F-H).

6.4.3. Add the calculated amount of original test samples to wells with 1x diluent. Use a new pipette tip for each well. A multichannel pipette might be used if possible. Repeat the same procedure until all desired wells have test sample added.

6.4.4. Using an appropriate size multichannel pipette, mix contents of row A. Without changing tips mix contents of rows B and C. Change tips, and then mix rows D, E and F (or rows F, G and H).

6.4.5. For further dilution, transfer desired volume from the lower dilution plate to a higher dilution plate (which has only 1x diluent). Mix contents as in Section 6.4.4.

6.4.6. Store the plates appropriately (Section 6.3.7).

6.5. ELISA standard preparation

6.5.1. Obtain sufficient amount of ELISA standard stock. At least 500 μl of the first working solution per plate is required.

6.5.2. Obtain sufficient amount of tubes (or 96-well dilution plate(s)) and put the appropriate amount of diluent in the each tube (or well).

6.5.3. Make an initial dilution from the stock standard, and then make 2-fold serial dilutions. Make total of 10 different 2-fold serial dilutions.

6.5.4. Transfer the serially diluted ELISA standard to a 96-well dilution plate (the highest concentration is in column 1 and the lowest is in column 10). Put diluent (without any antibody) in columns 11 and 12.

6.5.5. If possible, the ELISA standard may put in the same plate which has diluted primary antibody in row A-F (Section 6.4.6).

6.5.6. Store the plates appropriately (Section 6.3.7).

6.6. Blocking plates

6.6.1. Take desired number of plates out of refrigerator. Number each plate with a waterproof permanent marker.

6.6.2. Discard coating solution from plate(s) prepared at Section 6.2.6. Take care to ensure the plates do not completely dry before 5% Milk Buffer is added.

6.6.3. Using the multichannel pipette, fill each well with 300-350 μl of the 5% Milk Buffer and place the plate(s) into a humidified chamber (or stack the plate(s) with a blank plate on top) and leave for 2 hours ± 15min at room temperature.

6.6.4. Note: If needed, the plate(s) can be incubated with 5% Milk Buffer for a longer period than 2 hours. Record the deviation to a laboratory notebook.

6.7. Primary Antibody application

6.7.1. Decide upon a time interval between plates, generally 2.5-3.0 min. Wash plates at the same interval throughout the entire assay. Use a timer to measure the interval accurately.

6.7.2. Set up two timers; one for measuring interval between plates and the other for 2 hour primary antibody incubation.

6.7.3. Ensure that all test sera dilution plates (Section 6.4.6) and ELISA Standard plates (Section 6.5.5) are available and at room temperature.

6.7.4. Immediately, before adding primary antibody, wash the plates (Section 6.6.3) 4 times using Ultrawash Plus plate washer. When the plate washer start the process, start the timer to measure interval between plates.

6.7.5. Using a 50-300μL multichannel pipette, transfer 100 μL of primary antibody from the dilution plate to the ELISA plate. Without changing tips, transfer samples of rows A, B and C. Change tips, and transfer samples of rows D, E and F.

6.7.6. Change tips, and add 100 μl/well of standards to rows G and H.
6.7.7. When standards have been added to the first plate, start the 2-hour timer.
6.7.8. Continue washing plates at the same interval, as determined, adding the primary antibody and standards as in Section 6.7.5-6.7.6.
6.7.9. Place the plate(s) into a humidified chamber (or stack the plate(s) with a blank plate on top) and incubate for 2 hours at room temperature.

6.8. Secondary Antibody application

6.8.1. Several minutes before the end of the 2-hour primary antibody incubation period, prepare the secondary antibody. Dilute the secondary antibody with appropriate 1x diluent (5% Milk for human samples and BSA diluent for animal samples). Dilution factors might differ depending on the species, the batch of the secondary antibody, and the antigen tested, but usually 1:1000 dilution is selected.
6.8.2. Set up two timers; one for measuring interval between plates and the other for measuring the 2-hour secondary antibody incubation.
6.8.3. At the end of the primary antibody 2-hour incubation, begin washing plates as Section 6.7.4.
6.8.4. Add 100 □l/well of the secondary antibody to the ELISA plates.
6.8.5. When secondary antibody has been added to the first ELISA plate, start the 2-hour timer.
6.8.6. Continue washing plates at the same time interval and adding secondary antibody as Section 6.8.4.
6.8.7. Place the plate(s) into a humidified chamber (or stack the plate(s) with a blank plate on top) and incubate for 2 hours at room temperature.

6.9. Substrate application

6.9.1. Approximately 15-30 minutes before the end of the 2-hour incubation period, prepare the substrate solution. Use 10 ml of substrate solution per ELISA plate. To make 10 ml of substrate solution, dissolve 2 Phosphatase substrate tablets in 1x Coating Buffer.
6.9.2. The SpectraMAX plate reader and a computer should be turned on prior to the addition of substrate solution. Select a suitable SoftMax Pro .ppr program (or a pda file), and open it highlighting the bar for Plate 1.
6.9.3. At this time (or after Section 6.10.3), verify that the standards concentrations are correct in each plate and put the antigen name of the plate in Experiment Name or Section Name (e.g., plate #1 AMA1-3D7, etc).
6.9.4. Set up two timers; one for measuring the interval between plates and the other for 20-minute substrate incubation.
6.9.5. At the end of the secondary antibody 2-hour incubation, begin washing plates as in Section 6.7.4.
6.9.6. Add 100 □l/well of the substrate solution to the ELISA plates.

6.9.7. When substrate solution has been added to the first ELISA plate, start a 20-minute timer.
6.9.8. Continue washing plates at the same time interval and adding substrate solution as in Section 6.9.5.
6.9.9. Place the plate(s) into a humidified chamber (or stack the plate(s) with a blank plate on top).
6.9.10. Substrate incubation time is 20 minutes at room temperature.
6.9.11. Note: If there are more than 8 plates in the series and a 3-min interval has been used, the first plate(s) may require reading before all of the plates are washed/substrate solution added.

B.2 IM-6008-02 Membrane Feeding of Mosquitoes and Dissection of Midguts of *Plasmodium falciparum* Infected Mosquitoes

1. Purpose

This SOP describes the procedures for preparation of mosquitoes, mosquito membrane-feeding assay, and dissection of midgets of *P. falciparum* infected mosquitoes.

2. Revision history

2.1. The procedure of exfagellation evaluation was revised
2.2. Associated forms were revised
2.3. Minor modification and clarification in some procedures
2.4. Minor editorial revision

3. Definitions

3.1. MFA: Membrane-feeding assay
3.2. Pre-Feed Mixture: A mixture of *P. falciparum* mature gametocyte culture pellet (containing Stage V gametocytes), normal red blood cells, and normal human serum (with or without complement). The Pre-Feed Mixture has adjusted parasitemia and hematocrit to make the "Feed Mixture."
3.3. Feed Mixture: A mixture of "Pre-Feed Mixture" and test or control sample. The Feed Mixture is fed to mosquitoes.
3.4. RBC: Red blood cells.

4. References

4.1. Instruction Manual for Series II Water-jacketed Incubator, Thermo Electron Corp
4.2. Instruction Manual for Water Bath, Barnstead International Model No# 18002A

4.3. Safety protocol to work with mosquitoes infected with Plasmodia infectious to humans.
4.4. Maintenance of Plasmodium falciparum culture (IM-6030-XX)

5. Materials

5.1. See Appendix A: Specific Materials, Instruments and Equipments

6. Safety and Precautions

6.1. All culture work should be performed in the biological safety cabinet in accordance with Biological Safety Regulations unless it is needed to perform a procedure outside of the cabinet.
6.2. Wear vinyl or latex gloves and lab coat at all times while working with human blood, serum, or *P. falciparum* culture.
6.3. Handling of infected mosquitoes appropriately in accordance with 'Safety protocol to work with mosquitoes infected with Plasmodia infectious to humans'.

7. Procedures

7.1. General Recommendations

7.1.1. For the mosquito feed, use *P. falciparum* cultures with at least 0.5% stage V gametocytes and vigorous exflagellating centers.
7.1.2. Before preparing "Pre-Feed Mixture" (Section 7.9), have mosquitoes starved and distributed into pint containers (Sections 7.6), test sample prepared (Section 7.7) and feeders set up (Section 7.8).
7.1.3. Test/human serum control and sample preparations (Section 7.7) can be done in advance but less than a week before feed.
7.1.4. Monoclonal antibody 4B7 control sample (or other monoclonal antibody which is used as a control) should be freshly thawed for each feed.

7.2. Mosquito Container Preparation

7.2.1. Make a round hole (diameter approximately ¾ in) in the paper can wall using a cork-borer. Cover the hole with two squares (5 cm x 5 cm) of dental rubber with perpendicular-oriented cuts and attach 4 sides of each square separately to the paper can wall with filament-reinforced tape.
7.2.2. Fold autoclave tape lengthwise, sticky side out, and attach to the side of the pint container at the top, around the pint opening.
7.2.3. Put a 90 mm Ø filter paper inside to cover the bottom of the pint container (optional).
7.2.4. Place two net squares on a top (open) part of the paper can and put the metal ring over the net. Ensure that the net is taut.
7.2.5. Using scissors, cut outside part of the net leaving approx. 1 cm of it close to the metal ring.

7.2.6. Securely tape the net and the metal ring to the paper can with autoclave tape.

7.3. Determination of the Percentage of Stage V Gametocytes

7.3.1. Determine the percentage of mature stage V gametocytes in 14-18 day *P. falciparum* culture by Giemsa stained smear.

7.3.2. Count the number of stage V gametocytes in 10 fields at 1000 X magnification (the density of the RBC cells should be 150-350 cells per field). Use the formula:

7.3.3. % Stage V gametocytes = (total number of stage V gametocytes x 100%)/ (total number of RBC cells)

7.4. Evaluation of Exflagellation in Mature *P. falciparum* Gametocyte Culture

7.4.1. Using sterile pipette put 0.2 mL of mixed culture into an Eppendorf tube.

7.4.2. Spin down for 5 sec at 4000 x g in the Eppendorf centrifuge and aspirate supernatant but left same volume of supernatant to the pellet to make approximate 50% hametocrit suspension.

7.4.3. Add an equal volume of normal human O-positive heat-inactivated serum to the suspension (final hematocrit is 25%). Incubate the mixture for 20 minutes at 18ºC in micro-cooler.

7.4.4. Put 15 µL of pellet-serum mixture on a cellometer slide.

7.4.5. Using light microscopy at 400 X magnification, determine the average number of exflagellating centers per quadrant.

7.5. Mosquito Preparation

NOTES

- Female *A. stephensi Nijmegen* mosquitoes 3 - 8 days old (preferably 4 - 6 days old) are used for feeds.
- Mosquitoes are starved for 15 - 24 hours before the feed.
- Mosquitoes can be transferred to pint containers the day before feed.
- During and after the feed, mosquitoes should be kept in pint containers.

7.5.1. Put a bottle with warm water (40ºC – 60ºC) next to the wall of the gallon paper can with mosquitoes to attract female mosquitoes.

7.5.2. Using the mouth aspirator, collect female mosquitoes, up to 20 mosquitoes at a time, from the warm wall of the gallon paper can.

7.5.3. Distribute female mosquitoes into pint containers. Determine the number of mosquitoes distributed in each pint according to the experimental purpose. Usually 50 - 70mosquitoes per container, not more than 100 mosquitoes per pint container.

7.6. Preparation of Test and Control Samples for the Feed

7.6.1. Dispense 60 µL each of samples into labeled 1.5mL tubes.

7.6.2. Transport these tubes to high security feeding room and keep the tubes at 4 ºC if they are not used for feeding in half hour.

7.7. Feeder Preparation in High Security Feeding Room

7.7.1. Connect glass feeders to each other with silicone tubing to form a continuous chain. Connect the chain of feeders to the circulating water bath using plastic tubing connectors.

7.7.2. Stretch Parafilm square (approx. 2.5 cm x 2.5 cm) in both directions and place a thin Parafilm membrane on a membrane feeder. Secure by gentle compression.

7.7.3. Keep the glass feeder chain lifted above the table to protect the integrity of the Parafilm membranes.

7.8. Preparation of a "Pre-Feed Mixture"

NOTES

- "Pre-Feed Mixture" should be kept in sterile conditions before mosquito feed.
- *P. falciparum* mature gametocytes culture and Pre-Feed Mixture must be kept at 37°C at all times to prevent from exflagellation.
- Pre-Feed Mixture should be used for feeds as soon as possible (not more than an hour).
- Human serum pool used for feed dilution is aliquoted into 1 - 5 mL aliquots and stored at -80°C. Freshly thaw the aliquot(s) for each feed.
- When human complement is required for a feed, use a freshly thawed non-heat-inactivated serum aliquot(s).
- All serum, RBC solution should be pre-warmed at 37°C before mixing with gametocyte culture.

7.8.1. Calculate volumes and dilution factors.

7.8.2. Total volume of "Pre-Feed Mixture" needed for the feed:

7.8.3. Minimum volume will be: (200 µL x number of mosquito pints) + 600 µL.

7.8.4. Dilution factor for *P. falciparum* mature gametocyte culture.

7.8.5. Parasitemia of mature Stage V gametocytes in "Pre-Feed Mixture" is 0.15% ± 0.05%.

7.8.6. Calculate volumes of serum, RBC, and mature gametocyte culture pellet needed.

7.8.7. Hematocrit of "Pre-Feed Mixture" is 50%.

7.8.8. Estimate the Hametocrit of the gametocyte culture on the day of feed as 1.5% (or actually measure the culture pellet if needed).

7.8.9. When human complement is required for a feed

7.8.10. Half of the total liquid volume in a "Feed Mixture" should be non-heat-inactivated human serum pool

7.8.11. Depends on the number of samples need to be tested with complement, either 1) make a Pre-Feed Mixture with 20% of heat-inactivated serum and 80% of non-heat-inactivated human serum or 2) make a Pre-Feed Mixture with 100% of heat-inactivated serum and then replace some volume of serum later as Section 7.9.8.

7.8.12. Centrifuge enough volume of normal RBC (50% hematocrit) for 10 min at 2500 x g at room temperature. Remove supernatant and kept at 37°C.

7.8.13. Thaw enough volume of serum pool aliquots at 37°C water bath and kept at 37°C.

7.8.14. Warm centrifuge till 37 °C.

7.8.15. Collect calculated volume of mature gametocyte culture in a pre-warmed 50mL tube and centrifuge for 5 min at 530 x g at 37°C, set brake at level 1. Remove supernatant Keep the tube in a 37°C heat block .

7.8.16. Mix the infected RBC pellet (Section 7.9.5) with the calculated volumes of RBC and serum (7.9.1.3), gently vortex the pellet for 5 sec. (setting 7 of Vortex Genie -2, Diagger) and immediately put the tube back into a 37°C heat block.

7.8.17. Immediately aliquot Pre-Feed Mixture in pre-warmed (37°C) 1.5 mL tubes, 200µL per tube. Number of tubes is same as number of pints will be fed (test samples plus controls). The aliquots should be kept into a 37°C heat block.

7.8.18. When human complement is required for some samples:

7.8.19. Centrifuge required number of 200 µL aliquots of the "Pre-Feed Mixture" in 1.5 mL tubes at 1700 x g for 1 min.

7.8.20. Using a 200 µL, with a barrier tip, carefully take out 80 µL of supernatant (heat-inactivated serum). Discard the tip with the supernatant

7.8.21. With a new tip, add 80 µL of pre-warmed (37°C) non-heat-inactivated serum pool. Mix well.

7.9. Feeding Procedure

7.9.1. Turn on the circulating water bath and set the temperature at 40°C. Do not turn on more than 10 minutes before mosquito feed to avoid a leak from membrane during the feed.

7.9.2. Check the integrity of the Parafilm membrane of the feeders.

7.9.3. Put a warm glass feeder connected with the circulating water bath onto the pint container with mosquitoes.

7.9.4. Using a 200 µL Pipetman with a barrier tip, transfer 60 µL of test or control samples (Section 7.7) into 200 µL of the "Pre-Feed Mixture" (Section 7.9.). This is now "Feed Mixture." Mix by gentle pipetting (at least 10 times).

7.9.5. Put the "Feed Mixture" (260 µL) into the glass feeder.

7.9.6. Make sure that the "Feed Mixture" is distributed over the membrane surface. If not, slightly shake the feeder to let the "Feed Mixture" cover the membrane as completely as possible.

7.9.7. Secure the feeder containing the "Feed Mixture" to the pint container and to the feeding table with autoclave tape.

7.9.8. Place folded paper insulators between the warm silicone tubing and the netting on both sides of the feeder to avoid attracting mosquitoes to the warm tubing when necessary.

7.9.9. Feed all pints the same length of time (20-25 minutes). Use a timer to time each pint.

7.9.10. Immediately after the feed, pints with fed mosquitoes are placed into a high security room at 26°C and relative 75% humidity.

7.9.11. Mosquitoes are maintained on 10% Karo syrup solution in water soaked into cotton balls (changed daily by Insectary personnel)

7.9.12. Mosquitoes are kept up to 8 ±1 days after the feed for midgut dissections.

7.10. Feed Details Recording

7.10.1. Record date of the feed, parasite strain, lot numbers of blood, serum and culture used for the feed, strain and age of mosquitoes, and any comments in the MFA worksheet.

7.10.2. Save the worksheet in a study specific folder in "M:\Reference Center" folder.

7.11. Feeder Clean Up

7.11.1. After the feed, disrupt the Parafilm membranes with absorbent paper and place the chain of feeders into a 10% Clorox solution for at least 1 hour.

7.11.2. Wash feeders with tap water and remove residual Parafilm. Let feeders soak for a few hours or overnight. After a few washes with tap water, rinse the chain of feeders with distilled water and let them dry.

7.12. General Notes of Anesthetization / Inactivation / Decapitation of *P. falciparum* Infected Mosquitoes

7.12.1. Mosquito midgut dissection is usually manipulated in day 8 ± 1 post infection

7.12.2. If needed, a pint with infected mosquitoes can be placed at 4°C for up to 5 days in sealed plastic bags with moist cotton balls on the pints for later dissections

7.12.3. Before midgut dissections, mosquitoes must be inactivated or decapitation

7.12.4. None of mosquitoes will be taken out from the high security area without decapitation

7.12.5. In the most of cases, all of mosquitoes in a pint are anesthetized together, inactivated with 70% ethanol, and then dissected in the high security area. However, in some cases, only part of mosquitoes in a pint is anesthetized (Section 7.15) and/or mosquitoes are decapitated without ethanol inactivation (Section 7.16). Refer the specific section(s).

7.13. Anesthetization and Inactivation before Dissection

NOTE: If a part of mosquitoes in a pint is anesthetized/inactivated, skip to Section 7.15.

7.13.1. Bring one pint with infected mosquitoes at a time to the procedure room.

7.13.2. Pour 3 - 5 mL of chloroform inside a glass anesthesia jar.
7.13.3. Put some talc powder on the dental rubber covering the openings of the pints with mosquitoes to ensure that the aspirator tube moves freely in and out of the pint.
7.13.4. Place the pint with infected mosquitoes on its side into the glass anesthesia jar. Close the jar lid. Observe mosquitoes through the net.
7.13.5. Make sure all mosquitoes stop flying and fall to the bottom of the pint (usually 30sec -1min).
7.13.6. Open the jar and take the pint out.
7.13.7. Aspirate all mosquitoes by electronic aspirator (don't use mouth aspirator here!) through the rubber window and transfer all of them to a Petri dish containing 70% ethanol immediately.
7.13.8. Cover the Petri dish and shake it gently. Make sure all mosquitoes are fully covered by ethanol.
7.13.9. Transfer all mosquitoes on the whatman filter paper to remove extra ethanol, and immediately transfer all mosquitoes in another Petri dish containing 1x PBS.
7.13.10. Seal the Petri dish with the mosquitoes by parafilm and bring the dish to the dissection room in high security area

7.14. Anesthetization and Inactivation for a Part of Mosquitoes in a Pint

NOTE: This section is only applied when a part of mosquitoes in a pint will be anesthetized/inactivated.

7.14.1. Prepare an anesthesia pint.
7.14.2. Prepare a pint paper can with a side opening covered with dental rubber as described in Section 7.3.
7.14.3. Place two net squares on a top (open) part of the paper can and put the metal ring over the net. Ensure that the ring fits tightly.
7.14.4. Bring one pint with infected mosquitoes at a time to the procedure room.
7.14.5. Put some talc powder on the dental rubber covering the openings of the pints with mosquitoes and the anesthesia pint to ensure that the aspirator tube moves freely in and out of the pint.
7.14.6. Pour 3 - 5 mL of chloroform inside a glass anesthesia jar
7.14.7. Transfer up to 10 mosquitoes at a time with the aspirator to the anesthesia pint, till the number of mosquitoes needed is reached. While holding the anesthesia pin, slightly pressing the metal ring to keep the anesthesia pint standing on a bench top. Constantly watch mosquitoes in the aspirator tube while aspirating (keep blowing in) and removing (keep blowing out)
7.14.8. Check that no mosquitoes are present in the aspirator tube before removing it from the anesthesia pint (keep blowing out). Count the number of mosquitoes transferred.

7.14.9. Place the anesthesia pint with transferred mosquitoes into the glass anesthesia jar with chloroform vapors (Section 7.15.4). Close the jar lid. Observe mosquitoes through the net.
7.14.10. Make sure all mosquitoes stop flying and fall to the bottom of the pint (usually 30sec-1min).
7.14.11. Open the jar and take the pint out.
7.14.12. Remove the net and transfer all mosquitoes to a Petri dish containing 70% ethanol immediately.
7.14.13. Cover the Petri dish and shake it gently. Make sure all mosquitoes are fully covered by ethanol
7.14.14. Transfer all mosquitoes on the whatman filter paper to remove extra ethanol, and immediately transfer all mosquitoes in another Petri dish containing 1x PBS.
7.14.15. Seal the Petri dish with the mosquitoes by parafilm and bring the dish to the dissection room in high security area.

7.15. Decapitation of Infected Mosquitoes without Ethanol Inactivation

NOTE: This section is only applied when mosquitoes will be decapitated without ethanol inactivation.

7.15.1. Place a piece of water moisture filter paper into each Petri dish.
7.15.2. Follow Section 7.15.1-7.15.9, except more than 10 mosquitoes should not be anesthetized at a time.
7.15.3. Remove the net and carefully place mosquitoes onto white filter paper.
7.15.4. Decapitate mosquitoes with scalpel. Move cut mosquito head away from mosquito body for each mosquito. Confirm the number of mosquitoes to be same as the mosquitoes transferred.
7.15.5. Check all decapitated mosquitoes one more time, moving them individually from one side of the filter paper to another side.
7.15.6. Place decapitated mosquitoes into a Petri dish with filter paper (Section 7.16.1).
7.15.7. Repeat Section 7.16.2 - 7.16.6, till decapitate enough number of mosquitoes.
7.15.8. Seal the side Petri dish with Parafilm to keep the lid attached and to prevent drying of mosquitoes.
7.15.9. Keep Petri dish with decapitated mosquitoes at 4 ^{o}C. The dish can be transferred to the outside of high security area. If dissections are not done immediately, keep the Petri dish at 4^{o}C for up to 3 days.

7.16. Preparation of Mercurochrome Solution

7.16.1. Make 2% mercurochrome solution (stock solution) in distilled water. Sterilize by filtering through 0.2 µm sterile Nalgene filter. Store at room temperature in the dark for up to 5 years.

7.16.2. Dilute the stock solution 1:40 with 1x PBS to the working concentration of 0.05%. For example, to make 40 mL of working solution, mix 39 mL of water and 1 mL of 2% mercurochrome solution. Store at room temperature in the dark for up to 2 months.

7.17. Dissection of Mosquitoes

NOTE: Mosquitoes must be inactivated and/or decapitated prior to dissection.

7.17.1. Have 0.05% mercurochrome solution ready prior to starting dissections.
7.17.2. Select mosquitoes with formed eggs. Only mosquitoes with eggs are used for midgut dissections.
7.17.3. Put a drop of 0.05% mercurochrome solution on a dissecting slide (depending on the operator preference, use regular or cavity microscope slides).
7.17.4. Take a mosquito from a Petri dish to the mercurochrome drop. Close the Petri dish during the dissection.
7.17.5. Carefully, using two dissecting forceps, pull the posterior part of mosquito with one pair of forceps, holding the anterior part of mosquito at the same time with the other forceps. Separate mosquito midgut and put it into a well filled with 0.05% mercurochrome solution on a cavity microscope slide. Stain for 20 - 45 minutes.
7.17.6. Transfer midguts onto a regular microscope slide in a drop of mercurochrome and cover with a cover slide.
7.17.7. Note 1: Depending on the operator preference, midguts can be transferred to a dry microscope slide, and then add a drop of mercurochrome before covering with a cover slide.
7.17.8. Note 2: Do not let the midguts dry. Add more mercurochrome if necessary.
7.17.9. Examine and count oocysts using the light microscopy at 100 X magnification. Use 400 X magnification in questionable cases.
7.17.10. Record the oocyst counts in MFA worksheet and save the worksheet in a study specific folder in “M:\Reference Center” folder.

7.18. Data Processsing

7.18.1. % inhibition of oocysts and of infected mosquitoes