“Establishment of a phage display platform for the isolation of *Plasmodium falciparum* specific monoclonal antibodies”

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1 INTRODUCTION

1.1 PREVALENCE, SEVERITY AND IMPACT ON WORLD HEALTH

Malaria is an important cause of morbidity and mortality, particularly in sub-Saharan Africa where it accounts for between 300-500 million infections per year with 1.5-2.7 million deaths, with more than 90% of the deaths occurring in children under the age of 5 [1]. More than 90% of these deaths occur on the continent of Africa, and are due to Plasmodium falciparum, one of the five main species of plasmodium that infects man [2]. Infected female anopheles mosquitoes transmit the parasite when they bite to take a blood meal. Latest estimates suggest that malaria kills more people than HIV [3].

In many developing countries and in Africa especially, malaria exacts an enormous toll on life, not only in medical costs, but also in days of labour lost. The goal of producing an effective malaria vaccine still remains elusive, several years after the cloning of the malaria genes due in part to the multistage life cycle of the parasite, each with stage specific antigens, and the genetic diversity of the Plasmodium falciparum parasite, coupled with the poor understanding of the complex nature of the immune response to the parasite [4].

Despite intense research, either to eradicate malaria or lessen its fatality and morbidity, an effective control strategy still remains to be achieved. The development of resistance to cheap and previously reliable drugs like chloroquine by the Plasmodium parasites, particularly falciparum require that new therapeutic measures be developed if the fight against the disease is to be won.

1.2 PLASMODIUM FALCIPARUM LIFE CYCLE

Plasmodium parasites encode for at least 5600 genes [5] and have complex life cycles that include obligate intracellular asexual growth within vertebrate hepatocytes (the pre-erythrocytic stages) and erythrocytes (the blood or erythrocytic stages). Sexual differentiation is initiated in the vertebrate host while fusion of gametocytes and further parasite propagation via sporogony occurs in the mosquito vector.

The sexual stage in man begins when the female anopheles bites to take a blood meal during which it injects, on the average, 10-100 motile spindle shaped sporozoites into the dermis (rarely
directly into the blood stream). In the dermis, the injected parasites actively move to escape phagocytosis by CD11b+ phagocytic cells [6].

While most of the inoculated sporozoites are removed, the remaining sporozoites are transported through the blood stream to the liver where through a series of ligand receptor interactions between antigens on the sporozoites surface and receptors on the hepatocyte, the sporozoites enter the hepatocytes to begin the process of pre-erythrocytic schizogony, an essential but clinically silent phase of the life cycle, culminating in the formation of numerous merozoite-filled vesicles called merosomes which are transported through the hepatic sinusoids to the lungs. The merozoites are ultimately released into the blood stream when the hepatocytes rupture. The merozoites will within 30 seconds, through a complex but highly efficient process, invade the red blood cells to start the erythrocytic schizogony. The invasion ligands are present on the merozoite surface or harbored in apical organelles like the micronemes and the rhoptries.

During the following 48 hours, the parasites run through an intra-erythrocytic cycle of asexual replication through ring forms, mature trophozoites and then schizonts which contain the numerous merozoites. It is the invasion and multiplication of the merozoites in the red cells, and the subsequent rupture of the red blood cells to release these merozoites together with parasite metabolites that is responsible for the signs and symptoms of the disease.
Fig 1-1. Life cycle of the Plasmodium, the causative organism of malaria. Injection of the sporozoites into the skin is followed by travel into the liver to start the hepatic stage which is important for the life cycle but clinically silent. The parasites undergo maturation (exoerythrocytic phase) and are released when the hepatocyte ruptures. The released merozoites then enter the erythrocytes (erythrocytic phase), are released after maturation with some developing into sexual forms, which are picked up when the mosquito bites to take a blood meal. (Credit NIAID; http://www.niaid.nih.gov/topics/malaria/pages/lifecycle.aspx)

The released merozoites infect new red blood cells to start another erythrocytic cycle.

Some of the merozoites, through an unknown trigger, develop into microgametocytes and macrogametocytes, the sexual forms, which are ingested by the mosquito when it bites to take
another blood meal. It has been indicated that morphological changes associated with the
development of the sexual parasite are accompanied by distinct biochemical events like the
regulation of stage-specific gene expression at either the transcriptional or translational level [7, 8].
They travel to the mosquito mid gut where the microgametocyte exflagellates into a mature male
gamete and then fertilizes the macrogamete resulting in the formation of a non-motile zygote.
The zygote then develops into a motile ookinete that passes through several membranes and ends
up on the outer wall of the stomach as an oocyst.
Sporozoites contained therein are released into the haemocoel and finally travel to the salivary
glands ready to be injected into the skin when the mosquito next bites to take a blood meal.

1.3 MALARIA PREVENTION AND CONTROL
The 2015 goals of the World Health Organization’s Roll Back Malaria Partnership are to reduce
global malaria cases by 75% from 2000 levels and to reduce malaria deaths to near zero through
universal coverage by effective prevention and treatment interventions [9]. To this end, various
strategies and measures have been employed over the years to effectively lessen its morbidity
and mortality.
This has included the widespread implementation of the use of insecticide-treated bednets,
household spraying of residual insecticides, and effective drugs to reduce mortality and interrupt
transmission. These measures have however been hampered by political instability, war or
economic underdevelopment.
Quite apart from optimizing existing methods, it will be important to introduce new
complementary strategies, like vaccines especially as malaria remains one of the major diseases
in the world for which a vaccine is unavailable.

1.4 MALARIA VACCINES
It is generally believed that different types of malaria vaccines may be necessary for different
populations represented in the extremes by (1) individuals from areas with no exposure who
travel to malaria endemic areas, who unlike the inhabitants of malaria endemic areas are usually
prone to the life threatening forms or complications of the disease, for which a passive vaccine
may suffice, and (2) infants and children with heavy exposure to Plasmodium falciparum who
are exposed on an almost daily basis to the scourge of the disease, who may require an active
vaccine. Within these two extremes lies a wide array of populations affected by malaria. A proper understanding of the epidemiology of malaria is thus crucial to the design of an effective vaccine that will address the specific requirements of all these population groups especially considering the complex genetically variable human immune response and differences in transmission dynamics.

There are three general approaches to malaria vaccine development that may be pursued; maximization of the magnitude and quality of immune responses to a single or a few antigens such as merozoite surface protein one (MSP1) and circumsporozoite protein one (CSP1), induction of good or optimum immune responses to multiple antigens from different stages and duplication of whole-organism-elicited immunity. Pre-erythrocytic vaccines (vaccines that target the parasite before it reaches the blood), in particular vaccines based on circumsporozoite protein one (CSP1) make up the largest group of research for the malaria vaccine. Other vaccine candidates include those that seek to induce immunity to the blood stages of the infection, those that seek to avoid more severe pathologies of malaria by preventing adherence of the parasite to blood venules and placenta, and transmission-blocking vaccines that would stop the development of the parasite in the mosquito right after the mosquito has taken a blood meal from an infected person.

1.5 VACCINE TARGETS

The proteins expressed on the surface of the merozoite appear to be important targets of host immunity and therefore could be potential candidates for vaccine development [10]. The arguments for merozoite surface proteins as prime targets are hinged not only on the accessibility of these antigens/proteins to plasma antibodies, or on the fact that the clinical manifestations of the disease, including fever, anaemia, and coma are thought to be due to the invasion and exponential multiplication of the merozoites in the red cell and the subsequent rupture of the red cell, but also on the fact that antibodies against merozoite stage antigens have tended to reduce the severity and frequency of disease, and in some studies have prevented individuals from getting the disease [11].

The blood stage is thus crucial, and inclusion of a blood stage component in a multistage malaria vaccine would not only prevent disease caused by “leaky” pre-erythrocytic immunity, but would also protect against epidemics in vulnerable populations.
1.6 CHOICE OF ANTIGENS

Studies have shown that adult residents of holoendemic malaria regions have a naturally acquired partial immunity to malaria that renders them more resistant to new infections, limits parasitaemia, and reduces the frequency and severity of illness. This semi immunity, which is achieved in essentially all individuals, including older children, after sufficient exposure, is based on (I) the presence of specific serum antibodies, presumably a broad variety of antibodies against many different Plasmodium falciparum antigens and (II) the presence of immune cells that react to certain antigens of Plasmodium falciparum and play a central role in cell mediated immunity [12].

The most convincing evidence for the role of antibodies in malaria has come from clinical studies in which hyper-immune serum or purified immunoglobulins from adults, substantially modified the course of infection, reducing peak parasitaemias and leading to spontaneous resolution when administered to sick children [13, 14].

The direction of malaria research has shifted towards questions of protein structure, function and interaction, in particular at the host-parasite interface. Antibodies can, in this regard, provide important insights, into the structure and function of key antigens, for example by identifying epitopes that are best suited for incorporation into vaccines. Antibodies and immune cells from immune residents of holo- and hyper-endemic malaria areas may also provide crucial information about the role of these target antigens in natural immunity to malaria and pave the way for in-depth evaluation in clinical trials. Antibodies can also be effective in controlling malaria by reducing the damage that results from excessive host inflammatory responses associated with the disease [15].

The antigens selected for this study were based on cumulative evidence that the presence of antibodies to these antigens may be associated with protection. Life threatening cases of malaria can in principle, be treated with these antibodies, implying that a readily available source of antibodies, with defined specificities is required. Again, successful immunization with irradiated sporozoites and the induction of partial immunity (also called premunition) to infection and high levels of protection against the clinical manifestations also suggest that a vaccine is feasible. Understanding the molecular and immunological mechanisms
of the interaction between the parasite and the host is pertinent for the rational discovery and development of a safe, affordable, and protective anti-malaria vaccine.

1.6.1 MSP1-19

Merozoite Surface Protein1 (MSP1) which constitutes the major protein component on the merozoite surface [16] is synthesized as an approximately 200 kDa protein precursor and is held on the surface of the developing merozoite by a GPI (glycosyl phosphatidyl inositol) anchor.

In late schizogony, merozoites undergo maturation during which MSP1 is cleaved proteolytically into 4 major fragments which remain non-covalently associated at the surface of the parasite.

At the time of erythrocyte invasion, a second proteolytic cleavage separates MSP1-19 from the rest of the complex and remains on the surface whiles the remaining complex is shed from the surface of the parasite.

MSP1-19 is a major surface protein and a leading vaccine candidate, and various experiments have shown that antibodies against MSP-19 are protective not only against monkey and rodent malaria parasites, but human parasites as well and that immunization with MSP1 affords antiparasite protection in experimental animals [17-19].

It is now known, that people living in malaria-endemic areas have among others, anti-MSP1-19 specific antibodies and that these antibodies play a critical role in the immunity against malaria by their inhibition of erythrocyte entry [20]. There is also good evidence that Abs against MSP1-19 provide the major component of the invasion-inhibitory activity of human immune sera [21].

1.6.2 AMA1

AMA1 is an integral membrane protein that is initially localised to the micronemes of the merozoite but spreads to the merozoite surface after schizont rupture. It is synthesized during the late schizont stage as an 83kDa precursor protein and is thought to be involved in the apical re-orientation of the merozoite, and possibly tight junction formation just before invasion occurs [22-24].

It is a promising vaccine candidate, antibodies to which are also found in the serum of malaria immune individuals. It is relatively well conserved within and between species and studies have demonstrated a strong correlation between anti-AMA1 antibody titres and levels of protection. It
undergoes processing into a 66kDa protein and is also known to play a role in erythrocyte invasion [25, 26] making it an ideal vaccine candidate. Since AMA1 is also thought to be expressed on sporozoites, an AMA1 vaccine could also have some pre-erythrocytic effects.

1.6.3 MSP3
MSP3 is a 48 kDa protein that is the product of a proteolytic processing of a precursor protein and associates with other proteins on the merozoite surface [27, 28]. MSP3 may have a role in erythrocyte invasion as parasites with a truncated form of the protein invade erythrocytes less efficiently [29].

1.7 ANTIBODY STRUCTURE, ANTIBODY FRAGMENTS AND THEIR GENERATION
Antibodies are the secreted form of the B-cell receptor and are identical to the B-cell receptor of the cell that secretes it. There are five major types of antibodies. These are IgG, IgA, IgM, IgE and IgD. The prototype antibody, IgG, is roughly Y-shaped, consisting of three equal-sized portions, loosely connected by a flexible link.

They are made up of two identical heavy, and light chains lambda and kappa. Each light chain is made up of one variable domain (VL) and one constant domain (CL), while each heavy chain is formed by one variable (VH) and three constant regions (CH1-CH3).

The high antigen affinity and specificity of the variable heavy and light chain segment is determined by three hypervariable complementarity-determining regions (CDR1-3), which are flanked by the relatively constant framework elements. Due to the antibody symmetry, the two antigen binding sites contain exactly the same molecular structure, resulting in two identical antigen binding specificities making these proteins bivalent. The constant region mediates the effector function.

Although a given antibody or immunoglobulin has either lambda or kappa light chain and not both, no functional difference has been found between antibodies having lambda or kappa light chains and either type of light chains may be found in antibodies of any of the five major classes in humans.

The protein fragments obtained after proteolysis of the antibody molecule are determined by where the protease cuts the antibody molecule in relation to the disulfide bonds that link the two
heavy chains which lie in the hinge region between the $C_H1$ and $C_H2$ domains. Papain for example cleaves the antibody molecule on the amino-terminal side of the disulfide bonds and releases the two arms of the antibody (Fab fragments).

In naturally occurring immunoglobulins (Igs), the $V_H$ and $V_L$ regions are held together by the $C_H1$ and $C_L$ domains as well as by an interchain disulfide bond. These four regions, together with the interchain disulfide bond, comprise a Fab.

In contrast to the smaller Fv fragment, Fabs are much more stable, making them more broadly applicable not only to research but also to immunodiagnostic and immunotherapeutic applications; however, this stability results from a relatively high molecular weight of 50 kDa.

![Schematic diagram of the antibody molecule showing the various fragments obtained after proteolysis by different proteases.](http://www.piercenet.com/browse.cfm?fldID=4E03B016-5056-8A76-4ECA-982DA6CAAC8A)

The high diversity of the antibody repertoire in humans (about $10^9$) is achieved by somatic or V (D) J recombination during B cell development. This describes the random combination of one, variable” (V), one, diversity” (D), one, joining” (J) element as well as three to four constant segments for the heavy chain and one V-segment, one J-segment as well as one constant segment for the light chain.

### 1.8 MONOCLONAL ANTIBODY GENERATION

For more than 100 years, polyclonal antisera have been produced by immunization of animals
With the advent of hybridoma technology, which is based on the fusion of antibody producing B-lymphocytes with plasmacytoma cells or lymphoblastoid cell lines, it became possible to produce monoclonal antibodies of defined antigen specificity [31] in mice and rats, but only very few human antibodies were generated. Currently, two alternative strategies are used for the generation of human antibodies: transgenic mice and by using in vitro selection technologies. Transgenic mice contain the human immunoglobulin gene repertoire instead of the murine, allowing the generation of human antibodies by hybridoma technology [32-34]. An advantage of transgenic mice is the in vivo affinity maturation after immunization. Transgenic mice have already yielded a significant number of antibodies under clinical evaluation although it is limited in respect of toxic and highly conserved antigens [35].

One major problem with the hybridoma technology is the development of the Human Anti Mouse Antibody (HAMA) response where antibodies are developed against the mouse derived therapeutic antibodies when they are applied to humans leading to an increase in clearance of the foreign antibody, a decrease in efficacy with repeated administration, and the potential for allergic reactions or serum sickness thus necessitating the development of an immunotherapeutic molecule that can be used in humans. Again the hybridoma technique like the EBV transformation has a relatively lower efficiency [36].

1.8.1 PHAGE DISPLAY TECHNOLOGY

The technology (phage display) involves the combinatorial display of proteins or peptides on the surface of bacteriophages and provides an invaluable tool for screening diverse libraries for polypeptides that have a high affinity for a given target.

It includes a powerful enrichment strategy that allows rapid selection of reagents with desired properties and allows for genes of the antibody fragments to be easily manipulated to introduce mutations that enhance the binding characteristics of the antibody reagents.

DNA encoding millions of variants of certain ligands is batch-cloned into the phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI, or pVIII). Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the ligand being presented on the phage surface; its genetic material resides within the phage
particle. This connection between ligand genotype and phenotype allows the enrichment of specific phage, e.g., using selection on an immobilized target.

The antigen of interest is immobilized to a solid surface, such as nitrocellulose, magnetic beads, column matrixes, or most widely on plastic surfaces with high protein binding capacity, such as polystyrene tubes or microtitre plate wells. The antibody phage is incubated with the surface-bound antigen followed by stringent washing to remove the vast excess of non-binding antibody phages. Bound antibody phage are then eluted and reamplified by infection of Escherichia coli. Successful panning relies on a single molecular interaction of the antibody phage with the antigen in a tremendous background of interfering molecules. Consequently, the quality of the surface-coupled antigen and the panning conditions (pH, salt concentrations, competitors and blocking reagents) determine the quality of the derived antibody. The selection cycle is repeated by infection of the phagemid bearing E. coli cells from the former panning round with a helper phage to produce new antibody phage, which can be used for further panning rounds until a significant enrichment of antigen-specific phage is achieved.

Although a single panning round can be carried out, because of the usually low titers of output phage and the low numbers of clones with inserts, 2-3 panning rounds are usually carried out after which an Enzyme Linked Immunosorbent Assay is carried out to determine Phage clones with Fab inserts that bind to the target protein of interest.

Depending on the use to which the antibody will be put, a full sized antibody can be generated.

1.9 EXPRESSION OF ANTIBODY GENES IN PLANTS

Expression of full sized antibodies in plants was pioneered in 1989 by Hiatt et al. and has subsequently brought in its wake, the expression of various antibody formats, including Fab fragments, scFv’s, bispecific scFv’s and dAb for industrial and diagnostic applications amongst others [37-42].

By exploiting the protein sorting and targeting mechanisms of the plant cells, accumulation and stability of the recombinant antibody can be optimized, although the antibody expression levels are largely dependent on the plant species and on the intrinsic properties of the antibody itself.

Transient expression in *Nicotiana benthamiana* leaves for example, is known to allow the production antibodies within a few days after the expression plasmid has been obtained,
demonstrating the promise for using plants as bioreactors for the molecular farming of recombinant therapeutics, diagnostics, and antibodies [43].

1.10 AIMS OF THE THESIS

The development of resistance to the artemisinin group of drugs by *Plasmodium falciparum* [44] requires not only measures to ensure the prudent use of these antimalarial medications, but also the development of new drugs and the adoption of new strategies like antibody therapy and vaccine development if the fight against the parasite is to be won. The antibodies could be used in passive therapy in infections in malaria-naïve individuals and as prophylaxis for travelers to malaria endemic areas.

The Fraunhofer MP3 Malaria project aims amongst other things to establish platforms and pipelines that will allow for the isolation of *Plasmodium falciparum* specific antibodies that will not only bind to specific antibodies but also most importantly inhibit merozoite entry into the erythrocytes, the most important step in the symptomatology of malaria. In this regard 3 different approaches are being worked on by different PhD candidates at the Fraunhofer Institute; EBV transformation, single cell sorting and the phage display technology all with similar aims and objectives.

The phage display part of the project, which is the subject of this thesis, involves the construction of a functional human immune library that will allow for the successful isolation of monoclonal binders to select merozoite stage antigens of *Plasmodium falciparum*. If established, it will be the first of its kind in-house.

The first part of the thesis will be an immunoepidemiological survey that will serve to characterize various plasma samples from two malaria endemic areas with respect to their reactivity to selected merozoite antigens and their ability to inhibit merozoite entry into erythrocytes. This will also serve as the basis, not only for the selection of the probes to be used in the construction of the phage library, but also as a basis for selection of which ones will be used in single cell sorting and Epstein Barr virus transformation.

The top scorers after characterization, will be selected, total RNA isolated from their whole blood samples and reverse transcribed into cDNA. This will be followed by amplification of the V-genes and another amplification step to introduce restriction sites that will allow for the
cloning of the V-genes into a Fab phage display phagemid. A two-step cloning strategy will be employed to clone the light and the heavy chains into the vector following which electrocompetent *E.coli* cells will be transformed to create a library. It is envisaged to create a library of functional size $10^7$. A subtractive panning strategy will then be employed to isolate high affinity binders to the merozoite antigen(s). A monoclonal antibody-Fab-phage ELISA will be carried out using clones from the last selection round to detect binders. Monoclonal antibody-Fab-phages will be selected and soluble Fabs expressed using *E.coli* after which the soluble Fabs will be applied in an ELISA to determine binding to the antigen(s). A full sized-monoclonal antibody will be constructed from the Fab(s) using the *Nicotiana benthamiana* expression system so chosen because its expression system has been perfected in-house. Characterization of the antibody generated will involve ELISA, SDS-PAGE, surface plasmon resonance, and growth inhibition assay.
Fig 1-3 Workflow schematic depiction of the steps involved in this thesis.
2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS AND CONSUMABLES
Chemicals and Consumables were purchased from: Bio-Rad (Munich), Biozym (Oldendorf), Eppendorf (Hamburg), Invitrogen (San Diego, USA), Merck (Darmstadt), Millipore (Eschborn), Roth (Karlsruhe), Sigma Aldrich (Taufkirchen).

2.1.2 EQUIPMENT AND SUPPLY

<table>
<thead>
<tr>
<th>Device</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Autoclave</td>
<td>Varioklav H+P 75 S, H+P Labortechnik (Oberschleißheim)</td>
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<tr>
<td>Centrifuges</td>
<td>Eppendorf (Hamburg)</td>
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<td></td>
<td>Heraeus Multifuge 3 S-R, Kendro (Osterode)</td>
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<td>Avanti TM J251, Beckmann Coulter (Krefeld)</td>
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<td>Electroporator</td>
<td>Electroporator 2510 (Eppendorf)</td>
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<td>Milli-Q Device</td>
<td>Membrane pure, Membra-Pure (Bodenheim)</td>
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<td>PAA-Gel electrophoresis</td>
<td>Mini-PROTEAN II electrophoresis cell, Bio-Rad (München)</td>
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<td>pH-Meter</td>
<td>Basic Meter PB-11, Sartorius (Göttingen)</td>
</tr>
<tr>
<td>Photodocumentation</td>
<td>Gel Doc XR system, Bio-Rad (München)</td>
</tr>
<tr>
<td></td>
<td>Epoch multi-volume spectrophotometer system, BioTeK (Bad Friedrichshall)</td>
</tr>
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</table>
NanoDrop ND-1000 PeqLab,

Pipettes Pipetman, Gilson (Middleton, USA)

Rotors JA-10, JA-25.50, Beckmann Coulter (Krefeld)

Scale TE31025, TE6101, TE12000 Sartorius (Göttingen)

Sequencer ABI Prism 3730 Capillar Sequencer, Applied Biosystems (Foster City, USA)

Surface BIACORE® 2000 (BIACORE, Uppsala, Sweden) + PC, Windows NT 4.0 operating system (Microsoft) and Software (BIAControl 1.3 and BIAEvaluation 3.0).

Spectrophotome Epoch multi-volume spectrophotometer system, BioTek (Bad Friedrichshall)

Thermocycler Primus 96 plus, Aviso (Jena)/ AmpliSpeed Slide Cycler, Beckman Coulter (Krefeld)/ Mastercycler Gradient, Eppendorf (Hamburg)

2.1.3 BUFFERS

**TAE Buffer** | **Orange-G Loading Buffer (6x)**
--- | ---
TrisBase | 2 M | 30 % (v/v) glycerine
KHCO₃ | 100 mM | 0,1 % (v/v) Orange-G
EDTA | 370 mg/L |  

**10x PCR Buffer (Invitrogen, USA)** | **10x NEBuffer 4 (NEB, Frankfurt am Main)**
--- | ---
Tris-HCl (pH 8.4) | 200 mM, | 0,5 M potassium acetate
KCl 500 mM 0,1 M Magnesium Acetate
10 mM Dithothreitol
pH 7.9

10x T4 Ligase Buffer (NEB, Frankfurt am Main)

1 M NaCl 0,5 M Tris-HCL
0,5 M Tris-HCl 0,1 M MgCl₂
0,1 M MgCl₂ 10 mM ATP
10 mM Dithiothreitol 0,1 M Dithiothreitol
pH 7.9 pH 7.5

Extraction Buffer: PBS supplemented with 0.19 % Na-disulfide; pH 7.4

Protein A

- 5x binding buffer: 1 M TrisHCl, pH 9.0
- 1x binding buffer: 0.2 M Tris HCl, pH 9.0
- Elution buffer: 0.2 M Na-Citrate, pH 2.5

- 2xTY-Medium/Agar (pH 7.4)
- Trypton 1.6 % (w/v)
- Yeast extract 1 % (w/v)
- NaCl 0,5 % (w/v)
- (Agar – Agar) 1.5 % (w/v)
- At a temperature of about 50°C 100 µg/ml ampicillin and 2% (w/v) glucose was added.
- YEB medium
  : 0.5 % (w/v) beef extract, 0.1 % (w/v) yeast extract, 0.5 % peptone, 0.5 % (w/v) sucrose,
  0.05 % (w/v) MgCl₂
2.1.4 ANTIGENS
AMA1 was produced under GMP conditions in *P. pastoris*, MSP1-19 was produced in HEK cells and MSP3 was produced in plants. They were all together with the 3D7 crude *Plasmodium falciparum* lysate a kind donation from Dr. Rolf Fendel.

2.1.5 BACTERIAL STRAINS AND MEDIA
Media were prepared using two-fold distilled Mill-Q water (Millipore GmbH, Schwalbach) and sterilized by autoclaving at 121°C for 21 min at 1 bar. Heat labile components were filtered and added to the autoclaved substances after cooling down to approximately 50°C. Adjustment of pH was with 10 M NaOH or 1 M HCl. Commercially prepared electroporation-competent *Escherichia coli* TG1_ [F’ traD36 proAB lacIqZ ΔM15] supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rK - mK -] (Lucigen, USA) and XL1-Blue MRF_ (Stratagene, Amsterdam,NL) (F::Tn10(Tetr) proAB+lacIq_(lacZ)M15/recA1 endA1 gyrA96 (Nalr) thihsdR1 (rK mK+)glnV44relA1lac) were grown in 2*TY media at 37 °C.

2.1.6 PHAGES
M13KO7 helper phage (New England Biolab) is an M13 derivative which carries the mutation Met40Ile in gIII, with the origin of replication from P15A and the kanamycin resistance gene from Tn903 both inserted within the M13 origin of replication.

2.1.7 PLANTS
*Nicotiana benthamiana* was used for transient protein expression after vacuum infiltration of recombinant agrobacteria

2.1.8 ENZYMES AND REACTION KITS
Buffers for ligation and restriction of DNA were obtained from NEB (New England Biolabs, Frankfurt am Main).

Restriction Enzymes: ApaL1, Not, SaLI, SfiI and, BstNI (New England Biolabs, Frankfurt am Main)

Ligase: T4 DNA Ligase (New England Biolabs, Frankfurt am Main)
Kits:

- Nucleo Spin Extract II (Macherey Nagel, Düren)
- Nucleo Spin Plasmid (Macherey Nagel, Düren)
- NucleoBond® Xtra Midi / Maxi (Macherey Nagel, Düren)

Vivaspin6 concentrating spin columns, 10.000 Da MWCO, (Sartorius Stedim)

Platinum®Taq DNA Polymerase (Invitrogen, Eggenstein)

2.1.9 HUMAN PHAGE DISPLAY PRIMERS

2.1.9.1 VH Primer  5’ to 3’ sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-Sequence</th>
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<tbody>
<tr>
<td>VH1a</td>
<td>5’-CAG GTG CAG CTG GTG CAG TCT GG-3’</td>
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<tr>
<td>VH1b</td>
<td>5’-CAG GTG CAG CTG GTG GAG TCT GG-3’</td>
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<tr>
<td>VH1c</td>
<td>5’-CAG GTC CAG CTT GTG CAG TCT GG-3’</td>
</tr>
<tr>
<td>VH2a</td>
<td>5’-CAG GTC ACC TTG AAG GAG TCT GG-3’</td>
</tr>
<tr>
<td>VH2b</td>
<td>5’-CAG ATC ACC TTG AAG GAG TCT GG-3’</td>
</tr>
<tr>
<td>VH3</td>
<td>5’-GAG GTG CAG CTG GTG TCG GG-3’</td>
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<td>5’-CAG GTG CAG CTG CAG GAG TCG GG-3’</td>
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<tr>
<td>VH5a</td>
<td>5’-GAG GTG CAG CTG TTT CAG TCT GC-3’</td>
</tr>
<tr>
<td>VH5b</td>
<td>5’-GAG GTG CAG CTG GTG CAG TCT GC-3’</td>
</tr>
<tr>
<td>VH5c</td>
<td>5’-GAG GTG CAG CTG TTT CAG TCT GC-3’</td>
</tr>
<tr>
<td>VH6</td>
<td>5’-CAG GTA CAG CTG CAG TCA GG-3’</td>
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2.1.9.2 VH-(SfiI)-Primer  5’ to 3’ sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-Sequence</th>
</tr>
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<tbody>
<tr>
<td>VH1a (SfiI)</td>
<td>5’-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG</td>
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<td>CAG</td>
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<tr>
<td>VH1b (SfiI)</td>
<td>5’- GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG</td>
</tr>
<tr>
<td>CAG</td>
<td>CTG</td>
</tr>
</tbody>
</table>
2.1.9.3 Vκ-Primer

Vκ1:  5´-GAC ATC CAG ATG ACC CAG TCT CC-3´
Vκ2a: 5´-GAT ATT GTG ATG ACC CAG ACT CC-3´
Vκ2b: 5´-GAT ATT GTG ATG ACC CAG TCT CC-3´
Vκ3a: 5´-GAA ATT GTG CTG ACA CAG TCT CC-3´
Vκ3b: 5´-GAA ATT GTG CTG ACT CAG TCT CC-3´
Vκ3c: 5´-GAA ATT GTG TTG ACA CAG TCT CC-3´
Vκ3d: 5´-GAA ATT GTG TTG ACT CAG TCT CC-3´
Vκ4:  5´-GAC ATC GTG ATG ACC CAG TCT CC-3´
Vκ5:  5´-GAA ACG ACA CTC ACG CAG TCT CC-3´
 Vk8:  5’-GAG ATT GTG ATG ACC CAG ACT CC-3’
 Vk10:  5’-GAC CAC GTG ATG ACC CAG TCT CC-3’

 2.1.9.4 Vk(ApaL1)Primer

 5’ to 3’ sequence

 Vk1 (ApaL1)  5’-TGA GCA CAC AGT GCA CGA CAT CCA GAT GAC CCA GTC TCC-3’
 Vk2a (ApaL1)  5’-TGA GCA CAC AGT GCA CGA TAT TGT GAT GAC CCA GAC TCC-3’
 Vk2b (ApaL1)  5’-TGA GCA CAC AGT GCA CGA TAT TGT GAT GAC CCA GTC TCC-3’
 Vk3a (ApaL1)  5’-TGA GCA CAC AGT GCA CGA AAT TGT GCT GAC ACA GTC TCC-3’
 Vk3b (ApaL1)  5’-TGA GCA CAC AGT GCA CGA AAT TGT GTT GAC TCA GTC TCC-3’
 Vk3c (ApaL1)  5’-TGA GCA CAC AGT GCA CGA AAT TGT GTT GAC ACA GTC TCC-3’
 Vk3d (ApaL1)  5’-TGA GCA CAC AGT GCA CGA AAT TGT GTT GAC TCA GTC TCC-3’
 Vk4 (ApaL1)  5’-TGA GCA CAC AGT GCA CGA CAT CGT GAT GAC CCA GTC TCC-3’
 Vk5 (ApaL1)  5’-TGA GCA CAC AGT GCA CGA AAC GAC ACT CAC GCA GTC TCC-3’
 Vk8 (ApaL1)  5’-TGA GCA CAC AGT GCA CGA GAT TGT GAT ACC CAG ACT CC-3’
 Vk10 (ApaL1)  5’- TGA GCA CAC AGT GCA CGA CCA CGT GAT GAC CCA GTC TCC-3’

 2.1.9.5 Constant Primer

 5’ to 3’ sequence

 IgG-C  5’-GTC CAC CTT GGT GTT GCT GGG CTT-5’
 IgG-C(SaLI)  5’-CGC CGA CGA GTC GAC GTC CAC CTT GGT GTT GCT GGG CTT-5’
 Ck-new  5’-ACA CTC TCC CCT GTT GAA GCT CTT-5’
 Ck-new(NotI)  5’- GAG TCA TCC TCG ACT TGC GGC CGC ACA CTC TCC CCT GTT GAA GCT CTT-5’
 Cλ.1  5’-TGA ACA TTC TGT AGG GGC CAC TGT CTT-5’
2.1.10 PRIMERS FOR COLONY PCR AND SEQUENCING:

2.1.10.1 Vector based Primer

<table>
<thead>
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<th>Primer</th>
<th>5’ to 3’ sequence</th>
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<tr>
<td>pRic.seq.int-for</td>
<td>5’-GTG AAA AAA TTA TTA TTC GCA-3’</td>
</tr>
<tr>
<td>pRic.seq.int-back</td>
<td>5’-GTG AGA ATA GAA AGG AAC AAC-3’</td>
</tr>
<tr>
<td>LMB 3’</td>
<td>5’-CAG GAA ACA GCT ATG AC-3’</td>
</tr>
</tbody>
</table>

2.1.11 THE PHAGEMID VECTOR

Phagemid pRfII, a derivative of pHEN1, with 2 light chain and 2 heavy chain restriction sites (ApaL1/Not) and (SalI/SfiI) respectively, was used in the library construction. By the his-tag the antibody fragments can be detected and purified using affinity chromatography. The phagemid carries an ampicillin resistance gene as well as an origin of replication for *E.coli* cells (col E1 ori) and for M13 phages (M13 ori).
2.2 STUDY POPULATION AND BLOOD SAMPLE COLLECTION.

136 Adult residents of Kumasi (Ghana) and Mahajanga (Madagascar) between the ages of 25 and 45 years, who had on the average been malaria free for a minimum of 2 years, were chosen for the study. There were 73 females and 63 males.

Pregnant and nursing mothers, as well as individuals with known disease conditions and inflammatory conditions were excluded from the study. So were individuals who were anaemic as per a general physical examination at the time of blood collection.

Written informed consent was obtained from all participants after the goals of the project had been thoroughly explained to them.

Ethical approval for the study was obtained from the Committee on Human Research Publication and Ethics (CHRPE) of the Kwame Nkrumah University of Science and Technology in Kumasi Ghana and the National Ethics Committee of the Ministry of Health and Family Planning of Madagascar.

Blood was collected into heparinised vacutainers and centrifuged to separate the plasma from the cells. Plasma was stored at -20 ºC until needed.

Fig 2-1 Schematic diagram of the pRFII phagemid vector used in the construction of the library.
2.3 METHODS

2.3.1 ENZYME LINKED IMMUNOSORBENT ASSAYS

Microtitre plates were coated with 1μg/ml (50ng) of the recombinant proteins (2.1.4) diluted in phosphate buffered saline (PBS), pH 7.4. After incubation overnight at 4º C, the plates were washed 3 times with PBS and 0.05% (w/v) Tween 20(PBST) and blocked with 2% (w/v) milk powder in PBS for 1hour at 37ºC. Dilutions from 1:200 to 1:6400 were prepared in a low binding ELISA plate for each plasma sample, and then transferred into the test plates, followed by incubation at 37ºC for 1 hour. The plates were washed 3 times with PBST. The bound IgG antibodies were detected with a 1:5000 dilution of goat anti human IgG AP conjugated, after incubation at 37º for 1 hour. Plasma from a malaria naïve individual was used as a negative control.

PNPP (P nitro-phenyl phosphate) was used as the substrate for all ELISA, after washing 5 times vigorously with PBST. The optical densities were read at 405nm after 15 minutes.

2.3.2 PROTEIN A COLUMN AFFINITY CHROMATOGRAPHY

300μl of plasma made up to 1ml with PBS was carefully layered on top of a previously equilibrated Protein A matrix in a column. The flow through was discarded and the column washed with 35x column volumes of washing buffer. The top of the column was layered with 5 column volumes of 0.1M glycine, pH2.5, and the flow through collected into an appropriate volume of 1M Tris HCl to neutralize the flow pH and to halt any degradation of the activity of the antibody. The eluate was transferred into a concentrating column and centrifuged at 300xg for 15minutes. The antibody concentrate was then used in the erythrocyte invasion inhibition assay.

2.3.3 TOTAL RNA EXTRACTION

Blood was collected into PaXgene RNA Extraction tubes and incubated for 2 hrs. at room temperature following which they were stored at -80 ºC until they were transported to Aachen. Total RNA was then extracted from each sample in the PaXgene tube (after allowing thawing completely on ice and incubation at room temperature for 2hrs) following closely the manufacturer’s instructions.
2.3.4 FIRST STRAND cDNA SYNTHESIS
First-strand cDNA was synthesized from 8μl of total RNA (2.3.3) using Superscript III cDNA synthesis kit (Invitrogen) and Oligo DT primers according to the manufacturer’s instructions.

2.3.5 AGAROSE GEL ELECTROPHORESIS
PCR products, plasmids and restricted DNA were separated by agarose gel electrophoresis. (0.8 – 1.2 % (w/v). Agarose gels were produced by dissolving the required amount of agarose in 1xTAE buffer by heating. 0.1μg/mL Ethidium bromide was added for visualization of DNA. Gels were run in a Sub-Cell GT chamber for agarose gels in 1x TAE buffer under constant potential until bands were separated well. As molecular weight standards 3μL of 2-log DNA Ladder (New England Biolabs, Frankfurt am Main) were used. Documentation and visualization after the gel electrophoresis was conducted with the Gel Doc XR system (Bio-Rad, München) and the software Quantity One version 4.6 (Bio-Rad, München) by transillumination of the gel at 302 nm.

2.3.6 PREPARATIVE AGAROSE GEL ELECTROPHORESIS
For preparative purposes agarose gels were viewed in the prep UV mode at 365 nm in order to prevent destruction of DNA while cutting out the desired bands with a scalpel. DNA from cut out bands was purified with the Nucleo Spin Extract II Kit (Macherey-Nagel, Düren) according to the manufacturer’s instructions.

2.3.7 AMPLIFICATION OF HEAVY AND LIGHT CHAIN GENES
PCR reactions were carried out in a total volume of 25 μl by preparing a reaction mix containing 2.5μl of PCR buffer, 0.75μl of 50mM MgCl₂, 0.5μl of dNTP’s, 0.5μl each of the 10 pmol forward and backward primers, 0.25μl of platinum Taq DNA Polymerase and 2μl of the first strand cDNA reaction. This was then made up to 25μl with milliQ water and the following PCR conditions applied: initial denaturation at 95°C for 5 minutes followed by 30 cycles at 95°C for 1 min, 55°C and 63°C (for kappa, and heavy chains respectively) for 1 min, 72°C for 2 min and a final elongation for 10 min at 72°C.
2.3.8 REAMPLIFICATION OF HEAVY AND LIGHT CHAIN GENES

In a second round PCR to introduce the respective restriction/cloning sites, a 1 to 10 dilution of the gel purified DNA from round one was used in a 50μl PCR reaction using the same components and reaction conditions as for the amplification except for the addition of 0.5μl of DMSO to the PCR reaction mix.

2.3.9 DNA RESTRICTION

Preparation of antibody light and heavy chain inserts for cloning was carried out by enzymatic double restriction using Apal1 and Not1 for light chain DNA and, Sfi1 and Sal1 for heavy chain DNA. The light and heavy chain DNA used for the restriction digestion was the restriction sites-appended and gel-purified DNA. All the light chain PCR products were pooled together as were the heavy chains. 2μg light chain PCR DNA was restricted using 1μl of the light chain restriction enzymes. The reaction was incubated at 37°C, overnight followed by PCI extraction and ethanol precipitation of the cut DNA as described below.

2.3.10 PHENOL-CHLOROFORM EXTRACTION

Phenol-chloroform extraction followed by ethanol precipitation was done after ligation to purify the DNA from interfering proteins e.g. the T4 DNA ligase. The ligation mixtures were inactivated at 65°C for 10 minutes and made up to 250μl with milliQ and an equal volume of PCI added. The mixture was vortexed and centrifuged at 13000xg for 5 minutes at room temperature. The upper aqueous layer was pipetted out into another Eppendorf tube and an equal volume of chloroform added, followed by brief strong vortexing and then centrifugation at the same speed. The upper aqueous layer (containing the DNA) was pipetted into a fresh Eppendorf tube.

2.3.11 ETHANOL PRECIPITATION

10μl of a 3M sodium acetate, (pH 5.2) and 1μl of glycogen was added to the mix. 2.5x volume of 100% ice cold ethanol was added followed by incubation at room temperature for 2minutes. The mixture was then centrifuged at 4°C for 30mins at 16000xg. The supernatant was carefully pipetted and discarded. The DNA pellets were washed with 500ul of 70% (w/v) ethanol for 5mins at the same speed and temperature. This step was repeated once. The DNA pellet was then resolved in an appropriate volume of milliQ water.
2.3.12 DNA LIGATION
The digested Kappa light chain PCR products (2.3.9) were cloned into the digested pRicfabII vector, in a vector: insert ratio of 1:5. 700ng of the vector was added to 385ng of the insert and made up to a volume of 89μl with milliQ water 10μl of T4 ligase buffer was added and the mix vortexed after which 1μl of the T4 ligase enzyme was added. The total ligation volume was 100μl. The ligation mixture was then incubated at 15°C overnight. After an initial inactivation at 65°C for 10 minutes, PCI extraction and ethanol precipitation was carried out as described (2.3.10, 2.3.11).

2.3.13 ELECTROPORATION OF ELCTROCOMPETENT E.COLI
Commercially prepared electrocompetent TG1 E.coli (Lucigen USA) was completely thawed on ice for 15 minutes and the resolved DNA (20μl) pipetted into it. The mixture was mixed gently by flicking the tube a few times. The mixture was pipetted into a pre-chilled electroporation cuvette and pulsed twice at 1800 volts. 1ml of recovery medium (Lucigen) was pipetted into the cuvette and mixed with the bacteria by gently pipetting up and down, and pipetted into an eppendorf tube. This was then incubated at 37°C while shaking at 250rpm. Dilutions of $10^4$ to $10^6$ were plated on a 2XTY agar/glucose/ampicillin plates whiles the remaining bacterial culture was plated on a 25cmsq 2XTY agar/amp/glucose plate and incubated overnight at 37°C.

2.3.14 TRANSFORMATION OF AGROBACTERIUM BY ELECTROPORATION
0.2-1.0 μg of plasmid DNA or ligation product (2.3.12) in sterile dH2O was added to a thawed aliquot of electrocompetent A. tumefaciens cells and incubated on ice for 3 min. The cell/DNA mixture was transferred into a precooled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 μF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of SOC medium in a 2.0-ml tube and incubated at 28°C with shaking (250 rpm) for 1h. Finally, 1-10 μl of the cells were plated on YEB agar containing 100 μg/ml rifampicin (Rif), 25 μg/ml kanamycin (Km) and 100 μg/ml carbenicillin (Carb) (YEB-Rif-Km-Carb) and incubated at 28°C for 2-3 days. As a control transformation of A. tumefaciens cells with H2O was performed.

2.3.15 COLONY PCR
Using 0.4μl each of “seqint.for” and “Ck new” as forward and backward primers respectively, a colony PCR with the following reaction components was prepared: 20μl PCR buffer, 0.8μl
MgCl₂, 0.4μl dNTP’s, and 0.2μl Taq DNA polymerase, made up to 20μl with milliQ water. 10 randomly selected clones were picked with the tip of a sterile 200μl pipettes and swirled each into one reaction tube and PCR carried out an annealing temperature of 52°C. The other conditions were as previously described. 5 μl of the PCR product was run on a 1.2 % (w/v) agarose gel to analyze the clones.

### 2.3.16 BstNI RESTRICTION ENZYME DIGESTION

The remaining 15μl of the PCR products (2.3.15) from clones that showed an insert as per the colony PCR were digested with 1μl of BstN1 at 50°C for 1 hour and then run on a 3% (w/v) agarose gel to provide a fingerprint of the light chain and heavy chain inserts.

### 2.3.17 SEQUENCING OF ISOLATED pRFII VECTOR CONTAINING LIGHT CHAIN GENES

Single, transformed colonies were inoculated in 5ml LB media containing ampicillin (100 µg/ml) and incubated overnight at 37°C. The culture was centrifuged for 1 min at 13000xg and a mini preparation using the NucleoSpin Plasmid Kit (Macherey-Nagel, Düren) was done according to the manufacturer’s instructions. 2 μl, “fdseq.int for” primer was added to 900ng of the plasmid DNA (2.3.15) and made up to a volume of 30μl with milliQ water. The sequences were analyzed using Clone Manager and SeqBuilder and compared with data in gene banks using the website of the National Center for Biotechnology Information, USA ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) by blast.

### 2.3.18 ISOLATION AND PURIFICATION OF PLASMID DNA FROM SUB LIBRARY

The quality controlled agar plates containing the bacterial colonies were pooled together by pipetting unto each plate 15 μl of 2xTY media and subsequently scraping the colonies into a 50 ml falcon tube. This was centrifuged at 5000xg for 5 min and the supernatant was discarded. A plasmid midi preparation to isolate the cloned pRFII vector containing the light chain insert was done using the Nucleobond Xtra-Mdi Kit (Macherey Nagel, Düren) according to the manufacturer’s instruction.
2.3.19 CLONING OF V<sub>H</sub> GENES INTO PRICFABII VECTOR CONTAINING LIGHT CHAIN INSERTS

The digestions, ligations and transformations as well as its quality control were performed as described for the creation of the light chain sub library except for the following modifications:

1. The amplified HC genes and the pRicfabII vector containing light chain genes were initially digested sequentially with SfiI (NEB) at 50°C and then with SaLI (NEB) in a 20µl volume over a 4hr period at 37°C. All restrictions were carried out using the optimal incubation buffer according to the manufacturer’s instructions (NEB).

2. For Colony PCR the forward primer, LMB3” and the backward primer, fdseq.back” was used.

3. The sequencing was done using the primer, LMB3”.

4. The total ligation volume varied between 20 µl and 100 µl depending on the concentration of the digested DNA.

5. To prevent religation of the vector after double digestion 1µl of Alkaline Phosphatase (CIP, NEB) was added to the vector and incubated for 1 h at 37°C.

6. For sequencing the heavy and the light chain inserts of 25 random colonies containing both inserts were sequenced.

2.3.20 LIBRARY PACKAGING

The antibody gene library was packaged by inoculating 50ml 2xTY-GA (2xTY containing 100mg/mL ampicillin and100mM glucose) with a library glycerol stock. The bacteria were grown to an optical density at 600nm (OD 600) of 0.4–0.5 at 37 °C and 250rpm. 5mL bacterial culture (1.25×10<sup>9</sup> cells) were infected with 2.5×10<sup>11</sup> helperphage particles incubated at 37°C for 30min without shaking followed by 30min at 250rpm. The infected cells were harvested by centrifugation for 10min at 3220×g. The pellet was resuspended in 25mL 2xTY-AK (2xTY containing 100mg/mL ampicillin and 50mg/ml kanamycin). The phages were produced at 30°C and 250rpm overnight. The bacteria were centrifuged for 30min at 12,000×g. Phage particles in the supernatant were precipitated with 1/5 volume of 20% (w/v) polyethylene glycol (PEG)/2.5M NaCl solution for one hour on ice and pelleted by centrifugation for 45mins at 12,000×g at 4°C. The precipitated phages were resuspended in 400ul PBS. Residual bacteria and cell debris were removed by additional centrifugation for 2min at 16,000×g at RT. The
supernatants containing the antibody phage were stored at 4°C. The phages were then titrated as previously described.

2.3.21 PANNING
The panning was performed in 96 well microtitre plates (Maxisorb, Nunc, Wiebaden, Germany). The antigens were coated in phosphate buffered saline (PBS) pH 7.4 overnight at 4°C. The concentrations were reduced each panning round (down to 100ng/ well). The antigen wells and wells for the preincubation of the library were blocked with MPBST (2% (w/v) skim milk in PBS with 0.1% (w/v) Tween20) 2.5×10^{11} phage particles of the library in PBST with 1 % (w/v) skim milk and 1% (w/v) bovine serum albumin (BSA) were preincubated for 1h. The supernatant, containing the depleted library was incubated in the wells with the immobilized antigen at RT for 2h followed by 10 washing steps with PBST. Afterwards the bound Fab phage particles were eluted with 200ul of 100mM glycine pH 2.2 at RT for 10min. The supernatant containing the eluted Fab phage was transferred into a new tube containing 1m Tris of pH 8.0 to neutralize the antibody phages. 2ul of eluted phage was used for titration. The remaining Fab phages were reamplified and used for the next panning round. The second, third and fourth panning rounds were performed with the following modifications: the amount of antigen was reduced in the fourth round whilst the washing cycles were increased to 20, 30 and 45 in the 2nd, 3rd, and 4th rounds respectively.

2.3.22 SOLUBLE EXPRESSIONS OF FAB FRAGMENTS AND DIRECT ELISA
Screening of Fab fragment libraries was performed after the fourth round of panning by small-scale induction of Fab expression from pRicfabII phagemid vector in ELISA plates. 96 recombinant clones of E. coli strain XL1Blue were randomly selected and inoculated in 100 μl of 2xTY containing 100 μg/ml ampicillin and 1% (w/v) glucose in microtitre plates. The plates (master plates) were grown overnight at 37°C. The next day, cells were transferred from the master plate to a second microtitre plate containing 125 μl 2x TY with 100 μg/ml ampicillin. Recombinant bacteria were grown at 37°C until the OD_{600nm} reached 0.8-1. Expression of soluble Fabs was induced at 30°C for 16-24 h by excluding glucose from the medium. The cells were removed by centrifugation and the supernatant was used for a soluble ELISA.
2.3.23 TRANSIENT EXPRESSION IN NICOTIANA. BENTHAMIANA

The plant expression vectors were introduced into *Agrobacterium tumefaciens* using a Multiporator, according to the manufacturer’s instructions. *A. tumefaciens* cells containing the plasmids (pTRAc-p19si, pTRAc_LC or pTRAc_HC) were cultivated in YEB medium containing 50 µg/ml carbenicillin, 25 µg/ml kanamycin and 25 µg/ml rifampicin. On the day of the infiltration, the cultures were prepared by dilution to an OD$_{600}$=1 using water. The diluted cultures were then mixed in equal parts with 2x infiltration medium (10% (w/v) sucrose, 0.398% (w/v) glucose, 0.01% (w/v) Ferty2 Mega (Planta Düngemittel GmbH, pH 5.6) and acetosyringone (200 µM final concentration) and incubated for 2 h at RT. The final infiltration suspension contained 80% of the induced agrobacteria containing pTRAc_HC-, pTRAc_LC and 20% of the induced agrobacteria containing pTRAc-p19si. Plants used for agrobacterial infiltration were grown in a greenhouse under a 16°C natural daylight photoperiod and a 25°C/day and 22°C/night temperature regimen. The plants were approximately eight weeks old at the time of infiltration. Whole *N. benthamiana* plants with roots in rock wool were immersed upside down into the infiltration suspension and were vacuum-infiltrated. After the infiltration, the plants were cultivated 5 days at 25°C with 16 hours of light per day (110 µE) and regular watering. Only the leaves were harvested leaving the roots and stems.

2.3.24 HARVESTING AND PURIFICATION OF ANTIBODIES FROM TOBACCO PLANTS.

Plants were harvested on the 5th day post infiltration and the leaf-mass determined and shredded in 3x volume of ice-cold extraction buffer. The mass was filtered through MiraCloth and 500 mM NaCl added to the filtrate whilst the pH was adjusted to between 8.0-8.5. After incubation for 30 min on ice to allow insoluble material to precipitate the filtrate was spun for 10 min at 4°C at a speed of 40000xg. The supernatant was again filtered through a glass fibre and a 0.45 µm filter to remove any residual insoluble material and plant debris. The filtrate was then applied in a protein A chromatography. MAb-Select Sepharose was equilibrated using 5 column volumes (cv) of 1x binding buffer and added to the extracts and incubated over night at 4°C while being stirred continuously. The extracts were then loaded onto a column, the flow through was collected and the matrix washed with 20 column volumes (cv) of 1x binding buffer. IgG were eluted using 12 fractions of 1 mL elution buffer into a tube containing. 660 µL of 1 M TrisHCl, pH 9.0 to immediately neutralize the pH. The concentrations were determined via NanoDrop and...
fractions were pooled according to their concentrations and dialyzed over night against PBS at 4°C while stirring. All fractions of the mAb-select chromatography including the flow through, and wash fractions together with the elution pools were analyzed via SDS-PAGE and Western Blotting (standard conditions) using reducing and non-reducing sample buffers. Elution fractions were completely pooled and stored in RPMI using Vivaspin6-concentrating tubes.

2.3.25 SDS-PAA GEL ELECTROPHORESIS AND COOMASSIE BRILLANT BLUE STAINING

Discontinuous SDS-polyacrylamide gels (for the stacking gel: T = 4%, C = 2.6%, pH 6.8; for the separating gel: T = 12%, C = 2.6%, pH 8.8) were used for separation of protein samples. Before loading onto the gel, protein samples were denatured where necessary, in the presence of SDS and β-mercaptoethanol. The proteins were separated electrophoretically with 20V/cm for 1 hour. Protein bands were revealed by staining with Coomassie brilliant blue or transfer to nitrocellulose membrane for immunoblot analysis (Ausubel et al., 1995). Proteins were detected after incubating the gel for 30 min in Coomassie staining solution at RT under constant rocking. Coomassie staining was removed by destaining solution until the protein bands was clearly visible.

2.3.25.1 SDS-PAGE running buffer (pH 8.3):
Tris 125 mM (w/v)
Glycine 960 mM (v/v)
SDS 0.5% (w/v)

2.3.25.2 Coomassie staining solution:
Coomassie brilliant blue G-250 0.25% (w/v)
Methanol 50% (v/v)
Glacial acetic acid 9% (v/v)

2.3.25.3 Coomassie destaining solution:
Methanol 10% (v/v)
Glacial acetic acid 10% (v/v)
2.3.26 WESTERN BLOT ANALYSIS
Separated proteins were transferred from an SDS-PAA gel to a nitrocellulose membrane (0.45 μm). After blotting the membrane was blocked with PBS buffer containing 3% (w/v) skim milk powder (MPBS). As primary antibody anti his6 used in a dilution of 1:5000 in 1xPBS. Attachment of the primary antibody was detected by addition of the secondary polyclonal antibody coupled to alkaline phosphatase (AP). Both, primary and secondary antibodies were diluted in blocking buffer. The target protein was finally revealed by addition of substrate BCIP/NBT.

2.3.27 CHARACTERIZATION OF FAB FRAGMENTS BY BIACORE
Biomolecular interaction analyses of binding of purified fabs to the AMA1 antigen was done by Surface Plasmon Resonance (SPR) on a BIACORE 2000 (Biacore). All injected samples were diluted in HBS buffer (10 mM HEPES, 150mM NaCl, 3.4 mM EDTA, 0.005% (w/v) Surfactant P20) and subjected to centrifugation prior to injection (14000 rpm/10 min/4°C) to remove insoluble components. The ligands were immobilized on a sensor chip using a coupling kit (BIAcore). The immobilization of proteins on the chip was performed at a flow rate of 5μl/min. After each binding experiment the surface was regenerated with 15-20 μl of 1.2 M Guanidine HCl followed by 30 sec injections of HBS. The data were analysed using the BIAevaluation (3.0) software.

2.3.28 GROWTH INHIBITION ASSAY (GIA)
Protein A-purified IgG fractions (2.3.2,) at a 2.25mg/ml final concentration, positive control rabbit anti-AMA-1 (6mg/ml final concentration), synchronised P.falciparum schizonts and culture medium were applied to half-area 96-well tissue culture plates in a total volume of 50μl/well. Each sample was tested in triplicate. After an incubation of 40-42h, the parasitaemia levels were determined using a parasite lactate dehydrogenase assay and the percent inhibition of parasite invasion calculated as described by Remarque et al, (2008).
3 RESULTS

3.1 GENERAL CHARACTERISTICS OF THE STUDY POPULATION

200 adult residents in Kumasi, Ghana, and Mahajanga, Madagascar who had had no clinical malaria infection for a minimum of 2 years despite having no preventive treatment were randomly chosen for this study. 64 of them were excluded from the study on account of positivity to HIV and/or hepatitis C leaving 136. Blood samples were taken from these subjects into heparinised vacutainers and plasma separated by centrifugation and stored at -20°C until needed. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from a second set of samples for each subject as described in Section II Materials and Methods and stored in dry ice for onward transport to Germany.

Through an ELISA, the responses of each sample to the recombinant antigens, AMA1 (produced under GMP conditions in P. pastoris), MSP1-19 (produced in HEK 293T cells), and MSP3 (plant produced) as well as the 3D7 crude Plasmodium falciparum lysate were tested using plasma from a non-immune European donor as a negative control. The response, (as determined by the OD₄₀₅ measured 15 minutes after development of the ELISA with pNPP) of the European donor was set at 1. Specific reactivity was defined as values greater than two standard deviations from the average response of the negative control.

Of the 136 subjects selected, 78, representing 57.4%, were from Ghana, with the remaining 58 coming from Madagascar. About half (46.3%) of the studied population were males whose mean age ± SD of 31.9 ± 6.6 years was lower than that of the total studied population (33.6 ± 6.8 years.). The mean age of the females in the study was 35.1 ± 6.8 years. The subjects from Ghana were significantly (p < 0.0001) younger (mean age 30.5 ± 6.1 years.) than their Madagascan counterparts (mean age 37.9 ± 5.8 years.). A similar trend (p=0.0065) was found when the male study subjects were compared to their female counterparts.

3.2 ANTIBODY REACTIVITY

The mean reactivity of the samples was highest in 3D7 lysate followed by AMA 1, through MSP3 and lowest in MSP1-19. The same trend was observed when the study subjects were stratified by country and gender (Fig 3-1).
As shown in figure 1A, the mean reactivity ± SD of the total study population to AMA 1 was 1.7 ± 2.6. This was lower than the mean reactivity of the Ghanaian study subjects (2.4 ± 3.1), which was significantly higher (p=0.0002) than that of the Madagascans (0.8 ± 1.5), and the mean reactivity of the males in the study (2.4 ± 3.4), which was also significantly higher (p=0.0033) than the mean reactivity of the females (1.1 ± 1.5). Also as shown in figures 1B, C and D the mean reactivity to MSP1-19, 3D7 lysate and MSP3 were significantly higher in Ghanaians in comparison to the Madagascans (p = 0.0103, 0.0006, and 0.0002 for MSP1-19, 3D7 lysate and MSP3 respectively), and in males in comparison to the females in the study (p = 0.0026, 0.0141, 0.0034, respectively).

![Bar graphs showing reactivity to various antigens stratified by country and gender.](image)

**Fig 3-1. Reactivity to the various antigens stratified by country and gender.** ELISA was carried out using the plasma samples from Ghana and Madagascar (2.3.1). The average response of the negative control plus 2 standard deviations was set as 1. All values above 1 were considered as reactive. The mean reactivity ± SD of all the samples...
together, the samples from Ghana alone and those from Madagascar alone are as shown above. There are statistically significant differences between the reactivities of the Ghanaians and the Madagascans as well as between all the males and the females. A = AMA1, B = MSP1-19, C = 3D7 lysate and D = MSP3.

### 3.3 Antibody Prevalence

The total number of samples with responses greater than 1 was expressed as a percentage and referred to as the prevalence In all, anti-3D7 plasmodium lysate antibodies were the most prevalent (94 out of 136, 69.1%) followed by AMA1 (66 out of 136, 48.5%) through MSP3 (50 out of 136, 36.8%) to MSP1-19 (29 out of 136, 21.3) as shown (Fig 3-2). The Ghanaians in general had significantly higher prevalence of the antibodies to all the antigens tested in comparison to the Madagascans.

A significantly higher number of males had anti-AMA1, -MSP1-19, -MSP3 and -3D7 lysate antibodies in comparison to the females. (Fig.3-2)

![Graphs showing antibody prevalence](image)

Fig 3-2. **Prevalence of antibodies to the various antigens tested.** The prevalence of antibodies to the various antigens (2.1.4) was also determined in the ELISA (2.3.1). The Ghanaian study subjects (2.2) had significantly higher prevalence of antibodies to all the antigens tested in comparison to their Madagascan counterparts; and the males in comparison to the females A = AMA1, B = MSP1-19, C = MSP3 and D = 3D7 lysate.
3.4 INVASION INHIBITION STRATIFIED BY COUNTRY AND GENDER

To prevent nonspecific inhibition of erythrocyte entry by the merozoites from heparin and other substances in plasma, the samples were purified in a protein A affinity chromatography as described, and the purified antibodies used in an erythrocyte inhibition assay. Although the median invasion inhibition of the Ghanaians was higher than the Madagascans, and higher in the females than the males, the differences were not statistically significant.

![Boxplot of percentage invasion inhibition stratified by country and gender](image)

Fig 3-3. **Boxplots of percentage invasion inhibition stratified by country and gender.** The percentage invasion inhibitions of the various samples were determined (2.3.28) and compared. The lower and upper margins of the box represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. There was no statistically significant difference between the median invasion inhibition of the Ghanaians in comparison to the Madagascans, and the males in comparison to the females.

3.5 CORRELATION BETWEEN REACTIVITY AND INVASION INHIBITION

Figure 4 below shows linear regression graphs of the tested antigens and the erythrocyte invasion inhibition. This was done to test the ability of reactivity to the antigens to predict erythrocyte inhibition.
invasion inhibition. For every unit increase in AMA1 reactivity ($r^2 = 0.0503; p = 0.0484$), and MSP3 reactivity ($r^2 = 0.061; p = 0.0429$) there was a significant increase of 0.012% (AMA1) and 0.007% (MSP3) in erythrocyte invasion inhibition. The percentage increase of 0.007% and 0.0038% for MSP1-19 and 3D7 lysate in the percentage erythrocyte invasion inhibition did not reach statistically significant levels. (Fig 3-4).

![Fig 3-4. Linear regression between the reactivity to the various antigens and erythrocyte invasion inhibition. (2.3.1, 2.3.28)](image)

Linear regression graphs of the reactivity and the invasion inhibition drawn to determine the ability of reactivity to specific antigens to determine invasion inhibition. There is a significant positive correlation between reactivity to AMA1/MSP3 and invasion inhibition. The positive relation between MSP1-19/3D7 lysate and invasion inhibition did not reach statistically significant levels.

### 3.6 INVASION INHIBITION AND BREADTH OF ANTIBODY RESPONSE

A chi-square for trend analysis done showed a gradual increase in the percentage erythrocyte invasion inhibition as the breadth of antibody response, defined as the number of antigens to which the plasma sample reacts to, increased (Fig 3-5). The increasing trend however did not reach a statistically significant level ($p=0.2005$)
Fig 3.5. Chi-square for trend showing the relationship between invasion inhibition (2.3.28) and breadth of antibody response (2.3.1). The average percentage invasion inhibition for samples that reacted to none of the antigens tested, those that reacted to only one, only two, three, and then to all four tested antigens were determined and a chi square for trend drawn. As the number of antigens to which the plasma sample reacts to increases, the percentage invasion inhibition increased. On the response axis, the numbers 0, 1, 2, 3, and 4 refer to the number of antigens (2.1.4) to which the plasma sample reacted.

3.7 EFFECT OF GENDER ON REACTIVITY AND PREVALENCE

The Ghanaian males were significantly (p=0.0361) younger (29.2 ± 4.7 years) than their female counterparts (32.2 ± 7.4 years) and had higher mean reactivities to the antigens, with the difference reaching significant levels in the case of MSP1-19, MSP3 and the 3D7 lysate (table 1). Although many more Ghanaian men had antibodies to the antigens tested than their female counterparts, the differences were only significant in the cases of MSP3 and 3D7 lysate.

The Madagascan males and females were of about the same age (38.7 ± 5.7 years / 37.5 ± 5.1 years respectively, p=0.4515). The mean reactivity of the Madagascan males to MSP1-19 and MSP3 (0.3 ± 0.3 and 0.4 ± 0.4 respectively), as well as the prevalence of antibodies to these antigens (11.1% and 16.7% respectively) was not significantly different from that of their female counterparts (0.4 ± 0.4;10.0%, and 0.7 ± 1.1;22.5% for MSP1-19 and MSP3 respectively). There was however a significant difference between the 2 groups with respect to the prevalence of antibodies to AMA1 and the mean reactivity to the antigen, with the males having a higher mean reactivity and antibody prevalence. Although the mean reactivity of the Madagascan males to the
3D7-lysate was significantly higher than their female partners, there was no significant difference between the prevalence of anti-3D7 lysate antibodies in the males and females.

When the Ghanaian females were compared with their Madagascan counterparts, there was a significant statistical difference between their reactivities to all the antigens tested except for MSP 3, with the Ghanaians having higher mean reactivities to all the antigens. Again many more Ghanaian females had antibodies to the 4 antigens tested compared to the Madagascans. This however only reached significant levels in the case of AMA 1 and 3D7-lysate but not in the case of MSP1-19 and MSP3.

As shown in table 1 below, not only were the Ghanaian males significantly more reactive to the antigens tested in comparison to the Madagascan males, the prevalence of the antibodies to all the antigens in the Ghanaian men were also significantly higher.
Table 3-1. **Reactivity to the various antigens tested stratified by country and gender.** The prevalence and reactivity of the samples to the antigens was stratified by country and gender and an ANOVA test applied.

Continuous data are presented as mean ± SD and analyzed using unpaired t-test whilst categorical data are presented as proportion and analyzed using Chi-square analysis. P = Ghanaian female vs. Ghanaian male, P* = Madagascan Female vs. Madagascan Male, P** = Ghanaian female vs. Madagascan female and P*** = Ghanaian male vs. Madagascan male.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ghana Female</th>
<th>Ghana Male</th>
<th>Madagascar Female</th>
<th>Madagascar Male</th>
<th>P</th>
<th>P*</th>
<th>P**</th>
<th>P***</th>
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<tbody>
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<td>Age (yrs)</td>
<td>32.2 ± 7.4</td>
<td>29.2 ± 4.7</td>
<td>37.5 ± 5.1</td>
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<td>AMA 1</td>
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<td>0.0428</td>
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**Prevalence of antibodies to the various antigens (%)**

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<td>27(81.8)</td>
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3.8 TOTAL RNA EXTRACTION AND FIRST STRAND PCR SYNTHESIS
Although the initial plan was to extract RNA from PBMCs, the idea was discarded as the viability of the cells reduced by 50% on thawing. The PBMCs were thus used for EBV transformation in another leg of the project. The study subjects were therefore visited again in Ghana and blood samples taken into PaXgene tubes. Total RNA was then extracted as per the manufacturer’s instructions.

Table 3-2. Selected samples for use in library construction and their scores as relates to their reactivity (2.3.1) and invasion inhibition (2.3.28).

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<thead>
<tr>
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<th>Inhibition Score</th>
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<td>G060</td>
<td>4</td>
<td>1.0002</td>
</tr>
<tr>
<td>G016</td>
<td>8</td>
<td>3.52</td>
<td>G093</td>
<td>5</td>
<td>2.00E-04</td>
</tr>
</tbody>
</table>
Total RNA of the top 50 Ghanaians (Table 3-2) selected on the basis of their scoring as per their reactivity and invasion inhibition ability was reverse-transcribed into cDNA using Oligo dT primers, for subsequent use in PCR and routine cloning purposes.

Fig 3-6. **Scoring of study subjects as per their reactivity and invasion inhibition ability.** The study subjects were scored on the basis of their reactivity to the tested antigens and the ability of their plasma to inhibit invasion of the erythrocytes. The top 50 scorers, except those lost to follow up (10 in total) were selected and total RNA was extracted (2.3.3) for subsequent cDNA synthesis (2.3.4). ScELISA-score on the ELISA; ScInh-score on the invasion inhibition.

To assess the quality of the total RNA extracted, 5µl of the extracted RNA samples were run on a 0.8% (w/v) agarose gel. Quality was assessed by the presence of a larger 28s band and smaller 18s band as shown in the gel picture of five different samples below. Where no bands were seen, the extraction was repeated.
3.9 AMPLIFICATION AND REAMPLIFICATION OF HEAVY AND LIGHT CHAIN GENES

The cDNA produced from all individuals via reverse transcription were pooled together. 5µl of the pooled cDNA was used as a template in a PCR to amplify all the V genes using the primers described in section II Materials and Methods. Amplification yielded bands (of varying intensities) of the expected size (660bp). The PCR products were electrophoresed on a 1.2% agarose gel (Fig 3-8). The desired bands of the heavy and light chain fragments were excised from the gel and purified.

Fig 3-8. Gel picture of the amplified VH and VK genes (2.3.7). 5µl of the pooled cDNA was amplified using primers for the various V-gene families after which the amplicons were run on a 1.2 % (w/v) agarose gel and the bands (660bp) cut and purified. A=gel picture of the heavy chain B=gel picture of the light chain (2.3.6)

The gel extracted PCR products were reamplified to incorporate restriction sites that will allow for the cloning of the V-genes into the pRFII vector( Fig 3-9).
Fig 3-9. **Gel picture of the reamplified VH and VK genes.** A 1:10 dilution of the purified amplicons from the amplification of the cDNA (2.3.7) was reamplified ((2.3.8) using restriction site appended primers for the various V-gene families after which the amplicons were run on a 1.2% (w/v) agarose gel and the bands (660bp) cut and purified. A=gel picture of the reamplified heavy chain B=gel picture of the reamplified light chain

### 3.10 LIBRARY CONSTRUCTION

#### 3.10.1 SUB LIBRARY

A 2 step cloning strategy was employed by which the double digested (Apal1 and Not1) and purified vector and Vk genes were ligated to produce a kappa sub library. Several transformations with electrocompetent TG1 E.coli eventually yielded a kappa sub library of size $7.6 \times 10^6$ whose quality was assessed by colony PCR and subsequent BstN1 digestion to contain inserts of the correct size with the different clones having different fingerprints.

#### 3.10.2 WHOLE LIBRARY

Sequential digestion of the pRFII-Kappa sub library was then followed with successful cloning of the restriction site-appended heavy chain genes. After electroporation and several (about 85) transformations a total library size of $1.9 \times 10^7$ was realized. 80% of randomly selected colonies from the finished library contained both the heavy and the light chain genes.(Fig 3-10) This gave the functional size of the library as $1.4 \times 10^7$.

Fig 3-10. **Analysis of Colony PCR products (2.3.15) to screen for heavy and the corresponding kappa chain insert.** Colony PCR of randomly selected clones from the final library that was done, showed about 80% of the clones having inserts of the right sizes (660bp) as shown. A=V_H B= V_K.
3.10.3 BstN1 ANALYSIS

Each of the clones assessed by colony PCR and found to contain inserts were analyzed by BstN1 restriction and found to have a distinct restriction pattern from each other (Fig 3-11) showing that the library was made up of different clones with diverse light and heavy chain gene usage.

![BstN1 Analysis](image)

Fig 3-11. **BstN1 analysis (2.3.16) of randomly selected clones from the final library.** Digestion of selected PCR products of selected clones was carried out at 50°C with BstN1 for one hour. Each of the selected clones had a distinct fingerprint showing diverse V-gene usage. A= heavy chain fingerprint, B=kappa chain fingerprint.

3.10.4 SEQUENCING

Twenty-five clones were selected at random from the final library (2.3.20) and sequenced (2.3.17) to ascertain the sequence diversity and v gene usage. The heavy chain sequencing revealed that each family was represented with a slight predominance of the VH1 family (41.6%), followed by clones using genes from the VH3 family (29.1%). The VH2 (12.5%), VH4 (4.2%), VH5 (8.3%) and VH6 (4.2%) families were observed in a lower frequency (Fig 3-12A).

For the kappa genes however, 5 of the 6 Vk families were represented, with a predominance of the Vk1 gene (41.6%), similarly as observed for the heavy chain genes followed by Vk4 (25%), Vk3 (12.5%), Vk2 (8.3%) and Vk6 (4.2%). Vk5 was not detected.(Fig3-12B).
Fig 3-12. Sequencing (2.3.17) of 25 randomly selected clones showing the V gene family distribution for the heavy chain and the kappa chain. Alignment of the sequences to their closest germline was done using Ig blast (NCBI: http://www.ncbi.nlm.nih.gov/igblast/) Except for Vk5, the entire V gene families are represented with VH1 being the most dominant for the heavy chain and Vk1 being the most dominant for the kappa chain. (Reproduced with permission from Stefan Schmitz).

The Vk4 family showed the closest alignment by Ig Blast (NCBI) showing 6 top matches in all. The protein sequences of these six clones were therefore aligned using the program “CLC Main Workbench 6.0.2.” The constant and framework domains showed a high conservation and homology, while most variations were seen in the CDR1 and CDR3 regions.
Fig 3-13. Protein sequence alignment of 6 clones showing identical germline gene matches. Protein sequences showing by IgG Blast an identical top match for the VK4 family were aligned using the program “CLC man workbench 6.0.2”. Amino acid position 1-110 represents the variable domain, with the rest represents the constant domain. The heights of the bars reflect the consensus of the sequences. CDR regions were determined by using the program “IMGT/V-Quest; http://www.imgt.org/IMGT_vquest/share/textes/). (Reproduced with permission from Stefan Schnitz).

To get an overview of the germline gene combinations of the library, the VH and VL pairs for each clone were compared. This showed a random Fab assembly. 13 of the 18 clones (containing a full size human Fab-fragment) showed a combination of different families. Four combinations dominated. (Table III.5.). The sequences however differed in their CDR or framework.
Table 3-3. **Overview of the germline gene combinations.** The germline gene pairs of 20 clones containing heavy and kappa chain inserts were compared. The most abundant top match was for VK4 which was present in 6 clones. # = additional combination showing a stop codon

<table>
<thead>
<tr>
<th></th>
<th>VH1</th>
<th>VH2</th>
<th>VH3</th>
<th>VH4</th>
<th>VH5</th>
<th>VH6</th>
</tr>
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<tr>
<td>VK1</td>
<td>2</td>
<td>2</td>
<td>3(#)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>VK3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>VK4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<td>VK5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VK6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### 3.11 PANNING OF THE LIBRARY

Three rounds of panning were carried out with AMA1 and MSP1-19, antigens to which the responders had demonstrated reactivity. Exponential increases were observed in the output titers.
Fig 3-14. Output titers of the first second and third selection rounds of panning with MSP1-19 and AMA1 on the TG1 library packaged with TG1 E.coli; showed an exponential increase in the selection rounds. Media and agar contained 1% (w/v) glucose except in the propagation steps where no glucose was added.

This notwithstanding, none of the clones selected had inserts of the right size after a colony PCR was done (Fig 3-15), irrespective of whether helper phages or hyper phages were used in the first selection round.

Fig 3-15. Colony PCR of clones from the third selection round after panning with MSP1-19 and AMA1 on the TG1 library showing no clones with the desired insert of 900bp. In these rounds of panning, 1% (w/v) glucose was used in all media and agar. PC=positive control, M=DNA marker.

Minipreps and subsequent sequencing showed varied recombination events and no Fab sequences. Elution was with TEA pH 9.0. Similar results were gotten when the experiments were repeated with a commercially available naïve library kindly provided by Dubel.
Given that the packaging of the library was done with TG1 *E.coli*, a recombinase A positive strain with the potential to destabilise inserts, the library was repackaged with XL1blue *E.coli* which is recombinase A negative and the panning and sequencing repeated. To ensure that all the clones in the original library were duly represented, a final library size of $4.0 \times 10^8$ was created. This way, over 95% of the original clones were represented. The repackaging notwithstanding, none of the selected clones had inserts of the correct sizes after panning.

### 3.11.1 EFFECT OF VARIATION IN GLUCOSE CONCENTRATION AND ELUTION CONDITIONS ON THE OUTCOME OF PANNING.

To ascertain the effects of increased glucose concentration on the outcome of the panning process, scrapings of the glycerol stock of both the new and old libraries were inoculated into 5ml of 2TY medium containing 2% glucose and another containing 1% glucose as used previously, and the cultures incubated at 37°C overnight. 10µl of a $1:10^3$ dilution in PBS of the overnight culture was then plated on a 2TY amp/glucose plate and incubated overnight. Five microliters of the culture was again inoculated into a fresh 2TYamp/glucose medium (2%). A colony PCR was then performed to check for the presence of inserts of the correct size. After the first cultivation, 12 out of the 15 clones had inserts (Fig 3-16A). This reduced to six and then nine after the second and the third sub cultivation for the new library and then nine through four to seven in the old library packaged with TG1 *E.coli*. (Fig 3-17). For the cultures with 1 % (w/v) glucose however, after the first subculture, subsequent cultures examined for the presence of inserts showed that none of the selected clones had inserts contrary to what was found in the cultures with the 2 % (w/v) glucose as shown (Fig 3-16).

Cultures incubated at 30°C however, took three days for the cultures to grow and so 37°C was chosen as the optimal temperature for the cultures as prolonged culture had the potential of causing deletions.
Fig 3-16. Colony PCRs of the TG1 unselected library after three sub cultivations with media and agar containing 1% (w/v) glucose. Although the first sub cultivation showed about 80% of the clones having the correct inserts, these were lost after the second and third sub cultivations in media and agar containing 1% (w/v) glucose. A=colony PCR after 1st sub cultivation. B and C=gel picture of colony PCR after 2nd and 3rd sub cultivation respectively.
Fig 3-17. Colony PCRs of the XL1Blue and TG1 unselected libraries after three sub cultivations with media and agar containing 2% (w/v) glucose. Colony PCR was carried out with clones from the unselected XL1Blue and TG1 libraries. Although there was a decline in the percentage of clones with inserts, the culture still maintained many clones with inserts of the right sizes. A=colony PCR after first sub cultivation. B and C=gel picture of colony PCR after second and third sub cultivation respectively.
3.11.2 PANNING OF XL1BLUE LIBRARY WITH THE AMA1 ANTIGEN WITH GLUCOSE CONCENTRATION OF 2% UNDER DIFFERENT ELUTION CONDITIONS

Four new selection rounds were carried out with all media and agar containing 2% glucose where necessary, and elution by (i) direct infection with logarithmic phase E.coli, (ii) 100mM TEA at pH 9.0, and (III) 100mM glycine at pH 2.2.

Except for the glycine, none of the randomly selected clones on the titer plates of the first and second rounds had inserts of the right sizes. The third and fourth selection rounds were thus carried out using only glycine at low pH (Table 3-3).

Table 3-4. Outcome of panning rounds with 100mM Glycine pH 2.2. Four new selection rounds were carried out at low pH with 100mM glycine at pH 2.2. A final enrichment of 1200 was obtained in the 4th selection round over the first with a progressive increase in the percentage of clones with inserts from the first to the fourth round. Enrichment was defined as the ratio of the output/input ratio in any panning round to the same ratio in the first panning round.

<table>
<thead>
<tr>
<th>Elution type</th>
<th>Selection round</th>
<th>Input titer</th>
<th>Output titer</th>
<th>Output/input</th>
<th>Enrichment</th>
<th>% with inserts</th>
</tr>
</thead>
<tbody>
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<td>1.6X10¹²</td>
<td>1.9x10⁶</td>
<td>1.19X10⁶</td>
<td>-</td>
<td>4 (2/24)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.2x10¹²</td>
<td>9.0x10⁶</td>
<td>7.5X10⁶</td>
<td>6.3</td>
<td>36 (7/19)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.5x10¹¹</td>
<td>3.0x10⁶</td>
<td>1.2X10⁶</td>
<td>1.01</td>
<td>50 (14/28)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.0x10¹¹</td>
<td>3.0x10⁸</td>
<td>1.5X10³</td>
<td>1200</td>
<td>99 (28/29)</td>
</tr>
</tbody>
</table>

Except for the first panning round, after each round of panning, colony PCR analysis as well as BstN1 digestion and sequencing were carried out to ascertain the presence of Fab fragments (Fig 3-18 &19).
Second and third selection round colony PCR and BstN1 digestion

A

Fig 3-18. Colony PCR (2.3.15) and BstN1 digestion (2.3.16) of randomly selected clones after the second and third panning rounds; showing seven out of 19 clones with inserts. After the third round of panning, 14 out of 28 randomly selected clones on the titer plates, on colony PCR, had inserts of the right sizes as shown. BstN1 digestion (2.3.16) showed the antibodies to be belonging to three main classes. A=colony PCR B=the corresponding fingerprints

B

Fig 3-19. Fourth selection round colony PCR (2.3.15) and BstN1 digestion (2.3.16). After the fourth selection round, 28 out of the 29 randomly selected clones had correct inserts on colony PCR. According to the subsequent BstN1 digestion only 2 different clones were represented.

After each panning round, the positive clones were sequenced and their closest germ line gene matches identified via IMGT blast. The most abundant clone was found in 22 out of the 28 sequenced clones after the 4th selection round with its closest germ line sequence being
IGHV1 69*11/IGKV1D 17*02. The same clone was present in the 2nd and 3rd rounds with increasing frequency nominally, from 1 out of 7 (14.3%), through 12 out of 14 (85.7%), to 22 out of 28 (78.6%)

Table 3-5. **Sequence diversity of the various selection rounds showing the closest germ line matches.** At the end of each selection round, positive clones were sequenced and their closest germ line matches determined via IMGT blast. The most abundant clones in the third and fourth selection rounds (12 out of 13, and 22 out of 28 respectively) bore closest similarity with IGHV1 69*11 and IGKV1D 17*02.

<table>
<thead>
<tr>
<th>Selection Round</th>
<th>VH</th>
<th>VK</th>
<th>Number present</th>
</tr>
</thead>
<tbody>
<tr>
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<td>IGHV5</td>
<td>IGKV1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td><strong>IGHV1 69*11</strong></td>
<td><strong>IGKV1D 17*02</strong></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IGHV4</td>
<td>IGKV1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IGHV3</td>
<td>IGKV1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IGHV2</td>
<td>IGKV3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IGHV1</td>
<td>IGKV3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IGHV3</td>
<td>IGKV1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td><strong>IGHV1 69*11</strong></td>
<td><strong>IGKV1D 17*02</strong></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>IGHV1 69*09</td>
<td>IGKV1D 17*02</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td><strong>IGHV1 69*11</strong></td>
<td><strong>IGKV1D 17*02</strong></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>IGHV1 69*09</td>
<td>IGKV1D 17*02</td>
<td>6</td>
</tr>
</tbody>
</table>

**3.12 MONOCLONAL PHAGE AND SOLUBLE ANTIBODY FAB ELISA.**

Ninety-six randomly selected clones from the titer plates of the 4th selection round of the glycine eluted plates were analysed for their reactivity to AMA1 in a monoclonal ELISA as described in Section II Materials and Methods. Only clones whose OD_{405} was greater than 0.8 (8 times the negative control) were selected for subsequent analysis. Analysis showed the
reactive clones (OD\textsubscript{405} above 0.8) belonged to one of 6 groups. Representative clones were chosen and soluble Fabs produced as described in Section II materials and Methods, following, which a soluble antibody Fab ELISA was performed.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Monoclonal Phage and Soluble Antibody Fab ELISA (2.3.22) of selected clones. 96 clones were randomly selected from the titer plate of the 4\textsuperscript{th} selection round and a monoclonal Phage ELISA carried out, with a substrate reaction time of 15 minutes. Clones with OD\textsubscript{405} of 10x greater than the negative control NC were selected for a subsequent soluble antibody Fab ELISA. A=Monoclonal Phage ELISA, B=Soluble antibody Fab ELISA, NC=negative control}
\end{figure}
Two out of the 6 clones showed high reactivity and were thus chosen for plant expression into full sized antibodies and to further characterize them. The clones were named Oche A and Oche B.

3.13 TRANSIENT TEST EXPRESSION OF OCHE A AND OCHE B IN NICOTIANA BENTHAMIANA PLANTS.

A test expression of Oche A and Oche B were carried out using 2 leaves of *N. benthamiana* per construct as described (2.3.23). The heavy and light chain of Oche A and Oche B were also shuffled to produce 2 different constructs; Oche C (consisting of the heavy chain of Oche A and the light chain of Oche B), and Oche D (consisting of the light chain of Oche A and the heavy chain of Oche B). An ELISA with the full sized antibodies was performed using MSP1-19, another merozoite antigen, and PBS as controls to determine the selectivity of binding. Except for Oche A which was found to bind selectively to AMA1, none of the other constructs bound.

![Response of clones to AMA1 at varying concentrations, MSP1-19 and PBS](image)

**Fig 3-21.** **Regular ELISA to determine the selectivity of the antibody constructs.** ELISA was carried out by coating the wells of an ELISA plate with 50ng and 100ng of AMA1 per well in triplicates and using MSP1-19 and PBS as controls. Oche A was found to bind selectively to AMA1. Oche C which contained the heavy chain of Oche A was also found to bind moderately to the antigen.
3.14 CHARACTERISATION OF OCHE A

3.14.1 SDS PAGE

Anti-AMA1 was successfully produced and purified from *N. benthamiana* leaves, although the yield of the recombinant protein was low (about 6µg/g of leaf). The purity and integrity of antibody was analysed by reducing and non-reducing SDS-PAGE with subsequent Coomassie staining and Western blot. The observed size of the proteins was in accordance with the expected values at 50 kDa (heavy chain) and 25 kDa (light chain), and about 150kDa for the full sized antibody.

![SDS PAGE](image)

**Fig 3-22. SDS PAGE (A) (2.3.25)) and the corresponding Western blot analysis (2.3.26) of the full-sized antibody**. Both reducing and non-reducing SDS Page was carried out. The bands observed corresponded to the heavy chain and the light chains and an almost 150kDa band for the full-sized antibody M-marker, F-flow through, W-wash, 1,2,3-high medium and low concentration pools respectively(reducing), 4, 5, 6- high medium and low concentration pools(non-reducing)

3.14.2 SURFACE PLASMON RESONANCE

The interactions of the various Fab constructs were analysed by real-time interaction analysis on a Biacore instrument, using a microsensor chip coated with the recombinant AMA1. Only Oche A was found to bind to AMA1 with an affinity constant (K_D) of 1.363x10^-7
Fig 3-23. Sensogram plots for Oche A and recombinant Fab binding experiments on immobilized AMA1. The reactivity of the antibodies Oche A, B, C and D and the reaction kinetics were measured by Surface Plasmon Resonance (Fig A, B, and C). Apart from the reactivity of Oche A, to the AMA1 antigen, none of the other reactivities to the antigens could be detected (A and B). The antibody reacted with a dissociation constant (KD) of $1.363 \times 10^{-7}$ (SE $1.18 \times 10^{-8}$) and a maximum response (Rmax) of 24.3 as highlighted by the arrow, (2.3.27).

3.14.3 GROWTH INHIBITION ASSAY

To evaluate the inhibitory potential of the antibody generated, on the invasion of Plasmodium into red blood cells, a GIA was performed on *P. falciparum* 3D7A. The anti-AMA1 was tested in triplicate at a final concentration of 2.25 mg/ml, while rabbit-anti-AMA-1 (positive control) was used at 6 mg/ml. The negative controls (unspecific IgG and unspecific IgG) were used to calculate the maximal growth of the parasite culture.

Inhibition was calculated using the following formula;

$$%\text{inhibition} = 100 - \left( \frac{(A655\ IgG\ sample - A655\ RBC\ control)}{(A655\ SZ\ control - A655\ RBC\ control)} \right)$$

The positive control demonstrated the expected growth inhibition whiles for the unspecific rabbit IgG, a growth inhibition of approximately 20% was observed, again expected. The anti-AMA1 antibody strongly inhibited invasion (64.5%) with the percentage inhibition decreasing with decreasing antibody concentration.
Fig 3-24. **Graph of the invasion inhibition by Oche A.** Varying concentrations of OcheA were applied in a growth inhibition assay and the corresponding invasion inhibition calculated from the formula described above. As the concentration decreased, the percentage invasion inhibition also decreased. The maximum inhibition to growth was detected when the concentration of OcheA was highest (2.25mg/ml). BG98, a polyclonal rabbit serum was used as a positive control whiles UnspecIgG and UnspecIgG were used as negative controls. UnspecIgG is a blood purified plasma IgG from a non-immunized rabbit and UnspecIgG is a 2G12 antibody specific for the gp120 protein of HIV. Both are non specific to merozoites. (2.3.28).

4 **DISCUSSION**

4.1 **INTRODUCTION**

The Phage display arm of the Fraunhofer MP3 Malaria project which forms the basis of this thesis had as its main aim, the construction of an immune antibody-Fab phage display library that will allow for the selection of various monoclonal antibodies against the merozoite stage of *Plasmodium Falciparum* the causative agent of malaria tropica.

Developing an effective malaria vaccine or new anti-malarial drugs would not only enhance the control of the disease, but will also improve the human- and economic development in endemic countries.

Antibodies, could then be entered into clinical trials, as therapeutic agents, after appropriate testing, and would eventually be used as therapy for non-immune travelers to malaria endemic areas.
To select which of the many samples to use in the construction of the library, it was necessary to profile the samples with respect to their reactivity to select merozoite stage antigens and to determine the ability of the samples to inhibit erythrocyte entry. The profiling was also to serve as a basis for the selection of samples for use in single cell sorting, and EBV transformation by the other PhD candidates on the project. The phage display arm of the project in this respect, served as the pivot of the entire MP3 malaria project.

The breadth and magnitude of antibody response to malarial surface antigens such as MSP1-19 and AMA1 in inhabitants of endemic areas have been used not only as serological correlates of clinical immunity, but also as markers of cumulative exposure to malaria [45]. The development of vaccines against malaria requires the characterization of antibody correlates of protection so that appropriate antigens can be used for immunization. Susceptibility to malaria changes over time, with episodes of illness declining in frequency and severity. The identification of antibodies responsible for acquired immunity to malaria will help to determine what constitutes protective immunity and how this develops naturally. Many previous studies have compared antibody responses within a population over time, particularly the antibody responses of children compared to adults, or males compared to females. These studies were carried out in endemic areas and many different countries have been investigated, including Myanmar, Tanzania, Ghana, Senegal, Kenya, Mali and Venezuela [46-54]. However, there has not yet been a study, which compares different populations in different endemic areas and also stratifies those populations by gender.

Generally in Africa, malaria transmission is lower in the urban in comparison to the rural areas due to factors like improved drainage, better quality housing and lower density of vectors in addition to improved access to healthcare facilities [55-57].

4.2 KUMASI COHORT

Kumasi the capital city of the Ashanti Region is the second largest city in Ghana and the most populous. Its population was counted in the 2009 census as 2.5 million persons, with children under five years accounting for about 22.6. (http://www.wsup.com/whatwedo/kumasi.htm)

There are two rainy seasons; a major one extending from April to August, and a minor one between October and November. The temperature varies from 20°C to 36°C. Subsistence farming and small scale trading are the main sources of income. Malaria is holoendemic in this area with an entomological inoculation rate (EIR) of about 400 infective bites per individual per year. The predominant *Plasmodium* species is *P. falciparum* which accounts for about 90% of malaria infections [58].
4.3 THE MAHAJANGA COHORT
Located off the eastern coast of Africa, Madagascar has a population of about 19 million. There are four distinct malaria epidemiologic zones with stable, perennial transmission on the eastern and western coasts, and unstable, seasonal transmission in the central highlands and in the southern region Mahajanga, where the study participants were picked lies on the western coast. Malaria is endemic, over 90% [59], with the entire population considered to be at risk. Almost one million clinical cases are reported per year, making malaria a major public health problem [60].

4.4 THE PRESENT STUDY
A population-based immuno-epidemiological study in the malaria-endemic regions of Kumasi, Ghana and Mahajanga, Madagascar was carried out to profile the immune response to three malaria asexual blood-stage antigens and the crude 3D7 Plasmodium falciparum lysate, within and between these populations. An Enzyme Linked Immunosorbent Assay was used to measure IgG antibody responses to MSP1-19, AMA1 and MSP3 in the stratified study population, and also to determine the prevalence of the antibodies and correlations between antibody reactivity and erythrocyte invasion inhibition efficacy, with all data stratified by geographical region and/or gender.

It is essential to determine whether specific antibodies correlate with protection against malaria because in vivo protection may be based on the cumulative synergistic effects of responses to different antigens rather than any single antigen [50], and different correlates of protection may have greater prevalence in different population strata, allowing the use of more targeted vaccination approaches.

4.5 REACTIVITY AND PREVALENCE
The variation in reactivity observed among the individuals and populations may reflect a number of underlying factors, such as different exposure levels and transmission intensity across the study areas [61], differences in host genetic factors and differences in the predominant parasite strains in the tested areas [62].

The presence of other infectious diseases, whose epidemiology may be regional or gender-biased, may also prevent the development of antibodies against malarial antigens. There may also be erythrocytic heterogeneity among the stratified populations, resulting in the selection of particular parasite ligands that can mediate successful invasion [23].
There was an age-dependent effect on mean reactivity, with younger adults showing significantly higher reactivity than their older peers. This may reflect the cultural behavior of different age-stratified populations, with younger adults in the African setting more likely to congregate nocturnally, coinciding with the period of maximum parasite dispersal and thus implying increased exposure. The tendency for more males than females to congregate at night might also explain the higher mean reactivities of the males in the study population although there is no gender-related difference in the efficacy of invasion inhibition.

In many parts of Africa it is not culturally acceptable for females to congregate deep into the night for social gatherings as females who do that are generally considered not traditionally cultured.

4.6 INVASION INHIBITION AND REACTIVITY

The observed significant positive correlation between reactivity to AMA1 and MSP3, and invasion inhibition is similar to that reported by Miura K et al [63] who affinity purified AMA1-specific antibodies from two cohorts of people; vaccinated individuals in the US, and non-vaccinated individuals living in malaria endemic areas of Mali, and performed an ELISA and growth inhibition assay, and to that reported by Dicko et al, [64] who compared the % invasion inhibition assay pre- and post- vaccination. In each instance they demonstrated a statistically significant correlation between antibody levels and % invasion inhibition.

Courtin D et al [47] while working with a number of antigens including AMA1 also determined significant correlations between invasion inhibition and response to AMA1, in agreement with the present results.

The relationship between invasion inhibition and reactivity to MSP3 corroborates the report by Singh et al [65] who tested different regions of the C terminal part of MSP3 in an ELISA using serum samples from individuals living in malaria endemic Dielmo, a village in Senegal, and tested the ability of these sera to inhibit erythrocyte invasion and established a statistically significant relationship between reactivity to MSP3 and invasion inhibition in agreement with the present results and that of Muellenbeck MF et al.[66]

The results provide strong support for the development of a vaccine designed to elicit antibody responses simultaneously to multiple asexual stage antigens of *P. falciparum*. Given the very high entomological inoculation rate and the higher prevalence of malaria in Kumasi in comparison to Mahajanga, samples from Ghana were selected to create a library that will be uniquely Ghanaian, but which nonetheless would allow for the selection of binders to a wide range of antigens.
4.7 LIBRARY CONSTRUCTION

The phage display technology which was pioneered over 25 years ago when a portion of the gene encoding the *EcoRI* endonuclease was fused to the gene coding for the pIII protein coat from an M13 virus [67], has had a substantial impact in studies of immune responses, vaccine design and therapeutics, and has proven to be a powerful tool for the isolation of monoclonal antibodies.

The pioneering work resulted in the formation of hybrid filamentous phages that expressed the product of the fusion gene into the pIII protein on the surface of the phage particle. This way the foreign DNA was expressed in association with the phage coat protein with the phage acting like an expression vector [68].

The phage display technology is largely based on the M13 filamentous phage which has a cylindrical shape and contains a single stranded DNA genome encoding 11 genes, five of which are coat proteins. It has a proximal end where the g6p and g3p proteins are present, and a distal end hosting the g7p and g9p proteins; these proteins are referred to as the minor coat proteins and are present as four to five copies. The g8p protein the major coat protein which is responsible for encapsulating the phage DNA on the other hand, is present in almost 2700 copies.

Infection of bacteria by the virus (M13) occurs when the g3p protein of the virus comes into contact with the pilus of the bacterium. While in the bacterium the virus replicates and are finally released from the host cell without lysing it. The g3p which is responsible for phage infection and release from the cell following assembly has two N terminal domains one of which is essential for infectivity. Although all the five coat proteins have been used as fusion proteins for phage display, the g3p is the most popular and the most studied [69]. The tendency for large molecules to compromise the structure and function of the protein coat [70] led to the development of phagemid display systems which when introduced into a bacterial host together with a helper phage, allow for the display of both fusion and wild type coat proteins reducing the possible defects on phage function [71].

The technology has become a successful alternative to the traditional hybridoma technology not only because of the ease of use of its protocol, but also because it is fast (allowing for the generation of antibody fragments within weeks), more reliable, and lends itself to subsequent genetic manipulations to improve the affinity of the selected binders where necessary [72-75].
One of the most successful applications of this technology has been the selection of monoclonal antibodies using naïve phage display libraries by Griffith and his colleagues who observed that the probability of isolating high affinity binders is a function of the library size as evidenced by a comparison between two libraries of different sizes (2.9x10^7 and 10x10^10) with an increase of the library to the 500-fold, resulting in a 100-fold increase in affinity [76]. A library, constructed based on the V-gene repertoire of malaria immune Ghanaian population should thus allow for the successful isolation of binders against merozoite stage antigens.

Through a 2-step cloning strategy, amplified VK-genes were cloned into the cut pRFII vector followed by the heavy chain, since it was believed that a reduction in the number of cloning steps would minimize diversity losses and prevent the formation of non-functional or dysfunctional Fabs that could arise from the digestion with the ApaLI restriction enzyme, since the constant domain of the heavy chain was found to contain an ApaLI restriction site.

Two important things that were also considered in the creation and isolation of antibodies from the phage library were (i) the type of library to be constructed and the source of B cells to be used; and (ii) the selection strategy employed in the biopanning procedure [77].

4.7.1 TYPE OF LIBRARY

There are several types of phage display libraries including peptide libraries, which as the name implies allow for the selection of peptides, protein libraries whose purpose is to allow for the selection of proteins with specific functions or affinity to other molecules of interest, and antibody libraries.

The main use of the phage display technology has been the selection of phage display antibodies from antibody libraries, which commonly express the antibodies commonly as scFv or Fab fragments. The first step in the generation of antibodies using the technology is the construction of a library using B cells of immunized or non-immunized donors. Whereas the antibody repertoire from immunization is generally restricted to generating antibodies against the antigen of the original immunogenic response in immune libraries, naïve libraries in theory allow for the selection of antibodies against a wide repertoire of antigens to which there have been no prior including antibodies against self, non-immunogenic or relatively toxic antigens [78].

Immune libraries on the other hand contain rearranged variable regions from immunized animals or humans with prior exposure to particular pathogens of interest. Whereas naïve libraries usually require larger sizes [78], immune libraries as small as 10^5 members have allowed for the selection of high affinity and specific monoclonal antibodies [79, 80].
 naïve and immune libraries, an animal source or a human source can be used. Where the final antibody has the potential of been used for immunotherapy, the HAMA response associated with antibodies from animal sources limits the choice of donors to humans. In both animals and humans, peripheral blood can be used as the source of B cells. Animals are usually sacrificed and their spleens used as the source of B cells. In humans, the bone marrow, whose use in antibody library construction allows the broadest variability in terms of B cells has also been used.[77].

Synthetic libraries which are formed by rearrangement of VH and VL gene segments in vitro and introducing artificial CDRs of varying loop length using PCR and randomized oligonucleotide primers [69] have the limitation that a significant proportion of randomized CDR sequences do not allow proper folding of the antibody variable region and, thus, reduce the number of functional members and overall performance of the library.[81]. Its advantage is that no immunization or tissue selection is required. In this thesis donors with prior natural exposure to the plasmodium falciparum parasite were used.

4.7.2 B-CELL SOURCE

Although many studies till date have used peripheral blood mononuclear cells [82, 83], human bone marrow [84, 85] and peripheral lymphoid tissue [86, 87] as sources of B-cells for library construction, whole blood was used for RNA extraction in the construction of this library after it was discovered that the frozen PBMCs lost about 50% of the viable cells on thawing. The relative advantages or otherwise of this approach is not known as literature on a comparative analysis of different B-cell sources is lacking [77].

4.7.3 SELECTION STRATEGY EMPLOYED

In this thesis the antigens were directly coated on the microtitre plates and incubated overnight at 4°C after which the phages were applied as described previously. The direct coating of proteins on plastic is usually more efficient but can also be problematic, because the passive adsorption on plastic at pH 9.6 is a mechanism of protein denaturation [75] with about 95% of adsorbed proteins being nonfunctional [88, 89].

The alternative is to biotinylate the antigens and coat indirectly, using streptavidin. This has been known to improve the sensitivity in most cases and allows for a more efficient selection of phage antibodies [76, 90]. The elution of phages with Fabs indicated that the coating was successful and thus no biotinylation was required. However, it will be important to experiment with the biotinylation to find out if it will improve the chances of antibody isolation.
Another factor that was considered in the selection process was the temperature. Gertenbruch S et al [91] carried out panning at 4°C, and at 37°C and observed that none of the clones selected after 3 rounds of panning bound to the target antigen in a phage ELISA. In contrast, panning at 37 °C during the initial three rounds resulted in enrichment of consensus sequences as judged by BstNI digest and sequencing, accompanied by an increase of phage titer in ELISA. Elution at room temperature and at 37°C gave similar results in this thesis. Elution at 4°C was not experimented. All subsequent elutions were therefore carried out at room temperature.

To prevent degradation of the eluted antibodies, elution was carried out with low pH glycine for a maximum of 10 minutes after which the pH of the eluate was brought to between 7.2 and 7.5 using Tris of pH 9, and stored at 4°C until use.

4.8 CHOICE OF FABS OVER scFv

Human recombinant antibody fragments, both scFv and Fab, have been successfully isolated against antigens of the gametocyte (Pfs48/45 and a variant of PfMSP1.19) and merozoite (MSP3) life stage of *P.falciparum*, from libraries constructed from the peripheral blood mononuclear cells of patients with acute malaria or from individuals immune to malaria [92-94].

The choice of Fabs as the antibody format in this library was based on evidence to the effect that Fab-fragments are more suitable for display on the phage surface because of its thermostability which is the same as full size antibodies, and their tendency to have the same binding efficiency to the antigen as their parental antibody [95, 96]. Again, in comparison to scFv fragments, Fabs lack the tendency to form dimers, trimers, and tetramers as is the case with scFv’s; [76] an accurate determination of the affinity is thus not easily possible with mixtures of mono- and multimeric (dimeric, trimeric and tetrameric) scFv fragments. As a consequence, the affinity assay used for assessing individual clones (such as BIAcore analysis) will often require time-consuming purification to obtain the monomeric fraction of the selected antibody fragments [97, 98]. Therefore, compared with scFv libraries, selections with Fab phage may be more governed by affinity rather than avidity, even when performing selections by panning on immobilized antigen [97] as was done in this PhD thesis.

4.9 LIBRARY SIZE AND QUALITY

Although generally not as large as naïve libraries, antigen biased libraries (immune libraries) contain a significantly larger proportion of antibody clones relevant to the antigens in question and should provide binders with higher specificities and/or affinities [99].
The constructed phage display library was not very large \((1.9 \times 10^7)\) in comparison to other libraries like that of Vaughan TJ et al., who created an scFv library of size \(1.4 \times 10^{10}\), however the functional size of \(1.4 \times 10^7\) compares to the libraries of Hammond F et al [100] who created three different libraries of size \(5 \times 10^7\), and favourably to that of Itoh K et al [101] with a library size of \(3 \times 10^6\) who in both instances successfully isolated high affinity binders to select antigens through biopanning.

Selection of specific and/or high affinity binders was anticipated from this library since an immune source was used for library construction where affinity maturation and clonal expansion was deemed to have taken place. Apart from that, the study subjects had been malaria-free for a minimum of 2 years despite having no preventive treatment in an area with a very high entomological inoculation rate [58]. Again, the percentage of positive clones carrying a full size Fab fragment was high (80%) and analyses by BstNI fingerprinting showed diverse RFLP pattern for all clones. In cases where the band patterns were similar, differences were seen in the band patterns of the corresponding light chain and vice versa. The diversity was further confirmed by the sequencing of 25 randomly selected clones which showed all gene families duly represented.

Apart from size, which is determined by the number of clones, another important factor that characterizes combinatorial libraries is the representativity (the number of different VH-VL combinations). To increase the representativity of the present library, cDNA from 50 different individuals were used for the V-gene amplification this was similar to the number of donors used by Vaughan TJ et al [78] who created an ScFv library from 43 donors.

Another criterion considered was the quality and complexity of the library. The higher the diversity of the clones within the library, the better the chances of selecting a fragment of high specificity and/or affinity,[102]. In the present library most of the clones selected for quality control analysis showed differences in the fingerprint analysis and/or sequencing.

4.10 USE OF DIVERSE GERM LINE GENES

The VH genes have been grouped into 6–7 families on the basis of nucleic acid sequence homology. Among the seven families, the VH3 family is the largest, and consists of 22 functional genes. The VH1 and VH4 families each contain approximately a dozen functional genes, and VH2, VH5, VH6, and VH7 families contain 3, 2, 1, and 1 functional gene, respectively [103].
When 25 randomly selected clones were sequenced, all the heavy and light chain V gene families were represented except for VK5. The VH1 family was the most predominant (41.6%), followed by the VH3 family (29.3%). The VH2 (12.5%), VH4 (4.2%), VH5 (8.3%) and VH6 (4.2%) families were present in much lower frequencies. This differed from the reported predominance of the VH3 family in the normal human repertoire. The VH 2, 5, and 6 in the normal human repertoire are used at much lower frequencies [104]. The VH4 family, in comparison to the normal human repertoire, was underrepresented in this library. The results were similar to that of Li L et al.[105] who worked on anti-HIV monoclonal antibodies and could result probably from compensation to reduced usage of the VH3 family genes or may be due to antigen requirements.

For the VK families, except for VK5, which was absent, all the other families were represented, with VK1 been the most dominant (41.6%), followed by VK4 (25%), VK3 (12.5%), VK2 (8.3%) and VK6 (4.2%). This is reflective of the present knowledge about the predominance of 4 main VK families in the normal human repertoire (VK1 (32%) VK2 (7%), VK3 (51%) and VK4 (10%) [106]. The absence of the VH5 family could be due to the generally low frequency of usage of the VK5 family or the small number of clones sequenced.

4.10.1 PANNING

The process of sorting the library to give a sub population with increased fitness of binding to a particular antigen has been described as panning. In practice therefore a large population of clones is inputted to give a resulting tiny population with increased fitness of binding. This new population is then amplified by infecting fresh bacterial host cells in their log phase of growth so that each individual phage in the sub population is represented by millions of copies in the amplified stock. The amplification is repeated a second and a third time and sometimes a fourth time as was done in this thesis to obtain an even fitter subset of the starting clones.

The two main parameters of selection that were manipulated to enhance the efficacy of selection were the stringency defined as the degree to which clones with higher fitness are favoured over those with lower fitness, done by varying the number of washes with PBST and the amount of antigen used to coat the wells of the microtitre plates and the yield, which is the fraction of particles that survive the selection process and are eluted. Increasing the stringency of washing generally reduces the number of false and low specificity binders [107, 108].

Although the ultimate aim of selection was to isolate clones with better binding characteristics, the inverse relation between stringency of selection and the yield [68] limited how stringent the selection process could be. In the first round of selection a higher yield was
favored over stringency as it was assumed that the better binding clones are usually not present in very high proportions. Unnecessarily stringent selection would have reduced the probability of selecting the desired clone. The plates were thus washed only ten times with PBST in the very first round resulting in one clone out of 24 with the desired insert. As the number of rounds increased, the stringency of washing was increased whiles the amount of protein per well in the final selection round was also reduced.

The enrichment of AMA1 specific binders was monitored by the ratio of input/output phage in each cycle. The loss of enrichment from the second to the third selection round can be attributed to the selective advantage of the non-binding clones over the binding ones, as the enrichment was retained when the stringency of selection was increased in the fourth round. Increasing the stringency above the 30 washes with PBST done in the third round would have lowered the yield of a specifically selected phage below the background yield of a nonspecifically selected phage irrespective of their affinity to the test antigen. When the stringency was increased in the final selection round, a 1200 enrichment over the first selection round was attained. The percentage of clones with inserts increased progressively from the first to the final selection round, with almost all the randomly selected clones in the final selection round having the right inserts.

4.10.2 ELUTION

In order to select for target antibodies with the desired characteristics, not only is an adequate phage antibody library required, the right elution strategy should be chosen. Although many elution reagents are present; from pH dependent (triethylamine, glycine/HCl) to those with enzymatic mechanisms (trypsin and chymotrypsin) or even competitive elution with soluble antigen, the choice of reagent to a large extent is dependent on the nature of the antigen since this influences how well a specifically bound phage is eluted [69]. It is for example known that a protein that has an overall positive charge may favor elution of specific phage at a low pH such as glycine/HCl at pH 2.2 as was used in this library.

It is crucial to try to maximize the sequence diversity accessed from the total repertoire at round one since it is this subset that is used for further selection rounds and its diversity will influence the success of selection. At round one, the number of clones that will not bind the antigen far exceeds the number that do. Limiting antigen concentration or lack of exposure of certain epitopes may result in the loss of specific clones, and potent clones present at low levels may be missed. In this thesis three different elution strategies were tried; low pH elution with 100mM glycine pH 2.2, high pH elution with 100mM triethylamine pH 9.0 and
direct elution with log phase *E.coli*. Except for the glycine no clones with the desired inserts were eluted after the first round for the direct elution. The TEA elution in the first round resulted in one clone out of 50 with the desired insert after the first round but subsequent rounds revealed bald clones although the same conditions as for the glycine elution were employed.

4.11 LOSS OF DIVERSITY

As the number of panning rounds increased, the diversity of the library reduced from the first up to the fourth where there were only two clones represented. Even at that the differences between the two clones were very minimal and could be attributed to errors in the PCR. This is however not surprising as the amplification of libraries, which is an essential step in phage display selection, has been shown to decrease the diversity of libraries [109-111].

Derda *et al* [109] who sequenced 40-50 clones after each round of amplification or panning observed that, after three rounds of panning less than 10 sequences dominated, with 70% of the clones being eliminated after each round. The best way to avoid the loss of diversity would have been to bypass the amplification step. However due to the generally low ratio of binding clones to non-binding clones the amplification step was not omitted in this thesis.

4.12 THE GLUCOSE EFFECT

Most phage display systems use vectors, like the pRFII, which are based on the lac-Z-promoter [72, 112]. Deletions of antibody genes and phagemid instability have been noticed during phage propagation studies. Increased levels of glucose in the culture media and agar plates along with other techniques like the insertion of transcription terminator sequences have been known to effectively repress product formation before induction [113], preventing the deletions and instability. In the presence of glucose (an easily metabolized monosaccharide), cAMP levels are low, so transcription from the *lac* promoter is low. This phenomenon is called the glucose effect or catabolite repression and is shared by a number of *E. coli* operons. In the present library, 1% (w/v) glucose was not sufficient to repress product formation before induction even in the case of a commercially available TG1 library, hence the observed loss of inserts after a second and a third sub cultivation. Increasing the glucose concentration to 2% (w/v) however effectively repressed product formation. Glucose in the growth media represses the *lacZ* promoter, preventing expression of *g3p*-fusion [69] which would inhibit super infection. Once the helper phage genome is incorporated into the cell, the glucose is removed and phage production commences.
4.13 PLANT EXPRESSION

The expression and purification of recombinant proteins has become a popular alternative to mammalian bioreactors because they are cheaper and are capable of producing high yields of antibody in a relatively short period of time, particularly if the proteins are expressed transiently in *Nicotiana benthamiana*. Again the growth of plants does not require animal or human derived nutrients, thus minimizing the risk of possible contamination with toxins or pathogens of human or animal origin.

The presence of an endomembrane system and a secretory pathway in plants, similar to those of mammalian cells also allows for post translational modifications of the recombinant problems which are critical to proper function [115].

Oche A was transiently expressed in *Nicotiana benthamiana* plants through an agrobacterium-mediated gene-transfer system. The use of the p19 silencing suppressor gene was to avoid gene silencing and to raise the expression levels of the antibody [116, 117]. Transient expression was chosen over stable transgenic expression because of the relatively shorter time and the higher antibody yield in comparison to stable transgenic expression [114].

4.14 GROWTH INHIBITION

To evaluate the inhibitory functions of the Oche A antibody, a growth inhibition assay was performed. Oche A showed a mean inhibition ± SD of 64.5±3.1 (%) of *P.falciparum* 3D7A in vitro. In comparison to anti-AMA1 antibodies, which are often used as a gold standard demonstrating over 80% inhibition, Oche A showed relatively strong inhibition; especially as this high inhibitory value relative to AMA-1 was obtained despite the amount of anti-AMA-1 antibody being over a 100% greater than the amount of Oche A antibody used in the assay (6mg/ml vs. 2.25mg/ml). This is contrary to a proposition by Saul A [118] that invasion inhibitory antibodies would require higher concentrations to effectively block invasion due to rapid invasion kinetics.

Given that the signs and symptoms of malaria are as a result of the invasion of merozoites into red cells and their subsequent multiplication and rupture, Oche A’s ability to strongly inhibit invasion may be an indication of its potential for use as a passive vaccine and supports the view that AMA1 antigen is a potent vaccine candidate. However, testing antibodies for activity in standard in vitro GIAs cannot clearly differentiate merozoite invasion inhibition from potential inhibitory effects on schizont rupture or merozoite egress and intra-erythrocytic parasite development [119].
It is thus not surprising that conflicting reports on the validity of GIA as a measure of protective immune function have been put forward; with [120, 121] reporting associations, whilst [122, 123] report no associations.

4.15 AFFINITY VS INVASION INHIBITION
Proteins have very particular chain configurations and conformations that promote high levels of specificity during chemical interactions [124].
The immobilization process at BIAcore may have affected the native conformational structure and by extension the bioactivity of the antibody, leading to steric hindrance and possible modification of the antigenic epitopes. Again in comparison to IgM which is multivalent, the recombinant full-sized antibody generated is IgG and has less pronounced biphasic dissociation [125]. These may explain the low affinity as shown by the low KD value of $1.363 \times 10^{-7}$.

This KD value however compares to the dissociation constants of approximately $1.5 \times 10^{-8}$ reported by Mahler SM et al [126] and the $10^{-7}$ reported by Lafaye P et al [127] who also isolated monoclonal antibodies using the phage display technology.
The strong inhibition of invasion may be a pointer to the fact that affinity and invasion inhibition are unrelated since a similar trend was observed in the immunoepidemiological analysis of the Ghanaian and Madagascan plasma samples; where despite the significantly higher reactivity of the Ghanaian samples in relation to the Madagascans, there was no statistically significant difference between the median invasion inhibition demonstrated by the Ghanaians and the Madagascans.

4.16 SEQUENCE ANALYSIS
The most abundant Fab fragment, was present in 28 out of the 29 of the clones sequenced (28/29) in the fourth selection round, 14 out of 28 of the selected clones in the third selection round (14/28) and 7 out of 19(7/19) in the second selection round. This could be due to its predominant presence in the immune response of humans or to its preferential amplification by PCR or phage multiplication [128] and or due to the over selection of the library.

The comparison of VH fragment of OcheA with its nearest V germline gene fragments, IGHV1 69*11, showed differences in the nucleotide and amino acid levels distributed in the CDRs (1, 2, and 3). These mutations that possibly arose through somatic hypermutations could have an important positive effect on antibody affinity and stability considering that the Oche A was the most abundant fab specific to AMA1 selected.
5 SUMMARY

Malaria still remains an important cause of morbidity and mortality in many parts of the world. Despite an enormous research effort, the goal of finding an effective vaccine still remains elusive. The antibody generation part of the Fraunhofer Foundation Project aims amongst other things to isolate human antibodies by antibody phage display from semi-immune individuals that might be used for short term protection or therapy. Human plasma samples were tested in an ELISA for their reactivity to MSP1-19, MSP3, AMA1 and crude plasmodium lysate and the responses scored. Purified IgG from these samples were then tested in vitro for direct invasion inhibition. From the highest responders, through a 2-step cloning strategy, an antibody Fab library of functional size \(1.4 \times 10^7\) was constructed and a subtractive panning strategy applied. The selected antibodies were expressed in plants based on a protocol for rapid expression and purified through an affinity chromatography. A monoclonal Fab antibody that selectively binds to AMA1 was isolated and processed into full sized antibody. Functionality was assessed by surface plasmon resonance, reducing/non-reducing SDS PAGE, Western Blot, ELISA and growth inhibition assay. Analysis of the plasma immune responses revealed a significant correlation between responses to AMA1 and MSP3 and invasion inhibition. From the Fab phage display one antibody binding to AMA1 was isolated. The recombinant antibody specifically binds to the antigen and also strongly inhibits the parasite invasion directly.

The results of this thesis is central to the antibody generation part of the Fraunhofer MP3 project as the selection of suitable samples for use in single cell sorting and EBV virus transformation by the other PhD candidates are both based on the immunoepidemiological characterization that was done at the start of the thesis. Indeed, a functional full sized recombinant antibody to MSP10, another merozoite stage antigen of Plasmodium falciparum has been produced (patent pending) through the EBV transformation and it is anticipated that many more such antibodies will be produced in the very near future.

The constructed phage display library is the first functional human immune antibody library to be created in the institute and has not only established a working protocol, but a pipeline and a platform for antibody isolation.

The results also proved the applicability of the antibody phage display methodology for the isolation of functional recombinant human antibodies and thus a platform has been established that should allow for the isolation of antibodies against other blood stage antigens.
6 OUTLOOK

The data presented in this thesis has lent support to the fact that immunity to malaria may not be dependent on the presence of antibodies to just a few antigens but to a wide range of antigens. This is seen in the increasing percentage inhibition of invasion with increasing breadth of antibody response and supported by the ability of some plasma samples to inhibit invasion although they did not react to any of the antigens tested. It will be important to test a wider range of antigens probably in a longitudinal assay in a given population to allow for a more complete picture as relates to malaria vaccine candidates.

A functional full-sized antibody that strongly inhibits merozoite invasion of erythrocytes has been produced, but with a low affinity as shown by the $K_D$. It will be important to improve on the affinity of the antibody by site directed mutagenesis and/or error prone PCR. Panning of the library with a large repertoire of antigens that are in stock to isolate more binders to AMA1 and the other antigens should be carried out. To improve on the chances of selecting binders, another approach to the library construction will be a preselection with select antigens to create a more “directed“ library that will tremendously improve the chances of selecting binders to the antigens used of interest.

Although two soluble Fabs were isolated that bound to AMA1, only one of them after conversion into a full sized antibody maintained its binding activity. It will be interesting to optimize the expression conditions for this clone or try mammalian CHO cell expression as the different glycosylation patterns in plants and mammals could be responsible.

Of the six clones selected after the monoclonal Phage ELISA, only two were expressed into full sized antibodies for further testing. It will be important to convert the others into full sized antibodies for further testing.

Presently, selection is ongoing with MSP1-9 and MSP3 and it is hoped that antibodies that bind selectively to these and are also able to strongly inhibit merozoite entry will be isolated.
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8.3 ACKNOWLEDGEMENT
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8.4 PUBLICATIONS
Addai-Mensah O et al. “Acquired Immune Responses to three malaria vaccine candidates and their relationship to invasion inhibition in two populations naturally exposed to Malaria” submitted

Addai-Mensah O et al. „Construction of a human immune Phage display library and the isolation of a monoclonal binder to the AMA1 antigen of *Plasmodium falciparum” In preparation


8.5 CV

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