Recombinant expression of *Plasmodium falciparum* histidine rich protein-2 (*Pf*HRP-2) and characterisation of chicken anti-*Pf*HRP-2 antibodies

by

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Preface

The work and experiments presented in this dissertation were conducted at the School of Life Sciences in the Discipline of Biochemistry, at the University of KwaZulu-Natal, Pietermaritzburg, from October 2015 to January 2019 under the supervision of Professor J. P. D. Goldring.

The study described here is original work by the author and have not been submitted to another university in any form. Where the use of another author's work has been made, it has been appropriately acknowledged.

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Mlondi Shezi (April 2019)

As the supervisor, I agree to the submission of this dissertation by the candidate.

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Professor J. P. D. Goldring (April 2019)

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Abstract

The diagnosis of malaria using rapid diagnostic tests (RDTs) targets three Plasmodial proteins namely, *Plasmodium falciparum* histidine rich protein-2 (*Pf*HRP-2); lactate dehydrogenase (LDH) and aldolase. Early diagnosis is important for malaria control and accurate diagnosis guides treatment. The diagnosis of malaria prioritises the detection of *P. falciparum* (*Pf*), the species with the highest mortality. As a result, *Pf*HRP-2-based RDTs are the most commonly used RDTs to detect *P. falciparum* infections. *Pf*HRP-2 is a *Plasmodium falciparum* protein expressed during the blood stages of the parasite infection and is abundant in the blood of patients. The *Pf*HRP-2 protein is stable in the blood, urine, saliva and cerebrospinal fluids of patients where it can be detected. Detection of *Pf*HRP-2 in the field is problematic due to gene deletion, *Pf*HRP-2 protein sequence variation, low heat tolerance of mammalian anti-*Pf*HRP-2 antibodies in RDTs, prozone effect, low quality assurance standards and low sensitivity of RDTs in some cases. False positive results caused by mammalian antibodies reacting with human rheumatoid factor also affect the antibody-based detection of malaria. This limits the diagnosis of *P. falciparum* malaria and undermines malaria control efforts. Optimising the performance of current *Pf*HRP-2-based RDTs could prevent some of these problems and would strengthen malaria surveillance. Most malaria RDT problems associated with defective or cross-reactive antibodies could be abated using a different species of antibodies. Chicken IgY antibodies are stable at room temperatures and do not react with human rheumatoid factor.

To raise anti- *Pf*HRP-2 chicken IgY antibodies, recombinant histidine rich protein-2 (r*Pf*HRP-2) was expressed in *E. coli* BL21 (DE3) at 30°C overnight, induced with lactose. The recombinant protein was affinity purified and analysed on SDS-PAGE gels, resolving as a single protein band of 54 kDa. r*Pf*HRP-2 induced high antibody titers after 13-weeks in immunised chickens. Purified IgY antibodies against r*Pf*HRP-2 detected the 54 kDa band, a r*Pf*HRP-2 dimer and trimer which were also detected by human anti-r*Pf*HRP-2 antibodies.

The thermal stability of anti-r*Pf*HRP-2 IgY antibodies was assessed by exposure to temperatures resembling the field were RDTs are deployed. IgY antibodies stored at room temperatures for sixteen weeks were shown to be stable and still detected r*Pf*HRP-2 in an ELISA after storage.

To determine epitopes or peptides detected by the anti-r*Pf*HRP-2 IgY antibodies, the recombinant protein was cleaved with trypsin and the fragments probed with IgY. Trypsin cleavage of r*Pf*HRP-2 produced fewer fragments than predicted and the fragments resolved larger than their estimated sizes on SDS-PAGE gels. The fragments were detected by the anti-r*Pf*HRP- 2 IgY antibodies.

Chicken IgY antibodies have potential for possible application in malaria diagnostics to detect Plasmodial proteins. The IgY used in this study was stable at room temperatures, and detected trypsin hydrolysed r*Pf*HRP-2 fragments whose identity requires further investigation to better understand the fragment's behavior and determine epitopes of anti-r*Pf*HRP-2 IgY antibodies.

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Abbreviations

2xYT	2x yeast extract tryptone
Ab	Antibody
ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACT	Artemisinin-based combination therapy
An.	*Anopheles*
BSA	Bovine serum albumin
cDNA	Complementary DNA
C-terminus	Carboxyl terminus
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallisable region
FISH	Fluorescent *in situ* hybridisation
GFP	Green fluorescence protein
h (hrs)	Hour/s
His-tag	Histidine tag
HRG	Histidine rich glycoprotein
Hrp	Histidine rich protein gene
HRPs	Histidine rich proteins
ICAM-1	Intercellular adhesion molecule-1
IEM	Immunoelectron microscopy
IF	Immunofluorescence
IFNɣ	Interferon gamma
IgG, IgM, IgA, IgD, IgE)	Immunoglobulin (G,M,A,D,E)
IgY	Immunoglobulin Y
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS	Indoor residual spraying
ITNs	Insecticide-treated bed nets
kDa	Kilodalton(s)

LAMP	Loop mediated isothermal amplification
LB	Luria-Bertani
LDH	Lactate dehydrogenase
min.	Minutes
NDH	National department of Health (South Africa)
Ni-NTA	Nickel-Nitrilotriacetic acid
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
pI	Isoelectric point
RBC	Red blood cell
RDT(s)	Rapid diagnostic test(s)
RNA	Ribonucleic acid
r*Pf*HRP-2	Recombinant *Plasmodium falciparum* histidine rich protein – 2
RT	Room temperature (about 25°C)
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T and B cells	Thymus cells and bone marrow- or bursa-derived cells
TB	Terrific broth
TEMED	Tetramethyl ethylenediamine
VCAM-1	Vascular cell adhesion protein-1
WHO	World Health Organisation

Chapter 1

Literature review

1.1. Introduction to malaria

The genetic material from malaria *Plasmodium* parasites has been found in 100 – 4000-year-old mummified human tissue samples (Nerlich *et al*., 2008). The disease has affected man for thousands of years (Lalremruata *et al*., 2013) and is still prevalent in more than ninety countries (WHO, 2018). In the 1900s, malaria transmission occurred all over the world and has since been reduced to the Tropics and Subtropical regions of the globe (Hay *et al*., 2004). "Malaria" comes from the Italian term "*mal' aria*" meaning "bad air" (Ross, 1911; Bruce-Chwatt, 1981). The disease was associated with the smelly air of swamps and marshes (Bruce-Chwatt, 1981). This was before the identification of malaria parasites by Laveran in 1880 (Cox, 2010). The protozoan parasites of the *Plasmodium* genus are causative agents of malaria, aided by female *Anopheles* mosquitoes as transmission vectors. There are over one hundred *Plasmodium* species affecting mammals, reptiles, and birds (Suh *et al*., 2004). *Plasmodium falciparum; vivax; malariae; ovale* and *knowlesi* are the five species of malaria infecting humans. Each species presents slightly different characteristics and clinical complications in the human host. *Plasmodium falciparum* accounts for the most malaria-related deaths and is mainly found in Africa. Children under the age of five years, pregnant women and non-immune travelers are the most severely affected by malaria. It is estimated that malaria takes a child's life every two minutes (WHO, 2018).

1.2. Malaria distribution and global prevalence

Malaria occurs in Central and Tropical South America; sub-Saharan Africa; the East Mediterranean region, India and South East Asia (Figure 1.1). About 3.3 billion people were at risk of malaria in 2017 (WHO, 2018). Malaria-related deaths decreased from an estimated 451 000 in 2016 to 435 000 in 2017 and children under 5 years of age accounted for 61% of all malaria-related deaths (WHO, 2018). But malaria cases increased from 217 million to 219 million. Two hundred million (92%) of these cases occurred in Africa, with 5% in South East Asia and 2% in the East Mediterranean region. Sub-Saharan Africa accounted for 93% of all malaria deaths in 2017 and a marked increase in malaria cases (WHO, 2018). The African countries of Nigeria, Madagascar and the Democratic Republic of the Congo reported an increase of more than half a million cases each. Other African countries like Rwanda and Ethiopia reduced their malaria burden by more than 670 000 cases between them (WHO, 2018), but the burden in Africa is still the highest compared to other regions.

Plasmodium falciparum accounted for 99.7% of estimated malaria cases in sub-Saharan Africa, 69% in the East Mediterranean region and 62.8% in South East Asia including India (WHO, 2018). *Plasmodium vivax* is the major Plasmodial species in Central and South America accounting for 74.1% of malaria cases and is the second most prevalent in sub-Saharan Africa.

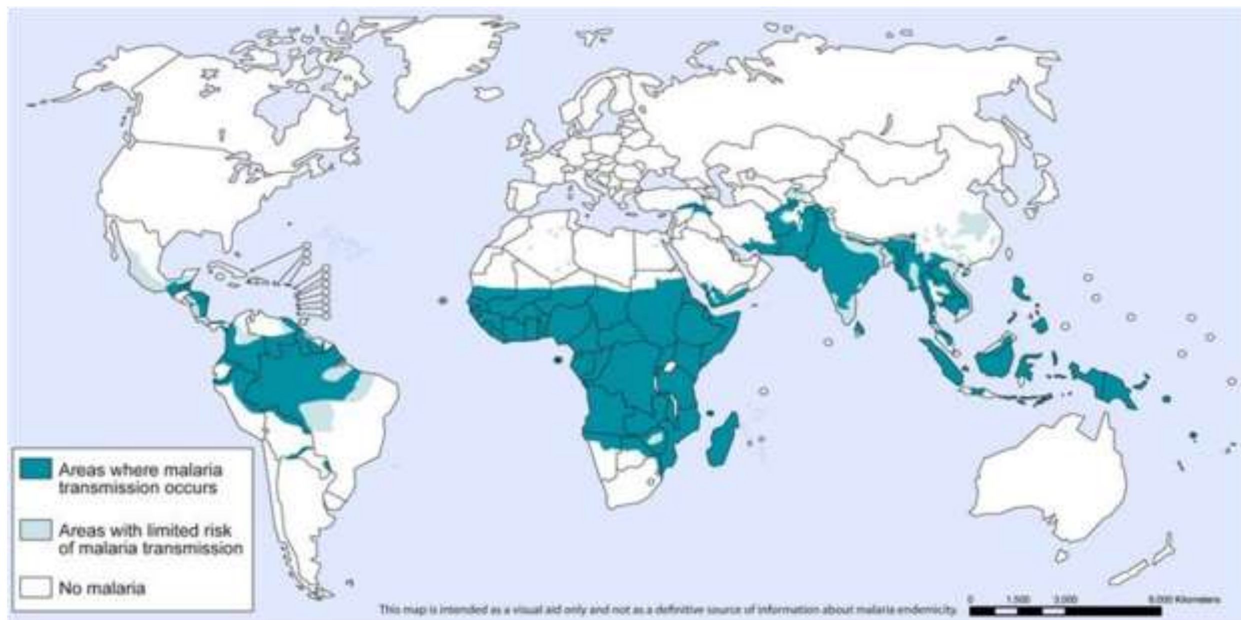

Figure 1.1. Areas of malaria transmission globally.
This includes areas of low and high malaria transmission in the WHO malaria regions. Data is according to the World Health Organisation. Adopted from Marks *et al.*, 2014.

1.2.1. Malaria in South Africa

In South Africa, malaria is seasonal and confined to the north-eastern parts of the country including the Limpopo, Mpumalanga and KwaZulu-Natal provinces (Burke *et al.*, 2017). Efforts are being made to eliminate the disease by 2020 (Hlongwana *et al.*, 2018; Mbokazi *et al.*, 2018). The risk of malaria transmission increases between September to May because of rainy weather conditions (NDH, 2017). According to the South African Department of Health, the malaria burden increased from 6 385 cases, 58 deaths in the 2015/2016 season, to 9 478 cases, 76 deaths in the 2016/2017 season (NICD, 2017). *Plasmodium falciparum* is the major malaria species in South Africa transmitted by several *Anopheles* mosquito vectors including *An. arabiensis*, *An. gambiae*, and *An. funestus* (Hargreaves *et al.*, 2000; Morris *et al.*, 2013; Burke *et al.*, 2017). Indoor residual spraying (IRS) for mosquitoes and malaria case management are the main control strategies in South Africa (Burke *et al.*, 2017). Indoor residual spraying is affected by insecticide resistance and outdoor malaria transmission and may not be enough for elimination. Thus, studies suggest additional malaria control strategies i.e. malaria surveillance, effective management of imported malaria, understanding of malaria

epidemiology and new malaria intervention tool to support malaria elimination in South Africa (Hlongwana *et al*., 2018; Mbokazi *et al*., 2018).

1.3. Malaria biology

The first demonstration of mosquitoes as vectors of malaria was shown by Ross in 1897 with avian malaria, and then for human malaria by Grassi and colleagues in 1898 to 1900 (Cox, 2010). Mosquitoes become carriers of malaria by feeding for a high protein meal on mammals or other animals already having the disease. The parasite multiplies sexually within the mosquito's gut and moves to the salivary glands where it is transmitted through saliva when feeding (Figure 1.2, (1)). The lifecycle of *Plasmodium* parasites consists of a liver stage, an asexual red blood cell stage and a sexual stage in the gut of a mosquito vector. The liver stage or "pre-erythrocytic stage" was first identified by Shortt and Garnham in 1948 (Shortt *et al*., 1948).

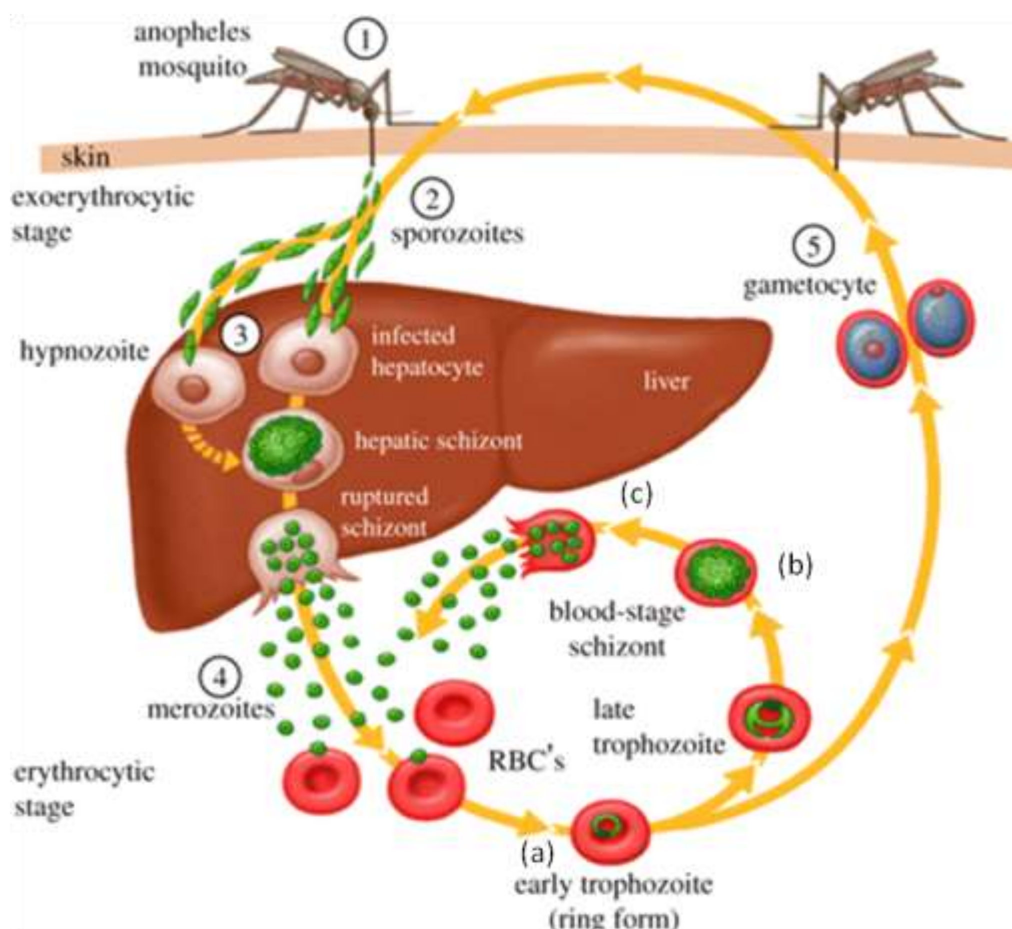

Figure 1.2. ***Plasmodium*** **parasites life cycle in humans.**
Malaria parasite stages are illustrated from an infected mosquito vector (1), the liver stage (2), the asexual blood stage (4a-c) and gametocyte uptake by uninfected mosquitoes (5) for further transmission. Figure adopted from Hill, (2011).

1.3.1. The asexual life cycle stage of *Plasmodium* parasites

Plasmodium sporozoites (Figure 1.2 (2)), travel from the salivary glands of the mosquito to the liver of the human host infecting liver cells (Figure 1.2 (3)). The sporozoites multiply and mature within the liver cells into schizonts (Hill, 2011). Mature liver-schizonts rupture to release merozoites (Figure 1.2 (4)), which infect red blood cells. Merozoites in red blood cells form rings (Figure 1.2 (a)) which mature during the trophozoite stage (Hill, 2011). Mature trophozoites progress to schizonts (b), which rupture releasing daughter merozoites in the blood (c). At this point, several parasitic proteins are released in to the blood of the host and can be detected for malaria diagnosis. The merozoites infect uninfected red blood cells, repeating the asexual life cycle (Hill, 2011). This invasion, multiplication and release cycle takes 24 h in *P. knowlesi*, 48 h in *P. falciparum; P. vivax* and *P. ovale* and 72 h in *P. malariae* (WHO, 2015_{b}).

1.3.2. The sexual lifecycle stage of *Plasmodium* parasites

During the erythrocytic stage of *Plasmodium* parasite development (Figure 1.2), some ring forms (a) mature to sexual forms called gametocytes (Figure 1.2 (5)). The male and female gametocytes are dormant in the human host and become active once taken up by the *Anopheles* mosquito vector (Hill, 2011). In the mosquito's gut the *Plasmodium* gametocytes produce gametes which combine to form a zygote, which develops to an ookinete. The ookinete develops into an oocyst which matures and ruptures releasing sporozoites. The sporozoites migrate to the salivary gland, containing mosquito coagulants where they can be injected with saliva during a blood meal (Hill, 2011).

1.3.3. Clinical malaria symptoms

Plasmodium parasites cause fever which can appear in 7 – 30 days after a malaria-infected mosquito bite. The clinical symptoms of malaria are common to other infections like flu and common cold and includes fever, chills, headaches and vomiting (WHO, 2018). Clinical symptoms are experienced during the asexual red blood cell stage. The symptoms are often categorised as uncomplicated or sever depending on the extent of the disease and are treated differently (discussed below).

1.3.3.1. Uncomplicated malaria

Uncomplicated malaria is a milder form of a malarial infection characterised by fever, hot and cold sensations, headaches, vomiting, and tiredness (WHO, 2018). It is marked by the absence of organ failure which could occur if the patient is not treated (Bartoloni and Zammarchi, 2012).

1.3.3.2. Severe malaria

Severe malaria is the most severe presentation of malaria and is 10 – 40% fatal even with treatment. The symptoms are often indicative of organ system failures leading to: cerebral

malaria, respiratory failure, acute renal failure and severe anemia (Bartoloni and Zammarchi, 2012).

1.4. Malaria control and treatment

Malaria prevention and control currently relies on the use of insecticide-treated bed nets, insecticide treatment of mosquito breeding sites, indoor spraying of insecticides and drug treatment of infected patients. The 2018 World Malaria Report by the World Health Organisation has indicated restricted progress in the fight against malaria in some regions. Ten high burden malaria counties experienced an increase of 3.5 million cases in 2017 and 10 out of 21 malaria eliminating countries reported an increase in indigenous malaria cases compared to previous years (WHO, 2018). Inadequate funding of malaria control programs, antimalarial drug resistance and insecticide resistance hamper malaria control and elimination in these regions. New control strategies are required to reduce the global burden of malaria and eliminate the disease in low transmission areas (Brooke *et al*., 2013; Killeen *et al*., 2016; WHO, 2018).

1.4.1. Vaccine development

Malaria vaccine development is an area of active research and several vaccine candidates are in preclinical and clinical phases of assessment (Table 1.1). Malaria vaccines target the pre-erythrocytic stage and the blood stages in humans, while transmission blocking vaccines target parasite development in the mosquito vector (Coelho *et al*., 2017; Laurens, 2018). Due to the slow progress in reducing the global malaria burden, the development of an effective malaria vaccine is essential.

Table 1.1. Several malarial vaccine candidates currently under development

Parasite stage	Vaccine classification	Current status
Pre-erythrocytic stage		
PfSPZ vaccine	Whole organism (radiation attenuation)	Phase II
GAP vaccines	Whole organism (genetic attenuation)	Phase I
RTS,S	Subunit	Phase IV
CVac	Whole organism (chemical attenuation)	Phase I
Blood stage		
Chemically attenuated parasites	Whole organism	Preclinical
AMA1-RON2	Subunit	Preclinical
PfRH5	Subunit	Phase I
Mosquito stage (TBVs)		
Pfs25	Subunit	Phase I
Pfs230	Subunit	Phase I
Pfs47	Subunit	Preclinical

Vaccine candidates under clinical trials: indicated as phase I-IV. Preclinical assessment indicates vaccine candidates being tested in mice, rodents and non-human primates. The data was adopted from Coelho *et al.*, 2017.

The World Health Organisation plans to license malarial vaccines with at least 75% protective efficacy by 2030, in line with a global malaria elimination goal (WHO, 2013; Laurens, 2018). The RTS,S/AS01 vaccine is the only malaria vaccine candidate in phase IV clinical trials and close to licensing for global distribution (Coelho *et al.*, 2017; Wilson *et al.*, 2019). RTS,S/AS01 shows partial 26% and 39% protective efficacy in infants 6-12 weeks old and children 5-17 months old respectively, and lasts for 48 months after four doses of immunisation (Coelho *et al.*, 2017; Draper *et al.*, 2018). The vaccine is associated with side effects such as meningitis, cerebral malaria and febrile seizures (Draper *et al.*, 2018). But, RTS,S/AS01 has been approved for pilot studies in Ghana, Kenya and Malawi commencing in 2019 (Coelho *et al.*, 2017; Laurens, 2018; WHO, 2018). This indicates some progress in the field of malaria vaccine development despite the low efficacy and short-lived protective ability of RTS,S/AS01 the leading vaccine candidate.

1.4.2. Vector control

The identification of mosquitoes as transmission vectors for malaria introduced ways of controlling malaria by targeting mosquitoes. These included the drainage of canals and the treatment of swamps with larvicides, which reduced mosquito breeding and eliminated malaria in some areas (Bruce-Chwatt, 1981). The spraying of residual insecticidal compounds like

dichlorodiphenyltrichloroethane (DDT) eliminated malaria in Italy, Greece, Venezuela and other countries (Bruce-Chwatt, 1981), and has established vector control as the main way of preventing and reducing malaria transmission (WHO, 2018). The four main classes of insecticidal compounds used for IRS and insecticide-treated mosquito nets (ITNs) are organochlorides, organophosphates, pyrethroids and carbamates (Ranson and Lissenden, 2016). The call to limit the use of DDT, the most effective organochloride and the reservation of pyrethroids for ITNs has reduced the scope of long-lasting insecticidal compounds for IRS (reviewed by Ranson and Lissenden, 2016). Increasing resistance to the four classes of insecticides has been indicated in Mali (Cisse *et al.*, 2015), Tanzania (Kisinza *et al.*, 2017) and Côte d'Ivoire (Edi *et al.*, 2012). The effect of *An. funestus* resistance to pyrethroids used for IRS in South Africa led to a malaria epidemic in the year 1999 to 2000 and forced the reintroduction of DDT in the country (Coetzee *et al.*, 2013). There is progress in the development of alternative vector control methods, such as biological control and genetic alteration of male and female mosquitoes (Kamaraju *et al.*, 2011).

1.4.3. Antimalarial drug treatment

Antimalarial drugs are used to prevent mortality from malaria as the disease is curable with early treatment. Drugs can prevent progression to sever illness by clearing parasitemia and reduce disease transmission. Thus, it is important to reduce the development and spread of antimalaria drug resistance (WHO, 2015_b). Combination therapy reduces antimalarial drug resistance. Artemisinin-based combination therapy (ACT) is recommended as the main method of treatment for *P. falciparum* infections (WHO, 2015_b). ACTs combine an artemisinin derivative with a long-lasting partner drug i.e. sulfadoxine-pyrimethamine or mefloquine. Artemisinins rapidly eliminate parasitemia and lead to patient recovery, while the long-lasting partner drug clears any remaining parasites leading to a cure (WHO, 2015_b). The long-lasting partner drug usually has a different mode of action to artemisinins to reduce the development of drug resistance (WHO, 2015_b). Intravenous treatment with artesunate an artemisinin derivative, is advised in severe malaria patients for at least 24 h before oral ACT treatment (WHO, 2015_b). The lack of safety data on ACT in the first trimester of pregnancy has excluded ACT treatment for malaria infected pregnant women (Moore *et al.*, 2016; Dellicour *et al.*, 2017). Chloroquine and quinine are still used in areas where the parasites are still susceptible to the drugs (Achan *et al.*, 2011) and are used for the treatment of non-*P. falciparum* malaria and first trimester pregnancy (WHO, 2015_b). The treatment of *P. vivax* and *P. ovale* also includes a dose of primaquine to prevent relapse from dormant liver stages (Ashley *et al.*, 2014). Low doses of primaquine are also given to *P. falciparum* infected patients to kill gametocytes, preventing disease transmission (Ashley *et al.*, 2014).

Antimalarials can be used to prevent malaria for pre-exposure prophylaxis and this practice is recommended in special risk groups i.e. during pregnancy; in newborns less than

a year old and in groups affected by seasonal malaria infections (WHO, 2015_b). Malaria prophylactic medicine prevent the liver stages of infection or suppress the asexual blood stage, and should be administered before, during and after the risk of a malaria infection (Schwartz, 2012; WHO, 2015_b; NDH, 2017). The objective of prophylactic treatment is to avoid a malarial infection by maintaining therapeutic levels of antimalarial drugs in the blood (Schwartz, 2012; WHO, 2015_b).

1.5. Malaria diagnosis

Early diagnosis of malaria is important for timely treatment and can prevent death (WHO, 2018). The detection of *Plasmodium* parasites during the diagnosis of malaria is mandatory for suspected malaria cases (WHO, 2015_a). This reduces the mismanagement of non-malaria fevers and can reduce parasite drug resistance (WHO, 2010). Parasitological diagnosis involves identifying products of *Plasmodium* origin i.e. proteins, nucleic acids or the parasites themselves (Makler, 1998; WHO, 2010).

Malaria is diagnosed by microscopy, antibody based diagnostic tests and nucleic acid based molecular techniques (Makler, 1998; Moody, 2002). A good diagnostic test must differentiate a true positive from a true negative infection, meaning that a test must be sensitive and specific (Altman and Bland, 1994). Diagnostic sensitivity is the percentage of infected persons accurately diagnosed positive and specificity is the percentage of uninfected persons accurately diagnosed negative (Altman and Bland, 1994; Saah and Hoover, 1997).

1.5.1. Detecting malaria parasites by microscopy

Microscopy is the reference standard for malaria diagnosis and allows visualisation of different intraerythrocytic stages of the infecting parasite (Bruce-Chwatt, 1981; Makler, 1998; Moody, 2002; Jan *et al*., 2018). A simple light microscope together with a thick-film Giemsa or other stain provides an effective tool for identifying malaria in infected blood samples at fifty parasites per microliter of blood (Makler, 1998; Moody, 2002). Microscopic examination of blood samples from malaria patients during treatment gives an indication of treatment success or failure, based on the presence or absence of parasites (Jan *et al*., 2018). The ability to distinguish between different malaria species and to quantify parasite densities (Makler, 1998; Moody, 2002; Jan *et al*., 2018) makes microscopy an ideal standard against which other malaria diagnostic methods are compared (WHO, 2000).

Microscopy-based diagnosis of malaria is most effective in the hands of trained experts (Mouatcho and Goldring, 2013) and is often not available in remote areas where malaria diagnosis and treatment are required (Jan *et al*., 2018). Microscopic diagnosis is time consuming, which can delay treatment (Moody, 2002; Jan *et al*., 2018); is unreliable for detecting low parasitemias (Leke *et al*., 1999; Bell *et al*., 2001; Bell *et al*., 2005) <50 parasites

per microliter in the field (Kilian *et al.*, 2000) and cannot detect sequestered parasites (Leke *et al.*, 1999).

1.5.2. Nucleic acid-based diagnosis of malaria

Malaria nucleic acid-based diagnostic techniques are centered around the amplification of targeted parasite DNA or RNA fragments to identify the presence of malaria DNA and hence parasites in patients (Makler, 1998; Moody, 2002; Kandie *et al.*, 2018). The techniques include loop mediated isothermal amplification (LAMP) and forms of polymerase chain reactions (PCR) such as nested, quantitative and reverse transcription PCR. These techniques are very sensitive as they can detect ≤ 5 parasites per microliter of blood (Snounou *et al.*, 1993; Kandie *et al.*, 2018). Their sensitivity allows the detection of low level parasitemia during parasite sequestration and can differentiate between malaria species (Moody, 2002). Malaria nucleic acid-based diagnostic techniques are not practical for field use because of the requirement for specialised equipment (Mouatcho and Goldring, 2013). Fluorescent *in situ* hybridisation (FISH) is a nucleic acid-based technique adapted for limited resource laboratories or clinics (Kandie *et al.*, 2018). This technique uses complimentary DNA or RNA labeled with fluorescent probes to detect *Plasmodium* nucleic material in samples of patients (Shah *et al.*, 2015; Kandie *et al.*, 2018). FISH has the sensitivity of PCR and the potential to be used in resource limited areas (Shah *et al.*, 2015).

1.5.3. Antibody based malaria diagnosis

Antibody-based malaria diagnostic tests include, enzyme linked immunosorbent assays (ELISA) and immunochromatographic diagnostic test strips. The tests use antibodies to detect Plasmodial proteins in samples from malaria patients. The detecting antibody in an ELISA assay is linked to an enzyme that indicate an immunological reaction in the presence of a suitable substrate. This amplifies the detection signal and allows detection of small concentrations of antigen. The ELISA assays used in malaria diagnosis have limits of detection in the rage of picograms of antigen per milliliter of blood sample (Jang *et al.*, 2018). This is important for the detection of asymptomatic malaria which could be essential for malaria elimination (Jang *et al.*, 2018). ELISA has a wide range of application in malaria research including quantifying the amount of malarial proteins in the blood, serum, saliva and cerebrospinal fluids of infected patients (Jang *et al.*, 2018; Nambati *et al.*, 2018; Thakur *et al.*, 2018); detection of all malaria species (Kim *et al.*, 2016) and can be used to correlate specific Plasmodial protein levels to blood parasitemia (Nambati *et al.*, 2018). This could help estimations or predictions of disease severity or parasite load which would assist treatment. ELISA is more sensitive than immunochromatographic diagnostic strips but is not suited for field application or point-of-care since it requires specialised laboratory equipment.

1.5.4. Rapid diagnostic test (RDTs)

Immunochromatographic test or rapid diagnostic tests (RDTs) are an attractive alternative to microscopy for field diagnosis of malaria and offer a simple, quick method of parasite confirmation before treatment (Makler, 1998; WHO, 2000). RDTs do not require specialised equipment or technology making them accessible in remove areas. Malaria RDTs are lateral flow immunochromatographic devices designed to detect and capture *Plasmodium* antigens in liquid samples i.e. blood (Moody, 2002; Mouatcho and Goldring, 2013). Usually a blood sample from a suspected malaria patient is loaded onto the RDT cassette (Figure 1.3) followed by a buffer containing *Plasmodium* specific labeled antibodies. The labelled antibodies interact with the *Plasmodium* antigen in the presence of malaria parasites in the blood forming a complex. The antibody-antigen complex migrates by capillary action on the nitrocellulose strip to interact with an antigen specific capture antibody bound on the nitrocellulose forming an aggregate test line. Some of the labelled antibodies migrate without the antigen and are captured by a specific antibody forming a control line (Figure 1.3).

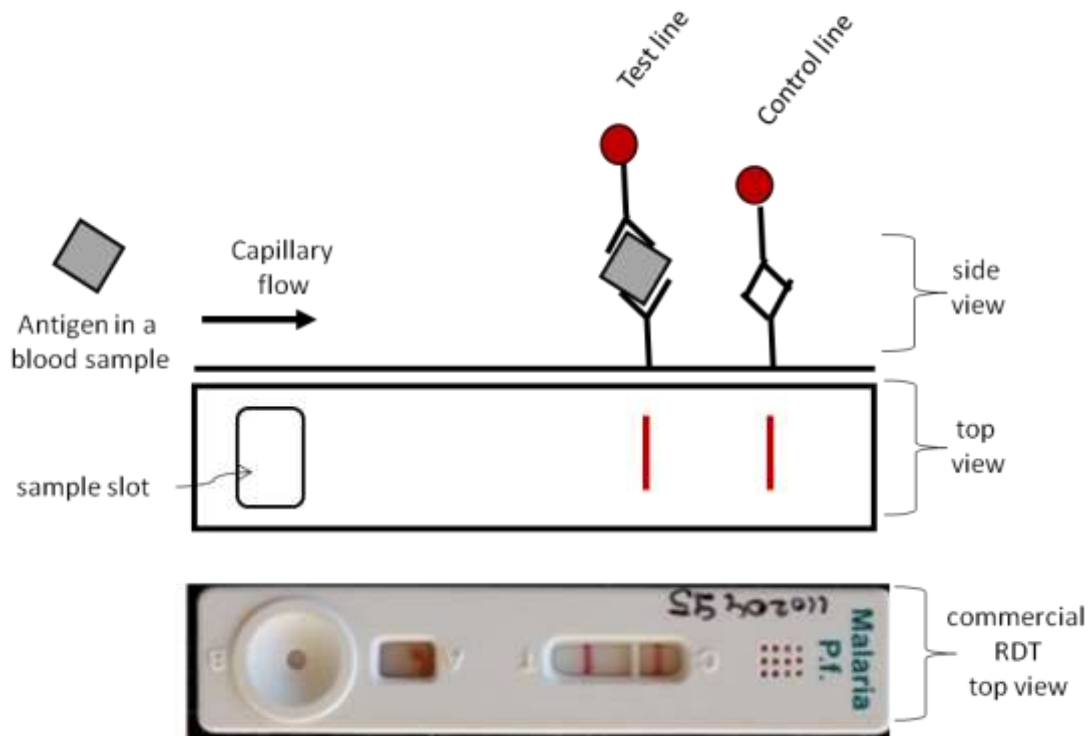

Figure 1.3. Schematic representation of a malaria rapid diagnostic test (RDT)
This picture was adopted from Gillet *et al*., 2010.

The current RDTs target three *Plasmodium* proteins *P. falciparum* histidine rich protein-2 (*Pf*HRP-2); *Plasmodium* lactate dehydrogenase (*P*LDH) and aldolase (Mouatcho and Goldring, 2013). LDH and aldolase are enzymes of the glycolytic pathway and can be detected in all *Plasmodium* species, unlike HRP-2 which is produced by *P. falciparum* alone. The detection of LDH and aldolase is useful in monitoring antimalarial treatment since these enzymes quickly clear from the blood after cure. Low levels of LDH can be detected in the

saliva of malaria infected patients (Nambati *et al*., 2018) like *Pf*HRP-2. *Pf*LDH-based RDTs have been shown to be as sensitive as *Pf*HRP-2 RDTs but more specific (Gatton *et al*., 2015). Thus, *Pf*LDH RDTs have been recommended as an alternative to *Pf*HRP-2 based RDTs in areas of *Pf*HRP-2 gene deletion (Berhane *et al*., 2018). *P. vivax* specific LDH based RDTs on the other hand, are associated with varying sensitivity and might require further development to be comparable to *Pf*HRP-2 and *Pf*LDH RDTs (Jimenez *et al*., 2017).

The current malaria RDTs use mammalian monoclonal antibodies which are usually IgG and IgM and the tests are temperature labile. IgG-based RDTs cross-react with human rheumatoid factor leading to false positive results in blood samples not containing *Plasmodium* parasites, but test positive for rheumatoid factor (Lee *et al*., 2014). RDTs are also affected by temperatures above 35°C and humidity which can reduce the reactivity of the antibodies on the test strip and this affects the detection of Plasmodia (Chiodini *et al*., 2007). A single chain recombinant antibody not reacting with human rheumatoid factor might address some of these challenges.

1.6. *Plasmodium falciparum* histidine rich protein (*Pf*HRPs)

*Pf*HRP-2 based RDTs are the most commonly used and more sensitive in the field compared to *P*LDH and aldolase RDTs (Batwala *et al*., 2010; Gatton *et al*., 2015; Alareqi *et al*., 2016; Jimenez *et al*., 2017). This study focuses on *Plasmodium falciparum* histidine rich protein-2.

Plasmodium falciparum parasites synthesise three histidine rich proteins (HRPs) during the asexual stage of the parasite life cycle (Leech *et al*., 1984; Wellems and Howard, 1986). Plasmodial HRP-1, HRP-2 and HRP-3 were identified by labelling *P. falciparum* proteins with ((^{3}H) Histidine) *in vitro* (Leech *et al*., 1984). HRP-1, is an 80-115 kDa membrane bound protein associated with parasite knobby protrusions (Kilejian, 1979; Howard *et al*., 1986; Rock *et al*., 1987). HRP-2 is soluble, has a molecular weight of about 60-105 kDa and is synthesized by both the knob positive and knob negative parasites (Howard *et al*., 1986; Rock *et al*., 1987). HRP-3 is homologous to HRP-2 (Wellems and Howard, 1986) and has the smallest molecular weight of 40-55 kDa (Rock *et al*., 1987). HRP-1 was thought to assist knobby adhesion of infected erythrocytes (Rock *et al*., 1987), while the functions of HRP-2 and HRP-3 are not yet clear.

1.6.1. *Plasmodium falciparum* histidine rich protein-2 (*Pf*HRP-2)

*Pf*HRP-2 is made up of 34% histidine, 37% alanine and 10% aspartic acid residues in its amino acid sequence (Wellems and Howard, 1986). The amino acid sequence predicts a 35 kDa protein (Wellems and Howard, 1986), but, *Pf*HRP-2 appears as a 60-105 kDa protein on SDS-PAGE gels (Rock *et al*., 1987). The protein consists of multiple tandem repeats of the

Ala-His-His and Ala-His-His-Ala-Ala-Asp peptides in its histidine rich region (Wellems and Howard, 1986) (Figure 1.4). *Pf*HRP-2 is glycosylated with galactose and this glycosylation is thought to aid the extracellular exportation of the protein (Panton *et al.*, 1989).

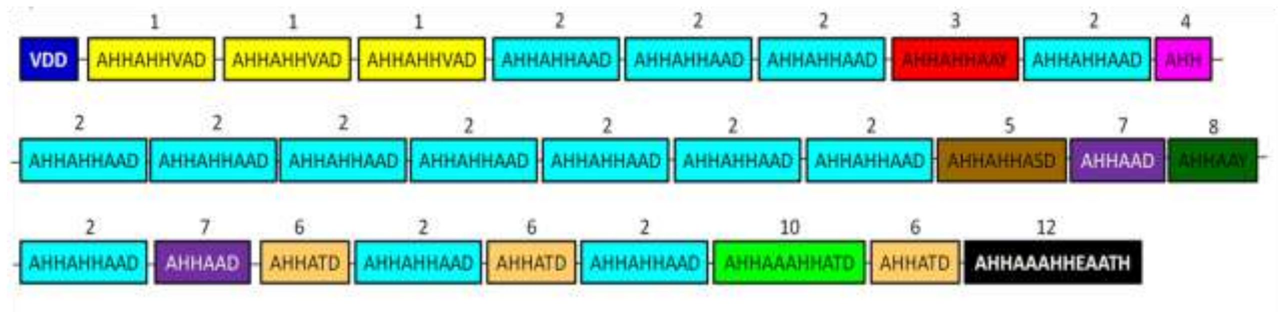

Figure 1.4. Tandem repeats in the histidine rich region of *Pf*HRP-2.
The histidine and alanine containing repeat types 1-12 of *Plasmodium falciparum* HRP-2. The repeat types frequencies vary between geographical isolates. Image adopted from Bharti *et al.*, 2017.

The expression of *Pf*HRP-2 by intraerythrocytic parasites is stage dependent (Desakorn *et al.*, 2005), with increased expression towards the late trophozoite and schizont stages of the parasite development (Akompong *et al.*, 2002). With the fast growth rate of *P. falciparum*, *Pf*HRP-2 can be detected within 2-8 h of the parasite ring development (Howard *et al.*, 1986). The majority, 90-97% of endogenous *Pf*HRP-2 is exported to the cytosol of the infected erythrocyte throughout the parasite's intraerythrocytic stages (Akompong *et al.*, 2002; Desakorn *et al.*, 2005). *Pf*HRP-2 clusters in the infected red blood cell cytosol (Desakorn *et al.*, 2005) and is found in the periphery of the infected cell (Parra *et al.*, 1991; Akompong *et al.*, 2002). It is not yet known why *Pf*HRP-2 is secreted by the parasite to the cytosol of the red blood cell but this could be linked to its function (Akompong *et al.*, 2002; Desakorn *et al.*, 2005). At the end of the intraerythrocytic stages of the parasite development, the red cell ruptures and *Pf*HRP-2 is released into the blood stream of the host.

1.7. Proposed functions of *Pf*HRP-2 based on experimental evidence

The precise function of *Pf*HRP-2 during a *P. falciparum* infection is not yet known. The protein is implicated in various roles during parasite-host interaction, including binding (Schneider *et al.*, 2005) and polymerisation of heme (Sullivan *et al.*, 1996) derivatives to non-toxic hemozoin (Lynn *et al.*, 1999). *Pf*HRP-2 is also implicated in immune suppression (Das *et al.*, 2006), exacerbating experimental cerebral malaria in mice (Pal *et al.*, 2017), compromising the blood-brain barrier (Pal *et al.*, 2016), inhibiting antithrombin (Ndonwi *et al.*, 2011) and binding Zinc (Zn^{2+}) (Panton *et al.*, 1989; Ndonwi *et al.*, 2011).

1.7.1. *Pf*HRP-2 heme binding and hemozoin formation

Hemozoin formation has been accredited to the HRP-2 in *P. falciparum* parasites (Sullivan *et al.*, 1996). *Plasmodium falciparum* parasites ingest haemoglobin from its host red blood cells during the intraerythrocytic stage of their life cycle (Goldberg *et al.*, 1990). Haemoglobin is digested and produces free heme in the parasite food vacuole (Sherman.,1979). The parasite lacks key enzymes to detoxify heme, such as heme oxygenase (Choi *et al.*, 1999). Free heme is toxic to the cell since it has the capacity to generate free radicals. Neutralisation of heme to inert, non-toxic hemozoin is essential for *Plasmodium falciparum* parasite.

Some experimental evidence has suggested that HRP-2 and HRP-3 initiate hemozoin formation under physiological conditions (Sullivan *et al.*, 1996). *Pf*HRP-2 was identified in purified *Plasmodium falciparum* food vacuoles by monoclonal antibodies and the native and recombinant *Pf*HRP-2 proteins were shown to promote hemozoin formation in the presence of heme (Sullivan *et al.*, 1996). *Pf*HRP-2-mediated hemozoin formation increased with time and protein concentration (Sullivan *et al.*, 1996). Other studies have shown that one *Pf*HRP-2 molecule can bind 15 (Schneider *et al.*, 2005) to 50 heme molecules (Choi *et al.*, 1999). *Pf*HRP-2 heme binding and hemozoin formation is pH dependent with an optimum at acidic pH, such as that found in the food vacuole (Sullivan *et al.*, 1996; Lynn *et al.*, 1999).

The following evidence does not support this role for *Pf*HRP-2. The parasite exports *Pf*HRP-2 to the cytosol of the infected red blood cell where 97% of the endogenous protein localises before extracellular release (Akompong et al., 2002). The parasite retains only 3% of the protein to mediate the essential process of hemozoin formation (Akompong *et al.*, 2002). This argues that more *Pf*HRP-2 would be in the food vacuoles if the proteins' function was heme detoxification. There is also no delivery system that has been identified for *Pf*HRP-2 transfer to the parasite food vacuole (Akompong *et al.*, 2002). *Plasmodium falciparum* isolates lacking the *hrp-2* and *hrp-3* genes exist (Gamboa *et al.*, 2010) and retain the ability to form hemozoin as do all malaria species that do not express HRP-2.

Unsaturated lipids (Fitch *et a*l., 1999) in the presence of hemozoin crystals can promote hemozoin formation in the absence of protein (Dorn *et al.*, 1995). Thus, it is possible that lipids could be involved in heme detoxification in malaria species without HRP-2. Furthermore, hemozoin has been identified in non-*Plasmodium* species that also digest haemoglobin (Chen *et al.*, 2001), without histidine rich proteins.

1.7.2. Immune suppressive effect of *Pf*HRP-2

*Pf*HRP-2 is found in high concentrations in the plasma of *Plasmodium falciparum* infected patients (Parra *et al.*, 1991), and remains detectable more than twenty-seven days after treatment (Kattenberg *et al.*, 2012). *Pf*HRP-2 is thought to be involved in regulating the host's immune responses during the erythrocytic stage of *P. falciparum* (Das *et al.*, 2006). The protein has been shown to bind human T and B cells in a concentration dependent manner

(Das *et al.*, 2006). This binding lead to suppression of T and B cell proliferation by 70% and 78.4% respectively, and a reduction in T cell interferon gamma (IFN-ɣ) levels (Das *et al.*, 2006). But the cell surface receptors associated with this binding were not identified.

It has been reported that histidine highly and tryptophan moderately neutralises singlet oxygen ($^1O_2$) species produced by activated neutrophils, thus suppressing targeted *P. falciparum* killing (Nnalue and Friedman,1988). This might explain the peripheral localisation of *Pf*HRP-2 within infected erythrocytes, as superoxide (O^{2-}) was shown to cross the erythrocyte membrane (Lynch and Fridovich, 1978). But, *Pf*HRP-2 has not been reported to neutralise reactive oxygen species and is not expressed by all *Plasmodium* species.

1.7.3. *Pf*HRP-2 blood brain barrier modeling and inflammation

*Pf*HRP-2 has been proposed as a virulence factor, contributing to malaria pathogenesis and cerebral malaria (Pal *et al.*, 2016; Pal *et al.*, 2017; Javaid *et al.*, 2017). Cerebral malaria is the deadliest, severe presentation of malaria, characterised by a loss of consciousness and parasite sequestration in the brain (Javaid *et al.*, 2017).

Physiological levels of *Pf*HRP-2 disrupt the integrity of the endothelial blood brain barrier (Pal *et al.*, 2016) and *Pf*HRP-2 treatment of endothelial cells increased expression of adhesion molecules (ICAM-1 and VCAM-1) aiding parasite sequestration (Pal *et al.*, 2017). The binding of *Pf*HRP-2 to the endothelial barrier redistributed endothelial junctional proteins, causing barrier leakage (Pal *et al.*, 2016) and parasite vascular sequestration. Mice injected with *Pf*HRP-2 exhibited cerebral endothelial barrier leakage and an increased progression to cerebral malaria like symptoms (Pal *et al.*, 2017). Endothelial barrier leakage is triggered by the signaling of the inflammasome caused by *Pf*HRP-2 binding (Pal *et al.*, 2017). The *Pf*HRP-2 receptor on the endothelial cell surface was not identified, but, caspase-1; interleukin-1β; MyD88 and NF-κB signaling molecules were involved.

The extended activation of endothelial cells and inflammation after a month of *Plasmodium falciparum* clearance (Moxon *et al.*, 2013), could be linked to the effect of *Pf*HRP-2 (Pal *et al.*, 2017).

1.7.4. Heparin binding and antithrombin inhibition of *Pf*HRP-2

*Pf*HRP-2 mimics the function of the human histidine rich protein, histidine rich glycoprotein (HRG) in plasma (Panton *et al.*, 1989). Both proteins have a high histidine content and are soluble and stable in plasma. HRG is involved in regulating coagulation by interacting with Zn^{2+} to bind heparin, which inhibits antithrombin (Lijnen *et al.*, 1983) and contributes to a "localised procoagulant state" (Kluszynski *et al.*, 1997).

*Pf*HRP-2 levels are high in the blood of *P. falciparum* patients and the protein has been shown to bind Zn^{2+} (Panton *et al.*, 1989). The interaction of *Pf*HRP-2 with Zn^{2+} increases the protein's affinity for heparin (Ndonwi *et al.*, 2011). This inhibited antithrombin from inactivating

thrombin and factor Xa, which promote coagulation (Ndonwi *et al.*, 2011). Zinc concentrations for *Pf*HRP-2 binding were physiological and *Pf*HRP-2 bound Zn^{2+} with a higher affinity compared to HRG (Ndonwi *et al.*, 2011). The procoagulant effect of *Pf*HRP-2 is thought to contribute to the microvascular blockage and organ failure associated with severe *P. falciparum* malaria (Ndonwi *et al.*, 2011). These findings suggest that *Pf*HRP-2 influences the host coagulation processes and might have a coagulant role in the blood of *P. falciparum* patients.

1.8. Challenges associated with *Pf*HRP-2 detection in the field

1.8.1. *Pf*HRP-2 gene deletion

The diagnosis of *falciparum* malaria is affected by *Pf*HRP-2 gene deletion which hinders diagnosis and treatment. False negative result from patients infected with *P. falciparum* have been reported in the field when detecting *Pf*HRP-2. False negative results lead to the misdiagnosis of *P. falciparum* malaria and can be fatal. *Pf*HRP-2 gene deletion (Gamboa *et al.,* 2010); low parasitemia (Tiono *et al.*, 2014; Laban *et al.*, 2015) and excess antigen or moderate concentration of antibodies in the test contribute to false negative results (Gillet *et al.*, 2009; Luchavez *et al.*, 2011). *Pf*HRP-2 gene deletion was first reported in the Amazonian region of Peru (Gamboa *et al.,* 2010). Since then, there has been gene deletion reports in Amazonian South America (Akinyi Okoth *et al.*, 2015; Rachid Viana *et al.*, 2017), South-East Asia (Li *et al.*, 2015; Bharti *et al.*, 2016) and in some parts of Africa (Koita *et al.*, 2012; Kozycki *et al.*, 2017).

1.8.2. *Pf*HRP-2 persistence in the blood of malaria patients

*Pf*HRP-2 persists in the blood of *P. falciparum* patients after treatment (Shiff *et al.*, 1993). Studies describe the detection of *Pf*HRP-2 after 7-14 (Shiff *et al.*,1993; Humar *et al.*, 1997; Mayxay *et al.*, 2001), 28 (Kattenberg *et al.*, 2012), 31 or more days after parasite clearance (Eisen and Saul, 2000; Swarthout *et al.*, 2007; Grandesso *et al.*, 2016). Why *Pf*HRP-2 persists is not well understood and excludes the detection of *Pf*HRP-2 for monitoring antimalarial treatment outcomes (Kattenberg *et al.*, 2012). Persistent *Pf*HRP-2 antigenemia is the major cause of false positive results in *Pf*HRP-2 based RDTs and overestimates malaria fevers, taking the focus away from the actual cause of the patient's symptoms (Swarthout *et al.*, 2007; Kiemde *et al.,* 2017). This redirects treatment, mishandles non-malaria fevers, reduces the specificity of *Pf*HRP-2 based RDTs and promotes the overuse of anti-malarial medicine (Swarthout *et al.*, 2007; Kyabayinze *et al.*, 2008; Grandesso *et al.*, 2016). False positive results are more frequent in high transmission areas compared to areas of low transmission and make it difficult to differentiate *Pf*HRP-2 persistence from reinfection (Kyabayinze *et al.*, 2008; Grandesso *et al.*, 2016).

For these reasons, the use of *P. falciparum* specific LDH based tests or a combination of *Pf*HRP-2 and *Pf*LDH RDTs has been suggested in affected regions (Berhane *et al.*, 2018).

The specificity and sensitivity of HRP-2-based RDTs is variable in the field and this has negative effects in the diagnosis of malaria. An example is false negative results caused by poor sensitivity leads to delayed treatment. IgG monoclonal antibodies used in HRP-2-based RDTs detect a single epitope of the protein to make the detection specific and universal. However, this could pose a problem if the epitope is not widely conserved across geographical isolates or if the targeted epitope has a low frequency within the protein. *Pf*HRP-2 is a polymorphic protein with wide variations in the frequency and arrangement of its repeating epitopes (Baker *et al.*, 2005; Lee *et al.*, 2006). This contributes to the variable sensitivity of *Pf*HRP-2-based RDTs observed in the field (Lee *et al.*, 2006). This limitation could be addressed by using polyclonal antibodies detecting multiple epitopes in the target protein.

RDTs using mammalian IgG antibodies have poor specificity in the presence of human rheumatoid factor in patients with rheumatoid arthritis (Lee *et al.*, 2014). This could promote antimalaria drug resistance since medication would be administered to patients without malaria as a result of a false positive RDT. This would take focus away from the underlying sickness which could be left untreated. Chicken IgY antibodies possess a Fc region that is different to IgG and is not recognised by the human rheumatoid factor receptor (Larsson *et al.*, 1991). Chicken IgY antibodies do not react with rheumatoid factor and their use in a RDT would reduce false positive results. Additionally, chicken IgY antibodies a raised as a polyclonal mix of antibodies that detect different epitopes of a protein target. This could improve the detection of a polymorphic protein like *Pf*HRP-2 with antibodies.

Chicken IgY antibodies have potential as an alternative to mammalian IgG antibodies employed in RDTs for the detection of malaria. This potential requires investigation in the context of current malaria RDTs. The IgY antibodies require characterisation to investigate their stability in warm and humid conditions likely to be encountered in the field where RDTs are deployed. This is important as studies have shown that antibodies in RDTs gradually lose their reactivity when exposed to temperatures above 35°C (Chiodini *et al.*, 2007). The epitopes of the target protein detected by the IgY antibodies would require identification to compare these epitopes with those detected by current HRP-2-based RDTs. A technique to identify the epitopes recognised by antibodies is the cleavage of the target protein by proteolytic enzymes. The fragments of digestion could be identified and incubated with antibodies to see which epitopes are detected by the antibodies. This technique has been referred to as protein foot-printing (Sheshberadaran and Payne, 1988).

1.9. Aims and objectives of the study

Malaria can be diagnosed with a wide range of methods, each with its own strengths and weaknesses. This diagnosis prioritises *P. falciparum* over the other human malaria species because of its effects on the infected individual. *Pf*HRP-2 RDTs are important in malaria control efforts, especially in the absence of a sterilising vaccine and are the most widely used RDTs in *P. falciparum* infected populations. But, anti-*Pf*HRP-2 antibodies in RDTs demonstrate limitations that affect the diagnosis of malaria and these include: variable specificity, sensitivity and lack of stability when exposed to high temperatures. Some RDTs cross react with human rheumatoid factor resulting in false positive diagnosis (Lee *et al.*, 2014).

Characterising other forms of antibodies that are non-mammalian and polyclonal could address some of these limitations. Chicken IgY antibodies have a long shelf life and do not react with human rheumatoid factor. These antibodies are specific, and their polyclonal nature might be useful in detecting a polymorphic protein like *Pf*HRP-2. The aim was to raise antibodies against recombinant *Pf*HRP-2 in chickens and to investigate the thermal stability of these antibodies and to determine their anti-r*Pf*HRP-2 epitopes using peptides produced by proteolytic enzymes.

The objectives of the study included: recombinant expression and affinity purification of *Pf*HRP-2; purification and characterisation of anti-*Pf*HRP-2 antibodies and epitope determination through protease digestion of r*Pf*HRP-2.

Chapter 2

Recombinant expression and affinity purification of the Plasmodium falciparum Histidine Rich Protein-2 (*Pf*HRP-2)

2.1. Introduction

2.1.1. Recombinant protein expression

Recombinant expression is a convenient and popular method for producing high amounts of protein which may be difficult to obtain from natural sources by routine protein isolation methods (Baneyx, 1999). This allows the study and characterisation of various proteins across multiple scientific disciplines and fields. The choice of an expression system depends on the protein, and its application after expression; or the interest in post-translational modification such as phosphorylation, acetylation or glycosylation which might aid protein folding and function (Demain and Vaishnav, 2009). Eukaryotic expression systems such as yeast, insects and mammalian cell lines are often selected for these characteristics (Demain and Vaishnav, 2009; Joseph *et al.*, 2015).

2.1.2. Prokaryotic *E. coli* expression

Prokaryotic expression systems lack some post-translational modification features, making the recombinant expression of functional and stable eukaryotic proteins difficult (Johnson *et al.*, 2010). Nevertheless, the *Escherichia coli* system is still the preferred expression system since it is easily accessible, the bacteria grow rapidly in inexpensive media and it produces high amounts of recombinant protein (Johnson *et al.*, 2010; Joseph *et al.*, 2015; Baneyx, 1999). The *E. coli* BL21 (DE3) strain is the most commonly used bacterial host and is genetically enhanced to improve the yield of complex proteins that might be difficult to express (Joseph *et al.*, 2015). The strain is devoid of proteases resulting in increased stability and survival of recombinant proteins and allows a simple Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction step, hence the DE3 designation (Gopal and Kumar, 2013).

2.1.3. Conventional *E. coli* expression conditions

The conditions for recombinant protein expression include expression with lysogenic broth (LB) media at 37°C for 4-6 hours and induction with IPTG. However, the conventional conditions for recombinant expression do not always produce high amounts of protein and thus there is often a need to optimise recombinant expression conditions (Studier, 2005; Fu *et al.*, 2006; Dvorak *et al.*, 2015).

Some proteins can be overexpressed in the soluble fraction at high temperature, 37°C for shorter times 4-6 hours; while other proteins form insoluble inclusion bodies under the same

conditions with the same host (Schein and Noteborn, 1988). Lower temperatures ≤ 30°C and longer times ≥ 16 hours can result in a more soluble protein expression (Vera *et al*., 2007), but this does not guarantee the solubility of all proteins.

2.1.4. Composition of *E. coli* growth media and influence on recombinant protein expression

The components of the growth media can influence recombinant protein expression (Fu *et al*., 2006). Glucose is a preferred carbon source for *E. coli* growth, leading to high cell densities (Epstein *et al*., 1966), but glucose also prevents the uptake of other sugars such as lactose. Metabolites of lactose promote recombinant protein expression (Inada *et al*., 1996; Studier, 2005). There is an important balance between cell growth and the induction of recombinant protein expression, and both are controlled by growth media components. Induction of protein expression channels most of the host cell resources to the overexpression of the recombinant protein, which may undermine essential host processes (Dvorak *et al*., 2015, reviewed by Glick, 1995). This metabolic burden reduces cell growth and could lead to low recombinant protein yields. Hence, the time of induction or growth phase optical density (OD) of the culture are important when inducing recombinant protein expression. It has been shown that low cell density with a high metabolic rate, and high cell density with a low metabolic rate tend to reduce the yield of the induced protein (Lim *et al*., 2004).

2.1.5. Yeast extract and autoinduction

Yeast extract has been suggested as the preferred nitrogen source for *E. coli* growth and promotes recombinant protein expression without induction (Fu *et al*., 2006). This is commonly referred to as autoinduction and has been reported to be better than manually-induced expression, since high cell densities and high amounts of recombinant proteins can be attained (Studier, 2005). Yeast extract contains lactose and other sugars which can be utilised for recombinant protein expression after glucose depletion (Studier, 2005). The addition of glycerol aids cell growth without affecting the induction of recombinant proteins (Studier, 2005). This means that the glucose content of the yeast extract rich autoinduction media can be optimised for recombinant expression without the need to monitor culture growth in a spectrophotometer (Faust *et al*., 2015).

2.1.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction

IPTG is the most widely used inducer of recombinant protein expression in *E. coli* BL21 (DE3) cells in partnership with the popular pET expression vectors (Joseph *et al*., 2015). IPTG is a lactose analogue that unlike lactose, is not hydrolysed following uptake during induction. IPTG permits high levels of protein expression without the need for frequently adding the inducer. However, IPTG can have a negative effect on cells and protein expression (Dvorak *et al*., 2015). Lactose on the other hand does not have a toxic effect on cells and is often used

as an alternative for IPTG (Lim *et al*., 2004; Dvorak *et al*., 2015), but it may be less efficient at inducing high levels of protein expression.

2.1.7. IPTG and lactose induction of lac-derived promoters

The induction of recombinant proteins in the *E. coli* BL21 (DE3) strain relies on the exhaustion of glucose in the growth media, which allows the uptake of lactose by cells via the lactose permease (Studier, 2005). Once in the cell, lactose is metabolised to allolactose by the β-galactosidase enzyme (Studier, 2005). IPTG mimics allolactose in the induction of the lac-promoter (Studier and Moffatt, 1986; Studier, 2005; Rosano and Ceccarelli, 2014). Allolactose and IPTG induce the expression of the T7 RNA polymerase under the control of lac-derived promoters i.e. lacUV5 (Studier and Moffatt, 1986; Studier, 2005). The T7 RNA polymerase encoded by the DE3 prophage of the host, induces the expression of the target gene/protein under the control of a T7 promoter (Studier and Moffatt, 1986) in the pET plasmid vectors, (reviewed by Rosano and Ceccarelli, 2014).

2.1.8. *Plasmodium falciparum* histidine rich protein-2

HRP-2 is a *Plasmodium falciparum (Pf.)* protein used in malaria diagnosis for point-of-care tests employed in malaria management and control. The protein is naturally produced by the parasite in infected erythrocytes (Rock *et al*., 1987) and is released by the parasite and present in the bloodstream of the host (Howard *et al*., 1986; Parra *et al.,* 1991; Akompong *et al*., 2002; Desakorn *et al*., 2005). The protein is produced by cultured parasites and can be harvested from cultures for study purposes (Howard *et al*, 1986; Panton *et al*., 1989; Parra *et al.,* 1991). Culturing malaria parasites is expensive, and cultures can be difficult to maintain. Recombinant protein expression is a cheap and readily accessible way to obtain protein for analysis.

Here, bacterial growth conditions including: temperature of the culture; incubation time; the type of growth media and the reagents used for induction to obtain optimum expression of the recombinant *Pf*HRP-2 protein were evaluated.

2.2. Materials and methods

2.2.1. Protein expression and optimisation of r*Pf*HRP-2 expression

Transformed, competent *E. coli* BL21 (DE3) cells containing the cDNA clone of *Pf*HRP-2 from *Plasmodium falciparum* parasites in the pET15b vector, were supplied by David J. Sullivan (John Hopkins university) (Sullivan Jr. *et al*., 1996; Ndonwi *et al*., 2011). Glycerol stocks maintained at -70°C were used to inoculate LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 2 g/L glucose and 1.5% (w/v) bacterial agar) and incubated overnight at 37°C. A single colony was picked and used to inoculate a 5 ml Luria-Bertani broth starter culture grown at 37°C overnight, 200 rpm with ampicillin, 50 µg/ml. The overnight culture was

diluted 1/100 in LB, 2xYT (16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl) or terrific broth (TB) (12 g/L tryptone, 24 g/L yeast extract, 4 ml/L glycerol, autoclaved and mixed with 2.31 g/L KH_2PO_4 and 12.54 g/L K_2HPO_4 filter sterilised salts) media in triplicate and grown to an OD_{600} between 0.5-0.6 at 37°C; before induction with 0.1 mM IPTG, or 0.1 mM lactose or sterile distilled water and grown at 37°C for 4 h or 30°C for 16 hrs at 200 rpm. The culture was centrifuged 4000x g, 10 min, and the bacterial pellet was resuspended in 10% of the original culture volume in phosphate-buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KCl, 1.02 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4, pH 7.2), sonicated 4x 30 s, with 2 min rest and centrifuged again at 12 000x g, 10 min. A sample of the resulting supernatant was mixed 1:1 with reducing treatment buffer (described below) and electrophoresed on SDS-PAGE gel.

2.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The SDS-PAGE Tris-glycine gels were prepared as described by Laemmli (Laemmli, 1970) with minor changes; i.e. glass plates for slab gels were used instead of glass tubes. The 12.5% SDS-PAGE gels were made from filtered 30% (w/v) monomer solution (A) (29.2% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide); 4x running gel buffer solution (B) (1.5 M Tris-HCl, pH 8.8) and 4x stacking gel buffer solution (C) (500 mM Tris-HCl, pH 6.8). A 10% (w/v) SDS solution (D) and 10% (w/v) ammonium persulfate solution (E) were also prepared. To make a single 12.5% SDS-PAGE gel the solutions were mixed as indicated on table 2.1.

Table 2.1. SDS-PAGE recipe for a 12.5% running and 4% stacking gel

Solution	12.5% running gel (5 ml)	4% stacking gel (1.42 ml)
A	2.08 ml	188 µl
B	1.25 ml	0
C	0	350 µl
D	50 µl	14 µl
E	25 µl	7 µl
Distilled water	1.58 ml	860 µl
Tetramethyl ethylenediamine TEMED)	2.5 µl	3 µl

The separating and stacking gel solutions respectively were mixed and poured into the BioRad gel casting system. The separating gel solution was overlaid with water ≥1 ml to separate the gel from oxygen and produce a flat surface after polymerisation. The water was removed, and the stacking solution was poured on top of the polymerised separating gel and left to polymerise. A 10 or 15 well gel-comb was inserted into the stacking gel. The cell culture lysate or protein samples were mixed 1:1 with reducing treatment buffer (500 mM Tris-HCl, pH 6.8, 2% (v/v) glycerol, 4% (v/v) of 10% SDS-solution and 1% (v/v) 2-mecaptoethanol, bromophenol blue). The samples were boiled for 5-minutes and allowed to cool before loading

10 µl in to the gel wells. The gel apparatus was assembled and electrophoresed at 20 mA with tank buffer (250 mM Tris-HCl, 192 mM1 glycine and 0.1% (w/v) SDS). After electrophoresis the gel was removed from the cassette and stained with Coomassie brilliant blue solution (50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) dH_2O and 1.5 mM Coomassie brilliant blue (R250) (825.99 g/mol)); and destained in (50% (v/v) methanol and 10% (v/v) acetic acid, 40% (v/v) dH_2O). Images of stained gels were taken with a Syngene G:Box XR5.

2.2.3. Affinity purification of r*Pf*HRP-2

The *Pf*HRP-2-(His_6) protein was expressed in TB media at 30°C for 16 h at 200 rpm, induced with 0.1 mM lactose when the cultures had an OD_{600} of 0.5-0.6. The cells were centrifuged at 4000x g, 10 min and the bacterial pellet was resuspended in 10% of the original culture volume in sodium phosphate buffer (6.0 g/L NaH_2PO_4, 17.53 g/L NaCl, and 10 mM imidazole, pH 8), sonicated 4x 30 s, 2 min rest and centrifuged again at 12 000x g, 10 min. The nickel-NTA agarose affinity matrix, 2 ml slurry was activated by washing with 10 times the matrix volume in 10 mM imidazole containing sodium phosphate buffer. The culture lysate containing soluble proteins was incubated with activated matrix beads in suspension for 1 h on an orbital rotator before loading into the column. The buffer and unbound proteins were eluted from the column and the column was washed twice with 10 ml sodium phosphate buffer containing 10 mM and 250 mM imidazole respectively, to remove bound *E. coli* proteins. Recombinant *Pf*HRP-2 was eluted from the matrix with phosphate buffer containing 500 mM imidazole at pH 8. The protein content of each fraction was analysed with SDS-PAGE and absorbance measurements at 280 nm.

2.3. Results

2.3.1. Recombinant expression of *Pf*HRP-2 in LB and 2xYT media at 37°C for 4 hours

Recombinant *Pf*HRP-2 (r*Pf*HRP-2) expression was optimised by comparing different expression conditions and induction reagents. Transformed *E. coli* BL21 (DE3) cells containing a plasmid expressing the *P. falciparum* HRP-2 gene (*pfhrp-2*) were grown at 37°C in LB and 2xYT media for 4 h. TB was not used because its autoinduction properties work best at lower temperatures for longer growth times. The cultures were induced with IPTG or lactose for recombinant expression of *Pf*HRP-2 before SDS-PAGE analysis. The experiment was repeated at least twice.

Figure 2.1a, shows the expression of *E. coli* proteins and r*Pf*HRP-2 in LB media. The intensity of individual *E. coli* protein bands was similar in all samples (Figure 2.1a, lanes 2-9). The protein band running at 135 kDa was absent in the non-induced samples (Figure 2.1a, lanes 2-3), and present in all the induced samples (Figure 2.1a, lanes 4-9). The recombinant

*Pf*HRP-2 expected at 54 kDa, was obscured by the presence of *E. coli* proteins of a similar size (Figure 2.1a, lanes 2-9). Purified r*Pf*HRP-2 (Figure 2.1, lane 10) shows the position of the recombinant protein in these gels and those of Figure 2.2, (lane 10).

E. coli cells expressing *Pf*HRP-2 were grown in 2xYT media. Figure 2.1b, shows that the intensity of each *E. coli* protein band was similar in all samples (Figure 2.1b, lanes 2-9). The high molecular weight protein band about 135 kDa was absent in the non-induced samples (Figure 2.1b, lanes 2-3) and present in the induced samples (Figure 2.1b, lanes 4-9). This protein was more darkly stained in 2xYT cultures (Figure 2.1b) compared to LB cultures (Figure 2.1a). A darkly stained protein of 54 kDa was present in the non-induced samples (Figure 2.1b, lanes 2-3) and lactose induced samples (Figure 2.1b, lanes 7-9) of 2xYT media. This protein was minimally expressed in the IPTG induced samples (Figure 2.1b, lanes 4-3). Perhaps these conditions promote plasmid leakiness which could account for r*Pf*HRP-2 expression in the non-induced sample. Thus, it is not clear whether the darkly stained protein was the recombinant protein or an *E. coli* protein of a similar size.

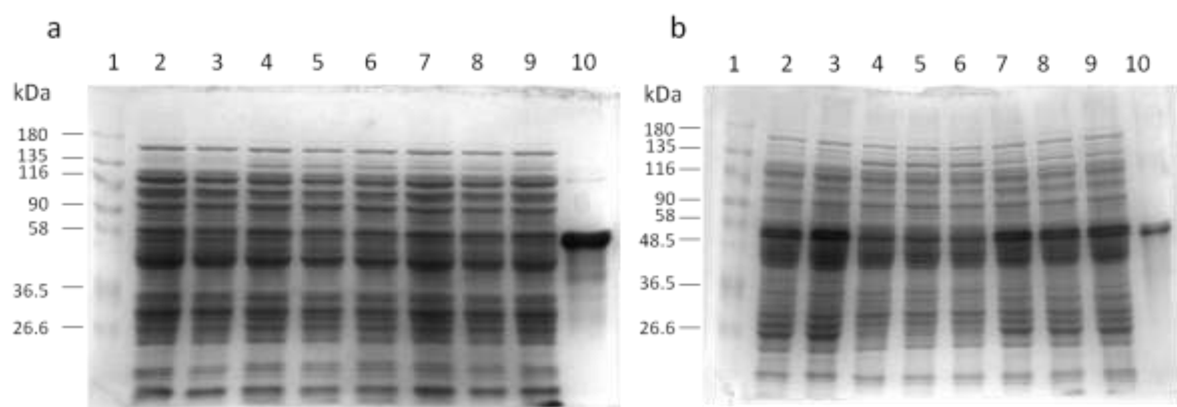

Figure 2.1. Recombinant expression of r*Pf*HRP-2 in cultures grown in LB and 2xYT media at 37°C.
E. coil BL21 (DE3) cells expressing r*Pf*HRP-2 were grown in (a) Luria-Bertani (LB) and (b) 2x yeast-extract tryptone (2xYT) media at 37°C for 4 hrs, uninduced (control) or induced with IPTG or lactose (at O.D~0.5). *E. coli* lysates were run on a 12.5% reducing SDS-PAGE gel and stained with Coomassie (R250). Lane 1: molecular weight markers; lane 2-3: non-induced cultures; lane 4-6: IPTG induced cultures; lane 7-9: lactose induced cultures and lane 10: affinity purified recombinant *Pf*HRP-2.

2.3.2. Recombinant expression of *Pf*HRP-2 in LB, 2xYT and TB media at 30°C for 16 hours

Recombinant *Pf*HRP-2 expression was evaluated at 30°C in LB, 2xYT and TB for 16 h. The *E. coli* cultures were induced with IPTG or lactose before protein expression was analysed with SDS-PAGE. The experiment was repeated at least twice.

The intensity of *E. coli* protein bands below the 36.5 kDa protein marker in the LB cultures was similar in all the samples (Figure 2.2a, lanes 2-9). The recombinant *Pf*HRP-2 protein at

54 kDa was minimally expressed in the non-induced samples (Figure 2.2a, lanes 2-3). The pattern of all stained proteins in the IPTG induced samples (Figure 2.2a, lanes 4-6) was consistent from the top to the bottom of the gel. The recombinant *Pf*HRP-2 protein was darkly stained and prominent in the lactose induced samples (Figure 2.2a, lanes 7-9).

The intensity of the *E. coli* proteins below the 36.5 kDa marker in the 2xYT cultures, was similar in all the samples (Figure 2.2b, lanes 2-9). There was some variation in the intensity of protein bands above 36.5 kDa (Figure 2.2b). The intensity of protein bands for two IPTG induced samples (Figure 2.2b, lane 4 and 5) was similar; while on the third sample (Figure 2.2b, lane 6) the intensity of protein bands appeared to be less when compared to proteins in the same lane and the same proteins in other lanes. The intensity of the r*Pf*HRP-2 protein band was less in IPTG induced samples (Figure 2.2b, lanes 4-6) compared to the lactose induced samples which seem to have more (Figure 2.2b, lanes 7-9).

The protein bands between 58 kDa and 36.5 kDa were lightly stained in lysates of cells grown in TB media, (Figure 2.2c, lanes 2-5). The band intensity of proteins below the 36.5 kDa marker appeared to be lower for the non-induced (Figure 2.2c, lane 2) compared to induced samples (Figure 2.2c, lanes 3-9). The r*Pf*HRP-2 protein was expressed in the non-induced samples (Figure 2.2c, lanes 2-3) and under IPTG induction (Figure 2.2c, lanes 4-6), but expressed in high concentrations in the lactose induced samples (Figure 2.2c, lanes 7-9).

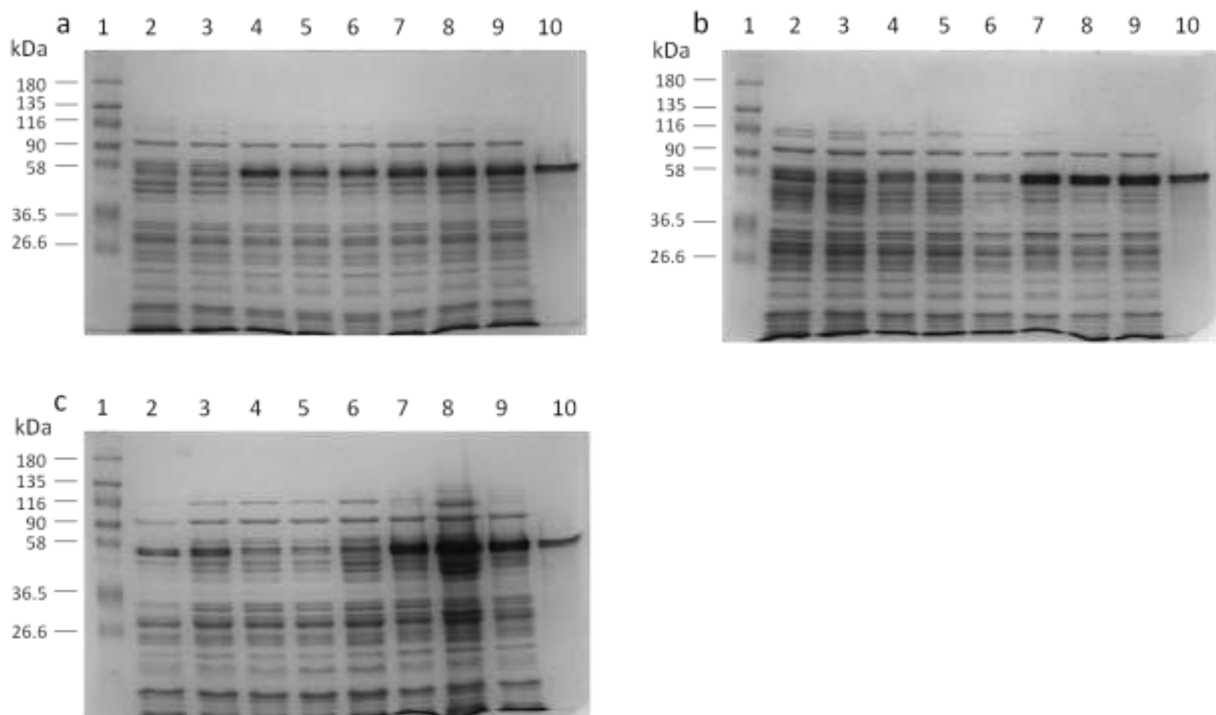

Figure 2.2. Recombinant expression of r*Pf*HRP-2 from cultures grown in LB, 2xYT and TB media at 30°C.
E. coli cells expressing r*Pf*HRP-2 were grown in (a) Luria-Bertani (LB), (b) 2x yeast-extract tryptone (2xYT) and (c) terrific broth (TB) media at 30°C for 16 hrs, uninduced (control) or induced with IPTG or

lactose (at O.D~0.5). *E. coli* lysates were run on a 12.5% reducing SDS-PAGE gel and stained with Coomassie (R250). Lane 1: molecular weight markers; lane 2-3: non-induced cultures; lane 4-6: IPTG induced cultures; lane 7-9: lactose induced cultures and lane 10: affinity purified recombinant *Pf*HRP-2.

SDS-PAGE gels were used to compare changes and trends in the pattern of proteins expressed at 37°C (Figure 2.1) and 30°C (Figure 2.2) and r*Pf*HRP-2. There were high molecular weight proteins above 116 kDa expressed by cultures grown at 37°C (Figure 2.1 a and b); these proteins were absent in cultures grown at 30°C (Figure 2.2 a-c). Recombinant *Pf*HRP-2 was expressed in the non-induced samples (Figure 2.1 and 2.2, lanes 2-3) due to plasmid leakiness; minimally expressed in the IPTG samples (Figure 2.1 and 2.2, lanes 4-6) and was more prominent in lactose induced samples (Figure 2.1 and 2.2, lanes 7-9). Overall, r*Pf*HRP-2 was poorly expressed in LB media compared to 2xYT media and the intensity of the r*Pf*HRP-2 protein band was darker in the TB lactose induced samples at 30°C compared to LB and 2xYT in the same conditions. Based on the prominence of r*Pf*HRP-2 expression in cultures grown at 30°C for sixteen hours compared to 37C° for four hours, 30°C was chosen for r*Pf*HRP-2 expression with TB media for sixteen hours in the presence of lactose.

2.3.3. Nickel affinity purification of recombinant *Pf*HRP-2

The r*Pf*HRP-2 protein in the *E. coli* lysate was purified using a nickel-NTA agarose affinity resin. Figure 2.3 shows the purification profile of recombinant *Pf*HRP-2. The protein bands in Figure 2.3a (lane 2 and 3) represent the proteins that do not bind to the nickel resin. The recombinant 54 kDa *Pf*HRP-2 protein was absent from this fraction. Some proteins (Figure 2.3a, lane 5) at about 72 kDa, between 52-42 kDa and 34-26 kDa had moderate affinity to the nickel matrix and eluted with 250 mM imidazole. The recombinant *Pf*HRP-2 protein has a high affinity for the nickel matrix and was eluted with 500 mM imidazole (Figure 2.3a, lane 7-10).

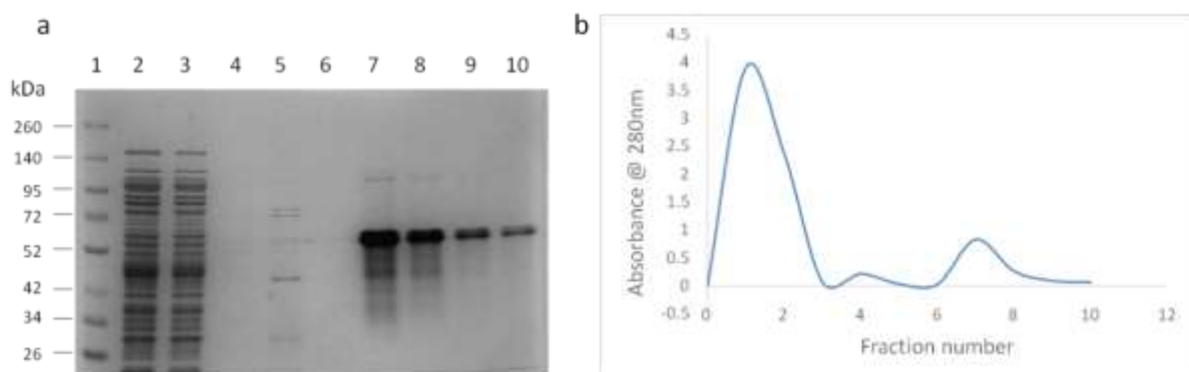

Figure 2.3. Purification of recombinant *Pf*HRP-2 on a nickel chelate affinity matrix.
E. coli cell lysate expressing r*Pf*HRP-2 was passed through a nickel NTA matrix. (a) Protein samples from the affinity matrix were run on a (12.5%) reducing SDS-PAGE gel and stained with Coomassie Brilliant blue (R-250). Lane 1: molecular weight markers; lane 2-3: unbound *E. coli* proteins; lane 4: wash; lane 5: 250 mM imidazole elution; lane 6: wash; lane 7-10: 500 mM imidazole elution. (b)

Absorbance readings of collected samples at 280nm. From left to right, peak 1: unbound proteins; peak 2: 250 mM imidazole elution and peak 3: 500 mM imidazole elution.

Figure 2.3b shows a large peak (fraction 1-3) for unbound *E. coli* proteins and proteins with moderate affinity for the nickel matrix (fraction 4) which were removed with 250 mM imidazole. The last peak (fraction 7, Figure 2.3b) indicates the elution of recombinant *Pf*HRP-2 at 500 mM imidazole. The absorbance readings of the purified protein seemed to be low compared to the prominence of the protein bands on the gel. Perhaps the Coomassie stain detects the protein more intensely because of lysine, arginine and histidine residues in the *Pf*HRP-2 sequence which outnumber the presence of tyrosine and tryptophan residues absorbing UV light at 280 nm.

2.4. Discussion

The ideal recombinant protein expression host should facilitate high amounts of soluble protein expression in small (millilitre) batch culture volumes, overriding the need for litres of culture to produce the protein. Often, *Pf*HRP-2 is recombinantly produced in large (litre) volumes of *E. coli* cultures (Lynn *et al.,* 1999; Ndonwi *et al*., 2011; Kang *et al*., 2015). *Plasmodium falciparum* HRP-2 is a diagnostic marker for falciparum malaria, and further characterisation of the protein could improve the performance of diagnostic tests.

Recombinant proteins are stable within the *E. coli* BL21 (DE3) strain and this bacterial host has been used for recombinant expression of Plasmodial proteins with IPTG induction (Reddy *et al*., 2014; Krause and Goldring, 2018). Recombinant protein expression often requires optimisation even with well-established expression hosts. LB media is relatively cheap and most commonly used for recombinant protein expression and offers a good starting point for protein expression optimisation. 2xYT is comparable to LB because of similar composition, while TB was selected for its autoinduction properties which work best at low temperature with long incubation times.

2.4.1. Recombinant expression of *Pf*HRP-2 in LB and 2xYT media at 37°C for 4 hours.

Bacterial growth conditions, to overexpress and purify recombinant *Pf*HRP-2 from small *E. coli* BL21 (DE3) cultures, were evaluated. Protein expression in *E. coli* is often performed at 37°C with Luria-Bertani broth in the presence of IPTG (Studier, 2005; Fu *et al*., 2006). Production of *Pf*HRP-2 at 37°C in LB and 2xYT media induced with IPTG or lactose produced a high molecular weight protein at 135 kDa (Figure 2.1). This protein was not detected by anti-r*Pf*HRP-2 antibodies against the whole protein (chapter 3). The expression conditions of this study promoted the accelerated expression of r*Pf*HRP-2 which burdens the *E. coli* host (Schneider *et al*., 2005). It is possible that the 135 kDa band is an *E. coli* protein expressed in

response to the metabolic burden imposed by recombinant expression (Dvorak *et al*., 2015, reviewed by Glick, 1995).

Recombinant *Pf*HRP-2 with a his-tag resolved at 54 kDa. The amino acid sequence of *Pf*HRP-2 is between 277 and 332 (P05227) forming a 30 kDa (Ndonwi *et al*., 2011) to 35 kDa (Wellems and Howard, 1986; Sullivan Jr. *et al*., 1996) protein, as predicted by mass spectroscopy (Schneider *et al*., 2005; Ndonwi *et al*., 2011). The *Pf*HRP-2 protein often resolves at a higher molecular weight on reducing SDS-PAGE gels (Wellems and Howard, 1986; Ndonwi *et al*., 2011), which was the same for the recombinant protein in this study.

In early *Pf*HRP-2 studies with cultured falciparum parasites, *Pf*HRP-2 resolved as multiple protein bands of 60 - 72 kDa on 5 -15% gradient gels (Howard *et al*, 1986; Panton *et al*., 1989; Parra *et al.,* 1991). Sullivan *et al*., (1996) observed a *Pf*HRP-2 doublet at 69 kDa on 8% reducing SDS-PAGE gels. A study employing recombinant, his-tagged *Pf*HRP-2 observed the protein as a 55 kDa band (Schneider and Marletta, 2005) in 12% reducing SDS-PAGE gels (Ndonwi *et al*., 2011), 25 kDa more than that predicted by the amino acid sequence. The current study found r*Pf*HRP-2 resolving 24 kDa more than that predicted by the amino acid sequence similar to the increase in size reported in the Ndonwi study in 2011.

The reason for the low mobility of *Pf*HRP-2 on reducing SDS-PAGE gels is not well understood. A possible cause is the high histidine content (Ndonwi *et al*., 2011) or large number of tandem repeat regions (Wellems and Howard, 1986) similar to the *P. knowlesi* (Ozaki *et al.,* 1983) and *P. falciparum* (Dame *et al*., 1984) circumsporozoite proteins and *P. falciparum* S-antigen (Cowman *et al*., 1985) which also show low mobility on SDS-PAGE gels. These *Plasmodium* proteins are thought to have a reduced affinity to SDS because of their multiple tandem repeats (Ozaki *et al.,* 1983). Proteins high in acidic amino acid residues such as glutamic acid (Lakoucheva *et al*., 2001) and aspartic acid (Armstrong and Roman, 1993) also show low migration on reducing SDS-PAGE gels. Aspartic acid makes up 10% of the *Pf*HRP-2 protein amino acid content (Wellems and Howard, 1986).

The expression conditions of Figure 2.1a did not show differences in the expression of proteins around the 54 kDa mark for the non-induced and induced samples, contrary to Figure 2.1b. The prominent protein band of the same size as the recombinant protein in the non-induced samples of Figure 2.1b, lanes 2-3 could indicate leaky expression of the plasmid genes. But, the lack of conclusive evidence in the form of an untransformed *E. coli* lysate or detection with anti-his antibodies do not exclude the possibility of the protein band being of *E. coli* origin.

2.4.2. Recombinant expression of *Pf*HRP-2 in LB, 2xYT and TB media at 30°C for 16 hours

Low incubation temperatures during protein expression, especially those below 37°C have been reported to reduce the rate of *E. coli* growth (Gadgil *et al*., 2005). This is caused by cellular processes slowing down in response to the low temperature, reducing the rate of protein transcription, translation and cell division (Gadgil *et al*., 2005; Chou, 2007). Reducing the rate of growth of the *E. coli* BL21 (DE3) cultures assisted the expression of recombinant *Pf*HRP-2. Expressing r*Pf*HRP-2 at 30°C reduced the expression of *E. coli* proteins, including the high molecular weight proteins. These findings are consistent with a study by Vera *et al.,* (2007), showing that the recombinant expression of the green fluorescent protein (GFP) in *E. coli* at low temperatures improved the solubility and yield of the protein. However, Vera *et al.,* (2007) argued that a seven-degrees Celsius drop in temperature may not be enough to observe significant changes. The low temperature is suggested to favour the expression of some recombinant proteins by reducing aggregate formation and protein degradation (Vera *et al*., 2007; Francis and Page, 2010; Rosano and Ceccarelli, 2014). The reduced level of recombinant protein at lower temperatures minimises the metabolic burden of recombinant expression on the host and the extended protein expression times of 16 – 24 h increase the yields. The data in this chapter shows low levels of *E. coli* protein expression at 30°C compared to 37°C and seemed to improve the recombinant expression of r*Pf*HRP-2, but without the quantification of protein yields supporting these findings it is difficult to ascertain that 30°C significantly improved r*Pf*HRP-2 expression compared to 37°C. The yield would indicate the level of improvement with quantitative data compared to the observed protein band intensities which are influenced by the protein amounts loaded on the gel.

It is likely that the high rate of expression of r*Pf*HRP-2 burdens the host, including the protein's high histidine requirement. Perhaps, the extra burden caused by the demand for His-tRNA is intensified at high temperatures (Schneider *et al*., 2005) affecting r*Pf*HRP-2 expression. Fast expression of r*Pf*HRP-2 introduces translational errors reducing protein yields (Schneider *et al*., 2005).

r*Pf*HRP-2 expresses better in the presence of lactose than IPTG in the *E. coli* cultures growing in the presence of 2xYT and TB media (Figure 2.2). Despite plasmid leakiness, lactose seems to improve expression of r*Pf*HRP-2 overall in the tested conditions. IPTG has been used to induce recombinant *Pf*HRP-2 expression in other studies (Schneider and Marletta, 2005; Schneider *et al*., 2005), but at a higher concentration and at room temperature (Choi *et al*., 1999; Ndonwi *et al*., 2011). IPTG has also been reported to be an adequate replacement of lactose for autoinduction media at low concentrations (Faust *et al*., 2015). However, some proteins do not express well with IPTG induction because of their biochemical properties, and stress on the host increases in the presence of IPTG (Dvorak *et al*., 2015). Haloalkane peptides showed host cell toxicity i.e. increased metabolic burden when recombinantly expressed in the presence of IPTG, but this effect was abated with lactose

(Dvorak *et al*., 2015). The benefit of IPTG over lactose induction is that IPTG is not metabolised or broken down when it is used to induce recombinant protein expression. This can be cheaper than using lactose which can be depleted by the growing culture during expression. But lactose has not been linked with *E. coli* cell toxicity or shown to increase metabolic burden in the host during recombinant expression which would affect the yields of the recombinant protein. Thus, lactose could be utilised in expressing other parasite proteins with unusual amino acid content.

2.4.3. Nickel affinity purification of recombinant *Pf*HRP-2

Plasmodium falciparum HRP-2 was expressed and affinity purified using nickel chelation chromatography. The nickel binding properties of the his-tag and the metal binding properties of histidine rich protein-2 (Panton *et al.,* 1989) were explored to purify the protein from the *E. coli* bacterial lysate. A few *E. coli* proteins were eluted with 250 mM imidazole (Figure 2.3). The recombinant *Pf*HRP-2-(His_6) fusion was eluted with 500 mM imidazole similar to other studies (Panton *et al.,* 1989; Lynn *et al.,* 1999; Ndonwi *et al*., 2011; Pal *et al*., 2016). *Pf*HRP-2 has been shown to bind zinc (Zn^{2+}) ions with a stronger affinity than the histidine rich glycoprotein (HRG) which binds Zn^{2+} in human blood (Panton *et al.,* 1989). A Zn^{2+} or Cu^{2+} assisted binding of recombinant *Pf*HRP-2 to heparin has been demonstrated indicating a strong interaction of HRP-2 with divalent cations (Ndonwi *et al*., 2011). Panton *et al.,* (1989), when purifying *Pf*HRP-2 from culture supernatant, observed a strong interaction of *Pf*HRP-2 with Cu^{2+}, reversible at 450 mM imidazole. These findings are relevant because a strong affinity between r*Pf*HRP-2 and nickel, a divalent ion was observed in this study. This was evident by the protein's elution with a high concentration of imidazole 500 mM which was double the concentration usually required for his-tagged recombinant proteins. It is possible that r*Pf*HRP-2 interacts with nickel via the protein's histidine residues regardless of the his-tag.

A small population of *E. coli* proteins were eluted at 250 mM imidazole from the affinity matrix (Figure 2.3). *E. coli* host proteins that bind metals have been observed in the BL21 (DE3) strains and the expression of these proteins increases in response to stress or the metabolic burden on the host (Bartlow *et al*., 2011) reviewed by (Bolanos-Garcia and Davies, 2006). The *E. coli* metal binding proteins interact with nickel in the presence of 20-50 mM imidazole, 10 mM was used in the matrix activation buffer of this study meaning that these conditions could also promote interaction of these proteins with nickel. These proteins have been identified and include catabolite gene activator protein (crp), bifunctional polymyxin resistance protein (arnA) and glucosaminefructose-6-phosphate aminotransferase (glmS) amongst others, and bind to activated metal chelated matrices through genuine metal binding pockets or topical metal binding amino acid residues brought together by protein conformation (Bolanos-Garcia and Davies, 2006; Bartlow *et al*., 2011; Andersen *et al*., 2013). These

proteins could be identified by detection with specific antibodies. It is possible that the recombinant expression of *Pf*HRP-2 causes an increase in the expression of metal binding proteins of the *E. coli* BL21 (DE3) strain. But these metal binding proteins have been previously observed after recombinant expression in LB media for 4 h induced with IPTG and not with TB for 16 h in the presence of lactose as indicated in this study. An *E. coil* lysate not expressing *Pf*HRP-2 i.e. without the plasmid containing *pfhrp-2* gene could be passed through an affinity matrix and its purification profile determined to further substantiate this idea.

2.4.4. Conclusion

The recombinant expression of *Pf*HRP-2 with lactose in *E. coli* BL21 (DE3) cells was optimised. Expression was better at low temperature of ≤ 30°C in the presence of lactose. r*Pf*HRP-2 eluted from a nickel chelate column at a high concentration of imidazole.

Chapter 3

Detection of recombinant *Pf*HRP-2 with chicken IgY antibodies

3.1. Introduction

3.1.1. Antibodies

Antibodies (Ab) are carbohydrate containing serum proteins (Schubert, 1970; Nose and Wigzell, 1983) and are part of the immune response in vertebrates with jaws (Sun *et al.*, 2012; Mashoof and Criscitiello, 2016) including mammals, birds and reptiles. Antibodies usually bind antigenic invaders through specific interactions. In mammals, antibodies are categorised into five classes i.e. IgG, IgM, IgA, IgD and IgE (Ceppellini *et al.*, 1964; Black, 1997).

3.1.2. Chicken (avian) antibodies and IgY

Avian species produce immunoglobulins in the blood and laying hens transfer the antibodies to their eggs for the growing offspring (Patterson *et al.*, 1962). Chickens produce three antibody isotypes i.e. IgA, IgM and IgY (Rose *et al.*, 1974), the existence of chicken IgD and IgE homologues is still disputable (Zhao *et a.*, 2000, reviewed by Michael *et al.*, 2010). Egg yolk immunoglobulin (IgY) is the most abundant antibody in the egg yolk (Rose *et al.*, 1974) and serum (Leslie and Clem,1969) of chickens. The uniqueness of IgY as a non-IgG antibody isotype was shown by Leslie and Clem (1969) who recommended the term IgY. IgY is composed of two heavy (H) and two light (L) chains, consisting of constant (C) and variable (V) regions, like mammalian antibodies. IgY has four heavy chain constant regions denoted (υ-upsilon) (Sun *et al.*, 2001; Zhao *et a.*, 2000), unlike IgG which only has three constant regions on its heavy chains. This makes IgY bigger than IgG (Leslie and Clem,1969) with a molecular weight of about 167-180 kDa (Sun *et al.*, 2001; Amro *et al.*, 2018) compared to 150 kDa for IgG.

3.1.3. Antibody applications

Antibodies are beneficial in protecting their host species against foreign antigenic substances and antibodies are an essential part of several biochemical techniques. Antibodies are an important resource in disease diagnostics and therapeutics, where they are employed to confirm the presence of infections before treatment e.g. malaria rapid diagnostic tests. Antibodies are useful in detecting and comparing recombinant proteins to their native counterparts in western blotting and can be used to purify protein antigens from complex mixtures through affinity purification. Antibodies have a wide application in mapping the location and co-localisation of proteins within cells by immunofluorescence (IF) and immunoelectron microscopy (IEM), (reviewed by Payne Jr. *et al.*, 1988). The broad applicability of antibodies stem from their ability to be joined to compounds i.e. enzymes,

fluorophores and particles while still preserving their specificity to ligands against which they were raised.

3.1.4. Monoclonal antibodies in disease diagnostics

Mouse and rabbit antibodies are often used in diagnostic testing and can be produced as monoclonal antibodies. A monoclonal antibody detects a single epitope of an antigen (reviewed by Payne Jr. *et al*., 1988). Malaria rapid diagnostic tests using the *P. falciparum* diagnostic marker HRP-2 utilise mouse IgG monoclonal antibodies and the tests are temperature labile (WHO, 2016), and can give inconsistent results in the field (reviewed by Bell *et al*., 2006). The cross-reactivity of anti-*Pf*HRP-2 mouse IgG antibodies with human rheumatoid factor leads to false positive results (Lee *et al*., 2014). Iqbal and colleagues showed that an RDT using mammalian IgG to detect *Pf*HRP-2 gave a false positive result in 26% of patients with rheumatoid arthritis and patients tested negative for malaria with microscopy (Iqbal *et al*., 2000). All patients tested RDT negative after the removal of rheumatoid factor from blood samples. Antigen variation in *Pf*HRP-2 (Baker *et al*., 2005) and reduced stability of the monoclonal antibodies in the test, exposed to high heat and humidity also contribute to this irregularity (reviewed by Bell *et al*., 2006).

3.1.5. Chicken IgY antibodies in malaria diagnostics

Chicken IgY antibodies are specific and stable, tolerating temperatures of up to 70°C (Shimizu *et al*., 1992). IgY can be stored for several months at 4°C (Goldring and Coetzer, 2003; Nilsson *et al*., 2012). Chicken IgY antibodies do not react with human rheumatoid factor (Larsson *et al*., 1991). The egg yolk of a single egg contains high amounts of IgY that can be easily isolated with cheap reagents (Goldring and Coetzer, 2003), making egg yolk IgY a good alternative to mouse IgG. Purified IgY polyclonal antibodies can be raised against multiple epitopes within a single protein and could improve the detection sensitivity of a diagnostic test.

IgY antibodies were raised in chickens against his-tagged r*Pf*HRP-2. The isolated and affinity purified IgY antibodies were characterised using ELISA and western blotting. The thermal stability of the affinity purified r*Pf*HRP-2 IgY antibodies was investigated. A human pool of antibodies against natural *Plasmodium falciparum* infection was used to characterise r*Pf*HRP-2.

3.2. Materials and methods

3.2.1. Immunisation and raising antibodies in chickens

Ethical approval for the animal experiments was granted by the University of KwaZulu-Natal animal ethics board (Ethics number 004/15/animal).

Two Hy-line silver brown chickens kept at the animal care unit of the University of KwaZulu-Natal, Ukulinga farm were immunised by a trained specialist in animal handling,

intramuscularly with 50 µg of recombinant *Pf*HRP-2-(His$_6$) i.e. 25 µg protein per breast muscle, triturated in an equal volume 1:1 with Freund's complete for primary injection or Freund's incomplete adjuvants for booster injections. Booster injections were administered every second week after the first immunisation. Eggs were collected before and after immunisation for 13 weeks and stored at 4°C until IgY isolation.

3.2.2. Isolation of chicken egg yolk IgY antibodies

Egg yolk IgY antibodies were isolated using the method of Polson *et al.* (1980). The egg yolk was separated from the white and the yolk sac punctured to release the yolk and the volume of the yolk determine with a measuring cylinder. Two egg yolk volumes of 100 mM sodium phosphate buffer containing 0.02% (w/v) NaN_3, pH 7.6 was added and thoroughly mixed. 3.5% (w/v) solid polyethylene glycol (PEG) was added and dissolved. The precipitate was removed by centrifugation at 4 500x g 30 min RT. The supernatant was filtered through absorbent cotton wool and its volume was determined. 8.5% (w/v) PEG was added and dissolved, the precipitated fraction was removed by centrifugation 12 000x g, 10 min RT. The resulting pellet was dissolved in the filtrate volume equivalent of 100 mM sodium phosphate buffer. 12% (w/v) PEG was added and dissolved, and centrifuged at 12 000x g, 10 min RT. The supernatant was discarded and the final IgY antibody pellet was dissolved in one-sixth of the original egg yolk volume and stored at 4°C until further use. Antibodies from eggs collected at week 4-8 and 9-13 were pooled.

3.2.3. ELISA to monitor antibodies in chicken eggs against r*Pf*HRP-2

96-well ELISA plates (Nunc-MaxiSorp^{TM} flat-bottom, from ThermoFisher Scientific) were coated with 1 µg/ml recombinant *Pf*HRP-2-(His$_6$) overnight at 4°C in PBS buffer. Excess, antigen was removed, and the wells blocked with bovine serum albumin (BSA) 0.5% (m/v) in PBS at 37°C for 1 h. The wells were washed with PBS containing 0.1% (v/v) Tween-20, three times and incubated with 100 µg/ml, 50 µg/ml and 25 µg/ml chicken IgY antibodies from week 0-13 at 37°C for 2 h, diluted in 0.5% (m/v) BSA-PBS. The wells were washed three times with PBS containing 0.1% (v/v) Tween-20 and incubated with rabbit anti-chicken IgY (Jackson Immuno-Research) antibody conjugated to horseradish peroxidase, diluted 1:10000 in 0.5% (m/v) BSA-PBS, at 37°C for 1 h. The wells were washed again and incubated with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate solution (0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2) in citrate-phosphate buffer (21 g/L citrate, 31.5 g/L Na_2HPO_4, pH 5), in the dark for 15-20 min. The signal was measured at 405 nm using the VersaMax Microplate Reader. Controls included: no coating antigen; no primary antibody, no secondary antibody and no block. Weeks with high antibody titers i.e. 4-13 were then pooled for affinity purification of antibodies.

3.2.4. Western blot of recombinant *Pf*HRP-2-(His$_6$)

Proteins separated on a 12.5% SDS-PAGE gels were electrophoretically transferred to nitrocellulose at 40 mA for 16 h in blotting tank buffer (6.05 g/L Tris, 14.4 g/L glycine, 1 g/L SDS and 20% (v/v) methanol). The membrane was stained with ponceau-S solution (0.1% (w/v) ponceau-S in 5% (v/v) acetic acid) and the molecular weight markers were marked with a lead pencil. The stain was removed by washing the nitrocellulose membrane with distilled water with a few drops of sodium hydroxide. The membrane was air-dried for 30 min and blocked with 5% (w/v) fat-free milk powder in tris-buffered saline (TBS) (2.42 g/L Tris and 11.69 g/L NaCl, pH 7.4) for 1 h at room temperature. The nitrocellulose was washed three times with TBS for 5 min and incubated with mouse anti-his tag antibodies diluted 1:1000 with 0.5% (m/v) BSA-TBS or affinity purified anti- *Pf*HRP-2-(His$_6$) IgY antibodies (1 µg/ml) diluted in 0.5% (w/v) BSA-TBS, incubated for 1 h RT. The nitrocellulose was washed three times with TBS for 5 min and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase or rabbit anti-chicken IgY conjugated to horseradish peroxidase, diluted 1:10000 in 0.5% (m/v) BSA-TBS, for 1 h RT. Excess/ unbound antibodies were washed off the nitrocellulose, three times with TBS for 5 min and the membrane was incubated with the substrate solution (0.06% (m/v) 4-chloronaphtol, 0.0015% (v/v) H_2O_2, 20% (v/v) methanol) in the dark for 15-20 min until the protein bands were visualised by the naked eye. The nitrocellulose was washed with distilled water and air-dried for 30 min before images of the protein bands were taken with a Syngene G:Box XR5 (UK).

To detect recombinant *Pf*HRP-2 with enhanced chemiluminescence (ECL) employing human antimalarial antibodies, the above protocol for western blotting was used except that all blocking steps included 8% (w/v) fat-free milk powder and all washes were for 8 min, with TBS containing 0.1% (v/v) Tween-20. The recombinant protein was probed with a pool of human antibodies against a natural malaria infection, diluted 1:200 with 0.5% (m/v) BSA-TBS and detected with rabbit anti-human IgG conjugated to horseradish peroxidase, diluted 1:1000 with 0.5% (m/v) BSA-TBS. The nitrocellulose was incubated with substrate solution (0.025% (w/v) p-iodophenol, 0.05% (w/v) luminol, 0.0075% (v/v) H_2O_2)) in 0.1 M Tris-HCl pH 8.5.

3.2.5. Affinity purification of anti-r*Pf*HRP-2-(His$_6$) IgY antibodies

Purified r*Pf*HRP-2-(His$_6$) was concentrated using the Amicon® Ultra-15 centrifugal filter (Merck Millipore) 10 000 Da cut-off. AminoLink® (aldehyde-activated) resin 2 ml slurry was equilibrated with 3x column volume coupling buffer (50 mM NaH_2PO_4, 17.53 g/L NaCl, 0.05% (w/v) NaN_3, pH 7.2). The AminoLink® resin was incubated with 1 mg of recombinant *Pf*HRP-2 and 40 µl cyanoborohydride (5 M $NaCNBH_3$) in 1 M NaOH solution for 6 h RT with rotation. The column was drained and washed with two column volumes of coupling buffer. The resin was incubation with quenching buffer (1 M Tris-HCl, pH 7.4) and 40 µl cyanoborohydride and mixed for 30 min. The column was drained and washed with 5x column volume of wash buffer (100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 6.5). IgY was filtered through Whatman #1 filter

paper and cycled overnight over the recombinant *Pf*HRP-2-(His_6) affinity column. The unbound IgY was removed and the column was washed with 5x column volume of PBS until the absorbance at 280 nm was between 0.01-0.02. The bound IgY was eluted with elution buffer (100 mM glycine, 0.02% (w/v) NaN_3, pH 2.8) and 1 ml factions collected in tubes containing 50 µl neutralisation buffer (0.05 M NaH_2PO_4, 0.02% (w/v) NaN_3, pH 8.5). The absorbance at 280 nm of the eluted fraction was measured before storage at 4°C.

3.2.6. Stability of purified *Pf*HRP-2-(His_6) IgY antibodies at different temperatures

The affinity purified anti-*Pf*HRP-2 chicken IgY antibodies 25 µg/ml or 125 µg protein in sodium phosphate buffer containing 100 mM glycine was incubated in a water bath at 23°C, 37°C and 45°C. The IgY antibodies stored at 4°C were used as the control. The detection of antigen by the IgY antibodies was determined using ELISA, every week for eighteen weeks. The ELISA 96-well plates (Nunc-MaxiSorp^{TM} flat-bottom, from ThermoFisher Scientific) were coated with 0.5 µg recombinant *Pf*HRP-2-(His_6) overnight at 4°C in PBS buffer. Unbound antigen was removed, and the wells blocked with 0.5% (m/v) BSA in PBS at 37°C for 1 h. Wells were washed with PBS containing 0.1% (v/v) Tween-20 three times and incubated with IgY at 37°C for 2 h, in 0.5% (m/v) BSA-PBS. The wells were washed again and incubated with rabbit anti-chicken IgY conjugated to horseradish peroxidase, diluted 1:10000 in 0.5% (m/v) BSA-PBS, at 37°C for 1 h. The wells were washed and the substrate (ABTS solution) was added as described above (ELISA). The signal was measured at 405 nm using the VersaMax Microplate Reader (USA) and readings done in triplicate.

3.3. Results

3.3.1. Anti-*Pf*HRP-2-(His_6) IgY antibody titers in the eggs of chickens

IgY antibody responses in chickens immunised with r*Pf*HRP-2-(His_6) were assed in eggs collected each week. IgY from eggs collected before immunisation were used as a control (Figure 3.1, week 0). Antibodies detecting r*Pf*HRP-2 were observed after week three (Figure 3.1a) and week two (Figure 3.1b) post immunisation. Chicken-2 had higher antibody titers compared to chicken-1. Unfortunately, chicken-2 stopped laying eggs at week 3-4 during the immunisation period (Figure 3.1b).

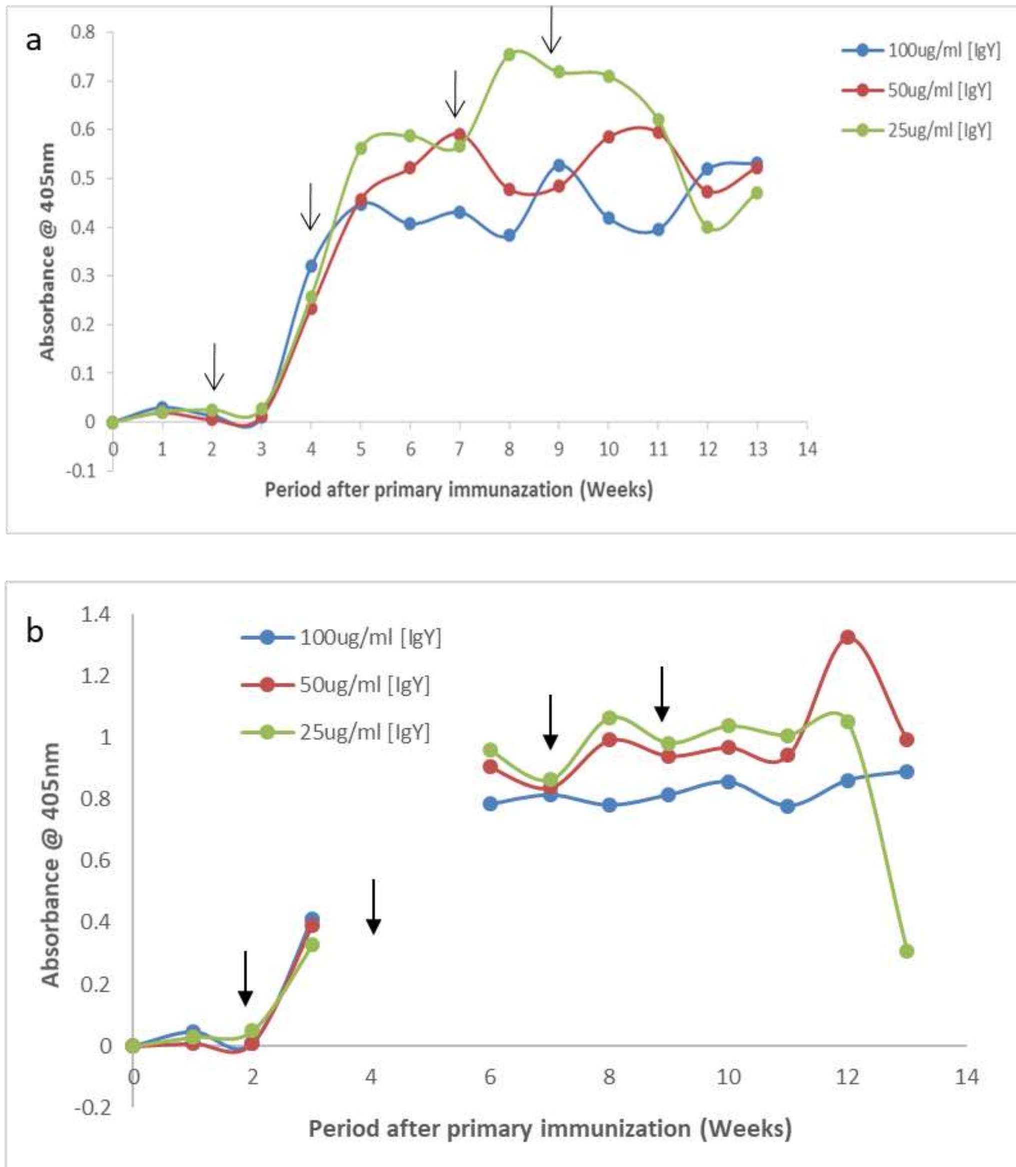

Figure 3.1. Antibody titers in eggs from chickens immunised with r*Pf*HRP-2.
IgY was isolated from the eggs of chickens immunised with r*Pf*HRP-2 and evaluated by ELISA. IgY was detected with a rabbit anti-chicken IgY conjugated to horseradish peroxidase. Booster immunisations were administered on week 2, 4, 7 and 9 (indicated with arrows). Two chickens (a and b) were immunised.

3.3.2. Detection of r*Pf*HRP-2 by affinity purified IgY antibodies

Figure 3.2a, show a 54 kDa purified r*Pf*HRP-2-(His$_6$) on a Coomassie stained SDS-PAGE gel (lane 2-3). The IgY antibodies against r*Pf*HRP-2 were affinity purified and used to probe purified r*Pf*HRP-2 on a western blot. The antibodies detected a 54 kDa r*Pf*HRP-2 band; a doublet about 116 kDa and a band above 116 kDa suspected as a trimeric form of *Pf*HRP-2 with a theoretical size of about 160 kDa (Figure 3.2b, lane 2-3). A mouse antibody against histidine residues detected the 54 kDa r*Pf*HRP-2 protein and a band estimated at about 112

kDa based on the size of the protein band compared to the molecular weight (Figure 3.2c, lane 2-3).

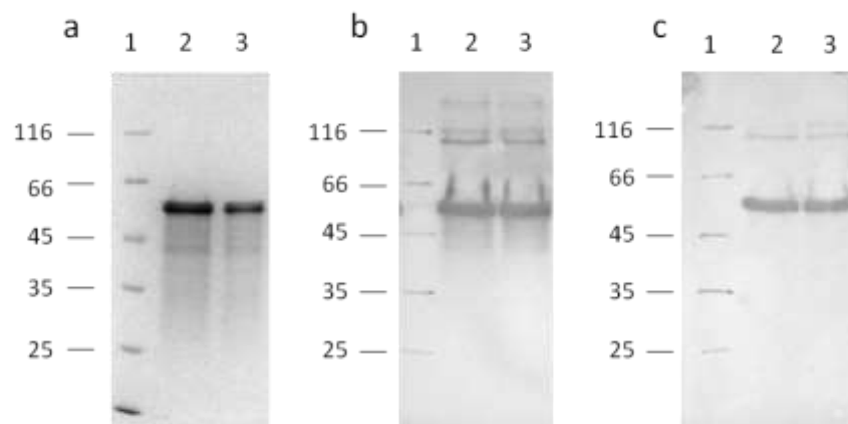

Figure 3.2. Detection of r*Pf*HRP-2-(His$_6$) with affinity purified IgY and anti-his tag antibodies on a western blot.
Purified r*Pf*HRP-2-(His$_6$) was run on 12.5% SDS-PAGE gels: (a) stained with Coomassie (R250) or (b-c) transferred to nitrocellulose. Lane 1: molecular weight makers; lanes 2 and 3: affinity purified recombinant *Pf*HRP-2-(His$_6$). In (b) the nitrocellulose was probed with purified anti-r*Pf*HRP-2 IgY and rabbit anti-chicken IgY conjugated to horseradish peroxidase. (c) The nitrocellulose was probed with mouse anti-his antibody and goat anti-mouse IgG conjugated to horseradish peroxidase.

3.3.3. Thermal stability of purified r*Pf*HRP-2 antibodies

Antibodies used for malaria diagnosis in rapid diagnostic tests need to be stable under tropical temperatures that the RDTs are exposed to in the field. IgY antibodies were incubated in a water bath at 23°C, 37°C and 45°C for eighteen weeks at the same time. Figure 3.3 shows the detection of antigen by the IgY after 1, 10, 16 and 18 weeks at the different temperature. The IgY antibodies detected r*Pf*HRP-2 after 1 – 16 weeks exposure at 23°C, 37°C and 45°C (Figure 3.3). The signal for the antibodies at 45°C declined after 10 weeks and by 16 weeks the absorbance values were below 0.5 (Figure 3.3 b and c). The antibodies incubated at 23°C and 37°C detected r*Pf*HRP-2 up to sixteen weeks. But the reactivity of the antibodies started to decline when the antibodies were stored at 37°C. At 18 weeks the IgY antibodies at 23°C, 37°C and 45°C no longer detected r*Pf*HRP-2 while antibodies stored at 4°C retained detecting r*Pf*HRP-2 for eighteen weeks (Figure 3.3d). It was not clear why the absorbance signals at 16 to 18 weeks fluctuated for the antibodies stored at 4°C, but the trend stayed the same regardless.

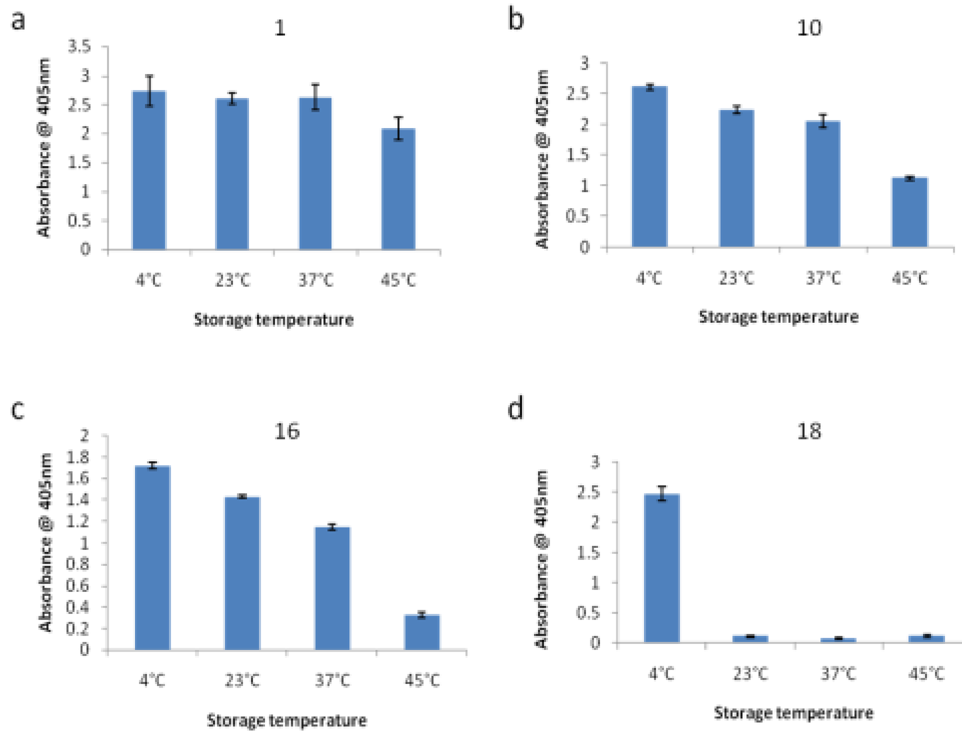

Figure 3.3. Thermal stability of purified anti-r*Pf*HRP-2 chicken IgY antibodies in solution. Chicken IgY antibody samples were incubated at 23°C, 37°C and 45°C for eighteen weeks in buffer. The stability of the antibodies was monitored with ELISA detecting r*Pf*HRP-2 (0.5 µg) with the antibodies (1 µg) after (a) week-1, (b) week-10, (c) week-16 and (d) week-18 of exposure to high temperatures. An IgY sample at 4°C was used as a control. A rabbit anti-chicken IgY antibody conjugated to horseradish peroxidase was used for detection. The error bars indicate the standard deviation. One sample stored at each specified temperature and experiments done in triplicates every second week.

3.3.4. r*Pf*HRP-2 detection with human antibodies

A lyophilised pool of antibodies from Malawian patients infected with malaria was used to probe r*Pf*HRP-2-(His_6) on a western blot (Goldring *et al.*, 1992). The human anti-malaria antibody pool detected the recombinant protein (Figure 3.4b). The high molecular weight proteins about 108 kDa and the 54 kDa protein were detected. This suggests that the human antibodies recognise r*Pf*HRP-2 dimers, similar to the chicken IgY antibodies.

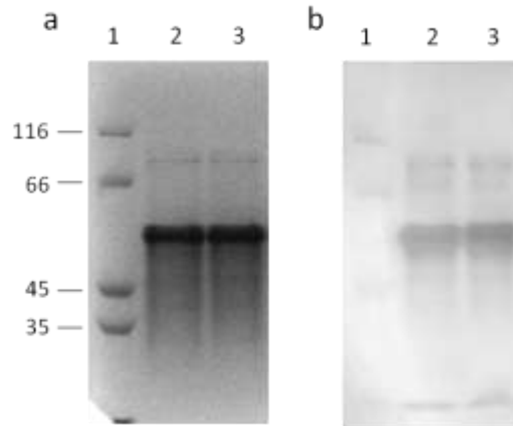

Figure 3.4. Detection of r*Pf*HRP-2-(His$_6$) by human antimalarial antibodies.
Purified r*Pf*HRP-2 was run on 12.5% SDS-PAGE gels: (a) stained with Coomassie (R250) or (b) transferred to a nitrocellulose. Lane 1: molecular weight makers; lanes 2 and 3: purified r*Pf*HRP-2-(His$_6$). The nitrocellulose was probed with a pool of human anti-malarial antibodies and detected with a rabbit anti-human IgG conjugated to horseradish peroxidase, with ECL.

3.4. Discussion

Chicken egg yolks are a good source of polyclonal antibodies, that can be easily isolated and purified (Polson *et al.*,1980; Goldring and Coetzer, 2003). Affinity purifying these antibodies, selects antibodies against paratopes of one specific antigen. The antibodies produced would recognise more than a single epitope of the target protein since they are raised against the whole r*Pf*HRP-2 protein. The recognition of multiple epitopes is likely to make the pool of antibodies more sensitive (Ascoli and Aggeler, 2018), because more antibodies can bind to the antigen increasing the detection signal. Polyclonal antibodies combine the affinity of an antibody to a specific epitope and the avidity of multiple antibodies detecting one protein, this could be useful in disease diagnostics. In this study, polyclonal IgY antibodies were raised in chickens, affinity purified and characterised against r*Pf*HRP-2. These antibodies were stable at high temperatures for up to sixteen weeks.

3.4.1. Raising anti-r*Pf*HRP-2 antibodies in chickens with Freund's adjuvants

Antibody responses in chickens often take days to accumulate (Beck *et al.*, 2003). The levels and duration of these responses can be enhanced with adjuvants (Sun *et al.*, 2008; Krause *et al.*, 2015), but can still vary between hosts (Figure 3.1). Freund's adjuvant was used in this study as it is tolerated by chickens without severe adverse effects and promotes a high immune response against the antigen by forming a deport to which immune responsive cells and molecules are recruited. The mycobacterial component of Freund's complete adjuvant is important for this role (reviewed by Billiau and Matthys, 2001). Chicken IgY in eggs correlate with IgY in the blood (Sun *et al.*, 2013) eliminating the need to bleed the animal for antibodies. Anti-r*Pf*HRP-2 antibodies in eggs remained high for both chickens after each booster injection.

The cost of raising polyclonal antibodies in chickens in terms of effort to results ratio i.e. obtaining high yields would be cheaper compared to monoclonal antibodies in mice. High yields of IgY antibodies are obtained in a single egg after isolation and purification compared to mice, where small volumes of blood are obtained.

3.4.2. IgY and mouse anti-his-tag antibody detection of purified r*Pf*HRP-2

The anti-r*Pf*HRP-2 IgY detected three forms of the r*Pf*HRP-2 proteins, corresponding to a monomer, a dimer and a trimer. The monomer is the most abundant of the three forms. The monomer and dimer were detected by the anti-his-tag antibody. The size of dimeric and trimeric r*Pf*HRP-2 is about 108 kDa and 162 kDa respectively. High molecular weight forms of *Pf*HRP-2 have been previously reported in the presence of heme (Schneider and Marletta, 2005), but not during recombinant protein expression. It is possible that the antibodies used in this study are very sensitive and enhance the detection of low occurring *Pf*HRP-2 forms during recombinant expression. But the sensitivity of the antibodies used in this study would have to be investigated to substantiate this idea. Another possibility is that a small fraction of r*Pf*HRP-2 is not fully and completely reduced in the conditions of this study and can hence form dimers and trimers not observed in other studies expressing the protein.

The chicken IgY antibodies raised in this study are shown to be specific to r*Pf*HRP-2, a protein to which the antibodies were raised. The detection of the recombinant protein amongst other proteins of *E. coli* or Plasmodial origin would have helped to further investigate the specificity of the chicken antibodies raised in this study. The specificity of the rabbit anti-chicken IgY conjugated to horseradish peroxidase could have also been investigated for western blotting.

3.4.3. Anti-r*Pf*HRP-2 IgY stability at room and higher temperatures

Affinity purified r*Pf*HRP-2 IgY stored at 23°C, 37°C and 45°C for up to sixteen weeks, about 112 days, detected r*Pf*HRP-2 (Figure 3.3c). Prolonged exposure of antibodies to temperatures above 23°C in solution, gradually reduced antibody detection of antigens as observed in this study (Chiodini *et al.*, 2007). The reactivity against r*Pf*HRP-2 after sixteen to eighteen weeks gradually decreased until it was lost by the antibodies at 23°C and 37°C. The biological reactivity of IgY in solution stored at 50-70°C can be preserved by the addition of sugars and complex carbohydrates as previously demonstrated by Jaradat and Marquardt (2000). Dried antibodies on the nitrocellulose strip of RDTs are also susceptible to heat-induced damage and this affects malaria diagnosis (Chiodini *et al.*, 2007).

Testing the stability of antibodies in solution is a good indicator of the temperature tolerance of antibodies for possible RDT applications. This study showed that prolonged IgY exposure in solution to temperatures as low as 25°C could affect detection. The heat tolerance of the IgY tested in this study is comparable to other studies where IgG thermal

stability was investigated (Chiodini *et al.*, 2007). The IgY antibodies in this study were exposed to warm and humid conditions in very dilute solution of 25 ug/ml, usually IgG antibodies are stored at concentrations ≥1 mg/ml during these studies (Szenczi *et al.,* 2006). This adds to the stability and long shelf life of the IgY antibodies tested in this study.

3.4.4. Human antimalarial antibody pool detection of r*Pf*HRP-2

The human pool of antimalarial antibodies detected the 54 kDa r*Pf*HRP-2 monomer and a dimer of 108 kDa (Figure 3.5b), similar to the chicken antibodies. *Pf*HRP-2 is found in blood of patient with *P. falciparum* malaria (Parra *et al.*, 1991) and is antigenic in humans (Biswas *et al.*, 2005; Das *et al.*, 2010; Ho *et al.*, 2014). A lack of anti-*Pf*HRP-2 antibodies in circulation has been reported in malaria-endemic regions of Cameroon (Taylor *et al.*, 2017). Including low antibody responses to *Pf*HRP-2 in parts of India (Das *et al.*, 2010). These indications suggest that *Pf*HRP-2 might not be highly immunogenic in humans leading to a low concentration of antibodies against the protein (Das *et al.*, 2010; Taylor *et al.*, 2017). Other studies suggest that *Pf*HRP-2 suppress T and B-cell function (Das *et al.*, 2006) which could translate to the observed low concentration of natural antibodies in some patients. But contrasting studies found the presence of anti-*Pf*HRP-2 antibodies in circulation and some forming immune complexes with the protein (Markwalter *et al.*, 2018). More studies are required to understand the antigenicity of *Pf*HRP-2 in humans, leading to the production of antibodies. The pool of human antibodies used in this study contained anti-*Pf*HRP-2 antibodies.

3.4.5. Conclusion

The use of polyclonal antibodies for detecting a polymorphic protein like *Pf*HRP-2 can be beneficial for malaria diagnostics. We show that chicken IgY is stable and tolerates room temperature for sixteen weeks in solution. Human antibodies against native *Pf*HRP-2 recognised r*Pf*HRP-2 which forms high molecular weight proteins, detected by both antibodies. More studies are needed to investigate the formation of the forms of r*Pf*HRP-2 detected in this study.

Chapter 4

Trypsin digestion of r*Pf*HRP-2

4.1. Introduction

4.1.1. Proteolytic enzymes

Proteolytic digestion of proteins is an important component in the field of proteomics. Proteolytic enzymes in the laboratory have a wide application including the generation of peptides for mass spectrometric analysis; cleavage of tags from recombinant proteins; breaking down of contaminating proteins in nucleic acid purifications (Mótyán *et al.*, 2013) and characterising antibodies by protein foot-printing (Sheshberadaran and Payne, 1988). Proteases are useful for studying protein structures, for example papain played a significant role in studies of immunoglobulin structures (Edelman and Benacerraf, 1962). Because the amino acid sequence of *Pf*HRP-2 is known, it could be possible to determine the epitopes that react with the anti-*Pf*HRP-2 antibodies if the fragments produced by proteolytic cleavage are identified.

Trypsin is a commonly used proteolytic enzyme in proteomic studies to generate peptides. This is because trypsin is efficient, accessible and specific, cleaves peptide bonds on the carboxy side or C-terminal end of arginine and lysine residues, that are not adjacent to proline residues (Tsiatsiani and Heck, 2015). Increasing evidence suggest that trypsin cleaves more frequently before proline than it does before cysteine residues (Rodriguez *et al.*, 2008). Arginine seems to be preferred over lysine meaning that arginine sites are cleaved faster than lysine sites (Perutka and Šebela, 2018). Additionally, missed cleavage can occur in peptides with successive lysine, arginine and arginine-lysine residues (Thiede *et al.*, 2000; Perutka and Šebela, 2018). Trypsin is active at pH 7-9 with optimum activity at pH 8.3 at 37°C (Olsen *et al.*, 2004). The cleavage of r*Pf*HRP-2 with trypsin would be specific and allow the identification of fragments formed based on predicted cleavage sites along the *Pf*HRP-2 sequence.

4.1.2. Disulfide bonds

Disulfide bonds are molecular interactions that form between two oxidised cysteine residues of polypeptide chains or proteins. These interactions can form within a single peptide i.e. intramolecular disulfide bonds or between two separate peptides i.e. intermolecular disulfide bonds. Disulfide interactions play a role in catalysis as part of the active sites of enzymes and a role in signaling in oxidative stress responses (Lee *et al.*, 2004; Landgraf *et al.*, 2017). Disulfide bonds are well known for their role in stabilising protein structures and protein conformation which can affect protein function (Landgraf *et al.*, 2017). Studies on RNase A not only show that disulfide bonds stabilise the tertiary structure of the protein, but that they are also involved in initiating protein folding (Landgraf *et al.*, 2017). Protein

monomers containing cysteine residues have been shown to form dimers and trimers through intermolecular disulfide bonding (Yang *et al*., 2016), which can be detected with SDS-PAGE. The formation of dimers and trimers within *Pf*HRP-2 has been shown to be assisted by cysteine residues in the presence of heme or other divalent cations (Schneider and Marletta, 2005). Thus, to possibly convert dimeric and trimeric *Pf*HRP-2 to its monomeric form, ethylenediaminetetraacetic acid (EDTA) a metal chelator could potentially remove heme or a metal possibly assisting its dimer formation from the protein and solution preventing *Pf*HRP-2 from forming dimers, assuming optimal conditions.

The histidine rich protein-2 of *Plasmodium falciparum* contains two cysteine (Cys33 and Cys306) residues in its amino acid sequence (Sullivan *et al*., 1996). Not much is known about the structure of *Pf*HRP-2 or the interactions of its cysteine residues. A random coil or loop structure has been suggested for *Pf*HRP-2 which is predicted to adopt a helical conformation (3_{10}-helixes) in oxidising environments (Schneider and Marletta, 2005). With this conformation *Pf*HRP-2 is thought to form intermolecular disulfide bonds which stabilises the protein's dimer and trimer forms (Schneider and Marletta, 2005). Investigating the intermolecular interaction of *Pf*HRPP-2 and its fragments might add insight to the overall knowledge of the *Plasmodium falciparum* HRP-2 protein as a diagnostic marker.

In this chapter r*Pf*HRP-2 was digested with trypsin and analysed in SDS-PAGE gels and western blotting with specific IgY antibodies to investigate possible peptides or epitopes interacting with the anti-*Pf*HRP-2 IgY.

4.2. Materials and methods

Affinity purified r*Pf*HRP-2 was incubated with trypsin in a 1:20 enzyme to protein ratio in PBS, pH 8 at 37°C for 4 h using 0.25 mg/ml of r*Pf*HRP-2 or with samples collected every 15 min for 2 h. The samples were mixed with reducing or non-reducing treatment buffer and run on 12.5% SDS-PAGE gels (described in chapter 2). The proteins from the gel were electrophoretically transferred with nitrocellulose membranes on both sides of the gel for 16 h. To bind proteins that may move towards or away from the positive electrode. The nitrocellulose membranes were probed with anti-r*Pf*HRP-2 IgY and anti-his tag antibodies, which were in turn detected by rabbit anti-chicken IgY conjugated to horseradish peroxidase and goat anti-mouse IgG conjugated to horseradish peroxidase respectively with western blotting using 4-chloronaphtol (described in chapter 3).

4.3. Results

4.3.1. Predicted trypsin digestion sites on *Pf*HRP-2 sequence

The *Pf*HRP-2 protein sequence (P90582_PLAFA) from UniProtKB was analysed with the ExPASy Peptide-Cutter tool for possible trypsin cleavage sites. The program predicted eight possible cleavage sites producing nine *Pf*HRP-2 fragments (Table 4.1). Two major fragments of *Pf*HRP-2 were predicted with molecular weight sizes of about 11 kDa and 17 kDa. One of the cysteine residues (Cys306) is located on the 11 kDa fragment, while Cys33 is located on a peptide predicted at 2.8 kDa as seen on Table 4.1.

Table 4.1. Predicted tryptic fragments of *Pf*HRP-2

Cleavage position	Resulting peptide Sequence	Peptide mass (kDa)
6	MVSFSK	0.7
8	NK	0.26
35	VLSAAVFASVLLLDNNNSAFNNNL**C**SK	2.80
38	NAK	0.33
44	GLNLNK	0.66
45	R	0.17
202	**LLHETQAHVD**DAHHAHHVADAHHAHHAADAHHAHHAADAHHAHHAADAHHAHHAADAHH AHHAAYAHHAHHAADAHHAHHASDAHHAADAHHAAYAHHAHHAADAHHAHHASDAHHAAD AHHAAYAHHAHHAADAHHAADAHHATDAHHAHHAADAR	16.7
305	HATDAHHAADAHHATDAHHAADAHHAADAHHATDAHHAADAHHATDAHHAADAHHAADA HHATDAHHAHHAADAHHAAAHHATDAHHATDAHHAAAHHEAATH**C**LR	11
Total		32.6

The cysteine residues are highlighted in grey. A 32 kDa protein was predicted from the *Pf*HRP-2 amino acid sequence. The predicted isoelectric point (pI) of *Pf*HRP-2 is (6.43); (6.37) for the 16.7 kDa fragment and (6.25) for 11 kDa. The *Pf*HRP-2 sequence was obtained from UniProtKB, (P90582_PLAFA) and fragments were generated using the ExPASy Peptide-Cutter tool. The underlined peptide in the sequence is the main difference between the 11 and 17 kDa fragment either than size.

4.3.2. Analysis of tryptic fragments of *Pf*HRP-2 on SDS-PAGE and western blotting

For the initial trypsin digestion experiments, r*Pf*HRP-2 was incubated with trypsin at 37°C for 4 h and the reaction was stopped by boiling the samples in the presence of reducing treatment buffer for 5 min. Figure 4.1a shows digested r*Pf*HRP-2 compared to the undigested 54 kDa protein on a reducing SDS-PAGE gel. Three r*Pf*HRP-2 fragments were observed at 40 kDa, 37 kDa and 30 kDa (figure 4.1a, lane 2-3). These fragments are different in molecular weight to the predicted fragments in Table 4.1, because the protein and its fragments migrate in an unusual manner and could not be matched. The r*Pf*HRP-2

fragments, the 54 kDa protein and the 108 kDa dimer were detected by the anti-r*Pf*HRP-2 IgY antibodies (figure 4.1b and c). All these proteins migrated towards both the negative and positive electrodes during western blotting (figure 4.1c). The protein molecular weight marker migrates towards the positive electrode.

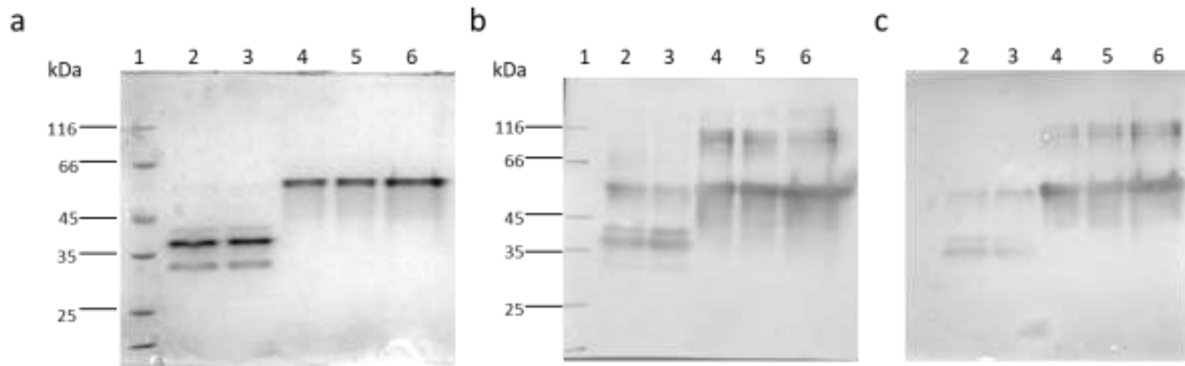

Figure 4.1. Trypsin cleavage of r*Pf*HRP-2.
Purified r*Pf*HRP-2 was digested with trypsin at 37°C for 4 h and run on reducing SDS-PAGE gels: (a) stained with Coomassie (R250) or (b-c) transferred onto nitrocellulose. Lane 1: molecular weight makers; lanes 2 and 3: digested r*Pf*HRP-2; lanes 4-6: undigested r*Pf*HRP-2 control. (b) positive electrode nitrocellulose and (c) negative electrode nitrocellulose probed with purified anti-r*Pf*HRP-2 IgY and rabbit anti-chicken IgY conjugated to horseradish peroxidase**.**

The trypsin digestion time with r*Pf*HRP-2 was decreased to 2 h with samples collected at 15 min intervals. The samples were boiled in reducing or non-reducing treatment buffer before being subjected to SDS-PAGE analysis and western blotting. Figure 4.2a and d (lane 2-6) shows the presence of the 54 kDa r*Pf*HRP-2 protein and its 40 kDa fragment in reduced samples digested for 15 min – 1 h and both proteins are completely digested after 2 h. The 37 kDa and 30 kDa fragments remain. Multimer forms of r*Pf*HRP-2 at 108 kDa (dimer) and proteins around 72 kDa were observed in non-reducing conditions, including the 54, 40, 37 and 30 kDa proteins (Figure 4.2a and d, lane 7-10). Two extra fragments were observed at 50 kDa and 47 kDa in non-reducing conditions at 15 min – 1 h of trypsin cleavage.

The anti-r*Pf*HRP-2 IgY and anti-his tag antibodies detected all the proteins except for the 30 kDa fragment and the 50 kDa - 47 kDa fragment (figure 4.2 b, c and e).

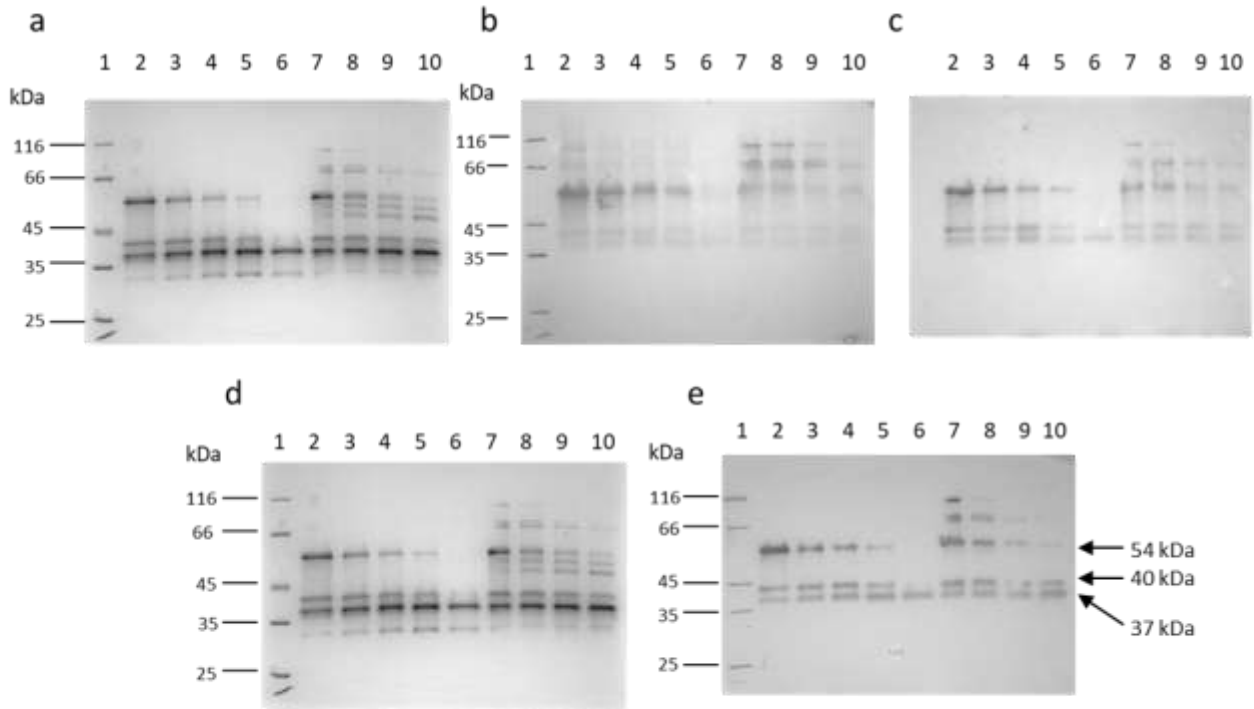

Figure 4.2. Tryptic fragments of r*Pf*HRP-2 in reducing and non-reduction SDS-PAGE.
Purified r*Pf*HRP-2 was digested with trypsin at 37°C for 15 min to 2 h and treated with reducing or non-reducing buffer before being run on SDS-PAGE gels: (a and d) stained with Coomassie (R250) or (b, c and e) transferred onto nitrocellulose. Lane 1: molecular weight makers; lanes 2-6: reduced r*Pf*HRP-2 fragments after 15 min, 30 min, 45 min, 1 h and 2 h of trypsin digestion; lanes 7-10: non-reduced r*Pf*HRP-2 fragments after 15 min, 30 min, 45 min and 1 h of trypsin digestion. (b) positive electrode nitrocellulose and (c) negative electrode nitrocellulose probed with purified anti-r*Pf*HRP-2 IgY and rabbit anti-chicken IgY conjugated to horseradish peroxidase. (e) positive electrode nitrocellulose probed with anti-his tag and goat anti-mouse IgG conjugated to horseradish peroxidase.

The addition of EDTA (Figure 4.3b) during the digestion of r*Pf*HRP-2 did not affect the profile of the fragments produced by trypsin or the formation of multimers in both reducing and non-reducing conditions as seen in the control (Figure 4.3a). EDTA was added to compete with the protein for heme or any divalent metal assisting its dimer formation. After 2 h of trypsin digestion the 47 kDa and the 37 kDa fragments remained in the gels of non-reduced samples (Figure 4.3a and b, lane 10).

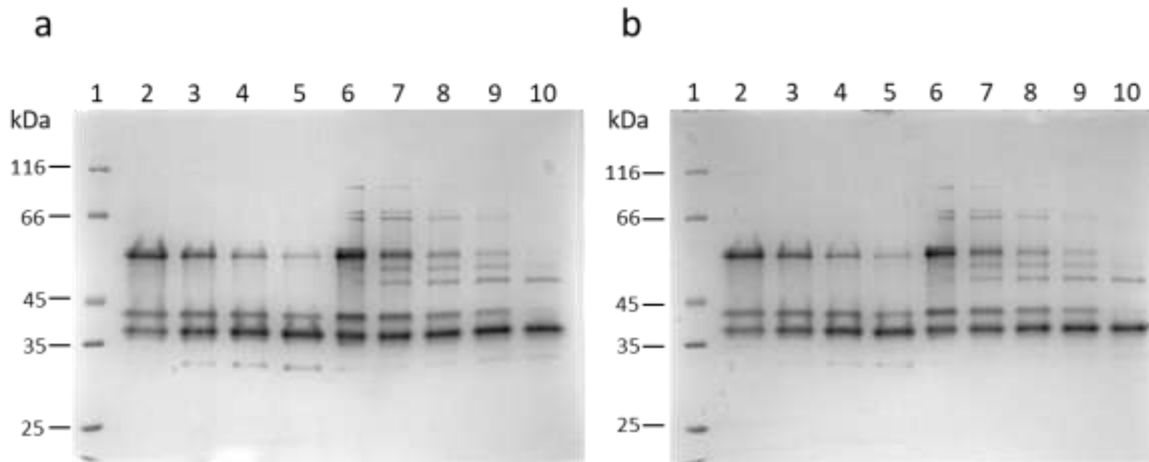

Figure 4.3. Digestion of r*Pf*HRP-2 with trypsin in the presence of EDTA.
Purified r*Pf*HRP-2 was digested with trypsin in the absence (a) or presence (b) of EDTA (50 mM) at 37°C for 15 min to 2 h and treated with reducing or non-reducing buffer before being run on SDS-PAGE gels stained with Coomassie (R250). Lane 1: molecular weight makers; lanes 2-5: reduced r*Pf*HRP-2 fragments after 15 min, 30 min, 45 min and 1 h of trypsin digestion; lanes 6-10: non-reduced r*Pf*HRP-2 fragments after 15 min, 30 min, 45 min, 1 h and 2 h of trypsin digestion.

4.4. Discussion

Products of protein digestion are useful in characterising proteins. The chymotrypsin fragments of *Pf*HRP-2 have been previously used to show amino acid substitution and translational modification during recombinant expression (Schneider *et al.*, 2005). In the present study, the behavior of r*Pf*HRP-2 trypsin fragments in reducing and non-reducing SDS-PAGE gels was analysed.

Trypsin was predicted to hydrolyse *Pf*HRP-2 at eight different sites leading to nine fragments ranging from 1 – 157 amino acids. The largest amino acid fragments were estimated at 11 kDa and 17 kDa (Table 4.1). Complete digestion of r*Pf*HRP-2 gave two fragments at 30 kDa and 37 kDa on reducing SDS-PAGE gels. Since *Pf*HRP-2 with a predicted size of 32 kDa resolves at 54 kDa, perhaps the 11 and 17 kDa fragments of *Pf*HRP-2 behave in a similar manner and are the 30 and 37 kDa bands. However, the 37 kDa fragment was detected by the anti-his tag antibody indicating the presence of the N-terminal histidine tag which is not present in the 17 kDa fragment according to Table 4.1. It is also interesting that the combined size of the two r*Pf*HRP-2 fragments at 30 and 37 kDa on the gels which is 67 kDa, is larger than the size of the whole protein 54 kDa. Usually, fragments of a cleaved protein are bellow or make up the size of the whole protein (Mihalyi and Szent-Gyorgyi, 1953), when analysed on SDS-PAGE gels (Klip *et al.*, 1980; Reece and Maxwell, 1989; Arias *et al.*, 1996). The reason for this strange behaver is not known but could be associated with the abnormal or low mobility of *Pf*HRP-2 fragments on SDS-PAGE gels. Another possibility could be that the fragments formed can also interact with each other forming disulfide bonds, but this would only apply to the two fragments predicted to contain cysteine residues in Table 4.1.

The pattern of r*Pf*HRP-2 fragments in non-reducing SDS-PAGE was different to the pattern under reducing conditions. Additional fragments at 47, 50, 72 kDa and a possible dimer about 108 kDa in non-reducing conditions, indicate the presence of disulfide bonds between r*Pf*HRP-2 fragments. Similar observations were made by Schneider and Marletta (2005) showing the behavior of *Pf*HRP-2 on native page gels. Fragments of different sizes could also be formed by selective r*Pf*HRP-2 hydrolysis since trypsin prefers cleaving after arginine (R) over lysine (K) residues (Perutka and Šebela, 2018). This means that if all lysine residues at position 6 to 44 (Table 4.1) were not cleaved, the trypsin hydrolysis of all arginine residues would produce fragments of 5, 11 and 17 kDa. Arginine residues within a protein sequence have varying susceptibility to trypsin hydrolysis and less susceptible sites would remain uncleaved or result in a small portion of the protein that is hydrolysed in that particular site (Arias, *et al*., 1996). This would also contribute to multiple fragments. An error in trypsin cleavage of arginine at position 45 of the r*Pf*HRP-2 sequence (Table 4.1) would produce fragments of 22 and 11 kDa, and these fragments would run larger than their predicted sizes of SDS-PAGE.

The anti-r*Pf*HRP-2 antibodies detected all the fragments except, the 30 kDa fragment in both reducing and non-reducing conditions. This fragment was present in low amounts and not detected in some gels (Figure 4.2). This fragment did not transfer well to nitrocellulose which could have contributed to the lack of detection by the antibodies. If the 30 kDa fragment is identified as the 11 kDa peptide (Table 4.1), it would be difficult to explain why it behaves differently to the 17 kDa (identified as 37 kDa) fragment with similar amino acid composition. Unless the unique peptide (LLHETQAHVD) underlined in Table 4.1 plays an influential role.

The anti-his tag antibody did not detect the 30 kDa, 47 kDa and 50 kDa fragments. The his-tag on the N-terminal end of r*Pf*HRP-2 might be absent in these fragments and the anti-his antibody does not react with the histidine rich region of *Pf*HRP-2. The r*Pf*HRP-2 protein and its fragments transferred in both directions from the gel during western blotting which was interesting because the isoelectric point (pI) of *Pf*HRP-2 and its fragments is similar ranging from 6.2 – 6.4.

The *Pf*HRP-2 protein is thought to form a helical structure in oxidising environments causing the formation of disulfide bonds and high molecular weight species (Schneider and Marletta, 2005). Heme (Fe^{3+}) is thought to be important in coordinating this interaction (Schneider and Marletta, 2005). To remove Fe^{3+} from the trypsin fragments of r*Pf*HRP-2 50 mM EDTA was added during the digestion. After treatment with trypsin EDTA did not affect the formation of dimers or multiple fragments of r*Pf*HRP-2. This could mean that the concentration of EDTA was not high enough to remove bound Fe^{3+} or that Fe^{3+} is not involved in disulfide bond formation in *Pf*HRP-2 (Schneider and Marletta, 2005).

This study gives insight into the trypsin digestion of r*Pf*HRP-2. Trypsin hydrolyses r*Pf*HRP-2 in to fragments that are further digested into smaller fragments as the end product.

Additional studies like mass spectral analysis of the digested fragments of r*Pf*HRP-2 would be useful in identifying the fragments observed on non-reducing SDS-PAGE gels. The utilisation of Tris-Tricine gels would able the analysis of low molecular weight fragments e.g. 2.8 kDa or lower of r*Pf*HRP-2 predicted in Table 4.1. and their antibody detection with western blotting. Mutation of *Pf*HRP-2 cysteines could have indicated the formation of intermolecular disulfide bonds which could be involved in the structure of r*Pf*HRP-2. More studies are required to understand the function, structure and the behavior of the *Pf*HRP-2 fragments in SDS-PAGE gels under reducing and non-reducing conditions, and after enzymic hydrolysis.

Chapter 5

General discussion

5.1. Introduction

Malaria killed 435 000 people in 2017 and 219 million cases were reported, which is 2 million cases more than the 2016 reports (WHO, 2018). These findings indicate the global burden of malaria. Most malaria occurs in developing countries in the tropics. Most of these countries lack the infrastructure and funding for sustained malaria control. The low efficacy of the leading malaria vaccine candidates will not be enough to alleviate the global malaria burden (Coelho *et al.*, 2017). Current malaria control measures such as the use of insecticide-treated bed nets and indoor residual spraying are still important (WHO, 2018).

Detecting the presence of *Plasmodium* parasites in patient samples is the basis of malaria diagnosis which in turn guides treatment. Parasitological diagnosis reduces the emergence of anti-malarial drug resistance and promotes the correct management of non-malarial fevers (WHO, 2010). Microscopy is the gold standard in malaria diagnosis for screening blood samples in hospitals and field clinics and is a reliable standard in the hands of trained experts (Mouatcho and Goldring, 2013). Rapid Diagnostic Tests (RDTs) or dipsticks are designed to be simple, cheap to operate and can be used in areas where microscopy is not accessible (Jan *et al.*, 2018). RDTs detect Plasmodial proteins such as *Plasmodium falciparum* Histidine Rich Protein -2 (*Pf*HRP-2), lactate dehydrogenase (LDH) and aldolase to indicate the presence or absence of malaria in patient samples (Mouatcho and Goldring, 2013). Because *Plasmodium falciparum* is the most fatal human malaria species (WHO, 2018), most commercial RDTs target this species for diagnosis. *Pf*HRP-2 is a *P. falciparum* protein produced during the red blood cell stages of human infection and is secreted into the cytosol of the red blood cell, where it is released into the blood when infected red blood cells rupture (Desakorn *et al.*, 2005). *Pf*HRP-2 is present in high concentrations in the blood of *P. falciparum* patients and is detectible in saliva, urine and cerebrospinal fluids (Parra *et al.*, 1991; Wilson et al., 2008; Mikita *et al.*, 2014; Castro-Sesquen *et al.*, 2016).

Studies on *Pf*HRP-2 aim at understanding the function of the protein in the parasite and the human host (Sullivan *et al.*, 1996; Pal *et al.*, 2017); testing antibodies against the protein (Verma *et al.*, 2017); monitoring the detection of *Pf*HRP-2 in the field (Gamboa *et al.*, 2010; Kiemde *et al.*, 2017) and optimising *Pf*HRP-2 detection (Castro-Sesquen *et al.*, 2016; Rogier *et al.*, 2017). The aim of this study was to raise antibodies against recombinant *Pf*HRP-2 in chickens and investigate the heat stability of the antibodies and their epitopes from trypsin cleaved fragments of recombinant *Pf*HRP-2.

5.2. r*Pf*HRP-2 recombinant expression

Recombinant *Pf*HRP-2 was expressed in *E. coli* and the optimal conditions involved culturing in terrific broth at 30°C overnight, and expression was induced with lactose. *Pf*HRP-2 is often recombinantly produced in large culture volumes in the presence of IPTG to achieve desired yields or high protein concentrations (Lynn *et al.,* 1999; Ndonwi *et al*., 2011). Optimising the production of r*Pf*HRP-2 in this study eliminated the need for large culture volumes and could save reagents. Lactose has not been reported to have negative effect on cells expressing proteins with unusual amino acid content that might require more resources from the host. *Pf*HRP-2 is a parasite protein with a high histidine content and lactose in terrific broth has the potential for the expression of parasite proteins that are difficult to recombinantly express.

5.3. Affinity purification and SDS-PAGE analysis

*Pf*HRP-2 binds nickel and other divalent metals such as copper and zinc (Panton *et al.,* 1989). This ability and the his-tag fusion partner were used to affinity purified r*Pf*HRP-2 from a bacterial culture lysate. The r*Pf*HRP-2 fusion protein had a high affinity for nickel, requiring four times the amount of imidazole usually needed to elute his-tagged recombinant proteins. Affinity purified r*Pf*HRP-2 with the his-tag resolved as a single protein band at 54 kDa on SDS-PAGE gels. This agrees with other studies on *Pf*HRP-2 since the protein often resolves bigger than the size predicted from its amino acid sequence (Wellems and Howard, 1986; Ndonwi *et al*., 2011). Perhaps the increase in size of the *Pf*HRP-2 protein on SDS-PAGE gels is due to the high histidine (Ndonwi *et al*., 2011) or aspartic acid (Armstrong and Roman, 1993) content of *Pf*HRP-2. It is possible that *Pf*HRP-2 does not interact well with SDS decreasing the number of negative charges on the protein making it less mobile in the gel. Further studies are required to understand this observation.

5.4. Characterisation of anti-r*Pf*HRP-2 IgY antibodies

5.4.1. Detection of polymorphic *Pf*HRP-2

Antibodies against purified r*Pf*HRP-2 were raised and isolated from chicken eggs. The recombinant protein induced high IgY antibody titers in eggs. The IgY detected dimeric and trimeric forms of r*Pf*HRP-2 in western blotting and these forms were not detected by Coomassie staining. The human antibodies against native *Pf*HRP-2 also detected recombinant *Pf*HRP-2 with western blotting. The detection of multiple *Pf*HRP-2 forms and the presence of IgY antibodies against different epitopes of the whole protein, suggest that IgY could detect polymorphic *Pf*HRP-2 from patient samples in the field. Variations in the epitopes

of *Pf*HRP-2 in different *P. falciparum* isolates are prevalent (Nderu *et al*., 2019; Fontecha *et al*., 2019). The frequency of conserved epitopes within *Pf*HRP-2 changes across different *P. falciparum* isolates and this affects malaria RDTs using monoclonal antibodies (Baker *et al*., 2005; Lee *et al*., 2006). The IgY antibody pool has a higher probability of detecting proteins with some conserved regions compared to monoclonal antibodies against one epitope. IgY used in this study could potentially detect *Pf*HRP-2 across different geographical isolates, without being affected by antigen variation and this could reduce false negative results (Lee *et al*., 2006). RDTs using antibodies with high avidity, like polyclonal IgY could be more effective in diagnosing *P. falciparum* malaria across different countries and this could improve the detection sensitivity of RDTs. Polyclonal antibodies have the potential to detect multiple epitopes and increase the signal. Monoclonal antibodies, on the other hand, might decrease the sensitivity, if the epitope targeted is present in low frequency in some of the isolates.

The epitopes detected by the IgY antibodies of this study were not identified. Identifying the epitopes could have allowed the comparison of IgY with other antibodies against *Pf*HRP-2.

5.4.2. Heat stability of anti-r*Pf*HRP-2 antibodies

The detection of malaria in remote areas of the tropics where microscopy is not available relies on RDTs. Transportation and storage of RDTs in these areas often does not involve refrigeration and indoor temperatures can exceed 45°C. Heat labile antibodies used in RDTs do not reliably diagnose malaria as they fail to detect Plasmodial proteins (Chiodini *et al*., 2007). To assess the stability of anti-r*Pf*HPR-2 antibodies at high temperatures the IgY antibodies were stored at 23 – 45°C for up to sixteen weeks. The antibodies were stable and able to detect r*Pf*HRP-2, but the efficacy was gradually reduced compared to the 4°C control.

5.4.2.1. Surveillance of *Pf*HRP2 gene deletion and the detection of *Pf*LDH

The increase occurrence of *Pf*HRP-2 gene deletion in *Plasmodium falciparum* isolates, has spread to previously unaffected regions. This is of concern for malaria control (Kobayashi *et al*., 2019). Isolates lacking *Pf*HRP-2/3 are not detected by *Pf*HRP-2 based RDTs and the patients would not be treated with the appropriated anti-malarial medication. To counteract the effect of *Pf*HRP-2 gene deletion, RDTs containig antibodies against *Pf*HRP-2 and antibodies against *Pf*LDH on a single RDT strip have been developed, but the antibodies in the tests are unreliable at detecting *Pf*LDH in regions of *pfhrp*-2 deletion. This is associated with the anti-*Pf*LDH antibodies losing efficacy after RDT exposure to humid and warm conditions. IgY antibodies as suggested in this study are relatively stable in warm-humid conditions and could be raised against *Pf*LDH to assist the detection of malaria in regions where *Pf*HRP-2 is not produced by the isolates. A negative test line for *Pf*HRP-2 and a positive test line for *Pf*LDH would indicate *Pf*HRP-2 gene deletion in these regions. *Pf*LDH is an

existing diagnostic marker that may replace *Pf*HRP-2 in diagnosing *P. falciparum* malaria (Alareqi *et al*., 2016).

5.4.3. Reduction of RDT false positive results

The use of IgY antibodies in RDTs could reduce false positive RDT results caused by mammalian antibodies reacting with human rheumatoid factor (Iqbal *et al*., 2000). This is because the Fc region of IgY is different to that of IgG and is not recognised by rheumatoid factor receptors (Larsson *et al*., 1991). A study by Larsson and colleagues investigated the effect of rheumatoid factor on sandwich ELISA tests using two mammalian IgG antibodies to diagnose other infections, showed that rabbit and human IgG antibodies reacted with rheumatoid factor (Larsson *et al*., 1991). This study suggested that the effect of false positive results can be avoided by using chicken IgY antibodies (Larsson *et al*., 1991). false positive results waste anti-malarial medicines.

5.5. Intermolecular interaction within the r*Pf*HRP-2 protein

*Pf*HRP-2 forms intermolecular bonding leading to an increase in the size of the protein on SDS-PAGE gels. These interactions have been identified as disulfide bonds formed between cysteine residues of two or more *Pf*HRP-2 monomers (Schneider and Marletta, 2005). The *Pf*HRP-2 disulfide bonding is pronounced under native gel conditions in the presence of an oxidising agent such as heme (Fe^{3+}) and can be detected with Coomassie staining (Schneider and Marletta, 2005). In this study, the increase in the size of r*Pf*HRP-2 was detected with antibodies in western blotting in the absence of heme.

r*Pf*HRP-2 high molecular weight proteins are indicative of dimeric and trimeric forms of r*Pf*HRP-2. These forms were observed under reducing conditions with western blotting. It is possible that a fraction of the protein was not fully reduced and able to interact with other *Pf*HRP-2 monomers. This could potentially occur because the dimerisation of *Pf*HRP-2 is said to be assisted by heme which brings cysteine residues close together for disulfide bond formation (Schneider and Marletta, 2005). This could enable the formation of dimers under reducing conditions, which can be investigated.

5.5.1. Trypsin digestion of r*Pf*HRP-2

*Pf*HRP-2 is highly polymorphic and consists of 24 repeated epitopes that vary in frequency and number based on isolates (Baker *et al*., 2005). Most commercially available RDTs and monoclonal antibodies detect type-2 and type-7 repeats. Thus, to compare the epitopes detected by the IgY antibodies in this study, the recombinant *Pf*HRP-2 protein was cleaved with trypsin and analysed on reducing and non-reducing SDS-PAGE gels and detected with IgY. The epitopes detected were not determined because it was difficult to identify the fragments formed.

The number of fragments formed was lower than predicted and higher in molecular weight. Some of the fragments were larger in size than the native *Pf*HRP-2 protein. It is possible that disulfide bonding occurs within the hydrolysed r*Pf*HRP-2 fragments increasing their size as observed on SDS-PAGE. It is also possible that the fragments behave in a similar manner as the *Pf*HRP-2 protein and resolve larger than their predicted molecular weight (Table 4.1). The smallest r*Pf*HRP-2 peptide after trypsin hydrolysis predicted at 11 kDa, thought to run at 30 kDa on SDS-PAGE gels was not detected by the anti-r*Pf*HRP-2 IgY antibodies and did not stain with ponceau-S on nitrocellulose. It is possible that this protein does not bind nitrocellulose under the blotting conditions of this study and hence the antibodies could not detect the protein. The reason for the protein's lack of interaction with nitrocellulose still needs to be resolved.

More work is required to understand the r*Pf*HRP-2 intrachain bonds which may involve mutating the protein's cysteine residues and characterising the mutants. This would give more insight into the behavior of r*Pf*HRP-2 monomers and the involvement of disulfide bonding. Mass spectral analysis of the r*Pf*HRP-2 fragments after trypsin hydrolysis would help to identify the trypsin fragments which could be important in understanding their behavior in the SDS-PAGE gels in this study.

5.6. Bidirectional transfer of r*Pf*HRP-2 during western blotting

The r*Pf*HRP-2 protein and the fragments formed after trypsin hydrolysis transferred to both the negative and positive electrodes during western blotting. The reason for this was not clear and requires further investigation.

5.7. Conclusion

The study optimised r*Pf*HRP-2 expression, eliminating the need for large culture volumes. The chicken IgY antibodies isolated here, could replace temperature labile, rheumatoid factor reactive mammalian monoclonal antibodies in *Pf*HRP-2 based RDTs for malaria diagnosis. The increase in size of r*Pf*HRP-2 forming dimers and trimers on SDS-PAGE gels and the behavior of trypsin hydrolysed r*Pf*HRP-2 fragments may be explained by disulfide bonding between r*Pf*HRP-2 monomers. More studies are required to better understand the nature of the disulfide bonding in *Pf*HRP-2. These findings contribute to the understanding of the use of IgY to detect *Pf*HRP-2 and the structure of *Pf*HRP-2.

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