

**Recombinant Expression, Purification, Analysis and Immunization of Mice with
Plasmodium yoelii Glyceraldehyde-3-Phosphate Dehydrogenase (rPyGAPDH) and
Lactate Dehydrogenase (rPyLDH) and Evaluation of Protection against *P. berghei*
Challenge**

by

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Abstract

The principal focus in malaria research is the development of an effective malaria vaccine which will help to prevent death and eliminate the disease. This study investigated two plasmodial glycolytic enzymes as possible malaria vaccine candidates. *Plasmodium yoelii* glyceraldehyde-3-phosphate (rPyGAPDH) and lactate dehydrogenase (rPyLDH) recombinantly as His-tagged fusion proteins were affinity purified using a cobalt affinity matrix. Ethanol has been reported to enhance expression of recombinant proteins in *E. coli* bacteria. Concentrations of 1%, 2% and 3% (v/v) ethanol were tested for enhancement of rPyLDH and rPyGAPDH expression in both lysogenic broth and terrific broth. Ethanol reduced the overall expression of proteins by inhibiting the growth of PyLDH and PyGAPDH *E. coli* host cells. The recombinant proteins were evaluated using a metal ion gel shift assay. Both rPyLDH and rPyGAPDH did not show a size shift on the gel following incubation with Cu^{2+} , Co^{2+} or Ni^{2+} . Both the reduced and non-reduced forms of rPyLDH and rPyGAPDH had similar migration on a 10%, 12.5% and 15% SDS-PAGE gels. Recombinant PyLDH and rPyGAPDH were prepared in Freund's adjuvants and mice were immunized to evaluate immunogenicity. Both rPyGAPDH and rPyLDH were immunogenic in mice and produced high antibody titers. Mice were immunized with rPyLDH or rPyGAPDH and challenged with *P. berghei* infection. All PyLDH immunized mice developed high parasitemia and 1/5 of the PyGAPDH immunized mice maintained low parasitemia below 5% throughout the study. Further work includes repeating the immunization and challenge experiments.

PREFACE

The research contained in this thesis was completed in the Discipline of Biochemistry, School of Biochemistry, Genetics and Microbiology of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa, from January 2017 to February 2019, under the supervision of Professor J.P.D Goldring.

The contents of this work have not been submitted in any form to another university and except where the work of others was acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Prof. J.P.D. Goldring Date

Date: February 2019

Sinothile Sementa Khuzwayo

DECLARATION: PLAGIARISM

I, Sinothile Sementha Khuzwayo, declare that:

- i. The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work.
- ii. This dissertation has not been submitted in full or in part for any degree or examination at any other university.
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Signed: Sinothile Sementha Khuzwayo

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ABBREVIATIONS AND SYMBOLS

ACT - artemisinin combination therapy

AMA1 - apical membrane antigen 1

ATP - adenosine triphosphate

CHMI - controlled human malaria infection

BSA - bovine serum albumin

CSP - circumsporozoite protein

DTT – dithiothreitol

ECL - enhanced chemiluminescence

ELISA - enzyme-linked immunosorbent assay

FVO - *P. falciparum* Vietnam-Oak Knoll strain

GAPDH - glyceraldehyde-3-phosphate-dehydrogenase

GLURP – glutamate rich protein

HRPO - horseradish peroxidase

HRP-2 - histidine rich protein 2

IgG - immunoglobulin G

IgY - egg yolk immunoglobulin

IPTG - isopropyl thioglucoopyranoside

ITNs - insecticide-treated bednets

IRS - indoor residual spraying

kD - kilo Dalton

LAMP - Loop-mediated isothermal amplification

LDH - lactate dehydrogenase

MSP – merozoite surface protein

mRNA - messenger ribonucleic acid

NAD - nicotinamide adenine dinucleotide (NAD⁺ oxidised form; NADH reduced form)

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PEG - polyethylene glycol

Pf - *Plasmodium falciparum*

Py – *Plasmodium yoelii*

Pb – *Plasmodium berghei*

Pc – *Plasmodium chabaudi*

RDT - rapid diagnostic test

RH5 - reticulocyte binding-like homology protein 5

SDS - sodium dodecyl sulphate

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SPZ - sporozoite

TEMED - N,N,N',N'-tetramethylethylenediamine

TM - trade mark

UV - ultra violet

WHO - world health organization

Chapter 1

Literature review

1.1 Malaria background

Malaria is a parasitic disease that is responsible for killing approximately 438 000 people annually (World Health Organization, 2016). Most of the deaths are reported in poor African countries, approximately 90% in tropical regions of Africa, while the remainder occurred in South East Asia and South America (World Health Organization, 2016). Malaria is caused by the protozoan parasite that belongs to the genus *Plasmodium*. The survival of *Plasmodium* parasites requires two hosts, the vertebrate host (including humans) and the mosquito which transmits the parasites. Out of hundreds of *Plasmodium* parasites, only five of these infect humans. These are *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. These parasites only infect humans apart from *P. knowlesi* whose host is the long-tailed and pig-tailed macaques (Luchavez *et al.*, 2008; White, 2008). *P. falciparum* causes most of the disease in sub-Saharan Africa, while *P. vivax* is mostly dominant in Asia and South America (World Health Organization, 2016). *P. falciparum* has a short erythrocyte cycle that leads to the production of a high number of merozoites and a high parasitemia associated with the disease (Bozdech *et al.*, 2003; van Dooren *et al.*, 2005).

During the feeding of an infected *Anopheles* mosquito on the human blood, the infectious sporozoites are injected into the skin (Cowman *et al.*, 2016). The sporozoites then pass through the bloodstreams to the liver where they infect liver cells. Sporozoites mature into merozoites in hepatocytes, merozoites are liberated in the bloodstreams to invade red blood cells in order to begin their asexual cycle (Cowman *et al.*, 2016). The asexual cycle of malaria inside the human erythrocytes causes the clinical symptoms of the disease. Depending on the species that causes the infection and the immunity of the infected individual, the disease symptoms typically appear between 8-14 days after infection (Cowman *et al.*, 2016; World Health Organization, 2016). Malaria manifests as fever symptoms, headache, chills, sweats, fatigue, nausea and vomiting (Cowman *et al.*, 2016; World Health Organization, 2016). *Plasmodium falciparum* is the most lethal parasite, which progresses to cerebral malaria, which can be deadly.

Animal models, commonly rodents are used when conducting clinical and laboratory studies of malaria. The reason for this is because there are ethical restrictions to use humans for many studies. Rodents are commonly used as they are cheap and easy to work with. They

also make it possible to investigate the disease progression which is a challenge in humans, and studying of the parasites sequester in organs e.g. brain, liver and spleen is possible in rodents (Wykes and Good, 2009). There are four plasmodia species that infect rodents, *Plasmodium berghei*, *P. chabaudi*, *P. vinkei* and *P. yoelii* (lethal and non-lethal strain). *Plasmodium berghei* and *P. yoelii* infects both mature red blood cells and reticulocytes, this allows for investigation of vaccine candidates (Wykes and Good, 2009). *Plasmodium vinkei* and *P. chabaudi* only infect mature red blood cells, these parasites are used when studying the immune responses of malaria and drug susceptibility (Wykes and Good, 2009).

1.1 Malaria life cycle

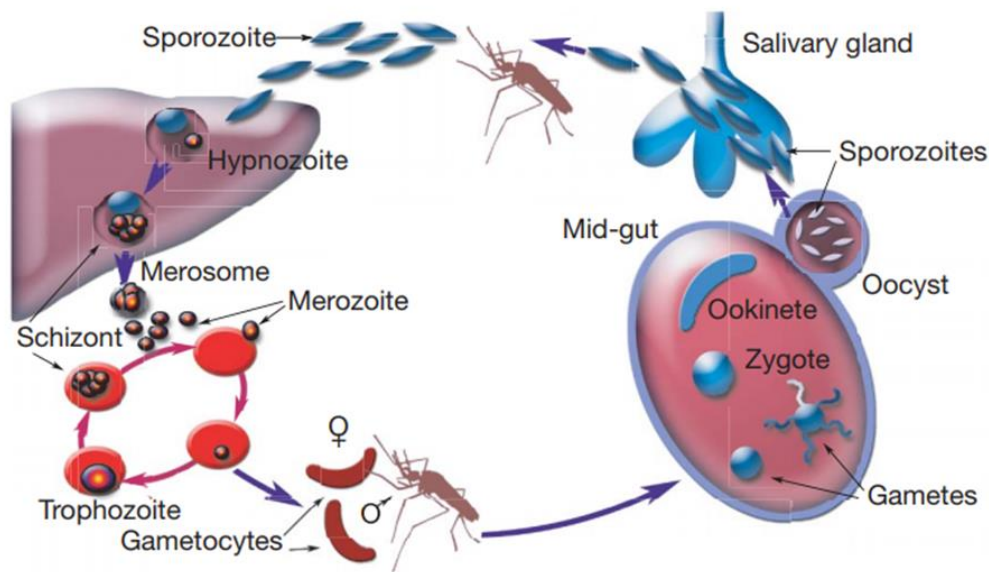


Figure 1.1: *Plasmodium* life cycle between the mosquito vector and a human host. The malaria life cycle begins with the mosquito vector which deposits the sporozoites into a human host. The sporozoites invade the hepatocytes where they develop and invade the erythrocytes to begin their asexual cycle. The asexual stage generates schizonts which infect more erythrocytes. Male and female gametocytes also develop from schizonts and are taken in by the mosquito when it feeds on a human host. Taken from Winzeler (2008).

1.1.1 Development of sporozoites inside the mosquito vector of *Plasmodium* species

Sporozoites develop from oocysts inside the mosquito midgut (Kappe *et al.*, 2003). Once the oocyst matures, it ruptures liberating sporozoites into the hemolymph. The sporozoites then travel to the mosquito salivary glands until a mosquito feeds on the vertebrate host (Kappe *et al.*, 2003).

1.1.2 Invasion of hepatocytes by plasmodial sporozoite

Human malaria infection begins when an infected *Anopheles* female mosquito bites a human host. The mosquito deposits the sporozoites in the skin. These sporozoites move to the liver and invade hepatocytes (Kappe *et al.*, 2003). During hepatocytes invasion, the sporozoites surface receptors which include the thrombospondin domains of the circumsporozoite protein (CSP) and the thrombospondin-related adhesion proteins (TRAP) attaches to the heparan sulfate proteoglycan on the surface of the liver cells (Kappe *et al.*, 2003). Immediately after the sporozoite receptors have successfully attached to the hepatocyte surface, sporozoites enter the hepatocytes and multiply into thousands of merozoites. Merozoites are then released to invade erythrocytes and initiate the asexual cycle (Figure 1.1).

1.1.3 Erythrocytes invasion by plasmodial merozoites

Erythrocytes invasion is initiated by an increase in pressure in the parasite, which causes the parasitophorous vacuole to break open to release the merozoites (Cowman & Crabb, 2006). The erythrocytes membranes are then disrupted by the Serine repeat antigens (SERA) which belong to the family of serine proteases (Cowman & Crabb, 2006). Four events are involved in erythrocytes invasion. Firstly, the merozoites bind to the erythrocyte surface; this binding is through the glycosylphosphatidylinositol (GPI) anchored membrane proteins found on the merozoite surface. Nine GPI anchored proteins which include Merozoite surface protein 1 (MSP1), MSP2, MSP4, MSP5, MSP10, *Pf12*, *Pf38*, *Pf92* and *Pf113* have been identified in *Plasmodium falciparum* (Cowman & Crabb, 2006). Following binding, the merozoite reorientates its apical end on the erythrocyte surface. Apical membrane antigen 1 (AMA1) facilitates the reorientation (Mitchell *et al.*, 2004). Invasion is sparked by the signalling pathways which implicate both calcium and potassium ions levels (Singh *et al.*, 2010). When merozoites are released in the blood which has low potassium ion concentration, the cytosolic calcium levels of the blood are elevated, and this triggers the movement of microneme proteins to the merozoite surface. These microneme proteins include AMA1 and erythrocyte binding antigen 175 (EBA 175) and their role involves stimulating the invasion process by interacting with erythrocyte receptors. EBA 175 attaches to glycophorin A on the red blood cell surface creating a junction for invasion (Singh *et al.*, 2010). Inside the erythrocytes, the parasites go through structural changes between the ring stage and the trophozoite stage. During the trophozoite stage, the parasites digest the erythrocyte cytoplasm and destroy haemoglobin followed by multiple nuclear divisions to produce schizonts that rupture erythrocytes and infect new erythrocytes (Figure 1.1) (Cowman & Crabb, 2006).

1.1.4 Plasmodial gametocyte development

During the development of parasites inside the erythrocytes, some parasites develop into sexual stages in a process called gametocytogenesis (Duffy & Avery, 2013). During this process, two sexual forms of parasites, the male and female gametocytes are produced. Gametocytogenesis is divided into five developmental stages (I-V) with distinct morphologies. In stage V of development, the gametocytes have fully developed, and male and female gametocytes can be easily differentiated (Baker, 2010). *Plasmodium falciparum* gametocytogenesis takes about 8-12 days (Sinden, 2009). During the feeding of an *Anopheles* mosquito on the blood of an infected host, matured gametocytes (male and female) are ingested and fertilization occurs in the mosquito gut forming an ookinete (Baker, 2010). In the mosquito gut, the ookinete matures forming an oocyst enclosing sporozoites which consequently move to the salivary glands of the mosquito where they fully develop. The sporozoites are subsequently deposited onto the skin of the next person the mosquito feeds on.

1.2 Transmission of *Plasmodium* parasites



Figure 1.2: Malaria transmission in South Africa. Pink areas are places of high malaria transmission and grey areas are places of low transmission. Taken from (Centers for Disease Control and Prevention, 2013).

There are hundreds of mosquito species that exist and about 30 species are malaria vectors (World Health Organisation, 2016). The disease is prevalent in tropical areas of the world including sub-Saharan Africa where the disease transmission is more robust than other parts of the world (Kiszewski *et al.*, 2004). Malaria transmission is reported to be contributed by the mosquito density, human activities, human and vector genetics and as well as climate conditions (Craig, 1999).

1.3.1 Influence of climate on malaria

Climate, particularly warm temperatures and rainfall provide favourable breeding conditions for mosquitoes. This results in an increased density of mosquitoes that transmit the disease. High density of complementary mosquito vectors is problematic as these vectors maintain malaria transmission throughout the year. Kiszewski *et al* (2004) reported *Anopheles gambiae* to breed during the wet season, while *Anopheles funestus* breeds during the dry season maintaining malaria transmission. Different Mosquito species live in different habitats; some prefer puddles e.g. *Anopheles gambiae*, while some prefer permanent water e.g. *Anopheles funestus*. These conditions are abundant during the rainy season in tropical countries and that is why 90% of malaria infections are reported in sub-Saharan Africa (Craig, 1999).

1.3.2 Human immunity to malaria

Human genetics is one of the crucial influences of malaria transmission. In malaria endemic areas where transmission occurs almost throughout the year, people who live in these areas acquire partial immunity that prevents the development of severe malaria. Immunity to malaria is acquired after multiple infections and it has been observed to increase with age leaving the younger children vulnerable to the disease (World Health Organization, 2016). Children under the age of five years, first time pregnant women and visitors to malaria-endemic areas are susceptible to malaria (Bloland & Organization, 2001). People of all ages are at risk in non-endemic areas e.g. Asia and Latin America as they do not have immunity to malaria since they are not continuously exposed to the disease (World Health Organization, 2016). In South Africa, malaria is seasonal and occurs during the rainy season, which is between September and May (World Health Organization, 2016). Areas at risk of malaria in South Africa include North-eastern Kwazulu-Natal, Limpopo and Mpumalanga, especially areas that are close to Zimbabwe, Mozambique and Swaziland borders (Figure 1.2). Almost all South African residents are non-immune and therefore are at high risk of getting severe malaria, which is deadly if it is left untreated (World Health Organization, 2016).

1.3.3 Human activities influencing malaria

Urbanization and intercontinental travelling are the major contributors to the transmission of malaria (Kiszewski *et al.*, 2004). Developing countries, particularly in sub-Saharan Africa, are being continuously urbanized. Urbanization results in high malaria transmission due to improper housing, hygiene and absence of proper plumbing. Usage of water tanks in developing countries increases the contact of people with mosquitoes and that increase malaria transmission (Kiszewski *et al.*, 2004). *Anopheles stephensi* has been reported in water tanks in India (Kiszewski *et al.*, 2004). Intercontinental travelling occasionally leads to the transportation of infected mosquitoes to non-endemic countries, thereby increasing malaria transmission (World Health Organization, 2016).

1.4 Malaria control

1.4.1 Mosquito vector control

The key approach of preventing malaria transmission is by minimising the interactions between infected mosquitoes and the people (World Health Organisation, 2016). The use of Dichlorodiphenyltrichloroethane (DDT) to spray homes in order to kill mosquitoes was implemented in the early 1960s. This practice had a huge influence on the complete elimination of malaria in most parts of the continent including Southern Europe, Russia and parts of Asia (Guerin *et al.*, 2002).

Mosquitoes usually bite during the night, therefore avoiding being out at night and using insecticide-treated bednets (ITNs) has been the best strategy to reduce human and mosquito contact in malaria-endemic areas. ITNs and indoor residual spraying (IRS) were introduced as early as the 1940s during World War II. Pyrethroids including permethrin and lambda-cyhalothrin are chemicals commonly used in ITNs and IRS that have shown high insecticidal activity and are not harmful to humans (World Health Organisation, 2016). Bhatt *et al* (2015) reported a decrease in *P. falciparum* infection in endemic African countries of 68% during 2000-2015 due to ITNs. Alonso *et al* (1991) showed that employing permethrin-treated bed nets reduced malaria infection and deaths of children by 30% during the malaria transmission season in Gambia. ITNs had been reported as a cost-effective approach for preventing malaria in a clinical trial in Gambia (Alonso *et al.*, 1991).

1.4.2 Mosquito vector resistance to insecticides

Though, malaria control had been difficult in tropical countries due to the lack of access to health services, poor health infrastructure and the occurrence of insecticide resistance

(Guerin *et al.*, 2002). Pyrethroid and DDT resistance by *Anopheles gambiae* has been reported in East and West African countries (N'Guessan *et al.*, 2007; Ranson & Lissenden, 2016). This makes it a challenge to control malaria as ITNs and IRS are the main methods of prevention. In South Africa, an invasion by *Anopheles funentus*, which was resistant to pyrethroid, was reported in 1999 (Barnes *et al.*, 2005). This resulted in a rapid increase in malaria transmission. ITNs have also been reported to be less effective in non-endemic areas and regions where mosquito vectors bite people in the late afternoon and morning (Kroeger *et al.*, 1999). Kroeger *et al.* (1999) reported a low protective efficacy of ITNs in Latin America where *Anopheles albimanus* the vector for *P. vivax* was observed to bite people during the late afternoon.

1.5 Diagnosis of malaria

Early and correct detection of malaria is important in order to administer the correct treatment to prevent deaths due to the disease (Amir *et al.*, 2018). Commonly used technique for diagnosis includes microscopy which is the gold standard, Polymerase Chain Reaction (PCR), Loop-mediated isothermal amplification (LAMP) and Rapid Diagnostic Tests (Amir *et al.*, 2018).

1.5.1 Microscopic diagnosis of malaria

Microscopic detection is based on the identification of malaria parasites inside red blood cells (Warhurst & William, 1996). Blood samples are obtained from the patient's finger by pricking it with a needle, and stained with Wright's, Field's or Giemsa stain (Warhurst & William, 1996). A thin or thick blood film is spread over a microscopic slide; the parasites are stained and counted under 100 microscope fields (Warhurst & William, 1996; Amir *et al.*, 2018). Giemsa is the most commonly used stain for routine malaria diagnosis owing to its stability at different temperature (Warhurst & William, 1996).

For microscopic examination of parasites, thick and thin films are used to estimate the parasite burden (Amir *et al.*, 2018). Thick blood films have an enhanced sensitivity, which allows for the detection of 50 parasites/ μ l of blood and it is 20-30 times denser than the thin film (Payne, 1988). A thin film is a preferred method as parasites can be identified by their morphology which cannot be seen using a thick film preparation (Warhurst & William, 1996). Morphological identification of parasites enables a microscopist to accurately distinguish different *Plasmodium* species, as this is important for the administration of correct treatment (Warhurst & William, 1996). The asexual stages of the parasites are unique and can be identified under the microscope. Unlike other *Plasmodium* species, both *P. falciparum* and *P. malariae* have a uniform trophozoite cytoplasm and non-enlarged infected red blood cells.

These two malaria parasites are distinguished from each other by the presence of hemozoin pigment, which is a characteristic of *P. falciparum* (Noland *et al.*, 2003). The trophozoite cytoplasm of *P. vivax* and *P. ovale* appear irregular, while the infected red blood cells are enlarged and distorted. *P. ovale* has a unique trophozoite cytoplasm that is partially fragmented, while *P. vivax* is distinguished by its oval-shaped infected red blood cells. Microscopy disadvantages include the misdiagnosis of *P. knowlesi* parasites for *P. malariae* as they have similar morphologies (Antinori *et al.*, 2013). The technique is also expensive as it costs a lot of money to maintain microscopes and employ trained personnel. Consequently, it cannot be used routinely in remote areas of sub-Saharan Africa.

1.5.2 Polymerase chain reactions (PCR) to detect plasmodia parasites

The polymerase chain reaction (PCR) is used to amplify specific DNA to make multiple copies. This technique is employed when diagnosing *Plasmodium* malaria (Snounou, 1996). The circumsporozoite (CS) gene and small subunit 18S ribosomal RNA are commonly used as the target DNA for amplification when diagnosing malaria (Snounou, 1996). Following the amplification of genes in the infected blood, PCR results are evaluated by agarose gel electrophoresis stained with ethidium bromide (Tirasophon *et al.*, 1991).

PCR has been used to detect *P. vivax* infections by amplifying the VK210 and VK247 CS variants in a study in Thailand (Kain *et al.*, 1993). PCR has been reported to be useful when monitoring the response of treatment in *P. falciparum*-infected patients (Sethabutr *et al.*, 1992). Nested PCR (nPCR) has been observed to detect microscopically-negative samples and mixed infections of *P. vivax* and *P. falciparum* that was diagnosed as mono-infections by microscopy (Morassin *et al.*, 2002). PCR is the most sensitive malaria diagnostic method, detecting < 5 parasites/ μ l while microscopy detects 50 parasites/ μ l of blood (Moody, 2002). Tirasophon *et al.* (1991) reported PCR to detect 1 parasite/ μ l of blood. However, PCR cannot be used for routine malaria diagnosis as it is expensive, and the technique requires trained personnel (Amir *et al.*, 2018).

1.5.3 Loop-mediated isothermal amplification (LAMP) to detect plasmodia parasites

The Loop-mediated isothermal amplification (LAMP) technique detects malaria infections through the detection of 18S RNA gene (Poon *et al.*, 2006). The LAMP technique has been described to detect all human *Plasmodium* species with high sensitivity (Han *et al.*, 2007). A sensitivity of 95% and specificity of 99% has been described with LAMP assays. In contrast to PCR, LAMP does not require DNA amplification steps, hence does not require expensive DNA amplification reagents and it is more robust compared to PCR (Poon *et al.*, 2006). LAMP is

more sensitive than microscopy and up to 6 parasites/ μ l of blood can be detected (Poon *et al.*, 2006). LAMP assays are visualized with a naked eye by observing a magnesium pyrophosphate precipitate that forms as a by-product of the amplification. The technique does not require trained personnel or sophisticated equipment as the results are easy to interpret and can be used routinely in rural endemic areas (Amir *et al.*, 2018).

1.5.4 Rapid diagnostic tests to detect plasmodia parasites

Rapid diagnostic tests (RDTs) is a technique that is based on the interaction of the parasite antigen from the infected patient's blood and a monoclonal antibody against the protein of interest in the mobile phase. The monoclonal antibody in the mobile phase is conjugated to gold particles. A second monoclonal antibody on the nitrocellulose strip functions to catch the antigen-antibody complex in the mobile phase and the colour develops as a visible line (Figure 1.3). The test has a control line to show that the test is working (Murray *et al.*, 2008). Migration of the mobile phase on the nitrocellulose paper moves by capillary action which depends on the absorbency of the membrane. RDTs produce results in 10-15 minutes, unlike microscopy which takes almost a day to obtain accurate results (Murray *et al.*, 2008; Amir *et al.*, 2018). An efficient RDT detects 100 parasites/ μ l of blood samples. RDTs are easy to perform therefore they can be used by inexperienced individuals. The common malaria RDT's target Histidine-rich protein 2 (HRP-2) which is only found in *P. falciparum* and *P. lactate dehydrogenase* (pLDH) and aldolase which is expressed by all *Plasmodium* parasites (Amir *et al.*, 2018).

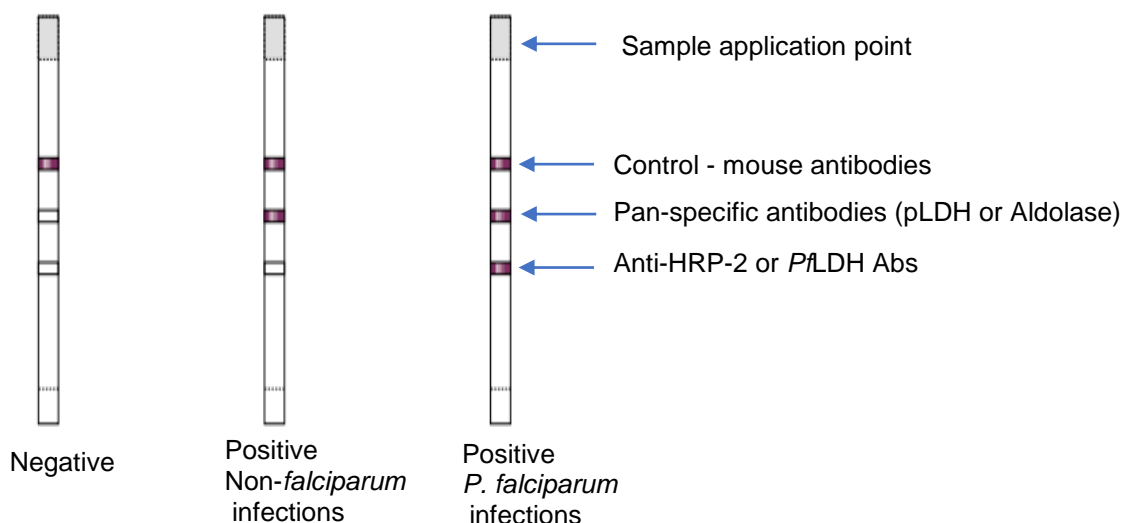


Figure 1.3: A representation of a malaria dipstick RDT. Adapted from World Health Organisation, 2000.

1.5.4.1 Histidine-rich protein 2 based RDTs to detect plasmodia parasites

Blood stage *Plasmodium falciparum* parasites produce Histidine-rich protein 2 (HRP-2); this protein is abundant in the blood during schizont rupture (Rock *et al.*, 1987). HRP-2 is only synthesized by *P. falciparum malaria* parasites and the levels of HRP-2 have been found to correlate with parasitemia (Rubach *et al.*, 2012). HRP-2 was the first plasmodial antigen to be used in RDTs and was targeted because of its abundance in *P. falciparum* infections (Iqbal *et al.*, 2004). Commercially available *P. falciparum* malaria RDTs targeting HRP-2 include Parasight-F test (Benton Dickinson), Paracheck® Pf (Orchard biomedical systems) and Diagnostic malaria Pf (ICT Diagnostics). The tests have been assessed in many clinical trials and the sensitivity and specificity range between 86.7% to 93.4% and 98.2% to 99.3% respectively (Ajumobi *et al.*, 2015; Azazy *et al.*, 2018; Shiff *et al.*, 1993). The HRP-2 based RDT may give false positives as the HRP-2 protein remains in the blood of the host even after the parasites have been cleared with anti-malarial drugs (Mayxay *et al.*, 2001). *P. falciparum* infections lacking HRP-2 protein have been identified in many African countries (Berhane *et al.*, 2018; Kozycki *et al.*, 2017), Brazil and Bolivia (Viana *et al.*, 2017) and in Indian isolates (Kumar *et al.*, 2013). The deletion of *Pfhrp-2* gene in patients results in a false negative diagnosis of *P. falciparum* infections with HRP-2 based malaria RDTs.

1.5.4.2 Lactate dehydrogenase based RDTs to detect plasmodia parasites

Plasmodial lactate dehydrogenase (pLDH) is an enzyme of the glycolytic pathway and it is found in high levels during the blood stages of plasmodial parasites (Vander Jagt *et al.*, 1990). The mRNA levels of the glycolytic enzymes including pLDH have been observed to increase during the blood stages of *Plasmodium* parasites (Le Roch *et al.*, 2003). The enzyme is present in all human malaria species and has a unique catalytic site compared to the human enzyme. Plasmodial LDH can use 3-acetylpyridine nucleotide (ADAP) substrate, which is the analogue of NAD (Makler & Hinrichs, 1993). This enables the detection of plasmodial LDH. The pLDH activity can be measured from plasma, serum and hemolysates of infected patients and the activity correlate with the levels of parasitaemia (Markwalter *et al.*, 2018). Unlike HRP-2, which remains in the blood of the host after parasite clearance, pLDH clears immediately following the parasite clearance (Oduola *et al.*, 1997; Markwalter *et al.*, 2018). Therefore, pLDH is a good diagnostic marker and can be used to accurately measure the effectiveness of antimalarial drugs. Piper *et al* (1999) developed monoclonal antibodies for an immunochromatographic test that detect pLDH (OptiMAL dipstick test). The OptiMAL dipstick tests uses a combination of pan-specific pLDH antibody that recognises the common epitope of

all four *Plasmodium* species and *P. falciparum* LDH specific antibody (Piper *et al.*, 1999). This RDT can diagnose and distinguish between the infections caused by *P. falciparum* and non-*falciparum* infections. The OptiMAL-IT test sensitivity has been reported to be low for *P. falciparum* infections compared to the HRP-2 RDT. A sensitivity of 83.6% - 95.3% for *P. falciparum* infections and 91.0% - 96% for *P. vivax* infections were reported for the OptiMAL test (Swarthout *et al.*, 2007). The pLDH test (HRP-2/pLDH combination) offers increased sensitivity of 94% for *P. falciparum* infections (Hawkes *et al.*, 2014). Singh *et al.* (2010) reported the First Response® Malaria Combo (pLDH/HRP-2) card test had a 94.7% sensitivity for *P. falciparum* and 84.2% sensitivity for non-*falciparum* infections. Reduced sensitivities of RDTs have been reported for HRP-2 /pLDH in pregnant women in Ghana (Kyabayinze *et al.*, 2016).

1.5.4.3 Aldolase based RDTs to detect plasmodia parasites

Plasmodial aldolase a glycolytic enzyme has been targeted for malaria diagnosis in RDTs (Cloonan *et al.*, 2001). Aldolase specific antibodies are pan antibodies that detect all *Plasmodium* species and in combination with HRP-2 monoclonal antibodies can differentiate between *P. falciparum* and non-*P. falciparum* infections (Murray *et al.*, 2008). An ICT *P.f/P. v* test has been assessed to be 95.5% sensitive and 89.8% specific for detection of *P. falciparum* infections and 94.8% sensitive and 98.2% specific for detecting *P. vivax* (Tjitra *et al.*, 1999). The sensitivity of detection of *P. vivax* and *P. falciparum* infection by the test correlates with the parasitemia as the overall sensitivity is 96% at parasites >500/μl and is reduced to 29% in parasites <500/μl (Tjitra *et al.*, 1999). The disadvantages of ICT *P.f/P. v* includes the inability to differentiate between non-*falciparum* when used in regions where all parasites prevail. The test has been reported to fail when detecting *P. malariae* infections in East Timor, Indonesia and Irian Jaya patients. BinaxNOW and HRP-2/Aldolase RDT have been reported to have 95.3% for *P. falciparum* and 68.9% for *P. vivax* malaria infections for >5,000 parasites/μl. The levels of *Plasmodium aldolase* decrease with infection during treatment (Tjitra *et al.*, 2001). Therefore, aldolase can be used to monitor parasite clearance. Although, the sensitivity of aldolase based RDTs is low compared to pLDH RDTs.

1.6 Treatment of malaria

Treatment of malaria should be started immediately after diagnosis to reduce sickness and death due to the disease. When treating malaria, it is important to know the *Plasmodium* species that caused the infection, severity of infection and host factors including pregnancy status and age (World Health Organization, 2015). The type of *Plasmodium* species is crucial as some species are resistant to certain antimalarial drugs and *P. falciparum* infections should be

treated instantly as they can rapidly progress to severe malaria. Chloroquine was initially used as the first-line antimalarial treatment (Ridley, 2002). Chloroquine-resistant *P. falciparum* parasites were identified in South America, Columbia and Africa (Young & Moore, 1961). Chloroquine resistance is associated with the *Pfcr*t gene mutation which results in an altered PfCRT protein with reduced chloroquine binding abilities (Sidhu *et al.*, 2002). The resistance of *P. falciparum* malaria to chloroquine, mefloquine, quinine and halofantrine have also been reported (Reed *et al.*, 2000). This resistance is associated with the polymorphism on *Pfmdr1* gene which encodes P-glycoprotein homologue 1 (Pgh1) protein of *P. falciparum*. Chloroquine *P. vivax* infections were also reported (Baird, 2004; Dua & Sharma, 1996; Schuurkamp *et al.*, 1992). Resistance of *P. falciparum* and *P. vivax* parasites against pyrimethamine-sulphadoxine, a commonly used replacement of chloroquine has been observed (Rønn *et al.*, 1996)

Consequently, artemisinin-based combination therapies (ACTs) is recommended for malaria treatment to reduce the development of resistant parasites (Koenderink *et al.*, 2010; World Health Organization, 2015). Artemisinin and its derivatives artesunate, artemether and artemotil decrease numbers of asexual parasites by 10000-fold in each life cycle, unlike antimalarial drugs which decrease asexual parasites by 100-fold to 1000-fold (World Health Organization, 2015). Artemether-lumefantrine, the main ACT has been an effective treatment for *P. falciparum* infections and prevention of re-infection (Mårtensson *et al.*, 2005). *P. falciparum* infections are treated with artemether-lumefantrine plus primaquine or quinine with doxycycline or clindamycin for pregnant women and infants (World Health Organization, 2015). Chloroquine alone is used for treating *P. malariae* and *P. knowlesi*, while it is used in combination with primaquine in *P. vivax* and *P. ovale* infections (World Health Organization, 2015). Mixed malaria infections of *P. vivax* and *P. falciparum* are treated with ACT and primaquine for *P. falciparum* and *P. vivax* infections respectively.

Primaquine is the only known antimalarial drug that acts against hypnozoites, high doses of primaquine are recommended to be taken for 14 days to prevent *P. vivax* malaria relapses (World Health Organization, 2015). Although, primaquine induces erythrocyte haemolysis which results in anaemia in individuals lacking glucose-6-phosphate dehydrogenase. Primaquine is not recommended for pregnant women (World Health Organization, 2015). The drug was also reported to clear gametocytes in cases of uncomplicated and acute *P. falciparum* infections in Indian patients (White, 2013).

1.7 Malaria vaccines

1.7.1 Pre-erythrocyte stage vaccines

1.7.1.1 Whole irradiated *Plasmodium falciparum* sporozoite vaccine

During malaria infections in the human host, the infectious *Plasmodium* sporozoites develop and invade liver cells. The liver stage of *Plasmodium* parasite is called the pre-erythrocyte stage and does not have clinical symptoms. Pre-erythrocyte stage vaccines could potentially prevent the development of erythrocytic stages of the disease thereby eliminating the parasites before they cause symptoms. The idea of developing a malaria vaccine that targets the pre-erythrocyte stage has been around for many decades. Nussenzweig *et al* (1967) observed that immunization of mice with X-ray irradiated *P. berghei* sporozoites offered protection against challenge infection with non-irradiated sporozoites from mosquito bites. The *P. knowlesi* irradiated sporozoites also protected monkeys from *P. knowlesi* sporozoite challenge (Gwadz *et al.*, 1979). Antibodies against the whole sporozoite and a circumsporozoite protein (CSP) the opulent antigen on the surface of sporozoites were induced by the vaccine (Egan *et al.*, 1993). Both anti-CSP antibodies and sporozoite specific CD8+ T cells inhibited the development of infections from sporozoite challenge thus affording protection. Protection was observed in human volunteers following immunization with irradiated *P. falciparum* sporozoites (Clyde *et al.*, 1973). This protective immunity persisted for 42 weeks (Hoffman *et al.*, 2002). The previously protected individuals sustained the immunity even from repeat exposure to infectious sporozoites.

1.7.1.2 Whole irradiated sporozoite chemoprophylaxis vaccine

Immunization of mice that were on chloroquine treatment with irradiated *P. berghei* sporozoites protected the mice from challenge infection (Beaudoin *et al.*, 1977). Chloroquine inhibited the blood stages of *P. berghei* parasites, which are reported to suppress the pre-erythrocyte stage immunity (Orjih *et al.*, 1985). Additionally, Orjih *et al* (1985) reported high antibody titers against sporozoites in immunized mice that are under chloroquine treatment compared to immunized mice that are not given chloroquine. Human volunteers were also vaccinated with non-irradiated *P. falciparum* sporozoites, while taking chloroquine during the immunization course (Roestenberg *et al.*, 2009). All the individuals (100%) under chloroquine treatment were fully protected and protection correlated with memory T cells, IFN γ , TNF α and IL-2 (Roestenberg *et al.*, 2009).

1.7.1.3 Sanaria® PfSPZ vaccine

Sanaria has produced a *Plasmodium falciparum* sporozoite (PfSPZ) vaccine also known as Sanaria® PfSPZ (Hoffman *et al.*, 2015). The PfSPZ vaccine was produced from irradiated, purified, aseptic, cryopreserved sporozoites (Epstein *et al.*, 2011). The PfSPZ vaccine-induced CD8⁺ T cells and gamma interferon (IFN- γ) response in monkeys and rodents (Epstein *et al.*, 2011). The PfSPZ vaccine was tested in humans, 6 volunteers were immunized four times with 1.35×10^5 PfSPZ and were all protected against controlled human malaria infection (CHMI) (Seder *et al.*, 2013). The PfSPZ vaccine has been assessed in phase 1 clinical trials and demonstrated safety and immunogenicity (Roestenberg *et al.*, 2013; Shekalaghe *et al.*, 2014). The PfSPZ vaccine-induced protective antibodies against sporozoites, Pf-specific T cells that lasted for 59 weeks (Ishizuka *et al.*, 2016). Lyke *et al.* (2017) observed the vaccine offer protection against heterologous strains after a repeat exposure to CHMI *P. falciparum* sporozoites 33 weeks post-immunization. The 48% vaccine efficacy was observed following the immunization with five doses of 2.7×10^5 PfSPZ in Malian healthy adults (Sissoko *et al.*, 2017).

1.7.1.4 RTS, S/AS01 malaria vaccine

Circumsporozoite protein (CSP) is found in the sporozoite surface of *Plasmodium* parasites and it is highly conserved (Zhao *et al.*, 2016). It is a membrane protein that is inserted in the surface of sporozoites by a GPI anchor in its C-terminus (Coppi *et al.*, 2011). Functions of CSP include the attachment of sporozoite to liver cells (Pinzon-Ortiz *et al.*, 2001) and the invasion of hepatocytes (Tewari *et al.*, 2002). Both functions aid in the establishment of the pre-erythrocyte stage infection. The RTS, S/AS01 is the first subunit vaccine that targets the pre-erythrocyte stage of *P. falciparum* infections (Stoute *et al.*, 1997). The vaccine is made up of PfCSP antigen combined with a hepatitis B surface antigen and is prepared with a liposome derived adjuvant AS01 (Stoute *et al.*, 1997). The RTS, S/AS01 vaccine has shown protection in malaria naïve adults and semi-immune adults (Stoute *et al.*, 1997), young children (5-17 months) and infants (6-12 weeks) (Gosling & von Seidlein, 2016; RTS, 2015). Phase 3 clinical trials of the vaccine showed the overall efficacy of 45.1% in young children and 31.3% efficacy in infants (RTS, 2015). Booster doses of the vaccine have been observed to increase the efficacy, although, the immunity provided by the vaccine lasts for up to 3-4 years (RTS, 2015). The vaccine has recently been approved by the European Medicines Agency for its use for vaccinating young children in endemic regions in Africa. The vaccine was not approved for vaccinating infants due to its low efficacy (Gosling & von Seidlein, 2016).

1.7.2 Blood stage malaria vaccines

Morbidity and mortality caused by human *Plasmodium* infections is the consequence of the erythrocytic stages of *Plasmodium* parasites (Good & Miller, 2018). A malaria vaccine that is directed against the erythrocyte stage could potentially reduce the parasite burden, thus prevent death. *Plasmodium* antigens expressed on the parasite surface during blood stages in the human host are the focus for vaccine development (Good *et al.*, 1998). This is because these antigens appear to be the target for the immune response against the disease (Good *et al.*, 1998). The leading blood-stage malaria vaccine candidates for *P. falciparum* includes an apical membrane antigen-1 (*Pf*AMA-1) (Thera *et al.*, 2011), merozoite surface protein-1 (*Pf*MSP1-42) (Ellis *et al.*, 2010), *Pf*MSP3 (Lusingu *et al.*, 2009), GMZ2 vaccine based on *Pf*MSP3 and glutamate-rich protein (*Pf*GLURP) (Esen *et al.*, 2009), and reticulocyte-binding protein homolog 5 (*Pf*RH5) (Jin *et al.*, 2018).

1.7.2.1 *Plasmodium falciparum* apical membrane antigen-1 (*Pf*AMA1)

Plasmodium falciparum apical membrane antigen-1 (AMA-1) is a type 1 integral transmembrane protein. *Pf*AMA1 is located on the merozoites surface during the blood stages of *Plasmodium* parasites and expressed by the infectious *Plasmodium* sporozoites that invade hepatocytes (Mitchell *et al.*, 2004; Polhemus *et al.*, 2007). The 83 kDa protein is synthesized and localises in the micronemes of developing merozoites (Nair *et al.*, 2002). During the erythrocyte invasion, *Pf*AMA1 is degraded by proteases into a 66 kDa polypeptide and released to the merozoite surface (Nair *et al.*, 2002). *Pf*AMA1 interacts with *P. falciparum* rhoptry neck protein (*Pf*RON2) and the complex initiates the formation of a junction for invasion (Srinivasan *et al.*, 2011). Antibodies against AMA1 alone and AMA1-RON2 complex abolish erythrocyte invasion (Srinivasan *et al.*, 2011). A relationship between anti-*Pf*AMA1 antibodies and protection against *P. falciparum* infections has been reported (Gray *et al.*, 2007; Osier *et al.*, 2007). *Pf*AMA1 protein is a target of acquired immunity and anti-*Pf*AMA1 antibody titres correlate with age (Kusi *et al.*, 2012). *Pf*AMA1 has been targeted for the development of an erythrocyte stage malaria vaccine. Vaccination of monkeys with *Pf*AMA1 prepared in Freund's adjuvant (Stowers *et al.*, 2002) or Montanide ISA 720 adjuvant (Dutta *et al.*, 2009) protected monkeys from challenge infection. Anti-*Pf*AMA-1 antibody in the vaccinated monkeys correlated with protection. Immunization of mice with *Pb*AMA1 or passive immunization with antibodies protected mice from challenge infection with *P. berghei* parasites (Crewther *et al.*, 1996).

The *Pf*AMA1 vaccine has been tested in multiple clinical trials since it showed protection in rodents and monkeys. The FMP2.1/AS02 vaccine is made from *Pf*AMA-1 from the 3D7 strain

in AS02 adjuvant. The vaccine was harmless and induced an immune response in phase 1 clinical trial in US adults (Polhemus *et al.*, 2007) and in Malian children (Thera *et al.*, 2010). The vaccine induced anti-*Pf*AMA1 antibodies, IFN- γ and IL-5. A 64.3% vaccine efficacy against a 3D7 *P. falciparum* infections and 17.4% efficacy against infections with FVO *P. falciparum* parasites was observed in Malian children (Thera *et al.*, 2011). The strain-specific efficacy of the vaccine was not sustainable as there was no efficacy after 24 months even though the anti-AMA1 antibody levels were still high (Payne *et al.*, 2016). The strain-specific immunity induced by FMP2.1/AS02 was due to the excessive genetic variation in the *Pf*AMA1 amino acid sequence (Duan *et al.*, 2008). Therefore, a multivalent vaccine could offer better protection. To overcome the strain specificity of AMA1 vaccine-induced antibodies, new AMA1 vaccines contain peptides that are highly conserved between *P. falciparum* strains. Diversity-Covering antigens (DiCo) AMA1 vaccine is composed of three AMA1 variants (Remarque *et al.*, 2008). The *Pf*AMA1 Dico-mix vaccine was shown to induce high antibody titres that inhibit the growth of many *P. falciparum* strains (Remarque *et al.*, 2008). The Dico mix vaccines formulated with CoVaccine HT™ or Montanide ISA 51 have shown to be safe and immunogenic in monkeys (Kusi *et al.*, 2011) and in European and African naïve adults when formulated with Alhydrogel or GLA-SE (Sirima *et al.*, 2017).

1.7.2.2 *Plasmodium falciparum* merozoite surface protein-1 (*Pf*MSP1)

Merozoite surface protein-1 (MSP1) is a 195 kDa glycoprotein that is found in all *Plasmodium* species (Holder *et al.*, 1992). MSP1 is the most abundant proteins amongst the nine GPI anchored membrane proteins found on the merozoite surface (Holder, 2009). MSP1 is produced as a 195 kDa protein in the schizont stage of the asexual parasites inside the infected erythrocytes and is expressed by merozoites (Holder *et al.*, 1992). The protein is cleaved into 83, 28-30, 38-45 and 42 kDa fragments during the late stages of schizonts before the merozoites are released from the infected erythrocytes (Holder & Freeman, 1984; Holder *et al.*, 1992). After processing, MSP1 fragments are expressed on the surface of merozoites where they interact with sialic acid on erythrocytes surface and facilitate the erythrocyte invasion by merozoites (Holder & Freeman, 1984). The 42 kDa (MSP1-42) fragment is further processed into 33 kDa (MSP1-33) and 19 kDa (MSP1-19) fragments before the invasion is completed (Pichyangkul *et al.*, 2004). The MSP1-33 resides on the merozoite surface whereas the MSP1-19 fragment is transported into the newly infected erythrocyte (Blackman *et al.*, 1990). The antibodies against the MSP1 and its fragments MSP1-19 and MSP1-42 have been reported in the serum of malaria-infected patients and antibodies against MSP1 and its fragments have

been observed in immune individuals in endemic areas (Daly & Long, 1993). Anti-*Pf*MSP1-42, demonstrated to be inhibitory against the *P. falciparum* parasites in *Aotus* monkeys (Chang *et al.*, 1996). Antibodies against *Pf*MSP1-19 have been demonstrated to inhibit erythrocyte invasion by *P. falciparum* parasites (Blackman *et al.*, 1990). The *Py*MSP1-19 vaccine prepared in Freund's adjuvant completely protected mice from lethal *P. yoelii* infections (Hirunpetcharat *et al.*, 1997; Ling *et al.*, 1994). Baculoviral expressed *Pf*MSP1-42 (Chang *et al.*, 1996) and *E. coli* expressed *Pf*MSP1-19 vaccines (Kumar *et al.*, 1995) protected monkeys against *P. falciparum* challenge. The vaccine induced antibodies blocked the invasion of merozoites into new red blood cells (Kumar *et al.*, 1995). These phenomena have led researchers to investigate *Pf*MSP1 as a human malaria blood-stage vaccine candidate.

Many *Pf*MSP1 vaccine designs have been investigated in phase 1 safety clinical trials. These vaccines include FMP1/AS02 vaccine based on 3D7 *Pf*MSP1-42 (Stoute *et al.*, 2007) and MSP1-42C1/Alhydrogel which is made from both FVO and 3D7 *Pf*MSP1-42 (Ellis *et al.*, 2010) have shown to be safe and immunogenic in Kenyan and United states healthy adults respectively. A strong T cell (CD4+ and CD8+) response and anti-*Pf*MSP1 IgG antibodies were induced by a *Pf*MSP1 vaccine formulated with chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) (Sheehy *et al.*, 2011). A multi-antigen vaccine JAIVAC-1 composed of *Pf*MSP1-19, C-terminus region of MSP1 and EBA-175 domain also demonstrated to be nontoxic and induced an immune response in phase 1 clinical trial (Chitnis *et al.*, 2015).

1.7.2.3 *Plasmodium falciparum* merozoite surface protein 3 (*Pf*MSP3)

Plasmodium falciparum merozoite protein 3 (*Pf*MSP3) is a soluble GPI-anchored transmembrane protein expressed on the merozoite surface (Tsai *et al.*, 2009). MSP3 was first discovered by McColl *et al.* (1994) in *P. falciparum* isolates in Papua New Guinea when it was observed as a membrane protein on the merozoite surface. MSP3 is produced as a 43 kDa protein in the parasitophorous vacuole of schizonts. It is then released to the merozoite surface during schizont rupture and it remains on the merozoite surface during the invasion of erythrocytes. The function of MSP3 is currently unknown. Anti-*Pf*MSP3 antibodies induced by natural *P. falciparum* infections in humans and vaccine induced mice antibodies have been found to promote monocyte dependent killing of parasites in an *in vitro* study (Oeuvray *et al.*, 1994). The protein has been reported to be a target for protective immunity against *P. falciparum* natural infections and the induced antibodies against MSP3 were independent of age unlike other malaria antigens (Roussilhon *et al.*, 2007). The *Pf*MSP3 vaccine produced from a full-length *Pf*MSP3 sequence has been reported to protect monkeys against *P. falciparum*

infections and protection was associated with anti-*PfMSP3* antibodies (Hisaeda *et al.*, 2002). Passive transfer of immune sera with anti-MSP3 antibodies reduced parasitemia in *P. falciparum* infected mice (Druilhe *et al.*, 2005).

PfMSP3 vaccine formulated in Montanide ISA 720 / aluminium hydroxide has been assessed in phase 1 clinical trial in adults (Druilhe *et al.*, 2005) and in children 12-24 months in Tanzania (Lusingu *et al.*, 2009). The vaccine was safe and induced both cellular and humoral immunity. The vaccine induced antibodies lasted for up to 1 year (Druilhe *et al.*, 2005). Strain-specificity has been observed in anti-MSP3 antibodies (Polley *et al.*, 2007). The vaccine has not yet been tested in phase 2 trial.

1.7.2.4 GMZ2 malaria vaccine

The GMZ2 malaria vaccine is composed of *PfMSP3* and glutamate-rich protein (GLURP). GLURP is a 220 kDa protein found in both the liver stage and blood stages of the malaria parasites (Theisen *et al.*, 1998). GLURP associates with matured schizonts on the infected erythrocytes. The function of GLURP is not yet known. Antibodies against *PfGLURP* have been associated with protection against *P. falciparum* infections (Amoah *et al.*, 2017; Theisen *et al.*, 2004). Anti-*PfGLURP* isolated from immune adults living in high malaria transmission regions have been shown to inhibit parasite growth in a monocyte-dependent manner like *PfMSP3* (Theisen *et al.*, 1998). A malaria vaccine targeting GLURP could potentially provide multi-stage specific immunity as the protein is produced in two life stages of the parasites (Theisen *et al.*, 1994). GLURP and MSP3 expressed together produced a vaccine that protected monkeys from *P. falciparum* infections and the protection was associated with high antibody titers against both proteins (Carvalho *et al.*, 2004). A human vaccine (GMZ2) has been produced from *PfMSP3* and *PfGLURP* and prepared in alhydrogel®. The vaccine has been tested in phase 1a clinical trials in German adults (Esen *et al.*, 2009) and in young children in Gabon (Belard *et al.*, 2011) and has shown to be safe and immunogenic. The GMZ2 vaccine induces high levels of antibodies that inhibit the *P. falciparum* parasite growth *in vitro* (Jepsen *et al.*, 2013). Memory B cells are also induced by the vaccine and have been reported to last for up to 12 months (Esen *et al.*, 2009). A GMZ2 vaccine-induced antibodies that reduced the incidence of both uncomplicated and severe malaria in a phase 2b clinical trial in African children, a 14% vaccine efficacy was reported (Sirima *et al.*, 2017).

1.7.2.5 *Plasmodium falciparum* reticulocyte-binding protein homolog 5 (*PfRH5*)

P. falciparum reticulocyte-binding protein homolog 5 (*PfRH5*) is expressed on the merozoite surface during erythrocyte invasion (Rodriguez *et al.*, 2008). *PfRH5* is the 6th member

of the *PfRH* family which consist of *PfRH1*, *PfRH2a*, *PfRH2b*, *PfRH3*, *PfRH4* and *PfRH5* (Rodriguez *et al.*, 2008). *PfRH5* is the smallest of them all and has a molecular weight of 62,5 kDa while the average *RfRH* proteins are 250-300 kDa (Baum *et al.*, 2009). *PfRH5* lacks the C-terminal transmembrane domain found in other *PfRH* proteins (Baum *et al.*, 2009). Therefore, *PfRH5* attaches to a *PfRh5*-interacting protein (*PfRipr*) and cysteine-rich protective antigen (CyRPA) to form a complex. This complex is critical for the formation of a crossing point for the merozoites to invade the erythrocytes (Reddy *et al.*, 2015). *PfRH5* interacts with Basigin on the erythrocytes surface (Rodriguez *et al.*, 2008). *PfRH5* is essential for the survival of the parasites and gene knockout studies have failed to produce *P. falciparum* parasites without the protein (Hayton *et al.*, 2008).

PfRH5 is not a target for acquired immunity in malaria-exposed individuals in endemic regions (Douglas *et al.*, 2011). Unlike other malaria vaccine antigens, *PfRH5* has limited polymorphism and anti-*PfRH5* vaccine-induced antibodies are not strain-specific (Douglas *et al.*, 2011). Vaccine-induced anti-*PfRH5* antibodies protected monkeys from both homologous and heterologous *P. falciparum* challenge (Douglas *et al.*, 2014; Douglas *et al.*, 2015). Passive transfer of anti-*PfRH5* antibodies protected humanized mice from developing blood stage infections when infected with a controlled human malaria infection (Foquet *et al.*, 2018). The RH5.1/AS01 vaccine based on *PfRH5* 3D7 is currently being tested in a phase 1/2a clinical trial for its safety, immunogenicity and efficacy in malaria naïve adults.

1.8 Glycolysis in *Plasmodium* parasites

Plasmodium falciparum, the deadliest malaria species has 48 hours to complete its asexual cycle inside human erythrocytes. This rapid development of *P. falciparum* requires an effective synthesis of adenosine triphosphate (ATP) to support their development (Roth *et al.*, 1988). It has been reported that *Plasmodium* parasites lack some metabolic pathways found in higher eukaryotes including an active oxidative phosphorylation pathway which release energy used to produce ATP. *Plasmodium* parasites have been reported to exclusively depend on glycolysis to produce ATP. Roth *et al* (1988) demonstrated the increased levels of glycolytic enzymes inside the malaria-infected erythrocytes compared to uninfected erythrocytes. Glyceraldehyde-3-phosphate and lactate dehydrogenase are amongst these highly elevated glycolytic enzymes (Roth *et al.*, 1988). The latter has brought a lot of attention to exploring the possibility of targeting glycolytic enzymes as therapeutic interventions for malaria.

1.8.1 *Plasmodium falciparum* glyceraldehyde-3-phosphate dehydrogenase (PfGAPDH)

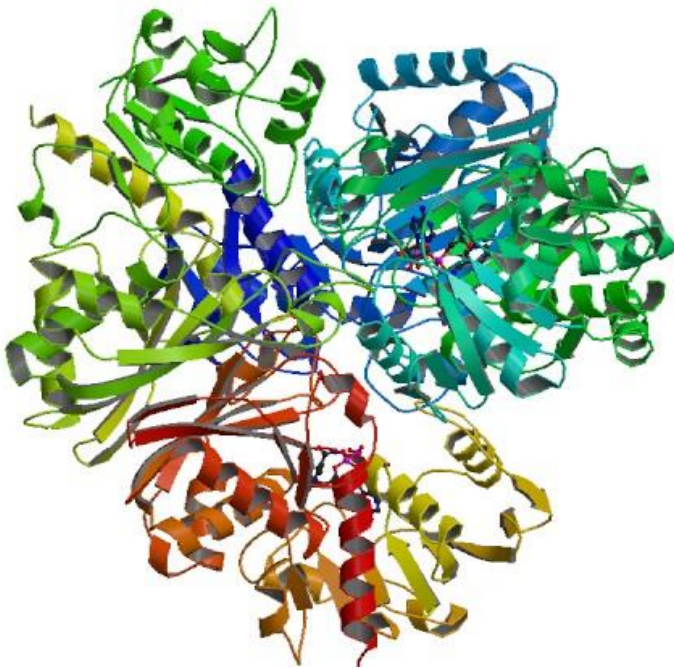


Figure 1.4: Ribbon model for GAPDH 3-dimensional structure. Adapted from Cowan-Jacob *et al.*, 2003. The subunits are represented by different colours (red, green, blue and yellow).

Glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.12) is a 150 kDa homo-tetramer of 36 kDa subunits. The enzyme is found in all living organisms and it catalyzes the 6th step of glycolysis. In this reaction, GAPDH catalyzes the phosphorylation of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate. The reaction involves a transfer of an electron from NAD⁺ to NADH (Daubenberger *et al.*, 2000). The active site of GAPDH contains a cysteine residue (Cys 149) where its sulfhydryl group is essential for the catalytic activity (Daubenberger *et al.*, 2000). The GAPDH 3-dimensional structure has a Rossmann fold conformation which is made up of twisted parallel β -sheets which are surrounded by α -helices (Figure 1.4). The catalytic site of the enzyme is made up of eight stranded α -helices which are combined with parallel β -sheets.

1.8.1.1 Comparison of *Plasmodium* GAPDH with human GAPDH

Glyceraldehyde-3-phosphate dehydrogenase from *Plasmodium falciparum* has been sequenced and the crystal structure has been determined (Satchell *et al.*, 2005). Unique amino acid residues have been observed in the amino acid sequence of *Plasmodium* GAPDH (Satchell *et al.*, 2005) that are not found in the human GAPDH (hGAPDH) sequence. *Plasmodium* GAPDH sequence has an insert of two amino acid residues lysine-194 and glycine-195 within the active site (Figure 1.5) (Satchell *et al.*, 2005). These inserted amino acid

residues could be targeted when developing antimalarial agents. Two unique *Pf*GAPDH epitopes have been identified and the antibodies against these epitopes differentiated *Pf*GAPDH from the GAPDH of other *Plasmodium* species (Krause *et al.*, 2017). The anti-pGAPDH antibodies could be potentially used for the development of a GAPDH based RDT that differentiates between *P. falciparum* infections and non- *P. falciparum* infections. The amino acid sequence alignment revealed 63 - 68% similarities between *Plasmodium* GAPDH and human GAPDH sequences. Therefore, antibodies made against *Plasmodium* GAPDH specific regions are unlikely to cross-react with hGAPDH.

1.8.1.2 *Plasmodium* GAPDH is a malaria vaccine candidate

*Pf*GAPDH has been reported to interact with merozoite Duffy binding-like and reticulocyte homology ligands and the interaction is important for the erythrocyte invasion by merozoite (Pal-Bhowmick *et al.*, 2012). The enzyme has also been identified on the surface of *P. falciparum* and *P. berghei* sporozoites where it interacts with CD68 on the Kupffer cell surface allowing the sporozoites to invade the Kupffer cells (Cha *et al.*, 2016). Antibodies against *Pf*GAPDH inhibits the sporozoite invasion and protects mice from *P. berghei* challenge (Cha *et al.*, 2016). Together, these findings support *Plasmodium* GAPDH as a potential target for the liver stage and blood-stage malaria vaccine candidate.

Trypanosoma	MGDDSMPICVGINGFGPIGRAVLFSFF-TDPLVNVTAINDASTSVVDYIAYVVQRESPLST	59
<i>Escherichia</i>	-----MSKVGINGFGRIGRLVLRLLLEVKSNDVVAIND-LTSPKILAYLLKHDSNYGP	53
<i>Leishmania</i>	-----MVKVGINGFGRIGRVVFRAAQ-MRPDIEIVGIND-LLDAEYMAYSKYDSTHGR	52
<i>Homo sapien</i>	----MGKVKVGVNGFGRIGRLVTRAAF-NSGKVDIVAINDPFIDLNYMVYMFQYDSTHGK	55
Rabbit	-----VKVGVNGFGRIGRLVTRAAF-NSGKVDVVAINDPFIDLHYMVYMFQYDSTHGK	52
<i>P. falciparum</i>	----MAVTKLGINFGRIGRLVFRAAF-GRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQ	55
<i>P. berghei</i>	----MAITKVGINGFGRIGRLVFRSAQ-ERSDIEVVAINDPFMDISHLIYLLKHDSVHGK	55
<i>P. yoelii</i>	----MAITKVGINGFGRIGRLVFRSAQ-ERSDIEVVAINDPFMDINHLIYLLKHDSVHGK	55
	*:**** * * * : : : . . * * * . : * . : * .	
Trypanosoma	EERASVIVVGDYVCIQGTHKIRVTQKHLVEIGWREAGVRYVVECSGLSSTRERCWGHIT	119
<i>Escherichia</i>	FPWSVD--FTEDSLIVDGKSI AVYAEKEAKNIPWKAKGAEI IVECTGFYTSAEKSQAHL D	111
<i>Leishmania</i>	FDGTVE--VIK GALVVNGKSI RVT SERDPANLKWDEIGVEVVVESTGLFLTQETAHKHIE	110
<i>Homo sapien</i>	FHGTVK--AENGKLVINGNPITIFQERDPSKIKWGDAGA EYVVESTGVFTTMEKAG AHLQ	113
Rabbit	FHGTVK--AENGKLVINGKAITIFQERDPANIKWGDAGA EYVVESTGVFTTMEKAG AHLK	110
<i>P. falciparum</i>	FPCEVT--HADGFL LIG EKKVSVFAEKDPSQIPWGKQVDV VCESTGVFLTKELASSHLK	113
<i>P. berghei</i>	FPCEVT--PTEGGIMVGNKVVVYNERDPAQIPWGKH AIDV VCESTGVFLTKELSN AHIK	113
<i>P. yoelii</i>	FPCEVT--PTEGGIMVGSKKVVVYNERDPAQIPWGKH AIDV VCESTGVFLTKELSN AHIK	113
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Trypanosoma	GGASGVVIAGQS-ADAPTVI I GANETELKKAYPVICAGAPIAVALAPLIRLLHEQYGVVEE	178
<i>Escherichia</i>	AGAKKVLISAPAG-EMKTIVYKVNDTLDGNDTIVSVASCTTNC LAPMAKALHDSFGIEV	170
<i>Leishmania</i>	AGARRVMTGPPKDDTPMFVMGVNHTTYKG-QPIISNASCTTNC LAPLAKVVNEKYGIVE	169
<i>Homo sapien</i>	GGAKRVIISAPS-ADAPMFVMGVNHEKYDNSLKIISNASCTTNC LAPLAKVIHDNFGIVE	172

Rabbit	GGAKRVIISAPS-ADAPMFVMGVNHEKYDNSLKIVSNASXTTNC	LAPLAKVIHDHFGIVE	169
<i>P. falciparum</i>	GGAKKIVMSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNC	LAPLAKVINDRFGIVE	173
<i>P. berghei</i>	GGAKKIVMSAPPKDDTPIYVMGINHEKYNSSQTIVSNASCTTNC	LAPIAKVIHENFGIVE	173
<i>P. yoelii</i>	GGAKKIVMSAPPKDDTPIYVMGINHEKYNSSQTIVSNASCTTNC	LAPIAKVIHENFGIVE	173
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Trypanosoma	CSYTAIHGMRTVE--PTAGRSKNTQDWRQTRAAADAIIPYSQTGNKTLEKIFPFLAERISG		237
<i>Escherichia</i>	GTMTTIHAYTGTQSLVDGPR--GKDLRASRAAAENIIPHTTGAAKAIGLVIPELSGKLG		228
<i>Leishmania</i>	GLMTTVHATTATQKTVDGPS--LKDWRGGRGASQNIIPSSTGAPKAVGKVYPALDGKLTG		227
<i>Homo sapien</i>	GLMTTVHAIATATQKTVDGPS--GKLWRDGRGALQNIIPASTGAAKAVGKVIPELNGKLTG		230
Rabbit	GLMTTVHAIATATQKTVDGPS--GKLWRDGRGAAQNIIPASTGAAKAVGKVIPELNGKLTG		227
<i>P. falciparum</i>	GLMTTVHASTANQ	LVVDGPSKGGKDWAGRGRSALSNIPASTGAAKAVGKVIPELNGKLTG	233
<i>P. berghei</i>	GLMTTVHASTANQ	LVVDGPSKGGKDWAGRGRSALLNIIPASTGAAKAVGKVIPELNGKLTG	233
<i>P. yoelii</i>	GLMTTVHASTANQ	LVVDGPSKGGKDWAGRGRSALLNIIPASTGAAKAVGKVIPELNGKLTG	233
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Trypanosoma	SAFQVPVTKGCAVDMLVRLAQPVSKELLDGTIKEAAGGQLREVLMYSGADLISRDCVPNG		297
<i>Escherichia</i>	HAQRVPVKTGSVTELVSILGKKVTAEEVNNALKQATTN--NESFGYTDEEIVSSDIIGSH		286
<i>Leishmania</i>	MAFRVPTPNVSVVDLTVRLEKPKATYKIDICAAIKAAAEGEMKILGYTDDEVVSSDFNGVA		287
<i>Homo sapien</i>	MAFRVPTANVSVVDLTCRLEKPAKYDDIKKVVVKQASEGPLKILGYTEHQVSSDFNSDT		290
Rabbit	MAFRVPTPNVSVVDLTCRLEKAAKYDDIKKVVVKQASEGPLKILGYTEDQVSSDFNSDT		287
<i>P. falciparum</i>	VAFRVPVIGTVSVVDLVCRLEKPAKYEEVALEIKKAAEGLLKGVLGYTEDEVVVSQDFVHDN		293
<i>P. berghei</i>	VAFRVPVIGTVSVVDLVCRLEKPAKYEDVAKKISQDFVHDSRSSIFYTEEEVVSQDFVHDS		293
<i>P. yoelii</i>	VAFRVPVIGTVSVVDLVCRLEKPAKYEDVAKKIKEASEGPLKILGYTEDEVVVSQDFVHDS		293
	* : ** : * : . . . : : . : * : : : * *		
Trypanosoma	KL-CYDTTS--SQCLRDEGVHKLKLLWFNFDGSYANRLLSLVPFLQKMNAAEEM		347
<i>Escherichia</i>	FGSVFDATQTEITAVGDLQLVKTVAWYDNEYGFVTQLIRTLKFAKL-----		333
<i>Leishmania</i>	LTSVFDVKAGIS---LNDHFVKLVSWYDNETGYSHKVLDLILHTSAR-----		331
<i>Homo sapien</i>	HSSTFDAGAGIA---LNDHFVKLISWYDNEFGYSNRVVDLMAHMASKE-----		335
Rabbit	HSSTFDAGAGIA---LNDHFVKLISWYDNEFGYSNRVVDLMVHMASKE-----		332
<i>P. falciparum</i>	RSSIFDMKAGLA---LNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN-----		337
<i>P. berghei</i>	RSSIFDLKAGLA---LNDNFFKIVSWYDNEWGYSNRLLDLAIHITKN-----		337
<i>P. yoelii</i>	RSSIFDLKAGLA---LNDNFFKIVSWYDNEWGYSNRLLDLAIHITKH-----		337
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Figure 1.5: Amino acid alignment of GAPDH from different species. The active site cysteine (Cys-149) (green), pGAPDH two amino acid insert –KG- (purple), S loop residues (188-203) for NAD⁺ binding (box). The alignment was generated by clustal omega on 17th of November 2018.

1.8.2 *Plasmodium* lactate dehydrogenase (pLDH)

Plasmodium lactate dehydrogenase (EC 1.1.1.27) is a 135 kDa tetramer of four identical 34 kDa subunits (Figure 1.6) (Vander Jagt *et al.*, 1981). The enzyme is involved in the final step of glycolysis (Berwal *et al.*, 2008). Glycolysis is crucial for *Plasmodium* parasites to generate energy to support its rapid development inside the host erythrocytes (Roth *et al.*, 1998). The reaction catalyzed by pLDH involves the conversion of pyruvate to lactate and the reduction of nicotinamide adenine dinucleotide (NADH) to NAD⁺. NAD⁺ generated by this reaction is used to generate more ATP (Brown *et al.*, 2004).

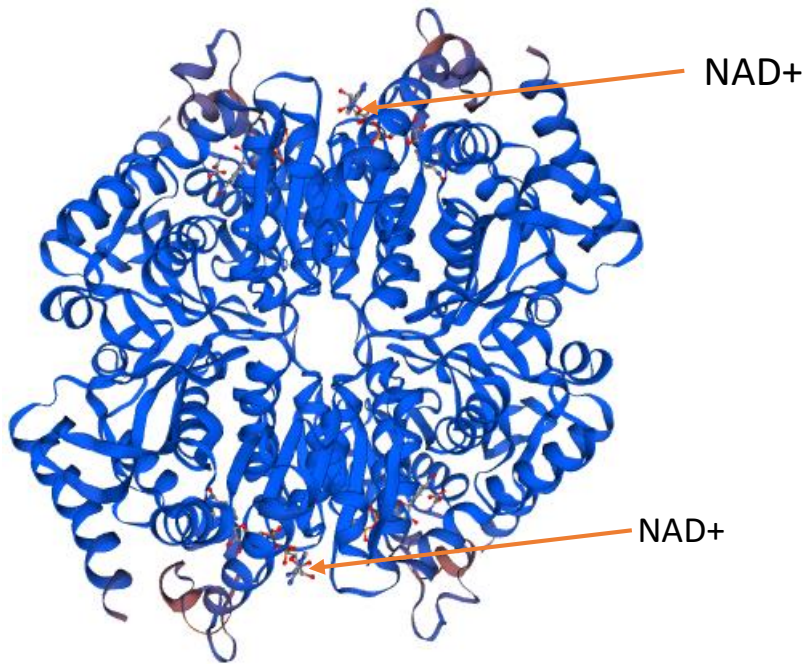


Figure 1.6: Ribbon model for *Pf*LDH 3-dimensional structure. *Pf*LDH homotetramer showing four NAD⁺ bound to the active site of the enzyme. Created using SWISS-MODEL. <https://swissmodel.expasy.org/>

1.8.2.1 Comparison of pLDH with human LDH

Lactate dehydrogenase from all human *Plasmodium* species exhibits 90-92% sequence identity to *P. falciparum* (Brown *et al.*, 2004). *Plasmodium* LDH possesses 33 out of 316 (10%) unique amino acids residues compared to the LDH from other organisms (Bzik *et al.*, 1993). The amino acid sequence of pLDH has a unique five amino acid insert in the active site and the NADH binding pocket of the enzyme (Figure 1.7) (Bzik *et al.*, 1993). The nicotine group in the NADH cofactor binding site of *Pf*LDH is slightly loosened compared to human LDH (Bzik *et al.*, 1993; Read *et al.*, 1998). These changes on pLDH have been reported to sustain the parasite in anaerobic conditions inside the erythrocyte and prevent pLDH from being inhibited by its substrate pyruvate unlike human LDH (Brown *et al.*, 2004).

Human LDH	MATIKSELIKNF AEEEEAIH HNKISIVGTG SVGVACAI SILLKGLSDELVLVDVDEGKLGK	60
<i>P. falciparum</i>	-----MAPKAKIVLVGSGMIGGV MATLIVQKNLGD-VVLFDIVKNMPHG	43
<i>P. knowlesi</i>	-----MAPKAKIVLVGSGMIGGV MATLIVQKNLGD-VVLFDIVKNMPHG	43
<i>P. vivax</i>	-----MAPKPKIVLVGSGMIGGV MATLIVQKNLGD-VVMFDDVVKNMPQG	43
<i>P. ovale</i>	-----MTPKPKIVLVGSGMIGGV MATLIVQKNLGD-VVMFDDVVKNMPQG	43
<i>P. reichenowi</i>	-----IVLVGSGMIGGLM ATLIVQKNLGD-VVMFDDVVKNMPQG	37
	* : ** : * : * : * . * . * : * : . * : . : *	
Human LDH	ETMDLQHGSPFMKM-PNIVSSKDYLV TANSNLV IITAGARQKKG ETR-----LDLVQRNV	114
<i>P. falciparum</i>	KALDTSHTNVMAYSNCKVSGSNTY DDLGADVVIVTAGFTKAPG KSDKEWN R DDLPLNN	103
<i>P. knowlesi</i>	KALDTSHTNVMAYSNCKVSGSNTY DDLGADVVIVTAGFTKAPG KSDKEWN R DDLPLNN	103
<i>P. vivax</i>	KALDTSHTNVMAYSNCKVTGSNSY EDLEGADVVIVTAGFTKAPG KSDKEWN R DDLPLNN	103

<i>P. ovale</i>	KALDTSHSNVMAYSNCKVTGSNSYDDLKADVVIVTAGFTKAPC KSDKE WNR DDLLPLNN	103
<i>P. reichenowi</i>	KALDTSHSNVMAYSNCKVTGSNSYDDLKADVVIVTAGFTKAPC KSDKE WNR DDLLPLNN	97
	:::* . * . : : : . * : * . : : : * : * : * : * : * : * : * : * : * : * : *	
Human LDH	SIFKLMIPNITQYSPHCKLLIVTNPVDILT ^Y VAWKLSGFPK ^N RVIGSGCNLDSARFRYFI	174
<i>P. falciparum</i>	KIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLHQHSGVPK ^N KIIGLGGVLDTSRLKYYI	163
<i>P. knowlesi</i>	KIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLHQHSGVPK ^N KIIGLGGVLDTSRLKYYI	163
<i>P. vivax</i>	KIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLFEHSGVPK ^N KIIGLGGVLDTSRLKYYL	163
<i>P. ovale</i>	KIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLFEHSGVPK ^N KIIGLGGVLDTSRLKYYI	163
<i>P. reichenowi</i>	KIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLFEHSGVPK ^N KIIGLGGVLDTSRLKYYI	157
	. * : : : * . : . * : . : : * * * * * : : . : : * * . * * : : * * * * * : : : *	
Human LDH	GQRLGIHSESCHGLILGEHGDSSVPVWSGVNIAGVPLKDLNPDIGTDKDPQWENVHKKV	234
<i>P. falciparum</i>	SQKLNVCPRDVNAHIVGAHG ^N KMVLLKRYITVGGIPLQEFIN ^N KLIS--DAELEAIFDRT	221
<i>P. knowlesi</i>	SQKLNVCPRDVNAHIVGAHG ^N KMVLLKRYITVGGIPLQEFIN ^N KLIS--DAELEAIFDRT	221
<i>P. vivax</i>	SQKLNVCPRDVNALIVGAHG ^N KMVLLKRYITVGGIPLQEFIN ^N KKIT--DEEVEAIFDRT	221
<i>P. ovale</i>	SQKLNVCPRDVNALIVGAHG ^N KMVLLKRYITVGGIPLQEFIN ^N KKIT--DEEVEGIFDRT	221
<i>P. reichenowi</i>	SQKLNVCPRDVNALIVGAHG ^N KMVLLKRYITVGGIPLQEFIN ^N KKIT--DEEVEGIFDRT	215
	. * : * : * : * * : . * : : : . : * * * * : : : : * : * : * : * : *	
Human LDH	ISSGYEMVKMKGYT ^S WGISLSVADLTESI ^L KNLRRVHPVSTLSKGLYGINEDIFLSVPCI	294
<i>P. falciparum</i>	VNTALEIVNLHASP ^Y VAPAAAIIEMAESYLKDLK ^K VLCSTLLEGQYGH-SDIFGGTPVV	280
<i>P. knowlesi</i>	VNTALEIVNLHASP ^Y VAPAAAIIEMAESYLKDLK ^K VLCSTLLEGQYGH-SDIFGGTPVV	280
<i>P. vivax</i>	VNTALEIVNLLASP ^Y VAPAAAIIEMAESYLKDIK ^K VLCSTLLEGQYGH-KNIFGGTPLV	280
<i>P. ovale</i>	VNTALEIVNLLASP ^Y VAPAAAIIEMAESYLKDIK ^K VLCSTLLEGQYGH-SNIFGGTPLV	280
<i>P. reichenowi</i>	VNTALEIVNLLASP ^Y VAPAAAIIEMAESYLKDIK ^K VLCSTLLEGQYGH-SNIFGGTPLV	274
	: . . . * : * : : . . . : : : : * * * * : : * * * * : * * * : * * * : * * * : * * * : *	
Human LDH	LGENGITDLIKV ^K LTLLEEAC ^L QKSAETLWEIQKELKL	332
<i>P. falciparum</i>	LGANGVEQVIELQLNSEEKAK ^F DEAIAETK ^R MKA-LA-	316
<i>P. knowlesi</i>	LGANGVEQVIELQLNSEEKAK ^F DEAIAETK ^R MKA-LA-	316
<i>P. vivax</i>	IGGTGVEQVIELQLTAE ^E KAK ^F DEAVAETK ^R MKA-LA-	316
<i>P. ovale</i>	IGGTGVEQVIELQLNAEEK ^T K ^F DEAVAETK ^R MKA-LI-	316
<i>P. reichenowi</i>	IGGTGVEQVIELQLNAEEK ^T K ^F DEAVAETK ^R MKA-LI-	310
	: * . * : : * : : * . * * : : : : : : : : : : * : *	

Figure 1.7: Amino acid sequence alignment of human LDH and *Plasmodium* LDH. The active site arginine-109 (red) and 5 unique pLDH amino acid insert (KSDKE) (box). The sequences were obtained from PubMed and PlasmoDB and aligned with Clustal Omega.

1.8.2.2 *Plasmodium* LDH is a drug and a diagnostic target

Plasmodium LDH has been targeted for the development of antimalarial drugs due to different amino acid residues compared to human LDH (Read *et al.*, 1999; Saxena *et al.*, 2018). Chloroquine was the first line antimalarial drug that is used all over the world (Yayon *et al.*, 1984). Chloroquine kills malaria parasites through binding to hemozoin in the malaria food vacuole preventing it from polymerisation to hemozoin (Chou & Fitch, 1992; Pulcini *et al.*, 2015; Yayon *et al.*, 1984). The accumulation of hemozoin has been reported to be toxic and resulting in the death of the parasites (Chou & Fitch, 1992; Pulcini *et al.*, 2015). Although, studies have

failed to demonstrate the existence of free hemozoin. *Pf*LDH showed that the enzyme binds to chloroquine and this binding prevents the binding of NADH, the cofactor of the enzyme (Read *et al.*, 1999). Human LDH binds with lower affinity to chloroquine, this makes chloroquine to be safe for human use. *Plasmodium* LDH level correlates with malaria infections and has been used for rapid diagnosis of malaria using immunocapture assays or in a dipstick format. The *Plasmodium* LDH can use 3-acetylpyridine nucleotide (ADAP) substrate unlike human LDH, this substrate is used in immunocapture assays (Moody, 2002). *Plasmodium* LDH RDTs are more efficient than HRP-2 RDTs as LDH clears with parasites and can be used to accurately monitor the response of parasites to treatment (Oduola *et al.*, 1997).

1.9 Motivation , aims and objectives

Many malaria blood-stage vaccine candidates have been presented over the years and none of the vaccines has demonstrated high efficacy in humans. Most blood stage malaria vaccines target the surface antigens that associate with the human erythrocytes during the blood stage of infection (Takala *et al.*, 2009). Targetting surface antigens results in malaria vaccines that block the establishment of the erythrocytic stage of malaria that produces clinical symptoms. The leading blood-stage malaria vaccine candidates include MSP1, MSP2, MSP3, AMA1, GLURP, RH5 etc (Cockburn & Seder, 2018). The surface antigens are exposed to the immune system during the blood stage infections. Many of these antigens have been identified to have a high degree of genetic diversity. The vaccines made against polymorphic antigens have been shown to have low efficacies (Takala *et al.*, 2009). *Plasmodium falciparum* apical membrane antigen 1 (*Pf*AMA1) has been observed to possess 62 polymorphic amino acid residues (Takala *et al.*, 2009) and they contribute to it inducing a strain-specific immune response with low efficacy (Thera *et al.*, 2011). The same applies to MSP1, MSP2 and GLURP (Mohammed *et al.*, 2018). In contrast, *Plasmodium falciparum* reticulocyte binding-like homology protein 5 (*Pf*RH5) only has 10 polymorphic amino acid residues and has been reported to have no strain specificity (Ouattara *et al.*, 2018). *Pf*RH5 is highly conserved amongst *Plasmodium* parasites (Hayton *et al.*, 2008) and RH5.1/AS0B is the most promising malaria blood-stage vaccine candidate that has been suggested for phase I/IIa clinical trials (Jin *et al.*, 2018).

Glycolysis is the critical energy-producing pathway in *Plasmodium* parasites. An increase in glycolytic enzyme activity has been observed in *Plasmodium falciparum* infected-erythrocytes when compared to uninfected erythrocytes. The levels of mRNA of glycolytic

enzymes have been reported to increase between the early ring stage and early schizonts stage of the parasite development (Le Roch *et al.*, 2003). The protein levels also increase during the stages of development. These enzymes included *Plasmodium falciparum* glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. This implies that the glycolytic enzymes are important in the parasite. These enzymes are highly conserved (Bzik *et al.*, 1993; Satchell *et al.*, 2005) and are both diagnostic targets (Krause *et al.*, 2017; Piper *et al.*, 1999). PfGAPDH is a pre-erythrocyte stage malaria vaccine candidate (Cha *et al.*, 2016).

Plasmodium falciparum is the leading cause of malaria in humans compared to other four human *Plasmodium* species. The main focus of the World Health Organization is to develop an effective vaccine against *P. falciparum* infections. Although, *P. falciparum* parasites cannot be used directly for initial vaccine studies as it is unethical to use humans in these studies. Rodent malaria is the alternative model for these studies. *Plasmodium berghei* and *P. yoelii* are the two malaria parasites that are widely used for investigation of vaccine candidates including the leading malaria vaccine candidate MSP1. The current study is going to use glycolytic enzymes from *Plasmodium yoelii* parasites to investigate them for potential malaria vaccine candidate. *Plasmodium berghei* parasites are used in this study to infect the PyLDH and PyGAPDH vaccinated mice since the enzymes in these two parasites share 95 and 99% amino acid identity..

Aim of the current study:

To investigate whether *Plasmodium yoelii* lactate dehydrogenase and *P. yoelii* glyceraldehyde-3-phosphate dehydrogenase could offer protection against blood-stage malaria infections in mice.

Therefore, the objectives of the current study were:

1. To optimise and evaluate different expression conditions for recombinant *Plasmodium yoelii* glyceraldehyde-3-phosphate dehydrogenase (rPyGAPDH) and lactate dehydrogenase (rPyLDH).
2. To study the polymorphisms in PyGAPDH and PyLDH sequences.
3. To evaluate the immunogenicity of rPyGAPDH and rPyLDH in mice.
4. To assess whether immunization with rPyGAPDH and rPyLDH protects the mice from challenge with *P. berghei* parasites.
5. To produce chicken antibodies against PfLDH.
6. To test Reactive black 5 for staining proteins on nitrocellulose membranes.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Equipment

Orbital shaking incubator (New Brunswick Scientific, USA), Autoflow Co₂-water Jacketed incubator, orbital LS and revolver (labnet international, USA), Shimadzu UV spectrophotometer (GE Healthcare, England), Avanti™ J-26 XPI (Bechman counter, USA), Olympus light microscope (Japan), VersaMax™ ELISA plate reader (Molecular Devices Corporation, USA), Virosonic™ cell disruptor (VirTis, USA), VersaDoc™ imaging system (BioRad, USA).

2.1.2 Reagents

The reagents purchased from Sigma-Aldrich (Germany) were 2-mercaptoethanol, 4-chloro-1-naphthol, acrylamide, ampicillin, bisacrylamide, sodium cyanoborohydride (NaBH₃CN), ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, imidazole, sodium dodecyl sulfate (SDS), formaldehyde, sodium carbonate (Na₂CO₃), sodium azide (NaN₃), sodium acetate, RPMI-1640 medium, Coomassie Brilliant Blue R-250, Freund's complete adjuvant, Freund's incomplete adjuvant, Ponceau S, Reactive black 5, N,N,N',N'-tetramethylethylenediamine (TEMED), Triton X-100, Tween-20 and ethidium bromide. The reagents purchased from Merck were potassium dihydrogen phosphate (KH₂PO₄), potassium hydrogen phosphate (K₂HPO₄), glycerol, agar bacteriological, sodium chloride (NaCl), bromophenol blue, acetic acid, sodium hydroxide (NaOH), silver nitrate and polyethylene glycol (PEG 6000). The reagents purchased from Saarchem were glucose, sodium dihydrogen phosphate (NaH₂PO₄) and gelatin. Tryptone, trisaminomethane (TRIS) and glycine were obtained from Melford laboratories. The reagents purchased from Fluka Analytical were yeast extract, kanamycin sulphate and Giemsa stain. Bovine serum albumin (BSA) was obtained from Roche Diagnostics (Mannheim, Germany), isopropyl β-D-1-thiogalactopyranoside (IPTG), ethanol and Aminolink® resin were purchased from ThermoFisher Scientific. Ammonium persulphate was purchased from Bio-Rad. Methanol was purchased from RAD CHEM and acetone was purchased from Associated Chemical Enterprises. The blotting filter papers were purchased from Separations Scientific PTY (South Africa) and the nitrocellulose blotting membrane was purchased from Life Sciences BioTrace™ (Mexico). The Whatman 1 filter papers were purchased from GE Healthcare limited (UK) and the Talon resin was obtained from Takara Bio (USA). The unstained protein molecular weight marker, containing: 116 kD β-

galactosidase; 66.2 kD bovine serum albumin; 45 kD ovalbumin; 35 kD lactate dehydrogenase; 25 kD REase Bsp981; 18.4 kD β -lactoglobulin and 14.4 kD lysozyme was purchased Fermentas (Vilnius, Lithuania). Low fat skimmed milk was purchased at Spar supermarket.

Molecular biology reagents were purchased from Solis Biodyne (Tartu, Estonia) and they include *Escherichia coli* host cells, BL21 (DE3) (glycerol stock). Mouse monoclonal anti-His-Tag IgG, peroxidase conjugated rabbit anti-chicken antibodies, peroxidase conjugated goat anti-mouse antibodies were purchased from Merck Biosciences (Germany)

2.2 Method

2.2.1 Expression vectors and expression host cells

Plasmodium yoelii genomic DNA was isolated from infected mouse blood, *PyLDH* and *PyGAPDH* were amplified using their specific primers and purified using the pegGOLD Purification Kit (Hurdayal *et al.*, 2010; Krause *et al.*, 2017). *Plasmodium falciparum* gDNA was isolated from an infected patient (Hurdayal *et al.*, 2010). The following purification, the product was ligated into pGEM-T easy vector and the vector was then propagated into *E. coli* JM 109 bacterial cells. The insert was removed from the isolated plasmid using EcoRI and NotI restriction sites for *PyLDH* and *PyGAPDH*, then ligated into pET-28a vector which was transformed into and expressed in *E. coli* BL21 (DE3) cells. *PfLDH* insert was removed from the isolated plasmid using EcoRI and PstI restriction sites and then ligated into pKK223-3 vector which was then transformed into and expressed in *E. coli* BL21 (DE3) cells.

Table 2.1: Plasmid vector, expression host cells, site of insertion of proteins for recombinant expression in *E. coli* cells.

Protein	Vector	Restriction sites	Antibiotic	Expression host
<i>PfLDH</i>	pKK223-3	EcoRI and PstI	Ampicillin	<i>E. coli</i> BL21 (DE3)
<i>PyLDH</i>	pET-28a	EcoRI and NotI	Kanamycin sulphate	<i>E. coli</i> BL21 (DE3)
<i>PyGAPDH</i>	pET-28a	EcoRI and NotI	Kanamycin sulphate	<i>E. coli</i> BL21 (DE3)

2.2.2 Glycerol stocks

The *rPyLDH*, *rPyGAPDH*, *rPflDH* and transformed *E. coli* cells were harvested from an overnight culture which was grown in a terrific broth (TB) media (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (w/v) glycerol, 0.231% (w/v) KH_2PO_4 , 1.254% (w/v) K_2HPO_4) which contained 25 $\mu\text{g/ml}$ kanamycin sulphate for *rPyLDH*, *rPyGAPDH* and 100 $\mu\text{g/ml}$ ampicillin for *rPflDH*. The antibiotic stocks were made in distilled water and filter sterilized. The cells were grown (16 h at 37°C) in a 200-rpm shaking incubator. One ml glycerol stocks of cells were made by adding 850 μl of overnight culture to 150 μl sterile 50 % (v/v) glycerol and stored at -80°C.

2.2.3 Agar plates

Luria broth (LB) agar was made by dissolving 1 % (w/v) tryptone; 0.5 % (w/v) yeast extract; 85 mM NaCl; 11 mM glucose and 1.5 % (w/v) agar prepared in distilled water and autoclaved (1 h at 120°C). The agar was supplemented with a final concentration of 25 $\mu\text{g/ml}$ kanamycin sulphate or 100 $\mu\text{g/ml}$ ampicillin and 3-way streaked with glycerol stocks of transformed *E. coli* cells and the colonies were grown overnight (37°C).

2.2.4 Recombinant protein expression

A single colony from the LB agar plates was inoculated into a terrific broth media (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (w/v) glycerol, 0.231% (w/v) KH_2PO_4 , 1.254% (w/v) K_2HPO_4) was supplemented with 25 $\mu\text{g/ml}$ kanamycin sulphate or 100 $\mu\text{g/ml}$ ampicillin and grown overnight (37°C, 200-rpm). The alternate media was Luria broth induced with 0.3 mM IPTG. The cells were obtained by centrifugation (4000 x g, 10 min at 4°C). The cell pellets were suspended in resuspension buffer (50 mM NaH_2PO_4 ; 300 mM NaCl at pH 8.0) to 5% of the original cell culture volume. No bacterial culture volumes exceeded 20% of the culture flask volume. The suspended cells were sonicated (6 x 30 sec) each cycle keeping the samples on ice between cycles. The pellet and supernatant were separated by centrifugation (12000 xg for 20 min at 4°C). The pellet was resuspended in phosphate buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 7 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.2) equivalent to 5% of the original culture volume and both the supernatant and pellet.

2.2.5 Talon® affinity resin preparation and recombinant proteins purification

A 2 ml suspension of Talon® resin was placed in a 15 ml falcon tube, centrifuged (4000 xg for 5 min) to remove the suspension buffer. The resin was washed twice with 5 column volumes of sodium phosphate buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH

8.0). The *E. coli* supernatant containing the recombinant protein was incubated with the resin and mixed using an end-over-end (1 h at RT or 16 h at 4°C). The unbound proteins were collected, and the resin was washed with a sodium phosphate buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole at pH 8.0) until the buffer absorbance at 280 nm reaches 0.02. The bound proteins were eluted from the resin with 5 ml sodium phosphate buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole at pH 8.0) collected in 1 ml fractions. Eluted fraction samples were analyzed by measuring the protein absorbance at 280 nm and on a 12.5% reducing SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie blue R-250.

2.2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A discontinuous Tris-Glycine buffer and the SDS-PAGE gel system described by Laemmli (1970) were used. The gels were prepared (Table 2.1) and poured between two glass plates: 81.5 mm x 101.5 mm and 72.5 mm x 101.5 mm. The gel was overlaid with 200 µl of distilled water and was removed once the running gel has polymerized. Stacking gel (1.5 ml) was added and a 15 well or 10 well combs were inserted. Afterwards, the gel plates were assembled in the casting gasket.

Table 2.2: Reagents and recipe for 12.5 % running gel and 3% stacking gel.

Reagents	12.5% running gel	3% stacking gel
Monomer solution (4.1 M acrylamide and 52 mM N, N'-methylenebisacrylamide)	6.25 ml	0.71 ml
4 x running buffer (1.5 M Tris buffer at pH 8.8)	3.75 ml	-
4 x stacking gel buffer (500 mM Tris buffer at pH 6.8)	-	1.75 ml
1.0% (w/v) SDS in distilled water	0.15 ml	0.075 ml
10% (w/v) ammonium persulfate in distilled water	0.070 ml	0.035 ml
Distilled water	4.75 ml	4.53 ml
TEMED	0.0075 ml	0.015 ml

The sample preparation was done as follows: an equal volume of sample reducing treatment buffer (125 mM Tris-HCl, 20 % (v/v) glycerol, 4% (m/v) SDS, (10% (v/v) 2-mercaptoethanol at pH 6.8, with bromophenol blue) were mixed and boiled for 5 min. Standard molecular weight marker was run along with the samples to estimate the molecular weights of the proteins. Electrophoresis was performed using a Bio-Rad system at 20 mA per gel with tank buffer (250 mM Tris-HCl, 192 mM glycine and 0.1 % (m/v) SDS, pH 8.3) for 1 h and the gels

were stained with Coomassie brilliant blue R- 250 (0.125% (w/v) Coomassie brilliant blue R-250, 50 % (v/v) methanol and 10 % (v/v) acetic acid) and destained with 50% (v/v) methanol and 10 % (v/v) acetic acid.

2.2.7 Standard curve calibration to estimate protein sizes on SDS-PAGE gels

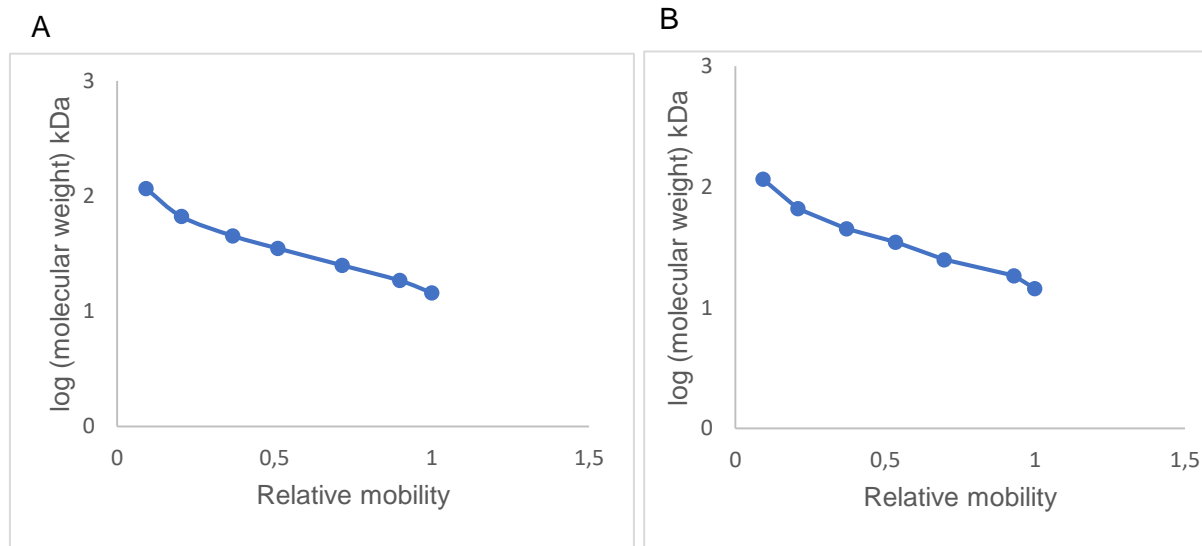


Figure 2.1: Standard curve of the log molecular weight against relative mobility of the protein standards. The molecular weight marker which consisted of β - Galactosidase (116), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), Rease Bsp 981 (25 kDa), β - Lactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa) was resolved on 12.5% reducing SDS-PAGE gel. (A) *rPyGAPDH* and (B) *rPyLDH* standard curve.

The molecular weights of proteins on SDS-PAGE gels were estimated using the standard curves (Figure 2.1).

2.2.8 Western blotting

Western blotting was performed as described by Towbin (1979). The proteins were first resolved on an SDS-PAGE gel. The proteins in the SDS-PAGE gel were transferred to the nitrocellulose paper by sandwiching the gel and the nitrocellulose paper together between six-layer filter paper in a transfer buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS, pH 8.3). Air bubbles were removed by rolling the surface using a glass rod and the transfer was done for 16 h at 40 mA. After the overnight transfer, the nitrocellulose paper was stained with 0.1 % (w/v) Ponceau S in 1% (v/v) acetic acid and molecular weight markers were marked with a pencil. The Ponceau S stain was washed off with distilled water with few drops of 1M NaOH and the nitrocellulose paper was blocked with 5 % (w/v) low-fat milk powder in TBS

(20 mM Tris; 200 mM NaCl, 0.1% (v/v) Tween, pH 7.4) solution (1 h at RT). All the washes were done with 0.1% (v/v) Tween 20-PBS (3 x 5 min). The nitrocellulose paper was washed and incubated in primary antibody prepared in 0.5% (w/v) BSA- 0.1% (v/v) Tween-TBS (2 h at RT or 16 h at 4°C). The nitrocellulose paper was washed and incubated in secondary antibody prepared in 0.5% (w/v) BSA- 0.1% (v/v) Tween-TBS (1 h at RT). The nitrocellulose was then washed and incubated with substrate (2 ml (0.3% (w/v) 4-chloro-1-naphthol in methanol, 8 ml TBS and 4 µl H₂O₂) and stored in the dark until colour develops after which it was washed with distilled water.

All the washes were done with 0.1% (v/v) Tween 20-PBS and repeated three times.

2.2.9 Thirty-minute silver stain

The silver staining method was performed according to Nesterenko *et al* (1994). The gel was fixed for 5 minutes in 50% (v/v) acetone, 1.25% (w/v) TCA, 0.015% (w/v) formaldehyde, rinsed (3 x 5 sec) and washed for 5 minutes with distilled water and incubated for 5 minutes in 50% (v/v) acetone. The gel was treated with (0.017% (w/v) sodium thiosulfate for 5 minutes and rinsed (3 x 5 sec) with distilled water. This was followed by incubation with 0.27% (w/v) silver nitrate, 0.37% (w/v) formaldehyde for 8 minutes. The gel was then rinsed (5 x 5 sec) with distilled water and developed for 20 seconds in 2% (w/v) sodium carbonate, 0.015% (v/v) formaldehyde, 0.004% (w/v) sodium thiosulfate. Band development was stopped by incubating the gel in 1% (v/v) acetic acid and kept in distilled water. The gel was visualized with the VersaDoc™ imaging system (BioRad, USA).

2.2.10 Chicken immunization with recombinant *Pf*LDH

Polyclonal antibodies were raised in Hy-line chickens against *rPf*LDH. Two chickens were immunised with *rPf*LDH (100 µg) Freund's complete adjuvant (1:1) whilst the second, third and fourth immunization was prepared in Freund's incomplete adjuvant (1:1). Each chicken was injected twice with 250 µl of an oil emulsion containing 50 µg *rPf*LDH in each breast muscle. The eggs were collected daily from the first day of immunization until week 12.

2.2.11 Isolation of IgY from chicken egg yolk

IgY was isolated from a chicken egg yolk according to Polson *et al* (1980). Egg yolk was separated from egg white by washing the egg under running water. The yolk sack was punctured, and the yolk was measured and suspended in sodium phosphate buffer (100 mM Na-phosphate buffer, 0.02% NaN₃, pH 7.6) twice the yolk volume. Polyethylene glycol (PEG

6000) 3.5% (w/v) was added and dissolved. The sample was centrifuged (4400 xg, 30 min, RT). The supernatant was filtered through cotton wool to remove all the fat cake. PEG 8.5% (w/v) was added and dissolved. The sample was centrifuged (12000 xg, 10 min, 4°C). The supernatant was thrown away, and the pellet was resuspended in sodium phosphate buffer equal to the supernatant volume after filtering. Twelve percent (w/v) PEG was added and dissolved by stirring gently. The sample was centrifuged (12000 xg, 10 min, 4°C). The pellet was resuspended in 1/6th of the original yolk volume and stored at 4°C.

2.2.12 Affinity purification of IgY using an Aminolink® column

The 50% (v/v) Aminolink® resin (4 ml) was equilibrated at room temperature and the storage buffer was drained. The resin was washed with 6 ml of coupling buffer (100 mM NaH₂PO₄, 0.05% NaN₃, pH 7.2). Recombinant rP_fLDH (5 mg) was added to the resin with 40 µl cyanoborohydride solution (5M NaBH₃CN in 1M NaOH) and incubated (16 hr, 4°C). The resin was washed with coupling buffer and incubated with 2 ml quenching buffer (1M Tris-HCl, pH 7.4) with 40 µl cyanoborohydride (30 min). The solution was drained, and the resin was washed with 10 ml of washing solution (100 mM NaH₂PO₄, 0.2% NaN₃, pH 6.5). The crude IgY was filtered through Whatman 1 filter paper and incubated with the Aminolink® affinity column (16 hr., RT) in an end-over-end mixer. The column was washed with PBS until the absorbance at 280 nm was 0.02. Chicken anti-rP_fLDH antibodies were eluted with 8 ml of elution buffer (100 mM glycine, 0.02% NaN₃, pH 2.8). One ml fractions were collected into 1.5 ml tubes containing 100 µl neutralization buffer (1M NaH₂PO₄, 0.02% NaN₃, pH 8.5).

2.2.13 Production of mouse anti-rPyLDH and anti-rPyGAPDH polyclonal antibodies

Polyclonal antibodies were raised in 8 weeks old Balb/c mice against rPyLDH or rPyGAPDH. Five mice in each group were immunised with rPyLDH or rPyGAPDH (100 µg) complete Freund's adjuvant (1:1) whilst the second and third immunization was prepared in an incomplete Freund's adjuvant (1:1). The immunizations were two weeks apart. All the injections were intraperitoneal. The mice were euthanized with isoflurane and the blood was collected through a cardiac puncture into heparin-coated tubes two weeks after the third immunization.

2.2.14 *Plasmodium berghei* parasite challenge

Mice were immunized for polyclonal antibody production against rPyLDH or rPyGAPDH (2.2.12). Mice immunized with PBS and rPkPMT served as vaccine control. Two weeks after the third immunization, mice were challenged with 1x10⁵ *Plasmodium berghei* parasites in the intraperitoneal site. The parasites were diluted in RPMI-1640 medium containing glutamate. The

parasitaemia was monitored every second-day post infection by bleeding the tail vein of each mouse. A microlitre of blood was smeared on a glass microscope slide and the parasites were stained and counted.

2.2.15 Giesma staining of parasites

A microlitre of blood was smeared on the glass slide. The parasites were fixed with 100% methanol for 30 sec and air dried. The parasites were stained with 10% (v/v) Giemsa stain in 6.7 mM phosphate buffer pH 7.1 for 20 min and washed with distilled water. The slides were air dried and viewed on an electron microscope (1000x).

2.2.16 Enzyme-linked immunosorbent assay (ELISA)

Antigens were prepared in PBS (1µg/ml) and 100 µl was pipetted into each well and incubated (16 h at 4°C) to allow for coating. All the washes were done with 0.1% (v/v) Tween 20-PBS and repeated three times. After coating, the wells were blocked with 150 µl of 5% (m/v) non-fat milk powder (1 h at 37°C). The wells were washed and incubated with 100 µl of primary antibody in 0.5% (m/v) BSA-PBS-Tween (2 h at 37°C). The wells were then washed and incubated with 100 µl of secondary antibody- HRPO conjugate prepared in 0.5% (m/v) BSA-PBS- Tween (2 h at 37°C). The wells were washed and incubated with 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (1 ml TMB diluted in 9 ml citrate-phosphate buffer at pH 5.0 and 2 µl H₂O₂) (1 hr at RT) and colour was developed in the dark. The plates were read at 370 nm and 652 nm with an ELISA-plate reader. The background controls included a no coat, no primary or secondary antibody. The ELISA for rPyLDH and rPyGAPDH was probed with 1/10, 1/100, 1/1000 and 1/10000 dilutions of mouse serum and the un-immunized mouse serum was used as a control. Goat anti-mouse antibody (1:6000) was used for detection.

2.2.17 Protein staining on SDS-PAGE gels and nitrocellulose

For staining of all SDS-PAGE gels and nitrocellulose membranes, a 40 ml staining solution was used, and the incubations were done with shaking at 60 rpm shaker at room temperature. Reactive black 5 and Ponceau S staining solution were prepared at 0.1% (m/v) and 1% (v/v) acetic acid in distilled water and Amido black 10B was prepared at 0.1% (m/v) in 30:10:60 methanol: acetic acid: distilled water and was filtered through Whatman 1 before used. SDS-PAGE gels were stained for 1 hour or overnight (16 h) and destained for 16 h or more in destain solution (50% methanol, 10% acetic acid, and 40% distilled water). The proteins on a

nitrocellulose membrane were stained for 5 minutes and the membrane was washed in running water for 2 minutes to remove the unbound stain.

2.2.18 Staining zymograms

Papain proteolytic activity was assessed in a zymogram. A 12.5% SDS-PAGE running gel containing 1% (w/v) gelatin was prepared with a 4% stacking gel. Papain samples were not reduced or boiled. After electrophoresis, the gel was washed with two changes of assay buffer (2.5% (v/v) Triton X-100, 100 mM sodium acetate, 1 mM EDTA, 0.02% (w/v) sodium azide, pH 5.0). The washing step was followed by a 3 h incubation (37°C) in assay buffer without Triton X-100. The gel was either stained with Reactive black 5 or Amido black 10B stain (1 h at RT). It was then destained in 30% (v/v) methanol, 10% (v/v) acetic acid in distilled water for 16 h.

2.2.19 Bioinformatics analysis of proteins

2.2.19.1 DNA and amino acid sequences

Protein and DNA sequences were obtained from PlasmoDB (<http://plasmodb.org/plasmo/>) or PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>). The multiple sequence alignments were made using clustal omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

2.2.19.2 3D modelling of protein structures

The protein structures were modelled using Swiss model (<https://swissmodel.expasy.org/>).

2.2.19.3 Immunogenic peptide prediction

B cell epitopes were predicted using ABCpred server (<http://crdd.osdd.net/raghava/abcpred/>) and T cell epitopes were predicted with NetMHCpan server <http://www.cbs.dtu.dk/services/NetMHCpan/>

Chapter 3

Recombinant expression, purification and analysis of *PyGAPDH*, *PyLDH* and *PfLDH*.

3.1 Introduction

3.1.1 Recombinant expression of proteins in *E. coli* bacteria

Bacterial systems allow genes to be cloned and expressed for purification and characterization studies without using large amounts of animal and plant materials (Rosano & Ceccarelli, 2014). *Escherichia coli* is the most popular bacterial system used for recombinant protein expression (Sivashanmugam *et al.*, 2009; Terpe, 2006). *E. coli* genetics are well studied and characterized and expressing recombinant proteins in *E. coli* is advantageous as bacteria grow fast in culture media and protein expression can be easily induced (Rosano & Ceccarelli, 2014; Terpe, 2006). High bacterial cell densities are easily reached with *E. coli* and the bacterial cells can be grown in an inexpensive culture media (Sørensen & Mortensen, 2005). BL21 (DE3) is the most commonly used *E. coli* bacterial host strain (Sørensen & Mortensen, 2005). The strain is deficient of OmpT and Lon proteases, which means the recombinant proteins are not degraded by proteases (Sørensen & Mortensen, 2005). *E. coli* cells allow for expression of high yields of proteins and an added advantage is the production of membrane proteins (Sørensen & Mortensen, 2005; Terpe, 2006).

3.1.2 Plasmids and their advantages for cloning target proteins

For recombinant protein expression, the genetic material of the target protein is cloned into the plasmid, which is then used to transform *E. coli* cells for over-production of the protein. The plasmid should have an origin of replication, a multiple cloning site which contains restriction enzyme sites where the gene can be inserted and an antibiotic resistant marker (Studier, 2005). The antibiotic resistance marker allows to selectively grow only the transformed *E. coli* cells without contamination. The plasmids used for *E. coli* systems include pET and pBAD vectors (Sørensen & Mortensen, 2005; Studier, 2005). There are about 40 different types of pET expression vectors which are commercially available (Sørensen & Mortensen, 2005). Expression of proteins in pET vectors is controlled by a T7 RNA polymerase, which is derived from bacteriophage T7 and the lac promoter in *E. coli* (Sørensen & Mortensen, 2005). The coding sequence of T7 RNA polymerase is present in the chromosome under the control of lacUV5 promoter which is found in *E. coli* DE3 host cells (Sørensen & Mortensen, 2005; Studier, 2005). T7 promoters are stronger compared to *E. coli* promoters and can lead to the production

of about 50% of the recombinant protein in the cell's total proteins (Sørensen & Mortensen, 2005). Another advantage of using pET expression systems is that they produce low plasmid copy number of 20–25 copies per cell which permits the highest production of T7 promoter without causing damage to the cells, which is caused by large copy number plasmids (Sørensen & Mortensen, 2005).

3.1.3 Comparison of IPTG inducible media and auto inducing media for the expression of proteins

Upon the transformation of *E. coli* cells with pET vectors, the cells are grown in liquid medium (Lysogenic broth) and the production of the recombinant protein is initiated by the addition of isopropyl-b-D-thiogalactoside (IPTG). IPTG is an inducer which works by attaching to the promoter, which liberates the LacI repressor and initiates the expression of proteins (Sivashanmugam *et al.*, 2009). Expression in an inducible media has disadvantages, which include continuous observing of the cell's growth by taking optical densities (OD 600) until they reach the mid-log phase where they are induced. This method requires lots of manual work, particularly when growing many cultures in parallel and the cells are also susceptible to contamination as they are opened every time when taking the OD (Studier, 2005). In contrast, the production of proteins in an autoinducing media (terrific broth) does not involve continuous monitoring of cells and no IPTG is added (Studier, 2005). Cells which are grown in terrific broth are only inoculated in media containing an antibiotic and are grown to their full capacity to yield the recombinant protein (Sivashanmugam *et al.*, 2009). Autoinducing media has been described to yield high amounts of proteins compared to the IPTG induced media (Studier, 2005). Additionally, lactose can be added in an autoinducing media to increase the yields of proteins that are produced in minimal amounts (Studier, 2005). Ethanol can also be added in both autoinducing and IPTG induced media enhance the production of recombinant proteins in *E. coli* cells (Chhetri *et al.*, 2015).

3.1.4 Production and isolation of IgY antibodies

IgY antibodies are the most abundant antibodies in chicken egg yolks, it's the chickens equivalent of human IgG (Yegani & Korver, 2010). IgY antibodies are widely used for immunotherapies and immunodiagnostics as they have limited cross-reactions with human serum proteins and their isolation methods are simple and non-invasive (Gassmann *et al.*, 1990). Chicken eggs have been described to produce IgY from 10 days after the initial immunization (Polson *et al.*, 1980). Precipitation methods including, water dilution, polyethylene

glycol, Xanthan gum and dextran sulfate have been described to isolate IgY from egg yolks. As described by Akita and Nakai (1993), water dilution method employs a 9:1 water and yolk dilution which isolates plasma proteins from granules and lipids. This step is followed by sodium sulfate precipitation and ultrafiltration, which purify IgY from plasma proteins. Polyethylene glycol (PEG) precipitation method involves the initial use of 3.5% (w/v) PEG to remove lipids and vitelline followed by 12% (w/v) PEG to precipitate IgY (Polson *et al.*, 1980). The dextran sulphate method involves the dilution of egg yolk in Tris-buffered saline and precipitating the IgY with dextran and sodium sulfate. Lastly, Xanthine gum precipitates the insoluble lipoproteins leaving IgY which is then achieved by salting out chromatography (Hatta *et al.*, 1990). Water dilution method has been reported to yield 9.8 mg/ml (Akita & Nakai, 1993), while PEG precipitation yields 6-12 mg/ml (Polson *et al.*, 1980), Xanthan gum yields 7.3 mg/ml and dextran sulfate yields 7.5 mg/ml IgY from a single egg yolk. PEG precipitation is the most commonly used IgY isolation method as this isolation procedure is simple, cost-effective and produces a high yield of IgY.

3.1.5 Aims of this chapter

This study focuses on the recombinant expression and characterization of *Plasmodium yoelii* Lactate dehydrogenase (*rPyLDH*), *P. yoelii* glyceraldehyde-3-phosphate (*rPyGAPDH*) and *P. falciparum* lactate dehydrogenase (*rPfLDH*). Recombinant *PyLDH*, *rPyGAPDH* and *rPfLDH* were expressed and purified as histidine tag fusion proteins in *E. coli* bacteria. Recombinant *PyLDH* and *rPyGAPDH* expression was evaluated in the presence of ethanol in the terrific broth and Luria broth. Mobilities of *rPyLDH* and *rPyGAPDH* in different percentage SDS-PAGE gels and in the presence of metal ions were evaluated. This was done to better understand the properties of the recombinant proteins as these proteins will later be used to vaccinate mice for protection against blood-stage malaria infections. Metal ions that are commonly used in metal-affinity chromatography were investigated for binding to the *rPyLDH* and *rPyGAPDH*. These metal ions include zinc (Zn^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}) and nickel (Ni^{2+}). Anti-*rPfLDH* antibodies were made in chickens and isolated from egg yolks using the PEG method (Polson *et al.*, 1980). Pure IgY was obtained following the use of a *rPfLDH* aminolink® column.

3.2 Results

3.2.1 Recombinant expression of *PyGAPDH* in Luria and terrific broth containing ethanol

Recombinant *Plasmodium yoelii* glyceraldehyde-3-phosphate dehydrogenase (*PyGAPDH*) was expressed in *E. coli* bacteria grown with or without ethanol in Luria and terrific broth (Figure 3.1A and B). The *E. coli* proteins were not affected by the presence of ethanol in the culture as indicated by the same intensity of *E. coli* protein bands in all ethanol concentrations (Figure 3.1A and B). Recombinant *PyGAPDH* was absent in the uninduced cultures and was present as a 40 kDa protein band in the induced cultures (Figure 3.1A). One percent (v/v) ethanol had no effect on r*PyGAPDH* expression while 2% and 3% (v/v) ethanol decreased the expression of r*PyGAPDH* (Figure 3.1A). A similar decrease of r*PyGAPDH* expression was observed when the cells were grown in terrific broth. Ethanol decreased the expression of r*PyGAPDH* in a concentration dependent manner (Figure 3.1B).

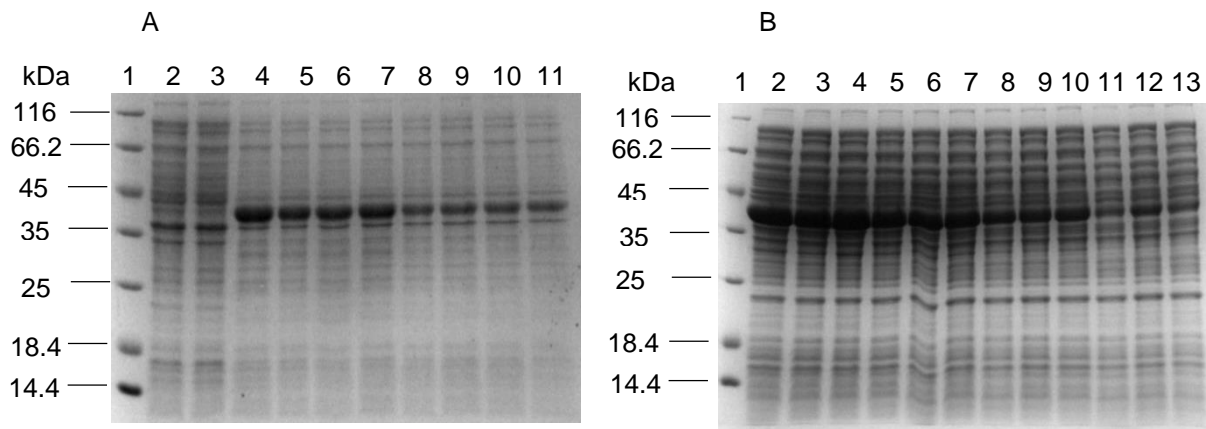


Figure 3.1: Recombinant expression of *PyGAPDH* in Luria or terrific broth supplemented with ethanol. Cultures were grown in Luria (A) induced with 0.3 mM IPTG and terrific (B) media with or without ethanol and r*PyGAPDH* expression was analysed on a 12.5% reducing SDS-PAGE gel. Lane 1: molecular weight marker, (A): lanes 2-3: uninduced culture, lanes 4-5, 6-7, 8-9, 10-11: 0%, 1%, 2% and 3% (v/v) ethanol. (B): lanes 2-4, 5-7, 8-10 and 11-13: 0%, 1%, 2% and 3% (v/v) ethanol. All samples were loaded in duplicates. The gels were stained with Coomassie brilliant blue R250.

3.2.2 Recombinant expression of *PyLDH* in Luria or terrific broth containing ethanol

Recombinant *Plasmodium yoelii* Lactate dehydrogenase (*PyLDH*) was expressed in *E. coli* bacteria grown in Luria and terrific broth with or without ethanol (Figure 3.2A, B and C). The 37 kDa r*PyLDH* protein band was absent in the untransformed cells and present in the induced cultures (Figure 3.2A and B). The *E. coli* proteins were not affected by the presence of ethanol

in the culture as indicated by a similar intensity of *E. coli* protein bands in all ethanol concentrations. Initially, *Py*LDH cultures with 2% (v/v) ethanol were observed to have a slight increase of *rPy*LDH (Figure 3.2A) which was not observed in triplicate cultures (Figure 3.2B). Although, a reduction in *rPy*LDH expression was observed in the presence of 2% (v/v) ethanol when the experiment was repeated in triplicates (Figure 3.2B). The *rPy*LDH expression decreased in terrific broth in the presence of ethanol in a concentration dependent manner (Figure 3.2C).

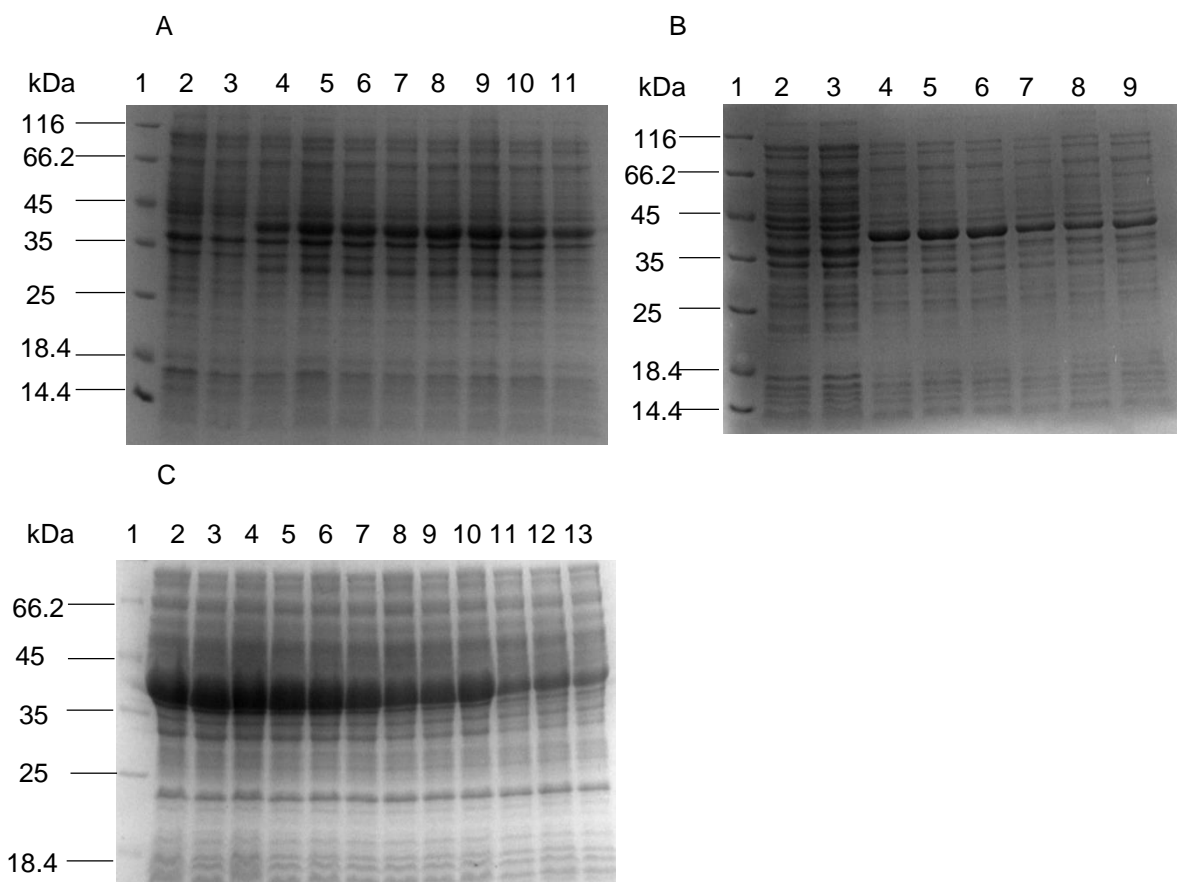


Figure 3.2: Recombinant expression of *Py*LDH in Luria or terrific broth supplemented with ethanol. Cultures were grown in Luria broth induced with IPTG (A and B) and terrific (C) media with or without ethanol and *rPy*LDH expression was analysed on a 12.5% reducing SDS-PAGE gel. Lane 1: molecular weight marker, (A): lanes 2-3: uninduced culture, lanes 4-5, 6-7, 8-9 and 10-11: induced with 0%, 1%, 2% and 3% (v/v) ethanol. (B): lanes 2-3: uninduced culture, lanes 4-6 and 7-9: induced 0% and 2% (v/v) ethanol. (C): lanes 2-4, 5-7, 8-10 and 11-13: culture with 0%, 1%, 2% and 3% (v/v) ethanol. The gels were stained with Coomassie brilliant blue R250. All samples were loaded in duplicates.

3.2.3 Comparison of *E. coli* bacterial growth in the presence of ethanol

E. coli bacteria expressing *PyGAPDH* or *PyLDH* were grown in Luria broth with or without ethanol (Figure 3.3A and B). At the first hour of their growth, there was no difference in the cell densities of ethanol treated and untreated cells for both *PyLDH* and *PyGAPDH*. Growth decreased from 2-3 hours (Figure 3.3A and B). The change in growth was concentration-dependent (Figure 3.3A and B). Together, these results suggest that ethanol hinder the growth of the bacterial cells resulting in the reduction of *rPyGAPDH* and *rPyLDH* expression.

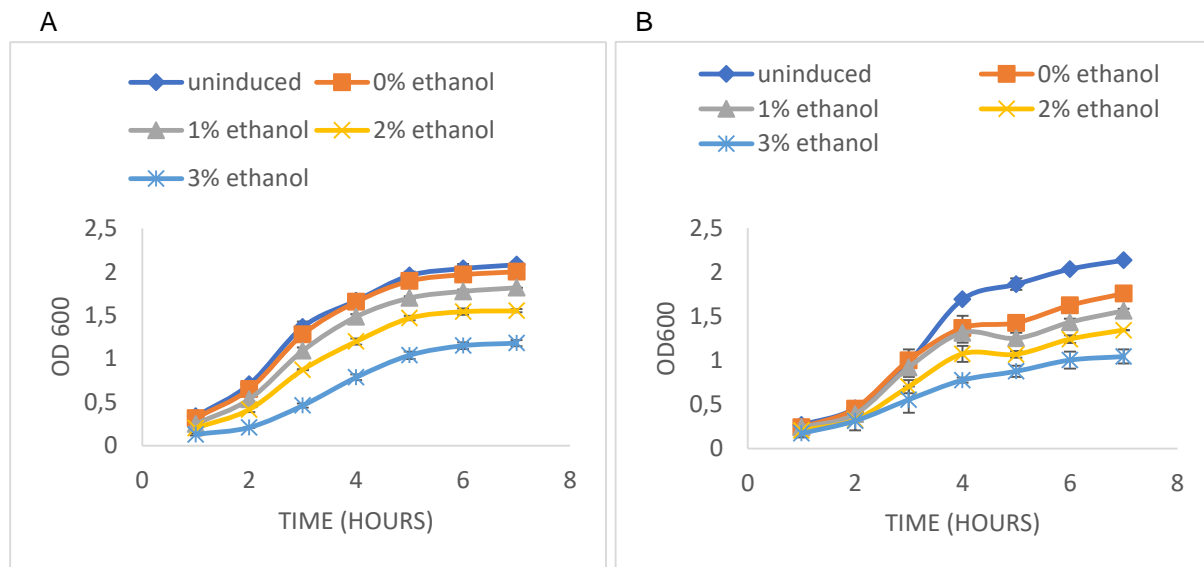


Figure 3.3: Effect of ethanol on the growth of *PyGAPDH* and *PyLDH* transformed *E. coli* cells in Luria broth. *E. coli* cells transformed with *PyGAPDH* (A) and *PyLDH* (B) plasmids were grown in Luria broth with or without ethanol in a 37°C continuous shaking incubator until they reached OD600 (0.5-0.6) and induced with IPTG for 4 hours. Samples were taken every hour to measure the OD600 for the construction of the growth curve.

3.2.4 Recombinant expression and affinity purification of *rPyGAPDH*

Plasmodium yoelii glyceraldehyde-3-phosphate dehydrogenase (*PyGAPDH*) was recombinantly expressed in *E. coli* BL21 (DE3) cells grown in a terrific broth. After expression of *rPyGAPDH*, the bacteria were lysed, and the soluble protein fraction was passed over a cobalt affinity column to purify *rPyGAPDH* with a hexa-histidine tag. The 40 kDa *rPyGAPDH* protein band was absent in the untransformed *E. coli* cells and was in the soluble fraction (Figure 3.4A). During the affinity purification, *rPyGAPDH* was eluted from the column with 250 mM imidazole phosphate buffer and a single protein band of 40 kDa was obtained (Figure 3.4A). The 40 kDa

rPyGAPDH band was consistent with the 40.328 kDa predicted from the genomic and predicted amino acid sequence with the histidine tag. Both anti-Histag antibodies and anti-rPyGAPDH antibodies detected the 40 kDa PyGAPDH protein in a Western blot (Figure 3.4B and C). Dimer and tetramer forms of rPyGAPDH with sizes of 80 kDa and 160 kDa were detected by the antibodies (Figure 3.4B and C). A yield of 12.48 mg rPyGAPDH was obtained from a 0.65 g of wet weight *E. coli* cell pellet.

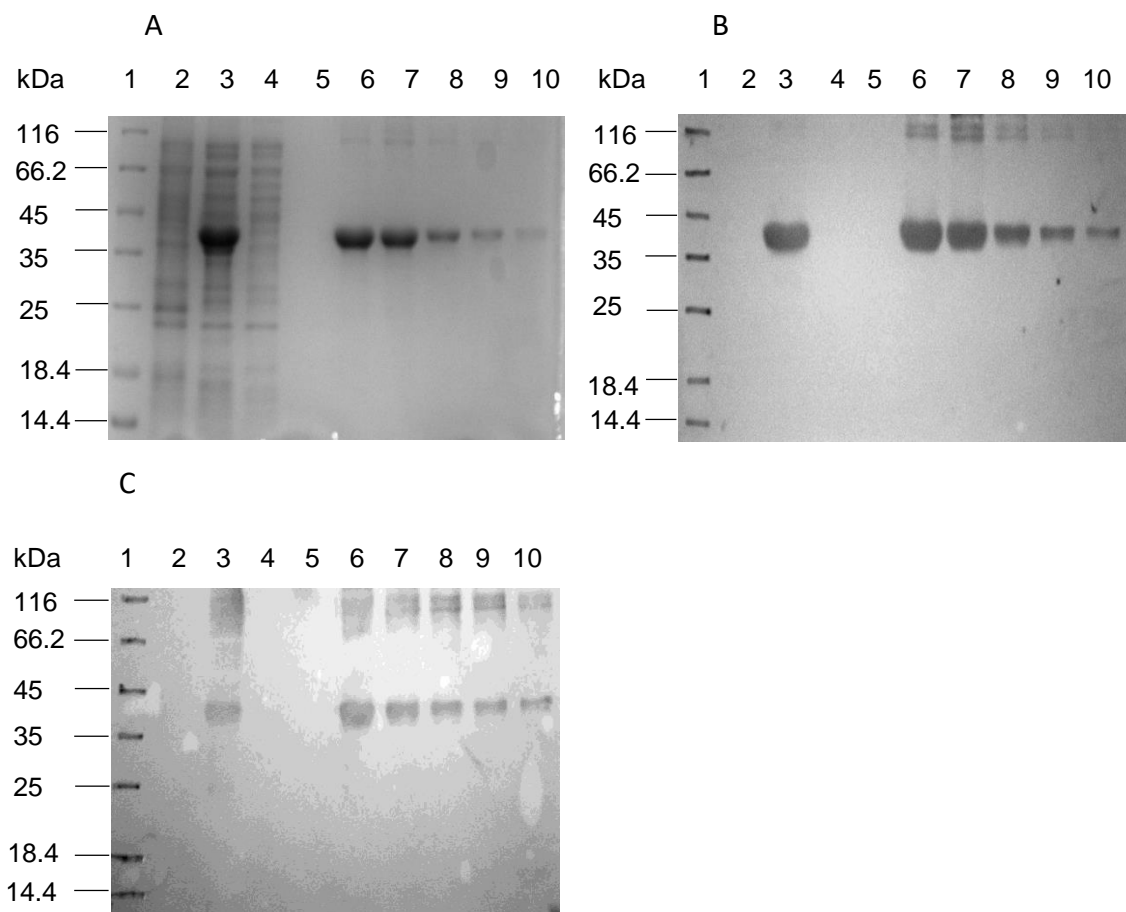


Figure 3.4: Recombinant expression and affinity purification of rPyGAPDH. Recombinant PyGAPDH was expressed in *E. coli* BL21 (DE3) cells grown in terrific broth and affinity purified with a cobalt affinity matrix. Samples were analysed on a 12.5% reducing SDS-PAGE gel (A) and Western blot (B and C). Lane 1: molecular weight marker; lanes 2-3: untransformed and transformed *E. coli* cells expressing PyGAPDH; lane 4: unbound proteins from the cobalt resin; lane 5: column wash. Lanes 6-10: rPyGAPDH eluted with 250 mM imidazole. The gel was stained with Coomassie brilliant blue R- 250. The Western blot was probed with mouse anti-Histag (B) and chicken anti-rPyGAPDH (C) antibodies.

3.2.5 Recombinant expression and affinity purification of rPyLDH

Plasmodium yoelii lactate dehydrogenase (PyLDH) was recombinantly expressed in *E. coli* BL21 (DE3) cells grown in a terrific broth. After expression of rPyLDH, the bacteria were

lysed, and the soluble protein fraction was passed over a cobalt affinity column to purify rPyLDH. The 37 kDa rPyLDH protein band was absent in the untransformed *E. coli* cells and was observed in the soluble fraction (Figure 3.5A). Recombinant PyLDH was eluted from the column with 250 mM imidazole and a single rPyLDH protein band was observed (Figure 3.5A). The 37 kDa rPyLDH band was consistent with the 37.06 kDa predicted from genomic and predicted amino acid sequence with the histidine tag. Recombinant PyLDH was detected by both anti-Histag and anti-rPyLDH antibodies in a Western blot (Figure 3.5B and C). A 74 kDa dimer form of rPyLDH was also detected (Figure 3.5B and C). We could not determine why the rPyLDH was not completely reduced during the SDS-PAGE procedure. A yield of 15.31 mg pure rPyLDH was obtained from a 0.61 g of wet weight *E. coli* cell pellet.

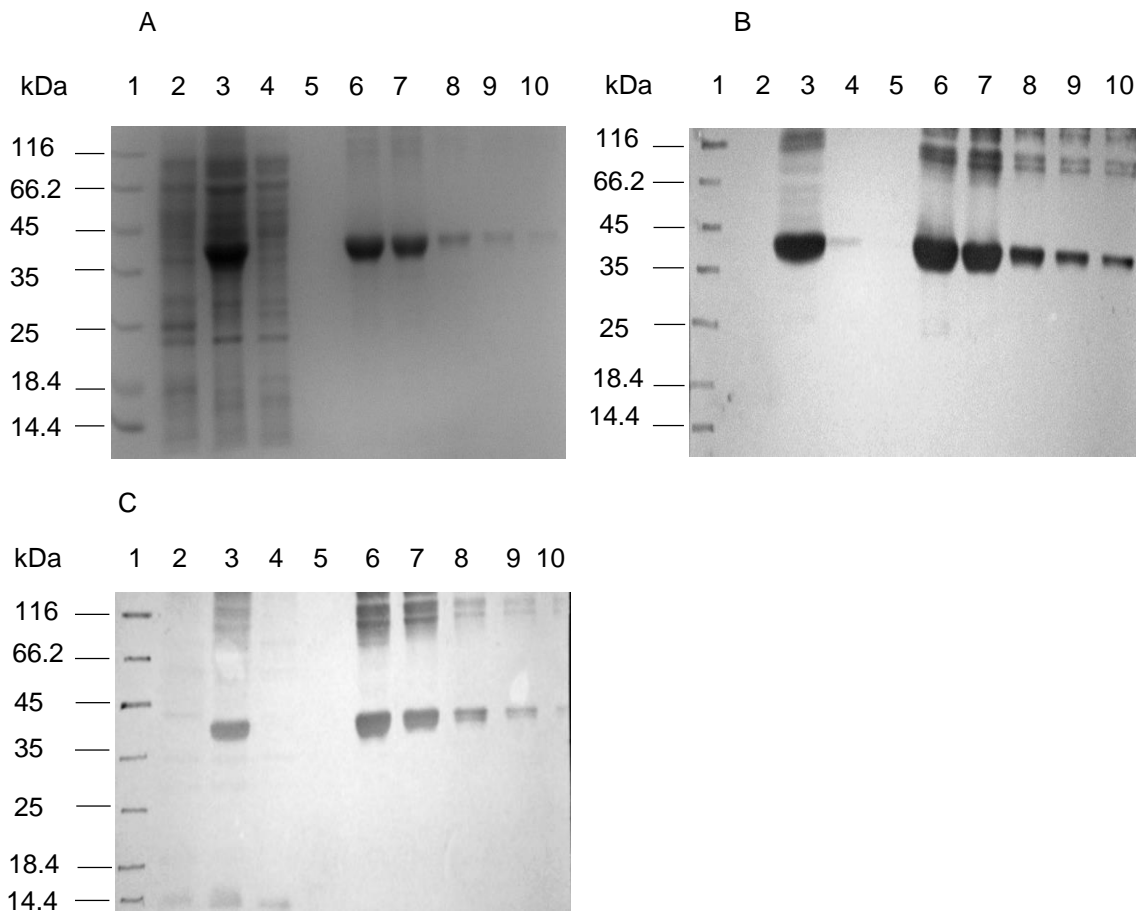


Figure 3.5: Recombinant expression and affinity purification of rPyLDH. Recombinant PyLDH was expressed in *E. coli* BL21 (DE3) cells grown in a terrific broth and affinity purified with a cobalt affinity matrix. Samples were analysed on and a 12.5% reducing SDS-PAGE gel (A) and Western blot (B and C). Lane 1: molecular weight marker; lanes 2-3: untransformed and transformed *E. coli* cells expressing PyLDH; lane 4: unbound proteins from the cobalt resin; lane 5: column wash. Lanes 6-10: rPyLDH eluted with 250 mM. The gel was stained with Coomassie brilliant blue R- 250. The Western blot was probed with mouse anti-Histag (B) and chicken anti-rPyLDH (C) antibodies.

3.2.6 Analysis of the purity of rPyGAPDH and rPyLDH

Following the affinity purification of recombinant proteins, we assessed the purity of proteins using an SDS-PAGE gel stained with silver, which detect 0.1 ng protein (Nesterenko *et al.*, 1994). Decreasing concentrations of rPyGAPDH and rPyLDH (200 ng – 5 ng) were analysed on an SDS-PAGE gel which was then stained with silver stain. The stain detected a single protein band of rPyGAPDH (Figure 3.6A) and rPyLDH (Figure 3.6B) which shows that the loaded protein was pure.

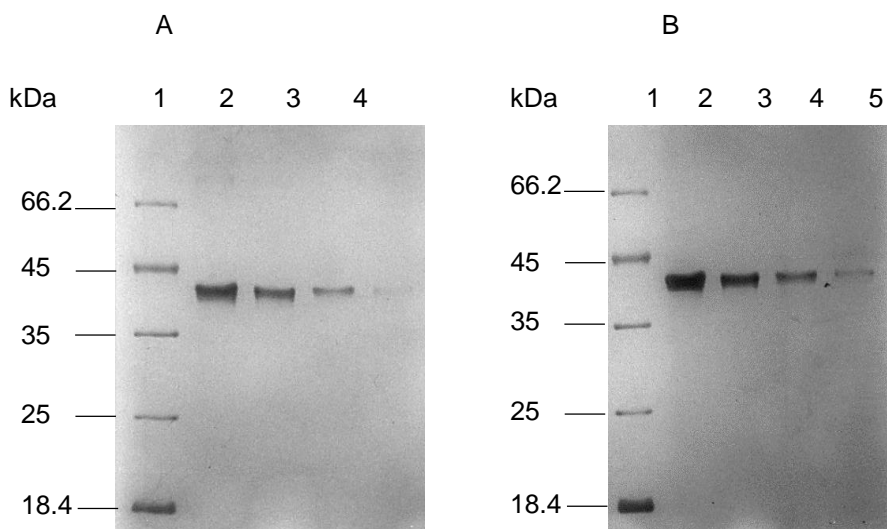


Figure 3.6: Assessment of the purity of affinity purified rPyGAPDH and rPyLDH with a silver stain. Recombinant PyGAPDH (A) and rPyLDH (B) were loaded on a 12.5% SDS-PAGE gel as follows: lane 1: molecular weight marker, lanes 2-5: 200 ng, 100 ng, 10 ng and 5 ng rPyGAPDH/rPyLDH. The gels were stained with silver stain.

3.2.7 Evaluation of the migration of rPyGAPDH and rPyLDH in presence of metal ions in SDS-PAGE gels made from different acrylamide concentrations

Proteins containing multiple histidine residues are reported to migrate slower on polyacrylamide gels and resolve with smaller sizes when treated with metal ions (Shakele *et al.*, 2017). Recombinant PyGAPDH and rPyLDH were assessed for metal ions binding as they contain a hexa-histidine tag. The recombinant proteins were incubated with CuCl₂, CoCl₂ and NiCl₂ and their migration was assessed on an SDS-PAGE gel (Figure 3.7). No differences in sizes were observed between untreated rPyGAPDH and rPyLDH and metal treated rPyGAPDH and rPyLDH (Figure 3.7A and B)

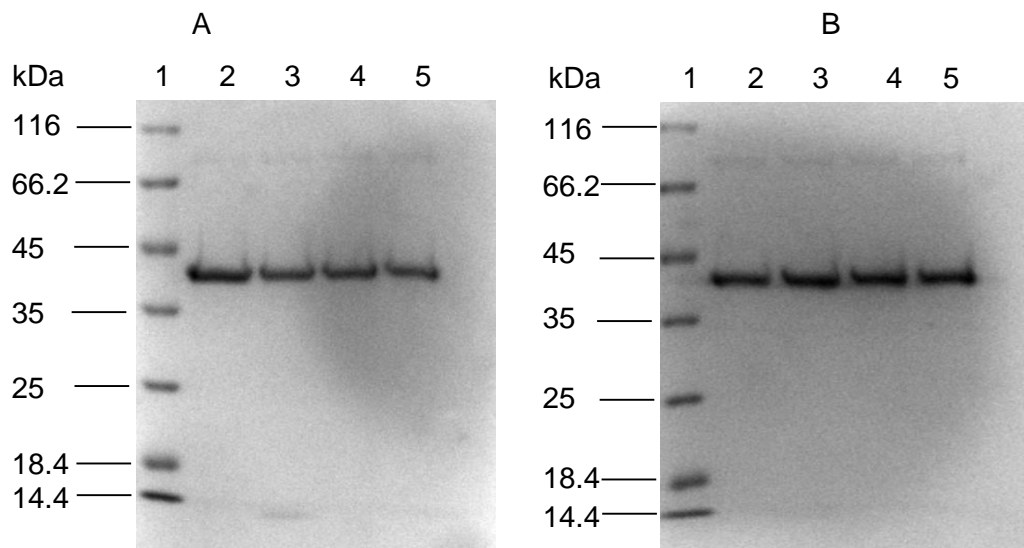


Figure 3.7: Effect of metals on rPyGAPDH and rPyLDH in SDS-PAGE gel. Recombinant PyGAPDH (A) and rPyLDH (B) were incubated in a 1:6 ratio with metal ions and resolved on a 12.5% reducing SDS-PAGE gel. Lane 1: molecular weight marker and lane 2: protein without metal ions. Lanes 3-5: proteins incubated with was CuCl_2 , CoCl_2 and NiCl_2 respectively. The gels were stained with Coomassie brilliant blue R-250.

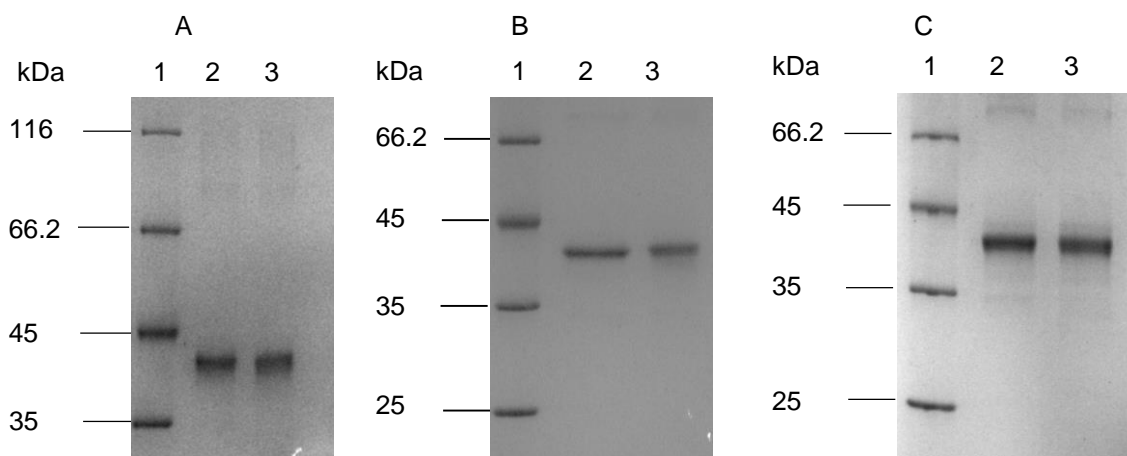


Figure 3.8: Evaluation of rPyGAPDH migration in different concentration of polyacrylamide gel. Recombinant PyGAPDH was resolved on a 10% (A), 12.5% (B) and 15% (C) SDS-PAGE gel. Lane 1: molecular weight marker, lane 2 and 3: reduced and non-reduced rPyGAPDH. The gels were stained with Coomassie brilliant blue R-250.

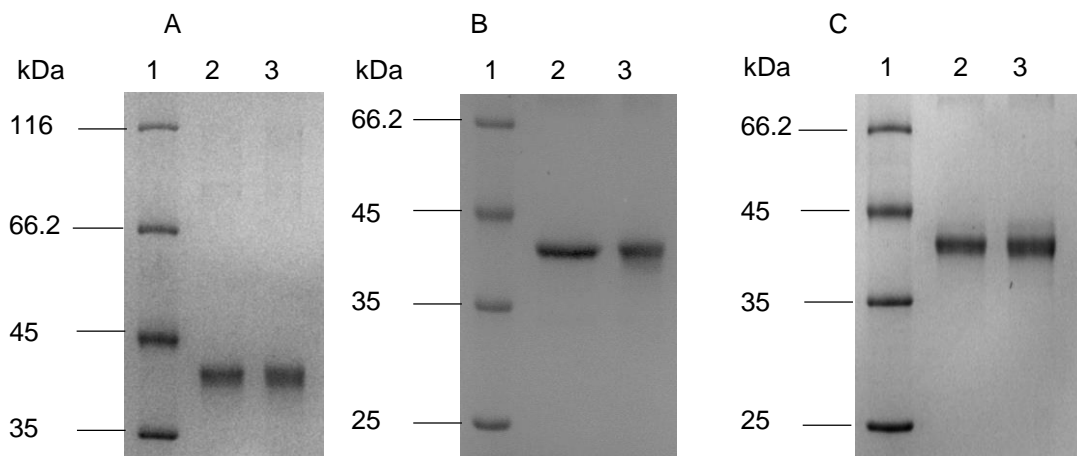


Figure 3.9: Evaluation of rPyLDH migration in different concentration of polyacrylamide gel. Recombinant PyLDH was resolved on a 10% (A), 12.5% (B) and 15% (C) SDS-PAGE gels. Lane 1: molecular weight marker, lane 2 and 3: reduced and non-reduced rPyLDH. The gels were stained with Coomassie brilliant blue R-250

3.2.8 Recombinant expression and affinity purification of rPflDH

Plasmodium falciparum lactate dehydrogenase (PflDH) was recombinantly expressed in *E. coli* BL21 (DE3) cells grown in a terrific broth. After expression of rPflDH, bacterial cells were lysed, and the soluble fraction was passed over a cobalt affinity column to purify rPflDH. Recombinant band was absent in the untransformed *E. coli* cells and the 33 kDa, 36 kDa, 75 kDa and 150 kDa rPflDH bands were present in the soluble fraction (Figure 3.10). Recombinant PflDH was eluted from the column with 250 mM imidazole and the resulting protein had four bands. The 36 kDa band is consistent with the size predicted from genomic and amino acid sequence of PflDH with the histidine tag. The 33 kDa is also a monomer of rPflDH. Dimer and trimer forms of rPflDH with sizes of 75 kDa and 150 kDa were observed (Figure 3.10A). All the forms of rPflDH were detected by anti-rPflDH and anti-Histag antibodies in a Western blot analysis (Figure 3.10B and C). A yield of 16.8 mg pure rPflDH was obtained from 0.7 g wet weight *E. coli* cell pellet.

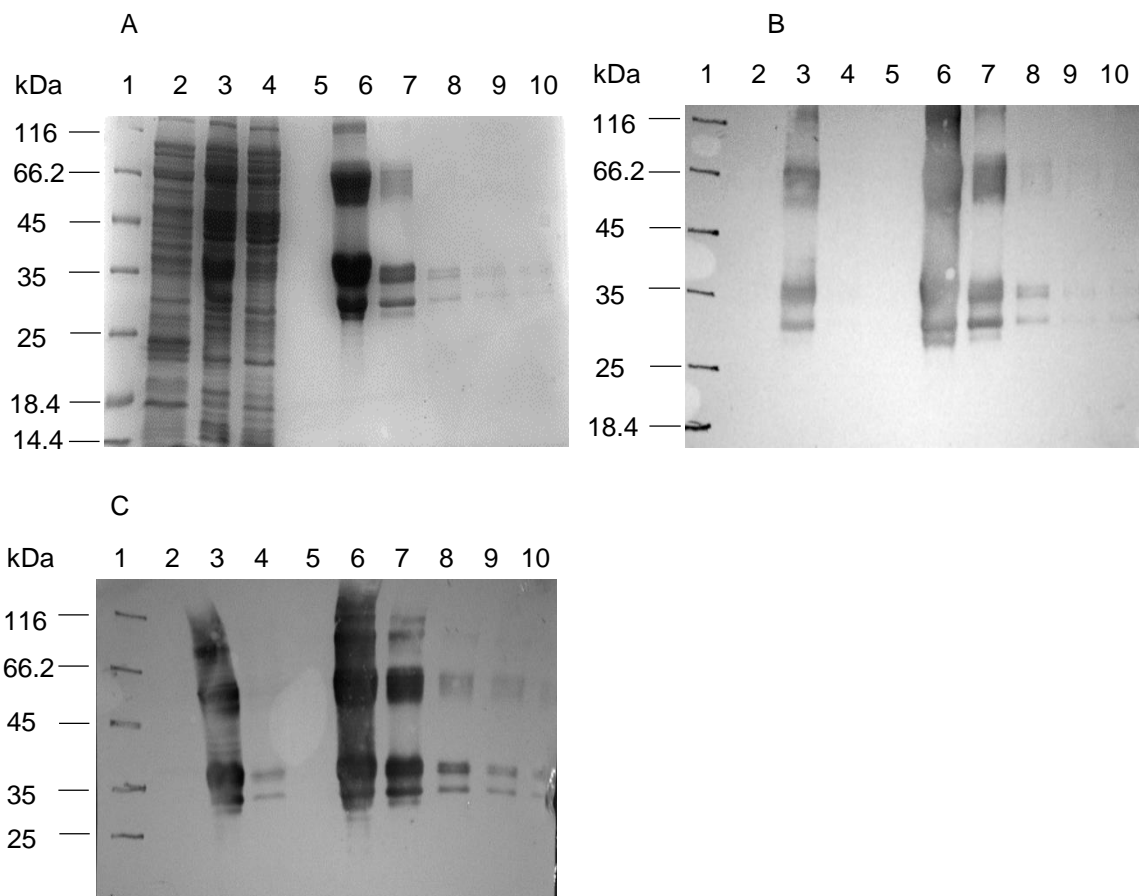


Figure 3.10: Recombinant expression and affinity purification of rPfLDH. Recombinant PfLDH was expressed in *E. coli* BL21 (DE3) cells grown in a terrific broth and affinity purified with a cobalt affinity matrix. Samples were analysed on and a 12.5% reducing SDS-PAGE gel (A) and Western blot (B and C). Lane 1: molecular weight marker; lanes 2-3: untransformed and transformed *E. coli* cells expressing PfLDH; lane 4: unbound proteins from the cobalt resin; lane 5: column wash. Lanes 6-10: rPfLDH eluted with 250 mM. The gel was stained with Coomassie brilliant blue R- 250. The Western blot was probed with chicken anti-rPfLDH antibodies (B) and mouse anti-Histag antibodies (C).

3.2.9 Purification of chicken antibodies against rPfLDH

Since two chickens were immunized with rPfLDH, the crude IgY isolated from both chickens were combined. The crude IgY was passed over an rPfLDH aminolink® column for affinity purification of anti-rPfLDH specific antibodies (Figure 3.11). A yield of 31 mg anti-rPfLDH antibodies was eluted from the column.

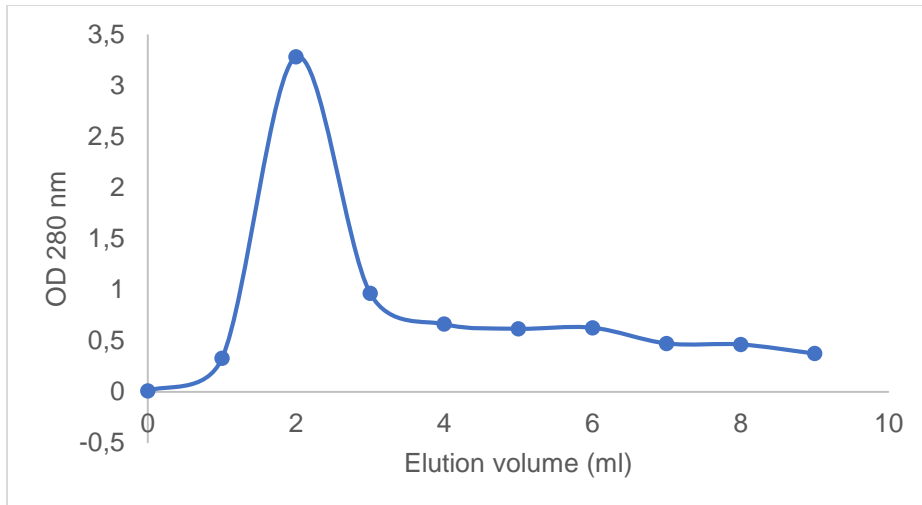


Figure 3.11: Elution profile after affinity purification of anti-*rPflDH* antibodies. Crude IgY was isolated from weeks following high anti-*rPflDH* antibody titres. Combined IgY from chicken 1 (week 1 to week 12) and chicken 2 (week 6 to week 12) were passed over an aminolink® column.

The affinity purified anti-*rPflDH* antibodies were assessed for the recognition of *rPflDH* on a Western blot (Figure 3.12). The chicken anti-*rPflDH* antibodies did not detect the *E. coli* proteins from untransformed *E. coli* bacterial lysate. The anti-*rPflDH* antibodies recognized *rPflDH* (Figure 3.12B). A high background signal was observed suggesting that the affinity purified anti-*rPflDH* antibodies has cross reacted with skimmed milk proteins.

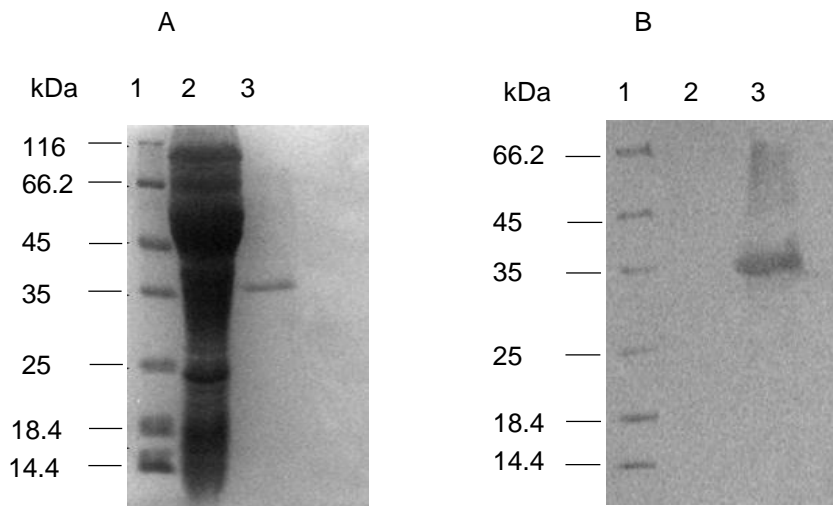


Figure 3.12: Evaluation of affinity purified chicken anti-*rPflDH* antibodies for *rPflDH* detection. Recombinant *PflDH* was resolved on a 12.5% reducing SDS-PAGE gel (A) and detected with chicken anti-*rPflDH* antibodies on a Western blot (B). Lane 1: molecular weight marker, lane 2: BL21 untransformed cells and lane 3: *rPflDH* (1 µg).

3.3 Discussion

3.3.1 Protein expression in the presence of ethanol

The recombinant expression of proteins is an important step to produce enough material for studying the protein of interest for vaccine or drug design or studying bacterial and parasitic infections like malaria (Sivashanmugam *et al.*, 2009). This study optimized the expression of high yields of *rPyGAPDH* and *rPyLDH* in *E. coli* bacterial cells by supplementing the growth media with ethanol. Ethanol has been described to enhance DNA synthesis in *E. coli* cells and consequently increasing the production of bacterial proteins (Basu & Poddar, 1994; Ingram & Buttke, 1985). Chhetri *et al* (2015) showed that the addition of 3% (v/v) ethanol in the growth media caused an increase of the expression of a wide range of yeast proteins. Our observations differed from the results by Basu & Poddar (1997) and Chhetri *et al* (2015). In the current study, ethanol decreased the expression of the recombinant proteins and the decrease was in a concentration-dependent manner (Figures 3.1 and Figure 3.2).

The decrease in recombinant protein expression by ethanol was explored by monitoring the growth of *E. coli* cells expressing both *PyLDH* and *PyGAPDH*. The inhibition of bacterial growth was proportional to the ethanol concentration. This suggests that ethanol impedes the cell growth leading to fewer cells expressing the recombinant proteins. Basu and Poddar (1997) found that high concentrations of ethanol reduced cell growth. They found that ethanol concentration above 5% (v/v) completely abolished the growth of *E. coli* cells (Basu & Poddar, 1997). Ethanol has been reported to instigate chemical stress in bacteria, which causes both the pH and temperature of the bacteria to increase to levels which kills the bacteria (Ingram & Buttke, 1985). Ingram and Buttke (1985) also reported that when the *E. coli* cells are grown in the presence of ethanol, their membrane lipid composition is altered, which is unfavourable for the survival of the bacteria. We suggest that the inhibition of *E. coli* bacterial growth resulted in fewer cells expressing both *PyLDH* and *PyGAPDH*.

3.3.2 Recombinant expression and affinity purification of *PyGAPDH*

Plasmodium yoelii GAPDH was recombinantly expressed in *E. coli* bacterial cells and purified using a cobalt affinity column. The expressed *rPyGAPDH* was soluble and the pure protein was obtained with an expected monomeric size of 40 kDa (Sangolgi, *et al.*, 2016; Krause *et al.*, 2017). A yield of 12.48 mg *rPyGAPDH* was obtained. This was higher compared to previous studies which obtained 8.95 mg pure *rPyGAPDH* per 50 ml culture using a cobalt affinity column (Krause *et al.*, 2017). Krause *et al* (2017) expressed *rPyGAPDH* at 30°C, while the current study expressed *rPyGAPDH* at 37°C. Higher protein yields are reported with higher

expression temperatures in *E. coli* cells (Rosano & Ceccarelli, 2014). However, high growth temperatures have been shown to result in the reduction of enzyme activity (de Groot & Ventura, 2006). Recombinant *PyGAPDH* activity was not measured here and we cannot rule out the possibility that the protein may have reduced activity.

3.3.3 Recombinant expression and purification of *PfLDH* and *PyLDH*

Plasmodium falciparum LDH was recombinantly expressed in *E. coli* cells and affinity purified. Recombinant *PfLDH* was obtained as two monomeric proteins of sizes 36 kDa and 33 kDa, a 75 kDa dimer and a 150 kDa tetramer (Figure 3.10). These sizes of *rPfLDH* were also observed in other studies (Berwal *et al* 2008; Turgut-Balik *et al.*, 2001). The *PfLDH* sequence has an internal methionine at position 19, which results in the production of two sizes of the protein, a 36 kDa full *rPfLDH* protein and truncated 32 kDa *rPfLDH* (Berwal *et al* 2008; Turgut-Balik *et al.*, 2001). *Plasmodium yoelii* LDH was recombinantly expressed and purified as a single 37 kDa protein band (Figure 3.5). Unlike *PfLDH*, *PyLDH* does not have an internal methionine, therefore it purifies as a single monomeric protein. The size of *PyLDH* was also comparable to previous studies which obtained a 39 kDa protein band (Hurdayal *et al.*, 2010). Recombinant *PyLDH* was also expressed and purified as a truncated protein with lower concentration. The truncated *rPyLDH* proteins were recognised by antibodies in a Western blot. This was also demonstrated by Hurdayal *et al* (2010). The yields of *rPfLDH* and *rPyLDH* in 50 ml cultures were 16.8 mg and 15.3 mg respectively. Previous studies obtained 15 mg of *rPfLDH* (Turgut-Balik *et al.*, 2001) and 12 mg of *rPyLDH* in 1-litre culture (Hurdayal *et al.*, 2010). The yields of *rPfLDH* and *rPyLDH* in the current study is converted to 336 mg and 306 mg in 1-litre cultures respectively, which is high compared to previous studies. The dimer and tetramer form of *rPfLDH* were not expected. They could be due to incomplete denaturation of the protein. Zhang *et al.*, 2019 reported that residual sizes of proteins including antibodies are observed when the proteins are not boiled at optimal temperatures and boiling time.

3.3.4 The influence of acrylamide concentrations and metal ions on protein migration in SDS-PAGE gels.

SDS-PAGE gels are widely used to evaluate the size of proteins (Rath *et al.*, 2013). Proteins with helical 3-dimensional structures migrate with lower sizes on SDS-PAGE gels (Rath *et al.*, 2013). These proteins include a 39 kDa bovine rhodopsin, which resolves as a 30 kDa on SDS-PAGE gels. Shelake *et al* (2017) reported a *Helicobacter pylori* (Hpn) protein that migrated faster following the binding to nickel ions, which could be followed by polyacrylamide

electrophoresis. The influence of copper, cobalt and nickel on *rPyGAPDH* and *rPyLDH* migration on SDS-PAGE gel was evaluated (Figure 3.7A and B); the metals did not affect protein migration. Acrylamide concentrations between 4-20% are commonly used for SDS-PAGE gels (Rath *et al.*, 2013). The influence of acrylamide concentrations on protein migration was evaluated. Both reduced and non-reduced forms of *rPyGAPDH* and *rPyLDH* migrated in a similar manner in different acrylamide concentrations (Figure 3.8A, B and C and Figure 3.9A, B and C). Unreduced proteins have been reported to migrate faster on SDS-PAGE gels, their fast migration is the consequence of sodium dodecyl sulfate (SDS) binding better to their compact structures with disulfide bonds (Rath *et al.*, 2013). This was not observed with unreduced *rPyGAPDH* and *rPyLDH*.

3.3.5 Production of chicken antibodies against *rPfLDH*

Plasmodial lactate dehydrogenase (pLDH) has been discovered to be an efficient target of malaria rapid diagnostic tests (RDTs) (Piper *et al.*, 1999). Mouse IgG antibodies are commonly used in RDTs (Moody, 2002). These antibodies have been shown to be unstable at high temperatures (Chiodini *et al.*, 2007). Chickens antibodies (IgY) have been observed to be effective for use in immunotherapies and immunodiagnosics (Gassmann *et al.*, 1990). This study produced chicken antibodies against *rPfLDH*. The IgY antibodies were functional in a Western blot and recognised *rPfLDH* and none of *the E. coli* proteins. A yield of 31 mg anti-*rPfLDH* antibodies were obtained from an aminolink® column.

Chapter 4

Evaluation of polymorphisms *Plasmodium* GAPDH and pLDH

4.1 Introduction

4.1.1 Polymorphisms in *Plasmodium* parasites

Malaria is a deadly disease that claims thousands of lives every year, particularly in sub-Saharan Africa. *Plasmodium falciparum* is the most virulent form of malaria and results in the majority of deaths in humans. *P. falciparum* has been studied and the most extensive genetic variation has been described in different strains and isolates from all over the world (Bolad & Berzins, 2000). Genetic diversity is particularly prevalent in regions of high malaria transmission (Nkhoma *et al.*, 2013). Genetic variation of *Plasmodium* parasites increases the ability of the parasites to attack the host immune system, creating a challenge to produce an immune response against many variants of the parasites (Ferreira *et al.*, 2004). There have been many efforts to develop malaria vaccines that could aid in the eradication of the disease. There is still no blood-stage malaria vaccine that prevents clinical symptoms and deaths due to the disease (Cockburn & Seder, 2018). The main contributing factor for this is a substantial genetic variation that exists in the parasite (Takala *et al.*, 2009).

4.1.2 Effects of polymorphisms in protein sequences of malaria vaccine candidates

Plasmodium falciparum proteins that have the greatest polymorphisms are the antigens that are expressed on the surface of infected erythrocytes and these antigens are mostly vaccine candidates (Nkhoma *et al.*, 2013). Merozoite surface protein (MSP1, MSP2) and glutamate-rich protein (GLURP) (Mohammed *et al.*, 2018; Pattaradilokrat *et al.*, 2018) and apical membrane protein (AMA1) (Takala *et al.*, 2009) are the leading blood-stage malaria vaccine candidates and are all reported to have a high degree of polymorphisms. Polymorphism results in an induction of strain-specific antibodies that are not enough to clear all the parasites. As a result, the antigens with polymorphism produce low efficacious vaccines and none of the blood stage malaria vaccines has progressed to phase II in human clinical trials (Cockburn & Seder, 2018).

4.1.3 Effects of polymorphisms in the protein sequences of malaria diagnostic targets for RDTs

Polymorphisms have been reported to influence the performance of rapid diagnostic tests (RDTs). *Plasmodium falciparum* histidine-rich protein (*PfHRP-2*) and *Plasmodium* lactate dehydrogenase (pLDH) are the common proteins that are targeted by RDTs that detect malaria infections (Moody, 2002). *Plasmodium* LDH is the most reliable antigen for RDTs as it is crucial for the parasite survival, present at high concentrations during plasmodial infections and the protein clears from the blood at the same time as parasites (Oduola *et al.*, 1997). A study performed by Baker *et al* (2005) revealed that genetic polymorphism in *PfHRP-2* results to the reduction of the sensitivity of the RDTs detecting it. Polymorphism has been identified in *PfLDH* in Thailand (Simpalipan *et al*, 2018) and *PfLDH* in Indian isolates (Keluskar *et al.*, 2014); these SNPs were observed to not influence the protein structure therefore, unlikely to influence the performance of pLDH base RDTs.

4.1.4 Aims of this chapter

Plasmodium lactate dehydrogenase (pLDH) and *Plasmodium* glyceraldehyde-3-phosphate dehydrogenase are both enzymes of the glycolytic pathway. The glycolytic pathway is crucial in *Plasmodium* parasites to produce energy for survival inside the host erythrocytes (Roth *et al.*, 1988). This study investigates the polymorphism in *P. falciparum* and *P. vivax* LDH and examines if the same polymorphisms are present in rodent plasmodial LDH including *P. yoelii* LDH. Polymorphisms on *P. falciparum* GAPDH are also analysed and investigated if they are present in rodent plasmodial GAPDH.

4.2 Results

4.2.1 Investigation of the polymorphisms shared by *PfLDH* with lactate dehydrogenases from rodent plasmodia species.

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KU869747.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
KX885919.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
KX885920.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
KX885921.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
EU330208.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
KX885922.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
KX885918.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
KU869736.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600
JN547219.1 GCTCATGGAATAAAAACGGTTCCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTA 600

KU869763.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KU869742.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KU869762.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KU869738.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KX885915.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KU869753.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KU869746.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
PF3D7_1324900 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KU869747.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KX885919.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KX885920.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KX885921.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
EU330208.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
KX885922.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660

KX885918.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
 KU869736.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660
 JN547219.1 CAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAATTAGAAGCTATATTTGATAGA 660

KU869763.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869742.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869762.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869738.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KX885915.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869753.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869746.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 JN547218.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 PF3D7_1324900 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869747.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KX885919.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KX885920.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KX885921.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 EU330208.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KX885922.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KX885918.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 KU869736.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720
 JN547219.1 ACTGTTAATACTGCATTAGAAATTGTAACTTACATGCATCACCATATGTTGCACCAGCT 720

KU869763.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869742.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869762.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869738.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KX885915.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869753.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869746.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 JN547218.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 PF3D7_1324900 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869747.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KX885919.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KX885920.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KX885921.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 EU330208.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KX885922.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KX885918.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 KU869736.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780
 JN547219.1 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAGTATTAATTTGC 780

KU869763.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869742.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869762.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869738.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KX885915.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869753.1 TCAACCTTGTCAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869746.1 TCAACCTTGTCAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 PF3D7_1324900 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869747.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KX885919.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KX885920.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KX885921.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 EU330208.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KX885922.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KX885918.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 KU869736.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840
 JN547219.1 TCAACCTTGTTAGAAGGACAATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTT 840

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KU869763.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KU869742.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KU869762.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KU869738.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885915.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAGATAGTGAGGAAAAAGCT  900
KU869753.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KU869746.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
PF3D7_1324900  TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KU869747.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885917.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885919.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885920.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885921.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
EU330208.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885922.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KX885918.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
KU869736.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
JN547219.1      TTAGGTGCTAATGGTGTGAACAAGTTATCGAATTACAATTAATAAGTGAGGAAAAAGCT  900
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KU869763.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869742.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869762.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869738.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KX885915.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869753.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869746.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
PF3D7_1324900  AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869747.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KX885919.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KX885920.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KX885921.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
EU330208.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KX885922.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KX885918.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
KU869736.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
JN547219.1      AAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA  951
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Figure 4.1: Alignment of *Plasmodium falciparum* lactate dehydrogenase (*PfLDH*) from patients isolates against the 3D7 strain (PF3D7_1324900). The *PfLDH* from clinical isolates were obtained from PubMed and the 3D7 sequence was obtained from PlasmoDB and they were aligned using Clustal Omega.

The 951 bp DNA sequence of *Plasmodium falciparum* lactate dehydrogenase (*PfLDH*) translates to a protein sequence with 316 amino acid residues. Single nucleotide polymorphisms (SNPs) were investigated on *PfLDH* DNA sequences. Fifty-four sequences were obtained from PubMed and PlasmoDB and aligned using Clustal Omega (Appendix 1) and only 16 of them contained SNPs (Figure 4.1). Nineteen SNPs were identified (Figure 4.1). Nine of the SNPs detected at nucleotide positions 15 (GCA/GCG), 39 (GGT/GGC), 136 (GCT/GCC), 513 (CCA/CCG), 540 (GGT/GGC), 546 (CAT/CIT), 759 (GAT/GAC), 758 (GGA/GGG) and 858 (GTT/GTC) resulted in synonymous mutations at amino acid residues 5 (A), 13 (G), 45 (A), 171 (P), 180 (G), 265 (G) and 286 (V) respectively. Ten SNPs detected at nucleotide positions 143 (ACA/ATA), 334 (CAT/IAT), 361 (ITT/CTT), 557 (AIG/ACG), 571 (AGA/GGA), 711

(GCA/ACA), 770 (GTA/GAA), 791 (TTA/TCA) and 873 (AAT/GAT) resulted in non-synonymous mutations at amino acid residues 48 (T/I), 112 (H/I), 121 (F/P), 186 (M/T), 191 (R/G), 238 (A/T), 257 (I/T) and 263 (L/S) respectively. The SNP at 873 (AAT/GAT) did not correspond to any amino acid in the protein sequence. All the SNPs only occurred in (1/54) sequences except for the SNP at 791 codon which occurred in 2 sequences (2/54). Only two *PfLDH* SNPs are documented on PlasmoDB and they occur at positions 21 (C/T) and 272 (G/D) and the sequences containing these SNPs are not available on PubMed databases. Simpalian *et al* (2018) identified 7 *PfLDH* non-synonymous SNPs that were not observed from sequence alignments. These SNPs were located at positions 25 (Q/K), 29 (G/R), 87 (G/R), 136 (L/S), 151 (G/R), 187 (V/G) and 188 (L/P). Therefore, *PfLDH* has a total of 18 non-synonymous SNPs.

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PF3D7_1324900      MAPPAKIVLVGSGMIGGVMATLIVQNNLGDVVLFDIVKNMPHGALDTSHTNVMAYSNC 60
PCHAS_1344700.1   MAPPAKIVLVGSGMIGGVMATLIVQNNLGDVVMFDIVKNDMPHGALDTSHTNVMAYSNC 60
PBANKA_1340100.1  MAPPAKIVLVGSGMIGGVMATLIVQNNLGDVVMFDIVKNMPHGALDTSHTNVMAYSNC 60
PY17X_1344800.1  MAPPAKIVLVGSGMIGGVMATLIVQNNLGDVVLFDIVKNMPHGALDTSHTNVMAYSNC 60
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

PF3D7_1324900      VSGSNTYDDLGADVVIVTAGFTAPGKSDKEWNRDDLLPLNNIMIEIGGHIKKNCPNA 120
PCHAS_1344700.1   VSGSNTYDDLGADVVIVTAGFTAPGKSDKEWNRDDLLPLNNIMIEIGGHIKKHCPHA 120
PBANKA_1340100.1  VSGSNTYDDLGADVVIVTAGFTAPGKSDKEWNRDDLLPLNNIMIEIGGHIKKNCPNA 120
PY17X_1344800.1  VSGSNTYDDLGADVVIVTAGFTAPGKSDKEWNRDDLLPLNNIMIEIGGHIKKNCPNA 120
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

PF3D7_1324900      FIIVVTNPVDVMVQLLHQHSGVPNKIIGLGGVLDTSRLKYYISQLNVCPRDVNAHIVG 180
PCHAS_1344700.1   FIIVVTNPVDVMVQLLHQHSGVPNKIIGLGGVLDTSRLKYYISQLNVCPRDVNAHIVG 180
PBANKA_1340100.1  FIIVVTNPVDVMVQLLHQHSGVPNKIIGLGGVLDTSRLKYYISQLNVCPRDVNAHIVG 180
PY17X_1344800.1  FIIVVTNPVDVMVQLLHQHSGVPNKIIGLGGVLDTSRLKYYISQLNVCPRDVNAHIVG 180
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

PF3D7_1324900      AHGNMVLLKRYITVGGIPLQEFINNKILISDAELEAIFDRTVNTALEIVNLHASPYVAPA 240
PCHAS_1344700.1   AHGNMVLLKRYITVGGIPIQEFINNKIISDQLEAIFDRTINTALEIVNLHASPYVAPA 240
PBANKA_1340100.1  AHGNMVLLKRYITVGGIPLQEFINNKIITDQELDAIFDRTINTALEIVNLHASPYVAPA 240
PY17X_1344800.1  AHGNMVLLKRYITVGGIPLQEFINNKIITDQELDAIFDRTVNTALEIVNLHASPYVAPA 240
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

PF3D7_1324900      AAIIEMAESYLDLKVLICSTLEGQYGHSDIFGGTPVLGANGVEQVIELQLNSEEKA 300
PCHAS_1344700.1   AAIIEMAESYIRDLRKVLICSTLEGQYGHKDIFAGTPLVIGGNGVEQVIELQLNADEKK 300
PBANKA_1340100.1  AAIIEMAESYIRDLRKVLICSTLEGQYGHKDIFAGTPLVIGGNGVEQVIELQLNADEKK 300
PY17X_1344800.1  AAIIEMAESYIRDLRKVLICSTLEGQYGHKDIFAGTPLVIGGNGVEQVIELQLNADEKK 300
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

PF3D7_1324900      KFDEAIAETSRMALA 316
PCHAS_1344700.1   KFDEAVAETSRMALV 316
PBANKA_1340100.1  KFDEAVAETSRMALI 316
PY17X_1344800.1  KFDEAVAETSRMALI 316
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:

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Figure 4.2: Alignment of *PfLDH* (3D7_1324900) amino acid sequence with *PcLDH* (PCHAS_1344700.1), *PbLDH* (PBANKA_1340100.1) and *PyLDH* (PY17X_1344800.1). The positions of SNPs are underlined, and lysine residues are highlighted.

Many of the *PfLDH* sequences did not have SNPs including PF3D7_1324900 (Figure 4.1). This sequence (3D7) was used as a reference to examine whether the *PfLDH* SNPs are

found in rodent malaria LDH. The *Pf*LDH sequence was aligned with LDH sequences from *P. berghei* (*Pb*LDH), *P. chabaudi* (*Pc*LDH) and *P. yoelii* (*Py*LDH) (Figure 4.2). Two non-synonymous SNPs at positions 21 (C/T) and 272 (G/D) are conserved between *Pf*LDH, *Pb*LDH, *Pc*LDH and *Py*LDH.

Lysine residues play a crucial role in the protein structure and are the regions of post-translational modification. This study investigated the lysine residues on *Pf*LDH and compared them with rodent plasmodial species. A total of 26 lysine residues are found in *Pf*LDH sequence and 21 lysine residues are conserved amongst all four *p*LDH sequences (Figure 4.2). Four lysine residues are conserved amongst *Pb*LDH, *Pc*LDH and *Py*LDH but not found in *Pf*LDH. These lysine residues are at amino acid position 71, 208, 271 and 300 and are replaced by alanine, leucine, serine and alanine in the *Pf*LDH sequence respectively. Three lysine residues located at position 252, 255 and 310 are unique in *Pf*LDH. Lysine-252 and 255 are replaced by arginine which is conserved in all three-rodent *Plasmodium* LDH and lysine-300 is replaced with serine which is also conserved in all three-rodent plasmodia species. Lysine-115 is only found in *Pf*LDH and *Pc*LDH and is replaced with asparagine both in *Pb*LDH and *Py*LDH. Lysine-60 is replaced by glutamine in *Pc*LDH sequence but conserved between *Pf*LDH, *Pb*LDH and *Py*LDH. None of the lysine residues is polymorphic (Figure 4.2).

4.2.2 Investigation of the polymorphisms in *Pf*LDH DNA sequences

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JX865771.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC 60
PVX_116630.1    ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547223.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
KM226653.1      -----AAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
KM226660.1      -----AAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547224.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547222.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547226.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547221.0      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGAGGGGCC
FJ527750.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
DQ060151.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JX872280.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547225.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547220.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCC
JN547221.1      ATGACGCCGAAACCCAAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGAGGGGCC
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JX865771.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG 120
PVX_116630.1    ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
JN547223.1      ACGCTGATTGTGCAGAAGAACCTGCGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
KM226653.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
KM226660.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
JN547224.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
JN547222.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
JN547226.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
JN547221.0      ACGCTGATTGTGCAGAAGAACCTGTGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
FJ527750.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
DQ060151.1      ACGCTGATTGTGCAGAAGAACCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATG
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JX872280.1 AC^GCTGATTGTGCAGAAGAACTGGGGGACGTAGTGTGTTGACGTAGTGAAAAACATG
JN547225.1 AC^GCTGATTGTGCAGAAGAACTGGGGGACGTAGTGTGTTGACGTAGTGAAAAACATG
JN547220.1 AC^GCTGATTGTGCAGAAGAACTGGGGGACGTAGTGTGTTGACGTAGTGAAAAACATG
JN547221.1 AC^GCTGATTGTGCAGAAGAACTGTGGGACGTAGTGTGTTGACGTAGTGAAAAACATG

JX865771.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG 180
PVX_116630.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547223.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
KM226653.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
KM226660.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547224.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547222.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547226.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547221.0 CCCCAGGAAAGGCAC^TAGATATGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
FJ527750.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
DQ060151.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JX872280.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547225.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
JN547220.1 CCCCAGGAAAGGCAC^TAGATACGTCTCACTCGAATGTGATGGCTTATTCCAATTGCAAG
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JN547221.0 GTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGTATCGTCACTGCG
FJ527750.1 GTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGTATCGTCACTGCG
DQ060151.1 GTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGTATCGTCACTGCG
JX872280.1 GTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGTATCGTCACTGCG
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PVX_116630.1    TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
JN547223.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
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JN547226.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
JN547221.0      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
FJ527750.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
DQ060151.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
JX872280.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
JN547225.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
JN547220.1      TCCACTCTACTAGAGGGACAATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTT
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JN547223.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACT
KM226653.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
KM226660.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGATC
JN547224.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JN547222.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JN547226.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JN547221.0      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
FJ527750.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
DQ060151.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JX872280.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JN547225.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JN547220.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
JN547221.1      ATCGGGGGCACC GGAGTTGAGCAAGTCATCGAGTTGCAGCTGAATGCCGAGGAGAAGACC
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JN547223.1      AAGTTCGACGAGGCAGTTGCGGAAACTAAAAGGATGAAGGCGCTCATTTAA----
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JN547224.1      AAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA----
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DQ060151.1      AAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA----
JX872280.1      AAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA----
JN547225.1      AAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA----
JN547220.1      AAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA----
JN547221.1      AAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA----
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Figure 4.3: DNA sequence alignment of *Plasmodium vivax* lactate dehydrogenase from Cheorwon 4 isolate (JX865771.1) with PvLDH sequences from other strains and field isolates. The sequences were obtained from PubMed and PlasmoDB and aligned using Clustal Omega. SNP's are highlighted and underlined.

SNPs were investigated in *PvLDH* DNA sequences. *P. vivax* isolates Bucheon 4 (JX872279.1), Bucheon 2 (JX872278.1), Bucheon 1 (JX872277.1), Gangwha 4 (JX872276.1), Gangwha 3 (JX872275.1), Paju 3 (JX865774.1), Gimpo 4 (JX865779.1), Gimpo 3 (JX865778.1), Gimpo 2 (JX865777.1), Gimpo 1 (JX865776.1), Paju 4 (JX865775.1), Paju 1 (JX865772.1), Cheorwon 4 (JX865771.1), Cheorwon 3 (JX865770.1), Cheorwon 2 (JX865769.1) and Cheorwon 1 (JX865768.1) are identical and contain no SNPs. The *PvLDH* sequence from Cheorwon 4 isolate (JX865771.1) was used as a reference to identify SNPs from other *PvLDH* (Figure 4.3). Nineteen SNPs were detected at nucleotide positions 56 (ATG/AGG), 85 (GGG/GTC/GCT), 143 (ACG/ATG), 233 (GTC/GCC), 242 (GGA/GAA), 265 (AGC/GGC), 313 (ATT/ITT), 341 (AAG/ATG), 348 (TGC/CGC), 358 (GCC/ACC), 374 (GTG/GAG), 407 (CTC/CCC), 479 (AAA/ATA), 558 (ATG/ATA), 635 (GAA/GGA), 677 (TIG/TCG), 754 (AAG/GAG), 899 (ACC/ATC) and 935 (ATG/AAG) resulted in non-synonymous mutations at position 19 (M/R), 29 (G/V/A), 48 (T/M), 78 (V/A), 81 (G/K), 87 (S/G), 105 (I/F), 114 (K/M), 117 (C/R), 118 (A/N), 124 (V/E), 135 (L/P), 159 (K/I), 186 (M/I), 212 (K/G), 226 (L/C), 251 (K/G), 300 (T/I) and 311 (M/K). Five SNPs were detected at nucleotide positions 450 (TTA/TTG), 456 (GGT/GGC), 510 (TGC/TGT), 666 (GTG/GTC) and 696 (CTT/CTC) resulted in synonymous mutations at amino acid residue 138 (G), 150 (L), 152 (G), 170 (C), 222 (V) and 232 (L) respectively (Figure 4.4). The synonymous SNPs at 414 (GAG/GAA) and 924 (GAG/GAA) did not correspond with glycine in the protein sequence. All the SNP's only occurred in 1/30 and 2/30 sequences except for the SNP at 666 which occurred in 11/30 sequences. JN547221.1, JN547221.0, JN54722.1 sequence have the highest number of SNPs while KM2226660.1, KM226653.1 and JN547220.1 had only 1 SNP per sequence (Figure 4.3).

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JX865771.1      MTPKPIIVLVGSGMIGGVMATLIVQNLGDVVMFDVVRNMPQGGKALDTSHSNVMAYSNC 60
PCHAS_1344700.1 MAPKAIIVLVGSGMIGGVMATLIVQNLGDVVMFDIVRDMPHGKALDTSHTNVMAYSNCQ 60
PBANKA_1340100.1 MAPKAIIVLVGSGMIGGVMATLIVQNLGDVVMFDIVRDMPHGKALDTSHTNVMAYSNC 60
PY17X_1344800.1 MAPKAIIVLVGSGMIGGVMATLIVQNLGDVVLFDIVRDMPHGKALDTSHTNVMAYSNC 60
*:** *****:***:***:***:*****:*****:

JX865771.1      VTGSNSYDDLKGADVIVTAGFTKAPGSDKEWNRDLDLPLNKKIMIEIGGHIKLNCPNA 120
PCHAS_1344700.1 VGSNTYDDLKGADVIVTAGFTKAPGSDKEWNRDLDLPLNKKIMIEIGGHIKLNCPHA 120
PBANKA_1340100.1 VGSNTYDDLKDADVIVTAGFTKAPGSDKEWNRDLDLPLNKKIMIEIGGHIKNNCPNA 120
PY17X_1344800.1 VGSNTYDDLKDADVIVTAGFTKAPGSDKEWNRDLDLPLNKKIMIEIGGHIKNNCPNA 120
*:***:*****.*****:*****:*****:*****:*****:

JX865771.1      FIIIVTNPVDVMVQQLLFEHSGVPKNIIVGLGGVLDTSRLKYYISQNLNVCPRDVNAHIVG 180
PCHAS_1344700.1 FIIIVTNPVDVMVQQLLHQHSGVPKNIIVGLGGVLDTSRLKYYISQNLNVCPRDVNAHIVG 180
PBANKA_1340100.1 FIIIVTNPVDVMVQQLLHQHSGVPKNIIVGLGGVLDTSRLKYYISQNLNVCPRDVNAHIVG 180
PY17X_1344800.1 FIIIVTNPVDVMVQQLLHQHSGVPKNIIVGLGGVLDTSRLKYYISQNLNVCPRDVNAHIVG 180
*****:*****:*****:*****:*****:

JX865771.1      AHGKIMVLLKRYITVGGIPLQEFINNKIITDDEEVEGIFDRTVNTALEIVNLLASPYVAPA 240
PCHAS_1344700.1 AHGKIMVLLKRYITVGGIPIQEFINNKIISDQDLEAIFDRTINTALEIVNLHASPYPVAPA 240
PBANKA_1340100.1 AHGKIMVLLKRYITVGGIPLQEFINNKIITDQELDAIFDRTINTALEIVNLHASPYPVAPA 240

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PY17X_1344800.1  AHGNKMVLLKRYITVGGIPLQEFINNKITDQELDAIFDRTVNTALEIVNLHASPYVAPA 240
*****:*****:***:..*****:***** *****

JX865771.1      AAIIEMAESYLKDIKVLVLCSTLLEGQYGHNSNIFGGTPLVIGGTGVEQVIELQLNAEKT 300
PCHAS_1344700.1 AAIIEMAESYIRDLRVLICSTLLEGQYGHKDIFAGTPLVIGGNGVEQVIELQLNADEKK 300
PBANKA_1340100.1 AAIIEMAESYIRDLRVLICSTLLEGQYGHKDIFAGTPLVIGGNGVEQVIELQLNADEKK 300
PY17X_1344800.1 AAIIEMAESYIRDLRVLICSTLLEGQYGHKDIFAGTPLVIGGNGVEQVIELQLNADEKK 300
*****:***:***:*****:..**.******.*****:***.

JX865771.1      KFDEAVAETKRMKALI 316
PCHAS_1344700.1 KFDEAVAETSRMKALV 316
PBANKA_1340100.1 KFDEAVAETSRMKALI 316
PY17X_1344800.1 KFDEAVAETSRMKALI 316
*****.******:

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Figure 4.4: Alignment of *Pv*LDH (JX865771.1) amino acid sequences with *Pc*LDH (PCHAS_1344700.1), *Pb*LDH (PBANKA_1340100.1) and *Py*LDH (PY17X_1344800.1). The positions of SNPs on *Pv*LDH sequence are underlined and lysine residues are highlighted.

The *Pv*LDH sequence from Cheorwon 4 isolate (JX865771.1) was aligned with *Pb*LDH, *Py*LDH and *Pc*LDH to investigate whether the SNPs of *Pv*LDH are found in rodent plasmodial sequences (Figure 4.4). The DNA sequences of *Pv*LDH and *Pb*LDH shows that only the synonymous SNP at position 666 is conserved between *Pv*LDH and *Pb*LDH. This SNP results in valine residue at amino acid position 222 and is not found in *Py*LDH and *Pc*LDH sequences. All the non-synonymous substitutions that occurred in *Pv*LDH sequences did not occur in *Pb*LDH, *Py*LDH and *Pc*LDH (Figure 4.4).

Lysine residues in *Pv*LDH sequence were evaluated and 27 lysine residues were identified and only 23 are conserved between *Pv*LDH, *Pb*LDH *Pc*LDH and *Py*LDH (Figure 4.4). Lysine-252, 255 and 310 are unique in *Pv*LDH sequence. Lysine-252 and 255 are replaced by arginine residues and lysine-310 is replaced by serine residues in *Pb*LDH, *Pc*LDH and *Py*LDH. Lysine-271 and 300 are conserved in rodent plasmodial species and replaced by serine and threonine in *Pv*LDH respectively. Lysine-115 is only found in *Pc*LDH and it is replaced by asparagine in *Pv*LDH, *Pb*LDH and *Py*LDH. Lysine-60 is conserved between *Pv*LDH, *Pb*LDH and *Py*LDH and replaced by glutamine in *Pc*LDH. Lysine-114 is mutated to methionine in *Pv*LDH sequences and this mutation does not occur in rodent plasmodial LDH sequences (Figure 4.4).

4.2.3 Investigation of the polymorphisms in *Pf*GAPDH protein sequence and comparison with GAPDH from rodent plasmodia species

Seven *Pf*GAPDH SNPs are documented on PlasmoDB and they are located at position 50 (D/A), 51 (S/T), 140 (Q/C), 74 (V/C), 143 (T/C), 228 (N/A) and 277 (L/T). More *Pf*GAPDH SNPs were identified in this study. The DNA sequences of 17 *Plasmodium falciparum* strains; 3D7, TG01, GB4, GA01, GN01, CD01, 7G8, KE01, Dd2, SD01, KH01, KH02, IT, TG01 and

SN01 showed that the protein is 100% conserved. Four *PfGAPDH* sequences that contain SNPs were obtained from PubMed and aligned with *PfGAPDH* sequence from the 3D7 strain (Figure 4.5). Three non-synonymous substitutions were observed at positions 140 (Q/K), 272 (P/L) and 276 (I/V) and they occurred from mutations at nucleotide positions 418 (CAA/AAA), 815 (CCA/CTA) and 826 (ATC/GTC) respectively. Cha *et al* (2016) identified 7 non-synonymous SNPs in *PfGAPDH* sequences from field isolates and they included 51 (S/T), 53 (H/P), 59 (E/D), 61 (T/S), 71 (E/D), 140 (Q/K) and 142 (D/S). Together, these results suggests the presence of 12 SNPs (51 (S/T), 53 (H/P), 59 (E/D), 61 (T/S), 71 (E/D), 140 (Q/K) and 142 (D/S), 143 (T/C), 228 (N/A), 272 (P/L), 276 (I/V) and 277 (L/T)) in the *PfGAPDH* sequence.

```

PF3D7_1462800.1 MAVTKLGINGFGRIGRLVFRAAFGRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQFPCEV 60
AAD10249.1 MAVTKLGINGFGRIGRLVFRAAFGRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQFPCEV 60
AAK30144.1 MAVTKLGINGFGRIGRLVFRAAFGRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQFPCEV 60
ETW58610.1 MAVTKLGINGFGRIGRLVFRAAFGRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQFPCEV 60
EWC73547.1 MAVTKLGINGFGRIGRLVFRAAFGRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQFPCEV 60
*****

PF3D7_1462800.1 THADGFLDIGEKVSVFAEKDPSQIPWGKCQVDVVCESTGVFLTKELASSHLKGGAKKVI 120
AAD10249.1 THADGFLDIGEKVSVFAEKDPSQIPWGKCQVDVVCESTGVFLTKELASSHLKGGAKKVI 120
AAK30144.1 THADGFLDIGEKVSVFAEKDPSQIPWGKCQVDVVCESTGVFLTKELASSHLKGGAKKVI 120
ETW58610.1 THADGFLDIGEKVSVFAEKDPSQIPWGKCQVDVVCESTGVFLTKELASSHLKGGAKKVI 120
EWC73547.1 THADGFLDIGEKVSVFAEKDPSQIPWGKCQVDVVCESTGVFLTKELASSHLKGGAKKVI 120
*****

PF3D7_1462800.1 MSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVH 180
AAD10249.1 MSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVH 180
AAK30144.1 MSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVH 180
ETW58610.1 MSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVH 180
EWC73547.1 MSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVH 180
*****

PF3D7_1462800.1 ASTANQLVVDGPSKGGKDWRAGRCALSNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
AAD10249.1 ASTANQLVVDGPSKGGKDWRAGRCALSNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
AAK30144.1 ASTANQLVVDGPSKGGKDWRAGRCALSNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
ETW58610.1 ASTANQLVVDGPSKGGKDWRAGRCALSNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
EWC73547.1 ASTANQLVVDGPSKGGKDWRAGRCALSNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
*****

PF3D7_1462800.1 GTVSVVDLVCRLQKPAKYEEVALEIKKAAEGLKGVLGYTEDEVVSQDFVHDNRSSIFDM 300
AAD10249.1 GTVSVVDLVCRLQKPAKYEEVALEIKKAAEGLKGVLGYTEDEVVSQDFVHDNRSSIFDM 300
AAK30144.1 GTVSVVDLVCRLQKPAKYEEVALEIKKAAEGLKGVLGYTEDEVVSQDFVHDNRSSIFDM 300
ETW58610.1 GTVSVVDLVCRLQKPAKYEEVALEIKKAAEGLKGVLGYTEDEVVSQDFVHDNRSSIFDM 300
EWC73547.1 GTVSVVDLVCRLQKPAKYEEVALEIKKAAEGLKGVLGYTEDEVVSQDFVHDNRSSIFDM 300
*****

PF3D7_1462800.1 KAGLALNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN 337
AAD10249.1 KAGLALNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN 337
AAK30144.1 KAGLALNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN 337
ETW58610.1 KAGLALNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN 337
EWC73547.1 KAGLALNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN 337
*****

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Figure 4.5: Alignment of *PfGAPDH* from 3D7 strain (PF3D7_1462800.1) with *PfGAPDH* sequences from field isolates. The SNPs are underlined.

The *Pf*GAPDH amino acid sequence was aligned with GAPDH sequences from *P. chabaudi* (*Pc*GAPDH), *P. yoelii* (*Py*GAPDH) and *P. berghei* (*Pb*GAPDH) to compare whether the SNPs of *Pf*GAPDH are conserved across rodent plasmodial species (Figure 4.6). Only the SNP at position 140 (Q/K) is found in *Pb*GAPDH, *Pc*GAPDH and *Py*GAPDH sequences (Figure 4.6).

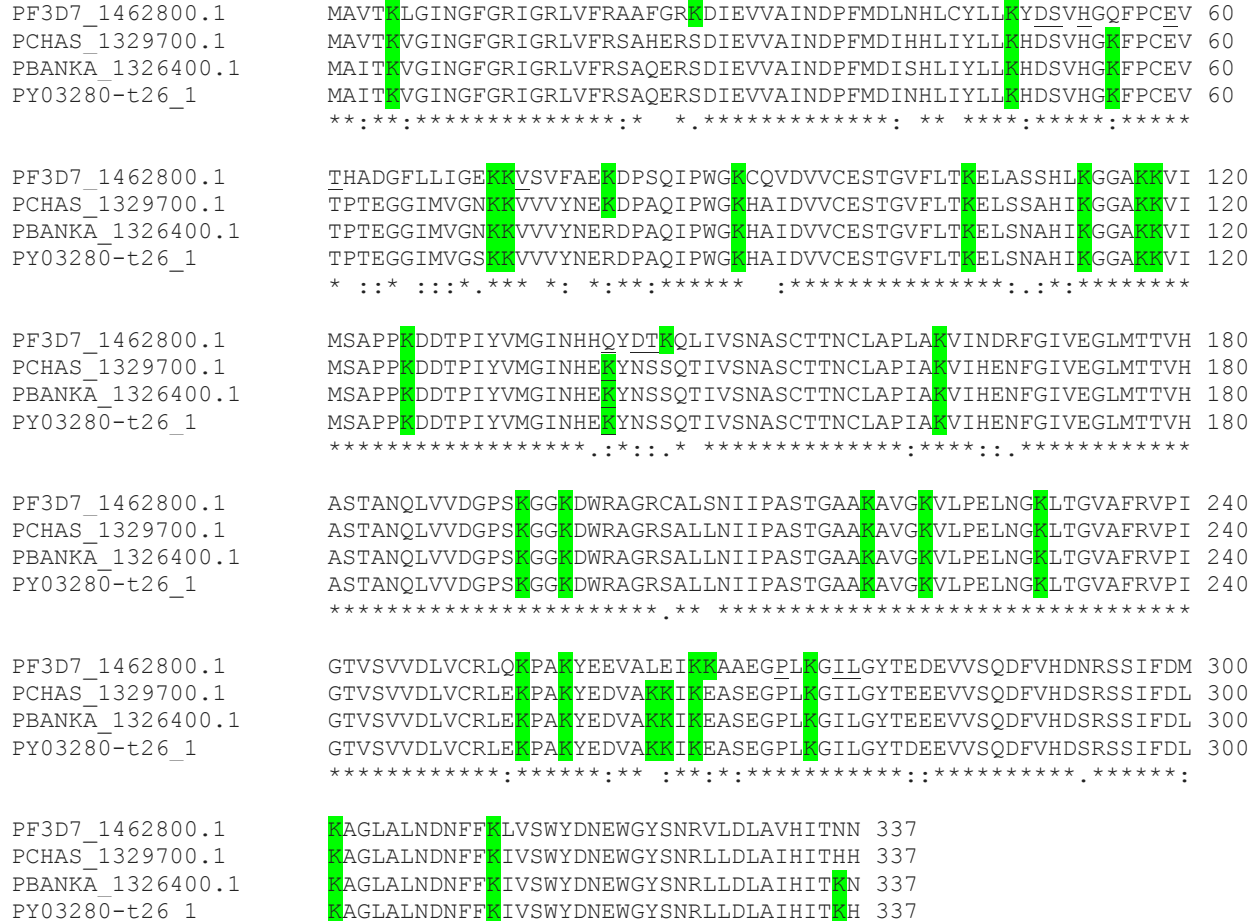


Figure 4.6: Alignment of *Pf*GAPDH (PF3D7_1462800.1) amino acid sequence with *Pc*GAPDH (PCHAS_1329700.1), *Pb*GAPDH (PBANKA_1326400.1) and *Py*GAPDH (PY03280-t26_1). The SNPs are underlined, and lysine residues are highlighted (green).

Lysine residues have influence on the structure of the protein. Plasmodial GAPDH has been reported to have post-translational modification, which results in moonlighting functions (Sirover, 2011). Lysine residues of proteins are often acetylated during post-translational modification of proteins (Yang & Seto, 2008). The current study evaluated lysine residues in *Pf*GAPDH amino acid sequence, and 26 lysine residues were identified (Figure 4.6). The *Pf*GAPDH sequence was aligned with *Pb*GAPDH, *Pc*GAPDH and *Py*GAPDH sequences to evaluate whether the lysine residues on *Pf*GAPDH are conserved in rodent plasmodial species

(Figure 4.6). Twenty-two lysine residues are conserved in GAPDH of all four plasmodial species. Four lysine residues were only found in *Pf*GAPDH and they were in position 26, 80, 144 and 267. Four lysine residues were absent in *Pf*GAPDH but conserved between rodent malaria GAPDH and were in position 55, 140, 263 and 264. Only *Pf*GAPDH and *Pc*GAPDH possessed lysine-80 while Lysine-336 was only found in *Pv*GAPDH and *Pb*GAPDH. Only one lysine residue (140) was polymorphic.

4.2.4 Investigation of the polymorphisms in *Pv*GAPDH protein sequence and comparison with GAPDH from rodent plasmodia species

Four SNPs have been identified in the *Plasmodium vivax* glyceraldehyde-3-phosphate dehydrogenase (*Pv*GAPDH) DNA sequence and are documented on PlasmoDB. The SNPs were identified on *Pv*GAPDH sequences using PO1 strain of *Plasmodium vivax* as a reference and these SNPs are located at nucleotide position 33, 87, 910 and 1002. The SNP at position 87 (GAG/ GAA) was identified on *Pv*GAPDH from Sal-1 strain (PVX_117322) and it results in a synonymous mutation at 29 (E). The SNPs at position 33 (TTC) and 1002 (ATA) resulted in synonymous substitutions at amino acid position 11 (F) and 334 (I) (Figure 4.6 and Figure 4.7). The SNP at position 910 (CTG) causes a non-synonymous substitution that changes leucine-304 (Figure 4.7). This mutation could either produce valine (GTG) or methionine (ATG). Unfortunately, the DNA sequences of *Pv*GAPDH isolates with these SNPs are not available on PubMed databases.

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PVP01_1244000.1 ATGGCCGTAACAAAGCTTGAATTAATGGATTCCGGACGTATCGGACGTTTAGTTTTTAGG 60
PCHAS_1329700.1 ATGGCAGTAACAAAAGTAGGAATTAACGGATTGGCCGTATCGGTCGTTTAGTATTCAGA 60
PBANKA_1326400.1 ATGGCAATAACAAAAGTCGGAATTAATGGATTTGGCCGTATCGGTCGTTTAGTATTCAGA 60
PY03280-t26_1 ATGGCAATAACAAAAGTAGGAATTAATGGATTTGGCCGTATCGGTCGTTTAGTATTCAGA 60
*****.*****. * ***** ***** ** *****:*****:*****:*** **

PVP01_1244000.1 GCTGCTTATGAAAGGAGTGACATCGAGGTAGTCGCGGTTAATGACCCATTTATGGACATC 120
PCHAS_1329700.1 TCTGCTCATGAAAGGAGTGACATCGAAGTAGTTGCTATCAATGACCCATTTATGGATATC 120
PBANKA_1326400.1 TCTGCCAAGAAAGAAGTGACATTGAAGTAGTTGCTATCAATGACCCATTTATGGATATT 120
PY03280-t26_1 TCTGCCAAGAAAGAAGTGACATTGAAGTAGTTGCTATCAATGACCCATTTATGGATATT 120
**** *:*****.***** ** ***** ** * ********** **

PVP01_1244000.1 AAGCACTTGTGCTACTTGCTCAAGTATGACTCCATCCACGGTGTATCCCAGCAGAGGTA 180
PCHAS_1329700.1 CATCACTTAATCTACTTATTAACATGATTCAGTTCATGGAAAATCCCATGTGAAGTA 180
PBANKA_1326400.1 AGTCACTTGATCTACTTATTAAGCATGATTCAGTTCATGGAAAATCCCATGTGAGGTA 180
PY03280-t26_1 AACCCTTGATCTACTTATTAAGCATGATTCAGTTCATGGAAAATCCCATGTGAAGTA 180
.. *****.: *****. *... ***** **..* ** **::***** :*.***

PVP01_1244000.1 ACCCCAGGCGATGGCTGCTTACCAGTTGGTAACAAGAAAATCTTTGTGCACAGCGAAAAG 240
PCHAS_1329700.1 ACCCCAACTGAAGGAGGTATAATGGTTGGAACAAAAAAGTCGTTGTTTACAACGAAAAA 240
PBANKA_1326400.1 ACCCCAACTGAAGGAGGTATCATGGTTGGAACAAAAAAGTCGTTGTTTACAACGAAAAGA 240
PY03280-t26_1 ACCCCAACTGAAGGAGGTATCATGGTTGGAAGCAAAAAAAGTCGTTGTTTACAACGAAAAGA 240
*****. **:*. * :*. *****:*.***** ** * *.******..

PVP01_1244000.1 GACCCAGCACAAATCCCATGGGGCAAATACGAAATTGATGTTGTGTGCGAATCCACTGGT 300
PCHAS_1329700.1 GACCCAGCTCAGATTCATGGGGAAAAACGCCATTGATGTTGTATGTGAATCAACTGGT 300

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PBANKA_1326400.1 GACCCAGCTCAAATTCATGGGGAAAAACACGCCATTGATGTTGTATGTGAATCAACTGGT 300
PY03280-t26_1 GACCCAGCTCAAATTCATGGGGAAAAACACGCCATTGATGTTGTATGTGAATCAACTGGT 300
*****:*. ** *****.*** ** .*****. ** *****.*****

PVP01_1244000.1 GTGTTCTTAACCAAGGAATTGTCCAATGCACATCTTAAGGGAGGAGCAAAGAAGGTTATC 360
PCHAS_1329700.1 GTATTTTAAACCAAGGAATTATCCAGTGCTCACATTAAGGGAGGTGCAAAAAAAGTTATC 360
PBANKA_1326400.1 GTATTTCTTAACCAAGGAATTATCCAATGCTCACATTAAGGGAGGTGCAAAAAAAGTTATC 360
PY03280-t26_1 GTATTTCTTAACCAAGGAATTATCCAATGCTCACATTAAGGGAGGTGCAAAAAAAGTTATC 360
.* ***.****.***:*. *****:*****.***.*****

PVP01_1244000.1 ATGTCCGCCCCACCAAAGGACGACACCCCTATTTACGTTATGGGTATTAACCATGATAAG 420
PCHAS_1329700.1 ATGTCAGCACCACCAAAGATGACACTCCAATTTATGTCATGGGTATTAACCACGAGAAA 420
PBANKA_1326400.1 ATGTCAGCACCACCAAAGACGACACACCAATTTATGTCATGGGTATTAACCACGAGAAA 420
PY03280-t26_1 ATGTCAGCACCACCAAAGACGACACACCAATTTATGTCATGGGTATTAACCACGAGAAA 420
*****.*.*****.* ** ***** **:***** * *****.***** ** **.

PVP01_1244000.1 TATGACCCAAAACAACCTCATCGTTTCAAATGCATCATGTACCACCAACTGTTTATCTCCA 480
PCHAS_1329700.1 TATAACAGTTCTCAAACCATCGTTTCCAATGCCTCATGTACTACCAACTGTTTACGACCA 480
PBANKA_1326400.1 TATAACAGTTCCCAAACCATCGTCTCCAATGCCTCATGTACTACAAATTGCTTAGCCCA 480
PY03280-t26_1 TATAACAGTTCTCAAACCATCGTTTCCAATGCCTCATGTACTACAAATTGCTTAGCCCA 480
.** .: .. ***** ** .*****.***** ** ** ** * **

PVP01_1244000.1 ATTGCAAAAAGTACTTCATGACAACCTTCGGAATTGTTGAGGGTTTGATGACCACTGTCCAC 540
PCHAS_1329700.1 ATTGCTAAGGTAATTCATGAAAACCTTGAATCGTTGAAGGTTTAAATGACCACTGTCCAT 540
PBANKA_1326400.1 ATTGCTAAGGTAATTCATGAAAACCTTCGGAATTGTTGAAGGTTTAAATGACCACTGTCCAT 540
PY03280-t26_1 ATTGCTAAGGTAATTCATGAAAACCTTCGGAATTGTTGAAGGTTTAAATGACCACTGTCCAT 540
*****:*. ** .*****.***** ***** ***** .*****.*****.*****

PVP01_1244000.1 GCTTCGACGGCCAACCAAGTTGGTAGTTGATGGACCATCCAAGGGTGGAAAGGACTGGAGA 600
PCHAS_1329700.1 GCCTCAACAGCCAACCAATTAGTTGTTGATGGACCATCAAAGGGAGGTAAGACTGGAGA 600
PBANKA_1326400.1 GCCTCAACAGCCAACCAATTAGTTGTTGATGGACCATCAAAGGGAGGTAAGACTGGAGA 600
PY03280-t26_1 GCCTCAACAGCCAACCAATTAGTTGTTGATGGACCATCAAAGGGAGGTAAGACTGGAGA 600
** **.*.*****.*.***:*****.*****.*****:*. ** .*****

PVP01_1244000.1 GCAGGCAGATGTGCATTGTCAAACATTATCCCAGCTTCAACTGGTGCAGCTAAGGCCGTA 660
PCHAS_1329700.1 GCAGGTAGATCAGCTTTGTTAAACATTATCCCAGCTTCAACTGGTGCAGCCAAAGCTGTA 660
PBANKA_1326400.1 GCAGGTAGATCAGCTTTGTTAAACATTATCCCAGCTTCAACTGGTGCAGCTAAGCTGTA 660
PY03280-t26_1 GCAGGTAGATCAGCTTTGTTAAACATTATCCCAGCTTCAACTGGTGCAGCTAAGCTGTA 660
***** ***** :*:***** ***** **.* ** .*****.***** **.* **

PVP01_1244000.1 GGTAAGGTCTTACCCGAATTGAATGAAAAGCTCACTGGTGTTCCTTCAGAGTCCCAATC 720
PCHAS_1329700.1 GGAAAAGTTTTACCAGAATTAACGGAAAATTAAGTGGTGTTCCTTTAGAGTTCCAATT 720
PBANKA_1326400.1 GGAAAAGTTTTACCAGAATTAACGGAAAATTAAGTGGTGTTCCTTTAGAGTTCCAATT 720
PY03280-t26_1 GGAAAAGTTTTACCAGAATTAACGGAAAATTAAGTGGTGTTCCTTTAGAGTTCCAATT 720
:*.* ** ***.*****.* **.*. * .*****.***** ***** **.*

PVP01_1244000.1 GGTACCGTGTGAGTTGTTGATTTAGTGTGCAGATTGAAAAACCAGCAGAAATATGAAGAA 780
PCHAS_1329700.1 GGTACCGTCTGTTGTTGATTTAGTATGCAGATTAGAAAAACCAGCAGAAATATGAAGAT 780
PBANKA_1326400.1 GGTACAGTCTCAGTTGTCGATTTAGTATGCAGATTAGAAAAACCAGCAGAAATATGAAGAT 780
PY03280-t26_1 GGTACAGTATCTGTTGTCGATTTAGTATGTAGATTAGAAAAACCAGCAGAAATATGAAGAT 780
*****.* ** *:***** *****.* ** *****.***** *****.*****.

PVP01_1244000.1 ATTGCCGCCCAAATGAAGAAAGCAGCAGAAGGACCACTCAAGGGAATATTGGGTTACT 840
PCHAS_1329700.1 GTTGCTAAAAAATTAAGAAGCATCTGAGGGACCACTAAGGGAATCTTAGGTTATACC 840
PBANKA_1326400.1 GTAGCTAAAAAATTAAGAAGCATCTGAGGGTCCACTAAGGGAATATTAGGTTATACC 840
PY03280-t26_1 GTAGCTAAAAAATTAAGAAGCATCTGAGGGACCACTAAGGGAATATTAGGTTATACC 840
.*:* * .*** ** .***** *:*.***:***** *****.*.***** **

PVP01_1244000.1 GAAGATGAAGTTGTTTCCCAAGATTTTCGTCACGACAAGAGGTCCTCCATTTTTGACTTG 900
PCHAS_1329700.1 GAAGAAGAAGTTGTATCTCAAGATTTTCGTCACGATGATAGCAGATCATCTATCTTTGACTTA 900
PBANKA_1326400.1 GAAGAAGAAGTTGTATCTCAAGATTTTCGTCACGATGATAGCAGATCATCAATCTTTGACTTA 900
PY03280-t26_1 GACGAAGAAGTTGTATCTCAAGATTTTCGTCACGATGATAGCAGATCATCTATCTTTGACTTA 900
.:*****:*. ** ***** ** *.* **.* ** ** *****.

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PVP01_1244000.1    AAGGCTGGTCTGGCATTGAACGACAACCTTTTTCAAAATCGTTTCATGGTACGATAATGAG    960
PCHAS_1329700.1    AAAGCTGGTCTTGCCTTAAATGATAACTTTTTCAAAATTTGTCTCATGGTATGACAATGAA    960
PBANKA_1326400.1    AAAGCTGGTCTTGCCTTAAATGATAACTTTTTCAAAATTTGTCTCATGGTATGACAATGAA    960
PY03280-t26_1      AAAGCTGGTCTTGCCTTAAATGATAACTTTTTCAAAATTTGTCTCATGGTATGACAATGAA    960
                    **.....**..**..**..**..**..**..**..**..**..**..**..**..**..**..
                    .....

PVP01_1244000.1    TGGGGATACTCAAACCGTGTGCTAGACTTGGCTGTCCACATAACAAAACATTAA    1014
PCHAS_1329700.1    TGGGGATATTCAAACCGTCTTTTAGATTTGGCTATCCACATCACCACCATTAA    1014
PBANKA_1326400.1    TGGGGATATTCAAACCGTCTTTTAGATTTGGCTATCCACATCACCAAAATTA    1014
PY03280-t26_1      TGGGGATATTCAAACCGTCTTTTAGATTTGGCTATCCACATCACCAAAACATTAA    1014
                    ***** ..***** * ..***** ..***** ..***** ..*****
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Figure 4.7: DNA sequence alignment of *PvGAPDH* (PVP01_1244000.1) with *GAPDH* sequences from *P. chabaudi* (PCHAS_1329700.1), *P. berghei* (PBANKA_1326400.1) and *P. yoelii* (PY03280-t26_1). The SNPs are underlined.

The *PvGAPDH* was aligned with *GAPDH* sequences from *P. chabaudi*, *P. yoelii* and *P. berghei* to evaluate whether the similar SNPs are found in rodent malaria *GAPDH* (Figure 4.7). Two SNPs at positions 87 and 910 are conserved between *PvGAPDH*, *PcGAPDH*, *PbGAPDH* and *PyGAPDH* (Figure 4.7).

```

PVP01_1244000.1    MAVTILGINGFGRIGRLVFRAYERSDIEVVAVNDPFMDIKHLCYLLYDSIHGVFPAEV    60
PCHAS_1329700.1    MAVTIVGINGFGRIGRLVFRSAHERSDIEVVAINDPFMDIHHLIYLLKHDSVHGKFPCEV    60
PBANKA_1326400.1    MAITIVGINGFGRIGRLVFRSAQERSDIEVVAINDPFMDISHLIYLLKHDSVHGKFPCEV    60
PY03280-t26_1      MAITIVGINGFGRIGRLVFRSAQERSDIEVVAINDPFMDINHLYLLKHDSVHGKFPCEV    60
                    **.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*
                    .....

PVP01_1244000.1    TPGDGCFTVGNKKIIVHSEKDPAQIPWGKYEIDVVCESTGVFLTKELSNAHLKGGAKKVI    120
PCHAS_1329700.1    TPTEGGIMVGNKKVVVYNEKDPAQIPWGKHVIDVCESTGVFLTKELSSAHIKGGAKKVI    120
PBANKA_1326400.1    TPTEGGIMVGNKKVVVYNERDPAQIPWGKHVIDVCESTGVFLTKELSNAHIKGGAKKVI    120
PY03280-t26_1      TPTEGGIMVGSKKVVVYNERDPAQIPWGKHVIDVCESTGVFLTKELSNAHIKGGAKKVI    120
                    **.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*
                    .....

PVP01_1244000.1    MSAPPDDTPIYVMGINHDKYDPKQLIVSNASCTTNCLSPIAKVLHDNFGIVEGLMTTVH    180
PCHAS_1329700.1    MSAPPDDTPIYVMGINHEKYNSSQTIVSNASCTTNCLAPIAKVIHENFGIVEGLMTTVH    180
PBANKA_1326400.1    MSAPPDDTPIYVMGINHEKYNSSQTIVSNASCTTNCLAPIAKVIHENFGIVEGLMTTVH    180
PY03280-t26_1      MSAPPDDTPIYVMGINHEKYNSSQTIVSNASCTTNCLAPIAKVIHENFGIVEGLMTTVH    180
                    ***** ..**.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*
                    .....

PVP01_1244000.1    ASTANQLVVDGPSKGGDWRAGRCSALLNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI    240
PCHAS_1329700.1    ASTANQLVVDGPSKGGDWRAGRSALLNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI    240
PBANKA_1326400.1    ASTANQLVVDGPSKGGDWRAGRSALLNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI    240
PY03280-t26_1      ASTANQLVVDGPSKGGDWRAGRSALLNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPI    240
                    ***** ..* ..***** ..***** ..***** ..*****
                    .....

PVP01_1244000.1    GTVSVDLVCRLEKPAYEEIAAQMKAAEGPLKGILGYTEDEVVSQDFVHDRSSIFDL    300
PCHAS_1329700.1    GTVSVDLVCRLEKPAYEDVAKIKEEASEGPLKGILGYTEEEVVSQDFVHDSRSSIFDL    300
PBANKA_1326400.1    GTVSVDLVCRLEKPAYEDVAKIKEEASEGPLKGILGYTEEEVVSQDFVHDSRSSIFDL    300
PY03280-t26_1      GTVSVDLVCRLEKPAYEDVAKIKEEASEGPLKGILGYTDEEVVSQDFVHDSRSSIFDL    300
                    ***** ..*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*
                    .....

PVP01_1244000.1    KAGLALNDNFFKIVSWYDNEWGYSNRLLDLAVHITH    337
PCHAS_1329700.1    KAGLALNDNFFKIVSWYDNEWGYSNRLLDLAIHITH    337
PBANKA_1326400.1    KAGLALNDNFFKIVSWYDNEWGYSNRLLDLAIHITN    337
PY03280-t26_1      KAGLALNDNFFKIVSWYDNEWGYSNRLLDLAIHITH    337
                    ***** ..**.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*
                    .....

```

Figure 4.8: Alignment of *Pv*GAPDH (PVP01_1244000.1) amino acid sequence with *Pc*GAPDH (PCHAS_1329700.1), *Pb*GAPDH (PBANKA_1326400.1) and *Py*GAPDH (PY03280-t26_1). The SNPs are underlined, and lysine residues are highlighted (green).

Lysine residues of *Pv*GAPDH were evaluated and 29 were identified (Figure 4.8). The *Pv*GAPDH sequence was aligned with the amino acid sequences of *Pb*GAPDH, *Py*GAPDH and *Pc*GAPDH to evaluate whether the lysine residues on *Pv*GAPDH are conserved between the *Plasmodium* species. Twenty-three lysine residues are conserved between all four *Plasmodium* species. Lysine-41, 144, 267 and 293 residues are unique in *Pv*GAPDH. Lysine-55, 263 and 264 are not found in the *Pv*GAPDH sequence but conserved between rodent plasmodial species. Lysine-80 is found only in *Pv*GAPDH and *Pc*GAPDH and is replaced with arginine in *Py*GAPDH and *Pb*GAPDH. None of the lysine residues is mutated, which implicates that they are all crucial in forming the structure of plasmodial GAPDH.

4.2.5 Genetic polymorphisms deduced for *Py*LDH sequences

Lactate dehydrogenases from *Plasmodium* parasites are highly conserved (Bzik *et al.*, 1993). Sequence alignments revealed a 90.19% identity between *Pv*LDH and *Py*LDH, while there was a 93.04% identity between *Pf*LDH and *Py*LDH. However, *Pf*LDH and *Py*LDH only share 2 SNPs while none of the SNPs of *Pv*LDH occurs in *Py*LDH (Figure 4.2 and 4.4). Therefore, the *Py*LDH sequence possesses two non-synonymous mutations at amino acid positions 21 (C/T) and 272 (G/D).

4.2.6 Genetic polymorphisms deduced for *Py*GAPDH sequences

Plasmodium GAPDH enzymes are highly conserved (Dauberger *et al.*, 2000). *Plasmodium falciparum* GAPDH has been found to share 1 SNP with *Py*GAPDH sequence. This SNP was located at amino acid position 140 (Q/K) (Figure 4.6). *Pv*GAPDH has been observed to share 2 SNPs with *Py*GAPDH. These SNPs include a synonymous and non-synonymous mutation at amino acid position 29 and 304 respectively (Figure 4.7). Therefore, the *Py*GAPDH sequence has 3 SNPs.

4.2.7 Genetic polymorphisms on B cell epitopes and T cell epitopes predicted from *Py*LDH and *Py*GAPDH sequences

Four CD8+ T cell epitopes were predicted in the *Py*GAPDH amino acid sequence and 2 in *Py*LDH amino acid sequence (Table 4.1). None of the *Py*GAPDH CD8+ T cell epitopes contained an SNP while 1 of the *Py*LDH CD8+ T cell epitopes had an SNP. The SNP results in a non-synonymous substitution at amino acid 272 (G/D).

Table 4.1: Prediction results of *PyGAPDH* and *PyLDH* CD8+ T cell epitope obtained by NetMHCpan Server using the HLA-A02:01 allele. The rank threshold for Strong binding peptides on MHC I was 0.5.

Antigen	Epitope	Start position	% Rank threshold
<i>PyGAPDH</i>	FMDINHLYL	37	0.03
	KLTGV <u>A</u> FRV	330	0.20
	ALNDNFFKI	305	0.09
	RLLDLAIHI	326	0.07
<i>PyLDH</i>	KDIFAGTPLV	271	0.50
	FII <u>V</u> TNPV	121	0.07

Table 4.2: *PyGAPDH* and *PyLDH* B cell epitopes using ABCpred server. A score of 0.85 was used as a cutoff. The polymorphic amino acid residues are underlined.

Antigen	Sequence	Start position	Score
<i>PyGAPDH</i>	FPCEVTPTEGGIMVGS	56	0.89
	KELSNAHIKGGAKKVI	105	0.89
	KEASEGPLKGILGYTD	266	0.88
	YVMGINHE <u>K</u> YNSSQTI	132	0.87
	KGILGYTDEEVVSQDF	274	0.86
	IVEGLMTTVHASTANQ	171	0.85
<i>PyLDH</i>	AGFTKAPGKSDKEWNR	80	0.95
	SGSNTYDDLKDADVVI	62	0.88
	MIEIGGHIKNNCPNAF	106	0.88
	HIKNNCPNAFIIVVTN	112	0.87
	IVLVGSGMIGGVMA <u>T</u> L	7	0.85
	AESYIRDLRKVLICST	247	0.85

Six B cell epitopes were predicted in both *PyGAPDH* and *PyLDH* sequence (Table 4.2). The *PyGAPDH* B cell epitope (Y132-I147) had one SNP which is 140 (Q/K). One of the *PyLDH* B cell epitopes (I7-L22) had an SNP at position 21 (C/T).

4.3 Discussion

4.3.1 Identification of polymorphisms in *Plasmodium yoelii* lactate dehydrogenase sequence

Plasmodium lactate dehydrogenase has been well studied and its crystal structure has been determined (Dunn *et al.*, 1996). *Plasmodium* LDH has been targeted for antimalarial compounds (Dunn *et al.*, 1996) and as a diagnostic biomarker for malaria rapid diagnostic tests (Piper *et al.*, 1999). The study of genetic polymorphisms of pLDH could determine whether the protein is an effective diagnostic and therapeutic target and could improve the effectiveness of malaria RDTs using pLDH. Genetic polymorphism of *Pf*LDH and *Pv*LDH were evaluated in this study in order to deduce the polymorphism in the *Py*LDH sequence. The DNA sequences were obtained from PlasmoDB and PubMed. The *Pf*LDH sequence revealed 17 non-synonymous substitutions. These SNPs were investigated in three rodent plasmodial LDH sequences (*P.chabaudi*, *P. berghei* and *P. yoelii*) and only 2 SNPs were conserved (Figure 4.2). Genetic polymorphism was also investigated in *Pv*LDH sequences (Figure 4.3). Twenty-six polymorphic sites were observed in the *Pv*LDH sequence and 19 of them were non-synonymous mutations (Figure 4.3). Keluskar *et al* (2014) described only a single non-synonymous mutation 15 (A/G) in *Pf*LDH sequence. Although, this SNP did not match any field isolate and *Pf*LDH strain. Simalipalan *et al* (2018) detected 15 SNPs of *Pf*LDH sequence and these did not include 15 (A/G). The *Pv*LDH sequence has been observed to contain more polymorphism than *Pf*LDH (Keluskar *et al.*, 2014; Talman *et al.*, 2007) and that was also observed in the current study. It was also observed that numerous *Pf*LDH and *Pv*LDH sequences from field isolates that have been reported in previous studies (Keluskar *et al.*, 2014; Simalipalan *et al.*, 2018) are not uploaded on the databases PlasmoDB and PubMed. Only 2 *Pf*LDH SNPs are documented on PlasmoDB and no genetic polymorphism has been documented in *Pv*LDH on PlasmoDB. Combining the SNPs shared by *Pf*LDH and *Pv*LDH with *Py*LDH, a total of 2 non-synonymous SNPs were deduced from *Py*LDH sequence.

4.3.2 Polymorphisms in pLDH do not affect the performance of the enzyme in RDTs

Talman *et al* (2007) described the polymorphisms in pLDH sequence to have no influence on the sensitivity of RDTs unlike *Pf*HRP-2 based RDTs. Hurdayal *et al* (2010) identified plasmodial LDH epitopes that could be targeted for malaria diagnosis and differentiate between *P. vivax* and *P. falciparum* infections. The common pLDH epitope (APGKSDKEWNRDDL), *Pf*LDH unique epitope (LISDAELEAIFDC) and *Pv*LDH (KITDEEVEGIFDC) epitope were identified and antibodies against the peptides were produced

in chickens. The common peptide is located between amino acid A82-97L; this region has a non-synonymous substitution at position 87 in both *Pf*LDH and *Pv*LDH. *Pf*LDH specific epitope (L108-120C) has a single SNP at position 112, while the *Pv*LDH epitope (K108-120C) has three SNPs at 114, 117 and 118 respectively. These SNPs are less frequent, occur in 1/54 sequences. The *P. falciparum* detection based on *Pf*LDH unique peptide and pan-malaria detection (common peptide) is unlikely to be affected by this mutation. The detection of *P. vivax* based on the *Pv*LDH specific peptide could be affected by polymorphisms since 3 of 13 amino acids on the peptide are polymorphic.

4.3.3 Identification of polymorphisms in *Plasmodium yoelii* glyceraldehyde-3-phosphate dehydrogenase sequences

Genetic polymorphisms were assessed in *Pf*GAPDH and *Pv*GAPDH sequences. The enzyme is essential in *Plasmodium* parasites as it catalyses glycolysis which is important for parasites survival in the host red blood cells. *Plasmodium* GAPDH has been identified as a diagnostic target (Krause *et al.*, 2017). *Pf*GAPDH is also a pre-erythrocyte vaccine candidate (Cha *et al.*, 2016). A study of genetic polymorphisms could provide information on whether the antigen is an efficient diagnostic and vaccine target. Twelve and four SNPs have been detected on *Pf*GAPDH and *Pv*GAPDH sequence respectively. Seven of *Pf*GAPDH SNPs are documented on PlasmoDB. A study by Cha *et al* (2016) identified five *Pf*GAPDH SNPs from field isolates, these SNPs included 53 (H/P), 59 (E/D), 61 (T/S), 71 (E/D) and 142 (D/S). These *Pf*GAPDH sequences are not available on the database PubMed. The *Pf*GAPDH sequence shares one SNP with *Py*GAPDH (Figure 4.6) and *Pv*GAPDH shares two SNPs with *Py*GAPDH (Figure 4.7). Therefore, a total of 3 SNPs were deduced for *Py*GAPDH sequence and they are located at positions 29 (E), 140 (Q/K) and 304 (V or M/L).

4.3.4 Polymorphisms in pGAPDH do not affect the performance of the enzyme in RDTs

Krause *et al* (2017) identified the pGAPDH common peptide (K126-H138) and *Pf*GAPDH unique peptide (A78-V92) for the detection of malaria infections using a pGAPDH based RDT's. Both the common and unique peptide are not located in the region with polymorphisms. Therefore, this suggests that the RDTs targeting pGAPDH will not be affected by genetic polymorphisms.

4.3.5 Lysine residues in *Py*GAPDH and *Py*LDH are not polymorphic

Lysine residues play a role in the formation of a 3D structure of proteins through the formation of hydrogen bonds and salt bridges to stabilize the protein (Sokalingam *et al.*, 2012). Lysine residues are the regions of post-translational modification, acetylation (Yang & Seto, 2008). Post-translational modifications on GAPDH from *Plasmodium* parasites and humans have been implicated with the moonlighting functions of the enzyme. The current study evaluated the lysine residues in *Pf*GAPDH, *Pv*GAPDH and *Py*GAPDH sequences and whether they have polymorphisms. None of the lysine residues on the *Py*GAPDH sequence was polymorphic. Lysine-140 was polymorphic and this non-synonymous polymorphism was conserved in *Pf*GAPDH and rodent plasmodial GAPDH. It is unlikely that a single SNP could have a major effect on the protein structure. No SNPs were identified on *Pf*LDH and *Pv*LDH and *Py*LDH lysine residues.

4.3.6 Effect of polymorphisms in *Py*LDH and *Py*GAPDH vaccine candidates

Genetic polymorphisms of malaria vaccine candidates have been shown to lower the efficacy of vaccines. As a result, there is still no licensed blood-stage malaria vaccine. Two non-synonymous substitutions were identified in both *Py*LDH and *Py*GAPDH sequences. The SNPs identified on *Py*GAPDH and *Py*LDH are low compared to the SNPs in *Pf*RH5 the most promising malaria vaccine candidate. *Pf*RH5 has 10 non-synonymous mutations (Ouattara *et al.*, 2018) unlike other malaria vaccine candidates with a lot of genetic variation. The low genetic variation of *Pf*RH5 causes the vaccine to produce an immune response that is not strain specific (Hayton *et al.*, 2008). As a result, this vaccine has high efficacy and it is currently being tested in a phase 1/2a clinical trial. Immunogenic peptides were predicted in *Py*LDH and *Py*GAPDH sequences. Four and two CD8+ T cell epitopes were predicted in *Py*GAPDH and *Py*LDH sequences respectively. Six B cell epitopes were predicted in both *Py*GAPDH and *Py*LDH sequences. The *Py*GAPDH CD8+ T cell epitopes did not have polymorphisms while one of the B cell epitopes had a single SNP. One CD8+ T cell and B cell epitope of *Py*LDH had a single SNP. The immune response produced by the *Py*GAPDH and *Py*LDH vaccine would not be strain specific since the proteins have minimal polymorphisms. Both *Py*GAPDH and *Py*LDH catalyses glycolysis, which is crucial for the parasite's survival and the enzymes are produced by the parasites during blood stage infections (Le Roch *et al.*, 2003). The vaccine targeting these proteins is hypothesized to produce immunity that would inhibit the growth of the parasites inside the erythrocytes. Both these vaccines are predicted to have high efficacies as the proteins have minimal genetic variation.

Chapter 5

Mice immunizations and challenge

5.1 Introduction

5.1.1 Pre-erythrocyte stage malaria vaccines

An effective malaria vaccine would contribute towards the control and the eradication of the disease that claims about 438 000 lives annually, particularly in sub-Saharan Africa (World Health Organization, 2016). RTS, S/AS01 vaccine is the only malaria vaccine that has been approved for use by the European Medicines Agency (RTS, 2015). The vaccine is based on the *Plasmodium falciparum* circumsporozoite protein (PfCSP) a sporozoite antigen and its targets the pre-erythrocyte stage of the infections (Stoute *et al.*, 1997). RTS, S/AS01 is only approved for its uses in malaria-endemic areas for vaccination of older children and not infants. It has been reported to have a low efficacy that declines in 4 years. Therefore, other vaccine candidates are required for the development of a malaria vaccine and many are under development. These include vaccines targeting the blood stages of malaria (Cockburn & Seder, 2018), which causes the clinical symptoms and deaths due to the disease (Miller *et al.*, 2002). Blood stage malaria vaccines could potentially decrease parasite development in the blood, therefore reducing the disease in young children and pregnant women living in endemic areas (Good & Miller, 2018). The leading blood-stage malaria vaccine candidates for *P. falciparum* include apical membrane antigen-1 (PfAMA-1) (Thera *et al.*, 2011), merozoite surface protein-1 (PfMSP1-19) (Murhandarwati *et al.*, 2009), GMZ2 vaccine based on PfMSP3 and glutamate-rich protein (PfGLURP) (Esen *et al.*, 2009), reticulocyte homolog 5 (PfRH5) (Jin *et al.*, 2018) and MSP3 (Lusingu *et al.*, 2009). Unfortunately, none of the blood stage vaccines has proceeded to Phase III clinical trials (Cockburn & Seder, 2018). New strategies for blood-stage malaria vaccine development are needed to produce an effective vaccine. *P. falciparum* depends entirely on glycolysis for development and large increases in glycolytic enzymes activities have been reported following the infection with *P. falciparum* (Roth *et al.*, 1988). These phenomena make glycolytic enzymes possible target for anti-malarial compounds.

5.1.2 Glycolytic enzymes as parasite drug and vaccine targets

Glycolysis is a 10 step energy producing pathway that is found in all living organisms (Pilkis & Granner, 1992). The pathway involves 10 enzymes and some enzymes including fructose 1, 6 -bisphosphate aldolase (aldolase), enolase, glyceraldehyde-3-phosphate

dehydrogenase (GAPDH), triose phosphate isomerase (TPI) and pyruvate kinase have been identified to have moonlighting functions (Sriram *et al.*, 2005). Enzymes with moonlighting functions have been reported to be associated with the pathogenesis of many human infections and some of these enzymes are vaccine and drug targets (Mendonça *et al.*, 2016; Srinam *et al.*, 2005). GAPDH has been reported to build up in the brain of Alzheimer's disease patients (Wang *et al.*, 2005). GAPDH is involved in the invasion of liver cells by *Plasmodium* sporozoites where the enzyme interacts with CD68 on Kupfer cells (Cha *et al.*, 2016). *P. falciparum* enolase has been identified to be involved in the invasion of erythrocytes by merozoites (Bhowmick *et al.*, 2009). Aldolase from *P. falciparum* and *Toxoplasma gondii* was reported to bind to thrombospondin-related anonymous protein (PfTRAP) and micronemal protein 2 (MIC2) respectively and this interaction is critical for the invasion to erythrocytes (Jewett & Sibley, 2003). Immunization with recombinant aldolase and rGAPDH of *Streptococcus pneumoniae* induced protection in mice against *S. pneumoniae* (Ling *et al.*, 2004). Aldolase has been identified as a potential diagnostic target for *Listeria* (Mendonça *et al.*, 2016). Antibodies against aldolase from *Onchocerca volvulus* were observed in immune individuals in endemic areas and the enzyme has been identified as a potential vaccine candidate for *O. volvulus* (McCarthy *et al.*, 2002). Vaccination of hamsters with TPI induced protective immune response against *Leishmania donoravi* infections (Kushawaha *et al.*, 2012). High levels of lactate dehydrogenase (LDH), an enzyme catalyzing the last step of glycolysis has been implicated with many diseases including cancer, leukaemia and malaria infections (Miao *et al.*, 2013; Piper *et al.*, 1999). LDH is a diagnostic target for these diseases. *Plasmodial* GAPDH has been identified as a diagnostic target for malaria (Krause *et al.*, 2017). *P. falciparum* LDH is a possible target for chloroquine, a first line anti-malarial drug (Waingeh *et al.*, 2013).

5.1.3 Aims of this chapter

The current study evaluated the immunogenicity of recombinant *P. yoelii* GAPDH and rPyLDH in mice and whether the immunization with the two proteins could offer protection against a *P. berghei* infection challenge in mice.

5.2 Results

5.2.1 Comparison of *P. yoelii* and *P. berghei* LDH and GAPDH amino acid sequences

PyGAPDH	MAITKVGINGFGRIGRLVFRSAQERSDIEVVAINDPFMDINHLIYLLKHDSVHGKFPCEV	60
PbGAPDH	MAITKVGINGFGRIGRLVFRSAQERSDIEVVAINDPFMDISHLIYLLKHDSVHGKFPCEV	60

```

*****
PyGAPDH   TPTEGGIMVGSKKVVVYNERDPAQIPWGKHAI DVVCESTGVFLTKELSNAIKGGAKKVI 120
PbGAPDH   TPTEGGIMVGNKKVVVYNERDPAQIPWGKHAI DVVCESTGVFLTKELSNAIKGGAKKVI 120
*****

PyGAPDH   MSAPPKDDTPIYVMGINHEKYNSSQTIVSNASCTTNCLAPIAKVIHENFGIVEGLMTTVH 180
PbGAPDH   MSAPPKDDTPIYVMGINHEKYNSSQTIVSNASCTTNCLAPIAKVIHENFGIVEGLMTTVH 180
*****

PyGAPDH   ASTANQLVVDGSPSKGGKDWGRAGRSALLNII PASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
PbGAPDH   ASTANQLVVDGSPSKGGKDWGRAGRSALLNII PASTGAAKAVGKVLPELNGKLTGVAFRVPI 240
*****

PyGAPDH   GTVSVVDLVCRLEKPAKYEDVAKKI KEASEGPLKGI LGYTDEEVVSQDFVHDSRSSIFDL 300
PbGAPDH   GTVSVVDLVCRLEKPAKYEDVAKKI SQDFVHDSRSSIFYTEEEVVSQDFVHDSRSSIFDL 300
*****
          .:          :. : **:*****

PyGAPDH   KAGLALNDNFFKIVSWYDNEWGYSNRLLDLAIHITKH 337
PbGAPDH   KAGLALNDNFFKIVSWYDNEWGYSNRLLDLAIHITKN 337
*****

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Figure 5.1: Amino acid sequence alignment of *PbGAPDH* and *PyGAPDH*. Developed using clustal omega on 20 November 2018.

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PyLDH     MAPKAKIVLVGSGMIGGVMATLIVQKNLGDVVLFDIVKNMPHGKALDTSHTNVMAYSNCK 60
PbLDH     MAPKAKIVLVGSGMIGGVMATLIVQKNLGDVVMFDIVKNMPHGKALDTSHTNVMAYSNCK 60
*****

PyLDH     VSGSNTYDDLKDADVIVTAGFTKAPGKSDKEWNRD DLLPLNNKIMIEIGGHKNNCPNA 120
PbLDH     VSGSNTYDDLKDADVIVTAGFTKAPGKSDKEWNRD DLLPLNNKIMIEIGGHKNNCPNA 120
*****

PyLDH     FIIIVTNPVDVMVQLLHQHSGVPKNKIVLGGVLDTSRLKYYISQKLNVCPRDVNAHIVG 180
PbLDH     FIIIVTNPVDVMVQLLHQHSGVPKNKIVLGGVLDTSRLKYYISQKLNVCPRDVNAHIVG 180
*****

PyLDH     AHGNKMVLLKRYITVGGIPLQEFINNKKITDQELDAIFDRTVNTALEIVNLHASPYVAPA 240
PbLDH     AHGNKMVLLKRYITVGGIPLQEFINNKKITDQELDAIFDRTINTALEIVNLHASPYVAPA 240
*****

PyLDH     AAI IEMAESYIRDLRKVLICSTLLEQG YGHKDI FAGTPLVIGNGVEQVIELQLNADEKK 300
PbLDH     AAI IEMAESYIRDLRKVLICSTLLEQG YGHKDI FAGTPLVIGNGVEQVIELQLNADEKK 300
*****

PyLDH     KFDEAVAETSRMKALI 316
PbLDH     KFDEAVAETSRMKALI 316
*****

```

Figure 5.2: Amino acid sequence alignment of *PbLDH* and *PyLDH*. Developed using clustal omega on 21 November 2018.

Amino acid sequences of *P. yoelii* and *P. berghei* LDH and GAPDH were obtained from PlasmoDB and aligned to compare the similarities between the sequences (Figure 5.1 and 5.2). The *PyGAPDH* and *PbGAPDH* sequences reveal a 94.96% identity. There are 4 single amino acid differences and a unique short peptide from 265-279 between *PyGAPDH* and *PbGAPDH*

sequences. The amino acid differences between *PyGAPDH/PbGAPDH* include asparagine 41 /serine 41, asparagine 71/serine 71, aspartate 282/ glutamate 282 and histidine 337/ asparagine 337 (Figure 5.1). An alignment of *PyLDH* and *PbLDH* amino acid sequences revealed a 99.37% identity between the sequences. Only two amino acid differences are observed between *PyLDH/PbLDH*, and they are leucine 33/ methionine 33 and valine 221/isoleucine 221.

5.2.2 Assessment of r*PyGAPDH* and r*PyLDH* immunogenicity in mice

To investigate the immunogenicity of the recombinant proteins, mice were immunized with the affinity-purified r*PyGAPDH* and r*PyLDH*. After 3 immunizations over 6 weeks, mouse anti-serum was obtained and evaluated for the presence of anti-r*PyGAPDH* or anti-r*PyLDH* antibodies in an ELISA (Figure 5.3A and B). Mouse serum taken 2 weeks after final immunization with r*PyGAPDH* and r*PyLDH* vaccines contained IgG antibodies that detected the corresponding recombinant proteins by ELISA at serum dilution greater than 1:10000 (Figure 5.3A and B). This was observed with all the serum of immunized mice. The serum taken from an unvaccinated mouse did not recognize r*PyGAPDH* and r*PyLDH* (Figure 5.3A and B).

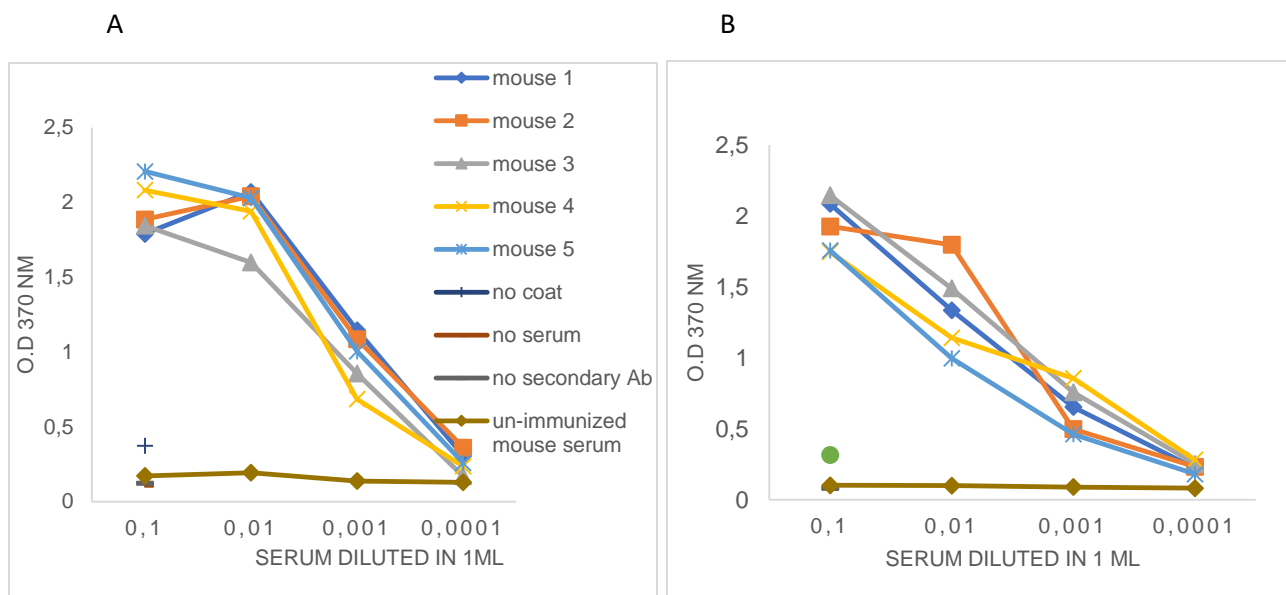


Figure 5.3: Examination of mouse anti-serum obtained from immunization of Balb/c mice with affinity purified r*PyGAPDH* and r*PyLDH*. Five Balb/c mice were immunized with r*PyGAPDH* (A) or r*PyLDH* (B). After 3 immunizations in 2 weeks intervals, mice serum was collected for the assessment of immunogenicity of the recombinant proteins with an ELISA.

5.2.3 Reactivity of mouse antiserum with r*PyGAPDH* and r*PyLDH*

Serum samples obtained from rPyGAPDH and rPyLDH immunized mice were tested for the recognition of rPyGAPDH (Figure 5.4B) and rPyLDH (Figure 5.4D) in a Western blot. The antisera detected the monomer, dimer and the tetramer forms of rPyGAPDH and rPyLDH (Figure 5.4B and 5.4D).

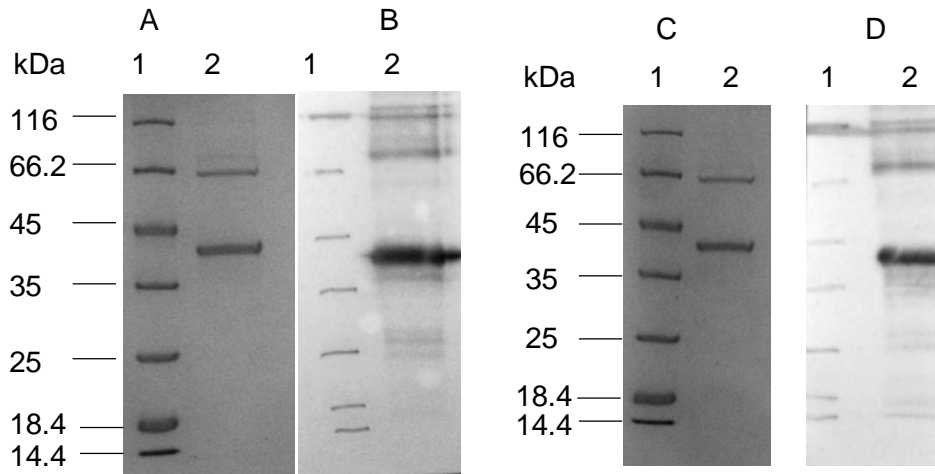


Figure 5.4: Reactivity of mouse anti-serum with rPyGAPDH and rPyLDH in a Western blot. Recombinant PyGAPDH (A) and rPyLDH (C) were analysed on a 12.5% reducing SDS-PAGE gels and a Western blot probed with rPyGAPDH (B) and rPyLDH (D) mouse antiserum. Lane 1: molecular weight marker and lane 2: rPyGAPDH (A and D) or rPyLDH (C and D).

5.2.4 Evaluation of the blood-stage parasites following a challenge infection

The progress of parasitemia was evaluated on mice immunized with PyLDH and PyGAPDH in Freund's adjuvant (Figure 5.5). All infected mice developed the blood stage infections and their parasitemia was monitored from day 2. Mice were euthanized when they showed fever symptoms and high parasitemia. Three mice in the PkPMT group were euthanised on day 9, one with 21% parasitemia and the other two with 8% and 3.8% parasitemia but with severe fever symptoms and paralysis (Figure 5.5A). The last two mice were euthanised on day 11 and 12 with 16% parasitemia and fever symptoms. Two mice in PyLDH group were euthanised on day 7 and 9 with 13% and 7.4% parasitemia respectively (Figure 5.5B). The last three mice survived until day 12, one of them had 26% parasitemia and showing severe fever symptoms and the other two had 16% parasitemia and were euthanised. Two mice in the PyGAPDH group were euthanised on day 8 and 9 both with 26% parasitemia and severe fever symptoms (Figure 5.5C). Three mice survived till day 12 and two of them had 12% and 14% parasitemia. One mouse in the PyGAPDH immunization group maintained the

parasitemia below 5% throughout the study and had no fever or any symptoms of discomfort on day 12 (Figure 5.5C).

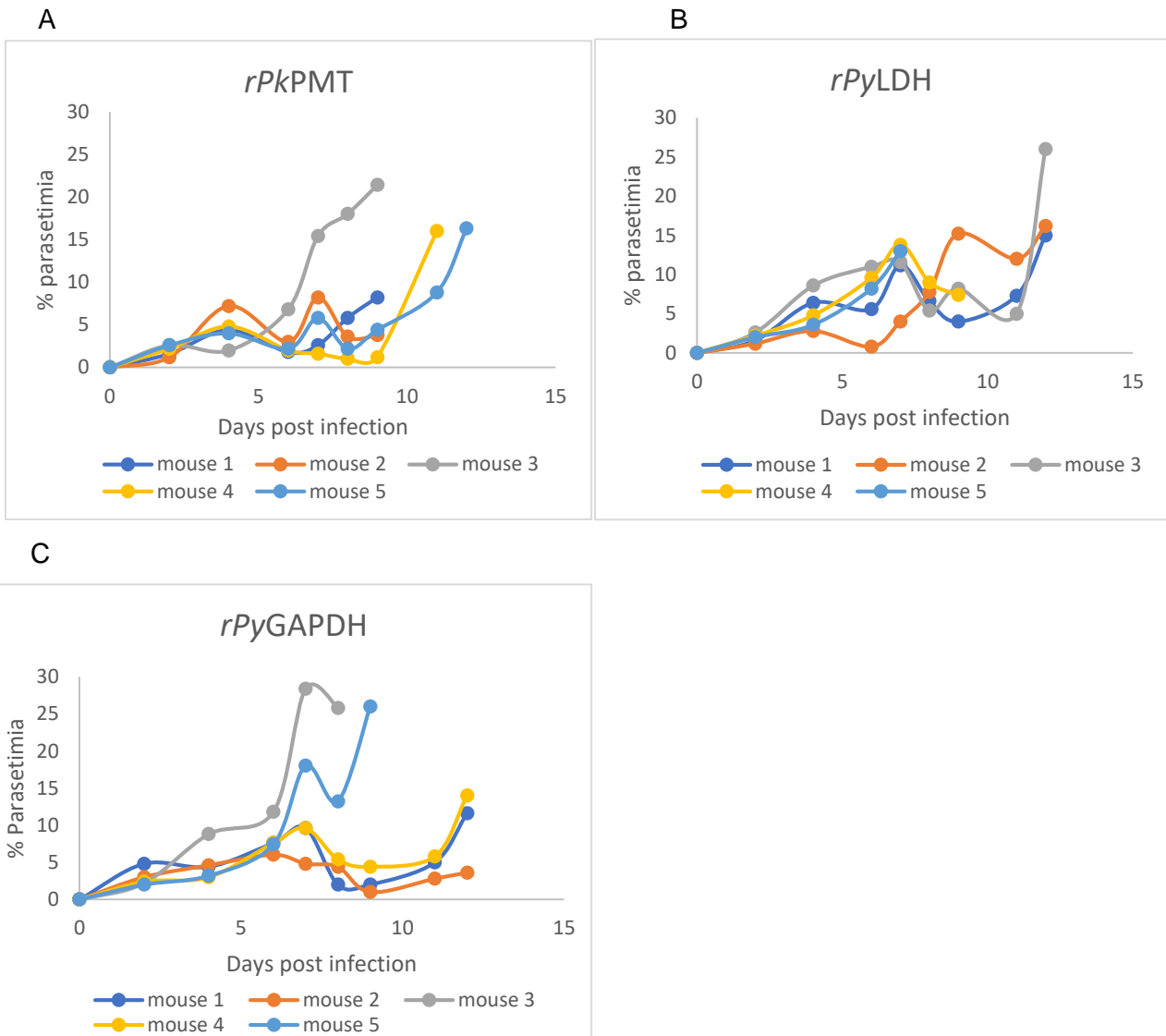


Figure 5.5: Evaluation of protection offered by *rPyLDH* or *rPyGAPDH* formulated in Freund's adjuvant against blood stage infections with *P. berghei* parasitized red blood cells. Mice were immunized with *rPkPMT* (A), *rPyLDH* (B) or *rPyGAPDH* (C) in Freund's adjuvant and challenged with 1×10^5 parasites/ μ l *P. berghei* infected red blood cells. Blood stage infections were monitored by staining blood with Giesma and counting parasites under 1000x microscope.

5.3 Discussion

5.3.1 Evaluation of immunogenicity of *rPyGAPDH* and *rPyLDH* in mice

Malaria is a disease that claims about 438 000 lives annually, in poor countries particularly in sub-Saharan Africa (World Health Organization, 2016). This study evaluated the

immunogenicity of GAPDH and LDH from *Plasmodium yoelii* in mice. *E. coli* produced rPyGAPDH and rPyLDH antigens were prepared in Freund's adjuvant and mice were immunized to evaluate the immunogenicity of the antigens. Antibody titers > 1/10000 were detected in mouse serum two weeks after the last immunization with rPyGAPDH and rPyLDH vaccines (Figure 5.3). Vaccination of animals with proteins in Freund's adjuvant generally produce high titers of antibodies against the proteins (Arevalo-Herrera *et al.*, 2005). Antibody titers between 1/5000 and 1/10000 in mouse serum have been shown to induce protection against sporozoite challenge following vaccination with a circumsporozoite protein immunogenic peptide (Wang *et al.*, 1995). Rabbit sera following immunization with *Plasmodium falciparum* reticulocyte-binding protein homolog 5 (PfRH5) exhibited antibody titers with the endpoint of 1/10000 which inhibited merozoite invasion (Douglas *et al.*, 2011). Therefore, immunization of mice with rPyGAPDH and rPyLDH produced antibody titers, which suggested that the proteins were immunogenic.

5.3.2 Challenge infection in immunized mice

The current study evaluated whether immunization with rPyLDH and rPyGAPDH could induce protective immunity against challenge with *P. berghei* blood-stage infections. *P. berghei* parasites were used for infections in this study. Amino acid alignments of GAPDH and LDH from *P. yoelii* and *P. berghei* (Figure 5.1 and 5.2) revealed 94.96% and 99.37% similarities, which suggest high levels of cross-reactivity between antibodies against the enzymes from the two parasites. Recombinant *Plasmodium knowlesi* phosphoethanolamine methyltransferases (rPkPMT), an enzyme involved in the biogenesis of lipids in human *Plasmodium* parasites was used as a control as the enzyme is not found in mammals and non-human *Plasmodium* parasites (Bobenchik *et al.*, 2011). There was no difference in parasitemia between PyLDH immunized mice and the control group. Four of the rPyGAPDH developed high parasitemia, while one mouse in this group showed mild protection and maintained the parasitemia below 5% throughout the study. Lactate dehydrogenase LDH has not been evaluated for protection against malaria infections. On the other hand, GAPDH from *P. berghei* has been shown to protect mice against sporozoite challenge (Cha *et al.*, 2016). Most malaria vaccine candidate studies generally challenge mice with 10^5 - 10^6 infected red blood cells (Hirunpetcharat & Good, 1998; White *et al.*, 1991). The current study challenged with 10^5 , which is often used and 1/5 mice in rPyGAPDH group appeared to not develop high parasitemia and did not show fever symptoms throughout the study. Future studies could challenge mice with a lower dose of parasites, this could possibly prevent the immune response from being overwhelmed by

parasites and protection might be observed in more animals. The serum of challenged mice following immunization should be evaluated for the presence of anti-rPyLDH and anti-rPyGAPDH antibodies.

Chapter 6

Reactive black 5, a novel stain for proteins on nitrocellulose.

6.1 Introduction

6.1.1 Protein staining in polyacrylamide electrophoresis gels

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used procedure for separating and calculating the molecular weight and analysis of proteins and for following purification steps. After resolving proteins on SDS-PAGE gels, anionic dyes that bind to proteins are used to visualize the proteins. Commonly used dyes include Coomassie brilliant blue (Meyer & Lamberts, 1965), Amido black (Wilson, 1979) and a very sensitive silver stain (Nesterenko *et al.*, 1994) to name a few. Coomassie brilliant blue is the most commonly used staining technique owing to its sensitivity and its simple staining process.

6.1.2 Coomassie brilliant blue staining of proteins in SDS-PAGE gels

Coomassie brilliant blue R-250 was discovered as a stain to detect proteins on cellulose-acetate strips by Groth *et al* (1963). The staining technique included fixing the proteins to the cellulose-acetate strip with sulfosalicylic acid. Coomassie brilliant blue R-250 was later modified and used to detect parotid saliva proteins in polyacrylamide gels (Meyer & Lamberts, 1965). The staining method was more convenient as the fixing and staining of proteins was done in a single procedure. The stain consisted of 0.25% (w/v) Coomassie brilliant blue R-250 in 5:1:5 (v/v) methanol/acetic acid/water. Coomassie brilliant blue R-250 stain detects as little as 0.1-0.5 µg proteins (Brunelle & Green, 2014). Coomassie brilliant blue G-250 in trichloroacetic acid or perchloric acid is more sensitive than Coomassie brilliant blue R-250 and it does not bind acrylamide so a destaining procedure is not necessary (Neuhoff *et al.*, 1985). The sensitivities of both stains can be enhanced by using their colloidal properties and colloidal Coomassie brilliant blue R-250 detects 0.7 ng of proteins on SDS-PAGE gels without any background staining (Neuhoff *et al.*, 1985).

6.1.3 Stains for staining proteins on nitrocellulose membranes

From the time the Western blot technique was developed (Towbin *et al*, 1979), there has been a need to detect proteins on the nitrocellulose paper to localize them after the transfer step (Hancock & Tsang, 1983). Stains including Coomassie brilliant blue R-250, Amido black 10B, Ponceau S and fast green are commonly used to stain proteins on nitrocellulose. Amido black 10B is an irreversible dye and has been reported to interfere with subsequent antibody

detection, while the reversible stains, fast green and Ponceau S allow for antibody-based detection (Dunn *et al.*, 1999).

6.1.4 Reactive black 5 dye

There are a wide variety of dyes, amongst them are azo dyes. Azo dyes are the biggest group of dyes used in the textile industry (van der Zee, 2002). These dyes are distinguished from other dyes by their possession of an azo group which is made up of two nitrogen atoms (-N=N-). Azo (mono, dis, tria or polyazo) dyes include anthraquinone, phthalocyanine and triarylmethane dyes (van der Zee, 2002). Reactive dyes contain reactive groups often fluoride or chloride which make covalent bonds with OH, SH or NH groups in fabric materials. Reactive black 5 has not been reported to stain proteins.

6.1.5 Aims of this chapter

We recognized Reactive black 5 dye in our laboratory and decided to investigate whether it could stain proteins. The dye appeared to stain proteins on nitrocellulose membrane and SDS-PAGE gels. We then conducted the current study to explore the staining properties of Reactive black 5 dye in order to determine if it could be an alternative of Ponceau S stain.

6.2 Results

6.2.1 Staining proteins in a polyacrylamide gel with Reactive black 5 stain

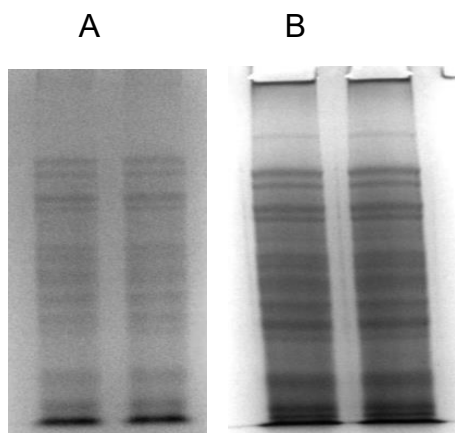


Figure 6.1: Comparison of staining the *E. coli* proteins with Coomassie brilliant blue R-250 and Reactive black 5 on SDS-PAGE gels. An *E. coli* BL21 (DE3) lysate was analyzed on a 12.5% reducing SDS-PAGE gel and stained with Coomassie brilliant blue R-250 (A) or Reactive black 5 (B) for 3 h and destained with 50% methanol and 10% acetic acid.

E. coli proteins on SDS-PAGE gel were stained with Reactive black 5 and staining was compared with Coomassie blue (Figure 6.1). Though the same proteins were stained, the staining intensity of proteins stained with Reactive black 5 is low compared to that of Coomassie

blue (Figure 6.1A and B). The destain solution did not completely remove the background staining of Reactive black 5.

To evaluate whether Reactive black 5 stains DNA on polyacrylamide gels, a DNA ladder and an *E. coli* lysate was analyzed on SDS-PAGE gel and stained with either Reactive black 5 or ethidium bromide. Ethidium bromide stained both DNA ladder and the DNA in the *E. coli* lysate (Figure 6.2A and B). Reactive black 5 did not stain the DNA ladder and only stained *E. coli* proteins on the gel (Figure 6.2B).

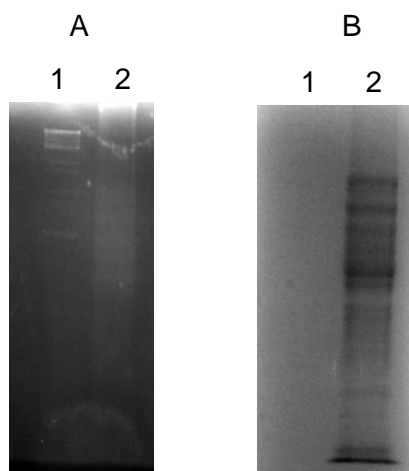


Figure 6.2: Reactive black 5 stains proteins and not DNA. DNA ladder (lane 1) and the *E. coli* lysate (lane 2) was analyzed on a 12.5% reducing SDS-PAGE gel which was stained with ethidium bromide (A) or Reactive black 5 (B).

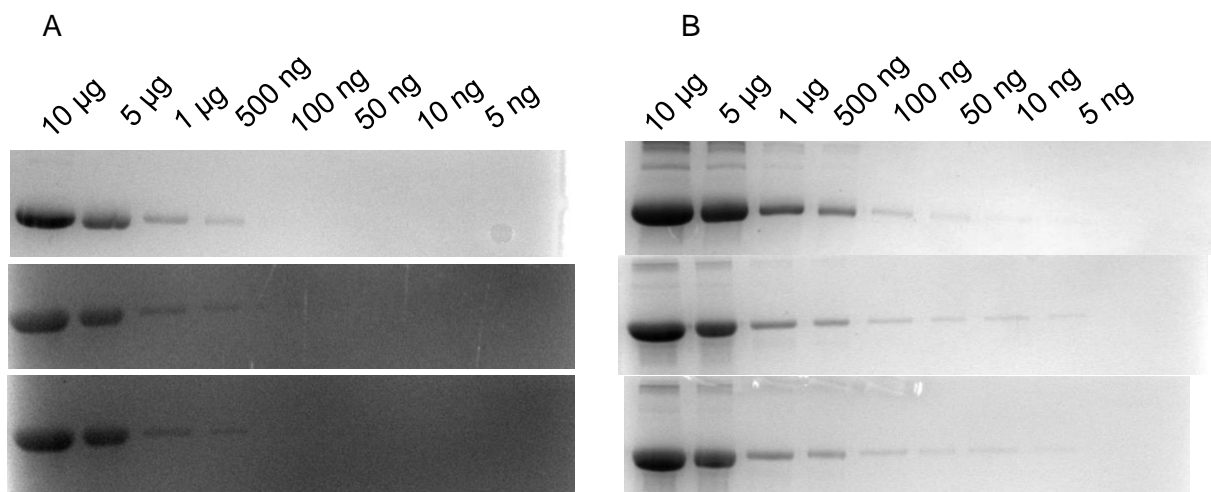


Figure 6.3: Comparison of the limit of detection of Coomassie brilliant blue R-250 and Reactive black 5. Decreasing concentrations of BSA (10 µg – 5 ng) were analysed on a 12.5% reducing SDS-PAGE gel and stained with Reactive black 5 (A) or Coomassie brilliant blue R-250 (B) for 16 h.

Reactive black 5 does not detect low concentration of proteins, it stained 500 ng of protein while Coomassie brilliant blue R-250 stained up to 5 ng (Figure 6.3A and B).

6.2.2 Staining zymograms with Reactive black 5

Zymography is a sensitive technique used to investigate the activity of proteolytic enzymes on a substrate containing SDS-PAGE gels. Amido black 10B is a commonly used stain to stain zymograms. We investigated whether Reactive black 5 can be used to stain zymograms and compared to Amido black 10B (Figure 6.4). Papain digestion was observed when staining with both Reactive black 5 and Amido black 10B (Figure 6.4A and B). The clear areas when the gel was stained with Reactive black 5 were more visible compared to Amido black 10B stained gels. The background staining for both Reactive black 5 and Amido black 10B was the same.

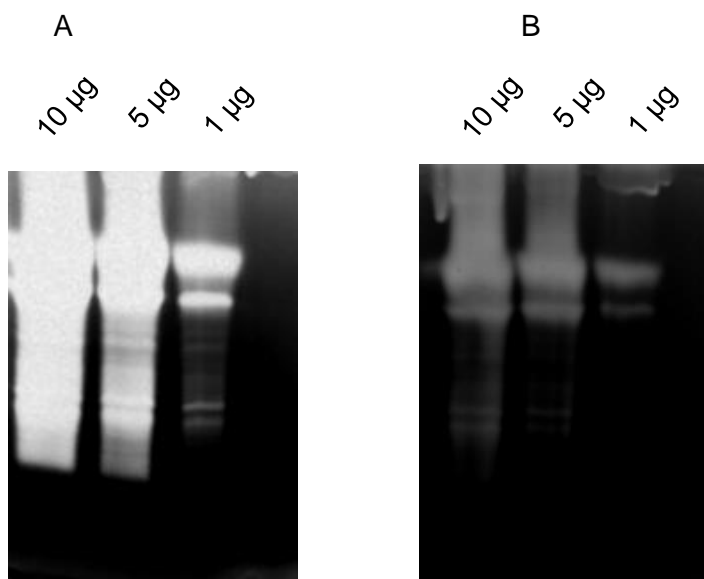


Figure 6.4: Comparison of Reactive black 5 and Amido black 10B staining of zymograms. Papain (10 µg – 1 µg) was resolved on a non-reducing 12.5% SDS-PAGE gel containing 1% (w/v) gelatin. The gel was stained with Reactive black 5 (A) and Amido black 10B (B).

The sensitivity of Reactive black 5 and Amido black 10B staining on a zymogram was investigated. Reactive black 5 detected 0.1 µg of Papain which was not detected by Amido black 10B (Figure 6.5A and 5B). Reactive black 5 digestion band intensity was higher compared to Amido black 10 B.

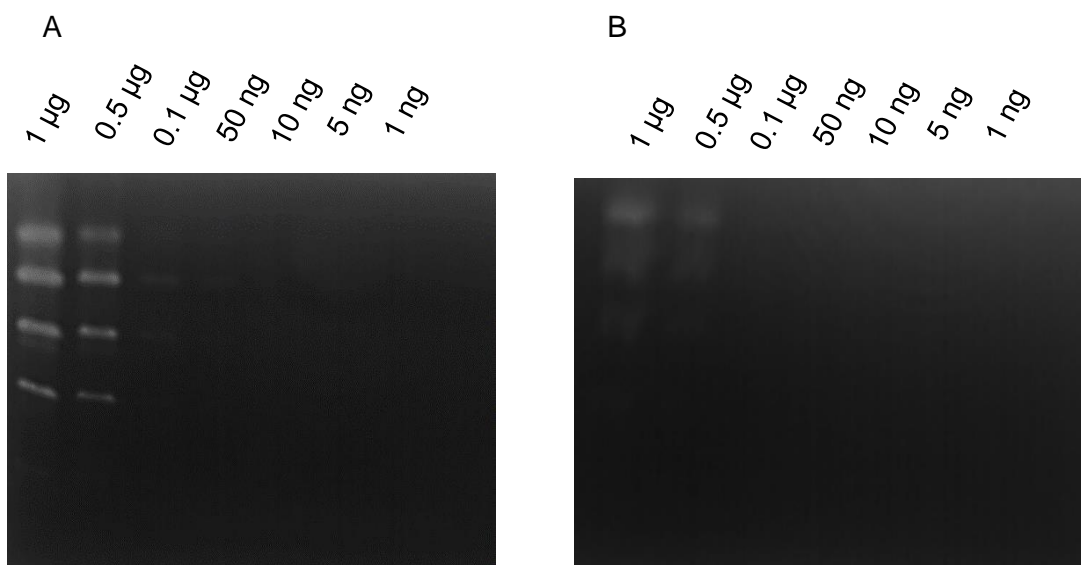


Figure 6.5: Comparison of the limit of protein detection of Reactive black 5 and Amido black 10B staining on zymograms. Papain (1 µg – 1 ng) was resolved on a non-reducing 12.5% SDS-PAGE gel containing 1% (w/v) gelatin. The gel was stained with Reactive black 5 (A) and Amido black 10B (B).

6.2.3 Staining proteins on nitrocellulose with Reactive black 5

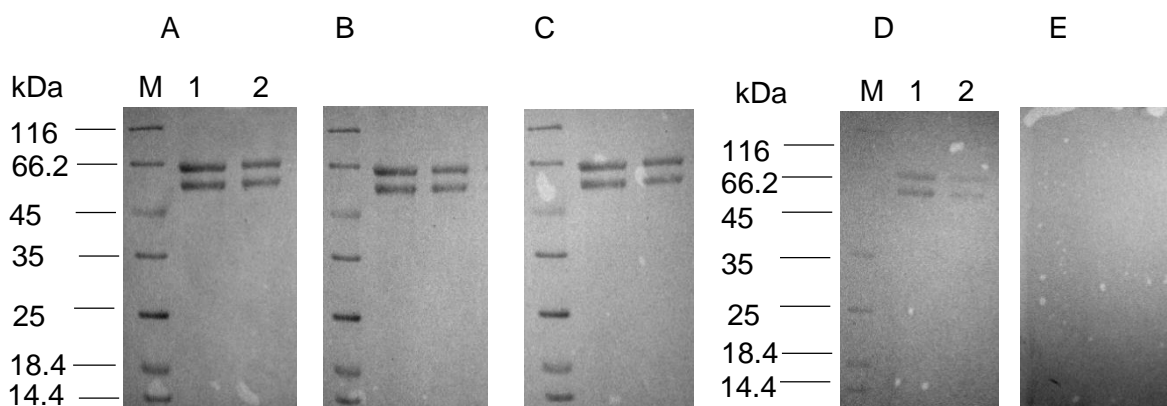


Figure 6.6: Comparison of protein staining on a nitrocellulose membrane with Reactive black 5 and Ponceau S. Protein molecular weight marker (lane M) and Rabbit albumin (lane 1 and 2) were resolved on a 12.5% reducing SDS-PAGE gel and transferred overnight on a nitrocellulose membrane which was stained with either Reactive black 5 (A) and washed with distilled water with 1M NaOH (B) or with destain I (C). Ponceau S stained nitrocellulose (D) and washed with distilled water with 1M NaOH (E).

To assess whether Reactive black 5 stained proteins on the nitrocellulose paper, protein molecular weight markers and rabbit albumin were resolved on an SDS-PAGE gel and transferred onto nitrocellulose and stained (Figure 6.6). Reactive black 5 stained all molecular weight marker proteins and rabbit albumin (Figure 6.6A). Ponceau S also stained all the

molecular weight marker proteins and rabbit albumin but with lower intensity compared to Reactive black 5 (Figure 6.6D). Reactive black 5 was not removed when washed with distilled water with NaOH or destain I (Figure 6.6B and C). In contrast, Ponceau S stain is removed by washing with distilled water with NaOH for 5 min (Figure 6.6E).

The sensitivity of Reactive black 5 and Ponceau S stain was compared using BSA. Different BSA concentrations were analyzed on a nitrocellulose membrane which was stained with either Reactive black 5 or Ponceau S stain (Figure 6.7). Reactive black 5 stained 5 ng of BSA on a nitrocellulose membrane (Figure 6.7A). The stain was not removed with multiple washes. Ponceau S detected 5 ng of BSA on a nitrocellulose membrane. Since Ponceau S is reversible, the washing step removes some of the stain that is bound to the proteins. This results in the reduced intensities of the protein bands (Figure 6.7B).

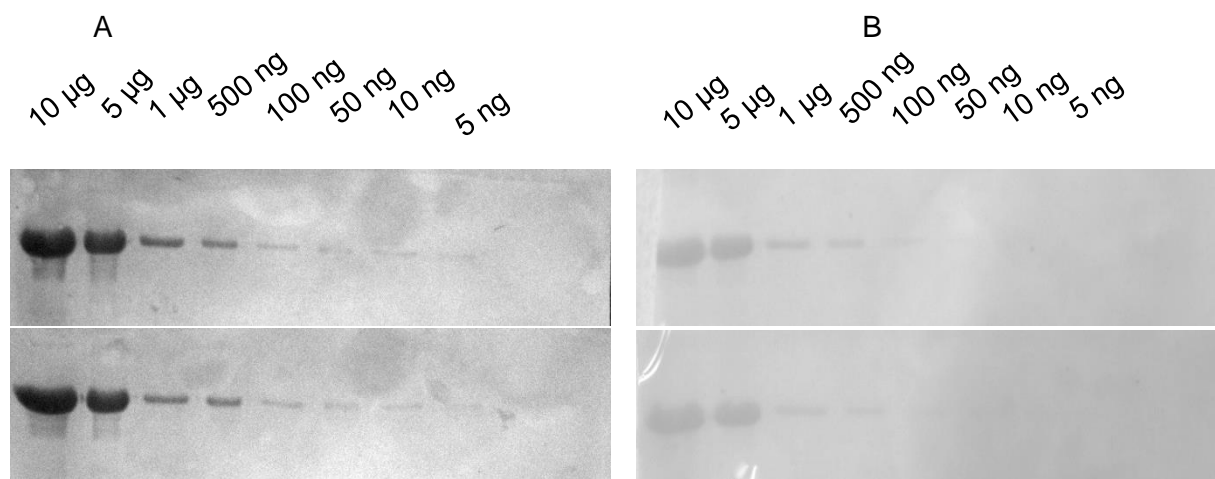


Figure 6.7: Comparison of the limit of protein detection of Reactive black 5 and Ponceau S stain. Decreasing concentrations of BSA (10 µg – 5 ng) were analyzed on a 12.5% SDS-PAGE gel, transferred onto the nitrocellulose and stained with Reactive black 5 (A) or Ponceau S stain (B) for 5 min.

Reactive black 5 and Ponceau S stains were compared with an *E. coli* lysate on a nitrocellulose membrane (Figure 6.8A and B). Both stains stained different proteins with different intensities. Both stains had a detection limit of 2.5 µg of *E. coli* proteins.

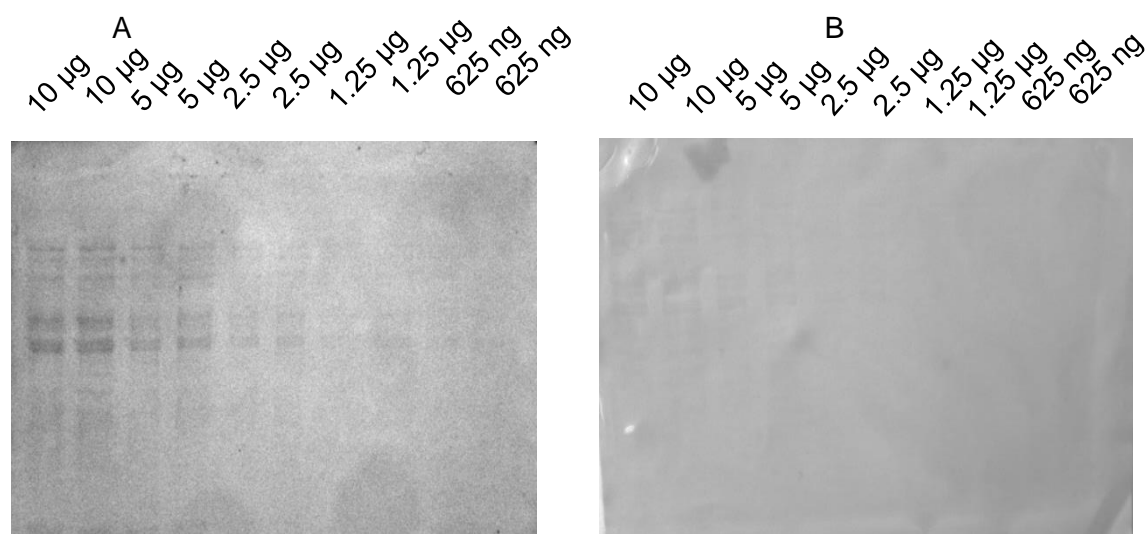


Figure 6.8: Comparing staining of an *E. coli* lysate with Reactive black 5 and Ponceau S stain. An *E. coli* lysate (10 µg – 625 ng) was resolved on a 12.5% SDS-PAGE gel and transferred overnight on nitrocellulose and stained Reactive black 5 (A) or Ponceau S (B) for 5 min.

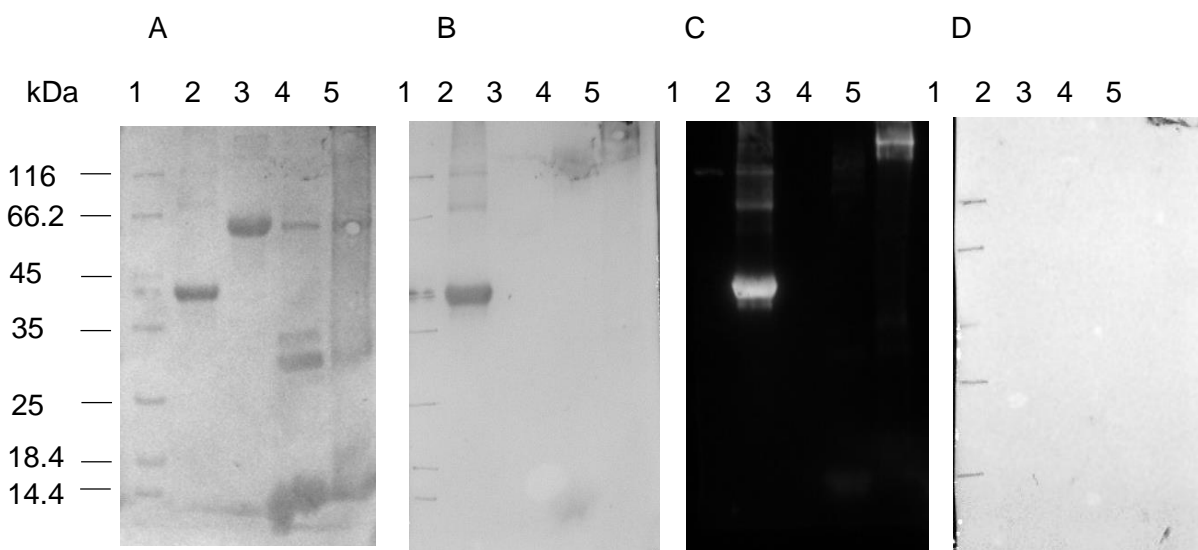


Figure 6.9: Staining proteins with Reactive black 5 does not affect the interaction with antibodies in a Western blot. Proteins were loaded as follows: lane 1: molecular weight marker, lane 2: rPyLDH, lane 3: BSA, lane 4: uninfected mouse red blood cell (RBC) and lane 5: *Plasmodium berghei* infected mouse RBC. Proteins were resolved on a 12.5% reducing SDS-PAGE gel and transferred to nitrocellulose and stained with Reactive black 5 (A). The obtained membrane was probed with anti-Histag antibodies and the signal was detected with chemiluminescence. B, C and D are the blot with ECL, ECL signal and nitrocellulose after antibody detection steps.

Reactive black 5 was assessed to evaluate if the stained proteins could be detected by antibodies. Proteins were transferred to nitrocellulose and stained with Reactive black 5 (Figure 6.9A). The stained proteins were probed with anti-Histag antibodies to detect rPyLDH (Figure

6.9B and C). Mouse proteins on lane 4 and 5 were also detected as the secondary antibody was a goat anti-mouse antibody. The stain did not interfere with the chemiluminescence signal. The figure also showed that nitrocellulose membrane stained with Reactive black 5 makes a good Western blot reference as the stain can stain both the molecular weight markers and the proteins being analyzed. After all, the antibody detection steps, Reactive black 5 stain dissociated from the proteins (Figure 6.9D).

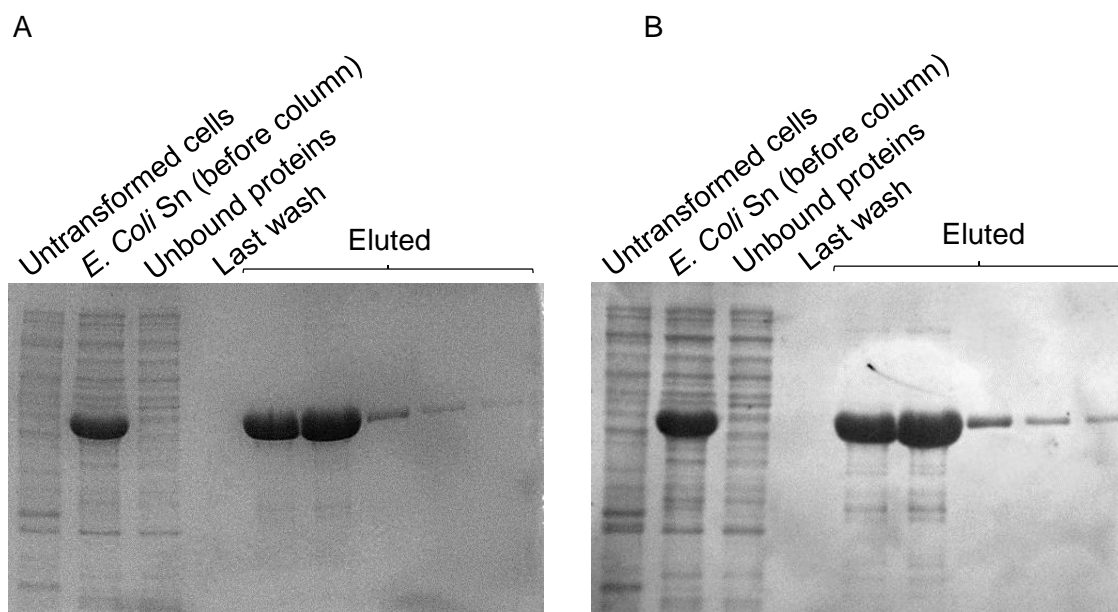


Figure 6.10: Comparison of Western blot reference with Coomassie brilliant blue R-250 stained SDS-PAGE gel and Reactive black 5 stained nitrocellulose. Recombinant *PyGAPDH* purification was analysed on a 12.5% SDS-PAGE gel and transferred overnight to a nitrocellulose membrane. Coomassie brilliant blue R-250 stained gel (A) and Reactive black 5 stained nitrocellulose (B).

Reactive black 5 stained nitrocellulose was compared with Coomassie brilliant blue R-250 stained SDS-PAGE gel for a Western blot reference (Figure 6.10). Reactive black 5 stains the same proteins on nitrocellulose as Coomassie brilliant blue R-250 stained SDS-PAGE gel (Figure 6.10A and B). Reactive black 5 stained nitrocellulose has proteins with higher intensities compared to Coomassie brilliant blue R-250 stained SDS-PAGE gel. Therefore, Reactive black 5 stained nitrocellulose membranes are alternative Western blot references.

6.3 Discussion

6.3.1 Staining proteins in polyacrylamide gels with Reactive black 5

Staining proteins with Reactive black 5 was evaluated. Reactive black 5 dye is used in the textile industry to stain fabrics. Reactive black 5 has not been documented as a protein stain on polyacrylamide gels or nitrocellulose membranes. Proteins in SDS-PAGE gels were stained with Reactive black 5 and the dye stained the same *E. coli* bacterial proteins as Coomassie brilliant blue R-250 but with poor intensity. Reactive black 5 had a high background on SDS-PAGE gels unlike Coomassie blue. Reactive black 5 showed to be a protein specific stain that does not stain DNA in SDS-PAGE gels and allows for staining zymograms. Zymogram staining with Reactive black 5 was more sensitive than staining with Amido black 10B stain.

6.3.2 Staining proteins on nitrocellulose with Reactive black 5

A Western blot (Towbin *et al.*, 1979) allows for transfer of proteins from SDS-PAGE to nitrocellulose where proteins are more accessible for further analysis. Various stains for proteins in nitrocellulose have been described. These stains include Coomassie blue, Amido black, fast green, India ink and Ponceau S. Ponceau S is the most commonly used stain owing to its reversible properties that allow antibody-based detection (Dunn *et al.*, 1999). Reactive black 5 stained rabbit albumin (Figure 6.6), bovine serum (Figure 6.7), proteins in an *E. coli* bacterial lysate (Figure 6.8), recombinant proteins and mouse red blood cell proteins (Figure 6.9). Reactive black 5 stained proteins in 5 min like Ponceau S. Washing Reactive black 5 stained nitrocellulose with water and sodium hydroxide did not remove the stain. Reactive black 5 had the same limit of detection as Ponceau S. Reactive black 5 staining did not prevent the antibody detection unlike other irreversible stains like Coomassie blue and Amido black 10B (Dunn *et al.*, 1999).

6.3.3 Reactive black 5 stained nitrocellulose as a reference for a Western blot

Stains for nitrocellulose membranes are important to ensure that the transfer of proteins was efficient before blocking the membrane for antibody detection and for localizing the molecular weight markers (Hancock & Tsang, 1983). Duplicate gels are stained to compare the size and position of the blotted proteins. SDS-PAGE gels increase in size after staining making it difficult to accurately compare proteins on nitrocellulose and the reference gel (Hancock & Tsang, 1983). Stained nitrocellulose membranes would provide an accurate comparison of protein bands and save reagents. India ink has been reported for staining nitrocellulose. India ink stains 80 ng proteins in 2 hr and its sensitivity varies for different proteins (Hancock &

Tsang, 1983). Reactive black 5 is sensitive, stains the molecular weight markers and the stained nitrocellulose is comparable with a stained SDS-PAGE reference gel (Figure 6.10).

6.3.4 Reactive black 5 decolorizes during the antibody detection step

Proteins on Western blots are often detected with a method that involves a specific antibody, followed by a secondary antibody conjugated to an enzyme like horseradish peroxidase (HRP) (Sennepin *et al.*, 2009). HRP detection requires hydrogen peroxide and a substrate to initiate an oxidation reaction that results in the formation of a coloured precipitate with a chromogenic substrate, or the emission of light with a chemiluminescent substrate (Haan & Behrmann, 2007). Oxidation involving hydrogen peroxide and ferrous ions have been reported to decolourize Reactive black 5 dyes in aqueous solutions (Lucas & Peres, 2006). The colour of the Reactive black 5 stain disappears after the enzyme reaction process (Figure 6.9D).

6.3.5 The Reactive black 5 staining mechanism

Reactive black 5 is an anionic dye like Coomassie blue, both dyes contain charged sulfonic groups (SO_3^-) (Lucas & Peres, 2006; Tal *et al.*, 1985). Coomassie blue binds to the amine groups of amino acids through its sulfonic groups when it is prepared in acidic conditions forming van der Waals, electrostatic interactions and hydrogen bonding (Tal *et al.*, 1985). Reactive black 5 dye forms covalent bonds with fabric when it is employed in textile staining (van der Zee, 2002). The ability of Reactive black 5 to stain nitrocellulose irreversibly could be afforded by the formation of covalent bonds with nitrocellulose.

6.3.6 The Reactive black 5 stain is economical

Reactive black 5, Amido black 10B and Ponceau S stains are all prepared in 0.1% (w/v) in 1% acetic and with 30% methanol for Amido black 10B. According to catalogue prices (Sigma-Aldrich, accessed 9/11/2018), Reactive black 5 is 2.2 times cheaper than Amido black 10B and 2.95 times cheaper than Ponceau S. Staining of zymograms is even cheaper as Amido black preparation requires additional methanol. Staining Nitrocellulose membranes with Reactive black 5 and taking images for Western blot reference could save a lot of money than having a duplicate reference gel.

Chapter 7

General discussion

7.1 Overview

Malaria is a disease that claims many lives in sub-Saharan Africa. *Plasmodium falciparum* is responsible for the majority of these deaths. People who are most affected are children who are 5 years old and younger, pregnant women and non-immune visitors to endemic areas (World Health Organization, 2016). Many strategies have been implemented in the past to control the disease. These strategies include insecticide-treated bed nets and antimalarial drugs. These methods have resulted in the clearance of the disease in Europe, Russia and some parts of Asia (Guerin *et al.*, 2002). The increase of insecticide-resistant mosquitoes and drug-resistant *Plasmodium* parasites have made it difficult to control the disease using insecticide-treated bed nets and common antimalarial drugs (Ranson *et al.*, 2011). New strategies to control the disease are required and a malaria vaccine could prevent the disease in young children and could also aid in eliminating the disease. The RTS, S/AS01 vaccine targets the liver stage of malaria infection, reducing parasites that develop and invade erythrocytes resulting in disease symptoms and death. RTS, S/AS01 is the only malaria vaccine that has been endorsed for use (RTS, 2015). Although, it has low efficacy and provides protection that diminishes over 3-4 years and it is not approved for infant vaccination. There are many vaccines that are currently being investigated for blood-stage malaria vaccine candidates including PfAMA1, MSP1, MSP3, GLURP and RH5 (Cockburn & Seder, 2018). These vaccines are based on the production of vaccine-induced antibodies to block the invasion of erythrocytes by malaria parasites, thereby, preventing the blood stage infection. None of these vaccines has made it to phase III clinical trials. More vaccine candidates and new approaches to developing a vaccine that could meet the World Health Organization goal to eliminate the disease are required.

7.2 Major findings, future work and conclusions

IgY is the most plentiful immunoglobulin in chicken serum and it's passed to the yolk for the embryo's immunity against infectious pathogens (Yegan & Korver, 2010). IgY does not interact with complement (Gardner & Kaye, 1982) nor Rheumatoid factor (Larsson & Sjöquist, 1988) and has been reported to not cross-react with mammalian proteins hence making it ideal for human immunodiagnosics (da Silva & Tambourgi, 2010). This study produced chicken IgY antibodies against recombinant *Plasmodium falciparum* lactate dehydrogenase (rPfLDH). The antibodies were isolated (Polson *et al.*, 1980) and affinity purified with rPfLDH coupled to an

aminolink® affinity column. High yields of anti-r*Pf*LDH IgY antibodies were obtained. The antibodies were specific for r*Pf*LDH and did not recognize *E. coli* bacterial proteins. The performance of RDTs is influenced by temperature (Chiodini *et al.*, 2007). Chiodini *et al.* (2007) reported a reduction of pLDH and HRP-2 RDTs sensitivity when the tests are stored and operated in high ambient temperatures. Mouse IgG antibodies are commonly used in RDTs (Moody, 2002). Further work could include a comparison of the stability of mouse IgG and chicken IgY antibodies at different temperatures.

Polymorphisms of diagnostic candidate proteins affect the performance of the proteins in rapid diagnostic tests (RDTs) (Baker *et al.*, 2005). The current study evaluated polymorphisms in plasmodial lactate dehydrogenase (pLDH) and glyceraldehyde-3-phosphate dehydrogenase (pGAPDH). The pLDH common and unique peptides identified for the detection of *P. falciparum* and *P. vivax* infections by RDTs had minimal polymorphisms which suggest that pLDH is a good diagnostic target. The pGAPDH common and *P. falciparum* unique peptide also had no polymorphisms. During the blood stages of *P. falciparum* parasite development, higher levels of *Pf*GAPDH are produced compared to *Pf*LDH (Bozdech *et al.*, 2003). Together these results suggests that the pGAPDH based RDTs could be potentially more sensitive than pLDH based RDTs. Many vaccine candidates have failed due to polymorphisms in their sequences which results in strain-specific immune responses (Takala *et al.*, 2009). Evaluation of polymorphisms in *Py*LDH and *Py*GAPDH sequences revealed only two synonymous mutations in both proteins which are low compared to the *Pf*RH5 vaccine which induces immunity that is not strain-specific. These observations imply that *Py*LDH and *Py*GAPDH based vaccines could be potentially effective and therefore should be further evaluated in animals.

Development of a malaria vaccine could aid in the prevention of morbidity and mortality due to the disease and facilitate elimination of malaria (Cockburn & Seder, 2018). The study evaluated *Py*GAPDH and *Py*LDH immunogenicity in mice and whether vaccination of mice with these proteins could be protective against *P. berghei* infection challenge. Immunogenicity of r*Py*GAPDH and r*Py*LDH was demonstrated from the mouse serum following vaccination. Although, vaccination with r*Py*LDH did not show protection against *P. berghei* infection when compared with r*Pk*PMT vaccinated mice. All the r*Py*LDH vaccinated mice developed high parasitemia and suffered from the disease. Only 1/5 r*Py*GAPDH vaccinated mice suppressed the blood stage parasitemia below 5% throughout the study. Further work includes repeating the r*Py*GAPDH and r*Py*LDH immunization and infecting mice with lower levels of parasitemia.

Passive immunization with purified anti-rPyGAPDH and anti-rPyLDH antibodies could be evaluated for protection against blood-stage malaria infection.

Reactive black 5 is a dye used in the textile industry to stain fabrics. This study evaluated protein staining with Reactive black 5. The dye stained a variety of proteins on SDS-PAGE gels, zymograms and nitrocellulose. Reactive black 5 stained the same proteins as Coomassie blue but with lower intensity on SDS-PAGE gels. The dye stained zymograms and the digestion bands were clearer than those of Amido black 10B stained zymograms. Reactive black 5 detected the same lowest concentration of proteins like Ponceau S on nitrocellulose. Unlike Ponceau S, Reactive black 5 binds strongly to proteins and the dye is not easily removed by changing pH and washing with water. Unlike Coomassie blue, Reactive black 5 did not interfere with antibody detection on nitrocellulose. An enzyme reaction involving horseradish peroxidase and hydrogen peroxide decolourized the dye on nitrocellulose. Reactive black 5 stain is economical, being 2.2 times cheaper than Amido black 10B and 2.95 times cheaper than Ponceau S.

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