THE POTENTIAL OF CYSTEINE PROTEASE-ENCODING-GENE AS CANDIDATE FOR DNA VACCINE AGAINST *PLASMODIUM BERGHEI* INFECTION IN MICE

BY

TIMOTHY BULUS
B.Sc.(UNN 1997); M.Sc. (ABU 2004)
(Ph.D/SCIE/45931/2004-05)

A DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

JANUARY, 2013

DECLARATION

I declare that the work in this Dissertation entitled, 'The potential of cysteine protease-encoding-gene as candidate for DNA vaccine against *Plasmodium berghei* infection in mice' was performed by me in the Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria under the supervision of Professor A.J. Nok (*MFR*, FAS, *NNOM*), Dr. E. Onyike and Dr. Sani Ibrahim. No part of this Dissertation, as it is, was previously presented for another degree or diploma at any University.

Information and excerpts cited from the work of others have been duly acknowledged by means of references.

_____ _____

TIMOTHY BULUS **DATE**
(Ph.D/SCIE/45931/2004-05)

CERTIFICATION

This dissertation entitled**,** "**THE POTENTIAL OF CYSTEINE PROTEASE-ENCODING-GENE AS CANDIDATE FOR DNA VACCINE AGAINST *PLASMODIUM BERGHEI* INFECTION IN MICE**" by Timothy Bulus meets the regulations governing the award of the Degree of Doctor of Philosophy (PhD) in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. A. J. Nok (*MFR*, FAS, *NNOM*) **Chairman, Supervisory Committee**	**Date**
Dr. E. Onyike **Member, Supervisory Committee**	**Date**
Dr. S. Ibrahim **Member, Supervisory Committee**	**Date**
Dr. Mrs H. M. Inuwa **Head of Department**	**Date**
Prof. A. A. Joshua **Dean, School of Postgraduate Studies**	**Date**

DEDICATION

I dedicate this work to JESUS CHRIST my Saviour and Lord, whose sacrificial death on the cross of Calvary made me all I am today and all I will ever be.

ACKNOWLEDGEMENT

God gave me life. He inspired this path and by His power and wisdom I walked this way. To Him I remain eternally grateful!

Prof. Andrew Jonathan Nok played an outstanding role here; he encouraged me to pick up a Ph.D form immediately I completed my M.Sc. degree. He gave me the direction for the work and provided the much needed supervision. His mentorship in the fields of Malaria and Biotechnology research is hereby acknowledged. The supervisory efforts of Dr. Sani Ibrahim and Dr. Elewechi Onyike both of the department of Biochemistry, Ahmadu Bello University were immense. For their professional guide, support, encouragement and above all their patience, I am very grateful.

I am particularly grateful to Prof. S. Kelm, the Dean, Department of Biology and Chemistry of the University Bremen, Germany, for his scientific and technical contribution to this study and especially for the permission to use the bench space in his laboratory to conduct the molecular aspect of the work. I also recognize the contributions of members of staff at the center for Biomoelecular Interaction, University of Bremen, Germany, especially Dr. Frank Dietz and Mr. Thaddeus Terlumun Gbem (Oga TT). Their assistance has made the realization of my objectives possible.

I appreciate the assistance of all the staff of the Center for Biotechnology Research and Training , A.B.U., Zaria; notable among them are Dr. Ela, Miss Gloria Dada Chechect, Dr. Afolabi-Balogun Nusrah Bolatito, Mr Silas, Malam Samir and Mr. Elisha. I acknowledge the various help I received from other postgraduate students working under the supervision of Prof. A. J. Nok- Mr. Azubike, Dr. Ishaya Longdet and Miss Helena Gwani.

Dr. James Sambo of the department of Veterinary Pathology assisted in the area of histopathology, Mr. Obemeata, J.A. of Data Processing Unit, I.A.R., A.B.U., Zaria, performed all the statistical analyses. I appreciate their patience and contributions.

I remain ever grateful to my father, Late Mr. Bulus Gambo (1941-2012) and my mother Mrs Laraba Bulus Gambo for their love and support. I am also grateful for the moral and financial support from my siblings- Mrs Helen Lekeys, Anti Abishag, Anti Esther, Anti Gloria, Anti Dorcas, Baban Sam (Mr Jonah Bulus), Emamanuel, Jenom and Iliya.

I thank the Head of department, Dr H.M. Inuwa, for her motherly leadership and I also sincerely appreciate the contributions of the members of staff of the Department of Biochemistry, A. B. U., Zaria, towards this study, particularly, Prof. H.C. Nzelibe, Dr. I.A. Umar Dr. A.B. Sallau and Mr. Njoku and Mr. Idowu.

The spiritual encouragement from brethren of the Chapel of Good News, Tafawa Balewa way, Kaduna, and Chapel of Redemption, A.B.U., Zaria, provided the much-needed divine momentum for the work to progress per time.

This work was partly sponsored by grant from Educational Trust Fund (ETF), Nigeria and grant from University Research, Kaduna State University, Kaduna.

Finally, I wish to thank my dear wife, Martha, for her love, patience, forbearance and support during this study, and my sons, Blessed and Joseph, for providing the fun at home, making the home atmosphere “charged” with inspirations and ideas. I love you.

Unto God who saw me through this study and made its completion possible, be all the glory now and through all eternity. Amen.

ABSTRACT

The potential of cysteine protease-DNA -vaccine- constructs to protect mice against *Plasmodium berghei* infection was investigated. The full-length genes encoding the orthologs of falcipain-1 and falcipain-2 were identified in the database, isolated and amplified by a nested polymerase chain reaction (PCR) using the genomic DNA of a rodent malaria parasite, *Plasmodium berghei, as* template. The oligonucleotides used to prime the PCR were designed based on the identified contigs at the Welcome Trust Sanger Institute containing the entire cysteine proteases reading frames. The gel purified amplicons were cloned into pcDNA3AmpStrepTag vector and the presence of the genes in positively transformed XL1-Blue *E. coli* strain were confirmed via colony PCR, restriction digest and sequencing. The Berghepain-1 (BP1) and Berghepain-2 (BP2) genes amplified contain 1560 bp and 1407 bp open reading frames encoding 519 and 468 amino acids with molecular masses of 60.31 kDa and 54.46 kDa, and isoelectric points of 7.52 and 6.01, respectively. Sequence analyses and alignments showed that both deduced proteins belong to peptidase_C1 superfamily, lacked signal peptides and possess high identity (up to 78%) with their corresponding cysteine protease orthologs from other malaria species and conservation of the Cys, His and Asn residues that constitute the catalytic triad. Immunoflouresence study with pcDNA3 plasmids harboring these genes and StrepTag sequences, in frame, confirmed the transient expression of these proteins in COS-7-transfected mammalian cells. The pcDNA3AmpBP1StrepTag and pcDNA3AmpBP2StrepTag midipreps were cut with two sets of restriction endonuleases (BamHI/XhoI and HindIII/XhoI) for onward ligation into pVax1 already cut with the same enzymes systems. The later strategies were adopted to allow for the subcloning into pVax1 in order to produce pVax1KanTransinBPStrepTag and pVax1KanBPStrepTag DNA vaccines for both enzymes with and without transin signal peptide respectively. Mean total plasmid DNA produced with transin was 2.73 mg while those without transin sequence was 3.02 mg. Seven groups of mice (ten mice per group) were each immunized with the vaccine constructs and all the animals were each sunsequently challenged with 1.43 x 10^7 *P. berghei*-iRBC. Mice in the positive and negative control groups were given membrane-filtered PBS as placebo in place of DNA vaccine and the groups were either challenged with the parasite or left unchallenged respectively. Parasitaemia, animal weights and red cell counts were determined during the experiment. Deaths were also closely monitored and survival rate of the experimental groups were noted. All values

observed were statistically compared with values of the three control groups (Negative, Positive and vector controls). Immunization with the DNA vaccine constructs lowered the parasitaemia in mice vaccinated with pVax1StreptagBP2, pVax1TransinStreptagBP1, and pVax1TransinStreptagBP2. This pattern in parasite burden also affected, in identical manner, the red cell counts, mean weight and ultimately the percentage population of healthy survivors seen. Animals vaccinated with bergheipain2 gene insert were better protected against the lethal infection than those vaccinated with Bergheipain1-encoding plasmids. Better protection were seen in groups of mice immunized with DNA vaccine with transin signal peptide than those without it. The possibility of plasmid DNA integration into the genomic DNA of experimental animals was also checked using PCR technique and results obtained did not show any possible integration. This study supports the immunogenicity of plasmodium cysteine protease (CP) and demonstrates the potential of bergheipain-based DNA vaccine to protect mice against infection due to *P. berghei*. Though the protection appeared to depend on the homologue employed, it was enhanced by the incorporation of signal peptide sequence at the 5′-end of the CP gene.

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LIST OF ABBREVIATIONS

ANOVA- Analysis of variance

BP1- Bergheipain1

BP2 - Bergheipain2

BSA- Bovine Serun Albumin

DMEM- Dulbecco's modified eagle medium

DNA- Deoxyribonucleic Acid

gDNA- genomic Deoxyribonucleic Acid

FCS- Foetal calf serum

iRBC- Infected Red Blood Cells

LB Medium- Luria-Bertani Medium

MHC- Major Histocompatibility Complex

PBS- Phosphate Buffer Saline

PBSG- Phosphate Buffer Saline Glucose

PCR- Polymerase Chain Reaction

RBC- Red Blood Cells

Th- Helper Thymus cells

TAE- Tris Acetate EDTA Buffer

NCBI - Center for Biotechnology Information

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Malaria is a life-threatening parasitic disease caused by various species of plasmodia and is transmitted via the bite of mosquitoes . It is thought to be man's most important parasitic disease. According to the World Health Organization there are 300 to 500 million clinical cases of malaria each year resulting in 1.5 to 2.7 million deaths (IDR centre report, 1996) with Sub-Saharan Africa known to be the region with the highest malaria infection rate. In fact, it is reported that 80 -90 % of malarial deaths occur in this region (IDR centre report, 1996).

Four plasmodium species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*) are responsible for malaria infection among human with *P. falciparum* infection accounting for most of the death cases encountered (Orjih, 2005) that is why research efforts on malaria has focused largely on *P. falciparum.* *Plasmodium berghei* is a causative agent of rodent malaria and usually presents similar cerebral infection equivalent to that by *P. falciparum* in human (Albay et al., 1999). The major features of *P. berghei* infection are anaemia, splenomegaly, fever and liver damage (Thurston, 1953; Sudhir and Saxena, 1980;).

The management of malaria requires the use of chemotherapeutic substances. Chloroquine and sulfodoxine-pyrimethamine are two of the commonly used antimalarial drugs. In recent time, however, drug toxicity and the increasing incidence of drug resistance (Rosenthal, 1998; Ekland and Fidock, 2007) have constituted the major challenges facing chemotherapeutic efforts against malarial infection and the molecular basis for this resistance is still highly speculative which makes it difficult to develop countermeasures (Salganik et al., 1987). These challenges are even more apparent in developing countries of the world where the purchasing ability of the majority

of the populace is quite low. The ultimate solution to the pandemic is the development of an effective vaccine against the disease.

The conventional approaches at developing effective malarial vaccine is largely carried out using isolated proteins or glycoproteins which had been implicated in the life cycle of the disease (Ramachandra et al., 2000). Most of these proteins are enzymes which catalyse key reactions which are important for the parasite's survival. Such approaches have required the extraction of large quantity of the immunogenic substance and so, recently, biotechnological tools have been utilized to identify genes coding for these proteins from where recombinant molecules are subsequently expressed, biochemically characterized (Eakin et al., 1990; Byoung-Kuk et al., 2004) and used during immunization regimen. Again, it has been observed that when isolated proteins are used to elicit immune response, the parasites exhibit an unusual mechanism of evading such response by a continual change of its surface coat (a phenomenon known as antigenic variation). This ability is a genetic property of the organism and so has made the development of an effective vaccine almost impossible.

DNA vaccine research, a relatively recent development in vaccine methodology, is a novel and powerful alternative to conventional vaccines efforts. This new technique has the potential of overcoming most of the difficulties faced in attempt to develop immunizing antibodies using conventional protein immunization methods. DNA vaccines, involves the deliberate introduction into tissues of a DNA plasmid carrying an antigen-coding gene that transfects cells in vivo and results in an immune response. DNA vaccines have several distinct advantages, which include ease of manipulation, use of a generic technology, simplicity of manufacture, and chemical and biological stability (Whalen, 1996). DNA vaccination results in the induction of specific Th1 and CD8 T cell responses and their ability to protect various host models against infections due

to viruses, bacteria and protozoans have been demonstrated (Jun-ming and Dao-yin, 2006; Carter et al., 2007).

The use of a wide variety of genes encoding particular pathogen's enzymes is a common feature being exploited in the design of DNA vaccine (Carter et al., 2007). Cysteine proteases [EC 3.4.22] (Dixon et al, 1979) are found among various protozoans (Byoung-Kuk et al., 2004; Nicolas et al., 2009; Caldeira et al., 2009). In malaria, cysteine proteases have been shown to play very important roles during the life cycle of the parasites which include; haemoglobin hydrolysis, erythrocyte rupture, erythrocyte invasion by the parasites and malarial sporozoite egress from oocystes (Rosenthal, 1998; 2004; Aly and Matuschewski, 2005). This makes cysteine protease a good target for vaccine development. Identifying cysteine protease gene in *P. berghei* and targeting it as a possible candidate for the development of model DNA vaccine against malaria is what this study intends to achieve.

1.2 Statement of Problem

The use of insecticides and bed nets plus current chemotherapeutic management of malaria, have not succeeded in eradicating malaria infection. Problems facing chemotherapy of malaria is largely due to observed growing incidence of resistance pose by plasmodium parasites against commonly used antimalarials (Ekland and Fidock, 2007). Despite several scientific efforts, to date, there is no effective malarial vaccine in use (Richie and Saul, 2002; Kaba et al., 2012). The development of effective malaria vaccine will provide a long term antidisease management alternative to chemotherapy .

1.3 Research Null Hypothesis

DNA vaccine based on cysteine protease gene will not confer protection against *Plasmodium berghei* infection.

1.4 Aims and Objectives of the Study

The aim of this study is to investigate if vaccination with DNA vaccine- encoding cysteine protease will protect mice against infection due to *Plasmodiun berghei*.

The objectives of this study are:

1. To identify and isolate the gene coding for cysteine protease in *P. berghei.*
2. To design and construct DNA vaccine encoding cysteine protease of *P. berghei*.
3. To evaluate the potential of the DNA vaccine construct to protect mice against *P. berghei* infection.

1.5 Justification for the Study

Every year, malaria is one of the leading causes of death and disease worldwide, especially in the developing world. It kills more than one million people each year, most of them in sub-Saharan Africa, with seventy-five percent of these deaths occurring in children under five. (http://www.malariaconsortium.org/pages/malaria.htm, 2012). In Africa malaria is the biggest killer of children under five years old, who account for with nearly 90 percent of all malaria deaths. It is estimated that a child dies every 45 seconds from the disease (http://www.malariaconsortium.org/pages/malaria_challenges.htm, 2012). The impact of malaria on children remains a serious obstacle to the achievement of many of the Millennium Development Goals (MDGs), including Goal 2 (universal primary education) and Goal 4 (the reduction of infant mortality) (http://www.malariaconsortium.org/pages/malaria_challenges.htm, 2012). There is a dare need to alter the existing sad statistics associated with malaria in Africa.

The management strategy adopted over the years against malaria infection has to be reviewed. This is in view of the fact that chemotherapy, which is a major component of the disease

management strategy, has suffered a lot of setback posed mainly by the increasing incidence of drug resistance observed with field use of most antimalarials (Ekland and Fidock, 2007).

It is thought that the development of effective malaria vaccine will provide a long term antidisease management alternative to chemotherapy. Several efforts at raising preventive antibodies in trying to develop effective malaria vaccine have over the years failed. The failure recorded with conventional approach to immunization using recombinant proteins suggests the need to try other alternatives in the search for the malaria vaccine.

DNA vaccination provides a new direction required for this deviation. DNA vaccine is a new and promising approach that is thought to have the potential of overcoming most of the challenges put forward by conventional protein immunization regimen (Whalen, 1996; Carter et al., 2007).

The gene of malarial cysteine protease is the chosen target for this DNA vaccine research because of the crucial roles the enzyme plays during the pre and intraerythrocytic stages of the parasite life cycle (Rosenthal, 1998; Byoung et al., 2004; Aly and Matuschewski, 2005). Furthermore, the gene has been considered as a good target for vaccine design (Nicolas at al, 2009). In Leishmania, cysteine protease has been identified as target for immune response in human leishmaniasis (Rafati et al., 2001) and the vaccine potentials of both the protein as well as plasmid DNA encoding the enzyme have been screened in experimental animals (Zadek-Vakili et al., 2004; Rafati et al., 2005; Doroud et al., 2011).

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Malaria is caused by various protozoan parasites belonging to the genus plasmodium. This parasitic disease is transmitted via the bite of plamodium-borne mosquitoe vector. Different species of plasmodium have been associated with different forms of malaria in human and animals.

The statistics associated with malaria is alarming; There are 300 to 500 million clinical cases of malaria each year with resultant 1.5 to 2.5 million deaths. Again, children aged one to four are the most affected group in terms of vulnerability to infection and death. Furthermore, The costs of malaria in Africa alone amounted to US $ 1.8 billion annually (IDR, 1996). It is believed that 40 % of the world's population, spanning about 90 countries, are at risk of the infection and that 80-90 % of malaria deaths occur in SubSaharan Africa where 90 % of the infected people live.

In Africa malaria is the biggest killer of children under five years old, who account for nearly 90 percent of all malaria deaths and it is estimated that a child dies every 45 seconds from the disease (http://www.malariaconsortium.org/pages/malaria_challenges.htm,2012). The impact of malaria on children remains a serious obstacle to the achievement of many of the Millennium Development Goals (MDGs), including universal primary educationand the reduction of infant mortality (http://www.malariaconsortium.org/pages/malaria_challenges.htm,2012).

Pregnant women are far more vulnerable to malaria than other adults: they are four times more likely to contract and twice as likely to die from malaria than other adults. This is due to the

typical immunosuppression associated with pregnancy and increased levels of the hormones cortisol and oestrogen (http://www.malariaconsortium.org/pages/malaria_challenges.htm,2012).

Studies (http://www.malariaconsortium.org/pages/malaria_challenges.htm, 2012) have shown that in Africa malaria in pregnancy accounts for 400,000 cases of severe maternal anaemia and 200,000 newborn deaths each year.

Placental infection, premature birth and low birth weight (a significant factor in infant mortality) are also caused by maternal malaria. In addition, severe maternal anemia increases the risk of perinatal complications. Malaria, therefore, is seriously hindering the achievement of improve maternal health (http://www.malariaconsortium.org/pages/malaria_challenges.htm, 2012).

The cost of malaria to Africa is estimated at $12.5 billion per year, which represents 1.3 percent of affected countries economic growth (GDP) (http://www.malariaconsortium.org/pages/malaria_challenges.htm,2012). In some countries, malaria accounts for up to 40 percent of total health expenditure and 20-50 percent of hospital admissions(http://www.malariaconsortium.org/pages/malaria_challenges.htm,2012).
Productivity is reduced and staff turnover increased by illness-related absenteeism and children's education is severely disrupted. Rural and poor populations carry the overwhelming burden of malaria because access to effective treatment is extremely limited (http://www.malariaconsortium.org/pages/malaria_challenges.htm,2012).

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The acquisition of drug resistance by plasmodium has severally prevented global chemotherapeutic control of malaria (Ekland and Fidock, 2007). Currently, the most effective malaria drugs are derived from artemisinin and used in combination with other drugs (http://www.malariaconsortium.org/pages/malaria_challenges.htm, 2012). The use of two different drugs together aims to ensure parasites resistant to one drug are killed by the other before resistance spread (http://www.malariaconsortium.org/pages/malaria_challenges.htm, 2012). The fact that malaria parasite is a complex organism with complicated life cycle and that the science of immune response associated with malaria is not fully understood by researchers are two major challenges to the development of effective malarial vaccine by conventional vaccine technology.

It is hoped that the new technique of DNA-based vaccination will provide the much needed effective management alternative to chemotherapy.

2.2 Classification of plasmodium

Plasmodium belongs to the family *Plasmodiidae* (Levine, 1988), order *Haemosporida* and phylum *Apicomplexa*.

Plasmodium berghei is a rodent malarial species belonging to the subgenus *Vinckeia* and has the following taxonomic classification:

KINGDOM	*Protista*
Subkingdom	*Protozoa*
Phylum	*Apicomplexa*
ORDER	*Haemosporida*
FAMILY	*Plasmodiidae*
GENUS	*Plasmodium*
SUBGENUS	*Vinckeia*
SPECIES	*berghei* (Garnham 1964)

2.3 Life cycle of Plasmodium

The life cycle of malaria (Figure 2.1) is a complex one for several reasons. First, plasmodium goes through several stages during its life cycle involving both invertebrate (mosquito) and vertebrate (mammalian) hosts. Secondly, in the process of development, the parasite experiences different intracellular and extracellular environments. In addition, the parasite possesses a large genome on 14 chromosomes encoding an approximately 5000 proteins (Doolan and Homan, 2002). It is believed that the complexity of malarial life cycle reflects a series of evolutionary adaptations that optimised its ability to exploit its hosts (Aravind et al., 2003).

Infectious sporozoites in mosquito's saliva enter the mammalian host's bloodstream as the mosquito feeds on host's blood. The plasmodium parasite usually differentiates into a series of morphologically distinct forms whether they are in the vertebrate host or in the vector. In each host, the parasite alternates between invasive (sporozoite, merozoite, and ookinete) and replicative stages (trophozoite, schizont, and oocyst) (Hall et al., 2005).

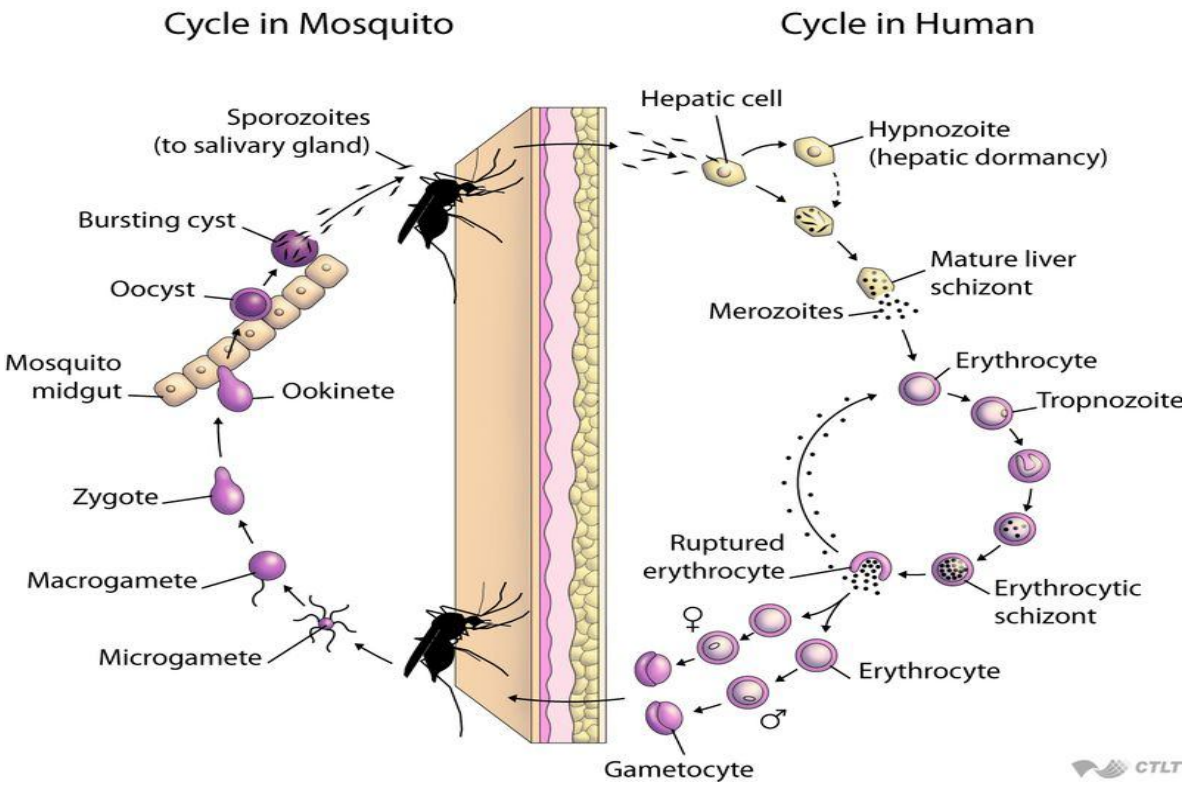


Figure 2.1 Life cycle of plasmodium
Source: http://www.malariasite.com/malaria/LifeCycle.htm

2.3.1 Sporogonic stage in mosquito vector: The *Anopheles* mosquito vector first picks up the male and female sexual forms of plasmosdium from the blood during a blood meal. In the vector, the sexual forms (gametocytes) recombine and generate genetically distinct sporozoites (Greenwood, et al. 2008). The parasites complete their sexual development to gametes when the gametocytes are taken into the mosquito midgut during blood meal. The gametes fuse to form a motile zygote termed the ookinete, which penetrates the chitinous peritrophic membrane surrounding the blood meal and then passes through the epithelial cells of the midgut lining to lodge in the basal lamina. There it grows into an oocyst that divides into numerous sporozoites. The oocyst bursts releasing sporozoites into the homocoel which then invade the salivary glands to complete the cycle (Aravind et al., 2003). The sporozoites are known to go through several metamorphosis during its passage through the Midgut and salivary glands of the infected mosquito and the vector can become infectious to another mammalian host in about two weeks after its blood meal infection. Development of plasmodium within the vector is influenced by ambiant temperature and this partly account for the preponderance of *P. vivax* infections outside tropical and subtropical regions (Greenwood et al., 2008).

2.3.2 Stages in mamalian hosts:

In the mammalian host, the cycle is divided into two main stages; the pre-erythrocytic and the erythrocytic stages.

2.3.2.1 Preerythrocytic stage

Upon entry into the bloodstream of the mammalian host, sporozoites travel by lymph and blood to invade specific tissues and develop as “exoerythrocytic” stages before infecting the blood cells. In mammals, these stages occur in hepatocytes, divide through a process termed

schizogony, and differentiate and mature into thousands of merozoites (Aravind et al., 2003). For *P. falciparum*, the development of the parasites into schizonts within the liver takes an average of 6.5 days (Moorthy et al., 2004). The schizonts then rupture releasing thousands of merozoites into the hepatic venous circulation and will invade any erythrocyte if it is not pick up by phagocytic cells. The liver stage is usually asymptomatic.

2.3.2.2 Erythrocytic stage

The asexual erythrocytic stage of the cycle starts when a single merozoite invade a red blood cell and is enclosed within a parasitophorous vacuole (Susan et al., 1997). Here, three morphologically distinct forms are easily seen; the first is the ring form which account for 24 hour of the intraerythrocytic phase in *P. falciparum* and is known to be metabolically nondescript (Susan et al., 1997). The ring stage is immediately followed by the trophozoite stage, a very active period during which most of the red blood cell cytoplasm is consumed. The last form is the schizont stage. The trophozoites undergo several rounds of binary divisions leading to the formation of schizont which actually contains numerous merozoites. The schizonts burst from the host cell releasing the merozoites which invade new erythrocytes and so initiate another round of the infection (Susan et al., 1997). The duration of erythrocytic cycle varies among plasmodium species: ranging from 24hr in simian *P. knowlesi*, 48 hr in *P. falciparum*, *P. vivax* and *P. ovale* to 72 hrs in *P. malariae* (Aravind et al., 2003).

2.4 Clinical and pathological features of malaria

The clinical manifestations of malaria result from schizont rupture and additionally, in the case of *P. falciparum*, from trophozoite adherence to endothelial cells. Fever, vomitting and/ or diarrhoea are the major signs observed in uncomplicated malaria. In adults, severe infection due

to *P. falciparum* in human is characterized by multiorgan damage, including renal failure. Severe malarial infection in children could come with prostration, respiratory distress, severe anaemia, and /or cerebral anaemia. Hypoglycemia, acidosis are some additional abnormalities associated with malaria (Greenwood et al., 2008).

Genetic factors, age of patient and intensity of transmission could influence susceptibility to cerebral and severe malaria (Greenwood et al., 2008). Malaria interact with other infectious diseases (like HIV) to modify the susceptibility and / or severity of either disease. Co-infection with Helminths as well as non-typhoid *Salmonella* spp could affect malarial patients differently (Greenwood et al., 2008).

2.5 Management of malaria

2.5.1 Vector Control

The epidemiology of malaria infection and disease are largely a function of seasonality, abundance and feeding of Anopheles mosquito vector (Greenwood et al., 2008). Mosquito vector control has been essential for successful malaria elimination programme across the world (Greenwood et al., 2008). Contemporary vector control strategies include the use of insecticide treated nets (ITNs), long-lasting ITNs (LLINs), and IRS. IRS with DDT was an essential component of the Global Malaria Eradication Programme in the past century and remains highly effective in regions where mosquitoes are sensitive to the insecticide. Resistance to pyrethroid insecticides has been identified as one of the most pressing research problems for vector biologists (Greenwood, 2008). Only pyrethroid insecticides are licensed for use in ITNs. Therefore, tools to rapidly detect the many genetic mechanisms that can underlie pyrethroid resistance are urgently needed.

2.5.2 Chemotherapy of malaria

The principal drugs currently in use to treat malarial infections include Chloroquine, Quinine, Amodiaquine, Mefloquine, Halofantrine, Artemisinin, Sulphadoxine-Pyrimethamine, Atovaquone-Proguanil, and Lumefantrine-Artemether. The old compounds (quinine, isolated from Cinchora bark in 1820) and the new compounds (artemisinin, purified from *Artemisia annua* in 1972) are products of ancient herbal therapies (Greenwood et al., 2008). Antimalarial drugs, singly or as combination regimen, interfere with certain aspects of parasites metabolism that differ significantly from that of the human host (Ridley, 2002). Plasmodium organelles such as lysosomal food vacuole, apicoplast and acristate mitochondrion with their associated metabolic roles in haemoglobin degradation, parasite transcription/translationa system and limited electron transport respectively, constitute the major drug targets of existing antimalarials. The development of drug resistance observed against antimalarials has led to a marked resurgence of research interest in the 1960s (Powell, 1972; Miller, 1992) and this has continued and is being encouraged by recent advances in human and plasmodium genomes' research outcomes (Patel et al., 2008).

2.5.3 Vaccine efforts against malaria

Malaria vaccine is one of the control efforts towards controlling the disease. The key goals of any vaccine research are thought to include the induction of strong immune response, the identification of stage-specific protective antigens and successful combination of candidate immunogens (Moorthy et al., 2004). Even though there are few vaccines at different trial stages, to date no effective vaccine against malaria is in circulation (Richie and Saul, 2002; Kaba et al., 2012). Efforts at developing such malaria vaccine had utilized whole attenuated strains, recombinant proteins and DNA vaccines targeted at different stages of plasmodium life cycle in

rodents and human models with various degree of efficacies (Richie and Saul, 2002; Moorthy et al., 2004). This progress could be accounted for, in part, by the increase in Worldwide funding for malaria from US$149 million in 2000 to almost $1·2 billion in 2008 (Moorthy et al., 2004; Pigott et al., 2012). Continued funding will no doubt speed up the production of effective malaria vaccine candidates.

DNA vaccination against malaria is a relatively new field with very few literatutes available but interestingly human clinical trial for a multi-antigen malarial DNA vaccine has been carried out between 1997 and 1998, and between 2000 and 2001 (Richie et al. 2012). The vaccine was found to be safe and well tolerated but did not evoke antibodies production and hence protection of volunteers (Richie et al. 2012). With approximately 5300 malarial antigens identified (Moorthy et al., 2004) in this post-genomic era, there is a wealth of information that could fast track development of vaccine, particularly, DNA vaccine against different species of plasmodium.

2.6 Review of enzymes involve in the metabolic activities of plasmodium.

A number of enzymes have been found in plasmodium and play key role in the parasite survival. Plasmodium parasites are highly dependent on glucose and very sensitive to oxidative stress. Preuss et al. (2012) have identified glucose-6-phosphate dehydrogenase (G6PD), a key enzyme of Pentose phosphate pathway (PPP) as a drug target. Both plasmodium parasites and the human host have complete PPP and they both seem to be important for the parasites. Major differences between parasite and human G6PD could be exploited for antimalarial drug design Preuss et al. (2012).

Protein kinases of plasmodium are another major class of enzyme relevant to the parasite metabolism. Most key enzymes in the inositol pathway are phosphorylated, which strongly suggests additional levels of regulation and crosstalk with other protein kinases that coregulate different biological processes (Talevich et al., 2011). cAMP-dependent protein kinase A- and phosphatidylinositol-signaling are involed in the regulation of host cell invasion (Lasonder et al., 2012). In addition, the second messenger cAMP has been shown to be important for the parasite's ability to infect the host's liver, but its role during parasite growth inside erythrocytes, the stage responsible for symptomatic malaria, is not clear. The P. falciparum genome encodes two adenylyl cyclases, the enzymes that synthesize cAMP, PfACα and PfACβ (Salazar et al., 2012) and it is thought that PfACβ plays an important role during the erythrocytic stage of the P. falciparum life cycle. Biochemical characterization of PfACβ revealed a marked pH dependence, and sensitivity to a number of small molecule inhibitors. These inhibitors kill parasites growing inside red blood cells (Salazar et al., 2012).

Histone deacetylases (HDACs) are another class of plasmodium enzymes. They effect post-translational modifications of proteins by altering the acetylation state of lysine residues (Andrews and Fairlie, 2012). HDACs control epigenetic changes that trigger cell transformation and proliferation of transformed cells associated with many diseases and they are thought to be validated drug targets for some types of cancer and are promising therapeutic targets for a range of other diseases, including malaria (Andrews et al., 2012).The absence of licensed vaccine for preventing malaria necessitating the search for new therapies. HDAC inhibitors are emerging as

a promising new class of antimalarial drugs with potent and selective action against Plasmodium parasites in vitro (Andrews et al., 2012). Recent studies on the effects of HDAC inhibitors on the growth and development of P. falciparum have provided important new information on transcriptional regulation in malaria parasites and have validated the potential of this class of inhibitors for malaria therapy (Andrews et al., 2012).

Another metabolic pathway crucial for the survival and replication of the parasite is the synthesis of the purine nucleoside monophosphates (Jersey et al., 2011). The identification and characterization of inhibitors of this enzymes as antimalarial drugs have been reviewed (Jersey et al., 2011). The acyclic nucleoside phosphonates (ANPs) are thought to be excellent drug candidates for many reasons: they are good inhibitors of the two Plasmodium enzymes, can be selective compared to the human enzyme, can arrest parasitemia in cell based assays, have low cytotoxicity to the human host cell, have stable carbon-phosphorous bond, and are stable within the cell (Jersey et al., 2011).

Plasmodium parasites also possess a single pyruvate dehydrogenase (PDH) enzyme complex that is localized to the plastid-like organelle known as the apicoplast. However, unlike most eukaryotes, Plasmodium parasites lack a mitochondrial PDH. The PDH complex catalyses the conversion of pyruvate to acetyl-CoA, an important precursor for the tricarboxylic acid cycle and type II fatty acid synthesis (FAS II) (Pei et al., 2010).

Infection of red blood cells (RBC) subjects the malaria parasite to oxidative stress. Malaria parasites adapt to the oxidative stress during their erythrocytic stages with the help of vital thioredoxin redox system and glutathione redox system. Glutathione reductase and thioredoxin reductase are important enzymes of these redox systems that help parasites to maintain an adequate intracellular redox environment (Kapoor and Banyal, 2009). Activities of glutathione reductase and thioredoxin reductase in P. berghei-infected host erythrocytes were found to be higher than those in normal host cells (Kapoor and Banyal, 2009). These enzymes being mainly confined to the cytosolic part of cell-free P. berghei may promise advances in chemotherapy of malaria (Kapoor and Banyal, 2009).

Closely related to these enzymes is another enzyme, gamma-glutamylcysteine synthetase (gamma-GCS). Plasmodium berghei parasites lacking expression of gamma-glutamylcysteine synthetase (gamma-GCS), the rate limiting enzyme in de novo synthesis of GSH, were generated through targeted gene disruption thus demonstrating, quite unexpectedly, that gamma-GCS is not essential for blood stage development.

Proteases of plasmodium are a class of enzymes that has received a lot of research attention. Facipains of Plasmodium falciparum, in particurly, has been extensively worked because of the promise it offer as target for antimalarials (Rosenthal, 1998; Rosenthal, 2004). Therefore, the next section will be devoted to different classes of malaria proteases.

2.7 Proteases and their biological roles

Proteases are enzymes that catalyze the degradation of peptide bonds in both proteins and peptides. Proteinases (or endoproteases) catalyze hydrolysis of internal bonds while exopeptidases are known to catalyse cleavage reactions involving one or two amino acids from either ends of a peptide chain. Exopeptidases could be aminopeptidase or carboxypeptidase depending on the terminus that is being acted upon (McKerrow et al., 1993). Depending on the pH range over which they are active, proteases have also been classified as acid, neutral or alkaline proteases (North, 1982). On the basis of their catalytic mechanisms, proteinases are either aspartic (EC 3.4.23), metalloproteinase (EC 3.4.24), serine proteinases (EC 3.4.21) or cysteine proteinases (EC 3.4.22). The later classification appears to be the most reliable and one that is most widely used by researchers (North, 1982).

Proteases are found in abundance in nature, in both plant and animal as well as in the lower kingdoms of the protists (North, 1982). Their biological roles in nature are diverse depending on their natural cellular location. These roles range from tissue penetration, digestion of host protein for nutrients, evasion of host immune responses, to other processes such as tissue catabolism and apoptosis (McKerrow et al., 1993). Other identified biological roles of proteinase involves their roles in posttranslational processing of primary translation products, activation and inactivation of specific proteins, their roles during morphogenesis and differentiation in microorganism as well as in several disease processes (pathogenesis) (North, 1982). These roles, in most cases, are stage specific in any parasite's life cycle.

2.7. 1 Cysteine proteases

Cysteine proteases derived their name due to the function of a catalytic cysteine, which mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon atom of a susceptible peptide bond (Rosenthal, 2004). Cysteine proteases are subdivided into clans. Different clan do not share sequence and structural identity and probably have independent ancestry. Clans share the use of a cysteine to catalyse the hydrolysis of peptide bonds and are subdivided into families based on sequence identities and similarities (Rosenthal, 2004). For example, proteases belonging to clan CA utilize catalytic Cys, His, and Asn residues that are invariably in this order in the primary sequence of the protease. Clan CA, Family C1 (papain-family) cysteine proteases are well characterized for many eukaryotic organisms and are the best characterized cysteine protease of *Plasmodium*. *Plasmodium falciparum* is thought to have up to four falcipains (the common name for its cysteine proteases) belonging to the CA clan. The second clan of interest in plasmodium is clan CD. Member of this clan utilizes a catalytic His-Cys dyad (in this order in the primary sequence) for activity. Clan CE , characterized by His, Glu (or Asp), Cys catalytic residues is also represented in the *P. falciparum* genome (Rosenthal, 2004).

2.7.1.1 Cysteine protease catalytic mechanism

Though structurally distinct, cysteine and serine proteases carry out analogous reactions and in having a catalytic triad features. While the catalytic triad in serine proteases consists of Ser195, His57, and Asp102, in cysteine proteases on the other hand, Cys 25, His159 and Asn175 constitute the putative catalytic triad. These amino acid are conserved in all members of the papain family (Higaki et al, 1989).

In papain, the δ-nitrogen of His 57 is within hydrogen-bonding distance of the thiol group of Cys 25. The third member of the triad, Asn175, can also form hydrogen bond with His 57, a similar role Asp102 play in serine protease. The catalytic state of papain was shown to exist as the ion pair where the negatively charged thiolate anion is paired with the positively charged (protonated) imidazole of His159 (Higaki et al, 1989).

The common feature in the catalytic mechanism of all cysteine proteases is the use of thiol group of cysteine as a nucleophile. The first step is deprotonation of the thiol in the enzyme's active site by an adjacent amino acid with a basic side chain, usually a histidine residue. The next step is nucleophilic attack by the deprotonated cysteine's anionic sulfur on the substrate carbonyl carbon. In this step, a fragment of the substrate is released with an amine terminus, the histidine residue in the protease is restored to its deprotonated form, and a thioester intermediate linking the new carboxy-terminus of the substrate to the cysteine thiol is formed. The thioester bond is subsequently hydrolysed to generate a carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme (Higaki et al, 1989).

2.7.1.2 Occurrence, Isolation and biochemical characterization of cysteine protease

In both plant and animal kingdoms, cysteine proteases exist abundantly in nature. Cysteine proteases have been found and in some instances isolated from various sources including Wheat (Dominiguez and Cejudo, 1996), the rhizome of Turmeric(*Curcuma longa*) (Nagarathnam et al., 2009), rice (Lee et al., 2004), the latex of *Carica papaya* (pawpaw plant), tissues of nematode (Yatsuda et al., 2006), worms such as *Entamoeba histolytica* (Luaces and Barrett, 1988) and in several pathogenic bacteria (Hotson and Mudgett, 2004; Liu et al., 2010) and protozoa such as African and south American trypanosomes, plasmodium and Leishmania (Boncertz and

Hungerer, 1978; McKerrow, 1993; Brooks, et al., 2000; Rosenthal, 2004). Cysteine proteases of typical papain-family type hydrolyse haemoglobin, casein, gelatin and different erythrocyte membrane proteins such as spectrin, protein 3, actin and gycophorin (Byoung-Kuk, 2004). They are known to require reducing conditions for their activities and to possess acidic pH optima, (Boncertz and Hungerer, 1978; Renard et al., 2000; Byoung-Kuk, 2004) .

2.7.1.3 Cloning of cysteine protease gene and molecular characterization of its recombinant protein

Recombinant DNA technology has been used extensively in the production of large amount of several proteins including cysteine proteases. The technique allows for the production of large amount of a protein whenever study requiring the protein in its pure or semi-pure state arises. Pamer et al. (1991) have produced a recombinant cysteine protease using cDNA isolated from *Trypanosoma brucei rhodesiense* and showed that the recombinant protein is identical to native enzyme in terms of substrates hydrolysis and response to inhibitors. Recombinant falcipains, cysteine proteases from *Plasmodium falciparum*, have been produced and characterized (Shenai et al., 2000; Kumar et al., 2007). Na et al. (2010) have also cloned gene of cysteine protease from *Plasmodium vivax* and have overexpressed the protein competent *E. coli* cells grown in LB medium by induction with 1mM isopropyl-1-thio-β-D-galactopyranoside.

Cysteine protease required for the molting of *Onchocerca volvulus* third stage larvae have also been cloned and characterized by Lustigman et al. (1996). It has been previously reported that the gene coding for enzyme domain of protozoan cysteine protease lacks an intron (Nene et al., 1990) and so a number of molecular study on cysteine protease gene had employed genomic DNA as template for PCR amplification.

2.7.1. 4 Biological functions of cysteine Protease

Cysteine proteases have been associated with several biological functions in nature few of which include: degradation of extracellular matrix during parasite penetration, involvement in molting processes, immunonoglobulins' hydrolysis as a way to escape host immune response (Renard, 2000). Cysteine proteinase in *Entamoeba histolytica*, hystolysin, is thought to be implicated in the parasite's infectiveness and invasiveness (Luaces and Barrett, 1988).

2.7.1. 5 Biological roles of cysteine protease in plasmodium

Valuable information reagarding the function of cysteine proteases in plasmodium are now available from several studies. Hydrolysis of haemoglobin is the best characterized biological function of plasmodium cysteine proteases (Rosenthal, 2004). Inhibition studies have shown that a trophozoite's cysteine protease plays key role in the degradation of erythrocyte haemoglobin in an acidic food vacuole to provide amino acids for parasite protein synthesis (Shenai et al 2000) even though only a fraction of the acids is utilized (KrugliaK et al., 2002) and the authors have associated such hydrolysis to falcipain-2. Falcipain-3 is another identified food vacuole hemoglobinase (Sijwali et al., 2004). The amino acids released also help to maintain the osmotic stability of the parasite and to provide space for the growing intraerytrocytic parasite (Rosenthal, 2004).

Another possible biological function of cysteine protease in malaria is in erythrocyte rupture; the enzyme appears to be very important in the release of merozoites, which rapidly invade other erythrocytes and so initiate another asexual cycle (Rosenthal, 2004; Sijwali et al., 2004).

Apart from haemoglobin hydrolysis and erythrocyte rupture, cysteine protease, in malaria, also have been associated with the process of red cells invasion by merozoites. Sijwali and his colleagues (2004) had ruled out the chance of falcipain-1 participating in erythrocytic hydrolytic event. The enzyme is now thought to be involved in erythrocytes invasion by merozoites (Greenbaum et al., 2002; Kumar et al., 2007).

During gametogenesis, a nonerythrocytic stage of malaria life cycle, the cleavage of gametocyte surface protein Pfs230 was found to be blocked by the membrane-permeant cysteine protease inhibitor E-64d, suggesting the possible role of cysteine protease in this stage. Knockout experiment involving falcipain-1 gene had led to marked decrease in oocyst production, suggesting the role of this protease in sexual stage of the infection (Rosenthal, 2004).

2.8 The science of DNA vaccination

Wolff *et al.* (1990) were the first to demonstrate that plasmid DNA after being injected in saline can be taken up and expressed in the cells of mouse skeletal muscle (Montgomery et al., 1997). They also, along with other studies, showed that the plasmid DNA exists as extrachromosomal molecules with no evidence of integration into mouse genomic DNA (Montgomery et al., 1997). Ulmer and his collegues in 1993 first demonstrated that protective immunity could be elicited by DNA vaccine technology (Montgomery et al., 1997).

It has long been established that the pathway of cellular immune response requires recognitionof antigen in the context of major histocompatibility complex (MHC) molecules by cytotoxic T Lymphocytes (CTLs) (Montgomery et al., 1997). DNA vaccine has been defined as a plasmid containing a viral, parasitic or bacterial gene that can be expressed in mammalian cells (in the case of infectious diseases) or a gene encoding a mammalian protein (in the case of

noninfectious diseases, such as cancer, autoimmunity, and allergy). Once admistered, the Plasmid, carrying the gene coding for the antigene of interest, uses the host machineries to express the protein and the resulting protein then evoke the needed immune response (Montgomery et al., 1997). Since the objective is not to raise antibodies against the DNA itself, DNA vaccine has also been described as DNA-mediated or DNA-based immunization (Whalen, 1996). The action of DNA vaccine is thought to be dependent on the means of delivery, the kind of an antigen encoded, the dose of DNA, and the accompanying vector-DNA sequences (Kofta and Wedrychowicz, 2001).

It is postulated that antigens produced inside the cells, for example muscle cells, are presented in the context of MHC I to antigen-presenting cells, with the released proteins being engulfed by macrophages that present immune peptides in the context of MHC II. These, in turn, induce the Th1 or Th2 types of immune response. The Th1 dependent response is characterized by the production of IgG2 antibodies (IgG1 in humans) and by the activity of cytotoxic T cells, the Th2 dependent response by IgM, IgG1 (IgG4 in humans), IgA and IgE responses. In general, DNA vaccination induces Th1-dependent rather than Th2-dependent immune responses (Kofta and Wedrychowicz, 2001). The outcome of vaccination often depends on the route of the administration: DNA vaccination induces the Th1-dependent response, while protein form generates the Th2 response (Kofta and Wedrychowicz, 2001).

DNA vaccines are thought to be efficient priming vaccines but do not boost efficiently. Suppression of immune response was seen when several DNA vaccines were administered into a single site in an animal (Sedegah et al., 2004).

The major advantages credited to DNA vaccine technology include: the ease of manipulation, the use of generic technology and simplicity of its production (Whalen, 1996). Others include their low cost, long shelf life due to their chemical and biological stability, lack of requirement for a cold chain and ability to induce good T-cell responses (Le et al., 2000; Carter et al., 2007).

Though recently introduced, DNA vaccine technology have been tried on a number of diseases that are of global significance. Protection of mice against influenza virus infection was observed after vaccination with DNA encoding viral protein (Ulmer et al., 1993). Several studies on the ability of DNA vaccination to induce protective immunity against Leishmania infection in rodents are available and have showed promising degrees of protection (Carter et al., 2007; Meby et al., 2001; Campos-Neto et al., 2002). Kurup and Tewari (2012) have demonstrated the induction of protective immune response in mice using a DNA vaccine encoding *Trypanosoma evansi* beta tubulin gene. Potent induction of $CD8^+$ T cells were also seen after vaccination with Plasmid DNA carrying the gene for circumsporozoite protein of *Plasmodium yoeli* alone or in combination with another DNA vaccine (Hoffman et al., 1997).

Initially, the major challenges facing DNA based vaccination are that of safety issues, tolerability and the chances of production of anti-DNA antibodies following vaccination (Kofta and Wedrychowicz, 2001). Scientists are also interested in knowing if persistent antibody production in small doses could induce immunological tolerance or if cells containing a vector could be identified and destroyed by cytotoxic T cells (Kofta and Wedrychowicz, 2001).

In human, production of antigene-specific antibodies has been quite difficult even when excellent CTL induction after malaria DNA vaccine trial was observed (Le et al., 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental Animals

BALB/c mice (20-25g, both sexes) were in house bred at Animal house of the Department of Biochemistry, Faculty of Science, Kaduna State University, Kaduna, Nigeria. The mice were used for *in vivo* maintainance of the *P. berghei* infection.

For the vaccination experiment, Eighty (80) female Balb/c mice were bought from the animal house of Nigerian Institute for Trypanosomiasis Research, Kaduna, Nigeria. All the animals were certified healthy by the animal scientist in the Institute before purchase and were never used for any scientific experimentation prior to purchase.

3.1.2 Parasite

Plasmodium berghei (ANKA strain), used for this work, was obtained from the Nigerian Institute for Medical Research (NIMR), Lagos, Nigeria.

3.1.3 Reagents/Chemicals

Molecular biology grade chemical compounds and reagents were used. All other reagents used were of analytical grade.

3.1.4 Labaratories used during the studies:

Preliminary studies involving infection of mice, collection and preparation of infected blood, isolation of parasite from infected blood, DNA extractions, immunization and post immunization analyses were all carried out at the Center for Biotechnology Research and Training, Ahmadu Bello University, Zaria, Nigeria. Molecular Biology studies including identification,

amplification, cloning of genes, design and production of DNA vaccine contructs were done using the facilities available at the Centre for Biomolecular Interaction, Department of Biology and Chemistry, University of Bremen, Germany.

3.1.5 Bioinformatics websites consulted and softwares utilized

The following websites and sofwares were employed for the bioinformatic studies: Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/), National centre for Biotechnology Information (http://www.ncbi.nlm.gov/), Plasmodium Genomic Resource–PlasmoDB (http://plasmodb.org/plasmo/), European Bioinformatics Institute (http://www.ebi.ac.uk), ExPasy (http://web.expasy.org/); Clustal Omega (1.1.0) available at the website of European Bioinformatics Institute (http://www.ebi.ac.uk) and Geneious Pro 5.5.3 sofware made available at the Center for Biomolecular Interaction, University of Bremen, Bremen, Germany.

3.2 Methods

3.2.1. Experimental Design

The chart below shows the diagrammatic sequence of activities designed towards the realization of set objectives.

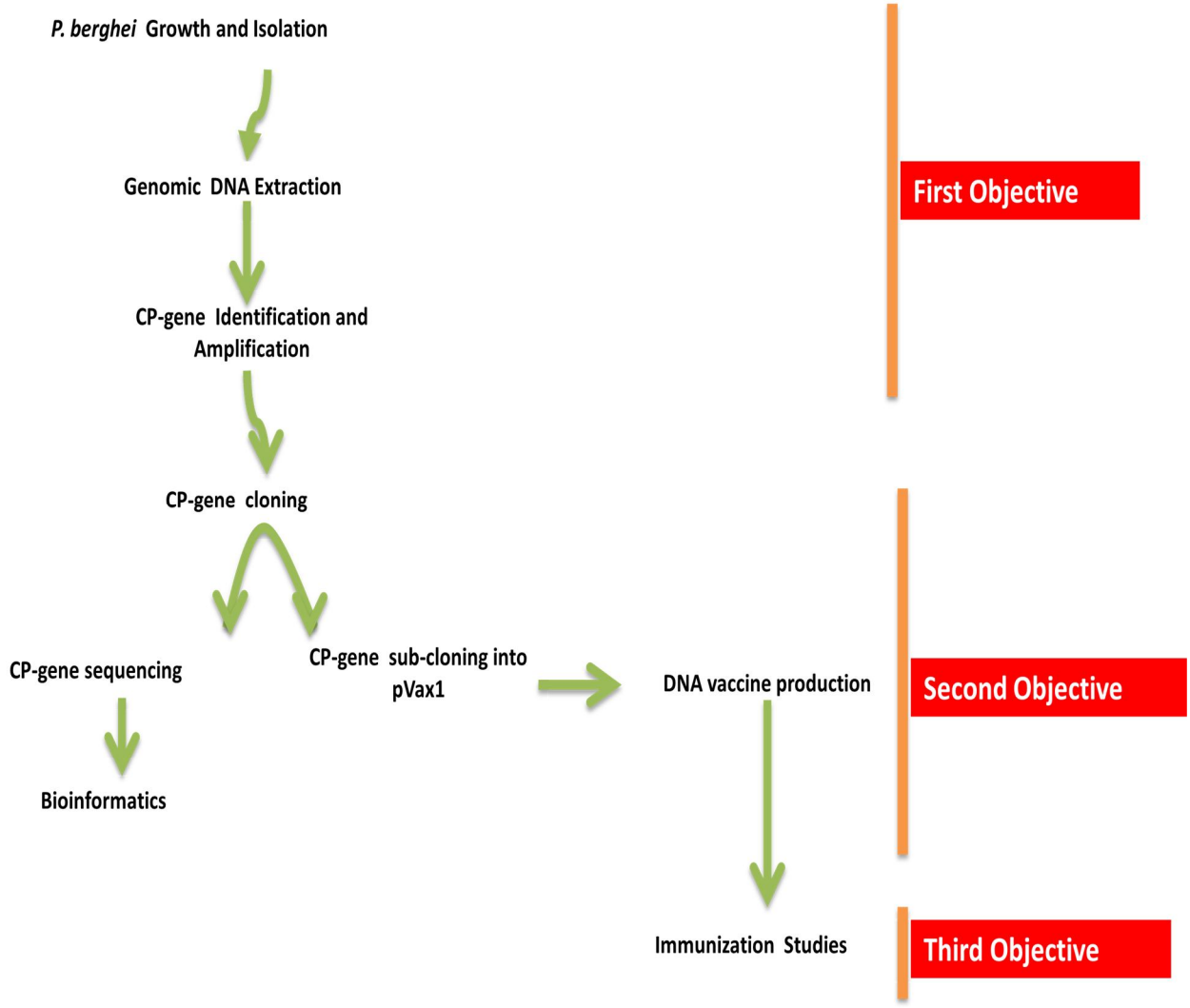


Figure 3.1 Flow chart of the experimental design

3.2.2 Parasite Inoculation

Blood from an infected mouse with rising parasitatemia (between 20 -40%) was collected in 3.8% (w/v) Sodium citrate solution (in PBS, pH 7.2). The suspension was administered to healthy mice intraperitoneally (0.2 ml per mouse) and the parasitaemia monitored thereafter.

3.2.3 Monitoring of Parasitaemia

The course of infection in infected animals was monitored via microscopic Giemsa-stained thin blood smears examination. The percent of infected red blood cells seen within a microscopic field, with evenly spread RBCs, was taken as a measure of infection.

3.2.4. Counting of Red Blood Cells

The number of red blood cells (for both infected and normal RBC) in animal whole blood was counted using Naeuber improved counting chamber as described by Turgeon (2007). For a dilution factor of 200, 10 µl of blood collected from animal's tail were added to 1990 µl of the counting fluid (containing no anticoagulant). After shaking, the mixture was then taken with a droping pipette for onward charging of the chamber. The red cells were viewed on X100 objective lense and then adjusted to X 400 objective for the count.

The number of red cells in a ml of blood was calculated using the formula blelow:

$$\text{Red cell density (Number of cells per ml of blood)} = C \times D \times 50 \times 10^6$$

Where,

C = microscopic count at x 400 magnification.
D = dilution factor

3.2.5 Removal of leucocytes and platelets, and separation of parasites from infected erythrocytes.

In order to reduce the chances of contamination of DNA from other sources, mainly from leucocytes and platelets, the infected blood was first treated with glass beads and dextran solution prior to erythrocyte lysis using the method of Zukerman et al. (1967), modified by Knox et al. (1977); Citrated (3.8% Sodium citrate in PBS, pH, 7.2) infected blood from newly sacrificed mice was mixed with equal portion of PBSG containing Grade A dextran (molecular

weight 200,000 – 275,000) and 0.2 % (w/v) glucose. The modification being that 3.0% of dextran was used instead of 3.6 % and 6% employed by the former and the later authors, respectively. The suspension was allowed to stand at 4 ^{o}C in tall glass cylinders for thirty minutes to allow for good separation of the white blood cells from the red cells. The supernatant was then discarded and the whole process repeated. The erythrocytes at the end of these processes was haemolysed and plasmodia released by treatment with 0.016 % (w/v) saponin as described by Spira and Zuckerman (1962) and Zuckerman et al. (1967); Infected erythrocytes were washed with PBS in a microfuge tube. The supernatant was removed and about 700 µl of the saponin solution added. After the cells were fully suspended in the saponin solution, the mixture was then incubated at 37 ^{o}C for 15 minutes. The lysed red cells were then centrifuged at 8000 rpm for one minute, the supernatant discarded and the saponin treatment repeated for another 10 minutes. The parasites (a thick brown pellet) were then washed three to four times by centrifugation at the same speed with PBS.

3.2.6.1 Isolation of Genomic DNA from *P. berghei* isolate

This was carried out using Genomic DNA extraction kit (genomic DNA Tissue MiniPrep Kit, Zymo Research) as described by the manufacturer using the general protocol of cell lysis, ethanol precipitation and DNA recovery as described by Rotureau et al (2005). The *P. berghei* suspension was adjusted to 100 µl with distilled water in each microcentrifuge tube and 95 µl of 2X Digestion Buffer and 5 µl of Proteinase K (20 mg/ml) were immediately added to it. The suspension was then mixed and incubated at 55 ^{o}C for 20 minutes. Seven hundred microlitres of Genomic Lysis Buffer was thereafter added to each tube and mixed thoroughly by vortexing. The mixture was subsequently transferred to Zymo-Spin^{TM} II column in a collection tube and centrifuged at 10,000 x *g* for one minute. To the spin column (in a new collection tube), 200 µl

of DNA Pre-Wash Buffer was added and centrifuged at 10,000 x*g* for one minute. This was followed by a washing step in which 400 µl of g-DNA Wash Buffer was added to the spin column and further centrifuged at 10,000 x*g* for one minute. Finally, the spin column was transferred to a clean microcentrifuge tube and the DNA material in the column was then eluted with 50 µl of DNA elution buffer or distilled water by adding the Elution Buffer to the spin column, incubating it for 2 to 5 minutes at room temperature, and centrifuging at 10,000 x *g* for 30 seconds. The eluted DNA was immediately quantified and stored at -20 ^{o}C for future use.

3.2.6.2 Isolation of Genomic DNA from organs of experimental mice

DNA was isolated from tissues of muscle, liver and spleen of representative mice selected from experimental groups using the Qiagen total DNA extraction kits as described in the handbook (http://www.qiagen.com/ 2012): Exactly 25 mg of liver (10 mg for spleen) tissues were cut into pieces with a clean scalpel and placed in 1.5 ml microcentrifuge tube. To each tube, 180 µl buffer ATL was added. Twenty micro liters of proteinase K (at 20 mg/ml) was then added and the content mixed thoroughly by vortexing, and was thereafter incubated at 56 ^{o} C for one to two hours or until complete lysis of the tissue was observed. During the incubation, the tube was shaken at 30 mins interval to disperse the sample. After the incubation, the lysate was vortexed briefly for 15 s and was followed by the addition of 200 µl of AL buffer and immediately mixed thoroughly by vortexing. Two hundred micro liters of 96-100% ethanol was the added to the sample and mixed again thoroughly by vortexing.

The mixture was then pipetted into DNeasy Mini spin column placed in a 2 ml collection tube (provided) and immediately centrifuged at 6000 x *g* (8000 rpm) for 1 min. The flow-through and the collection tube were discarded and the DNeasy Mini spin column placed in a new 2 ml collection tube (provided). Exactly 500 µl AW1 buffer was then added to the column and

centrifuged at 6000 x *g* (8000 rpm) for 1 min. The flow-through and the collection tube were again discarded and the DNeasy Mini spin column placed in a new 2 ml collection tube (provided). Exactly 500 µl AW2 buffer was then added to the column and centrifuged at 20, 000 x *g* (14 000 rpm) for 3 min to dry the DNeasy membrane. The flow-through and the collection tube were discarded, the DNeasy Mini spin column placed in a clean 1.5 ml microcentrifuge tube, and 200 µl of AE buffer was finally pipetted directly onto the DNeasy membrane and incubated for 1 min at room temperature before it was eluted by centrifugation at 6000 x *g* (8000 rpm).

3.2.7 DNA Quantification

All deoxyribonucleic acids (genomic DNA and Plasmid DNA) quantification were done by automated ND-1000 NanoDrop Spectrophotomer following the manufacturer's guidelines. Double distilled water was used to initialize the machine while elution buffer (Tris buffer at pH 8.0) was used as blank. Again, the absorbance values of the DNA solution at 260 and 280 nm was taken and their ratio ($A_{260nm}/A2_{80nm}$) noted.

3.2.8 Preliminary search of plasmodium data bank from PlasmoDB, Wellcome Trust Sanger Institute and National Center for Biotechnology Information (NCBI)

In an attempt to get at the sequence coding for Bergheipain, a cysteine protease from *Plasmodium berghei,* we found that there are two genes in *P. berghei* data base that are orthologs of Falcipain-1 and Falcipain-2 of *Plasmodium falciparum* respectively. Literature search showed that Bergheipain-2 exists in the PlasmoDB data with the gene ID PBANKA_093240. The gene is 1407 base pair long. The sequence for bergheipain -1, however,

exist in the PlasmoDB data base only as a P. berghei's putative gene. Its gene ID is PBANKA_132170 and has 1560 nucleotide bases.

Most works in the past focused on Falcipain-2 and its orthologs (bergheipain-2 inclusive) because of its potential role as malarial chemotherapeutic target. Though Falcipai-1 has since been identified, it is only recently that it has been linked functionally to erythrocyte invasion and so it was thought that this cysteine protease , rather than Falcipain-2, might be a likely candidate for vaccine research.

We here set out to construct plasmids carrying coding sequences for these enzymes with the hope of assessing their potentials as candidates for DNA vaccine against *P. berghei* infection.

Primer designs

Nested and standard sets of primers were designed for both enzymes by searching within their contigs for sequences flanking the 5'- and 3'- ends. The nested primers were simply nucleotide sequences outside (before and after) the genes. The standard primers, on the other hand, contain restriction sites for BamH1 at the 5'-end flanking sequences and that of EcoR1 at the 3'-end flanking sequences. The primers are given below:

Nested PCR Primers;

For Bergheipain-1

Sense: 5'-CTTTGGGGCAAGATG-3' (A total of 15 bp)
Antisense: 5'-GCTGCATGTTTAG-3' (A total of 13 bp)

For Bergheipain-2

Sense: 5'-GAAACATAATACATCATG-3' (A total of 18 bp)
Antisense: 5'-TTCCCTATCGGTAC-3' (A total of 14 bp)

Standard PCR Primers;

For Bergheipain-1

Sense (With BamHI site underlined and initiation codon marked bold red):
5'-CGGGATCC**ATG**ATAAACGATATAAGAC-3' (A total of 27 bp)

Antisense (With EcoRI site underlined):
5'-CGGAATTCTAAAATTGGAAAGAAAACG-3' (A total of 27 bp)

For Bergheipain-2

Sense (With BamHI site underlined and initiation codon marked bold red):
5'-CGGATCC**ATG**AATTACCATTCTAGCC-3' (A total of 26 bp)

Antisense (With EcoRI site underlined):
5'-CGGAATTCTTCAATTATAGGAGCAT-3' (A total of 25 bp)

These primers whose characteristics are given on Table 3.1, were ordered from Invitrogen (Germany).

Table 3.1 Primers for PCR amplification and their characteristics

Parameters	Bergheipain-1				Bergheipain-2			
	For Nested PCR		For Standard PCR		For Nested PCR		For Standard PCR	
	Forward (OSK2026)	Reverse (OSK2028)	Forward (OSK2027)	Reverse (OSK2029)	Forward (OSK2030)	Reverse (OSK2032)	Forward (OSK2031)	Reverse (OSK2033)
Length (bp)	15	13	27	27	18	14	27	25
MW(g/mol)	4648	3955	8325	8373	5499	4189	8219	7680
T_m (^oC)	47.8	36.0	61.9	58.9	44.6	42.0	65.0	58.1
GC (%)	53.3	38.5	40.7	33.3	27.8	50.0	48.1	36
Average T_m (^oC)	41.9		60.4		43.3		61.55	
Annealing Temperature (^oC)	**40.0**		**45.0**		**40.0**		**45.0**	

The PCR conditions and programme parameters are given in appendices (I-V). PCR products were analysed on 1.0 % Agarose gel after electrophoresis as described below.

3.2.9 Gel Electrophoresis

A 1% agarose gel was prepared by dissolving 1.5 g of agarose in a TAE buffer and heated in a microwave for 4 min. Immediately after removing the hot agarose liquid from the microwave, 3 µl of G-stain was added to it with a brief gentle swirling before the liquid was poured into a template with comb already set in it, and allowed to solidify. The PCR products (25 µl) were mixed with 3 µl of loading dye. The samples were placed in wells of gel and run in electrophoretic system (100 volt) for 1 hour. After 1 hr, the gel was taken to a transilluminator for viewing (Roe et al., 1996).

3.2.10 Preparative Gel Electrophoresis

For each gene, 110 µl of the PCR product was stained with 18 µl of the loading dye. The entire 128 µl mixture was then loaded into a single agarose well (casted specially for preparative purpose) and run for 1hr under 100 volt in an electrophoretic system. At the end of 1 hr, the DNA bands were identified under blue light transilluminator and were carefully excised out of the gel using a clean scalpel (sterilized with 70% Ethanol). The gel block containing the amplicons were separately cut into pieces and transferred into a clean microfuge tube and kept on ice before extraction (Roe et al., 1996).

3.2.11 Recovery of amplicon from the gel after agarose electrophoresis

This was carried out using GeneJET Gel Extraction Kit of Fermentas. The procedure involved adding 1:1 volume of binding buffer to the gel slice contained in microfuge tube and incubating the gel mixture at 50-60 ^{o}C until the gel slice is completely dissolved (around 10 min.). The tubes were inverted every few minutes to facilitate gel melting. The solubilized gel solution was then transferred into GeneJET purification column and centrifuged at 10000 rpm for one min.

The flow-through was discarded and the column placed back into the same collection tube. To the purification column, 100 µl of binding buffer was, again, added and centrifuged for one minute, the flow through was discarded and the column placed back into the same collection tube. Thereafter, 700 µl of wash buffer was added to the GeneJET purification column, centrifuged (1000rpm for 1 min.), the flow through discarded and the colum placed back into the same collection tube. The empty GeneJET purification column was centrifuged as above for additional 1 min to completely remove residual wash buffer. Finally, the GeneJET purification column was transferred into a clean 1.5 ml microcentrifuge tube and 50 µl of Elution Buffer was added to the center of the membrane of the purification column. The set up was then centrifuged for 1 min and the eluate (purified DNA was stored at -20 ^{o}C until required (Roe et al., 1996).

3.2.12.1 Strategy for the construction of DNA Vaccines without transin signal peptide

Nested PCR reaction protocol were employed to amplify the genes (berghepain 1 and Bergheipain 2) from *P. berghei* genomic DNA using *pfu* DNA polymerase. The internal primers used for both genes harbor BamH1 and EcoRI restriction sites. The genes were cloned into BamHI/EcoRI digested pcDNA3 Amp Strep-tag vector. Successful insertion and expression of the genes into the vector were checked through colony PCR and immunofluoresence technique on cos7 cells respectively. Sequencing techniques was used to confirm the presence of the insert and to check if they are in frame. Midipreps of recombinant Plasmids (pcDNA3BP1StrepTag and pcDNA3BP2StrepTag) that gave positive outcome were further cut with BamHI and XhoI restriction enzymes (in order to introduce the streptag detection peptide at the 3' terminus and were subcloned into pVaX1 vector that was previously cut with BamHI and XhoI enzymes. The chimeras so formed were transformed into XL1 Blue *E. coli* cells and colonies were screened using colony PCR. Plasmid midipreps were made from positive colonies and stored at -20 ^{o}C

until required for vaccination. Figure 3.2 gives the diagrammatic design of pVax1 vacine constructs without transin signal sequences.

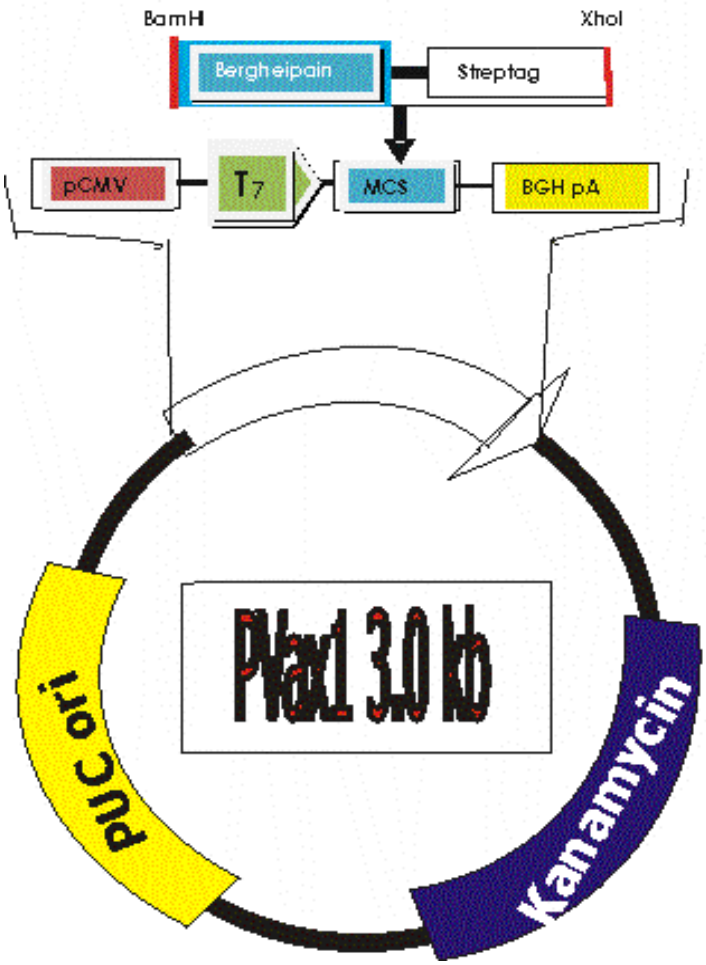


Figure 3.2 Design of pVax1KanBP1Streptag and pVax1KanBP2Streptag Constructs

3.2.12.2 Strategy for the construction of DNA Vaccines with transin signal peptide

Since preliminary online search has shown that the full genes of cysteine protease from *plasmodium berghei* do not have a signal peptide, it is thought that introduction of a signal peptide in the final expression vector will aid the enzymes' cellular secretion. For this, a new set of primers (one sense primer for bergheipain1 (with EcoRV restriction site), another sense primer for Bergehipain2 (also having EcoRV restriction enzyme site) and a third antisense primer (to be used for identification of streptag sequence on both genes during colony PCR) harbouring both a signal peptide sequence and site for XhoI restriction site were designed and ordered (eurofins mwg/operon, Anzinger Str. &a D-85560 Ebersberg, Germany). The primer pairs were used to amplify Bergheipain1 and 2 from midipreps (pcDNAIIIAmpStreptagBp1/2) made from colonies 5 and 6 of BP1 and 2 positive transformants obtained in section 3.2.12.1 above. For PCR, an pfu polymerase with annealing temperature of 45 oC and Primer elongation time of 3 minute were used as shown in appendix IV. The clean up copies of these amplicons were then cloned in frame into EcoRV/XhoI digested pcDNA3 Amp Transin *strep*-tag. Midipreps from positive transformant were then digested with HindIII and XhoI and subsequently subcloned into HindIII/XhoI digested pVax1 . Midipreps sufficient for the vaccination purpose were made and stored at -20 ^{o}C. Figure 3.3 gives the diagrammatic design of pVax1 vacine constructs with transin signal sequences.

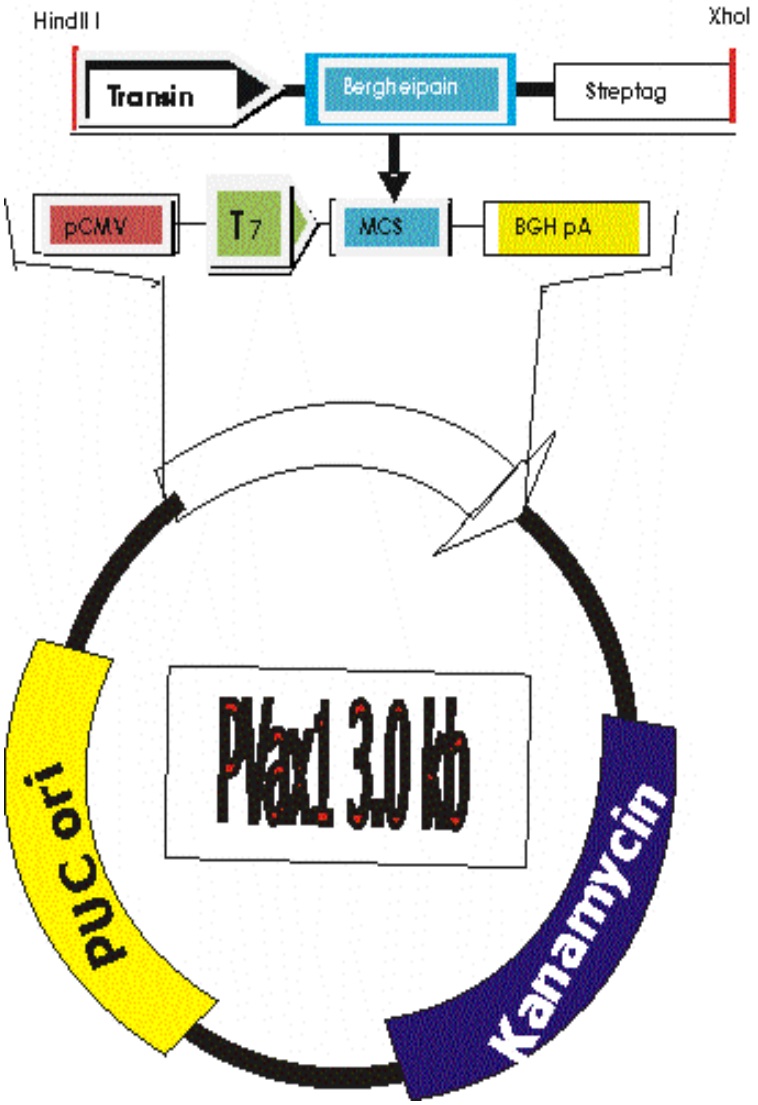


Figure 3.3 Design of pVax1TransinKanBP1Streptag and pVax1TransinKanBP2Streptag Constructs

3.2.13 Restriction Digest of PCR amplicon and the vector

Both amplicons and the eukaryotic expression vector, pcDNA3AmpStreptag (invitrogen), were digested using BamHI and EcoRI restriction enzymes (Roe et al., 1996). The vector contains a streptag and an ampicilin resistance region. The restriction digestion reaction (see appendix VI) of Bergheipain -1 amplicon is a 60 µl reaction mixture made up of 49 µl of the DNA (115.6 ng/ µl), 6 µl of X10 digestion buffer, 3 µl of ddH_2O, 1 µl of BamHI and 1 µl of EcoRI while that of Bergheipai-2 reaction mixture is a 40 µl volume made up of 29 µl of the DNA (44.5 ng/ µl), 4 µl of X10 digestion buffer, 5 µl of ddH_2O, 1 µl of BamHI and 1 µl of EcoRI. The mixtures , contained in 1.5 mL microcentrifuge tubes, were then placed into an incubator set at 37 ^oC for 1 hour. Thereafter they were removed for column purification of the digest.

3.2.14 Column Purification of product of Restriction Digest

After the restriction digest, the digest were mixed with equal volume of GeneJET binding buffer (i.e 60 µl of Bergheipain-1 and 40 µl of -2) . Each was transferred into separate GeneJET column and centrifuged at 13000 rpm for 1 min. The flow through was discarded and the column placed back into the collecting tube. Exactly 700 µl of washing buffer was then added to the column and centrifuged as above. The empty column was again re-centrifuged to completely get rid of residual washing buffer. Finally, the columns were transferred into clean microfuge tubes and the bound DNA eluted with 50 µl and 30 µl of elution buffer respectively.

3.2.15 Ligation Reaction

Each of the ligation mix consisted 8.5 µl ddH_2O, 2 µl of 10 ng/ µl of the insert, 2 µl of 10 ng/ µl the pcDNA3 vector, 2 µl of X10 T_4DNA ligase buffer and 2 µl of the T_4DNA ligase giving a

total volume of 16.5 µl mixture (appendices X and XI). The mixture was vortexed manually by gentle hitting with index finger and then given a short spin (approximately 3000 rpm for 5 second). The tubes were finally kept at room temperature for 45 minutes (for BamH1/Xho1) or were incubated at 16 ^{o}C overnight for the blunt ended EcoRV and the Xho1 ligation mix.

3.2.16 Transformation of Ligated vector into Competent cells

The ligated products were transformed into XL1-Blue chemically competent *E. coli* cells. Briefly, 200 µl of the iced competent cells were mixed with 12 µl of the ligation product and kept on ice for 10 minutes. The cells were heat shocked for exactly 90 seconds and immediately transferred to the ice (4 ^{o}C) for additional period of four minutes. After the four minutes, 1mL of SOC Medium, that was already warmed to 37 ^{o}C, was added to the cells and thereafter incubated at 37 ^{o}C with continuous shaking (250 rpm in) for 45 minutes. At the end of the 45 minutes incubation, the tubes were centrifuged for 30 sec at 800 rpm and then 800 µl of the supernatant removed and discarded. The pelleted cells were then resuspended in residual medium before being transferred with 100 µl-pipette into already prepared ampicilin/kanamycin culture plate. Each plate was prepared and labeled separately and were finally incubated overnight at 37 ^{o}C.

3.2.17 Replica Plating and colony PCR of transformed cells

At the end of 24 hr culture of the transformed cells, plates were taken and the colonies from each plate were simultaneously replica plated on a new amipicilin/kanamycin media plate as well as used for PCR. In this PCR reaction, Taq polymerase was used to catalyze DNA polymerization and in place of the template, the grown bacteria were used. The 24 µl PCR reaction mix consisted of 0.5 µl of each of the internal primer, 2.5 µl of Taq Polymerase Buffer, 0.5 µl of dNTPs, 1.5 µl of $MgCl_2$ (since the buffer was devoid of this salt), 0.2 µl of Taq

Polymerase, 18.3 µl of ddH_2O and a piece of bacteria, taken from bacterial plate with a pipette tip, dipped into the mixture, providing the DNA template (see appendix V for the detailed reaction mix). Positive (with appropriate Nested PCR products of bergheipain-1 and bergheipain-2) and negative control PCR (one without the primer and the other without DNA template) PCR controls were run side by side the PCR of the colonies. The Program used on the thermocycler with the Taq polymerase is given in appendix VI:

3.2.18 MiniPreparation (MiniPrep)-Amplification of positive transformants in small Amount

To amplify copies of plasmid containing gene of interest, positive bacterial clones were inoculated on Luria-Bertani (LB)-medium. For each of the genes, a clone was picked with a yellow pipette tip and was dipped into a liquid bacterial medium containing 5 mL of LB-medium and 5 µl of 50 mg/mL ampicilin solution in a 15 mL culture vial. The vials were taken to an incubator (Unifron) and left for the bacteria to multiply at 37 ^oC with slight shaking (250 rpm) overnight or for nine hours.

3.2.19 Midipreparation (MidiPrep)-Amplification of pVax1 in XL 1 Blue *E. coli* cells

In this procedure, copies of pVax1 vector were amplified after transforming into competent *E. coli* cells. Exactly 10 microliters of 1 ng/µl commercially purchased pVax1 were transformed into 200 µl of XL1 Blue® chemically competent *E. coli* cells as described for ligated plasmid above. Briefly, 200 µl of the iced competent cells were mixed with 10 µl of the 1ng/µl of pVax1© (Invitrogen) and kept on ice for 10 minutes. The cells were heat shocked (at 37 ^{o} C) for exactly 90 seconds and immediately transferred to the ice (4 ^oC) for additional period of four minutes. After the four minutes, 1 mL of Soc Medium, that was already warmed to 37 ^oC, was added to the cells and thereafter incubated at 37 ^oC with continuous shaking (250 rpm in) for 45

minutes. At the end of the 45 minutes incubation, the tubes were centrifuged for 30 sec at 800 rpm and then 800 µl of the supernatant removed and discarded. The pelleted cells were then resuspended in residual medium (approximately 200 µl) before being transferred with 100 µl-pipette into already prepared and warmed kanamycin culture plate. The plate was labeled and was finally incubated overnight at 37 ^{o}C. One of positive colonies was then use for the actual midiprep (i.e. the preparation of large amount or copies of the pVax1 in a pure form). For the midi prep, a positive colony was taken with a micropipette tip from positive plate and dipped into a 1000 ml-volumetric flask containing 200 mL of LB mixed with 200 µl of 50mg/mL Kanamycin solution. This inoculum was culture overnight at 37 ^{o}C with shaking (250rpm) in a Unitron incubator. Shortly before bacterial cells were harvested, the OD_{600} of the culture medium was determined using a Biophotometer (eppendorf) and was found to be around 2.563 (approx. 5 X 10^{11} cells per the 200 ml culture suspension used). The cells were transferred into a 300 mL centrifuge tube and were harvested by centrifugation at 6000 rpm for 15 minutes in Evolution RC centrifuge (Sorval).The plasmid DNA (pVax1) from these cells were extraction using the Macherey-Nagel High-copy Plasmid DNA purification kit as outline in the user manual.

3.2.20 Isolation of plasmid-containing inserts from bacterial colony (Plasmid DNA Minipreparation)

At the end of the bacterial growth period (9 hr), the tubes were removed from the incubator and were centrifuged at 4000rpm for 15 minutes. The plasmid were isolated from the bacterial cells using the GeneJET Plasmid Miniprep Kit of Fermentas. First, the supernatant in each tube was discarded and the cells were completely resuspended in 250 µl of the Resuspension Solution by pipetting up and down until no cell clumps remain. To these cells suspension, 250 µl of Lysis Solution was added and mixed thoroughly by inverting the tubes 4-6 times until the solution becomes viscous and slightly clear. At this point, care was taken not to vortex the solution and

that the tubes were not incubated for more than 5 min. The lysis step is immediately followed by the addition of 350 µl of Neutralization Solution with immediate and thorough mixing (by inverting the tube 4-6 times). The tubes were then centrifuged at 13000 rpm for 5 min to pellet the cell debris and chromosomal DNA. The supernatant (containing the plasmids) was gently transferred into the supplied GeneJET spin column by pipetting, being careful that the white precipitate was not pipetted along. The tubes were then centrifuged for 1 min, the supernatant discarded and the columns placed back into the same collection tube. The column was then washed twice by adding 500 µl of the wash Solution and centrifuged for 1 min. At the end of the second round of washing, the column was placed back into the collection tube and centrifuged empty at 13000rpm for 1 min. in order to remove the residual ethanol in the wash solution from the plasmid preparation. Finally, the columns were transferred into new 1.5 mL microcentrifuge tubes and 50 µl of Elution Buffer was carefully pipetted into the central of the silica membrane of the column, taking care that there was no contact between the pipette tip and the membrane. After about one minute of incubation at room temperature (22-25 ^{o}C), the tubes were centrifuged at 13000rpm for 1 min., the column were discarded and the flow-through (the plasmid extract solution) store at -20 ^{o} C until required for sequencing reactions and for subcloning.

3.2.21 pVax1 Plasmid DNA Midipreparation

The harvested cells were resuspended in 8 mL Resuspension Buffer (containing RNase A) by pipetting the cells up and down using 10 mL pipette attached to an eppendorf easypet. This was followed by the addition of 8 mL of lysis buffer, mixed gently by inverting the tube 5 times and then incubated at room temperature (18-25 ^{o}C) for 5 min. While the incubation was going on, the NucleoBond® Xtra Column was equilibrated by applying 12 mL Euilibration buffer onto the rim

of the column filter, wetting the entire filter. The column during equilibration, was allowed to empty by gravity flow. After the five minutes of the incubation mentioned earlier, 8 mL of Neutralization Buffer was added to the suspension and immediately mixed gently by inverting the tube 10- 15 times. The lysate is mixed well and immediately loaded on the already equilibrated column. Thereafter, the set up of NucleoBond® Xtra Column filter was washed by applying 5 mL of equilibration buffer to the rim of the filter. The column filter was then discarded by turning the column upside down in a waste container. The NucleoBond® Xtra Column was then washed with 8 mL of wash buffer and the plasmid eluted with 5 mL elution buffer into a 50 mL centrifuge tube. The next protocol that followed was the concentration of NucleoBond® Xtra eluates with NucleoBond® Xtra Finalizers; This is done by adding 3.5 mL of room-temperature isopropanol to the 5 mL eluates and leaving it to sit for 2 minutes. The plunger of a 30 mL syringe, provided, was removed and a NucleoBond® Finalizer attached to the outlet. The syringe was filled with the precipitated mixture, the plunger inserted back into the syringe. The syringe was held in a vertical position and the mixture pressed out of the syringe through the NucleoBond® Finalizer, by applying minimal pressure on the plunger. The flow- through was discarded, the NucleoBond® Finalizer removed from the syringe, the plunger pulled out and the NucleoBond® Finalizer reattached to the syringe outlet. The syringe was then filled with 2 ml of 70 % ethanol, the plunger inserted back, and while the syringe is held in a vertical position, the ethanol was slowly pressed out through the NucleoBond® Finalizer. The flow-through, again, was discarded. To dry the filter membrane, the NucleoBond® Finalizer was removed from the syringe, the plunger pulled out, the NucleoBond® Finalizer reattached to the syringe and air was pressed through the NucleoBond® Finalizer as strongly as possible while touching a tissue with

the tip of the NucleoBond® Finalizer to soak up ethanol. This is done for atleast three times until the membrane is dry.

To elute the DNA, the NucleoBond® Finalizer was removed from the 30 mL syringe, the plunger of a I mL syringe was also pulled out, 400 µL of redissolving buffer (5mM Tris/HCl, pH 8.5) was pipetted into the 1 mL syringe and the NucleoBond® Finalizer was now attached to the outlet of the I mL syringe and the NucleoBond® Finalizer outlet was placed over a 1.5 microcentrifuge tube in a vertical position and the plasmid DNA eluted drop by drop by inserting the 1mL plunger. The eluate was transferred back into the syringe and eluted into the same collection tube a second time. Finally, the NucleoBond® Finalizer was removed from the syringe, the plunger pulled out to aspirate air, the NucleoBond® Finalizer was reattached to the syringe and the air pressed out again to force out as much eluate as possible. The plasmid yied was determined using UV spectrophometer (the first yield was 759.3 µg/mL) and the plasmid integrity confirmed by agarose gel electrophoresis.

3.2.22 *In vitro* cultivation, maintenance and Transfection COS-7 cells with pcDNA3AmpBP1/BP2Streptag constructs

Aliquot of COS-7 cells, taken from a vial from liquid nitrogen, was thawed and used to prepare a culture plate; the cells were grown in 5 mL DMEM, pH 7.4 supplemented with 10% Foetal Calf Serum (FCS) (i.e 100 µL of FCS mixed with 900 µL of DMEM) on a petri dish. One microlitres of Gentamicin (50 mg/mL) was used as antibiotic for the *in vitro* cultivation, giving an effective antibiotic concentration of 50 µg/mL. After 24 hrs, the cells were viewed under an inverted microscope for growth and confluency. If the expected cell confluency was not attained, the spent medium was removed, cells were then washed twice with PBS(pH 7.4), the washing PBS removed with pasteur pipette and dislodged by wetting them with 1 mL of trypsin solution

for 2 min, the tripsin was removed by suction with Pasteur pipette, incubated for 2 minute at 37 ^{o}C in a CO_2-incubator and a fresh DMEM medium added to the culture petri dish. The Dish was then returned to the CO_2-incubator to continue cultivation for another 24 hr.

The cells were maintained in vitro by continuous subcultivation. Parent cells in a petri dish were taken to a lamilar flow and the spent medium removed using pateur pipette. The cells were then washed twice with PBS (pH 7.4), the washing PBS removed with pasteur pipette and dislodged by wetting them with 1 mL of tripsin solution for 2 min (the tripsin was removed by suction with Pasteur pipette), incubated for 2 minute at 37 ^{o}C in a CO_2-incubator. At the end of the two minute 10 mL of DMEM medium (supplemented with 10 % FCS) was added to the culture petri dish and the medium pipetted again into a sterile centrifuge tube with the dislodged cells in the medium. The clump of cells were separated into single cells by several rounds of tituration with a pipette. Therafter, the cells (now in 10 mL were splitted into two new sterile petri dishes (5 mL per dish). The Dishes were then returned to the CO_2-incubator for a fresh round of growth. This process is repeated whenever there was need for subculturing.

After the second 24 hrs of cultivation, the parent COS-7 cells which were still less than 50% confluent, were harvested as described above and were completely dislodged in a fresh DMEM medium by tituration with 10 mL pipette (attached to easypet) in a 30 mL plastic centrifuge tube. One mL of this stock cell suspension were seeded into each of 12 wells of 24-well microtitre plate. The plate was placed back into carbon dioxide incubator for another 24 hours.

The cells having attained 60-80 confluency, were taken from the incubator, the medium in them sucked out and 1 mL of fresh DMEM medium (supplemented with 2% FCS and 1µL of gentamicine per mL of medium) was added gently to the on top of the growing COS-7 cells in each well. The plate was then returned to the incubator. Immediately, aliquot of all the plasmid

DNA constructs were made ready for transfection in 1.5 mL microcentrifuge tubes by preparing, for each DNA, a 1.5 (μg/100 μL) DNA stock concentration in 150 mM sterile NaCl solution with 2.5 μL polyethylimine (PEI sterile stock is 1:750) per the total 100 μL. See appendices IX and X for the detailed preparation. During sample preparation the PEI was the last component added and usually as a drop (2.5) on the inner wall of the microfuge tube to allow for simultaeneous mixing. The mixtures were vortexed and kept at room temperature for ten minutes before they were each (100 μL) spray-released drop-wise into a well on the already labelled 24-wells plate. Finally, the plate was incubated for 18 hrs.

3.2.23 Immunofluoresence screening of the transfected COS-7 cells

After about 18 hrs of incubation, the medium in the wells were aspirated, the cells were washed twice with 1 mL of 1X PBS and were fixed with 300 μL (per well) of 4% Paraformaldehyde (PFA) for 10 minutes. The medium was aspirated and 250 μL of ice cold methanol was added to each well. The plate was left on ice for exactly 2 mins but the methanol was also aspirated and followed immediately with washing twice with 1 mL (per well) of 1XPBS (containing 3%BSA).

After the removal of washing solution, 500 μL of DMEM (supplemented with 10% FCS) was added to each well and the cells were incubated for 30 – 45 minutes.

After 45 minute incubation, each of the wells was washed twice with 1 mL of 1x PBS (containing 3%BSA) and immediately aspirated. Two hundred microliters of Antistrep antibody (primary antibody), prepared to 1:1000 dilutions with 1x PBS in 3%BSA, was then added to each well and left at room temperature for 1½ hr. After this incubation period, the cells were aspirated, washed twice with 1 mL (per well) of 1XPBS (containing 3%BSA) and immediately

incubated with 250 µL (1:500 dilution , antibody:1x PBA in 3%BSA) of the secondary antibody (Donkey antisheep Cy-3) at room temperature for another 1½ hr.

The cells were then washed twice with 1x PBS before viewing on a fluorescent microscope.

3.2.24 Sequencing of Bergheipain1 and Bergheipain2 genes

The BP1 and BP2 gene inserts were sequenced as part of midiprep plasmid DNA (pcDNA3) samples of some selected positive clones. The presence of the genes within the plasmid and whether the genes were in frame with Streptag sequence were ascertained by sequencing of the genes.

For the sequencing reaction, the 2 µL of the Big dye and 1 µL of a primer per reaction. Since the concentration of the plasmid DNA must be between 450 – 650 ng/µL, 500 ng/µL was used and the volume of the DNA sample used was calculated based on individual stock concentration. The volume of double distilled water used was also calculated such that it gives the total reaction volume of 11 µL. Again, each reaction PCR tube contained only one primer (either sense primer or strep specific primer as presented in appendices XIX and XII). Therefore, a total of twelve reactions were made (six per each gene).

The tubes were taken to the Thermocycler and run with the sequence programme overnight (see appendix XXIII). Alongside this, the sephadex (G-50 superfine) column (in 96-well microtitre plate) was wetted with 300 µL of ddH_2O and left wet at 4 ^oC overnight. The following morning, the columns were centrifuged at 2000 rpm for 4 minute (Eppendorf, *centrifuge* 5810 R). Exactly 200 µL of double distilled water was applied to the columns again and centrifuged as previously mentioned. Water was applied again and the plate centrifuged for the third time. The PCR reactions samples were made up to 20 µL with 9 µL of ddH_2O and were then applied to the

column after a new 96-wells plastic base was placed under the microtitre plate column. The centrifugation was carried for the last time at 2000 rpm for 4 minutes and the eluted DNA collected in the base 96-wells plate, labelled and taken to Marks Plank Institute, University of Bremen, for chromatography.

3.2.25 Screening of Plasmid DNA miniprep/midiprep for the presence of streptag sequence

The streptag sequence is a detection signal sequence. Its product, a streptag peptide, gives a fluorescent signal that could be used for detection of possible expression of genes of interests. Since the bergehipain gene constructs were made in such a way that they bear the streptag sequence at their 3'-end (not separated by a stop codon), a successfull gene expression is easily detected using a fluorescent microscope (OLYMPUS, IX 70). The aim of the step is, therefore, to check if the streptag sequence exist inframe as part of the gene constructs.

In order to do this, Tag Polymerase PCR reaction mix was made as previously described except that the primer combination in this case consists of an internal sense primer and strep-specific primer (in place of reverse primer).

3.2.26 Immunisation Studies

3.2.26.1 Experimental Design and Grouping

Eighty healthy female Balb/c mice weighing between 19 g to 23g were purchased from Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna, Nigeria. The mice were left to acclimatize to the animal room environment at the Center for Biotechnology Research and Training, ABU, Zaria. All animals were given sufficient feed (Pelletized Grower's marsh, pfizer company, Lagos-Nigeria) and were give water *ad libitum* throughout the study period. Sixty of these mice were randomly placed in seven special insect-proof cages (made in Germany) (see

appendix XXIV) representing the seven experimental groups: the first two groups (Negative and Positive controls) had 5 mice each while the remaining groups had 10 mice per cage. Body weights of all animals were determined before they were randomly divided into groups. The various experimental groups and their treatment are given below:

Group Number	Experimental Group (Cage-Tag)	Brief Description of Treatment
I	Negative Control	Mice were not vaccinated and were not challenged with *P. berghei*
II	Positive Control	Mice were not vaccinated but were challenged with *P. berghei*
III	Plasmid Control	Mice were vaccinated with naked plasmid DNA (pVax1) and were later challenged with *P. berghei*
IV	pVax1BP-1-No Transin	Mice were vaccinated with DNA vaccine (pVax1BP1Streptag) and were later challenged with *P. berghei*
V	pVax1BP-2-No Transin	Mice were vaccinated with DNA vaccine (pVax1BP2Streptag) and were later challenged with *P. berghei*
VI	pVax1BP-1-With Transin	Mice were vaccinated with DNA vaccine (pVax1TransinBP1Streptag) and were later challenged with *P. berghei*
VII	pVax1BP-2-With Transin	Mice were vaccinated with DNA vaccine (pVax1TransinBP2Streptag) and were later challenged with *P. berghei*

The remaining twenty mice (from the eighty that were purchased) were infected with *P. berghei*-infected red cells two weeks before the commencement of parasite challenge in order to serve as source for infected blood required for challenging fifty five experimental mice (groups II to VII).

3.2.26.2 Vaccination and challenge of experimental mice

All the mice in groups III to VII were primed i.m. with 50 μg of appropriate plasmid DNA. At two weeks intervals they were given the first (25 μg of plasmid DNA per mouse) and the second (10 μg of plasmid DNA per mouse) vaccine boosts. Animals in groups I and II were not given any vaccine but were given 50 μl of membrane-filtered PBS (pH, 7.4) instead. (see appendices XXV and XXVI for details of the vaccine administration).

Exactly twenty eight days after priming, all the experimental mice with the exception of those in group I, were each challenged with 1.43×10^7 P. bergehi-iRBCs (approximately 0.15 mL of PBS suspension containing 3.8% Sodium citrate as anticoagulant). Group I animals were left uninfected but were given 0.15 mL of the membrane-filtered PBS.

3.2.26.3 Collection of Blood and Sera from experimental mice

Five sets of blood samples (days o, 6, 13, 20, and 27) were collected before challenge. After challenge, more samples were also collected at 3 days intervals (days 31, 34, 37, 40, 43, 46, 49, and 52 post prime Vaccine. All samples were diluted with membrane-filtered PBS and subsequently stored in refrigerator (at 4 ^{o}C) until required.

3.2.26.4 Determination of animals' body weights

Weights of all animals were taken at days -28, 0, 15, 23, and 60 relative to the day of priming (day 0). For days post challenge, only surviving animals in a group were weighed.

3.2.26.5 Determination of Parasitaemia

Thin smears were made using drop of blood collected from animal's tail immediately before challenge and later at 3 days interval until day 24 post challenge. The smears were fixed with methanol for 30 seconds, stained with giemsa and were microscopically examined for the presence and percentage of infected red cells. For those animals that survived beyond the 24 day post challenge, the determination of parasitaemia continued until day 60 post challenge when the experiment was terminated.

3.2.26.6 Collection of tissues from representative mice and gDNA Extraction

On day 12 post challenge (i.e 40^{th} day after prime vaccination) a representative mouse from each group was sacrificed after chloroform anaesthesia and blood and organs (Muscle, liver and Spleen) collected in 2 mL sterile plastic containers. Genomic DNA was isolated from these tissues and were screened for BP1 and BP2 genes using their respective specific primers to run PCR.

3.2.26.7 Observation of experimental animals

Animals were observed for possible sign of toxicity throughout the duration of the experiment. Deaths were recorded, and whenever death occurred, the dead animal was opened up for gross observation of sign of malaria infection (darkened liver and enlarged spleen).

3.2.27 Histopathology

On the twenty fourth day post challenge, representative mice (a mouse from each group) were sacrificed and the internal organs (muscle, liver, spleen, cerebral cortex, heart, kidneys, and lungs) collected in sample containers and fixed for twenty four hours by preserving them in neutral phosphate buffered saline formalin (10% formalin). Smears from such tissues were then prepared and stained with haematoxylin and eosin. The histopahatological pictures were examined for the presence of parasites and possible tissue infiltrations.

3.2.28 Statistical Analysis of Data

The data generated from this study (Parasitaemia, RBC count and body weight) were analyzed using SPSS (version 17.0). Analysis of variance (ANOVA) was used to test variations between various experimental groups while DUNCAN multiple test was used to check for variation within each group. The statistical analyses were done at the Data Processing Unit of the Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria.

CHAPTER FOUR

RESULTS

4.1 Bioinformatic Identification of Bergheipain homologues and primer design

Various online Bioinformatics tools were used to identify genes in *Plasmodium berghei* that were orthologous to Falcipain 1 and Falcipain 2 of *Plasmodium falciparum*. Figures 4.1 and 4.2 presents the complete nucleotide sequences coding Bergheipain 1 and Bergheipain 2 respectively. Figures 4.3 and 4.4 are the identified contigs harbouring Bergheipain 1 and Bergheipain 2 genes respectively and from where the primers used for amplification were designed. Table 4.1 present the nucleotide sequences used for the nested PCR protocols.

ATGATAAACGATATAAGACGAATAAACATTACCACAAGCAGTATCGAAAGTTTGAA
TGAAAATAGCAAATATTTAAAAAGAAATCACAAGAGGACTATAAAAATATGTGCAT
ATGCAATTACAACATTTGCATTATTTTTTATTGTTGTAGTATATTTTAAAAATCAAAC
AAATGTAAATGATGCAAATAGAAATACTTTAGCAGCAATCGATGAAACAAGCTTAA
TGAATAAAGAAATAGCATATTTAAGAGAAATTTTGAAAAAATATAAAACAAAAACA
AATGAAAATAATGAATATGCATACGAAAAAAACGACGATATTAACGGAGATGGCGA
AGACGAGCACGAATTGCTATTAATGTTACATAAATTTTTAAAAAATAAAGGTAATCC
TAATAAAATAGATAGGTTTGATATTAACAATAATGACTCAAATAAAAATCGTGGTA
ATGAAAATATTGATCAAATAAACATACTTAGTCAAAAACTTGAAAGCATGCATGAT
AACATAAAATATGCATCAAAATTTTTCAAATATATGAAAGAATACAATAAAAAATA
CAAAAATATAGATGAACAATTAGTAAGATTTGAAAACTTTAAAACGAATTATATGA
AAGTTAAAAAACACAATGAAATGGTAGGTAAAAATGGTATTACATATGTACAAAAA
GTAAACCAATTTAGTGATTTTTCTAAGGAAGAACTTGATAGTTATTTTAAAAAATTA
TTACCTATTCCACACAATTTGAAAACTAAACATGTAGTACCACTAAAAACACATTTA
GACGATAATAAAATTAAACCAAAAGAAGGTGTATTAGACTATCCAGAACAGCGGGA
TTATAGAGAATGGAATATATTACTTCCTCCAAAGGATCAAGGTATGTGTGGGTCTTG
CTGGGCATTTGCTAGTGTAGGAAATTATGAAGCTCTTTTTGCAAAAAAATATTCTAT
ATTACCTATAAGTTTTAGTGAACAACAAGTTGTTGATTGTTCTAGTGACAATTTTGGA
TGTGATGGCGGTCATCCATTTTTATCATTTTTATATTTTCTTAATAATGGGGTATGCTT
TGGCGATAATTATGAATACAAAGCTCATGATGATTTTTTCTGCTTAAGTTATAGATGT
GCGTATAGAAGTAAATTAAAAAAAATTGGCAACGCATATCCATATGAATTAATTAT
GTCCTTAAATGAAGTAGGGCCAATAACTGTTAATGTTGGTGTATCCGACGAATTTGT
ATTATATTCGGGTGGTATATTTGATGGAACATGTGCTTCGGAGTTAAATCATTCTGTT
TTATTAGTGGGATATGGTAAAGTTAAAAGGAGCTTAGTATTTGAGGATAGTCATACT
AATGTAGATAGCAATTTGATAAAAAACTATAAGGAAAATATCAAAGACAGCGATGA
TGACTATTTGTATTACTGGATCATTAGAAATTCATGGAGTTCAACTTGGGGAGAAGG
TGGCTATATAAGGATTAAAAGAAACAAATTAGGAGACGATGTTTTTTGTGGAATTGG
TATTGACGTTTTCTTTCCAATTTTATAA

Figure 4.1 Nucleotide sequence of Bergheipain-1 obtained from gene data bank of PlasmoDB. [The start and stop codons are coloured blue and red respectively; Acession Number: PBANKA_132170; Sequence length:1560].

ATGAATTACCATTCTAGCCATCATATTAGACCCGAGGAGGAAATATTTGTTGATAAG
GGTATACAAAATGTACAGTTACGAAGAAAAAATAAAATGCTTATAGTTACCCTGGC
TATAGTTTTAGGTATGTTTGGTTTTACCGTCATATATTTTAATAGAACAAATAAATCA
TCATTTAATAATGGAAATGTTGAAAATTATTCGAATGATGACTATTTAATAAATTAC
TTATTAAAAAGCAAAGCAGTAAAGAAATTTATGGGATCTAAAATTGAAGAACTTAT
AGTAGAAAGTGAACAAAATGAAAAGAATAGCATTATCGTTAAAGATAATAAAAATA
ATGACTATAACGAAAAATCTGTATTATTTAATAAAAATAACGACAGCAAATCATTCA
CAACAAATTTACATGATATGCAATCTATTATGAACAATTTAGAATCTGTGAACATTT
TTTACAATTTCATGAAAGAATATAACAAACAATATAATTCAGCTGAAGAAATACAA
GAGAGATTTTATATCTTTTCTGAAAATTTAAAAAAAATTGAAAAGCATAATAAAGAA
AATCATTTGTATACAAAAGGTATTAATGCATTTAGTGATATGCGTCATGAAGAATTT
AAAATGAAATATTTAAATAATAAACTAAAAGAAAACCACAGCATTGATCTCCGACA
TTTAATTCCTTATACTACTGCAATTAGTAAGTACAAGTCTCCAACCGATAAAGTTAA
CTATACAAGTTTTGATTGGAGAGATTATAATGTTATTATAGGCGTTAAAGATCAACA
AAAGTGTGCTTCATGCTGGGCATTTGCTACCGCTGGTGTTGTCGCAGCCCAATATGC
TATTAGAAAAAATCAAAAGGTTTCTTTAAGTGAACAACAATTAGTTGATTGTGCACA
AAATAATTTTGGATGTGAAGGAGGTATACTTCCATATGCTTTTGAAGATCTTATAGA
CATGGATGGTTTATGTGAAGATAAATATTATCCATATGTAAGTAATGTTCCAGAATT
ATGCGAAATTAACAAGTGTACCGAAAAATACTCAATTTCAAAATTTGCATTAGTACC
ATTCAATAATTATAAAGAAGCTATTCAATATTTAGGTCCAATCACAATAGCTGTAGG
TGTAGATGATGATTTTGAATCTTACAATGGTGGTATATTCGATGGAGAATGTACAGA
TTTTGCAAATCATGCAGTTATGCTTATTGGATATGGTGTTGAAGAGGTATATGATAA
GCGTCTTAAAAAAAATGTTAAAGAATATTATTATATAATTAGAAACTCTTGGGGTGA
AGACTGGGGAGAACGTGGTTACATAAGACTTAAGACTAACGAATCAGGAACACTCA
GAAATTGTGTGTTAGTACAAGGTTATGCTCCTATAATTGAATAA

Figure 4.2 Nucleotide sequence of Bergheipain-2 obtained from gene data bank of PlasmoDB. [The start and stop codons are coloured blue and red respectively; Acession Number: PBANKA_093240; Sequence length:1407].

TTTTTATTACTTTATTATTATTAATTTTTTTTTATAGCAAAAAAATTTCGTAACGAAAT
AAATTTTTTAAATGATATTTTATTATATATATATTTATTTATAAAAATAATACACAAA
CGTAAGAAAGTTTTTATTTCTTTGGGGCAAGATGATAAACGATATAAGACGAATAA
ACATTACCACAAGCAGTATCGAAAGTTTGAATGAAAATAGCAAATATTTAAAAAGA
AATCACAAGAGGACTATAAAAATATGTGCATATGCAATTACAACATTTGCATTATTT
TTTATTGTTGTAGTATATTTTAAAAATCAAACAAATGTAAATGATGCAAATAGAAAT
ACTTTAGCAGCAATCGATGAAACAAGCTTAATGAATAAAGAAATAGCATATTTAAG
AGAAATTTTGAAAAAATATAAAACAAAAACAAATGAAAATAATGAATATGCATACG
AAAAAAACGACGATATTAACGGAGATGGCGAAGACGAGCACGAATTGCTATTAATG
TTACATAAATTTTTAAAAAATAAAGGTAATCCTAATAAAATAGATAGGTTTGATATT
AACAATAATGACTCAAATAAAAATCGTGGTAATGAAAATATTGATCAAATAAACAT
ACTTAGTCAAAAACTTGAAAGCATGCATGATAACATAAAATATGCATCACAATTTTT
CCAATATATGAAAGAATCAATAAAAAATACAAAAATATAGATGAACAATTAGTAAG
ATTTGAAAACTTTAAAACGAATTATATGAAAGTTAAAAAACACAATGAAATGGTAG
GTAAAAATGGTATTACATATGTACAAAAAGTAAACCAATTTAGTGATTTTTCTAAGG
AAGAACTTGATAGTTATTTTAAAAAATTATTACCTATTCCACACAATTTGAAAACTA
AACATGTAGTACCACTAAAAACACATTTAGACGATAATAAAATTAAACCAAAAGAA
GGTGTATTAGACTATCCAGAACAGCGGGATTATAGAGAATGGAATATATTACTTCCT
CCAAAGGATCAAGGTATGTTGGGTCTTGCTGGGCATTTGCTAGTGTAGGAAATTATG
AAGCTCTTTTTGCAAAAAAATATTCTATATTACCTATAAGTTTTAGTGAACAACAAG
TTGTTGATTGTTCTAGTGACAATTTTGGATGTGATGGCGGTCATCCATTTTTATCATT
TTTATATTTTCTTAATAATGGGGTATGCTTTGGCGATAATTATGAATACAAAGCTCAT
GATGATTTTTTCTGCTTAAGTTATAGATGTGCGTATAGAAGTAAATTAAAAAAAATT
GGCAACGCATATCCATATGAATTAATTATGTCCTTAAATGAAGTAGGGCCAATAACT
GTTAATGTTGGTGTATCCGACGAATTTGTATTATATTCGGGTGGTATATTTGATGGAA
CATGTGCTTCGGAGTTAAATCATTCTGTTTTATTAGTGGGATATGGTAAAGTTAAAA
GGAGCTTAGTATTTGAGGATAGTCATACTAATGTAGATAGCAATTTGATAAAAAACT
ATAAGGAAAATATCAAAGACAGCGATGATGACTATTTGTATTACTGGATCATTAGA
AATTCATGGAGTTCAACTTGGGGAGAAGGTGGCTATATAAGGATTAAAAGAAACAA
ATTAGGAGACGATGTTTTTTGTGGAATTGGTATTGA**CGTTTTCTTTCCAATTTTA**TA
AACATGCAGCAAAAAAAATTTAATGAAAAAAAAATTAATACTGAATAATGATAAAA
GCTAAATTGTGAAAAAGGGAAAATTGCATTTTATTACAAATACCTTCTACACCCGTA
TATAATACGTTCATAAAATAGTACGACATACTATATTTTTTTTATCTAACTAAAAAA
AATGAACGT

Figure 4.3 Contig containing Bergheipain-1 Nucleotide sequence.

[The sequence of Bergheipain 1 gene is coloured blue; the external primers are in yellow box while part of the internal primers are bolden blue and underlined. The restriction sequences used for cloning purpose are not indicated]

TTTTTATTAAAAAATAATTAAATTAATGTTTTTTTTTAATTAATGTGGAGTTTTATATT
TATATATAAATTTAATTCATCATAATATTTTGCAAAAAAAAATTAACATAGAAACAT
AATACATCATGAATTACCATTCTAGCCATCATATTAGACCCGAGGAGGAAATATTT
GTTGATAAGGGTATACAAAATGTACAGTTACGAAGAAAAAATAAAATGCTTATAGT
TACCCTGGCTATAGTTTTAGGTATGTTTGGTTTTACCGTCATATATTTTAATAGAACA
AATAAATCATCATTTAATAATGGAAATGTTGAAAATTATTCGAATGATGACTATTTA
ATAAATTACTTATTAAAAAGCAAAGCAGTAAAGAAATTTATGGGATCTAAAATTGA
AGAACTTATAGTAGAAAGTGAACAAAATGAAAAGAATAGCATTATCGTTAAAGATA
ATAAAAATAATGACTATAACGAAAAATCTGTATTATTTAATAAAAATAACGACAGC
AAATCATTCACAACAAATTTACATGATATGCAATCTATTATGAACAATTTAGAATCT
GTGAACATTTTTTACAATTTCATGAAAGAATATAACAAACAATATAATTCAGCTGAA
GAAATACAAGAGAGATTTTATATCTTTTCTGAAAATTTAAAAAAAATTGAAAAGCAT
AATAAAGAAAATCATTTGTATACAAAAGGTATTAATGCATTTAGTGATATGCGTCAT
GAAGAATTTAAAATGAAATATTTAAATAATAAACTAAAAGAAAACCACAGCATTGA
TCTCCGACATTTAATTCCTTATACTACTGCAATTAGTAAGTACAAGTCTCCAACCGAT
AAAGTTAACTATACAAGTTTTGATTGGAGAGATTATAATGTTATTATAGGCGTTAAA
GATCAACAAAAGTGTGCTTCATGCTGGGCATTTGCTACCGCTGGTGTTGTCGCAGCC
CAATATGCTATTAGAAAAAATCAAAAGGTTTCTTTAAGTGAACAACAATTAGTTGAT
TGTGCACAAAATAATTTTGGATGTGAAGGAGGTATACTTCCATATGCTTTTGAAGAT
CTTATAGACATGGATGGTTTATGTGAAGATAAATATTATCCATATGTAAGTAATGTT
CCAGAATTATGCGAAATTAACAAGTGTACCGAAAAATACTCAATTTCAAAATTTGCA
TTAGTACCATTCAATAATTATAAAGAAGCTATTCAATATTTAGGTCCAATCACAATA
GCTGTAGGTGTAGATGATGATTTTGAATCTTACAATGGTGGTATATTCGATGGAGAA
TGTACAGATTTTGCAAATCATGCAGTTATGCTTATTGGATATGGTGTTGAAGAGGTA
TATGATAAGCGTCTTAAAAAAAATGTTAAAGAATATTATTATATAATTAGAAACTCT
TGGGGTGAAGACTGGGGAGAACGTGGTTACATAAGACTTAAGACTAACGAATCAGG
AACACTCAGAAATTGTGTGTTAGTACAAGGTTATGCTCCTATAATTGAATAAAAAT
AATATTATGTACCGATAGGGAAAATAATAAGCACCGCTATCCGTATTCAAATATATA
TATATATATATAGTTGCACTTTATGGACGAATA

Figure 4.4 Contig containing Bergheipain-2 Nucleotide sequence.
[The sequence of Bergheipain 2 gene is coloured blue; the external primers are in yellow box while part of the internal primers are bolden blue and underlined. The restriction sequences used for cloning purpose are not included]

Table 4.1. Sets of Nested PCR primers designed for the amplification of Bergheipain 1 and Bergheipain 2 homologs

Gene		Primers design	
		External	**Internal**
Bergheipain 1	**Sense**	5'-CTTTGGGGCAAGATG-3' (A total of 15 bp)	5'-CGGGATCC**ATG**ATAAACGATATAAGAC-3' (A total of 27 bp)
	Antisense	5'-GCTGCATGTTTAG-3' (A total of 13 bp)	5'-CGGAATTCTAAAATTGGAAAGAAAACG-3' (A total of 27 bp)
Bergheipain 2	**Sense**	5'-GAAACATAATACATCATG-3' (A total of 18 bp)	5'-CGGATCC**ATG**AATTACCATTCTAGCC-3' (A total of 26 bp)
	Antisense	5'-TTCCCTATCGGTAC-3' (A total of 14 bp)	5'-CGGAATTCTTCAATTATAGGAGCAT-3' (A total of 25 bp)

BamHI sites (underlined) were added to sense primers while EcoRI sites (underlined) were added to the antisense primers. Initiation codons were marked bold red.

4.2 Preliminary Preparation and isolation of *P. berghei* isolates

Plasmodium berghei was grown in mice and the infected animals were sacrificed when the parasitaemia was between 20 to 35 %. In order to get rid of other possible sources of DNA contamination like white blood cells and platelets, the harvested blood was treated with 3.6 % dextran solution. This approach removed substantial amount of leucocytes and platelets as revealed by microscopic observation (Plate 4. 1)

4.3 Amplification of Bergephain 1 and Bergephain2 genes

Two genes for cysteine protease were identified from literature survey and Blast search on some DATA banks. External and internal primers were designed and used to amplify these genes using a nested PCR protocol with the gDNAs as templates. Both genes were successfully amplified using genomic DNA isolated from the *P. berghei isolate*. When the PCR product were run on 1 % agarose gel the genes appeared around 1500 bp region with BP 2 running ahead of BP1 (Plate 4.2) indicating that BP1 is longer than BP2.

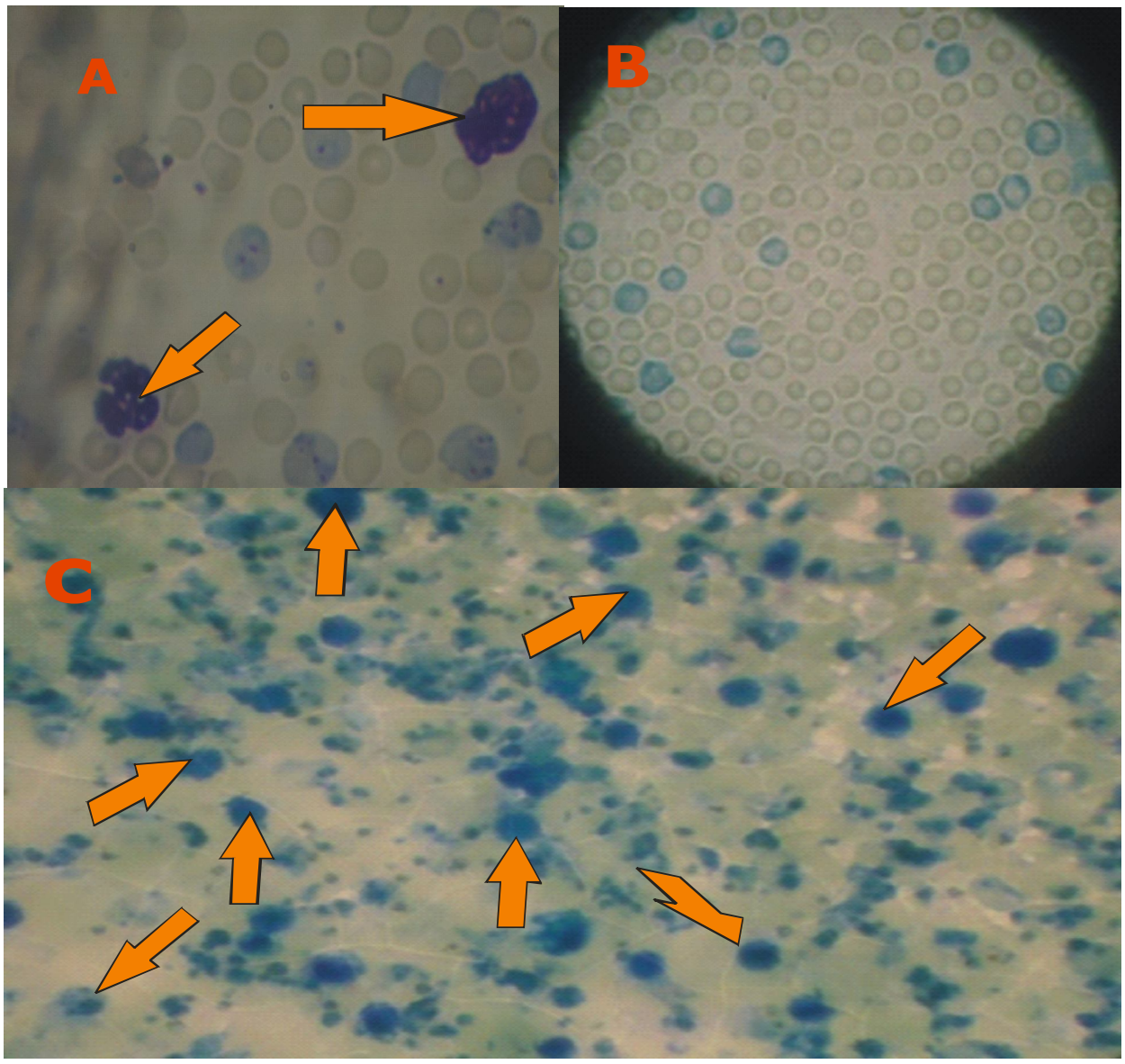


Plate 4. 1. Giemsa stained pictures of infected red cell.

[Before (A) and after (B) the removal of platelets and leucocytes by treatment with dextran solution; and the pooled leucocytes (C). Arrows identify the leucocytes.]

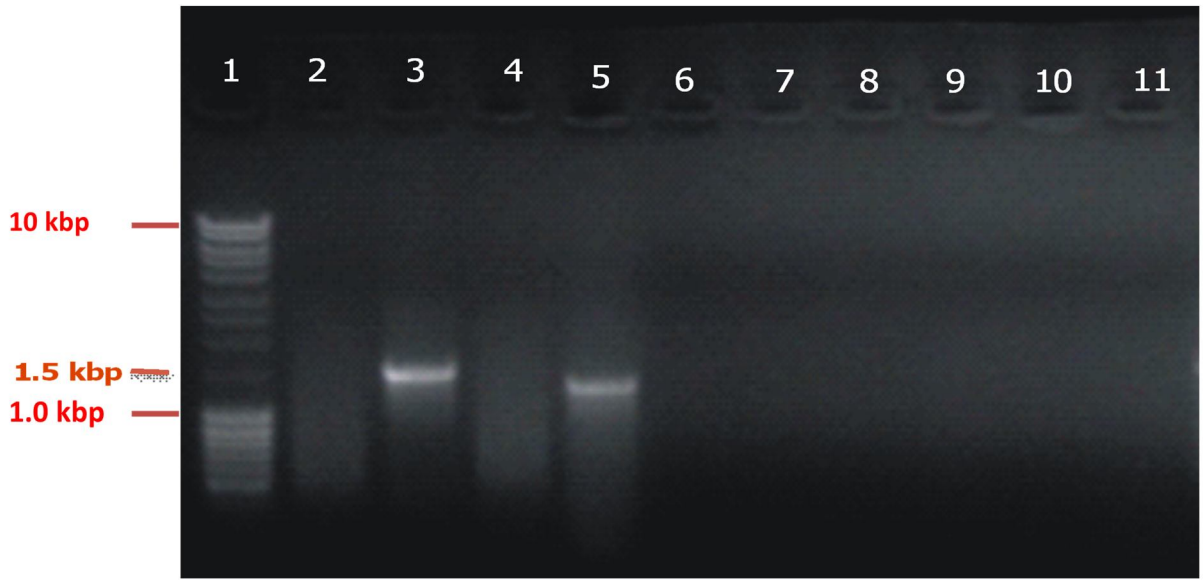


PLate 4.2. Analysis of Bergheipain genes amplified by nested polymerase chain reaction using genomic DNA isolated from *P. berghei* as template.

[pfu polymerase was used with reaction conditions as outlined in the methods. Electrophoresis was carried out on 1% agarose gel.]

KEYS:

Lane 1 (DNA marker)
2 (Amplification carried out using external primers of Bergheipain1)
3 (Amplification carried out using internal primers of Bergheipain1)
4 (Amplification carried out using external primers of Bergheipain 2)
5 (Amplification carried out using internal primers of Bergheipain 2)
6(control amplification without DNA template)
7(control amplification without BP1 primers)
8(control amplification without BP2 primers)
9(control amplification without pfu DNA polymerase)
10(control amplification using genomic DNA of *Trypanosoma congolense* with BP1 primers)
11 (control amplification using genomic DNA of *Trypanosoma congolense* with BP2 primers)

4.4 Cloning of Bergheipain1 and Bergheipain2 genes into pCDNA3Streptag Vector and their transient expression *in vitro*

The genes' bands on agarose gel were excised, melted and column purified using GeneJET purification kit. They were then subjected to restriction digest with EcoRI and BamHI before they were ligated to pCDNA3 vector previously cut with the same restriction enzyme system. After transforming XL1 blue *E. coli* cells with the ligation products, some positive colonies on LB Ampicilin plates were screened for the presence of the recombinant plasmids of both genes and replica plates were at the same time prepared. Colony PCR for both genes (Plates 4.3 and 4.4) revealed greater than 90% of the transformed cells had picked up the recombinant plasmid. Table 4.2 gives the yield and parameters of the midipreps of some selected colonies that gave positive amplification during colony PCR. Agarose gel electrophoresis (Plate 4.5) of the minipreps (appendix XIII) and midipreps show that they are indeed plasmids DNA of the expected base length. Since the gene inserts in the plasmids have streptag sequences at their 3' end, in order to confirm that the midipreps carry the genes with the required streptag, each gene was amplified using gene specific primer as the sense primer and strep specific primer as the reverse primer. Plate 4.6 shows that all the midipreps had the genes and the streptag sequence in them were in the required position. Restriction digest confirms the presence of an insert of approximately 1500 base pair in the plasmids (Plates 4.7 and 4.8).

Attempt towards *in vitro* expression of these genes on mammalian COS7 cells was carried out using immunoflouresence technique with Donkey antisheep Streptagged antibody as the primary antibody and conjugate donkey antisheep cys-3 antibody as

secondary antibody. Cells were transfected with the pCDNA3BP1/2Streptag plasmids when cells had attained about 60% confluency and immunoflouresence carried out 24 hr after transfection. Plate 4.9 gave positive *in vitro* expression of each gene in COS7 cells.

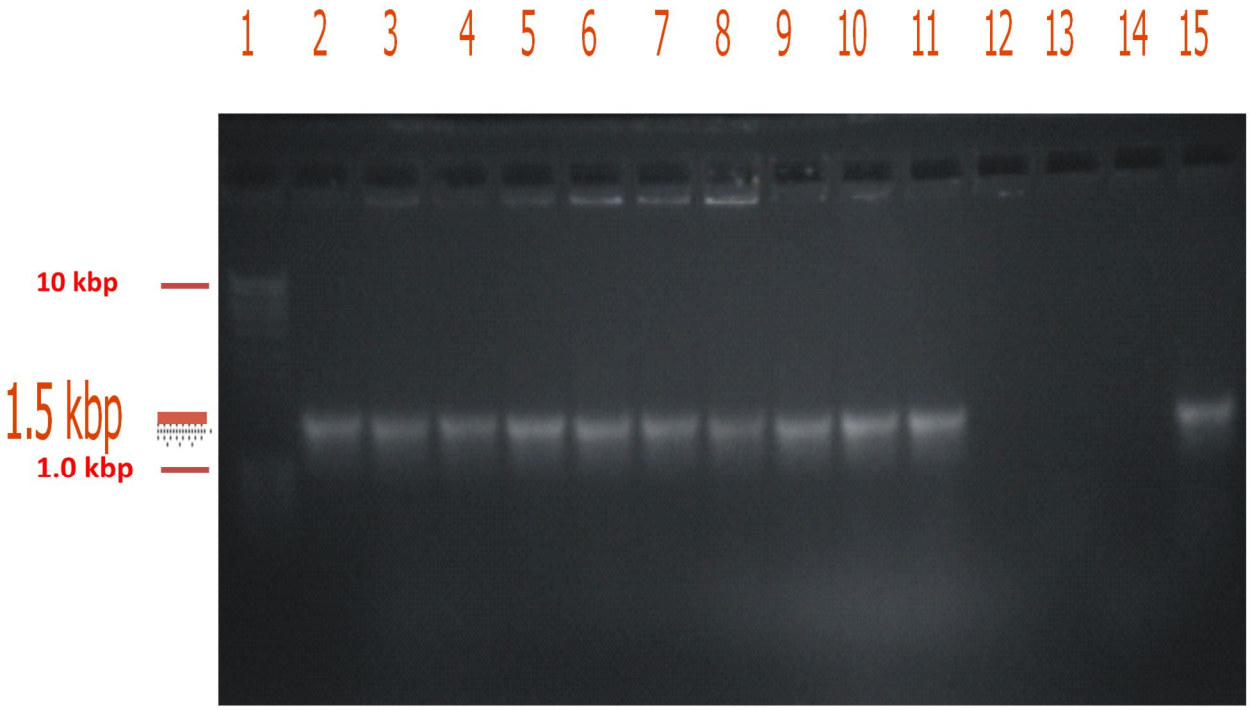


Plate 4.3. Result of the colony polymerase chain reaction after XL1 Blue *E.coli* cells were transformed with pcDNA3AmpBergheipain1Streptag recombinant plasmid and allowed to grow on Ampicilin LB-plate.

KEY:

Lane 1 (10 kbp DNA Markers)

2-12 were positive colonies screened,

13 (Negative controls without primers)

14 (Negative controls without Taq polymerase)

15 (Positive control- bergheipain 1amplicon was used as template for the PCR)

[For all reactions, Taq polymerase was used with the sense internal primers (OSK2027/29) of Bergheipain].

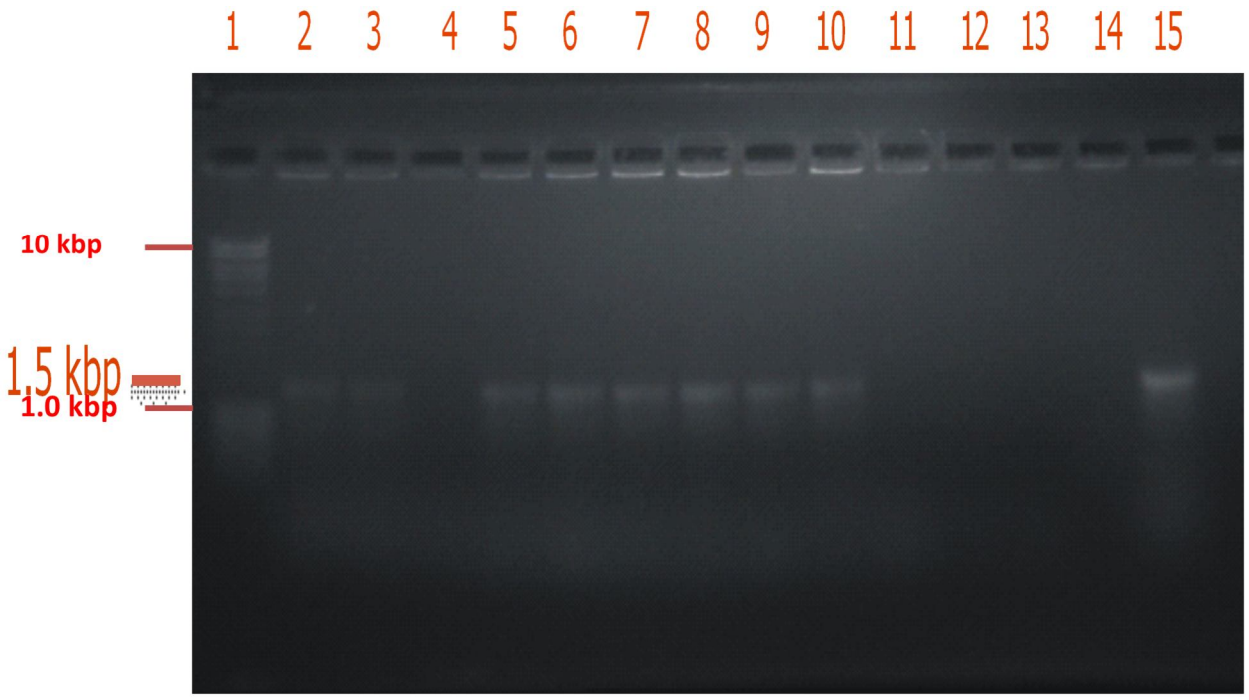


Plate 4.4. Result of the colony polymerase chain reaction after XL1 Blue *E.coli* cells were transformed with pcDNA3AmpBergheipain2Streptag recombinant plasmid and allowed to grow on Ampicilin LB.

Lane 1 (10 kbp DNA Markers)

2-12 were positive colonies screened,

13 (Negative controls without primers)

14 (Negative controls without Taq polymerase)

15 (Positive control- bergheipain 2 amplicon was used as template for the PCR)

[For all reactions, Taq polymerase was used with the sense internal primers (OSK2031/33) of Bergheipain2].

Table 4.2. Yield for pcDNA3Bergheipain1Streptag and pcDNA3Bergheipain2Streptag Midiprep from representative positive colonies

Gene	Colony number	Plasmid Nomenclature	Plasmid DNA Conc. (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}
Bergheipain-1	5	pCDNA3BP1Streptag	1311.1	1.87	2.35
	6	pCDNA3BP1Streptag	1494.6	1.87	2.35
Bergheipain-2	5	pCDNA3BP2Streptag	1299.3	1.88	2.34
	6	pCDNA3BP2Streptag	1815.9	1.87	2.33

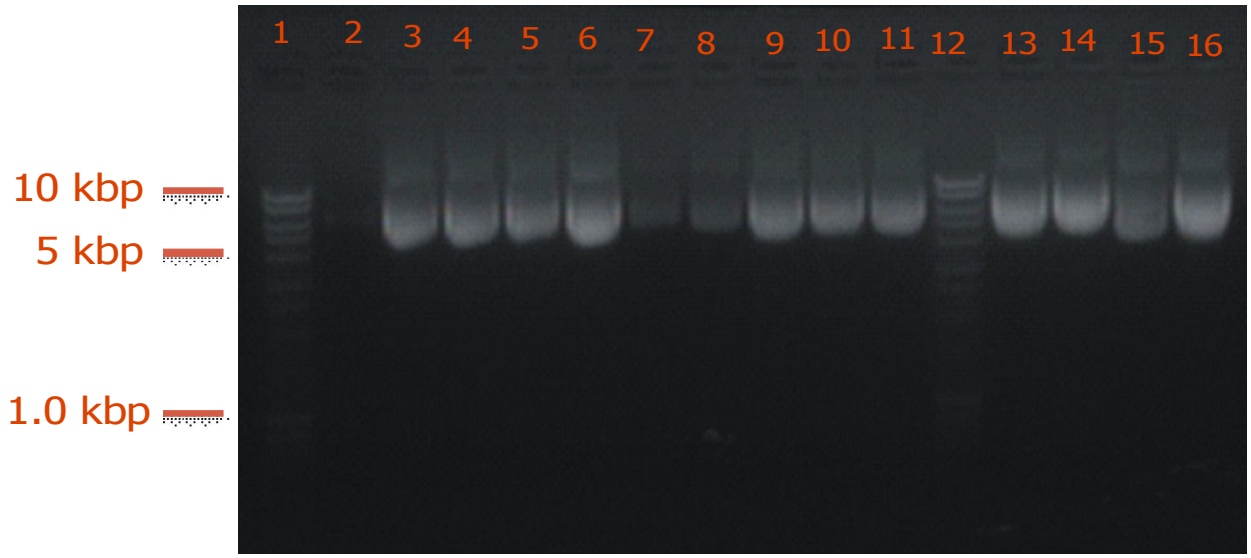


Plate 4.5. Electrophoregram of minipreps and midipreps of pcDNA3AmpStrepTagBP1 and pcDNA3AmpStrepTagBP2

KEY:
Lanes 1 and 12 (DNA-Ladder);
2 to 6 (minipreps from positive colony with pcDNA3AmpStrepTagBP1)
7 to 11(minipreps from positive colony with pcDNA3AmpStrepTagBP2)
13 and 14 (midipreps from Colonies 5 and 6 with pcDNA3AmpStrepTagBP1 respectively)
15 and 16 (midipreps from Colonies 5 and 6 with pcDNA3AmpStrepTagBP2 respectively).
[Electrophoresis was carried out on 1 % Agarose gel].

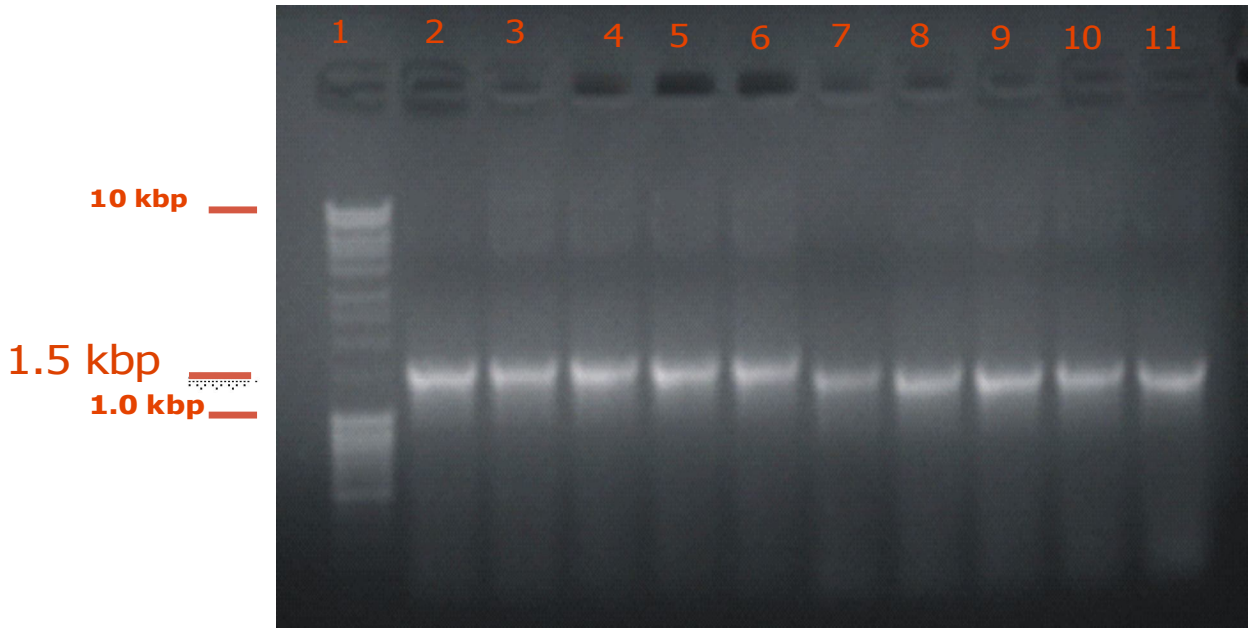


Plate 4.6. Screening of pcDNA3AmpStrepTagBP1 and pcDNA3AmpStrepTagBP2 midipreps for the presence of Bergheipain 1 and Bergheipain 2 genes having Streptag sequence downstream of the 3' ends of the genes.

KEY:

Lane 1(DNA Ladder)

2-6 (pcDNA3AmpStrepTagBP1 construct used as template for PCR)

7-11(pcDNA3AmpStrepTagBP2 constructs used as template for PCR). [Amplifications were done using Taq polymerase with gene specific internal primers (OSK2027 for Bergheipain1 and OSK2031 for Bergheipain 2) as the sense primer and Streptag-specific primer (OSK997)) as reverse primer. Electrophoresis was carried out on 1 % agarose gel.]

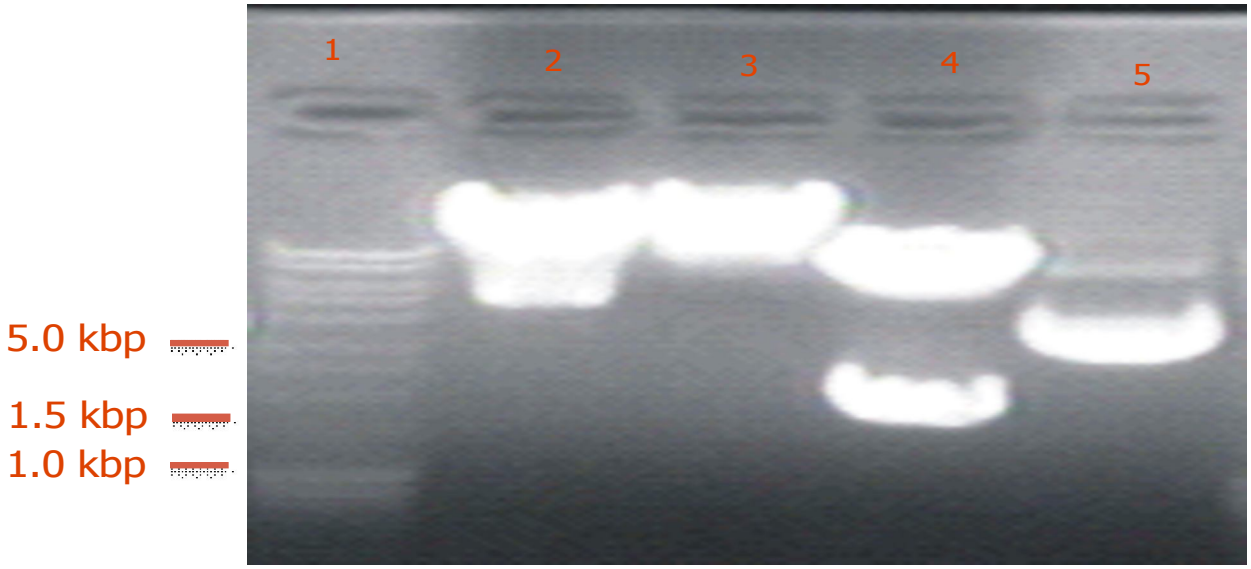


Plate 4.7. Analysis of Restriction Digest of pcDNA3AmpStreptagB1

KEY:
Lane 1 (DNA Ladder)

2 (Single digestion with BamH1)

3 (Single digestion with EcoR1)

4 (Double digestion with BamH1 and EcoR1)

5 (Intact pcDNA3AmpStreptagB1 vector without digestion)

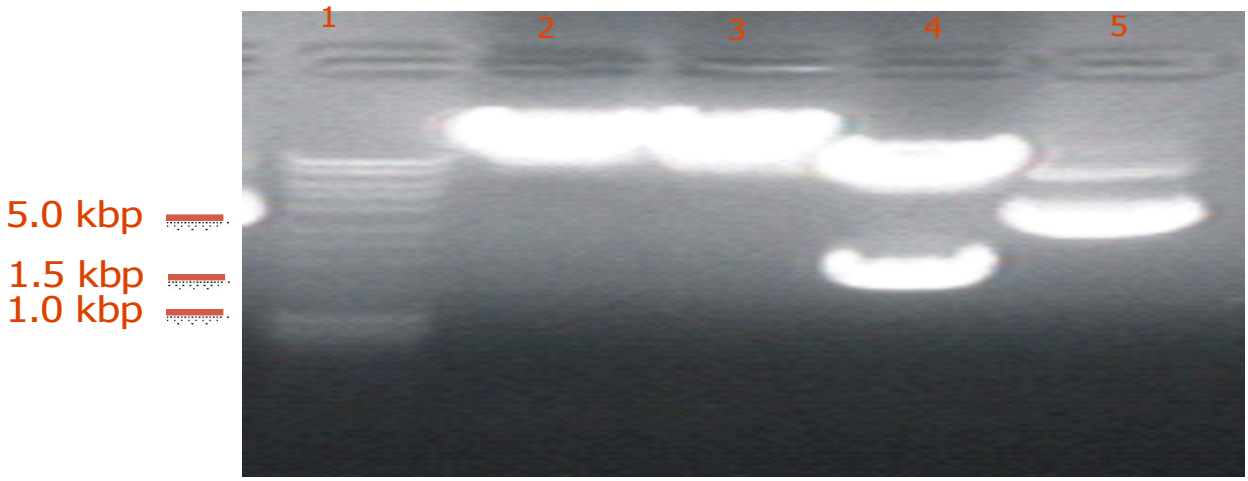


Plate 4.8. Analysis of Restriction Digest of pcDNA3AmpStreptagB2

KEY:
Lane 1 (DNA Ladder)

2 (Single digestion with BamH1)

3 (Single digestion with EcoR1)

4 (Double digestion with BamH1 and EcoR1)

5 (Intact pcDNA3AmpStreptagB2 vector without digestion)

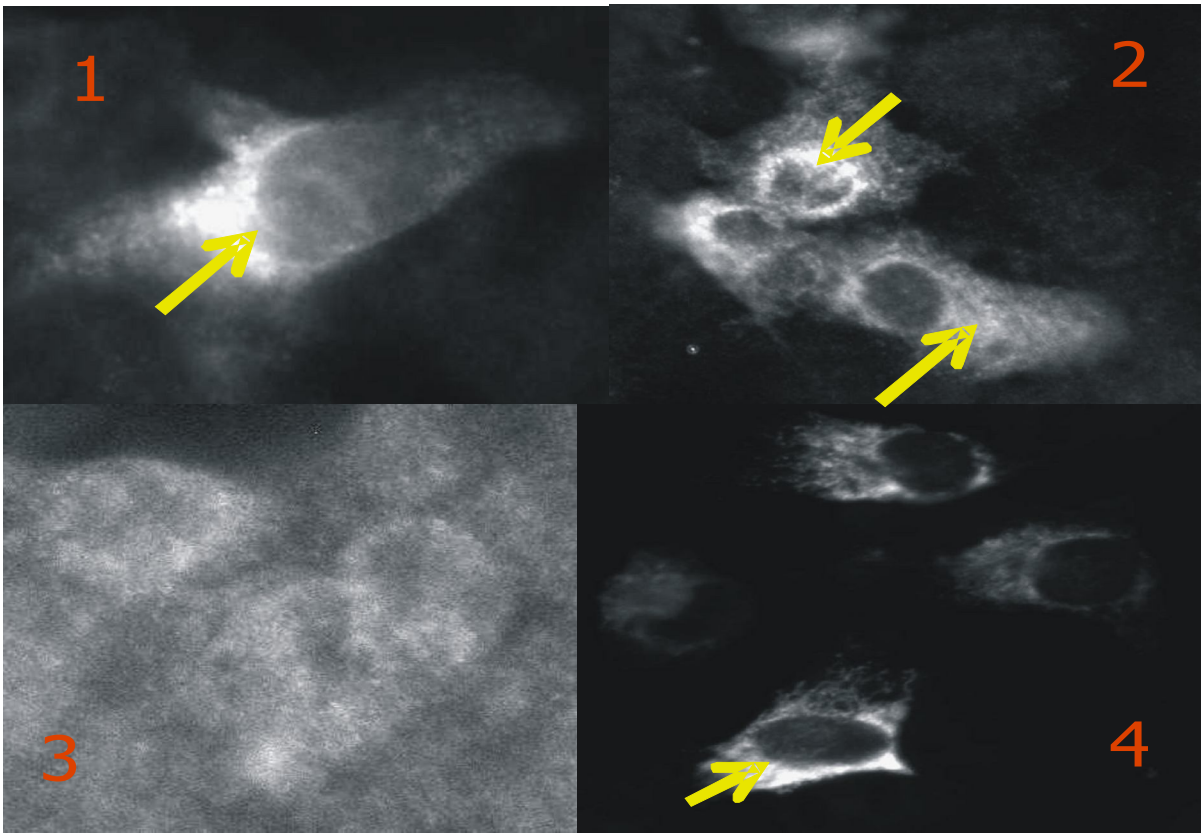


Plate 4.9. Outcome of *in vitro* expression of Bergheipain genes using immunoflouresence analysis.

KEY:

Plate 1 (COS7 cells were transfected with pCDNA3BP1Streptag)

Plate 2 (COS7 cells were transfected with pCDNA3BP2Streptag)

Plate 3 (Negative control; COS7 cells were transfected with PBS in place of the recombinant vector)

Plate 4 (Positive control; COS7 cells were transfected with Transialidase gene (TS1pDEF))

[Yellow arrows indicate regions of fluorescence].

4.5 Sequence Analysis and Bioinformatic studies of Bergheipain1 and Bergheipain2

Following nested PCR reactions with genomic DNA extracted from *P. berghei* isolate and subsequent ligation into pcDNA3Streptag vector, sets of sequencing primers were designed and used to sequence full genes of bergheipain1 and bergheipain2 in both directions. The nucleotide and deduced amino acid sequences of both genes are given in Figures 4.5 and 4.6 respectively. Nucleotide and decduced amino acid sequences extending beyond the 3' end of the genes into the plasmid show that both genes were in frame with the streptag sequence (Figure 4.7 and Figure 4.8) in place.

The sequenced Bergepain-1 (BP1) and Berghepain-2 (BP2) genes contain 1560 bp and 1407 bp reading frames encoding 519 and 468 amino acids with molecular masses and isoelectric points of 60.31138 kDa and 54.45853 kDa, and 7.52 and 6.01, respectively (Table 4.3). Sequence analyses and alignments showed that the deduced proteins belong to peptidase_C1 superfamily (Figure 4.9) and lack signal peptide (Figure 4.10 and Figure 4.11). Multiple sequence allignment using Clustal Omega 1.1.0 soft ware available at the website of European Bioinformatics Institute (http://www.ebi.ac.uk) confirmed that the sequenced genes are cysteine protease belong to the papain family (Figure 4.12); apart from exhibiting strong identity (up to 78%) with their corresponding cysteine protease orthologs from other malaria species (Table 4.4), they were found to possess conservation of the Cys, His and Asn residues that constitute the catalytic triad (Figure 4.12). Figure 4.13 presents the phylogenic relationship among these plasmodium cysteine proeases (BP1 and BP2 sequenced inclusive).

```
  1 atgataaacgatataagacgaataaacattaccacaagcagtatc
    M  I  N  D  I  R  R  I  N  I  T  T  S  S  I
 46 gaaagtttgaatgaaaatagcaaatatttaaaaagaaatcacaag
    E  S  L  N  E  N  S  K  Y  L  K  R  N  H  K
 91 aggactataaaaatatgtgcatatgcaattacaacatttgcatta
    R  T  I  K  I  C  A  Y  A  I  T  T  F  A  L
136 ttttttattgttgtagtatattttaaaaatcaaacaaatgtaaat
    F  F  I  V  V  V  Y  F  K  N  Q  T  N  V  N
181 gatgcaaatagaaatactttagcagcaatcgatgaaacaagctta
    D  A  N  R  N  T  L  A  A  I  D  E  T  S  L
226 atgaataaagaaatagcatatttaagagaaattttgaaaaaatat
    M  N  K  E  I  A  Y  L  R  E  I  L  K  K  Y
271 aaaacaaaaacaaatgaaaataatgaatatgcatacgaaaaaaac
    K  T  K  T  N  E  N  N  E  Y  A  Y  E  K  N
316 gacgatattaacggagatggcgaagacgagcacgaattgctatta
    D  D  I  N  G  D  G  E  D  E  H  E  L  L  L
361 atgttacataaatttttaaaaaataaaggtaatcctaataaaata
    M  L  H  K  F  L  K  N  K  G  N  P  N  K  I
406 gataggtttgatattaacaataatgactcaaataaaaatcgtggt
    D  R  F  D  I  N  N  N  D  S  N  K  N  R  G
451 aatgaaaatattgatcaaataaacatacttagtcaaaaacttgaa
    N  E  N  I  D  Q  I  N  I  L  S  Q  K  L  E
496 agcatgcatgataacataaaatatgcatcaaaatttttcaaatat
    S  M  H  D  N  I  K  Y  A  S  K  F  F  K  Y
541 atgaaagaatacaataaaaaatacaaaaatatagatgaacaatta
    M  K  E  Y  N  K  K  Y  K  N  I  D  E  Q  L
586 gtaagatttgaaaactttaaaacgaattatatgaaagttaaaaaa
    V  R  F  E  N  F  K  T  N  Y  M  K  V  K  K
631 cacaatgaaatggtaggtaaaaatggtattacatatgtacaaaaa
    H  N  E  M  V  G  K  N  G  I  T  Y  V  Q  K
676 gtaaaccaatttagtgatttttctaaggaagaacttgatagttat
    V  N  Q  F  S  D  F  S  K  E  E  L  D  S  Y
721 tttaaaaaattattacctattccacacaatttgaaaactaaacat
    F  K  K  L  L  P  I  P  H  N  L  K  T  K  H
766 gtagtaccactaaaaacacatttagacgataataaaattaaacca
    V  V  P  L  K  T  H  L  D  D  N  K  I  K  P
811 aaagaaggtgtattagactatccagaacagcgggattatagagaa
    K  E  G  V  L  D  Y  P  E  Q  R  D  Y  R  E
```

Figure 4.5 Nucleotide sequence and the deduced amino acid sequence of Bergephain 1 after cloning into pcDNA3AmpStretag Vector.

```
 856 tggaatatattacttcctccaaaggatcaaggtatgtgtgggtct
     W  N  I  L  L  P  P  K  D  Q  G  M  C  G  S
 901 tgctgggcatttgctagtgtaggaaattatgaagctctttttgca
     C  W  A  F  A  S  V  G  N  Y  E  A  L  F  A
 946 aaaaaatattctatattacctataagttttagtgaacaacaagtt
     K  K  Y  S  I  L  P  I  S  F  S  E  Q  Q  V
 991 gttgattgttctagtgacaattttggatgtgatggcggtcatcca
     V  D  C  S  S  D  N  F  G  C  D  G  G  H  P
1036 tttttatcatttttatatttcttaataatggggtatgctttggc
     F  L  S  F  L  Y  F  L  N  N  G  V  C  F  G
1081 gataattatgaatacaaagctcatgatgatttttctgcttaagt
     D  N  Y  E  Y  K  A  H  D  D  F  F  C  L  S
1126 tatagatgtgcgtatagaagtaaattaaaaaaaattggcaacgca
     Y  R  C  A  Y  R  S  K  L  K  K  I  G  N  A
1171 tatccatatgaattaattatgtccttaaatgaagtagggccaata
     Y  P  Y  E  L  I  M  S  L  N  E  V  G  P  I
1216 actgttaatgttggtgtatccgacgaatttgtattatattcgggt
     T  V  N  V  G  V  S  D  E  F  V  L  Y  S  G
1261 ggtatatttgatggaacatgtgcttcggagttaaatcattctgtt
     G  I  F  D  G  T  C  A  S  E  L  N  H  S  V
1306 ttattagtgggatatggtaaagttaaaaggagcttagtatttgag
     L  L  V  G  Y  G  K  V  K  R  S  L  V  F  E
1351 gatagtcatactaatgtagatagcaatttgataaaaaactataag
     D  S  H  T  N  V  D  S  N  L  I  K  N  Y  K
1396 gaaaatatcaaagacagcgatgatgactatttgtattactggatc
     E  N  I  K  D  S  D  D  D  Y  L  Y  Y  W  I
1441 attagaaattcatggagttcaacttggggagaaggtggctatata
     I  R  N  S  W  S  S  T  W  G  E  G  G  Y  I
1486 aggattaaaagaaacaaattaggagacgatgtttttgtggaatt
     R  I  K  R  N  K  L  G  D  D  V  F  C  G  I
1531 ggtattgacgttttctttccaatttataa 1560
     G  I  D  V  F  F  P  I  L  *
```

Figure 4.5 Nucleotide sequence and the deduced amino acid sequence of Bergephain 1 after cloning into pcDNA3AmpStretag Vector (continuation).

```
  1 atgaattaccattctagccatcatattagacccgaggaggaaata
    M  N  Y  H  S  S  H  H  I  R  P  E  E  E  I
 46 tttgttgataagggtatacaaaatgtacagttacgaagaaaaaat
    F  V  D  K  G  I  Q  N  V  Q  L  R  R  K  N
 91 aaaatgcttatagttaccctggctatagttttaggtatgtttggt
    K  M  L  I  V  T  L  A  I  V  L  G  M  F  G
136 tttaccgtcatatattttaatagaacgaataaatcatcatttaat
    F  T  V  I  Y  F  N  R  T  N  K  S  S  F  N
181 aatggaaatgttgaaaattattcgaatgatgactatttaataaat
    N  G  N  V  E  N  Y  S  N  D  D  Y  L  I  N
226 tacttattaaaaagcaaagcagtaaagaaatttatggatctaaa
    Y  L  L  K  S  K  A  V  K  K  F  M  G  S  K
271 attgaagaacttatagtagaaagtgaacaaaatgaaaagaatagc
    I  E  E  L  I  V  E  S  E  Q  N  E  K  N  S
316 attatcgttaaagataataaaaataatgactataacgaaaaatct
    I  I  V  K  D  N  K  N  N  D  Y  N  E  K  S
361 gtattatttaataaaaataacgacagcaaatcattcacaacaaat
    V  L  F  N  K  N  N  D  S  K  S  F  T  T  N
406 ttacatgatatgcaatctattatgaacaatttagaatctgtgaac
    L  H  D  M  Q  S  I  M  N  N  L  E  S  V  N
451 atttttacaatttcatgaaagaatataacaaacaatataattca
    I  F  Y  N  F  M  K  E  Y  N  K  Q  Y  N  S
496 gctgaagaaatacaagagagattttatatcttttctgaaaattta
    A  E  E  I  Q  E  R  F  Y  I  F  S  E  N  L
541 aaaaaaattgaaaagcataataaagaaaatcatttgtatacaaaa
    K  K  I  E  K  H  N  K  E  N  H  L  Y  T  K
586 ggtattaatgcatttagtgatatgcgtcatgaagaatttaaaatg
    G  I  N  A  F  S  D  M  R  H  E  E  F  K  M
631 aaatatttaaataataaactaaaagaaaaccacagcattgatctc
    K  Y  L  N  N  K  L  K  E  N  H  S  I  D  L
676 cgacatttaattccttatactactgcaattagtaagtacaagtct
    R  H  L  I  P  Y  T  T  A  I  S  K  Y  K  S
721 ccaaccgataaagttaactatacaagttttgattggagagattat
    P  T  D  K  V  N  Y  T  S  F  D  W  R  D  Y
766 aatgttattataggcgttaaagatcaacaaaagtgtgcttcatgc
    N  V  I  I  G  V  K  D  Q  Q  K  C  A  S  C
811 tgggcatttgctaccgctggtgttgtcgcagcccaatatgctatt
    W  A  F  A  T  A  G  V  V  A  A  Q  Y  A  I
856 agaaaaaatcaaaaggtttctttaagtgaacaacaattagttgat
    R  K  N  Q  K  V  S  L  S  E  Q  Q  L  V  D
901 tgtgcacaaaataattttggatgtgaaggaggtatacttccatat
    C  A  Q  N  N  F  G  C  E  G  G  I  L  P  Y
```

Figure 4.6. Nucleotide sequence and the deduced amino acid sequence of Bergephain 2 after cloning into pcDNA3AmpStretag Vector.

```
 946 gcttttgaagatcttatagacatggatggtttatgtgaagataaa
     A  F  E  D  L  I  D  M  D  G  L  C  E  D  K
 991 tattatccatatgtaagtaatgttccagaattatgcgaaattaac
     Y  Y  P  Y  V  S  N  V  P  E  L  C  E  I  N
1036 aagtgtaccgaaaaatactcaatttcaaaatttgcattagtacca
     K  C  T  E  K  Y  S  I  S  K  F  A  L  V  P
1081 ttcaataattataaagaagctattcaatatttaggtccaatcaca
     F  N  N  Y  K  E  A  I  Q  Y  L  G  P  I  T
1126 atagctgtaggtgtagatgatgattttgaatcttacaatggtggt
     I  A  V  G  V  D  D  D  F  E  S  Y  N  G  G
1171 atattcgatggagaatgtacagattttgcaaatcatgcagttatg
     I  F  D  G  E  C  T  D  F  A  N  H  A  V  M
1216 cttattggatatggtgttgaagaggtatatgataagcgtcttaaa
     L  I  G  Y  G  V  E  E  V  Y  D  K  R  L  K
1261 aaaaatgttaaagaatattattatataattagaaactcttggggt
     K  N  V  K  E  Y  Y  Y  I  I  R  N  S  W  G
1306 gaagactggggagaacgtggttacataagacttaagactaacgaa
     E  D  W  G  E  R  G  Y  I  R  L  K  T  N  E
1351 tcaggaacactcagaaattgtgtgttagtacaaggttatgctcct
     S  G  T  L  R  N  C  V  L  V  Q  G  Y  A  P
1396 ataattgaataa 1407
     I  I  E  *
```

Figure 4.6. Nucleotide sequence and the deduced amino acid sequence of Bergephain 2 after cloning into pcDNA3AmpStretag Vector (continuation).

```
  1 atgataaacgatataagacgaataaacattaccacaagcagtatc
    M  I  N  D  I  R  R  I  N  I  T  T  S  S  I
 46 gaaagtttgaatgaaaatagcaaatatttaaaaagaaatcacaag
    E  S  L  N  E  N  S  K  Y  L  K  R  N  H  K
 91 aggactataaaaatatgtgcatatgcaattacaacatttgcatta
    R  T  I  K  I  C  A  Y  A  I  T  T  F  A  L
136 ttttttattgttgtagtatattttaaaaatcaaacaaatgtaaat
    F  F  I  V  V  V  Y  F  K  N  Q  T  N  V  N
181 gatgcaaatagaaatactttagcagcaatcgatgaaacaagctta
    D  A  N  R  N  T  L  A  A  I  D  E  T  S  L
226 atgaataaagaaatagcatatttaagagaaattttgaaaaaatat
    M  N  K  E  I  A  Y  L  R  E  I  L  K  K  Y
271 aaaacaaaaacaaatgaaaataatgaatatgcatacgaaaaaaac
    K  T  K  T  N  E  N  N  E  Y  A  Y  E  K  N
316 gacgatattaacggagatggcgaagacgagcacgaattgctatta
    D  D  I  N  G  D  G  E  D  E  H  E  L  L  L
361 atgttacataaatttttaaaaaataaaggtaatcctaataaaata
    M  L  H  K  F  L  K  N  K  G  N  P  N  K  I
406 gataggtttgatattaacaataatgactcaaataaaaatcgtggt
    D  R  F  D  I  N  N  N  D  S  N  K  N  R  G
451 aatgaaaatattgatcaaataaacatacttagtcaaaaacttgaa
    N  E  N  I  D  Q  I  N  I  L  S  Q  K  L  E
496 agcatgcatgataacataaaatatgcatcaaaatttttcaaatat
    S  M  H  D  N  I  K  Y  A  S  K  F  F  K  Y
541 atgaaagaatacaataaaaaatacaaaaatatagatgaacaatta
    M  K  E  Y  N  K  K  Y  K  N  I  D  E  Q  L
586 gtaagatttgaaaactttaaaacgaattatatgaaagttaaaaaa
    V  R  F  E  N  F  K  T  N  Y  M  K  V  K  K
631 cacaatgaaatggtaggtaaaaatggtattacatatgtacaaaaa
    H  N  E  M  V  G  K  N  G  I  T  Y  V  Q  K
676 gtaaaccaatttagtgatttttctaaggaagaacttgatagttat
    V  N  Q  F  S  D  F  S  K  E  E  L  D  S  Y
721 tttaaaaaattattacctattccacacaatttgaaaactaaacat
    F  K  K  L  L  P  I  P  H  N  L  K  T  K  H
766 gtagtaccactaaaaacacatttagacgataataaaattaaacca
    V  V  P  L  K  T  H  L  D  D  N  K  I  K  P
811 aaagaaggtgtattagactatccagaacagcgggattatagagaa
    K  E  G  V  L  D  Y  P  E  Q  R  D  Y  R  E
856 tggaatatattacttcctccaaaggatcaaggtatgtgtgggtct
    W  N  I  L  L  P  P  K  D  Q  G  M  C  G  S
901 tgctgggcatttgctagtgtaggaaattatgaagctctttttgca
    C  W  A  F  A  S  V  G  N  Y  E  A  L  F  A
```

Figure 4.7. Nucleotide sequence and the deduced amino acid sequence of Bergephain 1 gene after cloning into pcDNA3AmpStretag Vector showing the position of Streptag (Underlined)

```
 946 aaaaaatattctatattacctataagttttagtgaacaacaagtt
     K  K  Y  S  I  L  P  I  S  F  S  E  Q  Q  V
 991 gttgattgttctagtgacaattttggatgtgatggcggtcatcca
     V  D  C  S  S  D  N  F  G  C  D  G  G  H  P
1036 tttttatcatttttatattttcttaataatggggtatgctttggc
     F  L  S  F  L  Y  F  L  N  N  G  V  C  F  G
1081 gataattatgaatacaaagctcatgatgatttttttctgcttaagt
     D  N  Y  E  Y  K  A  H  D  D  F  F  C  L  S
1126 tatagatgtgcgtatagaagtaaattaaaaaaaattggcaacgca
     Y  R  C  A  Y  R  S  K  L  K  K  I  G  N  A
1171 tatccatatgaattaattatgtccttaaatgaagtagggccaata
     Y  P  Y  E  L  I  M  S  L  N  E  V  G  P  I
1216 actgttaatgttggtgtatccgacgaatttgtattatattcgggt
     T  V  N  V  G  V  S  D  E  F  V  L  Y  S  G
1261 ggtatatttgatggaacatgtgcttcggagttaaatcattctgtt
     G  I  F  D  G  T  C  A  S  E  L  N  H  S  V
1306 ttattagtgggatatggtaaagttaaaaggagcttagtatttgag
     L  L  V  G  Y  G  K  V  K  R  S  L  V  F  E
1351 gatagtcatactaatgtagatagcaatttgataaaaaactataag
     D  S  H  T  N  V  D  S  N  L  I  K  N  Y  K
1396 gaaaatatcaaagacagcgatgatgactatttgtattactggatc
     E  N  I  K  D  S  D  D  D  Y  L  Y  Y  W  I
1441 attagaaattcatggagttcaacttggggagaaggtggctatata
     I  R  N  S  W  S  S  T  W  G  E  G  G  Y  I
1486 aggattaaaagaaacaaattaggagacgatgttttttgtggaatt
     R  I  K  R  N  K  L  G  D  D  V  F  C  G  I
1531 ggtattgacgttttctttccaattttagaattcgatatcggtacc
     G  I  D  V  F  F  P  I  L  E  F  D  I  G  T
1576 tggagccacccgcagttcgaaaaataa 1602
     W  S  H  P  Q  F  E  K  *
```

Figure 4.7. Nucleotide sequence and the deduced amino acid sequence of Bergephain 1 gene after cloning into pcDNA3AmpStretag Vector showing the position of Streptag [Underlined] (continuation).

```
  1 atgaattaccattctagccatcatattagacccgaggaggaaata
    M  N  Y  H  S  S  H  H  I  R  P  E  E  E  I
 46 tttgttgataagggtatacaaaatgtacagttacgaagaaaaaat
    F  V  D  K  G  I  Q  N  V  Q  L  R  R  K  N
 91 aaaatgcttatagttaccctggctatagttttaggtatgtttggt
    K  M  L  I  V  T  L  A  I  V  L  G  M  F  G
136 tttaccgtcatatattttaatagaacgaataaatcatcatttaat
    F  T  V  I  Y  F  N  R  T  N  K  S  S  F  N
181 aatggaaatgttgaaaattattcgaatgatgactatttaataaat
    N  G  N  V  E  N  Y  S  N  D  D  Y  L  I  N
226 tacttattaaaaagcaaagcagtaaagaaatttatgggatctaaa
    Y  L  L  K  S  K  A  V  K  K  F  M  G  S  K
271 attgaagaacttatagtagaaagtgaacaaaatgaaaagaatagc
    I  E  E  L  I  V  E  S  E  Q  N  E  K  N  S
316 attatcgttaaagataataaaaataatgactataacgaaaaatct
    I  I  V  K  D  N  K  N  N  D  Y  N  E  K  S
361 gtattatttaataaaaataacgacagcaaatcattcacaacaaat
    V  L  F  N  K  N  N  D  S  K  S  F  T  T  N
406 ttacatgatatgcaatctattatgaacaatttagaatctgtgaac
    L  H  D  M  Q  S  I  M  N  N  L  E  S  V  N
451 atttttacaatttcatgaaagaatataacaaacaatataattca
    I  F  Y  N  F  M  K  E  Y  N  K  Q  Y  N  S
496 gctgaagaaatacaagagagattttatatcttttctgaaaattta
    A  E  E  I  Q  E  R  F  Y  I  F  S  E  N  L
541 aaaaaaattgaaaagcataataaagaaaatcatttgtatacaaaa
    K  K  I  E  K  H  N  K  E  N  H  L  Y  T  K
586 ggtattaatgcatttagtgatatgcgtcatgaagaatttaaaatg
    G  I  N  A  F  S  D  M  R  H  E  E  F  K  M
631 aaatatttaaataataaactaaaagaaaaccacagcattgatctc
    K  Y  L  N  N  K  L  K  E  N  H  S  I  D  L
676 cgacatttaattccttatactactgcaattagtaagtacaagtct
    R  H  L  I  P  Y  T  T  A  I  S  K  Y  K  S
721 ccaaccgataaagttaactatacaagttttgattggagagattat
    P  T  D  K  V  N  Y  T  S  F  D  W  R  D  Y
766 aatgttattataggcgttaaagatcaacaaaagtgtgcttcatgc
    N  V  I  I  G  V  K  D  Q  Q  K  C  A  S  C
811 tgggcatttgctaccgctggtgttgtcgcagcccaatatgctatt
    W  A  F  A  T  A  G  V  V  A  A  Q  Y  A  I
856 agaaaaaatcaaaaggtttctttaagtgaacaacaattagttgat
    R  K  N  Q  K  V  S  L  S  E  Q  Q  L  V  D
901 tgtgcacaaaataattttggatgtgaaggaggtatacttccatat
    C  A  Q  N  N  F  G  C  E  G  G  I  L  P  Y
946 gcttttgaagatcttatagacatggatggtttatgtgaagataaa
    A  F  E  D  L  I  D  M  D  G  L  C  E  D  K
```

Figure 4.8. Nucleotide sequence and the deduced amino acid sequence of Bergephain 2 gene after cloning into pcDNA3AmpStretag Vector showing the position of Streptag (Underlined)

```
 991 tattatccatatgtaagtaatgttccagaattatgcgaaattaac
     Y  Y  P  Y  V  S  N  V  P  E  L  C  E  I  N
1036 aagtgtaccgaaaaatactcaatttcaaaatttgcattagtacca
     K  C  T  E  K  Y  S  I  S  K  F  A  L  V  P
1081 ttcaataattataaagaagctattcaatatttaggtccaatcaca
     F  N  N  Y  K  E  A  I  Q  Y  L  G  P  I  T
1126 atagctgtaggtgtagatgatgattttgaatcttacaatggtggt
     I  A  V  G  V  D  D  D  F  E  S  Y  N  G  G
1171 atattcgatggagaatgtacagattttgcaaatcatgcagttatg
     I  F  D  G  E  C  T  D  F  A  N  H  A  V  M
1216 cttattggatatggtgttgaagaggtatatgataagcgtcttaaa
     L  I  G  Y  G  V  E  E  V  Y  D  K  R  L  K
1261 aaaaatgttaaagaatattattatataattagaaactcttggggt
     K  N  V  K  E  Y  Y  Y  I  I  R  N  S  W  G
1306 gaagactggggagaacgtggttacataagacttaagactaacgaa
     E  D  W  G  E  R  G  Y  I  R  L  K  T  N  E
1351 tcaggaacactcagaaattgtgtgttagtacaaggttatgctcct
     S  G  T  L  R  N  C  V  L  V  Q  G  Y  A  P
1396 ataattgaagaattcgatatcggtacctggagccacccgcagttc
     I  I  E  E  F  D  I  G  T  W  S  H  P  Q  F
1441 gaaaaataa 1449
     E  K  *
```

Figure 4.8. Nucleotide sequence and the deduced amino acid sequence of Bergephain 2 gene after cloning into pcDNA3AmpStretag Vector showing the position of Streptag [Underlined] (continuation).

Table 4.3. Some deductions made from Bergheipain1 and Bergheipain2 Open Reading Frames

Cysteine protease	Deduced Parameters			
	Number of basepairs	Number of amino acids	pI	Molecular weight (kDa)
Bergheipain1	1560	519	7.52	60.31138
Bergheipain2	1407	468	6.01	54.45853

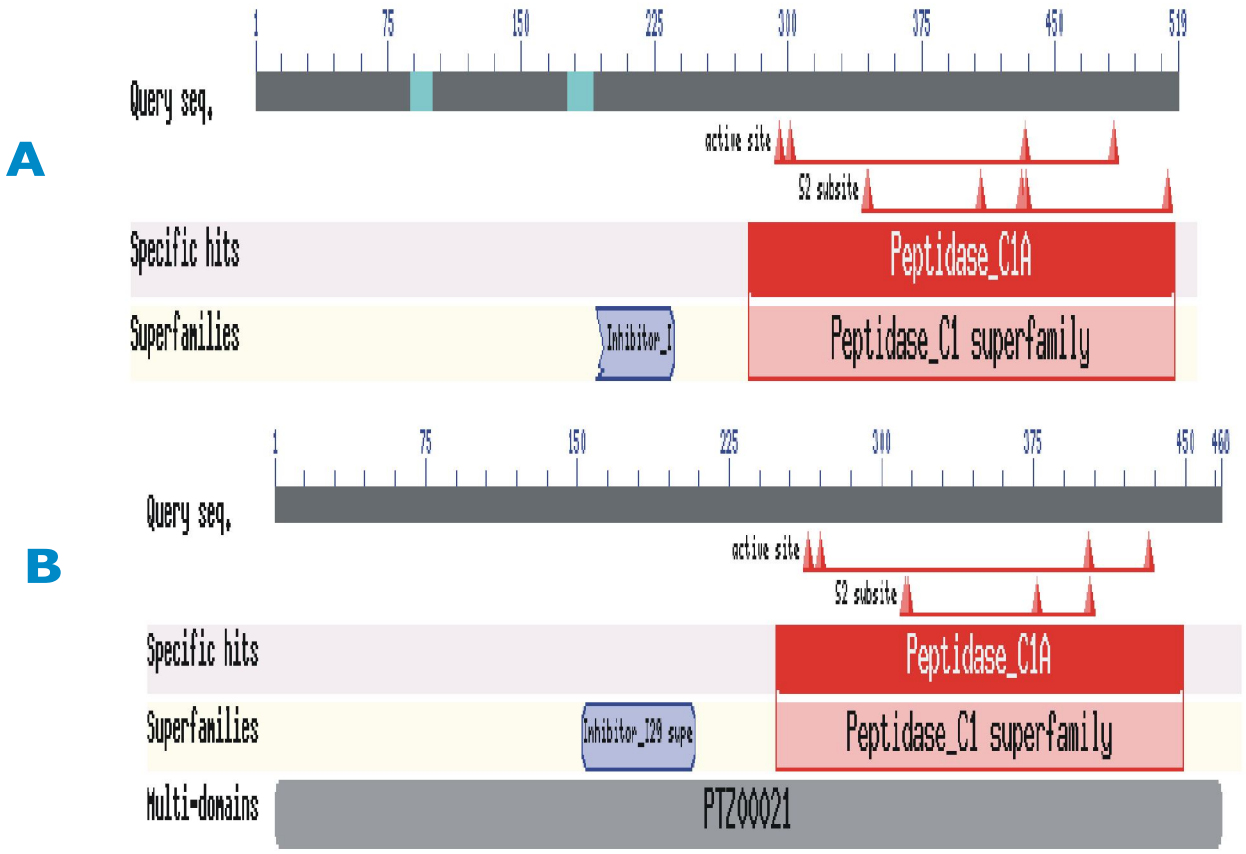
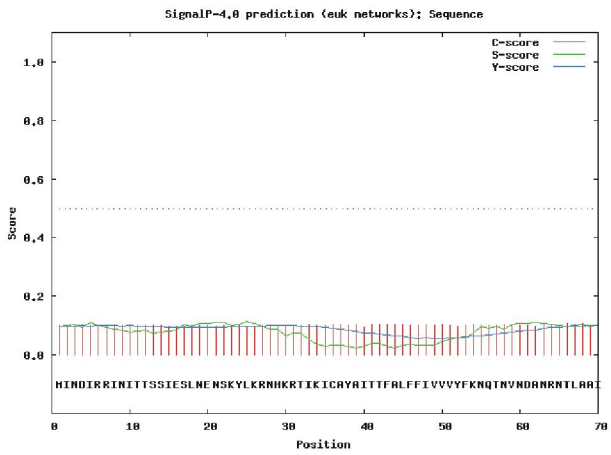


Figure 4.9. Blast outcome showing enzyme family of Bergheipain 1 and Bergheipain 2.

KEY:

A (Blast of Bergheipain 1 nucleotide sequence against NCBI data bank)

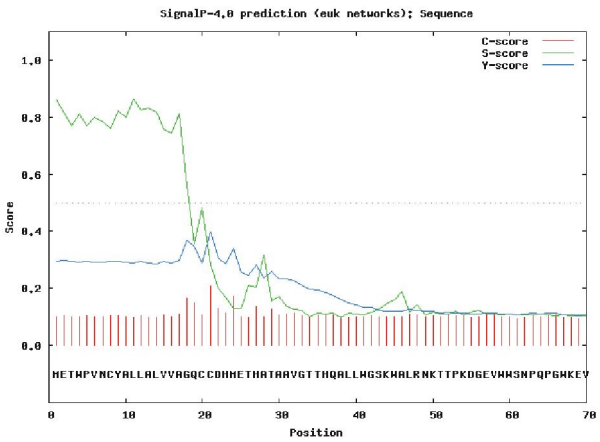
B (Blast of Bergheipain 2 nucleotide sequence against NCBI data bank)



Bergheipain1 from P. berghei

Measure	Position	Value	Cutoff	signal peptide?
max. C	68	0.106		
Max.Y	68	0.103		
max. S	25	0.113		
mean S	1-67	0.077		
D	1-67	0.092	0.500	NO

Name=Sequence SP='NO' D=0.092 D-cutoff=0.500 Networks=SignalP-TM



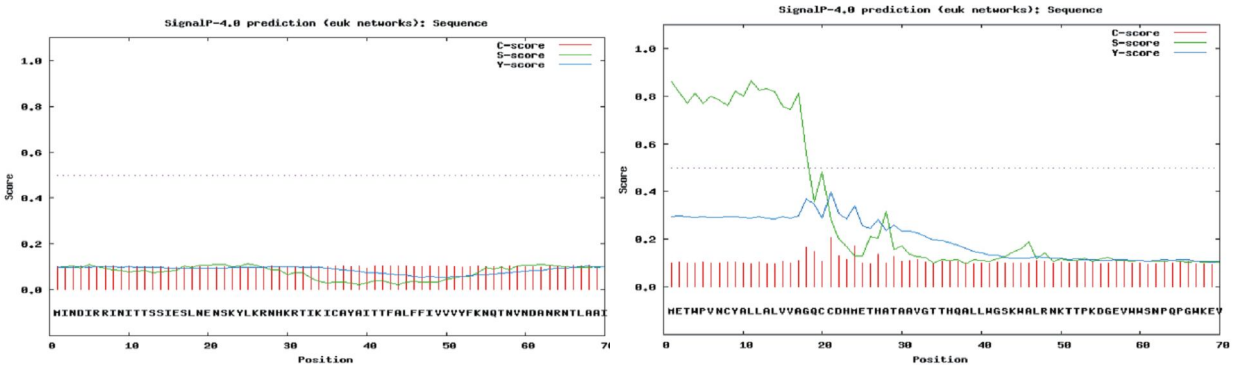
Transialidase from T. congolense

Measure	Position	Value	Cutoff	signal peptide?
max. C	21	0.209		
max. Y	21	0.399		
max. S	11	0.865		
mean S	1-20	0.752	0.590	YES
D	1-20	0.450		

Name=Sequence SP='YES' Cleavage site between pos 20 and 21: GQC-CD D=0.590 D-cutoff=0.450 Networks=SignalP-noTM

Figure 4.10 Outcome of signal peptide prediction of the amino acid sequence of Bergheipain 1.

[Transialidase sequence (right) was used as standard reference of protein having the signal peptide].



Bergheipain 2 from P. berghei

Measure	Position	Value	Cutoff	signal peptide?
max. C	68	0.106		
Max.Y	68	0.103		
max. S	25	0.113		
mean S	1-67	0.077		

D 1-67 0.092 0.500 NO Name=Sequence
SP='NO' D=0.092 Dcutoff=0.500 Networks=SignalP-TM

Transialidase from T. congolense

Measure	Position	Value	Cutoff	signal peptide?
max. C	21	0.209		
max. Y	21	0.399		
max. S	11	0.865		
mean S	1-20	0.752	D 1-20 0.590 0.450 YES	

Name=Sequence SP='YES' Cleavage site between pos. 20 and 21: GQG-CD D=0.590 D-cutoff=0.450 Networks=SignalP-noTM

Figure 4.11 Outcome of signal peptide prediction of the amino acid sequence of Bergheipain 2.

[Transialidase sequence (right) was used as standard reference of protein having the signal peptide].



Figure 4.12. Alignment and comparison of amino acid sequences of Bergephain1 and Bergephain2 seuences with those of other Plasmodium cysteine proteases

```
Falcipain-3             RT------------TGA-------------------------------------------
Vivapain-3              LP------------AQHG------------------------------------------
Vivapain-2              AK------------ASPS------------------------------------------


BP1Query-sequence       GED-EHELLLMLHKFLKNKGNPNKIDRFDINNNDSN--------------K-NRG----N
Bergheipain-1           GED-EHELLLMLHKFLKNKGNPNKIDRFDINNNDSN--------------K-NRG----N
chabaupain-1            SEDGEHQLLLMLHKLLKNNA--NKVNTFGINNE----------------------S----N
Falcipain-1             NEENRKELSNILHKLLEINKLILREEKDD--------KK-VY---LINDNYDEKG-ALEI
Vivapain-1              SEDNKEQLAKMLRELLKKKINERKKKREDPNGNNEEGKE-VINISVPSFNYKRVS-ANQD
BP2Query-sequence       ----------------------------NKNNDY---------------NEKSVLFNKN
Berghepain-2            ----------------------------NKNNDY---------------NEKSVLFNKN
Chabaupain-2            -------------------------ANDEHNKNY---------------NKKTPLFKKN
Falcipain-2a            -------------------------GNNNNNADFKGL---------SLFKEN-TPSNNF
Falcipain-3             ----------------------------EENNMNMNGIDDKDNKSV-SFVNKKNGNLKVN
Vivapain-3              -------------------------GTYKRFAKR-DQCTRNNCSVSPY-GKNAAQV-EV
Vivapain-2              -------------------------KEGPT-----GA---HSTSVATVSRQKQGNLK--


BP1Query-sequence       ENIDQINILSQKLESMHDNIKYASKFFKYMKEYNKKYKNIDEQLVRFENFKTNYMKVKKH
Bergheipain-1           ENIDQINILSQKLESMHDNIKYASKFFKYMKEYNKKYKNIDEQLVRFENFKTNYMKVKKH
chabaupain-1            TNTDNTYIFTQKLESMQDNIKYASKFFKYMKKYNKKYENMDEQLERFENFKISHMKVKKH
Falcipain-1             G---MNEEMKYKKEDPINNIKYASKFFKFMKEHNKVYKNIDEQMRKFEIFKINYISIKNH
Vivapain-1              DSDDEEEVSVAQIEGLFVNLKYASKFFNFMNKYKRSYKDINEQMEKYKNFKMNYLKIKKH
BP2Query-sequence       NDSKSFTTNLHDMQSIMNNLESVNIFYNFMKEYNKQYNSAEEIQERFYIFSENLKKIEKH
Berghepain-2            NDSKSFTTNLHDMQSIMNNLESVNIFYNFMKEYNKQYNSAEEIQERFYIFSENLKKIEKH
Chabaupain-2            NDGKKFSANLYDMKFIMSNLESVNIFYNFMKKFNKQYNSAEEMQERFYIFTENLKKVEKH
Falcipain-2a            IHNKDYFINFFDNKFLMNNAEHINQFYMFIKTNNKQYNSPNEMKERFQVFLQNAHKVNMH
Falcipain-3             NNNQVSYSNLFDTKFLMDNLETVNLFYIFLKENNKKYETSEEMQKRFIIFSENYRKIELH
Vivapain-3              VAQVNLVNPLVDTKFLMANLETVNSFYLFMKEHGKEYSTADEMQQRYLSFAENLAKIKAH
Vivapain-2              -VPKKIEINFADSRFLMINLEKVNAFYLFMKEHGKKYKTEEEMQQRYLAFTENLARINSH
                                  . .    * :  . *: :::   : *.  :*   ::  *  .   :: *
```

Figure 4.12. Alignment and comparison of amino acid sequences of Bergephain1 and Bergephain2 seuences with those of other Plasmodium cysteine proteases (continuation)



Figure 4.12. Alignment and comparison of amino acid sequences of Bergephain1 and Bergephain2 seuences with those of other Plasmodium cysteine proteases (continuation)



Figure 4.12. Alignment and comparison of amino acid sequences of Bergephain1 and Bergephain2 seuences with those of other Plasmodium cysteine proteases (continuation)



Figure 4.12. Alignment and comparison of amino acid sequences of Bergephain1 and Bergephain2 seuences with those of other Plasmodium cysteine proteases (continuation)

Table 4.4. Alignment of predicted Bergheipain amino sequences from PCR-purified gene fragments against common malarial cysteine proteases.

Query squences		Subject sequences									
		Berghepains		Falcipains				Vivapains		Chabaupains	
		1*	2	1	2A	2B	3	1	2	1	2
Bergheipain-1	Maximum Identity (%)	94	35	51	33	33	34	41	NABO	65	NABO
	Query coverage(%)	100	62	72	63	63	63	100	NABO	99	NABO
	E - value	0.0	4e-47	0.0	4e-42	1e-42	2e-45	1e-134	NABO	0.0	NABO
Brgheipain-2	Maximum Identity		99	32	45	44	43		43		78
	Query coverage(%)		100	77	100	100	100		98		99
	E - value		0.0	4e-53	1e-128	3e-128	4e-132		9e-129		0.0

*:Values were obtained from NCBI data bank and are expressed as percent
NABO: Not available on blast outcome

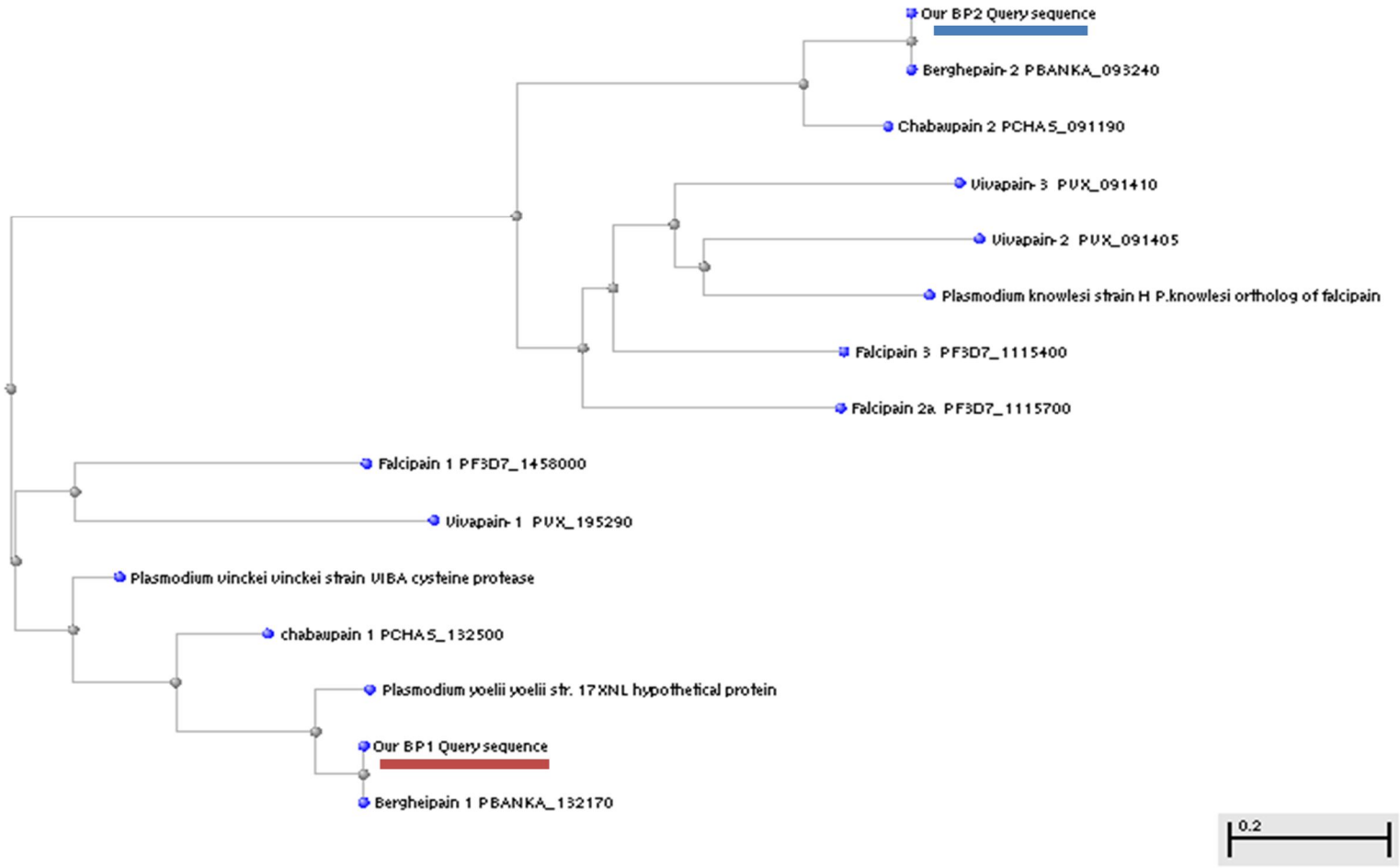


Figure 4.13. Dendogram showing combined phylogenic relationship between BP1 and BP2 genes with those of other related plasmodium cysteine protesases.

4.6 Subcloning into pVax1 vector and production of DNA vaccines

Two sets of DNA vaccine were required for the vaccination and so two different strategies were employed for the ultimate transfer of the genes into the DNA vaccine vector (pVax1). The first set of vaccine possesses no signal peptide but have the streptag sequence while the second set of vaccines has both the signal peptide as well as the streptag sequence. In order to produce the first set of vaccines, the midipreps having the gene inserts were cut with BamHI and XhoI and the inserts ligated into pVax1, already cut with the same BamHI/XhoI enzymes. The positive transformants will habour pVax1BP1Streptag or pVa1BP2Streptag recombinant DNA molecule which were identified via colony PCR. Plates 4.10 and 4.11 gave the electrophoregram of the colony PCR for such set of vaccines. Positive colonies were then picked from Kanamycin LB plate and grown in 200 mL of LB medium from where midipreps (the vaccines) were made. The Midipreps and their optical density properties are given in Table 4.5.

Introducing the transin signal peptide at the 5' end of the bergheipain genes necesitated the design and use of a different set of primers bearing EcoRV (blunt end cutter) and XhoI (sticky end cutter) restriction sites. OSK2034/OSK997 primers and OSK2035/OSK997 primers for BP1 and BP2 respectively were used for this cloning strategy. Amplicons generated with these primers were cut with EcoRV/XhoI enzyme system, gel purified and ligated into pcDNA3 vector which had been cut previously with the same enzymes and gel purified (see appendix XIV for the percentage yield of the gel purification). Plate 4.12 show that only 1 in 19 colonies had the required plasmid for Bergheipain1. No single positive colony was seen for Bergheipain2, indicating a very low transformation efficiency. The TransinBP1Streptag was then cut out of the pcDNATransinBP1Streptag by cuting with HindIII and XhoI restriction enzymes and transfered into pVax1 previously cut with the same enzymes. Plate 4.13 gave the outcome of the colony

PCR done when transformation was carried out with the ligation products. For this, the efficiency of transformation improved greatly. Screening the midipreps from some selective positive colonies on the kanamycin plate for the presence of Bergheipain1 gene gave a positive result (Plate 4.14). Ligation step for BP2 in required several adjustment of ligation conditions. When the ligation was done at 15 ^{0}C for 6 hrs and the ratio of the insert to the cut plasmid raised, the ligation was successful, however, with very low efficiency of transformation as shown in Plate 4.15. The corresponding colony PCR on kanamycin LB plate for pVax1 carrying the TransinBP2Streptag sequence was also successful (Plate 4.16).

All the positive colonies were finally picked from the replica plates and midipreps of the vaccines were made. Table 4.5 presents the data of the midipreps of all the four DNA vaccine constructs produced. The amount of the vaccine produced ranged from 1.6 mg for control plasmid (pVax1) to 3.4 mg for pVax1KanBP2Streptag.

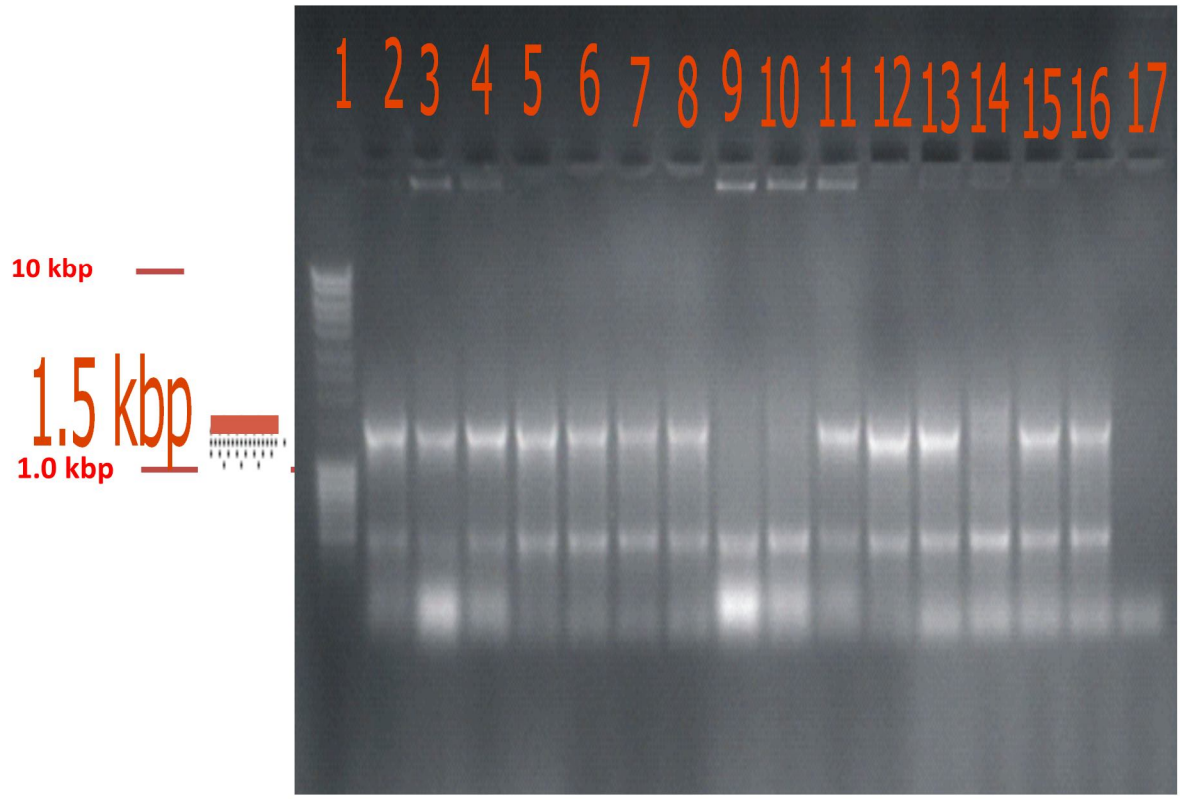


Plate 4.10. Result of the colony polymerase chain reaction after XL1 Blue *E.coli* cells were transformed with pVax1KanBP1Streptag recombinant plasmid and allowed to grow on Kanamycin LB plate.

KEY:

Lanes 1 (DNA ladder)
2-8 (from pcDNA3StreptagBP1-colony 5)
9-16 (from pcDNA3StreptagBP1-colony 6)
17(control well; amplification carried out with BP1 antisense primer (OSK2029) in replace of Strep-specific primer (OSK997).

.

[Amplification, except the controls was carried out with Internal sense primers and antiStrep primer having XhoI site.]

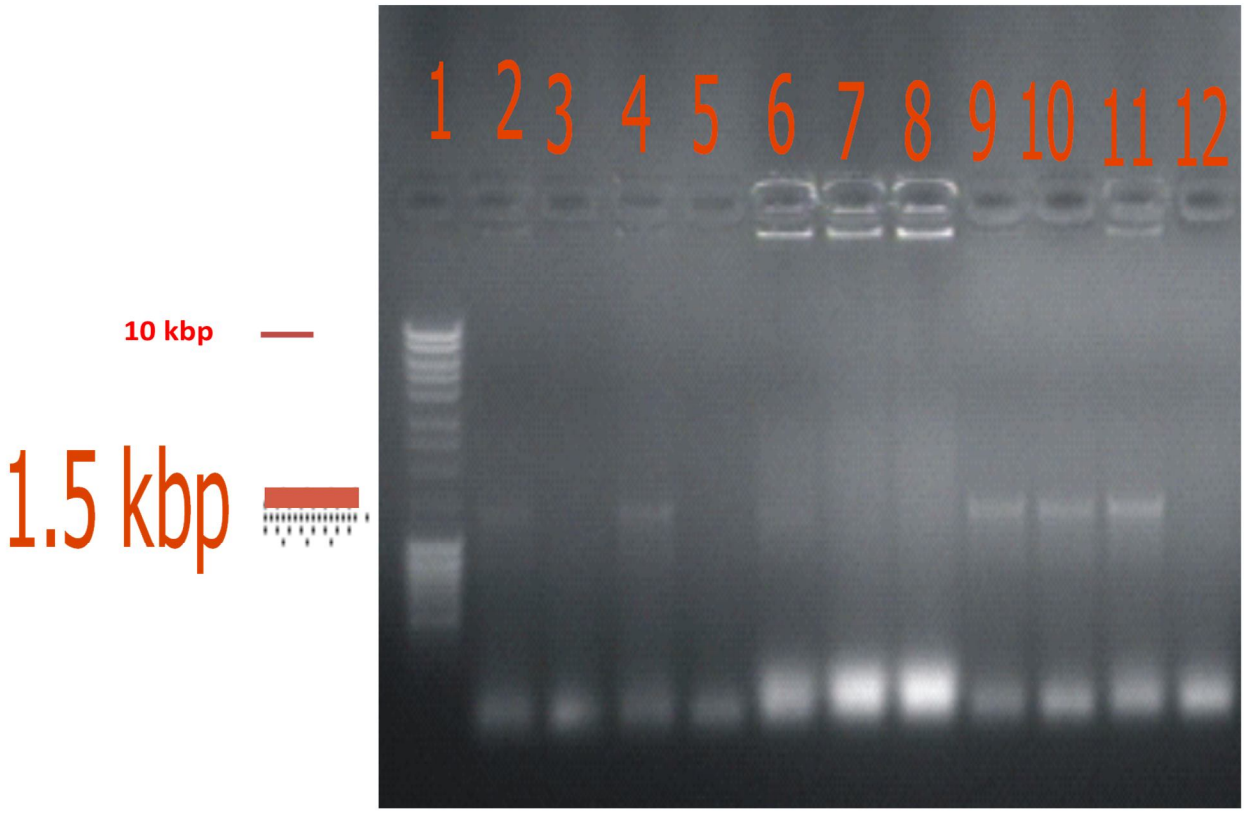


Plate 4.11. Result of the colony polymerase chain reaction after XL1 Blue *E.coli* cells were transformed with pVax1KanBP2Streptag recombinant plasmid and allowed to grow on Kanamycin LB plate.

KEY:

Lanes 1 (DNA ladder)
2-5 (from pcDNA3StreptagBP2-colony 5)
6-11 (from pcDNA3StreptagBP2-colony 6)
12(control well; amplification carried out with BP2 antisense primer (OSK2033) in replace of Strep-specific primer (OSK997).

[Amplification, except the controls was carried out with Internal sense primers and antiStrep primer (OSK997) having XhoI site.]

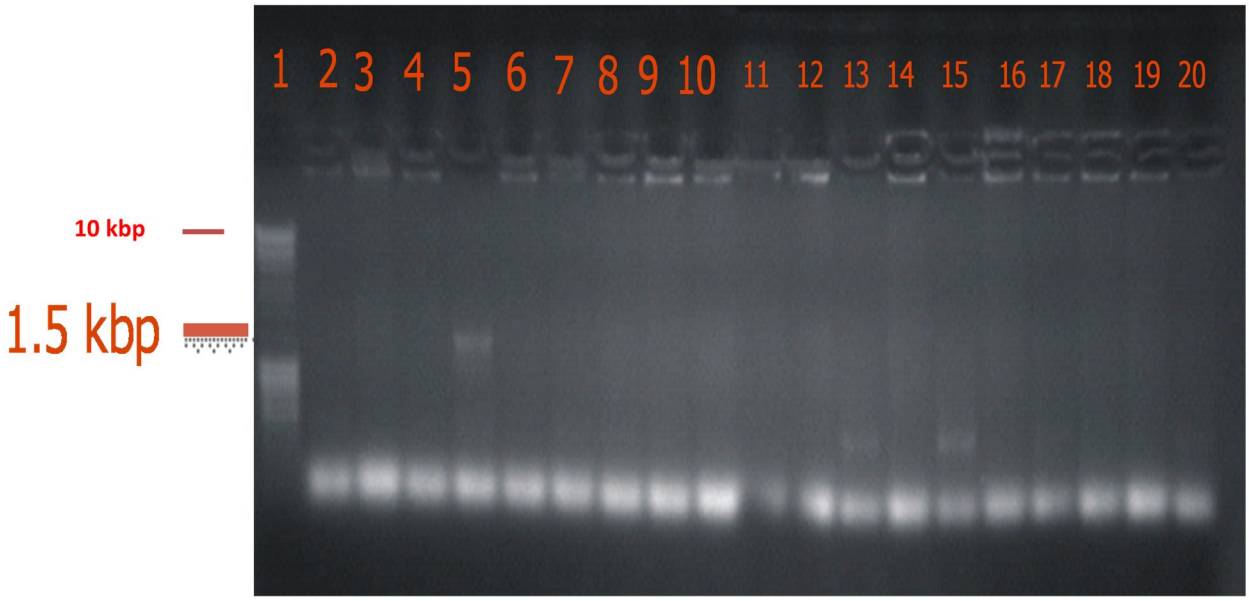


Plate 4.12. Result of the colony polymerase chain reaction after XL1 Blue *E. coli* cells were transformed with pcDNA3AmpBP1Streptag recombinant plasmid and allowed to grow on Ampicilin LB plate.

KEY:

Lanes 1 (DNA ladder)
2-20 (positive colonies from the ampicilin LB plate)

[Both inserts and pcDNA3 vector were cut with EcoRV and XhoI to allow for addition of transin signal peptide.]

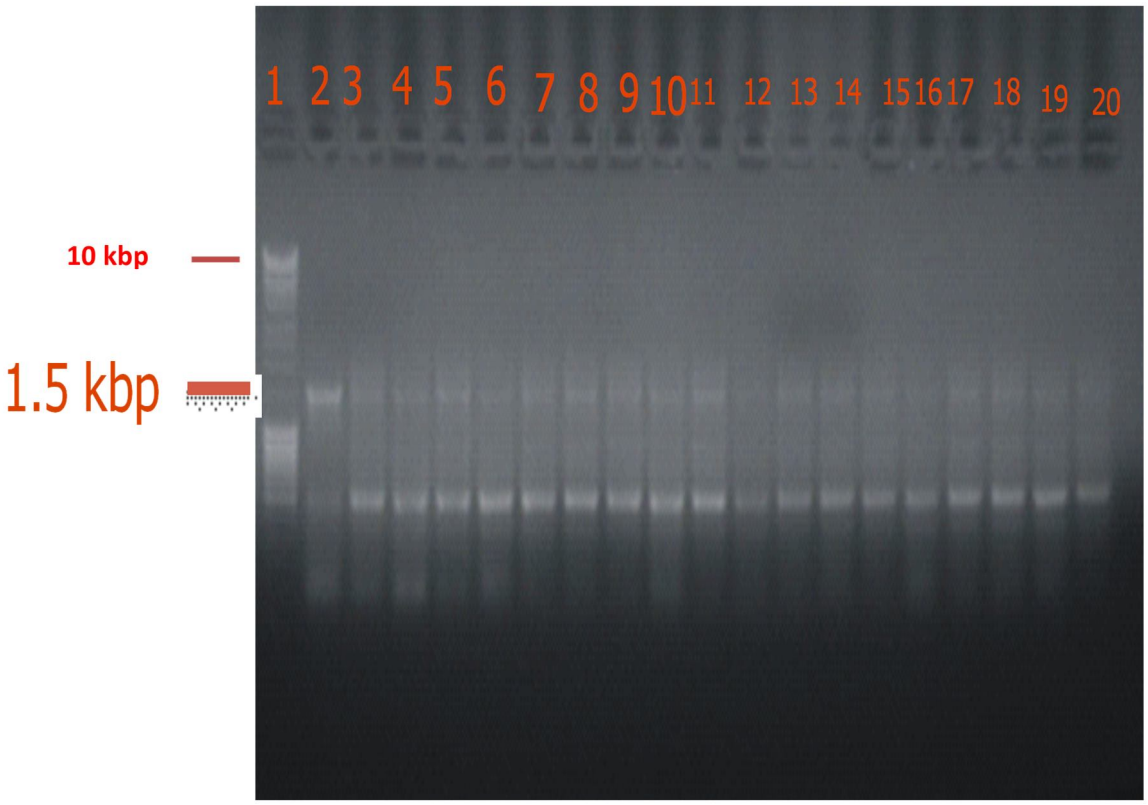


Plate 4.13. Result of the colony polymerase chain reaction after XL1 Blue *E. coli* cells were transformed with pVax1KanTransinBP1Streptag recombinant plasmid and allowed to grow on Kanamycin LB plate.

[Amplifications were carried out with OSK34 (having EcoRV site) and OSK997 (strep-specific, having XhoI site) primers. TransinBP1Streptag recombinant insert was cut from pcDNA3TransinBP1Streptag midiprep with HindIII and XhoI and introduced into pVax1 previously cut with the same set of restriction enzymes.]

KEY:
Lane 1 (Ladder)
Lanes 2-20 (positive colonies on Kanamycin plate).

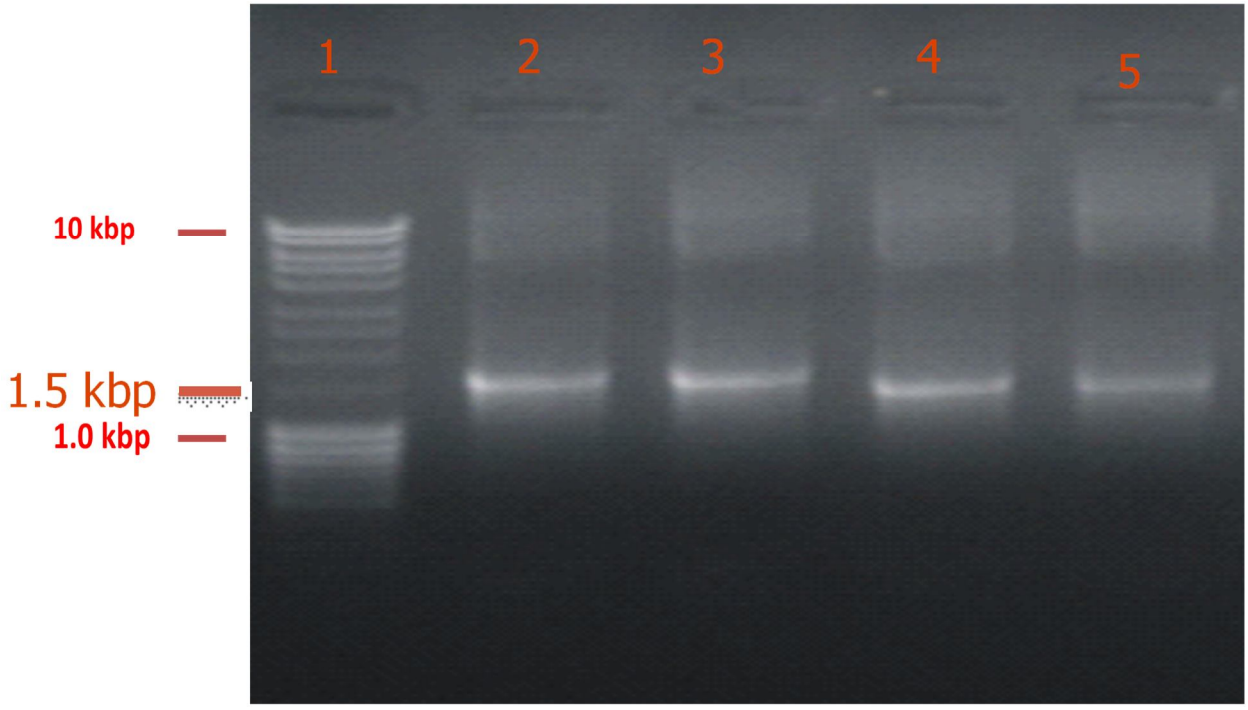


Plate 4. 14. Screening of pVax1KanTransinBP1Streptag midipreps from representative positively transformed XL1 Blue *E. coli* colonies for the presence of Bergheipain 1 gene.

[Amplifications were carried out using OSK2034 and OSK977 primers as the sense and reverse primers respectively. XL1 Blue *E. coli* colonies were grown on Kanamycin LB plates.]

KEY:
Lane 1 (Ladder)
Lanes 2-5 (PCR was carried out with four representative positive colonies on kanamycin plate).

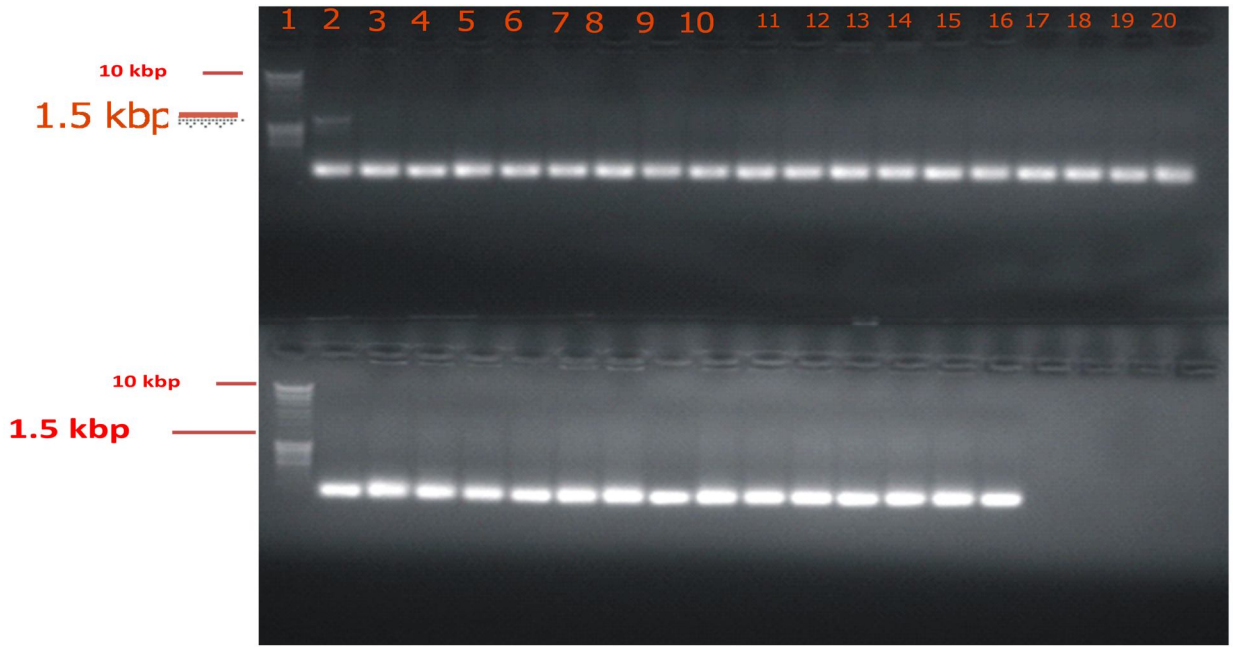


Plate 4.15. Result of the colony polymerase chain reaction after XL1 Blue *E. coli* cells were transformed with pcDNA3AmpBP2Streptag recombinant plasmid and allowed to grow on Ampicilin LB plate.

KEY:

Upper gel
Lanes 1 (DNA ladder)
2 to 11(colonies 1 to 10 of BP2-5 III)
12 to 20 (colonies 1 to 9 of BP2-6III)

Lower gel
Lanes 1 (DNA Ladder)
2 (colony 10 of BP2-6)
3 & 4(colonies 1 &2 of BP2-5 II)
5&6 (colonie 1 &2 of BP2-6II)
7 to 11(colonies 1 to 5 of BP2-5 I)
12 to 16 (colonies 1 to 5 of BP2-6 I).

[Both inserts and pcDNA3 vector were cut with EcoRV and XhoI to allow for addition of transin signal peptide. Amplification was carried out with OSK2035 (having EcoRV site) and OSK997 (having Xho1 site and streptag sequence**)** primers. Various Ligation reactions (see Methods section) were tried for transformation and I, II and III represents the various ratios of concentrations of insert and that of the vector (pCDNA3), both digested with EcoRV and Xho1 enzymes**.**]

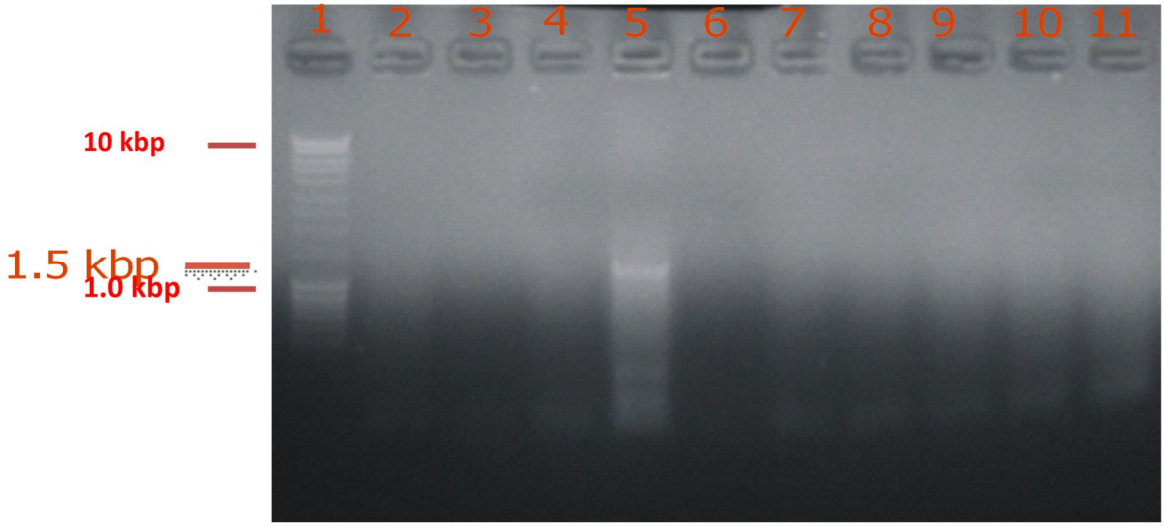


Plate 4.16. Result of the colony polymerase chain reaction after XL1 Blue *E. coli* cells were transformed with pVax1KanTransinBP2-5 III (1)Streptag recombinant plasmid and allowed to grow on Kanamycin LB plate.

[Amplification were carried out with OSK35 (having EcoRV site) and OSK997 (strep-specific, having XhoI site) primers. TransinBP1Streptag recombinant insert was cut from pCDNA3TransinBP1Streptag midiprep with HindIII and XhoI and introduced into Pvax1 previously cut with the same set of restriction enzymes.]

KEY:
Lane 1 (Ladder)
Lanes 2-11 (positive colonies on kanamycin plate).

Table 4.5. Plasmid DNA from Midipreps of positive colonies

Plasmid DNA	Plasmid concentration (µg/ml) (A_{260}/A_{280})				Total Plasmids produced (mg)
	Vial-1	Vial-2	Vial-3	Vial-4	
No Transin					
1. pVax1KanBP1streptag	**1325.6 (1.85)**	**1085.0 (1.92)**	**1364.5 (1.87)**	**1544.7 (1.88)**	**2.6379**
2. pVax1KanBP2streptag	**1753.9 (1.85)**	**1784.1 (1.87)**	**1889.7 (1.86)**	**1379.1 (1.87)**	**3.4034**
With transin signal peptide					
1. pVax1TransinKanBP1streptag	**1481.7 (1.86)**	**987.8 (1.89)**	**1146.3 (1.88)**	**1430.2 (1.90)**	**2.523**
2. pVax1TransinKanBP2streptag	**1429.9 (1.90)**	**1460.9 (1.90)**	**1437.0 (1.91)**	**1532.4 (1.91)**	**2.9301**
Control plasmid (pVax1)	**759.3 (1.89)**	**725.5 (1.9)**	**835.1 (1.89)**	**1018.5 (1.88)**	**1.6682**

Note: Each vial contains 500 µl of the eluted plasmid.

4.7 Results of immunisation studies with DNA vaccine

For *in vivo* test of the effectiveness of the DNA vaccines to protect mice against infection due to *Plasmodium berghei,* mice were immunized with separate vaccine construct. The experimental design consists of seven groups of ten mice each except the positive and the negative control groups which had 5 mice each. All the animals, with the exception of the negative controls, were thereafter challenged with same inoculums of iRBC. Samples of aminls' tail blood were taken at intervals throughout the study programme and used to determine parasitaemia and red cell counts. Animals weights were taken and deaths were also closely monitored and survival rate of the experimental groups were noted. All values observed were statistically compared with values of three control groups (Negative, Positive and plasmid controls).

Immunisation with the DNA-based vaccine constructs lowered the parasitaemia in mice vaccinated with pVax1StreptagBP2, pVax1TransinStreptagBP1, and pVax1TransinStreptagBP2 (Figure 4.14). Parasitaemia in group treated with pVax1StreptagBP1, however, remained progressively high and was not found to be significantly different with that in the positive control mice (group 2). Figure 4.15 gave the pattern of red cells counts observed in the experimental groups. Animals vaccinated with pVax1BP2Streptag, pVax1TransinStreptagBP1 had counts that were not significantly different from that seen in the unvaccinated noninfected (negative control) group but which were statistically different from the values obtained in the positive control group. The group which was vaccinated with pVax1StreptagBP2 had the next higher red cells count. Animals which were vaccinated with pVax1StreptagBP1 had very low mean red cells count as the mean value was not significantly different ($p > 0.05$) from the positive control group. Figure 4.16 shows the changes in animal mean weight pre and post challenge period. All groups showed increase in mean weight from the time of prime vaccination up to the time the

animals were challenged with life parasites. From the point of challenge forward variation in mean weights among groups became conspicuous; there was significant drop in weight in challenged animals which previously received PBS alone or naked pVax1 (plasmid control) and those given pVax1StreptagBP1. Figure 4.17 and appendix XXVII give the survival rate for all the experimental groups. By the 60^{th} day post challenge groups vaccinated with pVax1BP2Streptag, pVax1TransinBP1Streptag and pVax1TransinBP2Streptag had 37.5%, 25% and 25% survival rates respectively. With the exception of mice in the negative control group which had 100% survivl, animals in all other groups died by 24^{th} day post challenge.

Plates 4.17A and 4.17B are the photomicrographs of the liver cells from the experimental groups. The organs of representative mice taken from all the experimental groups were fixed in 10% Formalin before taken for histopathology. Groups with high parasitaemia (2, 3 and 4) visibly had hemosiderin deposits and showed large areas of necrosis. Their blood vessels seem to lack blood in them. Hepatocytes from three groups of mice immunized with the DNA vaccines (groups 5, 6 and 7) had low parasitaemia and were not as deranged as those of the positive and pVax1 controls, indicative of some level of protection. Inflammatory cells were also seen in liver of infected animals.

The safety usage of these DNA vaccines *in vivo* was studied by checking the possibility of plasmid integration into the genomic DNA of experimental animals. When the genomic DNA of immunized mice in all groups were scrutinized for possible integration by the plasmids, results obtained (Plate 4.18) gave no amplicon for all the experimental groups.

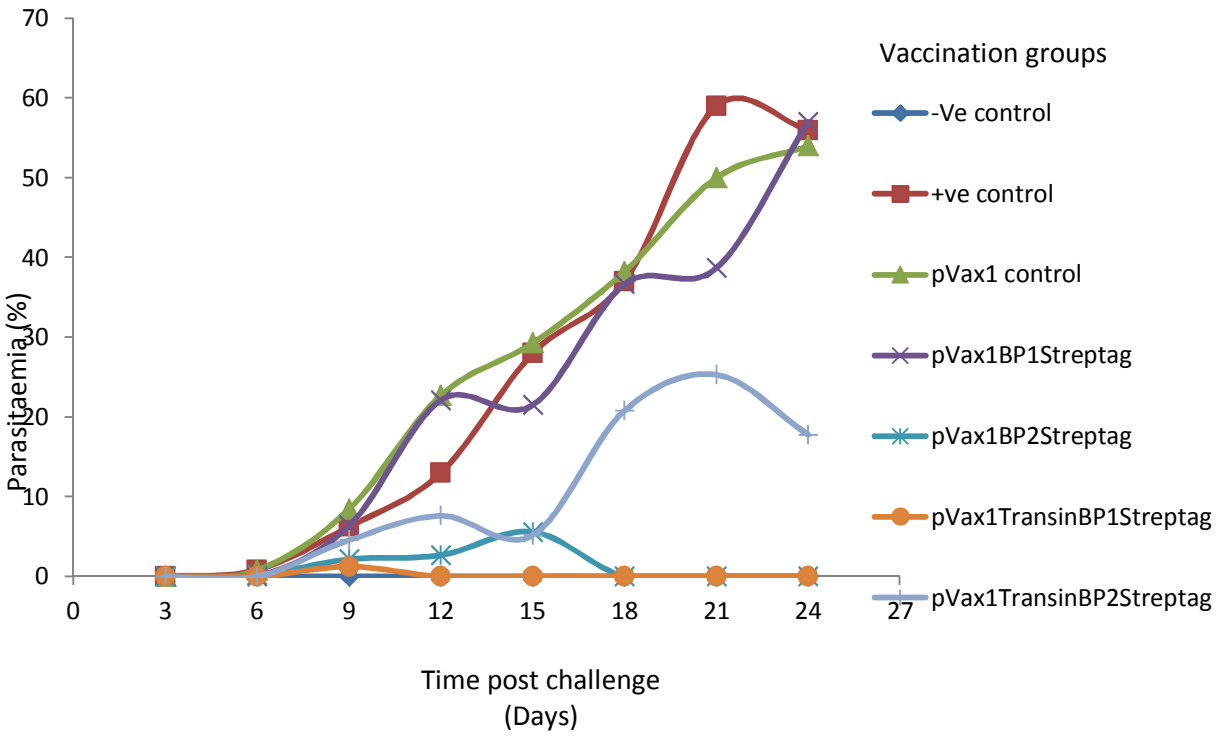


Figure 4.14 The effect of vaccination with DNA vaccine constructs on parasitaemiaof experimental mice.

Vaccination groups

-ve control (mice in this group were not vaccinated with DNA material but were given PBS as placebo and were not subsequently challenged with *P. berghei*).

+ve control (mice in this group were not vaccinated with DNA material but were given PBS as placebo but were subsequently challenged with *P. berghei*).

pVax1 control (this is the vector control group in which mice were vaccinated with the pVax1 vector not harbouring any gene insert).

pVax1BP1Streptag (mice in this group were vaccinated with pVax1BP1Streptag).

pVax1BP2Streptag (mice in this group were vaccinated with pVax1BP1Streptag).

pVax1TransinBP1Streptag (mice in this group were vaccinated with pVax1TransinBP1Streptag).

pVax1TransinBP2Streptag (mice in this group were vaccinated with pVax1TransinBP2Streptag).

[Each point is a mean value except were only one mouse survived and is monitored]

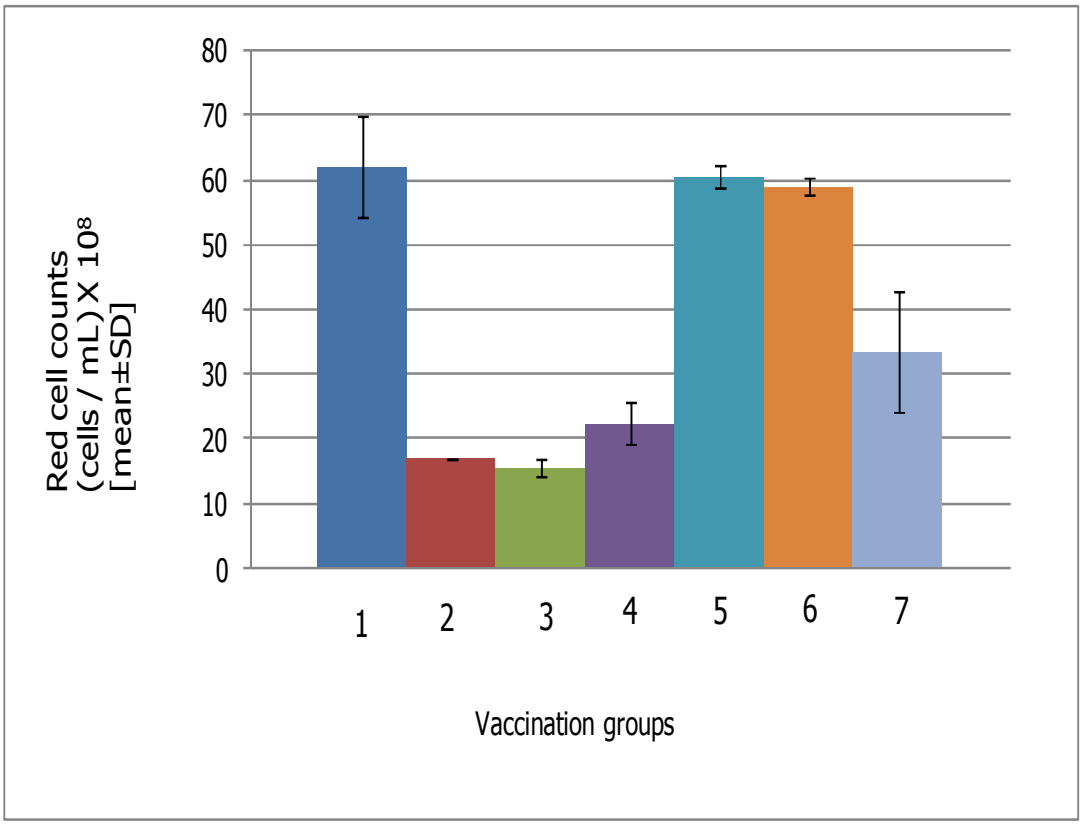


Figure 4.15. The effect of vaccination with DNA vaccine constructs on red cell counts of experimental mice on the 24th day post challenge.

Vaccination groups

1 (mice in this group were not vaccinated with DNA material but were given PBS as placebo and were not subsequently challenged with *P. berghei*).

2 (mice in this group were not vaccinated with DNA material but were given PBS as placebo but were subsequently challenged with *P. berghei*).

3control (this is the vector control group in which mice were vaccinated with the pVax1 vector not harbouring any gene insert).

4(mice in this group were vaccinated with pVax1BP1Streptag).

5 (mice in this group were vaccinated with pVax1BP1Streptag).

6 (mice in this group were vaccinated with pVax1TransinBP1Streptag).

7 (mice in this group were vaccinated with pVax1TransinBP2Streptag).

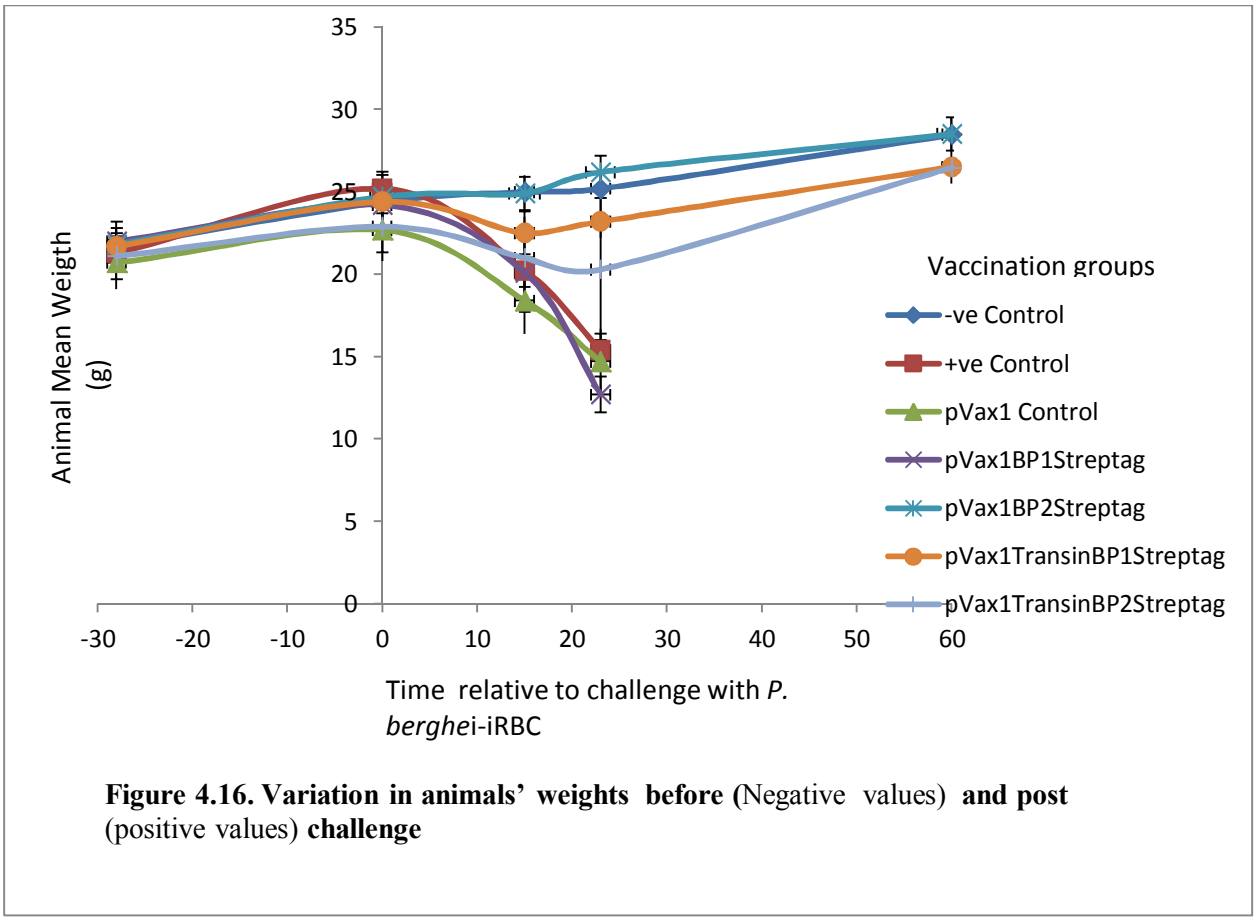


Figure 4.16. Variation in animals' weights before (Negative values) **and post** (positive values) **challenge**

Vaccination groups

-ve control (mice in this group were not vaccinated with DNA material but were given PBS as placebo and were not subsequently challenged with *P. berghei*).

+ve control (mice in this group were not vaccinated with DNA material but were given PBS as placebo but were subsequently challenged with *P. berghei*).

pVax1 control (this is the vector control group in which mice were vaccinated with the pVax1 vector not harbouring any gene insert).

pVax1BP1Streptag (mice in this group were vaccinated with pVax1BP1Streptag).

pVax1BP2Streptag (mice in this group were vaccinated with pVax1BP1Streptag).

pVax1TransinBP1Streptag (mice in this group were vaccinated with pVax1TransinBP1Streptag).

pVax1TransinBP2Streptag (mice in this group were vaccinated with pVax1TransinBP2Streptag).

[Animals were challenged with P. berghei-iRBC on day 0. Each point is a mean value except were only one mouse survived and is monitored. Error bars were constructed with standard deviation]

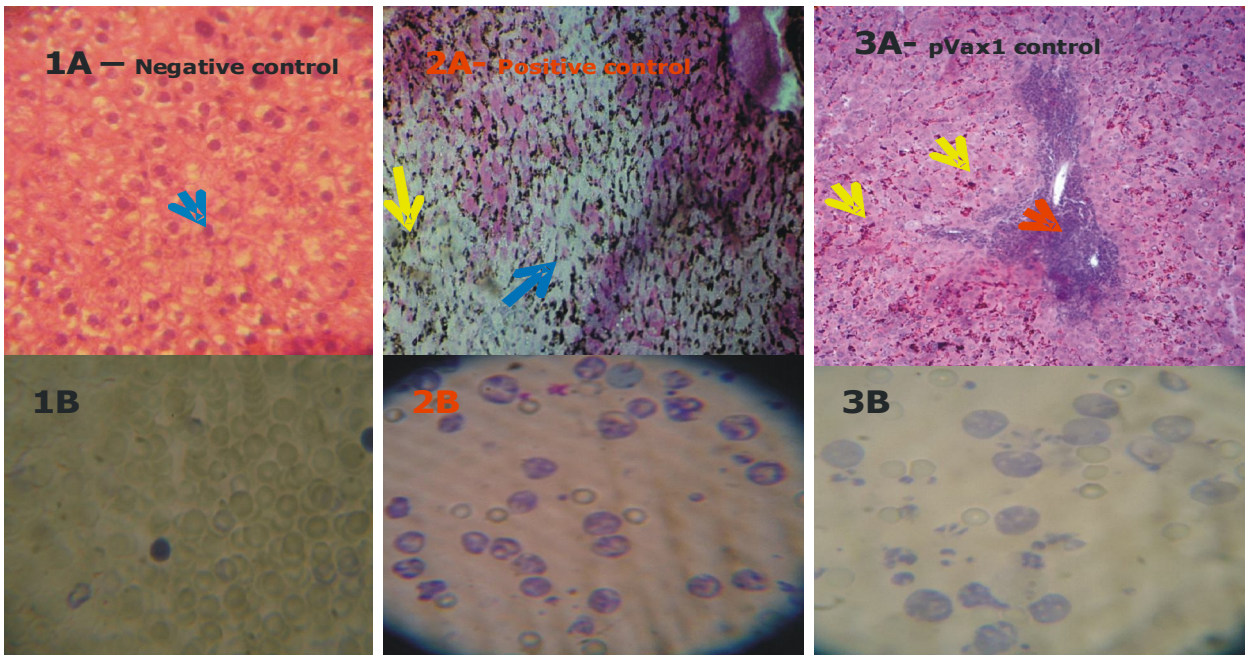


Plate 4.17A: Photomicrograph comparing the various liver pathology with parasitaemia from representative mice taken from experimental groups 1, 2 and3. H & E X 200-400.

[Group 1: not vaccinated and not challenged with *P. berghei*; Group 2: positive control- not vaccinated but challenged with the *P. berghei*; Group 3: pVax1 control group-animals were vaccinated with pVax1 and later challenged with *P. berghei.* Upper plates are the pictures of the hepatocytes while the lower plates are the corresponding giemsa stained blood smears. Blue arrow in 1A showing nucleus of intact hepatocytes, yellow arrows points to hemosiderin pigments, blue arrow in 2A points to region of necrosis while red arrow in 3A points to infiltration of perivascular inflammatory cells].

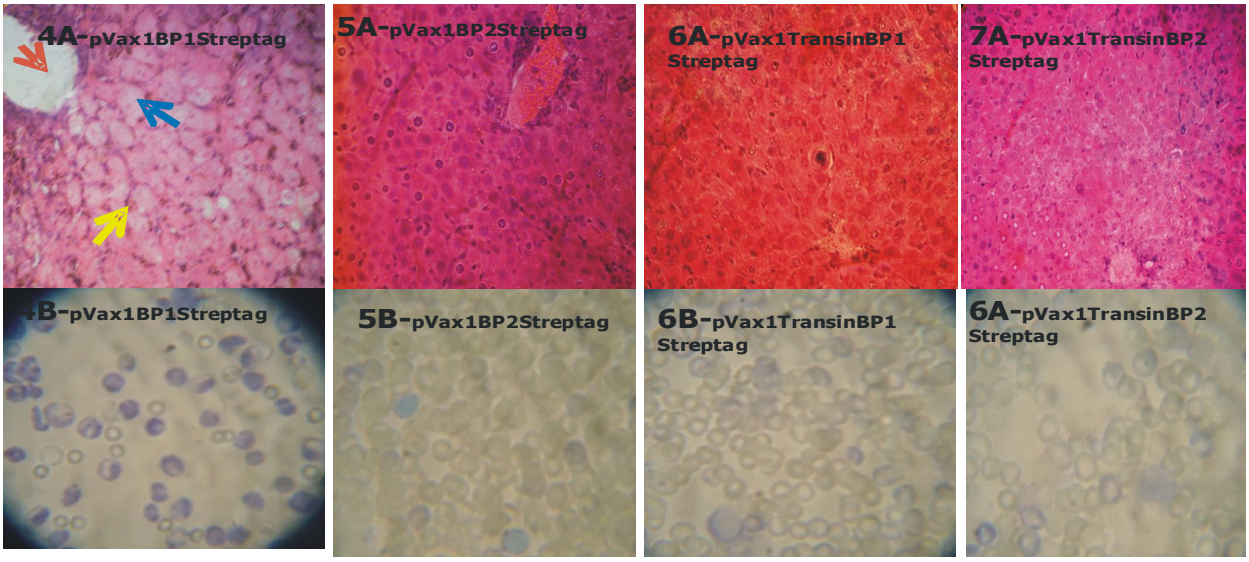


Plate 4.17B: Photomicrograph comparing the various degrees of pathology of liver cells with parasitaemia from representative mice taken from experimental groups 4, 5, 6 and 7. H & E X 200-400.

[Group 4: vaccinated with pVax1BP1Streptag and challenged with *P. berghei*; Group 5: vaccinated with pVax1BP2Streptag and challenged with the *P. berghei*; Group 6: animals were vaccinated with pVax1TransinBP1Streptag and later challenged with *P. berghei* ; Group 7: animals were vaccinated with pVax1TransinBP2Streptag and later challenged with *P. berghei.* Upper plates are the pictures of the hepatocytes while the lower plates are the corresponding giemsa stained blood smears. Red arrow show empty blood vessel, Blue arrow points to enlarged heppatocytes with with no nucleus, while yellow arrow points to hemosiderin].

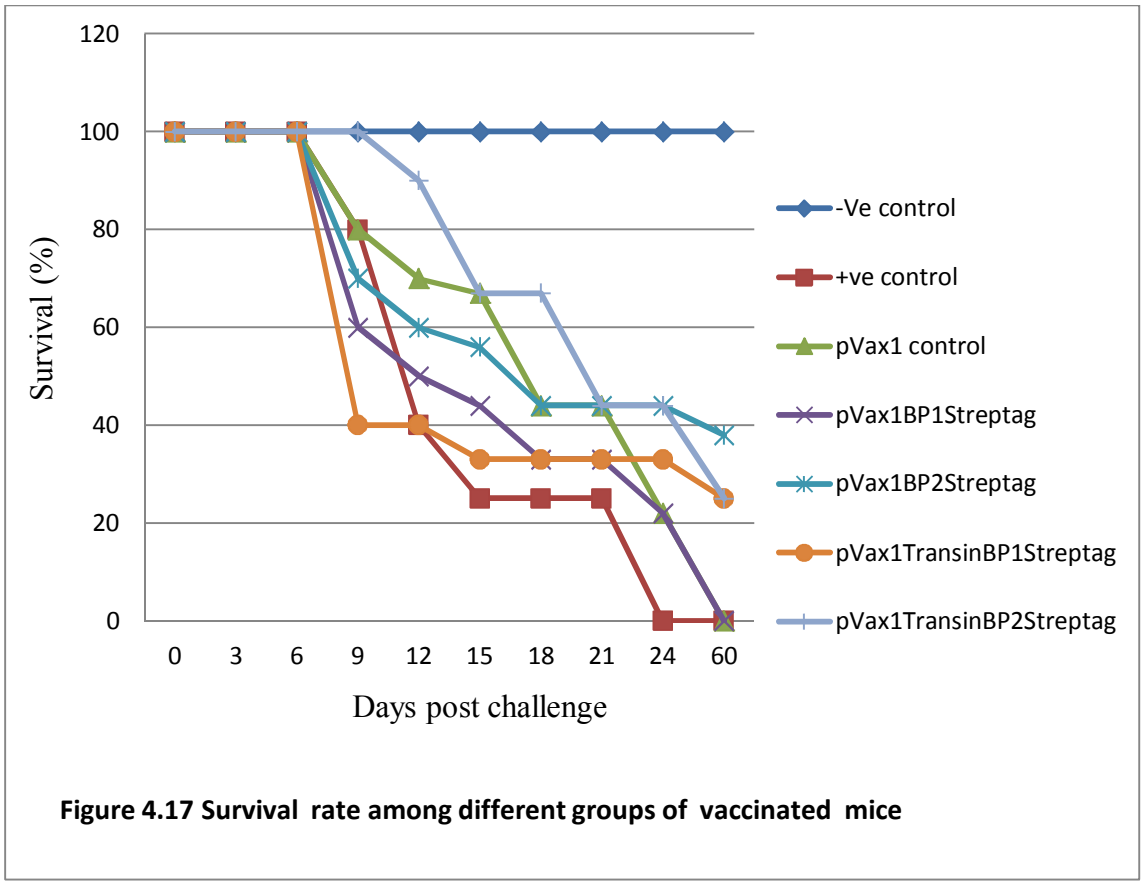


Figure 4.17 Survival rate among different groups of vaccinated mice

Vaccination groups

-Ve: (mice in this group were not vaccinated with DNA material but were given PBS as placebo and were not subsequently challenged with *P. berghei*).

+Ve: (mice in this group were not vaccinated with DNA material but were given PBS as placebo but were subsequently challenged with *P. berghei*).

pVax1 control: (this is the vector control group in which mice were vaccinated with the pVax1 vector not harbouring any gene insert).

pVax1BP1Streptag:(mice in this group were vaccinated with pVax1BP1Streptag).

pVax1BP2Streptag: (mice in this group were vaccinated with pVax1BP1Streptag).

pVax1TransinBP1Streptag:(mice in this group were vaccinated with pVax1TransinBP1Streptag).

pVax1TransinBP2Streptag:(mice in this group were vaccinated with pVax1TransinBP2Streptag).

Animals survival during the immunization period were expressed either as the ratio of the number of animals challenged with P. berghei-iRBC to the number of animals alive [3 to 24 days post challenge] or in terms of percentage of survivors [for 60th day post challenge].

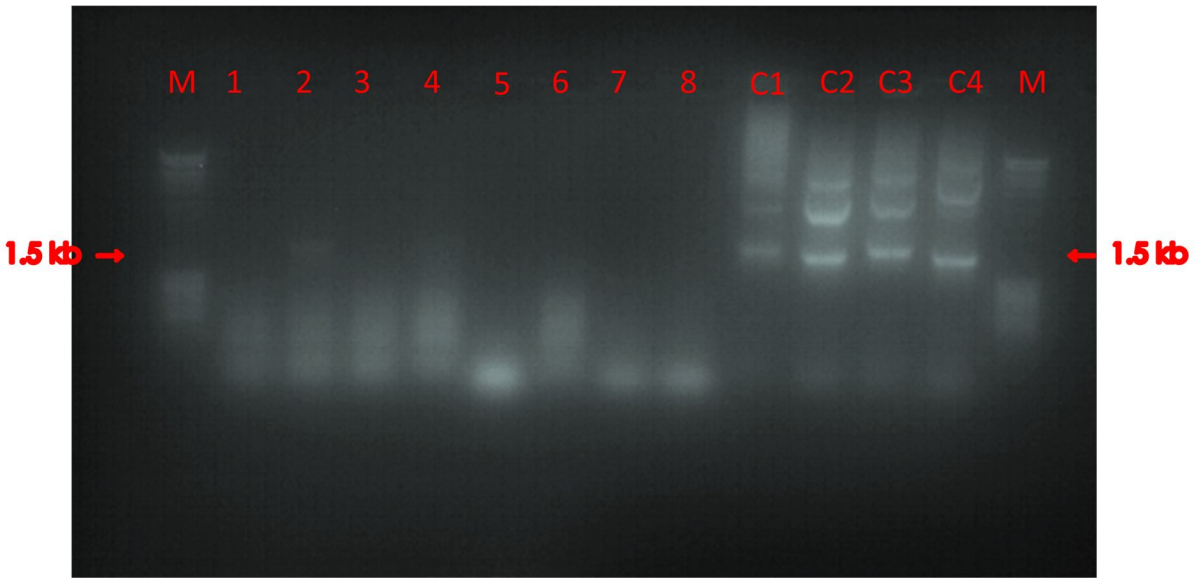


Plate 4.18. Screening for possible integration of plasmid DNA into genomic DNA extracted from liver of vaccinated mice from different experimental groups on the twelveth day post challenge (40 days post prime vaccination).

[PCR was carried out with gene-specific forward primer and streptag-specific primer as reverse primer. Lane1-DNA Ladder; Lanes 2 to 8-PCR results with genomic DNA from liver of representative mice taken from Groups 1(-ve control .i.e No infection ,no vaccination), 2(positive control i.e. vaccinated with PBS and Infected), 3(plasmid control, vaccinated with pVax1 and infected), 4(vaccinated with pVax1BP1), 5(vaccinated with pVax1BP2), 6(vaccinated with pVax1TransinBP1), 7(vaccinated with pVax1TransinBP2); Lane 9 (PCR result with genomic DNA from spleen of representative mice taken from group 7 i.e. vaccinated with pVax1TransinBP2; Lanes 10 to 13 were positive controls (PCR done with pVax1BP1, pVax1BP2, pVax1TransinBP1 and pVax1TransinBP2 as templates respectively); Lane 14 (DNA Ladder). Picture indicative of absence of genomic DNA integration.]

CHAPTER FIVE

DISCUSSION

5.1 Bioinformatic study preceeding gene amplification

The goal of this project is to study the vaccine potential of DNA vaccine encoding cysteine protease of *P. berghei* using animal model. The study started with bioinformatic search of the gene of the target enzyme following similar pattern employed by Caldeira et al (2009) for the amplification of Chaubaupain-1 and -2 from *Plasmodium chaubaudi*. Blast search on the available Plasmodium berghei genome database at the Sanger Institute and NCBI websites led to the identification of two putative cysteine proteases sequences (Bergheipain-1 and Bergheipain-2) that were orthogous to the previously described falcipain-1 and falcipain-2 respectively (Mohmmed et al., 2003) and their contigs. The contigs provided the required information for the design of primers used subsequently for gene amplification. In the past, cysteine protease genes from protozoan parasites have been amplified by designing degenerate primers based upon consensus sequences flanking the active site histidine, cysteine and asparagine residues (Eakin, et al., 1990; Renard et al., 2000; Byoung-kuk et al., 2004). For this study, however, the primers were designed based on already existing gene sequences.

5.2 Genomic DNA isolation, amplification and cloning of Bergheipain genes

Prior to DNA isolation, the infected mice blood were treated with glass beads and dextran in order to get rid of other sources of DNA such as leucocytes and platelets. This approach substantially increased the red cell/leucocytes ratio and provided parasite isolate that is largely free from other contaminating nucleic acids. DNA isolated from this source had appreciable yield and gave good A_{260}/A_{280} ratio. Amplification of cysteine protease genes from *Hevea brasiliensis* plant (Peng et al., 2008), *Paragonimus westermani* adult worms (Kim et al., 2000),

Boophilus microplus thick (Renard et al.,2000) and even in prokaryotes such as *Escherichia coli* (Joo et al., 2007) were carried out using cDNA as template for PCR. The bergheipain genes in this work were ampplified using genomic DNA instead. The use of cDNA in this study was thought to be unnecessary since protozoan genes have been reported to lack introns (Nene et al., 1990; Rosenthal and Nelson, 1992) and cysteine protease gene from *Trypanosoma cruzi* has been previously amplified with genomic DNA as the template (Eakin et al.,1990). The high fidelity *pfu*- DNA-Polymerase was employed for the PCR reactions since Taq polymerase is capable of misincoporating bases as it lacks 3′ exonuclease activity (Eakin, et al., 1990). The nested PCR adopted gave very clear and distinct bands on agarose electrophoregram. The nested PCR approach to gene amplification allowed for specific amplification of gene sequence that would otherwise have been mared by unspecific amplifications. Using the PCR conditions outlined in the Materials and method section, we have successfully amplified both Bergheipai-1 and Bergheipain-2 genes. Amplicon generated were around the expected size of 1500 base pairs and BP2 band visibly moved faster than BP1's indicating the greater size of the later.

The restriction sites for BamH1 and EcoR1 were neccessary for subsequent cloning into pcDNA3 plasmid and so were encoperated into the internal primers in the course of primer design. This allowed for successful cloning of the BP1 and BP2 genes into pcDNA3 plasmid vector. Colony PCR and restriction digest comfirmed the presence of the gene insert in the pcDNA3. The pcDNA3 was a strategy that would permit the monitoring of the gene expression *in vitro* on eukaryotic COS7 cell, the attachment of a strep tag at the carboxyl terminal end of the expresssed enzyme protein or encoporation of a transin signal peptide at its amino terminus. Similar strategy employing immunological detection of expressed protein using the strep tag has been used previously (Koliwer-Brandl et al., 2011).

Attempt in this study to transiently transfect COS7 cells with pcDNA3BP1 and pcDNA3BP2 gave positive expression of the genes as the presence of strep-tag was comfirmed using anti-Strep-tag rabbit polyclonal antibody with immunoflouresence technique. This result gives credence to the cloning strategy and presents optimism that the genes will be expressed *in vivo*.

5.3. Sequencing and bioinformatic studies of Bergheipain1 and Bergheipain2 genes

The locations of each bergheipain gene insert in the recombinant pcDNA vector were comfirmed following bioinformatic studies on the sequence generated using Genious software. The genes were found to be correctly inserted into the vector and were in frame with streptag sequence. The strep tag is an oligonucleotide that has been designed and was use for immunological identification of cloned genes (Koliwer-Brandl et al., 2011). The open reading frames of BP1 and BP2 were found to be 1560 bp and 1407 bp with predicted amino acid residues of 519 and 468 respectively. Similarly, their molecular masses and isoelectric points were predicted to be aproximately 60.31 kDa and 54.46 kDa; and 7.52 and 6.01, respectively.These values were identical to that predicted for cysteine protease from *Hevea brasiliensis* (Peng et al., 2008) and *Plasmodium falciparum* (Rosenthal and Nelson, 1992). BLASTsearches using the assembled contigs of the genes, show that both genes belong to Peptidase_C1 superfamily with specific hit on Peptidase C1A, where papain-like enzymes belong. The three residues, Cys25, His175, and Asn175, constitute the putative catalytic triad of papain (Higai et al., 1989) and all cysteine proteases belonging to papain family have nucleotides coding for these residues conserved. As expected, allignment with amino acid sequence of other plasmodium cysteine proteases (Figure 4.16) revealed that both genes possess the CGSCWAF, LNHSVL, and NSW motifs corresponding to the cysteine, Histidine and

Asparagine catalytic triad. Again, aligning our genes with reference BP1(Accession Number: XM 672551.1) and Bergheipain2 (Accession Number: AY06373763.1) located in PlasmoDb and NCBI data banks gave 94% and 99% maximum identity respectively to the reference genes, thus further confirming that our genes were actually the expected cysteine proteases from *Plasmodium berghei*. Again, the genes were phylogenetically related to their orthologues in other species of *plasmodium.*

5.4 Design and production of DNA vaccine constructs

The pVax1© forms the vector backbone of the DNA vaccine constructs designed and used in this study. The pVax1© is a 3.0 kb plasmids vector that has been designed for use in the development of DNA vaccines and its construction is reported to be consistent with the Food and Drug Administration (FDA) document of the United State of America (www.invitrogen.com). The plasmid possesses human cytomegaloirus (CMV) immediate-early promoter which permits efficient, high-level expression of recombinant protein (Andersson et al., 1987; Boshart et al., 1985; Nelson et al., 1987). The plasmid also has T7 promoter site which allows for *in vitro* transcription in the sense orientation and sequencing through the insert, multiple cloning site for gene insertion during cloning, Bovine growth hormone (BGH), polyadenylation signal for efficient transcription termination and polyadenylation (Goodwin and Rottman, 1992), and Kanamycin resistance gene which permits selection of vector in *E. coli* (Davies and Smith, 1978). The plasmid also has pUC origin to enable its high copy-replication and growth in *E. coli* (Bolivar et al., 1977a; Bolivar et al., 1977b). All these plasmid properties are critical in the design of DNA vaccine and their presence or absence have been found to affect the optimal function of DNA vaccines (Williams et al., 2009). Carter et al. (2007) and Muleke et al. (2007) have successfully employed pVax1 as vehicle for DNA vaccination. Having successfully cloned

the genes into pcDNA3, the gene inserts were then cut out of pcDNA3 and subcloned into pVax1© vector. Eventhough the cloning strategy adopted involving EcoRV (a restriction enzyme that produces blunt ends) did not allow for quick ligation, we have ultimately suceeded in ligating our gene inserts into the pVax1© (after incubating the ligation mix at 15 ^{o}C for six hours) and has produced the vaccine constructs in high enough amount that could be required for the *in vivo* immunisation studies.

5.5 Outcome of studies involving immunization with the DNA vaccine constructs

Immunization with DNA vaccine constructs lowered the parasitaemia in mice vaccinated with pVax1StreptagBP2, pVax1TransinStreptagBP1, and pVax1TransinStreptagBP2 and challenged with lethal inoculums of *P. berghei*-infected red cells. Lowering of parasitaemia was not observed in the group given pVax1StreptagBP1. Progression in the rise of parasitaemia is usually a sign of progression of the disease and animals normally come down with the disease if left untreated. Parasitaemia in the positive control group (those given PBS in place of vaccine and later challenged with the parasite) as well as the plasmid control group, continue to rise until they get to well over 50 % by the 24th day post challenge. Significant lowering of the parasite burden in vaccinated mice blood could easily be attributed to vaccination regimens administered. There is not clear explanation as to why animals vaccinated with bergheipain1 gene insert were not protected against the infection. One possible guess, however, could be that the *in vivo* expression of the CP-gene failed or that the expression was not strong enough to evoke significant rise in antibody titre against the protease since studies have shown that drop in parasitaemia correlated well with the level of antibodies in serum (Carter et al., 2007; Mor et al., 1995; Shalaby et al., 2003). It is worthy of note that transient expression of all the vaccine constructs on COS-7 cells gave positive results. Generally, it seems that animals vaccinated

with the plasmid carrying Bergheipain2 were better protected than those vaccinated with Bergheipain1 vaccines. This is contrary to our expectation; falcipain1 gene has been linked to the invasion of RBCs by merozoites and is therefore thought to be a better candidate for vaccine production (Mohammed et al., 2003) than its BP2 counterpart whose role as a haemoglobinase has long been established (Cheryl et al., 2005). Since anaemia and splenomegaly are major signs of infection due to *Plasmodium* (including *P. berghei*) (Thurston, 1953), the red cell counts of animals in all the experimental groups were determined and mean values compared on the 24th day post challenge, when the infection is thought to be terminal. Mean Values of positive, pVax1 controls and pVax1BP1Streptag were minimal while those belonging to groups vaccinated with PBS, pVax1StreptagBP2, pVax1TransinStreptagBP1, and pVax1TransinStreptagBP2 were maximal (Figure 4.26). This pattern in red cell counts among experimental groups followed that observed for parasitaemia. The mean weights for all groups, pre-immunization, were within the expected range (19-23g corresponding to 6-8 weeks age of mice) used by other researchers (Carter et al., 2007; Sukumaran et al., 2003). Even at the point of challenge, the mean weights of all the groups were not significantly different. However, the values clearly assumed variation as the infection progresses alongside variation in parasitaemia and red cell counts as earlier explained.

Histopathological studies showed that where vaccination appeared effective, there were lesser degree of liver damage. Though parasites were seen in some vaccinated liver, it is thought that this is part of the initial adaptation of such hepatocytes-infected cells as the parasites must first pass through the liver to get to the blood. It is expected that such liver will recover with time in animals that overcame the infection. Hepatocytic necrosis were seen in animals vaccinated with

pVax1BP1Streptag just as in those of the positive control and the pVax1 control group. The percentage survival of healthy mice seems to follow the pattern for the parasitaemia and red cell counts.

The protection of our experimental mice by the DNA vaccines could be largely due to antibodies production accompanying the transcription and translation of the gene inserts *in vivo*. Normally, the DNA vaccine coding for an antigen is transcribed and translated after its administration. The protein antigen so produced is then degraded into short peptides by ubiquination and proteolysis. This peptides are then taken up by the MHC Class I and II complexes, which are then recognized by the CD4+ T-helper cells and CD8+ cytolytic T lymphocytes (Viswanathan, 2005). Upon activation, these cells produce cytokines to stimulate the B cells to produce antibodies against the antigen (Viswanathan, 2005). This is a simplified mechanism for protection due to DNA-based immunization. In fact, it is well known that DNA-based immunization can stimulate all effector arms of the immune response, *i.e.* antibodies and CD4+ and CD8+ T cells (Grifantini et al., 1998**)**. Again, specific immune response has been associated with DNA vaccination; for example, vaccination with DNA vaccine encoding genes of *Leishmania major,* indicates that the protection observed with the Leishmanial DNA is mediated by acquired specific immune response rather than by the activation of nonspecific innate immune mechanisms (Campos-Neto et al., 2002). Protective immune responses against internal pathogens are known to require a balance between Th-2 and Th-1 type of cellular responses with the production of type 1 cytokines such as IFN and antibodies of the IgG2a isotype in mice (Qazi et al., 2005). Screening for the production of antibodies specific to Bergheipain-1 and 2 was not carried out in this work. The successful expression of these genes

in vitro on mammalian COS-7 cells in this study, however, gives hope that the protection observed could be associated to the genes' expression in the vaccinated mice and the possible production of antibodies by the B cells. Though the percentage population of healthy survivors was not very high, the results show that if other parameters are optimized, it is possible to enhance this survival rate.

On the safety usage of DNA vaccines, four major areas of concerns have been identified (Donnelly et al., 1997; Gurunathan, et al., 2000) to include: the possibility of plasmid integration into the host genome, thereby increasing the risk of malignancy (by activating oncogenes or inactivating tumor suppressor genes); the possibility of having induced responses against transfected cells, thereby triggering the development of autoimmune disease; the possibility of inducing tolerance rather than immunity; and the possibility of stimulating the production of cytokines that alter the host's ability to respond to other vaccines and resist infection Nichols et al. (1995). Of these four areas, we have only tried to study the possibility of plasmid DNA getting integrated into host genomic DNA using PCR tool. No possible integration was observed in this study. Nichols et al. (1995), using sensitive PCR methods, were also unable to detect integration after mice were injected i.m. with plasmid DNA encoding influenza nucleoprotein (NP).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

1. Bergheipain 1 and Bergheipain 2 genes were amplified, cloned into pcDNA3 vector, sequenced and subcloned into pVax1 expression vector. The gene were transiently expressed *in vitro* on COS7 cells.

2. Immunisation with the DNA vaccine constructs lowered the parasitaemia in mice vaccinated with pVax1StreptagBP2, pVax1TransinStreptagBP1, and pVax1TransinStreptagBP2

3. The pattern of parasitaemia seen to affected the red cell counts, mean weights, pathology of the liver and ultimately, animals survival.

4. Better protection was found among mice vaccinated with plasmids carrying Bergheipain 2 gene. In addition, those with transin signal peptides seems to give better protection than those without.

5. Integration of plasmids DNA into gDNA of the mouse was not observed.

6.2 Conclusion

Orthologoues of falcipain1 and 2 were amplified from *P. berghei*'s genomic DNA using a Nested PCR protocol. Both genes were cloned into pCDNA3 vector and used to transformed XL1 Blue *E.coli* cells. Midiprep were prepared from positive colonies on ampicilin-LB plate from where the full genes were sequenced. The genes belong to petidase_C1 papain-like cysteine protease family with all the catalytic triad residues well conserved. *In vitro* transfection of COS-7 cells with the pCDNA3BP1Streptag and pCDNA3BP2Streptag using immunofluoresence technique revealed transient expression of the genes.

The genes were successfully subcloned into pVax1 using strategies that yielded four vaccine contructs; two had transin signal peptide while the remaining two constructs do not.

Immunisation with the DNA vaccine constructs lowered the parasitaemia in mice vaccinated with pVax1StreptagBP2, pVax1TransinStreptagBP1, and pVax1TransinStreptagBP2. This pattern in parasite burden also affected the red cell counts, mean weight and ultimately the percentage population of healthy survivors seen in different experimental groups. Animals vaccinated with bergheipain2 gene insert were better protected from *P. berghei*-infection than those vaccinated with Bergheipain1 contrary to expectation. Better protection were seen in groups of mice immunized with DNA vaccines having transin signal peptide than those without it.

Integration of plasmids DNA into genomic DNA of the vaccinated animals was not observed, allaying the fears of any danger that the usage of this vaccine could lead to.

6.3 Recommendation

The general observation is that DNA vaccine are more effective when used as prime vaccine while recombinant proteins were known to boost better. It is recommended that further studies be carried out to study the effect of combine administration of pVax1BP as prime vaccine followed by boosting with BP recombinant molecule.

Again, several antigenic molecules have been identified at different stages of the life cycle of plasmodium. It is recommended that investigation be carried out to see the protective potential of vaccination with multigene DNA vaccine harbouring cysteine protease sequence and those of other molecules.

Sigh et al. (2007) observed that animal model parasites such as *P. berghei* contain cysteine proteases which are quite different from those of the key human parasite and have even

produced engineered mutant bergheipain2 that might lead to animal model parasite with a protease repertoir more similar to that of *P. falciparum*. It is recommended that such engineered sequences be used in the future as part of the design and construction of DNA vaccine and its efficacy tested in animal model. Alternatively, site-directed mutagenesis could be used to creat mutation on keycatalytic residues of the enzyme thereby producing catalytically inactive protease when expressed *in vivo*. The vaccine potential of DNA vaccine having such sequences could then be tested in mice.

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Appendix I: PCR Reaction Mix for Taq Polymerase

Component	Volume (µL)
	X1 Reaction
gDNA Template	1.0
Forward Primer	0.5
Reverse Primer	0.5
Taq Polymerase Buffer	2.5
dNTPs	0.2
$MgCl_2$	1.5
ddH_2O	18.3
Taq polymerase	0.5
Total	25

Appendix II : PCR Reaction Mix for pfu Polymerase

Component	**Volume (µL)**
	X1 Reaction
gDNA Template	1.0
Forward Primer	0.5
Reverse Primer	0.5
Pfu Poymerase Buffer	5.0
dNTPs	1.0
ddH_2O	41.0
Total	50

Appendix III: PROGRAM FOR NESTED PCR

CNTRL TUBE LID=105 ^{o} C		
	Temperature	Time(Hr:Min:Sec)
1	T = 95 ^{o} C	0:05:00
2	T = 95 ^{o} C	0:01:00
3	T = 40 ^{o} C	0:01:00
4	T = 72 ^{o} C	0:03:00
5	GO TO 2 REP 29	
6	T = 72 ^{o} C	0:05:00
7	Hold 4.0 ^{o} C ENTER	
end	^{o} C	

The program runs for 3.52 hr

Appendix IV: PROGRAM FOR STANDARD PCR with pfu polymerase

CNTRL TUBE LID=105 ^{o} C		
	Temperature	Time(Hr:Min:Sec)
1	T = 95 ^{o} C	0:05:00
2	T = 95 ^{o} C	0:01:00
3	T = 45 ^{o} C	0:01:00
4	T = 72 ^{o} C	0:03:00
5	GO TO 2 REP 29	
6	T = 72 ^{o} C	0:05:00
7	Hold 4.0 ^{o} C ENTER	
end	^{o} C	

Note that this same programme was used as STANDARD PCR programme amplification with OSK2034/2035 and OSK997 (Streptag reverse primer) primers and pfu as DNA Polymerase.

Appendix V: Colony PCR Reaction Mix Using Taq Polymerase

Component	**Volume (µL)**
	X1 Reaction
gDNA Template (in this case is positive clone)	A piece of the clone using yellow pipette tip
Forward Primer	0.5
Reverse Primer	0.5
Taq Polymerase Buffer	2.5
dNTPs	0.2
$MgCl_2$	1.5
ddH_2O	18.3
Total	25

Appendix VI: Program For Standard PCR With Taq Polymerase And Internal Primers

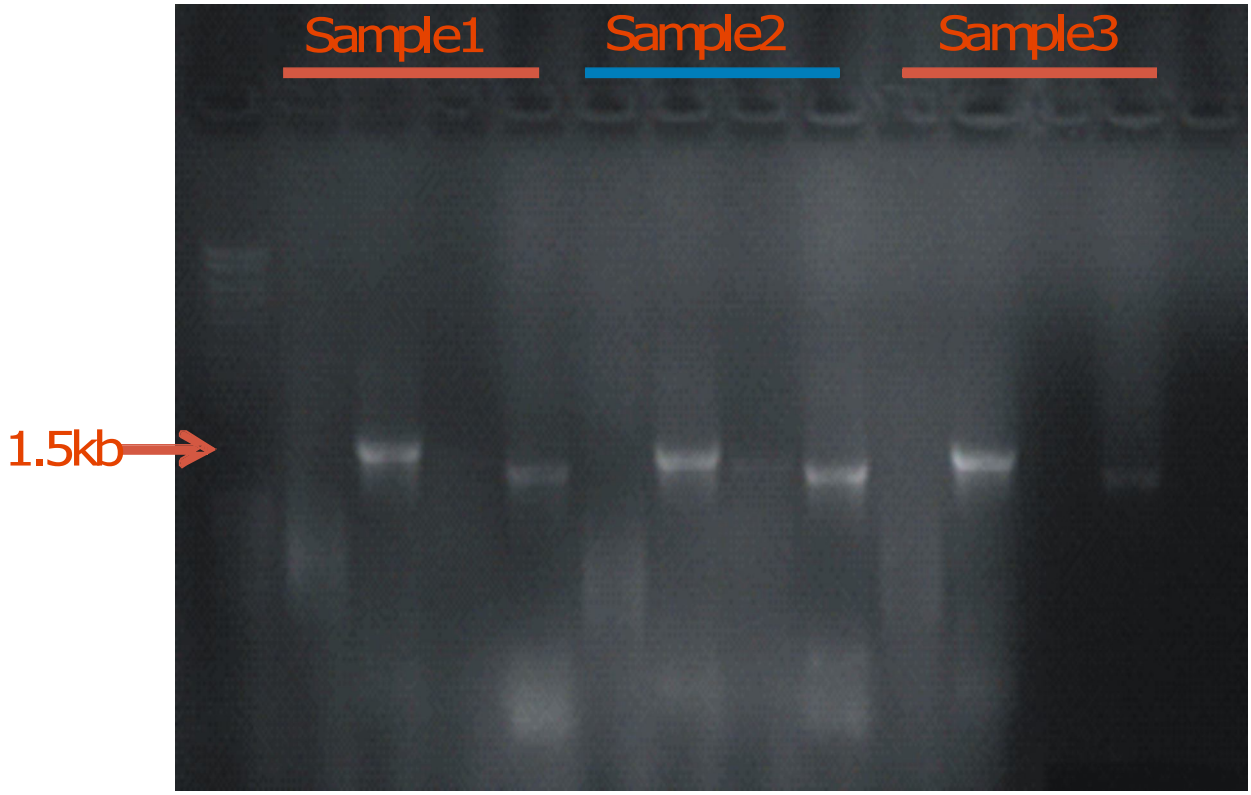
CNTRL TUBE LID=105 ^{o} C		
	Temperature	Time(Hr:Min:Sec)
1	T = 95 ^{o} C	0:05:00
2	T = 95 ^{o} C	0:01:00
3	T = 45 ^{o} C	0:01:00
4	T = 72 ^{o} C	0:01:30
5	GO TO 2 REP 29	
6	T = 72 ^{o} C	0:05:00
7	Hold 4.0 ^{o} C ENTER	
end	^{o} C	

The program runs for 2.57 hr.

Appendix VII Outcome of isolation of Genomic DNA from *P. berghei* isolates

P. berghei Sample	Concentration (ng/µl)	$A_{260/280}$
1	29.0	1.83
2	69.0	1.68
3	67.0	1.84

Note: that gDNA from sample 2 was chosen on the basis of gel picture when they were all subjected to Nested and standard PCR with bergheipain 1 and 2 primers. Sample 2 gDNA was used for preparative PCR.



Appendix VIII. Electrophoregram of Nested PCR amplification using three samples of genomic DNA extracted from different *P. berghei* isolates. Pfu polymerase was used with reaction conditions as outlined unders methods. Electrophoresis was carried out on 1% agarose gel.

Appendix IX: Reaction Mix for Restriction Digest

Component	**Bergehipain-1 X1 Reaction Volume (µL)**	**Bergehipain-2 X1 Reaction Volume (µL)**
Amplicon purified from agarose gel	49	29
X10 Restriction digest Buffer	6 (10% of total volume)	4(10% of total volume)
BamHI	1	1
EcoR1	1	1
ddH_2O	3	5
Total	60	40

Appendix X: Ligation Reaction Mix For BamH1/Xho1

Component	**X1 Reaction Volume (µL)**
Insert	2
Vector	2
X10 T_4 DNA ligase Buffer	1.5
T_4 DNA Ligase	1
ddH_2O	8.5
Total	15

Appendix XI: Ligation Reaction Mix For EcoRV/Xho1

Component	**X1 Reaction Volume (µL)**
Insert (10ng/µl)	2
Vector(10ng/µl)	4
X10 T_4 DNA ligase Buffer	2
T_4 DNA Ligase	2
ddH_2O	10
Total	20

Appendix XII. Yield from PCR preparative agarose gel electrophoresis before and after Restriction Digest with BamH1 and EcoR1

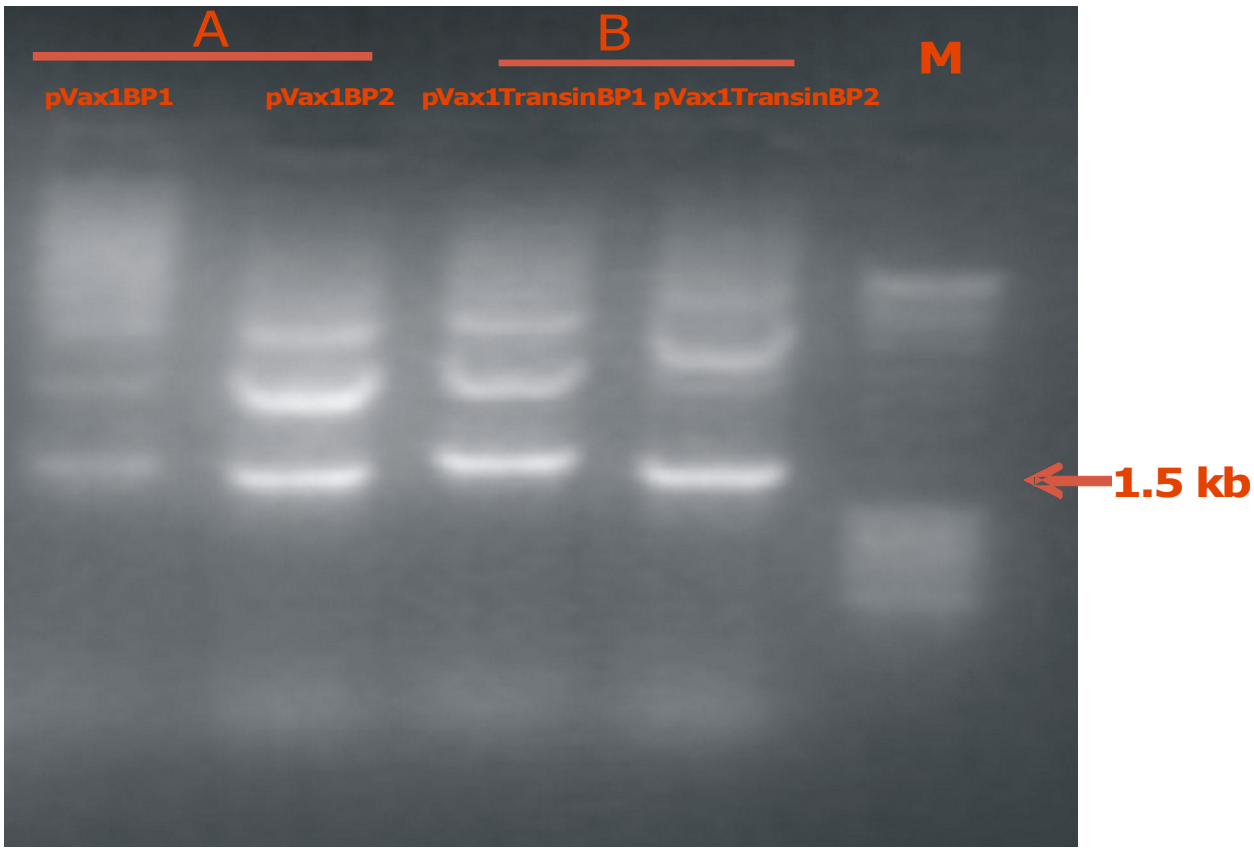
Digest		Gene	
		Bergheipain-1	Bergheipain-2
Before Restriction Digest	Total volume(µl)	50	30
	Concentration (ng/µl)	115.6	44.5
	$A_{260/280}$	1.88	1.86
After Restriction Digest	Total volume(µl)	50	30
	Concentration (ng/µl)	71.0	22.9
	$A_{260/280}$	1.93	1.74
	Recovery (%)	60.37	51.46

Appendix XIII. Yield for pcDNA3BP1/2Streptag Minipreps from representative colonies

Gene	Colony number	Plasmid Nomenclature	Plasmid DNA Conc. (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}
Bergheipain-1	1	pCDNA3BP1Streptag	169	1.89	2.22
	2	pCDNA3BP1Streptag	461.7	1.86	2.29
	5	pCDNA3BP1Streptag	410.3	1.87	2.26
	6	pCDNA3BP1Streptag	387.2	1.87	2.31
	8	pCDNA3BP1Streptag	492.9	1.86	2.30
Bergheipain-2	1	pCDNA3BP2Streptag	116.9	1.90	2.25
	2	pCDNA3BP2Streptag	150.2	1.90	2.22
	5	pCDNA3BP2Streptag	397.1	1.87	2.31
	6	pCDNA3BP2Streptag	345.3	1.87	2.30
	8	pCDNA3BP2Streptag	340.2	1.87	2.32

Appendix XIV. Yield of gene inserts recovered from agarose gels after the pcDNA3BP1/2Streptag plasmids were cut with EcoRV/ XhoI

DNA	Plasmid DNA Conc. (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}	A_{230}	A_{260}	A_{280}
Gel Extract of pcDNA3 cut with EcoRV/ XhoI	226.2	1.89	0.36	12.641	4.524	2.394



Appendix XV. Evaluation of the quality of the DNA vaccines with (B) and without (A) the transin signal sequence, after freezing and thawing associated with long distant transportation (from Bremen, Germany the place of production to Zaria, Nigeria where the vaccine was used). The PCR amplification were carried out using gene-specific forward primers and streptag specific primer as the reverse primer.

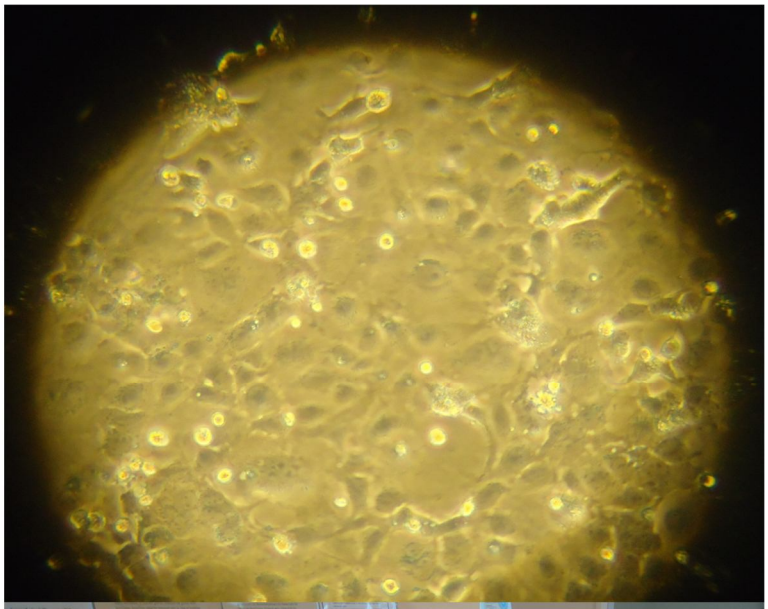
Appendix XVI: Reaction Mixture for the transfection of Cos-7 cells with Plasmids Miniprep (pcDNA3BP1Streptag& pcDNA3BP2 Streptag) constructs

Bacterial colony number	Stock Concentration of Plasmid DNA	Transfection concentration of the stock plasmid (µg/100 µL)	Amount (µL)of components of the 100 µL of transfection solution (µL)		
			Sterile NaCl (150 mM)	Plasmid DNA construct(volume of stock used)	PEI
Bergheipain-1					
1	169	1.5	88.62	8.88	2.5
2	461.7	1.5	94.25	3.25	2.5
5	410.3	1.5	93.84	3.66	2.5
6	387.2	1.5	93.63	3.87	2.5
8	492.9	1.5	94.46	3.04	2.5
Bergheipain -2					
1	116.9	1.5	84.67	12.83	2.5
2	150.2	1.5	87.51	9.99	2.5
5	397.1	1.5	93.72	3.78	2.5
6	345.3	1.5	93.16	4.34	2.5
8	340.2	1.5	93.09	4.41	2.5
Negative Control well-1	-	-	-	-	-
Negative Control well-2	-	-	-	-	-
Negative (PBS)Control well-3	-	-	92.5	5 of PBS	2.5
Negative (PBS) Control well-4	-	-	92.5	5 of PBS	2.5
Positive (TS-1) Control well-1	689	1.5	95.32	2.18	2.5
Positive (TS-1) Control well-2	689	1.5	95.32	2.18	2.5

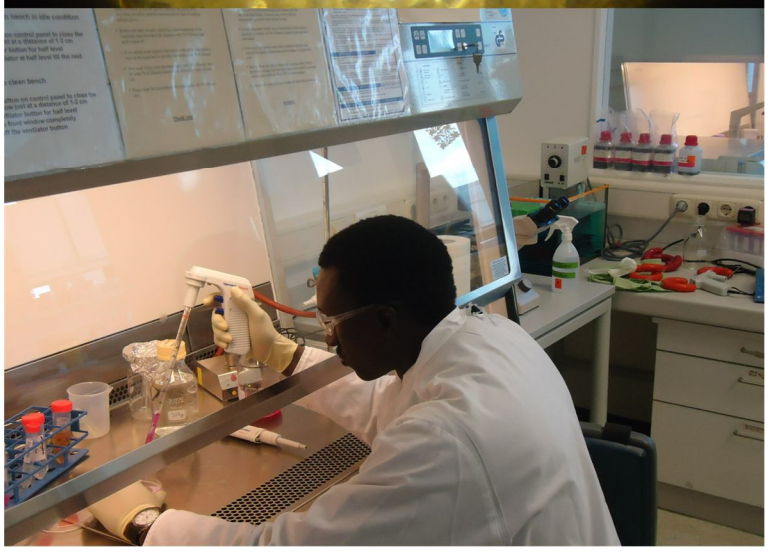
Appendix XVII: Reaction Mixture for the transfection of Cos-7 cells with midipre of the Plasmids (pcDNA3BP1Streptag& pcDNA3BP2 Streptag) constructs

			Transfection volume of component (µL)		
			Components (in 100 µL of Total volume)		
Positive Colony from where plasmids were Extracted	Stock Plasmid DNA concentration (µg/µL)	Plasmid DNA Transfection concentration (µg/100µL)	Sterile 150 mM NaCl	Amount of Stock plasmid DNA	PEI (µL)
BP-1					
5	1311.1	1.5	96.36	1.144	2.5
6	1494.6	1.5	96.50	1.004	2.5
BP-2					
5	1299.3	1.5	96.35	1.154	2.5
6	181.9	1.5	96.67	0.826	2.5
Positive Control (pDEF-TS2)	980	1.5	95.983	1.517	2.5
Negative Control	PBS	0	96	1.5	2.5

A



B



Appendix XVIII: Microscopic view of COS-7 cells grown on 24 Wells Titre Plate (A) And Tissue Culture work on COS-7 cells (B)

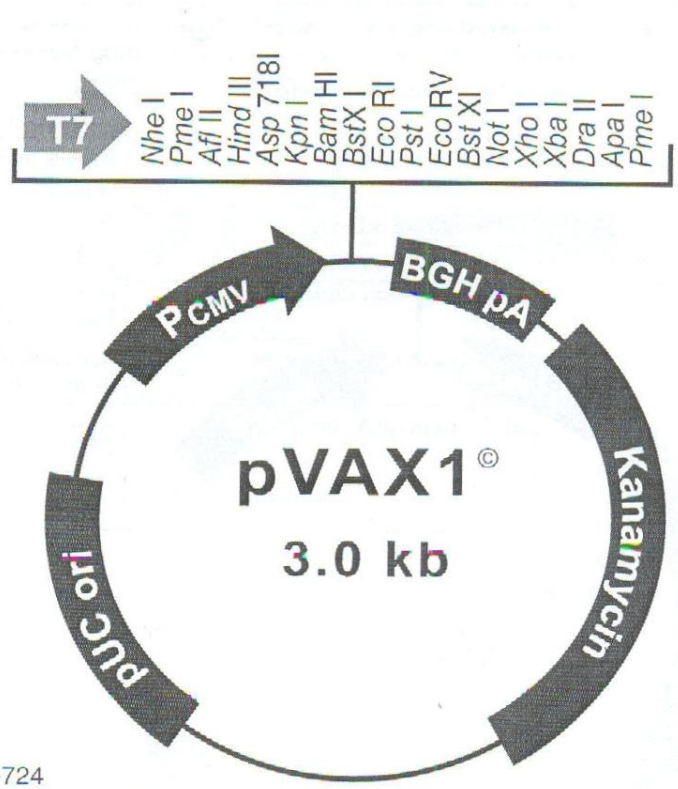
Appendix XIX: Sequencing primers for bergheipain1

Location on vector (5'→ 3')	**pCDNIIIAmpStrepT Primer**	Sequence (5'→ 3')
825 to 844; 63 Nucleotide from the BamH1 site	**Sense** (OSK2044) (20 bp; 50% GC; Tm=52^{o}C)	5'-**CTAACTAGAGAACCCACTGC**-3
1115 to 1134; 125 nucleotides from streptag's 3' terminus.	**Antisense** (OSK2045) (20bp; 60 %GC; Tm=56 ^{o}C)	5-**AGTGGCACCTTCCAGGGTCA**-3'
Location on gene (5→3)	I **Bergheipain-1** **Internal Primers**	Primer Sequence (5'→ 3')
1. 326 to 346	**Sense-1** (OSK2046) (21 BP; 62% GC; Tm=58 ^{o}C)	**5-ACGGAGATGGCGAAGACGAGC-3'**
2. 696 to 715	**Sense -2**(OSK2047) (20bp;30%GC;Tm=44 ^{o}C)	5'-**TTCTAAGGAAGAACTTGATA**-3'
3. 1116 to 1136	**Sense-3**(OSK2048) (21bp; 38% GC; Tm= 49 ^{o}C)	5'- **CTGCTTAAGTTATAGATGTGC**-3'
4. 1348 to 1368	**Sense -4**(OSK2053) (21bp; 331 %GC;Tm=47^{o}C)	5'-**GAGGATAGTCATACTAATGTA**-3'
1. 1280 to 1299	**Antisense-1** (OSK2049) (20BP;40% GC; Tm= 48^{o}C)	**5'-ATGATTTAACTCCGAAGCAC - 3'**
2. 823 to 841	**Antisense-2**(OSK2050) (19 BP;42% GC; Tm=47 ^{o}C)	**5'-GCTGTTCTGGATAGTCTAA-3'**
3. 574 to 595	**Antisense-3** (OSK2051) (22BP;27% GC; Tm=46 ^{o}C)	**5'-CAAATCTTACTAATTGTTCATC-3'**
4. 200 to 219	**Antisense-4**(OSK2052) (20BP;40% GC; Tm=48 ^{o}C)	5'-**TGTTTCATCGATTGCTGCTA**-3'

Appendix XX: Sequencing primers for bergheipain2

Location on gene (5→3)	I **Bergheipain-2** **Internal Primers**	Primer Sequence (5'→ 3')
1. 314 to 334	**Sense-1** (OSK2054) (21BP;24% GC; Tm= 43 °C)	**5-** GCATTATCGTTAAAGATAATA**-3'**
2. 660 to 680	**Sense -2**(OSK2055) (21bp;52%GC;Tm=54 °C)	5'-**CCACAGCATTGATCTCCGACA**-3'
3. 952 to 971	**Sense-3**(OSK2056) (20 BP; 35% GC; Tm= 46 °C)	5'- **GAAGATCTTATAGACATGGA** -3'
4. 1316 to 1336	**Sense -4**(OSK2057) (21 bp; 43 %GC;Tm= 50°C)	5'-**GAGAACGTGGTTACATAAGAC**-3'
1. 1175 to1194	**Antisense-1**(OSK2058) (20 BP;40% GC; Tm=48 °C)	**5'-ATCTGTACATTCTCCATCGA - 3'**
2. 838 to 858	**Antisense-2**(OSK2059) (21BP;43% GC; Tm= 50°C)	**5'-TCTAATAGCATATTGGGCTGC - 3'**
3. 417 to 440	**Antisense-3** (OSK2060) (24BP; 25 % GC; Tm=47 °C)	**5'- GCAATCTATTATGAACAATTTAGA - 3'**
4. 129 to 147	**Antisense-4**(OSK2061) (19 BP;37% GC; Tm=45 °C)	**5'- TATGACGGTAAAACCAAAC - 3'**

Appendix XXI: Map of pVax1© (http://www.invitrogen.com)



Comments for pVAX1©:
2999 bp

CMV promoter: bases 137-724
T7 promoter/priming site: bases 664-683
Multiple cloning site: bases 696-811
BGH reverse priming site: bases 823-840
BGH polyadenylation signal: bases 829-1053
Kanamycin resistance gene: bases 1226-2020
pUC origin: bases 2320-2993

Appendix XXII: Sequencing Reaction Mixture

Reaction Mixture	Bergheipain-1 clones			Bergheipain-2 clones		
	2	5	8	5	6	8
DNA stock concentration	461.7	410.3	492.9	397.1	345.3	340.2
Volume of ddH_2O (µL)	6.92	6.78	6.99	6.74	6.55	6.53
Volume of stock DNA (500 ng) used µL	1.08	1.22	1.01	1.26	1.45	1.47
Volume of Primer (sense/strep specific) (µL)	1	1	1	1	1	1
Volume of Big Dye (µL)	2	2	2	2	2	2
Total volume (µL)	11	11	11	11	11	11

Appendix XXIII: PCR PROGRAM FOR SEQUENCING REACTION

CNTRL	TUBE	LID=105 ^{o} C
	Temperature	Time(Hr:Min:Sec)
1	T = 95 ^{o} C	0:02:00
2	T = 95 ^{o} C	0:00:15
3	T = 60 ^{o} C	0:00:15
4	T = 60 ^{o} C	0:04:00
5	GO TO 2 REP 60	
6	T = 60 ^{o} C	0:07:00
7	Hold 4.0 ^{o} C	ENTER
END		

The program runs for 6.08 hr



Appendix XXIV: Picture of animals housed in special Experimental Cages

Appendix XXV: Amount (µg) and volume (µl) of vaccine administered to experimental mice

Plasmid Midiprep	Plasmid Storage Vial	Concentration of Plasmid DNA (µg/ml)	Amount of Plasmid DNA to be administered (µl) that will contain 50 µg of the Plasmid	Amount of Plasmid DNA to be administered (µl) that will contain 25 µg of the Plasmid	Amount of Plasmid DNA to be administered (µl) that will contain 10 µg of the Plasmid
A. Control Plasmid					
1. pVax1	A	725.5	68.9	34.46	13.78
	B	835.1	59.87	29.94	11.97
	C	1018.5	49.09	24.55	9.82
B. Without Transin					
1. pVax1BP1-5-colony6	A	1085.0	46.1	23.05	9.22
	B	1364.5	36.64	18.32	7.33
	C	1544.7	32.37	16.19	6.47
	D	1325.6	37.72	18.86	7.54
2. pVax1B2-5-colony3	A	1784.1	28	14	5.6
	B	1889.7	26.46	13.23	5.29
	C	1379.1	36.26	18.13	7.25
	D	1753.9	28.5	14.25	5.7
C. With Transin					
1. pVax1BP1-4-colony 1	A	987.8	50.62	25.31	10.12
	B	1146.3	43.62	21.81	8.72
	C	1430.2	34.96	17.48	6.99
	D	1368.4	36.54	18.27	7.31
2. pVax1BP2-5-colony4	A	1429.9	34.97	17.49	6.99
	B	1460.9	34.23	17.12	6.85
	C	1437.0	34.79	17.40	6.95
	D	1532.4	32.63	16.32	6.53



Appendix XXVI: Picture of the intramuscular administration of Vaccine on mice thigh muscle (quadricept). Disposable 1 ml insulin syringe, 29G x ½ (0.33 x 13 mm) (Agary pharmaceutical LTD China) was used for vaccine administration.

Appendix XXVII : The survival rate of mice in all experimental groups

Experimental Group	survival rate * Days (post challenge)								
	3	6	9	12	15	18	21	24	**60**
1	5/5	5/5	5/5	5/5	4/4	4/4	4/4	4/4	**100%**
2	5/5	5/5	5/4	5/2	4/1	4/1	4/1	4/0	**0%**
3	10/10	10/10	10/8	10/7	9/7	9/4	9/4	9/2	**0%**
4	10/10	10/10	10/6	10/5	9/4	9/3	9/3	9/2	**0%**
5	10/10	10/10	10/7	10/6	9/5	9/4	9/4	9/4	**37.5%**
6	10/10	10/10	10/4	10/4	9/3	9/3	9/3	9/3	**25%**
7	10/10	10/10	10/10	10/9	9/6	9/6	9/4	9/4	**25%**

*:Animals survival during the immunization period were expressed either as the ratio of the number of animals challenged with P. berghei-iRBC to the number of animals alive [3 to 24 days post challenge] or in terms of percentage of survivors [for 60th day post challenge].

Vaccination groups

1 (mice in this group were not vaccinated with DNA material but were given PBS as placebo and were not subsequently challenged with *P. berghei*).
2 (mice in this group were not vaccinated with DNA material but were given PBS as placebo but were subsequently challenged with *P. berghei*).
3 (this is the vector control group in which mice were vaccinated with the pVax1 vector not harbouring any gene insert).
4(mice in this group were vaccinated with pVax1BP1Streptag).
5(mice in this group were vaccinated with pVax1BP1Streptag).
6(mice in this group were vaccinated with pVax1TransinBP1Streptag).
6 (mice in this group were vaccinated with pVax1TransinBP2Streptag).