Localization of a dominantly expressed *Plasmodium berghei bir* gene

KORIR, PATRICIA JEBETT

W64-75965-2009

(Bsc. Medical Microbiology, HND Medical Parasitology and Entomology, Dip. Medical Laboratory Sciences)

Email- jebettagi@yahoo.co.uk

This dissertation is submitted in part fulfillment for the award of the degree of Masters of Science in Tropical and Infectious Diseases (MSc. TID)

University of Nairobi Institute of Tropical and Infectious Diseases (UNITID)

October 2011

23rd / Nov / 2011

SUPERVISORS

23rd November 2011

Prof. Walter Jaoko MB;Ch;B, MTropMed, PhD
Professor of Medical Microbiology
Department of Medical Microbiology
University of Nairobi

23rd of Nov. 2011

Dr. Steffen Borrmann. MD.
Group leader
Department of Infectious Diseases
Im Neuenheimer Feld 156
Universitätsklinikum Heidelberg
Germany

Declaration

This study represents an independent analysis within an ongoing project entitled "Disruption of a single member of the *hir* multigene family induces changes in disease outcome and improves host survival" by Yvonne Maier and the Borrmann group.

23rd/Nov/2011

Korir Patricia Jebett

Scholarship

My masters degree, travel to Germany and stay was sponsored by the Deutscher Akademischer Austausch Dienst (DAAD).

The laboratory work was sponsored and supervised by Dr. Steffen Borrmann, University of Heidelberg.

Acknowledgment

To Deutscher Akademischer Austausch Dienst (DAAD), for the scholarship and the opportunity to travel to Germany for this project.

To my supervisors Prof. Walter Jaoko and Dr. Steffen Borrmann for the guidance during the study.

To Yvonne Maier for the close supervision and guidance during the study and for taking time to teach me new techniques and most of all for your friendship.

To my Chairman Prof. Omu Anzala for giving me the opportunity to go back to school and for his support during the study period.

To Anja for taking time to teach me cell culture and for the time to be my friend.

To my colleagues, Mr. Eric Mbithi, Mr. Joel F. Kilonzo and Ms. Felista Wayua for taking up my duties during the period of my study.

Olivia and other UNITID staff.

Dedication

To Mali, Samuel Tagi, Vugutsa and my mother Margaret Korir Tagi.

To Antony Lugada Madoya

Abbreviations

µl- Microlitre

Amp- Ampicillin

bir – berghei interspersed repeats

BSA- bovine serum albumin

cir - chabaudi interspersed repeats

DNA deoxyribonucleic acid

ECM- Experimental Cerebral malaria

FCS- fetal calf serum

GFP- Green fluorescent protein

HRP- Horse radish peroxidase

ICAM-1 Intracelluar Adhesion Molecule 1

IFA- Immunoflourescence assay

IFN-γ- Interferon gamma

IL- Interleukin

kir - knowlesi interspersed repeats

LB – Luria Broth Medium

Min- minutes

o/n- over night

PBS- Phosphate buffer solution

PCR- Polymerase chain reaction

PfEMP-1 - *Plasmodium falciparum* erythrocyte membrane protein 1

*pir- Plasmodium interspersed repeat*s

RPM- revolutions per minute

RPMI- Roswell park memorial institute

RT- Room temperature

SP- Sulphadoxine-pyrimethamine

TNF-α- Tumor Necrosis Factor alpha

TSBT- Tris buffer saline tween

vir - vivax interspersed repeats

yir- yoelii interspersed repeats

Table of Contents

Abstract

Malaria is a major burden both to the economy and the health of the population in the tropics. In Kenya four species of *Plasmodium* occur, *P. falciparum, P. ovale, P. vivax* and *P. malariae*, but *Plasmodium falciparum* accounts for 98% of all the cases. *P. falciparum* is able to cause cerebral malaria through cytoadhesion, mediated by variant surface antigens, *Plasmodium falciparum* Erythrocyte Membrane Protein 1, (PfEMP1), which are encoded by the *var* genes.

Plasmodium berghei causes malaria in rodents. In this study *P. berghei*, ANKA strain which causes experimental cerebral malaria (ECM) was studied.

Antigenic variation in malaria parasites is achieved by the expressed antigens being switched frequently. In *P. falciparum* the process is mediated by PfEMP1 proteins, which are encoded by the *var* genes. The *var* genes are located at the subtelomeric regions. A large multigene superfamily, called the *Plasmodium* interspersed repeats (*pir*) which has striking similarities to the *var* genes were found in other *Plasmodium* species and the coding sequence of these genes are also located at the subtelomeric regions of the chromosomes. This family consists of the, *vir* genes in *P. vivax*, *kir* genes in *P. knowlesi*, *cir* genes in *P. chabaudi, yir* genes in *P. yoelii* and *bir* multigene family in *P. berghei.*

The aim of the study was to localize a dominantly expressed *bir* gene, called *BIRdom 1*, and study its localization in infected red blood cells using IFA. In a study by Maier *et al* they identified a dominant member of the *bir* gene, *BIRdom1,* and that is the gene chosen for the study.

In this study the *BIRdom* 1 gene was inserted into a vector and tagged with c-myc, so that the localization of the BIRdom 1 protein could easily be detected by immunoflourescence studies. Additionally it was used to investigate the protein expression of BIRdom 1 by western blot.

The insertion of the gene was successful and staining of the protein with the antibodies, mouse anti-cmyc and Alexa fluor anti mouse antibodies. The IFA showed a cytosolic expression of the BIRdom1 protein in blood stages. The uninfected red blood cells used as the control did not show any fluorescence. This showed that the expression of the BIR protein was cytosolic,

however we do not rule out that the gene could also be expressed on the surface and more experiments are needed.

This study was carried out at the University of Heidelberg, School of Medicine, Department of Infectious Diseases, Parasitology, under the Group of Dr. Steffen Borrmann.

1 Introduction

Malaria caused by the *Plasmodium* species of protozoan parasites is a major burden both to the economy and the health of the population globally. Malaria ranked 2nd and 8th highest in Africa and globally respectively in contributing to Disability Adjusted Life Year (DALY) with estimated 0.5- 2 million deaths each year (Snow *et al.,* 2003). Nearly one half of the world's population is at risk of malaria and approximately 1 million people mostly in the tropics, die annually from it (Hay *et al.,* 2009)

In Kenya, all the four species of *Plasmodium* that infect man, namely *P. falciparum*, *P. vivax*, *P.ovale*, and *P. malaria* are found. However, *P. falciparum* accounts for 98% of all the malaria cases (DOMC- Kenya).

P. falciparum causes the most deadly form of malaria and is responsible for >80% of deaths (Snow *et al.,* 1999). Among the most notable complications of *P. falciparum* malaria are cerebral malaria and anaemia, which account for most deaths (Marsh *et al.*, 1996).

A distinctive feature of *P. falciparum* compared with other human malaria parasites is its ability to sequester in the microvascular capillaries of various organs by the adherence of infected red blood cells (IRBCs) to the endothelial cell surface, these characteristic is shared with *P. berghei* and *P. chabaudi* (Cunningham *et al.,* 2010). Sequestration leads to obstruction of the capillaries, inflammation, endothelial damage, and organ dysfunction and failure. Hence, cytoadherence is central to the development of cerebral, placental and other organ-related severe pathological conditions (Ho and White, 1999; Cook and Zumla, 2009). Cytoadhesion in *P. falciparum* is mediated by PfEMP1 proteins which are encoded by a multigene family called the *var* genes (Scherf *et al.*, 2008; Montgomery *et al.,* 2007).

Antigenic variation is a process that enables parasites to evade the immune system. For *P. falciparum* this process is mediated by the variant surface antigens, PfEMP1, which are changed frequently. Beside the *var* gene family a large multigene superfamily is found in six different species of *Plasmodium*, called *Plasmodium* interspersed repeats, *pir*, which includes the *rif* gene in *P. falciparum, vir* genes in *P. vivax*, *kir* genes in *P. knowlesi*, *cir* genes in *P. chabaudi, yir*

genes in *P. yoelii* and *bir* multigene family in *P. berghei.* The *pir* multigene family shows similarities to the *var* genes of *P. falciparum.* Like the *var* genes, the *pir*s are localized at the subtelomeric regions of the chromosomes (del Portillo *et al.*, 2001; Janssen *et al.*, 2002, 2004; Cunningham *et al.*, 2009)

Antigenic variation in *Plasmodium falciparum* parasites pose a major challenge to vaccine development, since the repeated switching express different surface antigens making it difficult to target the erythrocytic stage of the parasite which is also the stage of the lifecycle that causes pathology. In malaria this antigenic variation is accomplished by expression of a single variant member of the large antigene gene family one at a time by the parasites (Janssen *et al.*, 2002).

The study of host pathogen interactions often relies on the use of adequate models. For *P. falciparum* study the ideal should be one that is capable of sequestration, only *P. berghei* and *P. chabaudi* are capable of doing that. The laboratory model chosen has to be closely related to the study parasite although from a different strain, but with relevance to the pathogenesis. Although *P. chabaudi* is able to sequester it is only *P. berghei* that is able to cause experimental cerebral malaria. The *pir* multigene family and the *var* genes are predominantly found in the subtelomeric regions (Janssen *et al.*, 2004) and the fact that the *var* genes encode for the PfEMP1, which is linked to cerebral malaria led to the choice of using *Plasmodium berghei* in this project to study antigenic variation by doing localization and expression of a dominantly expressed *bir* gene. *Plasmodium berghei* has been used in a lot of laboratory studies of experimental cerebral malaria to study immunology, genetics and drug resistance among others.

It is thought that the *pir* genes play an important role in the survival of the parasite and its development. Several BIR proteins have been linked to lipid raft, which could imply an involvement in signaling, trafficking and folding (Cunningham *et al.*, 2010).

The rodent malaria parasite *Plasmodium berghei* with its natural host being the thicket rat *Grammomys surdaster*, is able to cause experimental cerebral malaria (ECM) in mice. It is not yet clear if the ECM in rodents can be used as model for *P. falciparum* cerebral malaria in humans however murine models have been useful in studying the molecular mechanisms underlying the human cerebral malaria (Lovegrove *et al.*, 2006). Looking at the expression and

localization of the dominant expressed genes, would give us more insights in the mechanisms of antigenic variation and cytoadhesion in severe *P. falciparum* malaria.

1.1 Justification

Antigenic variation is the major reason that malaria infection results in chronic parasitemia leading to complications of the disease. Understanding the role of antigenic variation in *P. berghei* in the rodent model will help us understand its role in human malaria and thereby help in designing better prevention and treatment strategies.

Studies show that *P. berghei*, *P. falciparum* and *P. chabaudi* express adhesins and hence are able to sequester (Cunningham *et al.,* 2010). However, of the two rodent malaria, it is only *P. berghei* which causes ECM. For this reason, this study decided to use *P. berghei* ANKA strain.

1.2 Objective

1.2.1 General objectives

The general objective of the study was to localize a dominantly expressed *bir* gene, the *BIRdom* 1, and study its localization in the infected red blood cells, using IFA during different stages of the erythrocytic cycle.

1.2.2 Specific Objective

1. To localize the *BIRdom1* gene
2. To describe the localization and expression in infected red blood cells during different stages of the erythrocytic cycle

2 Literature review

2.1 Malaria

The malaria parasite belongs to the genus *Plasmodium,* which are sporozoan parasites of the red blood cells, transmitted through a bite of an infective *Anopheles* mosquito. *Plasmodium species* infect mammals, reptiles and birds. Five species have been found to infect humans. *P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi* (Cook and Zumla, 2009; Vythilingam *et al.*, 2008). Initially only four species were thought to occur in man, then cases of a simian malaria parasite, *P. knowlesi*, that infects the long tailed and pig tailed macaque monkeys, were reported in humans in South East Asia (Vythilingam *et al., 2008;* Cox-Singh and Singh, 2008; Cook and Zumla, 2009).

2.1.1 Transmission

Transmission of malaria mostly occurs from a bite of an infected *Anopheles* mosquito. *Anopheles* is the only species that is able to transmit malaria. *Anopheles* mosquitoes breed in shallow water collection of freshwaters. Climatic conditions like rainfall, temperature and humidity affect transmission and mosquito survival (WHO fact sheet 94). Malaria is endemic in 109 countries and found throughout the tropics. In Africa *P. falciparum* is the most wide spread while *P. vivax* is found in Central and South America, Indian subcontinent, Middle East and North Africa. *P. malariae* is distributed throughout Africa while *P. ovale* is common in West Africa (Cook and Zumla, 2009).

The optimum transmission conditions are high humidity, and temperature of between 20^0 and 30^0 C. At temperatures below 16^0C and above 33^0C there is no transmission, because this is unconducive for the development of sporogony. For transmission to occur the mosquito must survive for a period to allow sporogony to take place, after a blood meal from an infected person, while the sporogony is dependent on the ambient temperatures (Cook and Zumla, 2009). Other conditions also affect transmission of the parasite, the availability of the human reservoir with viable gametocytes and the presence of the right vector. There are estimated close to 400 species of Anopheline mosquitoes, with approximately 80 species being able to transmit malaria and 45 are thought to be important vectors (Cook and Zumla, 2009).

In addition malaria can also be transmitted congenitally, from an infected mother to the unborn child (Lesi *et al.*, 2010; Mali *et al.*, 2011). Malaria can be transmitted by blood transfusion of infected blood from a donor to a recipient (Mali *et al.*, 2011).

2.1.2 Lifecycle of *Plasmodium falciparum*

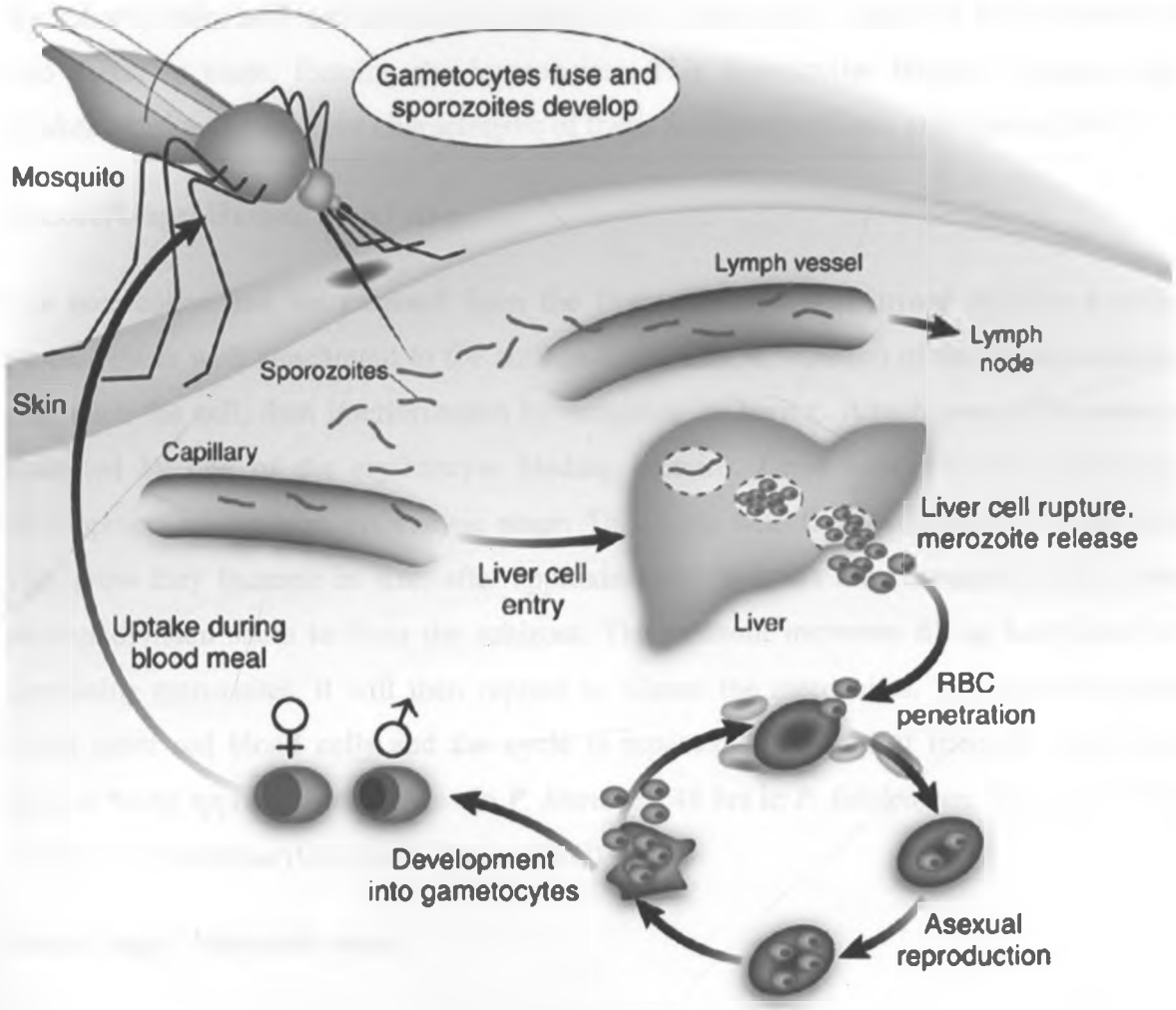

Figure 1 Life-cycle illustration by Jones and Good (2006)

The life cycle of malaria is made up of 3 stages, the pre-erythrocytic, asexual and sexual stage, with the first two stages occurring in the human host and the last stage in the mosquito vector.

Pre- erythrocytic/ human liver stage

The female anopheline mosquito will inoculate sporozoites during feeding to the human host. After inoculation they enter the circulation either directly or via the lymph channels, and target the liver parenchymal cells. After approximately 45 minutes the sporozoites have either entered the liver cells or have been cleared from circulation, once they gain entry into a hepatocyte they begin the asexual reproduction. Then the hepatic schizont ruptures and releases thousands of merozoites into the circulation, this depends on species and takes approximately 5.5 days in *P. falciparum*, while in *P. malariae* it is 15 days. In *P. ovale* and *P. vivax,* the liver schizonts can go into a resting stage, forming the hypnozoites. This hypnozoites become dormant and later awaken causing the relapses characteristic of these two species (Cook and Zumla, 2009).

Asexual stage/ Human blood stage

The merozoites that are released from the liver schizonts will invade red blood cells. This process starts with attachment to the surface of the cell, orientation of the apical complex so it can touch the cell, then interiorization by wriggling or boring. Attachment of the merozoite is mediated by one of the erythrocyte binding proteins. Once inside the red cell, they start development of the intra eythrocytic stage. This starts with the development of ring forms, as they grow they increase in size, after approximately 36 hours after invasion of the merozoite, nuclear division starts to form the schizont. The schizont increases filling the whole cell and containing merozoites, it will then rupture to release the merozoites. The released merozoites infect other red blood cells and the cycle is repeated. The asexual lifecycle varies with the species being approximately 24 hrs in *P. knowlesi*, 48 hrs in *P, falciparum, P.ovale, P vivax* and 72 hrs in *P. malariae* (Cook and Zumla, 2009).

Sexual stage/ Mosquito stage.

After a few asexual life cycles some parasites develop in the sexual form, the gametocytes, these are then picked up by the female anopheline mosquito during the next blood meal. After the ingestion of the gametocytes they become activated inside the gut of the mosquitoes, the male gametocyte undergoes rapid nuclear division and each of the 8 nuclei formed has a flagellum. The motile microgametes seek the female macrogametes. When fusion occurs a zygote is

formed, this develops into an ookinete which penetrates the wall of the mosquito midgut to encyst and form an oocyst. The oocysts increases in size and when mature bursts to release sporozoites, into the coelomic cavity of the mosquito. The sporozoites migrate to the salivary gland and wait to be released during the next blood meal and the cycle is repeated (Cook and Zumla, 2009).

2.1.3 Clinical Presentation of *P. falciparum*

Malaria comes from the Italian word meaning bad air. The terminology dates prior to the discovery of the causative agent, when it was associated with living near the swamps and the smell from the swamp was thought to be responsible for the illness. Malaria's first symptoms are "flu"–like, with fever, headache, muscle ache and joint pains. These symptoms begin approximately 2 weeks after an infective bite. The disease presents with the chills and shaking, followed by the hot phase with fever of up to 40^0C, this will resolve for 24- 48 hours depending on the causative malaria species (CDC; Nester *et al.,* 2004; Cook and Zumla, 2009).

Uncomplicated disease may be associated with anemia and jaundice, if untreated it can lead to severe/ complicated malaria.

2.1.3.1 Complicated / severe malaria

P. falciparum is the most severe form of malaria. It causes most of the complications of malaria, which include severe anemia, cerebral malaria, respiratory distress and low birth weight (Snow *et al.,* 2003; Marsha *et al.,* 1996). Severe malaria is a complex of syndromes affecting many organs, these include but are not limited to, coma, renal failure, pulmonary oedema, severe anemia, respiratory distress, coagulopathy and thrombocytopenia, black water fever, splenomegaly, liver dysfunction, acidosis, hypoglycemia and complications in pregnancy (Maitland and Marsh, 2004; Cook and Zumla, 2009).

Metabolic acidosis manifesting as respiratory distress is a component of severe malaria. Metabolic acidosis is demonstrated as a predictor of fatal outcome (Maitland and Marsh, 2004). Respiratory distress has been used to identify high risk outcome in children with metabolic acidosis being predominantly found. Severe malaria falls into three overlapping distinct syndromes, which are respiratory distress, impaired consciousness and severe anemia (Marsh *et*

al., 1995). Hypovolaemia has been linked strongly to acidosis of severe malaria (Maitland and Marsh, 2004). Hypoglycemia is a risk of death in complicated malaria and associated with poor prognosis (English *et al.,* 1998)

The renal injury in severe malaria results from acute tubular necrosis, this is presumed to be due to renal microvascular obstruction and cellular injury as a consequence of sequestration in the kidney, but there is recovery in the malaria survivors. Pulmonary oedema in severe malaria results from a sudden increase in the pulmonary capillary permeability, which is not reflected in other vascular beds. Severe anemia in malaria is multifactorial, resulting from destruction of red cells that contain the parasites at the merogony stages, splenic clearance of infected cell and clearance of non parasitized cells, compounded by bone marrow dyserythropoeisis (Cook and Zumla, 2009).

Black water fever is a condition in which there is massive intravascular haemolysis resulting in hemoglobinuria which can be seen through passage of Coca-Cola coloured urine, the condition is poorly understood. Tropical splenomegaly syndrome is manifested as an enlarged spleen, it occurs as a result of cellular multiplication and structural changes, and also increased capacity to clear red blood cells, from the circulation by immune mechanism and by filtration. Increased filtration by the spleen contributes to severe anemia, increased splenic clearance also causes thrombocytopenia (Cook and Zumla, 2009).

Malaria in pregnancy, causes anemia and low birth weight, and most of the babies are lower than <2.5 kg. Low birth weight in malaria is due to intrauterine growth retardation, in areas of high transmission there is premature deliveries. The anemia in pregnancy is a risk factor for maternal mortality. Hypoglycaemia and acute pulmonary edema are complications of malaria in pregnancy (Cook and Zumla, 2009).

2.2 Cerebral Malaria

Severe malaria affects mostly children under five years. Cerebral malaria has been associated with cognitive impairment and other neurological disability in children and also with retinopathy (Idro *et al.,* 2010; Bangirana *et al.,* 2006; Maude *et al.*, 2009).

Cerebral malaria is thought to be due to sequestration of infected red blood cells within the capillaries of the cerebral cortex (Grau *et al.,* 1989). Adhesion of infected cells results in activation of the endothelial cells leading to over expression of different mediators like cytokines contributing to the pathology. Although the mechanism of the adhesion is not well understood, it appears however that the adhesion of parasitized red blood cells leads to activation of molecules to which they bind (Mazier *et al.,* 2000).

2.2.1 Cytoadhesion

In the pathologenicity of *P. falciparum* cytoadhesion, occurs due to the ability of infected red blood cells to adhere to capillary and postcapillary venular endothelium during the second half of the 48hr lifecycle (Ho and White, 1999). Cytoadhesion is mediated by binding of the ligands on infected red blood cells to receptors on the surface of vascular endothelial cells (Marsh and Snow, 1997). Cytoadhesion presents the parasite with survival advantages due to the microaerophilic venous environment and provides an escape from clearance of the spleen (Ho and White, 1999). The stage and host cell specificity of cytoadherence suggests the involvement of specific host or parasite ligands expressed at the surface of infected red blood cells (Berendt *et al.,*1989; Ho and White, 1999) and a number of endothelial receptor molecules identified, these receptors are thrombospondin (TSP), CD36, ICAM-1, VCAM-1, PECAM-1, P-selectin, E-selectin and thrombomodulin (Berendt *et al.,*1989; Ho and White, 1999; Mazier *et al.,* 2000; Marsh *et al.,* 2011). Cytoadhesion does not occur in the placenta although there is sequestration, this is because the infected red blood cells are free in the intervillous spaces but there is no adherence to the endothelial surfaces (Ho and White, 1999).

2.2.2 Roles of Cytokines and Nitric oxide

Increased levels of TNF-α, IFN-γ, IL-1β, IL-4 have been demonstrated in cerebral malaria. These cytokines have an effect on cytoadhesion (Mazier *et al.,* 2000; Nahrevanian, 2004). Nitric oxide has been shown to affect the production of some cytokines which include TNF-α and IFN-γ (Nahrevanian, 2004). The nude mice are unable to develop cerebral malaria because they lack the T-lymphocyte, hence the importance of it for the development of these complications (Grau *et al.,* 1989). TNF α is released in large amounts by activated macrophages, while reduced levels of TNF α prevents the focal accumulation of macrophages in the brain vessels (Grau *et al.,* 1989). TNF α induces endothelial changes which include increase in adhesiveness, hence its

release plays a role in the pathogenesis of cerebral malaria or disease severity in malaria (Berendt *et al.,* 1989; Grau *et.al.*, 1989). The proinflammatory cytokines, TNF-α, IFN-γ and IL1 can upregulate the expression of ICAM-1 (Berendt *et al.,* 1989; Ho and White, 1999) which shows the importance of cytokines in the pathogenesis cerebral malaria.

2.2.3 PfEMP 1

There are at least five parasite proteins that are associated with the cell membrane of infected red blood cells at the different stages of the lifecycle, three of them are associated with knobs, PfEMP-1 PfEMP-2 and PfHRP-1 and of these only PfEMP1 extends beyond the cell surface to mediate cytoadhesion (Ho and White, 1999). The receptors on the endothelial space are known to interact with parasitized red blood cells, via PfEMP1, these receptors are thrombospondin (TSP), CD36, ICAM-1, VCAM-1, PECAM-1, P-selectin, E-selectin and thrombomodulin (Ho and White, 1999; Mazier *et al.,* 2000; Marsh *et al.,* 2011).

2.3 Antigenic Variation

Antigenic variation is the reason why people who reside in malaria endemic areas lack continuous immunity to the parasite, and if they develop any immunity it is partial (Bull *et al.,* 1998; del Portillo *et al.*, 2001). In a study by del Portillo (2001) they showed that in natural infection VIR protein are immunogenic and that patients are exposed to parasites displaying different VIR variants which implying that the proteins are immunovariant (del Portillo *et al.,* 2001).

Antigenic variation is a process that enables the parasite to evade the immune system. This process is also mediated by the variant surface antigens, PfEMP1, expressed on the surface of infected red blood cells. The *var* genes are changed frequently, and the parasite expresses one gene at a time. It is speculated that PfEMP1 proteins are encoded by 60 *var* genes, expressing a gene at the time while the rest are kept silent (Scherf *et al.*, 2008; Montgomery *et al.,* 2007). Cerebral malaria is caused by cytoadhesion which occurs due to infected red cells sequestering in the capillaries leading to reduced blood flow. Cytoadhesion is mediated by PfEMP1 proteins (Jane *et al.*, 2011; Kraeme and Smith, 2006). ICAM1 is a receptor expressed on brain endothelial cells, and it is associated with cytoadhesion and pathogenesis of cerebral malaria (Chakravorty and Craig, 2005; Fernandez *et al.,* 1997).

Beside the *var* gene family a large multigene superfamily was found in six different species of *Plasmodium*, called *Plasmodium* interspersed repeats, *pir*, which includes the *vir* genes in *P. vivax*, *kir* genes in *P. knowlesi*, *cir* genes in *P. chabaudi, yir* genes in *P. yoelii* and *bir* multigene family in *P. berghei.* The *pir* multigene family shows similarities to the *var* genes of *P. falciparum.* Like the *var* genes, the *pir*s are localized at the subtelomeric regions of the chromosomes (del Portillo *et al.,* 2001; Cunningham *et al.,* 2005, 2010; Janssen *et al.,* 2004). Based on this fact together with the finding that the PIR proteins are expressed on or near the plasma membrane of infected red blood cells it is considered that the members of the *pir* multigene family could play a role in antigenic variation as well (Janssen *et al.*, 2002). *Yir* genes found in *Plasmodium yoelii,* have been shown to be transcribed during erythrocytic cycle of the infection, and YIR proteins are expressed at the surface of parasitized red blood cells (Cunningham *et al.*, 2010).

2.4 Plasmodium berghei

Plasmodium berghei causes rodent malaria and is found in central Africa. It infects the thicket rat, *Grammomys surdaster,* which was described by Vincke and Lips in 1948. *P. berghei is* used in the laboratory to study immunology, molecular parasitology and other aspects of disease due to its ability to cause ECM in laboratory animals.

The *bir* gene family is located in the subtelometric region, and it contains 245 genes (Cunningham *et al.*, 2005). In studying the transcription profile of *bir* genes (Maier *et al.*, unpublished), showed that there is a dominantly expressed *bir* gene in blood stages of *P. berghei* infected mice and rats and that the one gene *BIRdom1* was more dominantly expressed than the other *bir* genes (Maier *et al* unpublished). After the findings by Maier *et al.,* (unpublished) the next step in the study was to characterize the dominant gene by localizing and studying the expression.

3 Materials and Methodology

3.1 Study Design

This was an experimental laboratory study, where 3 months were spent in the laboratory, from 1st April 2011 to 30th June 2011.

3.2 Study Population

The study was done using the NMRI (Naval Medical Research Institute) mice, at the University of Heidelberg, Department of Infectious Diseases.

3.3 Study methods

The *BIRdom* 1 gene was inserted into a vector and tagged with c-myc, so that the localization and expression of the BIRdom 1 protein could easily be detected by immunofluorescence studies and western blot.

In the study we used the genomic DNA of the *P. berghei BIRdom* 1 gene. *BIRdom* 1 gene was cloned into the PB279 vector, which was carried out in two steps because of the construct size which was 2.5 kb. The construct was divided into 2 fragments. The 1st fragment included a 1.35 kb part of the promoter amplified with primers, and contained restriction sites for Sac II and Not I, for integration into the vector. The 2nd fragment contained a short part of the promoter (100 b) and restriction sites for Not I and Xba together with the open reading frame of the *BIRdom1* gene

The 1st fragment of the *bir* gene was inserted into the vector's multiple cloning site using the Sac II and Not I restriction enzymes. After the 1st fragment was inserted, the 2nd fragment was then inserted at the Not I- Xba I restriction sites (sequences are in the PCR methodology). The stop codon of the gene was skipped so that the c-myc tag which was fused at the 3' end of the gene was transcribed together with the gene, leading to a BIRdom1-cmyc fusion gene.

PCR was used to amplify the fragments and purification of the product done using QIAquick PCR purification kit. Ligation was done using the T4 ligase. Transformation was then done using competent *E. coli* XL1 blue and the ligation added to the *E. coli* cells and mixed gently. To check for the success of the insertion of the 1st fragment and the construct plasmid DNA isolated using the QIAprep Miniprep kit, test digest and gel were done and pictures done.

For confirmation of the full construct a test digest was done using Sac II and Xba I which cuts the whole construct (1st and 2nd fragment) out of the vector. Since Not I is in the middle of the promoters, it was not used, because using it in combination with one of the previous enzymes would have shown either the 1st or the 2nd fragment but not the full construct.

Once the construct was ready re-transformation was done to multiply the plasmid DNA followed by alcohol precipitation of the plasmid DNA for transfection. A donor mouse was then infected with the *P. berghei* ANKA strain. Transfection was done using electroporation and naive mice were infected with the transgenic parasites we had created. The vector contained features that were important for the experiments. The *B-Lactamase* gene was for resistance to ampicillin, and this played an important role during transformation, the *E.coli* that were not transformed did not grow on the culture plate. The *Toxoplasma gondii* dihydrofolate reductase thymidylate synthase (*Tg*DHFR/TS) gene which provided resistance to pyrimethamine. After transfection the mice were given pyrimethamine in the drinking water to clear parasites that do not possess the vector that was transfected, since only the ones that had the cloned were drug resistant. After infection had been confirmed and the mice were blood slide positive for *P. berghei* they were sacrificed and the samples studied for localization and expression using IFA and Western blot. This was then repeated with 2 additional mice.

3.4 Ethical considerations

According to the Belmont report of 1979, where beneficence has to be considered during research, it was not possible to use human subjects, this is because the study required infection of study population with live parasites. In this study mice were used.

The mice used in this study were treated as per the animal regulations requirement for research animal and relevant guidelines of animal protection approved by the relevant German authorities.

The experiments were approved by and conducted according to the University of Heidelberg, ethics guidelines.

3.4.1 Animal keeping

The mice were kept in Makrolon- cages, 5 animals per cage. The cages were kept in a special room with constant room temperature of 22° C and humidity of 50- 60%.

3.4.2 Food

The mice were fed with Standard-Food (dry pellets) SSNIFF, they were also given water.

3.5 Biological Material

3.5.1 Escherichia coli- strain

XL-1 Blue (Stratagene)

*sup*E44, *hsd*R17, *rec*A, *gyr*A46 t*hi*, *rel*A1, *lac*-, F'[*proAB*+,*lacI*q, LacZ M15, Tn10(*tet*r)].

3.5.2 Vectors and Plasmids (*Plasmodium berghei*)

P. berghei transfection vector PB279 was used. This vector contains the selection marker *DHFR/TS (Dihydrofolate-Reductase* thymidylate-synthase) from *Toxoplasma gondii.* Additionally the vector includes sequences of the *β-Lactamase-Gene* from a *pBluescript-Plasmid.* This gene provides resistance against Ampicillin.

The transcription of the marker is in the opposite direction from the gene of interest which was cloned into the multiple cloning site of the vector.

3.5.3 Mouse strains

The mice used in this study were Naval Medical Research Institute, (NMRI), Charles River Laboratory, Sulzfeld, outbreeding mice.

3.5.4 Parasite strains

The rodent *Plasmodium berghei* ANKA clone 507 Green fluorescent protein (GFP) were used in the study. This strain imitates many features of cerebral malaria in humans causing ECM, in rodents.

3.6 Procedures

3.6.1 Overnight culture for competent *E.coli* cells

This work was done under flame to avoid contaminations. Briefly a yellow pipette tip was dipped into an aliquot of competent *E. coli* XL1 blue cells (-80^0 C) and the tip put into 3 ml of LB medium (without Amp). The next day: 350 µl of overnight culture was added to 50 ml of LB medium in an Erlenmeyer flask (without Amp!) and left to grow for at least 1.5 hrs at 37^0 C shaking. It was then measured at OD_{600} = 0.3-0.4 (photometer) using LB medium as a blank. The culture was then centrifuged at 2400 rpm 10 min 4^0 C, the supernatant was carefully discarded. The pellets were resuspended in 15 ml TBF 1 (cold) and incubated on ice for 1 hr. It was afterwards centrifuged 2000 rpm 5-10 min 4^0 C, the supernatant was discarded. The pellet was resuspended in 900 µl TBF 2 (ice cold) and 50 µl aliquots were made (on ice) and stored at -80^0 C.

3.6.2 Digests

To insert the 1st fragment, double digests of the vector and the fragment were done, with Sac II and Not I. For insertion of the 2nd fragment a double digest with Not I and Xba I was done.

Vector digestion was done by mixing, 5 µl of the vector DNA, 32.4 µl of water, 5 µl of buffer, and 5 µl of 10x BSA 1.3 µl of the 1st enzyme and 1.3 µl of the 2nd enzyme. For the PCR product which is the fragment to be inserted a mixture was also done. Seven (7) µl of water was mixed with 30 µl of purified PCR product, 5 µl of buffer two, 5 µl of 10X BSA and enzymes 1 and 2 each 1.3 µl. The digestion was then incubated at 37^0 C overnight. The next day ligation was done.

3.6.3 Ligation

Ligation was done using 1 µl of T4 ligase, 1 µl of T4 buffer, 6 µl of the PCR product and 2 µl of the vector. The ligation was left to stand at room temperature for 1 hr. After ligation transformation was done.

3.6.4 Transformation of competent cells

The competent *E. coli* XL1 blue cells (50 µl aliquots at -80° C) were thawed on ice for 5 to 10 minutes. Two (2) µl of the ligation was added to the cells and mixed gently. It was then incubated for 30 min on ice. During incubation the following preparation was done. Two heating

blocks were set at 42^0 C and at 37^0 C (shaking) respectively. The LB/Amp plates were put at 37° C and LB medium put at 37^0 C (1 ml for each reaction).

The bacteria were then heat shocked for 45 sec at 42^0 C, followed by incubation for 2 min on ice. One (1) ml of pre-warmed LB medium was then added and incubated at 37^0 C for 1 hr while being shaken. After 1 hr, the mixture was centrifuged for 1 minute full speed and the supernatant discarded by decanting. The pellet was then resuspended and plated on pre-warmed LB/Amp plates and incubated overnight at 37^0 C.

3.6.5 Bacterial overnight culture:

Fresh LB/ Amp medium was prepared by mixing 100 ml of LB medium and 100 µl of Amp Stock. Three (3) ml of LB/Amp medium for each reaction was added in a Greiner 14 ml PP Tube. Colonies were picked using yellow pipette tips and the tip put into the tubes containing medium, then incubated over night at 37^0 C shaking. The next day, extraction of plasmid DNA was performed using Qiagen Miniprep™ Kit, as outlined in the handbook.

3.6.6 Confirmation of the transformation

To confirm whether the transformation was successful, Minipreps were done as indicated in the QIAprep™ Miniprep handbook. After the plasmid DNA was isolated the test digests were done. For the test digests, 2 µl of plasmid DNA, 1 µl of enzyme 1, 1 µl of enzyme 2, 2 µl of buffer, 2 µl of 10x BSA and 10 µl of water were mixed and incubated at 37^0 C overnight. The mixture was then loaded onto a gel and run. Gel photos were later taken.

3.6.7 Linearization

After the integration of both fragments was confirmed, Qiaprep ™ mini prep was done followed by a single digest at the introduced Not I restriction sites to cut the vector into a linear shape. Linearization of the plasmid was done so as to introduce it into the merozoites of *P. berghei* for transfection. Test digest was done as follows; 70 µl of the mini prep product was mixed with 2 µl of enzyme, 10 µl of buffer 3, 10 µl of ten times BSA and 8 µl of water. This mixture was incubated at 37^0 C overnight. A gel was run the next day to confirm and photos taken.

3.6.8 Infection of mice:

Female NMRI mice were injected intraperitoneal (i.p.) with 100 µl of cryopreserved samples of blood stages of the *P. berghei* ANKA strain. Daily monitoring of parasitemia was done by collection of 10 µl of tail blood on slides and blood smears made. After 1 min fixation in Methanol, the slides were stained for 15 min in Giemsa solution. Parasites were detected using a light microscope (100x, oil) and parasitemia was calculated as follows:

$$\frac{\text{No of parasites}}{\text{No of erythrocytes}} \times 100 = \text{Parasitemia in \%}$$

3.6.9 Collection of blood

Mice were sacrificed when the level of parasites had reached the required parasitemia or if they showed symptoms of illness. The blood was collected by cardiac puncture using a heparinized syringe under anesthesia

3.6.10 Transfection

In vitro culture of schizonts for transfection.

Transfection medium was prepared by mixing 160 ml of RPMI, 40 ml of FCS and 40 µl of gentamycin. The mixture was sterilized using filtration. Ten (10) ml of transfection medium was mixed with 0.25 ml of heparin and incubated at 37^0 C. Overnight cultures were prepared by transferring 100 ml of transfection medium into 500 ml Erlenmeyer flasks and pre-warmed at 37^0 C. Blood from the donor mice was added into the falcon containing the 10 ml transfection medium/ heparin, it was then centrifuged for 8 min at 1000 rpm. Working under a sterile hood, the supernatant was discarded and pellets resuspended in 20 ml of transfection medium. With the pipette boy switched off, the solution was slowly dropped into the Erlenmeyer flask containing transfection medium. This was then incubated at 37^0 C for 15 min, the shaking was switched on and the culture left shaking for 18 hr.

Transfection

Blood from the culture was read after 18 hr. The culture was split in 4, 50 ml falcon tubes and underlayed with 10 ml of 55% nycodenz. The tubes were balanced exactly and centrifuged for 25 min at 1000 rpm without break. The schizonts formed a dark red ring between the medium and the nycodenz, this was collected using a Pasteur pipette. The schizonts were placed into 2, 50 ml

falcons, and transfection medium added to balance. This was centrifuged at 1500 rpm for 8 min without break, after which the supernatant was discarded and pellets resuspended in transfection medium. One (1) ml was transferred into eppendorf tubes and centrifuged at full speed for 20 seconds. Ten (10) µl of plasmid DNA was mixed with 100 µl of nucleofactor solution. The schizont pellets was resuspended in nucleofactor/ DNA solution and transferred into Amaxa cuvette and transfection done. Fifty (50) µl of transfection medium was added into the cuvette and 150 µl taken out using special Amaxa pipettes and transferred into new eppendorf tubes. Two (2) NMRI mice were injected intravenous with 75 µl of the tranfection product.

Selection of parasites

After injection of the transfected parasites, the next day the mice were check for parasite by making blood smears, staining, and examining under a light microscope. The mice were given drinking water containing pyrimethamine to clear non transfected parasites.

3.6.11 PCR

The purpose of polymerase chain reaction (PCR) was to make a huge number of copies of the gene. The restriction site of the restriction enzymes are underlined. The primer sequences are in red.

P1 for Sac II for PB279

TCCCCGCGGGAGTTATTATTATATAACTATAATATAATC

P1 rev Not I for PB279

ATTTGCGGCCGCCGCAAATGCTTTAACCCTATATATAA

P2 for Not I for PB279

ATTTGCGGCCGCTCACATATTGGGGATAAGATATATC

Open Reading Frame (ORF), rev XbaI without stop. PB279

GCTCTAGATTCATTTTCTTCTTTATTTTTTAGC

Tg forward Primer

CCATCTGGATTCGTCCGTGCGGG

Test forward Primer

CATAAGGTATAATAATAGATTAATCAC

BIRdom1 forward Primer

CAAGAATGATTGCACAAAATGTTC

PCR products of less than 2kb

The master mix was made by using 5 µl of 10x buffer, dNTP 5 µl, MgCl. 4 µl, forward primers and reverse primers each 0.5 µl, 32 µl of H_2O and1 µl of Taq Polymerase (fermentas). Two microlitre (2 µl.) of the DNA to be amplified was added to make a total of 50 µl. The Taq was added last in the mix because it is temperature sensitive and requires to be frozen always. A control was run with every test. This protocol was used for the genomic DNA before transformation to confirm the presence of the 1st and 2nd fragment which was 1.35 kb and 1.2 kb respectively. It was used again later for the episomal construct which was 1.7 kb. For testing the episomal construct the *Birdom1* forward primer and *Tg*-forward primers for reverse.

Long range PRC for products more than 2 kb

For PCR products of more than 2 kb the long range PCR (Qiagen) was used. The master mix was made by adding 2.5 µl of buffer, 1.25 µl of dNTP, 0.5 µl of reverse and forward primers, 19.05 µl of H_2O and 0.5 µl of the LongRange PCR Enzyme Mix making a total of 24 µl. One (1) µl of the DNA to be amplified was then added. The running time for the long range was 3.5 hrs.

The long range PCR was used to check the integration, which was 3.9 kb. Using test forward as the forward primer and *Tg* forward as the reverse primer.

Gel preparation and loading

Before the PCR product was used, confirmation was done on agarose gel. Two (2) µl of the PCR product was mixed with 10 µl of the loading dye and loaded on to the gel. Ten (10) µl of the 1 kb DNA ladder was also loaded on to the gel, and then run at 130 volts for 1 hr. After 1hr the results were read and the pictures taken.

3.6.12 Isolation of blood stage parasites

3.6.12.1 Preparation of column:

A 5 ml syringe was used and columns prepared as follows: 1 cm cotton, 2-3 cm Cellulose powder (Whatman), 1 cm Glass beads unwashed. To equilibrate the column, 1xPBS was used, and the blood was added to the column. When the colour of the eluted drops turned red, the

drops were collected in a 15 ml falcon. Fourteen (14) ml of eluat was collected and centrifuged for 8 min at 1500 rpm (erythrocyte pellet). The supernatant was discarded, and the pellet resuspended in 10 ml of 0.2% Saponin/PBS to lyse the erythrocytes, then centrifuged for 8 min, at 2800 rpm (parasite pellet). The supernatant was discarded, and pellet resuspended in 1ml 1x PBS and transferred into a 1.5 ml Eppendorf tube, then centrifuged for 2 min, at 7000 rpm. The supernatant was discarded and the pellet was resuspended in 200 µl 1x PBS. Isolation of **genomic DNA** using the QIAamp DNA blood mini Kit was done or sample stored at -20° C.

For **Protein** extraction the above procedures were followed, but instead of the last step, the pellet was resuspended in approx. 100 µl of 2x SDS Sample Buffer.

3.6.13 Western Blot procedure

SDS Polyacrylamide gel electrophoresis

The erythrocyte proteins were resolved on a SDS polyacrylamide gel with a 12% resolving SDS gel. The gel was cast between two glass plates of the Mini-Protean 3 Cell with a spacing of 1mm. After polymerization of the resolving gel, a 5% stacking gel was prepared and cast and allowed to polymerize for 30 min.

Denaturation of Protein samples

Ten (10 µl) of the erythrocyte lysate sample was mixed with an equal volume of SDS sample buffer (2x) and the sample heated for 5 minutes at 95^0 C. Ten (10) µl of each sample and 7 µl of the marker were then loaded onto the gel. The electrophoresis was run at 200 volts for 1 hr in 1x SDS running buffer.

Blotting (Semi-dry)

The gel was blotted on a PVDF transfer membrane with a pore size of 0.45 µM. Before the blotting the membrane was cut according to the gel size, soaked in Methanol and then in transfer buffer for 20 minutes. Eight (8) layers of Whatman paper were also cut according to gel size and soaked in transfer buffer. Four (4) layers were placed on the plate of the blotting machine (Bio-Rad Trans- Blot Semi dry) and the air bubbles were rolled out. The membrane was placed on Whatman paper stack, and the gel carefully placed on top of the membrane and 4 layers of

Whatman papers placed on top and air bubbles removed. The gel was blotted onto the membrane at 24 volts for 50 minutes and after blotting the membrane was incubated overnight in 5% milk at 4^0 C.

Detection

The next day the membrane was washed for 10 minutes in TBST buffer then incubated with the 1st antibody, mouse anti-cmyc for 1 hr at room temperature shaking. After incubation the membrane was washed 3 times for 10 minutes per wash shaking with TBST buffer. The secondary antibody anti-mouse HRP (horse radish peroxidase) was diluted 1:1000, in 10 ml TBST buffer. The membrane was then incubated with the 2nd antibody, for 1hr. After incubation it was washed 3 times, each was using TBST lasting 10 minutes. The bound antibodies were detected by incubating the membrane with ECL detection reagent, which comprised of detection reagent 1 and 2 mixed in ratio of 1:1 for 5 minutes. The membrane was quickly transferred, with its protein side facing up into a development folder in a plastic bag and taken to the dark room. In the dark room an Amersham Hyperfilm ™ ECL, a high performance chemiluminescene film was cut to the appropriate size and exposed to the membrane for 10 minutes. Afterward the film was passed through a developing machine. When the film was developed it was left to dry and the bands examined.

3.6.14 Blood Stage IFA

Thick drops of erythrocyte pellets (diluted in PBS) were made on Poly-L Slides and the cells left to settle for 60 min. This was followed by washing carefully with PBS in a Petri dish. The smears were then fixed for 30 min at RT with 4% PFA/0.0075% GA without shaking then washed three times with PBS. Blocking with 3% BSA in PBS was done for 30 min at RT. Circles were marked on the slides using a PAP Pen and after it had dried, the 1st Antibody, Mouse anti-cmyc, was added and incubated in the dark, at 4° C overnight. The next day it was washed three times, each wash lasting 10 min with PBS or PBS+0.01% tween.

After the first antibody, addition of 2nd Antibody, Alexa fluor anti-mouse 546 nm (red) or 488 nm (green) in 3% BSA/PBS (Alexa fluor diluted 1:300), was added and incubate for 1 h, RT,in the dark. Followed by washing three times for 10 min with PBS or PBS+0.01% tween.

Finally, Hoechst nuclear stain (1:1000 in PBS) was added and incubate for 5 min at RT in the dark, then washed 3x with PBS or PBS+0.01% tween. Mounting was done with 10% glycerol in PBS, and sealed with nail polish. The IFA were then examines using the laser microscope and photos taken.

4 Results

4.1 Infection of Mice

In this study naive (non-infected) female NMRI mice were used. Two (2) of the mice were used as the donors (**D**) 1 and 2, to prepare parasites for transfection. Both mice developed high parasitemia and they were sacrificed before they showed signs of paralysis.

Two (2) mice were injected with the transgenic parasites and were referred to as the parent (**P**) 1 and 2, since they were injected with the parental parasite generation directly after transfection.

The other 2 were used as transfer mice (direct blood transfer from the parent mice) to get more parasites that had been transfected and selected for a longer time. These animals were named transfer (**T**) 1 and 2.

Table 1- Outcome of infection among the mice used in the study

Mice	**Parasitemia**	**Illness**
Donor 1	3.1%	No symptoms
Donor 2	2.5%	No symptoms
Parent 1	1.5%	No symptoms
Parent 2	1.2%	No symptoms
Transfer 1	2.4%	No symptoms
Transfer 2	3%	No symptoms

4.2 Insertion of the fragments

Figure 2 and 3 shows the gel confirming the insertion of the 1st fragment and the full construct. The construct was inserted in two steps, because of the size which was 2.5 kb.

Figure 2 below shows the gel confirming successful insertion of the 1st fragment into the vector. Three mini preps were done and labeled Mini 1, 2 and 3. They all confirmed the insertion was successful by showing two bands in the same row, for the vector (7800 kb) and the 1st fragment (1300 kb).

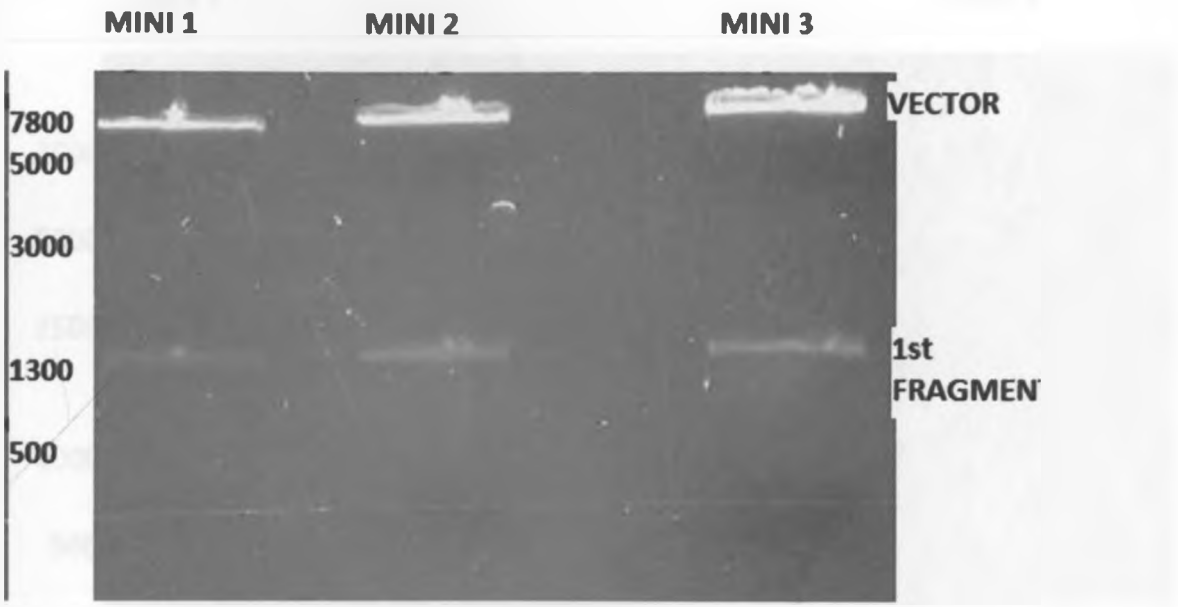

Figure 2: Gel showing two bands, confirming the insertion of the 1st fragment into the vector

4.2.1 Full construct

Figure 3 shows the confirmation of the full construct. After the insertion of the 1st fragment, the 2nd fragment was inserted following the same steps as for the 1st fragment. A gel was done to confirm that the full fragment was inserted (figure 3)

The insertion was successful and this is shown in figure 3. Two mini preps were done, namely mini 1 and mini 2. Mini 2 showed a better result than mini 1 and was chosen for further experiments.

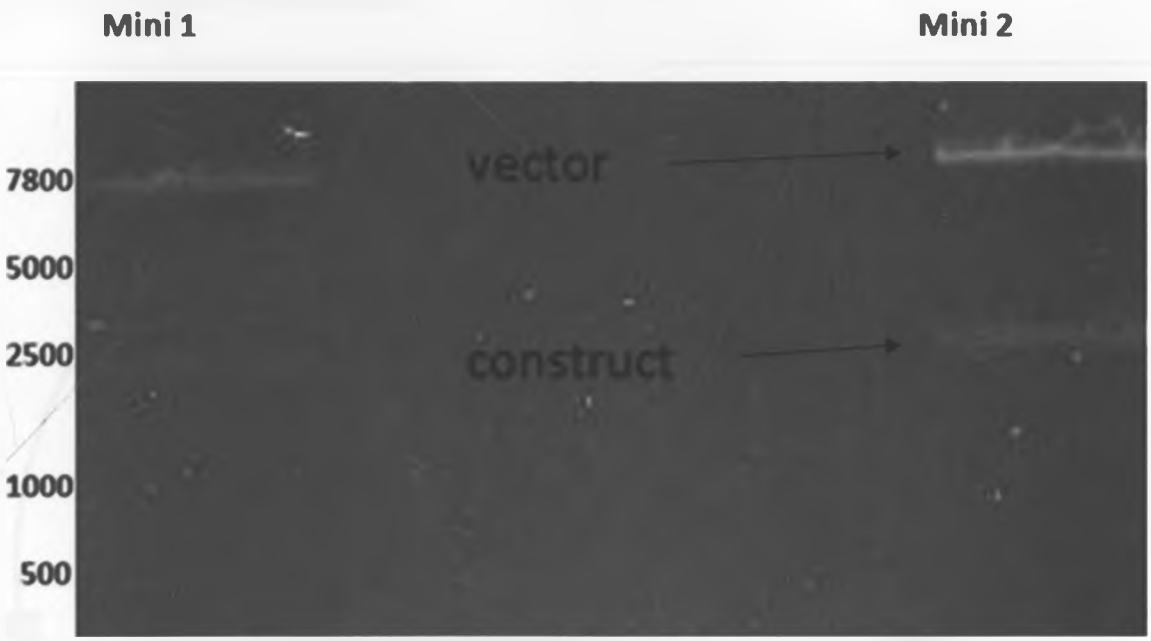

Figure 3: Gel showing the insertion of the full construct into the vector.

4.2.2 Illustration of the vector plus construct

Figure 4 below shows an illustration of how the vector looked like after insertion of the construct. It shows the 1st fragment (promoter part 1, P1) and the 2nd fragment (promoter part 2, P2) with the open reading frame (ORF) of *BIRdom1* and the c-myc tag following the *BIRdom1* ORF.

The illustration also shows the features of the vector that were important during the experiment. The *Tg*DHFR gene which provided the resistance to pyrimethamine. The *β-Lactamase* gene (AMP) for resistance to ampicillin.

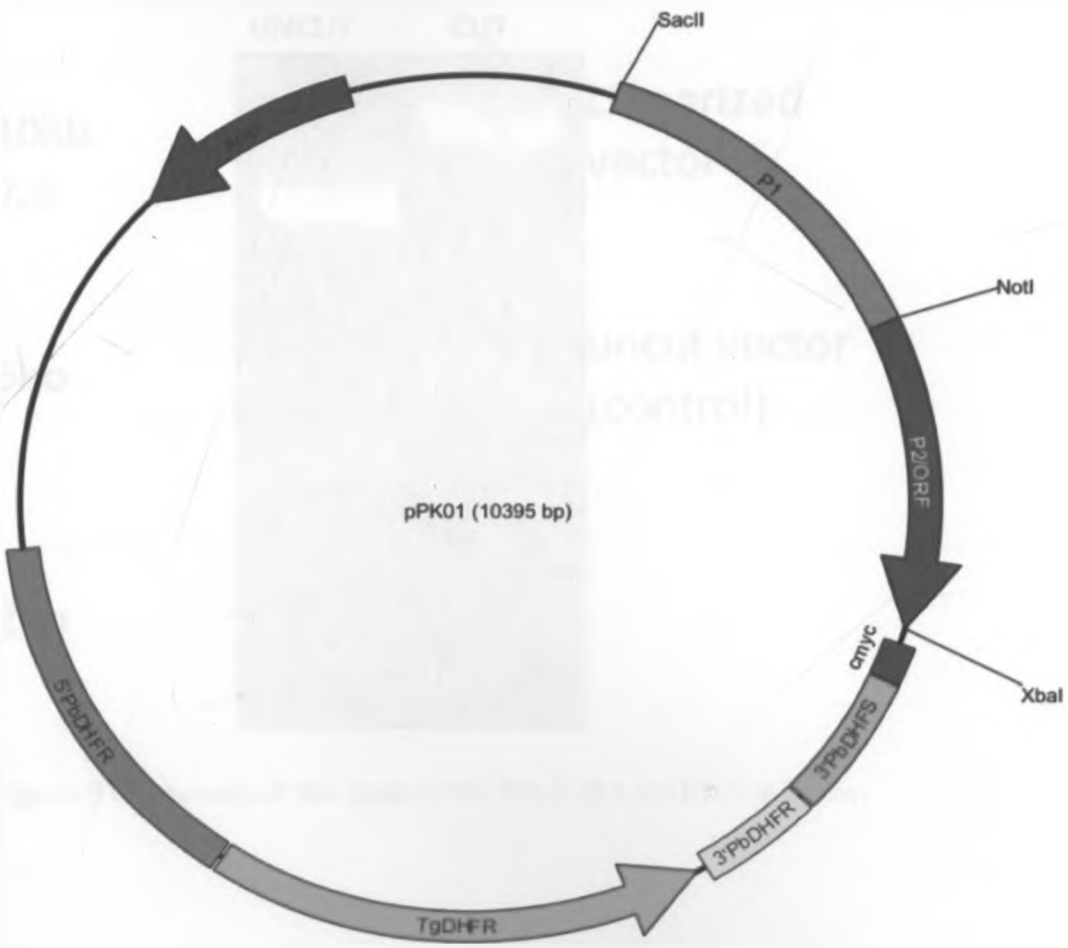

Figure 4: A diagrammatic illustration of how the vector looks like with the construct inserted.

4.3 Linearization

Figure 5 shows the results of linearization of the construct using Not I. An uncut vector was run as control showing a bright band for super coiled DNA which runs faster on the gel than the linear DNA. On the right side, the cut vector is shown. The linearized vector runs slower in the gel (upper band). The lower band is most likely vector that was not properly digested and stayed uncut.

Linearization of the plasmid was done so as to introduce it into the merozoites of *P. berghei* for transfection.

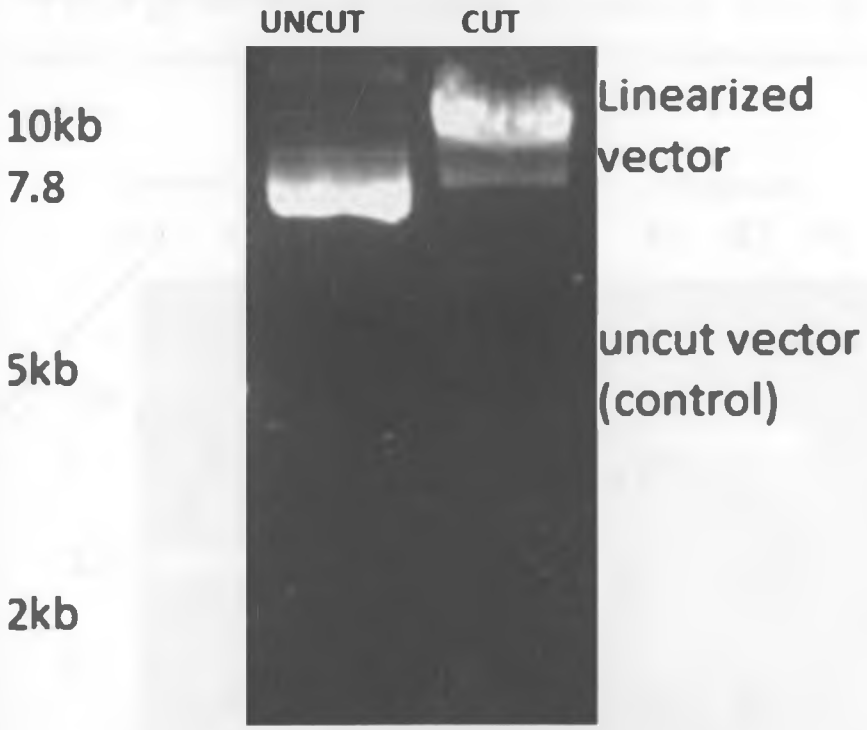

Figure 5 Gel results of the digest with Not I, the cut DNA is linear.

4.4 PCR

Figure 6 shows the gel picture of PCR for confirmation of the presence of an episomal and/or integrated construct. For each PCR 4 tests were run, two samples from the parental generation of mice directly after transfection and two from transfer mice. The transfer mice were labeled T1 and T2 and the parental mice were labeled P1 and P2

The episomal construct is 1.7 kb, from the start of the *BIRdom1* primer to the end of the *BIRdom1* ORF.

The long range PCR, was done to confirm the integration of the construct leading to a size of 3.9 kb. A gel was then run to confirm this (Figure 6). As the gel shows both PCR reactions showed a product, that is, the construct is present in both episomal and integrated version in all samples.

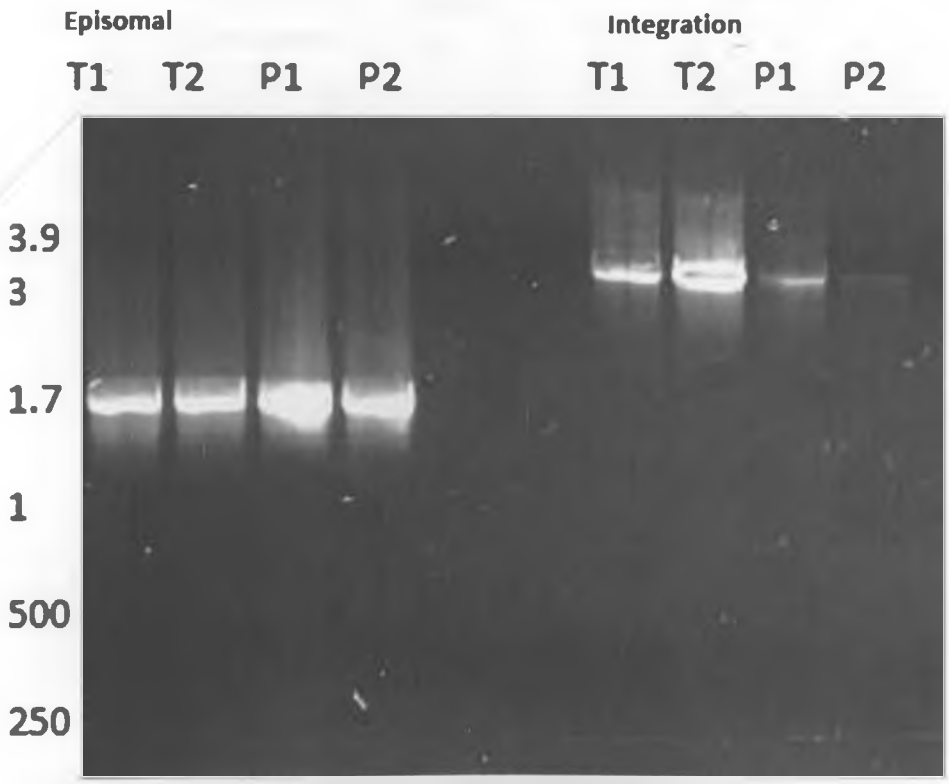

Figure 6: Gel picture showing the PCR results for confirmation of integration

4.4.1 Integration after Transfection

The figure 7 below shows an illustration of the endogenous locus after successful integration of the construct. The endogenous gene is shown in black and white; the exogenous gene is in orange. The *Tg*DHFR/TS codes for the resistance to pyrimethamine in (green). The test forward/*Tg* forward primer combination was used to test for the integration. The P2 forward and *Tg* forward primers were used to test for episomal construct (primer sequences in methodology).

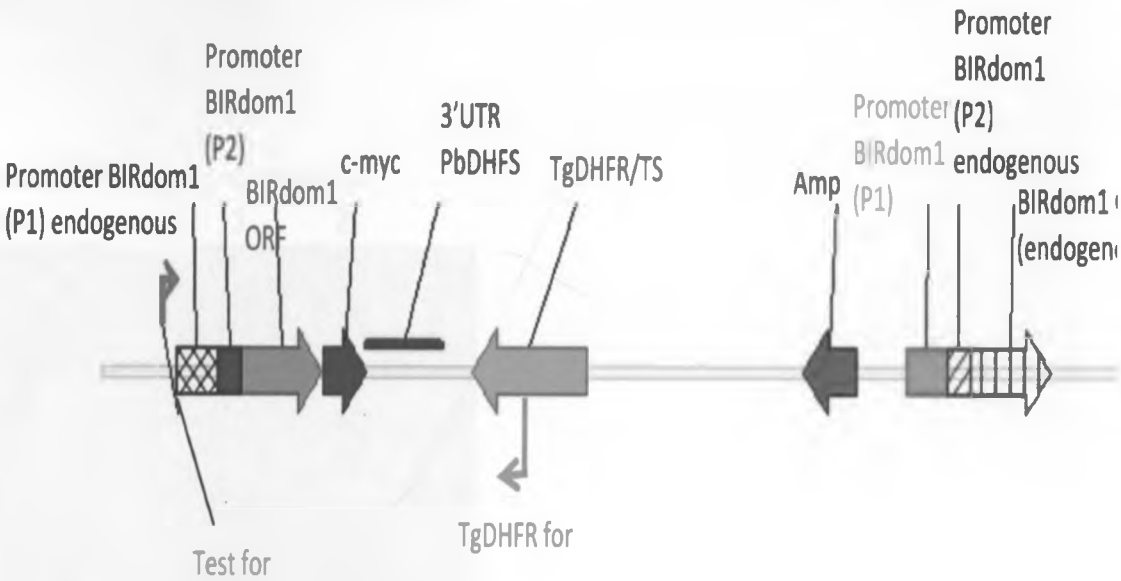

Figure 7 Illustrative diagram of the integration

4.5 Western blot

Figure 8 shows the western blot results, which were done to check for the expression of the protein in blood stages. Four tests were done, one with the wild type (WT), one with the BIRdom1 protein, and 2 controls provided by colleagues of the department. The positive controls are done to confirm that the antibody is working in case no signal will be detected in our sample.

The western blot showed an expression of the BIRdom1-cmyc fusion protein in blood stages, which confirmed that the designed construct was working and was properly expressed in these stages. There was no band for the BIRdom1-cmyc tag in the wild type confirming that this was a specific expression.

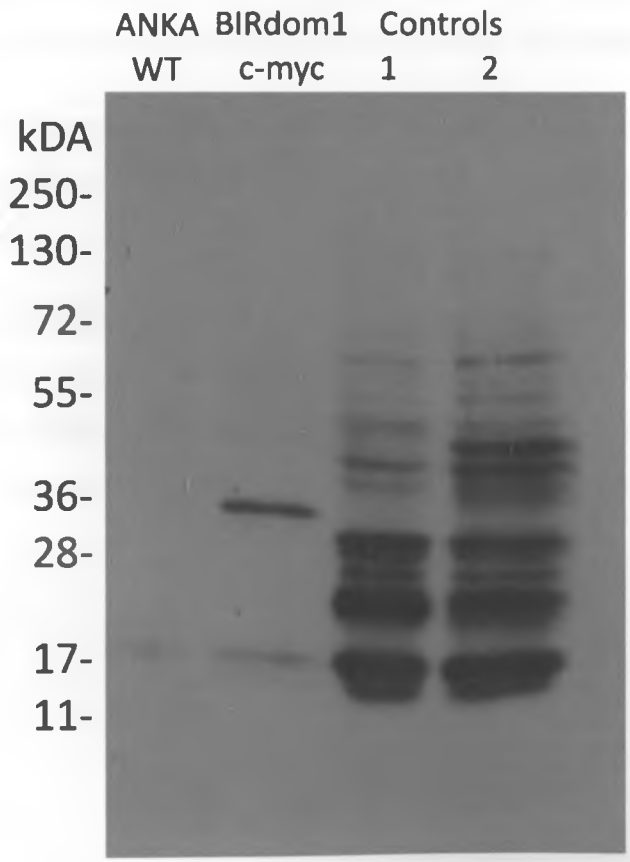

Figure 6 Western blot

4.6 Localization

Immunofluorescent assay and Confocal laser scanning immunofluorescence of *P. berghei* infected red blood cells

Following the western blot results confirming that the designed construct was working and was properly expressed. localization studies were performed as described in the methodology.

Figure 9 shows:

the laser scanning photos illustrating that the expression of the protein was cytosolic.

R1 and **R2** were the first IFA experiments done, using an antibody resulting in a red signal. Since the signal was really weak (**R2**) or even absent (**R1**) we repeated the experiment with a different antibody (R= Red signal).

G1 and **G2** were the repeat experiments with the green signal and they appeared very clear and the expression was well seen in the cytosol of the infected red blood cells (G= green signal).

C was the control (uninfected erythrocyte) stained with the green signal. There was no staining of the nucleus or of the protein confirming that uninfected erythrocytes have no fluorescence and the signal we saw in infected cells is specific.

The bright field shows the images of the infected red blood cell without any fluorescence. The BIRdom 1 shows the fluorescent staining of the protein which is cytosolic. The nucleus photo shows staining of the parasite nucleus which is an indication that the red blood cell is infected. The Merge is an overlay of the nucleus staining and the protein stains.

IFA images

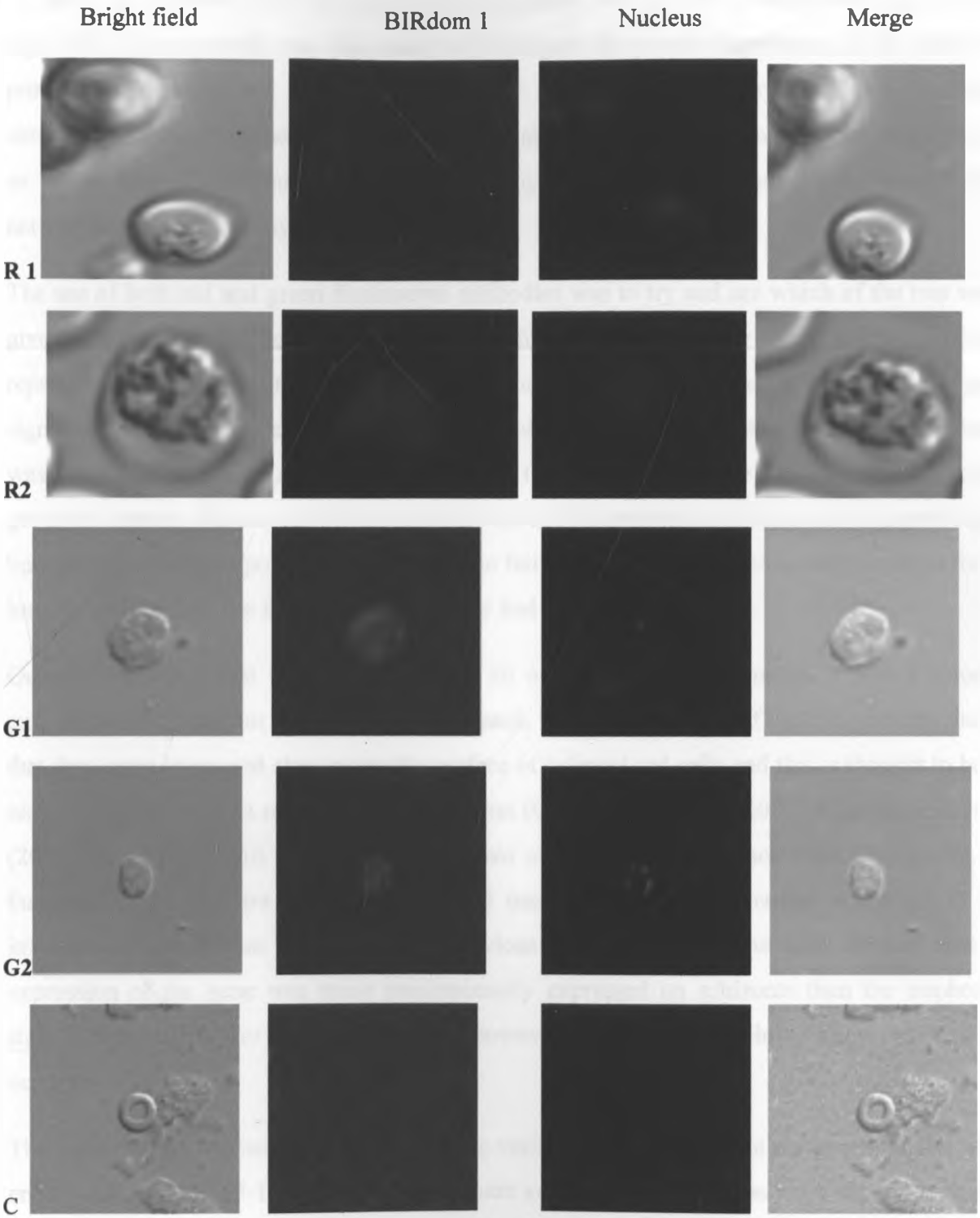

Figure 9 IFA images. R=red signal, G green signal C= control

5 Discussion

In this study we looked at the localization and the protein expression of a dominantly expressed *bir* gene, the *BIRdom*1. The *BIRdom 1* ORF was cloned into the PB279 vector and tagged with c-myc. This fusion protein was also used to investigate the protein expression, of the BIRdom1 protein, by western blot analysis. The tagging with c-myc is a good tool since anti-cmyc antibodies are easy to produce and to handle. In absence of antibodies against the study protein, as in the case of BIRdom1, addition of a c-myc tag allows the study of the protein using antibodies against the c-myc epitope.

The use of both red and green fluorescent antibodies was to try and see which of the two would give a brighter signal. The use of the red signal failed and gave a poor signal and we decided to repeat the experiment using green fluorescent antibodies. Lavazec *et al.,* (2006) used the green signal in which they tagged a *P. falciparum* stevor gene with a c-myc epitope and the expression was good. Girolamo *et al.,* (2008) in his study of the *bir* genes used the red flourescence and also got good signals. We can only speculate why the red fluorescent antibodies showed poor signal, but can't conclusively point to the cause of the failure. Maybe the parasites were exposed for too long to the laser and the fluorescence probably had weakened.

Our study showed that the expression was all over the cell which meant it was a cytosolic expression. This was not what we had anticipated, since other studies of the PIR proteins showed that they were expressed close or on the surface of infected red cells and this is thought to be the reason why they play a role in immune evasion (Cunningham *et al.*, 2009). Cunningham *et al.*, (2005) showed that YIR proteins are expressed on the surface of infected red blood cells, and that many different *yirs* are transcribed and transcription pattern changes according to host immunity (Cunningham *et al.*, 2005). Previous studies of *pir* gene also showed that the expression of the gene was more predominantly expressed on schizonts than the trophozoite stages (Cunningham *et al.*, 2005, 2010), however we were not able to show whether this occurred.

The most studied and best described antigenic variation in malaria is the *var* genes family, which encode for the PfEMP-1 antigens (Cunningham *et al.,* 2010) and it has been shown to mediate cytoadhesion (Jane *et al.*, 2011; Kraeme and Smith, 2006). Brown *et al.,* (1967) noted antigenic

variation in *P. knowlesi*, by using schizont infected cell agglutination test which showed that different variants are expressed at different times leading to the persistence of the disease.

The cytosolic expression of the BIRdom1 protein does not rule out the probability that the proteins may also be expressed on the surface of the infected red cell. Nevertheless the cytosolic expression could also hint to other important functions beside antigenic variation and/or cytoadhesion. The VSA in *P. falciparum* have been implicated in the development of cerebral malaria through cytoadhesion (Scherf *et al.*, 2008; Montgomery *et al.,* 2007). Surface expression of the protein in PIR, is thought to act as receptors or communication channels (Cunningham *et al.,* 2010). However it is not clear what this means to the parasite when the expression is cytosolic. Nevertheless, the expression of the protein and its presence in the parasite cytosol means that they must be playing an important role for the parasite.

The expectation was that the expression would be on the surface but instead we saw the expression being cytosolic. A control was set to see whether the non infected red cell would show any fluorescence and there was none confirming a parasite specific signal in our samples. There is need for more work to be done on the *bir* genes, to identify their importance. More analysis and studies are needed on the *bir* expression in other stages of the lifecycle of *P. berghei* and the location of BIR proteins within different parasite stages.

6 Recommendations and conclusions

The results of this study are inconclusive and call for more studies on the *bir* genes in order to:-

- Determine the expression of the protein at the different stages of the lifecycle.
- The diversity of the BIR protein and its localization.

7 References

Bangirana P, Idro R, John CC, Boivin MJ. **2006** Rehabilitation for cognitive impairments after cerebral malaria in African children: strategies and limitations. *Tropical Medicine and International Health*. 11(9):1341-9.

Berendt AR., Simmons D L., Tansey CI., and Marsh K. **1989**. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum. Nature* 341(6237):57-59

Brown N, Brown L and Hill L. (**1968**) Immunity to malaria: The Antibody response to Antigenic Variation by *Plasmodium knowlesi. Immunology.* 14 (1):127-138

CDC- www.cdc.org

Chakravorty SJ, Craig A. **2005.** The role of ICAM-1 in *Plasmodium falciparum* cytoadherence. *European Journal of Cell Biology*. 84(1):15-27

Cook G. and Zumla A., 22nd Edition, **2009**, Manson's Tropical Diseases. Elsevier publishers. 1201-1229

Cox Herbert, (**1959**) A study of relapse in Plasmodium berghei Infections Isolated from White Mice. *The Journal of Immunology*. 82(3): 209-214

Cunningham D, Jarra W, Koernig S, Fonager J, Fernandez-Reyes D, Blythe JE, Waller C, Preiser PR, Langhorne J. **2005** Host immunity modulates transcriptional changes in a multigene family (*yir*) of rodent malaria. *Molecular Microbiology*. 58(3):636-647.

Cunningham D, Lawton J, Jarra W, Preiser P, Langhorne J. **2010.** The pir multigene family of Plasmodium: antigenic variation and beyond.*Molecular and Biochemical Parasitology.* 170 (2):65-73.

Cunningham D., Fonager J., Jarra W., Carret C., Preiser P and Langhorne J. **2009**. Rapid Changes in Transcription Profiles of the *Plasmodium yoelii yir* Multigene family in Clonal Populations: Lack of Epigenetic Memory. *PLoS ONE* 4(1) e4285.

del Portillo HA, Fernandez-Becerra C, Bowman S, Oliver K, Preuss M, Sanchez CP, Schneider NK, Villalobos JM, Rajandream MA, Harris D, Pereira da Silva LH, Barrell B, Lanzer M. A. **2001** superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. *Nature*. 410(6830):839-42.

Division of Malaria Control, Kenya Malaria fact sheet 2009.

English M, Wale S, Binns G, Mwangi I, Sauerwein H, Marsh K. **1998.** Hypoglycaemia on and after admission in Kenyan children with severe malaria. *Quarterly Journal of Medicine*. 91(3):191-197.

Fernandez-Reyes D, Craig AG, Kyes SA, Peshu N, Snow RW, Berendt AR, Marsh K, Newbold CI. **1997.** A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya. *Human Molecular Genetics*. 6(8):1357-1360.

Grau, G.E., Piguet P.F., Vassalli P and Lambert P.H. **1989**. Tumor-Necrosis factor and the cytokines in cerebral malaria: experimental and clinical data. *Immunological reviews* .112: 49-70

Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, *et al*., (**2009**). A world malaria map: *Plasmodium falciparum* endemicity in 2007. *PLoS Med* 6(3):e1000048.

Ho, M and White NJ. **1999**, Molecular Mechanisms of cytoadherence in malaria. *American journal of physiology* 276 (cell physiology 45):C1231-C1242.

Idro R, Marsh K, John CC, Newton CR. **2010.** Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome. *Pediatric Research*. 68(4):267-274.

Janes JH, Wang CP, Levin-Edens E, Vigan-Womas I, Guillotte M, Melcher M, Mercereau-Puijalon O, Smith JD. **2011** Investigating the Host Binding Signature on the Plasmodium falciparum PfEMP1 Protein Family *PLoS Pathogen*.7(5) e1002032

Janet Cox-Singh and Balbir Singh, **2008**. Knowlesi malaria: newly emergent and of public health importance? *Trends Parasitology* 24(9): 406–410.

Janssen C.S., Barrett M., Turner C., Phillips R. **2002**, A large gene family for putative variant antigens shared by human and rodent malaria parasites, *Proceedings of the Royal Society London Biological Sciences* (269) 431-436.

Janssen C.S., Phillips S.R., Turner M.R. and Barret M.P., **2004**, Plasmodium Interspersed repeats: the major multigene superfamily of malaria parasites. *Nucleic Acids Research.* 32(19):5712-5720

Jones MK & Good MF (**2006**) Malaria parasites up close. *Nature Medicine.* 12(2):170-171.

Kraemer SM, Smith JD.**2006** A family affair var genes, PfEMP1 binding, and malaria disease. *Current Opinion in Microbiology.* 9(4):374-80.

Lavazec C, Sohini Sanyal S, and Templeton T.**(2006**) Hypervariability within the Rifin, Stevor and *Pf*mc-2TM superfamilies in *Plasmodium falciparum. Nucleic Acids Reseaerch*; 34(22): 6696–6707

Lesi F.E., Mukhtar M.Y, Rroha E.U, Egri-Okwaji M.T. **2010** , Clinical presentation of congenital malaria at the Lagos University Teaching Hospital. *Niger Journal of clinical practice.* 13(2):134-138

Lovegrove FE, Pena-Castillo L, Mohammad N, Liles C, Hughes T and Kain K.(**2006**) Simultaneous host and parasite expression profiling identifies tissue-specific transcriptional programs associated with susceptibility or resistance to experimental cerebral malaria. *BioMed Central Genomics* 7:295.

Maitland K. and marsh K. Pathophysiology of severe malaria in children.**2004.** *Acta Tropica* 90(2);131-140.

Mali S., Tan K.R., Arguin P.M. 2011. Division of Parasitic Diseases and Malaria, Center for Global Health ; Centers for Disease Control and Prevention. Malaria Surveillance- United States, 2009. *Morbidity and Mortality Weekly Reports Surveillance Summary.* 60(3): 1-15

Marsh K and Snow RW.**1997.** Host- Parasite interaction and morbidity in malaria endemic areas. *Philosophical Transactions of the Royal Society London* B, 352(1359): 1385- 1394

Marsh K, Forster D, Waruiru C, Mwangi I, Winstanley M, Marsh V, Newton C, Winstanley P, Warn P, Peshu N, et al **1995.** Indicators of life-threatening malaria in African children. *New England journal of Medicine.* 332(21):1399-1404.

Marsh K, English M, Crawley J, Peshu N. **1996** The pathogenesis of severe malaria in Africanchildren. *Annals of Tropical Medicine and Parasitology* 90(4):395-402.

Maude RJ, Dondorp AM, Abu Sayeed A, Day NP, White NJ, Beare NA **2009.** The eye in cerebral malaria: what can it teach us? *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 103(7):661-664.

Mazier D., Josiane N., and Idrissa-Boubu M. **2000** Cerebral malaria and immunogenetics,. *Parasite immunology* 22(12): 613-23

Montgomery J, Mphande FA, Berriman M, Pain A, Rogerson SJ, *et al.*. **2007**. Differential *var* gene expression in the organs of patients dying of *Falciparum* malaria. *Molecular Microbiology* 65(4): 959–967

Nahrevanian Hossein, **2004**, Nitric Oxide involvement during malaria infection; Immunological Concepts, Mechanisms and Complexities: a novel review. *Journal of Tropical Medicine and Parasitology.* 27(2): 93-101

Nester *et al.* 4th Edition, **2004.** Microbiology a Human Perspective. Mc Graw Hill publishers. 731-735

Noor, A. M.,Gething P.W, Alegana VA., Patil AP.Hay S.I, Muchiri E, Juma E and Snow R.W. **2009**. The risk of malaria infection in Kenya in 2009. *BioMed Central Infectious Diseases*.9:180

Scherf A, Lopez-Rubio JJ, Riviere L **2008** Antigenic variation in *Plasmodium falciparum. Annual Review of Microbiology.*62:445-70.

Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Peterson DS, Pinches R, Newbold CI, Miller LH. **1995** Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell.* 82(1):101-110.

Snow R., Craig M., Newton C., Steketee R. **(2003).** The public health burden of *Plasmodium falciparum* malaria in Africa: Deriving the numbers. Working Paper No. 11, Disease Control Priorities Project. Bethesda, Maryland: Fogarty International Center, National Institutes of Health. August 2003.

Snow RW, Craig M, Deichmann U, Marsh K **(1999)** Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bulletin of the World Health Organization 77(8):624–640

Vincke, I.H. and Lips, M. **1948** Un nouveau *Plasmodium* d'un rongeur sauvage du Congo: *Plasmodium berghei* n.sp. *Annales de la Société Belge de Médecine Tropicale* 28(1): 97-104

Vythilingam, I., Noorazian,Y., Tan Cheong Huat, Adela Ida Jiram, Yusof M Yusri, Abdul H *et al* **2008.** *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia *Parasites & Vectors*. 1(1): 26.

World Health Organization, Malaria fact sheet. N. 94 April **2010.**

ANNEX

Molecular biology work

Enzyme

I.	T4-DNA-Ligase	Fermentas; St. Leon-Rot
II.	*Taq*-DNA-Polymerase	Fermentas; St. Leon-Rot

Restriction enzyme:

I.	*NotI*	New England Biolabs; Frankfurt
II.	*SacII*	New England Biolabs; Frankfurt
III.	*XbaI*	New England Biolabs; Frankfurt

Nucleic acids

Primers for PCR were ordered from, Eurofins MWG Operon, Ebersberg.

The restriction site of the restriction enzymes is underlined. The primers sequences are in red.

P1 for Sac II PB279

TCCCCGCGGGAGTTATTATTATATAACTATAATATAATC

P1 rev Not I PB279

ATTTGCGGCCGCCGCAAATGCTTTAACCCTATATATAA

P2 for Not I PB279

ATTTGCGGCCGCTCACATATTGGGGATAAGATATATC

Open Reading Frame (ORF), rev XbaI without stop. PB279

GCTCTAGAATTCATTTTCTTCTTTATTTTTTAGC

Tg forward Primer

CCATCTGGATTCGTCCGTGCGGG

Test forward Primer

CATAAGGTATAATAATAGATTAATCAC

BIRdom1 forward Primer

CAAGAATGATTGCACAAAATGTTC

DNA and Protein marker and loading dye for Electrophoresis

I.	1 kb DNA-Ladder	MBI Fermentas; St.Leon-Rot
II.	6x Loading dye	MBI Fermentas; St.Leon-Rot
III.	Page ruler prestained Protein Ladder	MBI Fermentas, St.Leon-Rot

Reaction kits

I.	Plasmid DNA Isolation	QIAprep Spin Plasmid Miniprep Kit	Qiagen; Hilden
II.	DNA purification	QIAquick PCR Purification Kit	Qiagen;Hilden
III.	DNA Extraction:	QIAamp DNA Blood Mini Kit	Qiagen; Hilden

Competent cells (Hanahan)

TBF 1: **100ml**

- KAc 30mM (0,294g)
- $MnCl_2$ 50mM (0,809g)
- KCl 100mM (0,746g)
- pH 5,8 with 0,2M acetic acid (1:10 dilution of 100% aa)
- Glycerin 15% (15ml)
- Sterile-filtered

To be stored at 4°C in the dark

TBF 2: **100ml**

- MOPS/NaOH pH 7,0 10mM (0,209g in Aqua.dest, pH7 with NaOH)
- $CaCl_2$ 75mM (1,1g)
- KCl 10mM (0,075g)
- Glycerin 15% (15ml)
- Sterile-filtered

To be stored at 4°C

Laboratory equipment

1. Hera Cell incubator	Heraeus Instruments; Hanau
2. Incubator For *P. berghei* Transfection	Mytron; Heiligenstadt
3. Roller incubator	VWR; Darmstadt
4. Fluorescent Microscope	Leica Microsystems; Wetzlar
5. Gel chamber MiniSub-Cell	BioRad; München
6. GelDoc XR	BioRad; München
7. Heating Block	NeoLab; Heidelberg
8. Confocal Lasermicroscope LSM510	Zeiss; Jena
9. Light Microscope Axiolab	Zeiss, Jena
10. Microwave	LG; Willich
11. PCR-Thermocycler Mastercycler epgradient S	Eppendorf; Hamburg
12. Pipette Model Research	Eppendorf; Hamburg
13. Pipette controller Accu-Jet p ro	Brand; Wertheim
14. Shaking incubator	New Brunswick Scientific;
15. Power supply PowerPac Basic	BioRad; München
16. Spectrophotometer ND-1000 NanoDrop	Peqlab; Erlangen
17. Biohood, Herasafe	Heraeus Instruments; Hanau
18. Thermomixer Modell Compact	Eppendorf; Hamburg
19. Table Centrifuge 5415 R	Eppendorf; Hamburg
20. Vortex Mixer 7-2020	neoLab; Heidelberg
21. Balance, Adventurer Pro	Migge; Mannheim
22. Waterbath, Julabo 7A	Julabo; Seelbach
23. Centrifuge J2-MC	Beckman; Krefeld
24. Centrifuge Megafuge	Heraeus Instruments, Hanau

Consumables materials

1. Coverslips	Knittel; Braunschweig
2. Culture tubes, Polypropylen-12/75mm	Greiner Bio-one; Frickenhausen
3. Immersion oil	Zeiss; Jena
4. Needles 23G/ 27G	Becton Dickinson; Heidelberg
5. Cryotubes	NALGENE®; Denmark
6. Microscope slides	Menzel; Braunschweig
7. Parafilm	Pechiney Plastic Packaging; USA
8. Pasteur pipette	WU; Mainz
9. PCR-tubes	Greiner Bio-one; Frickenhausen
10. Petridishes	Greiner Bio-one; Frickenhausen
11. Pipette tips	Greiner Bio-one; Frickenhausen
12. Pipette tips, filter	Greiner Labortechnik; Nürtingen
13. Serology Pipettes (1,2,5,10 und 25 ml)	Corning Incorporation; Bodenheim
14. Sterile filter (0,2 µm, 0,4 µm)	Schleicher and Schuell; Einbeck
15. Sterile filtration Units	NALGENE®; Denmark
16. Syringe	Becton Dickinson; Heidelberg

Chemicals

The chemicals were supplied by the "Chemikalienlager" at the University of Heidelberg, School of Medicine.

1. Agarose	Invitrogen, Karlsruhe
2. Ampicillin	Roth, Karlsruhe
3. Bacto-Agar	Difco Laboratories, Augsburg
4. Bromophenol blue	Sigma, Taufkirchen
5. BSA (Bovine Serum Albumin)	Roth, Karlsruhe
6. DMSO (Dimethylsulfoxid)	Merck, Darmstadt
7. EDTA	Roth, Karslruhe
8. FCS (Fetal calf serum)	Gibco Invitrogen, Karlsruhe

9. FCS, certified (USA)	Gibco Invitrogen, Karlsruhe
10. Fibrose Cellulose Powder	Whatman, Great Britain
11. Formaldehyde	Merck, Darmstadt
12. Giemsa	BDH Laboratory Supplies
13. Glass beads 212-300 micron, unwashed	Sigma, Taufkirchen
14. Yeast extract	Difco Laboratories, Augsburg
15. Heparin	Braun, Melsungen
16. Magnesium chloride	Merck, Darmstadt
17. Nycodenz	Axis Shield, Oslo, Norway
18. PBS-Tablet	Gibco Invitrogen, Karlsruhe
19. Pyrimethamine	Sigma, Taufkirchen
20. RPMI 1640-Medium	GibcoBRL, Karslruhe
21. Saponin	Sigma, Taufkirchen
22. TEMED	Roth, Karslruhe
23. Trypsin-EDTA	GibcoBRL, Karlsruhe

Buffer, Media and Solutions

Antibiotics

Ampicillin Stock	100 mg/ml Ampicillin in ddH_2O

Bacteria-Media

Luria Broth (LB)-Medium	10 g/l Bacto-Trypton, 5 g/l Yeast extract, 5 g/l NaCl, pH 7,5
LB-Agar	LB-Medium, 15 g/l Agar

Production of competent Cells buffers:

I. TBF I	Potassium acetat 30 mM, $MnCl_2$ 50 mM, KCl 100 mM, Glycerin 15%, pH 5,8 Acetic acid 0,2 M

II.	TBF II	MOPS/NaOH pH 7,0, 10 mM, $CaCl_2$ 75 mM, KCl 10 mM, Glycerin 15%

Buffers

3 M NaAc- solution	24.06 g add 100 ml ddH2O; pH 4.8
TAE-Puffer 50x	2 M Tris, 250 mM Sodium acetate, 0.5 mM EDTA; pH 7,8
PBS (phosphat buffered saline) 10X	0,01M KH_2PO_4; 1,37 M NaCl, 0,027 M KCl, pH 7.0
Freezing-Solution	Glycerin:Alsever's Solution (1:9)

Medium for Transfection

1. Transfection-Culture medium — 160 ml RPMI-Medium, 25 mM HEPES, L-Glutamin (Gibco), 40 ml FCS, 50 µl Gentamycin
2. Nycodenz Stock solution: — 138 g Nycodenzpowder in 500 ml Buffermedium
3. Buffer medium: — 5 mmol/l Tris/HCL; 3 mmol/l KCL; 0,3 mmol/l $CaNa_2EDTA$
4. Human T-cell Nucleofactor solution
5. Pyrimethamin in DMSO for Selection of Parasites in Mice:
    - 25 mg in 10 ml DMSO; 0,1 ml pro Mouse with 25 g body weight

Western Blot

Buffers:

10x SDS running buffer

- 30,3g Tris Base
- 144g Glycine
- 10g SDS
- fill to 1l with Aqua dest

Transfer buffer:

- 5,81g Tris
- 6,52g Glycine
- 200ml Methanol
- 0,37g SDS
- fill to 1l with Aqua dest

10x TBS:

- 60,57g Tris Base
- 87,66g NaCl
- adjust to pH 8 with HCl
- fill to 1l with Aqua dest

1xTBST Buffer

- 1x TBS Buffer
- 0,1% Tween

1.5M Tris pH 8,8:

36,34g in 200mlTris Base, adjust pH with HCl

1M Tris, pH 6.8:

24,23g in 200ml

2x SDS sample buffer

- 2ml 0,5M Tris HCl pH 6,8
- 1,6ml Glycerol
- 1,6ml 20% SDS
- 1,4ml Aqua dest
- 0,4ml 0.05% bromophenol blue
- 7µl beta-mercaptoethanol

Transformation of competent cells

Ampicillin Stock:

100mg in 1ml Aqua.dest (store at -20°C)

LB Medium: 1l

- Yeast extract 5mg
- NaCl 5mg
- Bacto Trypton 10mg
- Autoclave!

LB/Amp plates: 1l

- Yeast extract 5mg
- NaCl 5mg
- Bacto Trypton 10mg
- Agar 15mg
- Amp Stock 1ml
- Autoclave!

Ligation

- T4 Ligase 1µl
- PCR Product 6µl
- Vector 2µl
- Ligation at least 1h RT or o/n at 4°C

KEY- Fragment that was inserted

- The red highlight shows the test forward, primer in the endogenous locus of BIRdom1 which was used for the long range PCR
- The green highlights encircle the 1st fragment which is 1.3kb, with the two green parts being the primers for cloning of the 1st fragment and the yellow showing the promoter.
- The 2nd fragment which was cloned into the vector using the restriction site for Not I and Xba I and has the size of 1.2kb. It is bordered by the cloning primers shown in purple and includes a small part of the promoter (yellow) and the ORF of BIRdom1. The BIRdom 1 forward primer (CAAGAATGATTGCACAAAATGTTC) is shown with no highlight.

FRAGMENT THAT WAS INSERTED

TATGATTTATATCATATTTATTTTGATTTAAATCGTGTTATCCAATTGAACTATAATAATATA
GATTCACAAAATATCGGTCGATATTCGACAATACAACGTTATCTATAAAAGGCATTTTATGC
ATCTAACATTTTAGTAATACATAAAAAATGCACTATAGATATATTATAATAAATTTATAAAT
TATAAATATATATAATACTCACTTTTTTCATTTCAATAATTTACATTTATGGTAATGTCTAATT
TATATTGATAGAACTACTTATATATATATTAATTTAT[redacted]
[redacted]TATTAATTTTAAATTTTAAAGATTTGAGGTATAAATATATTATTTTTAAAATAGTTAAAAAA
TAAAATATAAGTATATTAATAAATTTCATGTTATAGTTTGTTTTAATAATTATAAATAATATG
ATAGATAAAATGAGTTATTATTATATAACTATAATATAATCTAATAAAATACTCTACTAATA
ACTAATATTAAATATTAATTTTATCGAAAAGGCACATAAAATACATTATAATGATATAATTT
TTATATATTTGTTAAAGATACAATTTGGGATTTGTAGTTTTATATTATAAAAAATTGGATAAA
TACAGAAAAGGTTAAAGACTCAATTCATGTTAAATTGCATTTTATTATTCCACATTAGCATGC
ATTATATTATCATTGTTTAATGAATCATCATTTTGTAATATAAATTAGGATTTTTTACTATAG
AATTAATAAAACATAAATTTTTTAATAAATTATTTGAATGTATATACATTGACAACATAACA
ATAAAATATATAAAACTATACAATTCAGAACATTTAGCGGATATTTATCTTAATTTATATATT
AAAGAGAAAATATATCTTATCTCTCTCCTCTTAAAGTACACTATAACAATTTAATCAAACAT
AGTTTTCTATTATACTTTAAAATTTAAATCAATAGTTATTTATGTGCTAATATTTAATATTTGC
TATATATTATTTTAAAAGCAATCTACATTCAAAGGGTTATTTAAACTTATAAATAGCTTAATA
AATGCGGTTCATTGCTAATTGTGGTTTTAAACCGTGGAAAAGATGTGCAAAATATATTATAA
AATCACCCAATAATGTATATTTTAAAATTGATGCGCGTATTAATAAAATTAGAGCTATTGAG
TTTATTAAAATGGATTATGAATTTGTCCATATTAAATAAAAATAATATTAAAATTATGTATAT
GGAATTAAAAAAGGTAACAATGGAGATTATTTTGTAAATGCTATAGTTTTAATAAAATATTG
GTATATAGTATTAGAAACAACTATATAAATATTTAATATATCTTAAAAAATAACTATTTATAT
ACTTTTAAGGATTATAGTAATAATATAACTTTTTATTTTTTAGTTATATATAGTATTTAAGTTT
TAGAAAGGTAATCATTAAAAAATAAGATATAAATATATTCCTCTTCTATGTTCAAGCATAAA
TATAAGGAAATCTACATTAAATTCTTTATAAAAGTATATTAATTTTTATAATAAAGTTATACC
AGATGTTAGTGTGAGTAGCATTTTTTGTATATAATTCATTATATATATATTTAATTAAAATGT
ATTAAGTAACTCTCTATTTAAGGTAATTTAGCTAATTATGATAAGCGGAAATAGAACGTTTC
TTTACTAATAATATTGTTTTATTATGTATAATAATTTAATTATTAAACAGTTATAATACATTTA
ACTACAAACAGTTGTTATATATAGGGTTAAAGCATTTGCG[redacted]
[redacted]AAACAACATGATTTTACTCAATTTACAAATTTAAAATGAAATTTCATCACAATGGATGC
CGAAATAGTATATGCATTTTTTAATACTAATAATTTCCTTTACATTTTTTGATATTGTATTATA
TATAAATATCATTAAACTTTATTTTAACAAAATTTTCAATAATGCACATTTCTAATAATTGTT
TTTAAATATTTTTTTTTAGTGTAAGAACTTCCTTTTAGTAAGGACGAACTTTCCCGATCAATTG
GACAATAATGGAAACTATAAAATTGAAGATGATACACATTTCAAAGAGTATTGTAGTAATC
AAAAATTGTGTTAATGAGCTCGAAAAAATTAGTGCTGGATGTTTATATTTGTTTAATGAATTCT
TTAAGGATTCTTCTGTCTTTAATTCTGTTGCAAAAAGTAACATCAATATTGTTGATTACATTA
TAATATGGTTAAGTTATATGTTAAACCTAAAGGAAAATATTGATAACAACAGTCTAAGTTAT
TTTTATACTACAAATATAAATAATGATAAGTATACTAATACTATAGATGGTGTAGAGGGGTA
TACTAGTTACAAGGATCTTATAGATAAAACAAATATGATGAATATGGATATTAAAGATATAT
CTAAATTTTATGATGCATTTATATTATTATGTGAAATGTATTCTGTATTTGATGTAGACAAGA
ATGATTGCACAAAATGTTCGGATAAAGCTAACCAATTTGTTGAAAAATATAAAAATTTGAAT
GAAAATTCTAATAATACTGAAGGTAGTCCATATAGAAAAATATTGTCTACATTATCAACTGA
TTATAATAATTTAAAAAATAAATGTAGCGATAGTTCATCCTTTCCATCGATAGAAACAACAC

AAATTTCTGTAAAAGGTTCTGAACAAACTGTGCAAAATTCCGCACAAACATCTGAAGTTGCA
TTATTAAGTTCGTCGATAGGAAACAAATTAATTCCAGTTTTATCGATATTTGGTGCAATAGCA
TTTTTTTTAGGAATTGGATATAAGGTAAATAATAAGCCAATTAAAATATATTCAACAAGTAA
TTTATGAATATAAGTAAATTATACATTTTTTTTAATTTTTATATTAGTATTCATTATTTAAGTC
TCGAAAACGATCTCGAAAACAACATTTAAGAGAAAA[redacted]
TAATTAATATAAGATTCGAAAATAGTGACACGTTCAAGAATATTAATAATGATTGATATAT
GTTAAGAAATTGTCTATTTCGAAGTAATTTTTTACCATAATTTTTATATAGTTTTTATGTTGTG
GGTCAGGGTTATGTTTGCGGATCCCATATTCGGGTTAGGGTTAAGTATTA

Vector

PB279-MYC_CT_7841bp