TRANSFECTION AND CHARACTERIZATION OF PLASMODIUM KNOWLESI MALARIA PARASITES GENETICALLY MODIFIED TO EXPRESS HOST INTERFERON GAMMA (IFN-γ) IN BABOON (PAPIO ANUBIS)

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Transfection and characterization of
DECLARATION

I, James Muli Munyao, declare that this thesis is my original work and has not been presented for a degree in any other University or for any other award.

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DEDICATION

Dedicated to my wife Sabina, son Victor and daughter Annastacia for their passion, support and encouragement. To my parents, John Munyao Nzioki and Annah Munyao, for their love and investment in my education and my brothers and sisters for their encouragement, financial and moral support during the course of this study.
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ABBREVIATIONS AND ACRONYMS

AP – Alkaline Phosphatase.
BSA – Bovine Serum Albumin
DHFR/TS- Dihydrofolate Reductase Thymidylate Synthase
DMSO – Dimethyl Sulphur Oxide.
DNA – DeoxyriboNucleic Acid
EDTA – Ethylene Diamine Tetra Acetic Acid
ELISA – Enzyme-Linked Immunosorbent Assay
GFP – Green fluorescent Protein.
gm – grams
HRP – Horse radish peroxidase.
IFN-γ – Interferon gamma.
ml – Millilitres.
O.D – Optical density.
OPD – Othophanyl diamine.
Pan – *Papio anubis*
Pbama – 1 – *P. berghei* apical membrane antigen – 1
Pbef-Iα – *P. berghei* elongation factor – I alpha
PCR – Polymerase chain reaction.
PCV – Packed cell volume.
pH – Hydrogen-ion exponent.
P-NPP – Para-Nitrophenyl Phosphate.
RBC’s – Red blood cells.
TBE – Tris boric acid with EDTA
ABSTRACT

Despite efforts to control malaria, it still remains a major health problem in endemic countries. With the development of transfection technology, it is now possible to determine the structure-function relationship of vaccine candidates. In addition, transgenes relevant in malaria vaccine development can now be expressed and host-parasite interface determined. This study involved characterization of genetically modified *P. knowlesi* malaria parasites that express host IFN-gamma, in order to develop a baboon (*Papio anubis*) model for transfection and analysis of host-parasite interface.

One olive baboon was infected with wild-type *P. knowlesi* to generate parasites for transfection. At day 9 post inoculation, the baboon developed acute malaria with a parasitaemia of 7.6% and on day 10 post inoculation it was totally bled out. The blood was processed by centrifugation and 500 ml of top brown layer harvested. This layer contained $0.65 \times 10^9$ parasites enriched with mature schizonts. The parasites were resuspended in 300 ml of cytomix, mixed with 50 μl of plasmids DNA construct. The construct contained pyrimethamine resistant form of *dihydrofolate reductase thymidylate synthase* (*dhfr-ts*) from *Toxoplasma gondii*, IFN-gamma gene under control of *P. berghei* promoters and enhancer sequences. The mixture was electroporated in a Biorad gene pulsar in a pulse of 2.5 KV, at a capacitance of 25 μF and a resistance of 200 Ω for 0.9 ms, chilled on ice for 5 minutes and injected into two olive baboons. The baboons were given pyrimethamine orally for drug selection of transfected parasites. This was done 24 hours post inoculation. Pyrimethamine resistant *P. knowlesi* appeared from day 10 post inoculation. On day 15 post inoculation, blood was collected from the two baboons, and subjected to plasmodipur filtration to remove leucocytes before DNA isolation. The parasites were cultured *in vitro* for 48 hours, supernatant harvested and analysed by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for IFN gamma. Antibody response against transfected and passaged parasites in baboon was determined by ELISA from serum collected from baboons. Molecular characterization of isolated DNA from pyrimethamine resistant parasites by PCR revealed presence of selectable marker gene (*dhfr-ts*) and IFN-gamma gene. Supernatant of *in vitro* culture of transfected parasites showed release of high levels of IFN-γ, suggesting presence of IFN-gamma gene in transfected parasites. Antibody response against transfected *P. knowlesi* and passaged transfected parasites, suggested primary response with rise in IgM production at 0.34% and 0.33% parasitaemia respectively. These results showed for the first time that it is possible to genetically modify *P. knowlesi* to express host IFN-γ and induce immune response in baboon. These studies have developed an *in vivo* transfection protocol for *P. knowlesi* in the baboon. This opens possibilities for using the *P. knowlesi*-baboon model in vaccine development using cuttingedge technology.
CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria is a public health concern in over 90 countries of the world where 40% of the world population live (WHO, 1998). Annually there are approximately 300-500 million cases of malaria and upto 2.7 million deaths (WHO, 1998). In Africa alone, malaria is responsible for an estimated 200-450 million cases of fever in children and upto 175 deaths per 1000 live births occur annually before the age of five years (Penelope and Kuile, 1994). Africa, accounts for 90% of the global malaria cases (Breman et al., 2001). The total days of labour lost coupled with cost of treatment and high mortality associated with the disease makes malaria a serious obstacle for development (WHO, 1996). The persistence of endemic malaria in the tropics, and particularly in Africa, is the major contributory to the perpetual state of depressed economic growth in these regions (Breman et al., 2001).

Human malaria is caused by four species of an intracellular parasitic protozoon of the genus *Plasmodium* (*P. falciparum, P. vivax, P. malariae* and *P. ovale*) and is transmitted by female mosquito of the genus *Anopheles* (Garnham, 1966). *Plasmodium falciparum* is the most lethal as it is responsible for over 90% of malarial associated deaths. Among all the parasitic diseases known today, malaria is not only the greatest killer, but is also the most widely spread (Breman, 2001).

The global campaign to eradicate malaria has been far from optimal due to parasite, mosquito, environment and human related factors (Sachs and Melanie, 2002). Malaria
parasites have evolved mechanisms such as antigenic variation (Smith et al., 1995) for evading host defenses, subsequently complicating vaccine development (Richie and Saul, 2002). The complex life cycle of *Plasmodium* and rampant resistance to first line drugs (Wellems and Plowe, 2001; White, 1998), have also constrained vaccine development and raised the cost of malaria treatment. Insecticide resistance and population ecology are some of the issues preventing mosquito control due to emergence of new species (Chandre et al., 1999; della Torre et al., 2002). Factors in the environment including climatic changes such as global warming and El Nino (Lindblade et al., 1999; Hay et al., 2002) have very often frustrated malaria control efforts by increasing mosquito breeding sites, thus precipitating epidemics. Socially, conflicts (Pitt et al., 1998), mobility of people (Sawyer, 1993) and population increase do complicate planning for malaria intervention. Very often malaria infected people have other precipitating infections (Snow et al., 1999) requiring modified control approaches.

The search for new methods to combat malaria has been intensified (Gwadz and Green, 1978), this is because, the number of malaria cases may double by the year 2020 if new methods of control are not devised and implemented (Breman, 2001). The current research is multifaceted, geared towards the development of better means of mosquito control, new chemotherapeutic drugs and an effective vaccine (Gwadz and Green, 1978).

Theoretically, the most viable and cost-effective option for malaria control is the development of an effective vaccine. However, in order to facilitate vaccine development, there is need for deeper understanding of the biology of malaria parasites.
Research with human malaria parasites is difficult as it represents a major constraint on in vivo studies, therefore a significant amount of research has been carried out using animal models. The animal model offers the only means to experimentally investigate host-parasite interaction in vivo (Ozwara et al., 2003a). There are three Plasmodia-groups that are mainly used in experimental studies on host-parasite interactions they are rodent, avian and primate. Rodent malaria parasites are used to study parasite biology (Cox, 1988). However, these parasites are phylogenetically distant from human plasmodia (Escalante et al., 1998), and do not easily allow investigations of natural host-parasite interactions. Although P. gallinaceum and P. lophurae, which are the most widely used avian malaria parasites, are closely related to Plasmodium falciparum, their development in nucleated cells and the wide phylogenetic distance between birds and humans limits their applicability to study important questions on host-parasite interactions relevant to human malaria.

Primate Plasmodia such as Plasmodium knowlesi, which is a natural parasite of macaques, have a comparable phylogeny and host-parasite relationship to human malaria parasites (Escalante et al., 1998; Coatney et al., 1971). The parasites are used to identify, develop and evaluate vaccine and drug candidates (Wengelnik et al., 2002; Deans et al., 1984; Kocken et al., 1999) and to characterize host responses (Deans et al., 1988; Rogers et al., 2001; Gwadz and Koontz, 1984).

Plasmodium knowlesi is a natural parasite of Macaca fascicularis and M. nemestrina. In addition, P. knowlesi, infection has been experimentally induced in a number of other
non-human primates such as *M. mulatta, M. radiata, M. assamensis, Presbytis entellus, Callithrix jacchus, Aotus trivigatus* and *Saimiri sciureus* and baboon (Garnham, 1966; Coatney *et al.*, 1971; Collins *et al.*, 1978; Langhorne and Cohen, 1979). In baboon, infection has been induced in *Papio cyncephalus, P. doguera, P. jublieaus* and *P. anubis* (Coatney *et al.*, 1971). The parasite is phylogenetically close to *P. vivax* (Escalante *et al.*, 1995), and many genes identified in *P. vivax* have homologues in *P. knowlesi*, and this allows determination of function by analogy.

The assessment of a candidate vaccine for use in human subjects can be facilitated and expedited by prior testing in non-human primates (Strickland and Kenneth, 1982). Similarities in biological mechanisms between humans and non-human primates underlie the value of non-human primates as the final test system for the safety and efficacy of drugs and vaccines developed in studies with other laboratory animals and systems (WHO, 1988; King *et al.*, 1988; Stower and Miller, 2001). Non-human primates are widely used in malaria drug and vaccine development (Butcher, 1996; Stower and Miller, 2001; Wengelnik *et al.*, 2002). The baboon is attractive as an animal model, because it is well characterized and is frequently used in biomedical research (King *et al.*, 1988). Studies have shown that *P. anubis* infected with *P. knowlesi* display various clinical characteristics that are also seen in human malaria including cerebral involvement (Ozwara *et al.*, 2003a). Recently, protocols for long term *in vitro* culture and genetic modification of *P. knowlesi* have been developed (van der wel *et al.*, 1997; Kocken *et al.*, 2002). These are powerful tools for understanding parasite biology, especially gene function.
In this study characterization of genetically modified *Plasmodium knowlesi* that express host IFN-γ in baboon was carried out. The expression of IFN-γ is immunologically important because it is a key effector cytokine in protection against malaria particularly during the liver stages (Hoffman *et al.*, 1997; Schofield *et al.*, 1987; Sedegah *et al.*, 1994; Seguin *et al.*, 1994; Nardin and Nussenzweig, 1993). Studies in human and animal models looking at endogenously produced and exogenously administered IFN-γ have shown that the cytokine is also required for protection against blood stage infection (Herrera *et al.*, 1992; Luty *et al.*, 1999; Su and Stevenson, 2000; Yoneto *et al.*, 1999; Clark *et al.*, 1987; Shear *et al.*, 1989).

### 1.2 Statement of the Problem

The recent development of a protocol for the stable transformation of malaria parasites (Goonewardene *et al.*, 1993; Wu *et al.*, 1995; van Dijk *et al.*, 1995; Wu *et al.*, 1996; Crabb and Cowman 1996) facilitated genetic approaches to the understanding of the biology of malaria parasites. A proper understanding of the biology of malaria parasites will facilitate development of new drugs and vaccines against malaria. Stable transfection of the human parasite *P. falciparum* (Wu *et al.*, 1995) rodent parasite *P. berghei* (van Dijk *et al.*, 1995) was achieved through introduction of plasmids carrying the gene encoding the bifunctional enzyme dihydrofolate reductase thymidylate synthase (dhfr-ts), either obtained from *Plasmodium* species or from *Toxoplasma gondii* (Donald and Roos, 1993) as selectable marker. Although the value of transfection in the study of *P. berghei* and *P. falciparum* is highly recognized, these parasites do not easily allow investigations of interaction between parasites and their natural host (van der Wel *et al.*, 1997).
The rodents available for infection with *P. berghei* are phylogenetically distant from the natural host and the few animal models are susceptible to *P. falciparum* infection (new world monkey and chimpanzees; van der Wel *et al.*, 1997). These hosts are unnatural and have characteristics distinct from the human host (van der Wel *et al.*, 1997).

The *P. knowlesi* system provides a unique malaria transfection system that allows fast and simple *in vitro* genetic manipulation of the parasite and provides an opportunity to perform *in vivo* studies in a non-human primate that is closely related to humans (Kocken *et al.*, 2002). This fact makes *P. knowlesi* highly suitable as an experimental system for the development of new antimalarial drugs and vaccines, as well as for studying basic biology questions in the post genomic era and at the parasite host interface (Kocken *et al.*, 2002). Recombinant pathogenic micro-organisms that could express host cytokines such as IFN-γ have been shown to modulate immune responses, leading to enhanced protection (Gherardi *et al.*, 1999; Giavedoni *et al.*, 1992; 1997; Gurunathan *et al.*, 2000; Perera *et al.*, 2001; Steidler *et al.*, 2000; Tobin *et al.*, 1993). This indicates that *in vivo* expression of host cytokines by pathogens can manipulate the host-pathogen interaction and generate protective host responses.

It is now feasible to generate malaria parasites that could express bioactive host cytokines, to characterize the capacity of the parasite to immunomodulate the infection and the role of the expressed cytokine in host response to malaria using rhesus monkey (Ozwara *et al.*, 2003c). Although baboons are widely used for research (Moore *et al.*,...
their use has not been extended to characterization of genetically modified *P. knowlesi* malaria parasites that could express host IFN-\(\gamma\). Baboons and rhesus monkeys are close and hence rhesus monkey Interferon gamma gene can be transfected in *P. knowlesi* and tested in baboon.

The use of baboon was encouraged is feasible since they are locally available in Kenya, reagents for analyzing baboon system are readily available commercially, and IPR has been researching on the baboon for over 20 years. Use of Chimpanzees in research is outlawed hence baboon remains the only non-human primate phylogenetically closest to humans that is available for research. In addition, it is important to use baboon which is phylogenetically related to human to characterize the genetically modified *P. knowlesi* system that can express host IFN-\(\gamma\), so as to harness the benefit offered by the emergence of transfection technology in a long term endeavour to come up with a vaccine against malaria.

1.3 Research Question

Can genetically modified *P. knowlesi* malaria parasites expresse host IFN-\(\gamma\) in baboon model and induce an Immune response?

1.4 Null Hypothesis

Genetically modified *P. knowlesi* malaria parasites do not express host IFN-\(\gamma\) in baboon model and do not induce any immune response.
1.5 Objectives of the Study

1.5.1 General Objective
To transfect and characterize *P. knowlesi* malaria parasite genetically modified to express monkey IFN-γ in the baboon.

1.5.2 Specific Objectives
a) To transfect *P. knowlesi* with DNA-construct carrying selectable marker gene and monkey IFN-γ gene.

b) To monitor *in vivo* development and selection of transfected parasites in the baboon model.

c) To determine molecular characteristics of transfected *P. knowlesi*.

d) To monitor parasite produced IFN-γ and resulting host immune responses in the baboon.

1.6 Justification
Malaria is responsible for an annual death toll of up to 3 million people mainly children below the age of five years in ninety of the world’s poorest countries, including Kenya (Breman, 2001). Malaria control has failed in part due to parasite resistance to commonly used drugs such as chloroquine. The number of malaria cases may double by 2020 if new methods of control are not devised and implemented (Breman, 2001). The only viable long-term option for malaria control is the development of an effective vaccine. If a vaccine is developed, it is the residents of the area where medical care is rudimentary who stand to benefit most. Vaccines are being developed with the aim of blocking transmission from the host to mosquito, and vice versa (anti-sporozoite), blocking intra-
hepatic and intra-erythrocytic development (the stages that cause clinical malaria; Richie and Saul, 2002). Several trials of asexual blood stage vaccines have used synthetic peptide conjugates or recombinant proteins and there has been at least one trial of transmission blocking vaccine recombinant Pfs 25 (Carter, 2001). Upto now no immune protection induced in volunteers has attained the levels that are required in order to manufacture a vaccine (Richie and Saul, 2002). It seems likely that any ultimate malaria vaccine will comprise multiple components.

The proper understanding of the biology of malaria parasites will facilitate development of new drugs and vaccines against malaria. *Plasmodium knowlesi*, infects animals on multiple occasions before immunity develops and interactions can be evaluated in an immunological and metabolic environment similar to humans. This parasite has been utilized in discovery and characterization of malaria blood stage vaccine candidate AMA-1. The same parasite has been successfully used to identify, develop and evaluate vaccine and drug candidates (Wengelnik *et al.*, 2002; Deans *et al.*, 1984; Kocken *et al.*, 1999). The protocols for genetic modification of *P. knowlesi* have recently been developed (van der Wel *et al.*, 1997, Kocken *et al.*, 2002). These are powerful tools for drug and vaccine development because they enable the function of target drug and vaccine candidate’s genes to be determined. Homologues of *P. knowlesi* genes can be identified and developed in *P. falciparum* and *P. vivax*.

Baboons are widely distributed in many habitats in Africa (Zinner *et al.*, 2001) and are highly susceptible to *P. knowlesi* infections (Coatney *et al.*, 1971; Ozwarra *et al.*, 2003a).
Baboons infected by *P. knowlesi* display comparable disease profile to that observed in Rhesus monkeys following experimental infection with the same parasite (Ozwara *et al*., 2003a). This demonstrates that the virulence of the strain is similar in both models. In addition, when olive baboon (*Papio anubis*) is infected with *P. knowlesi*, it shows various clinical characteristics that are also seen in human malaria, including cerebral involvement (Aikawa, 1988; Aikawa *et al*., 1990). The baboon therefore, is an attractive experimental model that is well characterized and would be useful in studying host parasite interactions as required for vaccine design and formulation.

The use of baboons for malaria research will increase the number of models available for drug and vaccine development. It is important to use the baboon for malaria research since human studies have a number of limitations and some questions can only be addressed in suitable animal models. Baboon as a model for analysis of host-parasite interaction of wild-type and transfected *P. knowlesi* has not been described, since the development of transfection technology for malaria parasites (Goonewardene *et al*., 1993; van der Wel *et al*., 1997; van Dijk *et al*., 1995; Wu *et al*., 1995).

The development of transfection technology for malaria parasites enables expression of recombinant host proteins such as cytokines in *Plasmodium*. Interferon gamma is an effector cytokine in protection against malaria, particularly during liver stages (Hoffman *et al*., 1997; Nardin and Nussenzweig, 1993; Schofield *et al*., 1987; Sedegah *et al*., 1994; Seguin *et al*., 1994). Studies in human and animal models looking at endogenously produced and exogenously administered IFN-γ showed that the cytokine is also required for protection against blood stage infection (Clark *et al*., 1987; Shear *et al*., 1989; Su and
Stevenson, 2000; Yoneto et al., 1999). Therefore, *in vivo* expression of host cytokines by pathogens can manipulate the host pathogen interaction and generate protective host responses.

*In vitro* expression and bioactivity of *P. knowlesi* expressed rhesus monkey IFN-γ (rh IFN-γ) has been characterized in malaria parasites applying transfection technology (Ozwara et al., 2003c). In this study transfection technology was used for the first time to characterize genetically modified *P. knowlesi* that express rhesus IFN-γ. The molecular characterization of the transfected *P. knowlesi* provided important baseline data on the possibility of *in vitro* transfection of *P. knowlesi* to express host IFN-γ. Furthermore, the study provides basic information that *P. knowlesi* has the capacity to express cytokine genes from non-human primates. Cytokine expressing parasites can be tested in *P. knowlesi* hosts such as baboon in order to determine antibody responses. The studies on the characterization of transfected *P. knowlesi* that expresses host IFN-γ in baboons, as described in this thesis provide the prerequisite data required for further investigation on the bioactivity of the expressed cytokine in baboon model. Expression of immunomodulatory molecules such as cytokines in malaria parasites has the potential of generating immunopotentiated parasites. Transfected parasites will offer invaluable information in formulation of attenuated malaria vaccine.
CHAPTER TWO

LITERATURE REVIEW

2.1 The Malaria Problem

Malaria remains a major threat throughout the tropics since more than 300-500 million people are estimated to be infected annually. Each year the disease kills up to 3 million people the majority being children. Africa accounts for over 90% of the deaths. Of all the parasitic diseases, malaria is not only the greatest killer but also the most widely spread. Malaria is a threat to approximately 40% of the human population in over 90 countries in the tropics and sub-tropics (Breman, 2001).

Malaria is caused by unicellular protozoa of the genus *Plasmodium* that belong to the phylum Apicomplexa. The parasite is mainly transmitted by mosquitoes of the genus *Anopheles*. Human malaria is caused by four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*). Of the four species of *Plasmodium*, *P. falciparum* is the most pathogenic, accounting for over 90% of malarial associated deaths. Efforts to control the disease have mainly focused on chemotherapy and mosquito control. These efforts are seriously hampered by the development of insecticide resistant mosquitoes, the rapid emergence of drug resistant parasites, failing of health programmes, unsuccessful effective mosquitoes control in developing countries (van Dijk *et al.*, 1997) and lack of an effective vaccine. Further research aims at the development of transgenic mosquitoes, unable to support normal parasite development, new drugs and vaccines. The progress has been slow due to complexity of the parasite’s life cycle (Weber, 1988; Perkins, 1990). This is due to the ability of the parasite to generate enormous numbers of variants
upon which natural selection acts leading to selection of variants that may evade specific elements of the immune system (antigenic variation) or are resistant to antimalarial drugs. The complexity of the immune response, as well as the weak immune response to *Plasmodium* blood stage parasites, and lack of adequate knowledge on the basic biology of the parasite and mosquitoes, has complicated malaria control.

Malaria parasites use antigenic variation to escape the host immune response. Recently, a large family of genes (Var genes) that encode variant antigens on the surface of *P. falciparum* infected erythrocytes was identified of which the members are differentially expressed (Borst *et al.*, 1995; Su *et al.*, 1995; Smith *et al.*, 1995). This extremely diverse family of genes encodes 200-350 KDa proteins known as *Plasmodium falciparum* Erythrocytic Membrane Protein 1 (PFEMP1) and are secreted by the parasite to the surface of host erythrocyte, where they are concentrated in the membrane in structures known as Knobs. The differential expression of the var genes is thought to be linked to antigenic variation of the adhesive protein PFEMP1 on the red blood cell surface (Su *et al.*, 1995; Smith *et al.*, 1995; Baruch *et al.*, 1995). The rate of antigenic switching is as high as 2.4% per generation (Smith *et al.*, 1995) but the precise mechanism underlying antigenic switching is still unknown.

Drug resistance against malaria parasite was documented for chloroquine in 1960s in Asia and South America and in 1970s in Africa (Foote and Cowman, 1994; Wirth, 1995). The mechanisms involved in drug resistance are still under investigation.
Attempts to develop a vaccine against malaria have been going on for along time but no effective vaccine has been developed against malaria. This is because partial immunity to malaria can be established under natural conditions, but involves repeated infections, that take years to develop and is usually of short duration (Moelans, 1995). The only malaria vaccine that underwent extensive field trials in Latin America, Africa, and Southeast Asia was the synthetic vaccine spf66 which is composed of short peptide sequences derived from three different blood stage antigens of *P. falciparum* connected by the pre-erythrocytic repetitive circumsporozoite (CSP) sequence (Patarroyo *et al.*, 1988). This synthetic vaccine showed 80% protection in Colombian children aged 1-14 years but when the trial was conducted under conditions of intense malaria transmission the mean protective efficacy was 31% in Tanzanian children of 1-5 years old (Valero *et al.*, 1993). In a subsequent study from the Gambia conducted in an area of lower transmission only 8% efficacy was observed in infants aged 6-11 months (D’Alessandro *et al.*, 1995). From the last reported trials spf66 could not protect children of the age 2-15 years from clinical *falciparum* malaria (Patarroyo *et al.*, 1992; Alonso *et al.*, 1994; D’Alessandro *et al.*, 1995; Nosten *et al.*, 1996; Maurice, 1995).

Genetic variation and lack of knowledge concerning the basic biology of the parasite remains the major obstacles in the development of a malaria vaccine and effective drugs with long-lasting effects.
2.2.1 *Plasmodium knowlesi*

*Plasmodium knowlesi* is a parasite of monkeys that has immune and metabolic systems similar to those in humans (King *et al*., 1988). It is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (Escalante *et al*., 1998). *P. knowlesi* is a natural parasite of *Macaca fascicularis* and *M. nemestrina*. In addition, *P. knowlesi* infection has been experimentally induced in a number of other non-human primates such as *M. mulatta*, *M. radiata*, *M. assamensis*, *Presbytis entellus*, *Callithrix jacchus*, *Aotus trivigatus* and *Saimiri sciureus* (Garnham, 1966; Coatney *et al*., 1971; Collins *et al*., 1978; Langhorne and Cohen, 1979). Recently, *P. knowlesi* was shown to experimentally infect baboons and the disease profile is either acute or chronic (Ozwara *et al*., 2003a).

2.2.2 Life Cycle of *Plasmodium knowlesi*

The life cycle of *P. knowlesi* begins when an infected mosquito injects sporozoites into a susceptible vertebrate host during a blood meal. The sporozoites migrate to the liver, cells, where they undergo larval stage development characterized by many cycles of replication and transformation into liver schizonts, through a process known as exo-erythrocytic schizogony (liver stage development; Fig 2.1).

The infected hepatocyte ruptures releasing merozoites, which infects circulating erythrocytes immediately. The intra-erythrocytic blood stage development is characterized by cyclical pattern of development in which the parasites develop into rings, trophozoites and schizonts. Once the schizonts mature, erythrocyts burst open releasing more merozoites that continue to invade erythrocytes and replicate within the
erythrocytes. Schizont infected erythrocytes rupture, releasing an average of 10-merozoites that continue the cycle of infection (Fig 2.1). The asexual blood stage cycle lasts 24 hours and starts when merozoite invades erythrocytes upto when the infected erythrocytes release merozoites. During the blood stage development, *P. knowlesi* invades both mature erythrocytes and reticulocytes (Hegner, 1938). Band forms are common in the trophozoite stage. Mature schizonts have 10-merozoites on average, but the number can go as high as 16. Regular rings have one or more accessory chromatin dots (Garnham, 1966).

Some of the intra-erythrocytic parasites differentiate into male and female gametocytes. The factors, which signal sexual development, are poorly understood, but at some signal, a proportion of re-invading merozoites start to differentiate into male and female forms (micro- and macro gametocyte respectively). The mature microgametocyte with a diameter of 7 µm stains brick red in giemsa while macrogametocyte measures 8.5 µm and stains light blue in giemsa. Gametocytes are taken up to a mosquito in a subsequent blood meal. Within the mosquito midgut, the haploid gametocytes differentiate into mature micro and macrogametes, which mate to produce a diploid zygote. The diploid zygote differentiates into a motile oikinete that invades the gut and develops into a multinuclear oocyst, which undergoes a meiotic reduction division to produce haploid sporozoites. A mature oocyst filled with sporozoites, ruptures releasing sporozoites into the haemocoel, from where they migrate into the salivary gland, ready to infect a vertabrate host during the next blood meal (Fig 2.1; Garnham, 1966, Gwadz and Green, 1978) and the cycle continues again.
The mosquito vectors of *P. knowlesi* include *Anopheles dirus*, *A. talabacensis* and *A. hackeri* (Garnham, 1966; Coatney et al., 1971), all of which are found in Asia. Other known vectors include: *A. freeboni*, *A. maculatus* and *A. quadrima-culatus* (Coatney et al., 1971). The development in the mosquito (sporogony) takes 10-12 days at a temperature of 26-28°C while the exo-erythrocytic schizogony phase lasts 5.5 days.

![Life cycle of *Plasmodium knowlesi* in a non-human primate](image)

**Figure 2.1: Life cycle of *Plasmodium knowlesi* in a non-human primate** (Coatney et al., 1971)
2.2.3 Animal Models of *Plasmodium knowlesi*

The natural vertebrate hosts for *P. knowlesi* are *Macaca fascicularis* and *M. nemestrina*.

Experimental infections can be induced in a number of monkeys including *M. mulatta*, *M. radiata*, *M. asamensis*, *Presbytis entellus*, *callithrix jacchus*, *Aotus trivigatu*, *Saimiri sciureus* and *Papio anubis* (Garnham, 1966; Coatney *et al.*, 1971; Collins *et al.*, 1978; Dutta *et al.*, 1978, 1981; 1982; Langhorne and Cohen, 1979). In baboons, the infection has been induced in *P. cynocephalus*, *P. doguera*, *P. jubileaus* and *P. anubis* (Coatney *et al.*, 1971). The *P. knowlesi* also infect humans (Chin *et al.*, 1965; 1968; Knowlesi and Gupta, 1932; Ciuca *et al.*, 1955).

The availability of natural and experimental hosts of *P. knowlesi* offers the possibility to study the biology of the malaria parasite and its antigens in a natural host parasite combination and in hosts whose systems are predictable of the human situation (King *et al.*, 1988). *Plasmodium knowlesi* is an attractive experimental system for malaria research. *Plasmodium knowlesi* is a parasite of monkeys that have immune and metabolic systems very similar to humans (King *et al.*, 1988). Secondly, *P. knowlesi* is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (Escalante *et al.*, 1998). Thirdly, the entire genome of *P. knowlesi* has been recently sequenced to a five-fold coverage (http://www.sanger.ac.uk/projects/P-knowlesi), providing more gene sequences for analysis of the parasites biology and for prospects of discovering vaccine candidate genes. Finally, tools for genetic manipulation in *P. knowlesi* are available and because the parasite has 24-hour life cycle, this allows rapid generation and analysis of transfected parasites.
2.2.4 Baboon model of malaria

Baboons are widely distributed in many habitats in Africa and are highly susceptible to *P. knowlesi* infections (Coatney *et al.*, 1971; Ozwara *et al.*, 2003a).

Baboons infected by *P. knowlesi* display comparable disease profile to that observed in Rhesus monkeys following experimental infection with the same parasite (Ozwara *et al.*, 2003a). This demonstrates that the virulence of the strain is similar in both models. In addition, when olive baboon (*Papio anubis*) is infected with *P. knowlesi*, it shows various clinical characteristics that are also seen in human malaria, including cerebral involvement (Aikawa, 1988; Aikawa *et al.*, 1990). The baboon therefore, is an attractive experimental model that is well characterized and would be useful in studying host parasite interactions as required for vaccine design and formulation.

The use of baboons for malaria research will increase the number of models available for drug and vaccine development. It is important to use the baboon for malaria research since human studies have a number of limitations and some questions can only be addressed in suitable animal models. Although great apes such as the chimpanzee and gorilla are phylogenetically closer to humans (1.5% genome difference), they are endangered species and therefore not widely used as experimental animal models. The baboon offers an attractive alternative, as it is not an endangered species but an agricultural pest in many Sub-Saharan African countries. Therefore, it is ethically acceptable to consider the baboon as a research model (D’Hooghe *et al.*, 2008). Baboon as a model for analysis of host-parasite interaction of wild type and transfected *P. knowlesi* has not been described, since the development of transfection technology for
malaria parasites (Goonewardene et al., 1993; van der Wel et al., 1997; van Dijk et al. 1995; Wu et al., 1995).

2.3 The Technique of Genetic Manipulation

Genetic manipulation once simply meant the application of mutagenic agents to create mutations at random in the genome of the organism of choice, followed by the identification of specific desirable mutations through their effects on the organism’s phenotype (“forward genetics”; Tuite, 1992; Boothroyd et al., 1995). Once the desired mutants had been obtained they would then be subjected to detailed biochemical analysis (Tuite, 1992). Therefore, forward genetics “proceeds” from phenotype to genotype (Boothroyd et al., 1995). Following the advent of recombinant DNA techniques in the early 1970’s new techniques to genetically manipulate an organism of choice allow the conceptual inversion of forward genetics and the introduction of mutation at specific sites in the genome of the organism of choice (“reverse genetics”) were developed (Boothroyd et al., 1995).

Reverse genetics starts by mutating a particular gene in vitro followed by the creation of a mutant organism expressing the mutated gene and assessing its phenotype thereby proceeding from genotype to phenotype. Forward genetics is important in identifying genes involved in a given phenomenon, whereas reverse genetics allows precise structure function determination to be made (Boothroyd et al., 1995). The reverse genetic studies enables one to introduce genetic materials into the organism of choice and obtain the expression of the introduced DNA, such technique is commonly referred to as transfection.
2.4 Transfection Technique

Transfection is the method by which DNA is introduced into a eukaryotic recipient cell. Once the introduced DNA has entered the nucleus and is expressed, it may be retained only temporarily (transient transfection), or the introduced DNA is maintained in a functional state for extended periods of time leading to an inheritable change of the cell (stable transfection; Smith, 1990; Bellofatto, 1990). In the vast majority of stable transfection studies, the introduced DNA is designed in such a way that it donates the host cell a conditional growth advantage for which one is able to select.

Transient transfection assays are widely used in eukaryotic molecular biology for the dissection of elements mediating gene expression and regulation (Ozwara et al., 2003b). The transfecting DNA consists of a reporter gene coding for an enzyme of which the host cell lacks endogenous activity and which is stable and easily assayed and is flanked by DNA sequences suspected to control the expression. Commonly used reporter genes are those encoding bacterial gene chloramphenicol acetyltransferase (CAT) β-galactosidase (βGAL), β-glucuronidase (βGUS), the firefly luciferase (LUC) or the Jellyfish green fluorescent protein (GFP) (Roos and Donald, 1994).

A stable transfection system is used when aspects of the biology of an organism are to be examined for several generations. The stable transfection can be divided into two, those which the introduced DNA is maintained as an extra chromosomally replicating episome (episomally based stable transfection) and those which the introduced DNA is integrated into the genome of the host cell (van Dijk et al., 1995).
The appropriate transfection vectors for the development of a stable transfection system are more complicated to construct, as the vector should contain a selectable marker gene, so that one is able to select the successful transformed cells. Normally, drug-inactivating enzymes are used as selectable markers, the marker gene. The selectable marker gene should be flanked by DNA sequences responsible for proper gene expression in the organism of study. In addition, if episomal maintenance of the transfection vector is desired, then functional autonomously replicating sequence should be present. When site-specific integration is preferred, DNA sequences homologous to the target sequence in the host's genome should be included (Tuite, 1992; Winston et al., 1983; Stearn, et al., 1990; Rothstein, 1991; Koller and Smithies 1992; Bronson and Smithies 1994).

The episomally based stable transfection is well suited for introducing expression vectors to study the (over) expression of homologous, heterologous and modified genes and to perform functional complementation studies whereas site specific integration dependent transfection is appropriate to study the effect of gene disruption, deletion, in site modification and single/multi-copy gene expression. The transfection techniques in genetic manipulation of an organism are largely preferred to the classical genetic methods, however that does not imply classical genetics is obsolete, and on the contrary, the combination of method for transfection and method that allow the classical genetic approach would be viewed as an ideal for studying the biology of an organism (Tuite, 1992; Winston et al., 1983; Stearn, et al., 1990; Rothstein, 1991; Koller and Smithies 1992; Bronson and Smithies 1994).
A regulatory DNA sequence for *P. knowlesi* transfection was achieved by using regulatory DNA sequences from *P. falciparum* and *P. berghei* (van der Wel *et al.*, 1997). This study provided the first evidence of heterologous promoter activity in *P. knowlesi*. Experiments showed that the *pbef-1α5′* UTR could function in *P. knowlesi*. The *pbef-1α5′* UTR consistently yielded high luciferase activity, suggesting that it is suitable for driving over-expression of genes in *P. knowlesi*. Furthermore, the *pbef-1α* intergenic region has promoter activity in two directions (Vinkenoog *et al.*, 1998), hence it can be used to drive simultaneous expression of two genes, for instance the gene for the selectable marker, and the gene under study. This could have a significant improvement in the design of constructs for stable transfection of *P. berghei* and *P. knowlesi* by reducing the size of the constructs.

### 2.8 Transgene Expression in *P. knowlesi*

Transgene expression is the expression of foreign genes in the parasites formed as a result of replication of genetically modified *P. knowlesi*. Transgene expression is a means by which critical aspects of *Plasmodium* biology such as mechanisms of drug resistance (Cowman, 2001) can be investigated. Stable and transient transfection protocols for *P. knowlesi* have provided the fundamental tools for transgene expression. The two procedures have been used to express various transgenes in *P. knowlesi*, such as rhesus IFN-γ expressing parasites and luciferase and GFP reporter molecules (Ozwara *et al.*, 2003b; 2003c).

Luciferase and GFP reporter molecules were expressed in *P. knowlesi* as transgenes (Ozwara *et al.*, 2003b). To characterise heterologous promoter activity in *P. knowlesi*,
transient transfection was evaluated using luciferase as the reporter gene. The heterologous stage specific *P. berghei* apical membrane antigen –1 (*pbama – 1*) promoter (de Koning *et al.*, 1999), and the heterologous constitutive *P. berghei* elongation factor –1 alpha (*pbef-1α*) promoter (de Koning *et al.*, 1999), in *P. knowlesi* were used to drive expression of reporter molecules. The construct for expressing luciferase under heterologous *pdbhfr-ts* promoter was tested along side since the *pdbhfr-ts* promoter is known to be functional in *P. knowlesi* (van der Wel *et al.*, 1997; Kocken *et al.*, 2002). Upon transfection of the appropriate constructs into asynchronous *in vitro* culture adapted blood stage parasites, luciferase assay showed that luciferase activity was measurable using either of the three heterologous promoters and was consistently the highest using the construct with the *pbef-1α* promoter.

The *pbef-1α* promoter drives expression in all parasite blood stages of *P. berghei* and *P. knowlesi* except mature schizonts (Vinkenoog *et al.*, 1998), while the *dhfr-ts* and the *pbama-1* promoters are active in late trophozoites/young schizonts (van Dijk *et al.*, 1997) and mature schizont stages (Narum and Thomas, 1994) respectively. Similar experiments for expression of GFP as a reporter molecule showed that the heterologous *pbama-1* promoter maintains tight stage-specific regulation of expression in *P. knowlesi* (Ozwara *et al.*, 2003b). The expression of both luciferase and GFP reporter molecules provides an effective means to study both gene expression and proteins trafficking in tagging experiments (Cheresh *et al.*, 2002; Natarajan *et al.*, 2001). For instance, parasite molecules can be fused to GFP for detecting trafficking pathways and their signals (Waters *et al.*, 1997). In addition, the ability to easily harvest GFP expressing parasites by
fluorescence activated cell sorting as described for *P. berghei* (Sultan *et al.*, 1999) and *P. yoelli* (Mota *et al.*, 2001) could be adapted to *P. knowlesi* for harvesting specific parasite stages for further analysis.

### 2.9 Role of Cytokines in Protective Immunity against Malaria

A stable transfection technique of *P. knowlesi* to generate rhesus IFN-γ expressing parasites has been developed (Ozwara *et al.*, 2003c). The expression of IFN-γ in malaria parasites is attractive because it is one of the central effector cytokines in host response to malaria infection particularly during liver stage (Ferreira *et al.*, 1986; Hoffman *et al.*, 1997; Nussler *et al.*, 1991; Schofield *et al.*, 1987; Sedegah *et al.*, 1994 Seguin *et al.*, 1994).

*In vitro* and *in vivo* studies in rodent models of malaria have demonstrated that IFN-γ plays a central role in protection against malaria liver stage infection, possibly by inducing the infected hepatocyte to produce nitric oxide that kills parasites (Mellouk *et al.*, 1991; Nussler *et al.*, 1991). In clinical vaccination studies with an attenuated sporozoite vaccine, vaccinated humans were protected from subsequent infection through IFN-γ dependent responses. In separate studies in mice and monkeys, sterile protection was achieved through IFN-γ dependent responses after exogenous treatment with interleukin-12 (IL-12; Sedegah *et al.*, 1994; Hoffman *et al.*, 1997). Studies using rodents and human malaria models have demonstrated that IFN-γ also plays a role in protection against malaria blood stages when either endogenously produced (De souza *et al.*, 1997; Herrera *et al.*, 1992; Luty *et al.*, 1999; Stevenson *et al.*, 1990; Su and Stevenson, 2000;
Yoneto et al., 1999) or exogenously administered (Bienzle et al., 1988; Clark et al., 1987; Curfs et al., 1993; Shear et al., 1989).

Although cytokines have been shown to mediate protection against malaria infections after exogenous delivery (Clark et al., 1987; Hoffman et al., 1997; Sedegah et al., 1994; Shear et al., 1989), systemically delivered cytokines are short-lived and require repetitive administration often in large doses that could be toxic to the host (Cantell et al., 1983; Curfs et al., 1993; Ijzermans et al., 1990), and only a small portion reaches the site of infection (Cantell et al., 1983; Kurzrock et al., 1985). Alternatively, cytokine expression by the pathogen itself will ensure that the cytokine is released where its activity is required, as the infection persist and in proportion to the level of infection.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Parasites

*Plasmodium knowlesi* H strain (Chin *et al.*, 1965) inoculum was used for infection. The original parasite inoculum was clone PK (A+), previously cloned by micromanipulation and passaged in rhesus monkeys (Barnwell *et al.*, 1983). The wild-type *P. knowlesi*, was used to infect Pan 2574 in order to allow development and generation of enough parasites for genetic modification. The cryopreserved parasites were quickly retrieved from liquid nitrogen thawed at 37°C in a water bath, washed twice with 3.5% NaCl and twice in RPMI 1640 by spinning at 1200 RPM for 10 minutes. The pellet was transferred into starting culture medium of 2.5% PCV, 20% baboon serum and the rest was RPMI 1640 with a concentration of 20 μl/ml of gentamicin. The parasites were cultured overnight parasitaemia determined and inoculum was worked out.

3.2 Baboons

Baboons used in this study, were trapped from Kajiado district Kenya and conditioned at Institute of Primate Research primate facility. Prior to the experiments, the baboons were screened and certified to be free of infection with *Plasmodium* by a Giemsa-stained thick blood film. All the baboons were fed on a standard diet for non-human primates, and water was provided *ad libitum* (Olobo and Reid, 1990). The experiment to use the baboons was approved by the institutional animal care and use committee (ACUC), and the institutions scientific and ethical review committee (ISERC) of the Institute of Primate Research. The baboons were housed individually in single cages in a bio-
containment facility to avoid possible transmission of *P. knowlesi* between experimental baboons. The biological characteristics of the baboons used in this study are indicated in Table 3.1.

**Table 3.1 Biological Characteristics of Baboons (*Papio anubis*) used in Transfection Experiments of *P. knowlesi***

<table>
<thead>
<tr>
<th>Baboon</th>
<th>Sex</th>
<th>Weight (kgs)</th>
<th>Experiment(s)</th>
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<tbody>
<tr>
<td>PAN</td>
<td></td>
<td></td>
<td>Source of baboon serum and erythrocytes</td>
</tr>
<tr>
<td>2601</td>
<td>male</td>
<td>23.0</td>
<td>Source of baboon serum and erythrocytes</td>
</tr>
<tr>
<td>2574</td>
<td>female</td>
<td>13.20</td>
<td>Generation of <em>P. knowlesi</em> parasites for Transfections</td>
</tr>
<tr>
<td>2838</td>
<td>female</td>
<td>14.0</td>
<td><em>In vivo</em> selection of transfected parasites.</td>
</tr>
<tr>
<td>2851</td>
<td>male</td>
<td>15.0</td>
<td><em>In vivo</em> selection of transfected parasites.</td>
</tr>
<tr>
<td>2593</td>
<td>female</td>
<td>12.0</td>
<td>Development of passaged transfected. Parasites</td>
</tr>
</tbody>
</table>

**3.3 Preparation of Baboon Serum for Culturing of *P. knowlesi***

Serum for culturing *P. knowlesi* was obtained from baboon blood. Fifteen milliliters of blood was collected aseptically from one baboon (Pan 2601) and kept at room temperature overnight. The blood was centrifuged directly at 2000 RPM for 10 minutes (Sorval centrifuge model). The serum was then harvested, heat inactivated by immersing in a water bath at 56°C for 30 minutes and then aliquoted into 5 µl vials and stored at -20°C until required.
3.4 Preparation of Baboon Erythrocytes for Culturing *P. knowlesi*

Erythrocytes were obtained from baboon blood for culturing *P. knowlesi*. Briefly, venous blood was collected in heparin tubes, centrifuged at 1500 RPM for 10 minutes (Sorval centrifuge model) and the plasma removed. The pellets were washed 3-times by adding twice the original volume of RPMI 1640 medium and then spinning at 1500 RPM for 10 minutes and removing the supernatants. After the last wash supernatant was discarded and an equal amount of RPMI 1640 added resulting into 50% PCV. The mixture was stored at 4°C and used within 2 weeks.

3.5 Infection of Pan 2574 with *P. knowlesi*

The parasitaemia of the overnight-cultured *P. knowlesi* was determined by counting 2000 RBC on a thin smear. A total of 1.2 x 10^6 parasites were used to inoculate Pan 2574. These parasites were suspended in 1000 μl RPMI, then transferred to 1000 μl syringe and intravenously injected into baboon. All materials used for parasites injection were disposed immediately.

3.6 Animal Handling After Infection

The infected baboon was monitored daily. The health of the animal was assessed through observation by a veterinarian for general agility, playing habit and appetite. Three days post-inoculation, finger prick was made and thick and thin smear prepared for monitoring parasitaemia. Briefly, finger pricking was done using a sterile needle and about 100 μl of blood collected into heparinized capillary tube. The blood was used for parasitaemia determination by microscopic examination of Giemsa-stained thick and thin films.
Parasites for transfection were prepared when the parasitaemia had the highest number of schizonts (schizonts were used for transfection).

3.7 Preparation of Parasites for Transfection

Nine days post inoculation the baboon was sacrificed and bled completely. Three hundred milliliters of blood was collected in 32 heparin tubes, thin blood smear was made to determine parasitaemia and percentage of schizonts in the smear. All the blood was spun at 1800 RPM for 10-minutes and the top brown layer of erythrocyte pellets enriched with schizonts harvested and parasitaemia determined. The erythrocytes were washed two times in RPMI 1640 at a ratio of 1:1. Five hundred microlitres of packed erythrocytes cells containing $0.65 \times 10^9$ schizonts were resuspended in 300 µl of cytomix (120 mM KCl, 0.15 mM CaCl$_2$, 2 mM EGTA, 5 mM MgCl$_2$, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, 25 mM Hepes, PH 7.6), a salt solution used to stabilize and increase the survival of schizonts during transfection by electroporation (van den Hoff et al., 1992).

3.8 Transfection

Transfection was carried out by electroporation process where appropriate voltage per electric field was applied to cells causing temporary breakdown of the cell membrane leading to formation of pores through which DNA entered the cells. The DNA constructs (Figure 4.3) were thawed under ice and 50 µl of DNA construct mixed with 800 µl of parasites (schizonts) in Cytomix according to the method described by van den Hoff et al. (1992). Briefly, the mixture was transferred into 0.4 cm gap cuvette (Biorad) which had been chilled under ice. The 800 µl mixture of parasites in cytomix plus 50 µl of DNA construct in the cuvette was carefully fixed in Bio-Rad gene pulsar. The mixture was
subjected to pulse of 2.5 KV, at a capacitance of 25 μF and a resistance of 200 Ω for 0.9 milliseconds (ms) as described by Kocken et al., (2002). Transfection was done three times as per electroporation conditions and time constants shown in Table 3.2.

### Table 3.2 Transfection Parameters

<table>
<thead>
<tr>
<th></th>
<th>1st Transfection</th>
<th>2nd Transfection</th>
<th>3rd Transfection</th>
</tr>
</thead>
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<tr>
<td>Schizonts</td>
<td>800μL</td>
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<td>800μL</td>
</tr>
<tr>
<td>Voltage</td>
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<td>2.5KV</td>
<td>2.5KV</td>
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<tr>
<td>Capacitance</td>
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<td>25μF</td>
<td>25μL</td>
</tr>
<tr>
<td>Resistance</td>
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<td>200Ω</td>
<td>200Ω</td>
</tr>
<tr>
<td>DNA Constructs</td>
<td>50μL</td>
<td>50μL</td>
<td>50μL</td>
</tr>
<tr>
<td>Time constants</td>
<td>0.9ms</td>
<td>0.9ms</td>
<td>0.9ms</td>
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</table>

The electroporated parasites were pooled and chilled on ice for at least 5-8 minutes. The electroporation process was repeated two times using the same number of parasites in the cytomix in the same amount of DNA-constructs. The electroporated parasites were mixed to make 2.4 ml of electroporated *P. knowlesi* (schizonts).

### 3.9 Infection of Baboons with Electroporated Parasites

The 2.4 ml of electroporated parasites were mixed with 1.6 ml of RPMI 1640. The 4ml were divided into two equal amounts and used to inoculate each of the baboons (Pan 2838 and Pan 2851). The baboons were maintained at the biocontainment facility described.
3.10 Development of Electroporated Parasites in Baboons and Selection of the Transformants

The health of Pan 2838 and Pan 2851 were monitored daily. Two days post-inoculation, finger prick samples of blood were collected daily for determination of parasitaemia as described before. Starting 40 hours after injection of baboons with electroporated parasites, each baboon was orally given 1 mg/kg body weight of pyrimethamine in a banana per day. This was supplemented once per week with 3.5 mg folinic acid to counteract the bone marrow suppression caused by pyrimethamine, (Schoondermark-van de ven et al., 1995). After 8 days of pyrimethamine pressure, the drug was given after every other day, in order to ease the drug pressure on the parasites to allow the *in vivo* selected transfected parasites to replicate. The pyrimethamine pressure was continued after every other day till when parasitaemia was sufficiently high and constant.

The two baboons were sacrificed on day 15 post-inoculation and 250 ml of blood collected from each baboon. The leucocytes were removed by Plasmodipur filtration (Eurodiagnostica, Apeldoorn, The Netherlands) and leucocytes free *P. knowlesi* infected erythrocytes were used to characterize the transformed parasites.

3.11 Preparation of Transfected Parasites Stock and Cryopreservation

An equal volume (2.5 ml) of blood from each baboon was separately processed for cryopreservation of parasite stock. Briefly, the blood was spun down at 1200 RPM for 10 minutes and the supernatant was sucked off. The pellets were suspended, 1 PCV equivalent of cryoprotectant added. The mixture allowed to equilibrate for 5 to 10
minutes at room temperature. The mixture was then aliquoted in 400 μl volume per vial. Vials containing the aliquots were snap frozen in liquid nitrogen until use.

### 3.12 Preparation and Preservation of Serum from Transfected Parasites

Five ml of blood from each baboon was processed for serum. The blood was spun directly in the serum tubes at 2000 RPM for 10 minutes. The clear serum was sucked off and transferred into two sterile labeled vials for each baboon. Four serum vials were cryopreserved at -20°C. The preserved serum was later used to assay for antibody response against transfected parasites and IFN-γ secretion by transfected parasites.

### 3.13 Culture of Transfected *P. knowlesi* and Preparation of Supernatants for IFN-γ Assay

Equal volumes (10 ml) of blood from both baboons containing transfected *P. knowlesi* free of leucocytes was processed separately and cultured in two T-25 culture flasks (Trager and Jensen, 1976). Briefly, the blood was spun at 1500 RPM for 10 minutes and supernatant removed. The RBC pellets were transferred to T-25 culture flasks. Each culture flask contained 2.5 ml of PCV. The parasites were incubated at 37°C under reduced oxygen conditions (5% CO₂, 5% O₂ and 90% N₂) in 4 ml of a complete culture medium containing 20% baboon serum, 80% RPMI, 0.4 μg/ml of gentamicine and pyrimethamine in the ratio of 1:10,000 (0.1 μg/ml) for *in vitro* selection of the transfected parasites. After 42-hours the cultures were separately transferred to 50 ml sterile centrifuge tubes and spun at 1800 RPM for 10 minutes and supernatant harvested and aliquoted into 500 μl vials and kept frozen until ready for IFN-γ testing. Supernatant from wild-type *P. knowlesi* were also prepared and served as controls.
3.14 DNA Isolation from Transfected *P. knowlesi*

Total *P. knowlesi* DNA, was isolated (Gentra System, Inc., Minneapolis, Minn.) directly from blood samples of the two baboons according to the manufacturer’s instructions. Briefly, blood samples in heparin and EDTA anticoagulants, which had been subjected to Plasmodipur (pur) filtration to remove all the leucocytes, was mixed with 0.2% saponin solution to lyse red blood cells. The mixture was incubated for 10 minutes at room temperature during which it was inverted gently 10 times to allow full mixing of saponin and erythrocytes. After incubation, the mixture was centrifuged at 2000 RPM for 10 minutes. The supernatant was removed and the pellets were vortexed vigorously to resuspend the cells in the residual liquid in order to facilitate cell lysing. Cell lysis solution was added to the resuspended cells and mixed thoroughly to lyse the cells and form cell lysate solution.

In order to remove /degrade DNase, Rnase solution was added to the cell lysate. The samples were mixed with Rnase solution by vortexing and then incubated for 1 hr at 37°C. The proteins in the cell lysate were precipitated, by addition of proteins precipitation solution and then vortexed the mixture vigorously at high speed for 20 seconds. The mixture was spun at 2000 RPM for 10 minutes. The supernatant that contained the DNA was transferred into clean sterile 50 mls centrifuge tubes while the precipitated proteins were discarded.

To obtain DNA from the supernatants, it was mixed with 100% isopropanol (2-propanol) inverted gently 50 times, spun at 2000 RPM for 3 minutes and DNA appeared at the bottom as small white pellets. The supernatant was poured off and the tubes briefly
drained on a clean absorbent paper. Ten mls of 70% ethanol was added, tubes inverted several times to wash the DNA pellet formed on the inner part of the tubes. The tubes were spun at 2000 RPM for 3 minutes and ethanol slowly and carefully poured off. The tubes were inverted downward on clean absorbent paper and air dried for 15 minutes. The DNA pellets were hydrated by mixing with DNA hydration solution shaken thoroughly, and stored at 4°C. The isolated DNA was used for DNA analysis to determine if transfected parasites had taken up plasmids carrying selectable marker gene and the gene that expresses the host IFN-γ.

3.15 DNA Amplification Assay (PCR) of Isolated DNA to Determine Presence of Plasmids Carrying Selectable Marker (DHFR/TS gene)

The amplification assay of isolated DNA of the transfected *P. knowlesi* was done over two days. The first day was used to prepare the PCR products of the isolated DNA. The samples used for PCR included control samples, the plasmid DNA, the positive control and wild-type *P. knowlesi* as the negative control. The PCR programmes were set-up as follows file-Z1-Time at 95°C for 1 minute, at 52°C for 1 minute and at 72°C for 2 minutes, file 34 time delay at 72°C for 7 minutes, finally file 8 soak temperature 4°C at which PCR products are stable (Maniatis *et al.*, 1989). Using sterile pipettes mixes were prepared for 8 DNA samples in PCR tubes. The constituents of the mixes were 40 µl of 10x buffer, 32 µl of dNTPs, 10 µl of MgCl₂, and 305.5 µl of water nuclease free, 2.5 µl of Tag polymerase and 10 µl of primers (5 µl of Tox-3 forward primer and 5 µl Tox-4 Reverse primer) to make a total of 400 µl of mixes. The base sequences of primers used were 5'-cgtgatcaatgcataaaaccggtgtgc-3' and 5'-cgtgatcaaagcttctgtatctccgc-3'. Fifty microlitres of mixes were transferred to each of the 8 PCR tubes. The tubes were labeled
as F PK-H negative control, A 2851 heparin ppur (Plasmodipur filtered), B 2838- heparin ppur, C 2838 EDTA ppur, D 2838 culture, E 3851 EDTA ppur, G Plasmids positive control and H water negative control. Four microlitres of DNA samples was added into each corresponding tube containing the mixes except the controls. Two microlitres of PK-H -ve control DNA sample was added to PCR-tube F, while 1 μl of plasmid DNA sample +ve control was added to PCR tube G. All the 8-PCR tubes containing DNA-samples were spinned briefly and one drop of PCR-oil added. The tubes were then packed in the PCR Thermal Cycler Machine. The block-cover was replaced, power switched on and PCR Thermal Cycler Machine was left running overnight (Maniatis et al., 1989).

The PCR products were subjected to electrophoresis in 1% agarose gel medium in 0.5x TBE buffer the following day. To prepare 1% agarose gel in 0.5x TBE buffer, 1 g of agarose gel was weighed and placed in 250 ml conical flask and 100 ml of 0.5x TBE buffer was added. The mixture was shaken, heated for 2 minutes at 1000 watts in a microwave. The gel was cooled for sometime in a stream of cold tap water, after which 5 μl of stock ethidium bromide was added as the gel cooled. The gel was then poured in the tray, the comb fixed and 30 minutes allowed for the gel to cast. As the gel was casting the loading buffer was prepared. The molecular weight marker was thawed under ice. Five microlitres of DNA samples was carefully transferred into the 1 μl sample-loading buffer on the flat parafilm paper without mixing.

After casting of the gel, it was well submerged in 0.5x TBE buffer in the electrophoretic tank. The samples were then loaded into the wells carefully, to ensure that there was no
contamination and mixing of the samples. Five microlitres of the molecular weight marker was loaded first into the first well directly without loading/sample buffer followed by PCR samples. The power was switched on and 100V in Biorad power pack was used to run the gel for 45 minutes.

After 45 minutes the power was switched off and the electrodes were removed. The tray and the gel were removed and the buffer was drained off. The gel was viewed under UV light in UV box. To take the photograph, Polaroid camera was focused on the gel and main camera adjusted to stand accordingly. The camera diaphragm was pushed from right to left and 3 to 4 times, shots were made. On the photographic paper positions of different samples were labeled. The gel was finally dispensed into special wastebasket.

3.16 DNA Amplification Assay (PCR) for IFN-γ Gene from the Isolated DNA of the Transfected *P. knowlesi*

The procedure for the DNA amplification assay to determine the presence of IFN-γ gene in the transfected *P. knowlesi* was the same as that in section 3.15. The only difference was the primers used and running time.

The first day involved preparation of the mixes using constituents, 10x buffer, dNTPs, MgCl₂, water nuclease free, Taq polymerase and primers the same quantity and ratio was used as described in section 3.15. However, the primers used were different. The primers A (5'-ggcttttcagctctgcattg-3') and B (5'- ccgctcgaggctgggatgctcttcgacc-3') were used to detect IFN-γ gene (Maniatis *et al.*, 1989).
In order to amplify the IFN-γ gene, the running files on the PCR machines were set-up as follows file 21-time delay at 95°C for 3 minutes and during this time the coiled DNA is straightened. File 48 step cycles (3 cycles) at 95°C for 1 minute and during this time, DNA double strand separates into single strands, at 37°C for 30 seconds annealing of the unstable bases occurred, at 72°C for 1 minutes the extension of the annealed bases took place. File 36 the step cycle (35 cycles) at 95°C for 1 minutes DNA double strands separates to form single strands, at 55°C for 30 minutes annealing of bases, and at 72°C 1 minute for the extension of annealed bases. Finally file 8 soak temperature drops to 4°C, which remains constant, at which PCR products are stable. Fifty microliters of mixes were transferred into each 8 DNA-PCR tubes. Five microlitres of DNA samples and controls were added appropriately as described in section 3.15. All the 8 PCR tubes containing mixes and DNA samples were spinned briefly and one drop of PCR-oil added. The PCR tubes were arranged in thermal Cycler machine and left to run overnight as per running files set earlier. The procedure for gel-electrophoresis was done as described in section 3.15. The DNA gel-electrophoresis plan is shown in (Table 3.3).

Table 3.3. Plan for DNA gel-electrophoresis for the PCR-products

<table>
<thead>
<tr>
<th>Wells</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>M. wt marker</td>
<td>F</td>
<td>A</td>
<td>C</td>
<td>E</td>
<td>G</td>
<td>H</td>
</tr>
<tr>
<td>Description</td>
<td>PK-H</td>
<td>Pan</td>
<td>Pan</td>
<td>Pan</td>
<td>plasmid mixes</td>
<td>2851</td>
<td>2838</td>
</tr>
</tbody>
</table>
M. wt. marker-Molecular weight marker used to show the molecular weight of the amplified DNA.

PK-H- *Plasmodium knowlesi* H- strain negative control (DNA sample from wild-type parasites

**Pan 2851 Heparin**-DNA sample isolated from RBC from Pan 2851 infected with transfected parasites.

**Pan 2838 EDTA**-DNA sample isolated from RBC from Pan 2838 infected with transfected parasites.

**Plasmid positive control**-DNA sample of plasmid carrying the IFN-γ gene.

**Mixes water**-A negative control without any DNA sample.

### 3.17 Inoculation of Baboon (Pan 2593) with Passaged Transfected *P. knowlesi*

In order to determine the development of passaged transfected *P. knowlesi*, Pan 2593 was infected with cryopreserved transfected parasites. The transfected *P. knowlesi* used were from cryopreserved parasites of Pan 2838 and Pan 2851. The transfected *P. knowlesi* were retrieved from liquid nitrogen processed and cultured overnight as described before. An inoculum of $1 \times 10^6$ transfected parasites were used. Before infection the baboon was screened for malaria parasites and ascertained to be free of malaria infection. Pre-infection serum was prepared and frozen at $-20^\circ$C till required and the infection effected through femoral vein.

After infection the general health and parasitaemia of the baboon was monitored daily. The treatment of the baboon with pyrimethamine drug supplemented with folinic acid was effected as described in section 3.10. The pyrimethamine treatment was continued until when the parasitaemia was sufficiently high and stable, after which the baboon was
sacrificed and bled. Some of the blood obtained was used for preparation of stock of passaged genetically modified *P. knowlesi*. The remaining blood was used for DNA isolation and for serum preparation.

3.18 Enzyme-linked Immunosorbent Assay (ELISA) for IFN-γ Expressed by Transfected *P. knowlesi*

Enzyme-linked Immunosorbent Assay for expressed monkey IFN-γ by *P. knowlesi* was carried out using a macaque IFN-γ ELISA kit (U-Cytech, Utrecht, The Netherlands) according to the manufacturer’s instruction. Briefly, 250 µl of coating monoclonal antibody (MAb α to monkey IFN-γ) was reconstituted using double distilled water and aliquotted into 10-vials each containing 25 µl and kept at -20°C. To coat the ELISA plate, the 25 µl of the reconstituted coating antibody was diluted to 2.5 ml using PBS. Twenty five microliters of the diluted antibody was added per well followed by addition of 25 µl of PBS into every well to fill up to 50 µl/well. The ELISA plate was incubated overnight at 4°C. The following morning ELISA plates were washed six-times with 0.05% PBS tween 20 using ELISA washer and 200 µl of blocking buffer (3% BSA in PBS) was added to each well and incubated at 37°C for 1 hour. After 1 hour of incubation, blocking buffer was flicked off and 50 µl of the sample were transferred into each ELISA plate’s wells. The reconstituted standards (Rhesus monkey IFN-γ) was serially diluted and added to some set of wells parallel to samples being tested. The ELISA- plate was then sealed and incubated for two hours at 37°C.

The ELISA plate was then washed six-times using ELISA-washer as described above. Fifty microliters of the reconstituted detector antibody (MAb anti monkey IFN-γ) was
diluted in PBS-Tween to make 5ml. Fifty microliters of diluted reconstituted detector antibody was transferred to each well, ELISA plate sealed and incubated at 37°C for 1-hour. The plates were washed as described above and 50 µl of diluted conjugate (α monkey HRP) added into each ELISA-plate well sealed and incubated for 1 hour at 37°C.

The substrate was prepared by dissolving 1 TMB tablet in 1ml DMSO. A substrate buffer was prepared by dissolving 1 capsule in 100 ml distilled water. The 1 TMB tablet in 1ml DMSO was then added to 9 ml of substrate buffer thus making 10 ml of a substrate solution. After 1-hour of incubation of ELISA-plate with the conjugate, it was washed 10 times using washing buffer as described elsewhere. Hundred microliters of substrate solution was transferred into each well of ELISA Plate and left at room temperature for 30 minutes. Fifty microliters of 2MH₂SO₄ was added into each well to stop the reaction and the results read in an ELISA plate reader at an optical density (OD) of 450 nm.

3.19 Detection of Baboon Antibody Responses against Transfected *P. knowlesi*

In order to determine baboon antibody responses, to *P. knowlesi* crude antigen was prepared from 100 ml of heparinised blood obtained from an infected baboon, with a parasitaemia of 6.4%. The nucleated cells were removed using ficoll-hypaque (ICN/Cappel USA) by centrifugation at 3000 RPM for 30 minutes. The ficoll hypaque was washed off from the red blood cells three times using an equal volume of RPMI 1640 and spinning at 1500 RPM for 10 minutes. The red-blood cells with the parasites were then suspended in RPMI 1640 and spun at 1800 RPM for 10-minutes so as to concentrate the parasites. The supernatant was discarded and the topmost brown layer scooped into a separate tube.
The top brown layer had a parasitaemia of 13% and was used to prepare the crude whole parasite antigen. These cells were lysed using 0.2% saponin, incubated for 10 minutes at room temperature and then spun at 3200 RPM for 10 minutes. The parasite pellet obtained was washed twice with Phosphate Buffered Saline (PBS) by spinning at 1500 RPM for 10 minutes. The parasites were then homogenized, aliquoted at 1x 10^7 parasites in 100 μl of PBS and stored at -70°C until when required.

The prepared parasites antigen was used for IgM and IgG antibody detection. One hundred microlitres of soluble *P. knowlesi* homogenized antigen diluted at 1/625 in carbonate bicarbonate buffer was added to each well of polystyrene micro ELISA plate. The plate was covered and kept overnight at 4°C for effective coating. After overnight coating of the ELISA plate, it was washed in PBS tween three times as explained before. The plate was blocked with 3% BSA in PBS and incubated for 1 hour at 37°C. After incubation the plate was washed 4 times in PBS tween. One hundred microlitres of serum diluted at 1/25 or 1/125 in 1% BSA PBS/T was added into each well and incubated for 1 1/2 hours at 37°C. After the incubation period the excess serum was poured off and the plate washed four times in PBS/T.

To assay for IgG, 2.5 μl of Rabbit anti-monkey IgG alkaline phosphate was diluted in 5 ml of 1% BSA PBS/T. Fifty microlitres of the diluted conjugated antibody was added into each well and incubated for 1 hour. The plate was then washed six-times using PBS/T to remove excess conjugated antibody. One tablet of the P-Nitrophenyl phosphate (PNPP) substrate was dissolved in 5 mls of diethanolamine substrate buffer and 50 μl was added into each well and incubated for 30 minutes in the dark. Optical density (O.D)
was read at a wave length of 405 nm in a microplate reader (Dynatech laboratories, UK). To assay for IgM, the same experiment was repeated but Rabbit anti-monkey IgG AP conjugate was substituted with Rabbit anti-monkey IgM Horse raddish Peroxidase (HRP) (Cappel Organon Teknika, OR, USA). One hundred microlitres of horse radish peroxidase conjugated Rabbit anti-monkey IgM was diluted 1/2000 added to the serum and incubated for one hour at 37°C. One hundred microlitres Orthophenyldiamine (OPD) (Sigma, UK, final concentration of 0.4µg/ml) in phosphate citrate buffer was used as the substrate. Optical density was read at 450nm after 30 minutes of incubation.
CHAPTER FOUR
RESULTS

4.1 In vivo Development of P. knowlesi in Infected Baboon

To raise parasites for transfection, in vivo generation of large number of parasites was initiated by infection of a baboon (Pan 2574). Before infection, the appearance of RBC’S of blood smear of baboon was normal (Plate 4.1). After infection the in vivo development of P. knowlesi was closely monitored by observation of giemsa stained thin blood smear to examine different stages of parasites development.

Plate 4.1: Appearance of RBC before infection
Arrow A shows normal Red Blood Cells before infection

The baboon developed peak infection by day 9, which dropped by day 10 post-inoculation. At peak parasitaemia the erythrocytes were infected with mainly trophozoite stage of parasites (Plate 4.2).
Plate 4.2 RBC infected by different stages of *P. knowlesi*.
Arrow B shows Infected RBCs with trophozoites

4.2 Parasitaemia of the *P. knowlesi* in the Infected Baboon (Pan 2574)

The parasitaemia of infected baboon (Pan 2574) was monitored daily and various stages of parasitaemia were demonstrated at different days (Table 4.1).
Table 4.1: Parasitaemia profile of *P. knowlesi* infected baboon (Pan 2574)

<table>
<thead>
<tr>
<th>DAY</th>
<th>Thick smear</th>
<th>No. of parasites in each stage</th>
<th>Thin smear</th>
<th>Parasitemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ring</td>
<td>Trophozoites</td>
<td>Schizonts</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Rings Seen</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>“</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>“</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>“</td>
<td>1</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>8</td>
<td>“</td>
<td>5</td>
<td>97</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>“</td>
<td>20</td>
<td>105</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>“</td>
<td>0</td>
<td>13</td>
<td>90</td>
</tr>
</tbody>
</table>

No parasites were detected up to day 3 post-inoculation (Figure 4.1). However, from day 4 rings were seen in the thick smear and 1 schizont was observed in the thin smear with a parasitaemia of 0.05% equivalent to 5 parasites in 10,000 RBC’s (Table 4.1). The baboon developed acute infection with a peak level of about 800 parasites per $1 \times 10^4$ erythrocytes by day 9, which subsequently decreased to 500 parasites per $1 \times 10^4$ erythrocytes by day 10 post inoculation (Figure 4.1). At the peak parasitaemia, baboon showed severe clinical symptoms of malaria by day 8 and 9 post inoculation as it remained in a sitting position in the cage with ruttled fur looking thin and sickly,
(apathy). Therefore, at day 10 post-inoculation the baboon was totally bled out, blood collected and processed to provide *P. knowlesi* schizonts for transfection.

Figure 4.1: Parasitaemia profile of *P. knowlesi* in infected parasite donor baboon (Pan 2574)
4.3 Results of Preparation of *P. knowlesi* Schizonts for Transfection

At the peak parasitaemia (day 10 post-inoculation) parasitaemia was monitored on hourly bases (Table 4.2) to establish the time of peak schizont stage. On day 10 post-inoculation at about 16.30 hrs the baboon was bled and about 300 mls of blood was collected in heparinized 15 ml centrifuge tubes. The parasitaemia was established to be 13.4% with greater than 90% schizonts required for electroporation (Table 4.2). The blood was centrifuged and the top brown layer harvested. The layer had high concentration of schizont (Plate 4.3). From the parasitaemia of 13.4% of the top brown layer, the number of parasites required for successful electroporation was worked out. At least $0.5 \times 10^9$ schizonts/ml of parasites suspension were required for every electroporation.

**Table 4.2: Parasitaemia of PAN 2574 on day 10 post-inoculation at different times**

<table>
<thead>
<tr>
<th>Time</th>
<th>Source</th>
<th>Ring(s)</th>
<th>No. of parasites</th>
<th>Schizonts</th>
<th>Gam</th>
<th>Parasitemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:20 Hrs</td>
<td>A prick from the Pan 2574</td>
<td>0</td>
<td>13</td>
<td>90</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>15:30 Hrs</td>
<td>Heparin tube after bleeding</td>
<td>4</td>
<td>24</td>
<td>113</td>
<td>0</td>
<td>6.9</td>
</tr>
<tr>
<td>16:30 Hrs</td>
<td>Brown Layer</td>
<td>1</td>
<td>2</td>
<td>288</td>
<td>0</td>
<td>13.4</td>
</tr>
</tbody>
</table>
Plate 4.3: Schizonts before transfection by electroporation

Arrow C shows RBC infected by schizonts.

4.4 Results of the *in vivo* Development of Electroporated *P. knowlesi* in Baboons

Prior to inoculation the baboons were confirmed to be free of malaria. The first two days post-inoculation, no parasites were observed in both thick and thin smears. However, at 72-hours post-inoculation newly invaded parasites were readily detectable in both thick and thin smear in the two infected baboons with low parasitaemia (0.04%) (Table 4.3 and
4.4) After continuous treatment with pyrimethamine 1mg/kg body weight from day-2 post-inoculation for five days, parasitaemia rapidly dropped to undetectable levels. From day-0 up to day-6 post-inoculation both baboons appeared healthy. In order to ease the pressure of the pyrimethamine and allow replication of the transfected parasites, the drug administration was reduced and given only on alternative days, from day 6 post-inoculation. The parasitaemia rose to detectable levels from day 11 post-inoculation for Pan 2838 and from day-10 post-inoculation for Pan 2851 (Table 4.3 and 4.4 respectively). The parasitaemia of pyrimethamine resistant parasites in both baboons on day 11 post-inoculation was about 0.047% and was observed to rise steadily (Table 4.3 and 4.4). After day 12 post-inoculation the parasitaemia started fluctuating between a parasitaemia of 0.09% to 0.34% in both baboons up to day 15 when the baboons were sacrificed.
Table 4.3: Parasitaemia and *in vivo* selection of *P. knowlesi* in Pan 2838

<table>
<thead>
<tr>
<th>Day</th>
<th>Pyrimethamine orally given in (milligramme)</th>
<th>Thick Smear</th>
<th>No. of parasites in each stage</th>
<th>Parasitaemia %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No drug given.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&quot;   &quot; &quot;</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14 mg of drug + given daily up to day 6.</td>
<td>1</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>0</td>
<td>14 mg of drug given.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>No drug given.</td>
<td>+</td>
<td>0</td>
<td>0.047</td>
</tr>
<tr>
<td>2</td>
<td>14 mg of drug + given.</td>
<td>7</td>
<td>1</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>No drug given + upto the end of the experiment.</td>
<td>5</td>
<td>0</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>&quot;   &quot; &quot;</td>
<td>+</td>
<td>7</td>
<td>0.34</td>
</tr>
<tr>
<td>5</td>
<td>&quot;   &quot; &quot;</td>
<td>+</td>
<td>4</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Table 4.4: Parasitaemia and in vivo selection of *P. knowlesi* in Pan 2851

<table>
<thead>
<tr>
<th>Day</th>
<th>Pyrimethamine orally given in (milligramme)</th>
<th>Thick Smear</th>
<th>No. of parasites in each stage</th>
<th>Parasitaemia %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0 0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>+</td>
<td>1 0 0 0 0 0</td>
<td>0.048</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>0</td>
<td>0 0 0 0 0 0</td>
<td>0.04</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>+</td>
<td>0 0 0 0 0 0</td>
<td>0.047</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>+</td>
<td>5 0 0 0 0 0</td>
<td>0.25</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>+</td>
<td>6 0 0 0 0 0</td>
<td>0.29</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>+</td>
<td>2 0 0 0 0 0</td>
<td>0.09</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>+</td>
<td>3 1 2 0 0 0</td>
<td>0.29</td>
</tr>
</tbody>
</table>
4.5 Results of the Development of Passaged Transfected $P.\ knowlesi$ in Pan 2593

The transfected parasites were passaged into another baboon in order to characterize parasites that express monkey IFN-γ and the resulting host immune responses in the baboon (Pan 2593). The parasitaemia was monitored daily under pyrimethamine pressure (12 mg of pyrimethamine was administered on alternate days) throughout the period of experimentation. The passaged parasites could not be detected until after 4 days post-inoculation. However, from day 4 post-inoculation parasitaemia stabilised with low parasitaemia slightly higher than that in Pan 2838 and Pan 2851 (Figure 4.2). The parasitaemia rose to 1.35% by day 13 post-inoculation after which it started to drop and by day 20 post-inoculation the parasitaemia was as low as 0.04% (Table 4.5). This baboon (Pan 2593) had low parasitaemia and did not develop acute infection unlike to the donor monkey (Pan 2574), which had been infected with wild-type parasites (Figure 4.2).

This shows episomally transfected $P.\ knowlesi$ in baboon develop chronic infection.
Table 4.5: Parasitaemia and *in vivo* development of passaged transfected *P. knowlesi* in baboon (Pan 2593)

<table>
<thead>
<tr>
<th>Day</th>
<th>Physical parameters</th>
<th>Pyrimethamine Orally given</th>
<th>Thick smear</th>
<th>Ring</th>
<th>Troph</th>
<th>Schiz</th>
<th>Gam</th>
<th>Parasitemma %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Infected with $1 \times 10^6$ transfected <em>P. knowlesi</em> parasites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Normal</td>
<td>12mg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>12mg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.09</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>12mg</td>
<td>+</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.18</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.27</td>
</tr>
<tr>
<td>7</td>
<td>Normal</td>
<td>12mg</td>
<td>+</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.33</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>10</td>
<td>Normal</td>
<td>12mg</td>
<td>+</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>11</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.47</td>
</tr>
<tr>
<td>12</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.18</td>
</tr>
<tr>
<td>13</td>
<td>Normal</td>
<td>12mg</td>
<td>+</td>
<td>19</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>1.35</td>
</tr>
<tr>
<td>14</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.74</td>
</tr>
<tr>
<td>15</td>
<td>Normal</td>
<td>12mg</td>
<td>+</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>16</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.09</td>
</tr>
<tr>
<td>17</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>18</td>
<td>Normal</td>
<td>-</td>
<td>+</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 4.2: Parasitaemia Profile of Parasites in the Donor Monkey Pan 2574 and Transfected Parasites in Pan 2838, 2858 and 2593
4.6 Molecular Analysis of Transfected *P. knowlesi*

Molecular analysis of transfected *P. knowlesi* was carried out by polymerase chain reaction (PCR) analysis of DNA from transfected parasites. The results of polymerase chain reaction of the entire DNA sample isolated from transfected *P. knowlesi* from Pan2838 and Pan 2851 indicated positive results for selectable marker gene (*Tdhfr-ts*). The PCR analysis of the DNA sample displayed conspicuous bands for Pan 2838 and Pan 2851, while the negative control (wild-type *P. knowlesi*) had no band (Plate 4.4), showing that the transfected parasites had taken up the transfection plasmids. The results confirmed successful transfection of *P. knowlesi*.

The PCR analysis, which amplified the ORF of IFN-γ gene of the isolated DNA sample from transfected parasites showed the presence of plasmid carrying gene for IFN-γ (Plate 4.5). The positive results indicated that the gene for IFN-γ cytokine was intact and stable in the parasites.
Figure 4.3: DNA constructs used to genetically modify *P. knowlesi* to express baboon IFN-γ

![Diagram of DNA constructs](image)

**Key**
- Sequence of *Tgdhfr-ts* selection cassette
- *P. berghei* 5’ DNA regulatory sequence
- *rhIFN-γ* gene
- *P. berghei* 3’ *dhfr-ts* flanking sequence

**Plate 4.4 Plasmids in transfected *P. knowlesi***

<table>
<thead>
<tr>
<th><em>Tgdhfr-ts</em> PCR</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 3 2 1</td>
<td>2.0</td>
</tr>
<tr>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

**Plate 4.5 Interferon gamma gene in the transfected *P. knowlesi***

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Plate 4.4** lane 4 and 3 are bands for positive results for selectable marker gene from Pan 2838 and Pan 2851 respectively. Lane 2 is for negative control of DNA from wild-type parasites. Lane 1 is for Molecular weight marker. **Plate 4.5** lanes 4, 5, 6 and 7 show bands for positive results for IFN-γ gene from Pan 2838, 2851, 2838 and 2851 respectively. Lane 3 is for positive control, lane 2 is negative control and lane 1 is Molecular weight marker.
4.7 Results of *in vitro* Culture of Transfected and Passaged Transfected *P. knowlesi*

After 48 hours of *in vitro* culturing of the transfected parasites, the parasitaemia of Pan 2838 did not increase while that of Pan 2851 increased from 0.08% to 0.14%. The parasitaemia of passaged transfected parasites in Pan 2593 increased slightly from 0.12% to 0.16% (Table 4.6). The supernatant of the three cultures was harvested and kept at −70°C and later used for IFN-γ assays. The parasitaemia of parasites from Pan 2669 decreased from 0.14% to 0.09% but that of parasites from Pan 2698 increased from 0.09% to 0.19% (Table 4.7). After 48 hours of culturing the supernatant was harvested and kept at −70°C. The supernatant was used to assay for IFN-γ where it served as negative control of transfected parasites.

Table 4.6: Parasitaemia of *in vitro* culture of transfected parasites from (Pan 2838 and Pan 2851) and passaged transfected parasites in (Pan 2593)

<table>
<thead>
<tr>
<th>Time (HRS)</th>
<th>Baboon (PAN)</th>
<th>No. of parasites at each stage</th>
<th>Parasitaemia %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2838</td>
<td>0 2 0 0</td>
<td>0.09</td>
</tr>
<tr>
<td>2851</td>
<td>0 1 1 0</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>2593</td>
<td>1 1 1 0</td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td>48 HRS</td>
<td>2838</td>
<td>0 0 2 0</td>
<td>0.09</td>
</tr>
<tr>
<td>2851</td>
<td>0 2 1 0</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>2593</td>
<td>2 1 1 0</td>
<td></td>
<td>0.16</td>
</tr>
</tbody>
</table>
Table 4.7: The parasitaemia of in vitro culture of wild-type *P. knowlesi* in baboon serum

<table>
<thead>
<tr>
<th>Time</th>
<th>Baboon (Pan)</th>
<th>No. of parasites at each stage</th>
<th>Parasitaemia %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ring</td>
<td>Troph</td>
<td>Schiz</td>
</tr>
<tr>
<td>0-24HRS</td>
<td>2669</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2698</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>48 HRS</td>
<td>2669</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2698</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

4.8 Detection of IFN-gamma Secreted by Transfected *P. knowlesi*

Monkey IFN-γ was detected in culture supernatants from parasites episomally transfected with plasmid pDB.DTM.DB/AB.γMM.DB and also in the transfected passaged parasites, but not from control cultures of wild-type parasites (Table 4.8). The IFN-γ detected in Pan 2838 infected with transfected parasites was 31.143 pg/ml (parasitaemia of 0.09%), while IFN-γ concentration of 37.584 pg/ml was detected in Pan 2851 with a parasitaemia of 0.14%. Similarly, IFN-γ concentration of 40.182 pg/ml was detected in passaged transfected parasites Pan 2593 with a parasitaemia of 0.16%. The results showed that the higher the parasitaemia, the higher the concentration of IFN-γ produced in the in vitro culture of the transfected parasites.
Table 4.8: IFN-γ released from transfected and passaged transfected parasites and controls

<table>
<thead>
<tr>
<th>Culture</th>
<th>Parasitaemia %</th>
<th>Day post inoculation</th>
<th>Transfection Construct</th>
<th>IFN-γ Release in Pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type parasites</td>
<td>0.09</td>
<td>8</td>
<td>N/A</td>
<td>&lt;</td>
</tr>
<tr>
<td>Pan 2669</td>
<td>0.19</td>
<td>9</td>
<td>N/A</td>
<td>&lt;</td>
</tr>
<tr>
<td>Pan 2698</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td></td>
<td></td>
<td>N/A</td>
<td>&lt;</td>
</tr>
<tr>
<td>Transfected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parasites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pan 2838</td>
<td>0.09</td>
<td>14</td>
<td>pD_B. D_TM. D_B/A_B. γM_m. D_B</td>
<td>31.143</td>
</tr>
<tr>
<td>Pan 2851</td>
<td>0.14</td>
<td>14</td>
<td>pD_B. D_TM. D_B/A_B. γM_m. D_B</td>
<td>37.584</td>
</tr>
<tr>
<td>Pan 2593</td>
<td>0.16</td>
<td>14</td>
<td>pD_B. D_TM. D_B/A_B. γM_m. D_B</td>
<td>40.182</td>
</tr>
</tbody>
</table>

Pan 2593-was infected with passaged transfected parasites

<    Below detectable levels.

N/A    Transfection was not done.

4.9 Antibody Response against Transfected *P. knowlesi*

The cryopreserved serum at -20°C of transfected and passaged transfected parasites was used to assay for antibody immune response. Antibody response was positive for IgM isotype but not for IgG isotype in baboons infected with transfected and passaged transfected parasites. However, antibody response toward wild-type parasites (Pan 2574) which induced acute infection in the donor monkey was strong with rise in both IgM and
IgG isotype and the higher the parasitaemia, the stronger was the antibody response (Table 4.9)

**Table 4.9 Antibody production against transfected and passaged transfected *P. knowlesi* and controls in baboon**

<table>
<thead>
<tr>
<th>Day</th>
<th>Baboon (Pan)</th>
<th>Mean O.D 1gM</th>
<th>Mean O.D 1gG</th>
<th>Parasitaemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(2838)</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(transfected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>0.2</td>
<td>0.0</td>
<td>0.30</td>
</tr>
<tr>
<td>14</td>
<td>&quot;</td>
<td>0.2</td>
<td>0.0</td>
<td>0.34</td>
</tr>
<tr>
<td>0</td>
<td>(2851)</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(transfected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>0.1</td>
<td>0.0</td>
<td>0.08</td>
</tr>
<tr>
<td>14</td>
<td>&quot;</td>
<td>0.1</td>
<td>0.0</td>
<td>0.09</td>
</tr>
<tr>
<td>0</td>
<td>2593</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(passaged)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>0.1</td>
<td>0.0</td>
<td>0.12</td>
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<tr>
<td>14</td>
<td>&quot;</td>
<td>0.4</td>
<td>0.0</td>
<td>0.33</td>
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</table>
CHAPTER FIVE

DISCUSSION

5.1 Development of *Plasmodium knowlesi* in Baboon (Pan 2574)

The results of this study showed that the baboon developed acute parasiteaemia following infection with *P. knowlesi* (more than 700 parasites per $1 \times 10^4$ erythrocytes) with peak infection at day-9 post inoculation. Although the main purpose of the study was to raise large number of parasites for transfection, it also provided the opportunity to confirm that baboon with acute parasitaemia develops severe clinical symptoms characteristic of severe malaria (Ozwara *et al.*, 2003a). The same observation was made in rhesus monkeys (Ibiwoye *et al.*, 1993; Tatke and Malik, 1990; Spangler *et al.*, 1978; Rosen *et al.*, 1968).

However, the infection of baboon with *P. knowlesi*, so as to act as a donor for parasites for transfection is not fully reliable, since there are no parameters to tell if the *P. anubis* will develop acute parasitaemia or mild chronic infection. Secondly to bleed the baboon completely to obtain parasites is not always feasible for ethical reasons; hence more research on long term *in vitro* culture of *P. knowlesi* should be emphasized in order to obviate the need for monkeys as parasite donors for genetic manipulation. Nevertheless, the results of this study indicated that *P. anubis* is fully susceptible to experimental *P. knowlesi* H-strain infection since the baboon developed patent parasitaemia as previously established (Ozwara *et al.*, 2003a). The parasitaemia profile observed in Pan 2574 was comparable to those in rhesus monkey following infection with the same parasites (Kocken *et al.*, 2002), indicating that virulence of this strain is comparable in both monkeys. In contrast to rhesus monkey, *P. knowlesi* infected baboons developed clinical
symptoms at onset of patent parasitaemia. *Plasmodium knowlesi* infected rhesus monkeys frequently show minor clinical symptoms until they suddenly collapse due to massive parasitaemia (Butcher, 1996; Coatney et al., 1971; Tatke and Malik, 1990; Spangler et al., 1978).

### 5.2 Generation of Wild type *P. knowlesi* and Transfection by Electroporation

In order to carry out transfection large numbers of *P. knowlesi* were required, but they could not be obtained through *in vitro* *P. knowlesi* culture, since it had proved fruitless. Therefore, large number of *P. knowlesi* required for transfection was propagated in a donor baboon (Pan 2574). After processing the *P. knowlesi* infected blood from the baboon, the brown layer had 98.9% schizonts with a parasitaemia of 13.4%. At least $0.5 \times 10^9$ schizonts/ml were used for every electroporation. Large numbers of parasites were used in electroporation, since most of the parasites were expected to die due to shock of high voltage leading to the breakdown of cell membrane and pH-change because of electrolysis at the electrodes.

Transfection by electroporation using high-voltage electric shock was first initiated using fibroblasts (Wong and Neumann, 1982; Neumann et al., 1982) and since then the technique has undergone a lot of modification but it is still accompanied by large cell death. New mechanisms for delivering DNA into the parasites must be tested to identify those that enhance transfection efficiency using little number of parasites. One possible approach involves the use of polyamidoamine dendrimers (Mamoun et al., 1999b). Using this procedure, the efficiency for transfecting *P. falciparum* was estimated at 1 in 10. Conditions for intracellular transfection of *P. knowlesi* have not been optimized, since it
involves use of large number of *P. knowlesi*, in which the majority dies in the process of electroporation. The use of polyamidoamine dendrimers, would deliver DNA into all blood developmental stages thus improving transfection efficiency in *P. knowlesi*. However, it was not possible to test this transfection system due to limitation of resources.

The transfection procedure used in this study is only effective for mature schizonts of *P. knowlesi* when merozoites are mature and about to leave the cell, hence damage of the erythrocytes by electroporation would not affect the survival of the transfected parasites. Transfection by electroporation succeeded in this study as it was confirmed by *in vivo* selection of transfected parasites by pyrimethamine administration in baboon. This is because the parasites that had survived under pyrimethamine drug pressure, must have taken up plasmids carrying *T. gondii dhfr-ts* gene that conferred pyrimethamine resistance.

5.3 Development and Selection of Transfected and Passaged Transfected *P. knowlesi* and immune responses in baboon system

This study demonstrated that transfection of *P. knowlesi* by electroporation using transfection constructs containing a heterologous selection cassette based on mutagenised *T. gondii dhfr-ts* gene that confer pyrimethamine resistance, flanked by *P. berghei dhfr-ts* flanking sequences was effective. This is in agreement with the results obtained by Kocken *et al.*, 1998. This is because after 72-hours post-inoculation of transfected parasites in baboons, parasites were readily detectable in both thick and thin smear with low parasitaemia (0.04%). After initiation of pyrimethamine treatment continuously from day 2 post-inoculation the parasitaemia dropped to a level that no parasites could be
detected in thick smear for up to 10 days post inoculation. These results showed for the first time in baboons, that pyrimethamine administered orally in baboon had killed *P. knowlesi*, which had not taken plasmid having mutated *dhfr-ts* genes that conferred pyrimethamine resistance as selectable marker in the current transfection system. These results confirm that the parasites which had not taken the plasmids were selectively eliminated from baboon system, by pyrimethamine as previously observed (van der Wel *et al.*, 1997), when rhesus monkey were used. However, the results showed that by day 11 post-inoculation the few stable *P. knowlesi* detected, were resistant to pyrimethamine indicating they had taken the plasmid, hence pyrimethamine resistant parasite had been generated and selected.

This study showed that after day 6-post inoculation when the pyrimethamine drug was being administered in alternative days, the parasitaemia rose to detectable levels after reduced pressure of pyrimethamine which led to *in vivo* replication of the transfected parasites. The results further showed that the parasitaemia of the pyrimethamine resistant parasites was approximately the same in the two baboons (0.27% and 0.29%) in Pan 2838 and Pan 2851 respectively. A similar study in Netherlands (van der Wel *et al.*, 1997) using two rhesus monkey reported a higher parasitaemia of more than 1% by day 12 post-inoculation, while in this study parasitaemia was below 0.5% by day 15-post inoculation. The low parasitaemia observed was possibly due to the fact that only a small percentage of parasites had taken up the plasmid, hence were resistant to pyrimethamine and the rest were killed by pyrimethamine since they had not taken up the plasmid.
Close observation of the baboons infected by transfected parasites showed no clinical malaria symptoms. The baboons appeared healthy, active and had taken all the food cubes an indication that the transfected parasites that could express IFN-γ were too weak to cause clinical malaria symptoms and had possibly induced protective effect. This is contrasted with infection with wild-type parasites where classical malaria symptoms developed.

Although this study was limited by the small number of baboons used for selection and development of pyrimethamine-resistant transfected parasites, baboons were used for the first time to select the transfected *P. knowlesi*. The success of *in vivo* selection of the transfected *P. knowlesi* in baboons is a major step in understanding transfected parasite-host interaction in a permissive animal model, which is phylogenetically close to humans. The genetic manipulation of this parasite species (van der Wel *et al.*, 1997) offers the unique possibility to study parasites-host interactions in a system that is highly predictive for the human situation (Kennedy *et al.*, 1997; King *et al.*, 1988). For further analysis of pyrimethamine-resistant parasites red-blood cells of baboons infected with transfected parasites were collected for DNA isolation and PCR analysis to determine if the genetically manipulated parasites had taken plasmids carrying selectable marker and IFN-γ gene that could express host IFN-γ *in vitro*. The results of parasitaemia profile of *in vivo* transfected selected *P. knowlesi* in this study showed a lot of fluctuation from day 9 upto day 15 post-inoculation an indication that the baboons were actively modulating the replication of transfected parasites and developed a chronic infection. This was contrary
to what was observed in the wild-type parasites in the donor baboon that developed acute infection by day 9 post-inoculation and appeared extremely sick.

The poor development of passaged transfected *P. knowlesi* in the baboon, suggests that the passaged transfected parasites were well modulated in baboon system as previously established in rhesus monkey (Ozwara *et al.*, 2003c). The results further indicated that the transfected *P. knowlesi* could infect baboon, but could not induce acute malaria infection like in wild-type parasite infection.

In this study antibody responses assay indicated that IgM isotype antibody rose during infection with transfected *P. knowlesi*. The antibody response was mainly the primary response involving IgM as there was no significant production of IgG, which is the main antibody produced during secondary immune response. However, if the baboons were severely (acutely) infected, IgG antibodies could have risen significantly by day 14-post inoculation (Nyawira, 2004 unpublished data). This study showed that transfected *P. knowlesi* that expresses host IFN-gamma only induced mild infection as there was no significant rise in IgG and that explains possibly why both baboons infected with transfected *P. knowlesi* were able to control parasitaemia, since parasitaemia remained below 0.4% under pyrimethamine pressure and showed no clinical malaria symptoms.

The successful infection of baboon with passaged transfected parasites which could induce antibody response showed that transfected *P. knowlesi* that expresses host IFN-gamma could induce mild infection in olive baboons possibly because of
immunomodulation. Therefore, such result as demonstrated in this study, offers a new approach to explore the development of attenuated and immunopotentiating malaria vaccine.

5.4 Molecular Characterization of Transfected \textit{Plasmodium knowlesi} and Expression of IFN-γ

In this study blood collected from baboons infected by pyrimethamine-resistant \textit{P. knowlesi} was subjected to plasmodipur (ppur) filtration to remove nucleated leucocytes, so that infected RBC could be used for DNA isolation. DNA was isolated successfully from \textit{P. knowlesi} in the infected erythrocytes from each baboon.

The results in this study confirm that \textit{T. gondii} dhfr-ts gene was actually present in the transfected parasites and conferred pyrimethamine resistance to \textit{P. knowlesi}. The results showed that the parasites that failed to take up the plasmid carrying the selectable marker gene were eliminated by pyrimethamine, while the transfected parasites (pyrimethamine resistant) survived in the two baboons. These results are the first to demonstrate that \textit{T. gondii} dhfr-ts genes in \textit{P. knowlesi} can confer pyrimethamine resistant in baboons as previously established in rhesus monkey (van der Wel \textit{et al.}, 1997).

The results of this study are consistent with the findings that transfection of the mutant \textit{dhfr-ts} gene into \textit{P. falciparum} leads to generation of parasite lines that are resistant to pyrimethamine drug (Wu \textit{et al.}, 1996). The results have important implications in genetic manipulation of the parasites as it indicates that \textit{P. knowlesi} can be transfected \textit{in vitro} by
electroporation and the genetically modified parasites can be selected \textit{in vivo} using baboon system.

The results of PCR analysis for detection of IFN-gamma gene in this study showed positive results from both baboons infected with transfected parasites. This result further confirmed that the pyrimethamine resistant parasites had taken up the plasmids carrying IFN-\(\gamma\) gene that could express host-interferon gamma. The bands of DNA-gel electrophoresis of PCR products of DNA isolated from transfected \textit{P. knowlesi} from the two baboons confirmed that the plasmids had IFN-gamma gene which was intact and stable contrary to the negative control of wild-type parasites which showed no bands. The findings of this study are in line with the findings of transfected \textit{P. knowlesi} using rhesus monkey system which showed not only the presence of IFN-\(\gamma\) gene in the plasmids but also that the gene could express host interferon gamma cytokine in both episomally and chromosomally transfected parasites (Ozwara \textit{et al.}, 2003c).

In this study, host-IFN-\(\gamma\) was detected by ELISA in 48-hours culture supernatant. There was no detectable IFN-gamma in the wild-type parasites used as controls. The absence of IFN-gamma in the culture supernatant from the wild-type \textit{P. knowlesi} confirmed that, the cytokine detected was from IFN-\(\gamma\) gene of episomally transfected \textit{P. knowlesi}. These results showed for the first time that episomally transfected parasites with host IFN-\(\gamma\) gene could express host IFN-\(\gamma\) in baboon erythrocytes. This is in agreement with findings using rhesus monkey in which the parasites were transfected with both episomal and integration constructs (Ozwara \textit{et al.}, 2003c). The results showed for the first time that
the higher the parastaemia, the higher the concentration of IFN-gamma produced in the \textit{in vitro} culture of the transfected parasites. The results further showed that the production of host IFN-gamma had no marked effect on parasite proliferation. The results demonstrate that the malaria parasite transcription and translation machinery can effectively express host cytokine.

The secreted malaria parasite proteins have to be transported across three bilayer membranes before getting out of the infected erythrocyte (Sherman, 1985). This study was not able to establish the stage at which the transfected parasites secreted the cytokines as shown by (Ozwara \textit{et al.}, 2003c), this is because time course experiment could not be performed in our system. For example, Ozwara \textit{et al.} (2003c) showed that the secreted host IFN-\(\gamma\) coincides with schizont rupture.

The bioactivity of host IFN-gamma could not be demonstrated in this study, however Ozwara \textit{et al.} (2003c) showed that the \textit{in vitro} expressed host IFN-gamma was bioactive. However, since the kit used to assay for IFN-\(\gamma\) was for bioactive cytokine, this suggests that the IFN-\(\gamma\) secreted by transfected parasites was bioactive. Other studies have shown that the bioactivity of IFN-gamma depends on protease processing of the carboxyl end of protein (Arakawa \textit{et al.}, 1986). Furthermore, stable and bioactive IFN-\(\gamma\) exists as a non-covalent homodimer (Ealick \textit{et al.}, 1991). The results showed that passaged transfected parasites in baboon expressed host IFN-\(\gamma\) just like in primary infection with transfected parasites, suggesting that the passaged transfected parasites IFN-\(\gamma\) gene remained stable and active.
Interferon-gamma is a key effector cytokine in protection against malaria, especially during the liver stages (Hoffman et al., 1997; Nardin and Nussenzweig, 1993; Schofield et al., 1987; Sedegah et al., 1994; Seguin et al., 1994). Studies in humans and animal models looking at endogenously produced and exogenously administered IFN-γ have shown that the cytokine is also required for protection against blood-stage infection (Clark et al., 1987; Su and Stevenson, 2000, Yoneto et al., 1999). From this study it is now feasible to generate parasites that can express host interferon gamma in baboons, what remains is to characterize the ability of the parasite to immunomodulate the infection and characterize the role of the expressed cytokine in host protection against malaria.
CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The following conclusions can be made from the results of the study.

a) The *P. knowlesi* parasites were successfully transfected with DNA construct *in vitro* by electroporation process using 2.5 KV, 25 μF and 200Ω in 0.4cm cuvette (Biorad).

b) The pyrimethamine drug administered orally in a banana to a baboon infected with transfected parasites by electroporation was effective in selection of the transfected parasites.

c) Transfection by electroporation of *P. knowlesi* was successful because molecular characterization of transfected parasites by PCR analysis showed presence of plasmid carrying selectable marker gene *T. gondii dfrs-ts*, and also showed presence of IFN-γ gene.

d) Transfected parasites expressed host Interferon gamma *in vitro*.

e) Transfected and passaged transfected *P. knowlesi* induced production of IgM in baboons.
6.2 RECOMMENDATIONS

a) As the transfected *P. knowlesi* that expresses host IFN-γ induced mild infection and primary immune response leading to IgM production, there is need for further studies involving more baboons, which should be infected with transfected *P. knowlesi* parasites. After mild infection and recovery, the baboons can be challenged with wild-type *P. knowlesi*, in order to test vaccine potential of transfected parasites.

b) More experiments should be tried to make transfection by electroporation more efficient by applying different conditions of electroporation and examining the parasites under the microscope after each electroporation, so as to determine the most effective conditions.

c) There is need for concerted efforts in the modification of current protocols of *in vitro* culturing system for blood stage of *P. knowlesi* in order to facilitate *in vitro* transfection and expand experimental possibilities of the system.
REFERENCES


Penelope, P.H. and Kuile, F. (1994). Childhood malaria in Africa. In Africa Health, Jan,


APPENDICES

Appendix I

BUFFERS AND REAGENTS

Incomplete Cytomix

- 120mM KCl 0.8947g
- 0.15mM CaCl$_2$ 0.0022g
- 2mM EGTA 0.07608g
- 5mM MgCl$_2$ 0.1015g
- 10mM K$_2$HPO$_4$ 0.1360g
- 25mM Hepes 0.5955g.

All the above quantities were dissolved in distilled water to make 100mls of a solution. The solution was sterilized through filtration using filters.

10X TBE Buffer

- Tris base 108gm
- Boric acid 54gm
- EDTA 8.35gm

Three reagents were weight and dissolved in distilled water to make 1 litre of a solution PH = 8.5.

Loading Buffer or Sample Buffer

- Ficoll 1.5gm
- Bromophenol blue 0.02gm
- Xyleen cyanol 0.02gm.

Three reagents were accurately weight and dissolved in double distilled water to make 10-mls of sample buffer or loading buffer.

0.2% Saponin in RPMI-1640

- Saponin 1g
- RPMI 1640 500mls
1g of Saponin was weight accurately and dissolved in 500mls of RPMI 1640 to make 0.2% Saponin in RPMI.

**1% Agarose Gel in 0.5x TBE Buffer**

Agarose gel 1g

0.5x TBE buffer 100mls

1g of agarose gel was accurately weight and dissolved in 100mls of 0.5x TBE buffer. The mixture was shaken and heated for 2 minutes in 1,000 watt microwave, cooled briefly under cold-tap water, after which 5-μl stock ethydium bromide was added to the gel as it cooled.

**Elisa –Washing Buffer- 5x PBS**

Sodium chloride (NaCl) 40g

KH₂PO₄ 1g

Na₂HPO₄ 12H₂O 14.5g

KCl 1g

The four reagents were accurately weight and dissolved in 1 litre of distilled water to make 1L of 5x PBS solution.

**3.5% Sodium Chloride Solution**

NaCl 3.5g

3.5g of sodium chloride was accurately weight and dissolved in 100ml of distilled water to make 3.5% NaCl solution.

**70% Ethanol**

Absolute Ethanol 700mls

Distilled water 300mls

700mls of absolute Ethanol was dissolved in 300mls of distilled water to make 1-litre or 1000mls of 70% Ethanol.
20% Giemsa Stain

Concentrated Giemsa stain 10mls
Distilled water 40mls.

10mls of concentrated Giemsa stain was mixed with 40mls of distilled water to make upto 50mls of 20% Giemsa stain. The 20% Giemsa stain was sterilized by filtering using 45 μl filter.

2M H₂SO₄ (aq) Solution

From concentrated H₂SO₄.

Molecular weight of H₂SO₄=98.07g
Density of concentrated H₂SO₄=1.8g/ml.

1.84g of H₂SO₄ =1ML

2 MOLES H₂SO₄ (2x98.07) = ?

\[
\frac{196.14}{1.84} = 106.4\text{mls}
\]

106.4mls of conc. H₂SO₄ contains 196.14g of H₂SO₄ equivalent to 2. Moles of H₂SO₄.

∴ Concentrated H₂SO₄ - 106.4 mls.

106.4mls of concentrated sulphuric acid was carefully measured and dissolved in distilled water to make upto 1 litre (1000mls) of 2MH₂SO₄ (aq).

Carbonate Bicarbonate Coating Buffer (pH 9.6)

Na₂CO₃ 1.59g
NaHCO₃ 2.93g
NaN₃ 0.2g

The three reagents were accurately weight and dissolved in distilled water to make upto 1 litre of a solution PH = 9.6

3% BSA in PBS-Blocking Buffer

Bovine Serum Albumin (BSA) - 3g
Phosphate Buffered Saline (PBS) - 100mls
3g of BSA was accurately weight and dissolved in 100mls of PBS solution to make 3% BSA in PBS.

1% BSA in PBS-Diluting Buffer
Bovine serum Albumin (BSA) 1g
Phosphate Buffered Saline (PBS) 100mls

1g of BSA was weight accurately and dissolved in 100mls of PBS solution to make 1% BSA in PBS.

Diethanolamine Substrate Buffer pH 9.8

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethanolamine</td>
<td>97ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800ml</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>100mg</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.2g</td>
</tr>
</tbody>
</table>

1MHCL

Diethanolamine buffer (10%) was prepared by weighing accurately 100mg of MgCl₂.6H₂O and 0.2g of NaN₃ and dissolving them in a mixture of 97ml of diethanolamine in 800ml of distilled water. The PH of the solution was adjusted to 9.8 by using 1MHCL and distilled water was added to make up to 1 litre of Diethanolamine substrate buffer solution.
Transfection of *Plasmodium knowlesi* in baboon (*Papio anubis*) provides a new system for analysis of parasites expressed transgenes and host-parasite interface

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Abstract

Despite a lot of efforts to control malaria, it still remains a major health problem. With the new development of transfection technology, it is now possible to determine the structure-function relationship of vaccine candidates. The aim of this study was to develop a baboon (*Papio anubis*) model for transfection and analysis of host-parasite interface of transfected *P. knowlesi* parasites. One baboon was infected with wild-type *P. knowlesi* parasites for generation of parasites to be transfected. At peak parasitemia, the baboon was anaesthetized bled and blood stream parasites were harvested, transfected with DNA plasmid constructs containing pyrimethamine resistant form of dihydrofolate reductase thymidylate synthase (dhfr-ts) gene from *Toxoplasma gondii* as selectable marker and monkey interferon gamma (IFN-γ) gene as the transgene. Both the selectable marker and the transgene were engineered for expression under control of *P. berghei* DNA regulatory sequences. Equal volumes of electroporated parasites were injected into two baboons, followed by a daily oral administration of pyrimethamine. Transfected parasites were detected in peripheral blood at day 10 post-transfection. At day 15 post-transfection, blood was collected from the baboons, subjected to Plasmodiurp filtration to remove leucocytes and used for DNA isolation. Analysis of isolated DNA by PCR showed presence of *T. gondii* dhfr-ts and IFN-γ genes in transfected parasites. Enzyme Linked Immunosorbent Assay for IFN-γ showed release of significant levels of IFN-γ by transfected parasites. These studies have developed a *P. knowlesi* transfection protocol, which involves in vitro gene insertion and subsequent selection of transfected parasites in a baboon system. This opens new possibilities for using the *P. knowlesi*-baboon model in vaccine development using cutting edge technology.

Keywords: Transfection, *P. Knowlesi*, Baboon (*P. anubis*) and Interferon gamma