Transfecting *Plasmodium berghei* with the *Plasmodium falciparum* thiazole kinase gene

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Applied Parasitology of the University of Nairobi.
DECLARATION

This thesis is my original work and has not been presented for a degree in any University.

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DEDICATION

To my awesome parents and siblings, for their emotional and material support and for the many sacrifices they have made for this worthy cause. To my very special friends in Roshani and Sidai: a friend in need is a friend indeed.
I would like to express my heartfelt gratitude to The University of Nairobi for the scholarship they awarded me to pursue my studies. This project was funded by funds from Antimal, The World Federation of Scientists and The Association of African Universities. My profound gratitude goes to my supervisor, Prof. Francis Mulaa for sparing no effort to ensure this study came to a successful completion. I am deeply indebted to Dr. Hastings Ozwara for his advice, assistance and guidance during the whole project and Dr. Richard Mukabana for his encouragement and advice which was necessary throughout the project. I am humbled by the unfaltering support of Prof. Lucy Irungu and grateful for the timely advice she offered coupled with the sacrifices that she was ready to endure when situations presented themselves. I acknowledge the assistance accorded to me by Ms. Gladys Bichanga and Ms. Leah Tsuma of Biochemistry department (University of Nairobi). I would also like to register my appreciation for the help granted by the laboratory technicians of Biochemistry department and School of Biological Sciences. My sincere appreciation goes out to the malaria laboratory team at the Institute of Primate Research, Karen, Nairobi, Kenya (Esther, Maamun, Onkoba and Macharia) in assisting me in the parasite culture work. I would also like to recognize the help of the animal house attendants at the aforementioned institute-Jeffer and Walter. I am grateful to my fellow colleagues at the University of Nairobi, Lab B7-Nimo, Eva, Tirus and Beth for their great sense of humor, support and friendship. When all is said and done, all the glory goes to God.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>xi</td>
</tr>
</tbody>
</table>

1.0 CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Literature Review

1.1.1 Malaria as a world health problem

1.1.2 Evolution of chemotherapy against malaria

1.1.3 Novel drug targets

1.1.3.1 Hydroxyethyl thiazole kinase

1.1.3.2 Vitamin B1 biosynthesis pathway

1.1.4 Drug screening systems

1.1.5 The rodent malaria parasite *Plasmodium berghei*

1.1.5.1 Hosts of *P. berghei*

1.1.5.2 Life cycle of *Plasmodium berghei*

1.1.6 Transfection

1.1.6.1 Parameters for transfection system of *Plasmodium*

1.1.6.1.1 DNA transfer

1.1.6.1.2 Transfection constructs

1.1.6.1.3 Selectable markers
1.1.6.1.4 Regulatory DNA sequences ................................................................. 15
1.1.6.1.5 Stable transfection of \textit{P. berghei} ...................................................... 16

1.2 JUSTIFICATION AND SIGNIFICANCE OF THE STUDY ......................... 17

1.3 Main objective .............................................................................................. 18
1.3.1 Specific objectives ...................................................................................... 18

2.0 CHAPTER TWO: MATERIALS AND METHODS ......................................... 19

2.1 Chemicals and enzymes .............................................................................. 19

2.2 Human red blood cells ............................................................................... 19

2.2.1 Human sera ............................................................................................... 19

2.2.2 Culture media ............................................................................................ 20

2.3 Parasites, bacterial strains and plasmids ...................................................... 20

Table 1: Parasites and plasmid construct used in the study ............................... 20

2.4 \textit{In vitro} culture of \textit{Plasmodium falciparum} ............................................... 20

2.5 Phenol-chloroform extraction of total genomic DNA ................................. 21

2.5.1 Amplification of DNA by the Polymerase Chain Reaction (PCR) .......... 22

Table 2: The composition of a 1X PCR reaction mix ........................................ 22

2.5.2 Purification of DNA from Agarose gels ................................................... 23

2.6 Agarose gel electrophoresis ....................................................................... 23

2.7 Preparation of competent \textit{E. coli} cells ......................................................... 24

2.8 Cloning of thk fragment ............................................................................. 24

2.8.1 Ligation reactions ..................................................................................... 25

2.8.2 Transformation of competent cells ........................................................ 25

2.8.3 Isolation of plasmid DNA by the alkaline lysis method ............................ 25

2.8.4 Purification of plasmid DNA .................................................................... 26

Table 3: Composition of restriction digests reactions ....................................... 27

2.9.1 Expression of thiazole kinase protein on pQE-30 expression vector ........ 27

vi
2.9.2 Engineering of the transfection construct ................................................................. 28
2.9.3 Generating parasites for transfection ....................................................................... 29
2.9.4 Transfecting *Plasmodium berghei* parasites .......................................................... 29

3.0 CHAPTER THREE: RESULTS .................................................................................... 31
3.1 Culturing and isolating genomic DNA from *Plasmodium falciparum* ......................... 31
3.2 Characterization of Thiazole kinase (*thk*) from *Plasmodium falciparum* ..................... 31
3.3 Preparation of competent cells .................................................................................... 33
3.5 Expression of thiazole kinase protein in *Escherichia coli XLI* blue strain ................. 36
3.6 Sequence-to-structure alignment and homology modeling of *THK* protein ............... 38
3.6.1 Simple Modular Architecture Research Tool (SMART) analysis ............................... 38
3.6.2 *THK* amino acid signal peptide analysis ............................................................... 38
3.7 Engineering of the transfection construct .................................................................. 39
3.8 *In vivo* culture of *Plasmodium berghei* parasites in Balb/c mice .............................. 43
3.9 Transfecting *Plasmodium berghei* with pExpress-1-*thk* recombinant plasmids ....... 45

4.0 CHAPTER FOUR: DISCUSSION ................................................................................. 46
4.1 Conclusions ............................................................................................................... 50
4.2 Recommendations .................................................................................................... 50

5.0 REFERENCES ........................................................................................................... 51
LIST OF FIGURES

Figure 1: The reaction scheme of Hydroxyethyl thiazole kinase enzyme ................................ 8
Figure 2: Analysis of *Plasmodium falciparum* genomic DNA for PCR amplification .......... 31
Figure 3: ORF finder results ................................................................................................. 32
Figure 4: Agarose gel analysis of amplified *Pf thk* fragments for purposes of cloning .... 33
Figure 5: LB Ampicillin plate showing transformed *E.coli XLI* blue competent cells ......... 34
Figure 6: Analysis of p GEM-T-*Pf thk* recombinant plasmids ........................................ 35
Figure 7: PCR screening of p GEM-T recombinant plasmids ................................................ 35
Figure 8: Analysis of recombinant plasmids for expression of thiazole kinase protein .... 36
Figure 9: PCR screening for *thk* DNA fragment in recombinant p QE-30 plasmids ......... 37
Figure 10: *THK* amino acid sequence signal peptide sequence prediction ....................... 39
Figure 11: Gel analysis of digested and undigested forms of pExpress-1 plasmids ............... 40
Figure 12: PCR screening of recombinant pExpress-1-*Pf thk* plasmids ......................... 41
Figure 13: The transfection construct used to electroporate *P. berghei* ............................. 41
Figure 14: Analysis of restriction digests of the pExpress-1-*thk* transfection construct .... 42
Figure 15: Agarose gel analysis of purified plasmid DNA .................................................... 42
Figure 16: A thin blood smear of Giemsa-stained *Plasmodium berghei* parasites .......... 44
Figure 17: The growth curve of *P. berghei* ANKA strain parasites .................................... 44
LIST OF TABLES

Table 1: Parasites and plasmid construct used in the study ........................................... 20
Table 2: The composition of a 1X PCR reaction mix ....................................................... 22
Table 3: Composition of restriction digest reactions ....................................................... 27
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thk</td>
<td>4-Methyl-5-beta-hydroxyethylthiazole Kinase</td>
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<td>E-value</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethane Sulphonic Acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-Thiogalactopyranoside</td>
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<td>kDa</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
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<td>X-gal</td>
<td>5-Bromo-4-chloro-3-Indolyl-β-D-galactoside.</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal Broth</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>A₆₀₀</td>
<td>Absorbance at 600nm.</td>
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<tr>
<td>PCV</td>
<td>Packed Cell Volume</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>µF</td>
<td>Micro Faraday</td>
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<tr>
<td>Kᵥ</td>
<td>Kilo volts</td>
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<td><em>PF THK</em></td>
<td><em>Plasmodium falciparum</em> Thiazole kinase protein</td>
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ABSTRACT

As current antimalarial drugs become progressively ineffective due to parasite resistance there is need to develop and pursue new therapeutic strategies. Parasite-specific metabolic pathways promise to make an ideal source of novel drug targets and provide unique opportunities for chemotherapy. Vitamin B1 biosynthesis pathway in *Plasmodium falciparum* is one such pathway. The overall aim of this study was to transfect *Plasmodium berghei* to express the *Plasmodium falciparum* thiazole kinase (Pf thk) gene. Following successful DNA isolation and PCR, a 909 base pair gene fragment was isolated from *Plasmodium falciparum* genomic DNA. Upon expression of the gene in bacterial systems, the protein was found to be soluble with a molecular weight of 34.67 kDa. The thk was successfully cloned into a *P. berghei* expression vector, pExpress-1 thus generating a stable transfection construct. Parasites (2.5x10^9) were obtained from Balb/c mice (with a 5% parasitaemia of schizont stage) and upon overnight *in vitro* culture, the schizont stage grew to 5.0x10^9 parasites. For transfection of *P. berghei* blood-stage parasites, electroporation settings of 1 Kv, 25µF and 200 Ohms were used. Time constants of 0.7 ms and 0.8 ms were attained for two samples, showing a successful electroporation. Analysis of data showed that PFTHK protein is soluble and the amino acid signal peptide analysis revealed that the protein was non-secretory and without a cleavage site. These are characteristics of an attractive drug target. Further studies in the development of the protein as a drug target are recommended.
1.0 CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

An estimated 350–500 million clinical cases and over one million malaria deaths occur annually around the world. Most deaths occur among young children below five years in sub-Saharan Africa (WHO, 1997). The most important reason for the persistence of malaria in Africa is the presence of the mosquito vector *Anopheles gambiae*, which feeds preferentially on humans and lives long enough to effectively transmit malaria parasites from one person to another (Greenwood, 1987). Development of drug resistance by malaria parasites, insecticide resistance by insect vectors (Chandre, 1999), environmental (Ghebreyesus, 1996) and climatic changes (Brown, 1998) all contribute to the huge burden of malaria in Africa.

The two most commonly available antimalarial drugs sulphadoxine pyrimethamine and chloroquine have failed in most malaria endemic regions (Takechi, 2001). Emerging resistance by malaria parasites against the newly developed Artemisinin-based combination therapies has also been reported (Noedl *et al.*, 2008). The discovery and development of novel antimalarial compounds not encumbered by pre-existing mechanisms of drug resistance is implied. Efforts in drug discovery should focus on developing a large choice of drugs (and drug targets) to treat malaria. This will reduce the current dilemma of distributing the most effective drugs widely against the alternative of minimizing resistance development through controlled use. There is need to produce many effective and affordable antimalarials that meet and overcome the challenge of drug resistance.

Prospects for the control of malaria remain elusive even now that the *Plasmodium* genome sequence has been unraveled. The *Plasmodium* genome project has identified new drug targets. An example of the application of data from the genome project is the identification of potential drug targets on the metabolic pathway of the parasite that differ significantly from those present in the genome of human hosts (Macreadie *et al.*, 2000). Thiazole kinase (*thk*)
which is a second precursor of vitamin B1 biosynthesis is one such target. Malaria parasites are capable of synthesizing vitamin B1 de novo while human hosts are unable. Parasite metabolic pathways not present in the host can be used to design potent inhibitors that are non-toxic to humans.

Transfection technology will produce numerous tools to assist drug discovery and development (de koning-Ward et al., 2000). Transfection is the method by which foreign DNA is introduced into a eukaryotic recipient cell. This technology has provided modern genetic tools for investigating the relation of gene expression to the gene function, allowing the expression of transgenes in cells as well as elucidating the functions of proteins by disrupting, modifying or replacing genes encoding them (de koning-Ward et al., 2000). The development of transfection technology has enabled the expression of transgenes in Plasmodium species, so presenting a rational approach for vaccine and drug design at the molecular level (Ozwara, 2005). The aim of this work was to transfec...
impact of malaria has been estimated to cost Africa $12 billion US dollars annually. The
economic losses are occasioned by health care costs, working days lost due to sickness,
decreased productivity due to brain damage from cerebral malaria, loss of investment and
tourism (Greenwood *et al*., 2005).

Resistance to antimalarial drugs is one of the challenges facing malaria control today. This
has been implicated in the spread of malaria to new areas and re-emergence of malaria in
areas where the disease had been eradicated (Baird, 2002). Drug resistance also plays an
important role in the occurrence and severity of epidemics in some parts of the world. Apart
from reducing the problem of drug pressure in the affected areas, continuous supply of new
and affordable drugs in addition to development of effective and implementable control
measures can reduce the overall burden of malaria (Bloland, 2001).

1.1.2 Evolution of chemotherapy against malaria

Of several families of drugs used to treat malaria quinolines are widely used. Quinolines and
related aryl alcohols owe their origin to quinine, an active ingredient of the bark of the
*Cinchona* plant. Quinine has side effects associated with toxicity (such as tinnitus) and
because it requires a thrice daily regimen over seven days, its usage can result in poor
compliance (Jastreboff *et al*., 1991). Elucidation of the structure of Quinine led to the
development of synthetic 4-aminoquinolines, notably chloroquine and amodiaquine (Ridley
*et al*., 1998). The use of amodiaquine was limited since the mid 1980s after it was linked to
the occurrence of occasional agranulocytosis in adult travelers taking the drug
prophylactically (Bloland, 2001). However, amodiaquine retains a high degree of
effectiveness against all but the most highly chloroquine-resistant strains.
Mefloquine and halofantrine are structurally related drugs that are active against chloroquine-resistant strains, but resistance can develop rapidly to each of these drugs (Wellems et al., 2001). Additional limitations include occasional neuropsychiatry disturbances for mefloquine and restricted use of halofantrine by people with heart-disease. The antifolate class of antimalarial drugs owes their origin to compounds generated through knowledge of cell biology and synthetic medicinal chemistry. Fully reduced folate cofactors are essential for the key one-carbon transfer reactions needed for nucleotide biosynthesis and amino acid metabolism (Sherman, 1998). At present, the most common antifolate used to treat malaria is the combination of 2,4-diaminopyrimidine pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR), and sulphadoxine, a sulphanomide that interferes with the action of dihydropteroate synthase (DHPS), another enzyme in the folate pathway.

The two components of sulphadoxine-pyrimethamine synergize each other, so enhancing their activity and reducing the propensity for resistance development. The long half-life of the components may also account for the fact that sulphadoxine-pyrimethamine is used for intermittent treatment in pregnancy (Wolfe, 2001). Unfortunately, resistance seems to develop rapidly when this combination is used extensively (Takechi, 2001). Another reason preventing usage of sulphadoxine-pyrimethamine as a prophylactic agent is that hypersensitivity to the sulphur component occasionally gives rise to the Steven–Johnson syndrome, a toxic epidermal necrolysis that result in painful blistering of the skin (Bloland, 2001).

Atovaquone-proguanil is a fixed–dose combination whose development shows similarity to that of sulphadoxine-pyrimethamine. Hydroxynaphthoquinone atovaquone interferes with the mitochondrial electron transport chain. Rapid resistance develops against atovaquone owing to a point mutation in the cytochrome C reductase gene (Valdya, 2001). The addition of
proguanil to atovaquone results in a synergistic activity that prevents rapid development of resistance. At present atovaquone-proguanil is used primarily as a prophylactic agent and its price is too high for it to achieve widespread use in developing countries.

Common antibiotics acting against bacterial protein synthesis such as tetracycline, doxycycline and clindamycin inhibit parasite growth and are being used increasingly in combination with other antimalarials to augment their activity (Bloland, 2001). Quinine plus tetracycline and quinine plus doxycycline are commonly used antimalarial-antibiotic combinations in parts of South East Asia. The use of these combinations in Africa is limited because both antibiotics are contra-indicated in children under 8 years of age. Clindamycin is recommended in combination with other antimalarials in some situations. These antibiotics are thought to inhibit parasite growth through the inhibition of "prokaryote-like" protein biosynthesis in the apicoplast, an organelle that is unique to apicomplexan parasites such as *Plasmodium* (Fichera *et al*., 1997). Several semi synthetic derivatives of artemisinin, the active ingredient of the Chinese herb 'qinghao' (*Artemisia annua*), which was used traditionally for treating fevers, have been used increasingly over the past two decades (Li *et al*., 1998). These derivatives include artemether, arteether and artesunate, which are all metabolized to dihydroartemisinin, the main active agent in the body. These drugs are fast acting and also act against gametocytes, the sexual stages of the parasite that infect mosquitoes.

The short half-lives of both the parent semi synthetic derivatives and the dihydroartemisinin metabolite necessitate treatment over 5-7 days when these compounds are used alone. They are therefore being used increasingly in combination with longer half-life drugs to reduce treatment time and increase individual compliance (WHO, 2001). It is anticipated that rapid
clearance of the parasites by artemisinin derivatives will reduce the chances of resistance development to the partner drugs (White, 1999).

A few issues concerning artemisinin derivatives have arisen. These include the thinking that partner drugs with very long half-lives may be unsuitable for extended use in areas of intense transmission, where subsequent infections can occur before the drug has been cleared and that resistance is more likely to develop against the partner drug. Although the cost of the drug is inexpensive by international standards, artemisinin derivatives are significantly more expensive than traditional antimalarials such as chloroquine and sulphadoxine-pyrimethamine. Recently, resistance towards Artemisinin drugs has been reported in Cambodia (Noedl et al., 2008).

In view of all these, increased efforts in antimalarial drug discovery are urgently needed. The goal must be to counter the spread of malaria parasites that are resistant to existing agents and ensure that the antimalarials are very affordable to patients living in malaria endemic areas especially sub Saharan Africa. In spite of all these negativities, a lot of hope has been created by the huge increase in the understanding of the biochemistry, molecular and cell biology of the *Plasmodium* parasites. Attention is focused on the characterization of metabolic pathways and cell structures that are different between the parasite and host. That genomic sequence of *Plasmodium* parasites has been characterized opens doors to identifying metabolic differences that could result in defining potential drug targets. In addition, improved technologies for the transformation of the Plasmodium parasite will validate new drug targets, lead to new pharmacological paradigms and may be a source of innovative chemotherapy (Henri et al., 2001).
Antimalarial drug development follows two main strategies: (i) minor modifications of existing agents and (ii) design of agents that act against novel targets. Recent advances in knowledge of parasite biology as well as the availability of the parasite genome sequence data present a wide range of novel targets for drug development. Among the most remarkable antimalarial target proteins currently studied are: (i) proteases, like plasmepsins, falcipains, and falcilysin, (ii) protein kinases, (iii) glycolytic enzymes, and (iv) enzymes involved in lipid metabolism and DNA replication. In addition, redox active proteins like glutathione reductase, thioredoxin reductase, and glutathione-s-transferase (GST) have become increasingly interesting (Sahu et al., 2008).

Traditional approaches for identifying new therapies to combat malaria include: (i) optimization of current drug regimens and formulations, (ii) developing analogs of existing drugs, (iii) analysis of compounds from natural products, (iv) identifying resistance-reversal agents, utilizing combination chemotherapeutic approaches and (v) exploiting drugs indicated for other uses (Rosenthal et al., 2001). Two major approaches have been employed in the search for new antimalarial drugs (Wiesner et al., 2003). One entails development of chemically related analogs to existing antimalarial agents (Gelb, 2007) and the other centers on identifying novel drug targets and designing chemical entities acting on these targets (Woster, 2003). Several critical and unrelated biochemical pathways have been exploited for drug target identification (Cunha-Rodrigues et al., 2006). Parasite-specific metabolic pathways make an ideal source of drug targets and provide obvious opportunities for chemotherapy (Roos et al., 2002).
1.13.1 Hydroxyethyl thiazole kinase

The enzyme 4-Methyl-5-beta-hydroxyethyl thiazole kinase catalyzes the phosphorylation of the hydroxyl group of 4-methyl-5-beta-hydroxyethyl thiazole (Campobasso et al., 2000). 4-Methyl-5-beta-hydroxyethyl thiazole kinase belongs to the family of transferases specifically those transferring phosphorus-containing groups (phosphotransferases) with an alcohol group as acceptor. It participates in thiamine metabolism. Thiamine is an essential cofactor for several key enzymes of carbohydrate metabolism such as transketolase pyruvate dehydrogenase, pyruvate decarboxylase and 2-oxoglutarate dehydrogenase. Mammals have to salvage this crucial nutrient from their diet to complement their deficiency of de novo synthesis. In contrast, *Plasmodium falciparum* possesses a thiamine biosynthesis pathway (Wrenger et al., 2006). Hydroxyethyl thiazole kinase is a second precursor of vitamin B1 biosynthesis (thiamine biosynthesis).

![Reaction Scheme of Hydroxyethyl Thiazole Kinase](image)

**Figure 1:** The reaction scheme of Hydroxyethyl thiazole kinase enzyme.
1.3.2 Vitamin B1 biosynthesis pathway

The biosynthesis of thiamine involves the combination of two independently synthesized branches, a pyrimidine branch and 5-hydroxyethyl-4-methylthiazole (THZ). The pyrimidine branch starts with 4-amino-4-hydroxymethyl-2-methyl pyrimidine (HMP) which has to be phosphorylated in two steps to 4-amino-2-methyl-5-hydroxymethyl pyrimidine diphosphate (HMP-PP) by 4 amino-4-hydroxymethyl-2-methyl kinase or 4-amino-2-methyl-5-hydroxymethyl pyrimidine phosphate kinase. The second precursor of thiamine biosynthesis is 5-hydroxyethyl-4-methylthiazole (THZ), which is activated by THZ kinase to 4-methyl-5-(2-phosphoethyl)-thiazole (THZ-P). Finally the thiazole and pyrimidine moieties HMP-PP and THZ-P are merged to thiamine phosphate (Thi-P) by thiamine phosphate synthase (Thi E) (Taylor et al., 1998).

1.4 Drug screening systems

In vitro assays typically rely on simple interactions of chemicals with a drug target. However, in vitro results often poorly correlate with in vivo results because the complicated physiological environment is absent in the in vitro testing system. Although cell-based assays can provide some information, cultured cells do not provide the physiological conditions and complex interactions among different cell types and tissues. There is a growing trend of using human tissues for drug discovery research. Tissues, however, only provide an isolated ex vivo condition, which is not completely representative of in vivo responses because drug action often involves metabolism and interplay among different tissues. Therefore, results in animal studies are essential to validate high throughput screening and exclude compounds with unfavorable absorption, distribution, metabolism, excretion and toxicity properties which are responsible for more than half of compound attrition in costly clinical trials. With alternative small-animal models emerging (which are more cost-effective) it is now possible to carry out in vivo assays before the lead optimization stage (Chaoyong Ma, 2004).
Technologies for engineering the mouse genome have made it possible to create various disease models for use in screening corresponding therapeutic compounds. Knockout mouse models have been shown to be highly predictive of the effects of drugs that act on target genes and phenotypes of these knockout mice correlate highly to the effect of the corresponding drugs (Zambrowicz et al., 2003). Knock-in, conditional knock out and transgenic techniques have made it possible to create specific gene-sequence alterations and manipulate the levels and patterns of target-gene expression. Using these techniques, it is possible to generate specific disease models to validate targets as therapeutic intervention and screen drug candidates.

1.1.5 The rodent malaria parasite *Plasmodium berghei*

*Plasmodium berghei* belongs to a group of four *Plasmodium* species (*P. vinckei*, *P. chabaudi*, and *P. yoelii*) that infect murine rodents from Central Africa.

1.1.5.1 Hosts of *P. berghei*

*Plasmodium berghei* is principally a parasite of thicket rats, *Grammomys surdaster*. This association presents a practical model for the experimental study of human malaria parasites. The *Plasmodium berghei*-rodent host parasite system is recognized as a valuable model for the investigation of the developmental biology of malaria parasites, parasite-host interactions, vaccine development and drug testing. The basic biology of both the rodent and human parasites is similar. Recent studies demonstrate a high level of conservation of genome organization between rodent and human malaria parasites (Janse et al., 2006). *Plasmodium berghei* has two extra-nuclear DNA elements, mitochondrial DNA and plastid DNA.
Infection with *P. berghei* starts with the bite of an infected mosquito (e.g. *Anopheles stephensi*) leading to deposition of sporozoites into the blood stream of the rodent host. Sporozoites invade hepatocytes. Within the hepatocytes the sporozoites develop within 47-52 hours via the trophozoite stage into mature schizonts containing merozoites. When hepatocytes rupture haploid merozoites are released from the liver schizonts and invade reticulocytes but can also invade mature red blood cells. After rupture of the schizonts, the free merozoites invade new red blood cells, resulting in an increase in parasitemia. In each asexual cycle a small portion of parasites differentiate into sexual cells, the gametocytes. Haploid macrogametocytes (females) and microgametocytes (males) are the precursor cells of the female and male gametes, respectively. In *P. berghei*, the merozoites of liver schizonts are able to differentiate directly into gametocytes after invasion of the erythrocytes.

When a mosquito feeds on an infected host only mature gametocytes undergo further development in the mosquito midgut. Fertilization occurs by penetration of the haploid male gamete into the haploid female gamete resulting in a diploid zygote. The spherical zygote develops into a banana-shaped, motile ookinete. The ookinete possesses an apical complex which is used for penetrating and traversing cells of the midgut epithelium hence settling between the basement membrane and the basal lamina. The parasites rapidly round up to form oocysts that contain thousands of daughter cells (sporozoites). Oocysts rupture and the haploid sporozoites are released into the haemocoel migrating into the salivary glands from where they are released upon injection into a new host.

**1.1.6 Transfection**

Transfection is a method by which foreign DNA is introduced into a eukaryotic cell. Once the introduced DNA has entered the nucleus and become expressed, it may be retained
temporarily (transient transfection) or maintained in a functional state for extended periods of time resulting in heritable changes (stable transfection). Transient transfection assays are widely used in eukaryotic molecular biology for the analysis of elements mediating gene expression and regulation, while a stable transfection system is used when aspects of the biology of an organism are to be examined for several generations. Stable transfection can be distinguished into episomal and integration types. The former is where DNA introduced is maintained as an extra chromosomal replicating episome while in the latter, the DNA introduced is integrated into the genome of the host cell.

Initially, it was difficult to develop transfection constructs and introduce DNA into malaria parasites. This was because the DNA to be introduced had to cross four membranes between the external environment and the nucleus in intra-erythrocytic parasites, the limited number of markers available for positive selection in *Plasmodium* and the AT-rich nature of plasmodial DNA (Kim *et al.*, 1993). Strategies to circumvent the first of these limitations (the four membranes) include electroporation delivery of the DNA to the protozoan cell and the use of gametes or merozoites rather than intraerythrocytic asexual parasites (because gametes and merozoites have fewer membranes). The second limitation, the small number of selectable markers, has led to the use of the pyrimethamine resistance marker because it is the only naturally occurring marker in *Plasmodium* that permits the positive selection necessary to identify low-frequency transfection events. Strategies used to address the third limitation (the intrinsic instability of the AT-rich DNA of *Plasmodium* and the propensity for it to form rearrangements and deletions) include the development of plasmid constructs flanked by *P. falciparum* sequences known to be stable *in vivo* (Wu *et al.*, 1995). The use of these strategies has led to the successful transfection of *Plasmodium gallinaceum* (Goonewardene *et al.*, 1993), *P. falciparum* (Wu *et al.*, 1995), *P. berghei* (Kocken *et al.*, 1999) and *P. yoelii* (Mota *et al.*, 2001).
1.1.6.1 Parameters for transfection systems of *Plasmodium*

The application of transfection procedures is significantly influenced by the method of DNA transfer, the type of transfection construct, the regulatory DNA sequences driving gene expression and the nature of selectable marker(s) used (Ozwara, 2005).

1.1.6.2. DNA transfer

Different methods to mediate DNA transfer have been tested in *Plasmodium* like lipofection, the ballistic DNA gun and pre-loading of erythrocytes with the transfecting DNA before invasion of parasites. These have been attempted but proved to be unsuccessful or non-reproducible. Electroporation has come to be the most efficient means for introducing DNA into protozoan parasites because of its simplicity and efficiency and has been used in *Plasmodium* (Goonewardene *et al*., 1993).

1.1.6.3. Transfection constructs

Different DNA constructs are used in genetically modifying *Plasmodium* species, depending on the type of transfection. Vectors for transfection are based on a bacterial plasmid backbone for propagation in *Escherichia coli*. They are assembled in a cassette-like structure so that DNA sequences can be exchanged easily. Expression cassettes for transient transfection constructs consists of the gene to be expressed flanked by 5' and 3' untranslated sequences (UTR's) of *Plasmodium* origin. The cassette is cloned into a high copy number plasmid for cloning in *E. coli*. Transient transfection constructs are electroporated into *Plasmodium* parasites as circular plasmids. The design of constructs for transient transfection is remarkably similar in all *Plasmodium* systems. DNA constructs for stable episomal transfection are circular and contain a selection cassette and an adjacent expression cassette cloned either in a head to head or a head to tail orientation. The expression cassette contains
the gene of interest. The genes in both cassettes are flanked by UTRs of *Plasmodium* origin (Van der Wel *et al.*, 1997).

Replacement DNA constructs is based on vectors for selection and integration. They consist of homologous DNA targeting sequences disrupted by a selection cassette. In addition, an expression cassette can be cloned immediately downstream or upstream to the selection cassette. These constructs are designed for site specific integration by double crossover mechanism. Prior to electroporation, replacement constructs are linearised at two sites to extract bacterial DNA sequences. However, in *P. falciparum* circular constructs are used (Duraisingh *et al.*, 2002). Insertional DNA constructs are used for site specific integration of DNA by single cross over mechanism. The constructs are similar to replacement constructs, except that they have a single internal site specific homologous DNA segment for gene targeting. The construct is linearised within the targeting sequence prior to transfection. The structure and processing of constructs for integrating DNA into *P. knowlesi*, *P. berghei* and *P. yoelii* by single crossover mechanisms are similar. Thus far, the structures of *P. knowlesi* and *P. berghei* transfection constructs are identical (Mota *et al.*, 2001).

### 1.1.6.4. Selectable markers

The genus *Plasmodium* comprises of haploid organisms and so the availability of a single selectable marker has sufficed for the stable expression of transgenes and the knockout of nonessential genes by site directed integration (de koning-Ward *et al.*, 2000). Dihydrofolate reductase thymidylate synthase (*dhfr/ts*) genes of *Plasmodium* harbouring the single point mutation has been utilized as a selectable marker resulting in successfully transformed parasites (due to its selectable pyrimethamine resistant phenotype) in transfection experiments. Dihydrofolate reductase thymidylate reductase DHFR/TS is a vital enzyme involved in DNA synthesis and its function is competitively inhibited by the (substrate
analogue) antimalarial drugs pyrimethamine and cycloguanil. High level resistance to pyrimethamine appears to be correlated to a single point mutation in the \( dhfr \) region of the enzyme due to amino acid substitution (serine to asparagine), caused by a point mutation in the \( dhfr \) region the key to pyrimethamine resistance (van Djik et al., 1996).

However, to introduce or disrupt more than one gene within the same parasite clone or to genetically complement knockout parasites, additional selectable markers are required, a matter that has proven very difficult. The parasites are naturally resistant to most of the drugs that are used for the selection of other common eukaryotic markers. Recently, several new selectable markers for \textit{Plasmodium} species have been reported. Like pyrimethamine, the antimalarial drug WR99210 causes the selective and potent inhibition of malaria parasites’ \( dhfr \). However, the binding interactions in the folate substrate pocket are distinct for these two drugs. \textit{P. falciparum} parasites that are transfected with wild type or a variant form of the human DHFR gene are resistant to the antiparasitic effects of WR99210 or methotrexate, respectively (Fidock et al., 1997).

1.1.6.5. Regulatory DNA sequences

There are a number of regulatory DNA sequences available for controlling gene expression in \textit{Plasmodium} species. For some of the \textit{Plasmodium} transfection systems, there are several promoters that vary in strength and stage specificity providing the possibility to express genes at different levels at distinct stages of the life cycle. Some regulatory elements can also be used to drive gene transcription in more than one \textit{Plasmodium} species. For example the 5’UTR from the \textit{P. chabaudi} \textit{dhfr/ts} gene is transcriptionally active in \textit{P. falciparum} (Crabb et al., 1996), the \textit{P. falciparum} CAM and PFS25 5’UTRs can function in \textit{P. berghei} (Dechering et al., 1997) and \textit{P. gallinaceum}, respectively (de Koning et al., 2000), and several \textit{P. berghei} and \textit{P. falciparum} regulatory elements can function in \textit{P. knowlesi} (van der...
Wei et al., 1997). Although this suggests that common mechanisms control gene expression in the different malaria parasites several regulatory elements do not appear to function effectively in other Plasmodium species implying that promoter elements are not completely conserved within the genus. Nonetheless, the possibility of exchanging regulatory elements between Plasmodium species can be advantageous especially in gene targeting experiments.

1.1.6.6 Stable transfection of P. berghei

The first malaria species to be stably transfected was the rodent parasite P. berghei. The mature schizonts containing fully developed merozoites were targeted in order to introduce DNA as these stages are not dependent on erythrocytes for survival. This circumvents the problem of damage to the host cell during electroporation (host erythrocyte) on which other blood stages are completely dependent for survival. Another advantage of merozoites of P. berghei is that they are readily collectable in large numbers and appear to be more stable than merozoites of other malaria species. The conditions of electroporation are 800 V and 25μF which are similar to those used for free-living forms of unicellular parasites (Goonewardene et al., 1993). To select transgenic parasites, a transfection construct harboring a derivative of Toxoplasma gondii dihydrofolate reductase –thymidylate synthase (dhfr/ts) gene that possesses a Ser-110 →Asn point mutation known to impart pyrimethamine resistance is used (Cowman et al., 1990). This gene is flanked by the 5’ and 3’ UTRs of the wild type P. berghei dhfr-ts gene. These constructs additionally possess either a 1.8 kb fragment of the PBS21 gene which is not transcribed during the asexual blood stages (Paton et al., 1993) or a 2.2 kb fragment of the non-transcribed subtelomeric repetitive DNA element to target them into the parasites genome by homologous recombination.

Rodents infected with electroporated schizonts or merozoites are usually administered with pyrimethamine on the day after transfection and on each of the next 3 days to select for
transgenic parasites. Resistant parasites are then isolated at days 8-10 post transfection. In the absence of drug pressure, episomes replicating extra chromosomally disappear and parasites revert to pyrimethamine sensitivity (van Djik et al., 1995). Stable integration of transfected DNA into the genome of *P. berghei* is possible if linear DNA is used for electroporation (Van Djik et al., 1996). Linearization of the plasmids within the subtelomeric repeat sequence results in site directed integration into the subtelomeric loci of three different chromosomes by a double crossover recombination event.

### 1.2 JUSTIFICATION AND SIGNIFICANCE OF THE STUDY

Malaria is the most prevalent vector-borne disease in the world. It is caused by protozoan parasites belonging to the genus *Plasmodium*. Recent evidence indicates that resistance of *Plasmodium falciparum* parasites to till hitherto potent Artemisinin-based therapies has emerged. This predicament is likely to result in the rise of malaria cases, complicating the gains accrued against the disease through ongoing bilateral efforts. This state of affairs necessitates the development of alternative approaches for malaria control. The search for novel drug targets to act as substitutes to current drugs against which malaria parasites have developed resistance is implied. Strong involvement in the development and validation of drug targets by scientists from endemic regions will be enormously beneficial especially because such drug targets will ultimately have to be tested and validated in those regions.

This study was aimed at transfecting *P. berghei* with the *Plasmodium falciparum* thiazole kinase (*Pf tkh*) gene. Transfection provides a series of modern genetic tools for elucidating the functions of proteins by disrupting, modifying or replacing the genes that encode them. The preliminary results obtained from this study can be used to validate *Pf tkh* as a possible novel drug target.
1.3 **MAIN OBJECTIVE**

To develop a transgenic animal model by transfecting *Plasmodium berghei* with the *P. falciparum* thiazole kinase gene.

1.3.1 **Specific objectives**

1. To clone the *Plasmodium falciparum* thiazole kinase (*Pf thk*) gene and express it in *Escherichia coli*.

2. To engineer a stable transfection construct containing the *Plasmodium falciparum* thiazole kinase (*Pf thk*) gene.

3. To transfect *P. berghei* with the *Plasmodium falciparum* thiazole kinase (*Pf thk*) gene.
2.0 CHAPTER TWO: MATERIALS AND METHODS

2.1 Chemicals and enzymes

The restriction enzymes Bam HI, Eco RI and Hind III were sourced from Roche molecular biochemicals, Mbol was obtained from NRC Holland. The Klenow fragment T4 DNA ligase was obtained from the Promega Corporation, Tag DNA polymerase was supplied by Roche molecular biochemicals. All laboratory reagents and chemicals, unless otherwise stated, were purchased from Sigma chemical company. The reagents and chemicals were of analytical grade.

2.2 Human red blood cells

Fresh human red blood cells were obtained from a human blood group O' (positive) subject who had been screened for HIV and Hepatitis B infections. It was ensured that the donors had no history of malaria and that they had neither visited a malaria endemic area recently nor taken any antimalarial drugs or antibiotics three months before. The blood was spun at 1,500 rpm for 10 minutes and plasma removed to leave the Red blood cells (PCV). The PCV was transferred to a sterile 50 ml falcon tube and was washed thrice with RPMI 1640 in twice its volume. After the third wash, the RPMI 1640 was aspirated such that the volume left was equal to the blood (50% PCV). The blood was stored at 4°C.

2.2.1 Human sera

Blood was collected from each donor into a bag that had anticoagulant excluded from it. The blood was allowed to clot for one hour while standing at room temperature and at 4°C overnight. The serum was aliquoted into 50 ml tubes under sterile conditions, inactivated by heating at 56°C for 60 minutes and then stored at -20°C.
2.2.2 Culture media

*plasmodium* parasites were grown in RPMI-1640 supplemented with 25 mM HEPES, 20μg/ml gentamycin, 3.6nM Para-amino benzoic acid, 23 nM Folic acid, 2mM glutamine, 10 mM D-glucose. *E. coli* was grown in Luria Bertani (LB) broth (Becto Dickinson and company sparks, MD, USA) [Bactotryptone 10g/l, Bactoyeast 5g/l, sodium chloride 10g/l pH 7.0 supplemented with Ampicillin (Sigma chemical company) to a final concentration of 100μg/ml] at 37°C shaking at 175 rotations per minute (rpm) for 16 hours. On occasions that *E. coli* needed to be grown after transformation 15g/l of agar (Becto Dickinson and company sparks, MD, USA) was added to the broth media to solidify it.

2.3 Parasites, bacterial strains and plasmids

The parasites, bacterial strains and plasmids used in this study are shown in table 1.

<table>
<thead>
<tr>
<th>Parasite* / Plasmid</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>D6</td>
<td>Kenya Medical Research Institute, Nairobi, Kenya.</td>
</tr>
<tr>
<td><em>Plasmodium berghei</em></td>
<td>ANKA</td>
<td>Institute of Primate Research, Nairobi, Kenya.</td>
</tr>
<tr>
<td>p GEM-T</td>
<td>-</td>
<td>Promega corporation</td>
</tr>
<tr>
<td>p QE-30</td>
<td>-</td>
<td>QIAGEN Inc.</td>
</tr>
<tr>
<td>pExpress-1</td>
<td>-</td>
<td>Institute of Primate Research, Nairobi, Kenya.</td>
</tr>
</tbody>
</table>

2.4 In vitro culture of *Plasmodium falciparum*

*In vitro* culture was initiated with cryopreserved *P. falciparum* strain D6 obtained from the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. The parasites were cultured using the method described by Jensen (1983), a modification of the method of Rowe et al.,
1968. Parasites were grown in a static culture at 37°C. The culture was set at 0.2% parasitaemia and 2.5% haematocrit in complete culture medium comprising of RPMI-1640 supplemented with 3.6nM PABA, 23 nM folic acid, 2 mM glutamine, 25 mM HEPES, 10 mM D-glucose and 20μg/ml gentamycin and 10% pooled, heat-inactivated human serum. During the adaptation period, the medium was refreshed every 24 hours and the parasitaemia was determined by microscopic examination of giemsa-stained thin and thick films prepared from the cultured material. Fresh erythrocytes were added every four days to a maximum haematocrit of three percent. Once the parasites had established, the media was refreshed after every 24 hours and sub culturing carried out once parasitaemia exceeded five per cent. The culture-adapted parasites were adapted to grow in complete media serum, aerated with culture gas mixture in an incubator set at 37°C with medium changes every 24 hours. Parasites were cryopreserved at the ring stages of development using standard protocols.

2.5 Phenol-chloroform extraction of total genomic DNA

Total genomic DNA was extracted using the method developed by Sambrook et al (2003). Parasitized red blood cells (2.59 × 10⁵) were resuspended in PBS, centrifuged at 14,000 rpm for 5 mins at room temperature and the supernatant discarded. The pellets were resuspended in 400μl of TNE solution (25mM Tris-cl pH 8.0, 10 mM Nacl, 5mM EDTA pH 8.0 and 1% SDS), RNaseA (200 μg/ml) added and the preparation incubated at 37°C for 90 minutes. Proteinase K (100μg/ml) was then added to degrade protein. The genomic DNA incubated at 37°C for 3 hours. Malaria parasite pigments/proteins were eliminated by phenol/chloroform extraction. The DNA was precipitated overnight at -20°C by adding 0.1 volumes of ammonium acetate pH 5.3 and two volumes of absolute ethanol maintained at room temperature. The DNA was pelleted by centrifugation, washed with ice cold 70% ethanol and dried at room temperature for 5 minutes. Twenty microlitres of sterile Tris buffer (pH 8.0) was added to the DNA (avoiding pippeting). The DNA was then allowed to dissolve for one
hour at room temperature. The quality of the genomic DNA was analyzed by electrophoresis of an aliquot on 1% agarose gel. The genomic DNA was kept at -20°C for further use.

2.5.1 Amplification of DNA by the Polymerase Chain Reaction (PCR)
Specific oligonucleotide primers of 33-35 bases in length were designed based on sequences upstream and downstream of the start and stop codons for subsequent amplification of 4-Methyl 5-β-hydroxyethylthiazole kinase (thk). The primers flank the complete open reading frame (ORF). The sequences were retrieved from the genbank database. The accession number is AY166865. Computer software known as web primer was used to design the primers. Amplification of the thk gene was performed using the TPROFESSIONAL thermocycler. The gene thk of Plasmodium falciparum that encodes 4-Methyl-5-β-hydroxyethylthiazole kinase was amplified from genomic DNA with the primer pair: forward primer 5' agt cgg ata cat gag aaa ata taa ttt ttt tac 3' and reverse primer 5' tca gcc tag gtt atg cta ctt tgt aaa tat cta cg 3'. PCR composition of the reagents was as shown in Table 2.

Table 2: The composition of a 1× PCR reaction mix.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>d NTP mix (2.5Mm)</td>
<td>2.0</td>
</tr>
<tr>
<td>Forward primer (100ng/μl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Reverse primer (100ng/μl)</td>
<td>1.0</td>
</tr>
<tr>
<td>MgCl2 (25mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>Triple distilled water</td>
<td>11.0</td>
</tr>
<tr>
<td>Template DNA (100ng/μl)</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25.0</strong></td>
</tr>
</tbody>
</table>
The template strands were denatured at 95°C for three minutes in order to separate the two strands so that the primers could anneal to each separate template. This was followed by 35 amplification cycles comprising strand separation at 94°C for one minute, primer annealing at 60°C for one minute and primer extension at 60°C for one minute. The final extension was done at 60°C for ten minutes. The quality of the PCR product was analyzed by electrophoresis of an aliquot on an agarose gel containing ethidium bromide and thereafter purified in Illustra™ GFX PCR DNA and gel band purification kit.

2.5.2 Purification of DNA from Agarose gels

Different fragment sizes of DNA were purified from Agarose gel using GFX DNA and gel band purification kit. The DNA in solution or agarose gel was combined with a chaotropic agent and then transferred into a GFX column. It was bound to the silica membrane and then washed before eluting in 10mM Tris-HCl pH 8.0 solution or in sterile nuclease free water depending on the downstream applications.

2.6 Agarose gel electrophoresis

The quality of DNA fragments (depending on the fragment size), their separation and quantification were routinely done by performing (0.8%-1.0%) agarose gel electrophoresis in TAE buffer (40 mM Tris- acetate, 2mM EDTA Ph 8.0). One microlitre of 10mg/ml of ethidium bromide stock per 100 ml of gel was added and DNA suspended in TE was mixed with loading buffer (0.25% V/V Bromophenol blue, 0.25% V/V Xylene cyanol, 30% v/v Glycerol) and loaded in the wells in the gel. Molecular weight markers from Promega were used to estimate the DNA size. The electrophoresis was carried out in a Biorad electrophoresis chamber at 100 volts for 45 minutes.
Preparation of competent *E. coli* cells

Competent cells were prepared according to a previously described protocol (Hanahan, 1985). *E. coli* strains *X-LI* blue cells from a lyophilized stock were grown overnight on an LB (Luria-Bertani) media plate (per litre 10g Bactotryptone, 5g Bactoyeast extract, 5g Nacl and 15g agar, the pH adjusted to 7.5 with NaOH and autoclaved to sterilize). A single colony from the LB plate was used to inoculate 2.5 ml LB media (per litre 10g Bactotryptone, 5g Bactoyeast extract and 5g Nacl, the pH adjusted to 7.5 with NaOH and autoclaved to sterilize) and grown overnight at 37°C with shaking at 225 rpm. On the following day, the entire overnight culture was used to inoculate 250 ml of LB medium containing 20 mM MgSO₄ and the cells grown until the A₆₀₀ reached between 0.4-0.6. The cells were then pelleted by centrifugation at 4500g for five minutes at 4°C. The cell pellet was resuspended in 0.4 volume (based on original culture) of ice-cold TFB₁ (100 mM Rbcl, 50 mM Mncl₂, 30 mM Potassium acetate, 10 mM CaCl₂, 15% glycerol adjusted to pH 5.8 with 1 M acetic acid, filter sterilized). The resuspended cells were combined on ice and all pipette tips and flasks were chilled. The resuspended cells were incubated on ice for five minutes at 4°C. The cells were pelleted by centrifugation at 4500g for five minutes at 4°C. The cells were gently resuspended in 1/25 of the original culture volume of ice cold TFB₂ The cells were then incubated on ice for 60 minutes and then aliquoted into tubes for storage at -70°C.

2.8 Cloning of *thk* fragment

The amplified *thk* PCR product was ligated to pGEM-T vector using the procedure described in section 2.8.1 but without need for digestion as the vector was in a linearised form. The ligation reaction was then used to transform *XLI* blue *E. coli* cells as described in section 2.8.2 and the recombinant Plasmid DNA was isolated as described in section 2.8.3. The plasmids once isolated were subsequently purified as described in section 2.8.4.
2.8.1 Ligation reactions

Ligation reactions were set up according to previously described protocols (Kobs, 1996). The amount of 100ng/μl of plasmid vector was digested with the appropriate restriction enzyme and mixed with 300ng/μl of Pφθk, 10× DNA ligase buffer and three Weiss unit of T4 DNA ligase (Promega) to a final volume of 10μl. This was then briefly centrifuged and incubated for 16 hrs at 22°C. This ligation reaction was then used to transform competent cells.

2.8.2 Transformation of competent cells

Competent cells were transformed according to previously described procedures (Robles et al., 1994). The ligation reaction (10 μl) was added to a sterile Eppendorf tube placed on ice. The competent cells were, meanwhile, placed on an ice bath to thaw. Competent cells (50 μl) were carefully transferred to the ligation reaction and gently flicked then incubated in ice for 20 minutes. The reaction was then heat-shocked at 42°C for two minutes and the tubes immediately transferred to ice for two minutes, 900μl of SOC medium (2.0 g Bactotryptone, 0.5 g Bactoyeast extract, 1 ml 1M Nacl, 0.25 ml 1 M ACL, 1 ml 2M Mg^2+ stock and 1 ml 2M glucose up to 100ml) was added to the tubes which were then incubated at 37°C for three hours without shaking. The reaction was centrifuged for five minutes at 10,000 rpm to concentrate the competent cells before 750μl of the supernatant were aspirated leaving 250μl at the bottom. The residue was then spread on LB Ampicillin plates that had 50μl (100mM) IPTG and 40μl (50mg/ml) x-gal. The plates were incubated at 37°C overnight.

2.8.3 Isolation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method with a few modifications (Clark, 1988). This method exploits the difference in denaturation and renaturation characteristics of covalently closed circular plasmid DNA and chromosomal DNA. A single colony from the
freshly streaked LB Ampicillin plate was used to inoculate a starter culture of 5 ml LB broth media containing Ampicillin to a final concentration of 100μg/ml and incubated at 37°C overnight with vigorous shaking. On the following day, 1.5 ml of the overnight culture was centrifuged at 14,000 rpm for five minutes and the supernatant aspirated. The pellet was resuspended in 100μl of ice-cold resuspension solution (50mM Glucose, 25mM Tris-HCl pH 8.0 and 10mM EDTA). Upon resuspension 200μl of freshly prepared lysis solution (0.2N NaOH and 1% SDS) was added, mixed by inverting rapidly 5 times and placed in ice for five minutes. One hundred and fifty microlitres of ice-cold neutralization solution (5M Potassium acetate, glacial acetic acid and double distilled water) was added and the tube inverted for 10 seconds to dispense the neutralization solution before incubation in ice for 5 minutes. The tubes were then centrifuged at 12,000g for five minutes at 4°C and the supernatant transferred to a fresh Eppendorf tube. The supernatant was then incubated at 37°C for two hours after adding 2μl of 200μg/ml RNaseA. Plasmid DNA was extracted from the solution using the phenol chloroform procedure.

2.8.4 Purification of plasmid DNA

The Qiagen plasmid purification kit was used. The principle of purification was based on a modified alkaline lysis procedure where plasmid DNA was bound to the silica resin in a low pH condition then eluted in a high salt buffer and concentrated subsequently desalted by isopropanol precipitation. Three buffers were sequentially added to the bacterial cells to obtain a clear lysate which was applied to the spin cartridge to bind the silica resin followed by washing twice with wash buffer and elution in 10mM Tris-HCl buffer pH 8.0.
2.9 Subcloning of \textit{Pfthk} in \textit{pQE-30} Expression plasmids

The \textit{thk} fragment, upon successful cloning into \textit{p GEM-T} vector, was restricted and subcloned into the \textit{pQE-30} expression plasmid. The restriction digestion reaction (table 3) was followed by a ligation reaction between \textit{pQE-30} and \textit{thk} as described in section 2.8.1.

Table 3: Composition of restriction digest reactions

<table>
<thead>
<tr>
<th>Material</th>
<th>Experimental tube</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction enzyme (\textit{Bam HI})</td>
<td>1.0 ( \mu l )</td>
<td>1.0 ( \mu l )</td>
</tr>
<tr>
<td>Plasmid DNA (\textit{pQE-30} vector)</td>
<td>10 ( \mu l )</td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme buffer</td>
<td>1.5 ( \mu l )</td>
<td>1.5 ( \mu l )</td>
</tr>
<tr>
<td>DD \textit{H}_2\textit{O}</td>
<td>2.5 ( \mu l )</td>
<td>12.5 ( \mu l )</td>
</tr>
<tr>
<td>Total volume</td>
<td>15.0 ( \mu l )</td>
<td>15.0 ( \mu l )</td>
</tr>
</tbody>
</table>

2.9.1 Expression of thiazole kinase protein on \textit{pQE-30} expression vector

Thiazole kinase protein was expressed on \textit{pQE-30} expression vector using the method developed by Sambrook \textit{et al} (2003). Ten ml LB medium containing 100\( \mu g/ml \) Ampicillin in a 50-ml flask was inoculated with a single colony from the freshly streaked LB Ampicillin plate containing recombinant \textit{pQE-30-thk} plasmids and grown overnight with shaking at 225 rpm at 37\( ^\circ \)C. The following morning, ~50 ml of prewarmed media containing 100\( \mu g/ml \) Ampicillin was inoculated with 2.5 ml of the overnight cultures and grown at 37\( ^\circ \)C with vigorous shaking until the OD\(_{600}\) was 0.6. One ml sample was taken immediately before induction (non-induced control), the cells were pelleted and resuspended in 50\( \mu l \) \text{1x SDS PAGE} buffer. The sample was frozen at -20\( ^\circ \)C until needed for SDS PAGE analysis.
Expression was induced by adding IPTG to a final concentration of 1 mM. The cultures were grown for an additional 4-5 hours. A second 1 ml sample was collected (induced control) the cells pelleted and resuspended in 100μl 1× SDS PAGE sample buffer and frozen until use. The cells were harvested by centrifugation at 4000×g for 20 minutes. The cell pellet was resuspended in 5 ml of lysis buffer (50 mM NaH2PO4, 300mM NaCl, 100mM Imidazole) for native purification. Lysozyme was added to a 1 mg/ml final concentration and incubated on ice for 30 minutes. This was followed by sonication at 6×10 seconds with 10 second pauses at 200-300 watts. The lysate was kept on ice at all times. The lysate was centrifuged at 10,000×g at 4°C for 20-30 minutes. The supernatant was decanted (crude extract A, soluble protein) and saved on ice. The pellet was resuspended in 5 ml lysis buffer. This is a suspension of the insoluble matter (crude extract B, Insoluble protein). An SDS PAGE gel was run to determine the solubility of the protein.

2.9.2 Engineering of the transfection construct

The pExpress-1 transfection construct was engineered using the methods published by Ozware et al (2005). The pExpress-1 transfection construct contained a heterologous cassette based on a mutagenised *Toxoplasma gondii* dihydrofolate reductase/thymidine synthase gene (*Tg dhfr-ts*) conferring pyrimethamine resistance, flanked by *P. berghei* dhfr/ts flanking sequences. To construct the thiazole kinase expression vector, the open reading frame (ORF) of thiazole kinase was isolated by amplification from the thiazole kinase cloning vector, digested with *Bam*HI and subsequently cloned into the *Bam*HI site of the plasmid pD_b.D_Tm.D_b/D_.D to generate pD_b.D_Tm.D_b/D.thk.D (Figure 13). (The transfection vector was a gift from the Institute of Primate Research, Nairobi, Kenya).
2.9.3 Generating parasites for transfection

Parasites for transfection were generated using methods published in Janse et al (1985) with slight modifications. A vial of *P. berghei* parasite ANKA strain that had been preserved in liquid Nitrogen was retrieved and an equal volume of normal saline was added after thawing the vial in a 37°C water bath. Using an insulin needle 100μl of this PBS/parasite mixture was used to inoculate 3 *Balb c* mice intravenously. The mice were heart-bled with a 23-G needle containing 0.1 ml heparin stock solution after attaining a parasitaemia of 10-15%.

2.9.4 Transfecting *Plasmodium berghei* parasites

Transfection of *Plasmodium berghei* parasites was done according to the methods published in van Dijk et al (1995). The blood from all mice was pooled into a 15 ml tube containing three ml complete culture medium and spun at 1500 rpm for 10 minutes. The parasites were resuspended in complete culture medium such that the haematocrit was 3%. Culture flasks amounting to a volume of 20 ml were used to culture the parasites. Flasks were gassed with 5% CO₂, 5% O₂, and 90% N₂ for five minutes and incubated at 37°C for 18 hours on a shaker at minimal speed to keep cultures in suspension. Another culture flask containing parasites was incubated at 37°C overnight without shaking. After 18 hours had elapsed, the parasites were smeared by taking out 300μl of gently resuspended parasites, spinning briefly in 1.5 ml micro centrifuge tube, removing supernatant and resuspending parasites in residual medium. Smears were made and stained.

On confirmation that schizonts were well developed, the parasite cultures were split into 15 ml tubes so that each tube had 9 ml of culture material. Three ml of 55% Nycodenz solution was used to layer very carefully underneath the 9 ml culture material suspension. This was spun 20-30 minutes at 1370 rpm in a swing out rotor at room temperature. The brown layer containing schizonts was carefully collected. Four layers were present after spinning in the
The top layer was culture medium, the second layer from the top were schizonts, the third clear layer was Nycodenz and the final pellet was erythrocytes. Around two ml of culture medium from the top of this Nycodenz density gradient was added to help wash away the Nycodenz. Parasites were centrifuged at 1370 rpm for about 10 minutes. The medium was sucked off, the schizonts mixed with 450μl cytomix and added directly into the electroporation cuvette. Total volume in the cuvette was 500μl. The DNA solution consisted of 50μg of DNA in 50μl TE buffer. The cuvette was 0.4 cm from Biorad. Electroporation was done for three different plasmids with the following electroporation settings: 1 Kv, 25μF and 200 Ohms.

The DNA was immediately transferred on ice after electroporation and quickly transported to the mice room. Total time on ice was approximately five minutes. The mice were, meanwhile, warmed for 10 minutes with a heating source to make it easier to locate their blood veins. This also improved blood flow. The Balb/c mice were inoculated with the transfected parasites using an insulin needle. The widest circumference of the tail was selected.
3.0 CHAPTER THREE: RESULTS

3.1 Culturing and isolating genomic DNA from *Plasmodium falciparum*

To extract genomic DNA for *thk* gene isolation *P. falciparum* strain D6 obtained from KEMRI was cultured to a parasitaemia of $2.59 \times 10^5$ (see section 2.4). The culture was set at 0.2% parasitaemia. It was noteworthy that when folic acid and para-amino benzoic acid were added to a final concentration of 23nM and 3.6nM, respectively, to the culture medium, the parasites were healthier and grew faster than those in cultures lacking both components.

3.2 Characterization of thiazole kinase (*thk*) from *Plasmodium falciparum*

Genomic DNA (Figure 2) was extracted from the cultured *P. falciparum* parasites, using the phenol chloroform extraction method (see section 2.5), and frozen. The *thk* gene was PCR-amplified from *P. falciparum* genomic DNA using *thk*-specific primers (see section 2.5.1). The primers amplified the whole coding sequence (Figure 3). The primers amplified the gene without any hairpins or non-specific bands. A DNA fragment of 909 base pairs was generated (figure 4). The PCR reaction with a final MgCl₂ concentration of 2.0mM gave sharper bands when run on a 0.8% agarose gel than the one that had a final concentration of either 2.5mM or 3.0mM. An annealing temperature of 45°C gave better results compared to that of 60°C.

![Figure 2](image)

Figure 2: Analysis of genomic DNA prior to PCR amplification. Lane 1 represents the 1kb DNA ladder and lane 2 the *Plasmodium falciparum* genomic DNA.
ORF number 1 in reading frame 1 on the direct strand extends from base 1 to base 909.

ATGAGAAAATATAATTTTTTTACAAAAAAGTCGCTATTCTCTACCTT
AACCAAAAATAAT
AGGGTTAGGAATATCATGATGATATAAATAAAGATGCAATTGGGA
ATTCCGGTATTAAT
CCCTCTTTCTCATTGTATAACTAATAGAGTAAACCACTGAAAGGTT
CAAAACACCTTAT
GCTTCTGCTTCTCTCCGGCTATGATTGATAATCCTAAGGAAGTTGA
AGAATTGCTAAA
ATAGCTTCATGATATTTATTTCAACTTAGGTTACATACGACGCAGG
TAGAAAATATTAAT
TTATTAGAAAAAGTAAAAGAATGTATGAAAGATAAAATTTATGT
TAATATTAGATCCA
ATAGCTGTTGAGCAACAACCTATAGAACTATGTTATTAAGAGATA
TAATATTAAATGCA
CAACCTATATATAAAAAAGTAAATTTGCTGAAAATTTATTTAT
ATAAAGGAGAATT
TTGGGAGGGGTAGATAGAAAATAAACAATACCTAATAAGA
ACAGATGTAATTAAT
AGTGCCAGAAAATGGTGCATTAAAATATATTTGTCGTGAGTATGTA
CGTAAAAACAGAT
TATATTTGATGTTATGATTTCTACTCTATGAGCAAAATTAATATAT
TATAAAAAAAACTG
ACAAAAATTACTGGGTCAGGTTGTTCTGTTAGTGCCCTTTGTGCA
CAGCTACTTCAGTA
TATCTCAAAACACCATTACATGATGATAATCTGACTACTTTATATA
TAAATTTGGCAAGCA
TTCAAGACATATAAAAAAGAAGATCCAGGTTCCCTAAGTCATA
AAATTATTTGATGAT
ATTTATTACTATTACATAATCCTCATTTTCATTTCAAATCCTG
AGATATTTACAAA
GTAGCATAAA

ThiazBamhl forward primer: AGTCGGATCCATGAGAAAATATAATTTTTTAC
ThiazBamhl Reverse primer: CAGCCTAGGTTATGCTACTTTTGTTAAATATCTACG

Figure 3: ORF finder results. The above figure shows the results for 909 residue sequence "gi|27362862|gb|AY166865.1| Plasmodium falciparum 4-methyl-5-beta-hydroxyethylthiazole kinase mRNA, complete cds" starting "ATGAGAAAAT". The alignment of both the reverse and forward primers with the 909 bp sequence show that the primers amplified the complete coding sequence.
Figure 4: Agarose gel analysis of amplified *Pf thk* fragments. Lane 1 is a 1 kb DNA ladder while lane 2-6 represents the amplified *thk* fragments, lane 7 is the positive control generated by amplifying the same genomic DNA with a different set of primers. The *thk* fragment was digested with *BamH*I restriction enzyme resulting in products of different band sizes.

3.3 Preparation of competent cells

To clone the *thk* gene competent cells were required. *E. coli* cells of *XLI* blue strain were used to generate competent cells using the method described in section 2.7. The *XLI* blue strain of *E. coli* was preferred because although it grows slower than DH1 or DH5α it yields very high quality DNA and does not contain large amounts of carbohydrates or high levels of endonuclease activities. The competency of the *E. coli* cells was confirmed by transforming them with recombinant pGEM-T-*Pf thk* plasmids (Figure 5) using the method described in section 2.8.2. It was remarkable that rubidium chloride method of transformation gave rise to competent cells while the calcium chloride method of transformation resulted in non-competent cells. Cells harvested at *A*$_{600}$ of 0.5 resulted in higher efficiency during cloning compared to the cells harvested at *A*$_{600}$ at 0.6. Better results were obtained when the TFB$_1$ and TFB$_2$ buffer were freshly prepared. More colonies were observed on the LB Ampicillin
plate when these competent cells were eventually transformed using the heat shock method compared to when an automated program on the PCR machine was used and no colonies were observed when the cells were electroporated.

![Non-transformed cell (blue-colored) and Transformed cell (white-colored)](image)

**Figure 5:** LB Ampicillin plate showing transformed *E. coli XLI* blue competent cells

### 3.4 Cloning of *Pf thk* gene into a cloning vector

Upon successful transformation of the *XLI* blue competent cells with the recombinant pGEM-T-*Pf thk* plasmids, a few colonies from the LB Ampicillin plate were screened for the presence of the *Pf thk* insert (Figure 7) and the positive clones were used to inoculate LB media containing Ampicillin to a final concentration of 100\(\mu\)g/ml and grown overnight by shaking. The plasmids that were found to have successfully taken up the *Pf thk* insert (Figure 6) were subsequently extracted (refer to section 2.8.3).
Figure 6: Analysis of pGEM-T-Pf \textit{thk} recombinant plasmids. Lane 1 represents Lambda DNA/Eco R1+Hind III marker and lane 2-3 represents pGEM-T-Pf \textit{thk} recombinant plasmids.

Figure 7: PCR screening of pGEM-T recombinant plasmids. The above figure shows the PCR screening of the positive recombinant colonies. Lane 1 represents 1 kb ladder, lane 2-7 represents the positive screens of successful recombinants plasmids.
3.5 Expression of thiazole kinase protein in Eschecheria coli XLI blue strain

The *thk* DNA fragment of 909 base pairs amplified from the recombinant pGEM-T-Pf *thk* plasmid was sub-cloned into pQE30 expression vector for expression of the *thk* protein (Figure 8) using the method described in section 2.9.1. The transformed *E. coli* XLI blue cells were plated on a LB Ampicillin plate where white colonies were observed. The ligation reaction that had a 1:3 vector to *thk* insert volume ratio had the best results. The ligation reaction of 1:1 volume ratio had fewer successful recombinant colonies. The ligation reaction that was stored at 4°C for four nights gave similar growth as one stored at 22°C overnight.

![Figure 8](image)

Figure 8: Analysis of recombinant plasmids for expression of thiazole kinase protein. The figure above shows non recombinant and recombinant pQE-30-*thk* plasmids. The lane marked 1 represents a 1kb DNA ladder, lane 2-7 represents recombinant pQE30-*thk* plasmids and lane 8 contains the negative control plasmid of a non-recombinant pQE-30 plasmid. The recombinant plasmid contains a 909 bp fragment resulting in an invisible size difference between the two types of plasmids.

To confirm the presence of positive clones, PCR was done with confirmatory primers on the white colonies (Figure 9). The positive clones containing recombinant pQE-30-*thk* plasmids were subsequently used to inoculate LB medium containing 100μg/ml Ampicillin for *thk*
protein expression. The LB medium that contained both Ampicillin and Kanamycin antibiotics inhibited the growth of the bacterial cells and growth of the recombinant clones was observed when the LB media had Ampicillin only. When shaking was rapidly (~ 300 rpm), the log phase of growth was attained faster than when it was slower (~ 180rpm). The LB Ampicillin plate that had non-recombinant clones had a similar growth intensity as the one with recombinant clones. Upon attaining the log phase of growth, the recombinant cells were induced to express the \textit{thk} protein for further assay using SDS PAGE (see section 2.9.1). To determine its solubility and molecular weight, an SDS PAGE gel was run. The \textit{thk} protein was found to be 34.67kDa and soluble. Better results were observed when the cell pellet was incubated in ice longer than 30 minutes (3 hours) upon addition of lysozyme followed by sonication.

![PCR screening for thk DNA fragment in recombinant pQE-30 plasmids.](image)

Figure 9: PCR screening for \textit{thk} DNA fragment in recombinant pQE-30 plasmids. Lane1 indicates the 100bp DNA ladder, lanes 2-5 represent the amplified \textit{thk} fragment which demonstrates positive recombinant clones.
3.6 Sequence-to-structure alignment and homology modeling of THK protein

The sequence-to-structure alignment and homology modeling of the THK protein template was carried out so as to predict the protein structure of thk. The template for modeling the crystal structure of 4-methyl-5-beta-hydroxyethylthiazole kinase used was that of crystal structure of native thiazole kinase in the monoclinic form 1c3qC. The sequence identity [%] was 32.707 and the E value was 0.00e-1. The predictive study carried out on the protein shows that the overall fold is similar to that of ribokinase and adenosine kinase. The area of greatest similarity occurs in the ATP-binding site where several key residues are highly conserved (Campobasso et al., 2000).

3.6.1 Simple Modular Architecture Research Tool (SMART) analysis

Simple Modular Architecture Research Tool (SMART) analysis identified the presence of 4-methyl-5-beta-hydroxyethylthiazole kinase domain laying from the first base pair to the last i.e. the whole sequence code for an open reading frame.

3.6.2 THK amino acid signal peptide analysis

Analysis of the thk sequence using signalP 3.0 (Jannick et al., 2004) was carried out to establish whether it has a signal peptide (figure 10). Thk amino acid sequence signal peptide sequence prediction using SignalP 3.0 showed that thk is a non-secretory protein. With a signal peptide probability of 0.000 and a signal anchor probability of 0.000. It also lacked a cleavage site and had a maximum cleavage site probability of 0.000 between position 21 and 22.
3.7 Engineering of the transfection construct

Having ascertained that the \textit{thk} gene is capable of being expressed in an expression vector (see section 3.5) an episomal transfection construct was engineered using the pExpress 1 plasmid. The pExpress 1 plasmid and \textit{Pf thk} gene were both digested using the \textit{BamH}I restriction enzyme (Figure 11). During the restriction digestion process it was observed that it was easier to digest 2\(\mu\)g of the \textit{Pf thk} gene than it was to digest 5\(\mu\)g. The digested \textit{Pf thk} gene fragment was ligated into the digested pExpress1 plasmid and then used to transform \textit{E. coli XL1} blue cells. Incubation of the sticky-ended ligation reaction at 4\(^\circ\)C for four nights resulted in successful ligation but could not be compared to the growth resulting from incubation at 16\(^\circ\)C overnight which was higher. Sticky-ended ligation resulted in higher growth and successful cloning compared to blunt-ended ligation. Transformation of \textit{E. coli XL1} blue cells with 3:1 ratio of vector to DNA insert and 8% PEG-8000 added demonstrated a higher growth than the 3:1 ratio without 8% PEG-8000. Blunt-ended ligation at 22\(^\circ\)C for 4-16 hrs yielded a higher growth than at 4\(^\circ\)C for four nights.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{signalp_nn.png}
\caption{THK amino acid sequence signal peptide sequence prediction.}
\end{figure}
Figure 11: Gel analysis of digested and undigested forms of pExpress-1 plasmids. Lane 1 represents a 1 kb DNA ladder while lane 2 represents a negative control showing an undigested non-recombinant p Express1 plasmid, lane 3 represents linearised pExpress 1 plasmid and lane 4-8 represents recombinant pExpress 1-thk plasmid. The linearised plasmid lacks the supercoiled form of plasmid which is present in both the non-recombinant and the recombinant form.

After incubating the transformed E. coli cells overnight at 37°C on an LB Ampicillin plate, the resultant clones were screened for the presence of thk gene insert using confirmatory primers (Figure 12). The positive clones were grown in LB medium containing 100μg/ml Ampicillin followed by alkaline lysis. This resulted in 50μg of pExpress-1 recombinant plasmid that was to be used to transfect the Plasmodium berghei parasites in order to develop a transgenic animal model.
Figure 12: PCR screening of recombinant pExpress-1- *Pf thk* plasmids. Lane 1 represents a 100 bp marker; lane 2-7 represents the positive recombinant plasmids demonstrated by presence of the *thk* insert.

In order to ascertain the accuracy of the transfection construct, the non-recombinant plasmid was digested using different restriction enzymes (*Bam* HI and *Hind* III) resulting in different fragment sizes (Figure 14). Figure 13 shows a graphical representation of the resultant recombinant pExpress-1-*thk* transfection construct (pD.DTM.D./D.thk.D). The transfection construct contained *Tg dhfr-ts* gene controlled by *P. berghei dhfr-ts* to enable selection of transfected parasites on the basis of Pyrimethamine resistance. The expression of *Thk* was under control of 2.5-kb of *P. berghei dhfr-ts*.

![Diagram](image)

**Figure 13:** The transfection construct used to electroporate *P. berghei*
Figure 14: Analysis of restriction digests of the pExpress-1-thk transfection construct. Lane 1 represents a 1 kb DNA ladder while lane 2 represents an undigested form of the transfection construct, lane 3 shows the linearised form of the construct after digesting with BamHI enzyme and lane 4 contains different-sized fragments resulting from restriction of the construct by Hind III enzyme.

To ensure purity of the recombinant transfection construct, the resultant pExpress1-thk recombinant plasmids were purified using the Qiagen plasmid purification kit (Figure 15). The purified pExpress-1-thk recombinant plasmids were used to transfect Plasmodium berghei mature schizonts obtained from the passaged Balb/c mice.

Figure 15: Agarose gel analysis of purified plasmid DNA. Lane 1 represents lambda DNA/EcoRI marker while lane 2 contain cleared lysate. Lane 3 contains flow through fraction while lane 4 contains second wash fraction and lane 5 contained the eluate.
3.8 In vivo culture of Plasmodium berghei parasites in Balb/c mice

In order to generate parasites that would be electroporated with the recombinant pExpress-1-thk transfection construct, 300μl of Cryopreserved P. berghei parasites were revived and mixed with an equal volume of normal saline and, using a 23-G needle, the tail vein of three mice were injected with 100μl of this solution. The first mouse turned positive on day six and on day eight, one mouse had attained 15% parasitaemia while another had 10%. The third mouse achieved 8% parasitaemia on day seven. A total of three ml of blood was collected from the three mice by cardiac puncture under aseptic conditions into a heparinised syringe. The mice that had 15% parasitaemia had severe anemia. It also had the least amount of blood (0.5 ml) when cardiac puncture was performed. The mouse that had 10% parasitaemia gave one ml of blood while the one that had 8% parasitaemia gave 1.5ml of blood. Two ml of blood was cryopreserved by adding equal volume of freeze mix and storing in the nitrogen tank. One ml of the parasitized blood was used to infect naive mice. Five mice were injected with 200μl of this blood.

At day seven post inoculation, three mice attained peak parasitaemia of 10% with a 5% parasitaemia of schizont stage and two mice attained 8% parasitaemia with 3% schizont stage. Five millilitres of blood was obtained from cardiac puncture of the five mice and upon overnight in vitro culture the schizont stage grew to $5.0 \times 10^9$ parasites (Figure16). Balb/c mice that were 21 days old and had just been weaned acquired the infection much faster (within 4 days) than the mice that were much older (111 days old). These became positive at day eight.
Figure 16: A thin blood smear of Giemsa-stained *Plasmodium berghei* parasites showing young trophozoite stages and the mature schizont stage.

Parasitaemia count of three mice that were growing at a similar rate was done and a growth curve obtained (Figure 17). Different growth characteristics of *P. berghei* were observed in the *Balb/c* mice that were syringe-passaged from infected to naive mice compared to those that were passaged with cryopreserved stocks. The latter took longer to attain peak parasitaemia with parasites reaching detectable levels at day six while the former acquired parasites to detectable levels at day four and reached peak parasitaemia at day seven.

Figure 17: Growth curve of *P. berghei* ANKA strain parasites.
3.9 Transfecting *Plasmodium berghei* with pExpress-1-*thk* recombinant plasmids

In order to transfect *P. berghei* with the *Pf thk* gene, the parasites were cultured to the mature schizont stage and upon attaining a parasitaemia of 5% schizont stage, a PCV of 3% in a 20 ml overnight culture was prepared in order to synchronize the growth of the parasites. This resulted in mature schizonts of *P. berghei* with a parasitaemia of $5.0 \times 10^9$. It was evident that at synchronization stage, an overnight *in vitro* culture carried out without shaking resulted in $5.0 \times 10^9$ schizonts while when shaken fewer schizont-stage parasites were obtained $3.0 \times 10^5$. These parasites were electroporated with 50μg of recombinant pExpress-1 plasmids resuspended in cytomix buffer using a Biorad Electroporator with a setting of 1 Kv, 25μF and 200 Ohms. The electroporated parasites were then re-introduced into the tail vein of mice that had been warmed to make the veins more visible. Time constants of 0.7 ms and 0.8 ms were attained. The mice died of anaphylactic shock before the transfection outcome could be determined.
This study set out to transfect *Plasmodium berghei* so as to express *Plasmodium falciparum* thiazole kinase (Pf thk). *Plasmodium falciparum thk* is a significant enzyme given that it activates the second precursor of thiamine biosynthesis (Taylor *et al*., 1998) which is one of the parasite-specific metabolic pathways that can act as an ideal source of drug targets (Roos *et al*., 2002). The *Pf thk* gene was found to be composed of a single open reading frame and lacking an intron which means that the whole gene is a coding sequence. The *thk* gene shows a high level of conservation between *P. falciparum* and *P. vivax* indicating that the protein may serve similar roles in different species of *Plasmodium*. Previous work done by Tami *et al* (2003) and Wrenger *et al* (2006) shows the gene to be of a similar size as that isolated in this study (909 bp). The uniqueness of this gene to the parasite and its absence in the human host renders it a possible novel drug target since a good drug target lacks similarity to any human enzyme (Fatumo *et al*., 2008). To further characterize this gene, knock out studies should be carried out.

The *thk* gene proved to be unstable and hence very difficult to manipulate. The gene was absent upon reviving the glycerol stocks of the different plasmids that it had been cloned into (the reason seems to have been that the gene aborted from the plasmids). The gene was unstable both in the smaller vectors (such as pGEM-T and pQE-30 plasmids) and the larger plasmids (such as pExpress-1 plasmid). The instability of the gene was not dependent on the size of the plasmid that the gene had been cloned into but the reason could be that *Plasmodium* genes are intrinsically unstable because of their AT-rich nature and have a propensity to form rearrangements and deletions (Wesson *et al*., 1995).
The amplified PCR product of \textit{Pf thk} possessed an additional single, unpaired adenine residue at the end of the completed chain. The single 3’ Adenosyl extension generated by \textit{Taq} DNA polymerase granted a highly resourceful technique to clone the PCR product into the T vector containing a complementary unpaired 3’ thymidyl residue. This study therefore exploited the T- overhangs at the insertion site of a p GEM-T vector which greatly improved the efficiency of ligation of PCR products into the cloning vector by preventing recircularization of the vector (Mezei \textit{et al.}, 1994). Generally, it was easier to clone the \textit{thk} gene into the cloning vector successfully than it was to clone into the expression vector and the transfection vector.

It was possible to apply the blue white screening technique to screen for positive recombinant colonies because the pGEM-T vector has an \(\alpha\)-peptide coding region of the enzyme \(\beta\)-galactosidase. Insertional inactivation of the \(\alpha\)-peptide allowed identification of recombinants by blue/white screening on indicator plates. The pQE-30 plasmid lacked the \(\alpha\)-peptide coding region of the enzyme \(\beta\)-galactosidase and so PCR had to be performed to screen for the positive recombinants. At the same time, this expression vector contained ATG start codon and stop codons that allowed for the expression of the proteins. The pGEM-T cloning vector lacked these features and so could not be used to express the \textit{Pf thk} gene. The vector map of pQE-30 plasmid indicates that it contains Ampicillin resistance gene only and though previous studies show preference of growing the overnight culture in a medium that has both the Ampicillin and Kanamycin antibiotics, the presence of only one resistance gene could explain why growth could not be achieved when both antibiotics were included in the LB medium in this study.

Upon expression of the \textit{pfthk} gene, the protein was found to be soluble and of molecular weight of 34.67 kDa. Previous work found the molecular weight of thiazole kinase to be 34.73kDa (Tami \textit{et al.}, 2003) and 34 kDA (Wrenger \textit{et al.}, 2006) both of which are similar to the one observed in the study. These previous studies also showed that the protein is
expressed at both the asexual and sexual stages of parasite development. The expression of *thk* at both asexual and sexual stages of parasite development suggests that it is very essential for parasite development at all stages of *Plasmodium falciparum* and the predictive studies revelation that it is a mitochondrion enzyme makes it an interesting enzyme for study. This is due to the fact that the processes that occur in the mitochondria appear to be essential for survival and constitute potential targets for antimalarial chemotherapy (Painter *et al.*, 2007).

Thiazole kinase amino acid signal peptide analysis results imply that *Pf thk* is not produced as a pro protein but it is produced in its full functional state. Predictive studies carried out on *PFTHK* protein suggest that *Pf thk* clusters around other kinases thus proving that it belongs to the family of enzymes transferring phosphorus-containing groups.

The *Pf thk* gene was successfully cloned into pExpress-1 plasmid thus creating an episomal transfection construct. This study exploited the use of a transfection plasmid that had dihydrofolate reductase thymidylate synthase (DHFR/TS) gene of *Toxoplasma gondii* with point mutations that confer resistance to the antimalarial pyrimethamine. The *T. gondii* DHFR/TS<sup>R</sup> gene is generally preferred to *P. berghei* DHFR/TS as the former is known to reduce the possibility of unwanted recombination with the endogenous DHFR/TS gene and because it confers resistance to higher levels of pyrimethamine than the *P. berghei* DHFR/TS<sup>R</sup> (Waters, 1997). DNA constructs for stable episomal transfection are circular and contain a selection cassette and an adjacent expression cassette cloned either in a head to head or a head to tail orientation. The expression cassette contains the gene of interest. The genes in both cassettes are flanked by UTRs of *Plasmodium* origin. These characteristics of a transfection construct allow for the gene to be expressed while at the same time selecting for the parasites that bear the constructs with the insert. The circularized plasmid in its
unarranged form can be able to replicate episomally allowing elucidation of protein function and study of clinically relevant blood stage parasites (Van Dijk et al., 1995).

In the past, transfection of *Plasmodium* parasite species has faced several hurdles amongst them being the four membranes between the external environment and the nucleus in intra-erythrocytic parasites that has been difficult to penetrate (Wesson, *et al.*, 1995). This study circumvented this hurdle by using mature schizonts containing fully developed merozoites for introduction of DNA because these stages are not dependent on the erythrocyte for survival thus evading the problem of damage to the host cell during electroporation (host erythrocyte) on which other blood stages are completely dependent for survival. Another advantage of merozoites of *P. berghei* is that they are readily collectable in large numbers and appear to be more stable than merozoites of other malaria parasites.

Parasite culture media supplemented with folic acid and para aminobenzoic acid grew healthier and faster because both synthesize folate which is necessary for the production and maintenance of new cells as it is necessary for synthesis of DNA bases. It was easier to propagate *P. falciparum* parasites by *in vitro* culturing while *P. berghei* parasites could only be propagated by *in vivo* culture. Upon release of *P. berghei* haploid merozoites from the liver the schizonts invade reticulocytes but can also invade mature red blood cells. This renders a challenge to culturing the *P.-berghei in vitro*. On the other hand, *P. falciparum* can be cultured *in vitro* throughout its different life stages as their liver schizonts entirely invade red blood cells.
The transfection experiment showed that though available literature indicates that electroporation settings of 800 V and 25μF for *P. berghei* give good results (Goonewardene *et al.*, 1993), the results that were obtained from this study demonstrate that electroporation settings of 1 Kv and 25μF for *Plasmodium berghei* give better results. The electroporation experiment resulted in time constants of 0.7 ms and 0.8 ms. Published studies carried out for transfection indicate that apart from purity of plasmid DNA, a time constant of 0.8 ms is a useful indicator of likely success of electroporation (Tomas *et al.*, 1998). This strongly indicates that the results obtained in this experiment demonstrate the possibility of utilizing *P. berghei* as an animal model for screening *Pf thk* as a possible novel drug target.

Conclusions

1. Expression of *thk* protein has revealed that it is a soluble protein.

2. *PFTHK* amino acid signal peptide analysis showed that the protein is non-secretory and lacks a cleavage site.

3. The 3D model homology studies of *Pf thk* shows that the overall fold is similar to that of ribokinase and adenosine kinase.

4. *Pf thk* was successfully cloned into *P. berghei* expression vector, allowing for transfection and possible expression.

5. The *Balb/c* mice that have just been weaned acquire infection much faster than mice that are much older.

6. Electroporation settings of 1 Kv and 25μF give better results in *Plasmodium berghei* than that of 800 V and 25μF.

Recommendation

Further work on characterization of *Pf thk* gene should be initiated to further demonstrate the *Pf thk* gene as a possible novel drug target.
5.0 REFERENCES


Campobasso, N, I., Mathews, I.T., Begley, P and Ealick, S.E., 2000, Crystal structure of 4-methyl-5-beta-hydroxyethylthiazole kinase from Bacillus subtilis at 1.5 A resolution. Biochemistry, 39, 7868-7877


Chaoyong Ma, 2004, Animal models of disease. Model Systems, 7, 100-107

Crabb, B.S., Cowman, A.F., 1996, Characterization of promoters and stable transfection by homologous and non homologous recombination in Plasmodium falciparum, Proceedings of the National Academy of Sciences, USA 93, 7289-7294

Dechering, K.J., Thompson, J., Dodemont, J.J., Eling, W., Konings, R.N.H., 1997, Developmentally regulated expression of Pfs 16, a marker for sexual differentiation of the human malaria parasite Plasmodium falciparum, Molecular and Biochemical Parasitology. 89, 235-244


Henri, J; Rob, G and Alan, H, 2001, Drugs against Parasitic Diseases R & D Methodologies and Issues: 5.


Li, Y and Wu, Y., 1998, How Chinese scientists discovered qinghaosu (artemisinin) and developed its derivatives. What are the future perpespectives? Me’decine Tropicale 58, 9-12.

Macreadie, I., Mc Kie J.H., 2000, Antimalarial drug development and new targets, Parasitology Today, 16, 438-444

Current Innovations.


Ozwarra, H.O., 2005, Development and Application of a *Plasmodium knowlesi* Transfection System (PhD Thesis), Rijswik, Biomedical primate research centre


Snehal shah, M.D., Scott, F.M.D., Louise, M. and Causer, M.B.B., 2002, Malaria surveillance CDC –United States

Tami, G.G., 2003, Identification of *Plasmodium falciparum* transcripts differentially expressed or developmentally regulated during the sexual cell cycle, (PhD thesis), Nairobi, The University of Nairobi


Sherman, I., 1998, Parasite biology, pathogenesis and protection, Malaria, 177-184
Takechi, M., 2001, Therapeutic efficacy of sulphadoxine/pyrimethamine and susceptibility in vitro of P.falciparum isolates to sulphadoxine-pyrimethamine and other antimalarials drugs in Malawian children, Tropical Medicine and International Health, 6, 429-434


Wellems, T., Plowe, C., 2001, Chloroquine-resistant malaria, Journal of Infectious Diseases, 184,770-776


White, N, 1999, Delaying antimalarials drug resistance with combination therapy, Parassitologia 41, 301-308

WHO/CDS/CSR/DRS/2007 Geographic Distribution and Epidemiology


