Establishment of an Expression and Purification System for *Plasmodium falciparum* Multi Drug Resistance (pfmdr) Transporter

Honours Thesis in Biomedicine D01, 30 ECTS

(2007-02-09 - 2007-05-22)

ABSTRACT

Title: Establishment of an Expression and Purification System for *Plasmodium falciparum* Multi Drug Resistance (pfmdr) Transporter

Department: School of Life Sciences, University of Skövde

Course: Honours Thesis in Biomedicine D01, 30 ECTS

Author: Armanos Benjamin

Supervisor: Emily Crowly / Petra Pniesten

Examinator: Dennis Larsson

Date: 2007-02-09 – 2007-05-22

Keywords: Malaria; *Plasmodium falciparum*; Baculovirus; Drug resistance; Pfmdr 1; Pgh 1; ABC-transporter; Recombinant bacmid DNA.

Malaria is a life threatening parasite disease caused and transmitted by infected female anopheles mosquito. However, the parasite, P*lasmodium falciparum*, has become resistant to most anti malarial drugs, such as chloroquine, which contributes to fever and anaemia because of its ability to digest the haemoglobin in the red blood cells. The aims of this project were to establish whether "Bac to Bac" Baculoviral Expression System is suitable for expression of pfmdr 1 gene and for purification of the pgh 1 protein. The pfmdr 1 gene encodes an ABC transporter protein, pgh 1, fixed in the cell membrane of the *Plasmodium falciparuum* gut, which assist in elimination of drug compounds. Furthermore, "Bac to Bac" Baculoviral Expression System uses vectors with histidine tags to clone the pfmdr 1 gene and subsequently transform these into DH10Bac cells to produce the recombinant bacmid DNA. Since pfmdr 1 gene is an AT-rich sequence, PCR was optimized, by lowering the annealing and extension temperature to 47C^{o} and 66C^{o} respectively. The results show that "Bac to Bac" Baculoviral Expression System can be used to express the pfmdr 1 gene, though further experiments has to be performed.

Table of Contents

1 Introduction

1.1 Malaria

Malaria is a life threatening parasite disease (Le Bras & Durand, 2003) caused and transmitted by the infected female anopheles mosquito (Barillas-Mury & Kumar, 2005). The human specific parasite *plasmodium falciparum* (*P. falciparum)* lives in its mosquito vector till the mosquito, through its bet, transfer the parasite to humans (WHO, 2007). Furthermore, *P. falciparum* undergo its asexual phase (see figure 1) in man and contributes to fever and anaemia because of its ability to digest the haemoglobin in the red blood cells (RBC). In addition, *P. falciparum* promotes changes in the adhesive properties of the host cells (Cowman & Duraisingh, 2005). Malaria contributes to more than one million deaths annually (Reed et al 2000), which make it the second serious disease after tuberculosis (WHO, 2007).

1.2 *Plasmodium falciparum*

1.2.1 Life cycle

P. falciparum has an 80 % AT-rich genome, consisting of 5300 genes organized into 14 chromosomes (TIGR, 1999-2000). *P. falciparum* lives and proliferate in the epithelial cells in the mosquito gut (Mury-B & Kumar, 2005). However, the life cycle of *P. falciparum* consists of two phases; the sexual phase which occurs in mosquito gut and the asexual phase which takes place in humans (Manta, 1999), see figure 1. The life cycle starts with mosquito taking up female and male gametocytes, which fuses together and gives rise to the zygote. The zygote matures into an oocyst, which in turn ruptures and releases the sporozoites, which subsequently are transferred to humans, with mosquito saliva. Though, the kupfer cells in the liver terminate most of the parasites, a small part escape and infect the hepatocytes. In hepatocytes, *P. falciparum,* is transformed into schizonts and its genetic material is replicated till the hepatocyte ruptures and new form of the parasite, called merozoites, are released into the blood stream (Life Sciences Division & CXRO, 2001; Erwin, 2004), see figure 1. The merozoites enter the RBC, were they are transformed into trophozoites, which consumes haemoglobin to produce more merozoites (Life Sciences Division & CXRO, 2001; Bridges, 2002). However, jaundice, swollen liver and rapid breathing, are some of the symptoms specific to P. falciparum infection (Mohnot & Soni).

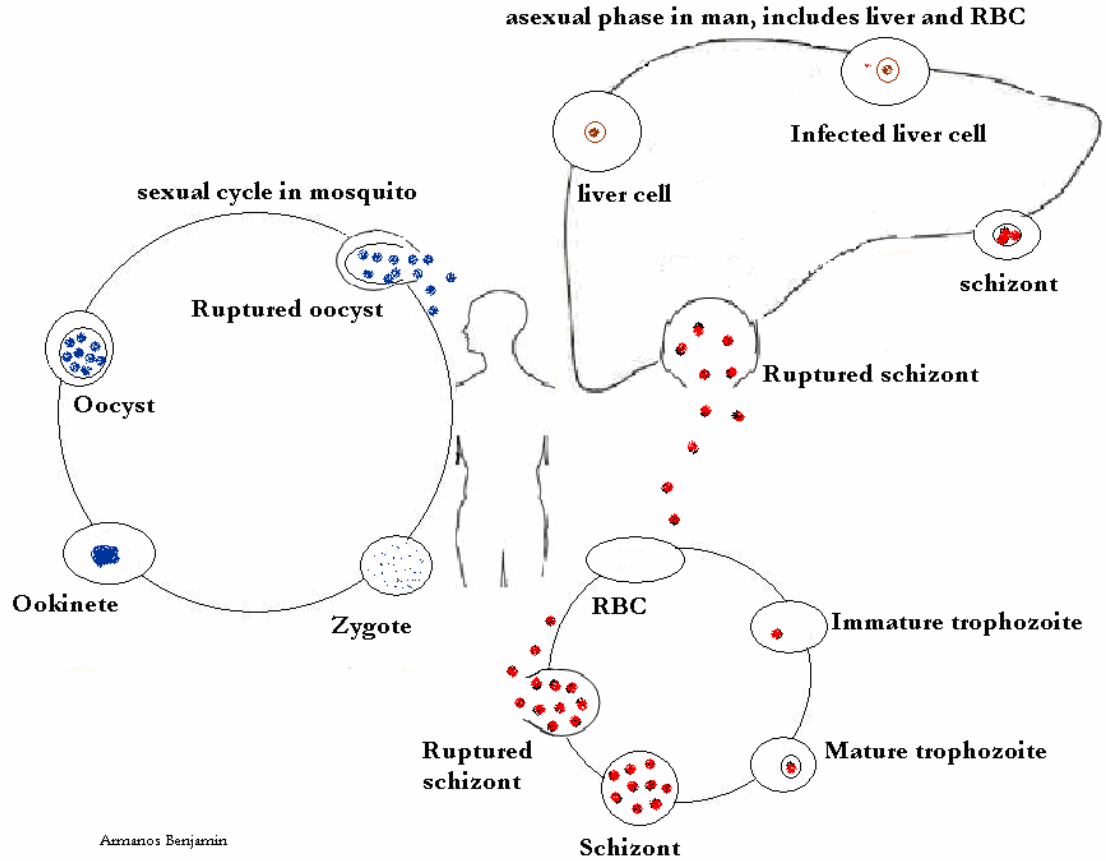

Figure 1. The life cycle of *P. falciparum.* During blood suction, the mosquito collects the gametocytes needed for *P. falciparum's* sexual phase. Subsequently, the mosquito transfers the parasite *P. falciparum* into man in the sporozoite form, which infects the liver and the red blood cell.

1.3 ABC-transporters

1.3.1 General

ABC (Adenosine triphosphate Binding Cassette) transporters are transport proteins fixed in the cell membrane. The function of the ABC-transporter proteins is to help in elimination of drug compounds from the cytosol, see figure 3. However, one example of ABC transporter protein in *P. falciparum* is pfmdr 1, also abbreviated pgh 1 (p glycoprotein homologue 1), since its homology to the p-glycoprotein, an ABC transporter in cancer cells (Holland et al, 2005).

1.3.1 Pfmdr 1 gene and Pgh 1 protein

Pfmdr 1 gene consists of 4260 base pairs (bp) (see appendix 2), is located on chromosome 5 (Warhurst, 2001) and encodes the pgh 1 protein. However, pgh 1 is a 162 kD protein

(Cowman & Duraisingh, 2005) that is located in the membranes of epithelial cells in the gut of *P. falciparum* (see figure 2).

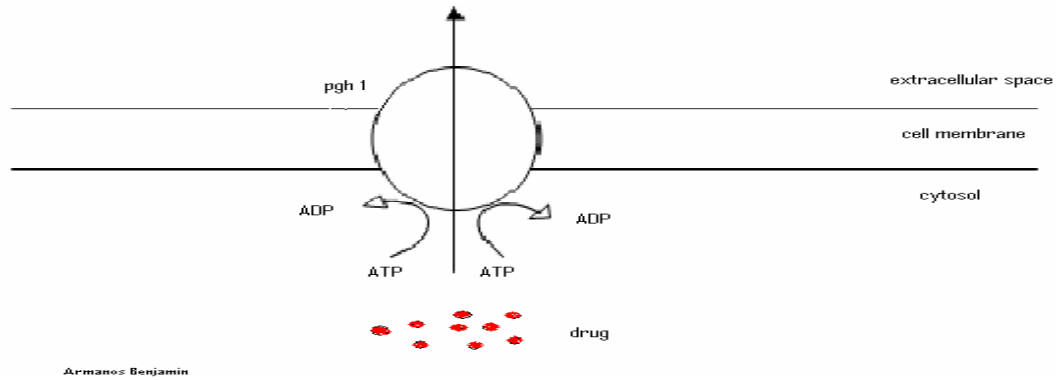

Figure 2. Pgh 1, an ABC transporter protein located in the *P. falciparum* gut, where it eliminates drug compounds from the cell.

Like all ABC transporter proteins, pgh 1 protein consists of two ATP cassettes and two domains linked via a liker region. Each domain consists of six transmembrane helixes, see figure 2 (Cowman & Duraisingh, 2005).

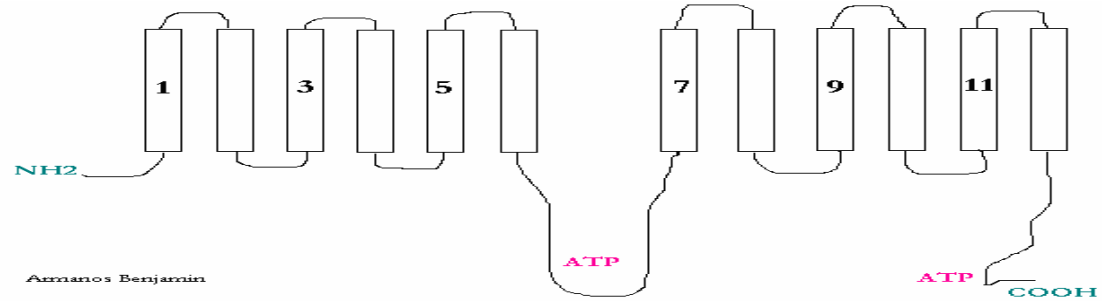

Figure 3. The structure of pgh 1 protein. Pgh 1 protein consists of two ATP cassettes and two domains, each with 6 transmembrane helixes.

1.4 Resistance mechanism

The active use of anti malarial drugs, such as chloroquine, has led to multi drug resistant *P. falciparum* (Le Bras & Durand, 2003). Multi drug resistance occurs when cells resistant to one type of drugs, becomes resistant to other structurally unrelated drugs. The mechanism is not clear yet but, increased mRNA expression, gene amplification and resistance in the pfmdr 1 gene are thought to be some of the factors (Cowman & Duraisingh, 2005).

1.5 Baculoviral Expression System

Bac to Bac Baculoviral Expression System (Invitrogen, 2002) uses Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) and was used during this project. Furthermore, the system uses pFastBac (PFB) vectors with six histidine tags to clone the gene of interest (GOI), pfmdr 1 gene, and subsequently transform the vector into DH10Bac or competent E-coli cells. The DH10Bac will incorporate the vector containing the pfmdr 1 gene in its genome and produce the recombinant bacmid DNA, which will be used to infect insect cells (see figure 4). Immediately, after transformation, the transposition takes place, which is a site specific process between the donor plasmid and the bacmid DNA. The pfmdr 1 gene will be incorporated into the vector between two flanking regions, Tn7 R at the right end of the gene and Tn7L of the left side, see figure 4. These flanking regions, called "mini Tn7" will help the vector to attach to its inverted copy "mini att Tn7" in the bacmid DNA. Hence, the attachment of mini Tn7 to mini att Tn7 will interrupt a gene, Lac Z, which encodes a protein that give rise to the blue colour formation in bacteria (Invitrogen, 2004).

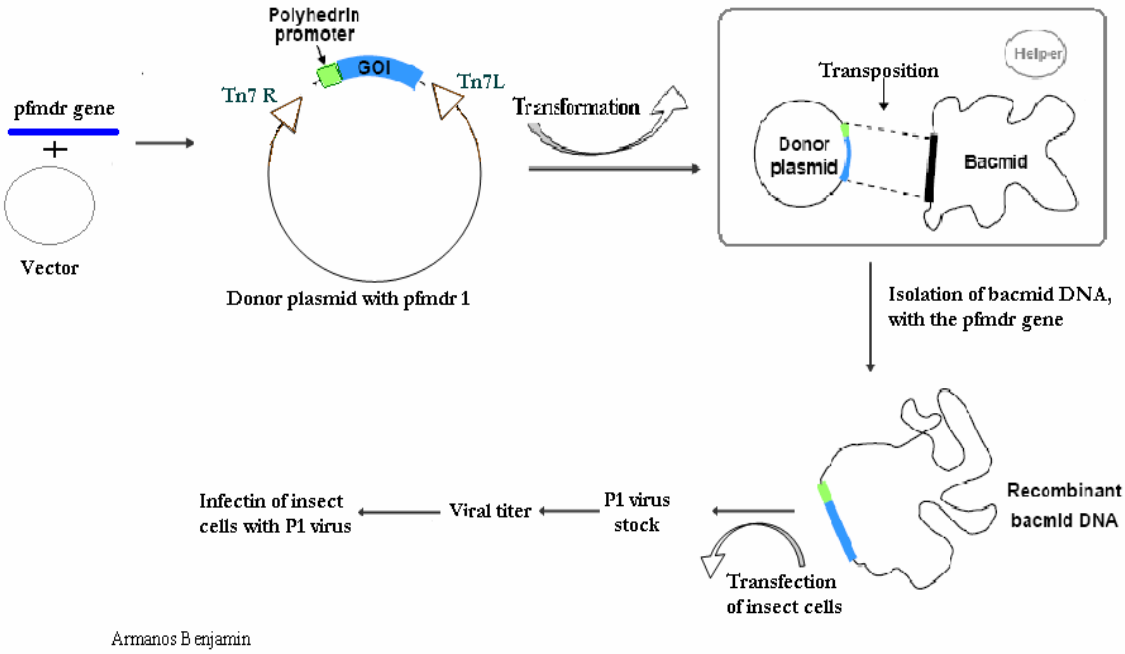

Figure 4. Bac to Bac Baculoviral Expression System, uses a donor plasmid, with the pfmdr 1 gene to transform into DH10Bac, competent *E-coli* cell.

The transposition makes it possible to distinguish between the parental and the recombinant bacmid cells. In the parental cells, no pfmdr 1 gene will be incorporated, thereby, no interruption of the Lac Z gene and hence blue colonies. Since the

incorporation of the pfmdr gene in the recombinant bacmid cells will interrupt Lac Z gene, no colour formation will occur. Thus, white bacterial colonies indicate recombinant bacmid cells. Baculoviral Expression System uses eukaryotic systems to produce the pgh 1 protein (Invitrogen, Life Technologies, 2004).

2 Aims of this Honours Thesis in Biomedicine

The aims of this project were to establish whether Bac to Bac Baculoviral Expression System is suitable for expression of pfmdr 1 gene and for purification of the pgh 1 protein.

3 Materials and methods

3.1 Materials

The following catalogues and protocols were followed, with the following changes:

Invitrogen, Life Technologies, Instruction Manual. *Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques.*

Oxford Drug Resistance Group, 2006, *ODRG INSECT CELL CULTURE HANDBOOK* (appendix 2). Page 5: protocol 2, Obtaining Bacmid DNA, stage 1, the 40% glycerol should be filtrated before use. Stage 2, a micro centrifuge was used at 14 000 rpm instead of 2500. Stage 4, the sample should be incubated at room temperature for 5 minutes maximum. Stage 5, when solution III is added, invert 4-5 times and at stage 7, invert when the supernatant is transferred to isopropanol.

QIAGEN (2004) QIAprep Spin Miniprep Kit Protocol. (p. 23)
Make glycerol stocks before starting with the protocol. The final concentration of RNase in P1 should be 100 µg/ml. Do not allow the lyses reaction, at stage 2, to proceed more than 5 minutes. Stage 7 (optional) was not performed, since XL-1 Blue cells were used. Stage 8 was performed twice and the PE buffer was left in the column for few minutes before centrifuge. At stage 10, only 30 µl of EB buffer was added.

3.2 Methods

3.2.1 PCR Optimization

PCR (polymerase chain reaction) was used to amplify different sequence fragments in the pfmdr 1 gene, vector and the bacmid DNA. Two types of primers were used, the pfmdr 1–6 forward and reverse, which can only attach at specific sites in the pfmdr 1 gene (see appendix 2). The second type of primer used was M13 forward and reverse, which is bacmid DNA specific. The amplified sequences were run in an electrophoresis to enable visualisation, see gel pictures in results. The negative control in the PCR did not contain any DNA, which was replaced by RNase free H_2O. Two types of positive controls were used. From the beginning, the positive control included DNA from pgp (p-glycoprotein), with M13 F/R primers or with M13F and T7R primers. After the optimization the positive control included DNA from the PFB, with pfmdr F1/R5 primers. Furthermore, Taq-polymerase was used for amplification of sequences with maximum 5 kb (kilo base pair). For longer sequences Phusion Polymerase was used. To optimise the PCR, the DNA concentration was measured in duplicates to check the contamination in the samples. Therefore, the spectrophotometer was used at 230 nm and 280 nm to measure the organic and protein contamination respectively and an average value was calculated from the duplicates. Hence, after isolating the bacmid DNA, the amount of DNA in the samples was measured and thereafter diluted, if needed, to a concentration of 0.1 µg/µl.

3.2.2 Restriction Enzyme Digest

No restriction enzymes were included in the positive controls and no DNA was included in the negative control. The samples were incubated at 37 degrees for 1 hour.

3.2.3 Western Blot

Western Blot was performed on cell lysate from H5 cells. Western Blot is a method that is performed to detect a specific protein in the cell lysate using antibodies. In this study only primary antibodies were used, since a conjugate was attached to these. Furthermore, the primary antibody was directed to detect the viral pgh 1 protein.

After transfection of H5 cells with recombinant bacmid DNA, H5 cells were lysed and a Western blot was performed on the cell lysate. The samples were prepared in three different concentrations, 10 ug, 15 ug and 20 ug

4 Results

4.1 Complementary Region Search

A complementary region search was performed, see appendix 4.

4.2 PCR optimization

The optimised PCR conditions in table 1 were applied on the longer DNA fragments, as can be seen in figure 1, were the expected fragment size is 3.1 kb. However, same conditions were applied for smaller fragments, 1 kb, see figure 2. Same setup was run at two different extension temperatures, 66 and 69 degrees.

Table 1. PCR conditions. The following PCR conditions were found to be optimal for PFB pfmdr 1 gene.

PCR-process	Melting temperature (Tm)	Melting temperature (Tm)	Time	
Initial denaturation	94 C	94 C	4 minutes	
Denaturation	94 C	94 C	45 seconds	35 cycles
Annealing	47 C	47 C	45 seconds	
Extension	69 C	66 C	x minutes	
Final extension	72 C	72 C	7 minutes	
Cooling	22 C	22 C	infinite	

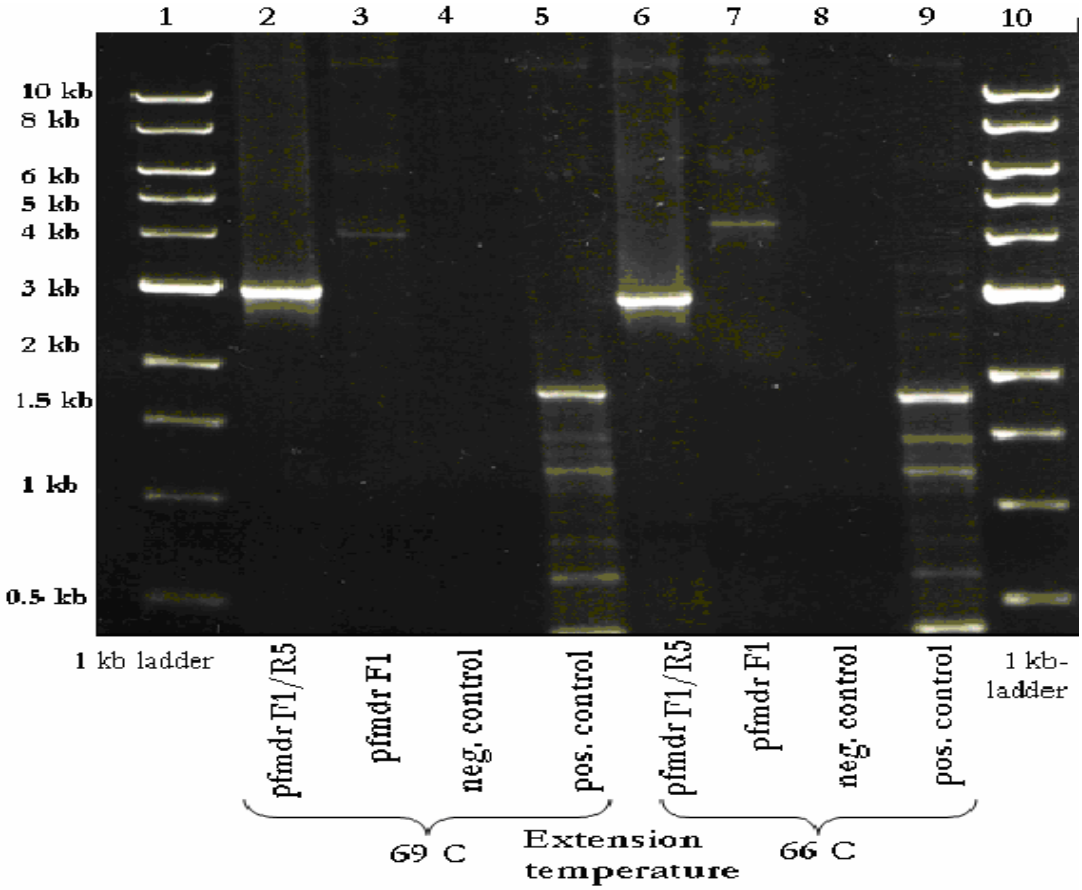

Figure 1. Digital picture on agarose gel containing two PCR setups. Well 1 and 11 contain 1 kb ladder, well 2 contains pfmdr F1/R5, well 3 and 8 contain only single primer (pfmdr F1), well 4 and 9 are negative controls. Well 5 and 10 are positive controls with the PGP 1 gene.

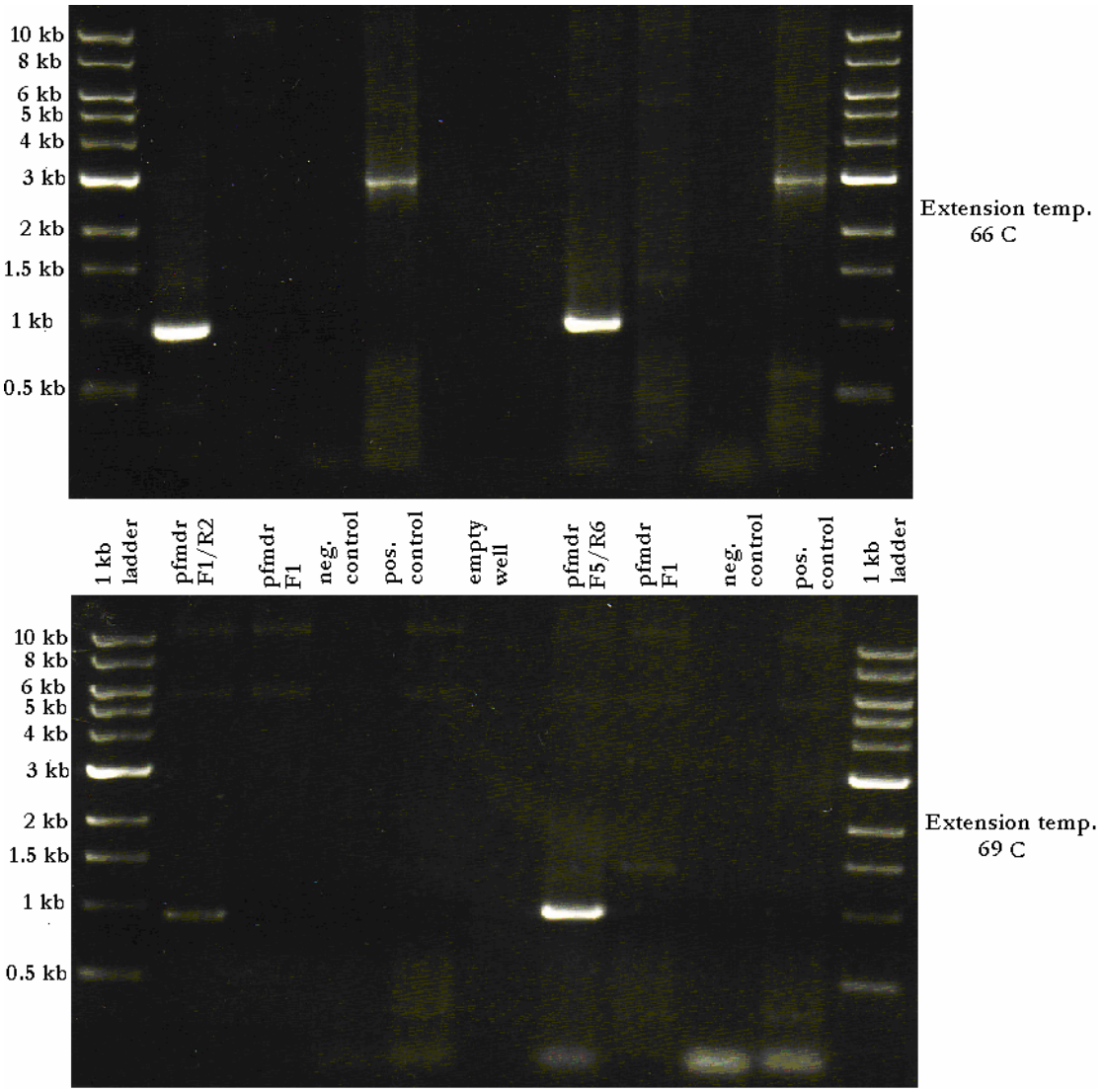

Figure 2. Two PCR setups, pfmdr F1/R2 and pfmdr F5/R6, run at two different extension temperatures, 66 and 69 degrees.

4.3 Restriction enzyme digest

The sequence of PFB pfmdr was run in NEBcutter (appendix 3) to predict were the restriction enzymes would cut and view the fragment sizes. Figure 3 shows the results from the RE digest with BamHI and EcoRI enzymes, which gave the best results.

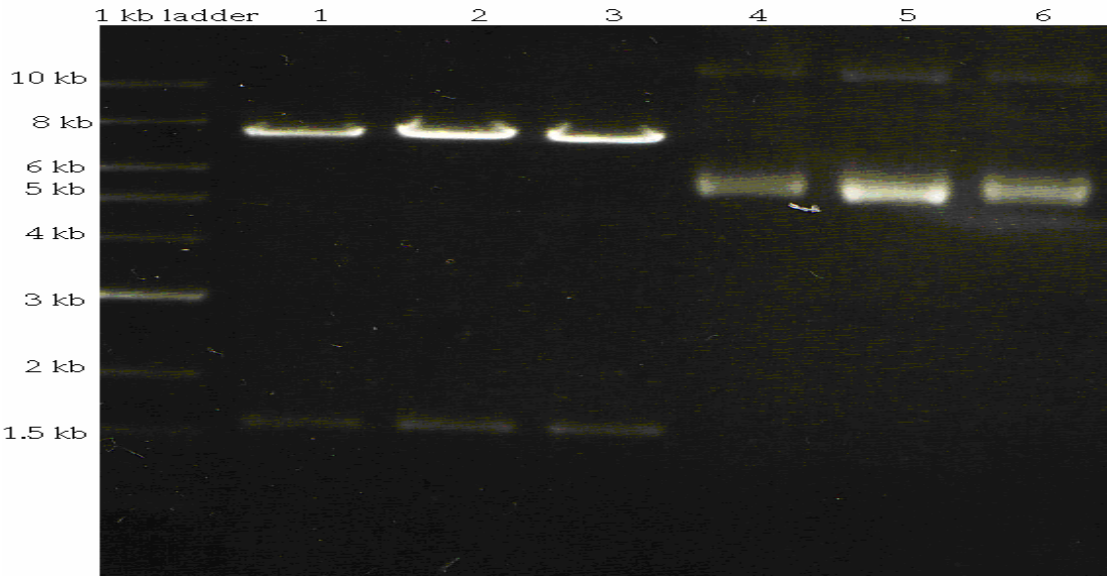

Figure 3. Restriction enzyme digestion with EcoRI and BamHI. Give two bands, 7.5 kb and 1.6 kb

4.4 Western Blot

No optimal results were obtained from the Western blot.

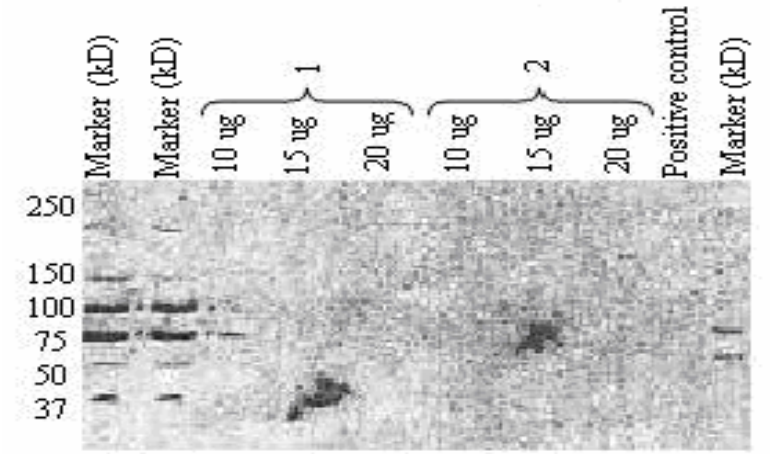

Figure 4. Western blot, showing two samples (1 and 2) in three different pgh 1 protein concentrations. The film was developed after 10 minutes exposure time.

5 Discussion

5.1 Complementary region search

The complementary region search was performed to check if there was any similarity between the pfmdr 1 sequence and the pfmdr primers. Furthermore, single primer (pfmdr forward 1) was used in the PCR (figure 1 and 2 in results) to check if the primer had sequence similarity to the pfmdr 1 gene. If similarities were observed, that could be the

underlying factor contributing to the non-specific bands. However, the similarities were not big enough to conclude that these were the reasons to the non-specific bands.

5.2 PCR optimisation for PFB pfmdr 1 and recombinant bacmid DNA

Samples containing bacmid DNA from blue colonies, should only give a band of fragment size 300 bp, since there is no pfmdr 1 gene inserted. The 300 bp band could be seen but the non-specific bands below 2 kb could also be seen, which could be an indication of contamination. Furthermore, one PCR setup did not work as planned, which probably was caused by an interruption of the PCR machine during the PCR reaction. The PCR optimisation was performed on PFB, were the number of cycles were increased from 30 to 35 and the annealing and extension temperature were lowered to 47 and 66 degrees, respectively, see table 1, in results. The positive control with the expected fragment size of 2 kb can be seen in figure 1. But, the PCR optimisation seems to have affected the positive control negatively, due to non specific bands below two kb. Therefore, the positive control sample consisting of pgp 1 was replaced by PFB pfmdr1 with pfmdr F1/R5 primers, were the expected fragment size is 3.1 kb. According to Dr. Ian Kerr (personal contact, University of Nottingham, 070410) lowering extension temperature has been proven to be sufficient for AT rich regions, such as pfmdr 1 sequence. Though, extension temperature 66 C was found to be more optimal than 69, as can be seen in figure 2, in results. The shorter sequences (pfmdr F1/R2 and F5/R6) are much clearer in the 66 degrees extension temperature. In addition, the positive control (pfmdr F1/R5, in figure 2), which should give a band of fragment size 3.1 kb, can be seen in the setup with 66 degrees, but not in 69 degrees extension temp. Therefore, the extension temperature for the optimized PCR conditions was concluded to be 66 degrees.

5.3 Restriction Enzyme Digest

RE digest was performed after cloning of pfmdr 1 gene into the PFB, to check if pfmdr 1 gene was correctly inserted into the vector. The enzymes, BamHI and EcoRI were best suited, due to less star activity, see appendix 3. However, the controls in the restriction enzyme digest gave a shorter fragment size than expected (see figure 3, well 4-6). This is because the plasmid DNA is super coiled and appears to be smaller than it is.

5.4 Western Blot

No god results were obtained from the Western blot, since the visualised band was too indistinct to draw any conclusions. The bands that were distinct were either too small or too big to match the size of 162 kD of the pgh 1 protein. However, a new western blot is required, which could present more optimal results. On the other hand, if no clear bands of the desired size of 162 kD appear, despite a new western blot, then it could be that the H5 cells were not prepared as required. The H5 cells should be remade to clear out all errors that could have made a significant change and prohibited the cells from producing the pgh 1 protein.

6 Acknowledgements

I would like to thank the ODRG group, Radcliff Hospital, in Oxford for giving me this opportunity to take part in the exciting research in malaria. Special thanks to Dr Ian Kerr in Nottingham, for his patience, understanding and great ability of collaboration. Furthermore, special thanks to professor Karin Klinga Levan and assistant professor and program director Dennis Larsson at Skövde University, for their great support during my thesis project.

7 References

Barillas-Mury, C; Kumar, Sanjeev (2005) *plasmodium-mosquito interactions: a tale of dangerous liaisons* Cellular Microbiology 7, 1539-1545. Blackwell Publishing Ltd

Cowman F, Alan., Duraisingh T, M (2005) *Contribution of the pfmdr1 gene to antimalarial drug-resistance. Acta tropica* 94: 181-190

Erwin, Van (2004). *Malaria* Available on: http://66.102.9.104/search?q=cache:PMawMjd3CNIJ:www.itg.be/itg/DistanceLearning/LectureNotesVandenEndenE/Teksten/sylabus/02_Malaria.doc+consumes+haemoglobin+to+produce+more+merozoites+Bridges,+2002&hl=en&ct=clnk&cd=1&gl=uk&client=firefox-a [Collected 240407]

Holland, Barry I; Cole P C, Susan; Kuchler, Karl; Higgins F, Christopher (2005) *ABC proteins From Bacteria to Man* Academic Press ISBN: 0-12-352551-9

Invitrogen, Life Technologies (2004) Bac to Bac Baculovirus Expression System Available at: http://www.invitrogen.com/content/sfs/manuals/bactobac_man.pdf [Collected 12/02/07]

Invitrogen, Life Technologies, instruction manual 2002 *Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques.* Available at: http://www.invitrogen.com/content/sfs/manuals/bevtest.pdf [Collected 12/02/07]

Le Bras, J; Durand, R (2003) The mechanism of resistance to antimalarial drugs in *Plasmodium falsiparum*. *Fundamental and clinical Pharmacology* 17: 147-153

Le Bras, J.; Durand, R (2003) *The mechanism of resistance associated with malaria parasite responses to chloroquine and quinine.* Fundamental & Clinical Pharmacology 17, 147-153. Blackwell Publishing.

Life Sciences Division and the Center for X-Ray Optics (CXRO). 2001. What is Malaria? Available at: http://www.lbl.gov/MicroWorlds/xfiles/malariawhatis.html [collected 23/03/07]

Manta, C (1999). *Artemisnin: is this the replacement for chloroquine in malaria chemotherapy* Pharmacology of Current Malaria Chemotherapy. Available at: http://homepages.uel.ac.uk/4474p/curre.htm [Collected 12/02/07]

MicrobiologyBytes (2004) Drug Resistance. Available at: http://www.microbiologybytes.com/introduction/malaria/Resistance.html [Collected 12/02/07]

Mohnot, A; Soni, S. Malaria in Sub-Saharan Africa Available at: http://www.stanford.edu/class/humbio103/ParaSites2005/Malaria/biology.htm [Collected 30/03/07]

Mu, J; Fredig T, M; Feng, X; Joy, D A; Duan, J; Furuya, T; Subramanian, G; Aravind, L; Cooper, R A; Wootton, J C; Xiong, M; Su, X-Z(2003) *Multiple transporters associated with malaria parasite responses to chloroquine and quinine.* Molecular Microbiology 49, 977-989. Blackwell Publishing Ltd.

New England BioLabs (2007) NEBcutter V 2.0. Available at: http://tools.neb.com/NEBcutter2/index.php [Collected 30/03/07]

New England BioLabs (2005-06) *Catalogue and Technical Reference* New England BioLab Inc.

QIAGEN (2004) QIAprep Spin Miniprep Kit Protocol. Available at: http://www.bio.indiana.edu/~chenlab/potocols/qiagenmini.pdf [Collected 30/03/07]

Reed B, M; Saliba J, K; Caruana R, S., Kirk, K., Cowman F, A. (2000) *Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum.* Nature, Vol 3

TIGR, The Institute for Genomic Research (1999-2000). *Introduction.* Available on: http://www.tigr.org/tdb/edb2/pfa1/htmls/intro.shtml [Collected 240407]

Warhurst, C. D. New England Journal of Medicine, Volume 344, No. 4, January 25, 2001, Available on: www.nejm.org [Collected 240407]

WHO, *Malaria* (2007). Available at: http://www.rollbackmalaria.org/cmc_upload/0/000/015/372/RBMInfosheet_1.htm [Collected 23/03/07]