Generation of knockout/knockdown of aspartate aminotransferase and malate quinone oxidoreductase in *Plasmodium*

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By

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DECLARATION

I hereby declare that this thesis entitled **"Generation of knockout/knockdown of aspartate aminotransferase and malate quinone oxidoreductase in *Plasmodium*"** is an authentic record of the research work carried out by me under the supervision of Prof. Hemalatha Balaram at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and that this work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on the findings of other investigators. Any omission, which might have occurred by oversight or misjudgment, is regretted.

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CERTIFICATE

This is to certify that the work described in this thesis entitled **"Generation of knockout/knockdown of aspartate aminotransferase and malate quinone oxidoreductase in *Plasmodium*"** is the result of investigations carried out by Ms. Arpitha Suryavanshi in the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

(Hemalatha Balaram)

Place:

Date:

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List of abbreviations

5-FC- 5 fluorocytosine

5-FU- 5 fluorouracil

AAT- Aspartate aminotransferase

ADSS- Adenylosuccinate synthetase

AMP- Adenosine monophosphate

ASL- Adenylosuccinate lyase

ATP- Adenosine triphosphate

BCKDH- Branched chain ketoacid dehydrogenase

BSA- Bovine serum albumin

BSD- Blasticidin deaminase

DHFR-TS- Dihydrofolate reductase-thymidylate synthase

DMSO- Dimethyl sulfoxide

ETC- Electron transport chain

FAD- Flavin adenine dinucleotide

FH- Fumarate hydratase

GFP- Green fluorescent protein

HA- Haemaglutinnin

hDHFR- human dihydrofolate reductase

MDH- Malate dehydrogenase

MQO- Malate quinone oxidoreductase

NADH- Nicotinamide adenine dinucleotide

Ni-NTA- Nickel nitrilotriacetate

PAGE- Polyacrylamide gel electrophoresis

Pb- *Plasmodium berghei*

PBS- Phosphate buffered saline

PCR- Polymerase chain reaction

PEPC- Phosphoenolpyruvate carboxylase

Pf- *Plasmodium falciparum*

PLP- Pyridoxal 5' phosphate

PM1KO- Plasmepsin I knockout

PNC- Purine nucleotide cycle

PVDF- Polyvinylidene fluoride

PVM- Parasitophorous vacuolar membrane

RFA – Regulatable fluorescent affinity

SDS- Sodium dodecyl sulphate

TCA- Tricarboxylic acid

yFCU- yeast cytosine deaminase and uridyl phosphoribosyltransferase

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Chapter 1

Introduction

1.1. Malaria

Plasmodium, a protozoan parasite is the causative agent of malaria. In 1880, Charles Louis Alphonse Laveran, a French army surgeon was the first to observe *Plasmodium* parasites in the blood of patients who suffered from malaria. In 1897, a British officer in India, Ronald Ross, found that parasites were transferred to Culicine mosquitoes from birds infected with *Plasmodium relictum*. Giovanni Battista Grassi, Angello Celli, Ettore Marchiafava, Camillo Golgi and Raimondo Filetti, in 1898, first introduced the names *Plasmodium vivax* and *Plasmodium malariae* for two of the malaria parasites that affect humans. They also found that human malarial parasite was transmitted by Anopheles mosquito. Henry Shortt and Cyril Garnham, in 1948 discovered that *Plasmodium* develops in liver before entering the bloodstream. In 1982, Wojciech Krotoski discovered the presence of dormant parasites in liver cells (Cox, 2010).

Plasmodium causes malaria in most vertebrates namely, reptiles birds and mammals (Hayakawa et al., 2008). Each *Plasmodium* species restricts infection to a particular host. Studies have revealed parasite-host co-evolution with diversification of malarial parasites coinciding with radiation of mammalian genera (Sherman, 1979). Malaria in humans, is caused by four different species of *Plasmodium*: *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovalae, Plasmodium vivax.* A recent report also indicates that the primate malarial parasite *Plasmodium knowlesi* is the fifth human malarial parasite (White, 2008). The symptoms of malarial infection include, fever, chill, severe anemia and metabolic acidosis.

1.2. Life cycle

The life cycle of malarial parasites is completed in two hosts with the asexual stages occurring in vertebrates and sexual stage in mosquito. Vertebrate hosts are infected with the parasite by the bite of mosquito. The sporozoite stage parasites are transferred to the vertebrate host during the intake of blood meal by female Anopheline mosquito (Fig 1.1(A)). The sporozoites circulate in the bloodstream of vertebrate host temporarily before the infecting the liver cells (Fig 1.1(B)). Here, the parasites undergo multiplication by asexual exoerythrocytic schizogony (Sturm et al., 2006). After 10-12 days, hepatocytes rupture to release daughter merozoites from the parasite filled vesicles (merosomes) that enter the bloodstream to infect erythrocytes (Fig 1.1(C)). The parasite embarks on asexual intra-erythrocytic cycle with each cycle lasting for 48 hours during which it divides asexually. The stages in the erythrocyte are ring ((Fig 1.1(D)), trophozoite (Fig 1.1(E)) and schizont. The trophozoite matures by schizogony, producing a number of merozoites (Haldar et al., 2006). The infected erythrocyte ruptures, releasing daughter merozoites that reinvade fresh erythrocytes (Fig 1.1(F)). The lysis of erythrocytes is characterized by periodic episodes of fever and chills. Some of the released parasites mature in erythrocytes to form gametocytes (Fig 1.1(G)). During the blood meal of an Anopheline mosquito, these gametocytes are taken in and the sexual cycle of the parasite is initiated. The gametocytes transform into gametes in the gut of the mosquito. Fertilization occurs to form worm-like zygote that penetrates the gut wall to come to the outer side of the stomach (Fig 1.1(H)). The zygote undergoes sporogony to generate numerous sporozoites and forms a cyst like structure termed as oocyst. Upon maturation of oocyst, sporozoites are released, which traverse to the salivary gland of the mosquito (Fig 1.1(I)). During the next blood meal of the mosquito, the sporozoites are transmitted from the salivary gland of mosquito to the blood vessel of the vertebrate.

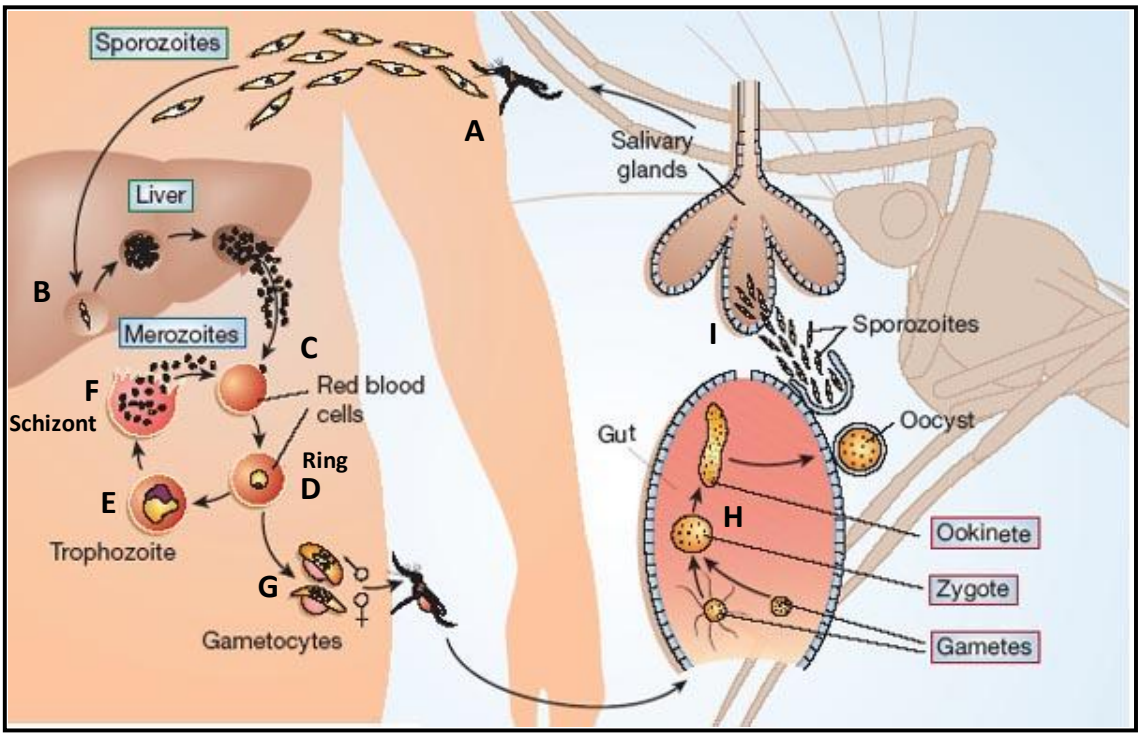


Fig 1.1. Life cycle of Plasmodium falciparum in human and mosquito hosts (A) *Sporozoites of P. falciparum are injected into the human host during the bloodmeal of female Anopheline mosquito;* ***(B)*** *The sporozoites invade the hepatocytes;* ***(C)*** *The matured merozoites released as a result of hepatic schizogony, enter the bloodstream to invade the erythrocytes;* ***(D)*** *The invaded merozoite forms a signet ring shape;* ***(E)*** *The ring stage parasite matures to form the trophozoite stage; (F) The parasites undergo schizogony, eventually rupture the erythrocyte to release merozoites;* ***(G)*** *A part of invaded parasite develops to form male or female gametocytes, which is taken up by a mosquito during a bloodmeal;* ***(H)*** *The gametocytes mature to form gametes in the mosquito, which fertilize to form the zygote and further develop to form ookinete, oocyst and release sporozoites;* ***(I)*** *Sporozoites traverse to salivary gland of mosquito. Image adapted from Ménard, 2005 with minor modifications and permission for reprint.*

Intra-erythrocytic asexual stage- This stage is clinically important due to the manifestation of the disease during this phase of the infection in the human host. As described before, this stage comprises of ring, trophozoite and schizont stage. A small fraction of growing parasites develops into gametocytes.

1) Ring Stage- The parasites appear as signet ring shaped cells in the erythrocyte. It feeds on the haemoglobin of the erythrocyte through its cytostome. The parasites also secrete proteins that aid in attachment to the endothelial walls of blood vessels (Bannister and Mitchell, 2003). The ring shaped parasite feeds to grow in size to form the trophozoite (Fig 1.2. (A)).

2) Trophozoite stage- At this stage, the parasites appears as a round cell covering the entire area of the erythrocyte. The parasite feeds on haemoglobin and is highly metabolically active in this stage. The trophozoite develops a large food vacuole where the heme liberated by proteolysis of haemoglobin is polymerized into the haemozoin crystals. During this stage, parasite proteins are exported to the surface of the erythrocyte which form knob-like structures on the membrane of the erythrocyte. This facilitates adhesion of infected erythrocytes to the blood vessels. Membranous sac like structures, called Maurer's cleft , are formed which aid in transport of necessary ions and other nutrients to the parasite (Bannister and Mitchell, 2003) (Fig 1.2. (B)).

3) Schizont stage- The nucleus of the trophozoite staged parasite divides to form 16-32 nuclei by several rounds of mitosis which generates multinuclear cells. This is followed by mass cytokinesis and the nuclei organize, to form daughter cells called merozoites (Sinden, 1991). Finally the merozoites are released from the erythrocyte by protease-dependent rupture of the parasitophorous vacuole membrane and host erythrocyte membrane (Salmon et al., 2001) (Fig 1.2. (C))..

4) Merozoites- A free merozoite is 1.2 µm long. Merozoites are egg-shaped structures having an apex. The merozoite consists of three secretory vesicles- a)rhoptries, b)micronemes and c)small rounded vesicles called dense granules.

Merozoites contain a thick adhesive bristly coat on their surface which. helps in infecting a fresh erythrocyte (Bannister et al., 2000) (Fig 1.2. (D)).

5) Erythrocyte invasion- The merozoite attaches to the erythrocyte membrane by its bristly coat. This attachment is random and reversible. A reorientation of the merozoite enables it to bring its apical prominence in contact with the erythrocyte membrane. At the point of contact of apical prominence with the erythrocyte membrane, there is a formation of pit in erythrocyte membrane. The merozoite glides into the erythrocyte with the dynamic action of actin and myosin (Pinder et al., 2000). Along with erythrocyte membrane secretions from the merozoite form the parasitophorous vacuole. The merozoite moves in to the erythrocyte with its posterior region reaching the pit and this eventually culminates in closing of the parasitophorous vacuolar membrane (PVM) and its detachment from the RBC membrane. The merozoite now becomes disc shaped to form the ring stage. The PVM expands in size to allow the growth of the parasite inside the erythrocyte. The asexual erythrocytic cycle repeats (Fig 1.2. (E))..

6) Sexual stage- Instead of forming schizonts by mitotic division, some parasites form gametocytes by gametocytogenesis. The key factor in determining the onset of gametocytogenesis is unknown. The main attribute to this in *in vitro* culture has been presumed to be environmental stress, high parasitemia and genetic variation (Baker, 2010). This process enables the parasite to form sexually competent stages, whereby male and female gametocytes are formed to undergo fertilization in the invertebrate host.

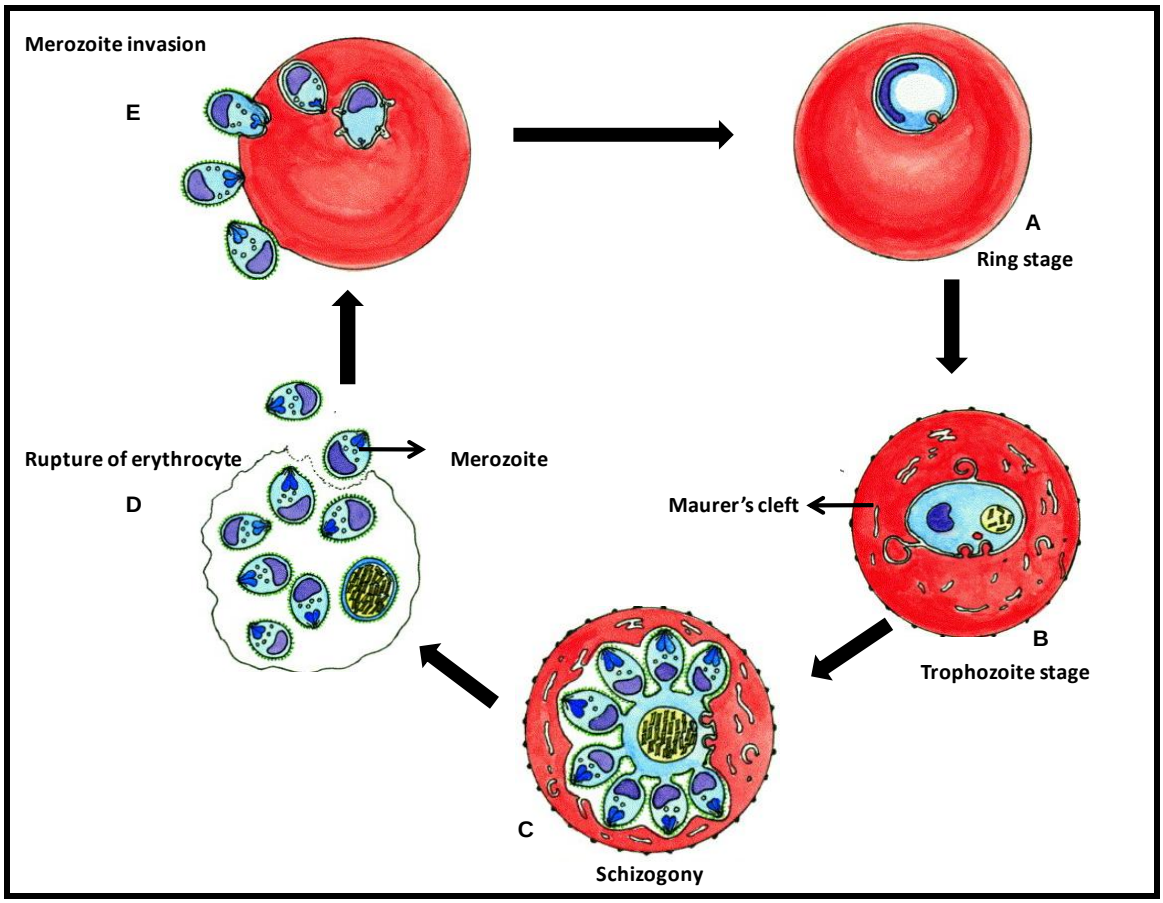


Fig 1.2. Erythrocytic stage of Plasmodium (Bannister and Mitchell, 2003)***. (A)*** *Ring Stage- Parasite appears as signet ring in the infected erythrocyte.* ***(B)*** *Trophozoite stage- Parasite grows in size. Presence of Maurer's clefts are visible.* ***(C)*** *Schizogony- Parasite divides mitotically to form daughter nuclei.* ***(D)*** *Rupture of erythrocyte- After multiplication, the parasite rupture the erythrocyte to release free merozoites* ***(E)*** *Merozoite invasion- The merozoite infects uninfected erythrocyte. Image adapted from Bannister and Mitchell, 2003 with minor modifications and permission for reprint.*

1.3. *Plasmodium* metabolism

Asexual erythrocytic stages and gametocyte stages of *Plasmodium* take up 75% more glucose than uninfected cells (Sherman, 1979). The parasites rely primarily on glycolysis for ATP, thereby generating the end product lactate, which contributes to acidosis in infected patients. Regardless of parasite's dependence on glycolysis, it possesses a mitochondrion. The essentiality of this organelle is lucid in the picture of transport of metabolites and proteins into the mitochondria (MacRae et al., 2013).

Plasmodium genome encodes all the enzymes of TCA cycle, but the gene encoding for mitochondrial pyruvate dehydrogenase was found missing. A single gene that encodes for pyruvate dehydrogenase, was found to be targeted to the apicoplast for fatty acid biosynthesis (Foth et al., 2005). It was also assumed that the TCA cycle is not functional in blood stages. But, a recent finding reported low flux of TCA cycle in asexual intraerythrocytic stages of parasite that is enhanced in the gametocytes (MacRae et al., 2013). An alternate gene called BCKDH (branched chain alpha- keto dehydrogenase) has been proposed as a likely candidate catalyzing the production of acetyl-CoA from pyruvate in the mitochondrion (Oppenheim et al., 2014).

1.3.1. Glycolysis

At trophozoite and schizont stages, that are metabolically highly active, there is 100 fold increases in consumption of glucose. Metabolite labeling studies have revealed large quantities of lactate are produced from glucose by the action of lactate dehydrogenase. A small fraction of glucose consumed is oxidized to carbon dioxide (Bowman et al., 1960). The normal growth of intraerythrocytic *P. falciparum* in microaerophilic conditions and its growth inhibition in normal atmospheric conditions implies that the parasite is a glucose fermenter. Interestingly, glycerol was also detected as another major metabolite, indicating the functionality of glycerol 3- phosphate shuttle in *Plasmodium* (Lian et al., 2009).

1.3.2. TCA cycle

Metabolite labeling studies by incorporating C-13 enriched glucose in the growth medium of *P. falciparum* divulged the parasite's emphasis on glycolytic pathway. Lactate, glycerol and pyruvate were the major C-13 enriched metabolites detected (Lian et al., 2009). Although, the genome sequence of *P. falciparum* revealed genes encoding for enzymes of TCA cycle (Gardner et al., 2002), the absence of label enriched TCA cycle intermediates indicated that the parasite lacks a functional TCA cycle (Lian et al., 2009). With the identity of the genes encoding for the enzymes involved in TCA cycle and electron transport chain, transcriptome profiling suggest an increase in expression of these genes during the switch from the asexual to sexual stages (Le Roch et al., 2003,

Bozdech et al., 2003). It was reported that there is a low flux of TCA cycle in asexual intraerythrocytic stages of *P. falciparum* (MacRae et al., 2013). Apicomplexan parasites target their single canonical pyruvate dehydrogenase to apicoplast as revealed by genetic and biochemical studies (Foth et al., 2005). Pyruvate dehydrogenase localized to the mitochondria has not been found. Hence, it was inferred that there is no conversion of pyruvate to acetyl-CoA in mitochondria for functional TCA cycle. A recent finding showed that the possible route of generation of acetyl CoA can be from mitochondrial branched chain α-keto dehydrogenase (Oppenheim et al., 2014). Maintenance of mitochondrial respiratory chain is essential in the light of dehydrogenases, that have important role in pyrimidine biosynthetic pathway apart from other functions.

It has been shown that less than 7 % of glucose consumed by the parasites is fed into TCA cycle (MacRae et al., 2013). The parasites display low flux of glucose and glutamine into the TCA cycle for generation of reducing equivalents for respiratory chain and heme biosynthesis (Mogi and Kita, 2010). There is an increased demand of energy requirement during gametocyte stage and hence, an up regulation of TCA cycle is evident in gametocytes. There is a notion that increased flux through glycolysis occurs to prepare for the post-fertilization step, where the glucose concentration is limiting in hemolymph of the mosquito (Talman et al., 2004).

The enzymes of TCA cycle are also expressed during the asexual stages (Bozdech et al., 2003). Isotopic labeling studies have revealed active synthesis of TCA cycle intermediates (Cobbold et al., 2013, MacRae et al., 2013). Glutamine and glucose are the major carbon sources that feed in to the TCA cycle. Vaidya and co-workers were successful in generating knockout of six of the eight enzymes of the TCA cycle in *P. falciparum* with no detectable growth defect (Ke et al., 2015). The group was unable to generate knockout of fumarate hydratase (FH) and malate quinone oxidoreductase (MQO) in *P. falciparum*. The study shows that the six enzymes are dispensable to the parasite at the asexual stage of the life cycle, but are required for parasite transmission. This study also reports metabolic plasticity allowing different carbon sources to feed into the TCA cycle.

The TCA cycle is essential for a) heme biosynthesis using succinyl-CoA as substrate, b) de novo pyrimidine biosynthesis using orotate (Painter et al., 2007), and c) iron-sulphur cluster biosynthesis (Van Dooren et al., 2006).

1.3.3 Electron transport chain

The electron transport chain (ETC) transfers electrons between four protein complexes through electron carriers. There are four dehydrogenases that form the four complexes of electron transport chain in *Plasmodium*. The four dehydrogenases are NADH dehydrogenase (Complex I), succinate:ubiquinone dehydrogenase (Complex II), dihydroorotate dehydrogenase (DHODH) and glycerol 3-phospphate dehydrogenase. Additionally, *Plasmodium* also has malate quinone oxidoreductase. The parsite has ubiquinol cytochrome c oxidoreductase (Complex III) and cytochrome c oxidase (Jayaraman et al., 2012). The transfer of electrons between the complexes is coupled to to the translocation of protons in the intermembrane space. This leads to the formation of proton gradient and membrane potential across the inner membrane of mitochondria. The proton gradient is harnessed for ATP generation through ATP synthase.

1.4. Genetic manipulation of *Plasmodium*

The wealth of revelation of the genome sequence can pave way for deciphering the functionality and essentiality of a gene. Gene manipulation is an important tool to investigate the significance of a gene to an organism. Experimental genetics is used to have an in-depth understanding of the biology of the parasites and its interactions with its hosts (Khan et al., 2013). Gene disruption knockdown in *Plasmodium* have shed light on function of many genes. Using reverse genetics, attempts to dissect the role of proteins expressed have been done.

Gene manipulation in *Plasmodium* relies on homologous recombination wherein, the gene is modified with a DNA construct that contains a selection cassette (Ménard and Janse, 1997). Genetic modification in *Plasmodium* is hard to achieve owing to the AT richness of the parasite genome and low transfection efficiency (Carvalho and Ménard, 2005). The transfected DNA needs to cross four membranes (erythrocyte membrane,

parasitophorous vacuolar membrane, cell membrane of parasite and nuclear membrane of parasite) to reach the genomic DNA of *Plasmodium*.

Plasmodium is a haploid organism. The advantage of haploid nature of the organism is that only a single allele has to be modified, given the shortage of *Plasmodium* selectable markers. The disadvantage of haploid nature is in obtaining a parasite with a modification in an essential gene. Attempts in abrogating/modifying an essential gene will not be successful as the modified parasite will die before selection. (Carvalho and Ménard, 2005).

Rodent malarial parasite namely, *P. berghei* and *P. yoelli* form important models to investigate vertebrate host-parasite interaction and parasite-vector relationship. Mouse is used as a model for vertebrate host and *Anopheles stefansi* for invertebrate host. Studies on sexual stages of parasite in the mosquito are achievable using rodent malaria parasites.

Genetic manipulation has been performed in *P. falciparum*, but *P. berghei* offers itself as an easier system. Rodent malaria parasites are genetically tractable. Higher transformation efficiency of *P. berghei* makes it a favorable model over *P. falciparum*. The efficiency of transfection of *P. berghei* is 10^{-2}-10^{-4}, while it is 10^{-6}-10^{-8} for *P. falciparum* and the frequency of desired integration is even lower. The genome of *P. berghei* is homologous to the human malarial parasite, *P. falciparum*. The limitations to this analysis include studies on prolonged *in vitro* culturing, invasion and egress from erythrocyte.

Different procedures are followed for transfection of *P. falciparum*. In order t obtain transfectants of *P. falciparum*, electroporation of ring staged parasites was initially done (Fidock and Wellems, 1997). Electroporation of erythrocytes with the DNA construct followed by the uptake of the DNA by schizont staged *P. falciparum* parasites has shown higher transfection efficiency as compared to other techniques (Deitsch et al., 2001). A recent technology called non-viral nulceofection technology has shown to increase the transfection efficiency of *P. falciparum* parasites from 10^{-6}-10^{-8} to 10^{-2}-10^{-4}.

This technology makes use of proprietary solution and unrevealed electrical parameters. A direct delivery of DNA into the nucleus is achieved by this tool (Janse et al., 2006a).

Erythrocytic stage parasites are transfected with the desired DNA construct and the recombinant parasites are allowed to multiply in rodent host without any selection. On checking the presence of parasites in the blood smear of the mouse, the parasites are subjected to positive selection. The presence of drug selectable marker in recombinant parasites confers resistance to the used drug. The two widely used positive selection drug markers are mutants of dihydrofolate reductase-thymidylate synthase (DHFR-TS) from *Toxoplasma gondii* or *P. berghei* that are resistant against pyrimethamine and human DHFR (hDHFR) that is resistant to both pyrimethamine and WR99210 (Fidock and Wellems, 1997). These markers can be used sequentially for generation of multiple gene modifications (Koning-ward et al., 2000). Due to the limitation of available positive selectable markers, a positive-negative selection procedure is widely used. This procedure enables recycling of positive selectable marker and permits successive modifications of the genome of parasite (Braks et al., 2006, Orr et al., 2012).

The positive-negative selection system most commonly used for gene targeting in *Plasmodium* employs the bifunctional yeast cytosine deaminase and uridyl phosphoribosyltransferase (yFCU) as a negative selectable marker. The yFCU enzyme converts the pro-drug 5-fluorocytosine (5-FC) to toxic compound 5- fluorouracil (5-FU). Firstly, the transformed parasites bearing the positive-negative selectable marker hDHFR-yFCU are selected using the positive selectable marker. The next step involves negative selection step for the removal of hDHFR-yFCU marker. Negative selection with 5-FC leads to death of the parasites bearing the hDHFR-yFCU marker and survival of parasites that have excised the marker via homologous recombination around the selection cassette.

1.5. Aims and objectives of the current study

With the focus on genetic modification of enzymes involved in metabolic pathways, the objectives for the study were laid with the given background.

There is a cross-talk between the purine nucleotide cycle (PNC) and the TCA cycle due to the involvement of common metabolites across the two pathways. Earlier studies have revealed that the fumarate generated by PNC cycle is not a metabolic waste (Bulusu et al., 2011). Upon incubation with 13-C labeled fumarate, buildup of 13-C labeled aspartate and pyruvate was seen. Fumarate generated from PNC cycle feeds into TCA cycle. The fumarate is converted to malate by the action of mitochondrial fumarate hydratase (FH). The malate is converted to oxaloacetate by malate quinone oxidoreductase (MQO). The oxaloacetate formed by this reaction is transported to the cytosol. By the action of aspartate aminotransferase (AAT) in the cytosol, oxaloacetate is converted to labeled aspartate (Fig 1.3). Hence, 13-C labeled aspartate was detectable when 13-C labeled fumarate was used. It was validated by using atovaquone inhibition, that by depleting ubiquinone pools, and thereby inhibiting MQO, there was no detectable labeled aspartate (Bulusu et al., 2011). This indicates that PfMQO catalyzes the conversion of malate to oxaloacetate. This study proves the functionality of MQO and AAT in *P. falciparum*. By genetic studies, the essentiality of these enzymes to the parasite needs to be addressed.

A recent report on phosphoenolpyruvate carboxylase (PEPC, an enzyme essential for CO_2 fixation) showed that the knockout of PEPC was possible only upon supplementation of malate or fumarate in culture medium of *P. falciparum* (Storm et al., 2014). This enzyme converts phosphoenolpyruvate to oxaloacetate. The oxaloacetate generated by PEPC reaction can be utilized by cytosolic aspartate aminotransferase (Fig 1.3). Aspartate is the precursor for pyrimidine synthesis and AMP synthesis by purine salvage pathway through the concerted action of adenylosuccinate synthetase and adenylosuccinate lyase (ASL). The oxaloacetate generated in the cytosol by PEPC or AAT can be converted to malate by the action of cytosolic malate dehydrogenase (MDH).

Plasmodium can also obtain aspartate by degradation of haemoglobin of the infected erythrocytes. Aspartate, thus obtained, is further converted to malate or fumarate by action of aspartate aminotransferase, malate dehydrogenase and mitochondrial fumarate hydratase. Hence, the growth defect of parasites seen in PEPC gene disruption could be a result of limiting supply of malate or fumarate. The function and essentiality of mitochondrial transporters for these metabolites is still uncertain

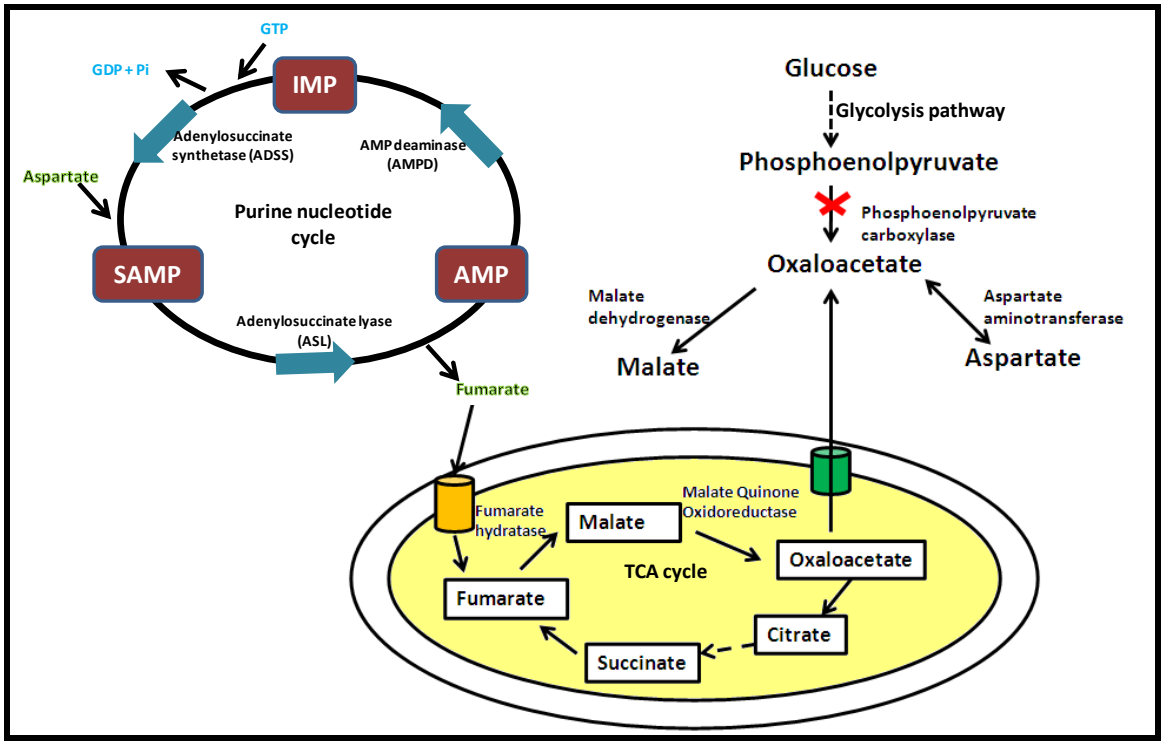


Fig 1.3. Cross-talk between purine nucleotide cycle, TCA cycle and glycolysis *. The fumarate produced by the action of ASL feeds into the TCA cycle. The fumarate is converted to oxaloacetate by sequential action of mitochondrial fumarate hydratase and malate quinone oxidoreductase. The source of oxaloacetate in the cytosol is from the glycolytic pathway by the action of PEPC, AAT and MDH.*

With this known background, an approach to study the essentiality and functionality of the two enzymes AAT and MQO has been initiated. By gene knockout and knockdown techniques using *Plasmodium berghei* and *Plasmodium falciparum* models, an attempt to generate the constructs and transfectants for the study has been done.

1.5.1. Aspartate aminotransferase

Aspartate aminotransferase (AAT) (EC 2.6.1.1) catalyzes the conversion of aspartate and alpha-ketoglutarate to glutamate and oxaloacetate. This is a pyridoxal 5' phosphate dependent enzyme. The PLP (pyridoxal 5' phosphate) abstracts the alpha amino group from aspartate to form pyridoxamine phosphate intermediate enzyme complex. The amino group from this intermediate is transferred to alpha-ketoglutarate to form glutamate (Fig 1.4). Structural characterization of this enzyme has revealed that it is active as a dimer. It consists of a N-terminal residue that helps in stabilization of dimeric structure, a small domain that forms a flexible region for open and closed conformations of the enzyme and a large domain that harbours the catalytic residues (McPhalen et al., 1992). *Plasmodium* aspartate aminotransferase has been classified in subgroup I of the aminotransferase superfamily (Mehta et al., 1993).

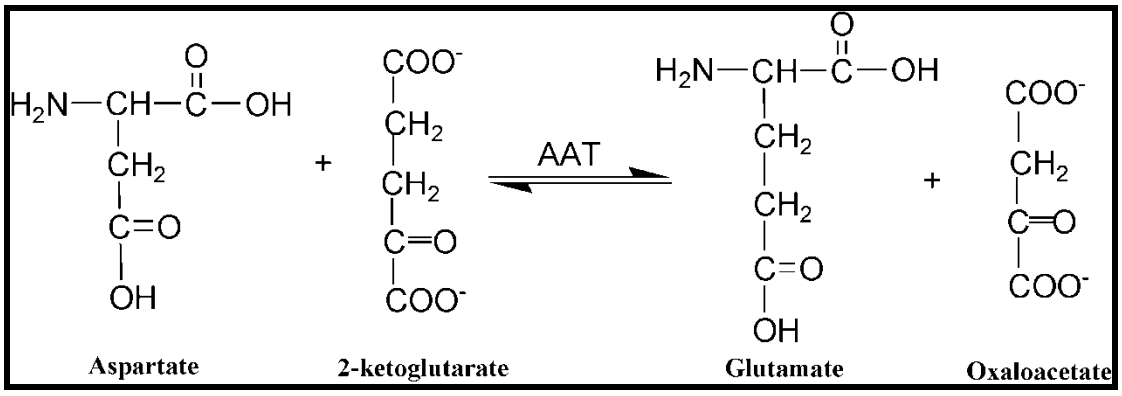


Fig 1.4. Reaction scheme of aspartate aminotransferase (AAT) (EC 2.6.1.1). *The enzyme aspartate aminotransferase is a PLP dependent enzyme that catalyzes the reversible transamination reaction of aspartate to glutamate. It catalyzes the transfer of α-amino group from aspartate to α-ketoglutarate to produce glutamate and oxaloacetate.*

Previous experiments on PfAAT have revealed the cytosolic localization of the enzyme (Wrenger et al., 2011, Bulusu et al., 2011). Wrenger et al., have demonstrated the inhibition of the PfAAT recombinant enzyme and in parasite lysates by the N-terminal fragment of the same enzyme. The N-terminal fragment does not affect the activity of cytosolic human aspartate aminotransferase. However, the effect of the N-terminal fragment of PfAAT on parasite growth was not examined. Therefore, the significance and

function of PfAAT is of question. This enzyme is predicted to participate in energy metabolism and *de novo* pyrimidine biosynthesis. The oxaloacetate generated by transamination reaction of PfAAT is critical in energy metabolism. The oxaloacetate is converted to malate by *P. falciparum* cytosolic malate dehydrogenase. The malate is presumed to be transported to the mitochondrion through the malate transporter. The malate is converted by PfMQO to oxaloacetate which reduces ubiquinone. Ubiquinone further participates in respiratory ETC (electron transport chain) to reduce the Cytochrome bc1 complex (Complex III). Increased sensitivity of PEPC knockout strain of *P. falciparum* to L-cycloserine (inhibitor of PLP-dependent enzymes) suggests the higher level of dependency on AAT for generation of oxaloacetate in the mutant (Storm et al., 2014).

Besides energy metabolism, PfAAT has been found to be involved in purine salvage pathway and *de novo* pyrimidines biosynthesis. The substrates and products of AAT are also important metabolites of TCA cycle. Hence, PfAAT is believed to be an important node for generation for metabolites which are involved in energy metabolism, purine, pyrimidine and protein biosynthesis pathways (Jayaraman et al., 2012). However, the presence of transporters is still an open question for the shuttle of metabolites in and out of mitochondrion.

Chapter 2 discusses the results obtained from our attempts to disrupt AAT gene of *P. berghei*. Using the final knockout construct procured from PlasmoGEM, transfection of cells of *P. berghei* was performed. By double crossover homologous recombination, the AAT gene was disrupted.

1.5.2. Malate quinone oxidoreductase

Malate quinone oxidoreductase (MQO) (EC. 1.1.5.4) is a FAD dependent and membrane associated enzyme. This enzyme catalyzes the oxidation of malate to oxaloacetate with ubiquinone as the electron acceptor (Fig 1.5.). The genome sequence of *Plasmodium* have revealed the presence of MQO gene (Gardner et al., 2002, Carlton et al., 2002) (http://plasmodb.org/plasmo/). This enzyme is absent in mammalian cells. MQO has been characterized from bacteria (Molenaar et al., 1998,Kather et al., 2000) though the studies reported are largely preliminary in nature. This enzyme generates the reduced form of ubiquinone (ubiquinol), at the end of the reaction. The ubiquinol is oxidized by complex III which accepts electrons. The electron transfer chain helps to maintain the membrane potential.

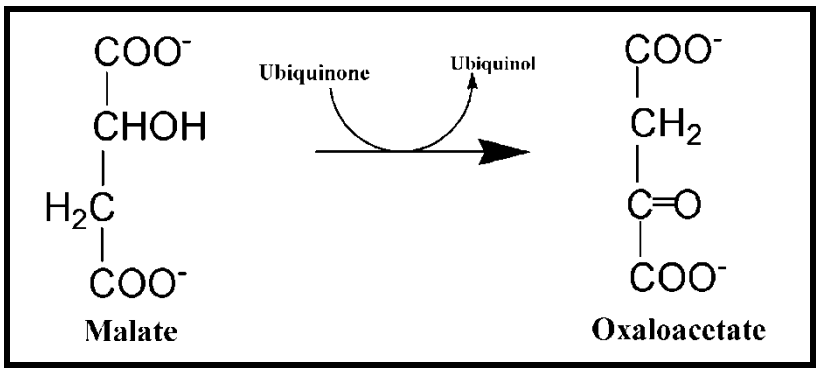


Fig 1.5. Reaction scheme of malate quinone oxidoreductase (MQO (EC. 1.1.5.4). *Malate quinone oxidoreductase catalyzes the oxidation of malate to oxaloacetate. The electrons from malate are transferred to mobile carrier of ETC, ubiquinone to form ubiquinol.*

The MQO enzyme present in bacteria has been found to localize at the cell membrane. Another enzyme that catalyzes the same reaction in other organism is NAD-dependent malate dehydrogenase (MDH). However, the advantages of the reaction catalyzed by MQO over that by MDH are that the catalysis by MQO is thermodynamically more favourable than the catalysis by MDH (Molenaar et al., 2000). Another advantage is that MQO is not sensitive to both oxaloacetate and NADH concentration in cytosol. PfMQO has been suggested to localize to the mitochondrion (Bender et al., 2003) and participate in TCA cycle. The presence of cytosolic NAD-

dependent malate dehydrogenase in *P. falciparum* has been revealed by genome sequence. Under oxidative conditions, such as during haemoglobin digestion by the parasite, there is dependence of MQO for catalysis than by NAD-dependent MDH (Tripathi et al., 2004). Bulusu et al, proved the functionality of MQO in *P. falciparum*. This study showed that Complex III inhibition by atovaquone depletes ubiquinone that is required for catalysis by MQO. According to a recent finding, PfMQO could not be deleted despite many attempts by the group (Ke et al., 2015). The group attributes the inability of disruption of PfMQO to its non-enzymatic essentiality in *P. falciparum*. Their studies propose that the gene could be essential for mitochondrial biogenesis. The essentiality and role of MQO in *Plasmodium* has not been deciphered. Towards this, an attempt has been made to check the localization of MQO in *P. falciparum.* To answer the essentiality of MQO in *P. falciparum* and the corresponding phenotypic change, a construct with MQO has been used for conditional knockdown study of MQO. Due to absence of study on MQO in rodent parasite, an attempt to obtain a knockout construct of MQO in *P. berghei* has also been carried out.

Chapter 3 highlights results from immunofluorescence towards localization of MQO in *P. falciparum*. Indirect immunofluorescence experiments using purified antibody against MQO was carried out. As the experiment failed to reveal mitochondrial localization of MQO, we resorted to GFP tagging of MQO. A vector construct comprising of MQO tagged at C-terminal end was used for transfection of *P. falciparum* cells.

Chapter 4 discusses the approach of conditional knockdown of PfMQO. A vector with a regulatable fluorescent tag containing GFP, HA and *E. coli* DHFR degradation domain tags was used for conditional knockdown study of MQO. PfMQO was successfully cloned using this vector for C-terminal tagging. *Plasmodium falciparum* PM1KO strain was transfected using the MQO tagged construct. By using different concentrations of trimethoprim, the expression of tagged MQO can be regulated in cells of *P. falciparum*.

Chapter 5 highlights the attempts to generate a knockout construct of MQO of *Plasmodium berghei*. Using recombineering and Gateway LR Clonase step, attempts to

obtain the final knockout construct were carried out. despite repeated attempts, we failed to obtain the required construct. However, the final knockout construct available in PlasmoGEM repository and this was procured and verified.

Chapter 2: Generation of aspartate aminotransferase knockout in *P. berghei*

Chapter 2

Generation of Aspartate aminotransferase knockout *P. berghei*

2.1. Abstract

Genetic ablation of aspartate aminotransferase (AAT) was performed by transfection of schizont staged cells of *Plasmodium berghei* with the available knockout vector construct of the gene. For the transfection of parasites, density gradient centrifugation was done to obtain parasites from whole blood of infected mouse. The parasites were transfected by electroporation with the knockout construct of aspartate aminotransferase. The transfected parasites were immediately injected into a healthy mouse. The parasitemia was monitored daily by microscopic examination of Giemsa stained smear of blood collected from the tail vein of the mouse. Genotyping by PCR of the transfected parasites revealed right integration of the knockout cassette. However, wild type AAT gene was also detected. Isolation of clones of AAT knockout in *P. berghei* is underway.

2.2. Introduction

Plasmodium berghei serves as a robust system for transfection owing to its high transfection efficiency (van Dijk et al., 1995). *P. berghei* is a rodent malaria parasite that permits *in vivo* study in vertebrate hosts, such as rats and mice and in the invertebrate host, *Anopheles stephensi*. This rodent parasite completes its intraerythrocytic life cycle in 24 hours. It matures to schizont stage that reinfects erythrocytes only *in vivo,* when present in the verterbrate host. However, the parasite can infect reticulocytes *in vitro*. Another advantage of using *P. berghei* to *P. falciparum* is the feasibility to follow the life cycle of *P. berghei* both in mice and mosquitoes.

Gene modification is done to examine the essentiality of the gene. Reverse genetics is a tool exploited to modify a gene. The endogenous gene is modified with the

incorporation of a selectable marker at the gene loci by double homologous recombination. Gene modifications include gene ablation, gene tagging or mutagenesis of a gene. Large sized homologous arms are proficient in crossover and replacement of the gene with selectable marker in *Plasmodium*. It is recommended to have a minimum of 1 kb length of homologous arm for efficient recombination in *Plasmodium*.

Plasmodium berghei preferentially infects reticulocytes (immature erythrocytes) (Cromer et al., 2006). Reticulocytes have large pool of ribosomal RNA and stain purple with Giemsa stain. Being young cells, these cells are rich in metabolites compared to normocytes. These cells mature further to form normocytes which contain less rRNA. *P. berghei* can infect reticulocytes *in vitro*, but not normocytes. Hence, it is not possible to maintain a continuous culture of *P. berghei in vitro*. To induce reticulocytosis in mice, an intraperitoneal dose of phenylhydrazine is injected. Phenylhydrazine damages the membrane of erythrocyte and causes anemia. Anemic condition leads to release of reticulocytes from the bone marrow of the mouse.

Transfection by nucleofection technology helps us in achieving high transfection efficiency. The DNA material has to traverse through four membranes: host erythrocyte membrane, parasitophorous vacuolar membrane, cell membrane of parasite and nuclear membrane of parasite.

2.3. Materials and methods

Institutional animal ethics and biosafety clearance for conducting animal experiments have been obtained. The final construct for knockout of AAT in *Plasmodium berghei* was obtained from PlasmoGEM. Restriction enzymes were bought from New England Biolabs, USA, plasmid preparation kits from Qiagen, Netherlands. Nucleofector kit was bought from Lonza Amaxa, Switzerland.

Reagents like pyrimethamine, Nycodenz, Penicillin-Streptomycin and heparin sodium salt were purchased from Sigma Aldrich, India. Fetal bovine serum was bought from Pan Biotech, South America.

2.3.1. Verification of knockout construct of aspartate aminotransferase by restriction digestion

The final knockout construct of aspartate aminotransferase of *P. berghei* was procured from PlasmoGEM (http://plasmogem.sanger.ac.uk/). The construct was confirmed by restriction mapping with the enzymes *Not*I, *Sac*I, *Xho*I, *Xba*I and *Bam*HI.

2.3.2. Phenylhydrazine treatment of mouse to induce reticulocyte formation

A BALB/c mouse was injected intraperitoneally with 0.1 mL of 25 mg/ml stock of phenylhydrazine hydrochloride in 0.9 % NaCl solution to induce reticulocyte formation. After two days, Giemsa stained smear of blood from tail vein was examined to check for the presence of reticulocyte.

2.3.3. Maintenance of *P. berghei*

The protocol for maintenance and transfection of *P. berghei* was carried out using protocols published earlier. (Janse et al., 2006b)

Three mice were infected with wild-type *P. berghei* ANKA from a glycerol stock by intraperitoneal injection. The parasitemia of the infected mice were checked daily by examining Giemsa stained smears blood obtained from tail vein.

2.3.4. *In vitro* culture of schizonts of *P. berghei*

Mice with parasitemia between 1 and 3 % were bled by inserting a capillary into the retro-orbital region. The infected blood was collected in a tube containing equal volume of heparin. The blood was transferred to a 250 mL tight capped Schott Duran bottle containing complete medium with 36 mL of RPMI with 25 mM HEPES, 1 ml of 0.5 M $NaHCO_3$, 12.5 mL of fetal bovine serum and 5 U/mL of penicillin and 5 µg/mL of streptomycin. The culture was gassed with a gas mixture containing 5 % CO_2, 5 % O_2, 90 % N_2 for two minutes before closing the bottle tightly. The culture was incubated on a shaker at 36.5 $^{\circ}$C and 110 rpm for 12-14 hours. The following day, 0.5 mL of the culture was taken and pelleted down. A Giemsa stained smear was made to check the health of the matured schizonts.

2.3.5. Purification of mature schizonts

The mature schizonts of *P. berghei* from the overnight grown culture were processed by density gradient centrifugation using 55 % Nycodenz in 1X PBS to enrich the parasites. 12.5 mL of culture was taken in a tube and 12.5 mL of 55 % Nycodenz was underlayed carefully in such a way that a well-defined demarcation was visible between two suspensions. This was centrifuged at 450 g for 20 minutes at room temperature. Thereafter, the brown-grayish layer of schizonts was removed using a pipette and collected in a tube. Uninfected cells pellet at the bottom after density gradient centrifugation. The mature schizonts collected were harvested by spinning again at 450 g for 8 minutes at room temperature. The supernatant was discarded and the parasite pellet was resuspended in 1 mL of complete medium. Nycodenz is preferred for density gradient centrifugation because it is less toxic to *P. berghei* cells as compared to Percoll.

2.3.6. Transfection of purified schizonts and injection into mice

The parasite pellet obtained from three infected mice was resuspended totally in 7 mL of complete culture medium. 1 mL of the resuspended parasites was distributed in 7 tubes. The tubes were centrifuged again at 450 g for 2 minutes to pellet the parasites. To four tubes of the seven, 100 µl of complete medium was added and the parasite pellet was resuspended in it. To the remaining three tubes, 100 µl of nucleofector solution containing 5 µg of *Not*I digested aspartate aminotransferase knockout construct was added and the parasite pellet was resuspended in it.

A mouse was anaesthesized by administering 4.5 mg/kg body weight of xylazine and 90 mg/kg body weight of ketamine intraperitoneally. The mouse tail vein was dilated by placing it under the IR lamp. 100 µl of the parasites resuspended in complete medium was injected into the dilated tail vein. Four control conditions were maintained for monitoring parasitemia upon infection with wild-type parasites. Of these, two wild-type conditions were maintained in phenylhydrazine treated mice and the remaining two in untreated mice.

The parasite pellet resuspended in 100 µl of nucleofector solution containing DNA was immediately placed in 2D Amaxa nucleoporator. U33 program was used to

electroporate the parasites. After the pulse was delivered, 50 µl of complete medium was added to recover the parasites. The solution containing electroporated parasites was immediately injected into dilated tail vein of a mouse. Three electroporations were performed. One lot of the electroporated parasites was injected in to a phenylhydrazine treated mouse and the other two into untreated mice.

2.3.7. Selection of transfected parasites

The following day after transfection was performed, the mice were fed with pyrimethamine in drinking water to select for transfectants. 7 mg/mL of pyrimethamine was prepared in DMSO and dissolved in 100 mL of acidified water (pH 3.5-5.5). Fresh water with drug was changed every 7 days.

2.3.8. Monitoring growth of parasites

A blood smear of all seven mice was made daily to check the parasitemia. The blood smear was stained with Giemsa and the parasitemia was counted under 100X oil immersion objective of an upright microscope.

2.3.9. Cryopreservation of parasites

Mouse with a parasitemia of approximately 5 % was bled by inserting a capillary in retro-orbital region. The infected blood was collected in a tube containing heparin. 0.2 mL of collected blood was mixed in 0.3 mL of 30 % glycerol in 1X PBS. This was collected in a cryotube and frozen in liquid nitrogen.

2.3.10. Genomic DNA isolation from parasites

1 mL of collected whole blood from the mouse was treated with 10 mL of cold 1X erythrocyte lysis buffer (10X erythrocyte lysis buffer- 1.5 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M Na_2EDTA, pH 7.4.). This was incubated for 3-5 minutes on ice. The parasites were harvested by centrifugation at 500 g for 8 minutes. The supernatant was discarded and the parasite pellet was used for genomic DNA isolation.

The parasite pellet was resuspended in 700 µl of TNE buffer (10 mM Tris HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl). To the resuspended pellet, 200 µg of RNase A and

1 % SDS were added and volume was made to 1 mL. This was incubated at 37 °C for 10 minutes. 200 µg of Proteinase K was added and incubated at 37 °C for 1 hour. To the lysed cells, buffered phenol was added and the upper aqueous phase was collected. The aqueous phase was collected again after extraction with phenol:chloroform:isoamyl alcohol at 25:24:1. To the collected aqueous phase, 0.1 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of 96 % ethanol were added. This was incubated overnight at -20 °C for genomic DNA precipitation. The following day, the precipitated DNA was centrifuged at 13,000 rpm for 10 minutes at 4 °C. The DNA pellet was washed with 70 % ethanol, air-dried and dissolved in water.

2.3.11. Genotyping of transfected parasites

The isolated genomic DNA was checked on 0.8 % agarose gel to estimate the concentration of DNA. The genomic DNA was used as a template for PCR using specific primers.

2.3.12. Selection of transfected parasites on WR99210

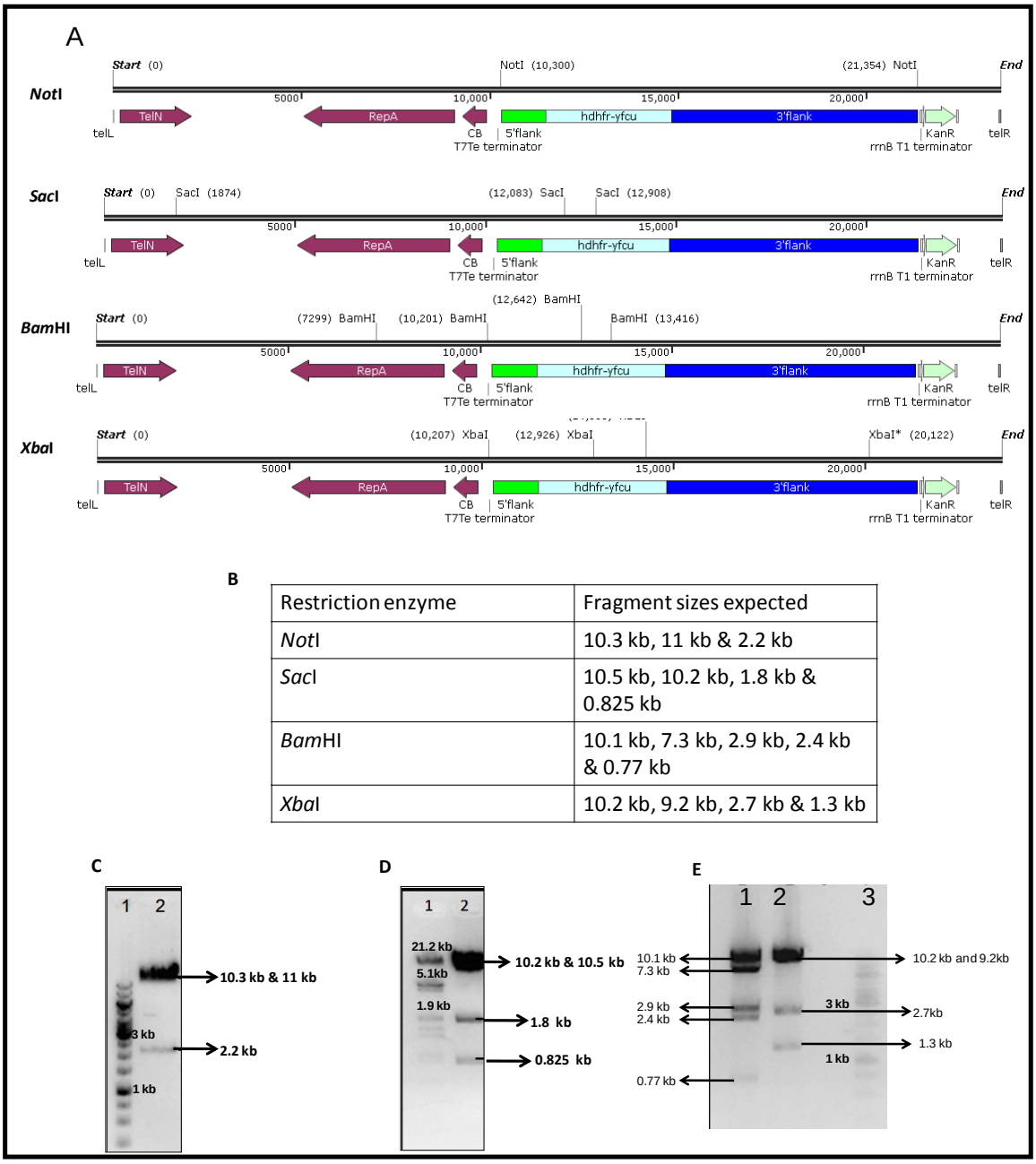
The aspartate aminotransferase knockout transfected parasites were selected on the drug WR99210 due to resistance conferred by hDHFR in the knockout construct. 0.1 mL of WR99210 from 20 mg/mL stock was injected subcutaneously in the neck of the infected mouse weighing 20 g.

2.4. RESULTS AND DISCUSSION

2.4.1. Verification of knockout construct of aspartate aminotransferase by restriction digestion

The final construct for knockout of aspartate aminotransferase in pJAZZ-OK vector was procured from PlasmoGEM (PbGEM-009046). The expected pattern and the sizes of fragments to be obtained upon restriction mapping is shown in Fig 2.1. (A). Expected sizes of DNA fragments to be obtained upon digestion is tabulated in Fig 2.1. (B). The construct was digested with five restriction enzymes and analyzed on

agarose gel. The size of fragments obtained was as expected (Fig 2.1 (C), (D), (E)). This construct was digested with *Not*I enzyme and used for transfection.



Restriction enzyme	Fragment sizes expected
*Not*I	10.3 kb, 11 kb & 2.2 kb
*Sac*I	10.5 kb, 10.2 kb, 1.8 kb & 0.825 kb
*Bam*HI	10.1 kb, 7.3 kb, 2.9 kb, 2.4 kb & 0.77 kb
*Xba*I	10.2 kb, 9.2 kb, 2.7 kb & 1.3 kb

Fig. 2.1. Verification of final knockout construct of AAT by restriction digestion. ***(A)*** *Scheme showing the digestion of final knockout construct of AAT in pJAZZ-OK vector. Digestion with enzymes NotI, SacI, BamHI and XbaI were performed.* ***(B)*** *Table indicating expected fragment sizes on digestion of pJAZZ-OK AAT KO construct with the mentioned enzymes.* ***(C)*** *0.8 % agarose gel showing fragments obtained on performing NotI digestion of final knockout construct of AAT. Lane 1, Marker; Lane 2, NotI digest of pJAZZ-OK AAT KO construct;* ***(D)*** *0.8 % agarose gel showing fragments obtained on performing SacI digestion of pJAZZ-OK AAT KO construct. Lane 1, Marker; Lane 2, SacI digested final*

knockout construct. ***(E)*** *0.8 % agarose gel showing fragments obtained on performing BamHI and XbaI digestion of pJAZZ-OK AAT KO construct. Lane 1, BamHI digested pJAZZ-OK AAT KO construct; Lane 2, XbaI digested pJAZZ-OK AAT KO construct; Lane 3, Marker.*

2.4.2. Phenylhydrazine treatment of mouse to induce reticulocyte formation

Phenylhydrazine administration to mouse resulted in reticulocyte formation. The presence of reticulocytes in blood was checked by making Giemsa stained smears (Fig. 2.2.). Purple stained cells larger than erythrocytes were visible in the microscopic field. The stain picked up by reticulocyte is due to the presence of ribosomal RNA that form a mesh-like network.

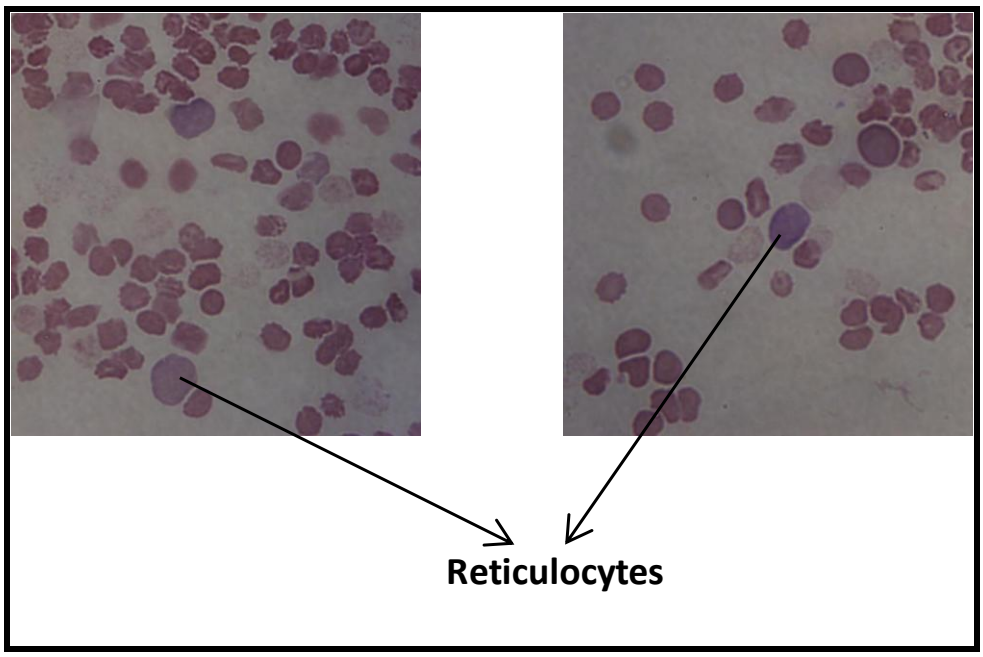


Fig 2.2 : Giemsa stained smear of blood from mice treated with phenylhydrazine to induce reticulocytosis. *Purple coloured cells are reticulocytes and the cells that appear less darly stained are normocytes.*

2.4.3. In vitro culture and purification of parasites to mature schizont stage

Three infected mice with parasitemia between 1 and 3 % were bled and the parasites were matured to schizont stage *in vitro* as described in methods. The following day, before proceeding with transfection, a Giemsa stained smear was made to check the morphology of the overnight grown mature schizonts. Healthy schizont staged parasites were visible (Fig. 2.3(A)).

After Nycodenz enrichment of the parasites, a smear was made again to check for the presence of parasites in the collected brown-grayish layer. Free merozoites and intact schizonts were visible (Fig. 2.3(B)). The transfection was performed after ensuring the presence of parasites with intact morphology after Nycodenz separation.

Table 2.1. Summarizes the experimental design for carrying out the knockout experiment of AAT in *P. berghei*.

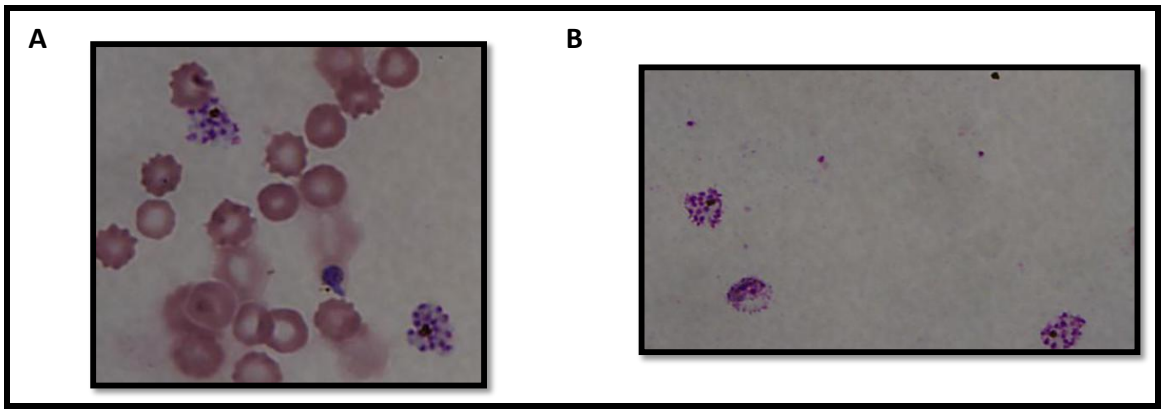


Fig. 2.3. Density gradient centrifugation on Nycodenz for enrichment of mature schizont staged P. berghei using Nycodenz. (A) *Giemsa stained smear of overnight grown P. berghei.* ***(B)*** *Giemsa stained smear of enriched parasites after Nycodenz density gradient centrifugation.*

Plasmodium berghei	Condition of mice	Number of mice	Pyrimethamine drug administration	Purpose
Wild-type	Normal	2	1-Treated, 1-Untreated	Pyrimethamine treated mice should not show infection indicating that drug is active. Pyrimethamine untreated mice should show increase in parasitemia with time
	Phenylhydrazine treated	2	1-Treated, 1-Untreated	Pyrimethamine treated mice should not show parasitemia indicating that drug is active. Pyrimethamine untreated mice should show increase in parasitemia with time But, a higher parasitemia must be seen earlier as compared to normal mice due to preferred growth in reticulocytes.
AAT KO	Normal	2	2-treated	Gradual increase in parasitemia or no parasitemia if knockout is essential.
	Phenylhydrazine treated	1	1-treated	Better growth of tranfectants in reticulocytes due to more availability of metabolites

Table 2.1. Experimental design for transfection of AAT KO construct in *Plasmodium berghei*. The number of mice used both in untreated and phenylhydrazine treated has been indicated.

2.4.4. Monitoring growth of parasites

Giemsa stained smears of blood collected from each mice was performed daily. The parasitemia observed in each condition was noted. Parasites were visible on 3rd day after the transfection in wild-type pyrimethamine untreated mice. The mouse infected with wild-type parasites and pyrimethamine treated, did not show presence of parasites in its blood. This indicates that all the wild-type parasites died on treatment with pyrimethamine. Increased parasitemia in phenylhydrazine treated mouse was seen in pyrimethamine untreated condition (Fig 2.4.). However, the phenylhydrazine treated mouse injected with the wild-type parasites and subjected to drug pyrimethamine pressure died few hours after injection of parasites. Hence, this control was unavailable for further experiments.

Parasites appeared on 12th day after transfection in AAT KO condition in normal mice. Few parasites were seen in AAT KO condition in phenylhydrazine treated mouse, but the mouse died due to unknown reason.

When the parasitemia of the transfectants reached 5 %, the parasites were harvested for genomic DNA isolation and genotyping analysis.

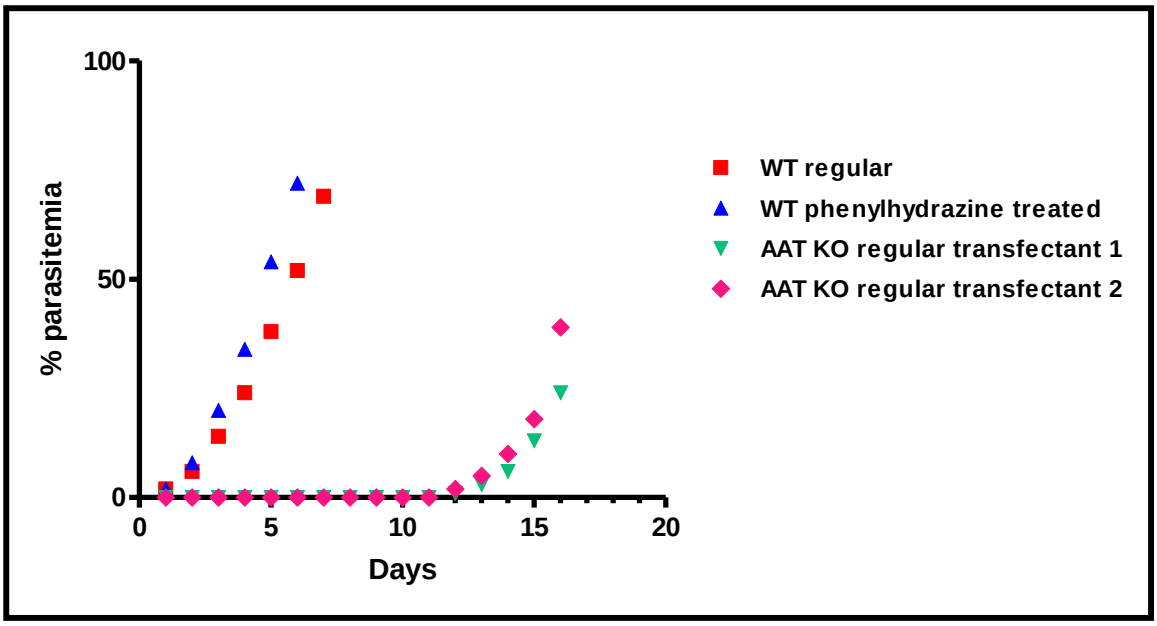


Fig 2.4. Graph indicating the percentage of parasitemia of parasites maintained in different conditions of mice. *The day zero marks the day the transfection was performed. The rise in parasitemia in wild-type conditions both in phenylhydrazine treated and untreated mice were evident from the first after transfection. However, the transfectants maintained in regular, phenylhydrazine untreated mice was apparent from 12th day of transfection.*

2.4.5. Genomic DNA isolation and genotyping of transfected parasites

The genomic DNA was isolated from untransfected and transfected parasites. The isolated genomic DNA of parasites from each condition was subjected to electrophoresis on 0.8 % agarose gel (Fig 2.5.) and further used as a template for genotyping by PCR. A table with expected sizes of amplicon is shown along with a figure indicating the location of the primer. (Fig. 2.6.A, B)

Figure 2.5. shows the presence of the genomic DNA isolated from the parasites from different conditions. However, transfected parasites injected into phenylhydrazine treated mouse did not show high parasitemia as seen in Giemsa stained smear. The mouse looked unhealthy and died due to unknown reasons as also demonstrated by higher number of leucocytes in blood smear of the mouse. Lane 6 in Fig 2.4. shows more amount of genomic DNA which may be contamination from the leucocytes of the mouse's blood.

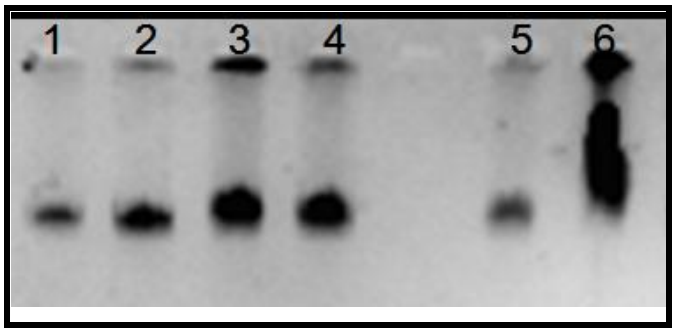
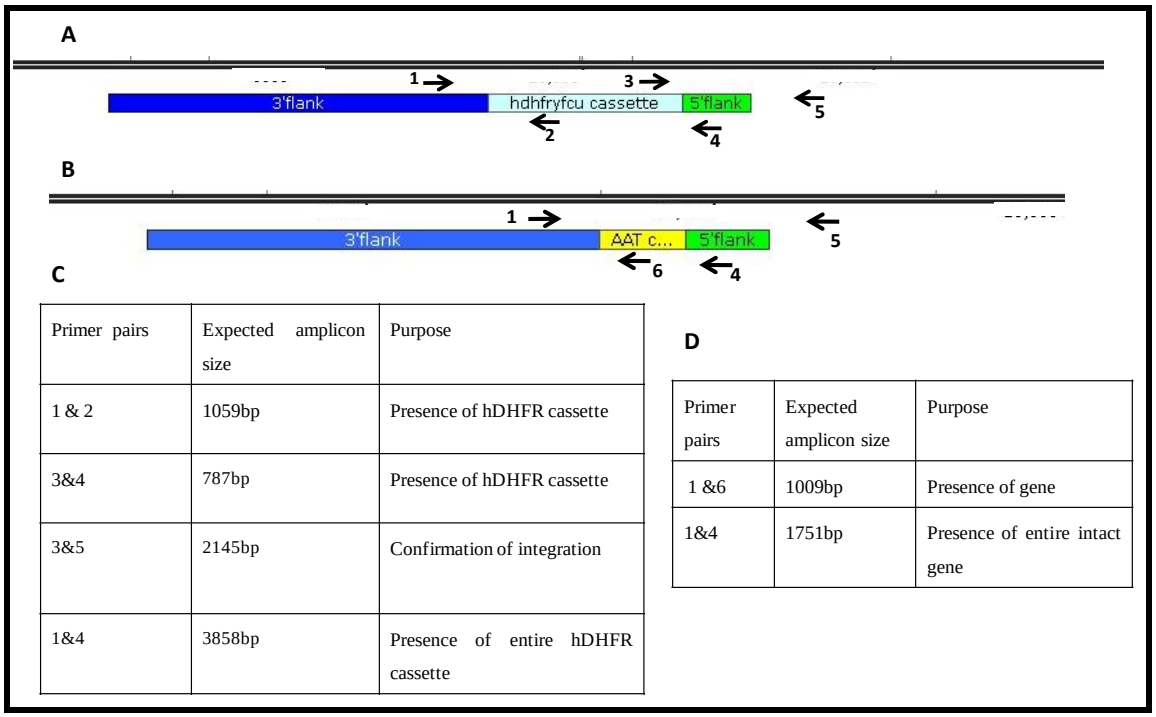


Fig. 2.5. Genomic DNA isolated from P. berghei. *Parasites were obtained from whole blood of infected mice from each of the conditions mentioned in Table 3.1. Genomic DNA was isolated and loaded on 0.8 % agarose gel. DNA from Lane 1, untransfected parasites grown in phenylhydrazine treated mice; Lane 2, untransfected parasites grown in normal mice; Lane 3, transfectants grown in normal mouse 2; Lane 4, transfectants grown in normal mouse 1; Lane 5, untransfected parasites grown in phenylhydrazine treated mice; Lane 6, Transfectants grown in phenylhydrazine treated mouse.*



C

Primer pairs	Expected amplicon size	Purpose
1 & 2	1059bp	Presence of hDHFR cassette
3&4	787bp	Presence of hDHFR cassette
3&5	2145bp	Confirmation of integration
1&4	3858bp	Presence of entire hDHFR cassette

D

Primer pairs	Expected amplicon size	Purpose
1 &6	1009bp	Presence of gene
1&4	1751bp	Presence of entire intact gene

Fig. 2.6. Primer combinations used for confirmation of AAT knockout and AAT gene in P. berghei genome. ***(A)*** *Scheme showing AAT gene replaced with hDHFR-yFCU marker with the locations of primers used. The flanked segments of DNA used in construct for knockout are indicated in blue and green colour.* ***(B)*** *Scheme showing wild-type gene AAT in P. berghei genome with the primers used.* ***(C)*** *Table showing the primer combinations used for PCR amplification in AAT KO condition.* ***(D)*** *Table showing the primer combinations used for PCR amplification of AAT gene in wild-type condition.*

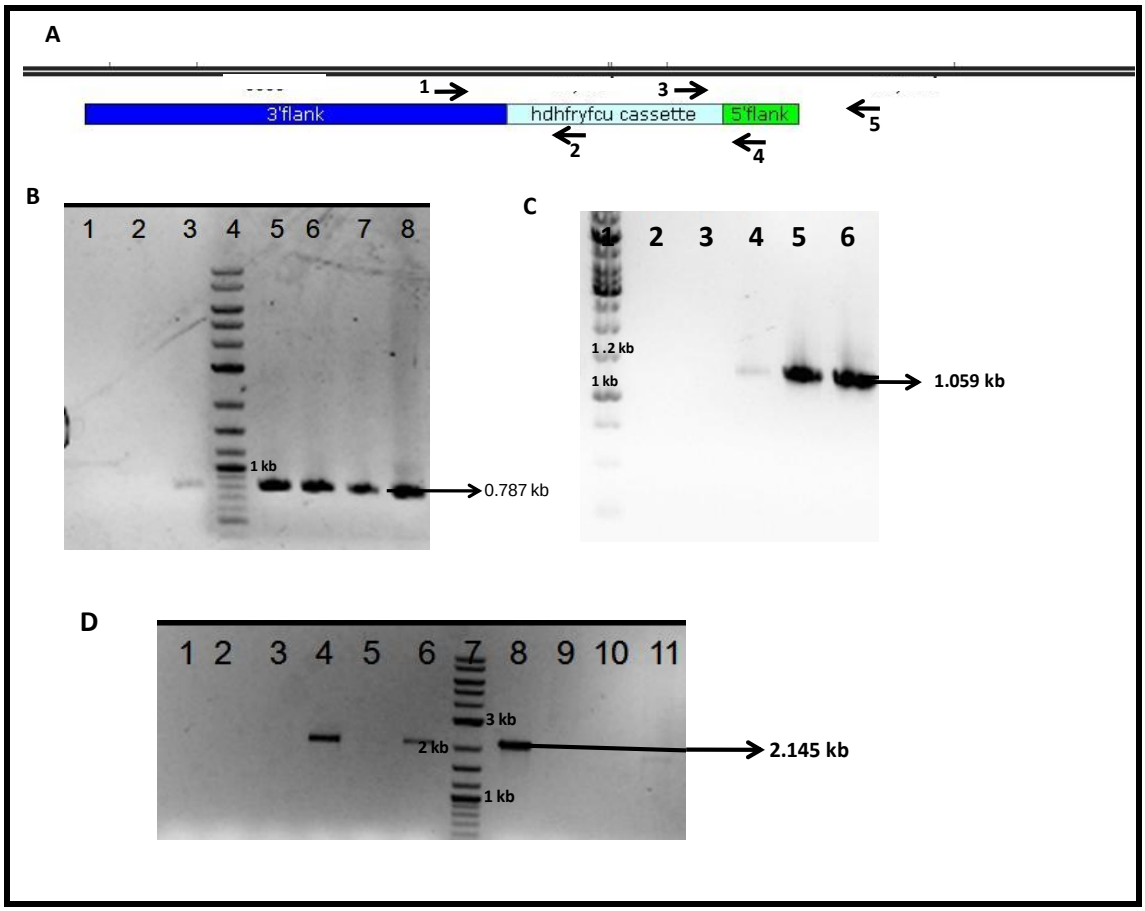


Fig. 2.7. Verification for the presence of hDHFR-yFCU marker cassette at the replaced AAT gene locus. ***(A)*** *Scheme showing the primer combinations for AAT gene knockout condition.* ***(B)*** *0.8 % agarose gel showing PCR amplicons obtained using primers 3 and 4 for verification of presence of marker cassette at the 5' flank end of the AAT gene. Genomic DNA isolated from parasites maintained under different conditions in mice were used as template for PCR. PCR amplification performed using genomic DNA templates isolated from Lane 1, Untransfectant grown in phenylhydrazine treated mouse; Lane 2, Untransfectant grown in normal mouse; Lane 3, Transfectant grown in phenylhydrazine treated mouse; Lane 4, Marker Lane 5, Transfectant regular mouse 1; Lane 6, Transfectant grown in normal mouse 2; Lane 7, Transfectant grown in regular mouse 1 and propagated in another normal mouse; Lane 8, Transfectant grown in normal mouse 2 and propagated in another normal mouse.* ***(C)*** *0.8 % agarose gel showing PCR amplicons obtained using primers 1 and 2 for verification of presence of marker cassette at the 3' flank end. Genomic DNA isolated from parasites maintained in different conditions of mice were used as template for PCR. PCR amplification performed using genomic DNA templates isolated from Lane 1,Marker; Lane 2, Untransfectant grown in normal mouse; Lane 3, Untransfectant grown in phenylhydrazine treated mouse; Lane 4, Transfectant grown in phenylhydrazine treated mouse; Lane 5, Transfectant grown in regular mouse 1; Lane 6, Transfectant grown in regular mouse 2.* ***(D)*** *0.8 % agarose gel showing the PCR amplicons obtained after using primers 3 & 5 for confirming the presence of marker cassette at the right loci towards 5' flank of DNA segment used in construct. The templates for PCR amplification used were from Lane 1, Wild-type P. berghei genomic DNA template; Lane 2- Untransfectant maintained in phenylhydrazine treated mouse ; Lane 3, Untransfectant grown in regular mouse; Lane4, Transfectant grown in regular mouse 2; Lane 5, Transfectant grown in regular mouse 1; Lane 6, Transfectant grown in regular mouse 2 with high parasitemia; Lane 7,Marker; Lane 8,*

Transfectant grown in regular mouse 2 and propagate in another regular mouse; Lane 9, Transfectant grown in phenylhydrazine treated mouse; Lane 10, Transfectant grown in normal mouse 1 and propagated; Lane 11, Vector construct for AAT knockout.

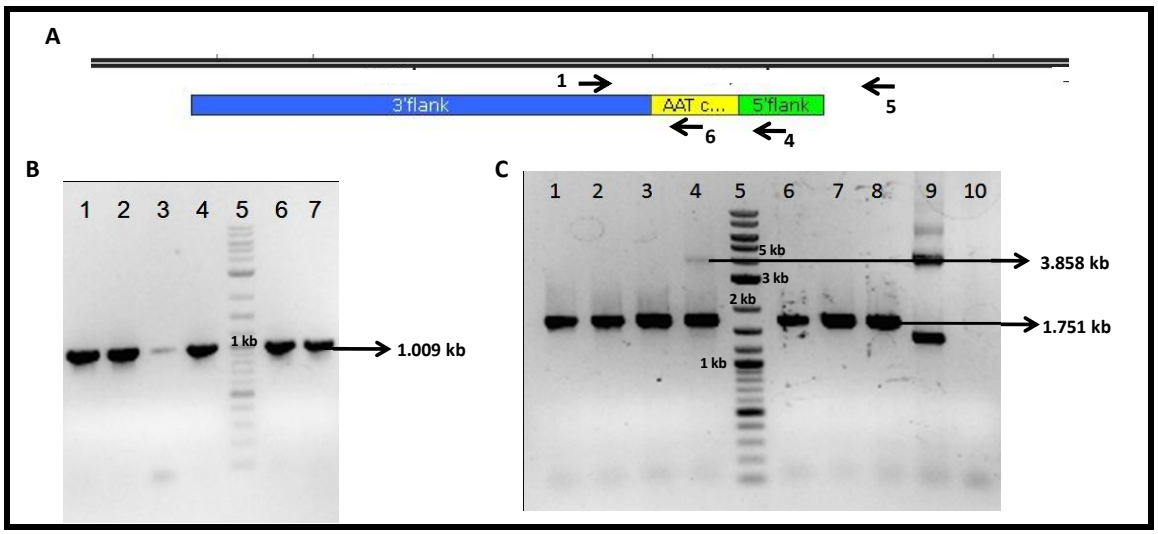


Fig. 2.8. Verification for the presence of AAT gene in wild-type genome of P. berghei . (A) *Scheme showing the primers for confirmation of presence of AAT gene in P. berghei .* ***(B)*** *0.8 % agarose gel showing the obtained amplicons on PCR amplification using primers 1 & 6. Genomic DNA isolated from parasites maintained under different conditions in mice were used as template for PCR . Lane 1, Untransfectant grown in phenylhydrazine treated mouse; Lane 2, Untransfected grown in normal mouser; Lane 3, Transfectant grown in phenylhydrazine treated mouse; Lane 4,Transfectant grown in normal mouse 1; Lane 5, Marker; Lane 6, Transfectant grown in normal mouse 2; Lane 7, Transfectant grown in normal mouse 1 and propagated in another normal mouse.* ***(C)*** *0.8 % agarose gel showing the obtained amplicons on PCR amplification using primers 1 & 4. Lane 1, wild-type P. berghei genomic DNA; Lane 2, Transfectant grown in normal mouse 1 propagated; Lane 3, Transfectant grown in normal mouse 2 propagated; Lane 4, Transfectant grown in normal mouse 2; Lane 5, 1 kb DNA ladder; Lane 6, Transfectant grown in normal mouse 1; Lane 7, Untransfectant maintained in normal mouse; Lane 8, Untransfectant maintained in phenylhydrazine treated mouse; Lane 9, Vector construct used for AAT knockout; Lane 10, Transfectant grown in phenylhydrazine treated mouse.*

PCR amplification using specific primers was performed as shown in the Fig 2.5. Refer appendix for primer sequences. Figures 2.7. (B) and (C) show the presence of hDHFR integrated in the genome of transfectants. The expected sizes of the amplicon were obtained, indicating the presence of hDHFR used in the construct for transfection in parasite. Figure 2.7. (D) confirms the right integration of knockout construct in the genome. The amplicon obtained was of expected size and this verifies the integration of hDHFR cassette at the 5' flank end of the construct used. However, in order to check the absence of the gene in this uncloned population of transfectants, PCR amplification with

AAT specific primers was carried out. With the combination of the mentioned primers, the AAT gene was detected in the uncloned transfectants as illustrated in Fig 2.8. B, C. Despite the presence of AAT in the transfected pool of cells is visible, the knockout of the gene was evident. This suggests that both right and wrong integration of the selectable marker into the genome could have taken place.

The knockout condition of AAT needs to be corroborated with Southern hybridization analysis. The presence of wild-type AAT gene in uncloned population of transfectants had to be answered. Certain reports have shown the presence of wild-type gene in uncloned transfectant population (Religa et al., 2014) (Ménard et al., 1997) (Masuda-Suganuma et al., 2012). The AAT KO transfectants were selected against pyrimethamine and the presence of wild-type parasite in uncloned transfectant population could be either due to spontaneous mutation of *Plasmodium berghei* endogenous DHFR to resist pyrimethamine or wrong integration of the knockout construct.

2.4.6. Selection of transfected parasites on WR99210

To investigate the spontaneous mutation of *P. berghei* endogenous DHFR to pyrimethamine, a selection on WR99210 was performed. Only hDHFR marker would impart resistance to WR99210. *Plasmodium* parasites bearing double mutations (C59R/S108N) and quadruple mutations (N51I/C59R/S108N/I159L) in DHFR are resistant to pyrimethamine and cycloguanil, but sensitive to WR99210 (Yuvaniyama et al., 2003). Pyrimethamine resistant *Plasmodium* DHFR is sensitive to WR99210 as crystal structures of *Plasmodium* DHFR show different sites for binding of pyrimethamine and WR99210. Hence, only those parasite cells of the uncloned population that could have undergone spontaneous mutation to resist pyrimethamine would die under WR99210 selection pressure. However, those parasites that would have acquired the DNA construct during electroporation will grow in WR99210 treated conditions due to the presence of hDHFR in the genome of the parasite. WR99210 selection pressure was introduced. The AAT KO transgenic parasites grew well with no reduction in parasitemia at any time period of the experiment. WR99210 pressure was mainrained for 5 days. The parasites were harvested for DNA isolation and PCR genotyping.

Genotyping by PCR was done to check for the presence or absence of wild-type gene. Presence of AAT gene was seen even on WR99210 selection (Fig 2.9. (A)). This ruled out the possibility of spontaneous mutation of endogenous PbDHFR. The presence of hDHFR was also seen (Fig 2.9. (B)).

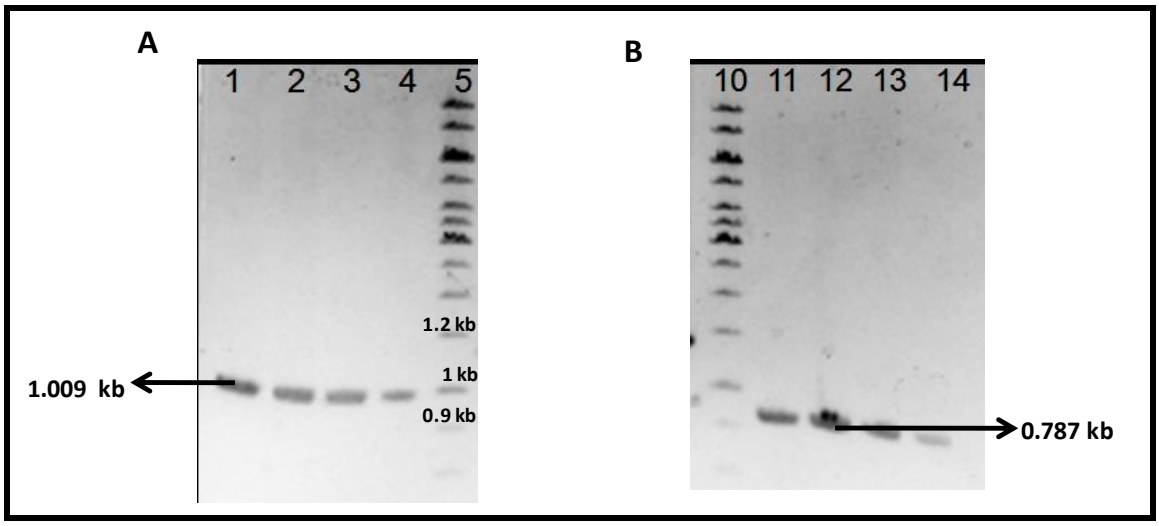


Fig. 2.9. Presence of AAT gene after WR99210 drug selection. (A) *0.8 % agarose gel showing PCR amplicons obtained. Genomic DNA isolated from transfectant parasites maintained in different conditions of mice were used as template for PCR Lane 1-4, PCR amplicons obtained for checking the presence of AAT gene using primer combinations 1 & 6. Lane 1, WR 99210 untreated in regular mouse; Lane 2, WR 99210 untreated and phenylhydrazine treated mouse; Lane 3, WR 99210 treated in regular mouse; Lane 4, WR99210 treated and phenylhydrazine treated mouse; Lane 5,Marker.* ***(B)*** *0.8 % agarose gel showing PCR amplicons obtained using primer combinations 3 & 4 for checking the presence of hDHFR gene in WR 99210 treated transfectants. Lane 1,Marker; Lane 2, WR 99210 untreated transfectants grown in regular mouse; Lane 3, WR 99210 untreated transfectants in phenylhydrazine treated mouse; Lane 4, WR 99210 treated maintained in regular mouse; Lane 5,WR 99210 treated transfectants maintained in phenylhydrazine treated mouse.*

2.5. Conclusion

Attempt to generate a knockout of AAT in *P. berghei* has been successful as checked by PCR genotyping. However, the presence of the wild-type gene AAT needs to be addressed. By PCR genotyping, we continue to see the presence of AAT gene even on WR99210 selections. In the studied uncloned population of disruptants, presence of desired disruptants in some cells and wrong integrants is evident from PCR genotyping results. This population of cells could have arisen from wrong integration of hDHFR marker cassette or integrated in to the AAT loci after an event of gene duplication thereby, showing positive results for the presence of marker as well as AAT. However, this possibility is being validated by southern hybridization. This will be followed by cloning by limiting dilution.

Chapter 3: Localization of malate quinone oxidoreductase in *P. falciparum*

Chapter 3

Localization of malate quinone oxidoreductase in *P. falciparum*

3.1. Abstract

Indirect immunofluorescence using polyclonal antibodies to malate quinone oxidoreductase (MQO) antibody was performed. The antibody was raised in rabbit previously in our laboratory. The antisera were purified by antigen affinity to obtain antigen specific antibody. The titre of the purified antibody was determined. Indirect immunofluorescence performed on asexual erythrocytic stages of *P. falciparum*. Failed to show a clear signal co-localizing with Mitotracker was not obtained. We had also made a GFP tagged construct of MQO in pGlux plasmid. Synchronized *P. falciparum* parasites were transfected with the GFP tagged construct. However, we failed to obtain parasites growing in the presence of WR99210 used for selection of transfectants.

3.2. Introduction

Malate quinone oxidoreductase (MQO) has been previously studied in bacteria. This enzyme is membrane associated that catalyzes the formation of oxaloacetate from malate. Higher organisms use malate dehydrogenase for the same reaction in cytosol and mitochondria. Malate quinone oxidoreductase localizes to cell membrane in bacteria (Molenaar et al., 1998 Molenaar et al., 2000). The origin of MQO in *Plasmodium* has been predicted to be from epsilon proteobacteria (Nosenko and Bhattacharya, 2007). Hence, MQO was assumed to be localized to mitochondria. Bioinformatic analysis on MQO of *Plasmodium* has a mitochondrial signal sequence. In this study, attempts have been done to examine the localization of MQO in *P. falciparum* by indirect immunofluorescence and GFP tagging.

3.3. Materials and methods

All reagents were obtained from Sigma Aldrich, USA and Fischer Scientific , USA.

Primers for PCR amplification of PfMQO gene were obtained from Sigma Aldrich, India. Phusion polymerase, restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, USA. Plasmid pGlux for GFP tagging was kind gift from Prof. Alan Cowman (University of Melbourne). *Escherichia coli* strain XL-10 gold was obtained from Novagen, India. Plasmid preparation kits, PCR clean up kits and gel extraction kits used were from Qiagen, Netherlands. Primary antibodies (raised in rabbit) against MQO protein were available in the laboratory. Alexa 488 tagged secondary antibody for indirect immunofluorescence was procured from ABcam, England. Ni-NTA agarose resins were obtained from Thermo Scientific, India. In-house Confocal Imaging facility was utilized for immunofluorescence studies.

Approval for use of human blood from healthy volunteers with informed consent has been obtained from the institutional bioethics committee. Consent is obtained prior to blood collection.

3.3.1. Antigen affinity purification of antiserum

The antiserum raised against MQO in rabbit was purified using antigen affinity method. The purified MQO protein along with a pre-stained ladder was subjected to electrophoresis on 12% SDS-PAGE and electroblotted on PVDF membrane. The blot was stained using Poncaeu S and the PVDF strip containing the protein band was cut. The PVDF membrane strips blotted with MQO were collected in a falcon tube and blocked using 5% skimmed milk in 1X PBS for 30 minutes. The blots were incubated with antiserum overnight at 4 °C. Thereafter, the blots were washed thrice using 1X PBS containing 0.1% Tween 20. The antibody bound to blots was eluted thrice using 100 mM Tris-glycine, pH 2.5. The eluate was immediately neutralized by adding 1M Tris HCl, pH 8.0. The three eluates were concentrated using centrifugal concentrator.

3.3.2. Western blot to determine titre of purified antibody

Dot blot was performed to determine the titre of purified antisera. The denatured MQO protein was electroblotted on PVDF membrane. The blot was blocked using 5% skimmed milk in 1X PBS for 30 minutes. To this, purified antisera at different dilutions were added and incubated at room temperature for 1 hour. The blots were washed with 1X PBS containing 0.1 % Tween 20 and incubated with goat anti-rabbit antibody for 1 hour at room temperature. The blots were washed again and developed using enhanced chemiluminescence kit (Thermo Scientific).

3.3.3. Parasite culture maintenance

The 3D7A strain of *P. falciparum* was cultured *in vitro* using a candle jar (Trager and Jensen, 1976). Parasites were maintained on human O+ erythrocytes at 5% hematocrit. Fresh medium containing RPMI 1640, 25 mM HEPES, sodium bicarbonate, 0.5 % Albumax II 11 mM glucose and 200 µM hypoxanthine with gentamycin (2.5ug/mL) was added daily. The culture was grown at 37 °C. The parasitemia of the culture was monitored every alternate day by making Giemsa stained smears.

3.3.4. Indirect immunofluorescence

The 3D7A strain of *P. falciparum* was used for immunofluorescence studies. 500 µl of the culture was taken and centifuged. The cell pellet was washed thrice with 1X PBS to remove excess media. The cells were fixed using 0.0075% of glutaraldehyde and 4% of paraformaldehyde in 1X PBS for 30 minutes. Following the fixation step, the cells were permeabilized using 0.08% Triton X100 in 1X PBS for 9 minutes. The cells were then washed with 1X PBS. Next, the cells were blocked for 1 hour at room temperature using 3% BSA in 1X PBS. Antigen affinity purified antibodies were added at different ratios; 1:200, 1:400, 1:600, 1:750 and 1:1000 to the blocking solution. This was incubated at room temperature for 1 hour. The cells were centrifuged and the obtained cell pellet was washed with 1X PBS to remove primary antibody. Goat anti-rabbit antibody tagged with Alexa 488 was used at 1:1000 dilution in 3% BSA in 1X PBS was used and cells were incubated in it for further 1 hour at 37 °C. The cells were washed

after incubation, and resuspended in 70% glycerol. 5 µl of this was mounted on a glass slide using coverslips and sealed using nail polish.

3.3.5. Cloning of MQO100 in pGlux plasmid

The first 300 bp of DNA of MQO gene corresponding to 100 amino acids was used for C-terminal GFP tagging in pGlux plasmid. The gene segment was amplified using specific primers MQO-pGluxFP and MQO-pGluxRP (appendix I for sequence) containing *Xho*I and *Kpn*I sites. The plasmid pGlux was isolated by a small scale preparation. The plasmid and PCR product were digested with the two restriction enzymes. The digested products were purified using gel extraction kit. The digested plasmid and PCR insert were ligated at 16 °C for 16 hours. The ligation mixture was used for transformation of *E. coli* XL 10 gold competent cells. The obtained clone pGlux-MQO100 was confirmed by PCR and insert release by digestion with restriction enzymes.

3.3.6. Synchronization of *Plasmodium falciparum* culture

Plasmodium falciparum culture 3D7A was synchronized using 5% D-sorbitol. Parasite culture at 5 % parasitemia and at the ring stage was washed with 1X PBS to remove medium. To one volume of culture pellet, five volumes of 5% sorbitol was added and incubated at room temperature for 5 minutes. The pellet was washed with complete medium to remove sorbitol and resuspended in medium to continue the growth of culture. This procedure lyses parasites at trophozoite and schizont stages with only ring staged parasites being resistant to sorbitol.

3.3.7. Transfection of *Plasmodium falciparum* by pre-loading of DNA in erythrocytes

Large scale preparation of clone pGlux-MQO100 was done and DNA pellet was resuspended in incomplete cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 25 mM HEPES). 100 µg of DNA was taken and volume was made to 400 µL using cytomix. 1 mL of 50 % human erythrocytes was taken and pelleted. The erythrocytes were washed with 5 mL of cold incomplete cytomix. Following this, the cell pellet was resuspended in the cytomix containing DNA. This

erythrocyte-DNA suspension was transferred to the bottom of pre-chilled 2 mm BioRad electroporation cuvette. This was immediately placed on ice for 5 minutes. The cuvette was placed inside the electroporation unit and pulsed using the exponential decay program set to 0.31 kV and 950 µF. The obtained time constant for the transfection was recorded. After the pulse, the cuvette was placed on ice and warm complete media was added. The cells were resuspended gently and collected in a tube. The volume was made upto 10 mL with complete medium and centrifuged. The supernatant containing lysed erythrocytes was removed and the cell pellet was resuspended in 10 ml of warm complete medium. To this, 1 mL of healthy schizont staged parasites at parasitemia of 5 % was added. The culture was maintained as described previously.

3.3.8. Drug selection of transfectants

The transfectants were selected against WR99210 drug. The resistance to this drug is conferred by human DHFR (dihydrofolate reductase) expressed from the plasmid. After two days of transfection, the parasites were put on 0.5 nM WR99210. The drug concentration was gradually increased to 0.75 nM and later to 1 nM.

3.4. Results and discussions

3.4.1. Antigen affinity purification of anti-MQO antisera

The purified MQO protein was subjected to SDS-PAGE gel and blotted onto PVDF membrane. The membrane was stained with Ponceau S and the protein band corresponding to 53 kDa was cut out (Fig. 3.1.). The antisera raised against the MQO protein in rabbit was added to the cut blots and purified as discussed in materials and methods section. The concentration of eluted antibody was determined by performing Bradford assay using BSA as a standard.

3.4.2. Western blot to determine the titre of purified anti-MQO antibody

A blot was performed to check the titre of the purified anti-MQO antibody. The hexahistidine tagged MQO protein was blotted on PVDF membrane and probed with the purified anti-MQO antibody (Fig. 3.2.) As a positive control, mouse anti-His antibody was used. Purified anti-MQO antibody at dilutions of 1:3000 and 1:5000 was used. A

strong chemiluminescent signal was obtained with 1:3000 dilution of purified antibody. A weak signal was seen when 1:5000 dilution of purified antibody was used. As a negative control, no primary antibody and pre-immune sera were used. This did not show any chemiluminescence signal. Another condition involved usage of 1:3000 dilution of unpurified antisera. This showed weaker signal, which indicates that the strong signal obtained with 1:3000 dilution purified anti-MQO antibody was a result of enrichment of antigen specific antibody.

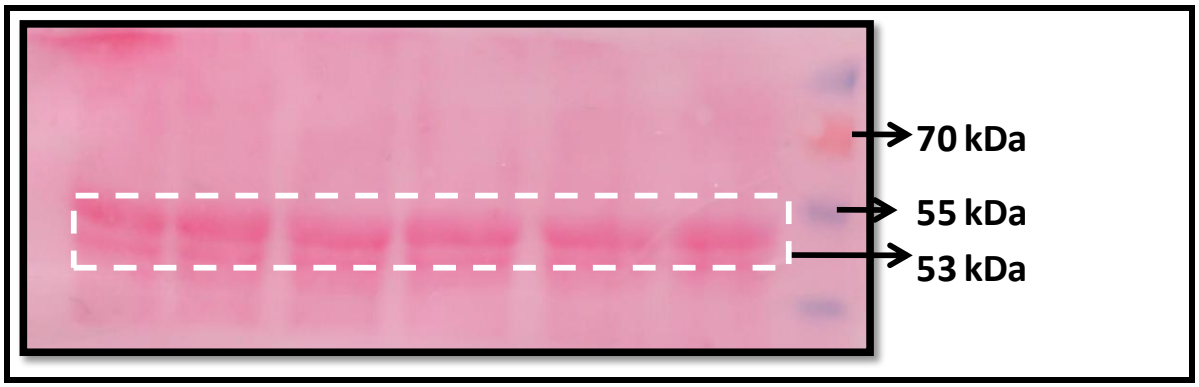


Fig. 3.1. Ponceau S stained blot showing the purified MQO protein. *The box shows protein bands corresponding to 53 kDa which indicates the region cut on the membrane for strip affinity purification*

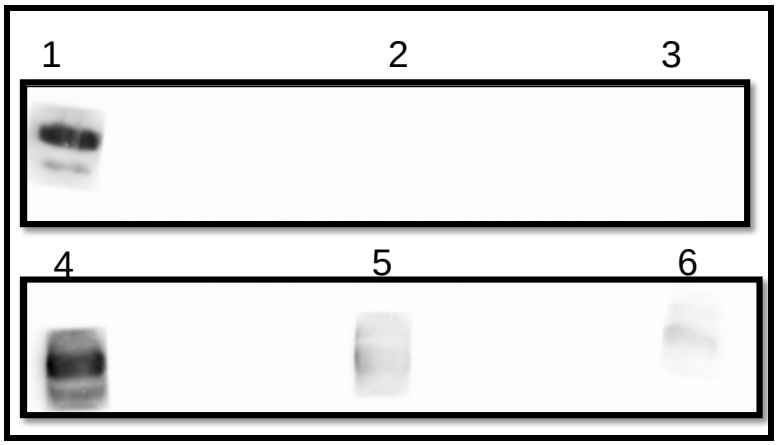


Fig. 3.2. Determination of titre of purified antibody *. PVDF membrane blots with His-tag purified MQO protein were used for determining the titre of purified antibody. Purified anti-MQO antibody at different concentrations were used along with anti-His antibody as positive control. Blot 1, anti-His antibody ; Blot 2, No primary antibody; Blot 3, pre-immune sera; Blot 4, anti-MQO antibody at 1:3000 dilution; Blot 5, anti-MQO antibody at 1:5000 dilution; Blot 6, unpurified antisera at 1:3000 dilution.*

3.4.3. Indirect Immunofluorescence

To determine localization of MQO in *P. falciparum*, an indirect immunofluorescence was conducted. The parasites were predominantly in trophozoite stage when they were used for performing immunofluorescence study.

No signal seen in pre-immune control. However, with the purified anti-MQO antibody, signal was seen throughout the cytosol. (Fig 3.3.). A perfect Pearson correlation co-efficient of Alexa 488 signal with Mitotracker was not obtained.

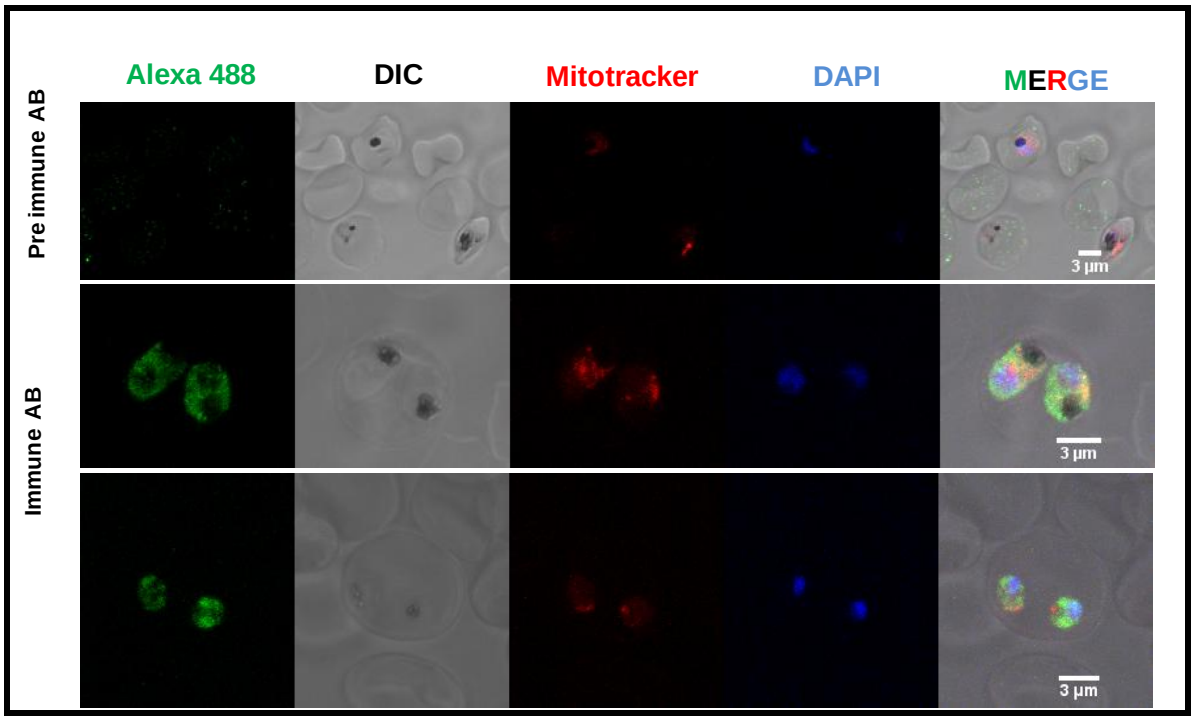


Fig 3.3. Indirect immunofluorescence. *Confocal images of indirect immunofluorescence using rabbit anti-MQO antibody. Secondary antibody used was goat anti-rabbit antibody that was conjugated to Alexa 488. The panel Alexa488 for using pre-immune sera shows no signal. Cytosolic localization signal is seen using anti-MQO antibody. For fluorescent staining of mitochondrion and nucleus, Mitotracker and DAPI were used, respectively*

3.4.5. Cloning of MQO100 in pGlux plasmid

PCR amplified MQO corresponding to 300 bp was cloned in pGLUX plasmid using *Xho*I and *Kpn*I sites. The clone was verified by insert release (Fig 4.4) and DNA sequencing.

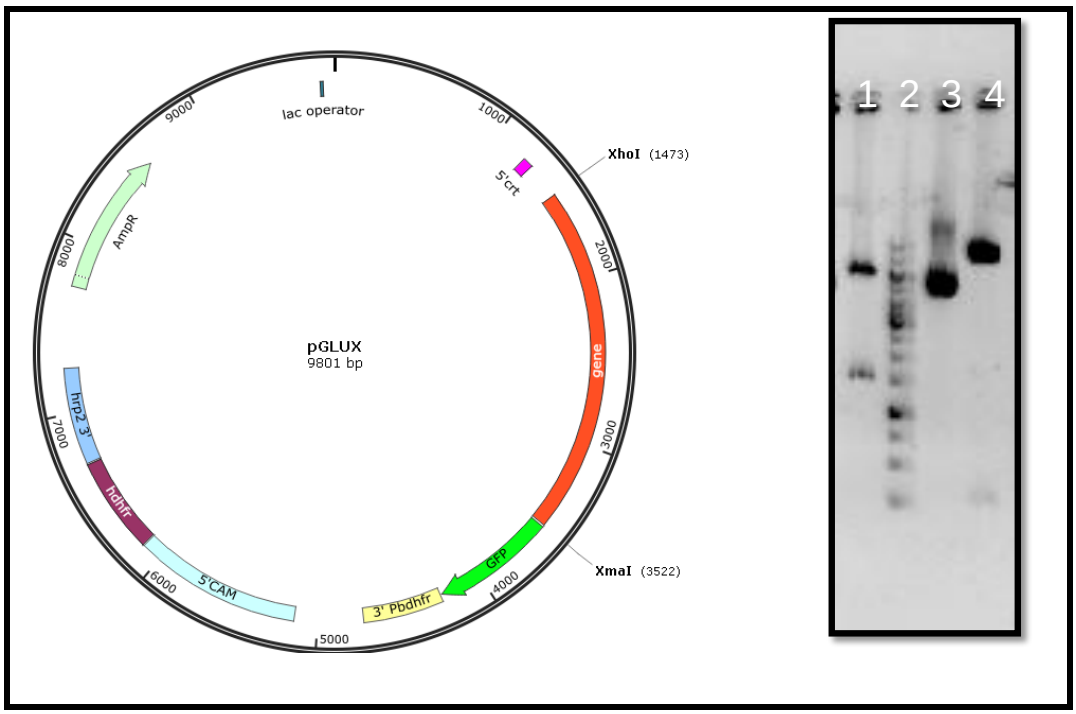


Fig 3.4. Insert release of pGlux-MQO100. *0.8 % agarose gel showing digested fragments of cloned MQO in pGlux. Lane 1, XhoI and BamHI digested pGlux-MQO100 clone; Lane 2, 1 kb DNA ladder; Lane 3, Uncut pGlux-MQO100 clone; Lane 4- XhoI and KpnI digested pGlux-MQO100 clone.*

Fresh erythrocytes were pre-loaded with the DNA construct. The exponential wave program was used for performing the electroporation of erythrocytes. The time constant obtained was 14 ms. Schizont staged *P. falciparum* 3D7A cells were added to infect the electroporated erythrocytes. After 2 days of recovery, WR99210 drug selection pressure was started to select for transfectants. The concentration of drug was gradually increased from 0.25 nM to 0.75 nM and then to 1 nM. At low WR99210 concentration of 0.25 nM, the parasites continued to grow normally and exhibited healthy morphology. Upon gradual increase of drug to 0.75 nM, the parasites continued to grow. However, the parasites died after subjecting to 1 nM of WR99210 drug pressure. Examination of the parasite uder microscope for GFP fluorescence failed to yield a signal even at low drug concentration.

3.5. Conclusion

Indirect immunofluorescence experiment did not give a clear signal localizing to the mitochondrion. The correlation co-efficient obtained on checking the colocalization signals between mitotracker and the antibody tagged to Alexa 488 was insignificant. Hence, an attempt to express GFP tagged MQO episomally in *P. falciparum* was done.

The gene MQO was successfully cloned in pGlux for GFP tagging. The plasmid was used for transfection of *P. falciparum.* At low concentrations of WR99210, the parasites persisted and continued to grow. However, transfectants failed to survive at the optimal concentration of 1 nM of WR99210.

Chapter 4: Conditional knockdown of malate quinone oxidoreductase in *P. falciparum*

Chapter 4

Conditional knockdown of malate quinone oxidoreductase in *Plasmodium falciparum*

4.1. Abstract

Study of essential genes through reverse genetics approach like knockout is not feasible due to haploid nature of *Plasmodium* genome. Hence, conditional knockdown is a beneficial tool to study the function of essential genes of the parasite. This study reports on our attempts to conditionally knockdown Malate quinone oxidoreductase (MQO) by tagging it with *E. coli* Dihydrofolate reductase degradation domain, where, the fusion protein is stable only when *E. coli* DHFR degradation domain is bound to trimethoprim. The gene was cloned in a vector for conditional knockdown and used for transfection of *P. falciparum* PM1KO strain. The parasites were transfected with the tagged MQO construct. The transfectants were selected against blasticidin and trimethoprim. The parasites appeared after 30 days of transfection. When the culture achieved a parasitemia of 5 %, blasticidin was withdrawn and growth was continued for 3 weeks only in the presence of trimethoprim. Thereafter, blasticidin was reintroduced in the culture to select for integrants. After this first round of drug cycling, the parasitemia dropped. The parasites appeared after 10 days of blasticidin reintroduction. GFP fluorescence was seen in the parasites after the first drug cycling, indicating the presence of right integrants in the culture. In addition, genotyping of the transfectants has to be performed to confirm the integration of the knockdown cassette.

4.2. Introduction

Conditional knockout or knockdown studies have been employed for studying the function and significance of essential genes. Conditional knockout using FLP/FRT (van Schaijk et al., 2010) or CRISPR/CAS9 (Ghorbal et al., 2014) systems are being widely

employed. Conditional knockdown by tetracycline inducible system was not successful (Meissner et al., 2005). The degradable domain of FKBP (FK506 binding protein) tagged to a protein of interest which is stabilized by Shield-1 ligand was less preferred due to the toxicity of the ligand to the parasite and its higher cost (Armstrong and Goldberg, 2007). Hence, an easier and alternative system used is the regulatable *E. coli* DHFR degradation domain.

The gene to be conditionally knocked down is cloned in the vector pGDB as a fusion with GFP (Green Fluorescent Protein), HA (hemagglutinin) and *E. coli* DHFR (dihydrofolate reductase) degradation domain to generate a regulatable fluorescent affinity (RFA) tag. The GFP helps in study of localization of the encoded protein and the HA tag aids in pull-down of the protein from the parasite cell lysate. The degradation domain of *E.coli* DHFR is stabilized in the presence of the folate analog, trimethoprim and prevents the encoded protein from proteolysis. On removal of the drug trimethoprim, the degradation domain gets destabilized and subsequently subjected to proteosome mediated degradation along with tagged protein of interest. Trimethoprim stabilizes the degradation domain in dose-dependent manner and therefore, conditional expression of the gene is achieved by adopting this strategy (Muralidharan et al., 2011). The plasmid pGDB also contains BSD resistance cassette that provides resistance against the drug blasticidin S.

The circular plasmid pGDB with the cloned gene for conditional knockdown undergoes a single-crossover homologous recombination via the homologous segment to shift the endogenous copy of the gene downstream of the inserted plasmid. A 3' ORF of the gene is cloned in vector, so that the crossover is mediated by the 3' ORF of the gene shifting the endogenous 3' ORF of the gene downstream. An insertion of the plasmid into the genome with the tagged 3' ORF of the gene is eventually obtained with the expression of the tagged protein regulated by the endogenous gene promoter (Fig 5.1.). The 3' ORF of MQO gene cloned in the vector has been used for conditional knockdown study.

A strain of *P. falciparum* called PM1KO is used in which, the non-essential Plasmepsin I gene is replaced with hDHFR (human dihydrofolate reductase). Due to the

presence of hDHFR gene in the parent strain, it is resistant to trimethoprim. This strain of *Plasmodium* is transfected with the cloned gene in pGDB. Transfectants obtained have the plasmid present episomally or integrated in the genome and thus, resistant to blasticidin S. The transfectants are grown in the presence of blasticidin S and trimethoprim.

Drug cycling using the two drugs, blasticidin S and trimethoprim is carried out for 2 cycles. Drug cycling with blasticidin S on and off is done to enrich the parasites containing the integrants and eliminate the parasites that have retained the plasmid episomally. To achieve this, the transfectants obtained are put off blasticidin for 3 weeks. The plasmids retained episomally are eliminated on removal of drug blasticidin S. Reintroduction of blasticidin S into the culture leads to a drop in parasitemia due to removal of parasites that could have retained the plasmid episomally (Fig 5.2). The parasites that have the plasmid integrated are retained and gradually replicate. This procedure of drug cycling is carried for another around to enrich the population of parasites that have the plasmid integrated in the genome.

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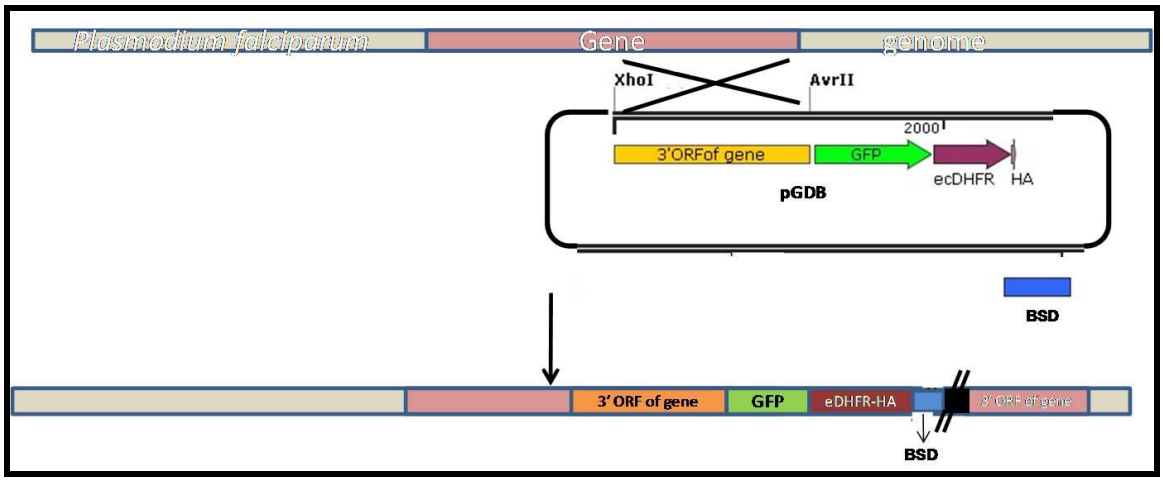

Fig 4.1. Single crossover strategy for conditional knockdown of MQO in P. falciparum. *By single crossover mediated by the cloned 3'ORF of MQO gene, the entire plasmid integrates into the genome of the parasite. The blasticidin deaminse gene is integrated in the genome, thereby, conferring resistance against the drug blasticidin S*

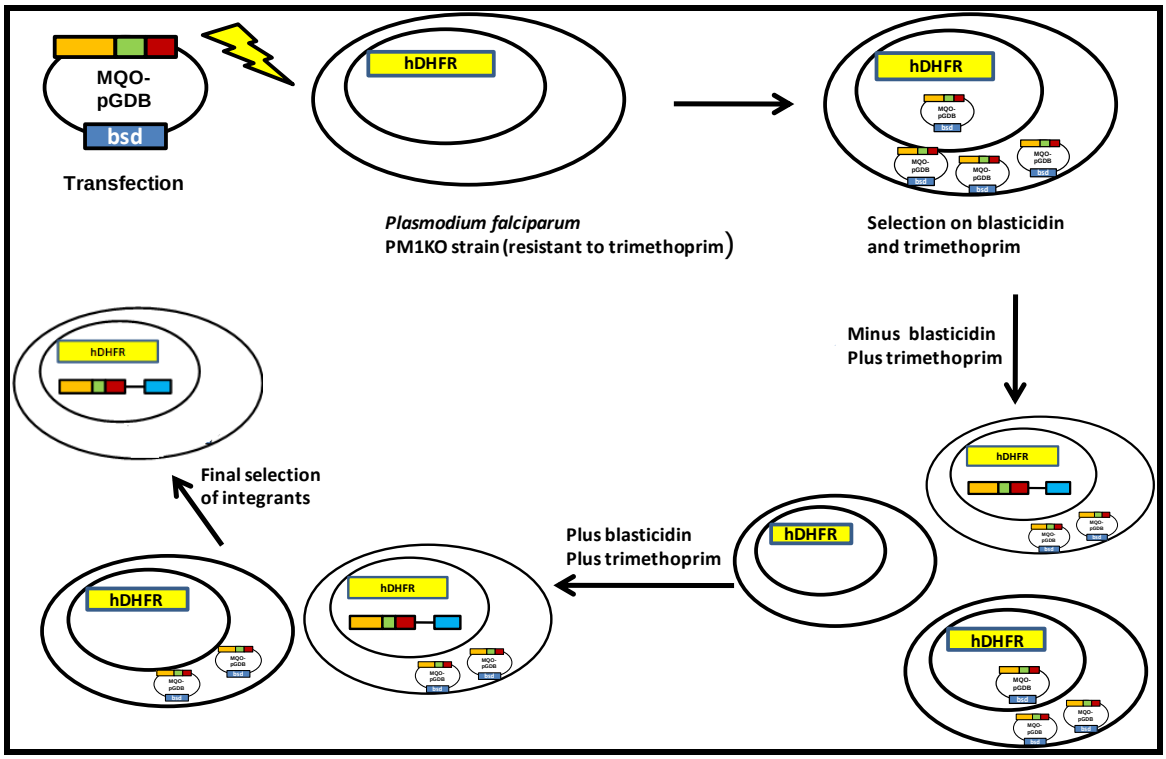

Fig 4.2. Blasticidin drug cycling for enrichment of integrant population of parasites. *Transfectants are selected in the first step by blasticidin and trimethoprim. On obtaining the transfectants, the culture is off blasticidin for removal of episomal copies of the plasmid. On re-introduction of blasticidin, integrants are selected*.

4.3. Materials and methods

Restriction enzymes were bought from New England Biolabs, USA. Plasmid pGDB and *P. falciparum* PM1KO strain were kind gifts from Prof. Daniel Goldberg Washington University School of Medicine, St Louis, MO. Primers for cloning were bought from Sigma Aldrich, India. Plasmid preparation kits, gel extraction kits were bought from Qiagen, Netherlands. Electroporator and 0.2 mm electroporation cuvettes used were from Biorad, USA.

4.3.1. Cloning of C-terminal of MQO in pGDB plasmid

The C-terminal part of MQO gene corresponding to 1.188 kb was used for cloning in pGDB plasmid. The gene was amplified using primers MQO-pGDBFP and MQO-pGDBRP (appendix I) with *Avr*II and *Xho*I sites. The plasmid pGDB was isolated

by a small scale preparation. The plasmid and PCR product were digested with the two aforementioned restriction enzymes. The digested products were purified using gel extraction kit and ligated using T4 DNA ligase at 16 $^{\circ}$C for 16 hours. The ligation mixture was used for transformation of *E. coli* XL 1 blue competent cells. The clone was confirmed by PCR, insert release by digestion with restriction enzymes and DNA sequencing.

4.3.2. Synchronization of *Plasmodium falciparum* culture

P. falciparum PM1KO culture was synchronized using 5% D-sorbitol. Parasite culture at 5% parasitemia, predominantly at ring stage, was washed with 1X PBS to remove medium. To one volume of culture pellet, five volumes of 5% sorbitol was added and incubated at room temperature for 5 minutes. The pellet was washed with complete medium to remove sorbitol and resuspended in complete medium to continue the growth of culture. This experiment lyses parasites at trophozoite and schizont stages. Only ring staged parasite are resistant to sorbitol treatment.

4.3.3. Transfection of *Plasmodium falciparum* PM1KO by pre-loading of DNA in erythrocytes

Large scale isolation of MQO-pGDB clone was done and the DNA obtained was resuspended in incomplete cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 25 mM HEPES). 100 µg of DNA was taken and volume was made up to 400 µL. 1 mL of 50% erythrocyte suspension was centrifuged and washed with 5 mL of cold incomplete cytomix. Following this, the cell pellet was resuspended in MQO-pGDB DNA suspension. This erythrocyte-DNA suspension was transferred to the bottom of a pre-chilled 2 mm electroporation cuvette. This was immediately placed on ice for 5 minutes. The cuvette was placed inside the electroporation unit and pulsed using the exponential decay program set at 0.31 kV and 950 µF. The obtained time constant for the transfection was 13.3 ms. After the pulse, the cuvette was placed on ice. Warm complete media at 37 °C, was added to the cuvette. The cells were resuspended gently and collected in a tube. The volume was made upto 10 mL and the tube was centrifuged. The supernatant containing lysed erythrocytes was removed

and the erythrocyte pellet was resuspended in 10 ml of warm complete medium. To this, 1 mL of healthy schizont staged parasites at parasitemia of 5% was added. The culture was maintained as described in Chapter 3.

4.4. Results and discussion

The purpose of this study was to generate conditional knockdown system for PfMQO. The PCR amplified insert of C-terminal of MQO was successfully cloned in pGDB. The clone was confirmed by insert release and sequencing (Fig 4.4 A, B).

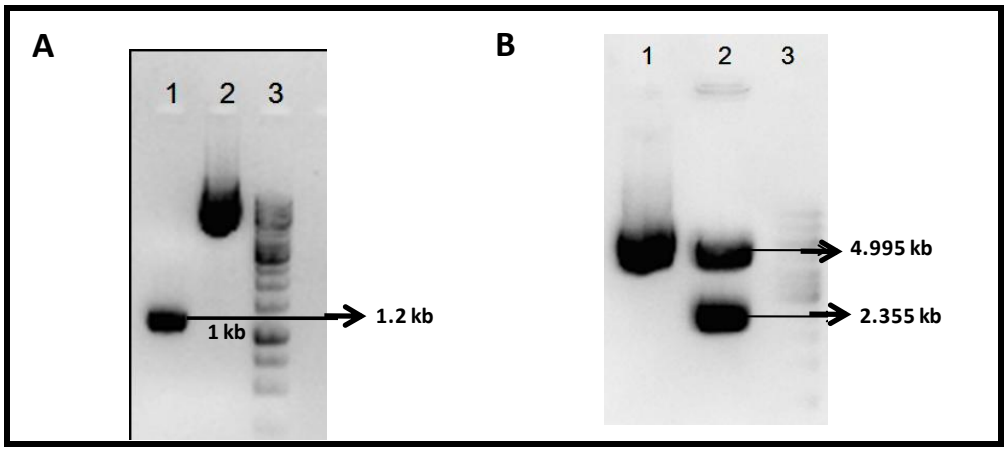

Fig 4.3. Cloning of 3' ORF of MQO in pGDB. ***(A)*** *PCR amplification of insert Lane 1, PCR amplicon of 3' ORF of MQO. Lane 2, Plasmid pGDB, Lane 3, 1 kb DNA ladder.* ***(B)*** *Insert release for confirmation of clone: Lane 1, undigested ligated product; Lane 2, Xho I and EcoRI digested ligated product; Lane 3, 1 kb DNA ladder*

The *P. falciparum* PM1KO strain was transfected with the cloned MQO-pGDB construct using the exponential decay program of the electroporator. The time constant obtained for the pulse was 13.3 ms, which falls in the recommended range 12-20 ms for successful transfection. The parasitemia reached to 6 % after 4 days of transfection, in which the parasites had undergone two life cycles. Healthy parasites were seen. Media was chaged everydays and giemsa stained smears were made daily. After two life cycles of the parasites, the transfectants were selected using 2.5 µg/mL of blasticidin and 10 µM trimethoprim. The transfectants appeared after 30 days of electroporation and were subsequently subjected to the first round of drug cycling. Parasites that appeared after reintroduction of blasticidin S after first round of drug cycling were checked for the

presence of GFP fluorescence. Parasite cells with GFP fluorescence were seen as shown in Fig 4.4. The presence of GFP fluorescence in cells indicates that the construct used has integrated into the genome of the parasite. To enrich for the integrants, the parasites are currently under second round of drug cycling.

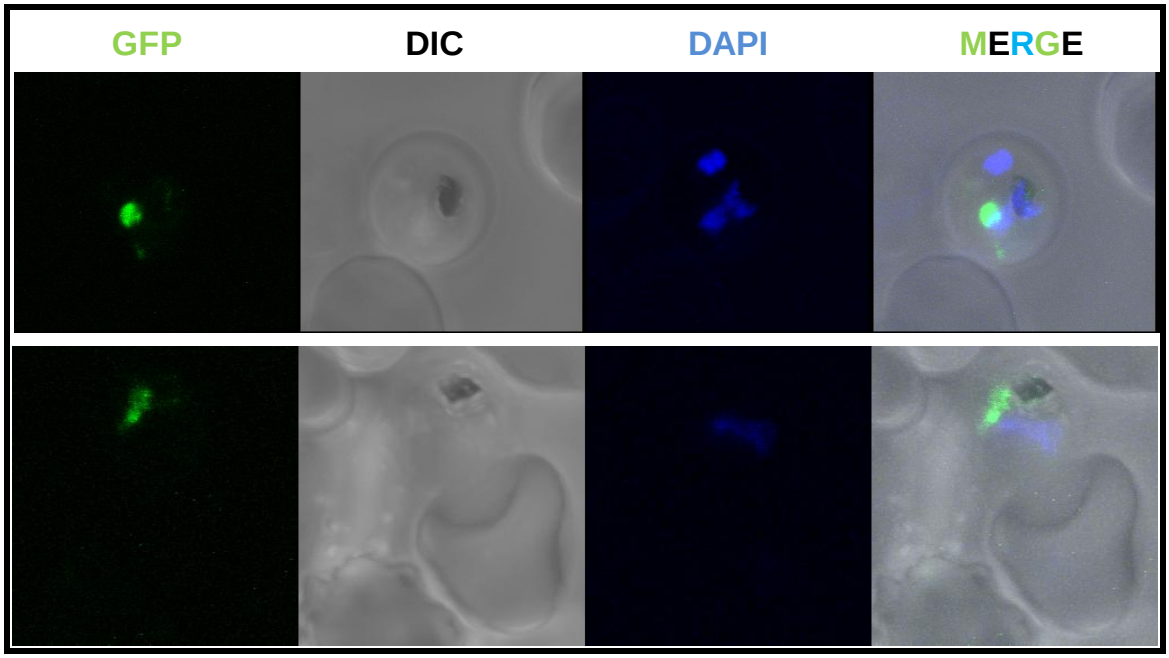

Fig 4.4. Confocal images of integrants expressing GFP. *The first panel shows GFP signal seen in transfectants after first round of drug cycling. Second panel shows the DIC images. Third panel indicates the nuclear staining of parasites by DAPI. The fourth panel shows merged panels.*

4.5. Conclusion

The C-terminal part of MQO was successfully cloned as confirmed by insert release from the vector and sequencing. The transfectants were obtained 4 weeks after transfection. The transfectants were subjected to first round of drug cycling. Presence of parasite cells expressing GFP indicates that the plasmid has integrated into the genome of these cells. Second round of drug cycling is currently underway. Confirmation of integration of the plasmid will be verified by PCR amplification using genomic DNA isolated from the transfectants. After confirmation of integration of the plasmid into the parasite genome, limited dilution cloning will be performed. The parasite cells will be

imaged using mitotracker to check for colocalization signal with the GFP. Phenotypic analysis of parasites will be done simultaneously.

Chapter 5: Generation of construct for knockout of malate quinone oxidoreductase

Chapter 5

Generation of construct for knockout of malate quinone oxidoreductase

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5.1. Abstract

Gene targeting in *Plasmodium* is efficient under situations where the homology arms in the target construct are greater than 1 kb. Owing to the AT richness of *Plasmodium* genes and further more in non-coding DNA segments, there is difficulty in cloning of *Plasmodium* DNA by conventional restriction-ligation methods. During cloning, assembling such large DNA segments in *E. coli* is often hard to achieve. As an alternative to restriction-ligation mediated cloning, recombineering is used, this facilitates exchange of genetic material in *E. coli* genomic DNA or plasmid by lambda phage encoded proteins. Genomic DNA library of *Plasmodium berghei* in a linear vector called pJAZZ-OK in *E. coli* TSA was procured from PlasmoGEM.. Malate quinone oxidoreductase of *P. berghei* that was cloned in the linear vector pJAZZ-OK at *Not*I sites in was *E. coli* replaced by a recombineering step with a bacterial marker to form the intermediate construct. This step is followed by replacement of bacterial selection marker by a *Plasmodium* selection marker to obtain the final knockout construct for transfection into *P. berghei*. An *in vitro* step using Gateway LR Clonase aids in replacing the bacterial selection marker with *Plasmodium* marker. In our attempts to generate the final construct of *P. berghei* MQO, we were successful in obtaining the intermediate construct. The final knockout construct could not be obtained using Gateway LR Clonase. When this project for generating knockout of MQO was initiated by recombineering in E. coli TSA and followed by an *in vitro* step using Clonase, the final knockout construct was not available in the PlasmoGEM repository. During the course of standardization of Clonase step to generate the final knockout construct of MQO, the required construct became available in the PlasmoGEM repository. Therefore, the final construct was obtained from

PlasmoGEM and verified by restriction mapping. The final knockout construct of *P. berghei* MQO is ready to be used for transfection.

5.2. Introduction

Recombineering is an *in vivo* genetic engineering technique that is enabled in *E. coli* cells that express recombinase protein upon induction. This system comprises of transient expression of recombinase complex and proof-reading. Recombineering is mediated by bacteriophage proteins either RecE/RecT from Rac prophage or Redαβγ from lambda bacteriophage. This technique of gene modification can be done with just 50bp of homology arm in either genomic DNA or episomal plasmids in *E. coli* cell. The proteins required for recombineering are encoded by lambda phage genes cloned in a vector under an inducible promoter (Zhang et al., 1998). Recombineering is an efficient system as it prevents the problematic step of restriction-ligation cloning.

In conventional protocols, the genome of *P. berghei* is modified using 0.8-1 kb long homologous arm. Greater length of homology arm is needed for higher recombination frequency (Pfander et al., 2011). Due to high AT richness of *Plasmodium* gene, cloning of large homology arms in bacterial vector by restriction-ligation is often difficult to achieve. As a result, recombineering has been used to generate the necessary plasmid constructs. A project group called PlasmoGEM (http://plasmogem.sanger.ac.uk/) generated genomic DNA library of *P. berghei* in a linear and low copy number plasmid called pJAZZ-OK in *E. coli* TSA cells. This genomic DNA library was used to generate appropriate construct for gene knockout of *P. berghei* by recombineering (Pfander et al., 2011).

E. coli TSA cells are used to obtain high yield and high quality plasmid. These cells have *end*A1 and *rec*A1 mutations which minimize non-specific recombination. These cells also have mutations in *mrr* and *mcr*, which helps in cloning methylated DNA isolated from other organisms. The cells contain *bla* gene inserted in genome that imparts resistance to ampicillin. The cells also contain genes from phage N15. The N15 genes are *tel*N, which encodes pro-telomerase for efficient replication of the linear plasmid,

pJAZZ-OK, the *sop*AB genes for stable inheritance, and the *ant*A gene to regulate copy number.

The pJAZZ –OK vector is N-15 phage derived that has covalently closed hair pin looped telomeres. The plasmid contains telomerase gene, kanamycin resistance gene, replication factor gene, origin of replication and multiple cloning sites. Large genomic DNA inserts of 30-40 kb long can be cloned in this vector. This plasmid can be easily maintained in *E. coli* TSA cells (Ravin and Ravin, 1999).

Large segments of DNA, as long as 10-20 kb of *Plasmodium* DNA, cloned in this linear vector can be modified by recombineering. This genomic DNA construct in linear vector is amenable to easy modification in *E. coli* TSA cells by encoded proteins of Redγβα operon of lambda phage and bacterial RecA protein under arabinose-inducible promoter in vector pSC101gbdA-tet. Nearly 30-50 bp of homology arms are sufficient for recombineering.

The desired gene in bacterial host cell is replaced by bacterial selection marker by recombineering. This recombination step is mediated by the encoded lambda red recombinase wherein, the marker that replaces the gene is flanked by 50 bp of homologous DNA segments. A general scheme for the replacement of the gene in *E .coli* mediated by recombineering is shown in Fig 5.1 (A).

A PCR amplification of the bacterial bicistronic marker, Zeo-PheS is done with 50 bp of DNA segment that shares identity with the 5' and 3' flanking region of the gene that needs to be knocked out. By PCR, the bacterial bicistronic marker and its flanking attachment sites *attR2* and *attR1* is amplified along with 50 bp of homology arms for recombination, referred to as recombineering PCR in Fig 5.2. *E. coli* TSA cells bearing the pJAZZ-OK parent vector are transformed with the amplified product containing the bacterial marker and attachment sites with homology arms. The cells are induced with arabinose for the expression of lambda phage genes for recombination. An *in vivo* recombineering step occurs wherein; the gene construct in pJAZZ-OK vector is replaced with bacterial bicistronic positive-negative selection marker that is flanked by Gateway *attR2* and *attR1* sites. The above step yields an intermediate construct with the bacterial

selection marker Zeo-PheS. This marker confers resistance against zeocin through the bleomycin resistance gene and sensitivity to 4-chloro- DL –phenylalanine through a mutant allele of phenylalanine t-RNA synthetase. Positive selection to select for the intermediate construct is performed on zeocin antibiotic containing plate. Negative selection is performed on plate containing 4-chloro- DL –phenylalanine to kill cells that have the intermediate construct. By recombineering, we generate the intermediate construct which serves as the template for next reaction to replace the bacterial marker with *Plasmodium* selection marker.

The next step involves the replacement of the bacterial selection marker with *Plasmodium* marker. The *in vitro* Gateway LR Clonase reaction exchanges the bacterial marker with *Plasmodium* marker by exploiting the attachment sites on vector containing *Plasmodium* marker referred to as entry clone and intermediate construct called as the destination vector (Landy, 1989). The vector that contains *attL* sites is the entry clone and the one that contains *attR* sites is the destination vector. (Fig 5.1(b)). The construct obtained after Gateway LR Clonase reaction is the pJAZZ-OK vector backbone containing the 5' and 3' flanking regions of the gene with the bacterial selection marker replaced by the *Plasmodium* marker.

The Gateway technology uses the lambda site-specific recombination system. Naturally, these sites allow the integration of lambda gene in the *E. coli* chromosome and the switch between lytic and lysogenic pathway. The attachment sites serves as the site for binding of recombination proteins. Attachment sites *attL* and *attR* sites undergo recombination with Integrase, IHF and Excisionase proteins to give *attB* and *attP* sites during lytic cycle of lambda phage. The three proteins are present in *in vitro* LR Clonase reaction. Lambda phage encoded Integrase enzyme and Integration Host Factor (IHF) are the proteins that facilitates recombination for lysogeny. These two proteins are present in *in vitro* BP Clonase reaction. Mutations in attachment sites have been introduced to eliminate secondary structures and stop codons and also make the *in vitro* Clonase reaction irreversible.

In our attempt to generate the final knockout construct with the *Plasmodium* marker, we used the attachment sites for exchange of our cassettes. The intermediate construct in pJAZZ-OK vector containing the bacterial bicistronic marker Zeo-PheS flanked by *attR2* and *attR1* sites served as the destination vector. A circular plasmid containing the *Plasmodium* bicistronic marker hDHFR-yFCU flanked by *attL2* and *attL1* sites was the entry clone. The two plasmids were set up in an *in vitro* reaction with Gateway LR Clonase at room temperature. A final construct in pJAZZ-OK vector with the *Plasmodium* marker hDHFR-yFCU flanked by attachment sites *attB2* and *attB1* is obtained. (Fig 5.2). The construct is released from the vector by *Not*I digestion and *Plasmodium* cells are transfected with this final construct. The marker hDHFR (human dihydrofolate reductase) confers resistance to pyrimethamine and WR99210. The yFCU (yeast cytosine deaminase and uracil phosphoribosyl transferase) marker can be used for negative selection, to excise the marker cassette from *Plasmodium* genome (Orr et al., 2012).

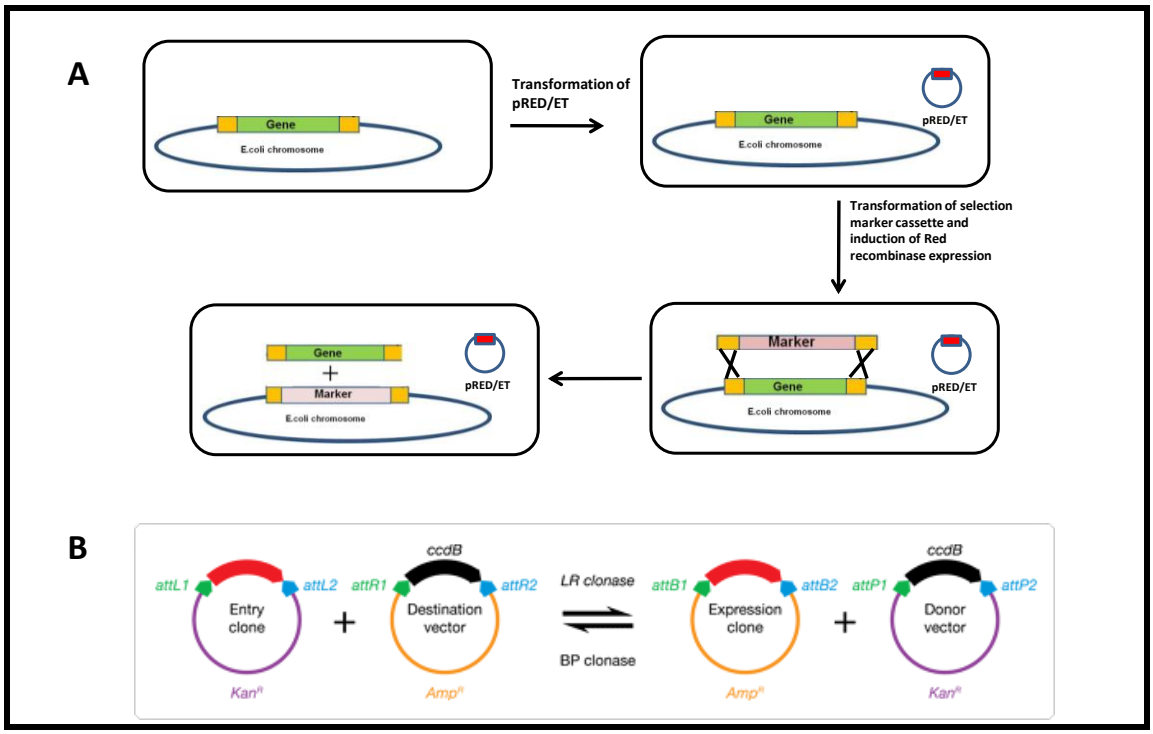

Fig 5.1: Scheme showing strategy for generating knockout of a gene *by* ***(A) Recombineering*** *(mediated by lambda red recombinase. The gene to be replaced is present episomally in pJAZZ-OK parent vector in E. coli host cell. The host cells were transformed with plasmid encoding lambda red recombinase which*

was under inducible arabinose pBAD promoter. The transformed cells carrying the plasmid coding lambda red recombinase is also transformed with bacterial selection marker flanked with 50 bp homologous arms for recombination in E. coli cell. Upon induction of expression of recombinase by arabinose, the gene is replaced by the bacterial selection marker. ***(B) Gateway LR Clonase.*** *This is in an vitro reaction, where, the bacterial selection marker is replaced by Plasmodium selectable marker by using the attachment (att) sites. The bacterial selection marker is flanked by attL2 and attL1 sites. The Plasmodium selection marker is flanked by attR2 and attR1 sites. On performing in vitro reaction with LR clonase, the Plasmodium marker replaces the bacterial marker.*

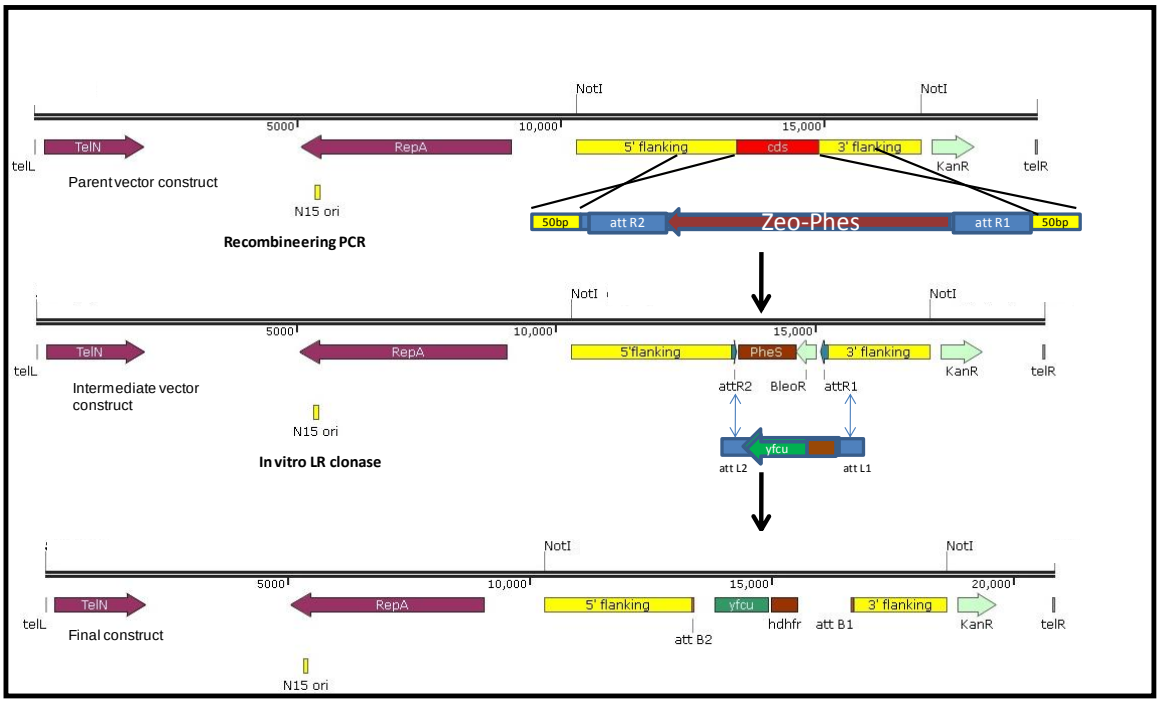

Fig 5.2. Scheme showing generation of final knockout construct of malate quinone oxidoreductase *Plasmodium berghei malate quinone oxidoreductase gene along with 1.2 kb of 5' flanking DNA segment and 6.5 kb of 3' flanking DNA segment was cloned in linear vector pJAZZ-OK using NotI sites. This parent vector was obtained from PlasmoGEM. Recombineering PCR amplification of bacterial selection cassette was done with 50 bp homologous DNA segments that served for double crossover for replacing the MQO gene with the bacterial marker cassette. The intermediate construct pJAZZ-OK Zeo-PheS consists of bacterial selection cassette with the flanking DNA segments of MQO. The bacterial selection cassette is flanked by lambda attachment sites attR2 and attR1. An in vitro Gateway LR clonase step is done to replace the bacterial marker with the Plasmodium selection marker hDHFR-yFCU flanked by attL2 and attL1 sites. The final knockout construct consists of Plasmodium marker with the flanking DNA segments of MQO. For transfection of Plasmodium berghei, the DNA fragment can be obtained by NotI digestion of final construct.*

5.3. Materials and methods

Restriction enzymes were obtained from New England Biolabs, USA. *E. coli* TSA electrocompetant cells were bought from Lucigen, U.K. and Gateway LR Clonase from Invitrogen, USA. Plasmid preparation kits were obtained from Qiagen, Netherlands. Gene Pulser Xcell and 1-mm electroporation cuvettes were from Bio-Rad, USA. *P. berghei* genomic DNA constructs in pJAZZ- OK linear vector were obtained in *E. coli* TSA cells from PlasmoGEM. The plasmids, pSC101gbdA-tet, pR6K attR1-zeo-PheS-attR2, pR6K attL1-hdhfr-yFCU-attL2 for recombineering and Gateway LR Clonase reactions were obtained from PlasmoGEM. Kanamycin (30 µg/mL) and tetracycline (5 µg/mL) were purchased from USB, USA. Zeocin (50 µg/mL) was purchased from Invitrogen, USA.

5.3.1. Verification of pJAZZ-OK MQO clone by restriction mapping and PCR amplification.

The pJAZZ-OK MQO clone was obtained from PlasmoGEM. The plasmid was isolated and digested with *Not*I and *Xho*I to verify the obtained clone. PCR amplification was also done to verify for the same using primers QCup and QCdown (appendix for sequence).

5.3.2. Recombineering

Electrocompetent cells of *E. coli* TSA cells having pJAZZ-OK MQO gene clone were prepared. The cells were electroporated with pSC101gbdA that carries the gene for red recombinase. The cells were recovered after electroporation by growing at 30 $^{\circ}$C for 70 minutes. Thereafter, the cells were plated on LB-agar containing kanamycin and tetracycline to select for transformants having recombinase plasmid and the parent pJAZZ- OK MQO vector.

For recombineering PCR, amplification of Zeo-PheS cassette with 50 bp homology arm to the flanking ends of MQO gene was performed using primers RecupR2 and RecdownR1 primers (appendix I for sequence). The amplified product was treated with *Dpn*I for 1 hour at 37 °C to remove the methylated plasmid template. The obtained

PCR amplified product was cleaned up using column filters and subsequently used for electroporation.

The amplified product was used for *in vivo* recombination that is mediated by recombinase, leading to replacement of MQO gene with Zeo-PheS marker. To achieve this, the TSA cells containing the parent pJAZZ-OK MQO vector and pSC101gbdA recombinase plasmid were grown at 30 °C until it reached 0.2 OD at 600 nm. Induction for expression of recombinase gene was done by adding 80 µl of 10 % arabinose and was grown at 37 °C for 35 minutes.

The induced cells were pelleted and washed with water and 10% glycerol and resuspended in 50 µl water containing 500 ng-1000 ng of the amplified product. This was electroporated at 1.8 kV, 10 µF, 600 Ω and cells were recovered by adding 3 mL of Terrific Broth medium. This was incubated at 37 °C for 70 minutes for recovery of electroporated cells and was plated on zeocin containing plates. In parallel, an overnight culture of the parent vector clone without recombinase plasmid was set up as a negative control.

Colonies were obtained on zeocin plate. The colonies were inoculated for screening by PCR amplification to check for the presence of modified intermediate construct. The primers used for screening were Qcup and PheSR2 (appendix I for sequence)

5.3.3. Gateway LR Clonase

The intermediate constructs for pJAZZ-MQO KO obtained from recombineering step served as a template for the next step, Gateway LR Clonase. The intermediate vector containing Zeo-PheS marker flanked by *attR2* and *attR1* sites as obtained from previous recombineering step and circular plasmid having hDHFR-yFCU flanked by *attL2* and *attL1* sites were isolated. These two vectors were used in the next step for *in vitro* LR Clonase step for exchange of Zeo-PheS marker with *Plasmodium* hDHFR-yFCU marker mediated by attachment sites *attR2-attR1* and *attL2-attL1*. The reaction mixture consisted of two vectors and Gateway LR Clonase mix. The reaction was set up at 25 °C for 12 hours.

After incubation, the reaction was terminated by the addition of Proteinase K provided in Gateway LR Clonase kit. The Proteinase K digestion of Gateway LR Clonase was carried out at 37 °C for 10 minutes. The reaction mix was dialyzed against water on Millipore 0.025 µm membrane discs for 10 minutes at 25 °C. The reaction mixture was then retrieved and was used for electroporation of *E. coli* TSA cells. Electroporation was carried out at 1.25 kV, 10 µF, 600 Ω. The electroporated cells were recovered and plated on medium containing 4-chloro-DL-phenylalanine. This medium aids in eliminating the cells containing the intermediate construct. The intermediate construct contains PheS mutant allele that encodes phenylalanine t-RNA synthetase. This uses 4-chloro- DL - phenylalanine, which stalls translation process in *E. coli* cell. Hence, by exchange of bacterial selection marker with *Plasmodium* marker that occurs in Gateway LR Clonase step we eliminate cells containing the bacterial selection marker by growing on YEG-Cl medium. As a consequence of negative selection, cells bearing the plasmid that would have lost the bacterial marker grow.

5.4. RESULTS AND DISCUSSION

5.4.1. Verification of pJAZZ-OK MQO parent vector by restriction digestion and PCR amplification.

The pJAZZ-OK MQO clone was found to be of right size as verified by restriction mapping with *Xho*I and *Not*I digestion (Fig 5.3(b)).

The clone was also verified by PCR amplification of the MQO gene in pJAZZ-OK vector. (Fig 5.3(c)).

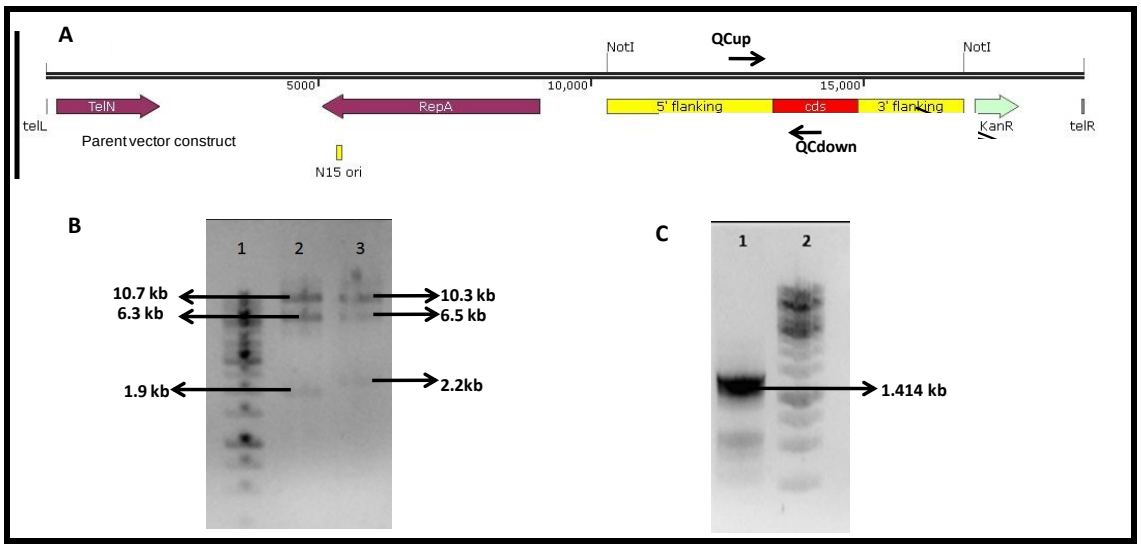

Fig 5.3. Verification of parent vector construct of MQO pJAZZ-OK-MQO : (A) *Scheme showing MQO gene with its flanking segments cloned in pJAZZ-OK vector using NotI sites.* ***(B)*** *0.8 % agarose gel showing DNA bands obtained on restriction digestion of parent vector MQO; Lane 1, 1 kb DNA ladder; Lane 2, XhoI digested pJAZZ-OK-MQO parent vector ; Lane 3, NotI digested pJAZZ-OK-MQO parent vector.* ***(C)****PCR amplification of parent vector construct of MQO using diagnostic primers QCup and QC down(panel A). The amplified product of 1.4 kb is of expected size.*

5.4.2. Recombineering

The bacterial selection marker cassette Zeo-PheS along with 50 bp homology arms of flanking segments of *P. berghei* MQO gene for recombination was amplified by PCR using primers RecupR2 and RecdownR1 (appendix I for primer sequence) (Fig. 5.4 A,C). The PCR product was cleaned up and used for transforming TSA cells carrying the parent vector pJAZZ-OK-MQO and pSC101gbdA recombinase plasmid were transformed with the PCR product. The cells were induced with arabinose for the expression of recombinase. Different amounts of the amplified DNA construct, 500 ng, 750 ng and 1000 ng, were used for electroporation of the cells. The electroporated cells were recovered and plated on zeocin containing TB-agar plates.

Colonies from the zeocin plates were screened for the presence of intermediate construct pJAZZ-OK-Zeo-PheS by PCR amplification. On performing PCR amplification, replacement of MQO by Zeo-PheS cassette in the pJAZZ-OK vector backbone was confirmed. The presence of intermediate construct pJAZZ-OK-Zeo-PheS was confirmed by PCR amplification (Fig 5.4 D). The intermediate construct pJAZZ-

OK-Zeo-PheS was successfully obtained in all used conditions; 500 ng, 750 ng and 1000 ng of used amplified product.

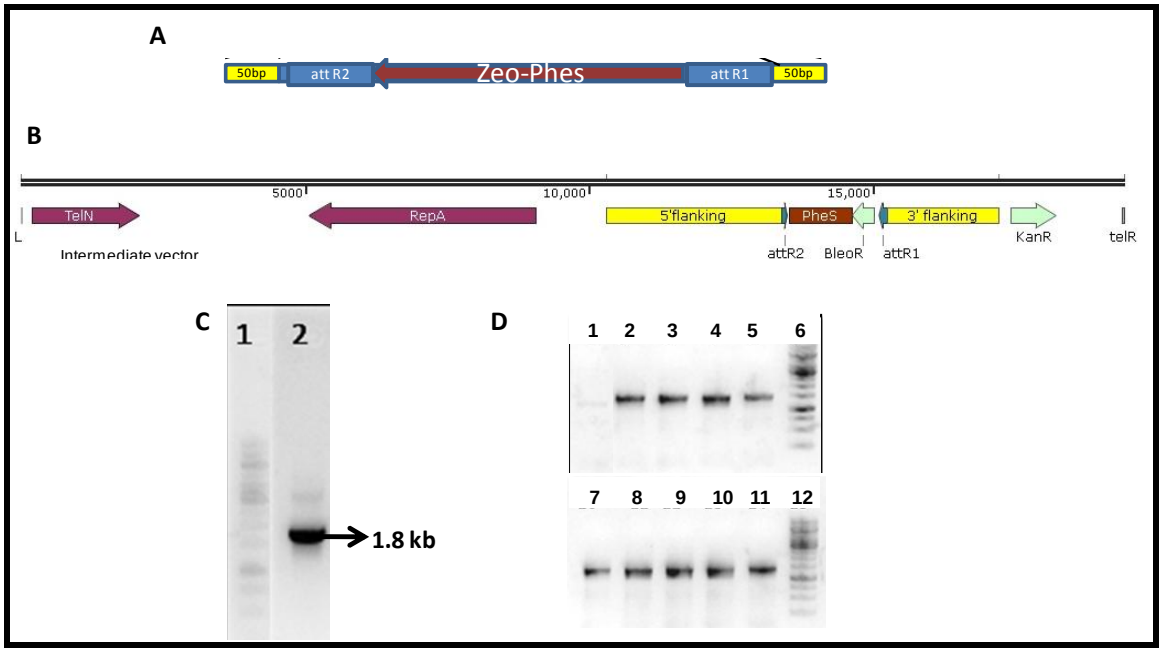

Fig 5.4. Generation of intermediate construct pJAZZ-OK-Zeo-PheS (A) *Scheme showing bacterial selection marker Zeo-PheS amplified product with 50 bp homology arms to flanking DNA segments of PbMQO gene;* ***(B)*** *Intermediate construct obtained by replacing MQO gene with the bacterial Zeo-PheS marker in pJAZZ-OK vector;* ***(C)*** *0.8 % agarose gel showing the amplified product for recombineering, Lane 1,1 kb DNA ladder; Lane 2, PCR amplicon with the amplified bacterial selection marker;* ***(D)*** *0.8 % agarose gel showing amplified product using templates as , Lane 1, parent vector pJAZZ-OK-MQO as template; Lanes 2-5 , Template obtained after recombineering performed using 500 ng of amplified Zeo-PheS cassette.; Lanes 7-10,Template obtained after recombineering performed using 750 ng of amplified Zeo-PheS cassette; Lane 11, Template obtained after recombineering performed using 1000 ng of amplified Zeo-PheS cassette; Lanes 6& 12, Marker.*

5.4.3. Gateway LR Clonase step:

The intermediate construct was isolated and used for setting up an *in vitro* Gateway LR Clonase reaction. Electroporation of cells with the reaction mix failed to yield colonies on YEG-Cl plate. The Gateway LR Clonase step tried was not successful in replacing the bacterial selection marker with the Plasmodial hDHFR-yFCU marker. This final step for generation of knockout construct of *P. berghei* MQO failed despite repeated attempts. From the examination of all the steps, we concluded that the Gateway

LR Clonase reaction had not worked due to loss of activity of the enzyme mix. During this period, the final knockout construct of MQO was deposited and available in PlasmoGEM repository. We procured the final construct from PlasmoGEM and verified. However, the standardization of all the steps described, enabled us to generate knockout constructs of other genes in our laboratory. Replacement of Gateway LR Clonase by the vendor enabled us to generate final constructs of other genes.

5.4.4. Verification of final knockout construct of MQO

The final knockout construct of MQO obtained from PlasmoGEM was verified by restriction digestion using enzymes, *Kpn*I and *Xba*I. The clone was confirmed to be of right size (Fig 5.5 (b)).

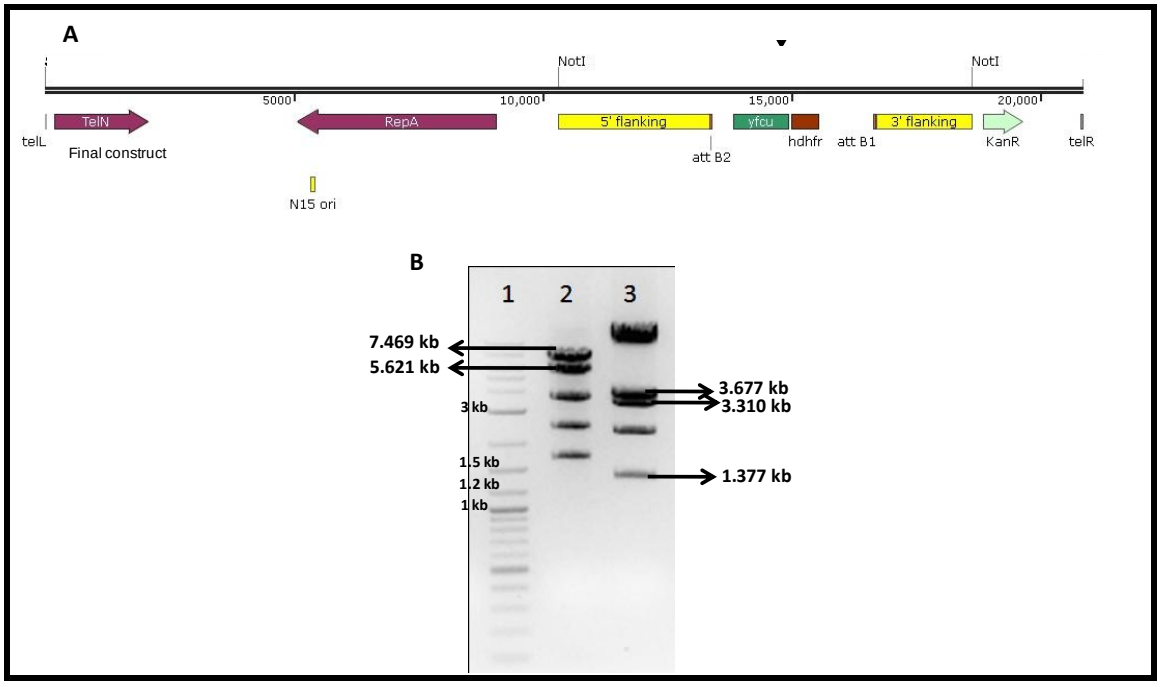

Fig 5.5. Verification of final knockout construct pJAZZ-OK-hDHFR-yFCU (A) *Scheme showing final knockout construct of MQO* ***(B)*** *0.8 % agarose gel showing digested products of final knockout construct of MQO. Lane 1,1 kb DNA ladder; Lane 2, KpnI digested final knockout construct of MQO pJAZZ-OK-hDHFR-yFCU; Lane 3-, XbaI digested final knockout construct of MQO pJAZZ-OK-hDHFR-yFCU.*

5.5. Conclusion

Recombineering step was successfully done to obtain intermediate construct. The presence of the intermediate construct was also verified by PCR amplification. The intermediate construct was attempted to be modified to generate the final knockout construct by *in vitro* Gateway LR Clonase reaction. However, during our failed attempts of obtaining the final knockout construct of MQO in pJAZZ-OK, the final construct was available from PlasmoGEM repository. The final construct has been procured and verified to be of the right size. Transfection of *P. berghei* cells with this final construct to generate a knockout of MQO is underway.

Summary thus far and future directions

Summary thus far and future directions

Aspartate aminotransferase

Aspartate aminotransferase of *Plasmodium falciparum* was found to be functional as demonstrated by Bulusu et al., 2011. This study showed the conversion of labeled fumarate to labeled aspartate by the action of mitochondrial malate quinone oxidoreductase and subsequently aspartate aminotransferase. Increased sensitivity of phosphoenolpyruvate carboxylase knockout strain of *P. falciparum* to L-cycloserine indicated the increased dependence on aspartate aminotransferase for oxaloacetate formation (Storm et al., 2014).

With the given background, we attempted to abrogate *Plasmodium berghei* aspartate aminotransferase. *P. berghei* serves as a model for easy manipulation of genes. The gene was successfully knocked out and replaced with hDHFR-yFCU cassette. Verification by PCR of uncloned population of AAT KO transfectants was performed to determine the presence of integration of marker cassette at the right locus. Proper integration of the marker at the 5' flank of homologous arm used for disruption of the gene was confirmed. However, presence of wild-type AAT gene was also seen in this uncloned population of AAT disruptants. Previous work reported presence of wild-type gene in uncloned gene disruptants which could be either due to spontaneous mutation of endogenous PbDHFR or wrong integration of marker cassette. In order to eliminate the possibility of occurrence of wild-type parasites due to spontaneous mutation of PbDHFR, the transfectants were selected against drug WR99210. Only hDHFR, used in our marker cassette, is resistant to WR99210. However, presence of wild-type AAT gene was still persistent even after WR99210 selection..

Presence of wild-type AAT gene in uncloned population of transfectants along with the disruptants could imply that there could be a population of wrong integrants. To validate this, southern hybridization will be carried out using Pb3'UTR of DHFR as

probe. The hDHFR-yFCU marker used for knockout has Pb3'UTR of DHFR in the cassette.

On validation of presence of right integrants in uncloned population of AAT gene disruptants, cloning by limiting dilution will be performed. This is done to obtain a clone of a single parasite cell lacking AAT gene and this will be done by injecting a single parasite in to a mouse. Genotyping will be performed before proceeding for phenotypic analysis of AAT KO transfectants.

Malate quinone oxidoreductase

Malate quinone oxidoreductase catalyzes the conversion of malate to oxaloacetate. This is membrane bound and FAD dependent enzyme. This enzyme has been characterized from bacteria, also revealing that it is bound to the membrane. The MQO transfers its electrons to ubiquinone and reduces it to ubiquinol , that is further oxidized by Complex III of the electron transport chain. Bioinformatic analyses have revealed that the *Plasmodium* MQO is originated from epsilon-proteobacteria (Nosenko and Bhattacharya, 2007) (Kabashima et al., 2013). However, there are no biochemical studies on PfMQO, due to inability of obtaining the protein in soluble fraction. The significance and essentiality of this enzyme to *Plasmodium* is also unknown.

Previous studies in our lab have shown the functionality of PfMQO in catalyzing the conversion of malate to oxaloacetate. Inhibition of Complex III by atovaquone depletes ubiquinone and consequently prevents the catalytic activity of PfMQO (Bulusu et al., 2011). A recent study has also reported the inability to generate a knockout of MQO in *P. falciparum* (Ke et al., 2015).

An attempt to investigate the localization and importance of MQO in *Plasmodium* has been initiated in this study. Indirect immunofluorescence using affinity purified anti-PfMQO antibodies did not indicate unambiguous localization to the mitochondrion and also did not agree with the initial predictions and studies. Another attempt done was GFP tagging of PfMQO. The PfMQO was successfully tagged with the GFP at C-terminal end, but the transfectants of *P. falciparum* with the plasmid could not be obtained.

Following transfection, the parasites did not survive in the presence of the drug WR99210.

The other approach for GFP tagging undertaken was the usage of regulatable fluorescent tag. This tag can serve three purposes; GFP tagging for localization, *E. coli* DHFR degradation domain for conditionak knockdown and HA tag for pull-down. The MQO- pGDB construct was used for these purposes. The 3' ORF of PfMQO gene was successfully tagged at the C-terminal end. Transfection of cells of *P. falciparum* was successfully done with the PfMQO tagged construct. Transfectants were obtained after 30 days and were subjected to drug cycling to enrich in cells that have the plasmid integrated in the genome. After the first round of drug cycling, the parasites reported GFP fluorescence, indicating that the plasmid had successfully integrated in the genome. However, confirmation of integration of the plasmid by genotyping needs to be performed.

Ensuing with the study further, the presence of integrants will be confirmed by genotyping. Fluorescence microscopy will be done to check for the localization of expressed GFP tagged PfMQO. To perform conditional knockdown study, the parasites will be grown in the presence of different concentrations of trimethoprim and checked for effect on its growth phenotype.

In conclusion, despite the low transfection efficiency and poor recombination efficiency of *P. falciparum*, we have successfully obtained integrants where, the endogenous MQO gene in the *P. falciparum* genome is tagged with GFP, *E. coli* DHFR degradation domain and 3X HA. The next step will involve genotypic characterization followed by phenotypic analyses and targeted metabolomics.

Phenotypic study of transgenic parasites

Successful gene targeting in *Plasmodium* will enable us to investigate the effect of growth phenotype of the transgenic parasites. Validation of the observed change in phenotype in parasites with disrupted gene is checked by complementing with *P. berghei* or *P. falciparum* gene.

Complementation assay in *P. berghei*

In order to validate the observed phenotype as a result of gene modification, complementation has to be performed (Goldberg et al., 2011). By complementation for the gene loss from *P. berghei* or *P. falciparum*, the transgenic parasites must restore the phenotype to wild-type condition. Sexual crosses can also be performed for complementation in transgenic *P. berghei* (Raine et al., 2007). Sometimes *in vitro* metabolite supplementation can be performed to rescue the observed phenotype of the transgenic parasites.

Invasion assay in *P. berghei* and *P. falciparum*

The ability to invade erythrocytes by the transgenic parasites AAT KO in *P. berghei* and MQO knockdown in *P. falciparum* will be monitored by flow-cytometry based assay using Vybrant-Green stained schizont staged parasites and DDAO-SE labeled erythrocytes (Pino et al., 2012). For checking the invasion of AAT KO *P. berghei* parasites, these parasites will be incubated at 37 ℃ and allow to mature to schizont stage *in vitro*. The parasites will be purified using Nycodenz gradient centrifugation. The parasite DNA will be stained using Vybrant Green. The target erythrocytes will be labeled with amine-reactive fluorescent dye DDAO-SE. The DNA stained parasites and the labeled erythrocytes will be mixed at 1:100. The schizonts will be ruptures mechanically by vigorous shaking. The released merozoites will be left to invade erythrocytes by incubating at 37 °C for 20 minutes. A negative control will be set up, where the schizonts will be treated with Cytochalasin D, which will inhibits actin polymerization and thereby inhibits invasion of erythrocytes. These samples will be fixed and analyzed by FACS. The *P. berghei* parasites will be labeled with Vybrant Green and will be allowed to invade mouse erythrocytes labelled with DDAO-SE. Additionally for AAT KO in *P. berghei*, the same assay will be done using Vybrant-Green stained schizont staged transgenic parasites and DDAO-SE labeled reticulocytes.

For MQO knockdown in *P. falciparum*, the transgenic parasites will be synchronized and allowed to mature to schizont stage. The parasites will be labeled with

Vybrant green and mixed with DDAO-SE labelled human erythrocytes. This will be incubated to check for invasion and will be analyzed by FACS (Theron et al., 2010).

Targeted metabolomics of genetically modified parasites

The metabolite pool from parasite lysates and the spent medium will be checked by using isotopomers. By the combination of genetic and metabolomic approaches, we will be able to decode the importance of different pathways in the cell.

Previous metabolomic studies have demonstrated and strengthened our knowledge on the parasites's dependence on different metabolic pathways. Parasites display metabolic plasticity wherein; these cells use different carbon sources to feed into the TCA cycle. There are three points in TCA cycle where malate, pyruvate and α-ketoglutarate serve as the entry points to drive the cycle.

Under PbAAT knockout condition, the parasites will be grown *in vitro* to schizont stage and incubated with metabolites for 6 hours. The spent medium and parasite cell lysates will be checked for metabolite levels by NMR or mass spectrometry. Metabolite labeling studies will be carried out using ^{13}C-U glucose, ^{13}C-U-glutamine and ^{13}C malate. By the usage of ^{13}C-U glucose, a buildup of 13-C enriched metabolites is expected in glycolytic and TCA cycle intermediates. Due to knockout of AAT, 13-C enriched oxaloacetate generated from ^{13}C-U glucose by glycolysis cannot be converted to 13-C enriched aspartate. The only source of aspartate for the parasite would be through haemoglobin degradation. Phenotypic changes and alterations in the level of metabolites will be checked by exogenous supply of aspartate. Similar levels of build of 13-C enriched metabolites are also expected from the usage of ^{13}C-U-glutamine and ^{13}C malate.

Under PfMQO knockout condition, $^{13-}$C –aspartate will be added to increase the supply of oxaloacetate pool in the cytosol through the action of AAT. However, studies conducted by Ke et al., 2015 suggest that oxaloacetate cannot be transported into the mitochondrion. Malate can be imported into the mitochondrion via malate/α-ketoglutarate antiporter. The oxaloacetate in the cytosol will be converted to malate by cytosolic malate dehydrogenase followed by the import of malate into the mitochondrion.

The flux from malate imported into mitochondrion needs to be followed as the TCA cycle cannot progress in forward direction. Similar studies will be conducted by using ^{13}-C-U glucose and ^{13}C-U-glutamine. An advantage of using this knockdown of PfMQO is that the levels of the enzyme in parasite cell can be regulated by using different concentrations of trimethoprim. The flux of TCA cycle will be studied in each case.

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PRIMER	SEQUENCE 5’ to 3’
QC up	GGATAGCTATAGTTCCCTTCTTTTATTATATCTCAATAATTTTTGC
QC down	GTAAAGCAGTACCTGTAACACCACCACC
PheSR2	TCATTCTTCGAAAACGATCTGCG
Primer 2- Hdhfr-yfcu	ACTTCTTAAACCTAATCTGTAGTAAGGAAGGGATTG
RECupR2	GAAAATTTTATATTTCAAAAAAAATACATGAATATATCAGCTTATTAAAAA+ CCGCCTACTGCGACTATAGA
RECdownR1	GATAATACAACTCTTTTTTTTCTGCAAAATTTTGAAATGTCATAACTTGG+ AAGGCGCATAACGATACCAC
Primer 1- *P. berghei* AAT 3’ flank	CTTGCTCCATATTCACGAATTCAAATATG
Primer 6- Pb AAT gene	CAAGGGTTTGGAAATGGAGAAATAGATAAC
Primer 4- PbAAT 5 flank forward primer	CAAAAGGCAACGATTATACATTATCGAAATTCAATTTC
Primer 5- Pb AAT chrome	CAACAGACTGTTCAGCAAAACTCAAAATATTG
Primer 4- hDHFR	GTGGATGAAAATATTACTGGTGCTTTGAGGGGTGAGC
MQO-pGLUX FP	ATACCGCTCGAGATGATATGTGTTAAAAATATTTTGAAAAGATATAAAAATAGTC
MQO-pGLUX RP	AGTCAGGGTACCGGCTAGCTTTTTTAAATTAGTAAATTTAGATAATAAAAAAAAC
MQO-pGDBFP	CGTAATCTCGAGATTGAAACCAATTATTCTTTTGAAAAAGC
MQO-pGDBRP	CGTGCTCCTAGGTAAATAATTAACGGGATATTCGCCTTC

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