Role of Amino Acid Transporter in the Survival and Transmission of Malaria Parasite

Dissertation submitted in partial fulfilment for the degree of

Master of Science in Biotechnology

Submitted By

Amrita pani

School of Biotechnology (Campus 11)
KIIT University
Bhubaneswar, Odisha, India

Under the Supervision of

DR.V.Arun Nagaraj
Scientist - D, Infectious dieases biology lab
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DECLARATIONS

I hereby declare that the dissertation entitled **"Role of Amino Acid Transporter in the Survival and Transmission of Malaria Parasite "** submitted by me, for the degree of Master of Science to KIIT University is a record of *bona fide* work carried by me under the supervision and guidance of **DR.V.Arun Nagaraj, Scientist-D, Institute of life science, bhubaneswar, odisha, india.**

Date:
Place:

Amrita Pani

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(An Autonomous Institute of the Department of Biotechnology, Govt. of India)

CERTIFICATE

This is to certify that the dissertation entitled "**Role of amino acid transporters in the survival and transmission of malaria parasite**" submitted by ***Amrita pani*** in partial fulfillment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No. *1662002* & Registration No.*16530150266* is a *bona fide* research work carried out by her under my guidance and supervision from 11th December 2017 to 11th May 2018.

12/05/2018

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This is also to certify that the above said work has not previously submitted for the award of any degree, diploma, fellowship in any Indian or foreign University.

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ABSTRACT

The intraerythrocytic malaria parasite derives much of its requirement for amino acids from the digestion of the hemoglobin of its host cell.it also takes up amino acids from the extracellular medium. It involves a numbers of transport pathways across the erythrocytic membrane. The movement of nutrients, metabolites and inorganic ions into and out of the intraerythrocytic parasite, as well as between subcellular compartments within the parasite, is mediated by transporters. These pathways contribute to the efflux or influx of important ions,nutrients and solutes that are essential to meet the metabolic demands of the parasite thus contributing in its survival. Genes encoding transporters and channels account for at least 2.5% of the parasite genome. In vitro studies have proved that the amino acid, purines and other metabolites required by the malarial parasite are translocated from the host cell and extracellular sources. However, the role of transporters that are involved in many of the uptake process still remain obscured. It is believed that the malaria parasite utilizes endogenous transporters and new permeation pathways to meet its demands. These transporters are also known to be involved in drug resistance by pvm(parasitophorous vacuolar membrane) and the fv (food vacuole). The main objective of my project was to study the significant of these transporter molecules in the sustainability of malarial parasite inside the host.

s

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DATE:

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ABBREVIATIONS

Pb- Plasmodium berghei

EtBr- Ethidium bromide

TBE- Tris boric acid EDTA

LB Broth- Luria bertani broth

NBT- Nova blue singles

RT- Room temperature

dNTPs- Deoxynucleosidetriphosphates

ddNTPs- Di-deoxynucleosidetriphosphates

PBS- Phosphate buffer saline

AAT- Amino acid transporter

RPMI- Roswell park memorial institute medium

CCMB- Competent cell modified buffer

Kb- Kilo base

DMSO- Dimethyl sulfoxide

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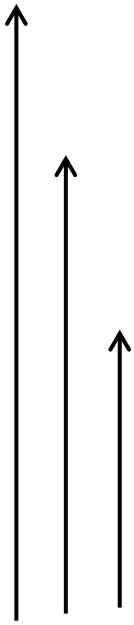
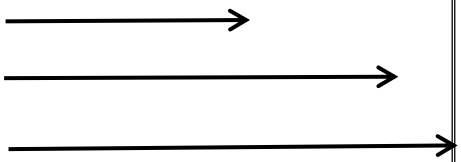
INTRODUCTION



CHAPTER - 1



Malaria has been showing a profound effect on human lives for thousands of years and remains one of the most serious, life-threatening infectious diseases. The disease is caused by protozoan pathogens of the Plasmodium sp.,*Plasmodium falciparum, Plasmodium vivax,plasmodium malariae,plasmodium ovale and plasmodium knowlesi* for which humans are the mammalian hosts,and *P.falciparum* are the most common species and are responsible for the largest public health burden. Malaria is transmitted by the bite of Plasmodium spp. infected female mosquitoes of the Anopheles genus. During a blood meal, infected mosquitoes inject sporozoites along with their anticoagulating saliva, which are the infective, motile stage of Plasmodium spp. Sporozoites travel through the skin to the lymphatics and into hepatocytes in the liver. Inside the hepatocyte, a single sporozoite can generate tens of thousands of merozoites (the stage that results from multiple asexual fissions (schizogony) of a sporozoite within the body of the host), which are released into the bloodstream where they enter red blood cells to replicate (erythrocytic schizogony). A fraction of merozoites release from RBCs also differentiate and mature into male and female gametocytes, which is the stage that infects the mosquito host when it takes a blood meal.

Five types of malaria parasite are known to infect a person:

Plasmodium falciparum **(P.f)**

The plasmodium parasite is recognised as the most lethal parasite that causes most infections and deaths related to malaria. This type of malaria is found in Africa, South America, and South East Asia. A Study held in 2002 showed that about 2.2 billion people in the world were at risk of getting affected by P.f. 25% of these events occurred in South East Asian region and almost 70% in Africa. The individual infected by this parasite experiences fatigue, dizziness, abdominal pain, aching muscles, enlarged spleen, seizures, sore back, joint pain, vomiting, nausea, fever, headache, anaemia and some neurological symptoms as well. Since it is the severest of all the four malaria types, it become important that this be checked, diagnosed and treated on time. This infection also has an adverse affect on brain and the central nervous system. Many times, changes in the levels of consciousness,paralysis and convulsions can also occur.

Plasmodium vivax **(P.v.)**

It has the widest distribution around the globe. Approximately 60% of infections in India are caused by P.v. Although it seldom causes death or other serious problems, it can still cause major illness. Some of the common symptoms of P.v are fatigue,

diarrhoea, bouts of fever and chills. Flu-like symptoms are commonly observed in the person affected by P*lasmodium vivax.*

***Plasmodium malariae* (P.m)**

This type of malaria is not as wide spread as the other types and is known to have less than 1 percent infections in the Indian subcontinent. The tropical and subtropical regions of South and Central America, South East Asia and Africa are witnessing its affects since long. Although it is not lethal, it still has varied distributions and is ranked third in prevalence. It manifests itself in the form of high fever and chills

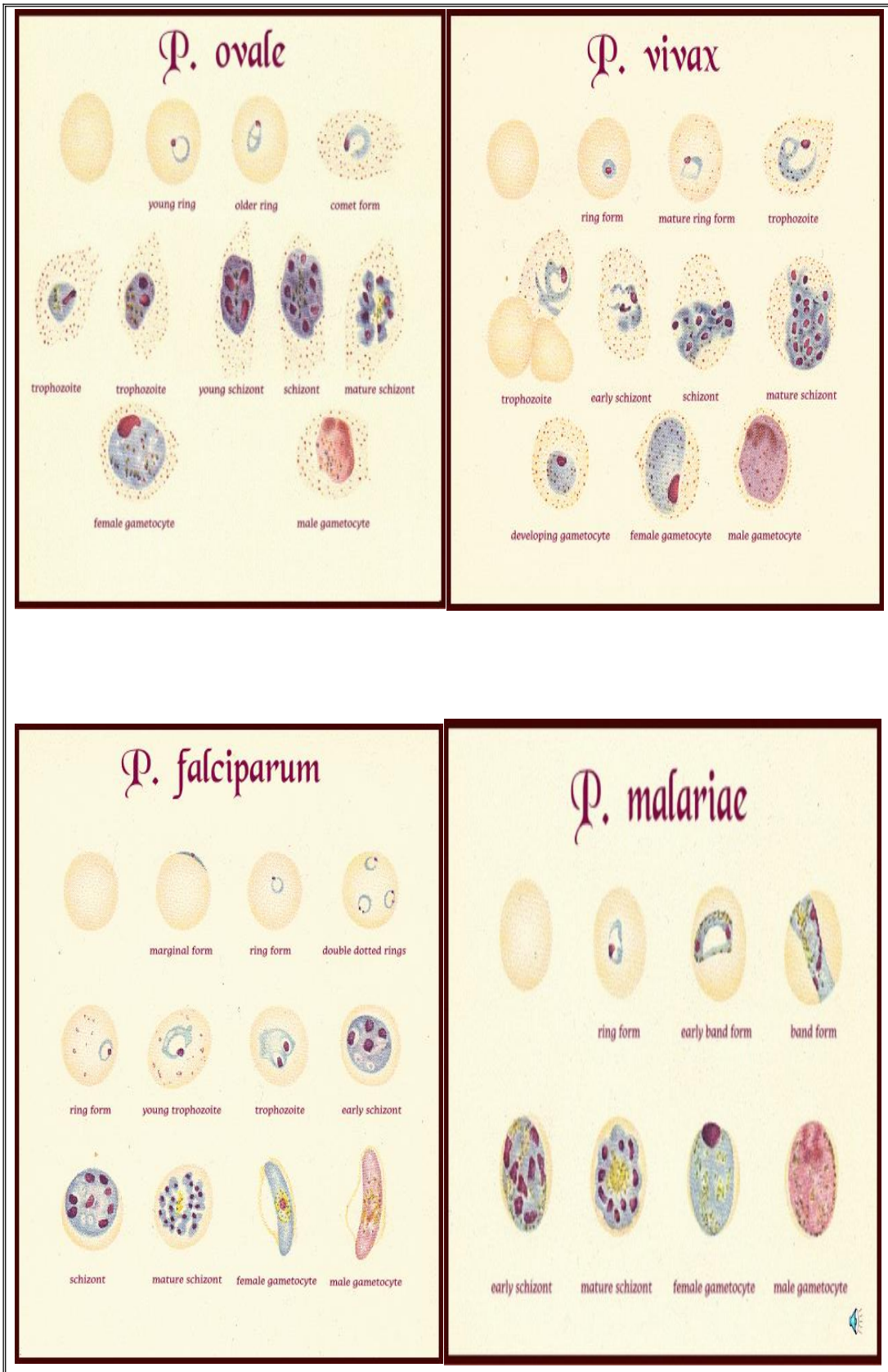
***Plasmodium ovale* (P.o)**

This is the rarest of all the malaria types and is mostly found in Ghana, Liberia, Nigeria and the tropical West African region. *Plasmodium ovale* reoccurs in some cases because the parasite can rest in the liver of a patient for a few months to 4 years after getting infected by the mosquito carrying malaria. These parasites are most likely to relapse and invade RBCs and making the patient sick again.

***Plasmodium knowlesi* (P.k)**

Plasmodium knowlesi was initially identified in 1930s as a natural Plasmodium of Macaca fascicularis monkey also capable of experimentally infecting humans also. P. knowlesi can cause severe malaria with a rate of 6–9% and with a case fatality rate of 3%. Respiratory distress, acute renal failure, shock and hyperbilirubinemia are the most frequently observed complications of severe P. knowlesi malaria. Chloroquine is considered the treatment of choice for uncomplicated malaria caused by P. knowlesi.P. knowlesi uses the Duffy blood group antigen as a receptor to invade human erythrocyte.

In vitro model for human malaria - *Plasmodium berghei*. It was first isolated from blood of a thicket rat. The parasite is readily grow in laboratory mice and rats where it shows a preference for reticulocytes infection. The parasite may be transmitted in the laboratory by *Anopheles stephensi* mosquitoes. It has been widely used as an in vitro rodent parasite model for human malaria.



[FIG 1 -The above figure showing the asexual stages of malaria parasite and gametocytes]

1.1Background and Context:

Malaria is an ancient disease which is caused by infection of parasites belonging to genus *Plasmodium* and it is transmitted by female *Anopheles mosquito* species. In 1880 Alphonse Laveranthe discovered the parasites in the blood of malaria patients. William MacCallum discovered the sexual stages in the blood in birds infected with a related haematozoan. In 1897 Ronald Ross elucidated that the whole of the transmission cycle in culicine mosquitoes and birds are infected with *Plasmodium relictum*. In 1898, Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava demonstrated that human malaria was transmitted by mosquitoes. In 1948 Henry Shortt and Cyril Garnham discovered that malaria parasites mature in liver before entering into the blood stream. In 1982 Wojciech Krotosk discovered the final stage in the life cycle of the mosquito and also the presence of dormant stages in the liver.

Hippocrates was the the first malariologist, who was described the various malaria fevers in humans. The Hippocratic corpus was the first document in which it was mentioned that splenic change in malaria is attributed to ingestion of stagnant water. In the first century Roman scholar Marcus Terentius Varro suggested that swamps breed certain animalcula which cannot be seen by the eyes and breathe through the nose and mouth into the body, where they are cause grave maladies. In 1716, Lancisi was first described black pigmentation of the brain and spleen in malaria. Lancisi linked malaria with the poisonous vapours of swamps or stagnant water on the ground. Lancisi postulated two ways in which malaria might be spread by mosquitoes. In the first one, the insects are deposit microscopic organisms in uncovered food and drink. And the human consumption of this contaminated food and drink produces the disease. The second one mechanism was the correct one for malaria mosquitoes. He writes always inject the mosquitoes salivary juices into the small wounds which are opened by the insects on their body surface. In 1816, Giovanni Rasori while suffering from malarial fever in prison, doubted on the “bad air” theory and suggested that a microorganism is responsible for the disease. Ronald Ross was discovered the malarial parasites under the microscope. In 1897 Ross was observe the true fate of the flagella. Within a blood smear he was saw two parasites near to each other. On 1898, Ross discovered that Malaria was transmitted to the birds from the mosquito’s saliva during the time of biting. In 1911, Brown discovered melanin from the malarial pigment by deducing the hematin origin of the latter and said that the black colour malarial pigment could be the pure hematin.

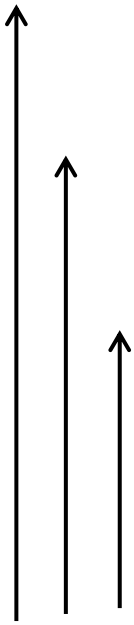
1.2.Scope and Objective:

Transporter represent transmembrane pumps ,transporter, escort protein,acid transport protein,cation transport protein, anion transport protein etc. Which is help in the exchange of nutrients and essential molecules which plays an important role in growth of living organisms. Membrane transport proteins are move hydrophilic substrates across hydrophobic membranes and also play a vital roles in most of the cellular functions in the organisms. Transporters also represents a diverse group of proteins that differ in topology,substrate specificity and also energy coupling mechanism. Transport proteins are also classified into channel or pore proteins,electron carriers,active transporters,electrochemical transporters, group translocators. Transport proteins are involved in the transportation of anions,amino acids,water, hormones, sugars, cations, mRNAs, electrons,proteins. Transporters can also transport various substrates.Multiple transporters are may be associated with the transport of a particular substrate across the cell membranes. The importance of membrane transport proteins to cells is illustrated by the fact that transporters typically make upto 5–15% of the total gene content of sequenced organisms. Transporters bring essential nutrients into the cell, and also determine the environments in which cell growth is possible. Seven classes of transporters are exclusive to a particular substrate, i.e.,protein/mRNA transporters, amino acid transporters/oligopeptides, cation transporters , sugar transporters, electron transporters, anion transporters and other transporters.

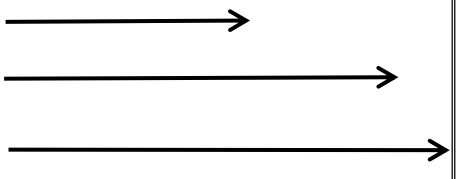
1.3.Overview of Dissertation:

In this study isolation of parasite from blood and parasite genomic DNA preparation and its cloning was performed. Plasmid isolation and digestion and release check for confirmaion gene for transfection. The amino acid transporter genes are knockout through double homologous recombination with hdfr gene to know the effect of amino acid transporter in parasite growth. In vitro study of malarial parasite to know the asexual stages of its after the knockout of the amino acid transporter gene. Effect of knockout gene are shown on their asexual stages of life cycle on the basis of in vitro study.

REVIEW OF LITERATURE



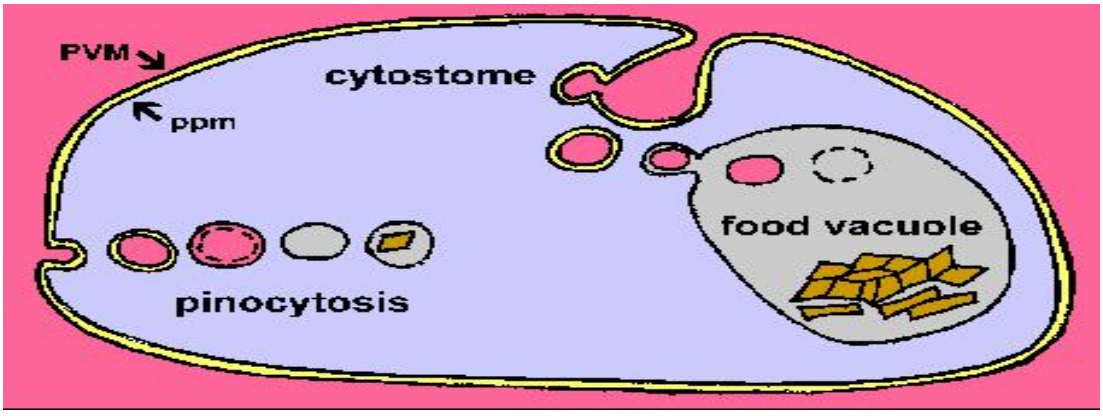
CHAPTER - 2



2.1.Hemoglobin degradation:-

The malaria parasite requires amino acids for the synthesis of its proteins. Malaria parasite lacks biosynthesis pathway for amino acid in asexual stages(1). The two main sources of amino acids are: Import from host plasma, and digestion of host hemoglobin. Hemoglobin is an extremely abundant protein in the erythrocyte cytoplasm and serves as the major source of amino acids for the parasite. Hemoglobin is broken down into peptides and amino acids within a vacuolar compartment known as the food vacuole(2).The parasite digests up to 65% of the total host hemoglobin into amino acids. However, most of these amino acids are effluxed from the infected erythrocyte and only 16% of the digested hemoglobin is incorporated into parasite proteins.

During the early ring stage, the parasite takes up the host cell stroma by pinocytosis resulting in double membrane vesicles(2). The inner membrane, which corresponds to the PVM, rapidly disappears and the digestion of hemoglobin takes place within the small vesicles during the early trophozoite stage. As the parasite matures, it develops a special organelle, called the cytostome, for the uptake of host cytoplasm and the small pigment-containing vesicles fuse to form a large food vacuole(1). Double-membrane vesicles pinch off from the base of the cytostome and fuse with the food vacuole. The inner membrane is lysed and the hemoglobin is released into the food vacuole.

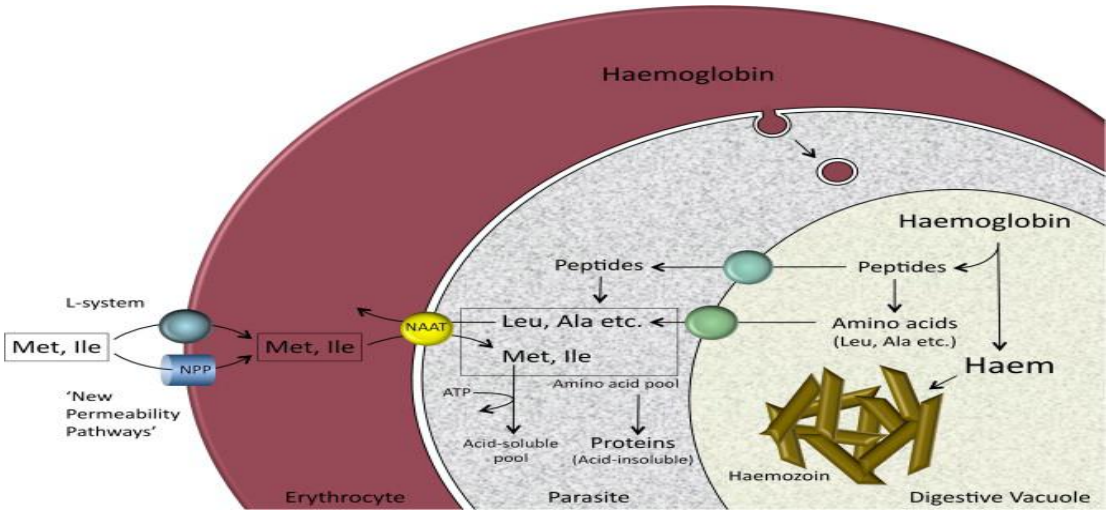


(ppm-parasite plasma membrane,pvm-parasite vacuole mambrane)

[FIG2-Ingestion of Host RBC Cytoplasm By malaria parasite]

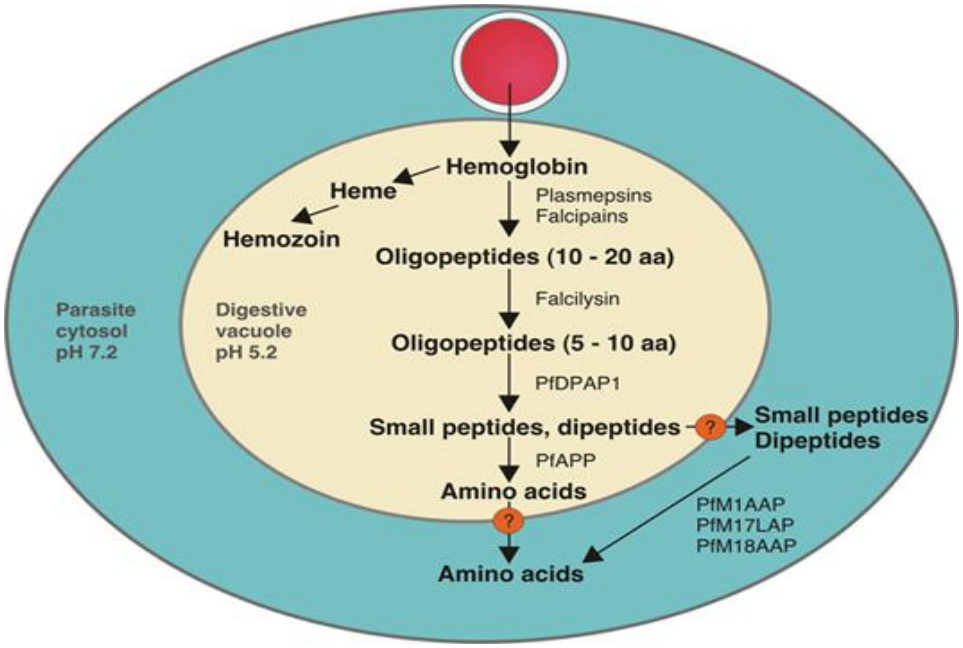
The food vacuole is an acidic compartment (pH 5.0-5.4) that contains protease activities(4). In this regard the food vacuole resembles a lysosome,

except that other acid hydrolases (eg., glycosidases and nucleases) have not been identified(4). Presumably other acid hydrolases are not needed since the micro environment of the erythrocyte is almost exclusively at one protein, hemoglobin. The acidic pH of the food vacuole is maintained by a H^+-translocating ATPase and a H^+-translocating pyrophosphatase, both of which are homologous to V-type transporters found in plants(5). Thus the food vacuole is probably homologous to the tonoplast found in plants and other protozoa. Several distinct protease activities, representing three of the four major classes of proteases, have been identified in the food vacuole(6). Multiple plasmepsins and falcipains have been identified. The digestion of hemoglobin probably occurs by a semi-ordered process involving the sequential action of different proteases. Several plasmespsin genes have been identified in the genome of P. falciparum and four of these apprear to function in the food vacuole(6). Plasmepsin-1 and plasmepsin-2 are the best characterized and both are capable of cleaving undenatured hemoglobin between phenylalanine and leucine residues located at positions 33 and 34 on the alpha-globin chains. These residues are located in a conserved domain known as the hinge region, which is believed to be crucial in stabilizing the overall structure of hemoglobin. Cleavage at this site presumably causes the globin subunits to dissociate and partially unfold(7). This unfolding will expose additional protease sites within the globin polypeptide chains. The other plasmepsins, as well plasmepsin-1 and plasmepsin-2, and the falcipains are then able to further degrade these large globin fragments. It has been suggested that falcipain-2, and possibly falcipain-3, are capable of digesting either native hemoglobin and therefore may also participate in the initial cleavage of hemoglobin.



[FIG3- The overall view of amino acid transport in malaria parasite]

Falcilysin cannot digest either native hemoglobin or denatured globin, but readily cleaves the small polypeptide fragments (up to 20 amino acids) generated by the action of falcipain and plasmepsin(8). The site specifity of falcilysin complements the plasmepsins and falcipains and leads to the formation of peptides 6-8 amino acids in length. Therefore, the digestion of hemoglobin is a semi-ordered process involving the initial degradation to large fragments followed by subsequent degradation to small peptides(9). The proposed pathway of hemoglobin digestion involves an initial cleavage by plasmepsin-1 (and possibly falcipain-2) followed by the combined actions of several plasmepsins and falcipains(10). The peptide fragments produced by these digestions are then digested into smaller peptides by falcilysin.



[FIG4- Enzyme involved in the digestion of Hemoglobin]

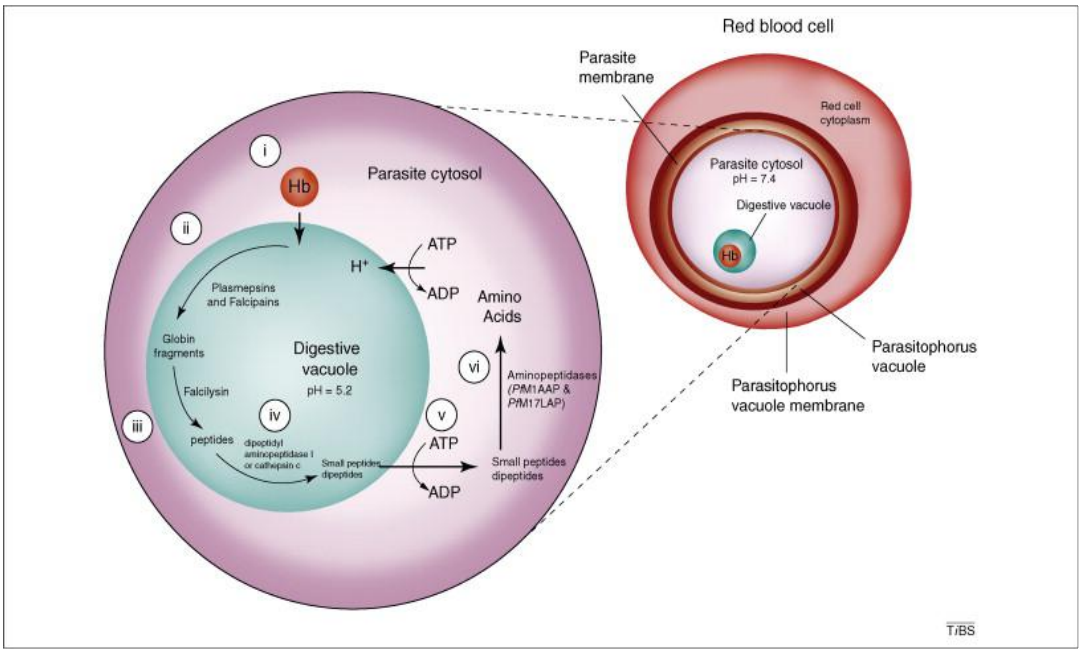
2.2.Detoxification of heme:-

Hemoglobin digestion releases heme. The ability to destabilize and lyse of the membrane causes free heme to be toxic to the cells. Heme can also inhibit the activity of several enzymes(11). The possible mechanisms by which heme is detoxified have been identified in malaria parasite.

- Sequestration of the free heme into hemozoin, or the malarial pigment.
- A degradation facilitated by hydrogen peroxide within the food vacuole.

- A glutathione-dependent degradation which occurs in the parasite's cytoplasm.
- And possibly a heme oxygenase which has been found in *P. berghei* (rodent parasite) and *P. knowlesi* (simian parasite), but not in *P. falciparum*.

The degradative pathways and the hemozoin formation pathway function simultaneously with 25-50% of the free heme converted into hemozoin and the remaining being degraded(12). Some studies suggest that in hemozoin up to 95% of the free iron released during hemoglobin digestion. Hemozoin has the same structure as β-hematin which was confirm by the analysis of X-ray crystallography and spectroscopic(13). β-hematin is a heme dimer which is formed by the the iron atoms of two heme molecules and reciprocal covalent bonds between carboxylic acid groups on the protoporphyrin-IX ring. These dimers are formed crystals of hemozoin, interact through with hydrogen bonds(14). Therefore, pigment formation was also known as a biocrystallization process.

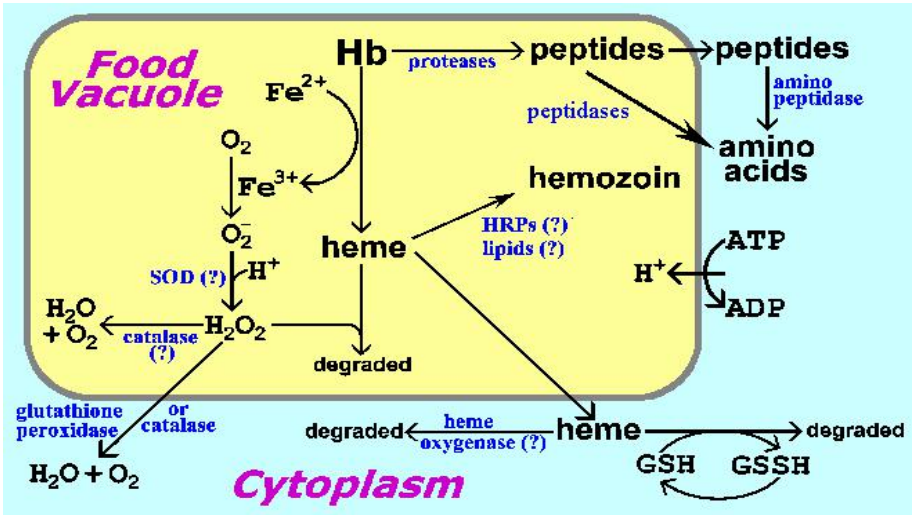


[FIG5-Overview of hemoglobin digestion in the malaria parasite]

The mechanism of hemazoin formation is not completely known. The heme detoxification protein (HDP) binds to the two molecules of heme with high affinity and promote the formation of the β-hematin dimer(15). The dimer is then released to initiate the crystallization process.The lipids may also participate in the process and lipid bodies have been observed within the food vacuole. Hemozoin is also found to

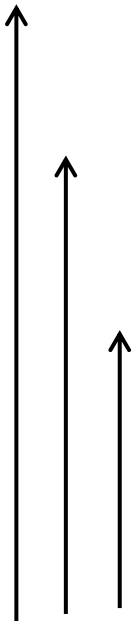
be associated with lipids(16). Some portion of the free heme may be degraded into non-toxic metabolites. Three potential processes have been described: in the food vacuole a hydrogen peroxide mediated oxidation of the porphyrin ring leads to its opening and subsequent breakdown; some of the heme translocates across the food vacuole membrane into the host cytoplasm where it is oxidized by reduced glutathione (GSH); and a heme oxygenase activity has been identified in some non-human malaria parasites(17). However, the extent of role of these processes play in the degradation of heme is not known.

The iron bound to hemoglobin is primarily in the ferrous state (Fe^{2+}). Release of the heme results in iron being oxidized to the ferric state (Fe^{3+})(18). Electrons liberated by this oxidation of iron promote the formation of reactive oxygen intermediates(ROI) such as superoxide anion radicals and hydrogen peroxide. ROI can cause cellular damage. Superoxide dismutase (SOD) and catalase are cellular enzymes that function to prevent oxidative stress by detoxifying the superoxide and hydrogen peroxide, respectively(19). Both of these activities are found in the food vacuole and may have been obtained from the host during ingestion of the erythrocyte cytoplasm. Hydrogen peroxide can also be exported into the parasite cytoplasm where it is detoxified by catalase and glutathione peroxidase(20). Some of the hydrogen peroxide produced as a result of the Fe^{2+} to Fe^{3+}conversion may also used for the peroxidative degradation of heme.

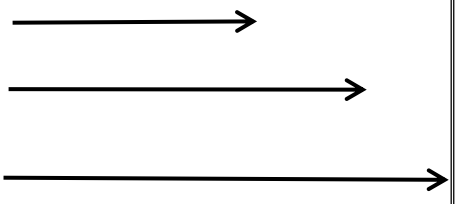


[FIG6-Summery of metabolic events occuring in food vacuole]

AIMS AND OBJECTIVE



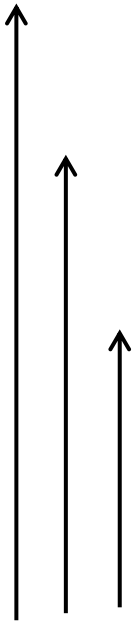
CHAPTER - 3



This study aimed at role of amino acid transporter in survival and transmission of malarial parasite. Also we wanted to check effect of knockout gene on malaria parasite growth and transmission. The major objective that were fulfilled to achieved the aim of study are as follows:

- ✧ Isolation of parasite and cloning of its genomic DNA.
- ✧ Double homologous recombination occur for knockout of amino acid transporter genes.
- ✧ In vitro study of asexual stages of malaria parasite.
- ✧ Survival growth of knockout parasite.

MATERIALS AND METHODS



CHAPTER - 4

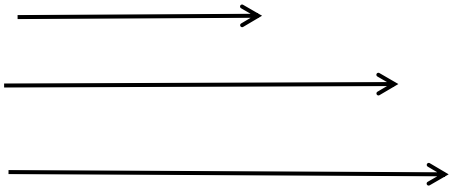


Table1: list of chemicals used

Chemicals	Company
Saponin	SIGMA
RNA extraction kit	Qiagen
Gel elution kit	SIGMA
Pb AAT2,3,4 Reverse primer	SIGMA
Pb AAT2,3,4 Forward primer	SIGMA
Random primer	Thermoscientific
RT buffer	Thermoscientific
DNTPs	NEB
RT enzyme	Thermoscientific
GC buffer	NEB
$MgCl_2$	NEB
DMSO	NEB
Phusion polymerase	Thermoscientific
5'UTR forward primer	SIGMA
5'UTR revrse primer	SIGMA
3'UTR forward primer	SIGMA
3'UTR reverse primer	SIGMA
Agarose	SIGMA
EtBr	Himedia
DNA loading dye	Thermoscientific
Ethanol	CSC
Qiagen gel purification kit	Qi quick
Cut smart buffer	NEB
ApaI	NEB
BglII	NEB
Pl0006 vector	NEB
Ligase buffer ligase	NEB
Tetracycline	SRL
Tryptone	HIMEDIA
NaCl	HIMEDIA

Yeast extract	HIMEDIA
Agar	HIMEDIA
Ampicillin	SRL
10X Taq buffer	NEB
Taq polymerase	Thermoscientific
Plasmid isolation kit	Qiagen
NaAc	NEB
NotIF	NEB
Heparin	Neon
RPMI culture medium	Tharmo
Nycodenz buffer	Himedia
Nucleofection solution	lonza
Supplement	lonza
Ketamine/xylazine solution	Neon
Supplement buffer	lonza
Trizol	SIGMA
chloroform	MERCK
Isopropanol	Merck
Platinum buffer	Thermoscientific
Platinum enzyme	Thermoscientific
1kb DNA Ladder	NEB
Geimsa stain	SIGMA
Emersion oil	Sigma
Acetone	Himedia
Pyrimetamine	Sigma

4.1.Instrument:-

Table2: list of instrument used

Instrument	Company
Centrifuge	Thermofisher
PCR	Proflex
Nanodrop	Thermofisher
Incubator	Labnet scientific
Nucleofector	Lonza
Gel electroporasis apparatus	Bio-bee
Hemocytometer	supeorior
UV Illuminator	Cleaver scientific
Microscope	Laben
Inverted flourosence Microscope	Olympus

4.1.Parasite isolation:-

2.5ml of whole blood was collected from mice in a 15 ml falcon tube. It was centrifuged at 2000rpm,5minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 6-7ml of 1X PBS. Again the solution was centrifuged at 2000rpm,5 minutes at RT. The suparnatant was again discarded and the pellet was retained. The centrifugation step was repeated 3-4 times in order to remove the RBC's,WBC's and get the parasite only. 1ml of 0.15% saponin was added to the pellet and vortex edit properlythe falcon tube containing pellet was incubated at 37^0C for 20 minutes with continuous tapping at the regular interval of 5 minutes. The falcon tube was immediately kept in ice after taking out of the incubator. All the procedures are done in ice from here onwards. 3.5ml of 1XPBS was added to the falcon tube and resuspended properly. 1ml each was dispensed to 5-6 eppendorf tubes. The eppendorf tubes were then centrifuged at 10,000g for 10 minutes at 4^0C. The supernatant was discarded and the pellet were resuspended in 1ml of 1XPBS. They were centrifuged at 3000g for 10 minutes at 4^0C. The supernatant was discarded and the pellet was again resuspended into 1ml of 1XPBS. A neat parasite pellet was obtained after washing 3-4 times with 1XPBS. The pellet was stored at -20^0C for further used.

4.2.ISOLATION OF RNA:

- **QIAGEN RNA EXTRACTION KIT METHOD:**

The parasite pellet was taken and resuspended by adding 350ul of RLT buffer(RLT buffer containing 3.5µl of B.mercaptoethanol). It was vortex and centrifuged for 30 second. The the lysate was transferred to gDNA eliminator mini column and the column was placed into a 2ml collection tube. Centrifuge it at 12000rpm for 30 second. Through the gDNA eliminator mini column and add 350µl of 70% ethanol and resuspended it for 7-8 times. 700µl of sample was added to the RNeasy spin mini column and the column was placed into a 2ml collection tube. Keep it 3min at room temperature then centrifuge it at 12000rpm for 30sec. The flow through was discarded and the mini column was placed back into a new collection tube then add 700ul of RW1 buffer for wash purpose.centrifuge it at 12000 rpm for 30sec. The flow through was discarded and the mini column was placed back into a new collection tube then add 500µl of RPE buffer centrifuge it at 12000rpm for 30 sec. Repeat the step with discarded the flow through and added 500µl of RPE buffer and centrifuge at 12000rpm for 2minute. A dummy spin was given at 12000rpm for 1minute in order to

remove the residual ethanol. The flow through was again discarded and the mini column was placed into a new collection tube. 35µl of autoclaved miliQ water was added to the mini column and was incubated for 5minute at room temprature. It was centrifuged at 12000rpmfor 1minute. Discard the mini column. The celuate was our RNA. It was stored at -20^0 C.

4.3.Primer preparation for cDNA synthesis:

- The primer concentration was 17pmol/µl.we have to prepare the stock for 0.17nmol/µl.
- PbAAT1 forward primer-37.2nmoles and PbAAT1 reverse primer-26.7nmoles.
- PbAAT2 forward primer-48.4nmoles and PbAAT2 reverse primer-41.9nmoles.
- PbAAT3 forward primer-35.8nmoles and PbAAT3 reverse primer-22.0nmoles.
- PBAAT4 forward primer-46.8nmoles and PbAAT4 reverse primer-46.9nmoles.
- To make 1:10 dilution add 10ul of primer and 90ul of water.

4.4.CDNA synthesis:

Take 3 PCR tubes and add 15 µl of autoclaved miliQ water to each of the tube. 2.5µl of Pb RNA was added to each of the tube. Then add reverse primer and random primer to the PCR tubes.

Table3: CDNA synthesis from RNA

Components	Pb RNA(µl)	Reverse primer(µl)	Random primer(µl)	Autoclaved MQ(µl)
PbAAT1	2.5	1	-	15
PbAAT2	2.5	1	-	15
PbAAT3	2.5	1	-	15
PbAAT4	2.5	1	-	15
Random primer	2	-	1	15

After added this to all the tubes go for 70RT PCR for 10 minutes. After PCR snapchill the tubes for 1minute.

Table4: Cocktail prepare for 25µl of reaction.

materials	5.5 reactions(µl)	Per reaction(µl)
RT buffer	27.5	5
DNTPS	5.5	1
RT enzyme	2.75	0.5
Total	35.75	6.5

Enzyme was added after adding all the product then go for 42RT PCR for 1hours. After PCR the tubes were stored at -20^{0}c.

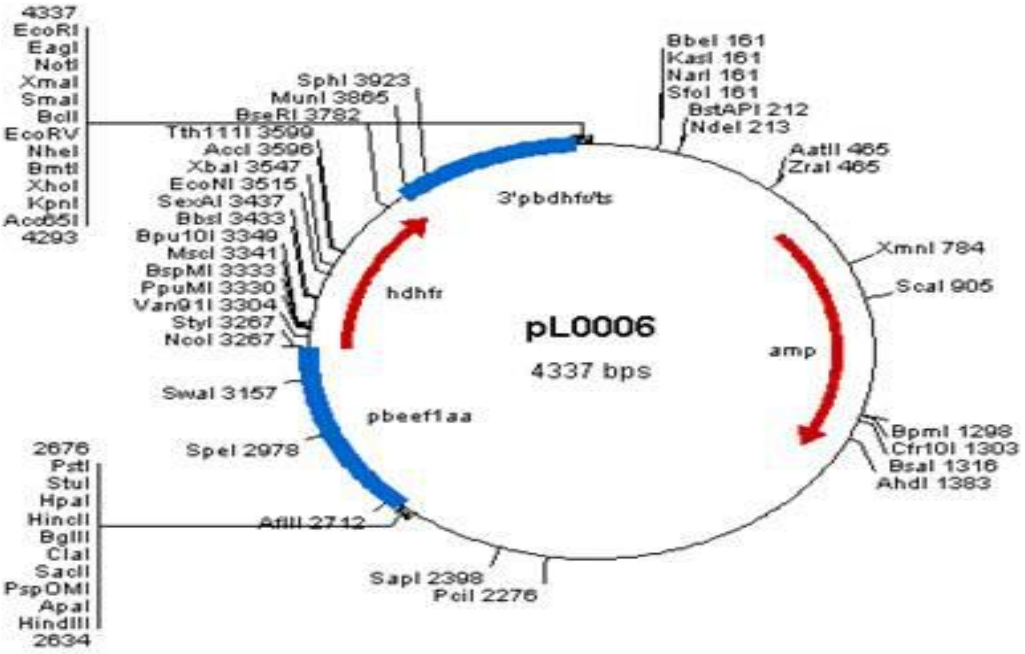
4.5.NANODROF QUANTITATION:-

Turn on the machine.select your sample(DNA/RNA/PROTEIN). Clean the instrument by adding 2-3 drops of autoclaved MiliQ water to its lower and upper portion. Repeat this for 2-3 times.Wipe the water with a tissue paper and add 1ul of autoclaved miliq in the nanodrop base. Close the lid.select blank.to confirm the blank again lift up the lid and put it down. Clean the nanodrop base and add 1ul of sample DNA to it. Close the lid and press measure. The value of the sample then display on the screen. After using the instrument clean the bases with the help of autoclaved miliq water and tissue paper. Thus the quantify of DNA was found to be

- **Cloning of human dhfr cassette:-**

- Plasmid used as vector:-pL0006. It contain human dihydrofolate reductase (Hdhfr) cassette that infers pyrimetamine resistance to the plasmid.

- **pL0006 vector map:-**



[Fig7:-pl0006 vector map]

- **Host for cloning:-**

Nova blue(NBT-E.coli). These cells have tetracycline as selectable marker. These host competent cells are isogenic with the IDE3 lysogene used for protein expression. The suitable vectors used are-PET,PRT Blue^{TM},pTRIEx^{TM}.

The major features are:-

- ✓ Chemically competent
- ✓ High efficiency
- ✓ Reproducible

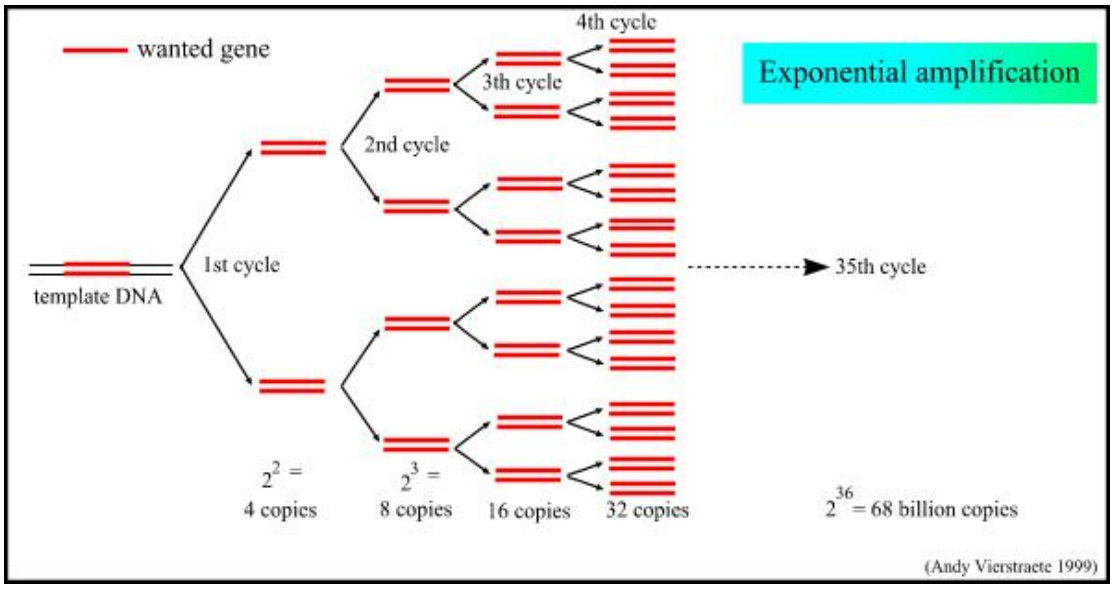
4 genes are selected for generation of knockout:-AAT1,AAT2,AAT3,AAT4

4.6.Polymerase chain reaction:-

- ❖ Principle:-

The main principle behind the PCR is the amplification of genomic DNA. Quantity of genomic DNA generated after a complete PCR cycle can be known from the formula 2^n where n=the no of cycles.there are three basics steps involves in:-

- ✧ Denaturation: The double stranded DNA is cleaved into single stranded DNA at a temperature ranging in between 94-96^{0}C.
- ✧ Annealing: Since the process of replication occur only in 5'-3',a stretch of 5-6 nucleotides complementary to the DNA sequence known as primers bind at the 3' position and start the process of replication.
- ✧ Extension: The DNA polymerase then start synthesizing the strand leading to the formation of milions copies of DNA.



[Fig8:- Polymerase chain reaction]

the forward and reverse primers were designed each for 5'UTR and 3'UTR for all the above mentioned gene.

4.6.1.Pcr standardisation of 3'UTR:-

All the components were kept out of -20^{0}C at room temperature except the enzyme before 15-20 minutes of performing the experiment. The components were immidietly transferred into ice one they got thawed. The following cocktail was prepared for 8 reaction.

Table5: Cocktail- 50ul reaction volume

reagents	Volume(µl)
GC buffer	85
$MgCl_2$	21.25
dNTPs	17
DMSO	12.75
Phusion polymerase	4.25
Autoclaved MiliQ Water	214.75
Template(genomic DNA)	2
Total	357

The enzyme was added after the addition of all other components. The cocktail was then equally divided(46µl) into 6 diiferent tubes. This was followed by the addition of individual 3'UTR forward and reverse primers to the tubes.

Table6: 3'UTR PCR reaction setup

genes	Cocktail(µl)	3'UTR forward primer(µl)	3'UTR reverse primer(µl)	Total(µl)
PbAAT1KO	46	2	2	50
PbAAT2KO	46	2	2	50
PbAAT3KO	46	2	2	50
PbAAT4KO	46	2	2	50

The tubes were then vortex and given a short centrifuge. The PCR reaction was setup in conditions suitable for Phusion Polymerase.

Denaturation: 98^{0}C/2minute

Final denaturation:98^{0}C/10second

Annealing:55^{0}C/30second

Extension:72^{0}C/1minute

Final extenstion:72^{0}C/10minute

Hold:4^{0}C,infinite hold

After pcr the tubes were stored at -20^{0}C.

4.6.2.PCR Standardisation of 5'UTR:-

All the components were kept out of -20^{0}C at room temperature except the enzyme before 15-20 minutes of performing the experiment. The components were immediatly transferred into ice one they got thawed. The following cocktail was prepared for 8 reaction.

Table7: Cocktail- 50ul reaction volume

reagents	Volume(µl)
GC buffer	85
$MgCl_2$	21.25
dNTPs	17
DMSO	12.75
Phusion polymerase	4.25
Autoclaved MiliQ Water	214.75
Template(genomic DNA)	2
Total	357

The enzyme was added after the addition of all other components. The cocktail was then equally divided(46µl) into 6 diiferent tubes. This was followed by the addition of individual 5'UTR forward and reverse primers to the tubes.

Table8: 5'UTR PCR reaction setup

genes	Cocktail(µl)	5'UTR forward primer(µl)	5'UTR reverse primer(µl)	Total(µl)
PbAAT1KO	46	2	2	50
PbAAT2KO	46	2	2	50
PbAAT3KO	46	2	2	50
PbAAT4KO	46	2	2	50

The tubes were then vortex and given a short centrifuge. The PCR reaction was setup in conditions suitable for Phusion Polymerase.

Denaturation: 98^{0}C/2minute

Final denaturation:98^{0}C/10second

Annealing:55^{0}C/30second

Extension:72^{0}C/1minute

Final extenstion:72^{0}C/10minute

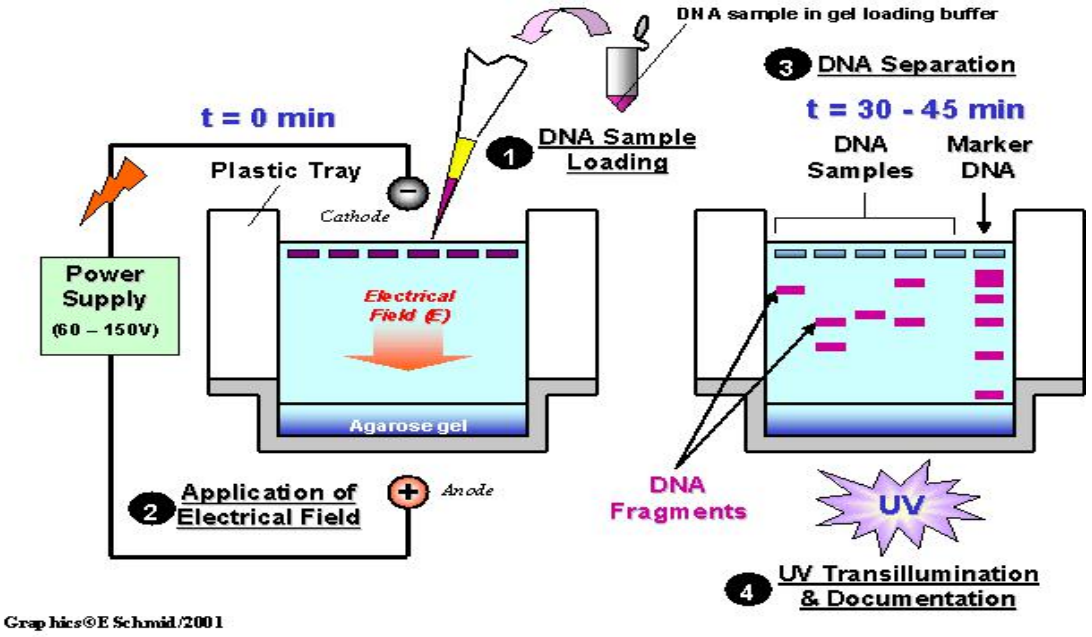
Hold:4^{0}C,infinite hold

After pcr the tubes were stored at -20^{0}C.

4.7.Agarose gel electroporation:-

❖ Principle:-

The principle behind this experiment is the separation of DNA fragments on the basis of their size. This separation can be visualize by the use of an interchalating agent EtBr. DNA being negatively charged propagates towards the positive electrode(anode). Generally 0.7%-2% agarose gel were casted to run DNA depending upon the pore size.



[Fig9:- agarose gel electrophoresis]

4.7.1.casting 1% agarose gel:-

A 250 ml conical flask was taken and rinsed thoroughly with tap water, miliQ water and then elix water. 10ml of 10X TBE was diluted into 100 ml of elix water(1X TBE). 1gm of agarose was weighted and dissolved into 100ml of 1XTBE. 1µl EtBr was added when the solution was lukewarm. It was then pored to the gel casting trey. The

gel was allowed to 45minutes to solidified. 350ml of 1X running buffer was prepared and poured in the gel tank. After the gel got solidified, it was shifted to the gel tank containing 1X running buffer.

4.7.2.Loading of sample:

1kb ladder was taken out from -20c andkept for thawing. A small parafilm paper was taken. Depending upon the no of samples 3µl each of 70% DNA loading dye was added to the parafilm paper. 7ul of sample was then mixed with the DNA loading dye and added to the gel. The electrodes are connected. The gel was then run at a voltage of 90 volts.

4.8.Gel purification:-

- Principle:-

The DNA isolated from the parasite may be conataminated with RNA or proteins despite of addition of proteinase K and RNAse. Proteins having less molecular weight as compaired to that of DNA will be passed through the pores of agarose gel into the buffer. Similarly the RNA and other non specific band which might have been amplified depending upon some similarities can also be separate from the band of our interest. This technique allow us to purify only the genetic material of our interest.

10X TBE was prepared by using 250ml of autoclaved measuring cylinder(1000ml,100ml) .1% agarose gel was prepared by using autoclaved 1X TBE. Autoclaved measuring cylinder (100ml) and autoclaved conical flask. The gel was then cover with aluminium foil to prevent any contaminants and allow to polymerize for 45minutes. The gel tank was thoroughly rinsed with miliQ water, elix water and autoclaved miliQ water. 350ml of 1X running buffer was prepared, using 1X TBE and and autoclaved MQ. The pooled respectives samples were taken out of -20^{0}C and were allowed to thaw.

- Sample preparation was done.

75ul sample+15ul of 70% loading dye

From here onwards the sample should not be kept in ice because the dye will precipitate. The gel apparatus was then kept in cold room. 90µl of sample is added to the wells in a 20µl pipette in the cold room only. 10ul of 1KB DNA ladder was added.

The gel was run in the cold room to prevent any DNAse activity. The gel was run at 90 volts. After the gel was run 3/4th of the casting trey, it was removed and the following procedure was followed to celuate the DNA from the gel.

4.9.Gel elution:-

The gel was sliced and the samples were retrived into a 1.5ml eppendorf tube. The blade was rinsed with 70% ethanol and elix water. The gel was then eluted using Qiagen gel purification kit.

- **Qiagen gel purification kit:**

The gels were sliced and kept in a 1.5ml of eppendroff tube. The blade was rinsed with 70% ethanol and then with elix water. The DNA was then eluted using **QIA quick gel extraction kit from Qiagen.** The weight of the gel slice was determined. Depending upon the weight of the gel, 3 volumes of QG buffer was added to 1 volume of gel. It was incubated at 50°C for 10 min. The tubes were vortexed in every 3 min to dissolve gel. 1 gel volume of isopropanol was added to the sample and mixed gently. A QIAquick spin column was taken. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min at 13000 rpm. The flow-through was discarded and the QIAquick column placed back into the same tube. 500 µl of QG buffer was added to the QIAquick column and kept for 2 minute. Then it was centrifuged for 1 minute at 13000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. 750 µl of PE wash buffer was added to QIAquick column and kept for 5 minutes. Then it was centrifuged for 1 min at 13000 rpm. The flow-through was discarded and the column placed back into the same tube. Dummy spin was done for 1 minute at 13000 rpm. The column was placed into a clean 1.5 ml microcentrifuge tube. 30 µl of autoclaved milliq water was added and kept for 5 minutes. Then it was centrifuged at 13000 rpm for 1 minute and the flow through was collected. The eluted DNA was stored at -20ºc.

4.10.5'UTR:-

4.10.1.Restriction digestion:-

❖ Principle:-

Restriction Digestion involves fragmenting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases commonly known as Restriction Enzymes (RE). Because of this property the restriction enzymes are also known as molecular scissors. Restriction enzymes cleave at specific sequences in the

double stranded DNA molecule, called restriction site to produce sticky end. While ligation, the insert is ligated to the vector in between these points and sealed with the help of DNA LIGASE.

BglII-A'GATCT
ApaI-GGGCC'C
NotI-

The temperature at which the above mentioned restriction endonuclease displayed optimum activity varied.ApaI(25^{0}C)and BglII(37^{0}C). Therefore inspite of double digestion,sequential digestion had be done for all the genes(insert)and pL0006(plasmid vector).

- The cocktail prepared in a reaction mixture of 50µl is as follows:

Table9: Cocktail for 50ul of reaction

Components	Per reaction(µl)
Cut smart buffer	5
ApaI	5
Autoclaved miliq water	2
Total	12

The mixture was then vortexed to allow the enzyme to be mixed and short centrifuge. It was then dispensed to the respective tubes lebelled as follows:

Table10: Restriction digestion PCR reaction

Genes(insert/vector)	5'UTR(µl)	Cocktail(µl)	Total(µl)
PbAAT1	38	12	50
PbAAT2	38	12	50
PbAAT3	38	12	50
PbAAT4	38	12	50
pl0006	20	8(4+4+2)	28(+12µl H_2O)=40

The cold water bath was set to 25^{0}C wit both its heating and cooling motors on such that if the temperature goes up and down the desirable one, it can troubleshoot. The tubes containing the mixture was again vortex and spin down and wrapped with a parafilm in order to prevent water from going inside. Thus the reaction was setup at25^{0}C for 12-15hours. After digestion with ApaI the insert and vector had to be purified. This can be done by column purification method.

4.11.Column purification:-

60µl of autoclaved MQ was added to TUBE1 and 80 µl of autoclaved MQ added to Tube2 to make the volume upto 100µl. 3 volume(300µl) of QG buffer was added to the two tubes. 1 volume(100µl) of isopropanol was added to each tube and mixed gently for 40 times.A QIAquick spin column was taken. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min at 13000 rpm. The flow-through was discarded and the QIAquick column placed back into the same tube. 500 µl of QG buffer was added to the QIAquick column and kept for 2 minute. Then it was centrifuged for 1 minute at 13000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube. 750 µl of PE wash buffer was added to QIAquick column and kept for 5 minutes. Then it was centrifuged for 1 min at 13000 rpm. The flow-through was discarded and the column placed back into the same tube. Dummy spin was done for 1 minute at 13000 rpm. The column was placed into a clean 1.5 ml microcentrifuge tube. 33 µl of autoclaved milliq water was added and kept for 5 minutes. Then it was centrifuged at 13000 rpm for 1 minute and the flow through was collected. The eluted DNA was stored at -20ºc.

- After column purification, the insert and vectors were sequentially digested with BglII. The cocktail was as follows:

Table11: Cocktail for digestion check

Components	Per reaction(µl)
Cut smart buffer	4
BglII	4
Autoclaved miliq water	2
Total	8

The cocktail was then vortex and spin centrifuge. It was dispensed into the following tubes. The complete reaction mixture was setup at 37^0C for 12 hours in CO_2 incubator.

Table12: Digestion reaction setup

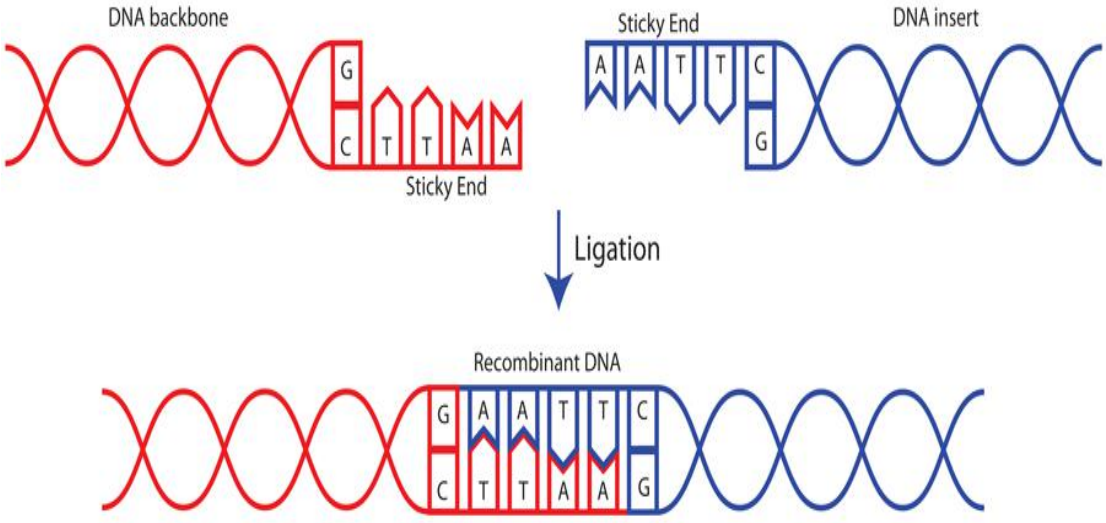
Genes(insert/vector)	5'UTR(µl)	Cocktail(µl)	Total(µl)
PbAAT1	32	8	40
PbAAT2	32	8	40
PbAAT3	32	8	40
PbAAT4	32	8	40
pl0006	20	8	40

After completing sequential digestion,the insert and vector again subjected to purified column purification followed by ligation.

4.12.Ligation:-

❖ Principle:-

Ligation of DNA is a critical step in molecular biology workflows. The main principle of ligation is to seal the sticky end produced by insert and vector by the action of the enzyme DNA Ligase in order to produce recombinant DNA.



[Fig10:- process of ligation]

The DNA ligase buffer and the eluted inserts and vector were kept out of -20°c to thaw. All the reaction mixtures were then vortex and short centrifuge and kept back in ice. The cocktail for ligation was prepared in total volume of 20µl.

Table13: Cocktail for 20ul reaction

Components	4.5 reaction (µl)	Per reaction(µl)
pL0006	13.5	3
Ligase buffer	9	2
Ligase	4.5	1
Total	27	5

The cocktail was then vortex and short centrifuge. It was dispensed to the following tubes.

Table14:- reaction setup for ligation reaction

Genes(insert)	5'UTR(µl)	Cocktail(µl)	Total(µl)
PbAAT1	15	5	20
PbAAT2	15	5	20
PbAAT3	15	5	20
PbAAT4	15	5	20

The reaction mixture was vortex properly and wrapped with parafilm. It was put in a floater and incubated in cold water bath at 16^0C until transformation was done in the next day.

4.13.1.Starter culture:-

The Laminar Air Flow was cleaned with 70% ethanol and all the materials were kept in UV for 20minutes. The LB broth was then kept at room temperature and Tetracycline in ice. 1 LB broth tube was taken and wiped with 70% ethanol. 3µl of Tetracycline was added to the tube. The NBT stock culture was then taken out of -80ºc and carried with liquid Nitrogen. A scoop of stock culture was then taken with the help of a 200µl pipette and was added to the LB broth. The NBT stock was immediately put back into liquid Nitrogen. The tube was incubated at 37ºc for 12hours at shaker incubator. The stock was then stored back at -80ºc.

4.13.2.1% inoculum preparation:--

The Laminar Air Flow was cleaned with 70% ethanol and all the materials were kept in UV for 20minutes. 2 LB broth tubes were taken. Each of them were wiped with 70% ethanol. 3ml of media was present in each tube and 3µl of Tetracycline was added to each tube. 30µl of the NBT starter culture was then added to each of the tubes. These tubes were then incubated at 30ºc for 4 hours. This was followed by the preparation of competent cells as follows:-

4.13.3.Competent cell preparation:-

UV was kept on with all the required materials inside it for 20 minutes. All the 4 LB broths containing 1% inoculum were taken out from the shaker incubator and kept in ice for 10minutes. 1.5ml of culture from each tube was taken in a different eppendrof tube and was centrifuged at 5000rpm, 5 minutes at 4^0C.The supernatant was then discarded inside the laminar air flow and the pellet was again resuspended with the

remaining 1.5ml of culture. This was followed by the centrifugation of tubes at 5000rpm for 5 minutes at 4^0C. The supernatant was discarded and the pellet was resuspended in 1ml of CCMB 80 buffer. The reaction mixture was then incubated in ice for 20 minutes and centrifuged at 3000rpm, 15minutes at 4^0C. The supernatant was again discarded and the pellet was dissolved in 250ml CCMB 80 buffer. The competent cells are now ready for transformation.

4.14.Transformation:-

The 20µl of ligated product was added to the competent cells.The ligated product was resuspended properly in the competent cells and were kept in ice for 30minutes.The reaction mixture was kept at 42^0C in water bath for 90seconds to give heat shock.It was then again kept in ice for 3 minutes.900µl of LB broth was added to the tube.The tube was sealed with parafilm and kept for 1 hour at 37ºc.Then centrifugation was done at 5000 rpm for 5 minutes.Supernatant was removed and resuspended properly.Then the product was spreaded over agar plate and kept at 37ºc for 15 hours.

4.15.Colony PCR:-

20 LB tubes were taken from the cold room and kept at room temperature. Similarly, 20 PCR tubes were taken and 35.5 µl of Milli Q water was added to each of the tubes. 3µl of Ampicillin and Tetracycline were added to the LB broth. This was followed by picking up of the colonies. Half of each colony picked was incoculated in the LB tube and half of the colony was inoculated in the PCR tube. These PCR tubes along with the inoculated tooth picks were then incubated in the laminar air flow for about 15 minutes.

Then Cocktail was prepared for the PCR.

Table15:-Cocktail for colony PCR

Materials	For 20 reaction(µl)	Per reaction(µl)
Forward primer	40	2
Reverse primer	40	2
10X Tag buffer	100	5
$MgCl_2$	50	2.5
dNTPs	40	2
Taq polymerase	20	1
Total	290	14.5

The cocktail was vortex and short spin. The cocktail was prepared 14.5ul for each tube and then vortexed and centrifuged properly. The tooth picks were then removed from the PCR tubes and the cocktail was then added. These PCR tubes were then vortexed and centrifuged for 1 minutes. The reaction was then setup at the conditions suitable for the Taq polymerase.

Denaturation:95^{0}C,10minutes
Final denaturation:95^{0}C,30seconds
Annealing:55^{0}C,30seconds
Extraction:72^{0}C,1minute 15 seconds
Final extension:72^{0}C,10 minutes

1% agarose gel electrophoresis was done to check the product .Only those LB tubes were retained in the shaking incubator for which clones are visualized on the gel. These tubes are incubated on the shaking incubator for 12-15hrs.

4.16.Conformation of clones:-

4.16.1.Plasmid isolation:-

The LB tubes containing grown culture of clones were centrifuged at 5000rpm for 5 minutes at 4^{0}C. The suparnatant was discareded and the pallet was the collected. This pallet was stored in -20^{0}C. The pallet was then kept at room temperature. 200µl of resuspended buffer was taken out of 4^{0}C by titling the eppendrof tube. The resuspension buffer containing EDTA, RNase therefore is stored at 4^{0}C. The pallet was resuspended gently with the help of 200µl pipette(15-20 times). The tubes were then incubated for 5 minutes at room temperature. 200µl of lysis buffer was added. The lysis buffer should be added only when the tubes reach room temperature in order to prevent its precipitation. The solution was then mixed 6-8 times and was incubated for 3 minutes. 350µl of nutralization buffer was added. The solution was then mixed 7-8 times. This was followed by centrifugation at 13000rpm for 10 minutes room temperature. The column was prepared. 500µl of column preparation solution was added to the mini column placed in a 2ml collection tube. It was incubated for 2minutes and then centrifuged at 12000rpm, 1minutes. The flow through was discarded and the mini column was placed back into the collection tube. The supernatant from the solution was then added to the mini column. It was allowed to stand for 2 minutes and was then centrifuged at 13000rpm for 1 minutes, RT. The

flow through was again discarded and 750µl of wash solution was added through the wall of the collection tube. It was the centrifuged at 12000rpm for I minute. The flow through was again discarded and the mini column was put back into the same collection tube. A dummy spin was performed at 13000rpm for 1 minute in order to allow the removal of residual ethanol. The flow through was again discarded and the mini column was placed into a new collection tube. 100ul of autoclaved miliQ water was then added to the center of the mini column and was then incubated for 2 minutes. This was followed by centrifugation at 13000rpm for 1 minute. The elute is our DNA of interest or clone. These tubes were then stored at -20^{0}C.

4.16.2.PCR of the Plasmid containing clones:-

The cocktail was prepared for 4.5 reaction:

Table16: Cocktail for 25ul reaction

components	Per reaction(µl)
$MgCl_2$	1.25
dNTPs	1
10x Taq buffer	2.5
Phusion polymerase	0.5
Autoclaved MQ	16.75
Total	22

The cocktail was then vortex and short centrifuged and dispensed in the respective tubes containing primers for respective genes.

Table17:- Reaction setup for colony PCR

Genes(5'UTR)	Forward primer(µl)	Reverse primer(µl)	5'UTR clone(µl)	Cocktail(µl)	Total(µl)
PbAAT1	1	1	1	22	25
PbAAT2	1	1	1	22	25
PbAAT3	1	1	1	22	25
PbAAT4	1	1	1	22	25

The tubes are again vortex and centrifuge and the PCR reaction mixture was set in the condition favourable for Phusion Polymerase. The clones are confirmed by appearance of clean band that could be visualized by 1% agarose gel electrophoresis.

4.17.Restriction digestion:-

The PCR products were then digested with ApaI and NotIHF in order to check the insert release.

- The cocktail prepared for 4 reaction.

Table18: Cocktail preparation for digestion reaction

Components	Per reaction(µl)
Cut smart buffer	6
ApaI	6
Total	12

The cocktail was then vortex and centrifuge. It was dispensed into the following tubes. The complete reaction mixture was setup at 25^0C for 12 hours.

Table19: Reaction setup for digestion

Genes	5'UTR 3'UTR(µl)	Cocktail(µl)	Total(µl)
PbAAT1	48	12	60
PbAAT2	48	12	60
PbAAT3	48	12	60
PbAAT4	48	12	60

After digestion with ApaI the insert and vector had to be purified. This can be done by salt precipitation method.

4.17.1.Salt precipitation:-

To 60µl of reaction mixture, 140ul of autoclaved miliQ water was added in order to makeup the volume upto 200ul. This was followed by addition of 20ul of 3m NaAc, PH 5.2(1/20) of the total solution. It was mixed in hand 20-30 times. 550ul of absolute ethanol was added to the mixture and was mixed well. Now the total volume of the reaction mixture become 770ul. The reaction mixture was dipped into liquid nitrogen. It was them centrifuged at 13000rpm for 15 minutes at 4^0C. The supernatant was discarded with the help of 200ul pipette. 70% ethanol was added to the pellet and it was vortex properly. The reaction mixture was then centrifuged at 13000rpm for 10 minutes at 4^0C to remove all the residual salts. The supernatant was again discarded and the pellet was retained. It was given a dummy spin at 13000rpm for 1 min at 4^0C to remove the residual ethanol. The rest of the ethanol was removed y the help of 20ul pipette. The eppendrof tube containing digested pellet were incubated at 37^0C for 30

minutes with their caps open such that any remaining ethanol was evaporate. Thereafter the pellet were resuspended in 51µl of autoclaved miliQ water. They are incubated in ice for 25 minutes with regular vortex at an interval of 5 minutes. Later these are stored in -20^{0}C.

- After salt precipitation, the insert and vectors were sequentially digested with NotIHF .The cocktail was as follows:

Table20: Cocktail for digestion reaction

Components	Per reaction(µl)
Cut smart buffer	6
NotIF	6
Total	12

The cocktail was then vortex and short centrifuge. It was dispensed into the following tubes. The complete reaction mixture was setup at 37^{0}C for 12 hours in CO_2 incubator.

Table21: Reaction setup for restriction digestion

Genes	5'UTR(µl)	Cocktail(µl)	Total(µl)
PbAAT1	48	12	60
PbAAT2	48	12	60
PbAAT3	48	12	60
PbAAT4	48	12	60

1% agarose gel electrophoresis was done to check the insert release for all the 4 genes.

4.18.Transfection:-

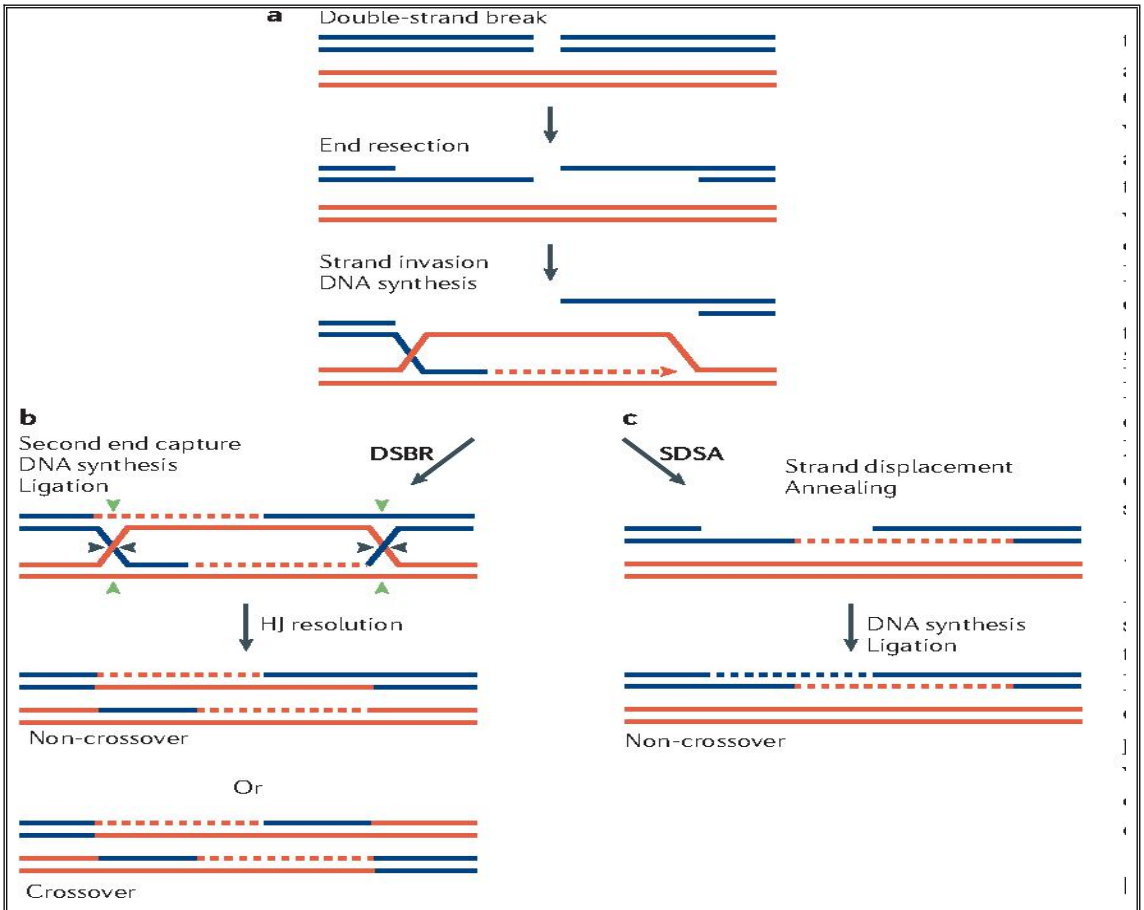
❖ Principle:-

Transfection is the process of deliberately introducing naked or purified nucleic acids into eukaryotic cells. Now, the plasmid that we had isolated from the colonies contained clone 5'UTR Followed by human DHFR cassette and then 3'UTR. Therefore there was a increase in its molecular weight, now this entire region was planned to be transferred into the mice. This would lead to double crossover homologous recombination in vitro leading to the production of gene knockout.

It was also be noted down that the double crossover homologous recombination would be successful only if the respective genes would not be essential for the asexual stage of malaria parasite.

4.19.Double cross over homologous recombination:-

Let us consider that we have a parental DNA duplex with regions A,B,C,D AND E and the duplex is broken by double stranded break in the C. To begin the repair process, the damaged duplex undergoes degradation by helicase in order to produce single stranded 3' end. These tails may be 100bp longs. In a process mediated by recombination one of the single stranded 3' ended tails from the parental duplex interact with the homologous duplex. It invades the homologous duplex in region of homology thus resulting in the formation of hetero duplex region. The hetero duplex region now contains the invading 3'single strand and the complementary homologous duplex. The displaced strand of the homologous duplex which has the same polarity as that of the invading 3' single strand, then forms a loop. This loop structure together with the invading strand called the displacement loop or D-Loop. The complementary strand of the homologous duplex serves as the template for the further synthesis of invading 3'strand. However, the replication in D-Loop does not form an extended region of hetero duplex but it goes on extending the 3'UTR of the invading strand considering the homologous strand as the template until and unless the other strand of the homologous duplex finds a region of homology in parental duplex. Once its finds the region of homology, the replication bubble dissociates and the newly formed strands goes and bind to the other 3' single strand break. Formation of the hetero duplex DNA containing newly synthesized strand and the strands created by double stranded breaks in the parental duplex. The 3' hydroxyl end of the broken duplex then serves as a primer for new DNA replication across the break extending its other side. Remaining gaps are the filled by replication and ligation. Now the C region was repaired by new DNA synthesis. Template for synthesis:-a duplex homologous to the broken /nicked DNA. The process of double crossover homologous recombination can occur only when the two DNA duplex are homologous to each other.



[Fig11:-Homologous recombination process]

The already prepared glycerol stock of WT *Plasmodium berghei* parasite was taken out of liquid nitrogen and was resuspended with 500ul of 1X PBS. 200µl of stock was injected into the 2 mice. After 8 days the mice was dissected and the blood was collected from it and was cultured it in culture medium. The blood was then washed with 1ml RPMI medium 3 times. Each time the blood was washed the supernatant was discarded. The pellet was then resuspended in *Plasmodium berghei* medium and the volume was made up upto 35ml. 10 ml of 50% autoclaved nycodenz solution with PBS (5ml nycodenz solution+5ml PBS) was then underplayed into the falcon tubes. This was followed by spinning down at 450g,20 minutes at RT. The layer in between blood and early trophozoite(the schizont layer) was collected and was resuspended into 12ml of *Plasmodium berghei* medium. It was then centrifuged at 450g for 8 minutes at RT. The supernatant was discarded and again the pellet was resuspended into 600ul of *Plasmodium berghei* medium. This solution was then transferred into different eppendrof tubes containing 1ml each. The nucleofaction solution and the supplemention solution were taken out of ice. Then it vortex and short centrifuge. For each reaction supplement buffer 81.8µl and supplement 3 18.2µl was taken. To this mixture was added 15µl of 5'3'UTR clone was added. This was followed by proper

mixing of the solution and spinning it down. The mice were taken and labelled. It was injected with ketamine/xylazine solution intraperitonially. The schinzoint layer along with the *Plasmodium berghei* medium was spin down at 80rpm, 1 minutes at RT. The supernatant was discarded and the solution containing supplement solution and 5'3'clone was added and mixed well. The whole solution was then transferred into the glass cuvette and the process of nucleofection was done by placing the glass cuvette along with the solution in the nucleofector. The solution was then transferred to the eppendrof tubes through dropper with adding 100ul of *Plasmodium berghei* medium. Then was injected intravenously(eyes)of the mice with an insulin syringe. The mice were then kept under observation of 7-8 days.

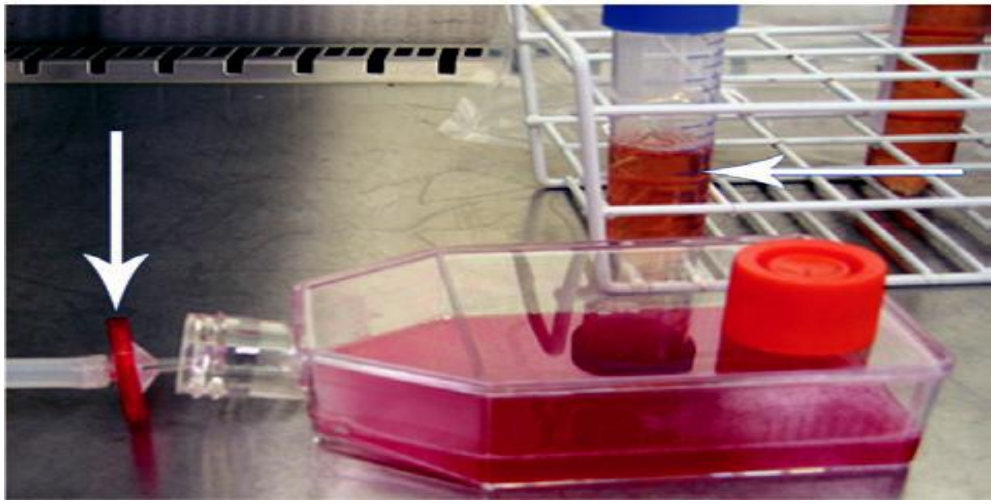
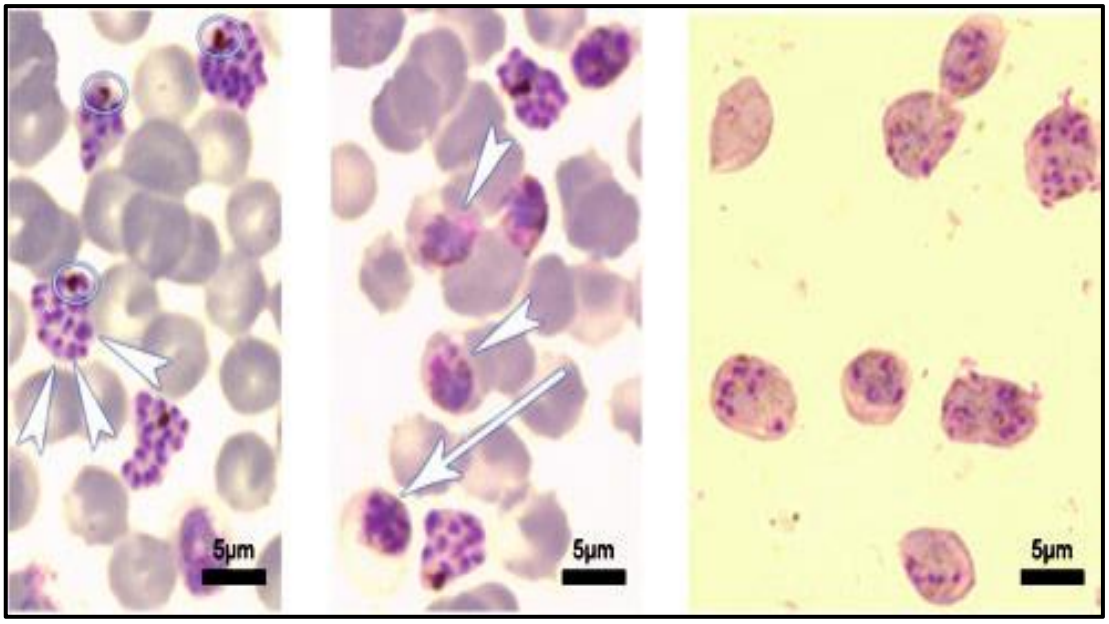


Fig12:-(250-ml cell-culture flasks containing *P. berghei*– infected blood in complete RPMI1640 medium with a gas mixture of 5% CO_2, 5% O_2, 90% N_2 using a 0.2-µm filter unit connected to the gas hose (arrow). The yellow-orange color of the culture medium in the 50-ml tube (arrow) is an indication of the correct pH. A more pinkish color indicates a pH that is too high and detrimental for the growth of the schizonts.)



(A) (B) (C)

[Fig13:-Images of cultured p.berghei schizonts in geimsa stain]

- A: Fully matured vaiable schinzonts(arrow head) are recognized by the presence of 6-7 merozoites with dots of clustered malarial pigment known as hemozoin (circles). These often brust at the time of preparing smear thus resulting in the presence of more or less clustered free particles.
- B: Immature schinzonts that are still in the process of merozoites formation and presence of gametocytes(arrow head).
- C: Immature schinzonts at the time of cell division that have been separated using the nycodenz density gradient.

(A) (B)

[Fig14:- Image showing schinzont layer in nycodenz buffer solution]

- A: The nycodenz gradient solution is layered under the culture suspension containing schinzont infected.
- B: After density gradient centrifugation, the schinzont infected erythrocytes are collected from the interface between the nycodenz solution and the culture suspension whereas the uninfected erythrocytes are at the bottom.

4.20.Clonal selection:-

The mice infected with 5'3'UTR of a perticular genes shows the occurence of parasite after 8-9 days. Not all the parasite undergoes transfection, at this step the wild type as well the parasite those who have under gone transfection will appear. For further selection of only parasite those who have gone recombination, the drug pyrimethamine was given. Three days after the treatment of the drug we will see a decrease in the parasitemia.all the non transfected ones and episomes would be destroyed. Only the parasites having knockout will appear. If at all no parasite will occure at this stage, then we can consider that previously appear parasite was wild type. By this time the parasite would have lost all its episome and what we can see under microscope is the parasite in whose genomic DNA, the gene of our interest has been knocked out.

❖ The process of clonal selection is as follows:-

The mice were anethasized with ketemine-xylazine solution.It was dissected and the blood was collected with a syringe of heparine-PBS. 2stock vials were prepared from this blood and stored in liquied N_2 in case the process does not work. Rest of the blood was diluted 2000 times.

4.20.1.For PbAAT1KO:-

- Dilution1-5ul of blood in 995ul of PBS.
- Dilution2-50ul of dilution1 in 450ul of PBS.
- The number of RBC's present in 1ul of blood was then counted with the help of hemocytometer.

  Let the number of cells in 12 out of 16 squares in 1st quadrants:

  (14+20+25+16+22+18+21+16+23+18+25+19)/12 = 20

  6.25nl contains 20 RBC

  1000nl(1ul) = (20/12)×1000 = 3200RBC

  Times of dilution = 2000

  Therefore the numbers of cells in 1ul 3200×2000= 6,400,000
- So 1ul of whole blood contain 6×10^6 cells.
- Smear was prepared to count the number of parasite.

  100 RBC contain 3 parasite

  1ul of dilution1 contain 3200 RBC's

  Dilution3 - 1:100 contains 32 RBC's
- Mice no 1-4:(1 parasite)

  312.5ul of dilution3 + 687.ul of 1X PBS
- Mice no 5-8:(5 parasite)

  3ul of dilution3 + 197ul of 1X PBS
- 200ul of of sample then injected into the mice.

4.20.2.For PBAAT2KO:-

- Dilution1-5ul of blood in 995ul of PBS.
- Dilution2-50ul of dilution1 in 450ul of PBS.
- The number of RBC's present in 1ul of blood was then counted with the help of hemocytometer.

  Let the number of cells in 12 out of 16 squares in 1st quadrants:

  (15+18+27+18+23+18+22+19+23+19+27+18)/12 = 21

  6.25nl contains 21 RBC

  1000nl(1ul) = (21/12)×1000 = 3360 RBC

  Times of dilution = 2000

Therefore the numbers of cells in 1ul 3360×2000 = 6,720,000

- So 1ul of whole blood contain 6×10^6 cells.
- Smear was prepared to count the number of parasite.

  200 RBC contain 5 parasite

  1ul of dilution1 contain 3360 RBC's

  Dilution3 - 1:100 contains 33 RBC's
- Mice no 1-4:(1 parasite)

  300ul of dilution3 + 700ul of 1X PBS
- Mice no 5-8:(5 parasite)

  3ul of dilution3 + 197ul of 1X PBS
- 200ul of of sample then injected into the mice.

4.20.3.For PBAAT3KO:-

- Dilution1-5ul of blood in 995ul of PBS.
- Dilution2-50ul of dilution1 in 450ul of PBS.
- The number of RBC's present in 1ul of blood was then counted with the help of hemocytometer.

  Let the number of cells in 12 out of 16 squares in 1st quadrants:

  (6+12+12+24+19+15+18+23+16+17+20+18+16+21+26+19)/16 = 18

  6.25nl contains 18 RBC

  1000nl(1ul) = (18/16)×1000 = 2880 RBC

  Times of dilution = 2000

  Therefore the numbers of cells in 1ul 2880×2000 = 5,760,000
- So 1ul of whole blood contain 6×10^6 cells.
- Smear was prepared to count the number of parasite.

  200 RBC contain 5 parasite

  1ul of dilution1 contain 2880 RBC's

  Dilution3 - 1:100 contains 28 RBC's
- Mice no 1-4:(1 parasite)

  69.5ul of dilution3 + 930.5ul of 1X PBS
- Mice no 5-8:(5 parasite)

  34.5ul of dilution3 + 965.5ul of 1X PBS
- 200ul of of sample then injected into the mice.

4.20.4.FOR PBAAT4KO:-

- Dilution1-5ul of blood in 995ul of PBS.
- Dilution2-50ul of dilution1 in 450ul of PBS.

- The number of RBC's present in 1ul of blood was then counted with the help of hemocytometer.

  Let the number of cells in 12 out of 16 squares in 1st quadrants:

  (19+26+22+20+17+18+32+18+22+23+25+20+20+15+24+20)/16 = 21

  6.25nl contains 21 RBC

  1000nl(1ul) = (21/16)×1000 = 3360 RBC

  Times of dilution = 2000

  Therefore the numbers of cells in 1ul 2880×2000 = 6,720,000
- So 1ul of whole blood contain 6×10^6 cells.
- Smear was prepared to count the number of parasite.

  100 RBC contain 2 parasite

  1ul of dilution1 contain 3360 RBC's

  Dilution3 - 1:100 contains 33 RBC's
- Mice no 1-4:(1 parasite)

  74.4ul of dilution3 + 925.6ul of 1X PBS
- Mice no 5-8:(5 parasite)

  37.5ul of dilution3 + 962.5ul of 1X PBS
- 200ul of of sample then injected into the mice.

4.21.Conformation of knockout:-

4.21.1.Isolation of Genomic DNA:-

The mice was dissected and 1ml blood was collected. This was then centrifuged at 2000rpm, 3minutes at room temperature. The supernatant containing plasma was then discarded and the packed cell was then resuspended in 1ml of 1X PBS. This was followed by centrifugation for 5 minutes at 2000rpm, RT. The supernatant was again discarded with the help of 200ul pipette. The packed cell was then resuspended with 200ul of 1X PBS.then add 2ul of RNaseA. Vortex it properly and incubate it 3minutes in RT. Then add 10ul of ProteinaseK vortex it after adding and incubate at room temperature for 10 minutes. Swich on the dry heater set the temperature at 56^0C. After incubation 200µl of AL buffer was added and mixed well with gently vortex then go for 10 minutes incubation at 56C heater .Cool down properly and then 200ul of ethanol was added to it. For mixing it vortex it properly. Then load it into the column and wait for 3 minutes. Centrifuged it for 1minutes at 8000 rpm, RT. Through the flow through and add 500µl of AW1 buffer to the column. Incubate it for 3 minutes at RT. Then spin it at 8000rpm for 1minutes, RT. Discard the flow through and add 500µl of AW2 buffer to the column and incubate it for 3minutes at RT. Centrifuged it at

13000rpm for 3minutes. Discard the flow through and go for a dummy spin to remove remain buffer at 13000rpm for 1 minutes. Take a collection tube place the column into it and add 100ul of autoclaved miliQ water into the column and incubate it 5 minutes at RT. Centrifuged it at 8000 rpm for 1 minutes then collect the sample in a new eppendrof and quantify it.

- Quantification of AAT1KO DNA:-C1-71.3ng/ul.C2- 58.2ng/ul.
- Quantification of AAT3KO DNA:-C1-61.2ng/ul.C2-69.7ng/ul.C3-50.9ng/ul.
- Quantification of AAT4KO DNA:-C1-115.5ng/ul.C2-56.0ng/ul.C3-56.4ng/ul.

4.22.Isolation of RNA:-

Before started the process, all the pipettes to be used were wiped with 70% ethanol. The PBS used for washing the packed cell volume was also filltered using a 0.22μm porosity and a 5ml syringe. 200μl of blood was spin down at 2000rpm for 3minutes ,RT. The supernatant was discarded and 1ml of filltered PBS was added. It was then spin down at 2000rpm for 5 minutes, RT. The supernatant was discarded and 1 ml of trizol reagent was added. The packed cell volume was resuspended immedietly and was incubated at RT for 5 minutes. 1/5 trizol volume chloroform was added to the mixture along the side of the wall. It was then mixed vigorously for 15 sec. the mixture was left at room temperature for 3 minutes. This was followed by centrifugation for 15 minutes at 12000rpm, 4^0C.The supernatant was shifted to another eppendrof tube and the pellet was discarded. 1/2 trizol volume isopropanol was added and mixed gently for 30-40 times.the mixture was left at room temperature for 10 minutes. It was then spin down at 12000rpm for 15 minutes at 4^0C. The supernatant was discarded and 70% ethanol was added.the pellet was dislodged from the wall where it would be sticking. The mixture was again centrifuged at 12000rpm for 10 minutes at 4^0C. The suparnatant was discarded and a brief spin was given to remove the remain ethanol. The pellet was ten kept for drying at the incubator for 20 minutes. The pellet was resuspended in 35μl of autoclaved miliQ water. The RNA was then quantify and stored in -20^0C.

- Quatification of AAT1KO:-C1-133.4NG/UL.C2-166,7ng/ul.
- Quantification of AAT3KO:-C1-143.6ng/ul.C2-148.2ng/ul.C3-80.2ng/ul.
- Quantification of AAT4KO:-C1-152.7ng/ul.C2-213.6ng/ul.C3-210.3ng/ul.

4.23.PCR using Genomic DNA:-

All the materials required except the enzyme were kept outside of -20^0C in RT and were allowed to be thawed. The following cocktail were prepared for testing the

expression of our gene and compare with a control which is GAPDH. The following cocktail was prepared for 4 reaction.

Cocktail (1):-

Table22: Cocktail for 25ul of reaction AAT1

components	For 2.5 reaction(µl)
PbAAT1FP	2.5
PbAAT1RP	2.5
dNTPs	2.5
MgCl2	3.125
DMSO	1.875
GC Buffer	12.5
Phusion polymerase	0.625
Autoclaved MQ water	31.875
Total	57.5

The cocktails were divided into 4 differents eppendrof tubes:-

- Tube1-PbWT gDNA + Cocktail (1)
- Tube2-PbWT gDNA + Cocktail (3)
- Tube3-PbAAT3C1 gDNA + Cocktail (1)
- Tube4-PbAAT3C2 gDNA + Cocktail (3)

Then these tubes are loaded into PCR for the amplification. The following reaction condition were followed:-

Denaturation: 98^0C/2minute

Final denaturation:98^0C/10second

Annealing:55^0C/30second

Extension:72^0C/2minutes

Final extenstion:72^0C/10second

Hold:4^0C,infinite hold

The conformation of knockout was done by running 1% agarose gel electrophoresis.

Cocktail (2):-

Table23: Cocktail for 25ul of reaction AAT3

components	For 2.5 reaction(µl)
PbAAT3FP	2.5
PbAAT3RP	2.5
dNTPs	2.5
MgCl2	3.125
DMSO	1.875
GC Buffer	12.5
Phusion polymerase	0.625
Autoclaved MQ water	31.875
Total	57.5

Cocktail (3):-

Table24: Cocktail for 25ul of reaction GAPDH

components	For 4.5 raction(µl)
PbGAPDHFP	4.5
PbGAPDHRP	4.5
dNTPs	4.5
MgCl2	5.625
DMSO	3.375
GC Buffer	22.5
Phusion polymerase	1.125
Autooclaved MQ water	57.375
total	103.5

The cocktails were divided into 4 differents eppendrof tubes:-

- Tube1-PbWT gDNA + Cocktail (2)
- Tube2-PbWT gDNA + Cocktail (3)
- Tube3-PbAAT3C1 gDNA + Cocktail (2)
- Tube4-PbAAT3C2 gDNA + Cocktail (3)

Then these tubes are loaded into PCR for the amplification. The following reaction condition were followed:-

Denaturation: 98^{0}C/2minute

Final denaturation:98^{0}C/10second

Annealing:55^{0}C/30second

Extension:72^{0}C/3minute

Final extenstion:72^{0}C/10second

Hold:4^{0}C,infinite hold

The conformation of knockout was done by running 1% agarose gel electrophoresis.

Cocktail(4):-

Table25: Cocktail for 25μl of reaction AAT4

components	For2.5 reaction(μl)
PbAAT4FP	2.5
PbAAT4RP	2.5
dNTPs	2.5
MgCl2	3.125
5X platinum buffer	12.5
Platinum polymerase	0.625
Autoclaved MQ water	33.75
Total	57.5

The cocktail were divided into 4 different eppendrof tubes.

- Tube1-PbWT + Cocktail(4)
- Tube2-PbWT gDNA + Cocktail(3)
- Tube3-PbAAT4 gDNA + Cocktail(4)
- Tube4-PbAAT4 gDNA + Cocktail32)

Then these tubes were loaded into the PCR for the amplification. The following reaction condition are followed.

Reaction condition:-

Denaturation: 98^{0}C/30second

Final denaturation:98^{0}C/10seconds

Annealing:60^{0}C/30second

Extension:72^{0}C/3minute

Final extenstion:72^{0}C/10second

Hold:4^{0}C,infinite hold

The conformation of the knockout was done by running 1% agarose gel electrophoresis.

4.24.Passaging:-

Take 4 c57 mice for the experiment. Infect these 4 mice with wild type parasite and AATKO parasite stock which was stored in liquid nitrogen .Add 500µl of PBS to each stock. Resuspended it well. 200µl of each stock inject into the mice. After post infection at 5^{th} day make the smear from mice tail and see the parasitemia percentage. If its very low in number then at 7^{th} day again make smear from the infected mice tail and count the parasite. If the parasite reaches more than 30% then go for exflagellation experiment.

4.24.1.Exflagellation:-

Disected the animal and prepared stock for future use and used blood for exflagellation experiment. For this experiment 8 BALB/C mice, 4 for wild type and 4 for AAT1KO were taken. For each mice 10^5 parasite were injected so the calculation was below:

10^5 parasite preparation;-

RBCs counting for 10^5 parasite

WT- (18+16+7+16+7+10+7+7+15+7+11+13+11+7+7+18)/16=11.06

AAT1KO - (17+16+21+13+24+20+13+24+17+19+17+16+14+13+15+25)/16=17.75

Parasite counting

WT- (59/114×4)+(66/131×4)+(71/136×4)=17.2

AAT1KO - (17/142×4)+(21/112×4)+(18/118×4)=3.76

Dilution1:995ul of PBS+5ul of blood

Dilution2:445ul of PBS+5ul of dil1

10^5 parasite

WT- 62.5/(11×0.172)=33.03µl of dil2

Total 165µl of dil2+835µl of PBS

AAT1KO- 62.5/17.75×0.0375=93µl of dil2

Total 465µl of dil2+535µl of PBS

At 7^{th} day start checking for the exflagellation,ookintes formation, growth curve of the parasite,survival curve.

For exflagellation:-

At the 7^{th} day take all 10^5 parasite infected wild type and AAT1KO mice for check the exflagellation. Take 8 eppendrof tube each containing 500µl of

exflagellation medium and 2.5ul of heparin PBS. Mixed it well in vortex. Then from mouse tail take 1.5ul of blood and add it into the medium. Mixed it gently and incubate it for 15minutes at 19^0C. After incubation take out the eppendrof tube from incubation and take cell culture plate. From the eppendrof tube take 220µl and put it into culture plate. Over it put coverslip and and go for flagella count in inverted flurosence microscope. Exflagellation was count for the 7th,8th,9th,10th,11th,12th,13th,14th days.

4.24.2.For growth curve:-

After the post infection every day the parasitemia will be checked till all the mice died. From the mice take 1drop of blood from their tail and make a smear. Fixed it with methanol. Then when it dry then stained it with 1ml of giemsa stain to each slide. After 20minutes wash the slides properly and let it dry then go for parasite counting in light microscope. According to the parasite growth in the blood day by day the growth curve was increased or decreased.

4.24.3.Survival curve:-

From the 1^{st} day of infection to the last day of the survival of the mice parasitemia was counted and the survival curve was plotted. Due to the increase and decrease of parasite in the blood causes the death and live of the mice.

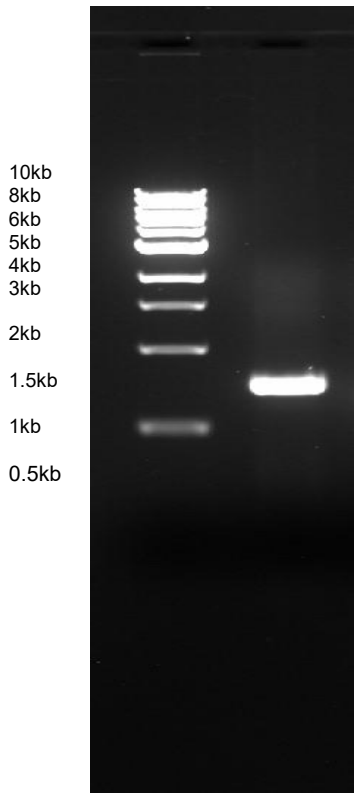
4.24.4.Ookintes formation:-

On the 7^{th} day take all 10^5 parasite infected wild type and AAT1KO mice to check the ookintes formation. Take 8 eppendrof tube each containing 500ul of exflagellation medium and 2.5ul of heparin PBS. Mixed it well in vortex. Then from mouse tail take 5ul of blood and add it into the medium. Mixed it gently and incubate it for 22hours at 19^0C. After incubation take out the eppendrof tube from incubation and centrifuge the eppendrof tube at 8000rpm for 3 minutes. Through the supernatant and resuspend the pellet take it out and make a smear on the slide. let it be dry then fixed it with methanol then after dry add 1ml of giemsa stain to stain the slide for 20 minutes. After 20minutes washed the slide properly and go for imaging and count the ookintes. Ookintes were counted on 7th,8th,9th,10th,11th,12th,13th,14th day of infection.

RESULTS

CHAPTER - 5

5.1.PCR Amplification of 5'UTR:-



Lane1:-1kb DNA ladder
Lane2:-PbAAT1

Fig:-15.The above figure shows the ethidium bromide stained 1% agarose gel PCR amplification of 5'UTR of PbAAT1 gene from the RNA isolation from the *Plasmodium berghei*. The PCR was done in the following condition:-$MgCl_2$, DMSO, 5XGC Buffer, Phusion polymerase and annaeling temeparture 55^0c. Clean band was obtained for PbAAT1 in between 0.5kb - 1kb in lane2. And in lane1 1kb DNA ladder was showing its band.

5.2.PCR Amplification of 5'UTR:-

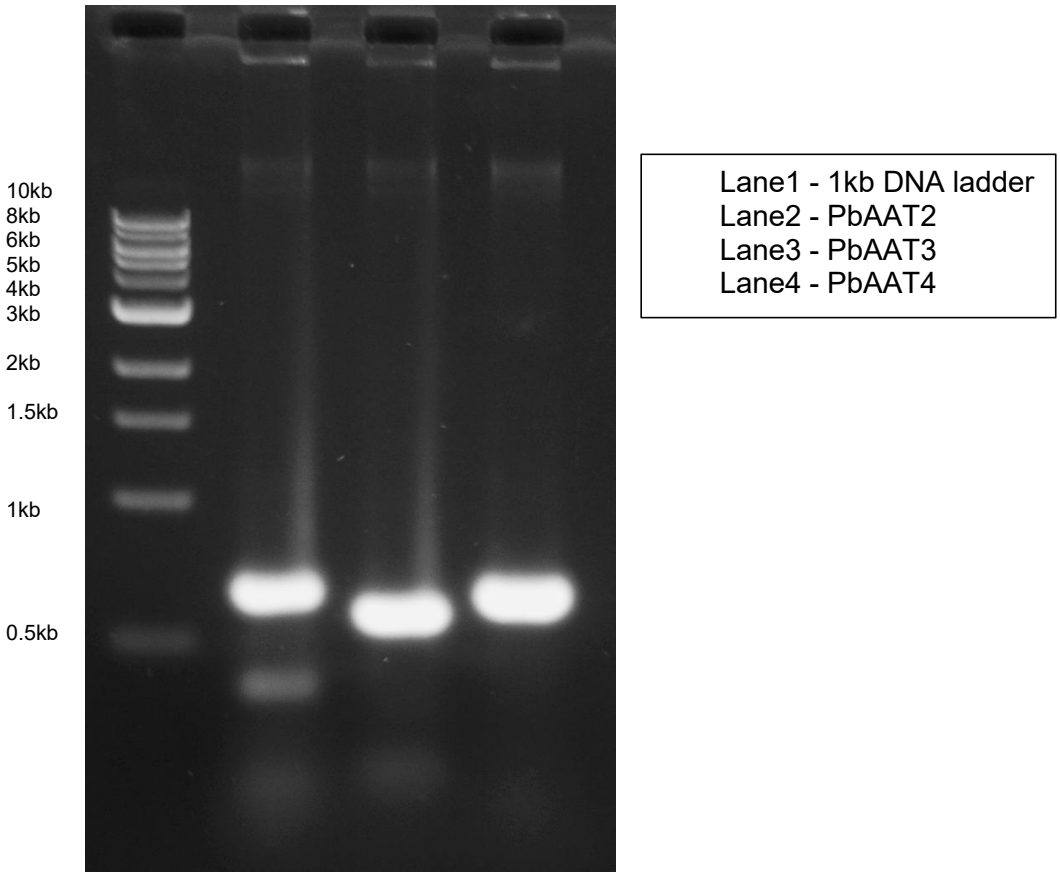
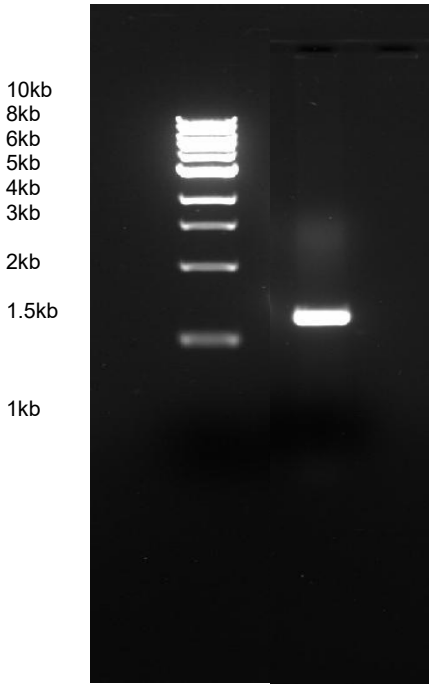


FIG:-16.The above figure shows the ethidium bromide stained 1% agarose gel PCR amplification of 5'UTR of PbAAT1,PbAAT2 and PbAAT3 gene from the RNA isolation from the *Plasmodium berghei*. The PCR was done in the following condition:- $MgCl_2$, DMSO, 5XGC Buffer, Phusion polymerase and annaeling temeparture 55^0c. Clean band were obtained for all the genes in between 0.5kb - 1kb in lane2,3 and 4.And in lane1 1kb DNA ladder was loaded.

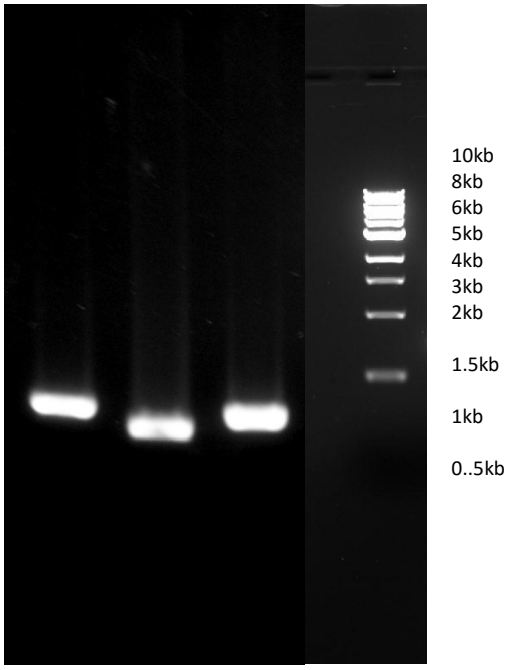
5.3.PCR Amplification of 3'UTR:-



Lane1:- 1kb DNA ladder
Lane2:- PbAAT1

[Fig:17.The above figure shows the ethidium bromide stained 1% agarose gel PCR amplification of 5'UTR of PbAAT1 gene from the RNA isolation from the *Plasmodium berghei*. The PCR was done in the following condition:- $MgCl_2$, DMSO, 5XGC Buffer, Phusion polymerase and annaeling temeparture 55^0c. Clean band were obtained for PbAAT1 in between 0.5kb - 1Kb in lane2. In lane1 showing 1Kb DNA ladder was loaded.]

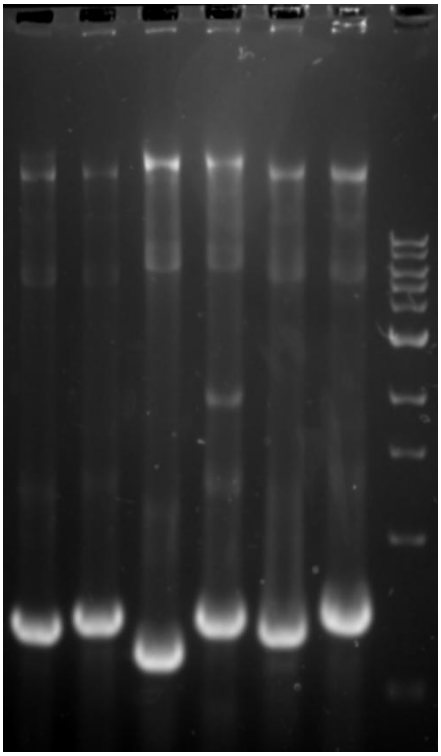
5.4.PCR Amplification of 3'UTR:-



Lane1-PbAAT2
Lane2-PbAAT3
Lane3-PbAAT4
Lane4-1Kb DNA ladder

[Fig:18.The above figure shows the ethidium bromide stained 1% agarose gel PCR amplification of 5'UTR of PbAAT2 and PbAAT3 and PbAAT4 gene from the RNA isolation from the *Plasmodium berghei*. The PCR was done in the following condition:-$MgCl_2$, DMSO, 5XGC Buffer, Phusion polymerase and annaeling temeparture for PbAAT2 and PbAAT3 is 55^0c and for PbAAT4 the anneling temperature is 60^0C. Clean band were obtained for PbAAT2 and PbAAT3 and PbAAT4 in between 0.5kb - 1kb in lane1,2 and 3. In lane4 showing 1Kb DNA ladder was loaded.]

5.5.PCR Confirmation of clones:-



Lane1:- PbAAT1
Lane2:- PbAAT1
Lane3:- PbAAT2
Lane4:- PbAAT2
Lane5:- PbAAT3
Lane6:- PbAAT4
Lane7:- 1Kb DNA Ladder

Fig:-19.Illustration of the plasmid clones containing the PCR products. Insertion of the PCR product in the cloning site of the plasmids was checked. 1ul of template was used.The UTRs of all the genes were amplified. The PCR reaction was done with Taq Polymerase.

5.6.Conformation of insert Release:-

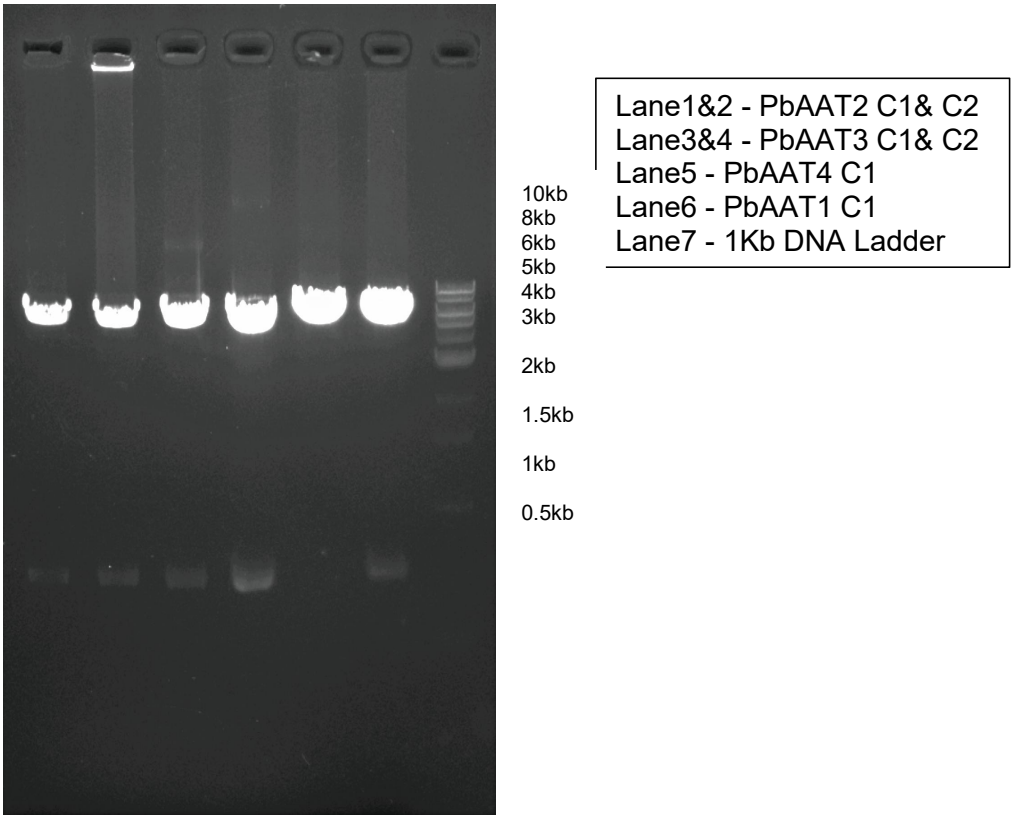
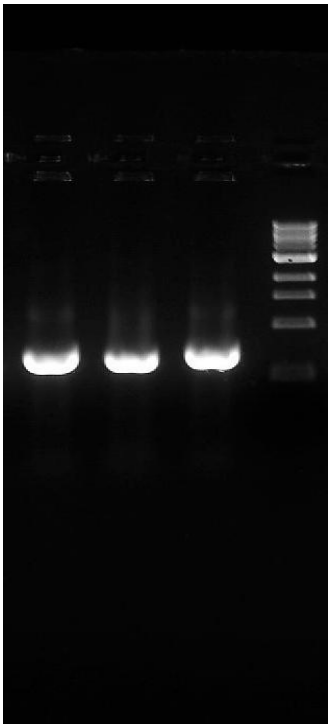


Fig:-20.Restriction digestion was done with the 5'UTR restriction enzyme NotI and BglII in a sequential manner.

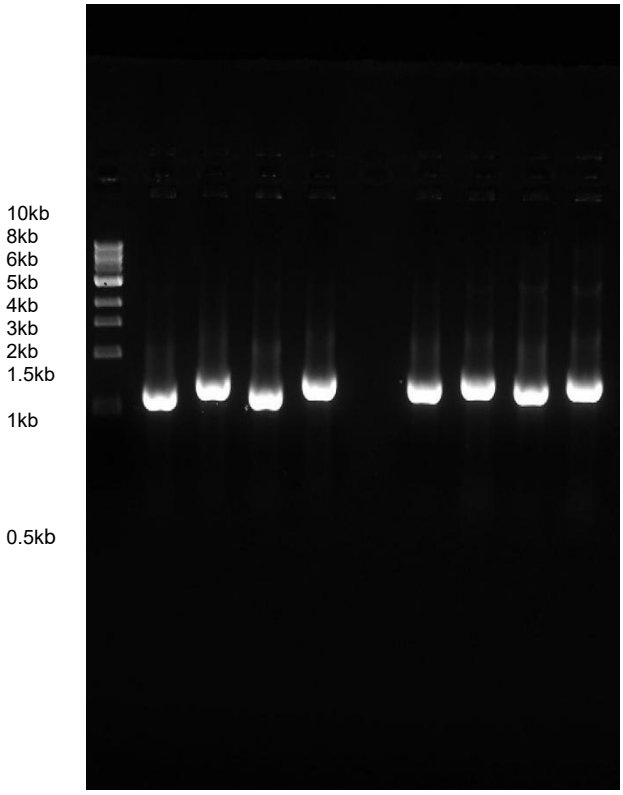
5.7.PCR Confirmation of 5'3'UTR clone:-



Lane1 - PbAAT2C1
Lane2 - PbAAT2C2
Lane3 - PbAAT1
Lane4 - 1Kb DNA Ladder

FIG:-21.These genes were transfected into swiss mice for the generation of knockout. The plasmid of these genes containing 5'UTR followed by the human DHFR cassette which infers pyrimethamine drug resistance followed by the 3'UTR. In order to confirm this, the plasmids were then digested with 5'UTR NotI and 3'UTR BglII.

5.8.PCR Confirmation of 5'3'UTR clone:-



Lane1 - 1Kb DNA Ladder
Lane2 - PbAAT3C1
Lane3 - PbAAT3C2
Lane4 - PbAAT3C3
Lane5 - PbAAT3C4
Lane7 - PbAAT4C1
Lane8 - PbAAT4C2
Lane9 - PbAAT4C3
Lane10 - PbAAT4C4

FIG-22.The Transfection was done with these four plasmids. The increase or decrease in the parasitemia was continuously monitored in an interval of 2-3 days before appearance of paresitemia.(3 days after administration of drug.)

1st the parasites were allowed to grow. When they started to appear and % was satisfied, pyrimethamine was administered orally. Then after the mice were monitored in an interval of 3 days to check an increase or decrease in the % of parasitemia.

This was followed by clonal selection and again drug treatment. These parasite were then allowed to increase in number. When the number of parasitemia was found to be high, dissection was done and blood was collected.

Table26: Infromation about the day after post transfection

gene	TF	AOP	DA	CS	AOP	DA	AOP	BC
Pbaat1	17/2/18	10th day	2nd day	15th day	22nd day	23rd day	25th day	25th day
Pbaat2	17/2/18	9th day	2nd day	19th day	29th day	30th day	35th day	35th day
Pbaat3	17/2/18	10th day	2nd day	17th day	24th day	25th day	27th day	27th day
Pbaat4	17/2/18	11th day	2nd day	18th day	26th day	27th day	29th day	29th day

TF-Transfection

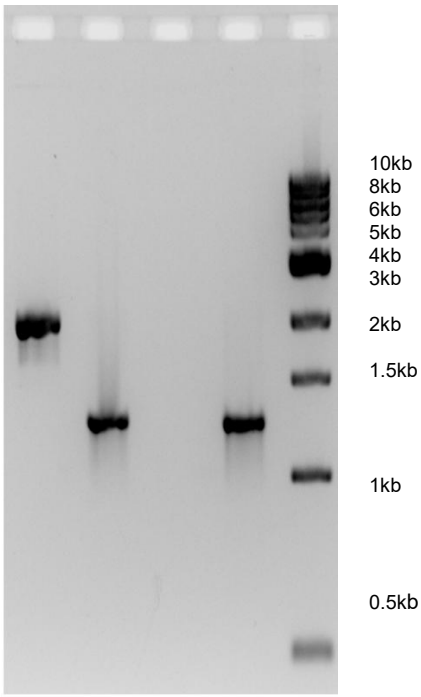
AOP-appearance of parasite

DA- drug treatment

CS- clonal selection

BC- blood collection

5.9.PCR of genomic DNA of AAT1KO using gene specific primer:



Lane1 - PbWT
Lane2 - PbGAPDH
Lane3 - PbAAT1KO
Lane4 - PbGAPDH
Lane5 - 1Kb DNA Ladder

Fig:-23.The above figure shows the knockout of PbAAT1 in lane3. In lane2 and 4 the band represent of GAPDH. And in lane1 the band showing wild type genomic DNA. AAT1 type genomic DNA size is 1.8 Kb.and GAPDH genomic DNA size is 1.3Kb. These all bands are showing clean product. lane3 donot showing any product because that lane having PbAAT1genomic DNA. The PCR was done in the following condition:-$MgCl_2$, DMSO, 5XGC Buffer, Phusion polymerase and annaeling temeparture for PbAAT1 and GAPDH is 55^0c and for Pb Wild type the annaeling temperature is 55^0C.

5.10.PCR of genomic DNA of AAT3KO using gene specific primer:-

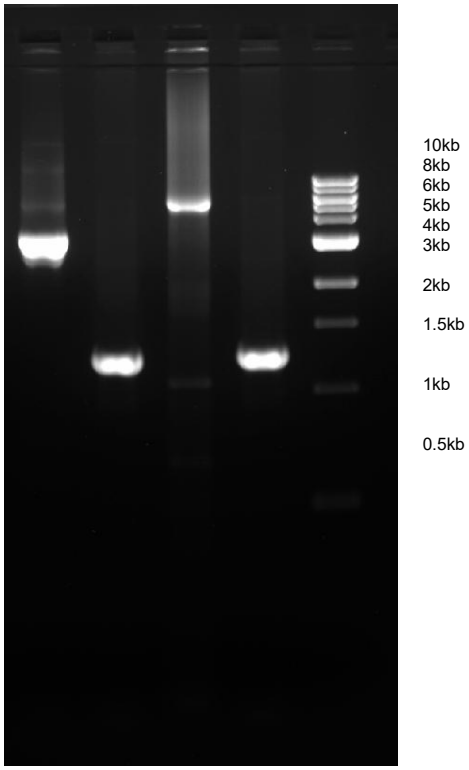
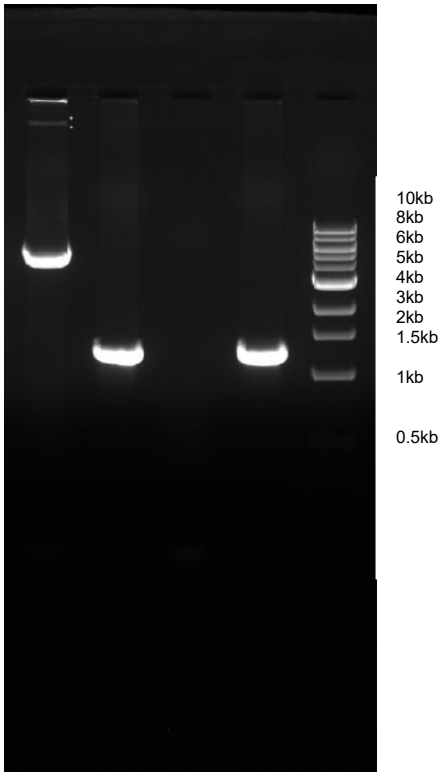


FIG:-24. The above figure shows the knockout gene of PbAAT3 in lane3. In lane2 and 3 the band showing the result of GAPDH genomic DNA. And in lane1 the band showing wild type genomic DNA. And GAPDH genomic DNA size is 1.3Kb. These all bands are showing clean product. lane3 showing one band but it didn't match the PbAAT3 genomic DNA size was 3.1Kb so we didn't said that this is the knockout gene band because that lane having PbAAT1genomic DNA with PbAAT3 specific primer so that this gene does not show any product. The PCR was done in the following condition:- $MgCl_2$, DMSO, 5XGC Buffer, Phusion polymerase and annaeling

temeparture for PbAAT3 and GAPDH is 55^0c and for Pb Wild type the annaeling temperature is 55^0C.

5.11.PCR of genomic DNA of AAT4KO using gene specific primer:-



Lane1 - PbWT
Lane2 - PbGAPDH
Lane3 - PbAAT4KO
Lane4 - PbGAPDH
Lane5 - 1Kb DNA Ladder

FIG:-25. The above figure shows the knockout gene of PbAAT4 in lane3.In lane2 and 3 the band showing the result of GAPDH genomic DNA. And in lane1 the band showing wild type genomic DNA. AAT4 genomic DNA size is 4.5 Kb. And GAPDH genomic DNA size is 1.3Kb. These all bands are showing clean product. lane3 do not showing any product because that lane having PbAAT3genomic DNA with PbAAT3 specific primer so that this gene does not show any product. The PCR was done in the following condition:- $MgCl_2$, dNTP's, Platinum superfamily Buffer, Platinum polymerase and annaeling temeparture for PbAAT3 is 60^0C and GAPDH is 55^0c and for Pb Wild type the annaeling temperature is 55^0C.

5.12.Exflagellation:-

Table27: Exflagellation growth rate day by day:-

	7th day	8th day	9th day	10th day	11th day	12th day	13th day	14th day
WT	9.1	5.28						
PbAAT1	0.29	0.52	0.11	0	3.2	7.696	8.824	6.59

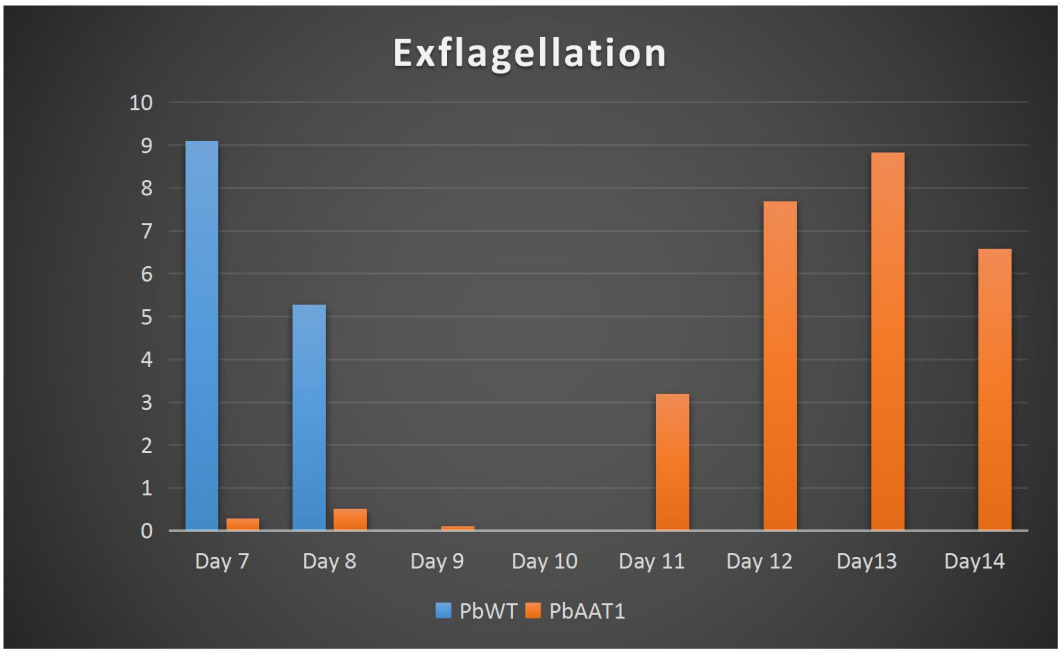


Fig:26.The above figure shows the exflagellation rate of PbWT and Pb AAT1KO.On 7th day the Pb WT exflagellation growth rate was much higher than the other days. After 7th day it become less. Because after 9th days the wild type mice were died.On 7th day to 10th day in PBAAT1 the exflagellation rate was very less.On the 10th day it was very less in number, but in 13th day it was high in number. After 14th days it was gradually decreased.

5.13.Growth curve:-

Table28: Parasitemia growth rate in day to day:-

	1	3	5	7	9	11	13	15	17
Pbwt1	0	0	6.27	28.2	16.33	57.83	63.6	56.2	78.8
Pbaat1	0	0	2.182	7.86	5.922	31.97	50	70.3	

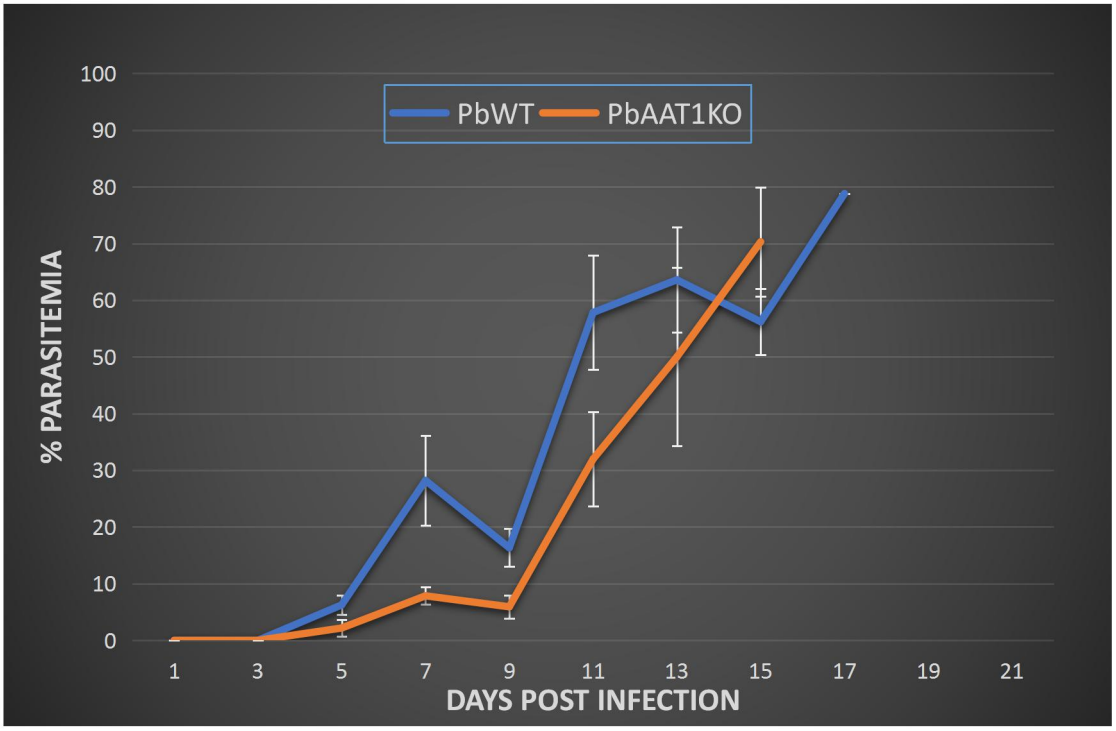


Fig:27. The above figure shows the % of parasite growth in different days. The parasite growth was monitored from day 5 in both WT and PbAAT1 type. The wild type parasite was grew very rapidly. But the knockout parasite grew slowly than the wild type. In wild type the growth was not continous till day7. On 9th day it decreased a little but after 2 days it become high again. But in PbAAT1 the growth from 9th day was continously high.

5.14.Survival curve:-

Table29: Mice survival day by day

Days	WT	WT %	AAT1	AAT1 %
0	4	100.00	4	100.00
1	4	100.00	4	100.00
2	4	100.00	4	100.00
3	4	100.00	4	100.00
4	4	100.00	4	100.00
5	4	100.00	4	100.00
6	4	100.00	4	100.00
7	4	100.00	4	100.00
8	4	100.00	4	100.00
9	3	75.00	4	100.00
10	3	75.00	4	100.00
11	3	75.00	4	100.00
12	3	75.00	4	100.00
13	3	75.00	4	100.00
14	3	75.00	4	100.00
15	2	50.00	3	75.00
16	2	50.00	3	50.00
17	1	25.00	0	0.00
18	1	25.00	0	0.00
19	0	0.00		

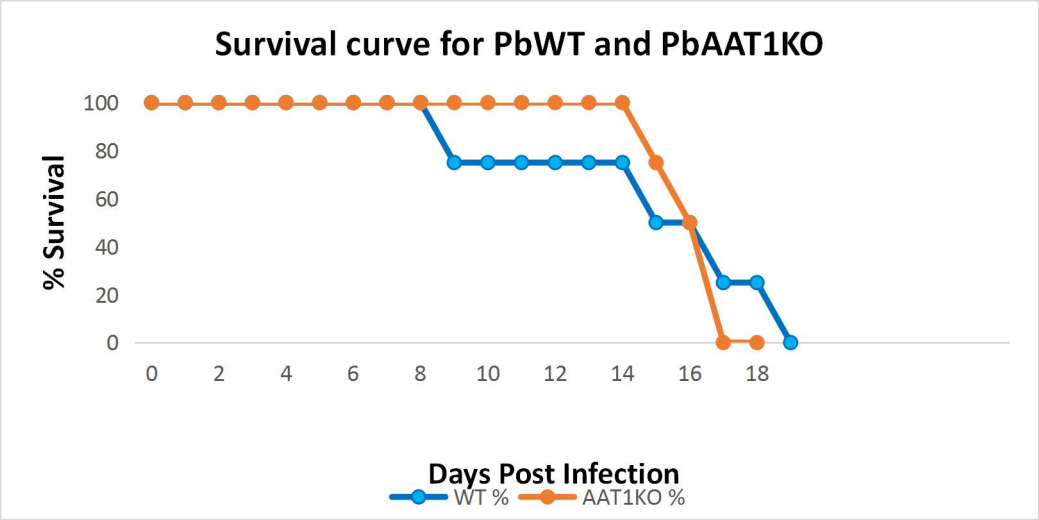


Fig:28.The above figure shows the mortality and the survival of the mice after post infection of WT and PbAAT1KO parasite. WT parasite infected parasite mice started died from 9th day onwards. But PbAAT1KO parasite mice were alive till 15th day after post infection. After that the mortality started.

5.15.Ookintes formation:-

Table30: Ookintes growth in parasite day by day

	Day 7	Day 8	Day 9	Day 11	Day 12	Day 13
WT	2.69	5.43	2.52	1.06	0.89	1.51
AAT1	1.06	2.91	1.2	4.54	7.79	6.68

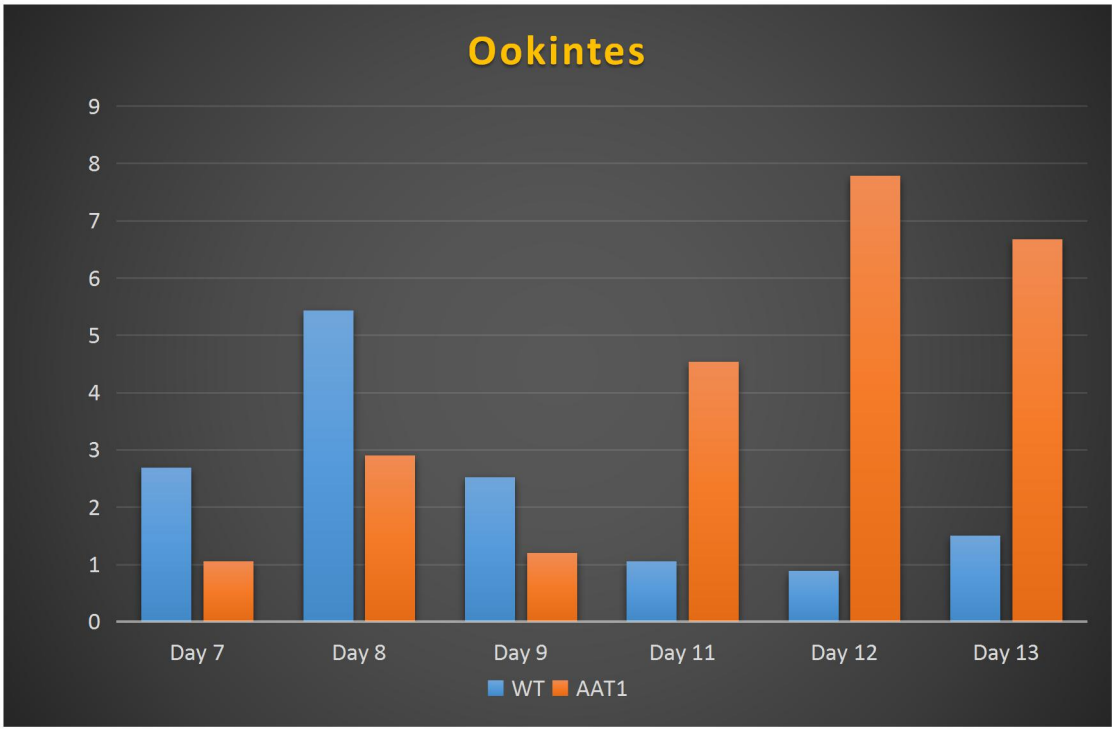
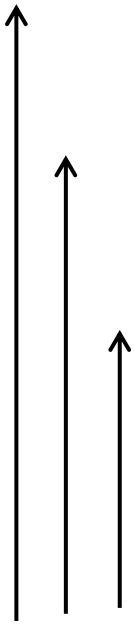
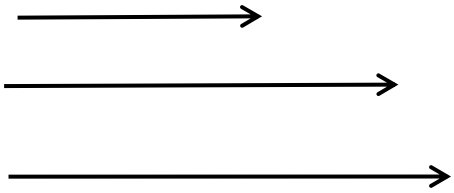


Fig:-29.The above figure shows the ookintes formation in parasite. From 7th day of post infection the ookintes formation started in both WT and AAT1KO. But in 7th day in WT the ookintes were more than AAT1KO. It became less after day by day in WT. From 11th day in AAT1KO the ookintes numbers were more till 13th day.

DISCUSSION



CHAPTER - 6



6.1.PCR Amplification of 5'UTR:-

The genomic size of 5'UTR of PbAAT1 is 0.75kb. The band appear between the 0.5kb and 1kb. It confirms that this is the band of pbAAT1 5'UTR. The band was very intense and clean it means which PCR condition we give for this reaction is favourable for the amplification of this 5' UTR prime. Taq polymerase enzyme was favourable for this reaction(fig5.1). In figure 5.2 the genomic size of pbAAT2, S3 and 4 is also 0.75kb. The bands were appear exactly in between the 0.5kb and 1kb DNA ladder. The bands were very intense. But in somehow its not clean so that means may be cause due to the presence of DMSO or it may be some impurities. Taq polymerase enzyme was favourable for this PCR amplification reaction.

6.2.PCR Amplification of 3'utr:-

The genomic size of 5'3'UTR of PbAAT1 is 0.75kb. The band appear between the 0.5kb and 1kb. It confirms that this is the band of PbAAT1 5'UTR. The band was very intense and clean it means which PCR condition we give for this reaction is favourable for the amplification of this 5' UTR prime. Taq polymerase enzyme was favourable for this reaction(fig5.3). In figure 5.4 the genomic size of PbAAT2,3 and 4 is also 0.75kb. The bands were appear exactly in between the 0.5kb and 1kb DNA ladder. The bands were very intense and clean. So that the PCR condition was favourable for this reaction. Taq polymerase enzyme was favourable foe this PCR amplification reaction.

6.3.PCR Confirmation of clones by insert release:-

Plasmid isolated from the NBT transformed cells were used for the restriction digestion to check either the knockout genes were inserted properly in the pl0006 vector or not. NBT is a k-12 strain ideally suited as an initial cloning host due to its high transformation efficiency.which result in high yields of excellent quality plasmid DNA.

In figure 5.5 it was shown that all bands appear clean that means all knockout genes were cloned successfully. The insert release was shown in figure5.6 which indicates that the insert release confirm the proper insertion of PbAAT1,2,3 and 4 in pl0006 vector.

6.4. Transfection:-

These four plasmids were then used to transfect WT parasite in swiss mice for the generation of knockouts. The plasmid of these genes now contain 5'UTR followed by the human DHFR cassette which infers pyrimethamine drug resistance

followed by 3'UTR. In order to confirm this the gens were then digested with 5'UTR BglII and 3'UTR NotI.

The main idea behind the restriction digestion was to elute the insert (5'UTR+hdhfr cassette+3'UTR) from 1% agarose gel and then transfect them into parasite. Since, the size of the vector and insert ware found to be nearly the same,the elution could not be done resulting in transfection of the 5'3' UTR as it is.the process of transfection included nucleofection of the 5'3'UTR clone into the schizonts of *Plasmodium berghei* isolated from mice and the injecting the entire solution.

6.5.PCR using gene specific primer for knockout genes:-

In figure 5.9.it shows the complete knockout of PbAAT1 gene in lane3. That means this gene was successfully knockout from the parasite genome. GAPDH(glyceraldehyde -3- phosphate dehydrogenase) is a protein of 37kDA that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. GAPDH genes is constitutively expressed at high levels in most tissues and cells including *Plasmodium berghei*. Thus it is known as a house keeping gene. By comparing the presence of GAPDH and the gene of our interest we could verify the knockout of gene from the entire genome. The size of GPADH genomic DNA was 1.25kb. In figure 5.10 the knockout of PbAAT3 was shown in lane3. In this lane one band was shown but it did not matched the size of the genomic dna of PbAAT3. The size of the PbAAT3 genomic DNA was 3.1kb. The band shown in lane3 would be some non specific band which was due to some impurities in the time of transfection. In figure 5.11 the knockout of PbAAT4 was shown in lane3. In this figure all band were very clean and intense without any additional band and impurities. This PCR reaction was set using Platinum enzyme which was 10x more efficiency than Taq polymerase and it also have platinum hot start technology designed for highest success in PCR reaction. It can amplify upto 20kb.

6.6.Exflagellation:-

Exflagellation of microgametocytes in malarial parasites is associated with the life cycle in the mosquito. Exflagellated microgametes of malarial parasites are know to occur in invertebrate host—mosquito. Exflagellation occurred in vitro facilitated by rise in pH of the blood and heparin by not altering the concentration of divalent cations. In this exflagellation process microgametes were formed which are fertilized to form zygotes. In figure26. the exflagellation started early in WT mice from the day 7. But after 9^{th} day mortality ofthe WT mice started. In AAT the exflagellation was started very late.Iit was seen from 11^{th} day. It gradually increase after day by day till 14^{th} day.

After 9th day all the WT animals were died so that their flagella formation was not monitored.

6.7.Growth curve:-

Growth curve was measured by the counting of percentage of parasitemia in the blood after the post infection. Parasitemia was checked on alternate days to know how the parasite grew after the post transfection. The parasitemia basically checked from 7th day onwards. In WT animal the parasite grew very rapidly. But in AAT animal the parasite grew very slowly. In figure27.the WT parasite growth was not constant in 9th day the growth was decrease and in 11th day it was again very high in percentages. But in AAT type animal the parasite growth after 9th day was very high than the WT animal.

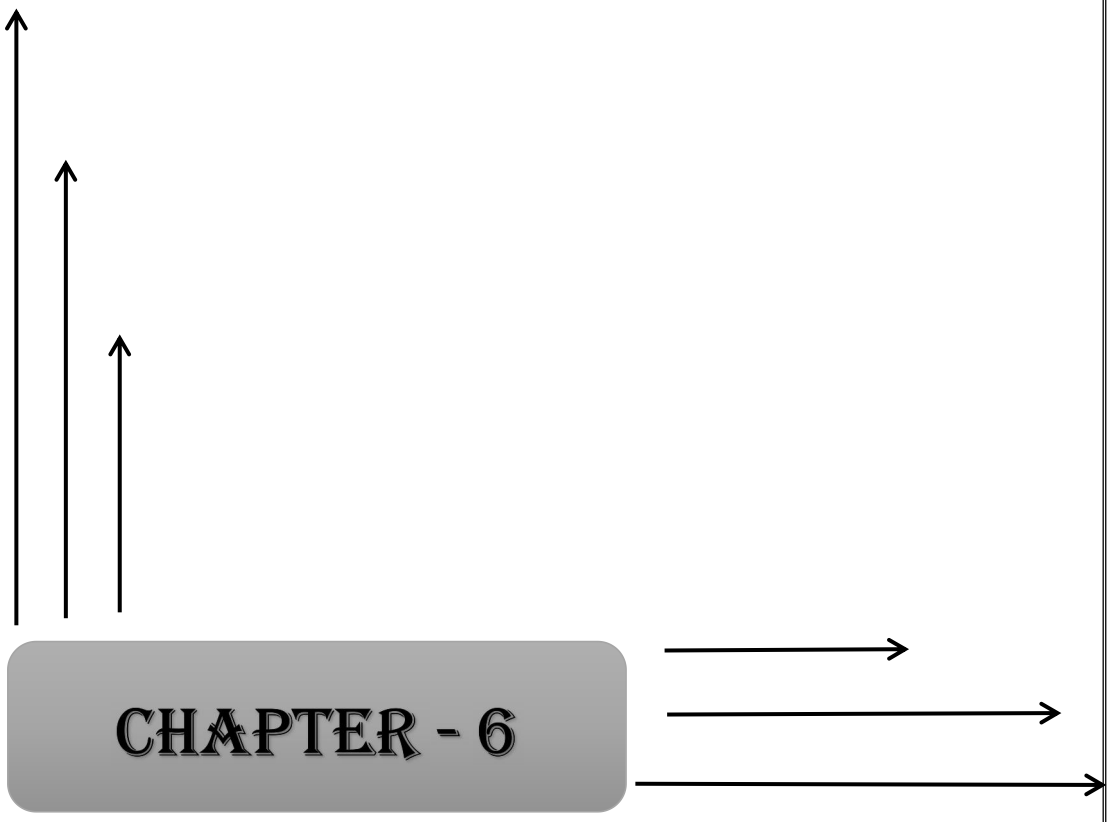
6.8.Survival curve:-

It was based on the survival of the infected mice after post infection. The WT mice were died early than the AAT1 type mice. From day 8th the WT mice were started dying. Till the 16th day all WT mice were died but AAT mice survived more days than the WT. They survive till 20th days after post infection. In their blood the percentage of parasite was high but they recovered from this and survive more days than the WT.

6.9.Ookinetes Formation:-

The oocyst is the only extracellular developmental stage of the malaria parasite life cycle. The microgamets are fertilize to form ookinetes. The motile zygote of a parasite of the malaria mosquito that forms an oocyst in the mosquito's gut was known as ookinetes. It is morphologically and biochemically distinct from the earlier sexual stages gametocytes and zygote, and from the later stages oocyst and sporozoites. In figure.29. the ookinetes formation was measured from day 7th.In the 1st 9 days WT animal the ookinetes formation was higher than the AAT type animal. After 10th days In AAT mice the ookinetes are more in number. In in vitro condition the ookintes formation was delayed in case of konckout genes.

CONCLUSION



CHAPTER - 6

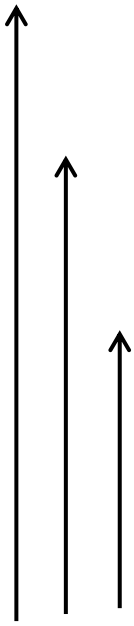
7.1.Summary:

The successful generation of knockouts for PbAAT1,PbAAT3 and PbAAT4 revealed that the transporters play a significant role in the other stages of the malaria parasite. The growth curve analysis show that the knockout genes may play an essential role in the asexual and liver stages growth of malaria parasite. The in vitro exflagellation analysis shows that the knockout parasite show very late exflagellation process. This suggest that it also hamper the formation of microgametes like male gametocyte and female gametocyte and also formation of the ookinetes. The ookinetes graph analysis shows the delay of ookinetes formation in knockout parasite. Ookinetes gives rise to oocyst and the oocyst undergoes multiple division to form sporozoites which plays a main role in infect the animal.The survival growth curve analysis show that the knockout genes animal are more motile in nature.they have that capacity to survive.All these analysis conclude that the knockout genes were shows delay in the exflagellation, ookinetes formation and growth of the parasite.

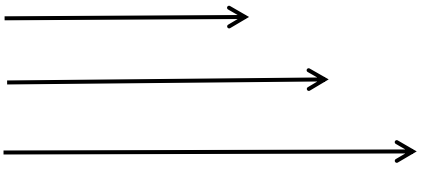
7.2.Future Work:

- ✓ Generation of AAT2KO parasite.
- ✓ Southern, Northern and Western analysis for PbAAT1KO, PbAAT2KO, PbAAT3KOand PbAAT4KO .
- ✓ Effect of asparaginase treatment on sexual and asexual stage of PbAAT1KO parasite.
- ✓ Heterologous Expression of PbAATs Transporter in Yeast system/ mammalian expression sytem to check amino acid uptake and antimalarial drug toxicity.

REFERENCE



CHAPTER - 6



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APPENDIX

1.**10X TBE:-**

COMPONENTS	AMOUNT
Autoclaved miliq water	350ml
Tric buffer	54gm
Boric acid	27.5gm
0.5M EDTA	20ml

2.70% DNA Loading dye:-

- ✓ 700ul of 100% glycerol.
- ✓ add 300ul of autoclaved miliq water.
- ✓ A pinch of BPB(Bromo Phenol Blue).

3.10mM dNTP's:-

COMPONENTS	VOLUME(µl)
Autoclaved miliq water	120
100mM dATP	20
100mM dTTP	20
100mM dGTP	20
100mM dCTP	20

4.1kb DNA Ladder:-

- ✓ 20ul of stock 1kb ladder
- ✓ 140ul of autoclaved miliQ water
- ✓ 40ul of 70 DNA loading dye

5.10X PBS:-(1000ml)

COMPONENTS	MASS(gm)
KCl	2
KH_2PO_4	2.4
NaCl	80
Na_2HPO_4	11.45

6.Giemsa stain:-

- ✓ 300ml of giemsa stain
- ✓ Dissolved in 25ml 100% glycerol

- ✓ Heat at 55^0C for 20 minutes
- ✓ Cool and let it come to RT
- ✓ Add 25 ml of methanol

6.Ketamine:Xylazine:-

COMPONENTS	VOLUME(µl)
1X PBS	850
Ketamine	100
Xylazine	50
Total	1000

7.Ampicillin and tetracycline stocks:-

- ✓ Taken 500mg of ampicillin and was dissolved in 5ml of autoclaved MQ.
- ✓ Then it was dispensed into 5 different eppendrof tubes.
- ✓ Taken 12mg of tetracycline and was dissolved in 1 ml of 70% ethanol.

9.LB Agar:-

COMPONENTS	MASS(gm)
Tryptone	1
NaCl	1
Yeast extract	0.5
Agar	1.5

Dissolved in 100ml of elix water.

10.**LB Broth:-**

COMPONENTS	MASS(gm)
Tryptone	1
NaCl	1
Yeast extract	0.5

Dissolved in 100ml of elix water.

11.**EtBr:-**

- ✓ Dissolve 1mg of EtBr in 1ml of MQ.
- ✓ Vortex it properly and stored it in 4^0C.

12.Resuspension Buffer:-

COMPONENTS	VOLUME(µl)
Tris pH 8.0	50
EDTA	100
SDS	50
NaCl	25
H_2O	775
Total	1000

13.CCMB Buffer 80:-

COMPONENTS	AMOUNT
$CaCl_2.2H_2O$	590mg
$MnCl_2.4H_2O$	200mg
$MgCl_2.6H_2O$	100mg
1M KCL	500µl
10% Glycerol	4.5ml
H_2O	45ml
Total	50ml

14.**Glycerol stock:-**

- ✓ Taken 15oul of glycerol
- ✓ 35oul of blood collected is added to it.
- ✓ The stock was then stored in liq.N_2.

15.Plasmodium berghei medium:-

COMPONENTS	AMOUNT
RPMI Medium	2.6gm
HEPES	1.49gm
$NaHCO_3$	0.53gm
FBS(CL)	50ml(20%)
Gentamycin	4 drops
H_2O	200ml

The whole solution was then filtered and stored at 40C.

16.Pyrimethamine drug(500ml):-

- ✓ 35mg of pyrimathamine was dissolved in 7ml DMSO.
- ✓ The volume was then made up to 500ml by elix water.
- ✓ The pH was set to 3.5-5.0 with 1M HCL.

17.Exflagellation medium(100ml):-

Components	Amount
RPMI Medium/HEPES	1.62gm
$NaHCO_3$	212mg
Xanthurenic acid	2mg
Complement heat inactivate	10ml
Gentamycin	2drops
Autoclaved miliQ water	90ml

18.HEPARIN PBS SOLUTION:-

- ✓ 995ul of PBS + 5ul of Heparin added and mixed it well.