Characterization and Localization of MAF1 Protein in *Plasmodium berghei*

A dissertation submitted to KIIT School of Biotechnology in partial fulfillment of the requirements for the Award of the Degree of

MASTER OF SCIENCE
in
Applied Microbiology

Submitted By

Gayatri Patra

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

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Under the Guidance of

Dr.V.Arun Nagaraj

Scientist-D, Infectious Disease Biology
Institute of Life Sciences, Nalco Sq.
Bhubaneswar, Odisha-751023
Website: www.ils.res.in

DECLARATION

I hereby declare that the dissertation entitled **"Characterization and Localization of MAF1 protein in the *Plasmodium berghei*"** 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.A.Nagaraj, Scientist-D, Institute of Life Science, Bhubaneswar,
Odisha, India.

Date:

Place:

Gayatri Patra

ABSTRACT

The artemisinin resistance in *Plasmodium falciparum* is emerging as one of the biggest problems because, artemisinin is more effective when parasites are actively metabolizing haemoglobin in the trophozoite stage. With the help of MAF1 protein , repressor of RNA polymerase III ,parasites enter the state of dormancy which results in the drug resistance. To overcome this drug resistance problem it is necessary to inhibit the function of MAF1 protein. The first step is to characterize and know the localization of MAF1 protein in *Plasmodium species. Plasmodium berghei* MAF1 was expressed as a recombinant protein in *E.coli* and polyclonal antibody was developed against MAF1 protein in mice. This antibody was used to determine the localization of MAF1 in *Plasmodium berghei.* We have performed large scale protein purification followed by immunization in mice. After 3 subsequent booster antibody was produced against PbMAF1 protein, which was used in Western analysis to confirm its reactivity and in immunofluroscence to study the localization of MAF1 in *Plasmodium berghei.*

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DATE:

PLACE: **Gayatri Patra**

CONTENTS

ABBREVIATIONS

Pb:*Plasmodium berghei*

A.M Water:Autoclaved Milli Q water

EtBr:Ethidium bromide

TBE:Tris Boric acid EDTA

LB broth :Luria bertani broth

NBT:NovaBlue singles

RT:Room temperature

dNTPs:deoxynucleosidetriphosphates

ddNTPs:di-deoxynucleosidetriphosphates

U:Uninduced

I:Induced

IPTG:Isopropyl β-D-1-thiogalactopyranoside

SDS-PAGE:Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TEMED:Tetramethylethylenediamine

APS:Ammonium persulfate

ALP:Alkaline phosphatase

NBT: Nitro-blue tetrazolium chloride

BCIP:5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

Ni-NTA:Nickel Nitrilotriacetic acid

CCMB:Competent cell modified buffer

DAPI:4',6-diamidino-2-phenylindole

FITC:Fluorescein Isothiocyanate

µl: Microlitre

Ml: Mililitre

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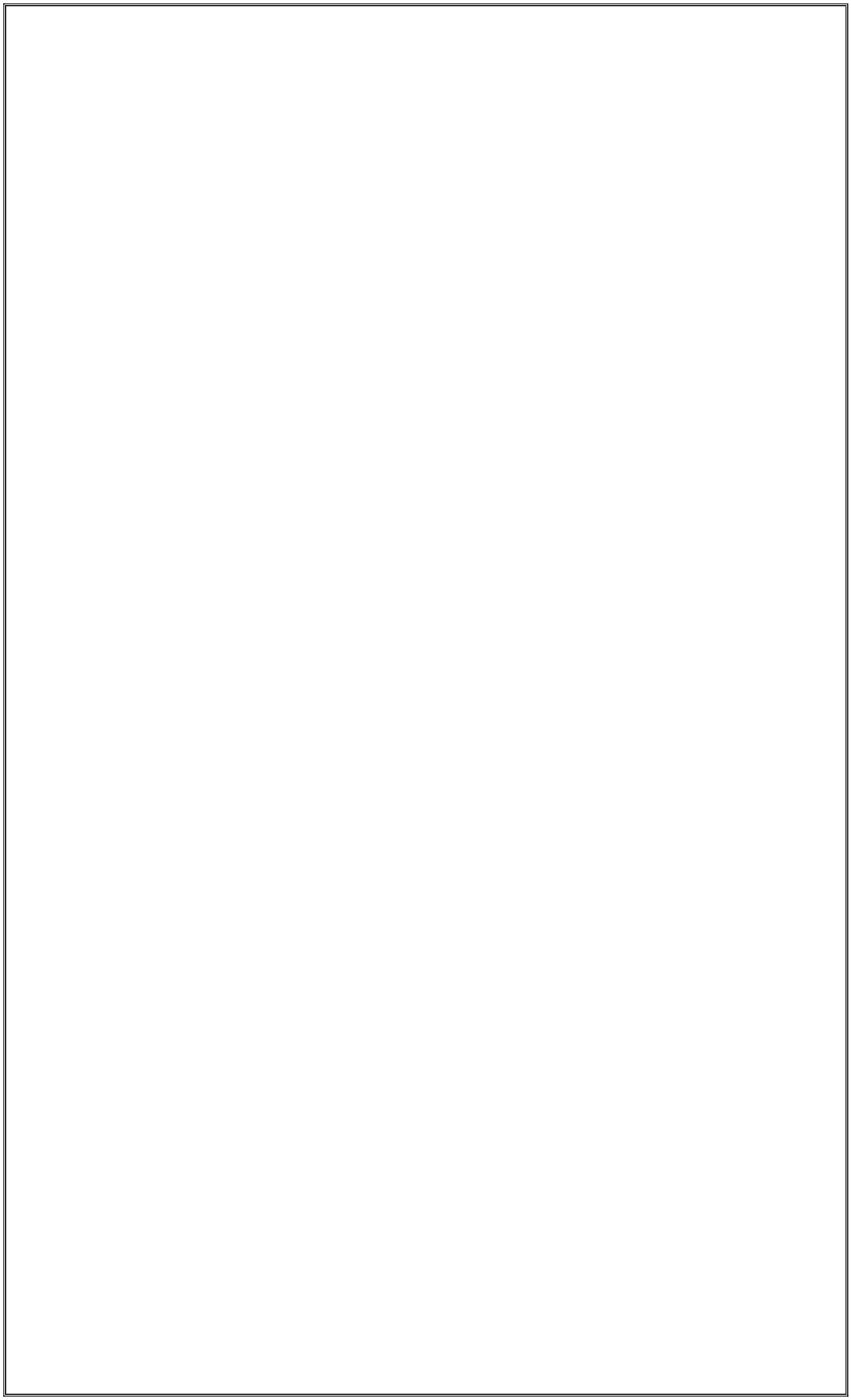


CHAPTER-1

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium* ,which are transmitted from one person to another by female *Anopheles* mosquitoes. When a malaria parasite infected mosquito bites a healthy person, the parasites are released into blood and infect the liver cell, which results in the burst of the liver cells and release of thousands of new parasites to enter the bloodstream and infect red blood cell. Five species of *Plasmodium* can infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae and P. knowlesi* . Infection is most commonly caused by *P. falciparum and P.vivax*, the former causing the most severe form of malaria. *P. falciparum* can cause severe malaria because it multiples rapidly in the blood, and results in severe blood loss (anemia). The infected parasites can also clog small blood vessels in the brain, which results in cerebral malaria. In case of *P. vivax*, it has dormant liver stages, known as hypnozoites, that can relapse and invade the blood several months or years after the bite of infected mosquitoes.

According to WHO, in 2016, about 445, 000 people died from the disease globally with African Region accounted for 91% of all malaria deaths in 2016, followed by South- East Asia Region (6%) [1].

Malarial infection begins when a female Anopheles mosquito bites a person, injecting *Plasmodium* parasite, in the form of sporozoites into the blood stream. The sporozoites travel to the liver, multiplying asexually over the next 7–10 days. During this time there are no symptoms. The parasites, now in the form of merozoites, emerge from the liver cells in vesicles and travel through the heart to the capillaries of the lungs. The vesicles eventually disintegrate, releasing the merozoites to enter the bloodstream where they invade and multiply in erythrocytes. When the cells burst, the fresh merozoites that are released invade more erythrocytes. Clinical symptoms, including fever, occur in synchrony with the rupture of infected erythrocytes and the release of erythrocyte and parasite debris, including malarial pigment (hemozoin) and glycophosphatidylinositol, the putative ‘malaria toxin’ [2].

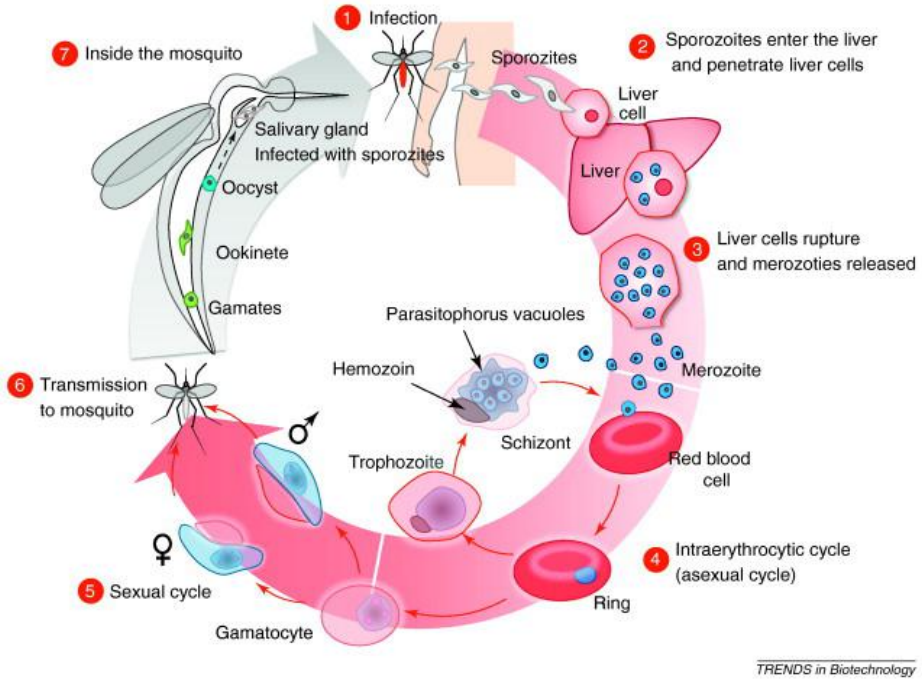


Fig-1:Life cycle of Malaria parasite

Several antimalarial drugs were utilized in the past to control the mortality and morbidity rate of malaria across the World. During 1990s, resistance to available antimalarial drugs such as chloroquine and sulfadoxine–pyrimethamine worsened across various parts of the world where malaria is endemic. As a consequence, morbidity and mortality associated with malaria increased. In 2005, the World Health Organization (WHO) recommended that artemisinin-based combination therapies to be used as first-line treatments for *falciparum* malaria in all countries where malaria was endemic [3]. The artemisinin resistance in *Plasmodium falciparum* is emerging as one of the biggest problems. Efficacy of the antimalarial artemisinin is decreasing throughout Cambodia and other parts of Southeast Asia [3]. The drug resistance phenotype presents as persistence, or delayed clearance, of ring-stage parasites in the peripheral blood after drug treatment that can clear susceptible parasites [4]. In fact artemisinin is more effective when parasites are actively metabolizing haemoglobin in the trophozoite stage. The mechanisms by which there occurs transient slow down in the cell cycle and how the resistant alleles allow recovery remain unclear [5].

The human malaria parasite *Plasmodium falciparum* is auxotrophic for most amino acids. The requirement of amino acids are met largely through the degradation of host erythrocyte hemoglobin. However the parasite cannot get isoleucine, because this amino acid is not present in adult human hemoglobin. The parasite depends on exogenous sources for the isoleucine. When there is no exogenous sources for

isoleucine, the parasites dramatically slow its cell cycle and exhibits decreased protein synthesis and metabolic activity. The parasite can remain in this state for several days and resume normal growth upon isoleucine supplementation [6]. A likely candidate to govern these responses is the target of rapamycin complex 1 (*TORC1*) pathway, as it is known to integrate a range of positive and negative growth signals, most notably the presence of amino acids, to drive or inhibit cellular growth [7]. The *TORC1* pathway is highly conserved throughout eukaryotes and was likely a central signaling hub in the last eukaryote common ancestor [8]. A factor associated with the TORC1 pathway is the RNA polymerase III regulator Maf1, which acts in nutrition depletion condition. Maf1 was originally discovered in budding yeast. In this organism, under optimal growth conditions, Maf1 is phosphorylated by a protein kinase A and TOR/SCH9-dependent manner, whereas under stress or in nutrient limiting conditions it becomes dephosphorylated and translocates to the nucleus to repress Pol- III transcription [9]. In yeast, Maf1 is one of the most important genes for maintaining viability during long-term starvation in stationary phase [10]. A putative *Maf1* ortholog appears to be conserved in the *Plasmodium* genus [11]. *P. falciparum* with defective *Maf1* expression is unable to regulate Pol III activity or to maintain viability during the dormancy-like state induced by isoleucine starvation. This mutant displays additional growth and recovery defects for a range of growth-inhibiting forms of stress. Furthermore, an artemisinin-resistant isolate displays more effective Pol III regulation and increased survival upon amino acid starvation, suggesting that *PfMaf1* remains a downstream effector of growth regulation pathways in *Plasmodium falciparum* despite its loss of *TORC1* [11].

To know the localization of Maf1 protein of *Plasmodium species*, we have taken *Plasmodium berghei* as the model organism. *Plasmodium berghei* is a rodent parasite that infects rodents . The aim of using rodent malaria parasites is that they are practical models for the experimental study of mammalian malaria. These parasites are analogous to the malaria of human parasite in most essential aspects of structure, physiology and life cycle [12]. Due to the characteristic features like (1)similarity in the basic biology of rodent and human parasite, (2) genetics, genome organization, housekeeping genes and biochemical processes are conserved between rodent and human parasite, (3) the molecular basis of drug sensitivity,resistance,the structure and function of vaccine candidate target antigens are conserved betwwen rodent and human parasite, (4) rodent parasite allow *invivo* investigations of parasite host

interactions and *invivo* drug testing,rodent parasites are recognized as valuable model parasites for the experiments.

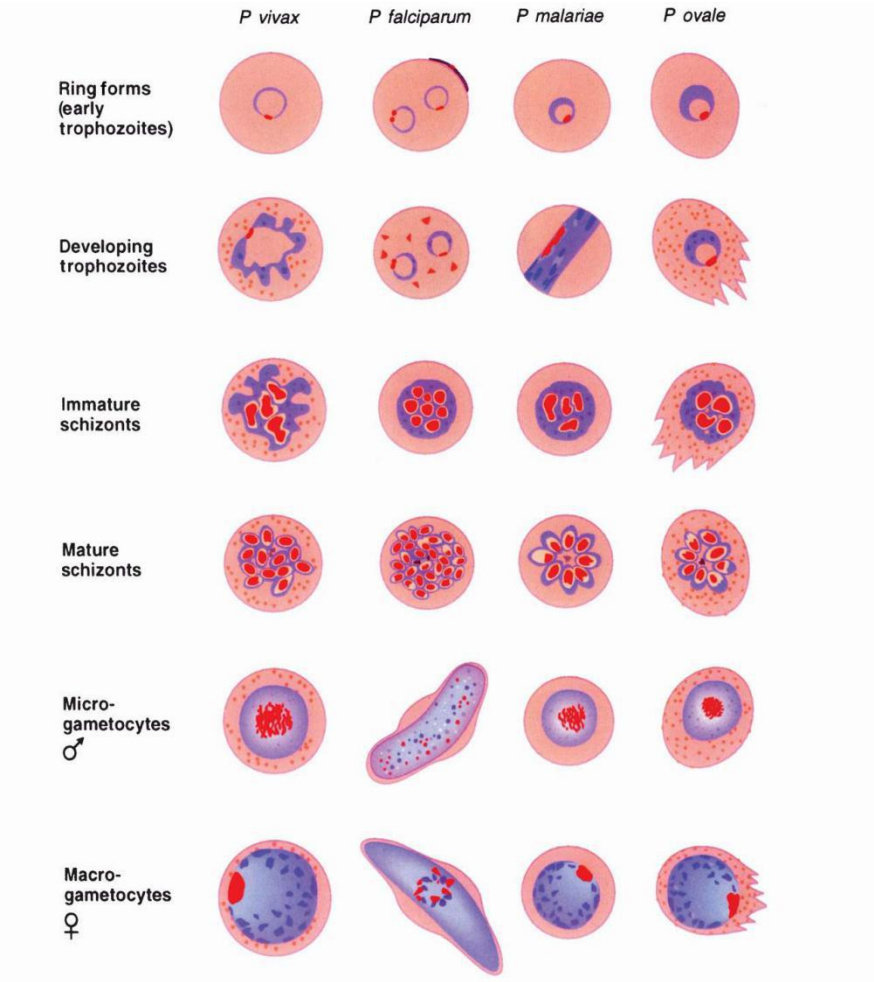


FIG 2:-Asexual stage and gametocytes of malaria parasite

1.1 History of malaria:

Malaria occupies a unique place in the annals of history. Over millennia, its victims have included Neolithic dwellers, early Chinese and Greeks, princes and paupers. In the 20th century alone, 150 million and 300 million people died due to malaria.This was approximately 2 to 5 percent of total deaths [13]. The symptoms of malaria were described in ancient Chinese medical writings. During 2700 BC, several characteristic symptoms of malaria were described in the *Nei Ching*[14]. Earlier it was believed that malaria was caused by bad air ("mala aria" in Italian) from marshlands [15]. On October 20, 1880,when Charles Louis Alphonse Laveran was looking through a crude

microscope at the blood of a febrile soldier, he saw crescent-shaped bodies that were nearly transparent except for one small dot of pigment. Later the brownish-black pigments were known as hemozoin, the product of hemoglobin digestion by the malaria parasite. He ultimately recognized four distinct forms in human blood, corresponding to different stages of malaria parasite lifecycle male gametocyte, schizoint and trophozoite stages. This discovery led him to be awarded with Nobel prize in 1907. Camillo Golgi linked the rupture and release of asexual malaria parasites from blood schizonts with the onset of every third- and fourth-day fever due to *P. vivax* and *P. malariae,* respectively [16]. Giovanni Batista Grassi and Raimondo Filetti, the Italian investigators, first introduced two malaria parasites, named *Plasmodium vivax* and *P. malariae* that affect humans. Laveran had believed that there was only one species that cause malaria, named *Oscillaria-malariae*. William H. Welch, reviewed the subject and in 1897, he named the malaria parasite as *P. falciparum.* In 1922, the fourth human malaria parasite, *P. ovale* was described by John William Watson Stephens. In 1931, *P. knowlesi* was first described by Robert Knowles and Biraj Mohan Das Gupta [14]. On 20 August 1897,Sir Ronald Ross made his landmark discovery. While dissecting the stomach tissue of an Anopheline mosquito, that was fed four days previously on a malarious patient, he found the malaria parasite and prove the role of *Anopheles* mosquitoes in the transmission of malaria parasites in humans [17]. The French chemists Joseph Pelletier and Jean Biename Caventou isolated quinine from cinchona bark in 1820. Quinine quickly became a major therapy for intermittent fever throughout the world. Despite of sporadic observations of quinine resistance, quinine remains as an important and effective malaria treatment nearly worldwide to the present day. Following World War II, chloroquine and DDT emerged as the two principal weapons in the WHO's ambitious “global eradication” malaria campaign [16].

1.2 Scope and Objectives:

The decrease in efficacy of antimalarial Artemisinin led to propose that *Plasmodium* may be able to enter the state of dormancy which results in programmed cell cycle arrest and drug resistance. During starvation parasite does not exit the cell

cycle but enters a state of slow growth.This change in growth rate occurs only if isoleucine is removed because parasite depends on exogenous sources for isoleucine. Parasites undergoing starvation is induced by Maf1, the global repressor of RNA polymerase III [28]. Maf1 acts as a key player to induce dormancy which results in drug resistance. To overcome the drug resistance,the initial step is to stop the parasites from entering into the dormancy stage. It can be possible if we inhibit the function of MAF1 by drug target. Drug can be targeted if the proper localization of the Maf1 is known. The major objective of present study is to determine the localization of the Maf1 protein in *Plasmodium.*

1.3 Overview of Dissertation:

In this study, total RNA was isolated from the parasite pellet followed by RT PCR to produce cDNA. cDNA was inserted into the plasmid and was transformed in NBT competent cell. Plasmid was isolated from the transformed cells and sequenced to check the alignment and DNA sequence.The Plasmid without any mutations was used for protein overexpression. Large scale protein induction was done, the recombinant protein was purified and injected into the mouse for the production of antibody. After the second booster small quantity of blood was collected to check the antibody by Western blotting. Western blotting confirmed the presence of PbMAF1 antibody and immunofluroscence was done to know the localization of PbMAF1 protein.

CHAPTER-2

REVIEW OF LITERATURE

Malaria remains as important public health concern in all over the world, specially where transmission occurs regularly. Malaria is a complex disease that varies widely in epidemiology and clinical manifestation in different parts of the world [18]. *Plasmodium* the single causative agent of malaria belongs to the phylum Protozoa and subphylum Apicomplexa. *Plasmodium* is a single-celled organisms that cannot survive outside of their host. More than 200 species of the genus *Plasmodium* have been identified that are parasitic to reptiles, birds and mammals. Four *Plasmodium* species have been well known to cause human malaria, namely, *P. falciparum, P. vivax, P. ovale, and P. malariae.* A fifth one, *P. knowlesi,* has been recently documented to cause human infections in many countries of Southeast Asia [19].

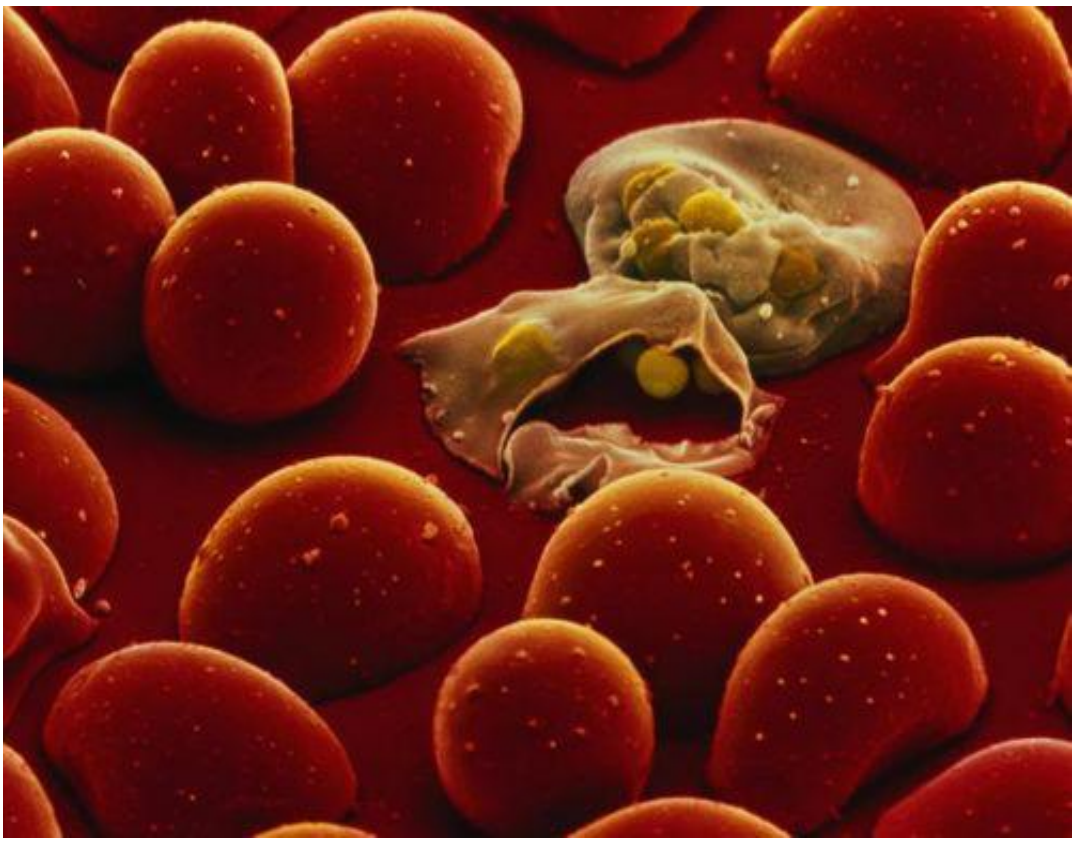


Fig 3:-Ruptured Red blood cell infected with *Plasmodium.*

Members of the genus *Plasmodium* belongs to the eukaryotic domain. Therefore, the cell and molecular biology of *Plasmodium* will be similar to other eukaryotes. The *Plasmodium* genus contains ~5500 genes distributed across 14 chromosomes and

approximately 80% is AT rich region [20]. Endoplasmic reticulum (ER) is attached to the nucleus, which functions similarly to the ER in other eukaryotes. Proteins are trafficked from the ER to the Golgi apparatus which generally consists of a single membrane-bound compartment in Apicomplexans [21]. *Plasmodium* contains three apical secretory organelles i.e micronemes, rhoptries, and dense granules, that are required for the infection of new host cells [22]. *Plasmodium* possesses two organelles of endosymbiotic origin: non-photosynthetic plastid (the apicoplast), and a mitochondrion, which together contribute to the parasites' metabolic needs. It possess a single mitochondrion whose biogenesis coordinates with the cell-cycle. The *Plasmodium* mitochondrion is capable of generating energy in the form of ATP via citric acid cycle; however, this function is only required for parasite survival in the insect host, and is not needed for growth in red blood cells [23].

The current scenario is very grave because malaria remains a major health threat as 446,000 people died in 2017 all over the globe.Till now there is no vaccine available for this disease. In addition to this, drug resistance is a major concern. The artemisinin resistance in *Plasmodium falciparum* emerge as one of the biggest problem because, artemisinin is more effective when parasites are actively metabolizing haemoglobin in the trophozoite stage [5]. Due to some factors parasites enter the state of dormancy, which results in the arresting the cell cycle and decreasing metabolic activity to limit damage and resume growth when drug concentration decreases below effective level. The most appreciated factor responsible for the dormancy is MAF1 protein, repressor of RNA polymerase III. The MAF1 protein is conserved from yeast to human [24]. MAF1 protein was first identified in yeast. Yeast MAF1 acts as a repressor of RNA Polymerase III in a TFIIIB dependent manner [25]. Maf1 proteins in all species share three regions of high similarity, named A, B, and C box. Across all species examined, Maf1 has been shown to localize to both the cytoplasm and nuclear cellular compartments. As the role suggests, Maf1 is as a negative regulator of transcription, it is expected that its primary function will be in the nucleus. This suggests that nuclear/cytoplasmic transport is one of the mechanisms of regulating Maf1 activity [25].. In growing yeast, Maf1 is phosphorylated and localized in the cytoplasm. Stress conditions lead to Maf1 dephosphorylation and nuclear import , which is directed by two nuclear localization signal (NLS) sequences . In the nucleus, Maf1 binds Pol III to prevent its interaction with TFIIIB and promoters and also binds Brf1, a subunit of TFIIIB [26]. CK2 kinase,

which is present directly on the Pol III complex and ensures a high rate of transcription via phosphorylation of both Maf1, TFIIIB and other Pol III components . When cells encounter unfavorable growth conditions, the CK2 catalytic subunit dissociates from the Pol III complex and is no longer able to stimulate transcription. Moreover, dephosphorylated Maf1 is imported from the cytoplasm increasing its concentration in the nucleus. This is the time when Maf1 takes over control and inhibits transcription [27].

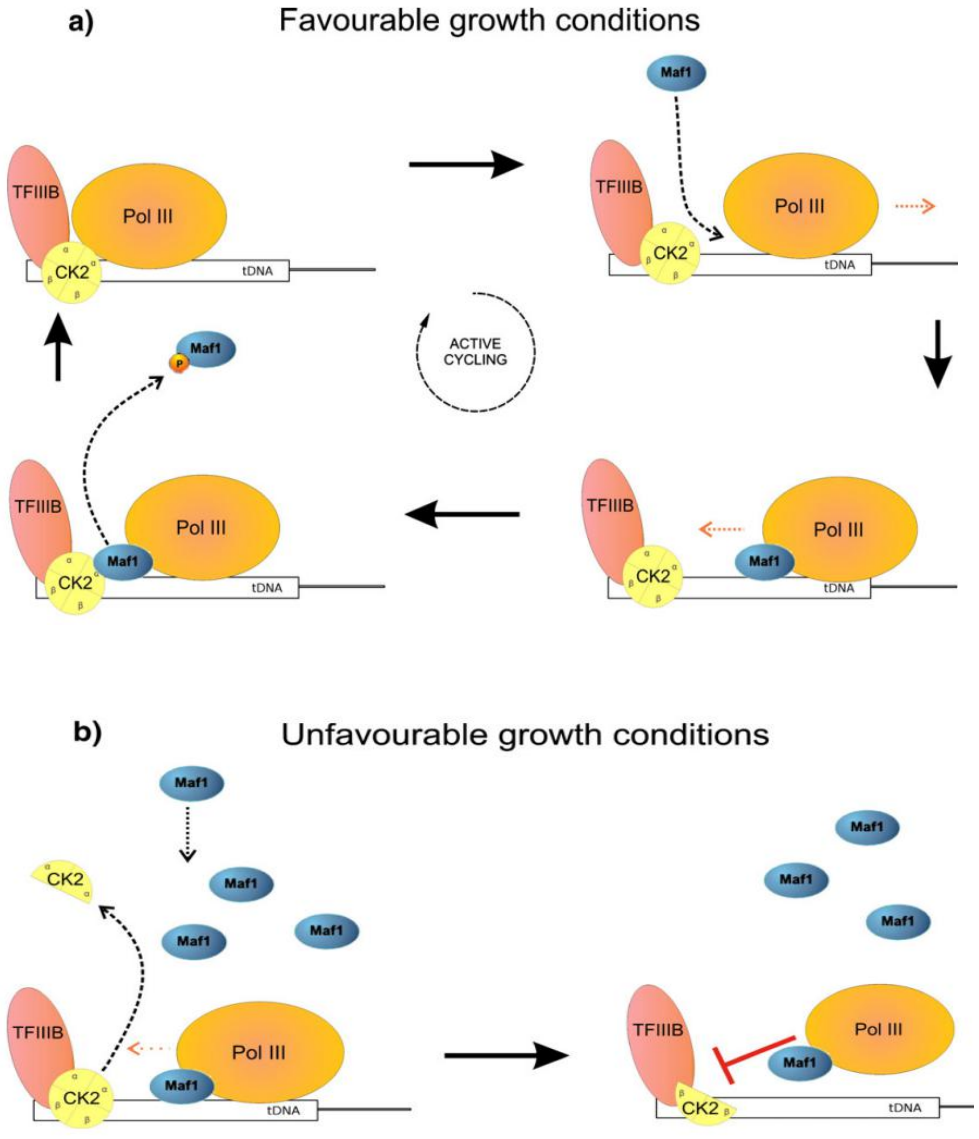


Fig 4-Mode of RNA III polymerase regulation by MAF1

P. falciparum gene is a functional ortholog of yeast *Maf1*.

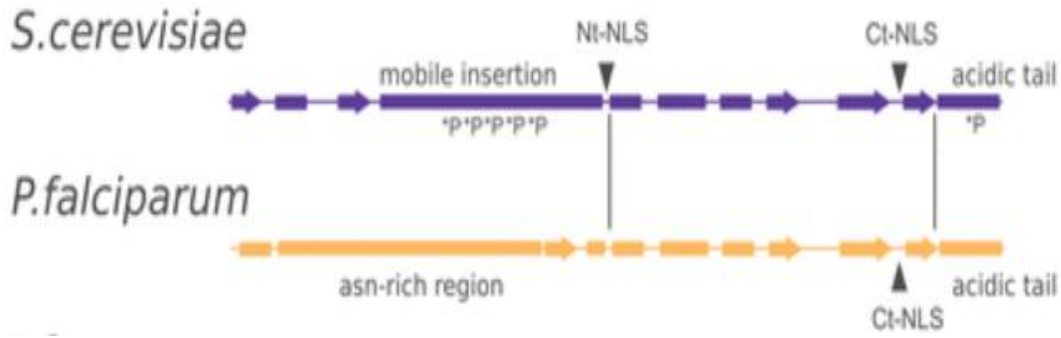


Fig 5:-Structural features of the Yeast and Plasmodium MAF1 orthologs.

Nt- N terminus,Ct- C terminus, NLS- nuclear localization signal.

Vertical lines indicate the homologous region exchanged to generate the chimera for complementation.

*P- Site of Phosphorylation in the yeast protein [11].

So,may be the mode of action of MAF1 in *Plasmodium falciparum* will be same as Yeast.

CHAPTER-3

AIMS AND OBJECTIVES

Aim of this study is to determine the localization of MAF1 protein in *Plasmodium berghei.* The major objectives that were fulfilled to achieve the aim of study are as follows:

- Isolation of mRNA from *Plasmodium berghei* followed by RT-PCR and amplification of PbMAF1 CDNA.
- Cloning of PbMAF1 CDNA in pRSETA vector followed by transformation.
- Isolation of plasmids from transformed colonies and overexpression of recombinant PbMAF1.
- Induction and purification of Large scale protein
- Injection of the purified fraction of the protein into the mice for the production of antibody against MAF1.
- Confirmation of antibody reactivity by Western blotting.
- Determination of localization of MAF1 protein in *Plasmodium berghei* by Immunofluroscence.

CHAPTER-4

MATERIALS AND METHODS

List of chemicals:-

Chemical Reagent	Company
Acrylamide	Sigma
Ammonium Persulfate	Merck
β -Mercaptoethanol	Sigma
Bis-acrylamide	Sigma
Comassie Brilliant Blue	Sigma
Bromophenol Blue	Sigma
Glycerol	Merck
Imidazole	MP biomedical
IPTG	MP biomedical
Prestained Protein Marker	Puregene
SDS	Sigma
TEMED	Sigma
Cutsmart buffer	NEB
BamHIHF	NEB
EcoRIHF	NEB
BCIP	MP biomedical
NBT	MP biomedical
RNA extraction kit	Qiagen
Ethidium bromide	Himedia
Unstained Protein Marker	Puregene
Phusion polymerase	NEB
Ligase enzyme	NEB
Ligase buffer	NEB
T7 Forward Primer	IDT
T7 Reverse Primer	IDT
DMSO	NEB
Agarose	Sigma
Gel purification kit	Qi quick

Tetracycline	SRL
Ampicillin	SRL
Chloramphenicol	SRL
Trypton powder	Himedia
Yeast extract powder	Himedia
NaCl	Himedia
Agar medium	Himedia
Gel elution kit	Qiagen
Plasmid isolation kit	Qiagen
GC buffer	NEB
$MgCl_2$	NEB

Instruments:

List of instruments:-

Name	Company
Thermo cycler	Proflex
Centrifuge	Thermoscientific
Incubator	Labnet scientific
UV illuminator	Cleaver scientific
Ultra Sonicator	Sonics vibra cell
SDS Gel apparatus	Bio-bee
Western blot apparatus	Bio-bee
Gel electrophoresis apparatus	Bio-bee
Nanodrop	Thermofisher
Inverted fluroscence microscope	Olympus

Bacterial cells:- *Escherichia coli* NBT and Rosetta strain

Expression vector:-pRSETA

PbMAF1(F):-5'GAAAGGATCCATGATAAGCTTAGATATCGAAAACCTTAATG3'

PbMAF1(R):-3'GCCCGAATTCTTATGAACTAGATGAATAATTTTCGTCATAAT5'

METHODS:-

4.1 RNA extraction from *Plasmodium berghei:*

Qiagen kit Method:

Parasite pellet was taken and resuspended in 350µl of RLT buffer. It was vortexed for 30 second. Lysate was transferred to gDNA eliminator and centrifugation was done at 12000 rpm for 30 sec.The flow through was transferred to another eppendorf.350µl of 70% ethanol was added and resuspended for 7-8 times immediately. 700 µl of the sample was added to RNeasy spin column and incubation was done for 3 min.Then Centrifugation was done at 12000 rpm for 30 sec. Flow through was thrown. 700 µl of RW1 buffer was added to the sample and centrifuged at 12000rpm for 30sec. Flow through was thrown. 500µl of RPE buffer was added and centrifugation was done at 12000 rpm for 2 minutes(2 times). Flow through was thrown.Dummy spin was done for 1 min.collection tube was changed and elution was done with 30µl of RNase free water. Centrifugation was done at 12000 rpm for 1 min.RNeasy spin column was thrown and RNA was extracted from the pellet.

4.2 CDNA synthesis from RNA by Reverse transcriptase PCR:

All the components required for the reaction were kept out of -20ºc at room temperature. The components were immediately transferred into ice once they got thawed . The following cocktail was prepared for 2.5 reaction.

Table 4.2.1 :- CDNA Synthesis from RNA

Cocktail :25µl reaction volume

Sample A(µl)	Sample B(µl)
PbRNA -2.5	PbRNA -2.5
Random primer -1	PbMAF1(R)-1
A.M Water -15.26	A.M Water -15.26

Components	For 2.5 reaction
5×RT buffer	12.5µl
dNTPs	2.5µl
RT enzyme	0.62µl
Total	15.62/2.5=6.24µl

First water(15.26µl) was added to 2 tubes(A & B). Then Pb RNA(2.5µl) was added to each tube. Random primer was added to sample A and PbMAF1(R) Primer was added to sample B.The tubes were then vortexed and given a short spin. The PCR reaction was set up for 10min at 70ºc. Then Snapchill was done. Cocktail(6.24µl) was added to each tube and PCR was done at 42ºc for 1hour.CDNA was synthesized. Then PCR was done to amplify CDNA.

4.3 Polymerase chain reaction:

The main principle behind the polymerase chain reaction is the amplification of DNA.There are generally 3 basic steps involved in PCR:-

- Denaturation
- Annealing
- Extension

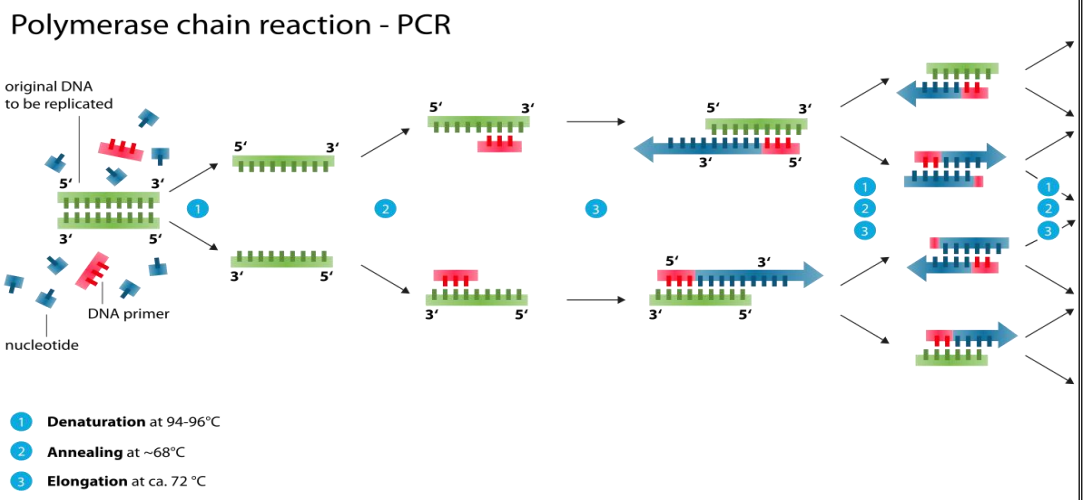


Fig 6:-Steps of Polymerase chain reaction

Protocol:-

Table 4.3.1:- Amplification of CDNA by PCR

PbMAF1-1	PbMAF1-2
Sample A-5µl	Sample B-5µl
PbMAF1(F)-1µl	PbMAF1(F)-1µl
PbMAF1(R)-1µl	PbMAF1(R)-1µl

All the components were kept out of -20ºc at room temperature. The components were immediately transferred into ice once they got

thawed. 2 eppendorf tubes were taken. Sample A was added to tube 1and sample B was added to tube 2. The following cocktail was prepared for 2.5 reaction.

Cocktail:-25µl reaction volume

Components	For 2.5 reaction
5×GC buffer	12.5µl
dNTPS	2.5µl
$MgCl_2$	3.125µl
DMSO	1.875µl
Phusion Polymerase	0.625µl
A.M Water	24.375µl
Total	45µl/2.5=18µl

It was added to each tube.

The tubes were then vortexed and given a short spin. The PCR reaction was set up in condition suitable for Phusion Polymerase:-

Denaturation :-98ºc,30 seconds

Final denaturation :-98ºc,10seconds

Annealing :-55ºc,30seconds

Extension :-72ºc,45seconds

Final Extension :-72ºc,10minutes

Hold :-4ºc,infinite hold

Then the Samples were run in agarose gel electrophoresis

4.4 Agarose Gel Electrophoresis:-

The principle behind this experiment is the separation of DNA fragments on the basis of their size. This separation can be visualized by the use of an intercalating agent EtBr. DNA being negatively charged moves towards the positive electrode(anode).

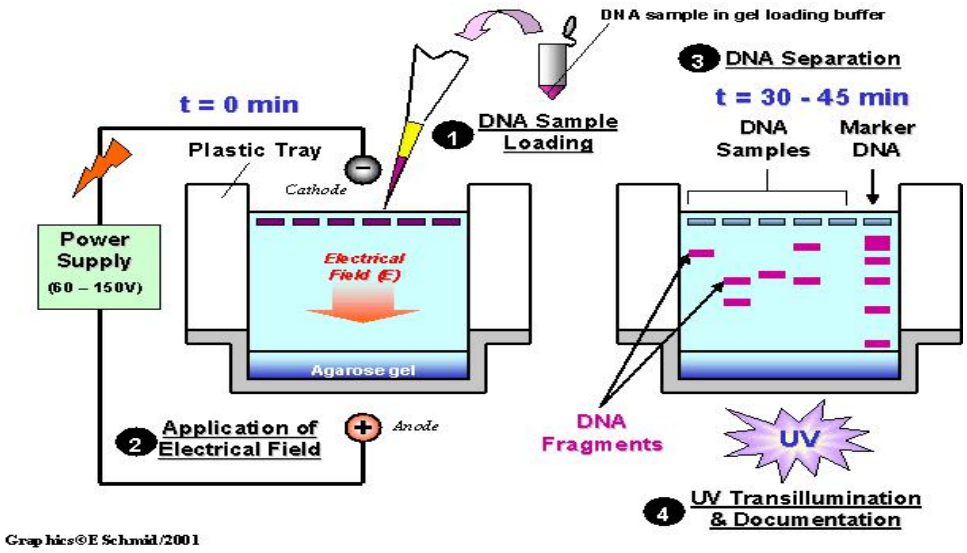


Fig 7:- Agarose gel electrophoresis

Protocol:-

Casting 0.7% Agarose gel:-

A 250 ml conical flask was taken and rinsed thoroughly with tap water,Milliq water and then by ELIX water. 7 ml of 10X TBE was diluted with 63 ml of elix water and 0.7 gm of agarose was taken and dissolved in 70 ml of 10X TBE and elix water solution. 1µl of EtBr was added when the solution was lukewarm. It was then poured to the Gel casting Tray and was allowed for 1hour to solidify. 350 µl 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

Loading of sample:-

1 kb ladder was taken out of -20ºc and kept for thawing.A small parafilm paper was taken and 3µl of DNA loading dye was added to the paper. Sample 1 & 3 were vortexed and spun down and 7 µl of sample was then mixed with the gel loading dye and added to the gel.The electrodes were connected and the gel was run at a voltage of 150 volts.

4.5 Gel Purification:-

Gel purification is used to recover DNA fragments after electrophoretic separation.

Protocol:-

1% agarose gel was prepared using autoclaved 10X TBE, autoclaved measuring cylinder(100ml) and autoclaved conical flask. The gel was then covered with aluminium foil to prevent any contamination and allowed to polymerise for 45 minutes. The gel tank was thoroughly rinsed with elix water, milliq and autoclaved

MQ. 350 ml of 1X running buffer was prepared using autoclaved 1X TBE,autoclaved MQ. The PCR samples were taken out of -20ºc and were allowed to thaw.

Sample preparation was done:- 75µl of sample+15µl of DNA loading dye.

The gel apparatus was then kept in cold room and 90µl of sample was added to the well. 10 µl of 1kb DNA ladder was added.The gel was run in the cold room to prevent any DNAse activity. It was run at 80 volts.After the gel was run 3/4th of the casting tray, it was removed and the following procedure was followed to elute the DNA from the gel.

Gel elution:-

The gels were sliced and kept in a 1.5ml of ependroff 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.**

QIA quick gel extraction kit method:-

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.

4.6 Restriction Digestion:-

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.

The two restriction endonucleases chosen for the digestion of Maf1 DNA were-*BamHI*-GGATCC(F)

EcoRI - GAATTC(R)

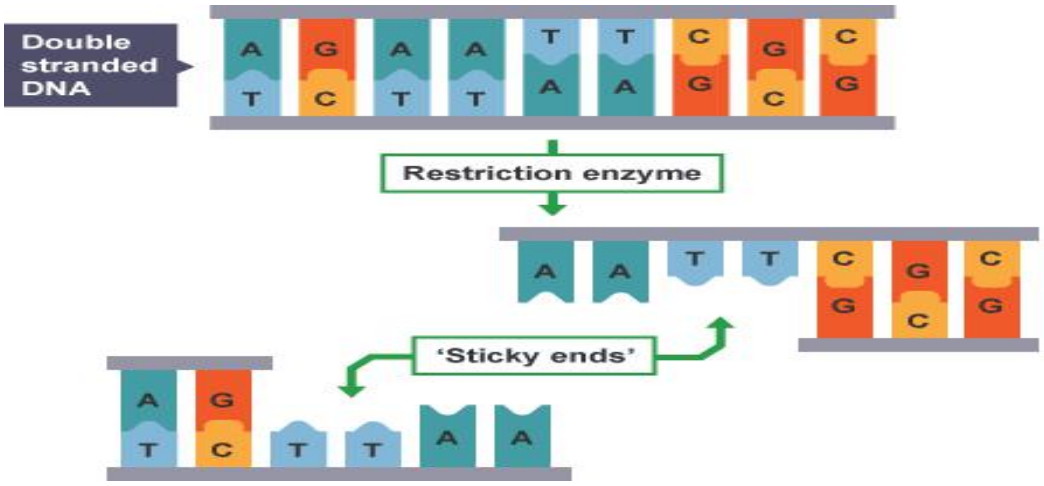


Fig 8:-Restriction digestion of double stranded DNA.

Protocol:-

After gel purification 26.5µl of PbMaf1 DNA was extracted. Double digestion was done for the DNA and vector. pRSETA vector was used for this process.Two ependroff tubes were taken. One for PbMaf1 and another for pRSETA.

<u>Table 4.6.1</u>:-Restriction digestion for Pb MAF1 and pRSETA vector.

Reagent	PbMaf1-26.5 µl	pRSETA-10µl
Cutsmart buffer	4 µl	2 µl
BamHIHF	2.5 µl	2 µl
EcoRIHF	2.5 µl	2 µl
Autoclaved milliq water	4.5 µl	4 µl
Total	40 µl	20 µl

Both the tubes were incubated at 37ºc for 12 hours.

4.7.Column purification:-

60µl of autoclaved MQ was added to Pb Maf1 tube and 80 µl of autoclaved MQ to pRSETA tube 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.

4.8.Ligation:-

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.

Protocol:

The DNA ligase buffer and the eluted inserts and vectors were kept out of -20ºc to thaw. All the reaction mixtures were then vortexed,spun down and kept back in ice.

One ependroff tube was taken and all the components were added to it.

Table 4.8.1:-Ligation set up for the inserts and vectors

Components	For one reaction(µl)
pRSETA vector	3
PbMaf1	14
Ligase buffer	2
Ligase enzyme	1
Total	20

Then the tube was incubated for 12 hours at 16ºc.

4.9.Starter culture preparation:-

Protocol:-

The Laminar Air Flow was cleaned with 70% ethanol and all the materials were kept in UV for 20 minutes. The LB broth was then kept at room temperature and Tetracycline in ice.1 LB broth tube was taken and wiped with 70% ethanol. 3 µl 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 12 hours at shaker incubator.The stock was then stored back at -80ºc.

1% Inoculum Preparation:-

Protocol:

The Laminar Air Flow was cleaned with 70% ethanol and all the materials were kept in UV for 20 minutes. 4 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:-

4.10.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 10 minutes. 1.5 ml of culture from each tube was taken in a different eppendrof tube and was centrifuged at 5000 rpm, 5 minutes at 4^0C. The supernatant was then discarded inside the laminar air flow and the pellet was again resuspended with the remaining 1.5 ml of culture. This was followed by the centrifugation of tubes at 5000 rpm for 5 minutes at 4^0C.The supernatant was discarded and the pellet was resuspended in 1 ml of CCMB 80 buffer. The reaction mixture was then incubated in ice for 20 minutes and centrifuged at 3000 rpm,15 minutes at 4^0C.The supernatant was again discarded and the pellet was dissolved in 250 ml CCMB 80 buffer.The competent cells are now ready for transformation.

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 90 seconds to give heat shock. It was then again kept in ice for 3 minutes. 900 µl 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.

4.11.Colony PCR:-

5 LB tubes were taken from the cold room and kept at room temperature.

Similarly, 15 PCR tubes were taken and 17.5 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.

Table 4.11.1:-Cocktail preparation for colony PCR

Materials	For 15.5 reaction(µl)
Pb Maf1 forward primer	15.5
Pb Maf1 reverse primer	15.5
10X Tag buffer	38.75
$MgCl_2$	19.375
dNTPs	15.5
Taq polymerase	7.75
Total	112.375/15.5=7.25 for each tube.

The cocktail was the 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:95^0C,10minutes

Final denaturation:95^0C,30seconds

Annealing:55^0C,30seconds

Extraction:72^0C,1minute 15 seconds

Final extension:72^0C,10 minutes

1% agarose gel electrophoresis was done in order to cheak the product. Only those LB tubes were retained in the shaking incubator for which clones were visualized in the gel.(Tube 4,5 and 14 showed intense band). These tubes were incubated in the shaking incubator for 15hours.

4.12.Conformation of clones:-

Plasmid Isolation:

The LB tubes containing grown culture of clones were centrifuged at 5000 rpm for 5 minutes at 4^0C.The suparnatant was discareded and the pallet was the collected.this pallet was stored in -20^0C.The pellet was then kept at room temperature.200µl of resuspended buffer was taken out of 4^0C by titling the eppendrof tube.The resuspension buffer containing EDTA, RNase therefore is stored at 4^0C.The pellet was resuspended gently with the help of 200 µl pipette(15-20 times).The tubes were then incubated for 5 minutes at room temperature. 200 µl 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 13000 rpm for 10 minutes room temperature. The column was prepared.500 µl of column preparation solution was added to the mini column placed in a 2 ml collection tube. It was incubated for 2minutes and then centrifuged at 12000 rpm,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 12000 rpm for 1 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. 100µl of autoclaved milliQ water was then added to the center of the mini column and was then incubated for 2 minutes. This was followed by centrifugation at 13000 rpm for 1 minute.The elute is our DNA of interest or clone.These tubes were then stored at -20^{0}C.

Quantification by nanodrop:-

Maf1 Clone 1-173.7ng/µl

Clone 2-208.7ng/µl

Clone 3-264.4ng/µl

Restriction digestion to check the insert release:-

The clones were then digested with *BamHIHF* and *EcoRIHF* in order to check the insert release.

Table 4.12.1:-Restriction digestion to check the insert release

Components	pRSETA Maf1 C1-7µl	pRSETA Maf1C2-7µl	pRSETA Maf1C3-7µl
CS buffer	1µl	1µl	1µl
BamHIHF	1µl	1µl	1µl
EcoRIHF	1µl	1µl	1µl
Total	10µl	10µl	10µl

The reaction mixtures were then vortexed and spin down .The mixture was kept in 37^{0}C incubator for 12 hours.1% agarose gel electrophoresis was done to check the insert release for these clones.

4.13.Sequencing:-

The classical chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal dNTPs, and modified ddNTPs, the latter of which terminate DNA strand elongation. These chain-terminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified

ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines.

Protocol:-

Table 4.13.1:Reaction mixture prepared for the sequencing

The following mixer was prepared for the sequencing.

components	Tube1(µl)	Tube2(µl)
T7 forward primer	2	-
T7 reverse primer	-	2
sample	3	3
total	5	5

4.14.Small scale Protein Preparation:-

Starter culture preparation:-

Protocol:-

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 Chloramphenicol in ice. 1 LB broth tube was taken and wiped with 70% ethanol.3 µl of Chloramphenicol was added to the tube. The Rosetta 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 200 µl pipette and was added to the LB broth.The Rosetta 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.

1% Inoculum Preparation:-

Protocol:

The Laminar Air Flow was cleaned with 70% ethanol and all the materials were kept in UV for 20minutes. 4 LB broth tubes were taken.Each of them were wiped with 70% ethanol. 3ml of media was present in each tube and 3µl of Chloramphenicol was added to each tube. 30µl of the Rosetta starter culture was then added to each of the tubes. These tubes were then incubated at 30ºc for 4 hours.

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 10 minutes. 1.5ml of culture from each tube was taken in a different eppendrof tube and was centrifuged at 5000 rpm, 5 minutes at 4^0C. The supernatant was then discarded inside the laminar air flow and the pellet was again resuspended with the remaining 1.5 ml of culture. This was followed by the centrifugation of tubes at 5000 rpm 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 3000 rpm, 15minutes at 4^0C. The supernatant was again discarded and the pellet was dissolved in 250 ml CCMB 80 buffer. The competent cells are now ready for transformation.

Transformation:-

The 20 µl 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 30 minutes. The reaction mixture was kept at 42^0C in water bath for 90 seconds 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.

Colony Picking:-

12 LB broth tubes were taken. 3µl of Ampicillin and Chloramphenicol were added to the LB broth.Single Isolated colonies were picked from the transformed plate and put into the LB broth tubes.The LB tubes were incubated at 37ºc for overnight.

Stock Preparation:-

4 ependroff tubes were taken and 200 µl of 100% glycerol was added. After overnight incubation, 800µl of culture was added to the ependroff and resuspended properly. Then stored at -80ºc.

1% Inoculum preparation:-

1% inoculum was prepared from the incubated tubes. Total 15 LB tubes were taken for three different temperature. 5 tubes for each temperature.From the 5 tubes,4 tubes were used for IPTG induction and 1tube was not induced.

At 37ºc:

5 LB tubes(1U,1I,2I,3I,4I) were taken and 3µl of ampicillin and chloramphenicol was added. 30µl of culture from the starter tubes were added to the tubes and incubated at 37ºc for 4 hours. After 4 hours,3µl of IPTG was induced to 4 LB tubes and 1 tube was kept uninduced and kept in incubator. After 2 hours centrifugation was done and pellet was stored in -20ºc.

At 30ºc:

5 LB tubes(5U,5I,6I,7I,8I) were taken and 3µl of ampicillin and chloramphenicol was added. 30µl of culture from the starter tubes were added to the tubes and incubated at 30ºc for 6 hours. After 6 hours, 3µl of IPTG was induced to 4 LB tubes and 1 tube was kept uninduced and kept in incubator. After 5 hours centrifugation was done and pellet was stored in -20ºc.

At 18ºc:

5 LB tubes (9U,9I,10I,11I,12I)were taken and 3µl of ampicillin and chloramphenicol was added. 30µl of culture from the starter tubes were added to the tubes and incubated at 30ºc for 8 hours. After 8 hours, 3µl of IPTG was induced to 4 LB tubes and 1 tube was kept uninduced and incubated in 18ºc for 15 hours. After 15 hours centrifugation was done and pellet was stored in -20ºc.

Table 4.14.1:-Lysis buffer preparation for small scale protein preparation

Components	
50mm tris(PH-8)	250µl
500mm NaCl	625µl
2% glycerol	100µl
0.5% Triton	25µl
Aprotinin	5µl
Leupeptin	5µl
Pepstatin	5µl
Milliq water	3.985ml

Pellet of Maf1 (1U to 12I tube) was taken and 200 µl of lysis buffer was added to each pellet. Then sonication was done.In case of sonication ultrasonic energy is applied to sample to agitate and disrupt the cell membrane . (Amplitude-25%,Time-4sec,Pulse-1 second on,1 sec off)

After sonication,centrifugation was done at 13000 rpm for 20 min. 21µl of Supernatant was taken and addded to another ependroff. 7µl of 4X SDS dye and 1µl of β-Mercaptoethanol was added to the supernatant and heated for 10 min at 95ºC.

Then SDS-PAGE was done.

SDS-PAGE:-

PAGE, is an analytical method used to separate components of a protein mixture based on their size. To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. For this different protein molecules with different shapes and sizes, needs to be denatured(done with the addition of SDS) so that the proteins lost their secondary, tertiary or quaternary structure. The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size. After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).

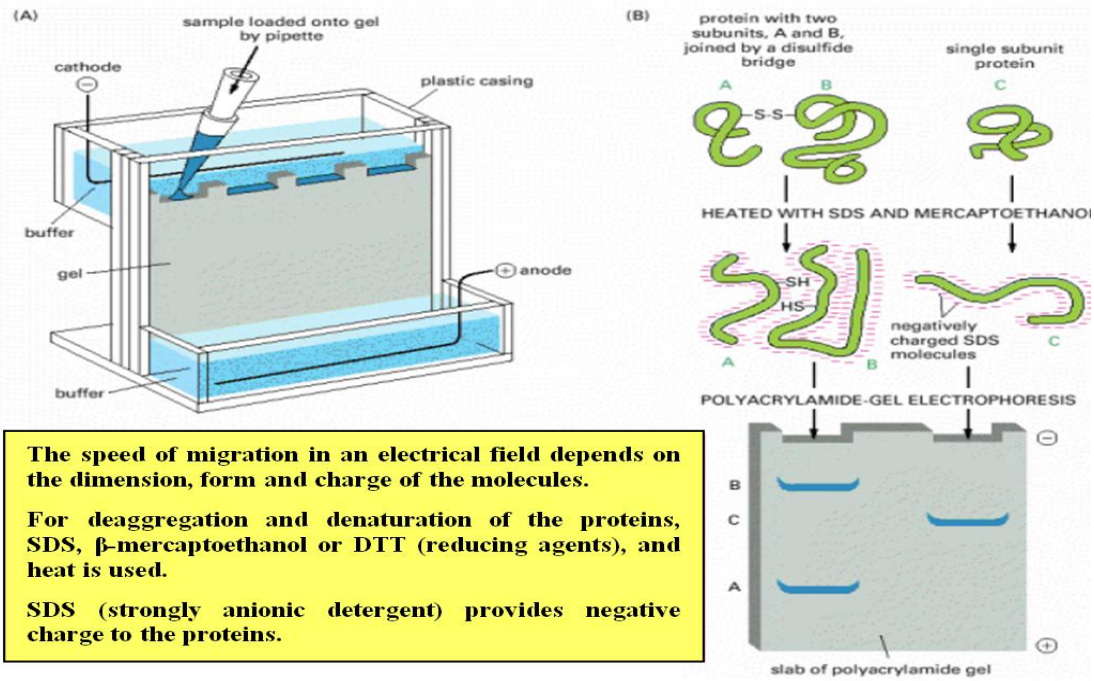


Fig 9:-Mechanism of SDS-PAGE

Protocol:-

Table 4.14.2:Composition of SDS gel

Components	Resolving gel(12%)	Stacking gel(5%)
Autoclaved Milli Q water	3.45ml	3ml
30% Acrylamide and Bisacrylamide	6ml	750µl
Tris(PH-8.8)	5.4ml	562µl
20% SDS	37.5µl	12µl
TEMED	19.5µl	9µl
10% APS	90µl	30µl

21 µl of sample was taken and addded to another ependroff. 7µl of 4X SDS dye and 1 µl of β-Mercaptoethanol was added to the sample and heated for 10 min at 95ºC.

10 µl of Protein prestain ladder was added to the first well of the gel and to the rest 21 µl of sample was added.The gel was run at 120 volt. After the gel run western blotting was done.

Western blot:-

It is an analytical method wherein a protein sample is electrophoresed on a SDS-PAGE and electrophoretically transferred to PVDF or Nitrocellulose membrane. The transferred protein is detected using primary and secondary labeled antibody. Antibodies bind specific sequence of amino acids, known as epitopes. A substrate that reacts with an enzymeis used to visualize the Protein/antibody complex.

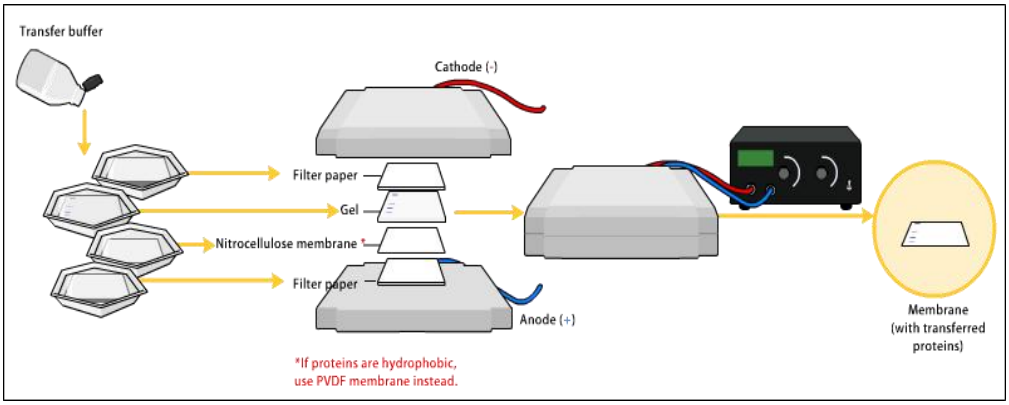


Fig 10:-Process of Western blotting

Protocol:-

The SDS gel was set for Western blot in a blotting apparatus and run for 3 hours at 10v. Then the membrane was blocked with skimmed milk (2gm skimmed milk powder in 40ml TTBS) for overnight in 4ºc. Membrane was then washed thrice with TTBS in the interval of 10 minutes. Primary antibody, anti-his tag antibody(1:3000) was added and stored for 3 hours. Then washed with TTBS for 3 times with the interval of 10 min each. 8µl of Goat-antimouse ALP conjugated secondary antibody and 20 ml of TTBS was mixed and added to the membrane for 2 hours. After 2 hours, washing with TTBS was done for 3 times with the interval of 10 minutes.

Signal Detection:-

The combination of NBT and BCIP yields an intense, insoluble black-purple precipitate when reacted with alkaline phosphatase enzyme,which is conjugated with the secondary antibody. BCIP is oxidized by NBT ,which forms an insoluble dark blue diformazan precipitate after reduction.First,10 ml of ALP buffer was added to the membrane and then 33 µl of NBT and 66 µl of BCIP was added.

After band was appeared ,the membrane was washed with Elix water.

4.15.Large scale Protein Preparation:-

Starter culture preparation:-

17 LB tubes were taken and 3µl of ampicillin and chloramphenicol was added to each tubes.A scoop of PbMaf1 pRSETA Rosetta stock was added.All tubes were incubated at 37ºc for 12 hours.

1% inoculum preparation:-

Four LB conical flask were taken.Each flask containing 1l of media. 1ml of ampicillin and chloramphenicol was added to each flask. 10 ml of starter culture was added to each flask. Incubated for 5 hours in 37ºc. After 5 hours,1ml of IPTG was added to each flask and kept in 37ºc for 2hours. After 2 hours,centrifugation was done at 7000 rpm for 5 minutes.The pellets were stored in -80ºc.

Protein Purification:-

Column Preparation:-

First the column was washed thrice by using Elix water and then with Milli Q water.

Then ethanol washing was done followed by Milli Q washing. Lastly,1.5ml of resin(Ni-NTA) was added to the column and kept overnight at 4ºc.Ni-NTA Provides a

simple method for purification of 6X-His tagged protein. Protein purification by Ni-NTA method based on the principle of Affinity Chromatography, where Ni-NTA resins act as stationary phase and 6X-His tagged protein from cell lysates are in the mobile phase.

The affinity of the Ni-NTA resin for 6X-His tagged protein is due to both the specificity of the interaction between Histidine residue and immobilized Nickel ion and the strength with which these ions are held to the NTA resin. Imidazole is the side chain of histidine amino acid and this binds to the Ni in the NTA resin. So,when imidazole is added to the column it competes with the His tagged protein for binding to the metal charged resin and protein is eluted out. A concentration of 300M imidazole in the buffer ensures complete elution of the His-tagged protein.

Buffer Preparation:-

Table 4.15.1:Lysis buffer composition for large scale protein preparation

Components	Column buffer	Lysis buffer	Wash buffer	Elution 1
50mmTris(ph-8)	2.5 ml	2.5 ml	1.25 ml	1.25 ml
500mm NaCl	6.25 ml	6.25 ml	3.125ml	3.125 ml
2% glycerol	1 ml	1 ml	500 µl	500 µl
1% Triton	500 µl	500 µl	250 µl	250 µl
Imidazole	-	-	500 µl(20M)	1.25 ml(50M)
Aprotinin	-	50 µl	-	-
Leupeptin	-	50 µl	-	-
Pepstatin	-	50 µl	-	-
A.M.water	39.75 ml	39.6 ml	19.375 ml	18.625 ml
Total	50 ml	50 ml	25 ml	25 ml

Components	Elution 2	Elution 3
50mm Tris(pH-8)	1.25 ml	1.25 ml
100mm NaCl	0.625 ml	0.625 ml
2% glycerol	500 µl	500 µl
0.1% Triton	25 µl	25 µl
Imidazole	2.5 ml(100M)	7.5 ml(300M)
A.M.Water	20.1 ml	15.1 ml
Total	25 ml	25 ml

Protocol:-

Cell pellet stored in -80^{0}c were resuspended in 50 ml of Lysis buffer and vortexed properly.Then sonication was done for 10 times (Pulse on-1 sec,Pulse off-1sec, Time-2min 20 sec,Amplitude-37%) and then centrifuged for 1hr 30 min, at 43000g at 4ºc and the supernatant was collected.

50ml of column buffer was passed in the Ni-NTA resin. Then 6xHis tagged protein was loaded in the column. When all the proteins were passed in the Ni-NTA resin,the Wash buffer, Elution 1,Elution 2 and Elution 3 buffer was loaded in the column serially and the flow through was collected. 25 ependroff tubes were taken and 5 ependroff tubes for each buffer (Wash buffer-Elution3) was taken and the flow through was collected.

For wash buffer-W_1,W_2,W_3,W_4,W_5

Elution 1-E_{11},E_{12},E_{13},E_{14},E_{15}

Elution 2-E_{21},E_{22},E_{23},E_{24},E_{25}

Elution 3-E_{31},E_{32},E_{33},E_{34},E_{35}

SDS-PAGE:-

Protocol:-

Composition of SDS gel:-

Components	Resolving gel(12%)	Stacking gel(5%)
Autoclaved Milli Q water	3.45 ml	3 ml
30%Acrylamideand Bisacrylamide	6 ml	750 µl
Tris(PH-8.8)	5.4 ml	562 µl
20% SDS	37.5 µl	12 µl
TEMED	19.5 µl	9 µl
10% APS	90 µl	30 µl

42 µl of sample was taken from each ependroff tube and addded to another ependroff. 14 µl of 4X SDS dye and 2µl of β-Mercaptoethanol was added to the sample and heated for 10 min at 95ºC. 10 µl of Protein unstain ladder was added to the first well of the gel and to the rest 29µl of sample was added. The gel was run at 120 volt. After that the gel was kept in Coomassie brilliant blue for overnight. Then Destaining was done to see the proper band.

Western blot:-

It is an analytical method wherein a protein sample is electrophoresed on a SDS-PAGE and electrophoretically transferred to PVDF or Nitrocellulose membrane. The transferred protein is detected using primary and secondary labeled antibody. Antibodies bind specific sequence of amino acids,known as epitopes. BCIP/NBT substrate that reacts with ALP to visualize the Protein/antibody complex.

Protocol:-

In another gel, 29 µl of sample and 7 µl of protein prestain ladder was added for western blotting. Then the gel was set for western blot in a blotting apparatus and run for 3 hours at 10v. Then the membrane was blocked with skimmed milk (2gm skimmed milk powder in 40ml TTBS) for overnight in 4ºc. Membrane was then washed thrice with TTBS in the interval of 10minutes. Primary antibody,Anti-his tag

antibody(1:3000) was added and stored for 3hours.Then washed with TTBS for 3 times with the interval of 10 min each.8µl of Goat-antimouse ALP conjugated secondary antibody and 20ml of TTBS was mixed and added to the membrane for 2 hours.After 2 hours,washing with TTBS was done for 3 times with the interval of 10 minutes.

Signal Detection:-

The combination of NBT and BCIP yields an intense, insoluble black-purple precipitate when reacted with alkaline phosphatase enzyme, which is conjugated with the secondary antibody. BCIP is oxidized by NBT, which forms an insoluble dark blue diformazan precipitate after reduction. First,10ml of ALP buffer was added to the membrane and then 33µl of NBT and 66µl of BCIP was added.

After band was appeared ,the membrane was washed with Elix water.

4.16.Preparation of sample for injecting mice:-

1.2 ml of protein was taken.Then 1.2 ml of Freund's complete adjuvent was added and vortexed for 20 minutes.

Immunization to mouse:-

Seven BALB/C mice was taken and anesthesized with ketamine xylazine solution. 200µl of ketamine xylazine solution was injected to each mice. Then 300µl of protein adjuvent complex was injected to each mice subcutaneously.

First booster:-

After 21 days first booster was given.Again large scale protein preparation was done. 1050µl of protein was taken and 1050 µl of Freund's incomplete adjuvent was added and vortexed for 20 minutes. Then 300µl of protein adjuvent complex was injected to each mice subcutaneously.

Second booster:-

After 7 days second booster was given. 1050µl of protein was taken and 1050 µl of Freund's incomplete adjuvent was added and vortexed for 20 minutes. Then 300 µl of protein adjuvent complex was injected to each mice subcutaneously.

After 3 days of second booster 200 µl of blood from each mice was collected. Blood was kept in cold room for overnight to separate the sera from the red blood cells. Serum from the above the blood was collected and the red blood cells were centrifuged at 2000 rpm for 5 minutes. Then the serum was collected and again the

red blood cells were centrifuged at 12000 rpm for 3 minutes.Serum was collected and tored in -22ºC.

After 3 days of partial bleeding third booster was given. 1050 µl of protein was taken and 1050 µl of Freund's incomplete adjuvent was added and vortexed for 20 minutes. Then 300 µl of protein adjuvent complex was injected to each mice subcutaneously.

Collection of blood from the mice:-

After 7 days of third booster, bleeding was done. Blood was collected and kept in cold room overnight to separate the sera from the red blood cells. Serum from the above the blood was collected and the red blood cells were centrifuged at 2000 rpm for 5 minutes.Then the serum was collected and again the red blood cells were centrifuged at 12000 rpm for 3 minutes.Serum was collected and tored in -22ºC.

4.17.Western blotting for the confirmation of PbMAF1 antibody:-

PbWT parasite pellet was taken and lysed in lysis buffer(Table-8) and sonicated for 2 times.Then centrifuged at 15000rpm for 20 minutes.

21 µl of supernatant was added to 7 µl of 4X SDS and 1µl of β-mercarptoethanol.

Sample was heated for 10 minutes at 95ºC. Then the samples were loaded in SDS-PAGE. Then the gel was set for western blot in a blotting apparatus and run for 3 hours at 10v. The nitrocellulose membrane was blocked with skimmed milk for 3hours in room temperature. The membrane was washed for three times with TTBS. Then primary antibody was added to the blot and kept in 4ºC for overnight. Membrane was washed for five times and secondary antibody was added and kept in room temperature for 3hours. After 3 hours the membrane was washed for five times and developed in dark room.ECL solution was used to develop the band. Above the nitrocellulose membrane ECL solution 800 µl was added and x-ray film was kept over it. After 2 min the x-ray film was developed in developer folllowed by washing in water and the fixed in the fixer.

4.18.Immunofluroscence:-

Immunofluorescence is a procedure to detect antigens in cellular contexts using antibodies. The specificity of antibodies to their antigen is the base for immunofluorescence.

Two cover slip was taken, 50 µl of Poly-L-lysine was added for coating and kept for 20 minutes. The cover slips were washed with autoclaved milli q water for 2 times in 10 minutes interval. Blood was collected from the wild type parasite infected mice in heparin. Blood was centrifuged for 3minutes at 2000 rpm to remove the Plasma, then washed for 3 times in filtered PBS. 1 ml of PBS was added and resuspended properly. Then the RBC was overlied on Poly-L-lysine coated cover slip and kept for 1hour. Unbound RBCs were removed and washed with 2ml PBS for 3times in the rocker. 2 ml of fixing solution was added to the coverslip and kept for 30min in dark. Then washed with 2ml of filtered PBS for 2 times in the interval of 5 minutes. 2ml of permeabilization solution was added and kept for 10 minutes. After 10 min washed with 2ml PBS for 2 times. Then 1.8ml of Quenching solution was added and kept for 10 min. After 10 min the coverslip was washed with 2 ml PBS for 2 times in the interval of 5 min. Then 2 ml of blocking solution was added to the coverslip on the rocker for 3 hours. 250 µl of primary antibody i.e Pb MAF1 antibody was added to the coverslip and incubated for 4 hours. After 4 hours the coverslip was washed with filtered PBS for 1 hour in every 10 minutes. Then secondary antibody (mouse IgG FITC) was added and kept for 3 hours. After 3 hours coverslip was washed for 3 to 4 times with PBS. 2 ml of DAPI was added to the coverslip and kept for 15 min. Then washed for 4 times with PBS. After washing the coverslip was kept for drying on parafilm. Then the cover slips was mounted on fluoroshield and observed in inverted fluroscence microscope.

CHAPTER-5

RESULT

5.1.Confirmation of cDNA by PCR:-

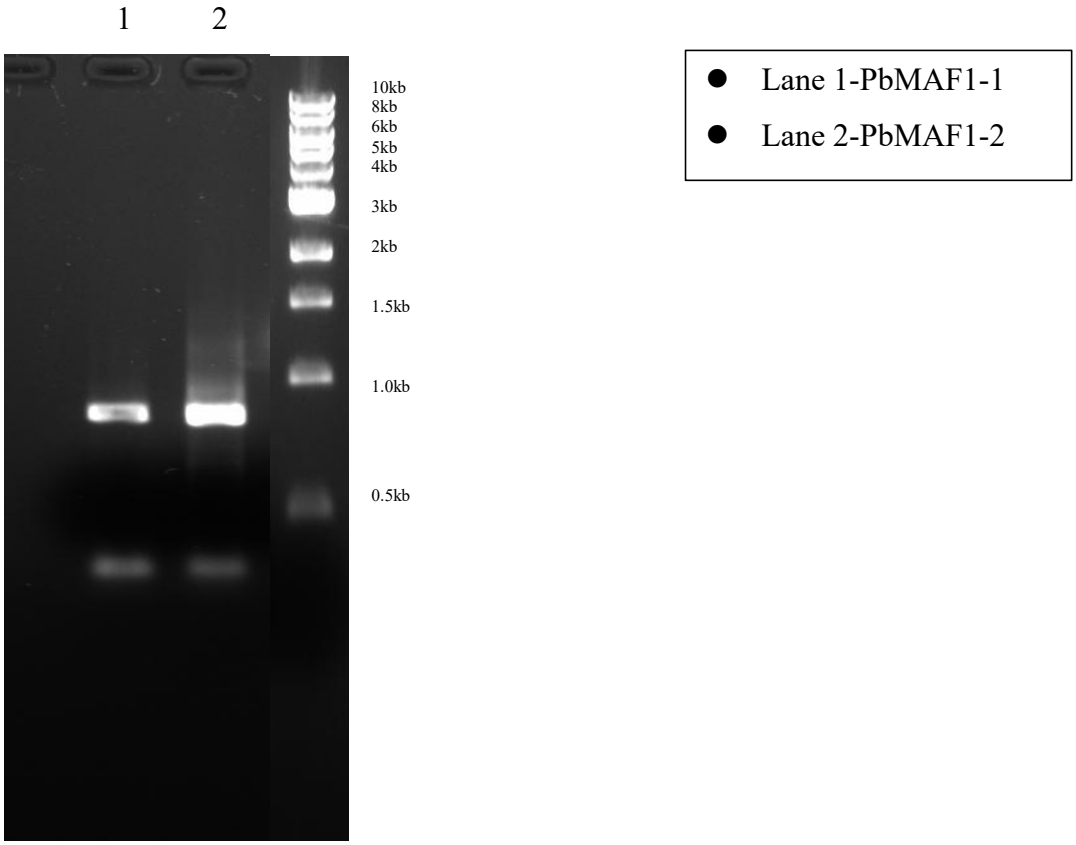
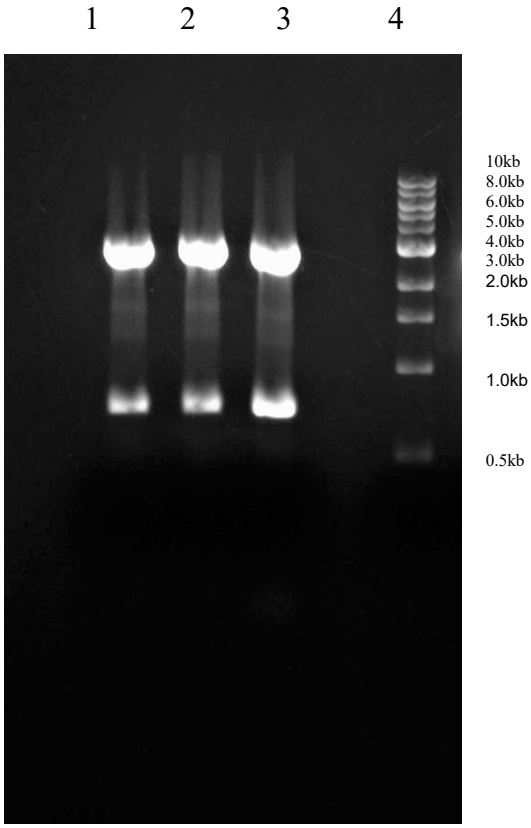


Fig 11:-The above figure shows the PCR amplification of cDNA of PbMAF1 gene from the RNA isolated from the *Plasmodium berghei.*The PCR was done in the following condition:-+MgCl2,+DMSO,+5X GC Buffer,+Phusion polymerase and annealing temperature 55°C. Clean bands were obtained for PbMAF1-1 and PbMAF1-2 in between 0.5kb-1kb.

5.2.Confirmation of clones by insert release:-



- LANE 1:-PbMAF1-pRSETA
- LANE 2:-PbMAF1-pRSETA
- LANE 3:-PbMAF1-pRSETA
- LANE 4:-1Kb DNA LADDER

Fig 12:-Restriction digestion was done with *BahmH1* and *EcoRI*. The insert release was seen in all the samples. The Plasmid pRSETA was found in between 2-3kb whereas the inserts were found in between 0.5-1kb.

5.3.Sequence alignment of PbMAF1 and pRSETA-MAF1

```
#
# Length: 927
# Identity:     730/927 (78.7%)
# Similarity:   734/927 (79.2%)
# Gaps:         193/927 (20.8%)
# Score: 3604.0
#
#
#=====================================
```

```
EMBOSS_001      308 ATGTTTTCCCAGATTATGAATTTAAATATTTAAACAACTCAAATTACAAA    357
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      451 ATGTTTTCCCAGATTATGAATTTAAATATTTAAACAACTCAAATTACAAA    500

EMBOSS_001      358 CCTATAAAAAATTTACACAATGTTATAGACAATATTAATTATAATTTATT    407
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      501 CCTATAAAAAATTTACACAATGTTATAGACAATATTAATTATAATTTATT    550

EMBOSS_001      408 TTATTTAGTTGAAAATATATATCGAGGATTTAATAAAAAAGTATGGAAAA    457
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      551 TTATTTAGTTGAAAATATATATCGAGGATTTAATAAAAAAGTATGGAAAA    600

EMBOSS_001      458 TATTAAAAGAATTAATTGATTTTAAATCTTGTGATATATATACATATTTA    507
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      601 TATTAAAAGAATTAATTGATTTTAAATCTTGTGATATATATACATATTTA    650

EMBOSS_001      508 AATAATACTGATAAAGACCCATATATAGATAAACAAAGTATTTCAAGTTT    557
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      651 AATAATACTGATAAAGACCCATATATAGATAAACAAAGTATTTCAAGTTT    700

EMBOSS_001      558 TAATTATTTTTTTTTTGCAAAAAAAACTAAAAGGATATTATTTATATCTT    607
                    |||||||||||||||||||||||||||||||||||||::|||||||||
EMBOSS_001      701 TAATTATTTTTTTTTTGCAAAAAAAACTAAAAGGATATTAKKTATATCTT    750
```

Fig 13:-The sequencing data was aligned with the *Plasmodium berghei* MAF1 sequence and similarity was 79.2%. So this clones were used for the expression of MAF1 protein.

5.4.Small scale protein preparation:-

18°C 30°C 37°C

12I 11I 10I 9I 9U 8I 7I 6I 5I 5U 4I 3I 2I 1I 1U

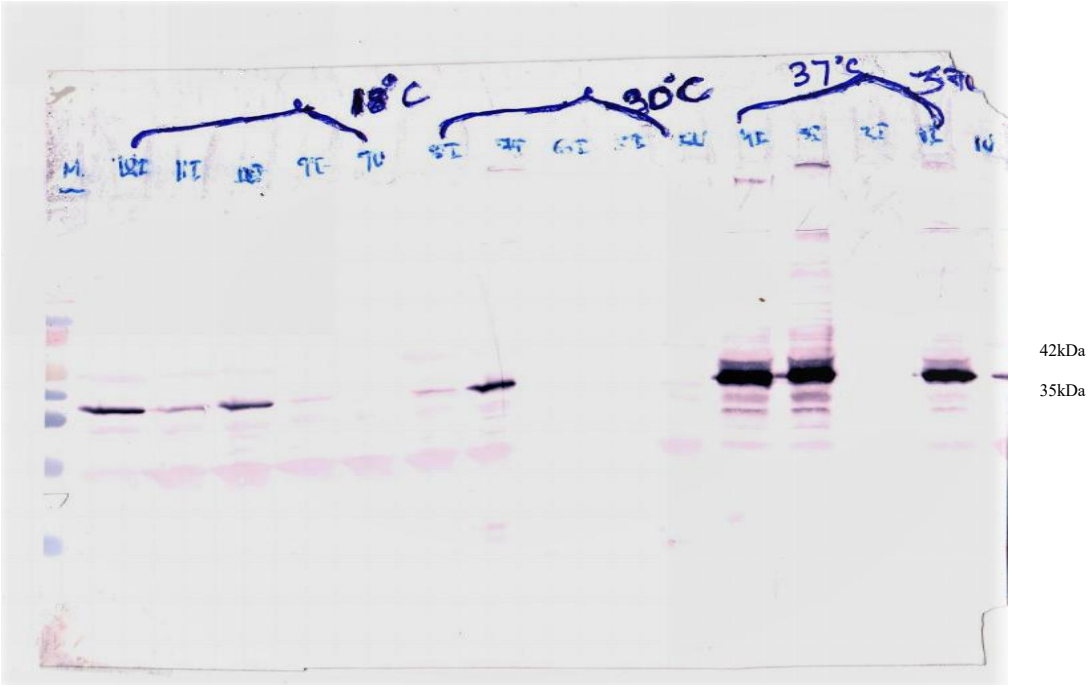
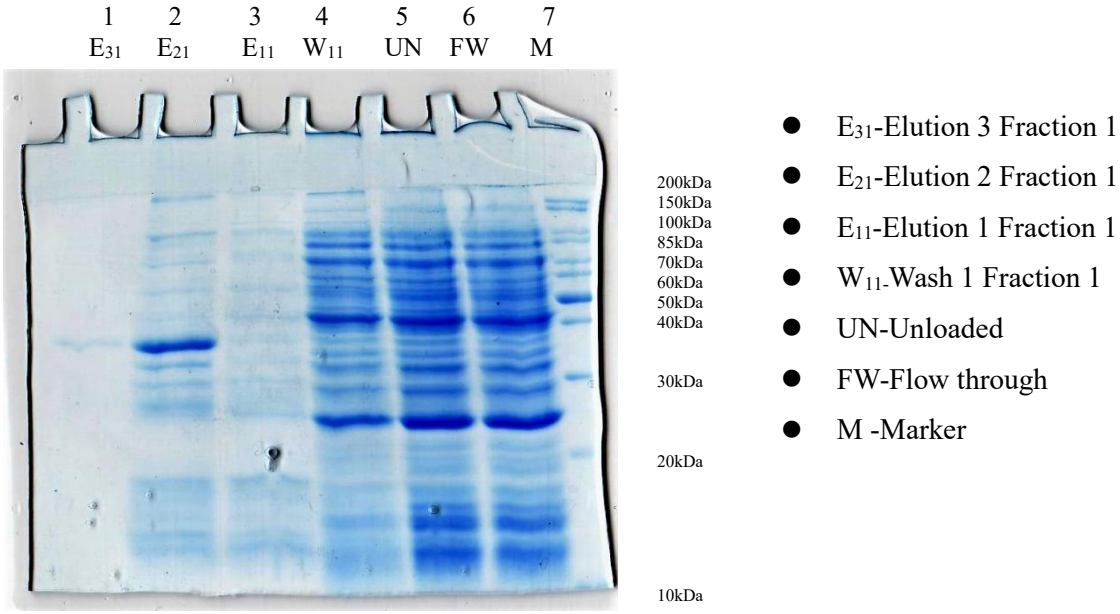


Fig 14:-In the small scale protein preparation, at 37°C the protein expression is good. 1I,3I,4I shows the most intense band while at 30°C it doesn't overexpress properly. At

18°C, protein is overexpressed but the intensity is very low. So,the stock prepared from 1I,3I and 4I are used for large scale protein preparation.

5.5.Large scale protein preparation:-

5.5.1.SDS-PAGE:-



- E_{31}-Elution 3 Fraction 1
- E_{21}-Elution 2 Fraction 1
- E_{11}-Elution 1 Fraction 1
- W_{11}-Wash 1 Fraction 1
- UN-Unloaded
- FW-Flow through
- M -Marker

Fig 15:-In the large scale protein preparation, the intense and specific protein was found in Elution 2 fraction 1. The band appears between 30kDa to 40kDa. In FW,UN,W_{11} many nonspecific intense bands were found. In E_{11} many nonspecific band were found,while in E_{31} less intense specific band was seen.

5.5.2.Western blotting:-

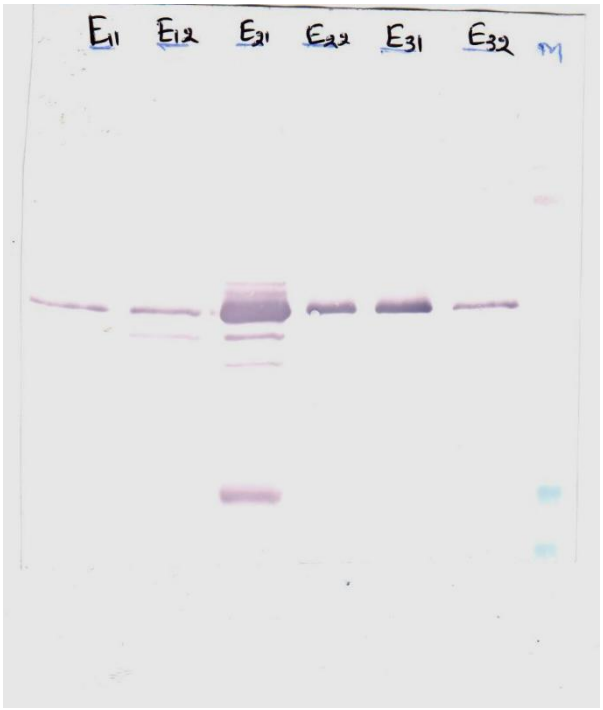


Fig 16:- Confirmation of identity of PbMAF1 protein via western blotting using anti-His tag primary antibody. In all the fraction band was seen just above the 35 kDa. In Elution 2 fraction 1 intense band was observed ,just below it the light bands are the degraded band of PbMAF1.So the E_{21} fraction was used for immunization.

5.6.Western blotting for the confirmation of PbMAF1 antibody:-

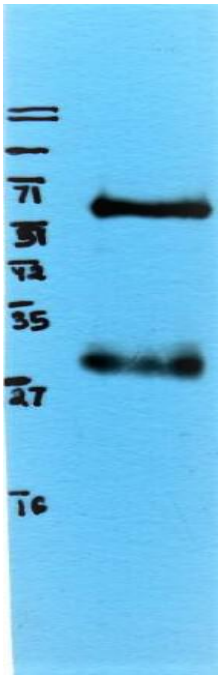


Fig 17:-The antibody produced against PbMAF1 binds to the PbMAF1 protein in the PbWT. The band was seen just above the 27 kDa i.e 29 kDa ,which is the size of MAF1 protein.

5.7.Immunofluroscence:-

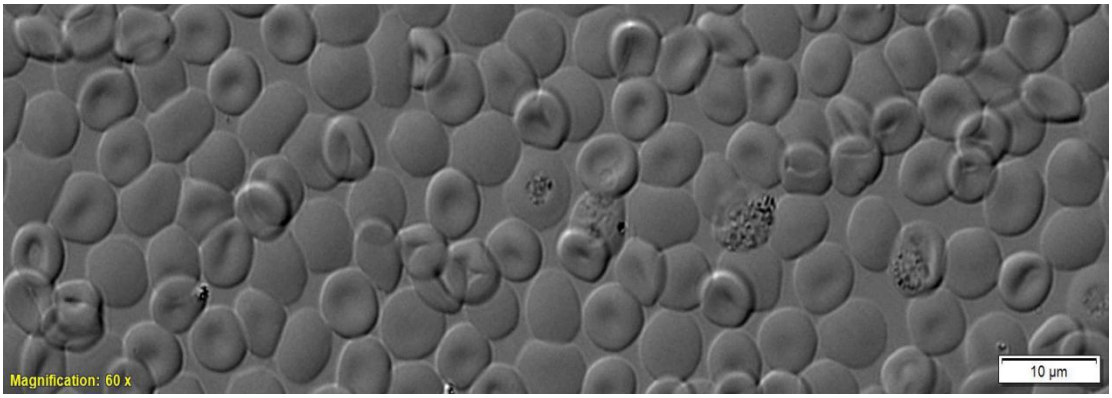


Fig 18(a):- In bright field parasitised RBCs were observed along with the normal RBCs.

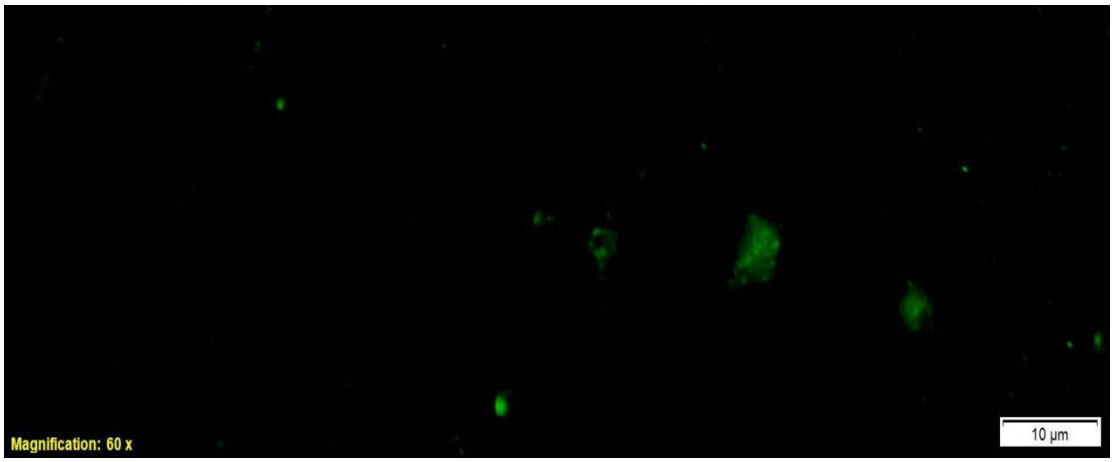


Fig 18(b):- Secondary antibody conjugated with FITC absorbs blue light causing molecules to become excited and emit a visible yellow-green light. In the above picture yellow-green light was observed because the FITC conjugated secondary antibody binds to the PbMAF1 primary antibody which indicates the localization of MAF1 protein in the cytosol of the parasite.

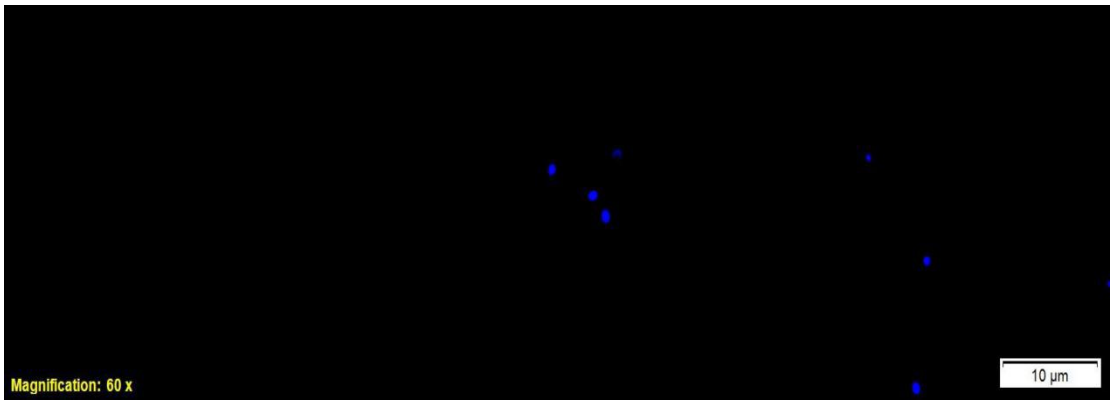


Fig 18(c):- DAPI nucleic acid stain preferentially stains dsDNA, it appears to associate with AT clusters in the minor groove. In the above picture DAPI staining was observed which indicates the presence of nucleus. In the nucleus no yellow-green light was observed which indicates the absence of Pb MAF1 protein in nucleus.

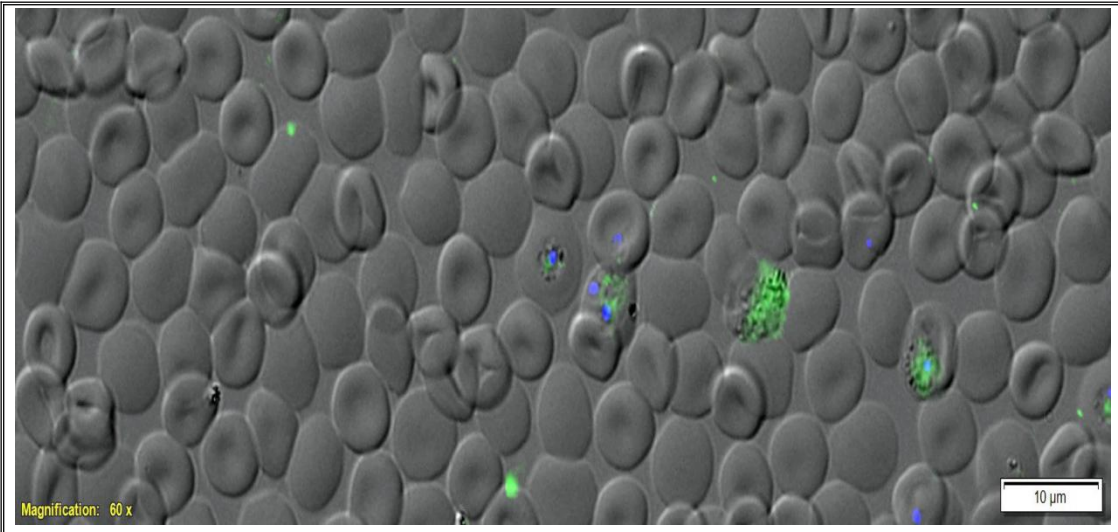


Fig 18(d):- The above picture was the merged picture of DAPI,FITC and Bright field. The nucleus was stained by DAPI, indicated by the blue colour. Yellow-green colour was observed in the cytosol of the parasite, which indicates the presence of Pb MAF1 protein in the cytosol and the bright field observed the normal RBCs and parasitised RBCs.

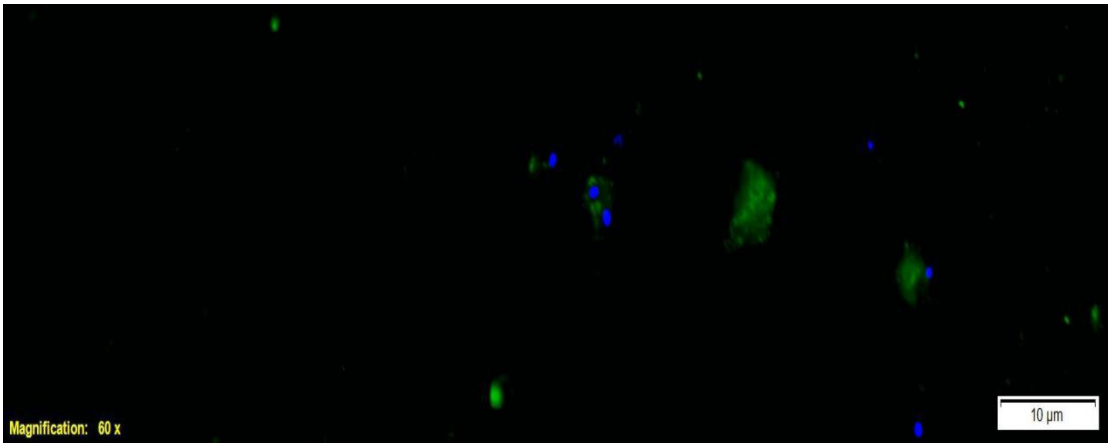


Fig 18(e):- In this figure merged DAPI AND FITC was observed. FITC stain indicates the presence of Pb MAF1 protein in the cytosol and DAPI indicates the presence of nucleus in the cell.

CHAPTER-6

DISCUSSION

MAF1 protein plays a critical role in the inhibition of RNA III Polymerase and ceasation of cell cycle,which ultimately results in the cells dormancy. To understood the molecular mechanisms, we need to characterize and study the localization of MAF1 protein. This study is mainly focused on the overexpression and the localization of MAF1 protein in *Plasmodium berghei.*

6.1.Confirmation of cDNA by PCR:-

The genomic size of the Pb MAF1 is 0.75kb. The band appears between 0.5kb and 1kb and it confirms that is the band of Pb MAF1. In both lane 1 and lane 2 the band is intense which implies that the PCR conditions used for this PCR worked well.

6.2.Confirmation of clones by insert release:-

Plasmids isolated from the NBT transformed cells are cubjected to restriction digestion to check whether the Pb MAF1 CDNA inserted properly in the pRSETA vector or not. NBT is a K-12 strain ideally suited as an initial cloning host due to its high transformation efficiency and recA endA mutations, which result in high yields of excellent quality plasmid DNA.

In the FIG:-12,it is seen that in all the 3 lanes the bands are clean.The lower band appears between the 0.5Kb - 1kb, that is near 0.75kb which indicates that is the genomic size of PbMAF1. The upper band appears between 2kb -3kb that is near 2.8kb, which is the size of pRSETA vector. So,this insert release confirms the proper insertion of PbMAF1 in pRSETA vector.

6.3.Sequencing:-

The similarity between the PbMAF1 genomic DNA sequence and the sequence obtained from pRSETA-Pb MAF1 clone was 79.2%. So this clone was used for the overexpression of protein.

6.4.Small scale protein preparation:-

Small scale protein induction was done in Rosetta strain because these strains are BL21 lacZY derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli.* Four IPTG induced tubes and 1 uninduced tube were kept in three different temperature each. IPTG is a molecular mimic of allolactose that initiate the transcription of lac operon and it is

therefore used to induce protein expression where the gene is under the control of the lac operator. At 18°c and 30°c the protein expression is very low suggesting that the protein cann't be overexpressed at low temperature. At 37°c intense bands were seen because it was the optimal temperature for the growth and overexpression of protein. In 1I,3I and 4I protein was overexpressed properly but in 2I, protein didn'tt overexpress.

6.5.Large scale protein preparation:-

1. SDS-PAGE:-

In W_{11},E_{11},E_{21},E_{31} different concentration of imidazole was used to elute the purified protein. Protein purification by Ni-NTA based on the principle of affinity chromatography,where Ni-NTA resin acts as a stationary phase and 6X-His tagged protein from the cell lysates are in the mobile phase. Imidazole is present in the side chain of histidine amino acid and it binds to the Ni. Imidizole was added in different concentrations to lysis buffers. When added to the column, it competes with the His-tagged recombinant protein and helps in the elution of recombinant protein. Imidazole also reduces the nonspecific binding of the protein to the column.

In the Fig-14, lane 5 and 6 contains unloaded and flow through protein respectively. Unloaded proteins were collected before it was added to the column and the flow through was collected after the protein passed through the column. In both the lanes number of non specific protein was very high because buffer containing imidazole was not used. In lane 4, W_{11},buffer containing imidazole was used, but in very low quantity i.e 500µl. So the number of non specific protein is very high. In lane 3, E_{11}, concentration of imidazole in buffer was 1.25 ml,which shows very low concentration of non specific protein. In lane 2, E_{21}, 2.5 ml of imidazole was used in buffer, which elute the maximum number of specific protein.The size of the PbMAF1 protein is 29 kDa but the band appeared between 30-40kDa.This increase in size occurs due to the presence of vector. Most of the specific protein was eluted in E_{21}, so in lane 1, E_{31} ,very low concentration of specific protein was seen. So, the E_{21} fraction was used for immunization to generate antibody specific to MAF1 protein.

2.Western blotting:-

The his-tag PbMAF1 recombinant protein was picked by anti-his tagged primary antibody. Goat anti-mouse ALP conjugated secondary antibody binds to the primary antibody. For the detection of protein, BCIP and NBT was used.BCIP is an chromogenic substrate used for the sensitive colorimetric detection of alkaline phosphate activity. It is used in combination with NBT. BCIP is oxidized by NBT which forms an insoluble dark blue diformazan precipitate after reduction. In between 35-42kDa dark blue band was seen. Size of the PbMAF1 protein is 29kDa.This increase in size may be due to the presence of vector. The most intense band was observed in E_{21} and small bands seen below could be the degraded products of Pb MAF1. The fraction having intense protein was used for immunization.

6.6.Western blotting for the confirmation of PbMAF1 protein:-

The antibody produced against PbMAF1 was used to detect the PbMAF1 protein in wild type *Plasmodium berghei.* The band was seen above the 27kDa i.e 29 kDa which is the exact size of PbMAF1. Another band was seen above the 51 kDA. It may be an ortholog of PbMAF1 protein. As we don't have knock out of PbMAF1, we couldn't confirm that it is an ortholog or not.

6.7.Immunofluroscence:-

For immunofluroscence,cover slip was coated with Poly-L-lysine because the cationic nature makes it an attractive molecular coating for the adhesion of negatively charged biomolecules. It is also ideally suited for the adhesion of cells, since the cell surface is known to be negatively charged. Fixing solution is used to maintain cellular structure as close as possible to the native state. Paraformaldehyde in the fixing solution used to maintain cellular structure as close as possible to the native state and the glutaraldehyde preserves the cellular structure very well but, causes strong autofluorescence. Permeabilization solution helps get the antibodies into the fixed cells. Quenching solution reduces the autofluorescence caused by the fixing solution.

Blocking is the most important part of the process. If blocking is proper the primary antibody strongly binds to the protein of interest. FITC is a fluorochrome dye used in conjugation with an secondary antibody . FITC absorbs blue light, exciting molecules which then emit a visible yellow-green light. When the excitation light is removed, the emission light ceases. DAPI binds to the AT rich region of the DNA ,so to know the position of nucleus DAPI stain was used.

Figure 18(a,b,c,d,e) indicates the presence of PbMAF1 protein in the cytosol of parasite. This experiment was done in the WT *Plasmodium berghei* and the parasite was in favourable growth condition with the supplement of essential amino acids, so the PbMAF1 protein was found to be localized in the cytosol.

CHAPTER-7

CONCLUSION

The major aim of present study was to overexpress PbMAF1 protein, raise polyclonal antibodies and determine its localization in *Plasmodium berghei* . The results give a preliminary indication of presence of MAF1 protein in the cytosol of *Plasmodium berghei* .

7.1. Summary:-

MAF1 protein is the key factor responsible for the repression of RNA Pol III. In our study we showed that MAF1 protein was found in cytosol because the wild type *Plasmodium berghei* was in favourable growth condition. The stress conditions lead to Maf1 dephosphorylation and nuclear impor , which is directed by two nuclear localization signal (NLS) sequences [26]. Therefore under normal conditions, MAF1 protein is found in the cytosol of PbWT.

7.2.Future work:-

Antibodies were successfully generated against PB MAF1 but an additional intense band was observed above the specific band,which may be an ortholog of MAF1, so knock out of Pb MAF1 will be attempted in future. In WT *Plasmodium berghei* MAF1 protein is found in cytosol, so in starvation condition immunofluroscence will be performed to analyze the translocation of MAF1 to the nucleus.

MAF1 is considered as a new player in dormancy and survival of the parasite. So the characterization of MAF1 and its localization in parasite will be helpful to explore its potential as a drug target to overcome the drug resistance problem by inhibiting the parasite from undergoing dormancy.

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APPENDIX

❖ **10Mm dNTPs:-**

Components	Volume(µl)
Autoclaved MQ	120
100Mm dATP	20
100Mm dTTP	20
100Mm dGTP	20
100Mm dCTP	20

❖ **10X TBE:-**

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

❖ **DNA Loading dye:-**

✓ 700ul of 100% glycerol.

✓ add 300ul of autoclaved miliq water.

✓ A pinch of BPB(Bromo Phenol Blue).

❖ **1kb DNA Ladder:-**

✓ 20ul of stock 1kb ladder

✓ 140ul of autoclaved water

✓ 40ul of 70 DNA loading dye

❖ **EtBr:-**

➢ Dissolve 1mg of EtBr in 1ml of MQ.

➢ Vortex it properly and stored it in 4^0C.

❖ **10x SDS:-**

➢ 800ml of A.M water was taken

➢ 26gm of Tris and 130gm of Glycine was added to it

➢ Autoclaved for 21min at 120ºC.

➢ SDS 10gm was added and volume was made upto 1lt.

❖ **30%Acrylamide and Bis-acrylamide:-(100ml)**

➢ Acrylamide 29gm and Bis-acrylamide 1gm was added to autoclaved milliq water.

❖ **WTB:-**

➢ 3gm of Tris and 14.4gm of Glycine was added to MilliQ water and volume was made up to 850ml

➢ Autoclaved for 21 mins at 120ºC,then cooled down and 150ml of methanol was added.

❖ **TTBS:-**

➢ 50ml of 1M tris(pH-8) and 37.5ml of 4M NaCl was taken and the volume was made upto 1lt.

➢ Autoclaved for 20min at 121ºc and cooled down.

➢ 1ml triton was added and shaked well.

❖ **ALP Buffer:-**

➢ 3.02gm of Tris,1.25ml of 1m $MgCl_2$ and 6.25 ml of 4M Nacl was added to Milli Q water and volume was made upto 100ml.

❖ **10ml of Chloramphenicol:-**

➢ 340mg of Chloramphenicol was taken and 9ml of Ethanol was added to dissolve it.

➢ Then the volume was made upto 10ml.

❖ **10ml of Ampicillin:-**

➢ 1gm of Ampicillin was taken and 9ml of water was added to dissolve it.

➢ Then the volume was made up to 10ml.

❖ **10ml of Tetracycline:-**

➢ 120mg of tetracycline was taken and dissolved in 9ml of ethanol.

➢ Then the volume was made up to 10 ml.

❖ **Ketamine:Xylazine:-**

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

❖ **LB Agar:-**

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

Dissolved in 100ml of elix water.

❖ **LB Broth:-**

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

Dissolved in 100ml of elix water.

❖ **CCMB Buffer 80:-**

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

❖ **Fixing solution(50 ml):-**

2gm of 4% Paraformaldehyde and 0.0075% of 15µl Glutarl dehyde

❖ **Permeabilizing solution(50ml):-**

50µl of Triton x was dissolved in 50 ml of PBS.

❖ **Quenching solution(50ml):-**

375gm of 0.1m glycine was added to 50ml of PBS.

❖ **Blocking solution(50ml):-**

1gm of 2% BSA was dissolved in 50ml of PBS.

❖ **DAPI solution(-20ºC):-**

50-100µl of aliquot was dissolved in PBS.