# Chapter III

## Identification and isolation of gene encoding cross-reactive antigen of *Plasmodium falciparum*

## Introduction:

*Plasmodium* parasites exhibit a complex life cycle living in two-host system i.e. human and mosquito host. The asexual multiplication occurs in the human host leading to all pathological conditions and its sexual cycle completes in mosquito host, which act as carrier to transmit the disease. The genome size of *Plasmodium* is 2 x $10^7$ base pairs spread in 14 chromosomes (Triglia *et al.*, 1992). The complete genome sequencing of *Plasmodium falciparum* has been published very recently (Gardner *et al.*, 2002), which has shown that it encodes about 5,300 genes in its 23-megabase nuclear genome. It will provide ample of information on the organization of the genes, proteins and help to isolate genes of interest and may play an important platform for vaccine and drug target identification. There are several methods to fish out a target gene from total genomic DNA. One way is the use of Polymerase chain reaction (PCR) and the other approaches screening of the genomic/expression libraries with sequence tags or antibodies. Here, the main objective is to identify and isolate the cross-reactive interspecies conserved antigen of *P. falciparum*. It was accomplished by constructing and immunoscreening of recombinant *P. falciparum* genomic expression library with anti-*P. yoelii* antibody probe. The *P. falciparum* protein shared by the *P. yoelii* was detected by probing the library with anti-*P. yoelii* sera. The recombinant clones identified by the sera were purified and re-probed with pooled normal human sera from malaria endemic area to identify cross-reactive as well immunogenic molecule.

## Experimental methodology:

### I. Parasite materials:

Parasite material is the prerequisite to study the molecular level mechanisms in any organism, which is obtained by maintaining a particular strain of parasite *in-vivo* or *in-vitro* or directly from the human host. In this study, we selected the most fatal human malarial parasite *P. falciparum* and a lethal rodent malarial parasite *P. yoelii*. *P. falciparum* was cultured *in-vitro* by using candle jar methods described by the Trager and Jensen (1976) and *P. yoelii* was obtained from *in- vivo*. The details of culture method and purification of the parasites are described below.

#### a. *In-vitro* culture of *Plasmodium falciparum:*

Indian isolate of *P. falciparum*, FCD-3 strain (Mehera & Bhasin, 1993) was *in-vitro* cultured using RPMI-1640 media supplemented with 10 % human sera using candle jar technique $AB^+$ sera were obtained from four to five normal individuals and pooled. The serum was heat inactivated at $56^0$C for 30 minutes and filtered through 0.45μm filter (Coaster, USA) It was stored at $-20^0$C for longer durations. Complete growth medium was prepared by the addition of 10% (v/v) heat inactivated pooled human serum, gentamycin (50 mg/liter) to RPMI-1640 media, sterilized by passing through 0.22 μm filter Only 100 – 200 ml of media was prepared at a time, stored at $+4^0$C and used within a week of preparation. Human uninfected red blood cells are required for the maintenance of the *P. falciparum* culture. O+ blood cells were collected with anticoagulant (Citrate-Phosphate-Dextrose) and stored at $4^0$C up to six weeks. Prior to use the cells were centrifuged (1500 rpm for 5 minutes), the plasma was removed along with the buffy coats. The lower layer containing red blood cells were suspended in incomplete media

(RPMI 1640 without serum) and centrifuged to wash the RBC. The washing was repeated twice and the washed cells were made 50% suspension with complete medium. The washed blood cells were stored at $4^0$C and used for up to four weeks. All the blood products were obtained from commercial blood banks and were tested negative for malaria parasites, hepatitis virus and human immunodeficiency virus. Sterility of Media, sera, and RBC were checked on LB Agar plates and by gram staining before using them in culture.

Suspension of previous *in-vitro P. falciparum* culture was taken after slide examination and counting the parasitemia percentage, centrifuged (1500 rpm for 10 min) and the pellet was washed once with incomplete medium. The washed pellet was added with equal amount of complete growth medium and 5% of uninfected red blood cell and transferred to a sterile Petri dish. The Petri dish was placed inside the jar and the candle was lighted. Initially the sleeve of the desiccator's was closed $2/3^{rd}$ of the opening and closed completely immediately after the candle extinguished to give a gas composition of 5% Oxygen, 5% Carbon-dioxide and 90% Nitrogen. The desicator was placed in the $37^0$C incubator. The medium was replaced daily. The growth of the culture was monitored by making a thin blood smear on a slide, stained in 5% Geimsa solution prepared in phosphate buffer (pH 7.4) for 30 minutes. The slide was washed under running water, air-dried and examined under oil immersion using a light microscope at a magnification of 1000X. The percentage of infected RBC and the stages of the parasites were recorded. When the parasitemia reached 15-20% the culture material was transferred to a 15 ml tubes, centrifuged and infected RBC pellet was used as source of

parasite material. Based on the requirement *in-vitro* culture was maintained in multiple Petri dishes, harvested and used for the study.

**b. Synchronization of *P. falciparum* culture:**

When the parasitemia of the *in-vitro* culture of *P. falciparum* reached 5% with a good number of early ring stages, the culture materials were collected from the Petri dishes into a sterile centrifuge tube (50 ml), centrifuged (2000g/7 minutes) and the supernatant was discarded. The pellet was suspended in 5 volumes of 5% D-sorbitol and incubated at $37^0$C for 5 minutes with intermittent shaking. The cell suspension was centrifuged and the cell pellet was washed twice with incomplete media to remove all the D-sorbital and once with complete growth media. The culture was established by adding fresh washed uninfected RBC and medium to give and 8% PCV with parasitemia of 0 1%. The culture was incubated in a candle jar at $37^0$C and the growth medium was changed daily After 35 hours of incubation, sorbitol treatment was repeated once as described earlier. The growth of the culture was monitored at regular intervals.

**c. *In-vivo* maintenance of *P. yoelii* in mouse:**

*P. yoelii nigeriensis* was maintained in inbred Balb/c mice aged 14 to 20 weeks (H-2d). The infected mouse was anaesthetized with ether and blood was drawn from retro-orbitally in to a 1.5 ml sterile tube containing acid citrate dextrose (75 mM Tri-sodium citrate, 38 mM Citric acid and 124 mM D-Glucose). The parasitized blood cells were further diluted with normal saline so that 200 µl of normal saline contained $10^4$-$10^8$ parasitized red cells. Mice were inoculated intra peritoneally with 0.2ml diluted infected blood and maintained in a standard animal house. The parasitemia of each mouse was

monitored by making a thin smear of blood collected from tail vein. When the parasite count reached 30% (at day 6-7), the blood was collected and subjected to column purification to remove white blood cells as descried below.

**d. Removal of white blood cells from the *P. yoelii* infected red blood cells:**

To obtain the pure parasite materials, it is important to get rid of leucocytes from the infected blood. The cellulose fibrous (CF-11) column procedure (Fluton and Grant 1956) is used widely to remove white blood cells. The infected blood collected in acid citrate dextrose was centrifuged at 250 g for 10 minutes and the plasma and buffy coat was aspirated out. The cell pellet was diluted with three volume of chilled PBS, centrifuged as before and finally suspended in double the volume of PBS. Two CF-11 cellulose columns were prepared and the column bed volume was made equal to that of blood pellet to be processed. The columns were equilibrated with three times the bed volume with PBS. The washed cell suspension was applied to the first column and the effluent was collected until $3/4^{th}$ volume of the cell suspension had eluted. The effluent was passed through a second column, the effluent centrifuged at 250 g for 10 min. The leucocytes depleted RBC pellet was washed twice with cold PBS. Then the pellet was suspended in 5 volumes of 0.15% of saponin prepared in PBS. The suspension was incubated at $37^0$C for 10 minutes with intermittent shaking to allow RBC to lyse completely. The lysed suspension was centrifuged (500 g for 15 minutes) at $4^0$C and supernatant was discarded. The pellet containing the free parasites was washed twice with cold PBS and isolated parasite was stored at $-70^0$C.

**II. Construction of *P. falciparum* genomic expression libraries in the bacteriophage vector lambda gt11 (λgt11):**

One of the most popular bacteriophage vectors in cloning history is the bacteriophage, λgt11. Its value for cloning lies in the fact that DNA can be inserted and packaged *in- vitro*. The cloning efficiency of the phage vector is very high compared to plasmid vector; hence it can be employed even with small amount of source material and ensures entire genomes representation. λgt11 is a replacement vector in which certain non-essential genes were removed to allow for insertion of foreign DNA molecules. The vector has been engineered to contain a Lac Z gene in this region with multiple cloning site into which a foreign DNA is inserted. The vector is capable of accepting up to 7 kb without affecting the vector's ability to package into a viable phage. One of the major additional advantages of the lambda gt11 vector system is that it is possible to control the expression of the Lac Z gene and trigger the production of the "fusion protein" by addition of Isopropyl β-D-thiogalactopyranoside(IPTG). The fusion protein is the product of encoded β-galactosidase gene fused with the open reading frame of the inserted foreign DNA Thus, not only it can be probed with DNA sequences but the production of fusion protein also allows the screening the library with antibodies. This allows the researchers to obtain a gene of interest by immunoscreeing. The step-wise method of construction of *P. falciparum* genomic library in λgt11 vector is presented below.

**a. Isolation of *P. falciparum* DNA:**

*In-vitro* cultured *P. falciparum* blood stage was the source of *P. falciparum* DNA. The DNA extraction method (Tungpradubkul and Panyim, 1985), was used based on lysis and organic extraction procedure, which is described below.

i. The pellet containing parasitized erythrocyte was suspended in double volume of 1% cold acetic acid to lyse the erythrocytes and centrifuged at 4000 rpm for 10 min.

ii. The pellet containing parasites was washed with PBS and centrifuged (4000 rpm for 10 min at $4^0$c), suspended in 1 ml of extraction buffer (10 mM Tris-Cl pH 8.0, 0.1 mM EDTA pH 8.0, 0.5% SDS and 20 mg/ml RNase A), proteinase-K (100µg/ml) was added and incubated at $56^0$C for overnight.

iii. The lysate was twice extracted with equal volume of Phenol: Chloroform: isoamyl alcohol (25:24:1 v/v) and centrifuged (5000 rpm/10 minutes). Finally, the aqueous layer was extracted with one volume of chloroform to remove phenol contamination.

iv To the aqueous phase, 0.1 volume of 3M NaOAc, pH 5.4 and then double volume of cold ethanol was added and the tube was placed at $-70^0$C for 1 hour. Then it was centrifuged at 14000 rpm for 15 min and the DNA pellet was washed with 70% alcohol.

v The pellet was air dried and dissolved in 100 µl of TE ( 10m M Tris, pH 8.0 and 1 mM EDTA) buffer. The quality of DNA was assessed by agarose gel electrophoresis and quantity was estimated by reading the OD 260/280.

**b. Insert DNA preparation:**

Approximately, 10 µg of *P. falciparum* DNA was digested with 2 µl EcoR I enzyme (200 units) for overnight at 37°C and the digested DNA was subjected to electrophoresis in 0.7% Agarose gel using 1X TAE (Tris-Acetate-EDTA) buffer. The

DNA fragments (0.5- 5.0 kb) were cut out from the gel and the purified using Qiax Gel extraction Kit (Qiagen, USA) and eluted in a final volume of 50µl of water.

**c. Ligation of EcoR I insert in to the EcoR I site of lambda gt11:**

The EcoR I digested λgt11 phage vector was obtained commercially (Promega, USA). The vector map is presented in Fig 3.2. The following reaction was carried out for the ligation.

| | |
|---|---|
| EcoRI insert DNA: | 10 µl (2 µg of DNA) |
| λgt11 vector- EcoR I arms: | 2 µl (0.5 µg) |
| T4 DNA ligase : | 1 µl (100 units) |
| 10x ligase buffer: | 2 µl |
| Sterile Water: | 4 µl |
| TOTAL : | 20 µl |

The above reaction was incubated at $16^0$C O/N in a water bath and finally the tube was briefly centrifuged and incubated at $65^0$C for 10 min to inactivate the enzymes. Then the mixture was packaged in to infectious phage particle as follows.

**d. *In-vitro* packaging of ligated DNA in to phage particle:**

Commercially available packaging extract, Gigapack II Packaging extracts (Stratagene, USA) was used in this experiment. The instruction of the manufacturer was strictly followed. One set of extract (yellow and red tube each) was removed from $-80^0$C and placed on dry ice. When the extract had just begun to thaw 4 µl of the ligated DNA was added immediately to the red tube. Quickly, 15 µl of sonic extract (yellow tube) content was added to the red tube and gently mixed with a pipette and briefly centrifuged.

The tube was incubated at room temperature for 2 hours and then 0.5 ml of SM buffer (20 mM Tris-HCl pH 7.4, 100mM NaCl and 10 mM $MgSo_4$) was added followed by 20 µl chloroform. Then the tube was centrifuged briefly to sediment the debris. The supernatant was transferred to a fresh tube and titer of the library was determined.

**e. Titration and amplification of recombinant λgt11 *P. falciparum* expression library:**

It was necessary to infect bacteria with an aliquot of packaging reaction to determine the number of plaque forming units (pfu) in the library and the percentage of pfu that are recombinant. Titration of the library and amplification of was carried out as per standard methods. Briefly, a single well isolated colony of plating host cells (Y 1090) was grown in LB medium supplemented with Ampicillin (100 µg/ml), 10mM MgSO4 and 0. 2% (v/v) maltose for 4-6 hours ($OD_{600}$ = 0.6-0.8). The cells were harvested (2000 rpm for 10 minutes) and the pellet was suspended in half the volume of original culture with sterile 10 mM $MgSO_4$ and used for plating the library. Serial dilution (1: 100, 1: 1000, 1: 5000 and 1: 10000) of library was carried out in SM buffer and 1 µl of the diluted library was added to 200 µl of plating cells. It was incubated at 37°C for 15 min to allow the phage to attach to the cells. Then, 3 ml of soft agar (48°C), 40 µl 0.1 M IPTG, 40 µl X-gal (2%) were added, mixed and plated immediately on pre-warmed LB plates. The plates were incubated at 42°C for six hours followed by 1 hour at 4°C. The recombinant plaques were counted and the titer of the library and percentage of recombinant was determined.

**III. Immunoscreening of the λgt 11- *P. falciparum* expression library:**

Immunoscreeing of expression library is one of the methods to identify the target antigen gene by using antibody probe. Using this approach many malarial antigens as well vaccine potential candidates were cloned and characterized (Lobo *et al.*, 1994; McColl *et al.*, 1994; Ma *et al.*, 1996; Zhang *et al.*, 1999; Galiniski *et al.*, 2001). Our specific aim was the identification of interspecies conserved antigens of *P. falciparum*, which was accomplished by screening recombinant *P. falciparum* DNA library with anti-*P. yoelii* antibody probe. The *P. falciparum* protein shared by *P. yoelii* was detected by probing the expression library with anti-*P. yoelii* sera. The cross reactive clones were picked up and reprobed with pooled immune human sera collected from malaria endemic areas to identify protein molecule which are also immunogenic in natural transmission area. Immunoscreening was carried out according standard method (Synder *et al.*, 1987).

**a. Preparation of convalescent – phase mouse anti-*P. yoelii* serum ( or Anti- *P.-yoelii* sera):**

Inbred mice were infected with *P. yoelii* parasites ($10^6$ parasites per mouse) intra peritoneally. After 5-6 days, when the parasitemia reached 10 to 15% the mice were cured by oral administration of 10µl of Reziz (0.25 mg of pyrimethamine and 0.5 mg of sulfadoxine) per mouse for 3 days. At day 10 from the beginning of drug treatment the mice were examined for infection. Once the mice were cured, they were again infected with $10^7$ parasites and cured with the same drug treatment. After three infection/cure cycles the mice became resistant to challenge doses of $10^8$ parasites, blood samples were collected, serum was separated and pooled together. The serum was heat inactivated at

$56^0$C and stored in aliquots at $-70^0$ C. This pooled serum (convalescent sera) was used for immunoscreening of library.

**b. Human serum from endemic areas:**

Cryo-preserved human serum samples, which were earlier, collected from the permanent resident (adult healthy individuals) of malaria endemic areas of Orissa were used in this work. The samples were highly reactive to *P. falciparum* antigens and characterized in the earlier publications (Ray *et al.*, 1994; Ma *et al.*, 1996). The serum sample of 10 such residents were pooled together and used for immunoscreening.

**c. Identification of immuno-reactive clones:**

1. Plating cells (Y1090) were prepared as described in titration of library. The phage library was diluted such that plaques were apart from each other. Around 10 µl of diluted phage was added to 100 µl of plating cells and stored at $37^0$C for 30 minutes. Three ml of molten agar ($48^0$C) was added to the above mixture, inverted quickly and poured on to LB-ampicillin plate (100µg/ml). The plates were inverted and incubated at 42°C for 4-5 hour (until the plaques appeared).
2. Once the plaque size appeared as pinpoint, nitrocellulose membranes (circles of 85 mm diameter, presoaked in 10 mM IPTG and dried) were gently overlaid on the top of the agar plates. Then, the plates were incubated for 3-5 hours during which the fusion proteins were induced and blotted on the membrane. The plates were kept for one hour at 4°C for hardening the agar to avoid peel off. Before removing the filters, the orientations were marked and membranes were carefully removed. Filters were washed in TBS (20 mM Tris-Cl pH 9.5, 100 mM NaCl) to

remove any traces agar particles. A second IPTG soaked filter was placed on the phage plate and lifted in the similar manner form each plate to obtain a duplicate membrane from each plate.

3. The filters were blocked in blocking buffer (5% Bovine Serum Albumin in TBS with 0.05% Tween 20) for 4-6 hours at room temperature on a slow rocking shaker. The blocked filters were washed twice with TBST buffer (20 mM Tris-Cl pH 9.5, 100 mM NaCl, 0.05% Tween 20) for 3 minutes each and incubated in 1: 300 dilution of primary antibodies (Convalescent- *P. yoelii* sera or human sera from endemic areas which were pre-adsorbed with *E. coli* lysate) in TBST supplemented with 1% BSA at room temperature for 2 hours.

4 Nitro-blue tetrazolium (NBT, 0.3 mg/ml), 5-bromo-4-chloro-3-indolyl phosphate reagent (BCIP, 0.15 mg/ml), were prepared in color development solution (NBT was added first then BCIP was added drop wise). The filters were immersed in the solution and covered with aluminum foil to allow the reaction continues in dark. The positive plaques developed purple color within 3-5 minutes of incubation and adding the stop solution terminated the reaction (20 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0).

5. The filters were aligned properly with the marked orientation on the corresponding plate and the positive plaques were picked up using a sterile Pasteur pipette and the agar plug was transferred to 1 ml SM buffer. Phage

particles were allowed to diffuse overnight at $4^0$C. The titer of the phage library was determined by serial dilution in SM buffer and plating.

6. The plating procedure was repeated with individual positive plaque to obtain reproducible result and a homogeneous purified immunoreactive recombinant phage was thus obtained. The purified positive plaques reactive against anti-*P. yoelii* sera were tested for their reactivity with normal human sera from malarious area. The plaques reactive to both anti-*P. yoelii* and human sera from endemic sera were picked up for further analysis.

## Results:

The development of *in-vitro* culture method of *P. falciparum* led to the enormous understanding of its biology and ultimately its genomics. *In-vitro* culture system utilized in the study is a simple technique, provided all the conditions of human host system and RBC for growth and multiplication of the parasites *P. falciparum*. The culture system yielded sufficient amount of parasite material necessary for all the experiments carried out in the present study. A representative Geimsa stained thin blood film from cultured FCD-3 parasites showing different stages is presented in Fig 3.1. Treatment of parasite culture with 5% D-sorbital resulted in the lysis of late trophozoites and schizonts, while rings and early trophozoites were allowed to grow. The repeated treatment of sorbital resulted a synchronized culture.

*P. yoelii nigerensis* was maintained by regular passage in Balb/c mice. Intraperitoneal inoculation of $10^4$ parasitized erythrocytes into a mouse resulted in about 1% parasitemia at day 4 and the parasitemia increased gradually to 60% by day 8 of

infection. Almost 100 % mice died at day 9 of infection. The blood obtained on day 6-7 was used to harvest parasites. The representative Geimsa slide showing various stages of *P. yoelii* was presented in Fig 3.2. The convalescent phase mouse sera (anti-*P. yoelii*) and pooled human sera from endemic area were adsorbed with *E. coli* lysate to remove anti-*E. coli* antibodies from the serum used for immunoscreening.

*In-vitro* cultured *P. falciparum* parasite pellet was used to extract parasite genomic DNA by organic extraction method. About 20 μg of pure DNA was obtained by this method and around 1 μg of DNA was resolved in agarose gel (Fig 3.3). The DNA was used for genomic library construction.

Choice of an appropriate cloning vector is crucial for successful cloning of any gene for a particular purpose. The requirement of the study was that recombinant clones obtained should be in frame, express and allow for immunoscreening. λgt series of vectors are designed to express the cloned fragment as a β- galctosidase fusion protein in frame with lacZ gene. Among those vectors, λgt11 (Young and Davis 1983) is a commonly used vector for immunological screening. The cloning capacity of λgt11 vector is up to 7.2 kb fragment size, which can be cloned into the unique EcoRI site located in lacZ. Insertion of DNA fragments at this site in proper orientation and reading frame resulted in the inactivation of β-galctosidase activity and the foreign DNA was expressed as fusion protein. Recombinant and non-recombinant phages were distinguished on the basis of colour with chromogenic substrate X-gal. Non-recombinants show blue and recombinants show white coloration when plated on lac indicator bacteria. It also contains a temperature sensitive repressor (cI857), which allows control of

bacteriophage replication and production of fusion proteins. The advantage of the λgt11 expression vector is that the expressed fusion protein will be still reacting with the antibodies and by immnoscreening procedures the target gene can be fished out. The restriction and structural map of λgt11 is presented in fig 3.4. λgt11 vector was used for construction of genomic expression library of *P. falciparum* and subsequent immunoscreening of the resulting library.

The titer of *P. falciparum* genomic expression library was 5 x $10^5$ pfu/ml with 80% recombinants (Fig 3.5). Around 0.2 million recombinants were immunoscreened with anti-*P. yoelii* sera and twenty positive plaques were found positive which were purified individually to homogeneity (Fig 3.6). The positive phage clones were then reacted with both anti- *P. yoelii* and pooled normal human sera from a malaria endemic area. Out of twenty clones one clone (KP1) was strongly reactive to both the sera (Fig 3 7) and it was taken up for further analysis, described in the next chapter.

## Discussion:

To identify the protein or gene of interest it is necessary to screen the genomic/cDNA libraries with either antibody or nucleic acid probe, chosen appropriately. cDNA libraries are highly advantageous over the genomic libraries when the expression of the genes are very high and genome size of the organism are large, but the library may not represent low level expressed genes. In order to clone any gene irrespective of its expression status genomic library are preferred but the drawback is introns may be present in the sequences. Both antibody as well as nucleic acid probe can be used to isolate the gene of choice, when expression vector is used for construction of library. As

mentioned earlier, λgt11 (Young and Davis, 1983) is a commonly used vector for immunological screening and it was used for construction of *P. falciparum* genomic expression libraries. Phage vector have an advantage over plasmid systems since the yield of recombinant phage per ng of vector DNA is more compared to the plasmid.

Kironde *et al* (1991) found that mice immunized with *P. yoelii* antigen extract were partially protected from challenge. However, the mice repeatedly infected with blood stage *P. yoelii* and cured with drugs acquired sterile immunity. The serum IgG from mice convalescent from *P. yoelii* infection inhibited the *in-vitro* erythrocytic growth of *P. falciparum* (Ray *et al.*, 1994). This convalescent serum recognized at least 15 polypeptides of *P. falciparum*, which were also recognized by human sera from endemic area

We used the sera of mice fully protected from *P. yoelii* infection (convalescent-phase mouse anti-*P. yoelii* sera) to screen the λgt11 genomic expression library of *P. falciparum* to identify the interspecies conserved protective epitope. Thus, the anti-*P. yoelii* serum contains the protective antibodies that recognize antigens of *P. falciparum* with cross reactive epitopes. These cross reactive antigens were probed with sera from individuals residing in malaria endemic areas in order to identify the antigens, which are cross reactive as well as immunogenic in human host living in malaria endemic areas. One recombinant cross-reactive clone (KP1) was identified out of 0.2 million recombinants and was selected for molecular characterization in the study.

**Figure 3.1.** Geimsa stained thin blood smear from *in-vitro* cultured *Plasmodium falciparum* showing various developmental stages viz ring, trophozoites and schizonts during its erythrocytic cycle

**Figure 3.2.** Giemsa stained thin blood film from *P. yoelii nigerensis* infected mice showing various asexual blood stages of the parasite and host lymphocytes

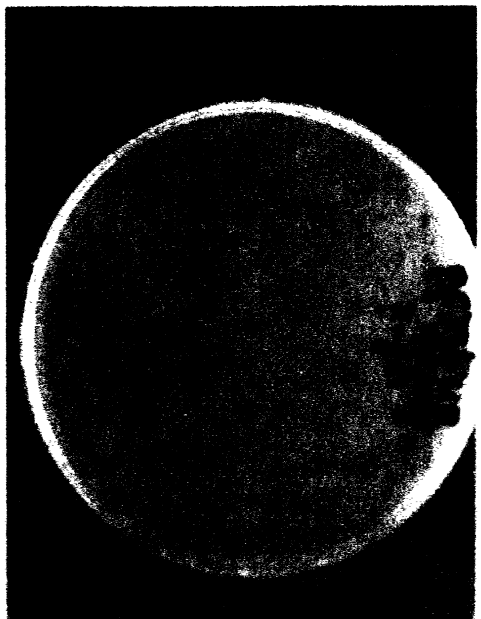
**Figure 3.3.** Agarose gel electrophoresis of *P. falciparum* genomic DNA. DNA was extracted from the parasite and subjected to electrophoresis in 0.7% agarose gel and stained with ethidium bromide. Lanes were loaded with Hind III digested λ DNA (lane M), *P. falciparum* genomic DNA (lane 1, 2)

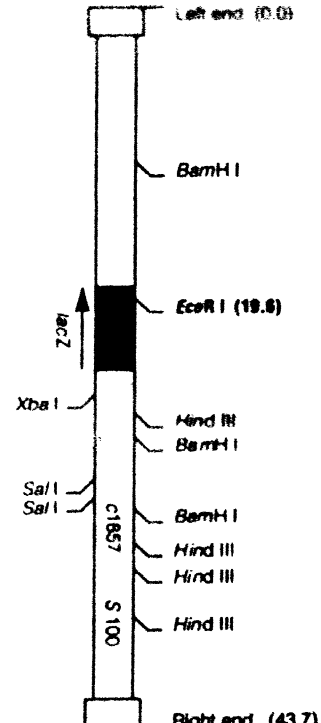
**TOP:**

**Figure 3.4.** Structural map of λgt11 vector showing the position of Lac Z gene, EcoR I cloning sites and some important enzymatic restrictions sites.

**Bottom:**

**Figure: 3.5.** *Plasmodium falciparum* genomic expression library constructed in λgt11 expression vector Titration of the expression library was carried out by incubating the diluted phage stock with Y1090 *E. coli* cells and plated on LB-agar plate (containing amplicillin, X-gal and IPTG), which shows blue (non-recombinant) and white (recombinant) phage plaque



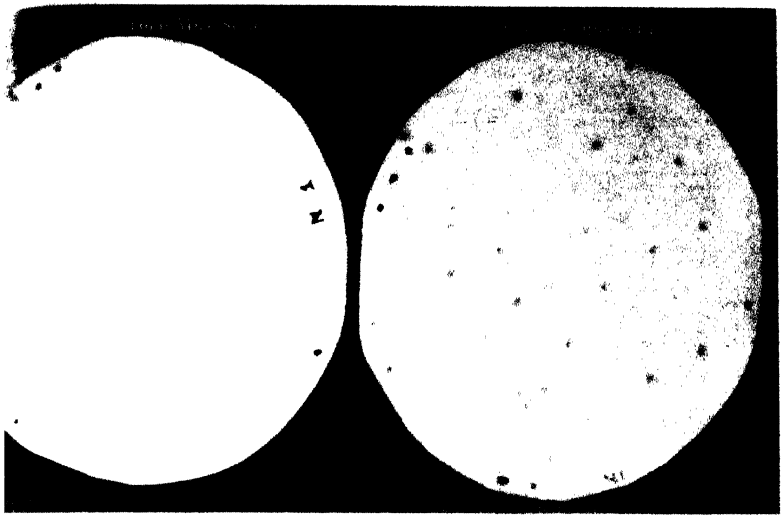

Left end (0.0)
BamH I
EcoR I (19.6)
lacZ
Xba I
Hind III
BamH I
Sal I
Sal I
BamH I
c1857
S 100
Hind III
Hind III
Hind III
Right end (43.7)

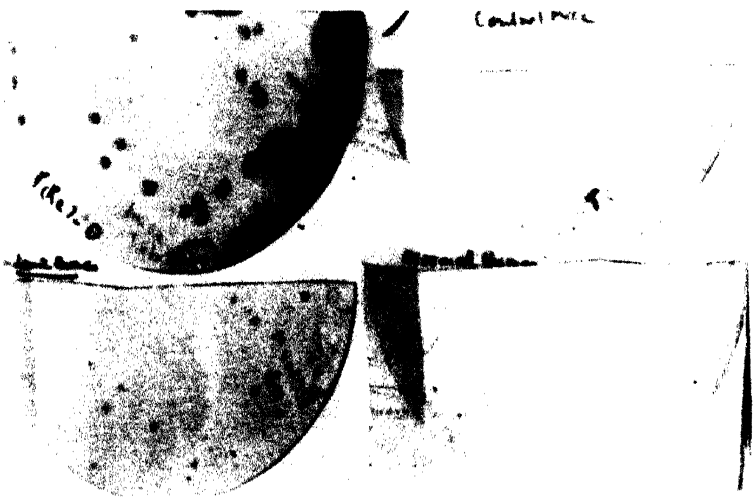
**TOP:**

**Figure 3.6.** Immunological screening of λgt11-*P. falciparum* genomic expression library using antibody probe Around 3000 phage plaques from the λgt11-*P. falciparum* genomic expression library were plated and overlaid with IPTG soaked nitrocellulose membrane to induce the recombinant fusion protein. The phage blotted membrane was lifted, probed with anti-*P. yoelii* sera followed by incubation with alkaline phosphatase conjugated anti-mouse antibody The bound antibody with the expressed plaque antigen was detected by NBT and BCIP substrate indicating brown coloration This figure shows, one of the strongest immnunoreactive clone (KP clone) was plaque purified and reconfirmed its immuno-reactivity with normal mice sera (control) and anti-*P. yoelii* sera.

**Bottom:**

**Figure 3.7.** Cross-immuno reactivity of purified positive plaque (KP clone) to anti-*P. yoelii* and immune sera from malaria endemic area The purified phages from the positive plaque (KP clone) were plated and duplicate phage membranes were lifted. Each circular membrane was cut in to two half in the middle, marked and probed with immune sera (anti- *P. yoelii* or immune human sera) and its respective control sera (normal mice or normal human sera) The filters were processed as mentioned above The results show the cross- reactivity of positive plaque (KP clone) with antibodies viz: Anti-*P. yoelii*, Normal Mice (Control), Pooled human sera from malaria endemic area (Endemic Normal) and human sera from non-malaria area (Control)





area Human Sera (Healthy subjects)