MALARIAL VECTORS & MOLECULAR BIOLOGY OF *PLASMODIUM* PARASITE

Submitted from

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Acknowledgement

I would like to express my heartfelt appreciation to the ICMR-NIMR Goa field station for providing me with the opportunity to embark on an incredible internship journey in Malaria research.

Completing an internship successfully requires the help and support of many individuals. I would like to take this opportunity to extend my gratitude to all those who have assisted me in preparing this report.

I am immensely grateful to the Department of Biotechnology (DBT) for offering us the invaluable opportunity to partake in a high-quality internship.

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Mosquito and Malaria Parasite:

Malaria is a life-threatening acute febrile illness which is caused by the parasite *Plasmodium*. This disease is spread by the bite of female *Anopheles* mosquito infected by *plasmodium* spp. Not all mosquitos are vector of the malarial parasite but there are 70 known *Anophenile* mosquitos spp. which are spreading malaria throughout tropical and sub-tropical region of the world.

The parasite *Plasmodium* is very destructive because it has 2 hosts - the mosquito and human. Though the male mosquitos cannot spread the parasite as they feed only on nectar from flowers, but the females are responsible for spreading the infection.

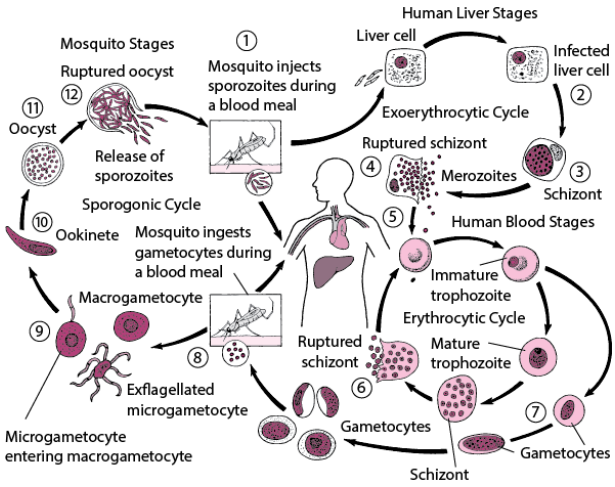
There are 5 spp. Of plasmodium which are known to infect mankind of which *Plasmodium falciparum* is the deadliest parasite occurred mostly in Africa. *Plasmodium vivax* is present in India and some other sub-tropical region. *Plasmodium ovale* and *Plasmodium knowlesi* are uncommon and *Plasmodium malaria* is the rarest type of malarial parasite.

Plasmodium is having its asexual stage in human body and sexual stage in mosquito.

In mosquito the parasite is present in midgut and saliva. In midgut the gametes are fertilized and make Ookinete and they accumulated in oocysts. Then after getting mature, they turned into Sporozoites and released from oocysts and diffuse towards salivary gland and retain inside it.

Infected mosquito when bites a human host it discharges the sporozoites in the blood and the sporozoites travel through blood and infect the hepatocyte cells. There in exoerythrocytic cycle Merozoites are produced and released in blood stream and attack the red blood cells. In RBC it turned into Trophozoites.

Then Trophozoites are undergo toward gametocytes and made gametes. Then when another mosquito bites the infected human, the gametes will come to mosquito midgut and the sexual cycle will resume again.



Reference: https://www.msdmanuals.com/-/media/manual/professional/images/i/n/f/inf_plasmodium_life_cycle.gif?thn=0&sc_lang=en

Image: Complete life cycle of Plasmodium parasite

Rearing & Maintenance of Different Species of Mosquito:

The mosquito which are carrying the *Plasmodium* sp. are called as Vectors. For study and research purpose we need to rear and maintain the different stages of their life. Here in the lab all life stages of vectors are well maintained in Insectary.

There are four stages of life of a Vector Anopheles – Egg stage, Larvae stage, Pupa stage, adult stage.

The egg is being laid by female mosquito and after several hours those eggs are hatched to larval stage. They generally lay 100 eggs at a time.

Larval stage has 4 stages which are called as instars. These instars are active form of larvae in water. During this stage, they should be provided with enough food. For their survival. They remain in this stage for 10-12 days.

After 10-12 days the larvae finally enter pupal stage.

The adult stage is the final step. It emerges from pupal case. The male mosquitos feed on nectar from flowers. The female vectors are feed on nectar and blood of human and animals.

Image: Egg stage

Image: Larval (4th instar) stage

Image: Pupa stage

Image: Adult

Among all the above stages only the healthy emerged Adult female infected mosquitos can transmit the malarial parasite. For this purpose, we need to have better understanding about the physiology and anatomy of the vector.

For this we need to rear and maintain a healthy adult mosquitos' population in lab.

The adult mosquitoes are kept in Percival. This instrument keeps the ideal condition like temperature, humidity, light which are ambient for growth of the mosquitoes. Food source for them includes glucose pads (which are cotton soaked in glucose solution) placed on top of meshed container.

In Goa two major vectors which are known to transmit malaria are *An. Stephensi* and *An. subpictus.* Of which An. Stephensi is the well-established vector in goa.

Works are going based on these 2 species of Malaria vectors.

Image: Percival

Mosquito Identification:

There are 70 Anopheline spp. Present worldwide and all of them differ in their morphology in order to identify them we use mosquito identification key.

Generally, we do identification of a full mosquito not a dissected mosquito. So, we first anaesthetize the mosquito with either Diethyl ether or Chloroform. Then we put the vectors under Binocular Microscope.

Identification Table:

Mosquitos	Identification Characters
Anopheles sp.	i) Elongated Palpi is equal to Proboscis. Palpi is slender in females and club shaped in males. ii) Dark spotted wings are present. iii) The Hind leg is darkly spotted.
Culex sp.	i) Palpi is not extended and shorter than Proboscis. ii) Transparent wings iii) Spotted legs are present here. iv) Abdomen is dark brown and thorax is light brown.
Aedes sp.	i)Short Palpi compare to Proboscis. ii) It has white scales on the dorsal surface of thorax. iii) Transparent wings and pointed legs are there.

Dissection of Mosquito:

Among all the mosquito species, a few Anophelines are responsible for the carriage of parasite of malaria i.e., *Plasmodium sp.* Dissection of mosquitoes is an important tool to detect and examine the parasite. For a successful identification of parasite with its various stages inside a vector mosquito body, mid gut and salivary gland dissection is the subject of immense importance. To understand the stages like Oocyst in mid gut we need to dissect it from a whole mosquito by a fine sharp needle (insulin syringe tip).

As we know the causing parasite is present in either in Midgut or in Salivary gland, we need to dissect the mosquito after anaesthetizing it. Then we need to separate the midgut and salivary gland as the oocyst and sporozoite stage of parasite are present here respectively.

The proboscis, legs, wings, are removed first that they cannot fly further and bite someone. Later, on a clear glass slide the mosquito sample is placed and view under binocular microscope. The head, thorax, abdomen is separated with clean sharp needle. The midgut is removed from abdomen and the salivary gland is removed from thorax to visualize the presence of parasite under phase contrast microscope. The midgut and salivary gland are kept with PBS (Phosphate Buffered Saline) buffer to maintain the physiological conditions. Then with Mercurochrome we stained the midgut and it is ready for visualization of the presence of parasite. The salivary gland need not to be stained and it can be visualized with PBS buffer only.

If we need to do molecular examination of the parasite, we require the thorax as a tissue sample for the DNA extraction of the parasite of the vector.

DNA Isolation from Mosquito:

If the mosquito is infected with Plasmodium Sp. Then we will get it from the thorax of the vector. So, dissection of thorax of *Anopheles stephensi* was performed first and then it was put into microcentrifuge tube. The next procedures are as follows

I. We put 50uL ATL buffer (All Tissue Lysis) to the tubes having vector thorax (25mg). It made tissues soft. Then we crush the thorax by pestle to bring out the DNA.

II. Then we add 130uL of ATL buffer by washing the pastle thoroughly. And the pestle was discarded.

III. Add 20uL Proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shacking water bath or on a rocking platform.

IV. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

V. Make mixture of AL buffer(200uL) and Ethanol(200uL) and add 400ul to tube.

VI. Carefully apply this mixture to QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.

VII. Carefully open the QIAamp Mini spin column and add 500uL Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube, abd discard the filtrate.

VIII. Carefully open the QIAamp Mini spin columnin and add 500 uL Buffer AW2. Close the cap and centriguge at full speed (14000 rpm) for 3 min.

IX. Place the QIAamp Mini spin column in a new 2 mL collection tube and discard the old collection tube with filtrate. Centrifuge at full speed (14000 rpm) for 1 min.

X. Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 50 uL Buffer AE. Incubate at room tempereture for 1 min and then centrifuge at 8000 rpm for 1 min.

XI. Repeat the last step

The DNA now can be stored in -20°C freezer.

DNA Isolation from DBS:

Dried blood spots are collection of blood of human on a filter paper. In the absence of a particular patient the DNA can be extracted from the dried blood spots for any purpose.

This is the process of storing someone's blood for future use and extracting DNA from the blood whenever required.

For short time storage of DBS it should be kept at 4°C (2 days) and -20°C (4-5 days).

For long time storage it should be kept at -80°C.

The extraction process of DNA from DBS is as follows

I. Punch and cut 4-5 small pieces of DBS and place into a microcentrifuge tube. Add 200uL of 1x PBS buffer and incubate 5-10 min at room temperature.
II. Add 20uL of Proteinase K solution to the microcentrifuge tube, mix by vortexing. Add 400uL of Lysis solution, mix thoroughly by vortexing or pipetting to obtain a uniform suspension.
III. Incubate the sample at 56°C for 10 min while vortexing occasionally or use a shaking water bath, rocking platform or thermos mixture until the cells are completely lysed.
IV. Add 200uL of ethanol (96-100%) and mix by pipetting.
V. Transfer the prepared mixture to spin column. Centrifuge for 1 min at 8000 rpm. Discard the collection tube containing flow through solution. Place the column into a new 2 mL collection tube.
VI. Add 500uL of Wash Buffer WB1 (with ethanol added). Centrifuge for 1 min at 8000 rpm. Discard the flow through and placed back into the collection tube.
VII. Add 500uL of Wash Buffer 2 (with ethanol added) to the column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube containing flow through solution and transfer the column to a sterile 1.5 mL microcentrifuge tube.
VIII. Add 50uL Elution Buffer to the centre of the column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 rpm.
IX. Discard the purification column. Use the purified DNA immediately in downstream applications or store at -20°C.

PCR of Isolated DNA:

Principle:

PCR is based on the enzymatic replication of DNA. In PCR, a short segment of DNA is amplified using primer mediated enzymes. DNA polymerase synthesises new strands of DNA complementary to template DNA. The DNA polymerase can add a nucleotide to pre-existing 3'-OH group only.

Protocol:

I. Add required reagents or mastermix and template to PCR tubes.
II. Mix and centrifuge.
III. Amplify in thermalcycler

There are different types of PCR using for various purpose. For malaria infection study mainly 3 types of PCR are focused.

ITS2 PCR: The internal transcribed spacer 2 region of nuclear ribosomal DNA is regarded as one of the candidate DNA barcodes as it possesses several valuable characteristics, such as availability of conserved region for designing the primers and ease of amplification.

The 18s sequence is very conserved for eukaryotes. In this PCR 18s sequence is commonly used.

Here we used ITS2 forward primer and reverse primer.

Reagents	Quantity(x1)	Quantity(x3)
PCR mixture	5uL	15uL
Forward Primer	1uL	3uL
Reverse Primer	1uL	3uL
Nuclease Free Water	1.5uL	4.5uL
Template	1.5uL	-
Positive Control	1.5uL	-
Negative Control	1.5uL	-

Multiplex PCR: More than one pair of primers are required in the same reaction. The primers can specifically combine with their corresponding DNA template, and more than one DNA fragment will be amplified in one reaction simultaneously.

For the sub species identification of malaria vectors this particular PCR is very useful.

Reagents	Quantity(x1)	Quantity (x3)
PCR Mixture	5uL	15uL
Forward Primer	1uL	3uL
Reverse Primer	0.5uL	1.5uL
Reverse Primer	0.5uL	1.5uL
Reverse Primer	0.5uL	1.5uL
Nuclease Free Water	1.5uL	4.5uL
Template	1uL	-
Positive Control	1uL	-
Negative Control	1uL	-

Nested PCR: This particular PCR involves two sequential amplification reactions, each of which uses a different pair of primers. The product of the first amplification reaction is used

as the template for second PCR, which is primed by oligonucleotides that are placed internal to the first primer pair.

Nested PCR involved in detection of infection status of malarial parasite in the body.

Reagents	Quantity(x1)	Quantity(x3)
PCR mixture	5uL	15uL
Forward Primer	1uL	3uL
Reverse Primer	1uL	3uL
Nuclease Free Water	1uL	4.5uL
Template	2uL	-
Positive Control	2uL	-
Negative Control	2uL	-

Gel Electrophoresis:

To visualise DNA, we have used gel electrophoresis. We made 1.5% agar. We took 0.75 gm of agar and dissolved it in 50 ml of TBE (Tris, Borate and EDTA) buffer.

The later procedure is as follows

Agar was melted in the TBE buffer in a conical flask and put inside microwave to be melted down. It should be done carefully as the buffer comes out of the flask upon boiling, so continuously it was put ON and OFF to prevent this problem.

Next, we brought out the agar solution from microvan and kept it for cooling. After little bit of cooling, we put SYBR Safe (4uL) and mix it gently. It is a visualising dye of DNA.

Then we allowed this solution to cool down little more. Then we put the solution into casting tray which was cleaned well in alcohol along with comb. Slowly poured that no bubble could form. Then this gel was leave for solidify for 15-20 minutes.

After gel was solidified, we removed the comb and the wells were visible clearly. Then we placed the gel into electrophoresis tank. We put TBE buffer and merged the gel completely. Make sure that wells are toward electrode.

Next, we loaded our samples inside every well 4ul. After that we turned the power supply ON at 70 volts. The sample started to migrate towards the anode (positive)

After 45 minutes we put OFF the power supply and put the gel inside Gel Doc. We turned UV light ON and visualize the DNA.

ITS2 product was amplified and visualised, the experiment was successful. Then we discarded the gel.

Image: gel electrophoresis

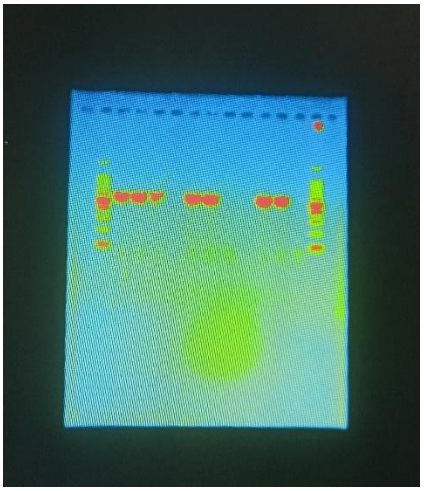


Image: gel electrophoresis

Microscopic view of blood smear:

Malaria suspected patient are examined multiple times with variety of tests. Among all the first test is based on microscopy. The presence of malarial parasite i.e., *Plasmodium sp.* will determine the primary detection of malaria. Microscopic visualization needs blood smear.

First, from finger of the patient, one drop of blood is taken by pricking. The blood is taken on a sterile glass slide. Now the blood can be smeared in 2 ways – thick smear and thin smear.

Thick smear is spherical spread of the blood drop on the slide whereas thin smear is the elongated spread of blood drop on the slide.

The blood drop speeded with sterile sharp edged glass slide. For thin smear there are some parameters for successful smear. The speed, pressure, angle (35°-40°) of the spreading slide should be perfect for a good smear. The end part of the thin smear should be tongue shaped.

Then slide is kept for drying completely. The slide then flooded with ethanol for fixation of ingredients of the blood. After 25-30 seconds we removed extra ethanol and let the slide to be air dried.

Next, we put giemsa dye (30% concentration) on the smeared slide and kept it for 25-30 minutes. After that we removed the stain and let the slide to be dried at room temperature.

Next, we have seen it under compound microscope in oil immersion objective lens at 100x.

Observation: We found the presence of malarial parasite i.e., *Plasmodium vivax.* There is also presence of different types of white blood cells (WBC) and platelets. RBCs were the dominating cell there. We found the parasite having 4 different stages or morphology separately.

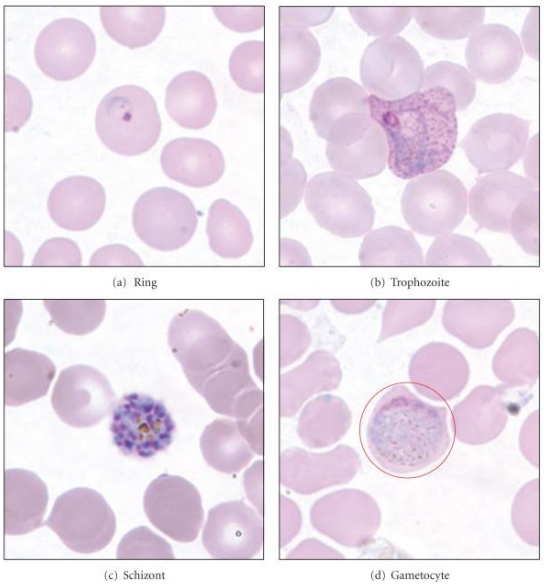


Image: Microscopic view of *P.vivax*

Reference https://www.researchgate.net/publication/232321364/figure/fig1/AS:214114317148160@1428060239793/The-malaria-images-for-the-four-life-cycle-stages-of-P-vivax-16.png

Ring stage of *P.vivax* is having a ring shaped structure where the head of ring like dot is there.

Trophozoite stage is not properly round shaped but having proper densely dyed nucleus.

Schizont stage has multiple densely dyed spots all over the body.

Gametocyte stage is not commonly found. It has oval like structure with scattered brown pigment.

References:

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Experience Certificate

This is to Certify that Mr. Abhranil Doloi, student of MSc Marine Biotechnology, Goa University, Taleigao, underwent training in skill and research practices in the Laboratory of ICMR-National Institute of Malaria Research, Field Unit Goa from 1st to 30th December, 2022. This certificate is being issued to Mr. AbhranilDoloion his request.

6/3/2023

(Officer in Charge)

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