METHODS
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IN VITRO CULTIVATION OF PLASMODIUM FALCIPARUM:

Plasmodium falciparum culture was maintained in 125 ml Erlenmeyer flasks using the modified procedure of Siddiqui et al. (1979). The human serum was substituted with rabbit serum (Sax & Rieckmann, 1980).

MEDIUM PREPARATION

To a premeasured RPMI 1640 packet of powdered medium, 2 gm of sodium bicarbonate and 5.95 gm of HEPES buffer were added in a volumetric flask containing slightly less than 1 litre of triple glass distilled water. The pH of the medium was adjusted to 7.45 and 25 mg of Gentamicin was added. The medium was made up to 1 litre and sterilized through a 0.45 um Millipore filter. The medium was then dispensed aseptically into flasks and stored at 4°C.

COLLECTION OF RABBIT SERUM

Rabbits were anaesthetized before taking blood from the heart. The blood was collected in 10 ml screw capped tubes under aseptic conditions and kept at room temperature for 4 hrs and then left overnight in the refrigerator. Serum was separated from the blood clot and centrifuged at 2000 x g for 30 min at 4°C. The serum was stored at 4°C in 10 ml screw capped tubes until used.
Figure 1: Photograph showing a laminar flow used for carrying out *in vitro* cultivation of *Plasmodium falciparum*.
CULTURE PROCEDURE

About 5 ml of venous blood was taken from a patient who had *P. falciparum* infection with a parasitomia of around 0.2%. The blood sample was collected in acid citrate dextrose (ACD). The infected blood was centrifuged at 1500 x g at 4°C for 10 minutes to separate the plasma from the cells. The packed cells were washed five times with RPMI medium and centrifuged each time at 1500 x g at 4°C for 10 minutes. During washing the top buffy coat containing the white blood cells were aspirated and removed. Packed and washed infected red cells were reconstituted with RPMI medium in the ratio of 1:1 containing 1 ml of the reconstituted blood along with 3 ml of RPMI medium and 1 ml of rabbit serum. The above mixture containing blood, serum and medium were put in an 125 ml volume Erlenmeyer flask. A rubber cork with two glass tubes was put on the top and a special gas mixture (Nitrogen 90%, carbon dioxide 8% and oxygen 2%) was passed. The flask was placed in a walk-in-incubator at 37°C. A slide smear (0 hour) from the starting culture was routinely made before it was incubated. Besides making such slides everyday, a record of parasite count from day 0 onwards was regularly maintained.

PREPARATION OF LEISHMAN STAIN

Leishman stain solution was prepared by dissolving 150 mg of Leishman powder in 100 ml of methanol. Glass beads
Figure 2 (A): Photograph showing a walk-in-incubator and the culture flasks fitted to the special gas cylinder.

(B): Diagramatic representation of Fig. 2(A).

(C): Culture flasks.
(A) Gas mixture: 2% O₂, 8% CO₂, 90% N₂

(B) Diagram:
- 2% O₂
- 8% CO₂
- 90% N₂
- Gas mixture
- Cotton plugs
- 125 ml Erlenmeyer flask
- 8 ml medium
- 1 ml serum
- Water seal
- Red cell layer
- Bubbler

(C) Setup with flasks and tubing.
were added to aid the dissolution of the powder during shaking. The solution was incubated at 37°C for 48 hours. Before use, the stain was filtered through a Whatman No.1 filter paper.

STAINING AND FIXATION OF BLOOD FILM

The dried blood film was first fixed in methanol for 4 minutes and then dried. The stain solution was made by mixing equal volumes of Leishman stain and phosphate buffer (0.01 M, pH 7.2). The stain mixture was then layered on the top of fixed and dried blood film. The slides were left for 30 minutes and then washed with phosphate buffer.

The stained films were examined in a microscope (Carl Zeis, Jena) under oil immersion.

COLLECTION OF EXHAUSTED CULTURE MEDIUM FOR ISOLATION OF SOLUBLE ANTIGEN

The exhausted culture media from parasite and uninfected, erythrocyte control cultures were collected at every 24 hours when parasitemia was around 5% or more. The collected medium was centrifuged at 2,500 x g for 30 minutes at 4°C, pooled and stored at -20°C until processed (Gabrielsen et al., 1983).

PREPARATION OF CRUDE S-ANTIGEN FROM POOLED EXHAUSTED CULTURE MEDIUM

The pooled parasite and erythrocyte control media were heated at 100°C for 5 minutes and the heat-aggregated protein
was removed by centrifugation at 12,000 x g for 20 minutes at 4°C. Then the supernatant was subjected to ammonium sulphate precipitation. The precipitate so obtained was dissolved in a minimal amount of distilled water (Anders et al., 1983). The resuspended parasite antigens were dialysed against PBS to remove ammonium sulphate.

CHECKING THE IMMUNOGENICITY OF S-ANTIGEN

The antigenicity/serologic reactivity of the crude S-antigen was tested against clinically positive human serum samples obtained from malaria patients using various immunological tests, like immunodiffusion, counterimmunoelectrophoresis and indirect haemagglutination (IHA) tests.

All the sera samples were first checked for the presence of specific antibodies against a reference, standard antigen sample in IFA tests. Immunizations of rabbits for checking the antigenicity of antigen isolates is still in progress.

PROTEIN ESTIMATION

Protein estimations were carried out according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Folin phenol reagent of AR grade was obtained from SISCO (Bombay).
PREPARATION OF COPPER REAGENT

Copper reagent was prepared by mixing the following reagents:

Four percent w/v sodium carbonate in double distilled water, 4 percent w/v sodium potassium tartarate and 2 percent w/v copper sulphate in the ratio of 100:1:1 (v/v/v).

An 0.1 ml of antigen or bovine serum albumin was brought to 1 ml volume by the addition of distilled water. A 5 ml sample of freshly prepared copper reagent was added to it and then it was incubated for 10 minutes at room temperature. After incubation, 1 ml of folin-phenol reagent was added to each tube. The tubes were shaken vigorously and left undisturbed for 30 minutes. The colour intensity of the reaction was read at a wavelength of 700 nm by using a Bausch and Lomb Spectronic-21 Spectrophotometer.

pH MEASUREMENTS

All pH measurements were made on an ELICO Model LI-10 pH meter. Sodium tetraborate (0.1M, pH 9.18, temp. 25°C) and potassium hydrogen phthalate (0.05M, pH 4.0, temp. 20°C) were used as standard buffer solutions to calibrate the pH meter.

SPECTROPHOTOMETRIC MEASUREMENTS

Spectrophotometric measurements were carried out on Bausch and Lomb Spectronic-21 Spectrophotometer.
IMMUNODIFFUSION

This test was performed according to the published procedure of Ouchterlony (1949). Agarose was dissolved in 0.05 M barbitol buffer, pH 8.6 containing 0.02% sodium azide. The concentration of agarose was 0.8%. The melted agarose was poured on glass slides to form an approximately 1.5 mm thick layer of uniform thickness. The slides were left for the gel to solidify. The wells were punched in the solidified gel.

The wells were loaded with antigen and antiserum samples. The test slides were kept for 4 hours at room temperature in a moist chamber and subsequently at 4°C for 72 hours. All the precipitin lines were thoroughly resolved and visible within 72 hours of incubation.

COUNTERIMMUNOELECTROPHORESIS

This test was performed according to the method of Bjerrum and BjHansen (1976). The method of slide preparation for CIE was the same as for immunodiffusion. On agarose coated slides, a set of wells was cut with a 2 mm punch. The centre to centre distance between the wells was 5 mm.

The cathodal wells were filled with the antigen, while
anodal wells were loaded with antiserum. Electrophoresis was performed for about 40-50 minutes. The slides were then incubated at room temperature for 4-5 hours in a moist chamber. After incubation, the resolved precipitin bands were later recorded.

INDIRECT FLUORESCENT ANTIBODY TESTS

The procedure outlined in World Health Organization Memoandum (1974) was followed with certain modifications.

(a) Preparation of Antigen

Smears were made from continuous in vitro cultures of P. falciparum when parasitemia was around 4-5%. The coverslips were air dried, fixed in acetone for 15 minutes and again air dried. The slides were wrapped in tissue papers and packed in air tight containers in small batches and stored at -20°C until used.

(b) Staining Procedure

Fluorescein isothiocyanate conjugated anti-human IgG was used as the conjugate in this test. Dilutions of the test sera were made in PBS (pH 7.2), a dilution of 1:8 was used as the starting point.

Antigen slides were removed from -20°C and brought to
room temperature. The antigen spots were covered with drops of successive serum dilution and incubated in a moist chamber for 30 minutes. After incubation the slides were rinsed off with PBS in three changes of PBS for 5 minutes each. The smears were blotted dry and the optimum dilution of the conjugate was applied to the antigen spots. The slides were incubated in a moist chamber for 30 minutes and later washed in PBS as above and dried with filter paper. The slides were then mounted with the coverslips using buffered glycerol. The edges of the coverslips were sealed with finger nail varnish. Two control slides were prepared by placing a drop of PBS buffer on one slide and a known positive antimalarial serum on the other and stained as above. The stained slides were examined immediately using fluorescent microscope (Olympus Co. Ltd., Japan) equipped with appropriate filters.

INDIRECT HAEMAGGLUTINATION TEST (IHA):

Indirect haemagglutination test was carried out for checking the IHA reactivity of the S-antigen. This test was done according to the method of Mathews et al. (1975). Microplates with U-shaped bottom (Cook U-plate, Cook Engineering Company, Alexandria, Virginia) were used for this test. Sheep red blood cells were washed four times in isotonic phosphate
buffer saline, pH 7.2, by centrifugation for 10 minutes at 1500 x g. A 3% suspension of sheep RBC was prepared in isotonic PBS. The diluted RBC suspension was mixed with an equal volume of 1/20,000 tannic acid and incubated at 4°C for 30 minutes with intermittent shaking. After incubation, the suspension was centrifuged at 1200 x g for 10 minutes and tanned sheep RBCs were washed three times with PBS containing 0.5% bovine serum albumin (BSA). The above was resuspended in PBS containing BSA to obtain a final concentration of 3% (v/v). The tanned sheep RBCs were sensitised with an equal volume of diluted antigen and incubated at 37°C for 30 minutes with intermittent shaking, so that the RBCs were uniformly coated with antigen. After incubation, they were centrifuged to remove the uncoated antigen supernatant. The pellet was washed 3-4 times with PBS containing BSA. A 1.5% (v/v) antigen coated RBC suspension was finally made. The antisera samples were serially diluted from 1:2 to 1:1024, undiluted antisera samples were also used. Each well in the plate received 25 ul of malaria positive serum along with an equal volume (25 ul) of tanned and antigen sensitized RBCs. The plates were rotated gently for five minutes, covered and sealed in a humid chamber and incubated at room temperature for 2 hours. The plates were kept overnight at 4°C. The formation of mat-like or carpet-like patterns indicated a positive reaction. The highest dilution of the serum giving a positive reaction was recorded.
The processed supernatant medium from parasitised as well as the uninfected control, were both tested for their IHA reactivity.

FURTHER PURIFICATION OF THE CRUDE S-ANTIGEN SAMPLE

The S-antigen samples obtained from continuous in vitro cultures of *P. falciparum* are being further purified using SP-tris acryl cation exchange chromatography (Thelu et al., 1985). The concentrated medium was dialysed against 0.01 M sodium acetate buffer (pH 3.7) for 48 hours at 4°C. Preliminary separation has been carried out by loading the crude S-antigen samples into a SP-trisacryl column (50 by 200 mm). The elution was done in 0.01 M sodium acetate buffer (pH 3.7), with an increasing stepwise salt gradient of 0.3 M NaCl at a flow rate of 300 ml/hour. One millilitre fraction samples collected from the initial runs have been sterilized by membrane filtration, and stored at -70°C until used for checking their immunogenecity.

IMMUNIZATION OF RABBITS USING CRUDE S-ANTIGEN PREPARATION:

Presently, rabbits are being immunized with the crude S-antigen samples for testing immunological responses through antibody detection by means of indirect fluorescent antibody test, indirect haemagglutination test and enzyme linked
immunosorbent assays. All immunizations are being carried out in healthy adult rabbits weighing about 1.5 kgs, of either sex. In all, eight rabbits were immunized with the supernatant medium derived from *in vitro* cultures of *P. falciparum* while five rabbits were immunized with the control medium derived from uninfected erythrocyte cultures. Immunogenicity of the antigen was fortified by the use of Freund's complete adjuvant (FCA). The supernatant medium concentrates were mixed with FCA and were injected intramuscularly in the hind quarters of the test animals. The immunization was completed over a period of four weeks with third week as the rest period. Booster doses were given in the fourth week in all animals. Weekly blood was drawn from the marginal ear veins for detecting the sequential appearance of anti-malarial antibodies. The protocol for animal immunization is indicated in Table I.
Table I. Immunization schedule.

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<th>Groups</th>
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<td>2. $U_{EC}$</td>
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Total inoculum in each injection was made up to 2 ml with the addition of sterile saline.

$S_{Ag}$ = S-antigen preparation, $U_{EC}$ = Uninfected erythrocyte control preparation, $F$ = Freund's complete adjuvant.