**MATERIALS AND METHODS**
Serum samples from the following groups of subjects were included in the present studies.

**Group - I** included slide positive cases of malaria. They gave a history of characteristic periodic high fever with chill. Most of these cases also had splenomegaly or hepatosplenomegaly. The samples from slide positive cases of malaria were collected from Distt. Mathura, PHC-Ral (41); Distt. Mirzapur, NTPC Shaktinagar (30); Distt. Unnao, PHC-Purva (18); Distt. Shahjahanpur, PHC-Kalan (55); Distt. Lucknow (11); Distt. Ambala PHC-Chaurmastpur (19); Distt. Karnal (20); Distt. Mathura, PHC-Mant (84) and Armed Forces Medical College, Pune (50).

**Group - II** comprised of 62 patients with pyrexia of varied origin.

**Group - III** was constituted by 234 random hospital patients who had no sign/symptoms or history of malaria.
Group - IV consisted of medical students apparently in good health and having no history of malaria.

For collection of serum, five to seven ml of venous blood from each subject was obtained aseptically by venipuncture and allowed to clot at room temperature for 45 to 60 minutes. Serum was separated from the clot immediately afterwards by centrifugation and inactivated at 56°C for 30 minutes. Thiomersal (British Drug Houses, U.K.) was incorporated at a final concentration of 1:10,000 in each serum sample and it was stored in a clean screw capped specimen tube (15 mm x 45 mm) at -70°C until used.

In addition, blood samples from 110 slide positive malaria cases, 257 random subjects from Sonepat (Haryana) and 40 normal healthy persons were obtained on filter paper discs (Whatman No. 3 MM chromatography paper) from the finger pricks. Each filter paper disc (10 mm diameter) was soaked in 0.2 ml of phosphate buffered saline, pH 7.2, at room temperature for one hour. The eluate was then tested for presence of malarial antibody using IHA and IFA tests.

Samples from 354 troops belonging to Central Command, Lucknow, were also collected from the finger pricks and tested by the IHA test alone.

Antigen

Four species of malaria parasites namely *P. knowlesi* (strain W), *P. cynomolgi* (strain B), *P. coatneyi* and
P. berghei were evaluated for their usefulness in the serological tests for human malaria. Adult rhesus monkeys (Macaca mulatta) of either sex, weighing about 3-5 kg were maintained on normal diet (pellets, bananas and soaked grass) in the animal house of this Institute. They were free from tuberculosis as shown by tuberculin tests and were kept under 12 hrs photoperiodicity with fluorescent lights on from 0700 hrs to 1900 hrs, which ensured synchronicity of infection with P. knowlesi. A strain of Plasmodium knowlesi, (W, kindly donated by Prof. Garnham) and the other two strains, namely, P. cynomolgi (B strain) and P. coatneyi which were obtained by the courtesy of Dr. W.E. Collins, Center for Disease Control, Atlanta, have been maintained by serial blood passage in normal monkeys or cryopreserved in buffered glycerol in liquid nitrogen and used for initiating infection from time to time.

The monkeys were inoculated generally with a standard inoculum of 1 x 10^6 parasitized erythrocytes intravenously through cephalic vein.

Blood smears from infected monkeys were prepared from ear vein and stained with Giemsa stain. The parasitaemia (percent infection) was determined by counting the number of parasites/10^6 erythrocytes. Infection became patent on day 3-4. Mean patent period was 4-5 days and monkeys infected with P. knowlesi which followed a fatal course died after attaining a maximum parasitaemia. The monkeys generally
remained in coma for 2-4 hrs before death whereas *P. cynomolgi bastianellii* and *P. coatneyi* infections are chronic in nature. For maintenance of *P. berghei* infection, white rats were used as host. The infection was maintained by passaging infected blood through the recipients.

**Preparation of antigen for IHA test**

Monkeys showing parasitaemia $> 50\%$ were bled at schizont stage by cardiac puncture. The blood collected in the prechilled acid citrate dextrose solution was centrifuged at 1500 rpm for 10 minutes in a refrigerated centrifuge and the supernatant was discarded. The cells were then resuspended in the phosphate buffered saline (pH 7.2, 0.15 M) to the original blood volume and centrifuged. This process was repeated twice. The buffy coat was removed after first and second centrifugation. The schizont infected erythrocytes which sedimented in a distinct, chocolate brown layer just beneath the buffy coat were aspirated and layered on a Ficoll-Conray-420 density gradient (10 ml of 9% Ficoll was mixed with 24 ml of 33% Conray-420). The schizont infected erythrocytes appeared as a diffuse band at the interphase, were aspirated and washed thrice with chilled phosphate-buffered saline (pH 7.2, 0.15 M) in a refrigerated centrifuge. After the final washing the sediment was suspended in double the volume of saponin saline solution.
(0.2% solution of saponin was made in normal saline) for half an hour in an icc bath. The liberated parasites were washed thrice with PBS (pH 7.2) in cold centrifuge to get rid of erythrocyte 'ghosts' and the final sediment was used as a source of antigen. It was divided into small aliquots and stored at -196°C in the liquid nitrogen. A part of this preparation was also stored at -70°C and -10°C and tested at different intervals of time to ascertain the stability of its antigenic reactivity in the IHA test.

Another part of the intact schizonts preparation was diluted 10 times with PBS- pH 5.3 and subjected to ultrasonication for 30 seconds with amplitude needle at mark 12 (Soniprep-150; MSE Scientific Instruments, Sussex, England). The ultrasonicate was centrifuged at 8900 g for half an hour at 4°C. The sediment which largely contained pigment granules was discarded. The supernatant was stored at -196°C, -70°C, -10°C and tested for antigenic reactivity at different intervals of time. Aliquots of intact schizont preparation and the supernatant obtained after ultrasonication of intact schizont, were lyophilized and stored at -70°C, -10°C and room temperature to check its antigenic reactivity in IHA test at different time intervals. Estimation of protein contents of the supernatant was carried out by the method of Lowry et al. (1951) using bovine serum albumin as standard.
Indirect haemagglutination test:

Indirect haemagglutination (IHA) test was performed according to the method of Meuwissen (1974).

Preparation of sheep red blood cells (SRBC)

Sheep blood was collected aseptically in the modified Alsever's solution in the proportion of 10 volumes of sheep blood and 12 volumes of the Alsever's solution and stored at 4°C.

Modified Alsever’s solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.50 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>8.00 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>4.20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
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</table>

The solution was sterilized by autoclaving at 15 lbs for 15 minutes.

In case the erythrocytes from a particular sheep agglutinated spontaneously during the process of tanning and sensitization, another sheep was substituted whose erythrocytes did not agglutinate during these processes.

Two or three day old SRBC’s collected as above, were washed three times in phosphate buffered saline, pH 7.2 and a 3% suspension of SRBC’s was prepared after the final wash.
washing (3 ml of packed washed SRBC's + 97.00 ml of PBS, pH 7.2).

**Phosphate buffered saline, pH 7.2 (PBS - pH 7.2, 0.15M)**

- 0.15M Na₂HPO₄ anhydrous: 380 ml
- 0.15M KH₂PO₄: 120 ml
- 0.15M NaCl: 1000 ml

**Tanning of SRBC's**

0.3 ml packed volume of the fresh SRBC was diluted with 9.7 ml of the working dilution of tannic acid and mixed together in a test tube or Erlenmeyer flask at 4°C for 70 minutes by gentle rotation.

**Tannic acid solution**

**Stock solution:**

- Tannic acid (E. Merck): 100 mg
- 0.15M NaCl: 100 ml

This solution was prepared weekly and stored at 4°C.

Working dilution of the tannic acid was prepared by mixing 1 ml of the above solution and 39 ml of PBS, pH 7.2. This solution was prepared fresh just before use.

After tanning, the cells were washed thrice with PBS - pH 7.2 and finally diluted to a 3% suspension in the phosphate buffered saline, pH 5.5.
Phosphate buffered saline, pH 7.2 (PBS - pH 7.2 - 0.15M)

- 0.15M Na$_2$HPO$_4$ .... 3 ml
- 0.15M KH$_2$PO$_4$ .... 47 ml
- 0.15M NaCl .... 30 ml

Sensitization of SRBC's

10 ml of the 3% SRBC suspension in PBS - pH 7.2, 0.15M was mixed with an optimal concentration of antigen and sensitization was carried out at 37°C for 30 minutes. The cells were kept in suspension by gentle rotation throughout the period of sensitization. Sensitized RBC's were washed thrice with 1% normal rabbit serum (NRS) in PBS - 7.2 and finally diluted in the same for use in the IHA test. Normal rabbit serum was examined for heterophile antibody against sheep erythrocytes and, if present, was removed by adsorption with an equal volume of sheep cells prior to use. It was also inactivated at 56°C for 30 minutes before use.

Test system

Micro-titre system was routinely used, using permanent lucito micro-titre plates with 96 U-shaped wells (Cooke No. 1-220-6 Dynatech Lab, Singapore). Each well received 0.05 ml of 1% normal rabbit serum in PBS - 7.2. Then 0.02 ml of each serum sample was picked with a micro-dilutor and serially diluted two-fold in the successive wells. Finally, 0.025 ml of the 1.3% suspension of sensitized RBC's was added to each
scrum dilution. The plates were covered with gummed transparent covers and shaken gently so as to mix the reactants uniformly. These were then allowed to settle for two hours at 37°C. Results were read according to Neuwissen (1974).

**Treatment of SRBC's with glutaraldehyde (Bing et al., 1967)**

Two or three day old SRBC's collected in the modified Alsever's solution, were washed three times with 0.15M NaCl solution at room temperature.

After the final washing, SRBC's were diluted to 3% suspension in 1% glutaraldehyde salt solution.

**1% Glutaraldehyde salt solution**

- 29% aqueous solution of glutaraldehyde (Riedal) .... 4.0 ml
- 0.15M NaCl .... 57.6 ml
- 0.15M Na₂HPO₄ .... 6.5 ml
- Distilled water .... 32.0 ml

pH of this solution was adjusted to 8.2 with 0.15 KH₂PO₄.

Prior to preparation of this suspension both the packed SRBC's and 1% glutaraldehyde salt solution were chilled to 4°C. SRBC's were, thus, treated with glutaraldehyde for 30 minutes at 4°C, keeping the cells in suspension by constant and gentle shaking.
Then, the SRBC's were washed five times with 0.15M NaCl solution and five times with distilled water, and finally a 30% suspension of the treated SRBC's in distilled water was prepared and stored at 4°C.

For tanning, SRBC's were diluted to 3% suspension with the working dilution of tannic acid. Tanning and sensitization of the SRBC's were carried out as already described.

**Treatment of SRBC's with chromic chloride** (Gold and Fudenberg, 1967)

Protein antigen in optimal concentration was mixed with equal volume of a solution of 0.1% CrCl$_3$ in 0.9% NaCl. An equal volume of thrice washed packed red cells was added immediately, since any delay interfered with the absorption of protein. The mixture was left at room temperature (approx. 20°C) for 4 minutes. Then the red cells were washed three times with saline and diluted to 1.3% suspension in the same. The suspension was stored at 4°C for 18 hours prior to use in the indirect haemagglutination (IHA) test.

**Preparation of formalin-treated erythrocytes** (Daniel et al. 1963)

Fresh sheep erythrocytes in Alsever's solution were washed five times in phosphate buffered saline (pH 7.2, 0.15M). One volume of washed, packed cells was mixed with 8 volumes of cold (approx. 10°C), 3% formaldehyde solution in phosphate buffered saline.
The mixture was gently agitated with the help of a magnetic stirrer at 4°C at a speed that maintained the cells in a suspension without foaming. After 24 hours the flask was opened and two volumes of cold, undiluted formaldehyde solution (approx. 40%) were added. Agitation was continued in the cold for another 24 hours. The cells were then centrifuged and washed 10 times in 8-10 volumes of saline and finally resuspended in 5-8 volumes of saline. Vigorous shaking was required to obtain adequate dispersion of packed cells during washings. Cells were stored at 4°C and merthiolate to a final concentration of 1:10,000 was added if prolonged storage before use was anticipated.

Tanning and sensitization

Formalinized cells were washed 4 times with isotonic saline and 2.5% cell suspension was prepared in it. One volume of this cell suspension was mixed with one volume of 1:2,000 tannic acid in saline and incubated at 37°C for 10 minutes. The preparation was centrifuged and the cells were washed with PBS, pH 7.2 and resuspended in PBS - pH 5.5, 0.15M to get 3% cell suspension. To this cell suspension was added antigen solution in appropriate concentration. This mixture was incubated at room temperature for 30 minutes and then centrifuged. After giving 5 washings with 1:200 normal rabbit serum in PBS pH 7.2, the cells were finally suspended in it to get a final concentration of 1.3%. The cells
prepared in this manner were stored at 4°C and successfully used in the IHA test.

**Indirect Fluorescent Antibody Test.**

**Antigen**

5 ml of the schizont infected blood from the monkeys injected with *P. knowlesi*, or *P. cynomolgi* or *P. coatnevi* or rats blood infected with *P. berghei* with a parasitemic level of 5-10% was drawn aseptically. The schizont rich brown layer of the parasitized cells was washed thrice with phosphate buffered saline (pH 7.2, 0.15M) by centrifugation at 4°C. After the final washings, the parasitized red cells were suspended to the original blood volume in PBS- pH 7.2. Eight drops of this suspension were placed on each of the clear, grease-free, microscopic slides and allowed to dry at room temperature. The dry antigen slides were wrapped individually in the filter paper and in order to study the effect of the storage condition on the antigenicity, batches of antigen slides were stored at -196°C, -70°C, -10°C and room temperature and tested against a battery of sera on different intervals of time.

**Fluorescent conjugate**

Antihuman IgG (Heavy and Light chains) labelled with fluorescein isothiocyanate was obtained from three different
commercial firms viz. Cappel Laboratories (Cochranville, USA), Pasteur Institut (Paris, France) and Nordic Immunological Laboratories, (Tilburg, The Netherlands) for use in the IFA test. Each of the sample was diluted 1:40 in PBS - pH 7.2 and compared for specific fluorescent staining in the IFA test. Subsequently, however, preparation from the Pasteur Institut was routinely employed.

**Performance of test**

The test was performed essentially as described by Collins and Skinner (1972) except that the antigen coated slides were fixed in dry acetone for 30 minutes at 4°C. The slides were air dried and used in the test as such or those were dehaemoglobinized by immersion in 0.1% HCl for 5 minutes, followed by one minute rinse each in distilled water and PBS - pH 7.2 and then used. For test, one drop each of the serial two-fold dilutions of serum were applied to the slide. The slides were incubated in a moist chamber at 37°C for 40 minutes. Excess serum was removed by rinsing in PBS - pH 7.2, three times for 5 minutes each. After air-drying an appropriate dilution (1:40) of FITC - conjugated anti-human IgG (Pasteur Laboratories) was placed on the slides and the slides were further incubated at 37°C for 40 minutes. After a final wash in PBS for 15 minutes, the slides were air-dried and mounted in 90% glycerol, buffered at pH 7.2.
Slides were examined under a fluorescence microscope (Fluorolume, American Optical, Buffalo, USA) using mercury vapour lamp HBO 200 W as light source with a combination of appropriate exciter and barrier filters. Reactions were graded as + to ++++ depending upon the intensity of fluorescence and the last dilution of a serum showing ++ reaction was considered the end point (Voller and O'Neill, 1971).

**Gel Precipitin Test**

Three ml of melted 1% agarose in phosphate buffered saline, pH 7.2 were poured uniformly on microscopic slides and allowed to solidify. Wells were punched in the agarose layer as required. Each well was 3 mm in diameter and 5 mm apart from the adjoining wells.

Antigen and antisera were filled in the respective wells, thrice at half hourly intervals so as to ensure an adequate quantity of the reactants in the test. The charged slides were incubated overnight at 4°C in a humidified chamber. Results were read next morning. A positive reaction usually appeared within 24 hours but slides were observed for 3 days for a maximum development of the precipitin bands as also for the negative results.