CHAPTER-III

3.0 Materials and Methods

Bonnet (Macaca radiata) and langur monkeys (Presbytis entellus) were procured from the forest area of Sagar, district, Shimoga, Karnataka State, South India. All these monkeys are maintained at the National Institute of Virology, Pune, Maharashtra State, India. All monkeys are healthy adult of either sex (Photo-1 and 2).

The bonnet monkey is a medium sized long tailed monkey. A whorl of long dark hairs on the crown radiates in all directions and forms a small cap. Dorsal parts are olive or gray-brown to deep brown. Arms and legs are greyish. The ventral parts are whitish. The face is bare pale pink to flesh coloured. The coat of the bonnet monkey is variable both among individuals and with the season. In cold weather, it is usually lustrous olive-brown, the underparts whitish. With the onset of the hot weather the coat loses its lustre, turns harsh scraggy, and fades to buffy grey.

The langur monkey is a large, black faced, gray body with long limbs and tail longer than head and body. Eye-brow are well developed. The body is silver gray. The hand and feet are black. Langurs living in the rain swept hill regions of the South West, India are generally darker than those from the drier Easter Zone of India.
Bonnet monkey (*Macaca radiata*)

Photo 1
Langur monkey (*Presbytis entellus*)

Photo 2
For Simian blood group testing, blood from 9 langur monkeys was collected. These monkeys were obtained from Jaipur, North India and were maintained at the Institute for Research in Reproduction, Bombay, India.

3.1 Collection of monkey blood

The bonnet and langur monkeys in the present study were not anaesthetised and were caught in the specially prepared nylon net for bleeding. The blood was collected from each monkey by puncturing femoral vein or artery and was mixed with the anticoagulant solution such as, Acid-Citrate-Dextrose (ACD) in the proportion of 10:1. The blood was centrifuged at 1000 g at room-temperature (28-30°C) for 15 minutes. The plasma was separated and stored at -20°C. The red cells were washed thrice with saline and prepared in the strength of 5 per cent in saline and stored at 4°C till tested.

3.2 Trypsinization of monkey red cells

The red cells of bonnet and langur monkeys were treated with freshly prepared Trypsin 1:250 (Difco) solution according to the method of Lis and Sharon (1972). Briefly, the monkey red cells were prepared in the strength of 5 per cent in phosphate buffer saline (PBS), PH 7.2. One per cent Trypsin solution was freshly prepared in phosphate buffer saline.
One volume of trypsin solution was mixed with 10 volumes of 5 per cent monkey red cell suspension. The mixture was incubated at 37°C for 60 minutes and was shaken intermittently. The mixture was centrifuged at 1000g at room temperature (28-30)°C for 5 minutes and the supernatant fluid was removed. The red cells were washed thrice in saline and were prepared in the strengths of 1.0 per cent and 0.5 per cent respectively in saline. The freshly trypsinized red cells were used for testing.

3.3 Collection of monkey saliva

Saliva was collected from bonnet and langur monkeys according to the method of Moor-Jankowski et al. (1964b). Briefly, a monkey was inoculated intramuscularly with 0.2 mg of aqueous solution of pilocarpine nitrate per kilogram body weight. Salivation started within 10 minutes after the injection. The mouth of the monkey was placed downward and the saliva was collected into a sterile test tube. The test tube was immediately kept into boiling water bath for 20 minutes, the following which it was cooled to room-temperature. The saliva was diluted 1:5 with saline and filtered through Whatman No.1 filter paper to remove solid particles. The saliva samples were stored at -20°C till tested.
3.4 **Human antisera and lectin**

The human anti-A and anti-B sera were obtained from the Associated Laboratories, Private Limited, Bombay, India. The human anti-Rh\(_0\), anti-rh', anti-rh", and anti-rh'" sera and the anti-M, anti-N sera and the anti-H lectin were purchased from the Behringwerke AG Marburg Lahn, West Germany.

3.5 **Human blood**

Human blood was obtained from the blood bank of B.J. Medical College and Sassoon General Hospital, Pune, Maharashtra State, India, and was tested for A-B-O and Rh blood groups.

3.6 **Solutions**

All chemicals used were of analar grade from British Drug House Company, India.

3.6.1 **Phosphate buffer saline (PBS), pH 7.2**

Contained 0.006 M Sodium dihydrogen phosphate, 0.012 M Disodium hydrogen phosphate, and 0.12 M Sodium Chloride in distilled water. It was autoclaved at 15 pounds pressure per square inch for 15 minutes.

3.6.2 **Acid-citrate-dextrose (ACD)**

Contained 0.038 M Citric acid, 0.135 M Dextrose, and 0.074 M Trisodium citrate in distilled water. The solution was autoclaved at 5 pounds pressure per square inch
for 5 minutes.

3.6.3 Sodium Chloride solution (Saline)

Contained 0.15 M Sodium chloride in distilled water. The solution was autoclaved at 15 pounds pressure per square inch for 15 minutes.

3.7 Preparation of heteroimmune antisera

Heteroimmune antisera were prepared in rabbits by inoculating red cells of bonnet and langur monkeys according to the method of Moor-Jankowski et al., (1965b). Briefly, blood was collected in acid-citrate dextrose (ACD) solution by puncturing the femoral vein or artery of a monkey. The blood was centrifuged at 1000 g at room-temperature for 15 minutes. The plasma was separated and stored at -20°C. The monkey red cells were washed thrice in saline and were prepared in the strength of 2 per cent, 5 per cent, and 10 per cent respectively in saline.

Two rabbits were inoculated with the erythrocytes of a single monkey. They were inoculated intravenously (i.v.) into the ear vein with 1.0 ml of 2 per cent red cells suspension in saline. Subsequently these rabbits were weekly inoculated intravenously with 1.0 ml of 5 per cent and 10 per cent red cells suspension in saline. The rabbits
were bled on the 10th day after the last injection. The serum was separated from the clotted blood. The serum was pooled and heated at 56°C for 20 minutes.

In the present work red cells from six bonnet monkeys and five langur monkeys were employed for inoculation into rabbits to produce heteroimmune antisera.

The antisera prepared in rabbits were called after the donor monkeys whose red cells were taken for immunization. Therefore the antisera prepared in rabbits by employing red cells of six bonnet monkeys in the present series were designated as anti-MR 299, anti-MR 421, anti-MR 422, anti-MR 423, anti-MR 424, and anti-MR 425. In general the antisera prepared in rabbits by inoculating red cells of bonnet monkeys were called as rabbit anti-MR sera.

Similarly antisera prepared in rabbits by employing red cells of five langur monkeys were designated anti-LR1, anti-LR9, anti-LR10, anti-LR12, and anti-LR15 according to the number tattooed on them. In general the antisera prepared in rabbits by inoculating red cells of langur monkeys were designated as rabbit anti-LR sera.
3.8 Preparation of isoimmune antisera

The isoimmune antisera were prepared in bonnet and langur monkeys by inoculating red cells of one monkey to another monkey of the same species according to the method of Moor-Jankowski et al. (1967b). The collection of blood and preparation of washed erythrocytes was as described in section 3.1. Another monkey of the same species was selected and inoculated intravenously with 5 ml of 5.0 per cent red cells suspension in saline. Three injections were given to the monkey with one week interval in each injection. After the last injection the monkey was given rest for a week. Further the same monkey was inoculated intramuscularly with 1.0 ml of washed and packed red cells of monkey (whose red cells were previously inoculated) mixed with equal volume of complete Freund's adjuvant (Difco) in small doses at multiple sites. Four injections were given to the monkey with four weeks interval after each injection. The monkey was bled on the 10th day after the last injection. The serum was separated from monkey blood and was heated at 56°C for 20 minutes and stored at −20°C till tested.

In the present work red cells from two bonnet monkeys and two langur monkeys were employed to produce isoimmune antisera against the red cells of one another.
monkey of the same species. The isoimmune antiserum was designated after the donor monkey whose red cells were employed for immunization. The isoimmune antisera produced in the two langur monkeys of the present series were designated as anti-LR13 and anti-LR19 after the donor monkey. The two bonnet monkeys MR31 and MR40 in the present series did not produce isoimmune antisera.

3.9 Preparation of lectin from the seeds of Phaseolus lunatus and Phaseolus vulgaris

The lectins were prepared from healthy dry seeds of Phaseolus lunatus and Phaseolus vulgaris respectively, according to the method of Galbraith and Goldstein (1972). The seeds were purchased from the local commercial market of Pune, Maharashtra State, India.

The seeds of the Phaseolus lunatus were ground in an electric mill. The finely ground meal was extracted with four times of its weight with 0.15 M sodium chloride solution at 4°C for 2 hours. The extract was filtered through cheese cloth and the residue was re-extracted as before. The extracts were pooled and centrifuged at 10,000 g for 20 minutes at 4°C to remove insoluble residues. The supernatant fluid of the extract was brought to pH 4.0 by adding 6 N HCl and was allowed to stand overnight at 4°C. The precipitated protein
of the extract was removed by centrifugation at 1000 g for 20 minutes at room temperature (28-30°C) and was discarded. The supernatant solution of the above extract was brought back to pH 7.0 by adding requisite amount of 7 N NaOH. The hemagglutinating protein fractions of the extract were precipitated with 40 per cent and 60 per cent saturated ammonium sulphate solutions respectively at 4°C. The extract was centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant fluid of the extract was discarded. The precipitated protein fractions were dissolved in 0.15 M NaCl and dialyzed against large volume of 0.15 M NaCl to remove ammonium sulphate ions. The dialyzing tube was covered with polyethylene glycol 6000 (Carbowax) pellets and kept at 4°C for 30 minutes to concentrate the protein fractions. Further dialysis was carried out against large volume of distilled water to remove the small amount of Carbowax that entered into the protein solution. The concentrated protein fractions of the lectin were stored at -20°C till tested.

The lectin from the seeds of *Phaseolus vulgaris* was prepared by identical method.

3.10 **Human-type A-B-0 blood groups of monkeys**

The human-type A-B-0 blood groups of bonnet and langur monkeys were determined on the basis of saliva
inhibition test carried out for the presence of A-B-H group specific substances. The test was done according to the method of Moor-Jankowski et al., (1964b). Briefly, the monkey saliva was mixed in equal volume (0.05 ml) with the anti-A, anti-B sera and anti-H lectin respectively. The mixtures were incubated for 60 minutes at room-temperature (28-30°C). Human group A, B, and 0 red cells were prepared in the strength of 1.0 per cent in saline. The human group A cells were added to the mixture of saliva and anti-A serum, group B cells were added to the mixture of saliva and anti-B serum, and group 0 cells were added to the mixture of saliva and anti-H lectin. All the mixtures were further incubated for 30 minutes at room-temperature (28-30°C). Agglutination of human red cells was observed under direct light and was confirmed under a microscope. The anti-A, anti-B sera and the anti-H lectin which were inhibited by the group specific substances present in the monkey saliva, failed to agglutinate red cells of group A, B and 0 respectively. Thus the inhibited antisera disclosed the presence of group specific substances viz., A-B-H in the monkey saliva. Therefore A-B-0 blood groups were determined among the bonnet and langur monkeys on the basis of saliva inhibition test.

The monkey sera were absorbed with the human group 0 cells to remove heteroagglutinins and the absorbed monkey sera
were tested with the human cells of group A and B for their content of anti-A and anti-B agglutinins. The reciprocal A-B-O blood groups were also determined in monkeys as per Landsteiner's rule of human A-B-O blood groups.

In the present study the human-type A-B-O blood groups have been determined among 25 bonnet monkeys and 18 langur monkeys on the basis of saliva inhibition and serum agglutinin test.

3.11 Testing of monkey red cells

The collection of blood from bonnet and langur monkeys and preparation of the erythrocyte suspension in saline was as described in section 3.1. For testing, the monkey red cells were prepared in the strength of 1.0 per cent in saline and were mixed with equal volume of antisera. The mixtures were incubated at room-temperature (28-30)°C for 30 minutes. Agglutination of monkey red cells was observed under direct light and was also confirmed under a microscope.

3.12 Human-type M-N blood groups of monkeys

Erythrocytes from 25 bonnet monkeys and 18 langur monkeys were tested as described above with the anti-M and anti-N sera respectively to determine their human-type M-N blood groups.
3.13 **Human-type Rh-Hr blood groups of monkeys**

Red cells from 12 bonnet monkeys and 12 langur monkeys were tested as described above with the human anti-Rh\textsubscript{b}, anti-rh', anti-rh''', anti-hr' and anti-hr''' sera to determine their human type Rh-Hr blood groups.

3.14 **Simian-type blood groups of monkeys**

Trypsinized as well as untrypsinized red cells from 12 bonnet monkeys were tested as described above with the six heteroimmune antisera such as, anti-MR 299, anti-MR 421, anti-MR 422, anti-MR 423, anti-MR 424, and anti-MR 425 sera to determine their simian-type blood groups.

Similarly erythrocytes from 17 langur monkeys were tested with the five heteroimmune antisera such as, anti-LR1, anti-LR9, anti-LR10, anti-LR12 and anti-LR15 sera, and isoimmune anti-LR-13 and anti-LR19 sera to determine their simian type blood groups.

3.15 **Polyacrylamide gel electrophoresis of phaseolus lunatus lectin.**

**Chemicals**: Acrylamide, N,N'-Methylene-bis-Acrylamide and Tris (Hydroxymethyl) aminoethane were obtained from Sigma Chemical Co., St. Louis, U.S.A. Glycine, Ammonium persulphate,
Tetramethylethylenediamine, Mercaptoethanol and Sodium dodecyl sulphate were obtained from Biorad Laboratories, Richmond California.

7.5% Acrylamide solution: 7.5 g Acrylamide, 0.161 g N,N'-Methylene-bis-Acrylamide, and 4.5 g Tris (Hydroxymethyl) aminoethane were dissolved in distilled water, pH was adjusted to 8.8 with HCl. The solution was filtered through Whatman No.1 filter paper to remove insoluble materials, and was stored in a dark bottle at 4°C until used.

Buffer: 6.0 g Tris(Hydroxymethyl)aminoethane, and 28.8 g glycine were dissolved in distilled water. pH was adjusted to 8.3 with HCl and diluted to 1000 ml with distilled water and stored at 4°C until used.

10% Ammonium persulphate: 1.0 g Ammonium persulphate was dissolved in 10 ml distilled water. It was freshly prepared and used.

N,N,N',N'-Tetramethylethylenediamine (TEMED): was used as such.

Stain: 200 mg Coomassie Brilliant Blue R250 (Sigma) was dissolved in 50% Trichloroacetic acid (BDH).
Destainer solution: 70 ml Glacial Acetic acid (BDH) was diluted up to 1000 ml with distilled water.

Preparation of electrophoresis gels: Glass tubes 12 cm long and of 0.5 cm diameter were employed for polymerization. To prepare 12 gels, 30 ml of 7.5% acrylamide solution was mixed with 0.2 ml of 10% ammonium persulphate and 0.02 ml of tetramethylethylenediamine (TEMED). The glass tubes were filled with the above mixture up to 10 cm height. The remaining part of the tubes were carefully layered with distilled water. The acrylamide solution was allowed to polymerize for about 40 minutes at room-temperature to form gel in situ.

Electrophoresis: The electrophoresis was carried out according to the method of Smith (1976). The gel tubes were fitted in an electrophoresis apparatus (Photo 3). The absorbed and unabsorbed Phaseolus lunatus lectin samples were mixed with bromophenol blue and were applied in the volume of 20 µl containing about 20 µg protein at the top of the gels. The anodic and cathodic chambers were filled with buffer. Constant electric current of 3 milliampere per gel was applied. Electrophoresis was carried out towards the anode at room-temperature (28-30°C) till bromophenol blue
Polyacrylamide disc gel electrophoresis of Phaseolus lunatus lectin
boundary reached the bottom of the tubes.

The gels were carefully removed from the glass tubes under the pressure of water from a syringe and were stained with Coomassie Brilliant Blue stain for 30 minutes at 37°C. The gels were removed from the stain and were washed with destainer solution with frequent changes until protein bands were clearly observed. Comparing the bands obtained with the unabsorbed lectin and the bands obtained after the absorption, the hemagglutinating protein fraction band of the lectin was identified. The molecular weight of the hemagglutinating protein fraction of the lectin was determined by SDS-Polyacrylamide gel electrophoresis method (Segrest and Jackson, 1972).

3.16 Determination of molecular weight of the hemagglutinating protein fraction of the Phaseolus lunatus lectin.

7.5% Acrylamide solution of resolving gel: Acrylamide solution was prepared as described above and 0.1 g sodium dodecyl sulphate per 100 ml solution was added.

3.0% Acrylamide solution for spacer gel: 3.0 g Acrylamide, 0.08 g N,N'-methylene-bis-Acrylamide, 1.5 g Tris (Hydroxymethyl)aminoethane, and 0.1 g sodium dodecyl
sulphate were dissolved in distilled water, pH was adjusted to 6.8 with HCL and diluted to 100 ml with distilled water. The solution was filtered through Whatman No. 1 filter paper to remove insoluble material and was stored in dark bottle at 4°C until used.

**Electrode buffer**: 6.0 g Tris(Hydroxymethyl)aminoethane, and 28.8 g glycine, and 1.0 g sodium dodecyl sulphate were dissolved in distilled water, pH was adjusted to 8.3 with HCL and diluted to 1000 ml with distilled water and stored at 4°C until used.

**Sample buffer**: 0.72 g Tris(Hydroxymethyl)aminoethane, and 2.0 g sodium dodecyl sulphate were dissolved in distilled water, 10 ml glycerine and 5 ml mercaptoethanol were added and diluted to 100 ml with distilled water. Two to three drops of bromophenol blue were added.

**Fixative Solution**: 50 g Trichloroacetic Acid (BDH) was dissolved in distilled water and diluted to 100 ml with distilled water.

**Stain**: Coomassie Brilliant Blue R250 stain was prepared in Trichloroacetic acid as described earlier.
Protein Molecular Weight Markers: Albumin (Bovine) M.W. 68000, Ovalbumin (2 X cryst) M.W. 45000, Cytochrome-C (Horse heart) M.W. 12,400, and Gamma Globulin (Human) M.W. 1,60,000 obtained from the Division of Becton, Dickinson and Co. B.D. Schwarz/Mann Orangeburg, New York, U.S.A. were used in the present study.

Preparation of resolving gel slab for electrophoresis: Two identical glass plates of 15 x 15 cm were separated by a plastic spacer of 2 mm thickness. Three sides of the glass plates were perfectly sealed with adhesive tape. About 45 ml of acrylamide solution was sufficient to prepare 15 x 12 square cm gel slab. To prepare a gel slab, 45 ml of acrylamide solution was mixed with 0.4 ml of 10% ammonium persulphate and 0.025 ml of tetramethylethylenediamine (TEMED). The space between the two glass plates was filled with the mixture up to the height of 12 cm from the bottom of the glass plate. The top surface of the acrylamide solution was carefully covered with distilled water and was allowed to polymerize for about 40 minutes at room-temperature (28-30)°C. After polymerization the water on the top surface of the gel was removed.
Preparation of spacer gel slab for electrophoresis:

To prepare spacer gel slab of 3 x 15 square cm, about 15 ml of 3.0% acrylamide solution was mixed with 0.1 ml of 10% ammonium persulphate and 0.01 ml of tetramethylethylenediamine (TEMED). The mixture was poured into the space of two glass plates in continuation of the resolving gel slab. A plastic comb was kept in the acrylamide solution to form wells of 2-3 mm width. The acrylamide solution was allowed to polymerize for about 30 minutes at room-temperature (28-30°C). The plastic comb was carefully removed.

Preparation of samples: The absorbed and unabsorbed *Phaseolus lunatus* lectin and the protein molecular weight markers solutions containing about 1 mg protein per ml were diluted (1:3) with the sample buffer. The diluted samples were kept in boiling waterbath for 1-2 minutes and were cooled to room-temperature. They were applied in the volume of 20 to 30 µl in each well.

Electrophoresis: The gel sandwich glass plate was fixed to electrophoresis apparatus (Photo 4). The samples of absorbed and unabsorbed lectin and protein
SDS-Polyacrylamide gel electrophoresis of Phaseolus lunatus lectin

Photo 4
molecular weight markers were applied in wells. Cathodic and anodic chambers were filled with electrode buffer. Constant electric current of 3 milliampere per track was applied. Electrophoresis was carried out at room-temperature (28-30)°C towards anode till bromophenol blue boundary reached the bottom.

The gel slab was carefully removed and placed into the fixative solution for 30 minutes. The slab was removed from the fixative solution and stained with Coomassie Brilliant Blue stain for 60 minutes at 37°C, following which the slab was removed from the stain solution and washed with destainer solution with frequent changes until clear protein bands were observed.

Electrophoretic mobilities of protein molecular weight markers and the hemagglutinating protein fraction of the lectin were calculated. A curve was plotted with the electrophoretic mobilities versus log molecular weights of the molecular weight markers. The approximate molecular weight of the hemagglutinating protein fraction of the lectin was determined from the curve.