CHAPTER III
MATERIALS AND METHODS
PART I: EPIDEMIOLOGY
CHAPTER - III
MATERIALS AND METHODS

I. EPIDEMIOLOGICAL METHODS:

For the purposes of this study, stray dogs were collected with the help of the dog catching squad of the Ahmedabad Municipal Corporation. The information from various sources was collected to locate furious dogs in various areas of the City. Efforts were made to collect such dogs. Totally 226 dogs were collected from city, cantonment and other places. A total of 224 sera samples and 178 cerebrospinal fluid (CSF) were obtained for study.

The dogs thus obtained were kept for observation for 11 days for any signs of illness, particularly their behaviour was noted. On day 12, the dogs were sacrificed. Before that a serum was collected by drawing blood from jugular vein, and a cerebrospinal fluid was collected by lumbar puncture or by passing a needle through atlanto-occipital joint and aspirating CSF from the canal. In this way 224 sera and 178 CSF samples were collected.

The dogs were euthanasised by injecting supersaturated magnesium sulphate solution through a jugular vein. Absence of corneal reflex was taken as sign of death. After euthanasia a cut was made behind the base of the ear and parotid salivary gland was exposed. Care was taken not to cut any blood vessels of the area. The gland was detached from its natural attachments, collected in a sterile petridish and was immediately transferred to deep freeze at -20°C.
Thereafter a mid line incision was made through the skin, fasciae and the muscles of the cranium beginning anteriorly just above the level of the eyes and extending posteriorly to the base of the skull. The skin, fasciae and the muscles were reflected laterally and the bone was exposed. The bone was cut by chisel and hammer taking full precautions that brain substance was not damaged. With fresh set of sterile instruments the brain was removed from the cranium. The brain was then transferred to sterile petridish and was kept at -20°C. 226 brains and 226 salivary glands were collected for study.

There are two Veterinary Hospitals in the city. The one at Prem Darvaja caters the need of the eastern part of the city and the other near Ellis Bridge caters the need of the western part of the city where the population of the pet dogs is concentrated. Pet dogs brought for antirabic prophylactic vaccination at these two hospitals were observed and the case history in each/about previous vaccination or a dog bite etc., was taken. A total of 214 sera and 36 CSF samples were collected from these dogs for the purpose of study.

**Collection of Human sera for epidemiological study:**

The Antirabic treatment unit at Civil Hospital Ahmedabad is giving post bite antirabic prophylactic treatment to persons who have received a dog bite. Persons from village and remote areas also visit this unit for treatment as there may be lack of facility or availability of the treatment.
The persons reporting for the AR treatments were bitten by a dog and therefore these persons were taken as representative samples of the population exposed to danger of rabies. Therefore sera samples from 572 persons were collected for a period of 17 months, for the purpose of study.

Collection of samples from rabies cases:

A circular letter was issued to all the Veterinary Hospitals of the state to send the materials for examination of a case suspected of rabies. During the campaign of locating clinical cases of rabies in the city, a dog was brought to the hospital and after few hours it died. The examination of smears from hippocampus revealed presence of Negribodies. The owner of the dog gave a history that few months ago another dog which was moving in the residential locality had bitten his dog. He was able to identify the dog and this dog was caught. It was kept under constant observation for study of carrier state.

The behaviour of the dog was being constantly observed. The serum was collected every week, as well as saliva was collected every day. The saliva was collected by pressing a clean sterile cotton-swab. This swab was transferred in deep freeze by placing it in a sterile glycerine-saline bottle.

The saliva collected in this way was used to study the presence of rabies virus. The serum was collected to detect the presence of antibodies against rabies. The dog survived for 302 days and died due to acute gastroenteritis.
The brain, salivary gland and intrascapular fat were collected to detect the presence of rabies virus.

Isolation of rabies virus from brain and salivary glands:

As soon as the brain and salivary glands were removed, and before transferring them to deep freeze at -20°C they were examined for the presence of Negribodies by using Seller's stain.

The entire brain was kept in a sterile petridish, and with a pair of sterile scissor and a pair of tongues, small transverse sections of about two to three mm in thickness from Ammon's horn, cærebrum and cærebllum were cut and placed on a clean white filter paper. Cut surface was put facing upward. A clean microscopic slide was touched against the cut surface of the tissue and was pressed gently over it enough to spread the tissue. It was kept in contact of the tissue for about 10 seconds, and such three impression smears were made on each slide from each of the cut pieces of tissue. These impression smears were stained by Seller's stain while moist. In a similar manner salivary gland was exposed and impression smears were prepared and stained.

Preparation of Seller's stain:

Stock solution:

A. 1. Methylene blue - 10 g.
2. Methanol - to make 1000 ml.
   (absolute acetone free)

B. 1. Basic Fuchsin - 5 g.
2. Methanol - to make 1000 ml.
   (absolute acetone free).
Staining solution:

Stock solution A - 2 parts,
Stock solution B - 1 part.

Mixed thoroughly and stored in ground glass stoppered or screw cap bottle, to use after 24 hours.

Staining procedure:

1. Smears are prepared.
2. Freshly prepared moist, smears are dipped in staining solution for about five seconds, depending upon thickness of smear.
3. Smears are taken out from staining solution and rinsed in running tap water.
4. After rinsing properly the smears are dried without botting.
5. Dried smears are examined.

Mice inoculation:

The pieces of Ammon's Horn, cerebrum and cerebellum which were cut for Seller's stain were collected by sterile pair of tongues, a piece from salivary gland from the centre was also cut and all these were triturated and made into ten per cent suspension in physiological buffer saline (PBS) (pH 7.0). This suspension is then centrifuged in a refrigerated centrifuge at 4°C at 3000 r.p.m. for 30 minutes. The supernatant was taken for mouse inoculation test.

Albino (white) mice obtained from National Institute of Virology and Vaccine Institute Baroda were bred at the laboratory. 21 day old healthy mice of either sex
were selected for the purpose. Since the brain and salivary glands were collected under strict aseptic precautions from freshly killed dogs in a laboratory, antibiotics were not added in the suspension, nor the serum was used as diluent as the inoculation was done on the same day.

Before inoculation mice were anaesthetised by inhalation of ether. A jar with a wire mesh bottom and absorbant cotton wool was used for the purpose. The anaesthetised mice were held by one hand between the first two fingers and a thumb. 0.03 ml of brain salivary gland 10% suspension was inoculated intracranially by using tuberculin syringe and 27 gauge tuberculin needle in each mouse and six mice were inoculated from each suspension. The seventh mouse was inoculated only PBS to serve as control. The syringe and needles were changed for every different suspensions for inoculation and the suspensions were kept packed in ice to maintain a temperature below 10°C. After each inoculation, suspensions of brain and salivary glands were transferred to deep freeze at -20°C for storage.

These inoculated mice were kept in a separate box and identification tags were put on each box. They were observed for 30 days after inoculation for any sign of illness. The mouse history card was prepared for record. The symptoms noted were ruffled fur (RF), tremours (T) when held in air by tail with a pair of forceps, lack of coordination (LC) of hind legs, gait (G) during walking when kept on table and made to move, paralysis (P) and prostration (PST).
Complement Fixation Test (CFT):

In most serological tests e.g. agglutination, precipitation etc. One antigen and one antibody system is used. In complement fixation test two separate test systems are used; one the test system consisting of test antigen or antiserum and known antiserum or antigen and complement, and the other; the indicator system, consisting of sheep erythrocytes (antigen) and haemolysin (antibody) against sheep erythrocytes. The union of antigen and antibody in the test system takes place through the intermediary action of complement after a period of incubation of 1 hour at 37°C or over night at 4°C.

This union is not visible and to detect this union indicator system is added to the test system after a period of primary incubation and a secondary period of incubation is carried out. If the test system has absorbed the complement, no haemolysis in the indicator system will occur and the test is positive. On the other hand failure of utilisation of complement by the test system will leave it free for utilization by indicator system and lysis of erythrocytes will occur and the test is thus negative.

There are three main methods of performing complement fixation test; the macro method performed in test tubes, the small volume method and the micro method. Reagents used, time temperature combination for reaction etc., are the same for all methods and all the three methods give similar end points on visual examination. Thus micro method is quite
suitable, satisfactory and economical for routine use and was used throughout these studies.

Chemically clean glasswares and haemagglutination slabs were used. Veronal Buffer Saline (VBS) pH 7.2 was used as diluent. Stock solution (5X) was prepared as follows.

- Sodium chloride - NaCl
- 5,5-diethyl barbituric acid
- Sod. 5,5 - diethyl barbiturate
- Magnesium chloride, MgCl₂, 6H₂O
- Calcium chloride - CaCl₂

5,5 diethyl barbituric acid was first dissolved in 500 ml warm distilled water and then sodium chloride (NaCl) and Sod. 5,5 - diethyl barbiturate were added and further 1000 ml of distilled water was added. After all these ingredients are dissolved, magnesium chloride and calcium chloride were added and the volume was made upto 2000 ml by addition of distilled water. The solution was autoclaved at 105°C for 20 minutes. The stock solution (5X) was stored at 4°C in refrigerator. It was diluted 1:5 before use.

Sheep erythrocytes:

Blood was obtained from jugular vein of a healthy young sheep using sterile 50 ml syringe and 18 gauge needle. The blood collected in this way was directly mixed gently with equal volume of Alsever's solution in a sterile screw cap bottle.
**Alsever's solution:**

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<td>Glucose</td>
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<td>Sodium chloride</td>
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<tr>
<td>Tri sodium citrate</td>
<td>0.80 gr.</td>
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<tr>
<td>Citric acid</td>
<td>0.055 gr.</td>
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<tr>
<td>Distilled water</td>
<td>100 ml.</td>
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</table>

All these ingredients were dissolved in distilled water and the solution was sterilized in autoclave at 10 lbs/4.5 kg pressure for 10 minutes.

About three ml of preserved blood was taken in a clean sterile centrifuge tube of 15 ml. VBS was added to the preserved blood up to 15 ml mark. It was centrifuged at 1500 r.p.m. for 10 minutes. Supernatant was discarded. The cells were resuspended in VBS again and washing was repeated three times. During the process of washing upper layer of leucocytes was removed. If the supernatant contained even traces of haemoglobin, the washing was repeated till the supernatant is clear of it. Finally pack cell volume was determined by centrifuging at 1800 r.p.m. for 10 minutes. Necessary dilution was made at the time of performing test.

**Haemolysin:**

Haemolysin was obtained from Haffkine Institute, Bombay. The titration of haemolysin was done immediately before performing the test.

Two rows of tubes were taken. The upper row of tubes was used for haemolysin dilution, the next row was kept blank and the lower row of tubes was used for titration.
In the first tube of the first row 0.2 ml of 1:100 haemolysin was taken and 1.8 ml of VBS was added to make it 1:1000 dilution. The dilution was mixed thoroughly. From tube 1 of row I 0.1 ml of haemolysin was transferred to tube 2 to tube 10. Then VBS was added from tube 2 to tube 10 in ascending order, that is 0.1 ml in tube 2, 0.2 ml in tube 3, 0.3 ml in tube 4 and so on. Thus dilutions of haemolysin from 1:1000 to 1:10,000 were available. These tubes were incubated at 37°C for 15 minutes in water bath.

In the second row of tubes, in each tube, 0.1 ml of each dilution of haemolysin was added, then 0.1 ml of one per cent. Sheep erythrocytes were added. All these tubes were shaken gently and incubation was carried out for 10 minutes at room temperature. Thereafter 0.2 ml of VBS was added in each tube, and 0.1 ml of 1:10 dilution of complement was added. Haemolysin control and sheep erythrocytes control tubes were kept. All the tubes were incubated at 37°C for 15 minutes. At the end of incubation period, the degree of haemolysis present in each tube was noted, and the controls were also seen. The Minimum Haemolytic Dose (MHD) is defined as the highest dilution of haemolysin that causes complete lysis (100%) of sheep erythrocytes.
## HAEMOLYSIN TITRATION

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Complement:

Complement used in the test was a pooled guinea pig serum obtained by collecting blood from ten laboratory bred healthy and young guinea pigs. Before the commencement of each day's work, complement was titrated in presence of antigen dilution to be used in the test proper and with the same stock of haemolysin and sheep erythrocytes which are to be used for the test.

The complement was diluted to 1:10 in VBS. The 4 MHD per 0.1 ml were calculated from the results of haemolysin titration. 4 MHD dilution was prepared for use in the complement titration. To prepare haemolytic system or sensitised sheep erythrocytes, 1.0 ml of 1 per cent sheep erythrocytes were taken in a test tube and 1.0 ml of haemolysin dilution containing 4 MHD units was added immediately. Taking into consideration the amount needed for the test, equal amounts of each were mixed and allowed to stand at room temperature for about 10 minutes.

The complement titration was done in a similar manner like haemolytic titration. Two rows of test tubes were used. In the first row VBS was added beginning from 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml, 0.7 ml and 0.8 ml in tubes 1 to 8 respectively. By using another pipette 0.1 ml of 1:10 dilution of complement was added in all the eight tubes of the first row. Thus the complement dilution was 1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80 and 1:90 in tubes.
1 to 8 respectively. By using a fresh pipette all the dilutions were mixed thoroughly. From each of the above dilutions, 0.1 ml was transferred to tubes 1 to 8 of the second row, and 0.2 ml of VBS was added to all the tubes 1 to 8 and in the tube 9, 0.3 ml of VBS was added. Thereafter 0.2 ml of 1% sensitised sheep erythrocytes (haemolytic system) were added to all the nine tubes. Then the entire rack containing test tubes was shaken gently for thorough mixing. The rack was kept in a water bath at 37°C for 45 minutes for incubation, and was shaken gently every 15 minutes.

The highest complement dilution that causes complete lysis (100%) is defined as one MHD (Minimum Haemolytic Dose) of complement per 0.1 ml.

The entire procedure of haemolysin titration and complement titration was carried out in test tubes for convenience of reading and interpretation of results.

**COMPLEMENT TITRATION**

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| Haemolytic system | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 |
Preparation of Antigen:

(Method of Ando et al. (modified) as described by Raichaudhari & Thomas, 1967).

Mice of about three weeks age and in healthy condition of either sex were selected and observed for two days. They were inoculated intracerebrally with 0.03 ml of 10 per cent challenge virus standard (CVS) of rabies virus obtained from Central Research Institute, Kasauli, taking all the necessary precautions for handling rabies virus. These mice were observed and any death which occurred during first 48 hours was not accounted for. When mice were found moribund after fifth to seventh and up to tenth day, they were sacrificed and their brains were harvested.

A batch of 5 gm mice brain was taken at a time and were grinded to a homogeneous paste in sterile mortar and pestle. Physiological saline pH 7.4 was added to make it 40 per cent suspension (7.5 ml for 5 gms). The suspension was collected in 15 ml centrifuge tube (sterile) and the virus was inactivated at 56°C for 30 minutes in a water bath. This was then centrifuged at 3000 r.p.m. for 30 minutes. The supernatant was collected and dialysed overnight for about 16 to 18 hours against citrate buffer pH 4.6 at 4°C. The dialysed antigen was collected in 15 ml centrifuge tube and was centrifuged at 2000 r.p.m. for 15 min. The supernatant was collected and stored at -20°C. This was Ando's antigen (D.S.) dialysed supernate antigen.
The deposit or the precipitate of the centrifuge tube was again suspended in two ml of Phosphate Buffer Saline (PBS) pH 8.2. The suspension was kept at 37°C for two hours with occasional shaking every 15 minutes to extract antigen. It was centrifuged at 3000 r.p.m. for 30 minutes at 4°C and supernatant was collected and stored at -20°C for use as (DP) Dialysed Precipitated antigen.

**Citrate Buffer pH 4.5:**

**Solution A:**
- 0.05 M sodium citrate $(\text{Na}_3\text{C}_6\text{H}_5\text{O}_7, 2\text{H}_2\text{O})$ - 14.7 g
- Distilled water to make - 1000 ml

**Solution B:**
- 0.05 M citric acid $(\text{C}_6\text{H}_8\text{O}_7)$ - 9.6 g
- Distilled water to make - 1000 ml

**Mixing in following ratio:**
- Solution A - 25.5 ml
- Solution B - 24.5 ml
- Distilled water - 50.0 ml

**Raising of antisera:**

Guinea pigs are best suited among the laboratory animals for raising antisera against rabies. Therefore about 10 guinea pigs healthy and weighing about 350 gms are chosen for the purpose. Before pre-immunisation bleeding, food was withdrawn but ample water was given for 18 to 24 hours.

By cardiac puncture, about two ml of blood was collected from individual guinea pig in sterile test tubes and labelled.
The serum was separated and centrifuged to make it free from residual erythrocytes. Then serum was stored and labelled in sterile stoppered vials.

Each sample serum was inactivated at 56°C for 30 minutes and was tested for nonspecific fixation against normal and rabid brain antigen and anticomplementary activity. Those guinea pigs whose serum showed non specific fixation and/or anticomplementary activity were discarded.

Preparation of immunising antigen:

With CVS - Kasauli strain the live virus antigen and inactivated antigen were prepared.

Two healthy guinea pigs were selected and after clipping off the hairs on the scalp, they were anaesthetised with anaesthetic ether. The scalp was painted with tincture iodine and a straight line incision of about one cm long was made. The parietal bone was exposed. A hole was drilled in the bone with the help of dental drill to reach cranial cavity. Through this hole one ml, of 10 per cent CVS rabies virus was inoculated with the help of sterile tuberculin syringe and 24 gauge needle. The cut surfaces were brought in opposition and wound was closed by applying adhesive tape. These inoculated animals were kept in labelled cages and were observed for clinical signs e.g. tremour, ataxia, followed by paralysis. The sick animals were sacrificed just prior to death and brains were collected in sterile petridishes and were kept at 4°C.
The 10 per cent emulsion was prepared from these brains in physiological saline. 12 ml portion was inactivated at a time at 56°C for 30 minutes. This was inactivated antigen. The rest of the 10 per cent emulsion was taken and equal volume of glycerol was added to dilute it to five per cent (1:20). After mixing well, it was distributed in two ml quantities in stoppered vials and stored at -20°C.

Immunisation:

The guinea pigs selected for the purpose were first immunized with 10 per cent heat inactivated fixed virus antigen by intraperitoneal injection of one ml. After one week, they were inoculated intraperitoneally with one ml of $10^{-4}$ dilution of live virus antigen twice in one week. In third week the inoculation was repeated in same manner with $10^{-3}$ dilution, and in fourth week with $10^{-2}$ dilution of live virus antigen. From fifth week onwards the guinea pigs were inoculated with intraperitoneal injection of one ml with a dilution of $10^{-1}$ live virus antigen for six weeks.

The final bleeding of animals is carried out on day 12 of the last injection. The food was withdrawn but ample of water was given prior to 24 hours of final bleeding to minimise lipid contents in serum. The animals were anaesthetised and a cut was made on the neck. The blood of each animal was collected separately in a sterile flask. The serum was separated and was centrifuged to remove erythrocytes, and other traces. The individual samples of serum collected from
each animal were tested for anticomplementary activity, and those sera showing anticomplementary activity were discarded. Rest of the sera were pooled after keeping an aliquot of one ml for serum virus neutralization test and were distributed in five ml quantities in stoppered glass vials and stored at -20°C.

Standardisation of antigen and antiserum:

The prepared antigen and antiserum were standardised by checkerboard titration and the optimal dilutions of antigen and antiserum were worked out. On the basis of the optimal dilutions obtained, further batches were standardised by using these antigen and antiserum as reference. The antigens and antisera were distributed in one ml aliquots and were stored at -20°C.

Two rows of test tubes and a plastic haemagglutination slab were taken. One ml of veronal buffer saline (VS) was added to both row of tubes. First row of tubes were used for antiserum dilutions. In the first tube, one ml of antiserum was added, so that the first tube contained 1:2 dilution of antiserum. With another pipette the contents of the first tube were mixed and one ml was transferred to second tube making the dilution of 1:4 and so on. From each of these tubes containing serial two fold dilutions, 0.1 ml of dilution was transferred to each of the eight cups of haemagglutination slab from top to bottom. Two fold dilutions were distributed in the cups of row a, b, c, d, e, f, and g. In row h antiserum dilution was not added as that was an antigen control.
The second row of tubes were used for antigen dilutions. One ml of antigen was added to first tube and the pipette was discarded. The antigen dilutions were made similarly like antiserum. A fresh pipette was used, serial two fold dilutions were made and were distributed in the cups till all the cups of the row A, B, C, D, E, F & G received 0.1 ml of respective antigen dilution. Row H was to serve as serum control therefore antigen was not added to it.

In the last row of slab marked as J complement titration with 2 units, 1 unit and 0.5 unit of minimum haemolytic dose (MHD) was carried out and next two cups were used for cell control. In all the cups including controls 0.1 ml of complement was added. This 0.1 ml contained 2 MHD/0.1 ml. In row J of complement titration and cell control, 0.1 ml of complement was added in first cup, 0.05 ml in second cup, 0.025 ml in third cup and no complement was added in fourth and fifth cups. Proportionately veronal buffer saline was added respectively from cups first to fifth as 0.2 ml, 0.25 ml, 0.275 ml, 0.3 ml and 0.3 ml. 0.1 ml of veronal buffer saline was added to first seven cups of row H (antiserum control) and row h (antigen control).

After the addition of complement, the slab was gently shaken for mixing. The aluminium foil was wrapped around the slab to prevent undue evaporation, and the slab was kept overnight in the refrigerator for incubation at 4°C.
Next morning, fresh sensitized erythrocytes suspension
was prepared by taking equal volumes of two per cent washed
sheep erythrocytes and four MHD haemolsin/0.1 ml in veronal
buffer saline. The mixture was allowed to stand for 10 to 15
minutes at room temperature for sensitisation. During this
period haemagglutination slab was kept at 37°C for 10 minutes
for warming up. 0.2 ml of sensitised erythrocytes were then
added to all the cups. The contents of the cups were then
mixed by gentle shaking and the slab was kept at 37°C for
45 minutes for incubation. At every 15 minutes interval the
slab was gently shaken during incubation period.

At the end of this secondary incubation the reading
was taken, according to the degree of haemolysis in the cups
as follows:

100% haemolysis or no fixation = 0
75% " " 25% " = 1
50% " " 50% " = 2
25% " " 75% " = 3
No " " 100% " = 4

Controls were read first and there was complete lysis
in all the cups of antigen control and serum control. Cups
containing 2 MHD and 1 MHD of complement showed complete lysis,
and there was no lysis in cell control. 0.5 MHD of complement
showed 50% lysis. Thus the test was suitable for reading.

One unit of antigen dilution (optimal) was taken as that
dilution which gave 100% or 75% fixation with the highest
dilution of antiserum. Similarly one unit of antiserum dilution (optimal) was taken as that dilution which gave 100% or 75% fixation with the highest dilution of antigen.

The scheme of Box-titration is given.

The test proper for rabies diagnosis was carried out with two units of rabies antiserum against serial two fold dilutions of antigen derived from brains and salivary glands of dogs collected for examination of a presence of rabies virus.

In case of sera and cerebrospinal fluids, the test was carried out by using two units of antigen prepared by a method of Ando et al. (cited) against serial two fold dilutions of sera and cerebrospinal fluid obtained for examination for presence of rabies antibody.

The primary incubation period was long of about twenty hours (overnight) for increased sensitivity. In cases where anticomplementary activity was found, reinactivation of sera was conducted and test was performed with a short incubation period.

Immunodiffusion:

The method suggested by Lepine (1973) was followed. A very high titer fixed virus (CVS - Kasauli) strain was inoculated in suckling mice brains (LD$_{50}$ $10^{-5.8}$). The antigen prepared from these brains was used to detect the presence of antibodies in serum and CSF of stray dogs. For detection of presence of rabies virus from brains and salivary glands obtained from stray dogs, hyperimmune antirabies serum was used.
### Scheme of Box Titration Antiserum Dilution

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<tr>
<th>Tube No.</th>
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<th>VS</th>
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<th>a</th>
<th>b</th>
<th>c</th>
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AS = Antiserum,    ES = Erythrocyte suspension,    C = Complement

A = Antigen,    VS = Veronal Buffered Saline.
The gel for immunodiffusion was prepared in the following manner:

- **Agarose** - 15 g
- **Methyl Orange** - 0.03 g
- **Merthiolate** - 0.20 g
- **Normal Saline Solution** - 1000 ml.

Agarose was used for better sensitivity; methyl orange was added to facilitate reading and merthiolate to inhibit bacterial growth. The medium was dispensed in 30 ml portions in large test tubes and stored at 4°C. Whenever required, the tubes were placed in a water bath and the melted medium was poured into petridishes. Those petridishes in which the clarity and homogeneity were slightly affected were discarded.

A circle of 14 mm radius was drawn on a white piece of paper. A glass tubing of 7 mm internal diameter and 8 mm external diameter was taken and one hole was punched in the centre by taking out a small vertical cylindrical piece of medium with the help of a glass tubing. More six such cylindrical holes were punched along the line of circumference of a circle, each hole was 5 mm apart. In all these seven holes, a drop of molten medium was pipetted to seal the bottom, thereby preventing the leakage of the test materials at the bottom of petridish.
Test procedure:

For testing the presence of antibodies in serum and cerebrospinal fluid collected from dogs, the peripheral cups were filled alternately with immune serum and suspected serum or cerebrospinal fluid and the central cup was filled with highly concentrated antigen prepared from suckling mice brains.

In case of detection of presence of virus in the brains and salivary glands collected from stray dogs, the peripheral wells were filled alternately with known rabies infected brain tissue and the suspected brain tissue and salivary glands. The central cup was filled with hyperimmune serum.

The petridishes were then placed in an incubator at 37°C. The cups of serum were refilled every twelve hours or as often as necessary for preventing evaporation and emptiness due to migration in the medium. The readings were taken after 12, 24, 48 and 72 hours which were dependant upon the appearance of precipitin lines. The reading of these lines were facilitated by use of dark ground illumination. The lines were completely discernible after 72 hours.
CHAPTER III

MATERIALS AND METHODS

PART II: DIAGNOSTIC METHODS
II. DIAGNOSTIC METHODS:

Clinically suspected cases of rabies were located and the material for diagnostic purposes was obtained. For this purpose a circular letter was issued to all the Veterinary Hospitals of the state to send brains and salivary glands from the animals which have died of rabies. The samples which were received in 10 per cent formaline were examined by staining techniques, and the samples which were brought on ice or glycerol saline were tested for biological test in mice along with other methods.

Personal approaches were made to Veterinary College Hospital at Anand and other places to get fresh brain material for study.

Fourteen samples of dog brains, two of salivary glands, one of cow brain, one of buffalo brain and one of horse brain were obtained. The street virus was isolated from these brains and salivary glands by inoculating three week old white mice. The brains were collected from such inoculated mice which showed the symptoms of rabies infection as stated in first topic of biological test, and when they were showing paralysis, just prior to their death.

The mice used for inoculation of rabies virus were obtained from National Institute of Virology, Poona, and from Veterinary College, Anand. These mice were bred in optimum laboratory conditions, and their progeny was used for inoculation. The mice were observed from their birth to third week and healthy growing mice of either six were used.
The brains collected from such mice inoculated with street virus, were put on white filter paper, were cut into two longitudinally and half of the brain was used for preparing paraffin embedded sections. The other half was used for preparation of impression smears and for the study of PCA reaction.

The paraffin embedded sections were prepared according to Culling (1963). The sections were cut to the thickness of three to four microns.

The impression smears were stained by Seller's stain, direct fluorescent antibody technique and direct immunoperoxidase technique.

Paraffin embedded sections were stained by:

(i) Massignani - Malferrari Method for staining Negribodies. (Eosin - Phosphotungstic Acid method)

(ii) Direct fluorescent antibody technique.

(iii) Direct immunoperoxidase technique

Massignani - Malferrari Method for staining Negribodies:

Fixation: 10 per cent buffered neutral formalin.

Technique: Paraffin sections are cut at three to four microns.

Solution-A: Harris Haematoxylin solution (without acetic acid):

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<th>Quantity</th>
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<tr>
<td>Haematoxylin crystals</td>
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<tr>
<td>Alcohol - absolute 100%</td>
<td>50 ml</td>
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<tr>
<td>Ammonium/Potassium alum</td>
<td>100 g</td>
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<tr>
<td>Distilled water</td>
<td>1000 ml</td>
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<tr>
<td>Mercuric oxide (red)</td>
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Haematoxylin is dissolved in alcohol and the alum in the water by gentle heat. The two solutions are mixed and are brought to boiling as fast as possible, by frequent stirring and is kept for one minute only. Slowly (red) mercuric oxide is added. Thereafter it is reheated to simmer till it becomes dark purple, removed immediately and the vessel is dipped in a basin containing cold water to cool it. The stain is ready for use as soon as it is cooled.

2.4 ml of glacial acetic acid per 100 ml of stain is added to increase the precision of nuclear stain.

It is to be filtered before use.

Solution-B: 0.5 per cent hydrochloric acid solution.

Hydrochloric acid conc. - 0.5 ml
Distilled water - 99.5 ml.

Solution-C: Lithium carbonate solution:

Lithium carbonate saturated aqueous - 1.0 ml
Distilled water - 200 ml.

Solution-D: Eosin-phosphotungstic Acid solution.

1.0 g of eosin and 0.7 g of phosphotungstic acid are ground together. 10 ml of distilled water is added first and is made up to 200 ml with absolute alcohol. 2 drops of saturated lithium carbonate is added and stirred for 10 minutes. The suspension is filtered.
Staining Procedure:

1. Sections are deparaffinised and hydrated to distilled water.

2. Sections are flooded with Harris haematoxylin solution for two minutes (Soln. A), taking care not to overstain.

3. Washed for 5 minutes in running water.

4. Dipped eight times in hydrochloric acid solution (Soln. B) and washed for five minutes in running water.

5. Slide is placed in diluted lithium carbonate solution for one minute (Soln. C).

6. Washed in running water for 10 minutes and dehydrated in 50 per cent alcohol, 70 per cent alcohol, 80 per cent alcohol, 90 per cent alcohol, 95 per cent alcohol and absolute alcohol by dipping ten times in each respectively.

7. Stained for eight minutes in eosin solution (Soln. D).

8. Wash thoroughly in water.

9. Dehydration is carried out by giving short dips in 50 per cent alcohol, 70 per cent alcohol, 80 per cent alcohol and 90 per cent alcohol respectively, and longer dips are given in 95 per cent alcohol.

10. Further dehydration is carried out by washing in absolute alcohol by giving two to three changes four minutes each.

11. Blotting is carried out on filter paper.

12. Cleared in two changes of xylene, four minutes each.

13. Mount and examine.

Results: Negribodies - Deep red.
Nuclei - Light blue.
(iii) **Direct Fluorescent Antibody Technique:**

The direct method of Fluorescent Antibody Staining Technique was used for study as it is quick, most satisfactory and practical for rabies diagnosis.

(a) **Preparation of antibody dye conjugate:**

(i) **Raising of antirabies serum:**

In order to minimise non-specific fluorescence, the rabies antigen of homologous origin was used, and the antiserum was readily prepared in adult guinea pigs by using rabies virus infected guinea pig brain as immunising antigen. The method is described in Part I. The serum was stored in deep freeze at -20°C till the conjugation procedures are carried out.

(ii) **Labelling of rabies immune serum:**

The purification of globulin from antirabies serum was carried out by precipitation with ammonium sulfate.

In a 250 ml capacity sterile centrifuge bottle, measured quantity of serum was taken and equal volume of physiological saline was added to it, and was chilled. The diluted serum was slowly stirred without frothing and an equal amount of saturated ammonium sulfate was added drop by drop with constant stirring. The stirring was continued for five more minutes after whole of the quantity of saturated ammonium sulfate have been added to it. The centrifuge bottle was stoppered and the precipitate was allowed to stand over night at 4°C.

Next day, the contents of the centrifuge bottle were centrifuged at 3000 r.p.m. for 30 minutes at 4°C and the
supernatant was discarded. The precipitate was then redissolved by slowly adding alkaline distilled water (pH 7.5 adjusted with N/10 NaOH) and by gentle stirring with a glassrod. Again equal volume of saturated ammonium sulfate was added to the dissolved precipitate and it was centrifuged at 3000 r.p.m. for 30 minutes at 4°C immediately. The supernatant was discarded. One more cycle of resolution and precipitation was carried out.

After third precipitation, the dissolved globulin in a minimum quantity of alkaline distilled water was dialysed at 4°C against sterile physiological saline in a clean plastic bucket, with magnetic stirring to keep the saline in continuous agitation to hasten dialysis. The saline in the bucket was changed every 12 hours and was checked for the presence of sulfate ion by testing with barium chloride and Nessler's Reagent. When the saline was free from sulfate ions, which usually was on the third day of dialysis, it was again changed and the dialysis was continued for another 8 to 12 hours. The total protein content of the globulin was determined by Biuret Method.

After the protein estimation was carried out, the globulin was diluted with physiological saline to contain one per cent of protein. It was chilled in a refrigerator and 0.5 M carbonate bicarbonate* buffer (pH 9) was added to it in an amount

* 0.5 M carbonate bicarbonate buffer pH 9.0 preparation.
Soln.-I Sodium Carbonate Na₂CO₃ = 55.0 g
Distilled water to make = 1000 ml
Soln.-II Sodium bicarbonate, NaHCO₃ = 42.0 g
Distilled water to make = 1000 ml
Distilled water to make 1000 ml. For pH 9.0 mix 4.5 ml of Soln.I and 100 ml of Soln.II. The pH is then checked, and can be adjusted by addition of Soln. I.
equal to 15 per cent by volume of globulin solution (15 ml in
100 ml globulin solution). This buffered globulin is chilled
and was stirred with a magnetic stirrer at 4°C. The stirring
was so gentle that no frothiness developed. At this stage
0.05 mg of fluorescein isothiocyanate was added at the rate of
one mg of protein in buffered globulin solution. This was then
allowed to combine overnight by magnetic stirrer at 4°C.

After overnight stirring for combination, the tagged
globulin was collected in a cellophane bag and dialysed
at 4°C for two days against physiological saline. Thereafter
the cellophane bag containing tagged globulin is transferred
to a 250 ml beaker containing 0.01 M phosphate buffer saline
(pH 7.5) and the dialysis was continued till the PBS was free
from dye. This was checked with UV lamp. After the
dialysate (PBS) was found free from dye, the dialysis was
continued for 12 hours more and then the tagged globulin
was centrifuged at 3000 r.p.m. for 30 minutes at 4°C. The
clear supernatant was collected and merthiolate at the
concentration of 1:10,000 was added. This was then dispensed
in 0.5 ml aliquots and stored in deep freeze at -20°C.
A small quantity was taken from the batch, was tested for
its staining property, particularly to ascertain whether any
nonspecific staining is seen, and if found proper, the 0.5 ml
aliquot distributed in flocculation tubes were sealed by
flame and stored at -20°C.
Preparation of (RMB) Rabid Mouse Brain:

Ten mice were inoculated intracerebrally each with 0.03 ml of CVS fixed virus with a dilution containing a titer of more than $10^{-3}$ LD-50 with tuberculin syringes. The brains from these mice were harvested when they were found sick and paralytic for about 12 to 24 hours. 20 per cent (w/v) emulsion was prepared in 10 per cent yolk of six day old chick embryo in 0.05 M sodium phosphate buffer pH 7.8. The emulsion was centrifuged at 1000 r.p.m. for 10 minutes at 4°C. The supernatant was collected and distributed in 0.5 ml aliquots and stored at -20°C.

Preparation of (NMB) Normal Mouse Brain:

Ten healthy mice about three weeks old of either sex and definitely free from infection were selected and their normal brains were harvested. 20 per cent emulsion in ten per cent yolk of 6 days old chick embryo was prepared in the same manner as mentioned above. The emulsion was centrifuged at 1000 r.p.m. for 10 minutes, supernatant was collected and was stored at -20°C in 0.5 ml aliquots.

Preparation of Sodium phosphate buffer (pH 7.8):

Solution A = 0.05 M NaH$_2$PO$_4$ in distilled water
Solution B = 0.05 M Na$_2$HPO$_4$ in distilled water

Mix:
Solution A = 13 ml
Solution B = 87 ml

Every batch of conjugate prepared was titrated before use to find out the highest dilution of the conjugate that
will give bright yellowish green fluorescence under fluorescent microscope. The titration of conjugate was carried out by taking three rows of test tubes in a rack. In the first row serial two fold dilutions were made of conjugate in normal mouse brain (NMB). From first row (I) 0.1 ml of each dilution was transferred to second row (II) and third row (III). The first row was then kept aside and subsequently discarded. In row II containing serial dilutions of conjugate, 0.1 ml of Normal mouse brain (NMB) was added, and in row III 0.1 ml of Rabid mouse brain (RMB) was added. Thereafter tubes of row II and III were kept at 4°C for 30 minutes before staining.

<table>
<thead>
<tr>
<th>Row No.</th>
<th>Tube No.</th>
<th>Initial dilution</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
<th>1/32</th>
<th>1/64</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NMB (ml)</td>
<td>0.45</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Conjugate (ml)</td>
<td>0.15</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>II</td>
<td>Conjugate dilution (ml) from row I</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMB (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Conjugate dilution (ml) from row II</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RMB (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Final dilution in Row I &amp; II</td>
<td>1/8</td>
<td>1/16</td>
<td>1/32</td>
<td>1/64</td>
<td>1/128</td>
<td></td>
</tr>
</tbody>
</table>
Staining:

The impression smears were prepared as mentioned in part I. The slides were airdried thoroughly at room temperature for about half an hour. These slides were transferred to Coplin jar containing chilled acetone and were transferred to deep freeze at $-20^\circ C$, and kept overnight for fixation. Next morning while in deep freeze the acetone was drained out from Coplin jar and the slides were allowed to dry and to allow evaporation of acetone in deep freeze only.

One tube containing 0.5 ml of conjugate was taken out of the deep freeze and was diluted with normal mouse brain suspension (NMB) to half the desired dilution depending upon the results of titration of the batch. Usually the optimum dilution of titration obtained was 1:32. Therefore 1:16 dilution was obtained by diluting the conjugate with NMB.

An aliquot from this dilution of pretreated conjugate was taken and was diluted with an equal amount of rabis mouse brain suspension - RMB. This was an RMB treated conjugate with a final dilution (1:32) desired.

Similarly rest of the aliquot was diluted with an equal amount of normal mouse brain NMB. This was an NMB treated conjugate with a desired final dilution (1:32).

Both these RMB and NMB treated conjugates were kept at $4^\circ C$ for 30 minutes.

The slides were then taken out from deep freeze, defrosted and dried at room temperature. Only thin and evenly distributed
smears were selected for staining. One of the smear on the slide, usually the first out of three or four on the slide was marked as control. These smears were ringed with vaseline for safety in staining. On the control smear, RMB treated conjugate after proper mixing was poured with a pasteur pipette enough to cover the smear. The other smears were covered with NMB treated conjugate in similar manner as above. These were test smears.

The slides were placed on a petridish having a moist filter paper at the bottom and two glass rods fixed on petridish about 3 cms apart with plastic clay. The petridish is then covered and incubated at 37°C for 30 minutes.

After the period of incubation, the slides were taken out of incubator and were placed in a rectangular jar filled with 0.01 M phosphate buffer saline and kept for five minutes. The buffer was replaced after five minutes and the jar was occasionally shaken gently. In this manner three washes of 0.01 M phosphate buffer saline were given. The last wash was given by distilled water for five minutes with occasional gentle shaking. The slides were allowed to dry at room temperature, were mounted in buffered glycerol, and were examined under a fluorescent microscope.

When the smears treated with NMB treated conjugate were examined under fluorescent microscope, the rabies antigen, when present looked like fine dusty particles varying in size and shape of round or oblong, emitting bright to dull yellowish
green fluorescence. The nerve cells at times were found outlined by fluorescent particles. The smear treated with RMB treated conjugate which was to serve as control smear, was next examined and no such fluorescing particles were seen.

The paraffin embedded sections were dewaxed in xylool and were brought to water level, and were stained with similar technique as applied to smears stated above.

(iv) Immunoperoxidase technique:

The labelling of antibodies with enzymes horse-radish peroxidase in particular was introduced in 1970's, and by substituting horse-radish peroxidase (HRP) for the fluoro-chrome label in the standard immunofluorescence methods, the HRP labelled antigen-antibody complex was revealed not only by fluorescent microscopy but by ordinary light microscopy as well. The brown reaction product which is obtained at the site of HRP activity by treatment with 3,3'-diaminobenzidine Hydrochloride (DAB) and hydrogen peroxide (H₂O₂), was stable and can be rendered electron opaque with osmium tetroxide (O₈).  

Labelling rabies antibody with HRP:

Two stage procedure with glutaraldehyde was adopted (Avrameas and Ternynck, 1971). Ten mg of horse-radish peroxidase (Type IV RZ 3.0 obtained from sigma - USA) was dissolved in 0.2 ml of 0.1 M phosphate buffer pH 6.8 and containing 1.25% glutaraldehyde. The solution was allowed to stand overnight at room temperature. Next day early
morning chromatography was carried out on a Sephadex G-25 column (60 x 0.9 cm burette) equilibrated with 0.15 M sodium chloride. The brown fractions—activated peroxidase—was collected. To this solution, one ml of 0.15 M sodium chloride containing five mg of IgG antibody against rabies was added and then 0.1 ml of carbonate bicarbonate buffer pH 9.5 was added. This was incubated at 4°C for 24 hours.

Next day 0.1 of 0.2 M lysine was added and allowed to stand for two hours. The dialysis was then started at 4°C against phosphate buffer saline (PBS) for 24 to 36 hours by changing PBS three times during the entire period at regular intervals. The conjugate was precipitated by addition of equal volume of saturated ammonium sulfate. The precipitate was washed twice with half saturated ammonium sulfate. The final precipitate was dissolved in a minimum volume of distilled water. This was dialysed for 48 hours at 4°C against physiological buffer saline 6 x 1 litres. The final product obtained was immediately stored in a deep freeze at -20°C.

Benzidine reaction (Kaplow, 1965) medium:

0.3 g benzidine dihydrochloride was dissolved in 100 ml of 30% ethyl alcohol. One ml of 0.132 M zinc sulfate, 1 g of sodium acetate, 0.7 ml of hydrogen peroxide (3%) and 1.5 ml of 1.0 N sodium hydroxide is added. 0.2 g of safranin 0 was dissolved in above solution to act as a counterstain. The solution was filtered and pH was adjusted to 6.1 with 1 N hydrochloric acid. This served as a stock solution and was found stable upto three months. It was stored at 4°C.
Staining technique:

Impression smears were prepared as mentioned in part I. They were fixed by the same method used for fluorescent staining in acetone.

Paraffin sections were dewaxed in xylol by giving two to three washes till all the paraffin is washed out. The sections were dipped in 70% alcohol for 30 minutes and then in 90% alcohol for another 30 minutes. The sections were brought out from 90% alcohol and the endogenous peroxidase activity is blocked with a fresh solution of 0.5 per cent hydrogen peroxide in methanol for 30 minutes and washed in water.

Non-specific background staining was reduced with normal guinea pig serum (NGPS) diluted to 1:5, for 5 to 7 minutes. Excess NGPS was removed but not washed off prior to next stage. The sections were then treated with horse radish peroxidase conjugated antirabies serum IgG diluted to 1:20 for 25 to 30 minutes and were incubated in a humidified chamber at 37°C by keeping moist filter paper dipped in phosphate buffer saline in the petridish and placing slides on two rods fixed by plastic clay about 3 cms apart on the petridish.

After incubation the slides were washed in physiological buffer saline for 15 minutes.

Thereafter the slides were stained with benzidine (Kaplow medium) for about 8 to 10 minutes, washed in water and examined under light microscope.
The controls for this direct method included following.

(i) Normal uninfected brain tissue section which is negative, was stained and tested for nonspecific reaction.

(ii) Blocking (inhibition test): Unlabelled antibody, in this case unconjugated antiserum from horse, which is not from the same species from which the HRP conjugate was prepared, was applied for 30 minutes to 60 minutes prior to the start of assay, and it diminished markedly and abolished in most of the cases the staining reaction (test for specificity). In order to confirm the specificity of viral antigen antibody reaction, the unconjugated antiserum from horse was used as the HRP conjugate was prepared from guinea pig antiserum.

(iii) Test for cross reactivity was performed by staining the slides with polio virus antibody conjugate, and no staining was found.

(iv) Test for endogenous activity was performed by direct incubation of tissue sections with enzyme substrate and no reaction was seen.

(v) Passive Cutaneous Anaphylaxis Reaction (PCA):

The PCA reaction was studied by using following antigens;

a) Street virus from dog brain.

b) Street virus from dog salivary gland.
c) Street virus from buffalo brain.
d) Street virus from cow brain.
e) Street virus from mice brains (Rabies positive horse brain one passage).
f) Rabies CVS (CRI - Kasauli).
g) Rabies CVS (IVRI).
h) Normal rabies infection free brains from all the above species.

The rabies infected brain were taken during postmortem and representative samples were taken. Ten per cent suspensions were prepared in physiological buffer saline in a pastile and mortar by keeping it over the ice in chilled condition. The triturate was then centrifuged at 2000 r.p.m. for 30 minutes at 4°C. The supernatant was collected and used for study.

In a similar manner normal brains were also processed and 10 per cent suspensions were made and used for study. Normal as well as infected suspensions were distributed in 1.0 ml aliquots and stored at -20°C in deep freeze.

The antisera used in PCA reaction are as follows:
a) Hyperimmune antirabies horse serum (purified gamma globulin IgG) for prophylactic use obtained from Central Research Institute, Kasauli.
b) Hyperimmune antirabies guinea pig serum prepared as mentioned in part I (CFT) and precipitated globulins prepared as mentioned in part II (IV) for FAT.
c) Hyperimmune antirabies dog serum:

It was prepared in following manner:

Three dogs apparently healthy and young were obtained from dog catching squad of Health Department of Ahmedabad Municipal Corporation. These dogs were observed for 12 days for any sign of illness particularly any symptom suggestive of rabies. They were then inoculated with Rabies vaccine (living) chick embryo origin Flurry (LEP) strain of rabies virus freeze dried, reconstituted in 3 ml of chilled distilled water intramuscularly. After one month, these dogs were again given the injection of the same vaccine in same dose intramuscularly. The blood was collected from these dogs after a period of three weeks. The serum was separated pooled and stored at -20°C.

d) It was decided to include hyperimmune antirabies serum from rabbits and the serum was prepared by the same method used for guinea pigs as mentioned in part I.

PCA reaction in mice:

Adult, about 10 to 12 weeks of age mice of either sex were used in the study. A batch of six mice were taken and hairs from both sides were clipped by shaving machine. Thereafter leather shaving cream was applied with a brush and the clipped portions of the back were shaved clear of hairs. Next day these mice were injected intradermally with 0.1 ml each of guinea pig, dog and horse antirabies serum on shaved back. Two mice were taken for each serum. On the corresponding
opposite side normal serum in the same quantity from the
same species was injected intradermally. After a lapse of
three hours these mice were injected with 0.25 ml of 0.1 per
cent Evans blue in normal saline mixed with 0.25 ml of rabid's
CVS mouse brain 10% suspension intravenously through tail
vein. After 10 minutes the mice were observed for blueing of
the skin at the site of the intradermal injection of sera.

The mortality in mice was observed probably due to
crude suspension of rabies infected brain tissue. The suspen-
sions were then prepared by making more homogeneous solution
and centrifuging at 3000 r.p.m. for one hour till it appeared
almost clear. The mortality reduced to some extent but it
was necessary to use four mice instead of two for the study.
However, guinea pigs were used in further studies.

PCA reaction in guinea pigs:

White laboratory bred guinea pigs weighing about 300
grams or more of either sex and healthy in condition were
used for the test. The method described by Mathew and Rao
(1973) was followed.

The hairs on both sides of guinea pigs were removed
by clipping and then by applying leather shaving cream and
shaving the site by safety razor. The test was performed
next day. 0.1 ml of guinea pig antirabies serum was injected
intradermally and by leaving some space normal guinea pig
serum was injected on the same side to serve as control.
Similarly on other side rabbit antirabies serum was injected,
and normal rabbit serum was injected intradermally. Second
guinea pig was used for horse antirabies serum and dog
antirabies serum in a similar manner, and the injections
were given in same way. 0.25 ml tuberculin syringe and 1/4
inch long 27 gauge needle was used for giving intradermal
injection. Blebs were formed on both sides at places where
serum was injected, which remained visible for a period of
four to five minutes.

After about three hours, each guinea pig was injected
with one ml of one per cent Evans blue in normal saline by
intracardiac route. The injection was given very slowly
taking about one minute or more for one ml of material.
After the injection, the animals were watched for about 10
minutes for any nonspecific blueing at the site of the i.d.
injections. Then one ml of 10 per cent suspension of rabies
CVS mouse brain prepared in physiological buffer saline was
again injected intracardially. The animals which showed
restlessness after the antigen injection were kept under a
fan or near air conditioner, and they recovered. In cases
where another cardiac puncture was not wise to repeat, ear
vein was used. In certain cases ear vein was used to inject
Evans blue as well as antigen. While using ear veins strict
precautions were taken because of the infective nature of the
material.

Three animals were used in each case and each were
euthanasised at an interval of 30, 45 and 60 minutes after
the injection of antigen and the skin was dissected out.
The underside was exposed to note the specific blueing reaction at the site of antiserum injected.

**PCA reaction with different rabies antigens:**

In an experiment with antirabies sera from different species, it was observed that except horse, dog, rabbit and guinea pig antirabies serum gave satisfactory results. These three sera were taken for further study of screening of rabies antigen obtained from different sources. The following rabies virus antigens were tested.

a) Street virus from dog brains.

b) Street virus from dog salivary glands.

c) Street virus from mice brains.
   (Rabies positive horse brain, one passage).

d) Street virus from buffalo brain.

e) Street virus from cow brain.

f) Rabies CVS strain mice brains (CRI - Kasauli).

g) Rabies CVS strain mice brains (IVRI).

In order to study the specificity of the test in diagnosis of rabies infection, the normal tissue antigens definitely free from rabies infection were also obtained from same species and were treated just like infected tissues.

Three guinea pigs were used to test each antigen, and each antigen was tested twice for confirmatory results.
The allergic nature of the reaction in area of blueing was confirmed by histopathology of skin in some of the representative cases.

The skin portion at the site of PCA reaction showed infiltration of polymorphonuclear cells, eosinophils and monocytes at the site indicating allergic reaction.

PCA reaction with antiserum dilutions:

It was observed that dog, rabbit and guinea pig anti-rabies sera gave satisfactory results with different rabies antigens. Therefore these three sera were further studied at a dilution of 1:2, 1:4, 1:8, 1:16 and 1:32 with antigens previously tested with these sera. In order to study the specificity of the test, such two fold dilutions of normal sera definitely free from rabies antibodies were also used in the test to serve as a control.

Three guinea pigs were used for each dilution of rabies antisera. However the test was done only once.

The skin portions dissected from guinea pigs tested above were cut, put to 10 per cent formaline and paraffin embedded tissue sections were prepared. The sections were stained with hematoxylene-eosin stain to study microscopic reaction in the skin tissue.