(VCI/CVE/SBT-I)

VETERINARY COUNCIL OF INDIA

(Statutory body of Government of India established under Indian Veterinary Council Act, 1984)

CONTINUING VETERINARY EDUCATION (CVE) PROGRAMMES

Training Module on Diagnosis of Rabies

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Preface

Consequent upon the decision of the Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India to implement the Continuing Veterinary Education (CVE) programmes, an activity of Professional Efficiency Development Scheme, through Veterinary Council of India as its nodal agency in the country, the skill based training programmes on diagnosis of livestock and poultry diseases having zoonotic importance have been initiated at the first instance. The primary objective of these trainings is to upgrade the knowledge and skills of the registered Veterinary practitioners aimed at improving quality of Veterinary services through efficient and effective diagnosis of the diseases.

Rabies is an acute, progressive and fatal encephalomyelitis caused by Rabies virus belonging to the family Rhabdoviridae and genus Lyssavirus. All warm-blooded animals including livestock of different species are susceptible to the disease, which has Zoonotic importance in terms of its transmissibility to human beings. As per the available literature, about 96% of the mortality and morbidity of this disease is associated with dog bites.

As there is no prescribed treatment for Rabies after onset of the clinical signs, quick and accurate diagnosis and immunisation play important roles in controlling the disease and transmission to humans. This Module for a three-day training programme developed and finalized by the experts in the subject emphasizes on the procedures for collection and despatch of laboratory materials, different diagnostic techniques and their interpretations, and the bio-security precautions to be followed by the Veterinarians while handling the suspected material.

The contents of this Module are also available on the website www.vci-india.in.

INTRODUCTION

Rabies is an acute, progressive, fatal encephalomyelitis caused by neurotropic virus. The word rabies has its origin in Sanskrit, 3000 years BC: “rabhas” means “to do violence”. In animals, this disease is called ***Rabies*** and in humans, it is called ***hydrophobia***. The disease is almost always transmitted by an animal bite that inoculates the virus into wounds. Very rarely, rabies has been transmitted by exposures other than bites that introduce the virus into open wounds or mucous membranes. All mammals are believed to be susceptible, but reservoirs are carnivores and bats. Although dogs are the main reservoirs in developing countries, the epidemiology of the disease differs sufficiently from region to region.

Epidemiology

- In India, it is estimated that almost 2. 3 million people annually receive post exposure prophylaxis against rabies following bite or exposure to rabid or suspected rabid animal.
- With the exception of Andaman and Nicobar and Lakshadweep Islands, human cases of rabies are reported from all over the country.
- About 96% of the mortality and morbidity is associated with dog bites.
- Cats, wolf, jackal, mongoose and monkeys are other important reservoirs of rabies in India.
- Bat rabies has not been reported from India.

Etiology

- Rabies virus (a single stranded RNA virus) belongs to the family *Rhabdoviridae* and genus Lyssavirus.
- The bullet-shaped virus is highly resistant against cold, dryness and decay.
- It is stable between pH 3 and pH 11 and may survive for many years at –70°C or when freeze-dried and kept at 0–4°C.
- By action of oxidizing agents, most organic solvents, surface acting agents, quaternary ammonium compounds, proteolytic enzymes, alcohol, ultraviolet rays and X-rays and pasteurization temperature rapidly inactivate the virus.

Transmission

- The virus circulates in two cycles namely Urban and Sylvatic.
- The most frequent way of transmission of the disease is through the bite of infected dog, cats and wild carnivores.
- In rabies infected dogs, the saliva contains rabies virus 2-3 days

before the onset of clinical signs.

- Cattle, horses, deer and other herbivores can become infected with rabies but they are unlikely to transmit the disease to humans.
- Transmission may also occur via aerosol.
- Transmission from person to person is extremely rare, and can happen through transplant surgery.

Pathogenesis

- The incubation period of rabies is variable (3 weeks to 3 months) and depends upon site of bite, the amount of virus introduced, severity of bite and the species of animals involved.
- With delivery of the virus at the site of bite, it replicates locally and spreads to neuromuscular junctions from where it reaches the peripheral nervous system, spinal cord and then the brain.
- Following replication within the CNS, the virus spreads centrifugally to salivary glands and other organs through peripheral nerves.
- Virus has been detected in adrenal glands, brown fat (interscapular gland) of bats, kidneys, bladder, ovaries, testicles, sebaceous glands, germinal cells of hair follicles, cornea, tongue papillae, intestinal wall.

Clinical Symptoms

- Initial symptoms of rabies are usually non-specific and suggest involvement of the respiratory, gastrointestinal and/or central nervous systems.
- In the acute stage, signs of hyperactivity (furious rabies) or paralysis (dumb rabies) predominate.
- In both furious and dumb rabies, paralysis eventually progresses to coma and death in all cases, usually due to respiratory failure.
- Death occurs during the first seven days of illness without intensive care.
- The stereotypical image of an infected (“rabid”) animal is a “mad dog” foaming at the mouth.

- Rabies may also be present in a so-called 'paralytic' form, rendering the infected animal unnaturally quiet and withdrawn.
- Rabies cannot be diagnosed reliably by an evaluation of behavior or clinical signs alone.

Dogs

- Incubation period lasts from 10 days to 2 or more months.
- Prodromal phase usually lasts for 2-3 days with apprehension, nervousness, anxiety, solitude and variable fever.
- Friendly animals become shy or irritable and may snap where as fractious ones may become more docile and affectionate.
- Most animals will consistently lick the site of viral inoculation.
- Pupillary dilatation with or without sluggish palpebral/corneal reflexes may become apparent.
- Some dogs may develop pruritus at the site of exposure and claw and chew at the area until it is ulcerated.
- Reflex excitability is enhanced, the animal being started by the least stimulus.
- There is anorexia, irritation in the region of bite, stimulation of the genitourinary tract and a slight increase in body temperature.

Furious form

- The furious stage of the disease in dogs usually lasts for 1 to 7 days.
- Animals become restless and irritable and have increased response to auditory and visual stimuli.
- They frequently become excitable, photophobic and hyper aesthetic and bark or snap at imaginary objects. As they become more restless, they begin to roam, usually becoming more irritable and vicious.
- Dogs may eat unusual objects, especially wood (pica).
- Rabid dogs often wander from their homes and travel long distances furiously attacking other dogs and animals along the way.
- When caged or confined, the dog often tries to bite or attack its enclosure.
- Salivation is profuse, since the animal does not swallow its saliva because of paralysis of deglutitory muscles. Bark changes to a prolonged and hoarse howl because of partial paralysis of the vocal

cords.

- In the terminal phase of the disease, generalized convulsions are frequently observed, followed by muscular in-coordination and paralysis of the trunk and extremities.

Dumb form

- The dumb or paralytic phase of the rabies usually develops within 2 to 4 days after the first clinical sign is noted.
- Cranial nerve paralysis may be the first recognizable clinical syndrome, if the bite occurs on the face.
- When the brain stem becomes affected, a change in the tone of the bark, resulting from laryngeal paralysis occurs.
- A “dropped jaw” develops as a result of paralysis of the masticatory muscles. Dogs may make a choking sound, which makes the owner to think that something is caught in the animals’ throat.
- Course of paralytic disease usually lasts two to four days.
- Animal often goes into coma and dies due to respiratory failure.

Cats

- Generally the disease is of furious type with symptoms similar to dogs.
- Paralysis of the posterior third of the body follows 2 to 4 days after the excitation symptoms appear.

Cattle

- Among farm animals, cattle are most commonly affected. Usual incubation period is about 3 weeks, which may vary from two weeks to several months.
- In the paralytic form, knuckling of hind fetlocks, sagging and swaying of hindquarters while walking, often deviation or flaccidity of the tail to one side are common early signs.
- Yawning is a very characteristic sign observed in cattle.
- Within eight hours of yawning the animal starts bellowing, which continues till the paralytic recumbent stage.
- Decreased sensation usually accompanies their weakness and is one of the best diagnostic criteria in the detection of rabies. It is most evident over the hindquarters.
- Tenesmus with paralysis of the anus, resulting in the suckling in and blowing out of air occur late in the in-coordination stages just before the animal becomes recumbent.

- Drooling of saliva is one of the most consistent findings.
- Bulls in this stage often have paralysis of penis.
- Death occurs usually 48 hours after recumbency and/or after a course of 6 to 7 days.
- In furious rabies, the animal has a tense, alert appearance, and is hypersensitive to sounds and movement and is attracted to noise and approach as though about to attack.
- Animal may violently attack other animals or inanimate objects.
- These attacks are often badly directed and are impeded by the in-coordination of gait.
- Loud bellowing is usual; sound is characteristically hoarse and actions are exaggerated.
- Sexual excitement is common.
- The termination of furious phase is often sudden.
- These signs may be evident for 24 to 48 hours and then animal collapses suddenly in a paralyzed state, dying usually within a few hours.

Sheep

- Clinical picture is similar to cattle.
- Sexual excitement, attacking humans or each other and vigorous wool pulling, sudden falling after violent exertion, muscular tremor and salivation.
- Excessive bleating does not occur.
- Most sheep are quiet and anorectic.

Goats

- Commonly aggressive and continuous bleating is common.

Horses

- Abnormal postures, frequent whinnying, unexplained aggressiveness and kicking, biting, colic, sudden onset of lameness in one limb followed by recumbency the next day, high stepping gait, ataxia, apparent blindness and violent head tossing.
- Lameness or weakness in one leg may be the first sign observed, but the usual pattern of death starts with lassitude.
- Sternal recumbency and lateral recumbency followed by paddling convulsions and terminal paralysis.

Pigs

- Excitement and a tendency to attack or dullness and in-coordination.
- Twitching of the nose, rapid chewing movements, excessive salivation and clonic convulsions.
- Backward walking.
- Paralysis and death occurs 12 to 48 hours after the onset of signs.

Man

Classical Rabies

- Most cases go through three stages.

I. Prodromal Stage

- Lasts from 2-10 days and presents in the form of fever, headache, malaise, fatigue, and also localized pain around area of initial infection.

II. Sensory Excitation Phase

- Hyper reactivity, hallucinations, disorientation, seizures and bizarre behavior.
- About 50% of infected individuals developed painful spasms of the pharynx and larynx resulting in a fear to eat or drink.
- Hydrophobia (fear of water) is pathognomonic feature of rabies.
- Increased salivation with drooling. This phase persists for 2-7 days.

III. Coma and paralysis phase

- Paralysis and respiratory problems.

Dumb rabies

- About 20% of cases have only two phases where the patient skips the sensory excitation phase
- Coma and paralysis phase.
- Dumb rabies is almost 100% fatal.

Treatment

- No treatment for rabies is available after the onset of the clinical signs.

- Treatment consists of thorough wound cleansing for a minimum of 15 minutes using water, soap and a virucidal antiseptic (e.g. povidone iodine or ethanol) followed by the administration of rabies passive immunization and cell culture or purified embryonated egg rabies vaccine of proven efficacy.

Prevention and Control

Vaccination

- Use of Cell Culture vaccines that will provide stable and long-lasting immunity is recommended.
- First vaccination of dogs and cats at the age of 3 months, the booster at the age of 12 months and subsequently, biannual vaccination is prescribed.

Control

- Canine rabies can be eliminated by regular vaccination of dogs.
- Vaccination coverage of 70% has been sufficient to control canine rabies in several settings.
- Oral vaccination of dogs offers a new approach that may significantly improve dog vaccination coverage.
- Three practical methods of dog population management are recognized: movement restriction, habitat control and reproduction control.

Post Exposure Vaccination

- Available vaccines are Human Diploid Cell Vaccine (HDCV); Purified Chick Embryo Cell Vaccine (PCEC); Purified Vero Cell Rabies Vaccine (PVRV) and Purified Duck Embryo Vaccine.
- Intramuscular administration of five injections on day 0, 3, 7, 14 and 28.
- The sixth injection (Day 90) should be considered as optional and should be given to those individuals who are immunologically deficient.
- Date 0 indicates date of first injection.
- The deltoid region is ideal for the inoculation of these vaccines. Gluteal region is not recommended because the fat present in this region retards the absorption of antigen and hence, impairs the generation of optimal immune response.
- Rabies immunoglobulin (RIG) should be given for all class III exposures, irrespective of the interval between exposure and beginning of treatment.

Pre- exposure vaccination

- One full dose of vaccine intramuscularly or 0. 1 ml intradermally on days 0, 7 and either day 21 or 28.

Laboratory diagnosis

- Definite diagnosis of rabies can only be obtained by laboratory investigations.

Biosafety considerations

- Biosafety level 2 safety practices are adequate for routine laboratory activities such as diagnosis and animal handling.
- Handling of infected animals for brain removal should be done with thick protective rubber gloves, sleeved rubber or plastic gowns and goggles or a polypropylene face shield. Rubber or strong plastic aprons that can easily be disinfected or discarded can also be used.
- High speed mixing, vortexing and centrifugation procedures should be carried out in tightly closed containers under a negative pressure safety hood to prevent aerosol infection.
- Quaternary ammonium disinfectants in 1: 500 dilution, 45- 70% ethanol, 1 % soap solution and 5-7% iodine solutions inactivate the rabies virus within 1 minute and are indicated for the treatment of wounds also.
- For pipette receptacles, a 1:1000 dilution of a quaternary ammonium compound, any iodine disinfectant with residual available iodine of at least 1:10,000, or a 1% concentration of soapy water or detergent can be used. The solution should be autoclaved and discarded after each use.
- Hot soapy water or detergent can be used for swabbing floors and tables.

- All the glass wares, plastic wares and instruments should be treated with one of the above-mentioned disinfectants followed by autoclaving for reuse or disposal.
- Carcass and animal tissues are best disposed using plastic bags and incinerated.

LABORATORY TECHNIQUES IN THE DIAGNOSIS OF RABIES

COLLECTION OF BRAIN SAMPLES

Materials Required

a) Necropsy tools – hammer, strong cutting blades, bone holding forceps and surgical bone saw

b) Petri dishes

c) Sterile vials

d) Glass slides

e) Blotting Paper

PROCEDURE I

- The brain has to be removed from the skull during postmortem of rabies suspected animals and dissected longitudinally to separate the two hemispheres.
- The cerebellum and medulla have to be detached from the hemispheres and a longitudinal incision is to be made externally in the posterior third of each hemisphere, about 1. 5 cm from the mid line. The incision is continued through the grey matter and white matter until a narrow space, the lateral ventricle, is reached.
- The hippocampus major could be seen as a semi-cylindrical white glistening body bulging laterally on either side from the ventricle floor.
- Using sterile scissors, parts of hippocampus and cerebellum are to be dissected out, impression smears made in clean glass slide, transferred to sterile storage vials and stored at –70°C. Brain samples should be properly labeled indicating the species and date.

PROCEDURE II

Collection of brain material without opening the skull

(a) Occipital foramen route: Brain samples may be taken through this

route by using a plastic pipette. Cut the skin and neck muscles over the joint between the occipital bone and atlas vertebra bend the head forward to give access to the occipital foramen, pass pipette through the foramen, screw it into the brain stem, cerebellum, hippocampus and cortex.

(b) Retro orbital route: Push the eyeball to one side, use a trocar to make an entry through the posterior wall of the eye socket, introduce through this hole a 2 ml disposable plastic pipette, screwing it in the direction of the occipital foramen; the straw or pipette will contain parts of cortex, hippocampus and cerebellum.

Preservation of specimens

- Refrigerate for 1-3 days; freeze at -20^0C 0r -70^0C or in liquid nitrogen for indefinite periods.
- For virus isolation the preservation is in 50% glycerol with streptopenicillin.
- In 10% formol saline for histopathology.

Shipment of samples

- Specimens for rabies diagnosis should be shipped according to the national and international regulations to avoid exposure hazards.
- Diagnostic specimens should either be refrigerated or shipped at room temperature in 50% glycerine-saline/10% formol saline.

General histopathology

- Mononuclear infiltration.
- Perivascular cuffing of lymphocytes or polymorph nuclear cells.
- Lymphocytic foci
- Babe’s nodules consist of Ting of glial cells.
- Negri bodies- i/c eosinophilic with a clear hallow

Materials

1. Light microscope
2. Glass slide (2.5 X 7.5 cm)
3. Scissors and forceps
4. Filter paper
5. Stock solution

Preparation and staining of slides

- Brain sample received in the laboratory may be unpreserved or preserved in 50% glycerol saline or 10% formol saline.

- For demonstration of Negri bodies the unpreserved specimen can be processed as such. The formalin preserved specimen can be used for histopathology. The glycerinated specimen can be used for making impression smears by immersing the tissue pieces in physiological saline for 30 min (three changes of 10 min each) to wash off the glycerine.
- With a pair of scissors make a longitudinal incision about 3-5 cms from the occipital pole into the dorsal surface of cerebral hemisphere approximately 2 cm lateral to midline of the brain.
- Cut through to lateral ventricle.
- Widen the opening to expose hippocampus on the ventricle floor.
- Hippocampus can be seen as white, glistening, semi cylindrical and curved body.
- Cut a small piece of hippocampus (0.5-1 cm) and place it on a spatula/filter paper with cut surface facing upwards.
- Place the filter paper on glass slide and lightly sponge the cut surface with the edge of a filter paper to remove blood.
- Press a clean microscope slide on the tissue piece on spatula/ filter paper to get an impression smear. Make at least 3 smears on each slide.

Staining

- Prepare working solution of stain by adding Solution I and II in water.
- To about 10 ml of water in a test tube add solution I, 2 to 3 drops, solution II 2 to 7 drops, mix well.
- Pour the stain over the impression smear.
- Gently heat the slide from under until steam emanates.
- Allow it for a minute or two and wash the slide with water.
- Blot and examine

Examination

- Screen the slide under low power to identify the area free from erythrocytes and for loops of ganglion cells. Place a drop of immersion oil on the smear. Examine under oil immersion.
    - The nuclei of nerve cells are stained blue in color with nucleoli taking dark blue stain.
    - Glial nuclei are stained in dark blue color and nucleoli are not appreciable.

- The Negri bodies are round or oval in shape located near the nuclei of nerve cells and are magenta red in color.
- Erythrocytes are coppery red in color.

Interpretation

- The presence of Negri bodies is a definite evidence of rabies. While the absence of Negri bodies does not rule out rabies.

Fluorescent Antibody Test (FAT)

Principle

- Rabies specific antibodies and antigen when mixed and kept under optimum conditions combine to form an antigen-antibody complex. This complex is not visible to naked eye. To detect this complex fluorescein isothiocynate (FITC) dye is used which emit fluorescence when exposed to ultraviolet rays. The FITC is tagged with antirabies antibody to form the conjugate. If the detection of antigen is a one step procedure, it is called a direct fluorescent antibody test (FAT).

Materials Required

a) Clean glass slides (2.5 X 7.5 cm)

b) Cover slip

c) Conjugate (Antinucleocapsid fluorescein/antirabies-antibody FITC conjugated)

d) Acetone

e) 10% glycerol and 0.8% normal saline solution

f) Humid chamber/box

g) Fluorescent microscope

Procedure

- Place the slides in cold acetone and keep it in the refrigerator for 45 - 60 minutes to fix the impression smear.
- Air dry the slides and add a drop of Conjugate (Antinucleocapsid fluorescein Conjugate) and incubate in a humid box, at 37°C for 45–60 min.
- Wash the slides in running tap water and dry the slides.
- Place a drop of 10% glycerol/saline solution and a cover slip and view under Fluorescent microscope.

Interpretation

- Positive reaction is characterized by bright apple green fluorescent inclusions.

Modified Counter Immuno Electrophoresis Test (CIEP)

Principle

- Antigen-antibody when permitted to diffuse through gels have a property of forming precipitin lines at the point of their meeting in optimal concentrations.
- Rabies antigen from suspected brain or salivary glands can be detected by using rabies specific serum, along with known positive rabies antigen and negative controls.

Materials required

a) Glass slide 2.5 X 7.5 cm

b) Barbitone buffer pH 8.2

c) Phosphate buffer saline (PBS) pH 7.2

d) Merthiolate

e) Agarose

f) Rabies hyper immune serum

g) Amido black stain

h) Glacial acetic acid

i) Glycerol

j) Electrophoresis apparatus

k) Water bath

Preparation of Agar gel slides

a) Prepare 1.2% Noble agar in 0.5 M Barbitone buffer (pH 8.2) containing 1:10,000 merthiolate

b) Apply a thin coat of the agarose on to clean 2.5 X 7.5 cm glass slides

c) Dry the slides at room temperature

d) Add 4 ml of melted agarose on to each slide with a 10 ml serological pipette with rubber bulb without any air bubble

e) Allow to solidify at room temperature

f) Place the slides in Petri dish and keep at + 4°C for 30 minutes

g) Cut four pairs of wells (5 mm diameter are cut 6 mm apart) in the agar.

Preparation of brain specimens

- Prepare a 20% (W/V) suspension of rabies suspected brain samples in 0.01M PBS (pH 7.2) with a clean pestle and mortar.

Test Procedure

a) The right–hand side is cathodal and the wells are filled with test samples or positive and negative antigen preparations.

b) The left-hand side is anodal and is filled with rabies hyper immune serum.

c) Samples are run for 60-120 minutes at a constant current of 10 mAmp/slide or a constant voltage of 6 volts/cm, at the end of the electrophoresis, precipitation lines are visible between the antigen and antiserum wells.

d) The slides may be washed and stained with 0.1% Amido black stain in acetic acid and glycerol for 10 min. De-staining is carried out using 7% acetic acid until the gel background is clear.

SCREENING FOR RABIES VIRUS ANTIBODIES

A number of serological procedures have been described for measuring rabies antibody. These include the Complement fixation test (CFT), heamagglutination test (HA), haemagglutination inhibition test (HI), plaque reduction test, gel diffusion test, counter immuno-electrophoresis test, mouse neutralization test, radio-immunoassay, rapid fluorescent focus inhibition test and ELISA.

ELISA FOR DETECTION OF ANTIBODIES

- Antirabies antibodies are measured in specialized laboratories in order to determine the degree of immunity of vaccinated subjects. A level of antibodies equal or more than 0.5 U/ml protects subjects exposed to the risk of contamination.

ELISA KIT

- ELISA (BIO-RAD, FRANCE) is an immuno-enzyme technique for the detection of rabies virus antiglycoprotein antibodies in human serum or plasma (including dog, cat, mouse, g. pigs, rabbit and monkey). The test is based on the use of solid phase, prepared with the glycoprotein extracted from the inactivated and purified rabies virus membrane, and an enzymatic conjugation (Protein A from *Staphylococcus aureus* coupled with peroxidase).

Method

1. The test serum as well the control serum are placed in the well of the precoated micro titre ELISA plates. If rabies virus anti-glycoprotein antibodies are present, they bind with the viral antigen fixed on the plate.
2. The protein A labeled with horseradish peroxidase conjugate is added after washing. It in turn bonds with the IgG retained by the solid phase.

3. The presence of the immobilized enzyme on the complexes is revealed by incubation in the presence of the substrate after the elimination of the unbound fraction of the conjugate.
4. A spectrometric reading is taken at 492 nm after the reaction has been stopped. Comparison with control serum titrated in IU/ml or direct reading on a reference series, provides the titer of the unknown serum in EU (unit equivalent to the international units defined by sero-neutralization).

MATERIAL OF THE ELISA ANTIBODY KIT

All of the reagents are intended for use with in-vitro antibody assay, enough reagents are provided for 2x 48 double assays.

Label	Type of reagent	Presentation code
R 1	Micro titration plate	2 plates
R 2	Washing solution concentrated 10 times	1 bottle (100 ml)
R 3	Human negative control serum	1 bottle (0.2 ml)
R 4	Human positive control serum	1 bottle (0.2 ml)
R 5	Diluents concentrated 5 times for the dilution of sera	1 bottle (2-0 ml)
R 6	Conjugate: Protein A-Peroxidase concentrated 10 times	1 bottle (2.5 ml)
R 7	Buffer for peroxidase substrate	1 bottle (60 ml)
R 8	Chromogen (Ortho Phenylene diamine 2 HCl)	8 tablets
R 9	Stopping solution (4 N sulphuric acid)	1 bottle (10 ml)
	Adhesive sheets for micro plates	6
	Plastic tongs for OPD tablets	1

RECONSTITUTION OF THE REAGENTS

Reagent 1 (R1): Sensitized strips. Open each pouch at the sealing point and bring to room temperature. Take the required rack out and immediately put the unused strips back in the pouch. Carefully close the pouch and store at +2 to + 8°C.

Reagent 2 (R2): Washing solution concentrated 10 times. Dilute the solution 10 times in distilled water. The washing solution is now ready for use. 500 ml is required for a plate.

Reagent 5 (R5): Diluents for sample, concentrated 5 times. Add 1 volume of R5 to 4 volumes of distilled water in order to

obtain the solution for the dilution of the serums.

Reagent 6 (R6): Conjugate concentrated 10 times. Add 1 volume of concentrated conjugate to 9 volumes of prepared washing solution. 11 ml is required for a full plate.

Reagent 7 (R7): Buffer for substrate. 10 ml are required for reconstitute one OPD tablet.

Reagent 8 (R8): Chromogen.

Reconstitution Table

Number of strips used (R1) (16 wells)	Number of OPD tablets (R8)	Volume of the buffer substrate (R7)
1	1	10 ml
2	1	10 ml
3	2	20 ml
4	2	20 ml
5	3	30 ml
6	3	30 ml

Method

1. Dilute the positive and negative control serum to 1/100th. Dilute by 2 in order to obtain the 1/200th, 1/400th, 1/800th, 1/1600th, 1/3200th, 1/6400th and 1/12800th dilutions.
2. Dilute the unknown serum or plasma to 1/100th (e.g. 10 µl of sample in 990 µl or 1000 µl of dilution solution).
3. Remove the required number of bars (R1) from the protective packaging. Wash twice with the washing solution by filling all of the wells. Empty and then dry the bars on their neck by turning over on a sheet of absorbent paper.
4. In the wells, place:

1A 2A 100 µl of positive control serum diluted to 1/100th

1B 2B 100 µl of positive control serum diluted to 1/200th

1H 2H 100 µl of positive control serum diluted to 1/12800th

3A 4A 100 µl of negative control serum diluted to 1/100th

3B 4B 100 µl of negative control serum diluted to 1/200th

3H 4H 100 µl of negative control serum diluted to 1/12800th
(Use the other wells on the bars for the dilute serum samples)

5A 6A 100 µl of the first diluted unknown serum

5B 6B 100 µl of the second diluted unknown serum

Cover with a self-adhesive film by firmly pressing on the whole surface in order to ensure sealing.

5. Incubate the bar or bars preferably in a water-bath or in a ventilated drying oven for micro plate set at 40°C.
6. Prepare the required conjugate solution before the end of the first incubation.
7. Remove the adhesive film. Empty or draw up the contents from all the wells and add 0.33 ml of washing solution to each of them. Draw up again. Wash two more times (3 washings in all) and then dry the plate by turning over on a sheet of absorbent paper. Use the same operating cycle with an automatic washer.
8. Place 100 µl of the conjugate solution in all the wells. Cover with a new film and incubate for 60 min + at + 40°C.
9. Remove the adhesive film, empty the contents of the wells by drawing up or turning over and wash 4 times as above. Dry the wells by turning over on a sheet of absorbent paper.
10. Prepare the substrate solution just before use.
11. Away from direct light, quickly distribute 100 µl of the enzyme activity detection (R7 + R8) in all the wells. Allow the reaction to develop in darkness for 30 minutes + 5 minutes at room temperature (18 to 25°C).
12. Add 50 µl of the stopping solution by using the same sequence and the same distribution rhythm as with the detection solution.
13. Carefully wipe the underside of the plates. Read the optical density at 492 nm by means of a plate reader within 30 min of stopping the reaction (the wells should always be kept in a light-free place before reading).
14. Before recording the results, check that the reading complies with the distribution and identification plan for the plates and samples.

Interpretation

- According to the WHO, a patient with a minimum level of 0.5 IU/ml of antibodies in his serum is immunized.

Mouse Inoculation Test

The test is simple but depends greatly on the accuracy of its performance for dependable results.

Mice

(a) Strain: Though white mice of any breeding strain may be considered suitable, Swiss albino mice are generally used since they are very susceptible to rabies virus and it is easy to maintain the breeding stock in the laboratory.

(b) Age: Though mice of all ages are susceptible to I/C introduction of rabies virus, suckling mice up to 3 days of age are more sensitive.

(c) Sex: Mice of both sexes are equally susceptible to rabies virus.

Preparation of the infected material for inoculation

Either the brain or the salivary gland tissue of a suspected rabid animal may be used for mice inoculation. The detection of the virus is more frequently possible in the brain than in the salivary gland. However, from the epidemiological and epizootiologial points of view, it is important to examine the salivary glands for the presence of virus. Usually Ammon's horn, the cerebellum and parts of the cortex and salivary gland particularly the submaxillary glands are those most likely to show the presence of rabies virus. Ten percent suspension of the brain is prepared in PBS using sterile sand or glass beads in Mortar and Pestle. 500 IU of Penicillin and 1560 IU of Streptomycin per ml of suspension are added to check the bacterial contamination. The suspension is centrifuged at 200 g for 5 min to remove the abrasive material.

Inoculation of mice: Mouse is inoculated @ 0.03 ml of suspension using 26 gauge needle 1-1.5 cm long I/C. Two imaginary lines are drawn by joining the inner canthus of the left eye with the base of the right ear and vice versa. The needle is inserted 0.1 to 0.2 cm into the brain at the point where the two imaginary lines stashing from base of the ear to the oppressive eyes cross each other.

Observation of inoculated mice: Although rabies virus will only rarely cause signs of illness in mice before the fifth day after I/C inoculation, it is advisable to observe daily. Death occurring 24-48 hours after I/C inoculation are attributable to causes other than rabies virus. The mice are observed for a minimum of 21 days after inoculation. The mice positive for rabies would show the symptoms of ruffled fur, tremors, in-coordination, paralysis and finally death.

PREPARATION OF REAGENTS/ STAINS

1. Vernol Buffer

Sodium barbitone	:	10.3 g
Diethyl barbituric acid	:	1.8 g

Distilled water : 1000 ml (pH 8.6)

2. Normal Saline Solution (NSS)

Sodium chloride : 0.85 gm

Distilled water : 100 ml

3. Phosphate Buffer Saline (PBS)

PBS (Solution A)

NaCl : 64 g

KCl : 1.6 g

$Na_2HPO_4, 7H_2O$: 17.36 g

KH_2PO_4 : 1.6 g

Distilled water : 6400 ml

PBS (Solution B)

Calcium chloride : 0.8 g

Distilled water : 800 ml

PBS (Solution C)

Magnesium chloride ($MgCl_2.6H_2O$) : 1.7 g

Distilled water : 800 ml

All the solutions (A, B and C) are sterilized separately at 20 lbs at 115°C for 30 minutes in an autoclave.

4. Seller's Stain

Solution A (1% Basic Fuchsin)

Basic Fuchsin : 1 g

Methanol : 100 ml

Solution B (1% Methylene blue)

Methylene Blue : 2 g

Methanol : 200 ml

Add solution A and B by mixing till proper color develops. Generally, 1:2 proportion gives excellent staining. Store over night at 4°C and check colour development.

5. Formol saline

35-40% formaldehyde	:	10 ml
Normal saline solution	:	100 ml

6. Formal sublimate

35-40% formaldehyde	:	10 ml
Saturated mercuric chloride to	:	100 ml

7. Buffered formalin

35-40% formaldehyde	:	10 ml
PBS (pH 7.2) to	:	100 ml

8. PBS- glycerine (FAT mounting media)

PBS (pH 7.2)	:	10 ml
Glycerine	:	90 ml

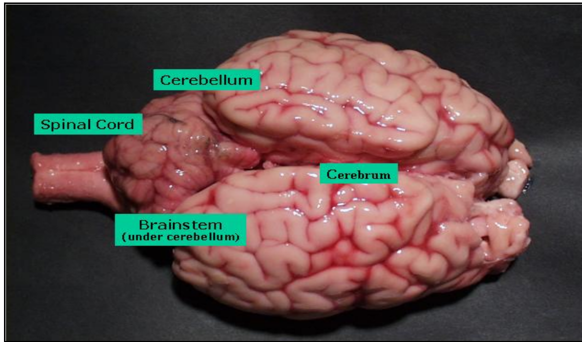

Fig. 1. Dorsal View of the Brain showing the different parts

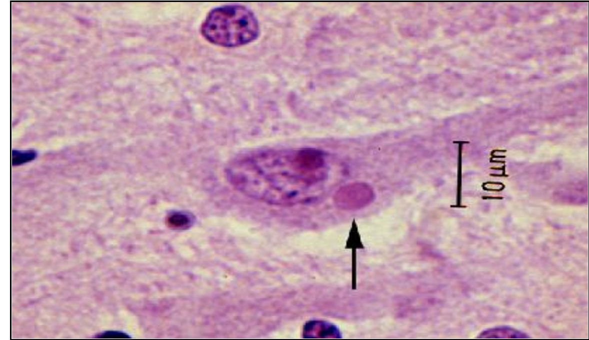

Fig. 4. Negri bodies- round or oval shaped magenta color inclusions

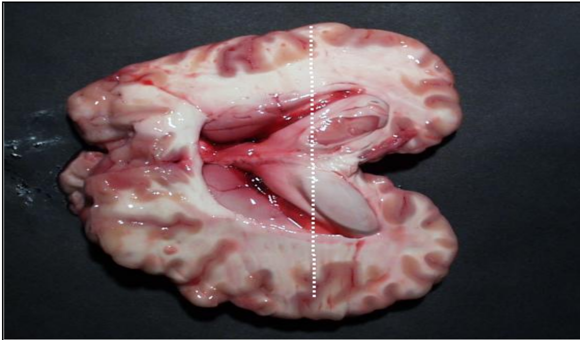

Fig. 2. Dorsal view of the two horn shaped protrusions of the hippocampus

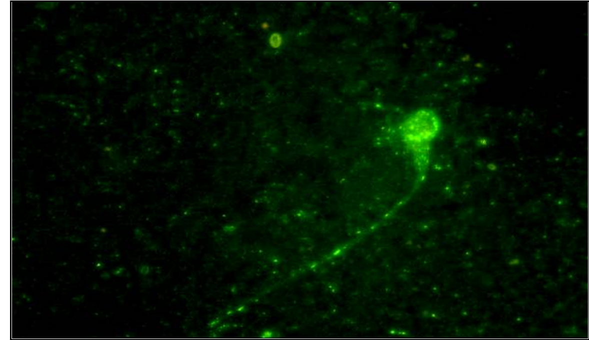

Fig. 5. Direct Fluorescent antibody test directed against the ribonucleocaspid of Rabies virus. Note the dust like fluorescent particles in the cytoplasm of the infected cells

Fig. 3. Two 15 mm tissue sections dissected from the cut surface of a brain stem cross section

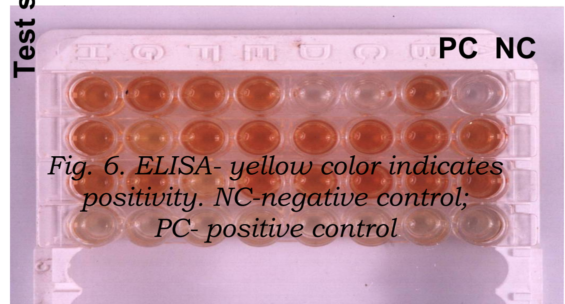

Fig. 6. ELISA- yellow color indicates positivity. NC-negative control; PC- positive control