

Southern and Eastern African Rabies Group

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RABIES DIAGNOSIS MANUAL

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FOREWORD

This booklet does not seek to replace any of the recognised text books on rabies. The rabies diagnostician is encouraged to be familiar with the OIE "Manual of Standards for Diagnosis Tests and Vaccines"(2000), the Technical Report of the WHO Expert Committee on Rabies (Eighth Report) and the WHO Handbook "Laboratory Techniques in Rabies" (Fourth Edition, 1996) before embarking on rabies work.

This booklet is produced by members of the Southern and Eastern African Rabies Group (SEARG). Its intention is to describe the minimum requirements necessary for a reliable rabies diagnosis laboratory, i.e. a robust diagnosis chain.

It is based on our practical experience and on papers presented at the different meetings of our group.

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INTRODUCTION : LYSSAVIRUS SEROTYPES AND GENOTYPES

Taxonomically, rabies viruses belong to the *Rhabdoviridae* family, a constituent of the Order *Mononegavirales*, in which the viruses are characterised by a non-segmented negative sense RNA genome and a bullet shape morphology. The family is divided into two serologically distinct genera, *Vesiculovirus* and *Lyssavirus*. The *Vesiculovirus* genus includes the viruses causing vesicular stomatitis and antigenically related viruses and the *Lyssavirus* genus includes rabies, the rabies-related viruses and others viruses which share only a distant relationship to rabies. By use of serological and molecular techniques, four Lyssavirus serotypes encompassing seven genotypes are currently recognised in the genus *Lyssavirus*. The distinction between viruses of serotype/genotype 1 (rabies), serotype/genotype 2 (Lagos bat), serotype/genotype 3 (Mokola) and serotype/genotype 4 (Duvenhage) (Table 1) was established by cross-immunisation experiments in animals. All of these serotype/genotype viruses have been isolated within Sub-Saharan Africa that includes the SEARG region. In addition, two distinct groups of the European bat lyssavirus (EBLs), detected by monoclonal antibody anti-nucleocapsid (Mab-N) reaction patterns, have been confirmed by molecular techniques and are now known as EBL 1 (genotype5) and EBL2 (genotype 6). Genotype 7 has been isolated in Australian bats (ABL).

The antigenic variation within African lyssaviruses has two main implications. Firstly, diagnostic tests that rely on detection of epitopes or gene sequences may be reliable for some sero/genotypes but not for others. When using a diagnostic method for the first time, it is important to validate it against the virus types expected in the area. Secondly, rabies vaccination may be less reliable against sero/genotype 2 and 3.

Table 1: Lyssavirus serotypes and genotypes, their sources and geographic distribution¹

Name (Sero/Genotype)	Source(s) of virus in nature	Known geographic distribution
Rabies (1/1)	Dog, cat, bat; wild carnivore, e.g. red fox, grey fox, bat-eared fox, skunk, raccoon, jackal, mongoose	World-wide except Australia, New Zealand, Antarctica, parts of Scandinavia, United Kingdom, several western European countries (Belgium, France, Luxembourg, Switzerland), Japan, Hawaii and some other islands
Lagos bat (2/2)	Frugivorous bat, cat, dog	Nigeria, Ethiopia, Senegal, Central African Republic, Zimbabwe, South Africa
Mokola(3/3)	Shrew, cat, dog, rodent	Nigeria, Ethiopia, Cameroon, Central African Republic, Zimbabwe, South Africa
Duvenhage (4/4)	Insectivorous bat	Zimbabwe, South Africa
European bat Lyssavirus 1 (-/5) = EBL 1	Insectivorous bat (chiefly serotines)	Germany, Poland, Ukraine, Netherlands, Denmark, France, Spain
European bat Lyssavirus 2 (-/6) = EBL 2	Insectivorous bat (<i>Myotis spp.</i>)	Netherlands, Denmark, Switzerland, Finland, United Kingdom
Australian bat Lyssavirus (-/7) = ABL	Frugivorous bats (<i>Pteropus spp.</i>) and insectivorous bats	Australia

¹ Adapted and updated from King and Crick (1988).

The intention of this booklet is to demonstrate simple techniques available in rabies diagnosis. It must be remembered, however, that specimens submitted for rabies diagnosis may contain pathogenic organisms other than rabies. Therefore the highest standards of safe working procedures are called for.

Access to the rabies *post-mortem* room and laboratory should be restricted to vaccinated personnel only. Within the *post-mortem* room protective clothing should be worn at all times and it should not be possible to wear this clothing outside of the room.

Contaminated paperwork associated with a rabies specimen may represent a hazard to unvaccinated staff. If packages are to be opened in the *post-mortem* room, registration documents should be completed and removed from the room before the commencement of autopsies.

A sterile set of instruments should be used for each diagnosis and contaminated instruments should be decontaminated by boiling in water. Potentially infectious material such as brain smears should be fixed before removal from the *post-mortem* room; it should be remembered that acetone fixation does not render the smears non-infectious.

All biological waste material should be decontaminated by incineration. Where this is not possible the waste may be buried in quicklime in an isolated place and at a depth that cannot be reached by carnivores.

Apparatus (table tops, vice, socket forceps etc.) used in the *post-mortem* room must be cleaned between specimens. *Post-mortem* room (floor, walls, tables, and other apparatus) should be washed down daily.

The chain of rabies diagnosis starts in the field with the collection of specimens and ends with a report to the sender and of "rabies bulletins" and collation of results into national and international statistics.

The different steps of this process are:

- Collection of specimens and of epidemiological data in the field.
- Transport of the specimen to the laboratory.
- Management of biological safety in the laboratory.
- Choice of the test(s).
- Laboratory diagnosis.
- Management of biohazardous waste.
- Submission of results.
- Inclusion into national/international statistics.

COLLECTION AND TRANSPORT OF SPECIMENS

For the reliable diagnosis of rabies the brain is required. Of the parts of the brain, the brainstem is the most reliable for the detection of antigen. Other tissues can also be used for rabies diagnosis, although not all are as reliable as brainstem. These include spinal cord, peripheral nerve tracts and salivary glands. The ante-mortem diagnosis of human rabies requires skin biopsies from the head region and corneal impression smears.

1 CHOICE OF SPECIMENS SENT FOR RABIES DIAGNOSTIC.

Whatever the specimen collected, it must be stressed that brain is required for a reliable rabies diagnostic.

Dogs that have contaminated people should always be examined when they die within 2 weeks after biting. For epidemiological studies, animals that are found dead or whose behaviour is unusual constitute the best sample to look for rabies.

2 WHICH INFORMATION SHOULD BE COLLECTED?

Every specimen submitted for rabies diagnosis should be accompanied by a submission form containing the following data:

1) Specimen description

- a) species
- b) date of death or collection, date of shipment
- c) location: name, geographical coordinates
- d) name and address of the owner or of the person who found the animal
- e) vaccinal status for domestic animals

2) possible human exposure

- a) type of exposure
- b) date of exposure
- c) location of the wound(s)
- d) name and addresses of exposed people

3) whenever possible, other information may be collected

- a) age, sex of the suspect animal
- b) clinical signs
- c) animal contacts

3 TRANSPORT OF SPECIMENS.

Whole carcasses, heads, brains or brain biopsies can be transported to the laboratory. Ideally, fresh material should be transported to the laboratory rapidly and under cold conditions. If this cannot be achieved, preservative solutions should be used. In this case the brain is extracted from the skull, one half is sent in a 50% glycerol-saline solution (see appendix) and the other half in formalin.

Brain extraction is described in page 8. If opening the skull is not possible, a sample can be collected by inserting a straw through the occipital foramen or through the posterior wall of the eye-socket. The section of straw containing the brain sample can then be detached into the 50% glycerol-saline solution for transport to the laboratory.

Straw sampling for histology, where the straw contents are immediately expressed into formalin is a "last resort" technique and is not recommended because of low sensitivity of histological tests.

MANAGEMENT OF BIOLOGICAL SAFETY IN THE LABORATORY.

Lyssaviruses are Group 3 pathogens - "organisms that may cause severe human disease and present a serious hazard to laboratory workers; they may present a risk of spread in the community but there is usually effective prophylaxis or treatment available". Human death has resulted from infection with each of the rabies serotypes and genotypes except serotype/genotype 2 Lagos bat virus, but even Lagos bat virus is known to cause infection in cats and dogs which is clinically indistinguishable from rabies. Therefore, it must be assumed to be capable of causing fatal encephalitis in humans.

The risk of acquiring a rabies virus infection in the laboratory is related to the techniques to be undertaken. Rabies diagnosis in field specimens may present a greater risk than the production of fixed strains of rabies on well defined substrates, because field strains may have undetermined characteristics and because these specimens may carry other unknown pathogens.

Every laboratory must develop its own working routine when dealing with pathogens, but there are some general principles that can be summarised here. These "risk limiting" measures include:

1 MAINTENANCE OF INTEGRITY OF "SAFE" AND "HIGH-RISK" WORKING AREAS.

High-risk areas, which may comprise only the necropsy room or include different rooms where rabies diagnosis is performed, must be clearly separated from safe areas. Access to the high-risk areas of the laboratory should be restricted only to those personnel who are vaccinated for rabies and who are familiar with the laboratory's safety protocols.

Staff should not enter a high-risk area without wearing protective clothing. This protective clothing must not be worn in a "safe" area. Clothing from a contaminated area must be decontaminated (e.g. by autoclaving) before removal for laundry. One method of ensuring that security is not breached is to wear colour-coded laboratory clothing, i.e. white clothing for safe areas and a coloured tag for the high-risk area.

Contaminated paperwork associated with a rabies specimen may represent a hazard to unvaccinated staff. If packages are to be opened in the post-mortem room, registration documents should be completed and removed from the room before the commencement of necropsies.

Apparatus (table tops, vice, socket forceps etc.) used in the post-mortem room must be disinfected using a suitable detergent and cleaned between specimens. A sterile set of instruments should be used for each specimen and contaminated instruments should be disinfected by boiling or autoclaving. All contaminated surfaces and instruments should be washed and disinfected after every diagnostic session and the floor, walls and other structures of the post-mortem room should be disinfected and washed down daily. Potentially infectious material such as brain smears should be fixed before removal from the post-mortem room, but it should be remembered that acetone fixation does not totally inactivate rabies virus.

2 IMMUNISATION OF STAFF.

WHO recommends the preventive pre-exposure immunisation of staff that work with live rabies virus. The immunisation protocol includes three injections at days 0, 7 and 28 and a boost injection one year later and then every 3 years in the absence of serological follow-up. Serological confirmation of protection should be carried out 10 days or more after the last injection. The subsequent testing of antibody level is recommended annually and staff whose antibody level declines below 0.5 IU/ml should be given booster vaccination.

This is particularly important in the SEARG region where rabies related viruses may be isolated since it has been shown in experimental animals that current vaccines for humans give a lower level of immunity against these viruses. Although it must be stressed that no properly vaccinated person and no unvaccinated person given proper post-exposure treatment has succumbed to the disease.

3 PHYSICAL PROTECTION FROM BIOHAZARDOUS AGENTS.

Good laboratory practice is the first line of defence against accidental infection.

Wearing of protective clothing, consisting of gowns, aprons, gloves, visors, masks, goggles and boots will protect the operator's skin and mucous membranes against splashing and spillage. All protective clothing is removed when leaving the rabies laboratory and kept near the exit.

Contaminated documents that contain information to be transmitted to other areas should be photocopied in a plastic envelope or decontaminated (for instance with ultra-violet light).

Storage and consumption of food or drink, smoking and applying cosmetics should be prohibited in the laboratories.

4 DISPOSAL OF BIOHAZARDOUS WASTE.

Rabies virus is easily inactivated by heat or chemical treatment. Iodine, quaternary ammonium compounds and Virkon are some of the most commonly employed disinfectants. Attention must be drawn on the decrease of activity of quaternary ammonium compounds in the presence of hard water or organic matters.

Diagnostic operations produce waste that is infected or contaminated with rabies virus. Safe disposal of these wastes is an important aspect of laboratory management. Although incineration is the most effective means of destroying animal and biohazardous waste, it requires a lot of energy. Waste can be autoclaved before it is removed from the laboratory. An alternative is to bury this waste in quicklime in a pit inaccessible to the public and to scavengers and in compliance with local legislation.

CHOICE OF LABORATORY TESTS

Whenever possible, all specimens should be examined with at least two different techniques: for example, the fluorescent antibody test (FAT) to detect antigen and the mouse inoculation test (MIT) as a back up.

The following tests can be performed according to the available specimens:

Specimen	Test
Fresh or frozen nervous tissue specimen.	FAT on smears Biological tests in mouse or mouse neuroblastoma cell cultures Polymerase chain reaction
Formalin fixed nervous tissue specimens embedded in paraffin.	Immunohistochemistry, histology* FAT following enzyme digestion**
Glycerol preserved specimen, after removing excess glycerol	FAT on smears Biological tests in mouse or mouse neuroblastoma cell cultures Polymerase chain reaction

* Very low sensitivity. Negative results must be interpreted with caution

** Not recommended as a routine procedure

The minimal required tests that should be stipulated for different situations should be the following:

- Human or animal exposure: FAT, followed by MIT if FAT is negative.
- Epidemiological surveillance: FAT alone is usually sufficient in enzootic areas.

LABORATORY DIAGNOSIS

This manual describes the fluorescent antibody and mouse inoculation tests as these would be the most appropriate for laboratories in Africa. Biological tests in cell cultures are appropriate if cell culture facilities are already available in a laboratory. Immunohistochemical tests are also good tests to use if the appropriate facilities are available. Histological tests using traditional stains are not recommended due to the unacceptably low sensitivity of the technique. PCR tests should only be used as backup tests to other techniques and the results, unless the operators have considerable experience, should be interpreted with caution.

1 BRAIN REMOVAL AND BRAIN DISSECTION.

These steps are detailed below pages 10 and 11.

2 FLUORESCENT ANTIBODY TEST (FAT).

The direct fluorescent antibody test involves the staining of a tissue smear (usually brain) with an FITC-labelled anti-rabies immunoglobulin, following acetone fixation. The stained smear is washed in buffer and read under blue-light fluorescence microscope to detect the characteristic green fluorescence associated with rabies antigen corpuscles.

A few quality control principles apply:

1. The quality of all reagents, including acetone, conjugate and washing buffers must be optimal. Storage conditions of the reagents must also be optimal, that is according to manufactures recommendations, and in clean vessels. Conjugate and buffers must be maintained and used at the appropriate pH. A common cause of poor fluorescence is formalin contamination of the acetone fixative.
2. Conjugate must be of broad spectrum and used at the appropriate dilution to recognize all locally occurring virus variants. Polyclonal conjugates are produced by the conjugation of fluorescein isothiocyanate with purified globulins from hyperimmune serum that is prepared by vaccination of appropriate animals (e.g. goat, guinea-pig) with purified rabies virus ribonucleocapsid proteins. They are usually provided in the freeze-dried form, but may also be provided as liquid concentrates.

The optimal working dilution must be determined before use by titration of the conjugate (at various dilutions, for example, between 1/100 and 1/2000) on representative and unusual antigenic types. The working dilution may differ between laboratories, depending on the microscope optical system used.

It is recommended that concentrate dilutions of conjugate be mixed with an equal volume of double distilled glycerol (for example to give dilutions of 1/20 or 1/40) and stored at -20°C. As the glycerol prevents freezing, the conjugate can be repeatedly retrieved from -20°C storage without the adverse effects of freezing and thawing, while maintaining it at the temperature optimal for preservation. Fresh conjugate must be prepared for each day's samples by dilution of the 50 % glycerol concentrate in sterile phosphate buffered saline (pH 7.2 – 7.4); unused conjugate should be discarded. However, if it is necessary to use conjugate remaining from a previous day it should be centrifuged above 5000g for 10-20 minutes before use to remove precipitates.

3. The routine use of positive and negative controls is essential. These may be prepared as explained below (page 13).
4. It is also important that the fluorescence microscope is functioning correctly and is properly aligned. A general way to do so is detailed in page 15.

The FAT can be conducted on formalin-fixed brain following a digestion step with proteolytic enzymes. This procedure is not as reliable as the FAT on unfixed brain and also results cannot be validated by

biological tests. For this reason it is not recommended as a routine procedure and negative results must be interpreted with caution. Brain tissue is cut into small pieces and ground in a pestle and mortar. After washing three times in PBS the brain homogenate is then mixed with 5 times the volume of 0.25% trypsin and incubated for 30 min at 37°C, followed by a similar incubation in 0.5 % pepsin solution. Smears are made of the homogenate and they are fixed and stained as described for the FAT.

3 HISTOLOGY.

In the past Mann's stain, Seller's stain and various other histological stains have been used to detect Negri bodies, the characteristic acidophilic intracytoplasmic inclusion bodies present in infected neurons. However, the reliability of these classical histological methods is strongly correlated to the non-autolysed state of the specimen. Because of their replacement with more reliable and easier tests that remain within affordable limits, these techniques are no longer recommended for the diagnosis of rabies.

4 MOUSE INOCULATION TEST.

Biological tests involve the inoculation of tissue suspensions into living systems, which amplifies virus present in the tissue. Biological tests can be done in live animals (*in vivo*) or in cell culture systems (*in vitro*).

The mouse is the most frequently used animal for the diagnosis of rabies because it is convenient, relatively cheap and sensitive to rabies viruses. For diagnostic purposes mice are normally inoculated into the brain, as this gives a highly reliable and relatively rapid test time. However, the MIT is unacceptably slow for the purposes of human exposure management and therefore cannot be considered a first-line diagnostic test for rabies. This test is detailed in page 17 and a proposed control form is page 22.

An alternative to the mouse inoculation test is the use of neuroblastoma cell culture test. This test is described in the appendixes (page 19).

IMMUNOHISTOCHEMICAL TEST.

This is not described in detail here. Diagnosticians who are interested in establishing the technique should consult the appropriate textbooks and procedures. The technique can be carried out on any tissue type according to routine procedures. The primary antibody must be selected carefully: it must recognise all the known local antigenic types and must be tested under immunohistochemical test conditions.

5 RESULT OF THE TESTS.

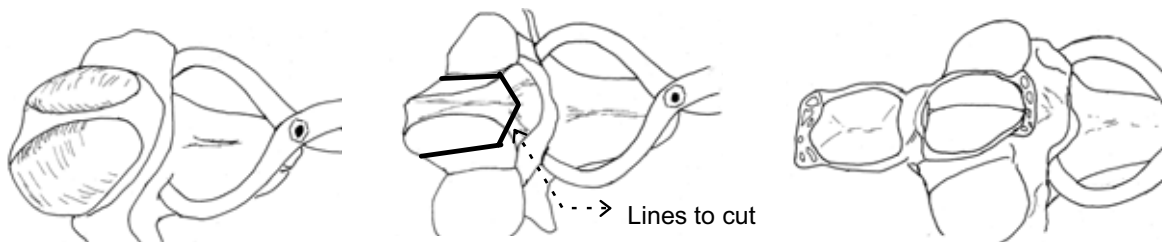
It is recommended that more than one test should be carried out on each specimen, particularly in the case of those involving human exposure. In most instances the results on all the tests done would agree with each other. In the case the results of the tests do not agree, a final decision must be taken by using the conflicting test results and the experience of the responsible of diagnosis. Where a diagnosis is required urgently, it is important to qualify initial results pending further tests.

This conflict does not arise if a single test is used, but this situation lacks a means of quality control. If more than one test is used, a positive result is given as soon as a reliable positive answer (e.g. a validated FAT result) is obtained. A negative answer to a test is sent when all tests are completed and when no positive answer has been obtained. In many instances it is useful to repeat the FAT in case of any doubt on an initial test.

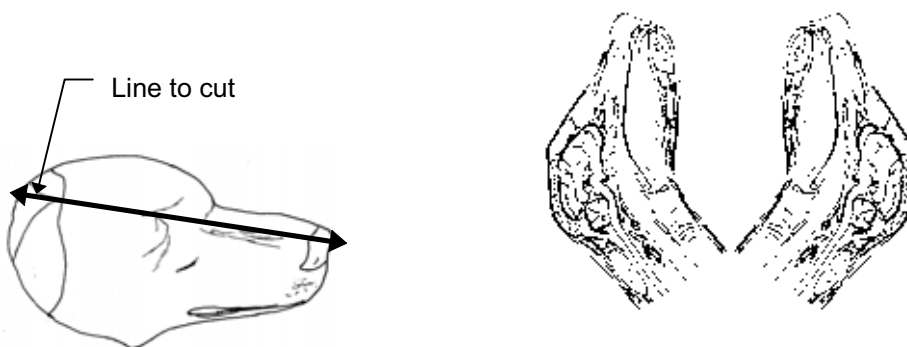
Regular bulletins should be prepared and exchanged with neighbouring countries. General data may be collected on the RABNET site (<http://oms.b3e.jussieu.fr/rabnet/>).

BRAIN REMOVAL AND SALIVARY GLANDS SAMPLING.

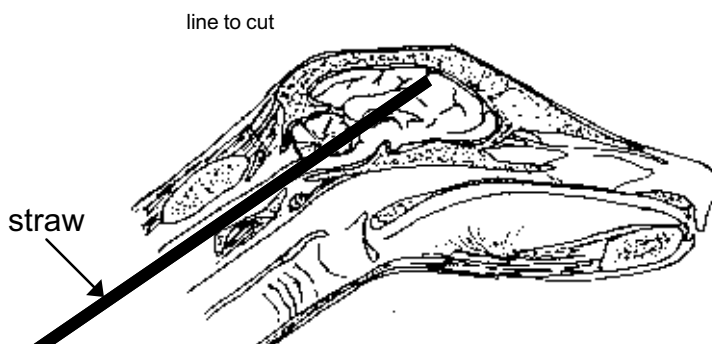
Method 1 : opening the skull.



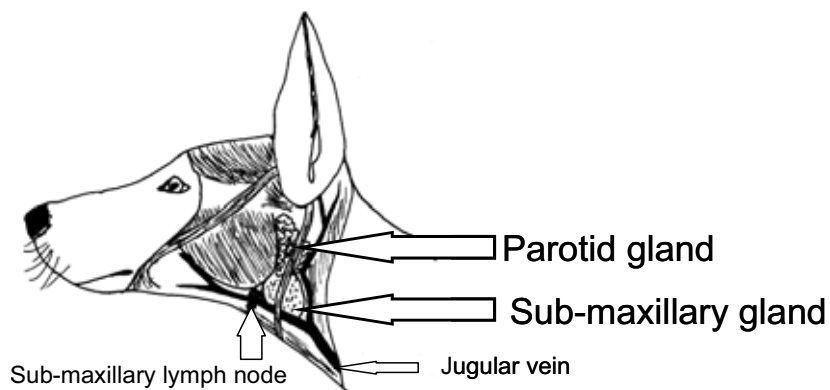
Method 2 : longitudinal section of the head.



Sampling without opening skull.



Salivary gland removal.



BRAIN SAMPLING FOR RABIES DIAGNOSIS.

1- Tools required.

One set of instruments per specimen.

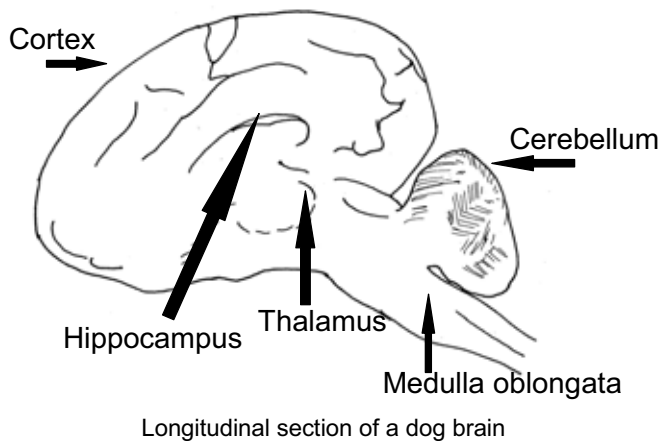
Dissection of brain :

- forceps and scalpel
- wood tongue depressor

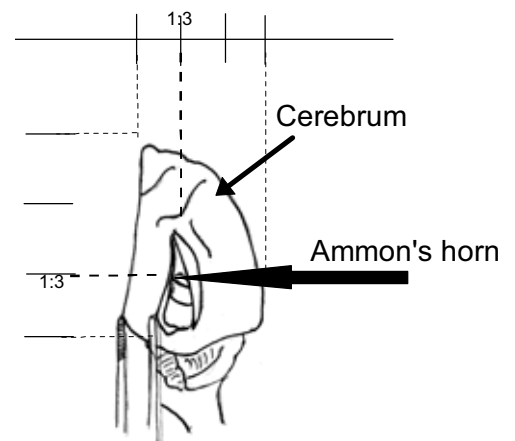
Collecting vials previously labelled with the registration number of the specimens :

- 10 ml tubes for mouse inoculation test, cell culture test.
- labelled microscope slides for FAT.
- 5 ml plastic tube to keep a part of brain frozen as a back-up sample.

2- Areas of the brain to be sampled.



3- Removal of Ammon's horn (hippocampus)



4- Choice of specimens.

The area of choice must include the brainstem. Traditionally, hippocampus and medulla oblongata are also traditionally recommended for diagnosis.

5- Back-up sample.

A piece of brain is kept frozen for 3 months if possible. This brain material is used in case the diagnostic procedure needs to be repeated.

FLUORESCENT ANTIBODY TEST.

1- Glycerin preserved specimen.

The specimen is treated as a fresh one after removal of excess glycerol.

2- Slide preparation.

1. Clean and degrease the slides using the solution described in the appendix.
2. Demarcate with a grease pencil two circles of approximately 1 cm in diameter on a labelled slide.

4- Acetone fixation.

1. Air dry the smear or the cell layer thoroughly.
2. Use high-grade acetone stored in containers dedicated to this use.
3. If the specimen is on a glass support use pure acetone; if it is on a plastic support use 80% acetone solution in distilled water.
4. Fix for 30min at -20°C.

If the smears will not be examined on the same day it is better to freeze them unstained.

6- Reading.

Controls and specimen slides are examined using a x8 or x10 eyepiece and a x20 to x25 objective at a total magnification of 250X. Fluorescence intensity is improved with a lower magnification eyepiece and a higher magnification objective.

Generally every slide should be examined independently by 2 experienced people.

3- Specimen preparation.

- Impression: a fresh section of hippocampus and medulla oblongata or any other available brain material is made by lightly pressing the slide on the brain pieces. It is dried on a piece of absorbent paper.

Positive and negative controls are included in every staining session; they are prepared and fixed regularly. Alternatively, control smears can be prepared at each staining session from stored brain material.

5- Staining.

1. Remove the slide from acetone, air dry it and allow it to reach room temperature.
2. Apply the conjugate at its working dilution onto the smears within each of the circles.
3. Place in a humid chamber and incubate 30min at 37°C.
4. Rinse in PBS at pH 7.2 to 7.4.
5. Drain slides.
6. Cover the smears with mounting medium.
7. Arrange the slides on plates for reading.

7- Result.

Positive controls must be positive and negative control sample must be negative for tests results to be acceptable.

The preparation of necessary solutions is detailed in appendices (page 18).

PREPARING THE REFERENCE CONTROLS FOR INOCULATION TESTS AND FAT.

1- Inoculation.

A group of 3 weeks old OF1 mice is inoculated intracerebrally with CVS or a street isolate. When the mice are in the terminal phase of the disease, they are euthanased and brains are harvested.

Note: when a control titration of CVS is made for other purposes (e.g. serology, vaccine control) the brains of these mice may be collected and used as reference controls.

Once collected, brains are paired in a 5 ml freeze dryer vial and frozen.

3- Rehydration of brains.

0.5 ml of sterile deionised water is added per vial of 2 mouse brains.

A vial is rehydrated every 2 weeks to prepare controls for FAT and cell culture tests.

5- Inoculation control.

The remaining brain material is homogenised at a 50 % (vol/vol) rate in grinding medium. The suspension is then diluted 1:2 in double distilled glycerol and kept in a -20°C freezer. In such conditions, the virus that is in the brain of a mouse inoculated with CVS strain remains infective for cells at least 4 months.

2- Freeze drying.

When all the brains have been harvested and frozen, freeze-drying is performed on the following day. At the end of the cycle, vials are closed under vacuum.

Each vial is then identified with the batch reference and the date.

4- Preparation of smears.

Fifteen to twenty control slides for FAT are prepared with the 2 brains contained in a vial.

The slides are then fixed as described in FAT section. They are kept at +4°C or frozen and will be used during the 2 next weeks. Old slides are discarded when the new slides have been found suitable as controls.

Note.

The same protocol may be used to prepare:

- negative control with mouse brains
- positive controls with pieces of positive brains of animals naturally infected with street strains of rabies.

When the incidence of rabies is high, positive controls should also be prepared from diagnostic samples, provided that they contain large amounts of antigen.

PREPARATION AND STORAGE OF CONJUGATE.

Storage of concentrate conjugate.

Read the manufacturer's instructions carefully regarding storage, reconstitution, dilution and use of the conjugate. Generally, freeze-dried conjugates must be reconstituted in sterile distilled water. Thereafter, dilutions of conjugate must be made in sterile phosphate buffered saline (pH 7.2 – 7.4).

It is recommended that concentrate dilutions of conjugate be mixed with an equal volume of double distilled glycerol (for example to give dilutions of 1/20 or 1/40) and stored at -20°C. As the glycerol prevents freezing, the conjugate can be repeatedly retrieved from -20°C storage without the adverse effects of freezing and thawing, while maintaining it at the temperature optimal for preservation. Fresh conjugate must be prepared for each day's samples by dilution of the 50 % glycerol concentrate in sterile phosphate buffered saline (pH 7.2 – 7.4); unused conjugate should be discarded. If it is necessary to use conjugate remaining from a previous day it should be centrifuged at high speed (above 5000g) for 10-20 minutes before use to remove precipitates.

Determination of the working dilution of a fluorescent conjugate.

1- Principle.

The optimal working dilution must be determined before use of every new batch of conjugate by titration on representative and unusual antigenic types of rabies virus that may be found during routine diagnostic, various dilutions are tested.

This working dilution may differ between laboratories, depending on the microscope optical system used.

2- Dilution of the conjugate.

Serial 1:2 dilutions of the conjugate are prepared in P.B.S. (pH 7.2 to 7.4). these dilutions can, for example, be from 1:25 up to 1:800 or higher.

3- Realisation of the test.

Smears are prepared using appropriate field specimen: one smear is prepared for each dilution of conjugate. Fixed slides are stained according to the routine staining procedures.

While reading, both non-specific background fluorescence and quality of specific fluorescence are estimated. The recommended working dilution is the highest dilution that gives bright specific fluorescence with the lowest background

FOCUSING THE LIGHT SYSTEM IN A MICROSCOPE.

FITC is a "blue light" fluorochrome. It is excited in blue light at 490nm and emits a greenish light at 510nm. A halogen bulb may be used for FITC fluorescence but a mercury vapour lamp gives a more powerful light and may also be used for u.v. fluorescence.

WARNING:

Ultraviolet light is hazardous to the eyes. UV light sources must be handled with caution. Xenon and mercury bulbs emit u.v. light which is hazardous for non protected eyes, the focusing of such bulbs must always be done through visible light fluorescence filters (blue, red or green fluorescence). Halogen, mercury or xenon lamps must not be touched with the bare hands.

These bulbs are very hot and therefore must be allowed to cool before changing. During replacement, the new bulb must never be touched by hand without thorough cleaning. Halogen bulbs should be thoroughly plugged in their socket. Mercury bulbs have to be inserted in up and down sockets, the maintaining screws should be tightly hand blocked to avoid electric arcs that reduce the life time of the bulb.

Whatever the light source of a microscope (halogen, mercury vapour or xenon), the following parts are present :

The source (B) fixed in a movable socket in the lamp housing which may also be equipped with a mirror (M).

A group of adjustable lenses (L) to create a parallel beam of light.

The field diaphragm (FD) that regulates the diameter of the observed illuminated field.

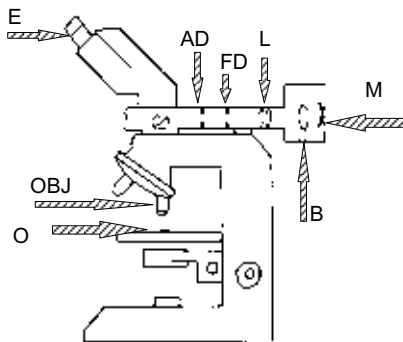
The aperture diaphragm (AD) that controls the intensity of the light and the quality of the observed image. It is included in the condenser (C) in trans-illumination light systems.

The object (O).

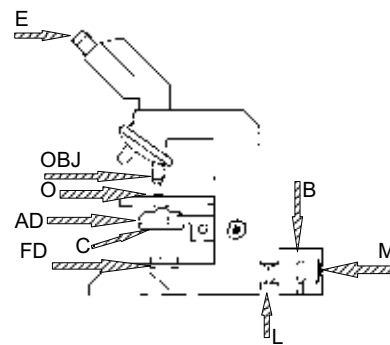
The objective (OBJ) is also used as a condenser in reflected light systems.

The eyepiece (E).

Epi-illumination system (reflected light).



Trans-illumination system (transmitted light).



LAMP HOUSING WITHOUT MIRROR OR WITH UNADJUSTABLE MIRROR

1- Centre the bulb in the housing.

1-Remove an objective from the turret. With the lamp on, put a paper on the stage in light path. Focus the arc or the filament with L and open the FD.



.WITH BLUE LIGHT
FLUORESCENCE FILTER



2-Fine adjustment: observing through the eyepiece, process as in step 1 with the objective that will be used and a slide in the light path.

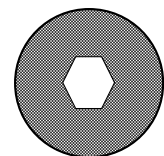
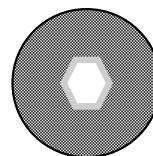
3-Put out of focus the arc or filament with L so that the field is uniformly illuminated.

2- Focus the condenser (if present).

1-Put a slide on the stage and focus the image.
2-Close the field diaphragm, adjust the height of the condenser to give a focused image of the field diaphragm :

out of focus

in focus



If the limit of the diaphragm is coloured, it is better to focus the condenser to give reddish rather than blue or violet edge.

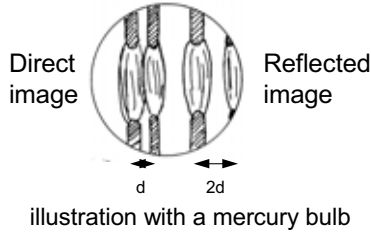
LAMP HOUSING WITH ADJUSTABLE MIRROR

1- Identification of the direct image.

1-Remove an objective from the turret. With the lamp on, put a paper on the stage in light path. After focusing, you should obtain:



2-After a movement of the lamp, the reflected image moves twice more than the direct image of the lamp.



3- Centre the reflected image of the bulb.

Classical observation.

Focus and place the reflected image by moving the mirror. Before unfocusing, it should look like this:



Microphotography: The direct image and the reflected one are overlaid in the centre of the field. It produces brighter light but it shortens strongly the lifetime of the bulb.



Put out of focus the arc or filament with L so that the illumination of field is uniform.

2- Centre the direct image of the bulb.

The procedure is identical to the one shown for microscopes with lamp housing without mirror, focusing is obtained by lamp movement.

Focus the arc or the filament with L while viewing the direct image on the stage (with objective removed).

Fine-focus through the eyepiece with the objective that will be used normally and a slide on the stage in the light path.

4- Focusing of the condenser (if any).

The procedure is identical to the one shown for microscopes with lamp housing without mirror.

1-Put a slide on the stage and focus the image.

2-Close the field diaphragm, adjust the height of the condenser to have a focused image of the field diaphragm :



END FOR BOTH TYPES OF MICROSCOPES

1- Adjust the field diaphragm (FD).

With the objective that will be used and a slide in the light path.

- 1-focus the image of the object
- 2-close FD
- 2-centre the light spot in the middle of the field
- 3-open FD just enough to illuminate the observed field.

This adjustment of the size of the illuminated area eliminates any extra light that could be diffracted inside the object and so reduce the quality of the observed image.

It should be checked every time the objective is changed.

2- Adjust the aperture diaphragm.

This diaphragm controls first the focus range. Neutral grey filters do better control the intensity of light than AD.

Some general rules are the following:

- With a dark field condenser: AD full opened
- With a phase contrast condenser: close AD 1/3 of diameter
- With a clear field condenser: close AD 1/3 of diameter.

MOUSE INOCULATION TEST.

1- Choice of specimens.

Fresh specimen should be used.

Treat glycerol preserved specimens as a fresh ones after a washing step in PBS to remove the glycerol preservative.

Formalin preserved specimen: virus is inactivated, inoculation tests cannot be used.

2- . Preparation of the homogenate.

Make a 1/10 dilution of brain material in cell culture medium or PBS with antibiotics.

3- Intracranial inoculation.

Volume:

Suckling mice are the most sensitive. Inoculate each mouse with 20µl.

Mice up to 3 weeks old (>21g) may be used. Inoculate each mouse with 30µl.

Inoculation site.



4- Result.

With a fox strain of rabies, deaths occur between the 9th and the 14th day after inoculation, but it may occur any time between the 4th and the 28th day.

Mice are examined daily for 28 days. Any found dead within four days are discarded as it is assumed they will have died of trauma or bacterial/toxic causes induced by the inoculation. The brains of those dying after four days post-inoculation are tested for rabies by FAT. If at the end of the 28-day observation period, all mice are still alive, they are euthanased and discarded.

It is possible to reduce the diagnostic lag period by using suckling mice, which can be sacrificed while healthy. Antigen can sometimes be demonstrated in the brains of healthy mice during the incubation period. From day 5 post-inoculation, it is possible to sacrifice and examine by FAT the brain of one mouse every 2 days.

APPENDICES

Mounting medium for FAT: buffered glycerin solution pH 8.5.

1- Concentrated solutions.

Solution A, pH≈4.2		
	KH ₂ PO ₄	89.82 g
	NaCl	8.5g
	Deionised water	1 litre

Solution B, pH≈8.8		
	K ₂ HPO ₄	114.96 g
	NaCl	8.5g
	Deionised water	1 litre

2- Buffer preparation

solution A	1ml
solution B	100ml

3- Mounting medium

bidistilled glycerol	9 vol
buffer	1 vol

Adjust pH to 8.5 with solution A or solution B.

The mounting solution should be kept refrigerated and a new batch is prepared 3 months.

Lens cleaning solution.

Isopropyl alcohol	50 ml
distilled or deionized water	150 ml
teepol	3 drops

50% glycerol-saline preservative solution.

Double-distilled glycerol	500 ml
Phosphate buffered saline (PBS) pH 7.4	500 ml
Merthiolate (not necessary)	0.1 g

Cleaning microscope slides.

1. Rinse in tap water
2. Overnight bath in 0.1N HCl
3. Tap water 20 min
4. Ethanol 20 min

CELL CULTURE TEST.

1- Choice of specimens.

Fresh specimen

Treat as a fresh specimen after a washing step in PBS to remove the glycerol preservative.

Formalin preserved specimen.

Virus is inactivated, inoculation tests cannot be used.

3- Cell production.

- Neuroblastoma cells (ATCC CCL131).

- Prepare a cell suspension with 1.5×10^5 cells/ml and distribute 400µl per chamber of a lab-tek slide. It is possible to realise the test with 96-well plates.

-Allow cells to settle for 30 to 60min at 37°C.

Cell culture medium is DMEM + antibiotics + 10% foetal calf serum.

6- Incubation.

Day 1 : slide is incubated 24 hours at 36 - 37°C with 5% CO₂.

Day 2, cell culture medium is changed.

Day 3, cell culture medium is discarded and cell layer is fixed and stained.

9- Validation of the test.

Results are acceptable if :

1. Controls give the expected result.
2. Cell have not been unduly damaged by specimen toxicity, etc...

2- Homogenate.

50% suspension in diluent (DMEM + 50% heat inactivated new born calf serum) prepared with mortar and pestle.

Freeze - thaw cycle to increase breakdown of cell walls.

Centrifugation :

3000g, 30min at +4°C.

4- Inoculation of specimens.

- 50µl of supernatant is inoculated per chamber.

- 1 chamber corresponds to 1 specimen

5- Controls.

Each test is controlled by the addition of known infected and non infected brain suspensions.

7- Fixation.

Thoroughly air dry before fixation.

Fix in cold acetone (-20°C) for 30min.

8- Staining.

See FAT section. (page 12)

10- Inactivation of cell culture medium.

- ◇ heat treatment (autoclave)
- ◇ chemical decontamination :
 - ◇ sodium hydroxyde 8/1000
 - ◇ virkon 1 to 3 %

MOUSE INOCULATION WORKSHEET.

Sample n°	Date of inoculation :	Strain :	Batch :
volume inoculated : µl	dilution :	route of inoculation :	
number of mice :	inoculated by :		

D(i)	FAT control P / N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Date																													
1																													
2																													
3																													
4																													
5																													

codes	
H	Healthy
C	Show clinical signs
D	Dead
E	Euthanasied