EXPERIMENTAL DIAGNOSIS OF RABIES.

ADAPTATIONS TO FIELD AND TROPICAL CONDITIONS

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Introduction

Clinical signs of rabies are generally not characteristic and may vary greatly from one animal to another, depending on the areas of the brain in which the virus is replicating and on individual responses to infection. As clinical observation may only lead to a suspicion of rabies, the only way to confirm the disease is to look for the presence of rabies virus or its components in samples collected from the suspected animal.

Several laboratory techniques may be used to detect rabies virus infection. These techniques include either histological or cytological methods to detect viral replication, or viral antigen, or the virulence and pathogenicity of non-inactivated virus, or viral nucleic acid.

This paper deals with sampling and shipment techniques and methods used for the routine diagnosis of rabies. Emphasis will be placed on techniques particularly applicable to hot or tropical countries. The subjects covered will be:

Collection of samples: occipital foramen and retro-orbital routes

Shipment of samples

Histological Techniques

Immunocytochemical Techniques

Immunoenzymatic Techniques

Virus isolation Techniques: souse inoculation and cell culture

Diagnostic Quality Control

Experimental diagnosis on preserved samples and

Choice of preservation techniques

Where relevant, attention will be given to the equipment required, technical training needs, test sensitivities, equipment costs and costs of individual tests.
Collection of samples

Brain sampling by opening the skull is hazardous when practised outside of laboratory or when technicians are not regularly trained. However, in field conditions, for example for epidemiological studies, one of two techniques may be used to collect brain samples without opening the skull:

A. Occipital foramen route brain sampling: (see Fig. 1. page 79).

Brain samples may be taken through the occipital foramen (Barrat and Blancou, 1988) by using a drinking straw (5mm in diameter) or a 2ml disposable plastic pipette (Bourhy and Sureau, 1990). The steps of this procedure are successively:

- cut the skin and neck muscles over the joint between the occipital bone (condylus occipitalis) and atlas vertebrae
- bend the head forward to give access to the occipital foramen
- pass a straw through the foramen and screw it into the brain, heading in the direction of an eye. This way the straw pierces the rachidian bulb, the base of the cerebellum, the Ammon's horn and the cortex.
- pinch straw between fingers and gently withdraw it from the head.

B. Retro-orbital route brain sampling: (Montano Hirose et al., 1991):

- 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 straw or a 2ml disposable plastic pipette, screwing it in, in the direction of the occipital foramen
- pinch straw between fingers and gently withdraw it from the head

The cerebral tissues sampled here are the same as those sampled via the occipital foramen, but they are taken in reverse order.

Shipment of samples

Ideally, the head or the entire animal should be kept cool during rapid transport to the laboratory. When these conditions cannot be followed, different preservation techniques may be used; the choice is governed by the laboratory techniques that will be used for diagnosis.

Formalin solution inactivates rabies virus. Virus isolation tests cannot then be used, but diagnosis can still be made by a modified fluorescent antibody test and by histology.

Glycerin solution does not inactivate rabies virus. It is a preservative that temporarily inhibits the growth of contaminants. As the virus is not inactivated, all laboratory techniques (including virus isolation tests) may be used on samples in glycerin.
Laboratory techniques used for diagnosis

The techniques cited here are detailed in the OIE "Recommended Techniques and Requirements for Biological Products" and in the WHO monograph (Kaplan and Koprowski, 1974). Therefore only improvements or adaptations to special conditions are detailed.

Histological Techniques

In 1903, Negri described eosinophilic inclusions in the cytoplasm of brain cells of rabid animals. Although these inclusions, which correspond to aggregates of viral proteins, are specific for rabies virus infection, the staining techniques used are not specific since they merely detect affinity for acidophilic stains.

Staining techniques

The two most frequently used are:

Sellers' technique, used on smears. Results can be obtained within one hour of sample receipt. With autolysed samples, even at the first stage of autolysis, interpretation of the results may be difficult.

Mann's technique involves tissue embedding in paraffin and section cutting. Results take longer but can be obtained within 4 days of sample receipt; interpretation of results is easier.

Equipment required

Both techniques require an incandescent-light microscope. In addition, the classical histology equipment for embedding and cutting is needed for Mann's technique.

Training of Technicians

Regular training is necessary to achieve good sections of embedded samples and their staining.

Test Sensitivity

The sensitivity of histology is closely related to the preservation of the sample: the result is more reliable when samples are fresh than when autolysis has begun. Sometimes non-specific inclusions may be observed and these are difficult to distinguish from Negri bodies. Depending on the freshness of the sample, Mann's technique detects 60 - 95 percent of positive samples.

Cost of histology techniques:

<table>
<thead>
<tr>
<th>Equipment for Mann's staining:</th>
<th>8800 us $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microtome</td>
<td>4000 US $</td>
</tr>
<tr>
<td>Light microscope</td>
<td>12000 US $</td>
</tr>
<tr>
<td>Embedding automatic system</td>
<td>1100 US $</td>
</tr>
<tr>
<td>65°C incubator</td>
<td>1500 US $</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>1300 US $</td>
</tr>
</tbody>
</table>
Immunocytochemical Techniques.
These are used to detect viral antigen whether or not the virus it inactivated.

Direct immunofluorescence:

Immunofluorescence was introduced to rabies diagnosis by Goldwasser and Kissling in 1958. Fluorescent conjugates may be prepared from either polyclonal or monoclonal antibodies. Polyclonal conjugates are prepared from antisera to native virus or purified nucleocapsids, which form the most abundant antigen in infected cells. Monoclonal conjugates, usually prepared against the nucleocapsids (Mab-Ns) may contain a single antibody but a mixture of Mab-Ns is usually employed.

Equipment required

Specimen examination requires blue-light fluorescence as fluorescein is excited by 490nm wavelength light and emits a greenish light of wavelength 510nm. This light may be obtained either with a halogen 12V 100W lamp or mercury vapour lamp. Both lamps have a life-time of about 200 hours, but the mercury vapour lamp is 5 to 15 times more expensive than the halogen lamp.

Training of technicians

Regular training in specimen examination is of particular importance. This is even more important when the sample is autolysed.

Test Sensitivity

When used by regularly trained persons, the direct immunofluorescence test may detect 97 - 99% of positive samples. Results may be obtained within three hours of sample receipt.

Cost of fluorescence techniques

Equipment costs:

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light microscope</td>
<td>4000 US $</td>
</tr>
<tr>
<td>Fluorescence equipment</td>
<td>5500 US $</td>
</tr>
<tr>
<td>370C incubator</td>
<td>1100 US $</td>
</tr>
<tr>
<td>Refrigerator and freezer</td>
<td>1200 US $</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>700 US $</td>
</tr>
<tr>
<td>Storage of slides</td>
<td>1300 US $</td>
</tr>
</tbody>
</table>

Test costs:

The cost of one test is 1 US $. Several tests may be used for a single diagnosis (confirmation of a negative result).
**Conclusion**

The fluorescent antibody test is the preferred technique for rabies diagnosis. It alone may provide a result and it may also be used to reveal the replication of rabies virus after cell culture or mouse inoculation. It may also be used on brain samples preserved in glycerin solution after washing in PBS, or, after enzyme treatment, on samples preserved in formalin solution.

**Immunoperoxidase on smears**

Immunoperoxidase may be used instead of immunofluorescence. Both techniques have the same sensitivity (Genovese and Andral, 1978), but as peroxidase tests take longer to perform and are more expensive, they are no longer used in routine laboratory diagnosis.

**Immunoenzymatic Techniques**

An ELISA technique has been developed (Perrin et al., 1986). This Rapid Rabies Enzyme Immuno-Diagnosis (RREID) detects the presence of rabies nucleocapsid in brain samples. In kit form, the RREID is commercially available and is distributed by Diagnostic Pasteur (ref. no. 72201). Plates may be read by eye, or more easily by the utilisation of a spectrophotometer. Results can be achieved within 4 hours of specimen receipt. The correlation between fluorescent antibody test and RREID is 96 - 99%.

**Training of technicians**

If the technician does not know any other ELISA technique, a few days training will be required. The interpretation of the test does not need any special training. Since positive and negative controls give clear-cut results, the threshold is easy to determine.

**Cost of Immunoperoxidase techniques:**

<table>
<thead>
<tr>
<th>Cost of Equipment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrophotometer</td>
<td>$7300</td>
</tr>
<tr>
<td>Titerplate washer</td>
<td>$3700</td>
</tr>
<tr>
<td>370C incubator</td>
<td>$1100</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>$550</td>
</tr>
<tr>
<td>Multichannel pipette</td>
<td>$750</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>$750</td>
</tr>
</tbody>
</table>

**Test costs**

The cost of one test is 4.5 US $ if it is made in duplicate. It is possible to use one single well per sample.

**Conclusion**

This simple and rapid technique is ideal for epidemiological studies. The immunocapture phenomenon allows easy and reliable diagnosis, even on autolysed samples.
The RREID has been improved by the creation of two new tests:

- RREID-biot which includes an amplification step using biotin-avidin complex
- RREID-lyssa which is also amplified and directed against PV, Mokola and EBL strains

**Virus Isolation Techniques**

These techniques detect viable rabies virus. They may be used in vivo (by mouse inoculation) or in vitro (on neuroblastoma cells).

**Virus isolation in mice:**

5 to 10 adult OF1 germ-free mice or 10x 1-2 day old new-born mice are inoculated intracerebrally. The mice are inspected every day for 28 days and the brains of any mice which die are checked for rabies by FAT. Because of the variability of rabies virus incubation periods, results may not be quickly obtained. For example, the incubation period for street fox rabies virus is 9 - 14 days.

The waiting period may be shortened by the sacrifice of one mouse every 3 days from day 5 onwards, followed by the examination of its brain by FAT. The correlation between FAT and mouse inoculation tests is good, both diagnostic methods detect rabies in 92 - 99 percent of samples from rabid animals.

**Equipment required**

A special room must be reserved for animals and an immunofluorescence laboratory is required to check for rabies in the brains of animals which die.

**Training of technicians**

Technicians must be used to taking care of animals and be able to perform immunofluorescence tests.

**Cost of mouse inoculation:**

**Cost of Equipment:**

The cost of the mouse room depends upon its degree of sophistication and the price of manpower. These costs begin at 450 - 650 US $ and can reach up to 44,000 US $ - 54,500 US $ for 2,000 mice in insulated boxes. To be added are the costs of an immunofluorescence laboratory.

**Test costs:**

The cost of five adult mice, their food and of the products needed for diagnosis is 14 US $ per specimen. If one uses new-born mice, the price is then 34 US

**Conclusion**

The mouse inoculation test does not give rapid results, but it has the advantage of being a simple and reliable method for the isolation of rabies strains for further typing.
**Virus isolation in cell cultures**

A suspension of the specimen is used to inoculate a cell monolayer. Most often, the replication of rabies virus in neuroblastoma cells does not induce any cytopathic effect, the presence of virus is revealed by a fluorescent-antibody test.

**Equipment required**

The cell line used here is a neuroblastoma cell line of murine origin obtained from the ATCC (catalogue no. CCL 131). For rabies diagnosis by cell culture a separate culture room with immunofluorescence facilities is required.

**Training of technicians**

The person who makes rabies diagnosis by virus isolation in cell cultures must be used to the manipulation of cells and the performance of fluorescent antibody tests.

**Test Sensitivity**

Tests performed in Malzéville show that after incubation for 48 hours, N2a cell diagnosis detects 96 - 97% of positive samples (Barrat et al., 1986). One cycle of virus replication in these cells takes at least 18 hours.

**Cost of cell culture virus isolation techniques:**

- Inverted microscope 5500 US $
- CO2 incubator 9000 US $
- Refrigerator and freezer 1300 US $
- Vertical Laminar flow hood 5500 US $
- Refrigerated centrifuge 12000 US $
- Liquid nitrogen cylinder 1400 US $
- Miscellaneous 1700 US $
- Fluorescence see above

**Test cost:**

One diagnosis costs 2.2 US $ per sample.

**Conclusion**

Diagnosis by cell culture techniques is at least as sensitive as the mouse inoculation test. Results are obtained more quickly (usually 3 days) and the tests are cheaper to perform.
Laboratory diagnosis quality control

In a study by the CDC, Atlanta, of the quality of experimental diagnoses made in different US laboratories, 129 laboratories were sent 10 test slides. 41 of the laboratories gave erroneous results. These laboratories were sent a further group of 10 slides from other cases and 6 laboratories again made interpretation errors.

These results were correlated with the number of diagnoses performed in the different laboratories. The most important factor was related to the number of diagnoses made in a laboratory - the more samples that were tested the fewer the test errors.

The five main sources of error were:

1. technical procedure not followed
2. no quality control, no control slides
3. incomplete or partial technical procedure
4. inadequate microscopes
5. irregularly trained technicians

This study emphasises the great importance of regular training in and practice of FAT. It also highlights the need for strict quality control. In fact, these two points indicate that diagnosis should be under a centralised structure.

Fig. 1. Occipital foramen route brain sampling.
**Experimental diagnosis on preserved specimens**

As stated above, if it is not possible in field conditions to keep samples cool and send them rapidly to the diagnostic laboratory, they may be preserved by using either formalin or glycerin preservation.

**Treatment of formalin preserved specimens:**

Histology (Mann's stain for example) may be performed directly on a portion of the already fixed sample.

Alternatively, FAT may be indirectly performed on formalin preserved samples. Treatment with proteolytic enzymes is necessary (Umoh and Blenden, 1981; Barnard and Vosges, 1982); pre-treatment with a trypsin digestion step is required (Barrat, 1986).

The samples to be treated consist of small pieces of brain (3 mm cubes) stored in a 10% formalin solution. The following procedure is used:

1. grind one piece in 5ml of Ca-free and Mg-free PBS pH 7.5
2. Centrifuge the tube and discard the supernatant fluid
3. resuspend the deposit in 5 ml of a 0.25% trypsin solution
4. keep the tube refrigerated (+4°C) overnight
5. centrifuge the tube and discard the supernatant fluid
6. wash the pellet twice in Ca-free and Mg-free PBS
7. make smears from the pellet and perform FAT

In our laboratory we have tested the capacity of this technique to recover positive samples after up to 20 days at ambient temperature (i.e. 20 – 25°C).

On day 0, naturally infected rabid foxes were selected by FAT. The FAT positivity of fresh samples was estimated for the quantity of fluorescence on a 5 point scale. Different Ammons' horn samples, as for fresh FAT, were fixed in formalin.

On days 2, 5, 8 and 20, a piece of fixed brain was treated according to the procedure described above and the results were recorded according to the defined scale.

**Results:**

<table>
<thead>
<tr>
<th>Days in 10% formalin at 20-25°C</th>
<th>2</th>
<th>5</th>
<th>8</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>with grinding</td>
<td>100%</td>
<td>97%</td>
<td>98%</td>
<td>91%</td>
</tr>
<tr>
<td>N=98</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without grinding</td>
<td>92%</td>
<td>75%</td>
<td>85%</td>
<td>62%</td>
</tr>
<tr>
<td>N=52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 80 -
Fluorescence was more abundant in samples which had been ground. This indicates that grinding allows better contact with trypsin, leading to improved test sensitivity.

**Histological examination**

Histological examinations were performed at days 2, 5, 8 and 20 using Mann's technique on other samples taken from the same foxes.

Tests were performed on 50 samples. Histology recovered 50 positive samples after 2 and 5 days in formalin and 49 after 8 and 20 days.

**Sensitivity of diagnosis using both FAT and histology**

The overall sensitivity of techniques available for formalin preserved samples was determined on 50 naturally infected rabid foxes. The results were as follows:

<table>
<thead>
<tr>
<th>Days in formalin at 20-25°C.</th>
<th>2</th>
<th>5</th>
<th>8</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAT alone</td>
<td>50</td>
<td>so</td>
<td>so</td>
<td>48</td>
</tr>
<tr>
<td>Histology alone</td>
<td>50</td>
<td>50</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>FAT + Histology</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**Conclusion**

The use of both FAT and histology allows identification of 100% (N=50) of positive samples after up to 20 days in formalin solution at ambient temperature.

**Occipital foramen sampling and formalin preservation**

Following trypsin treatment of 107 rabid fox brains fixed in formalin and held at room temperature for 8 days, 98 percent were FAT positive. Of 107 straw samples from the same brains held in formalin at room temperature for the same 8 day period, 102 (95.3 percent) were positive following trypsin treatment. However, only 14 of 24 (58.3 percent) of straw samples from these foxes which were held in formalin at room temperature for 8 days were positive following trypsin treatment of the entire sample instead of only a small 3 mm, cube (or 50 microlitres of the pellet obtained after the first washing step).

**Conclusion**

The use of formalin fixation with the straw technique, followed by trypsin treatment, considerably improves the accuracy of diagnosis from specimens which have not been kept cool and rapidly transported to the laboratory.
Treatment of glycerin preserved samples

Glycerin has an antiseptic action, but it also softens the brain tissues and makes their handling difficult. If animals are sampled with straw or pipette, whatever is the route, the sample must be kept inside the straw so that glycerin softens only those small parts of the sample at the opened ends of the straw.

In order to eliminate as much glycerin solution as possible, the samples are then washed in PBS before any other treatment. After washing, the glycerin preserved sample is processed according to the classically described techniques.

Influence of temperature on diagnosis in glycerin preserved samples

On day 0, 130 foxes received for diagnosis were sampled twice with a straw through the occipital foramen and diagnosis was performed classically on a fresh sample after opening of the skull. The straw samples were preserved in glycerin solution.

When the normal diagnostic test was positive, one "straw" sample was kept for 8 days at ambient temperature (i.e. 20°C) and the other straw was kept in an incubator at 37°C.

FAT and cell culture tests were performed on both fresh and preserved samples. Fluorescence quantity was recorded on a 5 point scale (fluorescence in 0%, 12.5%, 25-50%, 50-75% and 75-100% of fields).

Preservation out of the straw

After 8 days preservation in glycerin solution with sample outside the straw, 18% of the samples were negative. Fluorescence intensity was estimated on 10 samples during this kind of storage and the average fluorescence decrease was 12.5%.

After 8 days at 37°C, FAT was negative for 2 samples and cell culture for 1 of these same 10 samples; but no sample was negative to both FAT and cell culture. Fluorescence decrease was 50% in these conditions.

Preservation within the straw

All 130 samples which were kept within the straw in glycerin for 8 days at 20°C were positive by both FAT and cell culture. The average decrease of fluorescence was measured in 35 samples and was less than 12.5%. At 37°C also both tests were positive for all samples. The average decrease of fluorescence was measured on the same 35 samples and was less than 25%. The same comparison was made between fluorescence in cells when samples were preserved at 20°C and at 37°C. The average decrease of fluorescence was 12.5%.

Conclusion

When samples are to be preserved in glycerin solution, it is better to keep them protected within the straw. After 8 days in glycerin solution with the brain sample inside the straw, the recovery rate was 100% for positive samples determined on fresh specimens. The result was obtained whatever the temperature, 20°C or 37°C.
Choice of a preservation technique

If samples cannot be rapidly sent refrigerated to the laboratory, for example in the context of epidemiological field studies, they must be preserved. Two possibilities exist, each of which allows the use of different laboratory techniques. If samples are preserved in glycerin solution, they must be left inside the sampling straw. If samples are preserved in formalin solution, they must be pushed out of the sampling straw or pipette with a cotton swab or air by use of a rubber bulb.

The two preservative solutions offer the same recovery rate of 100% after 8 days at ambient temperature or even at 37°C. The choice of preservative therefore depends both on available laboratory techniques and on transport conditions.

References


Barrat J. and Blancou J. (1988). Technique simplifiée de prélèvement, de conditionnement et d'expédition de matière cérébrale pour le diagnostic de rage, doc WHO/Rab. Res./88.27


