Overview of OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

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Rabies Diagnostic Standards


**WHO**: Laboratory Techniques in Rabies (1996)

1. Collection of brain samples
2. Shipment of samples
3. Laboratory tests
   3.1 Fluorescent antibody test
   3.2 Immunochemical tests
   3.3 Enzyme-linked immunosorbent assay (ELISA)
   3.4 Cell culture test
   3.5 Mouse inoculation test
   3.6 PCR
   3.7 Fluorescent antibody virus neutralisation (FAVN) test
   3.8 Rapid fluorescent focus inhibition test (RFFIT)

Diagnostic tests are similar.
The test described by WHO is more detailed than OIE standard.
PART 1  GENERAL INFORMATION ( INTRODUCTORY CHAPTERS )

1.1. Collection and shipment of diagnostic specimens

   Collection of brain samples for rabies diagnosis—Ye Feng, Day 1

1.2. Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities

   All staff handling infected or suspect material should receive the preventive immunization.

   The laboratory should check their serum antibody every 6 months. Booster vaccination must be given when the titre falls below 0.5 IU per ml.

   Setting up of basic rabies laboratory –Changchun Tu, Day 5

1.3. Quality management in veterinary testing laboratories

   For laboratories seeking accreditation of testing, the use of ISO/IEC 17025 will be essential.

   Proficiency Test (PT)

   All laboratories should, where possible, participate in external proficiency testing.
PART 1  GENERAL INFORMATION ( INTRODUCTORY CHAPTERS )

1.5. Principles and methods of validation of diagnostic assays for infectious disease

Selection of methods

Direct diagnostic tests for rabies

Fluorescent antibody test (FAT)
-- recommended as ‘gold-standard’ test by both WHO and OIE

Direct, Rapid Immunohistochemical test (dRIT)
-- used as an alternative to FAT in routine rabies diagnosis

Enzyme-linked immunosorbent assay (ELISA)
-- used for large epidemiological surveys

Cell culture inoculation test (RTCIT)
-- used as confirmatory tests and used for virus isolation replace the mouse inoculation test

Reverse transcription-PCR (RT-PCR) & Real time TaqMan RT-PCR (FQ-PCR)
-- should be considered for use as confirmatory tests with standardization and very stringent quality control
PART 1  GENERAL INFORMATION ( INTRODUCTORY CHAPTERS )

1.5. Principles and methods of validation of diagnostic assays for infectious disease

Selection of methods
PART 1 GENERAL INFORMATION (INTRODUCTORY CHAPTERS)

1.3. Quality management in veterinary testing laboratories

Proficiency Test (PT)

Organized by OIE and EU reference laboratory, FANCE compliance with ISO 17043 international standard

PROFICIENCY TEST ON RABIES DIAGNOSIS (detection of *lyssavirus*)

FAT, RTCIT, RT-PCR, qPCR

Frequency: once every two years

At least one discordance: the participant fails the test

RABIES SEROLOGY PROFICIENCY TESTS

FAVN or RFFIT

Frequency: once a year

Registration form will be sent in December and PT will begin in April-May.
PART 1  GENERAL INFORMATION ( INTRODUCTORY CHAPTERS )

1.6. Principles of veterinary vaccine production

1.7. International standards for vaccine banks

PART 2  OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE

2.1 Multiple species

2.1.13 Rabies

2.2 Apinae

2.3 Aves

2.4 Bovine
CHAPTER 2.1.13.

RABIES

A. INTRODUCTION

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent
   a) Collection of brain samples
   b) Shipment of samples
   c) Laboratory tests
   d) Other identification tests

2. Serological tests

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS
A. INTRODUCTION

**Lyssavirus** (14 recognised species, 2 putative species)

- RABV is found worldwide. More than 99% human rabies was caused by RABV.
- Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats.
- All lyssaviruses tested to date cause the same clinical disease as classical rabies.

Phylogenetic reconstruction based on the nucleoprotein gene, including all representative lyssaviruses.
Rabies virus vaccines may not provide adequate cross-protection against all genetically divergent lyssaviruses.

**Lyssavirus** (Fourteen recognised species)

- Rabies lyssavirus (RABV)
- Lagos bat virus (LBV)
- Mokola virus (MOKV)
- Duvenhage virus (DUVV)
- European bat lyssaviruses type-1 (EBLV1)
- European bat lyssaviruses type-2 (EBLV2)
- Australian bat lyssavirus (ABLV)
- Aravan virus (ARAV)
- Khujand virus (KHUV)
- Irkut virus (IRKV)
- West Caucasian bat virus (WCBV)
- Bokeloh bat lyssavirus (BBLV)
- Shimoni bat lyssavirus (SHIBV)
- Ikomalyssavirus (IKOV)

**Two putative lyssavirus species**

- Lleida bat lyssavirus (LLEBV)
- Gannoruwa bat lyssavirus (GBLV)

**Phylogroup 1**
Conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination.

**Phylogroup 2**
Little or no cross-protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed.
Brain is required for a reliable rabies diagnosis
Sensitivity and specificity

Rabies virus is particularly abundant in the **thalamus, pons and medulla**.
1. Collection of Brain Samples

a. Opening the skull

- Complicated operation and difficult to operate in the field especially for some large animals
- Brain tissue is easy to splashing and spillage
- Operate in laboratory facilities
1. Collection of Brain Samples

b. Drinking-straw method

- Cerebral cortex
- Corpus callosum
- Ammon’s horn region
- Cerebellum
- Occipital
- Atlas
- Medulla oblongata

**Recommended by both WHO and OIE.**

- Suitable for epidemiological studies conducted in the field, no access to laboratory facilities
- Convenient operation
- Less contamination
- Prevent brain tissue to splashing and spillage
- Less materials
Drinking-straw method

Materials

• Protective clothing: gown, gloves and mask

• Drinking straws or disposable plastic pipettes

• Scissors and scalpel

• Collection tube
1. Bend the head downwards and cut the cervical muscles

2. Exposure of the occipital foramen
3. Insert the straw into the occipital foramen with a slight twisting movement towards one of the eyes. Before withdrawing the straw, pinch it between the fingers in order to prevent material escaping from the straw. Samples are taken from the medulla oblongata, the base of the cerebellum, the hippocampus and the cerebral cortex.
Drinking-straw method

4. Transferring a specimen

Apparatus (table tops, vice, socket forceps, scissors, forceps and grinder, etc.) must be disinfected and cleaned between specimens.
1. Collection of Brain Samples

Information to be sent with samples

The itemized list

• Specimen description
  – species
  – clinical signs
  – age, sex of the suspect animal
  – date of death & date of collection
  – location (geographical coordinates)
  – date of shipment
• Name and address of the owner or of the person who found the animal
• Vaccination status for domestic animals
• Possible human exposure or animal contacts
2. Preservation of Specimens

Majority of test results reliable on fresh specimens
The specimen should be rapidly cooled and kept cold during shipment, and then submitted to laboratory within 48h.

Not possible to send refrigerated samples:
- 4% Formaldehyde solution
- 50% Glycerol solution

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Positive Results of FAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days</td>
</tr>
<tr>
<td>20°C</td>
<td>100% (98/98)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Positive Results of FAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 days</td>
</tr>
<tr>
<td>20°C</td>
<td>100% (130/130)</td>
</tr>
<tr>
<td>37°C</td>
<td>100% (130/130)</td>
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</table>
2. Preservation of Specimens

Rabies diagnosis of samples in decomposition conditions

<table>
<thead>
<tr>
<th>Time of exposition</th>
<th>7 days</th>
<th>15 days</th>
<th>30 days</th>
<th>120 days</th>
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<tbody>
<tr>
<td>Sample</td>
<td>FAT</td>
<td>MIT</td>
<td>RT-PCR</td>
<td>FAT</td>
</tr>
<tr>
<td>537-03</td>
<td>++</td>
<td>P</td>
<td>P</td>
<td>+</td>
</tr>
<tr>
<td>909-04</td>
<td>+++</td>
<td>P</td>
<td>P</td>
<td>+</td>
</tr>
<tr>
<td>693-05</td>
<td>++</td>
<td>P</td>
<td>P</td>
<td>N</td>
</tr>
</tbody>
</table>

FAT: fluorescence antibody test; positivity degree of FAT: +, ++, ++++, +++++, ++++++; MIT: mouse inoculation test; RT-PCR: reverse transcriptase polymerase chain reaction; N: negative; P: positive

Beltrán et al. J Infect Dev Ctries 2014

✓ FAT and MIT are reliable on fresh specimens.
✓ RT-PCR can be useful for rabies diagnosis and testing samples stored in inadequate conditions.
3. The fluorescent-antibody test (FAT)

- Recommended by both WHO and OIE
- ‘Gold-standard’ test
- Highly sensitive (between 90% and 100%)
- **Reliable results on fresh specimens**
  - Glycerol-preserved specimens need a washing step.
  - Formalin-preserved specimens should be treated with a proteolytic enzyme before FAT. Digested samples are always less reliable than the fresh tissue.
3. The fluorescent-antibody test (FAT)

1. Place slides inside the cabinet and label it. Positive and negative control should be added.

2. Cut a small piece of the brain tissue with a tip. Place this brain tissue onto the tongue blade.
3. The fluorescent-antibody test (FAT)

Make an impression smear directly onto the slide. Slides may need to be re-blotted on the tongue blade to remove excess tissue.

Place all the slides into a coplin jar. Fix in 80% cold acetone at -20°C for 30 min, then wash with PBST for 3 times.
3. The fluorescent-antibody test (FAT)

5. Allow the slides to air dry

6. Circle the impressions by a marker pencil

7. Add 20 µL conjugate to each impression
   (4× anti-rabies fluorescent conjugates diluted with PBS)
3. The fluorescent-antibody test (FAT)

8. Place the slides in a humid box.

9. Incubate the slides at 37°C for 40 minutes, then wash with PBST for 3 times.

10. Observe on a fluorescent microscope. Excited at 490nm and re-emits at 510nm.
Results

Positive

- Bright apple green fluorescent inclusions in nerve cells.

Negative

- Only red brain tissue

Recommend:
- FAT slide should be read by 2 experienced operators.
Results - Positive

FAT is suitable for all the rabid animal diagnosis.

- dog brain
- cow brain
- pig brain
- camel brain
- fox brain
- mouse brain

The bright 'apple' green fluorescent inclusions can be seen clearly in these brain tissues.
Results - false positive

Yellow fluorescent granules rather than apple green inclusions. The FITC-conjugates bond to infectious tissues in a non-specificity way.
Recipe

<table>
<thead>
<tr>
<th>True positive</th>
<th>False positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point-like</td>
<td>Mass-like</td>
</tr>
<tr>
<td>Bright apple green</td>
<td>Yellow or dull green</td>
</tr>
<tr>
<td>Fluorescent will be quenched in a few mins when exposed with fluorescent microscope</td>
<td>No change</td>
</tr>
</tbody>
</table>
Comparative assay of fluorescent antibody test results using various anti-rabies conjugates

How to choose the anti-rabies fluorescent conjugate?

A. Bioveta (Ivanovice na Hane, Czech Republic)
B. Millipore (Livingston, United Kingdom)
C. Fujirebio (Malvern, United States)
D. SIFIN (Berlin, Germany)
E. Bio-rad (Marnes-La-Coquette, France)

Comparative assay of fluorescent antibody test results using various anti-rabies conjugates

No significant difference between RABV strains

<table>
<thead>
<tr>
<th>Lyssavirus</th>
<th>Millipore ($100/mL)</th>
<th>Fujirebio ($200/mL)</th>
<th>SIFIN ($700/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RABV</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>LBV</td>
<td>+ +</td>
<td>+</td>
<td>+ + + +</td>
</tr>
<tr>
<td>MOKV</td>
<td>+ + + +</td>
<td>+</td>
<td>+ + + +</td>
</tr>
<tr>
<td>DUVV</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EBLV-1</td>
<td>+ + +</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>EBLV-2</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>ABLV</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

Fujirebio conjugate is used in our laboratory for FAT.
The dRIT can be used as an alternative to FAT in routine rabies diagnosis as it has similar sensitivity and specificity. The dRIT will be recommended by OIE manual in 2018, used in laboratories that do not have access to a fluorescent microscope.
4. Direct Rapid Immunohistochemistry Test (dRIT)

Make an impression smear

- 10% buffered formalin for 10 min
- 3% hydrogen peroxide ($\text{H}_2\text{O}_2$) for 10 min
- Biotinylated anti-rabies monoclonal antibody for 10 min
- Streptavidin–peroxidase complex for 10 min
- 3-amino-9-ethylcarbazole (AEC) working solution for 10 min
- Gill’s hematoxylin for 2 minutes
- Apply water-soluble mounting medium and coverslip

Observe in light microscope
Positive results were based on the presence of magenta inclusions visible on a blue neuronal background.
Positive results were based on the presence of magenta inclusions visible on a blue neuronal background, while negative results were based on a lack of magenta inclusion bodies on the blue neuronal background.
4. Direct Rapid Immunohistochemistry Test (dRIT)

- **Recipe**
  1. The impression smear of brain sample on glass microscope slide for dRIT should not be too thick, although thick impression smear is acceptable for FAT. Stacked impression of brain tissue was not conductive to the observation of technicians. The blue neuronal background are easy to be recognized in thin brain tissue impression.

  2. The 3-amino-9-ethylcarbazole (AEC) working solution can be stored at 4 °C and should be used out in 3-4 days after preparation, otherwise red inclusions in positive brain tissue samples are not easy to recognized for unwell-trained technicians.

  3. The time of slides incubated with the hematoxylin should be strictly followed. Magenta inclusions will be covered by the background subjected to too long time incubation.
Practice

Four teams (3-4 person each team)

1. Collect brain tissue with the drinking straw
2. Make the impression smears on the slides for FAT and dRIT

- Protective clothing: gown, gloves and mask
- Marker pen & pencil, timer
- Scissors and scalpel
- Drinking straws
- Collection tubes
- Slides
- For brain tissue collection: One healthy dog head/per team
Practice

• Every trainee should make two slide with the impression smears today. One slide will be used for FAT and other slide with thinner impression smear will be used for dRIT. Make two impression smears onto one slide.

• Given to bio-safety, every trainee will get two sets of fixed slides including one positive control slide and one positive sample slide respectively for FAT and dRIT tomorrow morning.
Questions

Working together to make rabies history!