FLOURESCENT ANTIBODY TEST IN DIAGNOSIS OF RABIES

[1]S. RAJU &
[2]M.R. SASEENDRANATH
Department of Veterinary Epidemiology & Preventive Medicine,
College of Veterinary & Animal Sciences,
Mannuthy, Thrissur

Only few diseases cause as much anxiety as does rabies, this applies equally to the exposed people and the health authorities responsible for its prevention and control. The laboratory diagnosis occupies a central role in meeting the threat of rabies because upon its verdict often depend both the decision whether or not to proceed with a long course of treatment on the need to institute elaborate measures for controlling an epizootic in a community.

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another. As no clinical sign or gross post-mortem lesion can be considered to be pathognomonic, the diagnosis of rabies has to rely on laboratory testing. Serological evidence of infection is rarely obtained because of late seroconversion and 100% mortality rate of host species, although such evidence may be used in some epidemiological surveys. The only way to perform a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests. Specimens for rabies diagnosis must be sent to the laboratory rapidly under cold chain. Shipment conditions must be considered to be part of the 'rabies diagnosis chain'.

THE IMPORTANCE OF ROUTINE RABIES TESTS

Rapid and accurate laboratory diagnosis of rabies in humans and other animals are essential for timely administration of post exposure anti rabies therapy. Within a few hours, a diagnostic laboratory can determine whether or not an animal is rabid and inform the responsible medical personnel. The laboratory results may save a patient from unnecessary physical and psychological trauma, and financial burdens, if the animal is not rabid. In addition, laboratory identification of positive rabies cases may aid in defining current epidemiologic patterns of disease and provide appropriate information for the development of rabies control programs.

Several laboratory techniques are used. The methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied, though less effectively, to other organs (e.g. salivary glands). In the brain, rabies virus is particularly abundant in the Brain stem, Ammon's horn, Cerebellum and the Medulla oblongata. To reach these parts of the brain, it is necessary to remove the entire organ after having opened the skull in a necropsy room. Under some conditions (e.g. in the field or when sampling for large epidemiological studies), a simplified method of sampling through the occipital foramen, or through the orbital cavity, can be used

ESSENTIAL CHARACTERISTICS FOR ROUTINE RABIES TEST

The nature of laboratory tests be

1. Standardized,
2. Rapid,
3. Sensitive,
4. Specific,
5. Economical and
6. Reliable

2. IMMUNOCHEMICAL IDENTIFICATION OF RABIES VIRAL ANTIGEN

Direct Fluorescent antibody test (dFA)

The standard test for rabies testing is dFA, which is recommended by both WHO and OIE and has been thoroughly evaluated for more than 40 years. This is recognized as the most rapid and reliable of all the tests available for routine use. Sensitivity and specificity reaches 100%

This test may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis.

[1]Veterinary surgeon, Dist. Veterinary Centre, Kollam
[2]Associate Professor & Head

Fluorescent microscopy makes use of the property of some molecules to absorb light of a particular wavelength and then to re-emit the light at longer wavelengths (fluorescence). The primary (exciting) light is separated from the secondary (emitted) light by filters (coated glass elements) in the microscope.

The dFA is based on the observation that animals infected by rabies virus have rabies virus proteins (antigen) present in their tissues. The rabies antibody (flouresecent-labelled rabies antibody) used is primarily directed against the nucleoprotein (antigen) of the virus

INFECTED CELLS MAY CONTAIN

1. Large round or oval inclusions containing collections of nucleoprotein (N)
2. Smaller collections of antigen that appear as dust-like fluorescent particles if stained by the dFA procedure.

The ideal tissue to test for rabies antigen is brain. When labelled antibody is incubated with rabies-suspect brain tissue, it will bind to rabies antigen. Unbound antibody can be washed away .Areas where antigen is present can be visualized as fluorescent-apple-green areas using a fluorescence microscope.

Tests are performed on samples of

1. Saliva,
2. Corneal impressions
3. Spinal fluid,
4. Skin biopsies (or hair follicles) at the nape of the neck.

TEST PROPER
TISSUE SAMPLING

1. Brain stem.

The brainstem is anterior to the cerebellum and continuous with the spinal cord. The uppermost portion of the brain stem is the midbrain. The hindbrain portion of the brain stem is composed of pons and medulla oblongata. A cross (transverse) section of one of these areas is necessary for rabies testing. .

2. CEREBELLUM.

The visible portion of the cerebellum, the cerebellar cortex, is a thin layer of highly convoluted gray matter The core of the cerebellum contains the white matter. The cerebellum may be broadly divided into the midline structure called the vermis ("worm") and two lateral cerebellar hemispheres. A rabies test should include examination of tissue from a cross section through the hemispheres and the vermis. If only one hemisphere is available because the brain has been bissected for other tests, slides can be made by sampling multiple areas of the same hemisphere so that the total surface area examined is equivalent to that examined for a complete cerebellum.

3. AMMON'S HORN (HIPPOCAMPUS)

The hippocampus is burried deep in the temporal lobe near the center of the brain and is only visible when the brain is dissected The lateral horn-shaped protrusions of the hippocampus are the reason for its alternative name, Ammon's horn. If used for rabies testing, a cross section including areas of both horns is needed

4. COMBINED AREAS FOR VERY SMALL ANIMALS LIKE BATS.

Slides are prepared from tissue cut through the brain at the point at which the cerebral hemispheres overlay the cerebellum. If the cut is made properly, the section will include parts of the cerebellum and midbrain, as well as both cerebral hemispheres.

A duplicate impression from each area has to be made for staining with the second reagent.(dealt in section 7)

5. CONTROL SLIDES.

Positive and negative control slides are fixed in acetone at the same time as test slides to control for the effect of acetone fixation on test performance. Control brain material for slide preparation should be retained for this purpose from animals naturally infected with the most common variant of rabies virus in the submitting area for the laboratory. Slides are stored frozen at -20°C for up to one month or at -70°C for one year. Slides may be made from either brain stem or cerebellum.

6. DRYING AND FIXATION.

Impressions/smears must dry completely at room temperature prior to the fixation step. This may take 15 to 30 minutes. Do not use ovens or a hot air source to dry slides as this may denature antigen. Unless a biocontainment hood is available, do not direct air from fans over slides as this may create airborne tissue particles. When the tissue no longer appears wet and glistening, slides from an individual test animal are combined in one container for

fixation. Do not combine slides from different animals or combine test slides with control slides in the same container. Slides should be fixed for a minimum of 1 hr to overnight at -20°C.

7. STAINING, RINSING, AND MOUNTING.

After the acetone-fixed impression / smear control and test slides are air dried at room temperature and each anti-rabies conjugate(the antinucleocapsid conjugate and anti-glycoprotein conjugate) is added by using a micropipette. The slides are arranged so that the positive control slide is the first to receive the conjugate and the negative control slide is the last to receive the conjugate The slides are then incubated for 30 minutes at 37°C in a high humidity chamber. The use of two conjugates increases the specificity of the test. If both the slides are negative the sample is negative for rabies.

8. COUNTERSTAINS

If added to the working dilution of the conjugate, these provide contrast and lower background and also serve as a marker for accidental omission of the diagnostic reagent. Counterstain use is optional. Evans Blue counterstain (0.5% in PBS) can be aliquoted and stored at +4°C for up to 6 months and indefinitely at -20°C. Due to counterstain, the tissue will be noticeably red, but should not be so strongly red as to diminish the specific green fluorescence of rabies virus proteins. An Evans Blue concentration of 0.00125% works in many laboratories.

After staining, excess conjugate is drained from the slides or wicked onto absorbent paper and the slides are given a brief rinse under a stream of PBS, then immersed and soaked in PBS for 3 to 5 minutes (control slides and slides from each test animal in a separate rinse container). The PBS is discarded and replaced and the slides soaked for a second 3 to 5 minute interval. Slides are carefully blotted to remove excess liquid, and air-dried before mounting. Slides are mounted by dropping a small amount of 20 per cent glycerol - Tris buffered saline pH 9.0 onto coverslips arranged on absorbent paper.

Stained slides are inverted over the coverslips. Excess mountant is wicked into the absorbent paper by applying light pressure to the back of the slides. Slides should be read within 2 hours of mounting. Rabies-specific staining should be stable for at least 2 hours, and stained slides can be preserved for reference for weeks to months at refrigerator temperature or below.

8. Reading. A sample can be considered negative for rabies only when each area of the brain stained with each anti-rabies conjugate is scanned over approximately 40 fields at a magnification of approximately 200X or greater for fluorescing inclusions. Fluorescence suggestive of rabies is examined at a 400X magnification. A minimum of 160 total fields is to be examined

ANTIGEN DISTRIBUTION.

For each area of the brain examined, staining is graded by the amount of antigen present as follows:

1. +4, a massive infiltration of large and small inclusions of varying shape in almost every area of the impression.
2. +3, inclusions of varying size and shape are found in almost every microscopic field, the number of inclusions per field varies, but inclusions are numerous in most fields.
3. +2, inclusions of varying size and shape are present in 10 per cent to 50per cent of the microscopic fields and most fields contain only a few inclusions.
4. +1, inclusions of varying size and shape are present in <10 per cent of the microscope fields and only a few inclusions are found per field (usually only one or two inclusions per field).

TEST INTERPRETATION.

If the tissue sample submitted for testing was adequate and suitable for rabies diagnosis, results for a test animal are reported as positive or negative for rabies (test complete) or non-diagnostic (test incomplete) based on observed patterns of staining in test and control slides.

1. **Test complete / reportable result.** Test results are reported to the submitter as complete, samples of brain material are stored for reference, and the carcass and other material discarded, if the following observations are made:

Test controls: Both large and small antigen accumulations in positive control slide stain with +4 intensity and +3 to +4 antigen distribution. No staining is present on negative control slide.

Test samples: Required brain areas were present and no tissue deterioration or alteration was noted when slides were prepared. Samples are clearly negative (no specific staining in test slides) or clearly positive (at least +3 to +4 intensity and +2 to +4 distribution of antigen in slides made

from brain stem and cerebellum or hippocampus).

GENERALLY

1. If all test slides are negative, the animal is considered negative for rabies.

2. If additional test slides repeat the sparse staining observed with the first test and the reagent control indicates that the staining is specific (no staining of the specimen with the reagent control), the sample is considered positive for rabies.

3. If one reagent is negative and the inclusions in test slides stained with the other reagent are shown to be non-specific (i.e., control for that reagent also contains inclusions), the sample is considered negative for rabies.

4. If inclusions are found in test slides stained with only one reagent and the reagent control indicates that the staining is specific (no staining of the specimen with the reagent control), the sample is considered positive for rabies. This finding may indicate a new variant of rabies virus, the original brain tissue should be sent to a reference laboratory for confirmation and virus typing.

The FAT may be applied to glycerol-preserved specimens after a washing step. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with an enzyme

Skin biopsies are usually taken from the nuchal area of the neck, with hair follicles containing peripheral nerves. Corneal impressions (never scrapings) are taken from animals showing clinical symptoms by lightly touching the central part of the cornea with a microscope slide. The quality of the samples -both corneal impressions and skin biopsies - is paramount; they should be refrigerated immediately after collection and until the test is carried out.

Nevertheless, the sensitivity of the FA technique for intra vitam diagnosis is limited. Rabies antigen has been demonstrated in corneal impressions taken from infected animals. However, while a positive result is indicative of rabies, a negative result does not rule out the possibility of infection .Although rabies antigen may be detected in skin biopsies at the onset of clinical signs, the proportion of positive results tends to increase as the disease progresses. With nuchal skin biopsies, only some show positive results, especially during the early phase of clinical illness.

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However the extreme sensitivity can result in nonspecific positive result. Contamination with minute amounts of extraneous DNA will lead to generation of false positives and confusing results, which may hamper the introduction of this new technique in routine diagnosis.

PCR IN MOLECULAR EPIDEMIOLOGY

PCR is used extensively in genome characterization techniques. It can be applied to amplify genes specific to taxonomic groups of bacteria/ virus/ parasites and to detect their genes involved in virulence. DNA finger prints of clinical and environmental isolates thus obtained can be compared for species specific identification of the pathogens (Harmon *et.al.*,1997). This helps in identifying the single source of outbreaks and to detect mutations resulting in strain variation. The out breaks of FMD are investigated and the relationship of the field isolates with other strains is genotypically compared. PCR is widely used to trace the source of FMD out breaks (Meyers, *et.al.*, 1991).

CONCLUSION

Nucleic acid amplification techniques are labour intensive to perform. This limits their use to commercial laboratories or research oriented diagnostic laboratories. For primer selection, knowledge about the gene to be amplified is essential. Non –availability of this information becomes a limitation in the application of PCR. The need to automate PCR procedures is indispensable in near future. It is expected that this technique will lead to vast improvements in disease diagnostic capabilities and thus to a better understanding of infectious diseases of animals.

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