Advances in Diagnosis of Rabies

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Abstract

Rabies is a major zoonosis for which diagnostic techniques have been standardised internationally. Laboratory techniques are preferably conducted on central nervous system (CNS) tissue removed from the cranium. Agent identification is preferably done using the fluorescent antibody test. A drop of purified immunoglobulin previously conjugated with fluorescein isothiocyanate is added to an acetone-fixed brain tissue smear, preferably made from several parts of the brain, including the hippocampus, cerebellum and medulla oblongata. For a large number of samples, as in an epidemiological survey, the immunoenzyme technique can provide rapid results (the rapid rabies enzyme immunodiagnosis). FAT provides a reliable diagnosis in 98-100% of cases for all genotypes if a potent conjugate is used, while RREID detects only genotype 1 virus. Infected neuronal cells have been demonstrated by histological tests and these procedures will reveal aggregates of viral material (the Negri bodies) in the cytoplasm of neurones. However, the sensitivity of histological techniques is much less than that of immunological methods, especially if there has been some autolysis of the specimen. Consequently, histological techniques can no longer be recommended. As a single negative test on fresh material does not rule out the possibility of infection, inoculation tests, or other tests, should be carried out simultaneously. Newborn or 3-4-week-old mice are inoculated intracerebrally with a pool of several CNS tissues, including the brain stem, and then kept under observation for 28 days. For any mouse that dies between 5 and 28 days, the cause of death should be confirmed by FAT. Alternatively, a monolayer culture of susceptible cells is inoculated with the same material as used for mice. FAT carried out after appropriate incubation will demonstrate the presence or absence of viral antigen. Wherever possible, virus isolation in cell culture should replace mouse inoculation tests. The identification of the agent can be supplemented in specialised laboratories by identifying any variant virus strains through the use of monoclonal antibodies, specific nucleic acid probes, or the polymerase chain reaction followed by DNA sequencing of genomic areas. Such techniques can distinguish between field and vaccine strains, and possibly identify the geographical origin of the field strains. Virus neutralisation assays in cell cultures are the prescribed tests for international trade.

Keywords: Rabies, Zoonosis, Diagnosis, ELISA, CNS, DNA, Virus, Negri bodies.

Introduction

Rabies is caused by a neurotropic virus of the genus Lyssavirus of the family Rhabdoviridae, and is transmissible to all mammals. As it is transmissible to humans by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organisation (WHO).

Seven distinct genetic lineages can be distinguished within the genus Lyssavirus by cross-protection tests and molecular biological analysis, namely the classical rabies virus itself (RABV, genotype 1, serotype 1), Lagos bat virus (LBV, genotype 2, serotype 2), Mokola virus (MOKV, genotype 3, serotype 3), and Duvenhage virus (DUUV, genotype 4, serotype 4). The European bat lyssaviruses (EBLV), subdivided into two biotypes (EBLV1, genotype 5 and EBLV2, genotype 6) and the Australian bat lyssavirus (ABLV, genotype 7), recently isolated in Australia, are also members of the Lyssavirus genus, but are not yet classified into serotypes. Viruses of serotypes 2-4, EBLV and ABLV are known as rabies-related viruses. The use of monoclonal antibodies (MAbs) directed against viral nucleocapsid or glycoprotein antigens, and the sequencing of defined genomic areas has made possible the definition of numerous subtypes within each serotype. Lyssaviruses cause a clinical disease indistinguishable from classical rabies. Conserved

antigenic sites on the nucleocapsid proteins permit recognition of all lyssaviruses with modern commercial preparations of anti-rabies antibody conjugates used for diagnostic tests on brain tissue. For RABV, DUUV, EBLV and ABLV, conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. Little or no cross-protection against infection with MOKV or LBV is elicited by rabies vaccination and most anti-rabies virus antisera do not neutralise these lyssaviruses.

Humans working with suspect material must be vaccinated against lyssaviruses or other pathogens that may be present in diagnostic samples. The laboratory must comply with national biocontainment and biosafety regulations to protect staff from contact with pathogens. WHO recommends the preventive immunisation of exposed staff. The immunisation protocol includes three injections, e.g. at days 0, 7, and 28. The serological evaluation of immunisation is made 1-3 weeks after the last injection, and checked every 6 months in the case of laboratory workers or every 2 years for other diagnosticians. Booster vaccination must be given when the titre falls below 0.5 International Units (IU) per ml. In the absence of serological monitoring, the vaccination regimen should consist of a booster vaccination at 1 year and thereafter every 1-3 years.

As no clinical sign or gross post-mortem lesion can be considered pathognomonic in domestic or wild animals, the diagnosis of rabies has to rely on laboratory testing. Serological evidence of infection is rarely useful because of late seroconversion and the high mortality rate of host species, although such data may be used in some epidemiological surveys.

Diagnostic Techniques

1. Identification of the agent

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. The only way to perform a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests.

As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the 'rabies diagnostic chain'.

Several laboratory techniques may be 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 thalamus, pons and medulla. The hippocampus (Ammon's horn), cerebellum and different parts of the cerebrum have been reported to be negative in 3.9-11.1% of the positive brains. The structure of choice is the thalamus as it was positive in all cases. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested. 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.

a) Shipment of samples: During the shipment of suspect material for diagnosis (animal heads, brain or other tissue samples), no risk of human contamination should arise: brains must be placed in a leak-proof rigid container (animal heads will be wrapped in absorbent material) as prescribed in the International Air Transport Association (IATA) Dangerous Goods Regulations must be followed.

When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is closely linked to the tests to be used for diagnosis: Formalin inactivates the virus, thus the isolation tests cannot be used and diagnosis depends on using a modified and less sensitive direct fluorescent antibody test (FAT), immunohistochemistry or histology;

Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS). It does not protect against titre decline due to thermal conditions and therefore, because rabies is thermo-labile, the virus titre will decline during glycerol/PBS storage. Under normal transport conditions in the tropics, this protection may only be effective for a matter of several days. Therefore, whenever possible samples in glycerol/saline should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples.

b) Collection of samples: Usually the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected. This step may be hazardous if laboratory technicians are not fully trained, or under field conditions. In such cases, there are two possible methods of collecting some brain samples without opening the skull:

Occipital foramen route for brain sampling: A 5 mm drinking straw or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian

bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata.

Retro-orbital route for brain sampling: In this technique, a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

c) Routine laboratory tests: Laboratory diagnosis can be performed by using three kinds of procedure.

Histological identification of characteristic cell lesions: Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Immunohistochemical tests are the only histological test specific to rabies.

An unfixed tissue smear may be stained by the Seller's method, diagnosis is then obtained in under 1 hour. Generally, histological tests, such as Mann's test, are performed on fixed material after a paraffin-embedding step, and the result of the test is obtained within 3 days. These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. Whichever staining method is used, the evidence of infection is provided by intracytoplasmic acidophilic bodies. These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity and should be abandoned.

Immunochemical identification of rabies virus antigen

i) Fluorescent antibody test: The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. 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. The FAT gives reliable results on fresh specimens within a few hours in more than 95-99% of cases. The sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled, on the type of lyssavirus and on the proficiency of the diagnostic staff. Sensitivity may be lower in samples from vaccinated animals due to localisation of antigen, which is confined to the brainstem. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue, that includes the brain stem, are fixed in high-grade cold acetone and then stained with a drop of specific conjugate. Anti-rabies fluorescent conjugates may be prepared in the laboratory. Those available commercially are either polyclonal conjugates specific to the entire virus or specific to the rabies nucleocapsid protein, or they may be prepared from a mix of different MAbs. In the FAT, the specific aggregates of nucleocapsid protein are identified by their fluorescence. The specificity and sensitivity of these anti-rabies fluorescent conjugates for locally predominant virus variants should be checked before use.

The FAT may be applied to glycerol-preserved specimens. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme. However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue.

ii) Immunochemical tests: The antibody may be conjugated to an enzyme such as peroxidase instead of fluorescein isothiocyanate (FITC). This conjugate may be used for direct diagnosis with the same sensitivity as FAT, but attention should be paid to the risk of nonspecific false-positive results. This risk is considerably reduced by the thorough training of the technicians. It must also be emphasised that this technique needs one incubation step more than the FAT.

Peroxidase conjugate may be used on sections of formalin-fixed tissue for immunohistochemical tests.

An enzyme-linked immunosorbent assay (ELISA) that detects rabies antigen is one variation of the immunochemical test. This rapid rabies enzyme immunodiagnosis test (RREID) is available commercially. The correlation between the FAT and the RREID ranges between 96% and 99%. The 'routine' version of this test is not sensitive to rabies-related viruses as RREID only detects genotype 1 lyssaviruses.

Detection of the replication of rabies virus after inoculation

These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known human exposure.

i) Mouse inoculation test: Five-to-ten mice, 3-4 weeks old (12-14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. It is recommended, though not strictly essential, to use specific pathogen free (SPF) mice. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon's horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetised when inoculated. The young adult mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For street fox

rabies strains, deaths due to rabies generally begin 9 days post-inoculation. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation.

This in-vivo test is quite expensive, particularly if SPF mice are used, and should be avoided where possible. It does not give rapid results (compared with in-vitro inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes. Another advantage of this low-tech test is that it can be easily and practicably be applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

ii) Cell culture test: Neuroblastoma cell lines, e.g. CCL-131 in the American Type Culture Collection are used for routine diagnosis of rabies. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO_2. Its sensitivity has been compared with that of baby hamster kidney (BHK-21) cells. This cell line is sensitive to street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 hours (one replication cycle of virus in the cells); generally incubation continues for 48 hours or in some laboratories up to 4 days. This test is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results.

It is often advisable to carry out more than one type of test on each sample, at least when there has been human exposure.

d) Other identification tests: The tests above may be completed in specialised laboratories using MAbs, nucleic acid probes, or the polymerase chain reaction (PCR), followed by DNA sequencing of genomic areas for typing the virus. This enables a distinction to be made between vaccine virus and a field strain of virus, and possibly the geographical origin of the latter.

2. Serological tests

Serological tests are rarely used in epidemiological surveys, due to late seroconversion and the low percentage of animals surviving the disease and therefore having post-infection antibodies. Oral immunisation of rabies reservoirs is the method of choice for wildlife rabies control. For follow-up investigations in oral vaccination campaigns, virus neutralisation (VN) tests in cell culture are preferred. However, if poor quality sera are submitted, the VN tests in cell culture are sensitive to cytotoxicity, which could lead to false-positive results. For such samples, the use of an indirect ELISA with rabies glycoprotein-coated plates has been shown to be as sensitive and specific as the VN test on cells.

a) Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (a prescribed test for international trade): The principle of the fluorescent antibody virus neutralisation (FAVN) test is the neutralisation in vitro of a constant amount of rabies virus ('challenge virus standard' [CVS] strain adapted to cell culture) before inoculating cells susceptible to rabies virus: BHK-21 C13 cells.

The serum titre is the dilution at which 100% of the virus is neutralised in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralising dilution of a standard serum under the same experimental conditions (OIE serum of dog origin or WHO standard for rabies immunoglobulin [human]. Several publications have shown that the FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results.

b) The rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody (a prescribed test for international trade): Each well of an eight-well tissue-culture chamber slide contains 25-50 distinct microscopic fields when observed at x160-200 magnification. One unit of virus for the RFFIT is determined as the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells (the focus-forming dose, FFD_{50}). The stock virus suspension should contain at least 1×10^4 FFD_{50} per 0.1 ml (i.e. the well with cells infected with the 10^{-4} dilution of the virus should contain at least one focus of infected cells in 50% of the observed microscopic fields). A stock virus suspension of this titre can then be diluted to $10^{-2.3}$ to obtain a challenge virus containing 50 FFD_{50}.

Reference sera: A national or international reference serum standard diluted to a potency of 2.0 IU/ml should be included in each test. The reference serum used at the Centres for Disease Control and Prevention is the first international standard for rabies immunoglobulin, which may be obtained from the NIBSC. The reference serum should be maintained as frozen aliquots in amounts sufficient for 1 week of tests. A positive serum control standard diluted to a potency of 0.5 IU/ml and a negative serum control standard with a potency of <0.1 IU/ml should also be prepared by the laboratory and included in each test.

Test sera: Serum samples should be heated at 56°C for 30 minutes before testing in order to inactive complement. If sera are frozen, they should be reheated after thawing. Serial dilutions of test sera

may be prepared in an eight-well tissue-culture chamber slide. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy.

Calculation of virus-neutralising antibody titres: Residual virus is detected using a standard fluorescence microscope. The serum neutralisation end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralisation titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralised and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the national reference standard included in each test. It should be noted that it is also valid to perform the RFFIT using BHK-21 cells instead of neuroblastoma cells. A modified protocol for this has been published.

c) Virus neutralisation in mice: This method is no longer recommended by either OIE or WHO and should be discontinued.

d) Enzyme-linked immunosorbent assay: Commercial kits are available for indirect ELISA that allow a qualitative detection of rabies antibodies in individual dog and cat serum samples following vaccination. In accordance with the WHO recommendations, 0.5 IU per ml rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity that correlates with the ability to protect against rabies infection. The ELISA provides a rapid (~ 4 hours) test that does not require handling of live rabies virus, to determine if vaccinated dogs and cats have sero-converted. The sensitivity and specificity of any kit used should be determined by comparison with virus neutralisation methods. The ELISA is acceptable as a Prescribed Test for international movement of dogs or cats provided that a kit is used that has been validated and adopted on the OIE Register as fit for such purposes. Virus neutralisation methods may be used as confirmatory tests if desired.

ELISA methods are also useful for monitoring of vaccination campaigns in wildlife populations, provided the kit used has been validated for the wildlife species under study.

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