Diagnostics procedures in rabies

T. Malovrh, P. Hostnik

Rabies is a major zoonosis for which diagnostic techniques can only be performed in the laboratory. Laboratory techniques are preferably oriented on tissue removed from the cranium: hippocampus (Ammon's horn), cerebellum and the medulla oblongata or tissue liquids. Clinical observation may only lead to a suspicion of rabies. The only way to perform a reliable diagnosis of the disease is to identify the virus or some of its specific components using laboratory tests such as histological identification of characteristic cell lesions, immunological identification of rabies virus antigen and virus isolation. Serological tests are rarely used in epidemiological surveys but much more frequently in control of the vaccination programs (e.g. oral vaccination). Most commonly used serological tests are the virus neutralisation test on cell culture (FAVN), virus neutralisation in mice and ELISA.

Key words: rabies, diagnostics procedures

Introduction / Uvod

Rabies is a fatal zoonosis 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 the infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organisation (WHO). A part from the classical rabies virus, there are 6 other lineages of viruses of the *Lyssavirus* genus responsible for rabies-like diseases. These are the: Lagos bat virus, Mokola virus, Duvenhage virus, European bat viruses and Australian bat virus. 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. According to the OIE classification the disease is found under multispecies diseases list B. The distribution of Rabies ex-
tends over most of Europe, throughout Africa, the Middle East, most of Asia, and the Americas. United Kingdom, Ireland, parts of Scandinavia, Japan, Singapore, Australia, New Zealand, Papua New Guinea and the Pacific Islands are free. Recently, a rabies-like disease due to a previously unknown Lyssavirus was found in bats in Australia. Clinical signs of the disease can be very variable and potentially rabid animals should be approached with extreme caution. In all species the incubation period varies considerably from two weeks to several months, and death occurs 2 to 10 days after the onset of symptoms, rarely after 2 weeks. Recovery is exceptional. Usually the clinical signs change during the illness, different forms of the disease are described and may differ between species, but abnormal behaviour is a constant. Post-mortem findings are no characteristic. The stomach may contain abnormal objects (sticks, stones etc). Microscopic lesions are found in the central nervous system (Negri bodies). We should take into account the differential diagnosis, which includes bacterial, parasitic and mycotic diseases of the central nervous system (listerosis and cryptococcosis), poisons and heavy metals (such as lead), acute psychosis in dogs and cat, canine distemper and infectious canine hepatitis, Aujeszky's disease, equine viral encephalomyelitis, foreign bodies in the oropharynx or oesophagus and other traumatic injuries. 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.

**Diagnostics / Dijagnostika**

**The importance of routine rabies tests / Važnost rutinskih testova na besnilo**

Rapid and accurate laboratory diagnosis of rabies in animals and in humans is essential for taking regulated measurements and for timely administration of post exposure prophylaxis. 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, if the animal is not rabid. In addition, laboratory identification of positive rabies cases may aid in defining current epidemiologic patterns of the disease and provide appropriate information for the development of rabies control programs.

**Laboratory tests for rabies / Laboratorijski testovi na besnilo**

The standard test for rabies testing is the direct fluorescent antibody test (dFA). This test has been thoroughly evaluated for more than 40 years, and is recognized as the most rapid and reliable of all the tests available for routine use. All rabies laboratories perform this test (post-mortem) on animals suspected of
having rabies. Other tests for diagnosis and research, such as cell-culture isolation techniques, immunohistochemistry (IHC), histologic examination, RT-PCR, and electron microscopy (EM) are useful tools for studying the rabies virus. Serological tests, such as fluorescent antibody virus neutralisation (FAVN) and rapid fluorescent focus inhibition test (RFFIT), ELISA and mouse inoculation test (MI), are tools for controlling the efficacy of prophylactic vaccination of animals and humans and to control the effect of oral immunization of wild carnivores.

**Direct fluorescent antibody test (dFA) / Direktni test fluorescentnog antitela (dFA)**

The dFA technique is a highly sensitive method for detecting rabies antigen in fresh specimens and, it may also be performed on fixed specimens. The method was developed in 1958 by Goldwasser & Kissling. The dFA test is based on the observation that animals infected by rabies virus have rabies virus nucleoproteins (antigen) present in their tissues. Rabies virus replicates in the cytoplasm of cells, and infected cells may contain large round or oval inclusions containing collections of nucleoprotein (Negri bodies) or smaller collections of antigen. Because rabies is present in nervous tissue, the ideal sample to test for rabies antigen is the brain. Areas where antigen is present can be visualized as fluorescent-apple-green areas using a fluorescence microscope. The dFA test is now the most widely used method for diagnosing rabies infection in animals and humans. Apart from an appropriate microscope, the two main requirements for success in using this technique are well-trained personnel and conjugated monoclonal antibodies (mAb) or antiserum of good quality.

**Cell-culture isolation techniques / Tehnike izolacije čeljske kulture**

Successful in vitro cultivation of the rabies virus was first reported in 1936. This property has been used extensively in research on rabies. Tests for the isolation of street rabies in cell culture were first carried out in the mid 1970s using baby hamster kidney cells, line 21 (BHK-21), chick embryo-related (CER) and neuroblastoma cells (e.g. CCL-131). These studies demonstrated that rabies infection could be detected by immunofluorescence from as early as 4-5 hours up to 5 days following inoculation (the result is obtained after at least 18 hours, generally incubation continues for 48 hours or in some laboratories up to 4 days). Furthermore, it was found that BHK-21 cells were comparable in sensitivity to MI, whereas neuroblastoma cells were more sensitive than MI to infection by the street rabies virus. In addition, virus isolation in cell culture has been shown to be as efficient as the dFA test and the MI test for demonstrating small amounts of the rabies virus. However, specimens containing a small amount of rabies virus and which are negative by dFA and subsequently positive by virus isolation in cell culture require an incubation period of 4 days after inoculation of the cells. It is often advisable to carry out more than one replication of the test on each sample, at least when there has been human exposure.
Immunohistochemistry / Imunohistohemija

IHC methods for rabies detection provide sensitive and specific means to detect rabies in formalin-fixed tissues. These methods are more sensitive than histological examination, such as H&E. Like the dFA test, these procedures use specific marked antibodies to detect rabies virus inclusions. The techniques use enzyme-labeling systems that enhance sensitivity as good to be used to detect rabies virus variants.

Histological examination / Histološka istraživanja

Histological examination of biopsy or autopsy tissues is occasionally useful in diagnosis of unsuspected cases of rabies that have not been tested by routine methods. When brain tissue from rabies virus-infected animals are stained with histological stains, such as hematoxylin and eosin, evidence of encephalomyelitis may be recognized. Evidence of rabies encephalomyelitis in brain tissue and meninges includes mononuclear infiltration, perivascular cuffing or inflammation around a blood vessel, lymphocytic foci, Negri bodies (affinity of these structures for acidophilic stains) and nodules consisting of glial cells. Histological examination is non-specific and not considered diagnostic for rabies.

Virus detection by RT-PCR / Otkrivanje virusa putem RT-PCR

Samples containing small amounts of the rabies virus may be difficult to confirm as rabies-positive by routine methods. A relatively new method for amplifying the nucleic acid portion of the rabies virus uses biochemical methods. With this procedure, rabies virus RNA can be enzymatically amplified as DNA copies. Rabies RNA can be copied into a DNA molecule using reverse transcriptase (RT). The DNA copy of rabies can then be amplified using the polymerase chain reaction (PCR). This technique can confirm dFA results and can detect rabies virus in saliva and skin biopsy samples.

Electron microscopy / Elektronska mikroskopija

The ultrastructure of viruses can be examined by electron microscopy. Using this method, the structural components of viruses and their inclusions can be observed in detail. The rabies virus is in the family of Rhabdoviruses. When viewed negatively stained with an electron microscope Rhabdoviruses are seen as bullet-shaped particles.

FAVN and RFFIT / FAVN i RFFIT

For follow-up investigations in prophylactic vaccination and in oral vaccination campaigns, virus neutralisation tests in cell culture are preferred. Both fluorescent antibody virus neutralisation (FAVN) and rapid fluorescent focus inhibition test (RFFIT) are based on neutralisation of the inoculated virus by sample serum in defined cells. Examinated sera diluted (FAVN threefold dilutions, RFFIT
1/5 and 1/50 dilutions) were then mixed with a constant dose of the challenge virus. The sera/virus mixture was incubated at 37°C for an hour. After incubation, susceptible cells (BHK) at a concentration of 4x10^5 cells/ml for FAVN and for RFFIT at a concentration 5x10^5 cells/ml were added to the serum/virus mixtures. In RFFIT, cells were incubated in an eight-well tissue-culture chamber slide for 20 hours. FAVN requires a 48-hour incubation period in a 96-well microtiter plate. After the incubation period, slides for the RFFIT or the microtiter plate for FAVN were fixed with acetone and stained with FITC-conjugated anti-rabies antibodies and then observed under the fluorescent microscope. In the RFFIT the presence of non-neutralized virus (fluorescent foci) could be detected, by reading of 20 microscopic fields while the FAVN test uses a qualitative reading of the whole surface of the well (positive if there is at least one fluorescent cell). According to the serum dilutions the titer of antibodies is calculated.

The comparative study of the FAVN test and RFFIT has shown that the distribution of titres was statistically identical for vaccines whatever the test used. The FAVN test is superior to the RFFIT in allowing an effective discrimination between naive and immunised dog sera. According to these results, it is possible to detect positive sera at a threshold lower than 0.5 IU/ml with the FAVN test.

**ELISA / ELIZA**

Commercially available rabies ELISA allows a qualitative detection of rabies antibodies in individual serum samples following vaccination. Wells of microplates are coated with the rabies virus surface glycoprotein, while the detection system for specific anti-rabies antibodies is based on staphylococcus protein A or species-specific anti-immunoglobulin antibodies. Although this indirect ELISA has a lower sensitivity than the FAVN or RFFIT, it can be used as a rapid screening test, which does not require the handling of the live rabies virus, to determine the presence of antibodies in a sera sample. Due to the lower sensitivity of the test, negative results should be confirmed by FAVN or RFFIT.

In accordance with the WHO recommendations, 0.5 I.U./ml rabies antibodies is the minimum antibody titre to be sufficient for immunity against rabies infection.

**Mouse inoculation test / Test inokulacije miševa**

Mice, 3 to 4 weeks old (12 to 14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. 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 (e.g. 550 UI penicillin/ml and 1,560 UI streptomycin/ml). The animals were observed daily for a period of 28 to 30 days. The appearance of the symptoms (bristling of the fur, agitation, aerophobia, paralysis and death) was monitored. Every dead mouse is examined for rabies using the dFA. The incubation period of the sample was considered to be the first day of the appearance of the symptoms in one of the laboratory
mice. Once a cell culture unit exists in the laboratory, isolation of the rabies virus in cell culture should replace intracerebral mouse inoculation whenever possible. It should, however, be borne in mind that only laboratories where cell-culture techniques are currently used can successfully maintain cell cultures for diagnosis.

**Rabies diagnosis in humans / Diagnosa besnila kod ljudi**

Several tests are necessary to diagnose rabies ante-mortem (before death) in humans; no single test is sufficient. Tests are performed on samples of saliva, serum, spinal fluid, and skin biopsies of hair follicles at the nape of the neck. Saliva can be tested by virus isolation or reverse transcription followed by polymerase chain reaction (RT-PCR). Serum and spinal fluid are tested for antibodies to the rabies virus. Skin biopsy specimens are examined for the rabies antigen in the cutaneous nerves at the base of hair follicles.

References /Literatura

Besnilo je značajna zoonoz za koju se dijagnostičke procedure mogu da izvedu samo u laboratoriju. Laboratorijske tehnike se uglavnom fokusiraju na tkivo uklonjeno iz kranijuma: hipokampusa (Amonov rog), malog mozga i medule oblongate, ili tečnih tkiva. Kliničko posmatranje može da obezbedi samo sumnju na besnilo. Jedini način da se obavi pouzdano dijagnostikovanje bolesti jeste da se identifikuje virus ili neka od njegovih specifičnih komponenti putem laboratorijskih ispitivanja, kao što su histološka identifikacija karakterističnih cellijskih lezija, imunohemijijska identifikacija antigena virusa besnila i izolacija virusa. Serološka istraživanja se retko koriste u epidemiološkim pregledima, ali mnogo češće kod kontrole vakcinom. Serološki testovi koji se najčešće koriste su test neutralizacije virusa u cellijskoj kulturi (FAVN), neutralizacija virusa kod miševa, i ELIZA.

Ključne reči: besnilo, dijagnostičke procedure