VETERINARSKI ARHIV 71 (2), 65-73, 2001

Doubtful and discordant results in fluorescent antibody test for rabies diagnosing

Peter Hostnik*, Marjeta Štrancar, Darja Barlič-Maganja, and Jože Grom

Institute for Microbiology, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia

HOSTNIK, P., M. ŠTRANCAR, D. BARLIČ-MAGANJA, J. GROM: Doubtful and discordant results in fluorescent antibody test for rabies diagnosing. Vet. arhiv 71, 65-73, 2001.

ABSTRACT

A total of 7339 fox, dog, cat, cattle, sheep and other mammalian brains were tested to rabies virus antigen. Results of fluorescent antibody test (FAT) were evaluated by two microscopists. Doubtful and discordant results in FAT were analyzed again in virus isolation test (VIT) using mouse neuroblastoma (NA) cell line. Twenty-eight brain samples were determined as doubtful, while 9 brain samples were determined to be positive by one microscopist and negative by the other. Samples which were shown as doubtful and discordant in FAT were retested in VIT. Seventeen of these were positive in the VIT.

Key words: rabies, immunofluorescence, cell culture

Introduction

Rabies being an infectious zoonotic disease, it is still considered the most feared disease present on all five continents. Two epidemiological forms of rabies are known: urban rabies, where dogs are responsible for the maintenance and in many cases for the transmission of the disease to man. The other, wildlife (sylvatic) form of rabies spreads among wild animals. In Central European countries the main vector for sylvatic rabies is the red fox (*Vulpes vulpes*). In Slovenia, where urban rabies is under

* Contact address:

Doc. Dr. Peter Hostnik, DVM, Institute for Microbiology, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia; Phone: +386 61 4779 178, Fax: +386 61 4779 542, E-mail: Peter.Hostnik@vf.uni-lj.si

ISSN 0372-5480
Printed in Croatia

control through an effective dog vaccination programme, the rabies disease is still present among wildlife. Wild animals, particularly rabid foxes, could be a potential source of rabies infection to non-vaccinated domestic animals, and directly or indirectly to humans (PASTORET et al., 1985). The disease is caused by the Lyssa virus, which is a member of the family *Rhabdoviridae*, genus *Lyssavirus*. The virion is enveloped, bullet shaped, lipid-containing and consists of a single-stranded negative sense RNA (TORDO and POCH, 1988; VAN REGENMORTEL et al., 2000). This neurotropic virus is pathogenic and deadly to all warm-blooded animals, including humans. The clinical sign of the disease is characterized by viral encephalitis (CHARLTON, 1991).

Diagnosing rabies in animals is most important mainly in cases where the examined animal has bitten, harmed or been in any contact with humans prior to death. In cases where a rabies-positive animal is discovered, post exposure or follow-up rabies treatment for humans is essential. A sensitive and specific diagnostic test must be used. The mouse inoculation test (MIT), traditionally known as the "golden" method in diagnosing rabies, is used to confirm results of other techniques (KOPROWSKI, 1996). The major difficulty with this method is a two- to three-week waiting period while the test mice are observed. The fluorescent antibody test (FAT) is the most routinely employed method and recommended by WHO for detecting viral antigens in brain samples. The most important factors for a reliable result are: specific rabies immunoglobulin conjugate, a sophisticated microscope and trained technicians (ANONYMOUS, 1992). The correlation between both tests is good. However, FAT false-negative results at a level of 0.2% are described (HEMACHUDHA, 1989). The virus isolation test (VIT) on cell cultures has replaced the MIT in many laboratories as the test is more sensitive, easy to perform, less time consuming and more humane (WEBSTER and CASEY, 1996). The ELISA test is also employed routinely using commercially available kits (Diagnostics Pasteur), which can be especially useful for epidemiological studies (ANONYMOUS, 1992) Also, the polymerase chain reaction test (PCR) is the most commonly used method for epidemiological studies (WHITBY et al., 1997).

The first rabid fox in Slovenia was discovered in 1973 near the Hungarian border. This was followed by intensive fox hunting but which failed to halt the spread of the disease. The most explosive outbreak

occurred in 1981 in the north-western part of Slovenia near the borders with Austria and Italy. The occurrence rate increased and decreased in regular cycles. Vaccination against rabies is mandatory for all dogs and for farm animals that pasture every year; vaccination of other animals is on a voluntary basis (BIDOVEC et al., 1993).

In our laboratory the diagnosis of rabies in animals were earlier carried out by Negri body detection with use of Seller's stain, but it has been accomplished by use of the fluorescent antibody test since 1973. Samples of the hippocampus (horn of Amon) were stained in both tests. Tissue specimens with doubtful results on microscopic examination, or a specimen obtained from an animal that had bit a man, were all submitted for mouse inoculation test. The mice were observed for 4 weeks.

Post-exposure vaccination of humans following an animal bite or scratch is frequently based upon the results of laboratory diagnosis. False-negative results in IF test are not common but can occur (GRIFFIN, 1984; RUDD and TRIMARCHI, 1987), so WHO (ANONYMOUS, 1992) recommends mouse inoculation or the virus isolation test on cell cultures in suspicious cases or antemortem diagnosis in animals which bite or scratch man. RUDD and TRIMARCHI (1987) showed that the virus isolation test on cell cultures is equal to MIT in sensitivity, but most importantly it shortens the test period; in MIT the test takes 10-30 days and the virus isolation test takes 2-5 days.

This paper describes our retrospective study of 7339 brain samples using the IF test and analysis results of 37 samples, when variance between the results of two observers, or where unclear results were obtained. The virus isolation test on a neuroblastoma cell culture was used as a confirmation test.

Materials and methods

During our study period 7339 brain samples from different animal species were analyzed to rabies virus antigen by the FAT. The FAT was performed by the technique of LEPINE and ATANASIU (1996). Two impressions of Amon's horn and two impressions of cerebellum tissue were made and fixed for 10 minutes in cold acetone. The fluorescent anti-rabies conjugate (anti-rabies serum fluorescein-conjugated, Sanofi, Diagnostics Pasteur, Marnes l-al Coquette, France) was used for staining the impressions. After

incubation (30 minutes at 37 °C) the slides are washed in a phosphate buffer (PBS), pH 7.2, dried, covered with buffered glycerol (pH 7.4) and coverslip and examined. The two specialists evaluated the results with a epifluorescence microscope Opton axioscop (×400). FAT results for brain sample slides were graded on a reading scale, from negative to 3+ positive. The samples with bright green fluorescence particles were graded as 3+, dull fluorescence was graded 2+, and samples with dim fluorescence particles were graded as 1+. Slides with only a few fluorescence particles, and the slides with discordants in FAT results between both specialists, were declared as doubtful. The samples were diagnosed as negative when the fluorescence was not evident to both microscopers.

Brain samples were determined to be FAT positive by one mocroscopist and negative by the other (9 samples), and samples determined as doubtful (28 samples) were retested in the virus isolation test using mouse neuroblastoma cell line, clone NA, obtained by P. Schuster from Impfstoffwerk, Dessau, Germany. The cell cultures were used at passages 90 to 100. Brain samples from 37 animals (15 foxes, 13 cats, 6 dogs, 2 cows and 1 sheep) were mashed and pestled until a paste was formed. They were re-suspended in a cell culture medium (minimal essential medium, MEM, Gibco) to a final dilution of 1:10. The homogenate was centrifuged at 2.000 x g for 15 minutes and the collected supernatant was filtered through a 0.45 Millipore filter. Two-fold dilutions of samples were made. Each sample dilution was inoculated onto 24-hour-old NA cell cultures growing at 37 °C in 96-well cell culture microplate (Nunc) and on NA cell cultures growing at 37°C in tubes (Nunc). It continued to adsorbtion for 0.5 - 1 hour at 37 °C, the inoculate was discarded, Eagle's MEM added, supplemented with 5% foetal calf serum (0.05ml/well) and incubated. After a 72-hour incubation period at 37 °C in a humid chamber with 5% carbon dioxide (CO_2), the medium in wells was discarded and cells were fixed for 20 minutes in 85% acetone at –20 °C and stained with anti-rabies conjugate (Sanofi Pasteur, France). The microplates were washed in PBS, dried and examined under a fluorescent microscope (Zeiss, Axiovert 25, Germany).

Fluorescent inclusions could be found in the infected cell's cytoplasm. If results were negative the cells in the tubes were trypsinized. One part of

suspended cells were added in cell culture microplate wells for testing the rabies antigen and a second part into new tubes, and the passage of samples was carried out.

Results

During the study period 28 brain samples were determined to be FAT doubtful by both microscopists; 9 brain samples were determined to be positive by one microscopist and negative by the other microscopist. In this study period 15 (0.27%) of 5523 foxes, 6 (1.25%) of 478 dogs, 13 (1.97%) of 659 cats, 2 (2.56%) of 78 cattle and 1 (5.25%) of 19 sheep were determined as doubtful (Table 1). Of thirty-seven samples with doubtful or discordant results in FAT, 17 were positive and 20 were negative in VIT test. During retesting of samples in VIT, 3 fox, 3 dog, 10 cat and 1 sheep samples were declared positive.

Evaluation of the virus isolation test is simple as the fluorescent inclusions in the NA cell infected with rabies virus are clearly visible. Seventeen of the 37 samples (3/15 foxes, 10/13 cats, 3/6 dogs, and 1/1 sheep) declared as doubtful in FAT test became positive in virus isolation test. Fourteen samples were positive after 72 hours in the initial inoculation; only three samples became positive in first passage, and none in the second passage (Table 1).

Table 1. Suspicious results of 37 samples in FAT retested in the virus isolation test

Species	N° of sample	FAT	Virus isolation test	
			Negative	Positive (passage)
Fox	15	+/-	12	3 (2/1/0)*
Cattle	2	+/-	2	0 (0/0/0)
Dog	6	+/-	3	3 (2/1/0)
Cat	13	+/-	3	10 (9/1/0)
Sheep	1	+/-	0	1 (1/0/0)
Total	37		20	17 (14/3/0)

*(2/1/0) - positive in initial inoculation/positive in first passage/positive in second passage

Discussion

Rabies virus infection has persisted in Slovenian wildlife since 1973. The red fox (*Vulpes vulpes*) is the predominant host to the virus and is the main vector and reservoir of the disease. Rabies in Slovenia among domestic animals has been most often diagnosed in cats (HOSTNIK et al., 1999). Not surprisingly, as cats are not vaccinated against rabies regularly and in rural areas they quite often come into contact with rabid foxes. Rabies virus antigen is usually and routinely shown in brain tissues using antigen detection or virus isolation techniques. The histopathological technique for detecting eosinophilic cytoplasmic inclusions known as Negri bodies is not an accurate method for rabies diagnosis, (LEPINE and ATANASIU, 1996) as it gave 10-30% false negative results. In many laboratories the immunofluorescence test has been used for the rapid detection of the virus antigen and is a good method for the sensitive and specific detection of antigens (ANONYMOUS, 1992; TEPSUMETHANON et al., 1997) and is the recommended (but not the prescribed) test by WHO and O.I.E. (Office international des Epizooties, 1996)

The WHO recommendation is to perform mouse inoculation test or VIS on samples that gave FAT negative results, if an animal had had contact with man. Rabies virus can also be isolated and later determined using the virus isolation test (WIKTOR, 1996) or mouse inoculation test (KOPROWSKI, 1996). KULONEN et al. (1991) reported about 6901 specimens, which were in FAT test negative, except 32, all of them being positive in VIT. Mouse neuroblastoma cell line is more sensitive for the field rabies virus strain than other cell cultures. (UMOH and BLENDEN, 1983; RUDD and TRIMARCHI, 1987). IWASAKI and CLARK (1977) demonstrated that street rabies virus is more invasive on an NA cell line than on other lines.

The results of this present study confirm that the FAT is a simple technique, but evaluation of results can be also subjective. Many factors have influenced an objective reading of results. Nowadays, satisfactory equipment (mainly U.V. microscopes in European countries) cannot impede the decision. A very important factor is the immunofluorescent conjugate. The strong background of the visible field can cause errors in evaluation of results, and small particles may not be seen. The results of microscopic examinations were graded using four stages: strong positive, weak positive,

doubtful, and negative. The final results in 37 samples of the 7339 tested were not conclusive and a second test had to be performed. In these cases, only small atypical grains, or only a few grains, were found. The decomposed and bacterially contaminated brain tissues were declared as useless for use in the FAT. "Weak" positive samples and especially samples giving doubtful results in FAT also have to undergo a second test in virus isolation test. A relatively high incidence of FAT doubtful results (13/659, 1.97%) was found in cats. The immunofluorescence grains in brain tissue from cats are smaller than in foxes and is less evident in cat tissue. It is concluded that of 13 cats declared as doubtful in FAT, 10 were positive in VIT, while in foxes of 15 doubtful in FAT only 3 were positive in VIT. Another explanation could be that the cat samples were fresh, while the age of fox samples was often unknown. It is possible that the rabies virus in fox samples was inactivated, which resulted in a high grade of negative results in VIT, which have been determined as doubtful in FAT. Our results also indicate that FAT must be read by two specialists. Furthermore, a single inoculation without passage on neuroblastoma cells is not sufficient for the sensitive detection of rabies virus.

References:

ANONYMOUS (1992): Expert committee on rabies. 8th report. WHO Technical report series, N° 824. Genova, World Health Organization, pp.7-8.

ANONYMOUS (1996): OIE Manual of standards for diagnostic tests and vaccines 3rd ed., Paris, pp. 207-218.

BIDOVEC, A., Z. ŽELEZNIK, A. TOMAŠIČ (1993): Uporaba peroralne vakcinacije za preprečevanje širjenja stekline v Sloveniji. Zbornik. Prvi slovenski veterinarski kongres, Portorož, 18-20 nov., pp. 465-471.

CHARLTON, K. M. (1991): The pathogenesis of rabies. In: Rabies. (Campbell, J. B., K. M. Charlton, Eds.) Boston, Kluwer Academic Publisher. pp. 101-150.

GRIFFIN, C.W. (1984): Performance evaluation critique. Fluorescent rabies antibody test 1983-1984. Centers for Disease Control, Atlanta, pp. 2-4,

HEMACHUDHA, T. (1989): Rabies. In: Handbook of clinial neurology. Viral diseases. (Vinken, P. J., Ed.) New York, Elsevier Science Publishing, pp. 383-404.

HOSTNIK, P., A. BIDOVEC, J. GROM (1999): The decrease of rabies cases established in Slovenia. Vet. nov. 25, 301-308.

IWASAKI, Y., H. F. CLARK (1977): Rabies virus infection in mouse neuroblastoma cell. Lab. Invest. 36, 578-584

KOPROWSKI, H. (1996): The mouse inoculation test. In: Laboratory techniques in rabies. (Meslin, F. X., M. M. Kaplan, H. Koprowski, Eds.), 4th ed. Genova, WHO, pp. 80-87.

KULONEN, K., E. NEUVONEN, M. TOPI (1991): Methods for detecting artic rabies virus in dilution series of field samples. J. Virol. Meth. 32, 149-155.

LEPINE, P., A. ATANASIU (1996): Histopathological diagnosis. In: Laboratory techniques in rabies. (Meslin, F. X., M. M. Kaplan, H. Koprowski, Eds.), 4th ed. Genova, WHO, pp. 66-79.

PASTORET, P., B. BOLUANGER, B. BROCHIESR (1985): The rabies situation in Europe. In: The Veterinary Annual 35. (Mary-Elizabeth Raw, T. J. Parkinson, Eds.), Blackwell Sciences, pp. 1-17.

RUDD, J. R., C. V. TRIMARCHI (1987): Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of street strain rabies virus. J. Clin. Microb. 25, 1456-1458.

TEPSUMETHANON, V., B. LUMLERTDACH, C. MITMOONPITAK, R. FAGEN, H. WILDE (1997): Fluorescent antibody test for rabies: prospective study of 8987 brains. Clin. Infect. Dis. 5, 1459-1461.

TORDO, N., O. POCH (1988): Structure of rabies virus. In: Rabies. (Campbell, J.B., K. M. Charlton, Eds.). Boston, Kluwer Academic Publishers, pp. 25-45.

UMOH, K., C. BLENDEN (1983): Comparison of primary skunk brain and kidney and raccon kidney cells with established cell lines for isolation and propagation of street rabies virus. Infect. Immun. 41, 1370-1372.

VAN REGENMORTEL, M. H. V., C. M. FAUQUET, D. H. L. BISHOP, E .B. CARSTENS, M. K. ESTES, S. M. LEMON, J. MANILOFF, M. A. MAYO, D. J. McGEOCH, C. R. PRINGLE, R. B. WICKLER: Virus Taxonomy: Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses, edited by Virology Division, International Union of Microbiological Societies, Academic Press, San Diego, 2000, pp. 563-583.

WEBSTER, W. A., G. A. CASEY (1996): In: Laboratory techniques in rabies. (Meslin, F. X., M. M. Kaplan, H. Koprowski, Eds.), 4th ed., WHO, Genova, pp. 96-104.

WHITBY, J. E., P. R. HEATON, H. E. WHITBY, E. OSULLIVAN, P. JOHNSTONE (1997): Rapid detection of rabies and rabies related viruses by RT-PCR and enzyme-linked immunosorbent assay. J. Virol. Meth. 69, 63-72.

WIKTOR, T. L. (1996): Tissue culture method. In: Laboratory techniques in rabies (Meslin, F. X., M. M. Kaplan, H. Koprowski, Eds.), 4th ed., WHO, Genova, pp. 101-123.

Received: 17 April 2000
Accepted: 20 April 2001

HOSTNIK, P., M. ŠTRANCAR, D. BARLIČ-MAGANJA, J. GROM: Sumnjivi i nepodudarni rezultati u dijagnostici bjesnoće testom imuno-fluorescencije. Vet. arhiv 71, 65-73, 2001.

SAŽETAK

Na prisutnost antigena virusa bjesnoće bilo je pretraženo ukupno 7339 uzoraka mozgova lisica, mačaka, goveda, ovaca i drugih vrsta životinja. Rezultate izravnog imunofluorescentnog testa vrednovala su dva stručnjaka. Uzorci koji su u testu imunofluorescencije davali sumnjive rezultate, ili se rezultati stručnjaka nisu slagali bili su pretraženi izdvajanjem virusa na kulturi stanica. Rabljena je stanična linija neuroblastoma miševa. Dvadeset osam uzoraka bilo je proglašeno sumnjivim na bjesnoću. Rezultat prvog mikroskopskog pregleda u devet uzoraka bio je pozitivan, a drugog negativan. Od ukupno 37 uzoraka, koji su bili ponovno pretraženi izdvajanjem virusa, 17 je bilo pozitivnih na bjesnoću.

Ključne riječi: bjesnoća, izravna imunofluorescencija, stanična kultura