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Development of an immunoenzyme test system for diagnosis of rabies



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ABSTRACT

Rabies remains one of the most serious and pressing problems for veterinary science and medicine. According to the data of Rosselkhoznadzor Information and analytical center, the Russian Federation has been an area of natural focal endemicity for many years [13]. An important stage in the control and eradication of rabies in its laboratory diagnosis by detection of virus antigen in the pathological material. The aim of the study was to design an enzyme-linked immunosorbent assay (ELISA) test kit intended for the laboratory diagnosis of rabies in agricultural, domestic, and wild carnivores. Obtaining specific components for the kit, the GNKI "Sheep" master seed strain of rabies virus, the standard CVS strain of rabies virus, and white mice of the BALB line were used.

In order to obtain high-activity of specific immunoglobulins (Igs), the processes of antirabies serum production, immunoglobulin isolation, and purification have been improved as well as the conditions for immunoglobulin conjugation with peroxidase. It was found that the binding ratio should not be lower than 0.45–0.6.

A method of inactivation of rabies virus antigen has been developed, which eliminates the risk of being infected for laboratory workers and shows high specific activity in serological tests. The optimal concentrations of the reactants (immunoglobulin, antigen, conjugated) were determined; basic conditions for conducting the reaction were identified (pH of the carbonate/bicarbonate coating buffer, the optimum temperature, and duration of incubation) that ensure the required level of accuracy and sensitivity of the method.

As a result of the conducted studies, a "Kit of preparations for the laboratory diagnosis of animal rabies in animals by ELISA method" was developed. The correlation between results obtained by the immunofluorescence (IF) method and the enzyme-linked immunosorbent assay (ELISA) methods was determined; the compliance coefficient constituted 98.8%, the sensitivity threshold of ELISA method being 3.3 Ig LD₅₀ / ml. The sensitivity, specificity, and reproducibility of the developed test system were determined through laboratory and production tests.

Keywords: ELISA, IF, rabies, diagnosis

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INTRODUCTION

Animal rabies is one of the most important international criteria (WHO, FAO, OIE) for the assessment of the biological and ecological safety of the human environment.^{1,12}

Rabies is a highly dangerous 100% fatal disease which occupies a significant position among pathogenic infections affecting humans and animals both in the Russian Federation and throughout the world. Every year more than 10 million people around the world get various injuries inflicted by animals, and more than 4 million people receive specific anti-rabies treatment. Approximately 50,000 people die after being bitten by rabid animals worldwide annually. Therefore, eradication of rabies remains a big problem for human and veterinary medicine.⁶

According to the World Health Organization, rabies is among five infectious diseases common to humans and animals which cause the most economic damage resulting from losses due to work disability and mortality in the human population, animal deaths, reduced agricultural sector

productivity, costs of quarantine and preventive measures, trapping of stray dogs and cats, regulation of wildlife predator populations, conducting diagnostic tests, and the medical treatment of people who have been exposed to rabid animals.^{5,11}

In order to implement anti-epizootic measures and prescribe post-exposure rabies vaccination to people affected by animal bites, the quick and accurate diagnosis using modern laboratory testing methods is very important. At present, various methods are applied in practice: bioassays with the use of laboratory animals, morphological study, immunofluorescence method, and the precipitation reaction in agar gel.^{2,3} However, all of these methods, to a varying extent, have significant limitations: low sensitivity and insufficient specificity (light microscopy and the precipitation reaction), the considerable time is taken to obtain test results, and the labor input (bioassay and neutralization reaction performed on white mice). Therefore, there is an increasing need for improving existing

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methods and developing rapid test methods and efficient diagnostic tools for this zoonosis.^{8,9,10}

MATERIALS AND METHODS

The GNKI "Sheep" fixed master seed strain of rabies virus and the standard CVS strain of rabies virus were used in the experiments. Obtaining specific immunoglobulins, five sheep with body weights of 40–50 kg, and 20 rabbits with body weights of 3.0–3.5 kg were used; 300 BALB line white mice with body weights of 6–7 g were used to receive control antigens.

Diagnostic studies were carried out using test kits developed by FGBNU "FTSTRB-VNIVI": "Fluorescent anti-rabies globulin" (PS 9388-012-00492374-99), "Kit of preparations for the laboratory diagnosis of rabies by enzyme-linked immunosorbent assay (ELISA)" (PS 9388-012-00492374-99) registered in the Russian Federation.

In the testing process, a calibration kit of proteins for electrophoresis (Pharmacia Biotech, USA) was utilized containing phosphorylase b from rabbit muscle (94000 D), bovine serum albumin (67000 D), ovalbumin (43000 D), carbonic anhydrase of bovine erythrocytes (30000 D), soybean trypsin inhibitor (20100 D), alpha-lactalbumin (14400 D), sucrose, the blocking solution "NEWLAVBLOTIR2", 5X ("Sanofi diagnostics Pasteur, France"), and others.

The following equipment was used in the studies: a spectrophotometer SF-16; a scanning spectrophotometer "Titertek multiskan" (Switzerland); centrifuges (different kinds); a thermostat; refrigeration units; a light microscope (Biolam), a fluorescence microscope LUMAM-I2, and a pH meter.

In the course of ELISA design and testing, 250 samples of postmortem material were examined (brain, submandibular salivary glands) delivered from rabies-vulnerable areas of the subjects of the Russian Federation. Brain suspensions obtained from intact white mice (negative control antigens) and immunoglobulin (Ig) from non-immunized animal and human sera served as controls in the experiments. Brains cells from white mice infected with rabies virus (GNKI "Sheep" strain) served as positive control antigens. Brains from mice infected with the Aujeszky's disease virus (VGSKI strain) were used to obtain the heterologous antigen. All control and test samples were prepared as a 10% suspension from the brain or submandibular salivary glands with 0.85% saline solution supplemented with penicillin and streptomycin. Antigens had been previously clarified by centrifugation at 800g for 15 min and inactivated at 56°C for 30 min.

To obtain and purify the antigen, the method of viral material accumulation was applied by intracerebral administration of a 10% virus-containing suspension to outbred white mice with body weights of 4–6 g (1,000 animals), and white rats with body weight up to 40 g (10 animals). In order to receive cultural rabies virus (GNKI "Sheep" strain), RGGN-1 cell culture was infected by introducing 10% virus-containing suspension made from white mice brain to the cell culture suspension at a concentration of 5×10^5 cells / ml in a 1:1 ratio.

The liberation of viral material from cell debris was performed by the method developed by N. A. Khismatullina and R. H. Yusupov (1998), with consequent purification in linear sucrose density gradients (15–50%) according to B. Dietzschold (1996).

The degree of purification of the rabies virus and its protein fractions was examined by disc electrophoresis in 12.5% polyacrylamide gel (PAG) in the presence of sodium dodecyl sulfate by the method of U.K. Laemmli (1970) and B. Dietzschold (1996). A set of proteins of standard molecular weight (94.0, 67.0, 43.0, 30.0, 20.1, and 14.4 kDa) served as control. The specificity of protein fractions was detected by immunoblotting according to H. Towbin, T. Staehelin, and J. Gordon (1979) with the use of mouse anti-rabies polyclonal serum and the conjugate diagnostic antibodies against peroxidase-labeled mouse immunoglobulin produced by N.F. Gamaleya Research Institute of Epidemiology and Microbiology (Moscow).

The digital data received in the course of experiments were processed by the method of variation statistics using the Microsoft Excel 2000 software package. All experiments were carried out with the number of retries assuring the acquisition of reliable results.

RESULTS AND DISCUSSION

The effectiveness of enzyme-linked immunosorbent assay for detecting rabies virus antigen largely depends on the quality of conjugate. To prepare anti-rabies immune enzyme conjugate, 240 sets of experiments were conducted using Ig with different titers of specific antibodies.

The study showed that the activity of the conjugate is primarily determined by that of the specific Ig used for conjugation with peroxidase. It was established that the Ig activity expressed in the titers of complement-fixing antibodies, should be at least 1:320. To obtain highly active specific Ig, the method for preparation of anti-rabies blood serum of sheep has been improved. The method involves the use of an organosilicon liquid, polyethylsiloxanes (PES-3),

in the composition of the oil adjuvant for conducting sheep immunization with rabies virus, which makes it possible to increase its neutralizing activity 4 times as compared to the prototype, incomplete Freund's adjuvant (IFA), and 2 times as compared to the analogue (dextran sulfate).

The conjugate quality also depended on the Ig purity degree of Igs used for conjugation with peroxidase. Based on the outcomes of testing anti-rabies Igs isolated by ion exchange chromatography in DEAE-cellulose or by gel filtration in

Sephadex G-200 and globulins obtained through precipitation by ammonium sulfate or PEG 6000, the advantage of the first method was determined: ensuring the maximum activity of conjugate (1: 1600, $K_{sp} = 17.8 \pm 0.2$) and therefore a high sensitivity degree of ELISA method. The quality of conjugate was furthermore dependent on the optimal ratio between peroxidase and immunoglobulin (binding coefficient). It was found that the binding coefficient should be in the range 0.45–0.6.

Further research was aimed to develop a method for producing a rabies virus non-infectious antigen eliminating the risk of being infected for laboratory workers and having high specificity in serological tests: ELISA, prolonged complement fixation test (PCFT), etc. To this end, a 10% brain suspension from mice infected with the GNKI "Sheep" rabies virus strain or culture virus-containing fluids with an infectivity titer not lower than $5.731 LD_{50} / 0.01$ ml. Acrolein at various concentrations and modes of incubation with the virus was used as a test inactivator. Phenol served as a prototype; thermal exposure served as analog (Table 1).

The conducted studies resulted in determining the optimal concentration of acrolein allowing for efficient inactivation of rabies virus at 22°C for 1 hour with a constituent of 0.5%, and at 37°C for 30 minutes with a constitute of 1%. The specified method allows for obtaining antigen of high titers in ELISA (1: 3200–1: 6400), in contrast to the prototype and analog - 1: 800–1: 400. Therewith, the titer of rabies virus in ELISA before its exposure to inactivators was 1: 3200.

The inactivation of rabies virus antigen by acrolein enables its use in RCFT as well due to the fact that the antigen does not have anticomplementary properties after inactivation.

Subsequently, the optimal concentrations of reactants were identified (immunoglobulin, antigen, conjugate), and the basic reaction conditions were determined (pH of carbonate/bicarbonate coating buffer, the optimum temperature, and incubation duration) to ensure achievement of the required level of accuracy and sensitivity of the method (Table 2).

Thus, the maximum sensitivity of the direct sandwich ELISA format regarding the detection of rabies virus antigen can be achieved under the following conditions: sensitization of the plate by specific immunoglobulin for 18 hrs at 4°C at a concentration of protein of 10 mcg/ml, with the optimum pH of 0.01 M carbonate/bicarbonate buffer (CBB) accounting 9.6, and the incubation of test antigen and conjugate for 1 hour at + 37°C. The examination of the stability of Ig immobilized on the plate indicated that Ig retains its activity for 6 months (observation period) at + 4°C. The use of a pre-sensitized Ig plate reduced the reaction time to 3–4 hours without any loss in the sensitivity of the method.

A strong correlation between the results of IF and ELISA methods was established by testing brain samples obtained from different wildlife animals. The coefficient of concordance was 98.8%. The threshold for detection was 3.3 Ig LD_{50} / ml for ELISA method and 3.81 Ig LD_{50} / ml for IF method. A simultaneous use of these two rapid diagnostic methods, ELISA and IF, allows for higher diagnostic accuracy, in particular, if one of these tests provides a questionable result. Furthermore, visual estimation of test results in ELISA does not require specific equipment which enables the use of the method under field conditions and in laboratories which are not equipped with fluorescent microscopes.

CONCLUSION

As a result of the studies conducted, a kit of preparations for laboratory diagnosis of rabies by enzyme-linked immunoadsorbent assay (ELISA)

Table 1 Conditions of rabies virus inactivation (n = 5)

Conditions of inactivation	Inactivator			
	Acrolein	Acrolein	Formalin	t, °C
t, °C	22°C	37°C	37°C	56°C
%	0.5%,	1%	0.5%,	-
T,h	1 h	0.5	1 h	0,5
Virus titer in ELISA	3840.0±715.5	3520.0±876.3	880.0±219.1	800.0±244.9

Table 2 Optimal test conditions

Indicators	Parameters
Working dilution for immunoglobulins	1:3200
Working dilution for positive and negative control antigens	1:10
Conditions for sensitizing the plate by specific immunoglobulin	18 hrs at +4°C
Conditions for incubation of antigen under study	1 h at +37°C
Conditions for conjugate incubation	1 h at +37°C

method was developed. Laboratory and production tests of the designed ELISA test system indicate that according to its characteristics the test system is suitable for intended use, comparable to the method of similar purpose, as well as sensitive, specific and reproducible.

REFERENCES

1. Arai, Y.T. (2005) Epidemiology of rabies virus and other lyssavirus. *Nippon Rinsho* 12: 2167–2172.
2. Bingham, J., Mlambo P. (1995) Ante-mortem diagnosis of human rabies by the skin biopsy technique: three case reports from Zimbabwe. *J. Med.* 41: 258–260.
3. Cliquet F, McElhinney L., Servat A. (2004) Development of a qualitative indirect ELISA for the measurement of rabies virus - specific antibodies from vaccinated dogs and cats. *Viol. Methods* 117: 1–8.
4. Dietzschold B. (1996) Techniques for the purification of rabies virus, its subunits and recombinant products. Geneva.
5. Franka, R., Svrcek S., Madar M. (2004) Quantification of the effectiveness of laboratory diagnostics of rabies using classical and molecular-genetic methods. *Veter. Med.* 49: 259–267.
6. Khismatullina N.A., Yusupov R.H., Ivanov A.V., Chernov A.N., Zaydullin M.Sh. (2006) Epizootic situation and fox rabies prophylaxis in Tatarstan. *Dev.Biol.* 125: 41–49.
7. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage ac T4. *Nature* 227: 680–685.
8. Madhusudana S.N. (2004) Rapid diagnosis of rabies in humans and animals by a dot blot enzyme immunoassay. *Int. J.Infect. Dis.* 8: 339–345.
9. McElhinney L.M. (2008) Diagnostic tools for the detection of rabies virus. *EJCAP* 18: 224–231.
10. Sissoeff L., Mousli M., England P. (2005) Stable trimerization of recombinant rabies virus glycoprotein ectodomain is required for interaction with the p75NTR receptor. *Gen. Virol.* 86: 2543–2552.
11. Khismatullina N.A., Gulyukina A.M., Sabirova V.V., Gafarov A.Z., Elakov A.L. (2012) Development and application of the block - ELISA test system to monitor the effectiveness of rabies vaccination. *Veterinary Medicine* 96: 64–66.
12. Chernov A.N. (2013) Epizootological monitoring and diagnostic tools to develop rabies, Aujeszky's disease and brucellosis. Ufa.
13. Chernyshova E.V., Nazarov N.A., Metlin A.E. (2013) Epizootic situation on rabies in Russia and analyze the effectiveness of anti-rabies vaccination in animals exported abroad. *Veterinary today* 4: 49.



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