

Obtaining Rabies Virus Purified Antigen

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Abstract

Aim: Currently, in connection with the deterioration of the epizootic situation of rabies in the world, the question of improving the means of routine laboratory diagnostics of rabies remains highly controversial to obtain results of a high degree of reliability. Especially in demand diagnostic rapid test systems based on enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IFA) methods, the sensitivity and specificity of which depend on the degree of purification of antigenic components. **Materials and Methods:** In this article, a method is given for obtaining a rabies antigen and the results of an experimental study of the activity of its highly purified fractions were obtained by separation in a stepwise gradient of sucrose (20–60%) by ultracentrifugation at 30,000 g. **Results:** The study of electrophoretic profiles of antigenic fractions in 12.5% polyacrylamide gel showed that the major polypeptide fractions are located in the range of 44.6–70.0 kDa. According to the results of the tests in ELISA and IFA, Fraction 1 was the most active. We optimized the conditions for the formulation of the reactions, and the optimal working concentrations were determined. **Conclusion:** The revealed immunochemical characteristics of this fraction will allow using it not only as an antigenic component for diagnostic test systems but also as an immunizing material for obtaining highly specific rabies immunoglobulins.

Key words: Electrophoresis, enzyme-linked immunosorbent assay, immunoblot, rabies antigen, rabies virus

INTRODUCTION

The spread of rabies - acute lethal anthroozoonosis - among animals is one of the most important international criteria for assessing the biological and ecological safety of human habitat. In the world of rabies, from 55 to 70 thousand people die each year, and up to 6.5 million people turn for post-exposure care.^[1,2] Rabies is one of the most dangerous virus diseases common to humans and animals, causing the greatest social and economic damage. Given the exceptional nature of rabies with the inherent characteristics of many classic infectious diseases - the world nosoareal, natural foci, extreme contagiousity, vector transmission by carnivores, the susceptibility of most animal species, social and economic significance, the disease is included in the list of, especially, dangerous infectious diseases of the OIE (World Organization for Animal Health, 2015).^[3] Russia occupies a dominant position both in terms of the number of registered foci of furiousness and in the incidence of animals; in the past 20 years,

the highest death rate among people from rabies among developed countries has been recorded. Despite the anti-epizootic measures being carried out, natural foci of rabies intensified on the territory of Russia, the incidence of wild carnivores increased, and the animals are intensely involved in the epizootic process, creating a threat to the population, which predetermines the modern features of the epizootic disease and the species composition of the diseased animals. To date, the problem of fighting rabies is one of the most urgent due to the widespread prevalence of the disease and the lack of specific treatment.^[4]

The most significant means of controlling the spread of rabies along with specific prevention are accurate and reliable

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Received: 22-11-2018

Revised: 10-12-2018

Accepted: 15-12-2018

laboratory diagnosis.^[5] Modern laboratory diagnostic tools have a set of diagnostic tests, such as indirect hemagglutination test, neutralization reaction, complement fixation test, indirect immunofluorescence (IFA) reaction, diffusion precipitation reaction,^[6] however, in screening studies, they have a number of drawbacks due to their length and laboriousness. To solve such problems, enzyme-linked immunosorbent assay (ELISA) methods and their various variants have been developed, on the basis of which test systems for the quantitative determination of antibodies to the rabies virus in blood sera have been designed;^[7] however, not all of them differ in a sufficient sensitivity and specificity, which is determined by biological properties of antigens used in them.

In this regard, one of the most important tasks of preventing rabies is to improve the methods for isolating the highly purified antigen of the rabies virus for further use in the development of diagnostic tests. The use of improved methods for the purification of viruses implies an increase in the yield of virus mass, preservation of its serological activity, production of highly purified discrete, and monomeric structures of the pathogen without foreign impurities, with constant monitoring of each stage of purification by analytical electrophoresis, immunoblotting, and ELISA using hyperimmune blood sera of rabbits and sheep.

Thus, the aim of this study was the experimental preparation of highly purified fractions of the rabies virus antigen.

MATERIALS AND METHODS

As the primary viral material for purification, a 20% brain suspension of a 2-month-old lamb infected with an intracerebral production strain of the rabies virus “Ovechiy” GNKI (infectious titer $10^{5.25}$) was used. The suspension was preliminarily subjected to a single homogenization on a Fast-Prep 24 (MP Biomedicals) apparatus for 20 s in the presence of uniform silicon carbide particles. Deposition of the suspension was carried out on an Eppendorf® 5804 R centrifuge at 4200 g for 60 min.

The resulting supernatant was ultracentrifuged on an ultracentrifuge Optima L-90K (Beckman) at 37,000 g for 90 min, after which the precipitate formed was dissolved in 0.02 M Tris-HCl. Purification of the resulting virus-containing material was carried out by settling and floating density separation in a stepwise sucrose gradient of 20–60% on an Optima L-90K ultracentrifuge (Beckman) at 30,000 g for 240 min followed by gel filtration on an NGC discovery device (Bio-Rad) using the ENrich™ SEC 70 column. The purity of the isolated antigens was monitored by electrophoresis. Electrophoresis was performed in 12.5% polyacrylamide gel (PAG) in the presence of 0.1% sodium dodecyl sulfate according to Laemmli,^[8] followed by staining with a Coomassie G-250 colloid solution (DIA-M LLC). Electrophoresis was performed in a chamber for

vertical electrophoresis Mini-PROTEAN® Tetra cell (Bio-Rad) at a constant voltage of 210 V for 60–80 min. The results of electrophoresis were recorded on a Gel Doc XR + (Bio-Rad) instrument and processed using Image Lab Software 6.0. Transfer to a nitrocellulose membrane (Supported nitrocellulose membrane 0.45 μ m, Bio-Rad) was performed according to Towbin.^[9] Immunoblotting was performed using hyperimmune sera of rabbits (titer 1:12800).

The activity and specificity of the antigenic fractions most satisfying the requirements were evaluated in ELISA^[10] and IFA^[11] using certified diagnostic kits.

A 96-well polystyrene plate was used as the solid phase for the ELISA formulation. The antigens were tested at concentrations of 5, 10, 20, 30, and 40 μ g/ml, positive control was hyperimmune sheep serum (titer 1:12800), and negative - serum from intact sheep, in a 1:800 dilution. The peroxidase rabies conjugate (Federal Center for Toxicological, Radiation and Biological Safety [FCTRB-VNIVI], Kazan, Russia) was used in a 1:3200 dilution. The instrumental recording of the results of the reaction was carried out on a Bio-Rad 680 spectrophotometer at a wavelength of 490 nm.

Direct IFA with rabies virus antigen fractions was performed according to the instructions for the use of the “Set for laboratory rabies diagnosis by IFA” (FCTRB-VNIVI, Kazan, Russia). The colored prints were viewed in the field of view of the Nikon TS100 (Japan) fluorescent microscope.

The obtained numerical results were subjected to statistical processing using the STATISTICA 12.6 software package (StatSoft).

RESULTS AND DISCUSSION

As a result of the separation of the virus-containing material in the preformed sucrose gradient at 30,000 g, four opalescent rings forming the zones of sucrose solutions were visually noted, from which four main fractions were conventionally designated Fraction 1 (zone 20–30%), Fraction 2 (zone 30–40%), Fraction 3 (zone 40–50%), and Fraction 4 (zone 50–60%). The most intensive opalescence was observed in the zone of 20–30%. The results of electrophoretic analysis of the raw materials and isolated antigenic fractions [Figure 1a] were confirmed by immunoblot results using hyperimmune rabbit sera [Figure 1b].

Based on the electrophoretogram and the results of the immunoblot, the starting materials (brain suspension and supernatants after low-speed centrifugation) are characterized by heterogeneity and a significant admixture of ballast proteins. Antigenic samples obtained by separation in a graded sucrose gradient are distinguished by a much greater purity and differentiation of the zones of polypeptide fractions. Thus, the greatest activity is concentrated in the ranges of

molecular masses of 44.6–70.0 kDa, and also additional activity was detected in the range of 28.8–31 kDa, which is evident from the results of densitograms [Figure 2a-d].

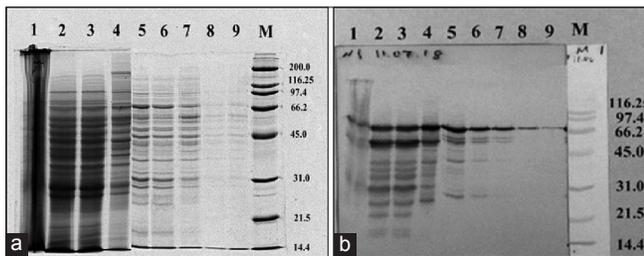


Figure 1: Analysis of activity of original material and antigenic fractions in: (a) electrophoresis in 12.5% polyacrylamide gel (Coomassie staining); 2 - Immunoblot with hyperimmune rabbit serum: 1 - original cerebral suspension; 2 - supernatant after sedimentation (4200 g); 3 - supernatant after double sedimentation (37,000 g); 4 - viral material before stratification in sucrose gradient; 5 - Fraction 1 (20–30% sucrose zone); 6 - Fraction 2 (30–40% sucrose zone); 7 - Fraction 3 (40–50% sucrose zone); 8 - Fraction 4 (50–60% sucrose zone); 9 - Fraction 5 (under 60%); M - marker of molecular masses Broad Range (Bio-Rad)

The next stage of our study was the determination of the serological activity of the investigated fractions in ELISA using hyperimmune and negative sheep serum [Table 1] to determine the optimum concentration for sorption of the material on the plates.

Based on the data of Table 1, the antigenic fraction 1 from the sucrose zone of 20–30% shows the greatest serological activity.

Thus, its activity at a concentration of 10 µg/ml 1.3 times ($P < 0.001$) exceeds that of the starting material and is 1.033 ± 0.011 OD, which is the optimal indicator for the activity of the component. This is because the range of location of its major polypeptide constituents is localized in the zone corresponding to the location of the nucleocapsid N-protein of the rabies virus, as well as a smaller content of secondary proteins. Significant differences in the optical density of the positive and negative control serum were obtained by studying the activity of the antigenic fraction 2 from the sucrose zone of 30–40% at a concentration of 20 µg/ml. Fractions 3 and 4 differ significantly in their activity, which is related to the corresponding distribution of the viral material along the floating density in the sucrose gradient.

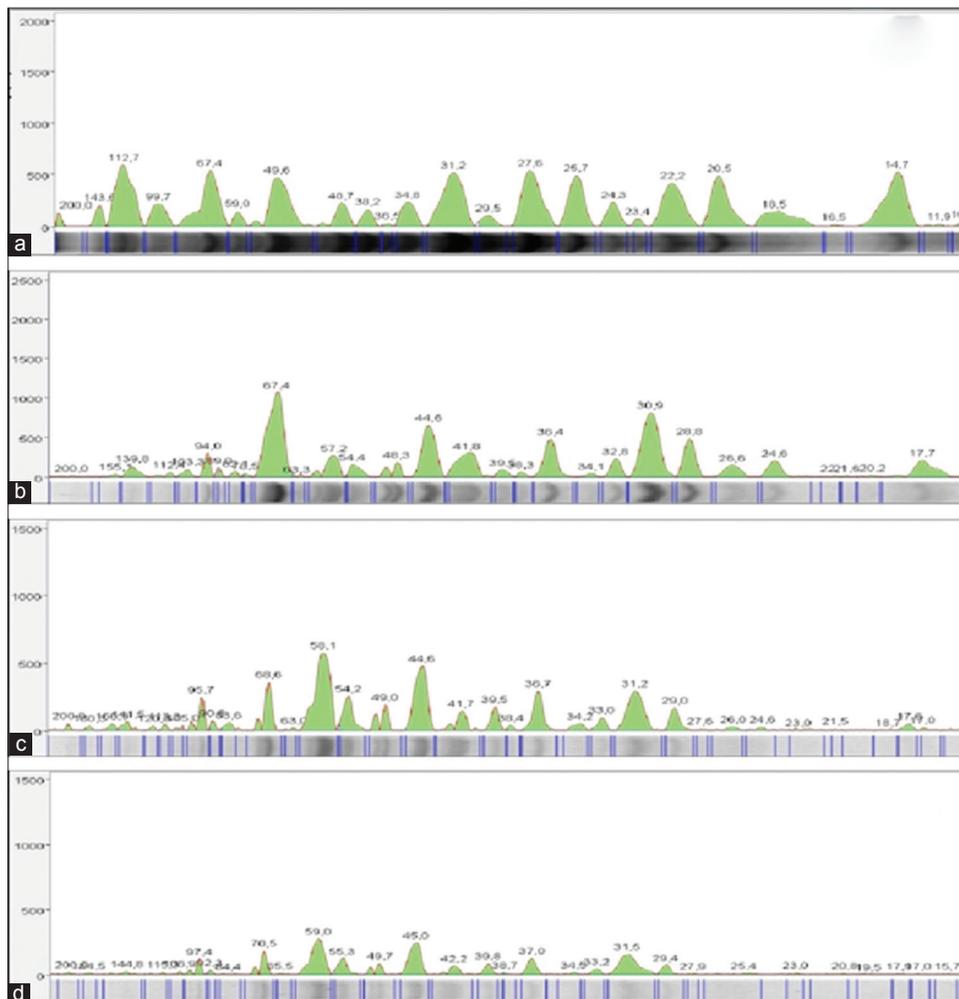


Figure 2: (a) - Viral material before stratification in sucrose gradient; (b) - Fraction 1 (20–30% sucrose zone); (c) - Fraction 2 (30–40% sucrose zone); (d) - Fraction 3 (40–50% sucrose zone)

Table 1: Serological activity of original viral material and purified antigen fractions in ELISA

Antigen fraction	Protein concentration, mcg/ml	Optical density at the wavelength 490 nm in the highest dilution (OD)	
		Hyperimmune sheep serum (1:6400)	Negative sheep serum
Viral material before stratification in sucrose gradient	5	0.522±0.006	0.058±0.002
	10	0.792±0.007	0.079±0.003
	20	0.837±0.005	0.136±0.004
	30	0.882±0.009	0.272±0.003
	40	0.967±0.012	0.311±0.004
Fraction 1	5	0.722±0.008	0.069±0.006
	10	1.033±0.011	0.072±0.005
	20	1.092±0.009	0.072±0.007
	30	1.224±0.008	0.077±0.006
	40	1.199±0.012	0.079±0.002
Fraction 2	5	0.671±0.006	0.081±0.002
	10	0.798±0.009	0.112±0.003
	20	0.961±0.011	0.178±0.003
	30	0.984±0.008	0.214±0.005
	40	1.057±0.0012	0.281±0.006
Fraction 3	5	0.354±0.007	0.058±0.003
	10	0.387±0.009	0.060±0.002
	20	0.418±0.008	0.082±0.003
	30	0.567±0.011	0.101±0.005
	40	0.681±0.016	0.154±0.006
Fraction 4	5	0.237±0.008	0.053±0.002
	10	0.288±0.006	0.082±0.003
	20	0.302±0.012	0.093±0.003
	30	0.416±0.010	0.137±0.002
	40	0.506±0.013	0.198±0.006

ELISA: Enzyme linked immunosorbent assay, OD: Optical density

Table 2: Activity and specificity of original viral material and purified antigen fractions in IFA

Antigen fraction	Titer in IFA with control fluoresceing antirabic globulin*/Intensity of glow
Viral material before stratification in sucrose gradient	64/3+
Fraction 1 (20–30% sucrose zone)	128/4+
Fraction 2 (30–40% sucrose zone)	64/3+
Fraction 3 (40–50% sucrose zone)	4/4+
Fraction 4 (50–60% sucrose zone)	4/3+

*Inverse values of antigen titers, IFA: Immunofluorescence

The determination of the activity and specificity of the antigens obtained was confirmed in IFA with control fluorescent and control negative rabies antibodies [Table 2]. For the optimal result, a luminescence was taken, visually estimated at 3–4 crosses.

Thus, the antigenic fractions 1 and 2 show the greatest activity in IFA, while dilutions of 1:64 and 1:32 were established

as the working titer for a clear visualization of the reaction results, respectively.

SUMMARY

In the course of the research work, we made the following conclusions:

1. The expediency of separation of a virus-containing material in a graded sucrose gradient (20–60%) was considered, which made it possible to selectively isolate four antigenic fractions of the rabies virus. By means of electrophoresis in PAG, the main range of localization of major polypeptide fractions was determined, which was 44.6–70.0 kDa.
2. As a result of analysis of the activity and specificity of the obtained fractions by methods of electrophoresis in PAG, immunoblot, ELISA, and IFA, the antigenic fraction 1, selected from the sucrose zone of 20–30%, was identified as the most active.
3. Optimum concentrations of Fraction 1 for routine laboratory diagnostics of rabies were determined. For ELISA, this was 10 µg/ml, which corresponded to 1.033 ± 0.011 OD; as a working caption for IFA, giving a visual result of the highest intensity is defined as 1:64.

CONCLUSION

Thus, the results of our research are aimed at finding a technological solution for obtaining highly purified and highly specific antigens of the rabies virus. The optimal conditions were selected by experimentally alternating low-speed centrifugation and ultracentrifugation schemes, forming a graded sucrose gradient, chromatographic purification conditions, and testing the obtained preparations at different concentrations using diagnostic kits based on the ELISA and IFA methods. The immunochemical characteristics of the final preparation revealed by us satisfy the requirements for the antigen components of the test systems. In the long term, the technology will be adapted to the production of this antigenic fraction on a production scale, which will significantly improve the reliability of the results obtained with the use of improved test systems.

Moreover, this component is a potential immunizing material for obtaining highly specific immunoglobulins for specific major polypeptide fractions.

Bioethics

All stages of the study were carried out in accordance with the ethical requirements for conducting animal experiments, all protocols for the use of laboratory animals were approved by the Ethical Committee of the Kazan State Medical University for the Protection of the Rights and Use of Animals for Experimental Purposes (No. 9–2013).

ACKNOWLEDGMENTS

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

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Source of Support: Nil. **Conflict of Interest:** The authors confirm the absence of a conflict of financial/non-financial interests related to the writing of the article.