Brief information about the project

Name of the project	AP14870256 «Development novel nanobodies for efficient neutralization and as specific and sensitive probe for rapid detection rabies virus»
Relevance	Rabies occurs worldwide in a variety of reservoir animals and is known to be a deadliest viral infection with nearly 100% fatality after symptom onset. Globally, rabies is still endemic in over 150 countries and territories causing an estimated 8.6 billion USD worth of economic loss and contributing to over 60,000 human deaths annually. The danger of rabies lies in the fact that there are no effective treatment and the disease usually leads to death. Human rabies presents a serious public health threat in Kazakhstan. Previous published data suggested that between 2007 and 2011, 44 cases of human rabies were recorded or a mean of 9 cases per year. The incidence of dog bites was reported as 3700 in 2010 and 4130 per million population in 2011. Post exposure prophylaxis was given to 57,000 individuals in 2009, 59,000 in 2010 and 67,000 in 2011. The economic costs of this disease in Kazakhstan exceed US $20 million per year. The problem of rabies in Kazakhstan remains unresolved, natural foci of the disease are constantly recorded, which requires an increase in the effectiveness of rabies prevention and control measures. The main way to combat rabies is specific prophylaxis with the use of vaccines and anti-rabies immunoglobulin (RIG). RIG is obtained from the sera of horses (ERIG), or humans (HRIG) immunized with rabies vaccine. However, due to adverse effects, ERIG are now used under the form of pepsin-digested Fab-fragments. The production of HRIG, however, requires great number of immune donors and raises concerns about the transmission of infectious agents. Alternative approaches using human monoclonal antibodies are being widely studied. However, antibodies produced in this way are expensive and require a long production time and are less effective due to low tissue permeability. The worldwide shortage and the high costs make these products poorly available, the reason why the WHO recommends developing alternatives. Therapy with nanobodies is now considered a very promising alternative for the treatment of dangerous viral infections. Nanobodies are the smallest functional fragments (15kDa) of heavy chain-only antibodies naturally occurring in *Camelidae* and represent the antigen-binding variable domain. Nanobodies have several advantages, including cost-effectiveness and ease of production in large quantities in bacteria, good solubility, resistance to significant temperature and pH fluctuations, and better penetration into tissue under *in vivo* conditions. Based on these features, nanobodies become a more promising tool for diagnosing and treating various diseases, including rabies, compared to conventional antibodies. Currently available tests for the detection of rabies virus (RABV) include virus isolation, ELISA, diffusion precipitation test, fluorescent antibody test. However, these methods are quite time consuming and are carried out using primary and secondary antibodies to which enzymes such as horseradish peroxidase are chemically bound, which ultimately reflected in the high cost of these

	test systems. Traditional antibodies have several disadvantages due to their limited quantity, difficulties with permanent storage, and the need to use secondary antibodies, as well as the problem of delivery to endemic areas.
Purpose	Development of novel nanobodies for the efficient neutralization of RABV. Engineering highly specific and sensitive nanobody-derived probe for rapid detection RABV in the immunoassays.
Objectives	1. Expression of rabies virus G protein as correctly folded recombinant protein and evaluation of antigenicity of the expression product. 2. Construction of a cDNA-VHH library by phage display methods and selection of RABV-G protein specific nanobodies. 3. Study of RABV neutralization efficacy of mono-, bi- and trivalent RABV specific nanobodies on mice challenged by a lethal dose of RABV.
Expected and achieved results	Based on the results of the first two years, a computer analysis of the extracellular domain of the G-protein of the rabies virus (RABV-G) was carried out. The G protein was optimized by replacing hydrophobic amino acids with linkers (G4S) between amino acid residues 73–79 and 117–125, and codons and GC content were optimized for optimal expression in yeast. The cDNA gene ExRABVG-GS, encoding RABV-G with a signal peptide at the N-terminus and c-Myc, 6xHis tags at the C-terminus, was cloned into the pBluescript II SK (+) vector. A yeast pGAPZα/ExRABVG-GS vector was constructed containing a signal peptide, a constitutive GAP promoter, and a cDNA gene for ExRABVG-GS. The expression and secretion of G protein by the yeast strain GS115 P. Pastoris into the culture medium was analyzed. The presence of a major protein band with a molecular weight of about 51 kDa, corresponding to the G protein, was shown by SDS-PAGE electrophoresis. Protein was purified from a three-day culture medium using affinity and ion exchange chromatography. Rabbits were immunized with the purified recombinant protein and a polyclonal antiserum was obtained containing antibodies against the extracellular domain of the G protein of the rabies virus. The titers of the obtained antiserum were determined using the indirect ELISA method. Polyclonal antibodies were precipitated and purified. The antigenic characteristics of the G-protein epitope of the rabies virus were analyzed using the indirect ELISA method using commercially available monoclonal antibodies specific to the G-protein. Subcutaneous immunization of two New Zealand white rabbits with purified recombinant ExRABVG-GS protein was carried out. The titer of polyclonal antibodies was determined by the indirect ELISA method. Antibodies were purified and concentrated by precipitation. They were further analyzed for immunogenicity using dot blot and western blot methods. Antibodies have been shown to recognize both undenatured and denatured proteins.

	Bactrian camels were immunized with the inactivated rabies virus strain CVS-11 and the recombinant ExRABVG-GS protein separately. Mononuclear cells were isolated from 150 ml of peripheral blood using Ficoll-Paque PLUS. From these, total RNA was subsequently isolated, from which cDNA libraries of nanoantibody genes were obtained. Construction of cDNA libraries encoding VHH (nanobodies) was carried out by cloning PCR products into the phagemid vector pADL-23c. E. coli TG1 transformants containing the pADL-23c/VHH vector were used to produce M13K07 phages encoding novel VHHs. Nanoantibodies specific to RABV-G were selected by bio-panning using an indirect ELISA method where the previously purified recombinant ExRABVG-GS protein and the Platelia® Rabies II kit (BioRad) were used as the coating antigen. Next, the selected nanoantibodies were sequenced, expressed in E. coli strain WK6, and His-tagged proteins were purified by affinity chromatography using the Akta start FPLC system.
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List of publications with links to them	Bayandy G.A., Akhmetsadykov N.N., Bisenbaev A.K. Molecular genetic characteristics of the rabies virus, pathogenesis and achievements in diagnosis and development of control agents [Rus: Molekulyarnaya geneticheskaya kharakteristika virusa beshenstva, patogenez i dostizheniya v diagnostike i razrabotke sredstv bor'by] // Experimental biology, - 2023. – Vol. 95, №2. – P.4-20. https://doi.org/10.26577/eb.2023.v95.i2.01