Cloning and expression of fragment of the rabies virus nucleoprotein gene in *Escherichia coli* and evaluation of antigenicity of the expression product

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Summary

Rabies virus nucleoprotein (N protein) encapsidates genomic RNA of the virus and forms the viral ribonucleoprotein complex. These N proteins represent highly organized structures which activate proliferation of B cells and production antibodies against the N protein. In addition to the B cell, the rabies virus N protein has been shown to induce potent T helper cell responses resulting in a long-lasting and strong humoral immune response. Rabies virus N protein is a molecular target of choice for development of tools to diagnose acute rabies infection. We produced a recombinant immune reactive C-terminal fragment of the rabies virus N protein which contains an antigenic determinant located between positions 360-389. Synthetic gene encoding the N protein was cloned into an expression plasmid to produce the recombinant antigen in *Escherichia coli* cells BL21 (DE3). SDS-PAGE showed presence of the product with expected molecular weight (44 kDa). The recombinant fragment of the N protein efficiently recognized antibodies in sera from mice immunized with an inactivated rabies virus. Thus produced recombinant antigen of the rabies virus N protein can be used in an enzyme-linked immunosorbent assay (ELISA) for diagnosis of the rabies infection.

Key words: Diagnostics, ELISA, Nucleoprotein, Rabies virus, Recombinant antigen

Introduction

Rabies virus belongs to the Rhabdoviridae family, genus Lyssavirus and is known for its ability to infect nervous system causing long-term injury or even death. The rabies virus causes encephalitis with severe neurological symptoms in almost all mammals and humans. Its genome is a single-stranded negative sense RNA molecule. Five viral proteins are translated from the viral antigenome (positive sense RNA), namely the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L) (Masatani *et al*., 2010).

The N protein is involved in formation of a viral capsid which is the internal component of virions comprising viral RNA. It was shown that recombinant N protein binds to various RNAs and such binding results in formation of spiral-shaped ribonucleoprotein complexes resembling the viral capsids (Guy *et al*., 2001). These particles have the same stoichiometry as the rabies virus capsids: one monomer of the N protein binds to and covers nine residues within a RNA molecule. During centrifugation in CsCl gradients these ribonucleoproteins exhibit buoyant density similar to that of the natural rabies virus' capsids (Guy *et al*., 2001). It was also shown that the N protein produced in infected cells is subject to post-translational modifications, e.g. serine residue at position 389 undergoes phosphorylation. Post-translational modifications affect formation of the ribonucleoprotein particles (Akihiko *et al*., 1999).

Although the N protein is thought to be internal component of the virion, mutations in the N protein sequence were described which influence the immunogenicity of rabies virus. Some described mutations also change pathogenicity of the virus. For example N proteins of the pathogenic strain Nishigahara and non-pathogenic strain Nishigahara-CE differ in three positions in their amino acid sequences (Phe/Leu at pos.273, Tyr/His at pos.394, and Leu/Phe at pos.395) (Masatani *et al*., 2011). In reverse genetics experiments changing the N protein sequence by introducing strain-specific residues modulated both virulence and immune response. Two positions in the N protein were found whose mutations allow the virus to block antiviral immunity: pos.273 and .394. The N protein is not superficially present on the viral particles and therefore antibodies against the N protein have no virus neutralization activity. Nevertheless these antibodies confer protective immunity. Moreover, protection against death from infection can be induced by vaccination of animals with the purified N protein (Hiroyuki *et al*., 1994).

In addition to the described properties, the N protein is highly evolutionary conserved. Genetic analysis of 30 isolates of the rabies virus from India has shown that all isolates had very similar sequences of the N protein's

gene. It was also demonstrated that the N protein has group-specific antigenic determinants characteristic to all studied rabies virus isolates and even cross-reacts with the corresponding antigen from other species of the genus Lyssavirus. Mapping with monoclonal antibodies identified a number of antigenic determinants on the N protein (Flamand *et al*., 1980). The N protein of the ERA strain has at least three immunodominant regions (Lafon *et al*., 1985) whereas four determinants (I-IV) were described on the homologous antigen of the RC-HL strain. The determinants I and IV contain linear epitopes and determinants II and III comprise several confor-mational epitopes (Minamoto *et al*., 1994). Some epitopes were precisely mapped to small sequences (10-25 residues in length) (Dietzschold *et al*., 1987).

Sequence conservatism of the N protein facilitates utilization of this viral antigen for immunochemical diagnostics of the rabies infection (Manjunatha *et al*., 2011). The N protein is produced to detectable levels early in infection, before the build-up of specific antibodies. At this stage of infection emergency diagnostics can be made by detecting the N protein in tissues. It must be noted, the N protein-based enzyme-linked immunosorbent assay (ELISA) was developed to test quality of vaccines (Katayama *et al*., 1998).

Antibodies to the N protein also have diagnostic value. In this article, we report development of the ELISA test which uses the recombinant rabies virus antigen. The recombinant antigen is a C-terminal fragment of the rabies virus N protein comprising known antigenic determinant at pos.360-389.

Materials and Methods

Bacterial strains, plasmids and primers

In this study we used *Escherichia coli* strains DH5α for genetic engineering and BL21 (DE3) for protein expression. Plasmid vector for protein expression is pET32a (Novagen). Table 1 lists PCR primers used to produce the gene encoding C-terminal fragment of the rabies virus β N protein.

Cloning and expression of the rabies virus fragment of nucleoprotein cDNA in *E. coli*

Gene encoding the C-terminal fragment of rabies virus N protein was synthesized from oligonucleotides in a constructive PCR. This method (*de novo* synthesis of DNA fragments) allows production of genes without use of the pre-existing long matrices such as viral cDNA. Used procedure is a two-round PCR. On the first round, mixture of fourteen partially overlapping oligo-nucleotides (N1-N14, Table 1) was subject to PCR amplification. No exogenous matrix was added to the first-round PCR reaction. On the second round, the PCR was performed using a pair of primers forwN and stopN (Table 1). Small volume (1 μL) of the first-round PCR reaction was added to the second round reaction mixture to serve as a matrix for amplification of the desired fragment (563 bp long). Product of the second round PCR was cloned into pGEM-T plasmid using a direct cloning kit. Inserts in plasmids from three randomly selected clones were subject to bidirectional sequencing for confirmation of the correct assembly. DNA fragment containing the gene of interest was excised from the pGEM-T clone with sequence-verified insert. The gene of interest was engineered into the expression vector pET32a under control of the T7 promoter. A pair of restrictases NcoI and XhoI and standard restriction-ligation procedures were used to assemble the expression construct. The resulting construct named pET32a/NRV encodes fusion protein, the schematic representation is: (the *E. coli* thioredoxin, Trx) − (hexahistidine tag) − (enterokinase cleavage site) − (fragment of the N protein of rabies virus). The plasmid was used to transform BL21 (DE3) to obtain the expression strain.

Cultures of the expression strain (BL21 (DE3)/ pET32a/NRV) were prepared by inoculating single colonies into 5 ml-cultures in LB broth supplemented with ampicillin (100 μg/ml) and incubating these cultures for 16 h at 37°C with shaking (250 rpm). Aliquots (100 μL) of the overnight cultures were transferred into 10 ml portions of fresh medium and incubated at 37°C to optical density $OD_{600} \sim 0.8$. Protein expression was induced by addition of the isopropyl-β-

Table 1: PCR primers used for construction of the N gene

Code	Sequence (5´→3´)
N1	GGCCATGGCGGCCGCGGGAATTCGATTGGGCCCAGGAAACCGCGGTGCCGCA
N2	GGCTTTTGCCGCTCAGGCCCAGGCTGCGAAAATGAATAAAATAGCTATGCGGCACCGCGG
N3	CCTGAGCGGCAAAAGCCCGTATAGCAGCAACGCGGTGGGCCATGTGTTTAACCTGATTCA
N4	TCAGGCTGCGCACCTGGCCCATATAGCAGCCCACAAAATGAATCAGGTTAAACACATGGC
N5	CAGGTGCGCAGCCTGAACGCGACCGTGATTGCGGCGTGCGCGCCGCATGAAATGAGCGTG
N6	GGTGCCTTTGCCAAAAAATTCTTCGCCCAGATAGCCGCCCAGCACGCTCATTTCATGCGG
N7	GAATTTTTTGGCAAAGGCACCTTTGAACGCCGCTTTTTTCGCGATGAAAAGAACTGCAG
N8	CGCCACATCGGTTTTGGTCAGTTCCGCCGCTTCATGTTCCTGCAGTTCTTTTTTCATCGCG
N9	ACCAAAACCGATGTGGCGCTGGCGGATGATGGCACCGTGCATAGCGATGATGAAGATTAT
N10	ATACACCGCTTCCGGGCTGCGGGTTTCGCCGCTAAAATAATCTTCATCATCGCTATGCAC
N11	GCCCGGAAGCGGTGTATACCCGCATTATGATGAACGGCGGCCGCCTGAAACGCAGCCATA
N12	GGCGCGCCTGATGGTTGCTGCTCACGCTCACATAGCGGCGAATATGGCTGCGTTTCAGGC
N13	ACCATCAGGCGCGCCCGAACAGCTTTGCGGAATTTCTGAACAAAACCTATAGCAACGATA
N14	GCTCGAGTTAGCTATCGTTGCTATAGGTTTTGTTCAG
forwN	GGCCATGGCGGCCGCGGGA
stopN	GCTCGAGTTAGCTATCGTTGCT

D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, followed by incubation at 37°C with shaking. Cells were harvested 4-5 h post-induction at 15000 rpm for 1 min. The cell pellets were suspended in 200 µL of 2^x Protein Sample Buffer (125 mM Tris-HCl pH = 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol; 0.002% bromophenol blue), heated to 95°C for 5 min and analyzed by SDS-PAGE.

Preparation of anti-rabies virus hyperimmune sera

Sera containing antibodies against rabies virus proteins were obtained from mice immunized with inactivated purified rabies virus. The inactivated rabies virus produced from infected cell cultures and purified was generously gifted by the LLP "Antigen" (Kazakhstan). The BALB/c mice (2-4 weeks old) were intraperitoneally injected with a mixture of 50 µg of inactivated virus with Complete Freund's Adjuvant. The boost was given 30 days later. 10-14 days after the second immunization, 100-200 µL of blood was collected from each animal to produce sera. The mouse hyperimmune sera were used in ELISA.

Western blotting

The obtained recombinant antigen was separated electrophoretically in 12% denaturing polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore). The membranes were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 1% bovine serum albumin (BSA). The following antibodies were used to probe the blots: anti-His-tag mouse monoclonal antibody (Sigma) and anti-(rabies virus) rabbit polyclonal antibody provided by the LLP "Antigen" (Kazakhstan). Signals in the western blot were detected by using either peroxidase-conjugated anti-rabbit IgG (Sigma) or peroxidase-conjugated anti-mouse IgG (Sigma) where appropriate. The coloring of bands was detected visually after processing by solution of 4-chloro-1-naphthol and hydrogen peroxide.

Indirect ELISA

In wells of 96-well immunological plates 100 µL of solution of the recombinant N protein used as the trapping antigen was added. To prepare the solution 5 µg of the recombinant N protein was dissolved in 10 ml of coating buffer (50 mM Na_2CO_3, pH = 9.6). Plates were incubated overnight at 4°C. The plates were blocked by incubating with solution of 1% BSA in PBS for 30 min at room temperature (RT). Upon three washes with PBS containing 0.1% Tween 20 (PBST), diluted mouse hyperimmune sera was added to wells. A set of serial dilutions starting from 1:100 were prepared from mouse sera for testing of the immunological reactivity of the polyclonal (anti-rabies virus) antibodies with the recombinant antigen in the ELISA. Pre-immunization (baseline) sera were used as negative controls. Plates were incubated for 1 h at RT followed by three washes in PBST. Secondary antibody, anti-mouse IgG conjugated with the horseradish peroxidase was added in dilution

1:5,000. The plates were incubated for 30 min at RT followed by three washes in PBST. Next, solution of o-phenylendiamine dihydrochloride (Sigma) was added to wells to produce coloring reaction. The reaction was stopped after 15 min by addition of 2 M sulfuric acid. Optical density (OD) in wells were read at 490 nm. OD in wells with dilutions of the hyperimmune sera >2 times that of the OD in wells with the negative controls were considered positive results indicting presence of specific antibodies. Titers of the antibodies were counted as the highest dilution of the hyperimmune sera which gives OD >2 times that of the OD of the same dilution of baseline sera.

Purification of the recombinant rabies antigen

400 ml culture of producing strain was induced for the protein expression in conditions similar to that described for small-scale experiments. Cell biomass was harvested by centrifugation at 6,000 rpm for 10 min at 4°C and the pellet was re-suspended in ice-cold TNE buffer (20 mM Tris-HCl; 1 mM EDTA; 100 mM NaCl, pH = 7.5) in the presence of 0.1 mM protease inhibitor (PMSF). Sonication on the Omni-Ruptor 4000 sonicator was used to lyse the cells. After centrifugation (11000 rpm, 60 min at 4°C) the pellet was suspended in 5 ml of buffer I for solubilization of inclusion bodies (1 M Urea, 20 mM HEPES pH = 7.5, 10 mM 2-mercaptoethanol) and the suspension was incubated for 30 min at RT, on a shaker (150 rpm). After next centrifugation (11000 rpm, for 30 min at 4°C) the pellet was re-suspended in 5 ml of buffer II for solubilization of inclusion bodies (8 M Urea, 20 mM HEPES pH = 7.5, 10 mM 2-mercaptoethanol) and incubated as described. The supernatant was decanted after final centrifugation (11000 rpm, for 20 min at 4°C). Solubilizates of the inclusion bodies were analyzed by SDS-PAGE. Purification of the recombinant N protein was performed using the metal affinity chromatography (IMAC) using columns 5 ml HisTrap HP (GE Healthcare) in accordance with the manufacturer's instructions.

Results

To produce diagnostic antigen of the important viral pathogen - rabies virus - we coupled bacterial expression with method to obtain synthetic gene. Synthesis of DNA fragment with desired sequence from oligonucleotides was used to obtain the gene for C-terminal part of the rabies virus N protein. The synthesized gene has frequencies of codons optimal for expression in *E. coli* (wild type) strain B which is an ancestor for the expression strain BL21 (DE3). The resulting recombinant protein is identical (except for the presence of starting methionine) to the homologous protein from the virus isolated in Mongolia. This isolate was selected to reproduce the N gene for heterologous expression because of the proximity of this country to Kazakhstan, which is our geographic region of interest. Unfortunately, to date there is lack of information on the genetic variability of Kazakhstan's isolates of rabies

Fig. 1: Multiple alignment of the amino acid sequences of rabies virus N protein from isolates obtained from several geographic regions in Asia. Sequence of the recombinant protein described in this paper is shown in separate lane of the alignment. Known immunodominant antigenic determinant is depicted within a rectangle

virus. Also, the recombinant antigen described in this paper has high (~100%) homology with the N proteins from rabies viruses isolated in various parts of Asia (Fig. 1).

The gene encoding the C-terminal fragment of the rabies virus N protein was constructed with a sequence which is codon-optimized for expression in *E. coli*. Sequencing of the cloned DNA fragment confirmed full agreement with the sequence engineered *in silico*. The obtained gene was cloned into expression vector pET32a. Genetic map of the resulting plasmid is shown in Fig. 2.
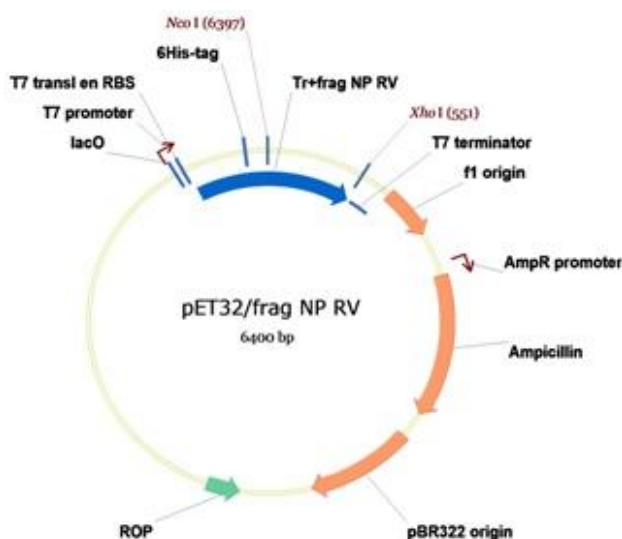
We engineered the synthetic fragment of N gene into a bacterial expression plasmid and produced the recombinant antigen in *E. coli* cells BL21 (DE3). SDS-PAGE of the soluble (cytoplasmic) fraction and insoluble fraction (inclusion bodies) from induced bacterial cells revealed that the recombinant protein localizes in the pellet containing inclusion bodies and cell debris (Fig. 2). Expression product with expected molecular weight (44 kDa) was found in the induced bacterial cells. The recombinant antigen accumulated to high amounts, up to ~25% of the total cellular protein (Fig. 3).
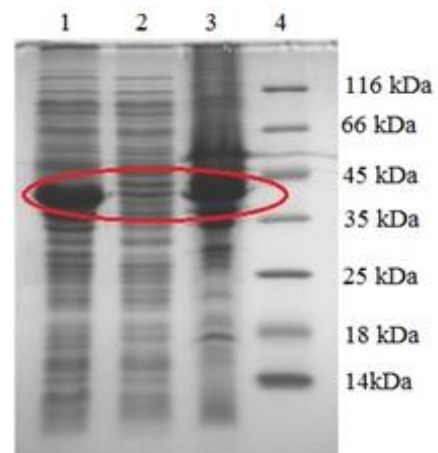

Fig. 2: Genetic map of the plasmid pET32a/NRV

Fig. 3: SDS-PAGE analysis of production of the recombinant C-terminal fragment of the N protein. Lane 1: Insoluble fraction (pellet) of cell lysate, Lane 2: Soluble fraction (supernatant) of cell lysate, Lane 3: Unfractionated cell lysate, and Lane 4: Marker of molecular weights. Molecular masses indicated next to marker's lane

Presence of the hexahistidine tag in the expression product was confirmed using western blot with anti-His-tag monoclonal antibodies (Figs. 4A-B).
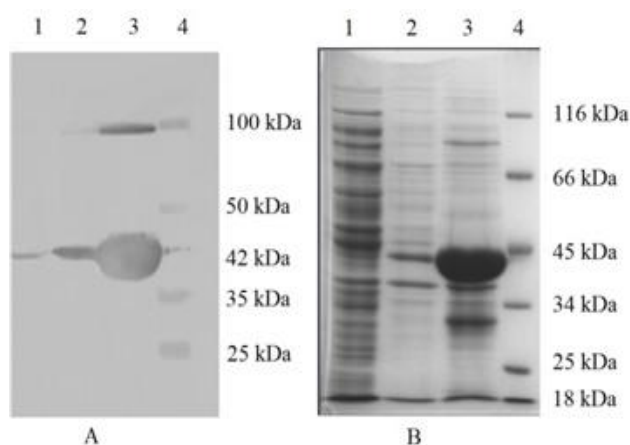

Fig. 4: Western blot and SDS-PAGE of the partially purified C-terminal fragment of the N protein. (A) Western blot with anti-His-tag mAbs, and (B) SDS-PAGE analysis of the expression products eluted from inclusion bodies. Lane 1: Soluble fraction, Lane 2: Proteins solubilized into 1 M urea, Lane 3: Proteins solubilized into 8 M urea, and Lane 4: Marker of molecular weights. Molecular masses indicated next to marker's lane

The recombinant antigen was obtained from inclusion bodies by elution into buffer for metal-affinity chromatography containing denaturing agent (8 M urea). The suspension was clarified by centrifugation and filtered through 0.45 um syringe filter. Purification of the recombinant antigen was performed using IMAC (Fig. 5).
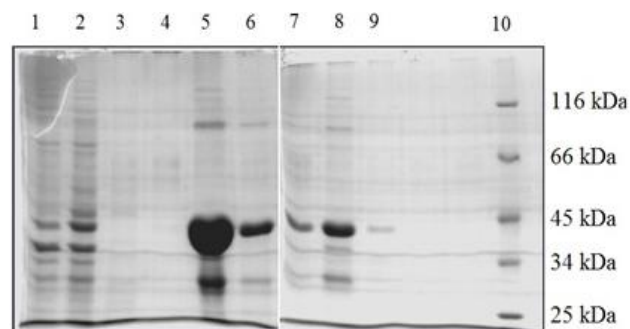

Fig. 5: Results of chromatographic purification of the recombinant C-terminal fragment of the N protein. Analysis of fractions in SDS-PAGE. Lanes 1-2: Flow through, Lanes 3-4: Samples eluted from column during wash with 20 mM imidazole, Lane 5: Recombinant protein eluted from column into 100 mM imidazole, Lanes 6-8: Recombinant protein eluted into 200 mM imidazole, Lane 9: Protein eluted into 500 mM imidazole, and Lane 10: Marker of molecular weights. Molecular masses indicated next to marker's lane

Analysis in chromatographic fractions in SDS-PAGE showed that upon elution from inclusion bodies the recombinant antigen effectively bound to the Ni-charged IMAC resin and the recombinant protein eluted from

column in the presence of sufficient concentration of imidazole. Peak of elution was observed at concentration of imidazole in the elution buffer 100 mM.

BALB/c mice were injected with inactivated rabies virus during two-week immunization to obtain hyper-immune sera. Antibodies in the sera reacted with the bacterially expressed fragment of N protein in western blot (Fig. 6). Thus immunization with the inactivated virus leads to development of antibodies against the N protein among other viral antigens.
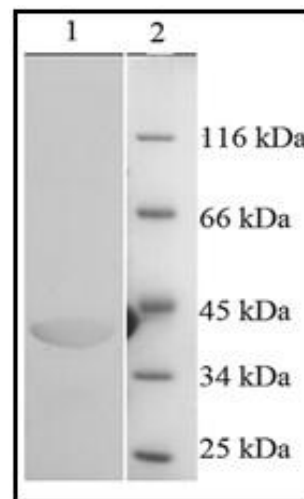

Fig. 6: Western blot of the recombinant N protein with mouse immune serum. Lane 1: Recombinant N protein, and Lane 2: Protein marker of molecular weights

Titers of antibodies reactive with the recombinantly expressed C-terminal part of the N protein were determined in sera from mice immunized with either the inactivated rabies virus, or the purified recombinant antigen. Indirect ELISA was used for this purpose. It was also shown that titers in ELISA correlate with the results of immunofluorescence which is routinely used to detect early rabies infection. In our experiments antibodies against the C-terminal fragment of the N protein were present in the sera of mice immunized with inactivated virus in high titers (1:6400). Optical density at 492 nm in ELISA with the hyperimmune serum was diluted to 1:6400 reached 1,500 (Fig. 7).

In ELISA with the recombinant antigen immobilized on the solid phase, sera from mice immunized with the inactivated virus showed higher titers compared to sera from mice immunized with the same recombinant antigen (Fig. 7A). Opposite results were obtained in ELISA using the inactivated virus as the immobilized antigen (Fig. 7B). In the latter case the titers were higher in serum from mice immunized with the inactivated virus. The pre-immunization (baseline) sera in both ELISAs showed low OD. Results of immunochemical reactions demonstrate both value of the recombinant fragment of the N protein for use as a diagnostic antigen for detection of rabies infection, and ability of the recombinant protein to elicit the anti-nucleoprotein antibodies during immunization.
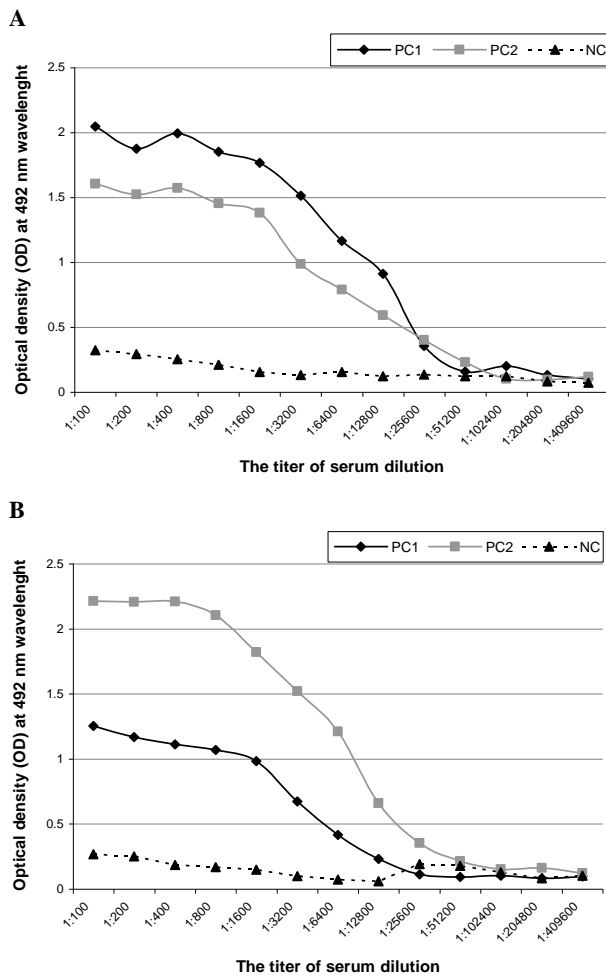
A

B

Fig. 7: ELISA of two antigens (the recombinant N protein and inactivated rabies virus) with antibodies present in sera of immunized mice. (A) Reaction in ELISA of the recombinant N protein and antibodies coming from positive control 1 (PC1): Sera from mice immunized with the purified recombinant N protein, positive control 2 (PC2): Sera from mice immunized with the inactivated rabies virus, and negative control (NC): Pre-immunized sera. (B) Reaction in ELISA of the inactivated rabies virus with the sera PC1, PC2, and NC

Discussion

Complete or partial N gene of rabies virus was produced *de novo* in a variety of works. Goto *et al.* (1995) synthesized part (having length 519 bp) of the N gene of strain RC-HL. The fragment reportedly carried the most immunogenic epitopes from N protein. Fanayi *et al.* (2014) synthesized the N gene of strain PV and developed a eukaryotic expression system. Xiangping *et al.* (2013) synthesized full-length N gene of strain ERA. The latter synthetic gene was codon-optimized for prokaryotic expression.

Our results are similar to the ones published for purification of the recombinant N protein of rabies virus strain CTN (Wangbin *et al.*, 2013) although different expression vector (pMal-c2x) and strain (JM109) were used in the cited work. Authors of (Wangbin *et al.*, 2013) emphasize that recombinant expression of the N protein

in *E. coli* has a significant advantage in yields and purity compared to traditional method of isolation of the N protein from cultured virus. In our experiments the recombinant N protein fragment accumulated in transformed bacterial cells to ~25% of the total cellular protein.

Goto *et al.* (1995) performed mapping of epitopes in antigenic determinants I and IV of the N protein of rabies virus. In the cited work, fragment of the N gene of rabies virus strain RC-HL was PCR amplified to obtain a product (519 bp) which was cloned into an expression vector pET3a. Then PCR-mediated mutagenesis was used to introduce mutations into selected sites of the protein. The resulting N proteins were expressed in *E. coli* BL21 (DE3) and purified using the IMAC.

The ELISA was shown to be a method of choice to check for the presence of anti-rabies IgG in vaccinated dogs and cats (Yan *et al.*, 2010). It was also shown that titers in ELISA correlate with the results of immuno-fluorescence which is routinely used to detect early rabies infection. In our experiments antibodies against the C-terminal fragment of the N protein were present in the sera of mice immunized with inactivated virus in high titers (1:6400). The results are in accordance with data from (Inoue *et al.*, 2003), who used the ELISA to rapidly screen sera from vaccinated and recovered animals.

The C-terminal fragment of the N protein of rabies virus was produced by recombinant expression in *E. coli*. The recombinant protein (44 kDa) carries known antigenic determinant of the N protein (pos.360-389). Sera from mice immunized with the inactivated rabies virus shown high-titer reactivity with the recombinant antigen in western blot and ELISA. The ELISA with recombinant N protein of rabies virus is a safe and convenient test, the use of which may improve the veterinary surveillance in Kazakhstan.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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