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OBTAINING RECOMBINANT NUCLEOCAPSID PROTEIN OF PPR VIRUS FOR DISEASE SERODIAGNOSTIC

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Abstract

Peste des petits ruminants is a highly contagious, acute or subacute viral disease of sheep and goats, characterized by fever, sores in the mouth, haemorrhagic gastroenteritis, lesions in lymphatic system and pneumonia. (E.P.J. Gibbs et al., 1979; A. Diallo et al., 1989; T.M. Ismail et al., 1995). Because of high morbidity of 50-100 % and mortality of 50-90 %, Peste des petits ruminants belongs to a number of emerging diseases, having a significant threat to livestock production in countries where the disease is notified (R.A. Kock et al., 2015; E.M.E. Abu-Elzein et al., 1990). The etiological agent of PPR is a Morbillivirus (PPRV) of *Paramyxoviridae* family (M.H.V. Van Regenmortel et al., 2000). The PPRV antigens are similar to antigens of other Morbilliviruses (G. Libeau et al., 2014). Severity of the clinical signs depends on different factors, e.g. PPRV line, animal species, breed, and immune status. Because of that, the final diagnosis must be confirmed by laboratory methods. In diagnostics and monitoring serological testing, the preference is given to sensitive and automated Enzyme-linked immunosorbent assay (ELISA). Modern methods of PPRV serodiagnostic are developed on the basis of virus-specific recombinant proteins and primary nucleocapsid N protein (A. Diallo et al., 1994; G. Libeau et al., 1995; M. Munir et al., 2013; N.V. Vavilova et al., 2006.), which is superior to the other Morbilliviruses's proteins in antigenic and immunogenic characteristics (P.C. Lefevre et al., 1991; M. Yunus et al., 2012). The other protein N advantage is that, as an antigen, it is the most conservative of the PPR virus proteins (M. Muhammad, 2013). The purpose of this paper was to obtain recombinant nucleocapsid N protein of PPR virus as an antigen and virus-specific antiserum of pigs as a source of antibodies for serodiagnostic of disease by competitive ELISA. A gene construct was designed which contained a sequence of protein N gene fragment of 1530 kb in length in the express plasmid vector pET32a. After polypeptide screening by SDS-PAGE and immunoblotting we found clones of *Escherichia coli* pET32a/N/10 which express 70 kDa virus-specific major polypeptide. It was shown, that in competitive ELISA the optimal dose of recombinant protein N purified by Immobilized Metal Chelate Affinity Chromatography method is 0.25 μg per well. The ratio of OD_{450} values for negative and positive control goat sera was 11.52. So the electrophoretically purified and immunochemically pure recombinant protein N can be used in competitive ELISA for PPRV serodiagnostic. For obtaining specific antisera, pigs were inoculated with purified PPRV. The antibody titers in antisera samples from the pigs in a neutralization test with PPRV were 1:64-1:128. These values are comparable with antibodies titers in sera of sheep and goats vaccinated against PPR (A.V. Konstantinov et al., 2017). However pigs' antisera were less effective in competitive ELISA than positive goat sera.

Keywords: peste des petits ruminants, *Morbillivirus*, diagnostic, recombinant proteins, immunosorbent assay

Peste des petits ruminants (PPR) is a highly contagious, acute or subacute viral disease of sheep and goats characterized by fever, sores in the mouth, haemorrhagic gastroenteritis, lesions in lymphatic system and pneumonia [1-3]. For the first time, the disease was described on the coast of Cote d'Ivoire in the West Africa [4]. Then, it was reported in the Africa South of the Sahara, on the Arabian Peninsula and in the Southwest Asia [5]. Due to high morbidity (50-100%) and mortality (50-90%), PPR belongs to a number of emerging diseases having a significant threat to livestock production in countries where the disease is notified [6, 7].

The etiological agent is a PPR virus (genus *Morbillivirus*, family *Paramyxoviridae*). The PPRV antigens are similar to antigens of other Morbilliviruses [8, 9]. The virus is transmitted by a close contact of susceptible animals (several species of gazelles, oryxes and white-tailed deers) through secretions and excretions of ill animals. Although cattle, buffaloes and pigs can be naturally or experimentally PPRV-infected, they are dead-end hosts because of absence of the disease clinical signs and impossible viral transmission to other animals [5]. Four PPR virus lines are reported nowadays. One of them (line 4) is common in Asia only, but others are wide-spread in Africa [10, 11]. The line division is based on differences between a nucleocapsid protein gene. As compared to a categorization centered around a glycoprotein gene F alterations, the arrangement highlights a geographical origin more clearly [12, 13]. Intensity of clinical signs depends on PPR virus line, species, breed and immune status of an animal. That is why a final diagnosis should be confirmed by laboratory data.

Coupled with a direct detection of the viral genome by a real-time polymerase chain reaction (RT-PCR), laboratory diagnostics applies some serological tests such as virus neutralization test (NT) [14], diffuse precipitation test [15], counter-immunoelectro-osmophoresis, indirect immunofluorescence test [16, 17], direct and indirect enzyme-linked immunosorbent assay (ELISA) [18], and monoclonal antibody-based competitive ELISA [19-23] to reveal virus-specific antigens and antibodies. NT and ELISA are the most common methods to study serum samples. NT is the most laborious method, which is not suitable for an investigation of great number of samples because of required handling with cell cultures. Therefore, ELISA, an advanced sensitive method, is a technique of choice to conduct diagnostic and monitoring studies.

Initially, an inactive PPR virus (PPRV) purified from infected cell lysates was used in ELISA to detect virus-specific antibodies. However, since the antigen preparations included cell culture proteins, the method specificity was low. The technology related to design of recombinant proteins with affinity labels enables their purification by a chromatographic method on metal chelate sorbents, withdrawal of a living PPRV use and simultaneous increase in ELISA specificity by means of highly purified antigens. Advanced PPR serodiagnostic tools are developed on the basis of virus-specific recombinant proteins. Above all, we mean protein N [24-27] whose antigenic and immunogenic properties are better than in other Morbillivirus proteins [28, 29]. Another protein N advantage is that, as an antigen, it is the most conservative protein of the PPR virus [30]. It worth mentioning that immune responses to protein N generated by susceptible animals during vaccination/disease are not protective tools because of its intravirionic localization.

The paper presents a technology related to production of a recombinant full-size PPRV nucleocapsid protein (N) producer. It was demonstrated that purified recombinant protein N is suitable to detect antibodies to PPRV in a competitive ELISA. We studied a potential use of positive PPRV vaccine strain-immunized pig sera as controls of test systems intended for the disease serodiag-

nosis for the first time ever. It was found that, according to competitive ELISA, they are inferior to positive goat sera.

The purpose of our study was to obtain recombinant PPRV protein N (i.e., an antigen) and virus-specific pig antisera (i.e., antibody source) intended for the disease serodiagnosis by a competitive enzyme-linked immunosorbent assay.

Techniques. Large White domestic pigs (*Sus scrofa domesticus*) weighed 30 kg (n = 2, Animal Preparation Sector, Federal Research Virology and Microbiology Center), green monkey kidney cell line (VERO) and PPRV vaccine strain (45G37/35-K; collections of Federal Research Virology and Microbiology Center) were involved in the study.

PPRV accumulation and infectious activity were evaluated in VERO cell culture in Eagle's Minimum Essential Medium (PanECO, Russia) with 2.5% cattle fetal serum in polystyrene flasks and 96-well plates (Costar, France), respectively. During titration, the virus-containing material underwent a successive 10-fold dilution in quadruplicate. Infected and control VERO cell cultures were exposed at 37 °C (5% of CO_2). The maintenance medium was refreshed every 2-3 days. Results were recorded considering a cytopathogenic effect (CPE) for 10 days. The viral titer was calculated as per Körber's method modified by I.P. Ashmarin. The result was expressed as $TCID_{50}/cm^3$ [31].

A neutralization test involved a 45G37/35-K strain. Antisera were subjected to 2-fold serial dilutions in the cultivation medium. An 100 µl aliquot of the virus (titer 10^3 $TCID_{50}/cm^3$; 10^2 $TCID_{50}/cm^3$ per a well) and 100 µl of diluted antisera test samples (4 wells per a dilution) were mixed. Then, the cultivation medium was removed from 96-well plates containing VERO cell culture and virus-antiserum mixtures were added. Virus-free and PPRV-infected (10^2 $TCID_{50}/cm^3$) wells were used as controls. Plates were incubated at 37 °C for 2 h (5% of CO_2). Liquid phase was removed from wells and cultivation medium was added instead. Antibody titer in serum was calculated according to dilutions neutralized PPRV in 50% of wells.

pJET1.2 (Thermo Fisher Scientific, Inc, USA) and pET32a (Novagen, USA) plasmid vectors were used to clone PCR-products and to create expression constructs, respectively. Sequencing was carried out by an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Inc., USA). Potential application of a recombinant protein N in a competitive ELISA was evaluated using several components of ID Screen® PPR Competition Kit (IDvet, France) (hereinafter — Kit) intended for detection of antibodies to PPRV nucleoprotein in serum and plasma of sheep and goats.

Some components of ID Screen® PPR Competition Kit (IDvet, France) were used in a competitive ELISA.

Electrophoretic separation of polypeptides was performed in 10% polyacrylamide gel by Laemmli method [32]. Polypeptides were electrotransported from the gel to a nitrocellulose membrane (Sigma, USA) in a semi-dry buffer system by Kyhse-Andersen method [33]. Total protein content in test samples was assayed according to Lowry et al. [34] with a KFK-2 photocolorimeter (Granat PGK, Russia) at λ = 750 nm.

Results. PPRV (45G37/35-K strain) was accumulated in the setting of multiple infection (10^{-1} $TCID_{50}$/cl) and titrated according to TCID in VERO cell culture. The virus demonstrated infectious activity of $10^{4.5}$-$10^{6.5}$ $TCID_{50}/cm^3$ at the fourth passage.

Typically, Paramyxoviruses can reproduce inside the body of most of animal species. For example, the better part of ground carnivores (e.g., *Canidae*, *Mustelidae*, *Procyonidea*, *Ursidae*, *Hyaenidae*, *Ailuridae*, *Viverridae)* infected with canine distemper virus (CDV) die or demonstrate clinical signs of the dis-

ease. Despite cats (*Felidae*) and pigs (*Suidae*) are also susceptible to CDV infection, they do not show its clinical signs [35, 36]. Titers of virus-neutralizing antibodies to CDV found in antisera of experimentally infected pigs are comparable to those in dogs and ferrets who had the disease [37]. Also, PPRV can reproduce in pigs [38]. We studied potential production of PPR-specific pig antiserum intended for serodiagnosis (i.e., competitive ELISA) because pig immunoglobulins can react with conjugates on the basis of protein A.

To reduce possible antibody response to cell components and virus-containing suspension proteins dissolved in the cultivation medium from, virions were purified by a differential centrifugation. PPRV suspension (titer of 10^5 $TCID_{50}/cm^3$, 30 cm^3 volume) was centrifuged at 5,000 g for 40 min. Cell debris was removed. Supernatant was recentrifuged through a 20% (w/v) sucrose cushion at 45,000 g for 4.5 h. At the end, supernatant was decanted, and precipitate was resuspended in phosphate buffer (10 cm^3). Purified virions (titer of 10^5 $TCID_{50}/cm^3$) were administered by 2.5 cm^3 to both pigs intramuscularly and intranasally as a single dose. Clinical signs of the disease were not detected. Animals were exsanguinated in 28 days after infection. In terms of NT, antibody titers in antiserum samples of both PPR-infected pigs were 1:64-1:128. The values were comparable to antibody titers in serum samples of PPR-vaccinated goats and sheep (39).

Nucleotide sequences of PPRV protein N gene were obtained from the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/). To design primers, Nigeria 75/1 (X74443.2) strain nucleotide sequence was used as a reference one. Primers flanking full-size copies of the viral protein N gene were designed with Oligo 7 and SnapGene v.4.1 software. Primer pair containing SalI and EcoRV restriction sites on 5′-ends was designed. Forward-primer (F-PPRV_N_atc) sequence is 5′-ATCTCGGGTT-CAGGAGGGGCCATCCGGGGG-3′, Revers-primer (R-PPRV_N_SalI) sequence is 5′-GGGTGCGTCGACTTAGCTGA-GGAGATCCTTGTCGTTGTAGATCTG-3′. Primers were designed to amplify a fragment of 1530 bp in length (positions nos. 4-1518 in a protein N gene). Specific hexameric sequences of SalI and EcoRV endonuclease restriction sites were built in oligonucleotide primers. RNA was extracted using TRIzol (Invitrogen Corp., USA). To synthesize the first chain of cDNA with a single-strand RNA matrix, M-MLV reverse transcriptase was used (α-Ferment, Russia). Prior to RT-PCR, annealing of primers was carried out at 70 °C for 5 min.

Protein N gene nucleotide sequence was synthesized by a polymerase chain reaction (PCR) with Pfu-polymerase (α-Ferment, Russia) with a Tertsik amplifier (DNK-Tekhnologii, Russia) as per the manufacturer's guidelines. Previously obtained cDNA was used as a matrix. The program included several stages such as hot start at 95 °C (pre-denaturation); 3 min at 95 °C (denaturation), 30 s at 95 °C (annealing of primers), 185 s at 68 °C (elongation) (25 cycles); 5 min at 72 °C (final elongation). To purify PCR products from agarose gel, GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Inc., USA) was used as per the manufacturer's instructions. The PCR product purified from agarose gel was cloned on the blunt end basis in pJET1.2 vector using T4 DNA ligase (Thermo Fisher Scientific Inc., USA).

Escherichia coli (Rosetta 2 pLysS strain) competent cells were transformed with obtained constructs as per the standard procedure using heat shock and further growing in a solid medium containing a selective antibiotic (i.e., ampicillin). PCR-positive plasmid clone sequencing demonstrated integrity of the recombinant gene open reading frame. The next stage represented EcoRV and XhoI restriction site-based recloning of a protein N gene from pJET1.2/N/4 plasmid to a pET32a expressing plasmid vector (Fig. 1). Presence of thioredoxin in the N-

end of a pET32a vector multicloning site increased solubility of recombinant protein. In turn, two polyhistidine areas enabled nickel sorbent-based purification of the expressed recombinant protein. Single positive clones were screened in PCR. Sequence of positive clones confirmed integrity of protein N gene (clones nos. 8 and 10) open reading frame. Further, plasmid pET32a/N/10 DNA was applied.

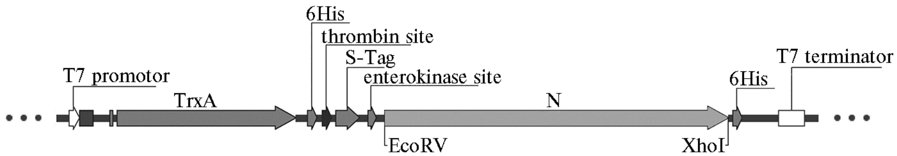

Fig. 1. Scheme of the open reading frame related to the recombinant gene with a cloned sequence of the fragment encoding PPRV protein N: T7 promotor — T7 bacteriophage RNA polymerase promotor; TrxA — thioredoxin gene A; 6His — polyhistidine encoding sequence; thrombin site — nucleotide sequence encoding thrombin cleavage site; S-Tag — RNase A N-end oligopeptide encoding sequence; enterokinase site — nucleotide sequence encoding enterokinase cleavage site; EcoRV and XhoI — restriction sites; T7 terminator — T7 bacteriophage RNA polymerase transcription terminator.

PET32a/N/10 plasmid was transformed into *E. coli*. Then, single clones were induced in different conditions (26, 30 and 37 °C in liquid SOB medium containing ampicillin, the selective antibiotic). Samples were evaluated in 4, 8 and 16 hours, respectively. It was found that the best possible induction was performed at 26 °C for 16 hours with IPTG inducer (isopropyl-β-D-1-thiogalactopyranoside) at 0.4 mM final concentration.

The estimated molecular weight of recombinant protein N fused to thioredoxin A and two 6His was about 70 kDa. SDS-PAGE polypeptide screening revealed clones of *E. coli* pET32a/N/10 nos. 3, 5, 6, 8 expressing a major polypeptide of 70 kDa absent in pET32a plasmid-transformed *E. coli* cell lysate without an insertion.

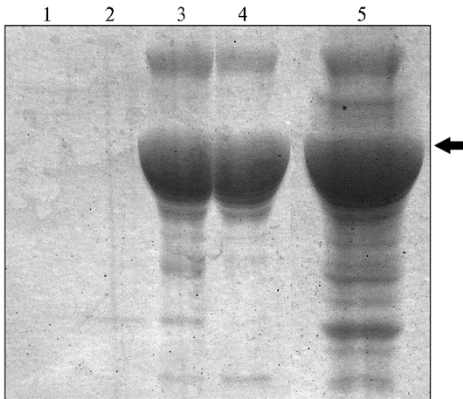

Fig. 2. Electrophoregram of fractions after chromatographic purification of recombinant PPRV protein N in the setting of a discrete increase in imidazole concentration: 1 — 50 mM, 2 — 100 mM, 3 — 250 mM, 4 — 500 mM, 5 — initial lysate of *Escherichia coli* induced cell. Position of purified recombinant protein N fraction is arrowed.

Recombinant protein N was purified by Metal Chelate Affinity Chromatography in native conditions. Ni Sepharose™ 6 Fast Flow (GE Healthcare Bio-Sciences Corp., USA) was used as a sorbent. Then, elution with aqueous imidazole solutions (50, 100, 250 and 500 mM) was performed. Eluates were screened by SDS-PA-GE method (Fig. 2).

After dialysis final concentration of recombinant protein N in eluate fraction with 500 mM imidazole was adjusted to 2 mg/cm^3. Its antigenic activity was confirmed by immunoblotting assay with antibodies of PPR-positive pig antisera. A major virus-specific polypeptide with molecular weight of 70 kDa was detected on the blottogram. Activity of recombinant PPRV protein N and PPRV pig antisera was calculated using several components of ID Screen® PPR Competition Kit (IDvet, France).

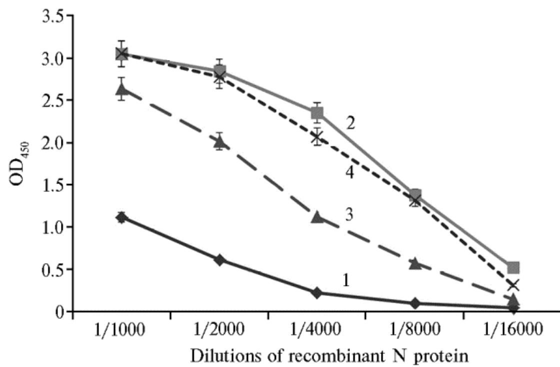

Fig. 3. Competitive enzyme-linked immunosorbent assay of recombinant PPRV protein N immobilized on strip wells (Eppendorf, Germany): 1 and 2 — positive and negative sera (ID Screen® PPR Competition Kit, IDvet, France); 3 and 4 — pig sera (positive and negative ones collected in an experimentally PPRV-immunized animal and an intact animals, respectively).

Instead of ID Screen® PPR Competition Kit strips (IDvet, France), free plastic strips (Eppendorf, Germany) were used in whose wells obtained recombinant protein N (by 50 µl in increasing 2-fold dilutions, 1:1000 - 1:16000) was immobilized. An aliquots of 100 µl of positive/negative sera form the kit or positive/negative pig sera were added to wells and incubated in a shaker at 37 °C for 1 h. The next stages were performed as per the kit manufacturer's guidelines.

The optimal concentration of recombinant protein N was 0.25 µg/well (1:8000 dilution). Considering the concentration, the ratio of OD_{450} values for negative and positive sera included into the kit was 11.52; in turn, the ratio for negative and positive pig sera was 2.22 (Fig. 3). The similar result (0.20 µg/well) was obtained while testing 50 kDa recombinant protein produced in *E. coli* BL21(DE3) pLysS cells transformed with protein N gene 5′-end 838 bp sequence [23].

In using sensibilized strips from the manufacturer's kit, the ratio of OD_{450} values for negative and positive sera was 16.03. Considering objective parameters, obtainer recombinant protein N is suitable for PPR serodiagnosis by competitive ELISA method.

It should be emphasized that absorbance values of negative pig serum reaction were comparable to those obtained with a negative serum included into the manufacturer's kit. Along with this, 5-fold difference was seen between the values resulted from tests involved positive serum included into the manufacturer's kit and positive antiserum collected in immunized pigs when recombinant protein dilution was 1:8000. It is likely to indicate lower concentration of antibodies to protein N in sera of PPRV-immunized pigs than in positive serum included into the kit and collected in recovered goats.

Recombinant PPRV protein N was used as an antigen in indirect/competitive ELISA to provide serodiagnosis for several studies. Previously, a great amount of the protein was extracted from cells of insects infected with a recombinant baculovirus containing PPRV nucleoprotein gene [25]. Competitive ELISA demonstrated activity of shorter and full-size PPRV protein N producer in *E. coli*. The best concentration of protein N to reveal antiviral antibodies was 0.16 µg/well [40]. We note that *E. coli* prokaryotic system is the most common in proteins that do not require post-translational modification because of simple expression and potential production of a great protein amount. Moreover, as compared with other heterologous systems, bacterial production of recombinant antigens is quite simpler and more cost-effective.

Thus, we obtained *Escherichia coli* pET32a/N/10 clone suitable for application as a producer of recombinant protein N of peste des petits ruminants (PPR) virus. A chimeric recombinant protein N contains a thioredoxin fragment to increase solubility in water and two polyhistidine regions to provide an efficient nickel sorbent-based purification. Underwent electrophoretic and immunochemical purification, pure recombinant protein N is suitable for PPR serodiag-

nosis by a competitive enzyme-linked immunosorbent assay (ELISA). Active in the neutralization test, specific anti-PPR pig antisera used in a competitive ELISA were less efficient than a positive goat antiserum (ID Screen® PPR Competition Kit, IDvet, France). At the next stage, we are going to obtain other active components of the developed test system suitable for diagnosis of peste des petits ruminants by a competitive ELISA.

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