https://doi.org/10.29326/2304-196X-2025-14-4-372-382

Recombinant antigens in serological diagnostics of transboundary and emerging bovine infections

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ABSTRACT

Introduction. Transboundary and emerging infections of cattle and small ruminants, such as peste des petits ruminants, Schmallenberg virus infection, etc., pose a serious animal health and economic threat in the context of developing globalization. Given the current geopolitical situation, the need for modern domestically produced diagnostic systems is particularly acute. Such systems can be developed using genetic engineering methods.

Objective. Analysis of domestic and foreign publications on the production of recombinant proteins of pathogens of transboundary and emerging infections of cattle and small ruminants. Creation of genetic constructs based on the processed data for further development of diagnostic tools, in particular enzyme-linked immunosorbent assay test systems.

Materials and methods. Using bioinformatics tools, codon composition of the sequences encoding the nucleocapsid proteins of peste des petits ruminants virus and Schmallenberg virus was analyzed and optimized. The optimized gene fragments were synthesized *de novo* and cloned into the pET-32b(+) expression vector. Successful insertion of the target sequence into the vector was confirmed by polymerase chain reaction and restriction analysis.

Results. Information on enzyme-linked immunosorbent assay test systems developed on the basis of recombinant antigens for the diagnosis of peste des petits ruminants and Schmallenberg virus infection is presented. The main technological aspects of obtaining recombinant antigens for their further use in a diagnostic system factored in the biological features of a particular pathogen are highlighted. Our proprietary methodology for creating protein expression vectors for the pathogens of the diseases under review is additionally described.

Conclusion. The most promising recombinant antigens for use in enzyme-linked immunosorbent assay test systems designed to detect antibodies against peste des petits ruminants virus and Schmallenberg virus are full-length and truncated virion nucleocapsid proteins. Furthermore, the biophysical properties and antigenic structure of these proteins enable their production in *Escherichia coli*. It should be noted that production of significant amounts of functional proteins in soluble form may require their expression as part of fusion proteins with tags enhancing solubility and facilitating correct folding.

Keywords: recombinant antigens, serological diagnostics, enzyme-linked immunosorbent assay (ELISA), cattle, peste des petits ruminants, Schmallenberg virus infection

Acknowledgements: This work was supported by the Russian Science Foundation (Grant Agreement 25-24-20116) and the Ministry of Economic Development and Industry of the Vladimir Oblast.

For citation: Tenitilov N. A., Yarygina N. A., Sprygin A. V. Recombinant antigens in serological diagnostics of transboundary and emerging bovine infections. *Veterinary Science Today.* 2025; 14 (4): 372–382. https://doi.org/10.29326/2304-196X-2025-14-4-372-382

Conflict of interests: The authors declare no conflict of interests.

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УДК 619:616.98:578:616-078

Рекомбинантные антигены в серологической диагностике трансграничных и эмерджентных инфекций рогатого скота

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РЕЗЮМЕ

Введение. Трансграничные и эмерджентные инфекции крупного и мелкого рогатого скота, такие как чума мелких жвачных животных, болезнь Шмаленберга и другие, в условиях развивающейся глобализации представляют серьезную эпизоотическую и экономическую угрозу. С учетом текущей геополитической обстановки необходимость в современных диагностических системах отечественного производства ощущается особенно остро. Подобные системы могут быть разработаны с использованием методов генной инженерии.

Цель исследования. Анализ отечественных и зарубежных публикаций, посвященных получению рекомбинантных белков возбудителей трансграничных и эмерджентных инфекций крупного и мелкого рогатого скота. Создание на основе обработанных данных генетических конструкций для дальнейшей разработки на их основе диагностических средств, в частности иммуноферментных тест-систем.

Материалы и методы. При помощи инструментов биоинформатики проведен анализ и оптимизация кодонного состава последовательностей, кодирующих нуклеокапсидные белки вирусов чумы мелких жвачных животных и болезни Шмалленберга. Оптимизированные фрагменты генов были синтезированы *de novo* и клонированы в экспрессирующий вектор pET-32b(+). Успешность вставки целевой последовательности в вектор подтверждали методом полимеразной цепной реакции и рестрикционного анализа.

Результаты. Представлена информация о разработанных на основе рекомбинантных антигенов иммуноферментных тест-системах для диагностики чумы мелких жвачных животных и болезни Шмалленберга. Освещены основные технологические аспекты получения рекомбинантных антигенов для дальнейшего их использования в диагностической системе с учетом особенностей биологии конкретного инфекционного агента, а также описана собственная методология создания векторов для экспрессии белков возбудителей обозреваемых болезней.

Заключение. Наиболее перспективными для использования в качестве рекомбинантных антигенов в иммуноферментных тест-системах, направленных на выявление антител к вирусам чумы мелких жвачных животных и болезни Шмалленберга, являются полные и усеченные нуклеокапсидные белки вирионов. При этом биофизические свойства и антигенная структура данных белков позволяют получать их в культуре клеток *Escherichia coli*. Следует отметить, что для получения значительных количеств функциональных белков в растворимой форме может понадобиться их экспрессия в составе слитых белков с повышающими растворимость и облегчающими корректный фолдинг тегами.

Ключевые слова: рекомбинантные антигены, серологическая диагностика, иммуноферментный анализ, рогатый скот, чума мелких жвачных, болезнь Шмалленберга

Благодарности: Исследование выполнено за счет средств гранта Российского научного фонда (соглашение № 25-24-20116) и при поддержке Министерства экономического развития и промышленности Владимирской области.

Для цитирования: Тенитилов Н. А., Ярыгина Н. А., Спрыгин А. В. Рекомбинантные антигены в серологической диагностике трансграничных и эмерджентных инфекций рогатого скота. *Ветеринария сегодня.* 2025; 14 (4): 372–382. https://doi.org/10.29326/2304-196X-2025-14-4-372-382

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

Для корреспонденции: Тенитилов Никита Алексеевич, аспирант, ветеринарный врач лаборатории молекулярных и генетических исследований ФГБУ «ВНИИЗЖ», ул. Гвардейская, 6, мкр. Юрьевец, г. Владимир, 600901, Россия, *tenitilov@arriah.ru*

INTRODUCTION

To date, the transboundary and emerging infections of cattle and small ruminants pose a serious threat. First of all, this is due to the significant impact on the economy and international trade.

Such infectious animal diseases as peste des petits ruminants (PPR) and Schmallenberg virus (SBV) infection are highly contagious and, in the context of the developing globalization and formation of tight international relations, they can easily spread beyond the borders of enzootic and infected regions. Outbreaks of these infections are accompanied by significant economic losses, which are associated with the animal disease eradication and prevention measures, decrease in animal performance and restrictions on the export of animals and animal products. At the same time, the need for reliable diagnostic tools becomes especially urgent.

The current level of molecular biology and biotechnology development, in particular, recombinant DNA technology, allows creating highly sensitive, specific and safe diagnostic tools.

The most valuable tool for the serological diagnosis of viral animal diseases is enzyme-linked immunosorbent assay (ELISA). Furthermore, the ability to produce recombinant proteins ensures that future ELISA test systems will be safe as there will be no need to handle infectious agents during their manufacture. Moreover, they will have much greater sensitivity and specificity due to a more efficient purification method used. The ability to synthesize recombinant proteins in the laboratory makes it easy to scale up the production of antigens, allowing production to be customized to the existing requirements.

It should be noted that recombinant protein-based ELISA test systems intended for the diagnosis of infectious animal diseases are being successfully developed abroad by such companies as IDvet (France), IDEXX Laboratories (USA), Ingenasa (part of Eurofins Scientific holding, Luxembourg). High sensitivity and specificity are crucial factors contributing to the use of these diagnostic tools, *inter alia* in domestic diagnostic laboratories.

Peste des petits ruminants (PPR) is an acute or subacute viral disease of sheep and goats, characterized by fever, conjunctivitis, rhinitis, necrotic stomatitis, gastroenteritis, pneumonia and mortality of the infected animals [1, 2]. The first documented reports of this disease date back to the early 20th century. From 1917 to 1929, outbreaks of a disease clinically similar to rinderpest were reported in sheep and goats in Senegal, Guinea and Nigeria. In the period from 1940 to 1942, L. Gargadennec and A. Lalanne described a disease affecting small ruminants in West Africa [3]. They detected a disease with clinical signs similar to rinderpest, but affecting only sheep and goats. Later, in 1968, peste des petits ruminants was established as an independent nosological entity [4].

The disease is caused by an enveloped RNA-containing pleomorphic virus belonging to the genus *Morbillivirus* of the *Paramyxoviridae*

family. It is closely related to the viruses causing rinderpest, canine distemper, phocine distemper, and human measles [1, 2]. The virion size can vary from 150 to 700 nm. It consists of an RNA molecule, which, together with a phosphoprotein and an L-protein, is covered with a nucleocapsid envelope, all housed within a supercapsid.

The PPR virus (PPRV) genome is composed of a single-stranded, non-segmented, linear RNA molecule with negative polarity. The genome is about 16,000 nucleotides in size and encodes six structural proteins such as nucleocapsid protein (N), phosphoprotein (P), polymerase protein (L), hemagglutinin (H), fusion protein (F) and membrane protein (M). Furthermore, two nonstructural proteins, C and V, are translated from the P-encoding transcript using an alternative reading frame [1].

Today, PPR is enzootic in most parts of Africa, Middle East, South Asia, and China. Due to the high morbidity and mortality in the primary outbreaks (up to 100%), the disease is of great economic significance for the enzootic regions [2]. It should be noted that in recent years, the PPR outbreaks have been reported in countries with which the Russian Federation maintains trade and economic relations. This is indicative of the threat of PPRV introduction into Russia from the disease infected countries [5].

The main source of the PPR causative agent are the diseased and/or convalescent animals. The most common transmission routes involve contact and airborne ones. Goats, sheep, and wild small ruminants are susceptible to the PPRV. Moreover, there are reports of the PPR causative agent detection in cattle, camels, pigs and buffaloes. It should be noted that such hosts are atypical for this virus, and in case of infection, further disease and spread of the pathogen do not occur [2, 6].

As soon as the virus enters the body of a susceptible animal through the oral cavity or nasopharynx, the virions are captured by cells of the monocyte macrophage system and transported to regional lymph nodes and lymphoid tissue clusters, where primary and secondary replication of the pathogen occurs. Next, the virus is disseminated to organs and tissues remote from the primary replication foci. It should be noted that the PPRV demonstrates pronounced tropism to lymphoid tissues. Its dissemination throughout the organism causes profound immunosuppression due to the destruction of a significant part of leukocytes. In addition, necrotic lesions are observed in the spleen, thymus, and pulmonary lymph nodes [1].

The PPR incubation period ranges from 2 to 7 days. Viremia develops during 1–2 days before the clinical signs onset, and the active virus shedding begins, which contributes to the spread of the infection [2]. The PPR clinical signs include pyrexia, erosive stomatitis, ocular and nasal discharge, and diarrhea. Death usually occurs within 4–6 days after the fever onset. It should be noted that the form of the disease and its severity can vary depending on the individual animal characteristics, such as species, age, breed and production type [7].

As soon as the PPRV enters the body of a susceptible animal, a strong virus-specific immune response is reported despite significant immunosuppression. The key protective cellular and humoral immune responses are targeted at H-, F- and N-proteins of the virion. Activation of the cytotoxic T-cells is observed, which destroy the virus-infected cells. Furthermore, a large quantity of virus-neutralizing antibodies is produced, primarily targeting the glycoproteins of the virion envelope – the H- and F-proteins. In case of recovery, the animal acquires a stable lifelong immunity against re-infection.

Successful control of the disease spread, in particular, prevention of the infection in previously PPR-free regions, includes the use of a number of diagnostic tools, aimed primarily at detecting specific antibodies.

Several methods have been developed for PPR serological diagnosis, such as virus neutralization test, diffuse precipitation test, indirect immunofluorescence test, direct and indirect enzyme-linked immunosorbent assay (ELISA), as well as competitive ELISA [8]. It is important to note that the virus neutralization test, while considered the most accurate method, has significant drawbacks despite its advantages, i.e. labor-intensive and lengthy procedure. ELISA is, therefore, most often used for the routine studies [7].

The first ELISA kits for the detection of PPRV antibodies utilized inactivated and purified virus. It is worth mentioning that this approach was characterized by low specificity due to the presence of cell culture-contaminating proteins. The use of recombinant protein technology has solved this problem. Furthermore, production safety has been significantly enhanced. For this reason, to date, the majority of the PPR serological diagnostic tools are recombinant protein-based [9].

Recombinant N-protein is used as an antigen for ELISA kits to detect the PPRV antibodies, owing to the high degree of conservation of the gene encoding this protein. The protein is involved in the formation of the virion nucleocapsid, therefore it is expressed in large numbers by the affected cells [10]. In addition, N-protein has different epitopes, enabling its use for the

detection of antibodies against PPR viruses of different genetic lineages [11], since all genetic lineages are serologically indistinguishable.

One of the first papers devoted to the production of the recombinant PPRV nucleocapsid protein was published in 1995 by G. Libeau et al. [12]. To produce the protein, they utilized an insect cell expression system using *Spodoptera frugiperda* (Sf9) cells. The protein-encoding sequence was derived from PPRV Nigeria 75/1 strain. The recombinant baculovirus *Autographa californica* served as the transfer vector, generated through recombination with the transfer vector pAcYM1 containing the inserted target gene under the control of a baculovirus promoter.

The competitive ELISA developed on the basis of a recombinant protein and monoclonal antibodies to it demonstrated high sensitivity and specificity. Moreover, a good correlation of the results ($r = 0.94$) was shown when comparing this method with the virus neutralization test [12].

In 2005, K.-S. Choi et al. [13] developed a competitive ELISA variant based on recombinant N-protein, which was specified by a short assay time of less than one hour. The recombinant antigen for this kit was also produced using Sf9 insect cell system. In addition, the antigen isolation included additional purification using affinity chromatography.

In 2006, employees of the Federal Centre for Animal Health N. V. Vavilova and A. V. Scherbakov developed an indirect ELISA version for PPR diagnosis. In contrast to the previously cited studies, the recombinant antigen for this diagnostic tool was produced in *Escherichia coli* culture. The N-protein-encoding sequence was amplified with the RNA of the native virus deposited in the Centre's collection of strains of microorganisms using reverse transcription polymerase chain reaction (RT-PCR) and inserted into the plasmid vector pQE under the control of the T5 promoter. Protein purification was performed using metal chelate affinity chromatography. The developed test system complied with the required sensitivity, specificity and reproducibility parameters. Over 200 sheep and goat serum samples from various regions of the Russian Federation were tested using this test system [8].

In addition to the full-length N-protein, its truncated forms were also tested for their suitability as antigens for various ELISA variants. In 2006, V. Yadav et al., having amplified the full-length and truncated N-protein-encoding sequences by RT-PCR, inserted them into the pET33b vector under the control of the T7 promoter. Using these genetic constructs, *E. coli* BL21 strain cultures were transformed and recombinant PPRV N-proteins were obtained, which showed successful results, when used for diagnostic purposes [10].

In the paper published in 2011 G.-R. Zhang et al. demonstrated the recombinant protein production method, which differed significantly from traditional ones. The PPRV N-protein-encoding nucleotide sequence was obtained from GenBank database (FJ905304), and its assembly was carried out using a set of 20 primer pairs through several overlapping PCRs. Furthermore, upon completion of the target sequence synthesis, several nitrogenous bases in the chain were replaced using site-directed mutagenesis. After verification of the assembled genetic construct, it was introduced into *E. coli* BL21 culture to produce a recombinant protein. The sensitivity and specificity of the indirect ELISA variant based on the obtained antigen was 96.7 and 96.1%, respectively, according to the results of 697 sera testing [11].

In 2019, D. Yu. Morozova et al. published a paper on the production of the recombinant PPRV N-protein. It should be noted that in addition to the encoding sequence, obtained by amplification of the native virus genome fragment, the resulting recombinant plasmid contained the thioredoxin gene. This allowed increasing the amount of protein synthesized by bacteria in the soluble form [9]. Later, an ELISA technique was developed based on the obtained protein, which demonstrated successful results [8].

Thus, the most promising antigen for routine diagnostics is the partial or full-length nucleoprotein N, produced using various genetic engineering systems, which is widely used in both international and domestic practice.

Schmallenberg virus (SBV) infection is an emerging viral vector-borne disease of ruminants, accompanied by depression, diarrhea, decreased milk production, as well as abortions, stillbirths, and congenital malformations in young animals [14, 15].

In 2011, outbreaks of an infection of unknown etiology were reported in cattle in Germany and the neighboring Netherlands. The disease was accompanied by a mild clinical course [16].

In herds where the new disease was reported, an increase in the number of calves with congenital pathologies was observed in the subsequent months. Severe neurological disorders, as well as musculoskeletal pathologies were noted. As a rule, calves with such abnormalities were inviable and died within a few days or weeks. In addition, the number of abortions and stillbirths increased [16, 17].

Using metagenomic analysis of blood samples from the diseased animals, a new virus was identified, named after the place where the first

disease cases had been reported – Schmallenberg virus [18]. Later, during experimental infection of calves with the blood of cows that showed a positive result when tested for the virus presence by RT-PCR, the disease clinical signs were reproduced [17]. Thus, it was established that the cause of the outbreaks was a new virus that had not previously been reported in Europe.

Phylogenetic analysis of the viral genome sequences allowed assigning SBV to the *Bunyaviridae* family, genus *Orthobunyavirus*, Simbu serogroup, which comprises over 170 viruses, including pathogens that cause diseases in both humans (Oropouche fever and La Crosse encephalitis viruses) and ruminants (viruses causing Akabane disease, Aino disease and Cache Valley fever). It is worth mentioning that most representatives of the Simbu serogroup are spread in the Middle Eastern, African and Oceanian regions [14, 19].

The virus genome is a negative sense single-stranded RNA and it includes three segments (large, medium, and small), which encode RNA polymerase, surface glycoproteins, and nucleocapsid proteins, respectively [14, 20].

It was demonstrated that domestic and wild artiodactyls are SBV susceptible. Herewith, the susceptibility does not depend on gender or age. It is important to note that among domestic artiodactyls, sheep are the most susceptible to the infection, followed to a lesser extent by cattle and goats. Moreover, SBV-specific antibodies were detected in domestic pigs, wild boars, dogs, and elephants by various methods [14, 19, 20].

The pathogen can be transmitted both horizontally and vertically. In the first scenario, the virus enters the body of a susceptible animal through the bites of blood-sucking insects, primarily midges of the genus *Culicoides*. In the second case, the pathogen is transmitted from the infected mother to the fetus [14, 15, 19]. When infected with the SBV, the incubation period can range from one day to 4–5 days. The infection spread efficiency and rate depends on the regional natural and climatic conditions as well as on the activity of insect vectors. Up to 90% of the animal population can be infected over the relatively short period. Mortality from the disease is low and rarely exceeds 3–5%. Herewith, the number of abortions and stillbirths can reach 60% of the total number of pregnant animals [2, 15, 21].

It is worth mentioning that after the disease discovery in Germany, the virus rapidly spread across Europe. It was detected in biological samples collected from cattle, sheep, goats, bison and roe deer. Antibodies to the virus were detected in the sera of alpacas, fallow deer, mouflons, deer, and water buffaloes [18].

It was established that SBV exhibits a pronounced tropism for the nervous tissue. Upon entry of the pathogen into the body of a susceptible animal, viremia develops, lasting for several days. At the same time, a stable protective immunity is formed in parallel. However, when a pregnant animal not previously exposed to the pathogen is infected, the virus can cross the hematoplacental barrier and affect the fetus. It is important to note that the susceptibility of developing embryos and fetuses depends on the gestational age and the stage of placental development [22, 23].

The disease is characterized by a subclinical or subacute course with mild clinical signs, including fever, diarrhea, and decreased milk production in dairy animals, followed by recovery to the herd average within 2–3 weeks. The number of stillbirths increases, as does the frequency of newborns with congenital developmental pathologies such as arthrogryposis, brachygnathia, ankylosis, torticollis, and hypoplasia of the brain and spinal cord [20].

To effectively control the spread of the new viral infection and further monitor the animal disease situation, a range of diagnostic tools has been developed, including several serological ELISA systems based on the recombinant nucleocapsid protein of the virus.

The N-protein was chosen as the target due to its highest prevalence – this antigen is found both in virions and in infected cells. In addition, recombinant N-proteins of SBV-related hantaviruses (*Hantaviridae*), produced in *E. coli* culture, insect cells, or yeast, are successfully used for serological diagnosis of human hantavirus infections [17].

In 2013, E. Bréard et al. published a paper on the development of the recombinant N-protein-based ELISA systems for detecting antibodies to SBV. The basis for producing the recombinant nucleocapsid protein was an artificially synthesized nucleotide sequence obtained from GenBank database (accession number HE649914). It was cloned into an expression vector under the control of the T7 promoter. The expression of this genetic construct in *E. coli* BL21 (DE3) pLysS culture resulted in the production of a recombinant protein. In addition to the amino acid sequence of the SBV nucleocapsid protein, this recombinant protein contained six histidine residues at its N-terminus. After expression, the protein was denatured in the presence of urea and purified by immobilized metal affinity chromatography. The antigen produced in such way was then used as an antigen for testing sera of the infected

and intact animals. It should be noted that the ELISA system based on the produced recombinant nucleocapsid protein demonstrated high sensitivity and specificity parameters. Moreover, the effectiveness of detecting positive samples was compared with the virus neutralization test, resulting in 98.9% agreement [16].

In the same year, 2013, a group of Chinese researchers led by Y. Zhang obtained the recombinant N-protein of the SBV. A fundamental difference in their work involved the use of the native nucleotide sequence of the produced protein. In addition, two types of genetic constructs based on pET-28a-c(+) and pMAL-c5X vectors were used to express the recombinant protein. They encoded proteins containing hexahistidine labels and maltose-binding protein, respectively. It should be noted that the expression of recombinant protein with maltose-binding protein increases its solubility. This greatly facilitates the subsequent isolation and purification of the protein. After expression, the proteins were purified under native conditions and subsequently used for monoclonal antibody generation and as antigens for ELISA [18].

Later, in 2014, J. Lazutka et al. [17] produced the SBV nucleocapsid protein in yeast cell culture. Similar to E. Bréard et al. [16] they used in their work an artificially synthesized sequence encoding the viral N-protein. The gene was inserted into the expression plasmid pFX7-SBV-6-HisN under the control of a galactose-induced promoter. The construct also contained an N-terminal hexahistidine tag for subsequent purification. The resulting protein was tested as an ELISA antigen on bovine sera. The sensitivity was 95%.

The published data allows for the conclusion that SBV infection is an emerging viral disease of ruminants posing high economic significance due to its pronounced impact on the reproductive performance of susceptible livestock.

For the SBV infection serological diagnosis, use of a recombinant antigen represented by a nucleocapsid protein is advisable. However, the accumulation of the recombinant protein during prokaryotic expression is accompanied by the formation of inclusion bodies, which necessitates the use of denaturing conditions for purification.

The work was aimed at the production of plasmid vectors based on the pET-32b(+) plasmid and codon-optimized gene sequences encoding the PPRV and SBV nucleocapsid proteins, for their expression in *E. coli* cells.

MATERIALS AND METHODS

The nucleotide sequences of the genes encoding the PPRV and SBV nucleocapsid proteins were received from GenBank database (accession numbers NC_006383.2 and NC_043582.1, respectively). Optimization of the codon composition for expression in *E. coli* was performed using the GenScript Rare Codon Analysis tool [24], taking into account the codon adaptation index (CAI). To eliminate mRNA secondary structure regions and restriction sites, the UGENE software was used [25]. The gene synthesis was made by "DNA-Synthesis" Company (Russia), followed by cloning into pUC57 vector.

E. coli XL1-Blue strain (Evrogen, Russia) of genotype recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F' proAB laclq ZΔM15 Tn10 (Tetr)] was used for cloning. The cells were cultured in LB medium (Helicon Company, Russia) in an incubator shaker at 37 °C and 180 rpm. For the selection of recombinant clones, ampicillin was added to the medium to a final concentration of 100 μg/mL.

The pET-32b(+) plasmid vector (Novagen, USA) was linearized using NcoI and HindIII restricting enzymes (TransGen Biotech, China) at 37 °C for 1 hour. The synthesized genes were amplified using high-fidelity DNA polymerase and primers containing restriction sites (Table).

Table
Specifications of the primers used

Infectious disease	Primer	Sequence 5´⟶3´	Enzyme
Peste des petits ruminants	PPRV Hind 3620	ATAT**AAGCTT**CTGCGAGGCAATCTCGCTAAC	HindIII
	PPRV NcoI 3620	AAAA**CCATGG**CTACACTGTTAAAATCGCTC	NcoI
Schmallenberg virus infection	SBV_HindIII_R	CTCT**AAGCTT**GTGTATATTTATCCCGAACTGTTGCAGGAATG	HindIII
	SBV_NcoI	ATAT**CCATGG**ATGAGCTCGCAGTTTATCTTCGAG	NcoI

Recognition sites for restriction enzymes are in bold.

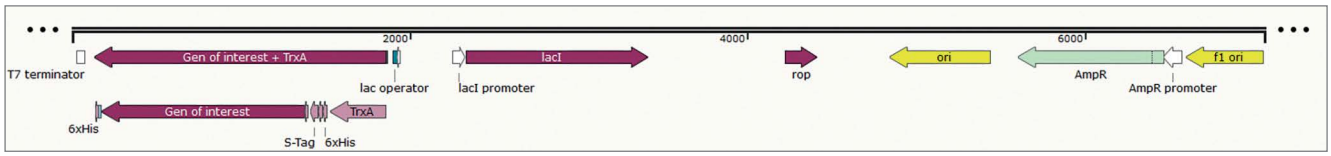

Fig. 1. Design of pET-32b(+) plasmid-based vector: T7 terminator – T7 phage transcription terminator; 6×His – histidine tag; Gen of interest – target gene encoding PPRV or SBV nucleocapsid protein; S-Tag – ribonuclease A fragment; TrxA – thioredoxin tag; Lac operator – lac promotor operator; LacI promoter – promotor of lacI protein; LacI – repressor protein; Rop – plasmid replication regulator; Ori – plasmid origin of replication; AmpR – ampicillin resistance gene; AmpR promoter – AmpR gene promoter. The scheme was constructed using SnapGene software[1]

The DNA fragments were purified using a gel extraction kit (Evrogen, Russia). Ligation was performed with T4 DNA ligase (Evrogen, Russia) with vector-to-insert molar ratio of 1:3 at 14 °C for 16 hours.

Competent cells were transformed using the heat shock method, after which 1 mL of LB medium was added and incubated for 1 hour at 37 °C. Selection was performed on plates with LB agar medium containing 100 µg/mL ampicillin.

For primary screening, bacterial colony PCR was used. For this purpose, primers complementary to the regions flanking the insertion site were used – T7 Promoter (TAA TAC GAC TCA CTA TAG GG) and T7 Terminal (GCT AGT TAT TGC TCA GCG G). The reaction was performed using Taq polymerase (Syntol, Russia). To confirm cloning, plasmid DNA was isolated using the CleanUp S-Cup kit (Evrogen, Russia) and restriction analysis was performed.

RESULTS AND DISCUSSION

Genetic constructs based on pET-32b(+) vector were developed for the expression of PPRV and SBV nucleocapsid proteins. The gene sequences obtained from GenBank database (NC_006383.2 and NC_043582.1) underwent codon optimization. The design of the developed vectors is shown in Figure 1.

After *de novo* synthesis of the target genes, they were amplified using high-fidelity polymerase and primers containing restriction enzyme recognition sites. The amplification results are shown in Figure 2.

The resulting amplicons were purified from the agarose gel and prepared for further restriction digestion alongside the vector. The restriction digestion results are shown in Figure 3.

The resulted DNA fragments, after purification and ligation via sticky ends using T4 DNA ligase, were used for the chemical transformation of competent *E. coli* XL-1 Blue cells. The presence of the insert in the transformant clones was verified by colony PCR using primers flanking the insertion region. The results are shown in Figure 4.

After the required amount of plasmid DNA containing the target gene inserts was accumulated, the restriction analysis was performed. The results are shown in Figure 5.

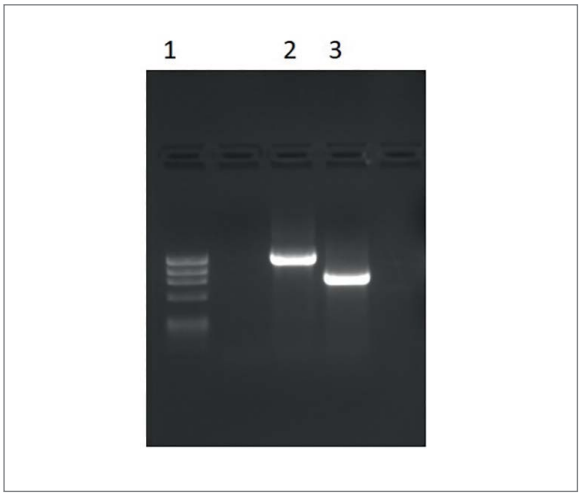

Fig. 2. Target PPRV and SBV gene amplification results: 1 – DNA molecular weight marker (310, 603, 872, 1,078, 1,353 bp); 2 – amplicon of the target PPRV nucleocapsid protein-encoding gene, estimated size – 1,053 bp; 3 – amplicon of the target SBV nucleocapsid protein-encoding gene, estimated size – 719 bp

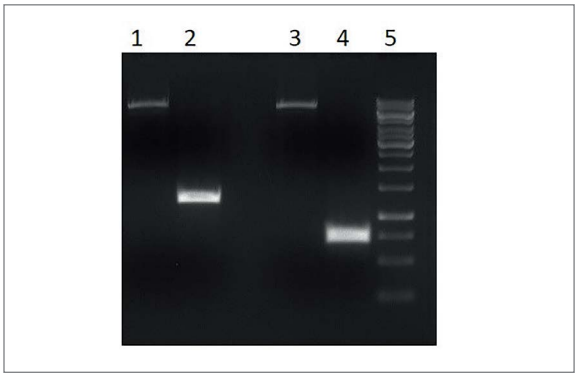

Fig. 3. Restriction digestion of target gene amplicons and pET-32b(+) vector: 1, 3 – pET-32b(+) vector, digested with NcoI and HindIII restriction enzymes, estimated size 5,861 bp; 2 – target gene amplicon encoding PPRV nucleocapsid proteins and digested with NcoI and HindIII restriction enzymes, estimated size 1,045 bp; 4 – target gene amplicon encoding SBV nucleocapsid protein and digested with NcoI and HindIII restriction enzymes, estimated size 710 bp; 5 – DNA molecular weight market (250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp)

[1] https://www.snapgene.com

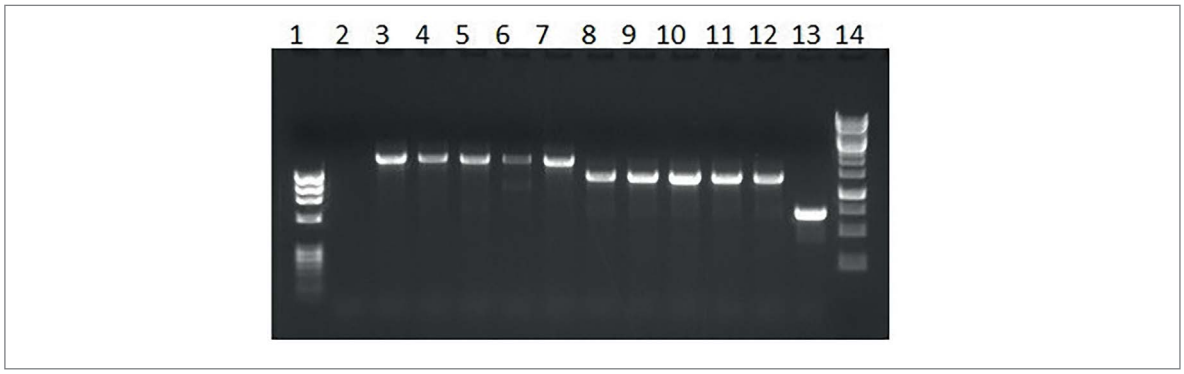

Fig. 4. Screening clones for target gene-containing plasmid inserts: 1 – DNA molecular weight marker (310, 603, 872, 1,078, 1,353 bp); 2 – negative PCR control; 3–7 – clones containing pET-32b(+) plasmid with PPRV nucleocapsid protein-encoding gene, estimated size 1,876 bp; 8–12 – clones containing pET-32b(+) plasmid with SBV nucleocapsid protein-encoding gene, estimated size 1,653 bp; 13 – positive PCR control (insertion-free pET-32b(+) vector); 14 – DNA molecular weight marker (250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp)

Thus, two recombinant plasmid vectors based on pET-32b(+) plasmid were obtained. They contained codon-optimized sequences encoding the PPRV and SBV nucleocapsid proteins.

It should be noted that the expression of codon-optimized genes in prokaryotic systems such as *E. coli* offers a number of key advantages due to the specifics of the bacterial translational machinery.

Firstly, the frequency of codon use in prokaryotes differs significantly from that in eukaryotes. This can lead to a shortage of corresponding tRNAs and, as a result, to translation delays, incorrect protein folding or premature termination. Codon optimization allows adaptation of a gene's nucleotide sequence to the preferred codons of the host, thereby increasing the translation speed and efficiency [26].

Secondly, it facilitates the increase in the yield of recombinant protein by reducing the likelihood of forming mRNA secondary structures that can interfere with the movement of the ribosome.

Thirdly, codon optimization minimizes the risk of amino acid misincorporation errors, which can occur when rare codons are used due to mispairing with non-canonic tRNAs. Furthermore, when expressing heterologous proteins (e.g., of human origin) in *E. coli*, codon optimization is often essential to achieve physiologically relevant production levels, as the native sequences may contain multiple codons that are rare in bacteria. Therefore, the use of codon-optimized genes in prokaryotic expression systems is an important tool for increasing the yield, stability, and functionality of recombinant proteins, which is particularly relevant for biotechnological and biopharmaceutical applications [27].

Such dangerous animal diseases as PPR and SBV infection, considering their current spread,

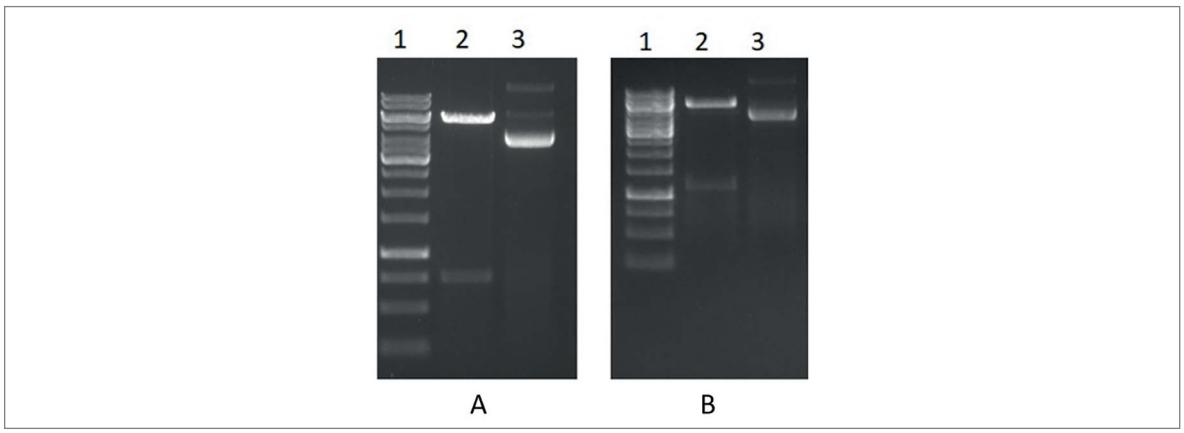

Fig. 5. Results of the restriction analysis of recombinant plasmids isolated from screening-positive clones. A: 1 – DNA molecular weight marker (250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp); 2 – plasmid containing SBV nucleocapsid protein gene and digested with NcoI and HindIII restriction enzymes, estimated size 5,861 and 785 bp; 3 – negative control, plasmid not digested with restrictases; B: 1 – DNA molecular weight marker (250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, 10,000 bp); 2 – plasmid containing PPRV nucleocapsid protein gene and digested with NcoI and HindIII restriction enzymes, estimated size 5,861 and 1,202 bp; 3 – negative control, plasmid not digested with restriction enzymes

remain highly relevant. International cooperation and trade in small ruminants and cattle create conditions for the transmission of the causative agents of these diseases. Currently, the Russian Federation is actively pursuing an import substitution program in the fields of human and animal health, particularly in the development and production of diagnostic test systems, driven by the lack of high-quality domestic equivalents. This leads to the challenge of timely and high-quality diagnostics using our own technological arsenal. Domestic manufacturers have developed many ELISA systems based on the use of native antigens. However, such systems can frequently demonstrate non-specific results. Recombinant proteins, in turn, demonstrate significant advantages over native antigens, including higher product purity, reproducibility of results, and capacity to produce large amounts of material. Furthermore, the ELISA systems based on recombinant proteins are safer for personnel, as there is no need to produce large quantities of infectious material to obtain a viral antigen preparation.

It should be noted that there are a number of limitations to the PPR serological diagnosis. This is related to the specific characteristics of the disease course. Often, the diseased animals die earlier than the virus-specific antibodies are produced. Thus, the direct ELISA variant is the most suitable for emergency tests of the animals. At the same time, the indirect variant, including the one based on recombinant antigens, allows differentiation of vaccinated, convalescent and immune animals, for example, during monitoring studies.

CONCLUSION

It should be noted that ELISA technologies based on recombinant proteins continue to evolve, enabling improvements in the test sensitivity and specificity, thereby opening up new opportunities for laboratory diagnostics and fundamental research. Particular attention should be given to optimizing the conditions for protein expression, purification, and conjugation, which directly impacts the quality of the final diagnostic product.

A significant advantage of ELISA based on recombinant proteins is that, if the virus cannot be cultured for any reason, one can quickly respond to newly emerging viral strains of existing pathogens, provided the gene sequence is available. In addition, the obtained recombinant antigens can be used to produce poly- and monoclonal antibodies and formulate appropriate vaccines.

The continued refinement of recombinant protein production technology will contribute to the advancement of veterinary medicine and enhance the efficacy of infectious disease diagnostics.

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Received 07.08.2025
Revised 17.09.2025
Accepted 15.10.2025

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Вклад авторов: Тенитилов Н. А. – подготовка текста статьи, проведение поисково-аналитической работы, проведение эксперимента; Ярыгина Н. А. – подготовка текста статьи, проведение поисково-аналитической работы; Спрыгин А. В. – научное консультирование, дизайн эксперимента, редактирование статьи, утверждение окончательного варианта.