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Preparation of recombinant SARS-CoV-2 nucleocapsid protein

Anastasia S. Yakovleva, Anzhelika V. Kanshina, Anna M. Timina

Federal Centre for Animal Health, Yur'evets, Vladimir 600901, Russia

ABSTRACT

Introduction. The new coronavirus infection (COVID-19) agent SARS-CoV-2 has become widespread in the world and has caused the pandemic that started in 2019. The virus is a zoonanthropotic infectious agent that causes infection in humans as well as in many mammal species. To date, SARS-CoV-2 has been reported both in domestic and in wild animals. Moreover, successful experimental infection of certain animal species was reported during the studies. There is also the evidence that infected animals can transmit the virus to other animals in natural settings through contact including virus transmission between animals of different species. Currently, some researchers fear that SARS-CoV-2 may spread to mammalian species in the wild that will become a natural reservoir responsible for this infection outbreaks in humans. Furthermore, the virus effect on potentially susceptible wild animal species, including endangered animal species, is currently not fully understood. Therefore, the infection spread in wild animals requires further study. This requires highly sensitive and specific diagnostic methods. Enzyme-linked immunosorbent assay (ELISA) using SARS-CoV-2 nucleocapsid protein as an antigen can be used for serological surveillance of the new coronavirus infection in animals. Recombinant protein used as an antigen is the most preferable because of its safety.

Objective. The study was aimed at preparing highly concentrated recombinant SARS-CoV-2 nucleocapsid protein and testing it for antigenic activity and specificity.

Materials and methods. The following was used for the study: SARS-CoV-2, pQE plasmid, *Escherichia coli* JM109 strain. The following was performed: reverse transcription and polymerase chain reaction, molecular cloning, recombinant protein synthesis, recombinant protein purification, indirect ELISA was used.

Results. Molecular cloning of SARS-CoV-2 N-gene was carried out using prokaryotic expression system. *Escherichia coli* clones producing 33 kDa recombinant SARS-CoV-2 nucleocapsid protein were prepared. Optimal expression and purification conditions for highly concentrated antigen preparation were determined. It was shown that optimal inducer concentration was 0.5 mM, optimal expression period was 4 hours. Urea at a concentration of 8 M as a denaturing agent and optimal imidazole concentration of 0.4 M in the elution buffer were selected based on the results of study of optimal conditions for recombinant antigen purification. Use of the optimal expression and purification procedure allowed us to prepare 1.5 mg of purified antigen from 100 mL of *Escherichia coli* culture. The recombinant protein demonstrated its high antigenic activity and specificity when tested with indirect ELISA.

Conclusion. Preparation of highly concentrated recombinant SARS-CoV-2 nucleocapsid protein enables its further use as an antigen for ELISA test system for detection of antibodies against SARS-CoV-2 nucleocapsid protein in animal sera.

Keywords: coronavirus, SARS-CoV-2, nucleocapsid protein, enzyme-linked immunosorbent assay (ELISA)

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For correspondence: Anastasia S. Yakovleva, Cand. Sci. (Biology), Senior Researcher, Reference Laboratory for Highly Dangerous Diseases, Federal Centre for Animal Health, Yur'evets, Vladimir 600901, Russia, yakovleva_as@arriah.ru

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Получение рекомбинантного нуклеокапсидного белка SARS-CoV-2

А. С. Яковлева, А. В. Каньшина, А. М. Тимина

ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), мкр. Юрьево, г. Владимир, 600901, Россия

РЕЗЮМЕ

Введение. Возбудитель новой коронавирусной инфекции (COVID-19) SARS-CoV-2 получил широкое распространение в мире, став причиной пандемии, которая началась в 2019 г. Вирус является зооантропонозным инфекционным агентом, вызывает инфекцию как у человека, так и у многих видов млекопитающих. К настоящему времени имеются сообщения о выявлении SARS-CoV-2 у домашних животных, а также у представителей дикой фауны. Кроме того, проведены исследования по успешному экспериментальному заражению некоторых видов животных. Имеются также доказательства того, что инфицированные особи могут передавать вирус другим животным в естественных условиях при контакте, в том числе между разными видами. В настоящее время ряд исследователей опасается, что SARS-CoV-2 распространится на виды млекопитающих в дикой природе, которые станут природным резервуаром, что может быть причиной вспышек инфекции в популяции людей. При этом воздействие вируса на потенциально восприимчивые виды животных дикой природы, в том числе исчезающие, в настоящее время до конца не изучено. В связи с этим необходимо проводить исследования

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по изучению распространения данной инфекции среди животных дикой фауны. Для этого требуются высокочувствительные и специфичные диагностические методы. Иммуноферментный анализ с применением в качестве антигена нуклеокапсидного белка SARS-CoV-2 может быть использован для серологического надзора за новой коронавирусной инфекцией среди животных. Применение в качестве антигена рекомбинантного белка является наиболее предпочтительным с точки зрения безопасности.

Цель исследования. Получение рекомбинантного нуклеокапсидного белка SARS-CoV-2 в высокой концентрации и проверка его антигенной активности и специфичности.

Материалы и методы. В работе использовали: SARS-CoV-2, плазмиду pQE, штамм *Escherichia coli* JM109; осуществляли обратную транскрипцию и полимеразную цепную реакцию, молекулярное клонирование, синтез рекомбинантного белка, очистку рекомбинантного белка, применяли непрямой вариант иммуноферментного анализа.

Результаты. Выполнено молекулярное клонирование N-гена SARS-CoV-2 с использованием прокариотической системы экспрессии. Получены клоны *Escherichia coli*, продуцирующие рекомбинантный нуклеокапсидный белок SARS-CoV-2 размером 33 кДа. Определены оптимальные условия экспрессии и очистки, обеспечивающие получение препарата антигена в высокой концентрации. Показано, что оптимальной концентрацией индуктора является 0,5 мМ, оптимальный период экспрессии – 4 ч. В результате исследования оптимальных условий очистки рекомбинантного антигена в качестве денатурирующего агента определена мочевины в концентрации 8 М, подобрана оптимальная концентрация имидазола – 0,4 М в элюирующем буфере. Использование оптимальной схемы экспрессии и очистки позволило получить 1,5 мг очищенного антигена с 100 мл культуры *Escherichia coli*. Показана высокая антигенная активность и специфичность рекомбинантного белка в непрямом варианте иммуноферментного анализа.

Заключение. Получение рекомбинантного нуклеокапсидного белка SARS-CoV-2 в высокой концентрации позволит в перспективе использовать его в качестве антигена при разработке иммуноферментной тест-системы для выявления антител к нуклеокапсидному белку SARS-CoV-2 в сыворотках крови животных.

Ключевые слова: коронавирус, SARS-CoV-2, нуклеокапсидный белок, иммуноферментный анализ

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INTRODUCTION

The new coronavirus (SARS-CoV-2) caused a pandemic of acute respiratory infection that swept the world and led to the deaths of several million people [1]. The infected person can develop either asymptomatic disease or severe pneumonia resulting in death in case of total lung damage. SARS-CoV-2 is an enveloped single-stranded RNA-containing virus of the *Coronaviridae* family, *Betacoronavirus* genus. The virus virion has characteristic crown-like appearance with spike (S), membrane (M) and envelope (E) proteins located in a two-layer phospholipid envelope. The new coronavirus has 30 kb RNA genome encoding 16 non-structural proteins and 4 main structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) [2].

Besides humans, SARS-CoV-2 is capable of infecting many mammalian species [2, 3, 4, 5, 6, 7, 8, 9, 10]. According to the World Organization for Animal Health (WOAH), the new coronavirus was detected in cats, dogs, rodents (hamsters), minks and ferrets, zoo animals (monkeys, tigers, lions, cougars, etc.), deer, foxes, horses. Since the beginning of the pandemic, 35 countries have reported SARS-CoV-2 in animals according to data given in the review prepared by the Rosselkhoz nadzor Information Analysis Center [11].

Currently, it is found that the virus can be transmitted from one animal species to another, from humans to animals, and from animals to humans [12, 13, 14, 15, 16, 17, 18].

In May 2023, the World Health Organization has announced the end of the pandemic, but some groups of scientists consider this announcement premature. They suggest that the virus could pose a threat to public health for decades. Even if the virus circulation in the human population is completely eliminated, SARS-CoV-2 will pose a danger to human health and domestic and wild animal health due to hidden reservoirs in the wild [13, 19, 20].

Some animal species could play the role of a natural reservoir of this virus. Serological monitoring for the new coronavirus infection in domestic and wild animal populations is carried out in some countries to study this possibility. For example, in France, more than 5,600 serum samples from cats and dogs were tested for antibodies to SARS-CoV-2, and in China, more than 20,000 samples from these two animal species were tested. Seromonitoring of the new coronavirus infection in wild fauna is actively carried out in the USA. High seroprevalence has been found in raccoons, squirrels, red foxes, opossums, skunks, white-footed mice, and white-tailed deer [21, 22, 23].

Investigation of SARS-CoV-2 spread in wild and domestic animals requires appropriate diagnostic tools. In 2021, enzyme-linked immunosorbent assay (ELISA) test-system for detection of antibodies to SARS-CoV-2 in animal sera was developed at the Federal Centre for Animal Health [24]. Inactivated coronavirus is used as an antigen in this test-system. However, the virus cultivation is required for

the preparation of such antigen that is associated with a high biological risk. Recombinant SARS-CoV-2 proteins produced in prokaryotic or eukaryotic expression systems can be a safer alternative to the antigen prepared from the native virus. Previously, the nucleocapsid protein was shown to be the most immunogenic SARS-CoV-2 protein having conserved amino acids [25, 26].

The study was aimed at preparation of recombinant SARS-CoV-2 nucleocapsid protein for further use as an antigen for ELISA test-system.

MATERIALS AND METHODS

Virus. Clinical samples (nasal swabs) from the patient with confirmed COVID-19 were used for SARS-CoV-2 RNA extraction.

The viral RNA was recovered using GF/F glass fiber filters according to O. G. Gribanov et al. method [27].

Reverse transcription polymerase chain reaction (RT-PCR). SARS-CoV-2 N-gene was amplified with RT-PCR. The RT-PCR products were analyzed with agarose gel-electrophoresis at 50 mA using 1.5% agarose gel containing 0.001% ethidium bromide.

Molecular cloning of RT-PCR products was performed with common methods [28]. Also, pQE plasmid (plasmid QIAGEN, Netherlands), *Escherichia coli* JM109 strain (Promega, USA) were used.

Recombinant protein synthesis. *E. coli* was cultivated in orbital shaker at 150 rpm and 37 °C. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the cell culture that reached the logarithmic growth phase. Recombinant protein was analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recombinant protein was purified with metal-chelate affinity chromatography using Ni-NTA-agarose (Thermo Fisher Scientific, CША).

Indirect enzyme-linked immunosorbent assay (ELISA) was carried out using Tris buffered saline with Tween 20 (TBS-T) for plate washing. 1% milk in TBS-T was used for blocking nonspecific binding sites and for dilution of sera and secondary antibodies. Protein A conjugate (KPL Company, Italy) was used for tests; ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was used as a substrate for peroxidase conjugate.

RESULTS AND DISCUSSION

The fragment of N-gene of SARS-CoV-2 was amplified by RT-PCR using primers containing the BamHI and Sall restriction sites.

The pQE plasmid and the gene fragment were digested with restriction enzymes. A ligase mix containing the treated amplicon and plasmid vector were transformed into chemically competent cells of *E. coli* strain JM109. The cell pellet was spread on LB-agar containing 100 µg/mL of ampicillin. After transformation, the resulting colonies were screened and several *E. coli* clones expressing the recombinant SARS-CoV-2 nucleocapsid protein containing a poly-histidine tag at the N-terminus were selected. The molecular weight of the protein was 33 kDa that corresponded to the estimated data.

To increase the concentration of recombinant protein expressed in the selected *E. coli* clone, the optimal concentration of the inducer and the optimal expression period ensuring maximum protein accumulation in bacterial cells were determined.

Solutions of 0.1, 0.2, 0.5, and 1.0 mM IPTG were used to determine the optimal concentration of the inducer. The protein expression level was determined visually in 12% SDS-PAGE. The accumulation of recombinant protein reached a peak at an IPTG concentration of 0.5 mM and did not change when the inducer concentration was increased by twofold. The concentration of 0.5 mM was determined as optimal, and subsequently all expression runs were carried out using this inducer concentration.

To determine the optimal period of recombinant protein expression, *E. coli* cell lysate was examined 2, 4, and 18 hours after induction. The protein accumulation level was analyzed with 12% polyacrylamide gel electrophoresis. The maximum level of recombinant protein expression was observed 4 hours after adding of the inducer (Fig. 1). The protein amount was lower 18 hours after induction that may be accounted for its destruction during long-term *E. coli* cultivation. Thus, the optimal time for recombinant protein synthesis was considered to be 4 hours after induction.

The recombinant protein was purified with metal chelate chromatography. When cell lysate was prepared under native conditions, the most of the protein remained in the cellular debris, so further lysis was carried out under denaturing conditions.

To prepare purified protein at maximum concentration, studies were carried out to determine the optimal composition of lysis and elution buffers.

The following was included in the buffer composition: 8 M urea – for lysis buffer No. 1, and 6 M guanidine

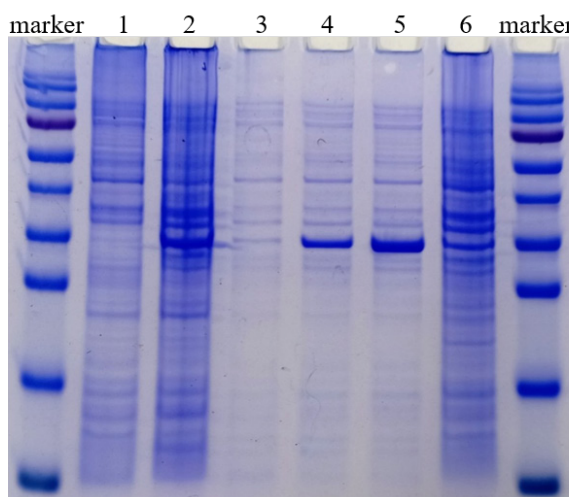


Fig. 1. Determination of optimal period of recombinant SARS-CoV-2 nucleocapsid protein expression:
protein molecular weight marker
(170, 130, 95, 72, 55, 43, 34, 26, 17, 10 kDa);
1 – *E. coli* JM109 cell lysate;
2 – cell lysate of SARS-CoV-2 N-gene-containing *E. coli* clone, 18 hours after incubation;
3 – cell lysate of SARS-CoV-2 N-gene-containing *E. coli* clone, before induction;
4 – cell lysate of SARS-CoV-2 N-gene-containing *E. coli* clone, 2 hours after induction;
5 – cell lysate of SARS-CoV-2 N-gene-containing *E. coli* clone, 4 hours after induction;
6 – cell lysate of SARS-CoV-2 N-gene-containing *E. coli* clone, 18 hours after induction

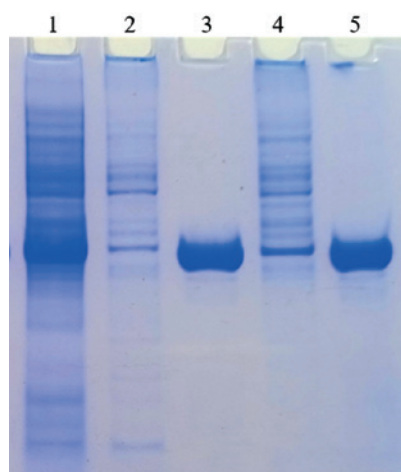


Fig. 2. Effect of lysing buffer composition on purified recombinant SARS-CoV-2 nucleocapsid protein solubility and yield:

- 1 – cell lysate of recombinant *E. coli* clone, 4 hours after induction;
- 2 – sediment of recombinant protein-expressing cells after treatment with lysis buffer No. 1 containing 8 M urea;
- 3 – purified recombinant protein when denaturing buffer containing 8 M urea was used;
- 4 – sediment of recombinant protein-expressing cells after treatment with lysis buffer No. 2 containing 6 M Gu-HCl;
- 5 – purified recombinant protein when denaturing buffer containing 6 M Gu-HCl was used

hydrochloride (Gu-HCl) – for lysis buffer No. 2. Under denaturing conditions, most of the protein was cleared from cellular debris by centrifugation. The protein yield was approximately the same when both 8 M urea and 6 M

GuHCl were used (Fig. 2). It was decided to use 8 M urea as a denaturing agent.

Cell lysate was purified by metal chelate chromatography using Ni-NTA (nickel-nitrile acetate) agarose. Four buffer variants were used for N-protein elution: buffer A (8 M urea, 0.1 M Na_2HPO_4 , 0.01 M tris-Cl, pH 4.0), buffer B (8 M urea, 0.1 M Na_2HPO_4 , 0.01 M tris-Cl, 0.2 M imidazole, pH 8.0), buffer C (8 M urea, 0.1 M Na_2HPO_4 , 0.01 M tris-Cl, 0.4 M imidazole, pH 8.0), buffer D (8 M urea, 0.1 M Na_2HPO_4 , 0.01 M tris-Cl, 0.5 M imidazole, pH 8.0). The protein concentration in each eluate was assessed visually based on electrophoregram. The highest concentration of recombinant protein was found in the eluate derived using buffer C (Fig. 3). Thus, the maximum yield of purified N-protein was achieved by denaturation using buffer containing 8 M urea and elution using buffer containing 0.4 M imidazole.

During experiments for optimization of expression and purification conditions, the following scheme for recombinant SARS-CoV-2 nucleocapsid protein preparation was determined. One milliliters of recombinant *E. coli* clone cell suspension collected after overnight incubation was added to 0.1 L of LB medium supplemented with 100 $\mu\text{g/mL}$ of ampicillin and incubated 37 °C at 150 rpm, until a density of $\text{OD}_{600} = 0.5$ was reached. For induction IPTG was added to a final concentration of 0.5 mM and the culture was incubated for another 4 hours at 37 °C and 150 rpm. The cell suspension was clarified at 5,000 rpm for 15 min. The pellet was resuspended with 5 mL of lysis buffer containing 8 M urea. The cellular debris was removed by centrifugation at 12,000 rpm for 5 min. The supernatant was used for metal chelate chromatography. The clarified lysate was mixed with 1 mL of sorbent (Ni-NTA agarose) for 15 min, then the resulting suspension was centrifuged at 12,000 rpm for 1 min. The pellet was washed twice with

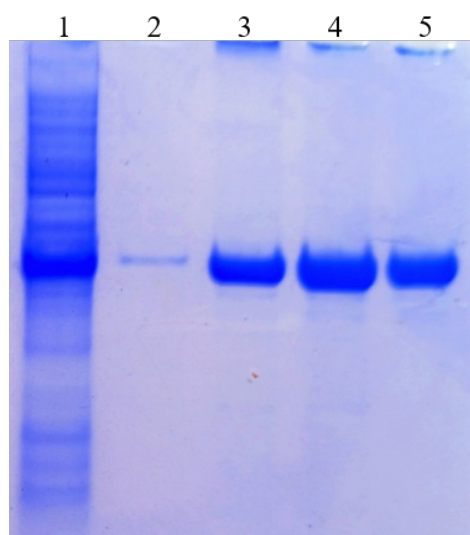


Fig. 3. Effect of elution buffer composition on purified recombinant SARS-CoV-2 nucleocapsid protein yield:

- 1 – cell lysate of recombinant *E. coli* clone, 4 hours after induction;
- 2 – purified recombinant protein when buffer A was used;
- 3 – purified recombinant protein when buffer B was used;
- 4 – purified recombinant protein when buffer C was used;
- 5 – purified recombinant protein when buffer D was used

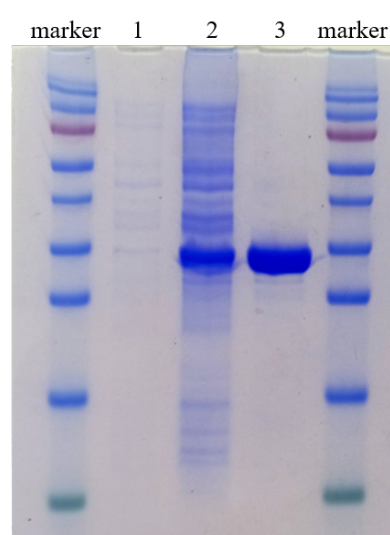


Fig. 4. Assessment of recombinant SARS-CoV-2 nucleocapsid protein size and purification level: protein molecular weight marker

- (170, 130, 95, 72, 55, 43, 34, 26, 17, 10 kDa);
- 1 – cell lysate of recombinant *E. coli* clone before induction;
- 2 – cell lysate of recombinant *E. coli* clone, 4 hours after induction;
- 3 – purified recombinant protein

Table

Results of tests of recombinant SARS-CoV-2 nucleocapsid protein for its antigenic activity with indirect ELISA

| Control serum dilution | ELISA OD value of controls | | | |
|------------------------|----------------------------|----------|----------|----------|
| | –C | +C No. 1 | +C No. 2 | +C No. 3 |
| 1:20 | 0.094 | 2.552 | 2.839 | 3.102 |
| 1:40 | 0.092 | 1.170 | 2.712 | 3.054 |
| 1:80 | 0.083 | 1.911 | 2.628 | 2.995 |
| 1:160 | 0.081 | 1.261 | 2.240 | 2.803 |
| 1:320 | 0.078 | 0.753 | 1.717 | 2.420 |
| 1:640 | 0.078 | 0.466 | 1.181 | 1.899 |
| 1:1,280 | 0.077 | 0.266 | 0.693 | 1.293 |
| 1:2,560 | 0.070 | 0.182 | 0.422 | 0.849 |

a lysis buffer. Elution was performed by adding 1 mL of elution buffer containing 0.4 M imidazole to the pellet, the pellet was stirred for 1 min, then centrifuged at 12,000 rpm for 1 min. The supernatant was tested for recombinant protein. The 12% polyacrylamide gel electrophoresis was performed to assess the degree of purification and the size of the resulting protein (Fig. 4). The protein concentration was measured by Bradford assay.

Optimization of parameters of recombinant SARS-CoV-2 nucleocapsid protein expression and purification allowed for preparation of highly concentrated protein. The yield of purified protein from 100 mL of *E. coli* culture was 1.5 mg.

The recombinant SARS-CoV-2 nucleocapsid protein was tested for its antigenic activity in indirect ELISA using control rabbit sera. For this purpose, the recombinant protein diluted to 1:800 with carbonate-bicarbonate buffer was added to ELISA plate wells and the plate was incubated overnight. After blocking of non-specific binding sites and subsequent washing, control rabbit sera diluted from 1:20 to 1:2,560 were added to the plate wells. Sera from three rabbits immunized with the SARS-CoV-2 (+C) antigen were used as a positive control, and serum from non-immunized rabbit (–C) was used as a negative control. Sera were removed after 1 hour, the plate was washed, and protein A peroxidase conjugate was added. After another 1 hour, the plates were washed and ABTS substrate was added for reaction visualization. ELISA results are shown in the Table.

The studies showed that the recombinant nucleocapsid protein demonstrated high antigenic activity against positive control sera and absence of nonspecific binding with negative control serum.

CONCLUSION

The molecular cloning of the gene encoding the SARS-CoV-2 nucleocapsid protein was performed using a prokaryotic expression system.

E. coli clones expressing recombinant nucleocapsid protein were prepared.

The expression and purification conditions ensuring a high yield of purified antigen were determined.

Indirect ELISA test results have shown that prepared recombinant protein has high antigenic activity and can be used for detection of antibodies against SARS-CoV-2 in animal sera.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Anastasia S. Yakovleva, Cand. Sci. (Biology), Senior Researcher, Reference Laboratory for Highly Dangerous Diseases, Federal Centre for Animal Health, Vladimir, Russia; <https://orcid.org/0000-0001-9211-9110>, yakovleva_as@arriah.ru

Anzhelika V. Kanshina, Cand. Sci. (Veterinary Medicine), Senior Researcher, Reference Laboratory for Highly Dangerous Diseases, Federal Centre for Animal Health, Vladimir, Russia; <https://orcid.org/0009-0004-2464-8183>, kanshina@arriah.ru

Anna M. Timina, Cand. Sci. (Veterinary Medicine), Senior Researcher, Reference Laboratory for Highly Dangerous Diseases, Federal Centre for Animal Health, Vladimir, Russia; <https://orcid.org/0000-0002-0109-3507>, timina@arriah.ru

Яковлева Анастасия Сергеевна, канд. биол. наук, старший научный сотрудник референтной лаборатории по особо опасным болезням ФГБУ «ВНИИЗЖ», г. Владимир, Россия; <https://orcid.org/0000-0001-9211-9110>, yakovleva_as@arriah.ru

Каньшина Анжелика Владимировна, канд. вет. наук, старший научный сотрудник референтной лаборатории по особо опасным болезням ФГБУ «ВНИИЗЖ», г. Владимир, Россия; <https://orcid.org/0009-0004-2464-8183>, kanshina@arriah.ru

Тимина Анна Михайловна, канд. вет. наук, старший научный сотрудник референтной лаборатории по особо опасным болезням ФГБУ «ВНИИЗЖ», г. Владимир, Россия; <https://orcid.org/0000-0002-0109-3507>, timina@arriah.ru

Contribution of the authors: Yakovleva A. S. – conceptualization, formulation of key goals and objectives, study design and conducting, obtained data analysis and interpretation, paper writing, key study result formulation, approval of final paper text; Kanshina A. V. – conceptualization, formulation of key goals and objectives, performing experiments, obtained data analysis and interpretation; Timina A. M. – conceptualization, formulation of key goals and objectives, performing tests, obtained data analysis and interpretation.

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