FGBI "FEDERAL CENTRE FOR ANIMAL HEALTH" (FGBI "ARRIAH")

FEDERAL SERVICE FOR VETERINARY AND PHYTOSANITARY SURVEILLANCE (ROSSELKHOZNADZOR)

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РУССКО-АНГЛИЙСКИЙ ЖУРНАЛ

JUNE | ИЮНЬ №2 [37] 2021

CORONAVIRUS DISEASE (COVID-19)

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A Word from the Editor-in-Chief

Dear readers! Your attention is invited to the second issue of the journal, which offers you the articles on the vital topic of Coronavirus infection.

In recent world history, 2019–2021 were marked by a pandemic of the novel coronavirus infection COVID-19, caused by the SARS-CoV-2. None of the previous epidemics recorded in the current century, including SARS (2002) and MERS (2012), has had such a global spread and such significant social and economic consequences. To date, more than 170 million cases of COVID-19 among people are already known; more than 3.5 million people have fallen victims to the disease. Having a pronounced zoonotic potential, coronaviruses are able to overcome the species barrier. This led to spillover events at the beginning of the pandemic: the SARS-CoV-2 spread to humans from bats, possibly through an intermediate host (pangolins), and then from humans to other species, primarily domestic ones (dogs and cats), to zoo cats (tigers and lions), as well as to minks. In the latter case, unprecedented measures were taken in a number of European countries. For example, Denmark culled almost all farm minks over fears of the SARS-CoV-2 mutation in these animals.

Reports about COVID-19 cases in animals urged infectious pathology and epizootology research institutions to take immediate response measures. In this regard, the FGBI "ARRIAH" and the FGBI "VGNKI", subordinated to the Rosselkhoznadzor, in a prompt manner developed diagnostic test kits, which allowed detection of two infected cats during screening tests. Taking into account the need to establish the true COVID-19 situation in animals in our country, amid a growing flow of data on cases in different species abroad, the FGBI "ARRIAH" boosted its screening activities in 2020–2021 among different species, and developed and registered the product, the first one in the veterinary world, aimed at prevention of this disease in carnivorous animals. Besides, employees of the FGBI "ARRIAH" and the FGBI "VGNKI" conducted advanced on-line training courses, including free ones, on the epizootic situation, diagnosis and prevention of COVID-19 in animals. Thanks to the work done, our veterinary specialists possess reliable tools for the infection diagnosis and prevention in animals.

Photo: Alexander Plonskiy

The issue of the journal that you are holding in your hands summarizes the results of the work done. Its purpose is to draw the attention of the scientific community to the need for further study of the problem related to the spread of COVID-19 among animals and to avoid the negative development of the situation to panzootic.

Best regards,
Editor-in-Chief
Doctor of Science (Veterinary Medicine)
Artem Ye. Metlin

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Development of Carnivac-Cov vaccine against coronavirus infection (COVID-19) in carnivores

T. S. Galkina[1], A. A. Nesterov[2], A. V. Borisov[3], I. A. Chvala[4], A. V. Kononov[5]
FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia
[1] *ORCID 0000-0001-9494-8537, e-mail: galkina_ts@arriah.ru*
[2] *ORCID 0000-0002-4288-1964, e-mail: nesterov@arriah.ru*
[3] *ORCID 0000-0001-9880-9657, e-mail: borisov_av@arriah.ru*
[4] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*
[5] *ORCID 0000-0002-5523-3261, e-mail: kononov@arriah.ru*

SUMMARY
Development of specific protection in susceptible carnivores against COVID-19 caused by zoonotic agent is of great importance for maintaining epidemic and veterinary favourable situation in the Russian Federation and prevention of new coronavirus infection spread in humans. Development of inactivated adsorbed whole-virion vaccine (Carnivac-Cov) against coronavirus infection (COVID-19) for carnivores intended for specific disease prevention in fur animals and pet animals (cats and dogs) and tests of the vaccine for its safety and effectiveness are described in the paper. The vaccine was developed and tested at the FGBI "ARRIAH" (Vladimir) subordinated to the Rosselkhoznadzor in accordance with the Russian Federation legislation requirements. Clinical trials were carried out on fur farms, in veterinary clinics and animal shelters. More than 330 animals (fur animals, cats, dogs) were involved in the preclinical and clinical trials. The trials have demonstrated that the vaccine is safe for target animals. Carnivac-Cov vaccine administered twice intramuscularly at the dose of 1.0 cm^3 induces anti-SARS-CoV-2 immune response 14 days after the second administration that lasts for at least 6 months. The vaccine transportation, storage and application do not require any specific protective equipment. The vaccine can be used on fur farms and in veterinary clinics without limitations. Carnivac-Cov is the first tool for specific COVID-19 prevention in animals. The vaccine has successfully passed comprehensive quality control and is registered in the Russian Federation.

Keywords: SARS-CoV-2, prevention, COVID-19, vaccine safety and effectiveness, fur animal farming, pet animals.

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For correspondence: Tatyana S. Galkina, Candidate of Science (Veterinary Medicine), Head of Laboratory for Pet Disease Prevention, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: galkina_ts@arriah.ru.*

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Создание вакцины против коронавирусной инфекции (COVID-19) плотоядных животных «Карнивак-Ков»

Т. С. Галкина[1], А. А. Нестеров[2], А. В. Борисов[3], И. А. Чвала[4], А. В. Кононов[5]
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия
[1] *ORCID 0000-0001-9494-8537, e-mail: galkina_ts@arriah.ru*
[2] *ORCID 0000-0002-4288-1964, e-mail: nesterov@arriah.ru*
[3] *ORCID 0000-0001-9880-9657, e-mail: borisov_av@arriah.ru*
[4] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*
[5] *ORCID 0000-0002-5523-3261, e-mail: kononov@arriah.ru*

РЕЗЮМЕ
Для обеспечения эпизоотического и ветеринарного благополучия Российской Федерации и предотвращения распространения новой коронавирусной инфекции среди людей первостепенное значение имеет вопрос формирования специфической защиты среди восприимчивых плотоядных животных против COVID-19, возбудитель которого обладает зоонозным потенциалом. В статье представлены результаты разработки, а также оценки безвредности и эффективности инактивированной сорбированной цельновирионной вакцины против коронавирусной инфекции (COVID-19) для плотоядных животных «Карнивак-Ков», предназначенной для специфической профилактики заболевания пушных зверей и животных-компаньонов (собак и кошек). Разработку

и контроль качества препарата осуществляли согласно требованиям законодательства Российской Федерации на базе подведомственного Россельхознадзору ФГБУ «ВНИИЗЖ» (г. Владимир). Клинические испытания проводили в условиях звероводческих хозяйств, ветеринарных госпиталей и приютов для животных. В общей сложности при проведении доклинических и клинических исследований участвовало более 330 голов животных (пушные звери, кошки, собаки). Проведенные испытания препарата показали его безвредность для целевых животных. Через 14 суток после двукратного внутримышечного введения иммунизирующей дозы (1,0 см^3) «Карнивак-Ков» вызывает формирование иммунного ответа против SARS-CoV-2 продолжительностью не менее 6 мес. Транспортировка, хранение и применение препарата не требует обеспечения особых условий. Вакцина может свободно использоваться в условиях звероводческих хозяйств и ветеринарных клиник. «Карнивак-Ков» является первым в мире инструментом специфической профилактики COVID-19 у животных. Препарат успешно прошел всесторонний контроль качества и зарегистрирован на территории Российской Федерации.

Ключевые слова: SARS-CoV-2, профилактика, COVID-19, безвредность и эффективность вакцины, пушное звероводство, животные-компаньоны.

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INTRODUCTION

The first human cases of COVID-19, the disease caused by new SARS-CoV-2 coronavirus, were officially reported in Wuhan, People's Republic of China, in December 2019. Since that time the disease has spread around the world, affected more than 170 mln people and caused more than 3.5 mln human deaths. Cases of COVID-19 transmission from human to animals, its agent circulation in animal population and its transmission back to humans were officially reported [1, 2]. COVID-19 infection agent is an enveloped single-stranded non-segmented RNA-virus, SARS-CoV-2, belonging to *Coronaviridae* family, *Betacoronavirus* genus. *Coronaviridae* family members are the major pathogens of mammals (including humans), amphibians and birds [3–5].

Coronaviruses have multiple virus subgroups. Alpha- and beta-coronaviruses (including coronavirus causing common cold in humans) as a rule affect mammals; gamma- and delta-coronaviruses generally cause infectious diseases in birds and fish. Many of the common coronaviruses causing diseases in domestic animals, such as feline enteral coronavirus, belong to alpha coronaviruses. SARS-CoV-2 affecting the human respiratory system belongs to beta-coronavirus group [4, 6–8].

The initial source of COVID-19 infection agent has not yet been identified, but the World Health Organization (WHO) and World Organization for Animal Health (OIE) are both of opinion that bats are the most probable environmental reservoir of SARS-CoV-2. However, the agent could undergo passages in an animal of other species that was an intermediate host, before it infected the human for the first time. Such intermediate host is supposed to be a livestock animal, wild animal or farmed wild animal. The role of animals in the infection spread is not clearly defined. However, according to the OIE, some animal species are susceptible to COVID-19. Zoonotic potential of the agent was confirmed for the first time by detection of SARS-CoV-2 genome in biomaterial samples collected from dogs in Hong Kong [9–13].

SARS-CoV-2-susceptibility of animals is currently not sufficiently understood, but there are some reports on experimental infection of ferrets, cats, dogs and pigs. It was demonstrated that SARS-CoV-2 effectively replicated in cats and ferrets but dogs were low susceptible [4, 11]. B. S. Pickering et al. showed that pigs were susceptible to nasal infection with SARS-CoV-2 at a high dose [12].

COVID-19 agent was identified and reported in mink population in 10 countries: Canada, Denmark, France, Greece, Italy, Lithuania, the Netherlands, Spain, Sweden and the United States of America. First two disease outbreaks on mink farms were reported in the Netherlands in April 2020. Most affected farms reported SARS-CoV-2 infection cases in their workers that supposed coronavirus transmission from humans (animal owner or handlers) to minks and was indicative of the agent ability to become a reverse zoonosis [1, 2, 13–15]. The situation was the most complicated in Denmark where more than 17 mln minks were culled on fur farms due to partially detection of SARS-CoV-2 mutation (cluster 5) variant that was able to transmit from animals to humans [1, 2].

Thereafter, cases of SARS-CoV-2 infection in animals of different species with respiratory and intestinal signs were detected in several countries.

Emerging infectious diseases often pose threat to wildlife and biological diversity. Fur animals escaping from the farm can become the virus-maintaining source and can cause agent spillover to sympatric wild animal species in case of susceptible species presence. Currently available information is not sufficient to assess the probability of SARS-CoV-2 reservoir establishment in susceptible wild animals.

Mink escaping from the farm has always been a problem for the countries where fur farming exists or has existed. It is believed that the number of escapes increases during mass culling. It was found in one region of Denmark that the majority of the minks living in the region were born on a farm and then escaped. This is indicative of the

role of the farms as an actual sources for wild population replenishment and maintaining of mink population at large size. Similar conclusions were made in other countries. Escaped minks come into contact with wild minks: hybridization between farmed and wild individuals in the wild were documented.

The first free-ranged local wild mink with confirmed SARS-CoV-2 infection was detected in Utah State (USA) in December 2020. Phylogenetic analysis of the recovered virus isolate confirmed that it was closely related to the virus isolated from the animals on a mink farm. This suggests that the wild mink has been infected through indirect or direct contact to the infected farmed minks [14, 15].

The risk of SARS-CoV-2 infection of humans by fur animals poses a serious threat to human health and socio-economic development. The agent can be transmitted from fur animals to wild animals through direct contact between wild and infected farmed animals as well as indirectly via infected carcasses, wastes and other contaminated objects, as a result of direct and indirect contacts between fur animals/contaminated objects and stray animals. Such stray animals could be an intermediate species and transmit the virus to susceptible wild animals. SARS-CoV-2 spread on mink farms creates new opportunities for its evolution due to facilitating the agent transmission to wild animal species that could become the virus reservoirs [1, 2, 13, 15].

Thus, development of specific anti-COVID-19 protection in susceptible carnivores is of utmost importance for maintaining favorable veterinary situation in the Russian Federation and prevention of the disease spread in humans.

Considering the abovementioned, the Rosselkhoznadzor-subordinated FGBI "ARRIAH" was tasked to develop safe and effective vaccine against coronavirus infection (COVID-19) for carnivores and register it in the Russian Federation.

MATERIALS AND METHODS

Carnivac-Cov vaccine was developed and tested for its quality in accordance with Federal Law No. 61-FZ on circulation of medcines and Order of the Ministry of Agriculture of the Russian Federation No. 101 on approval of the rules for preclinical testing of veterinary medicinal products, clinical trials of veterinary medicinal products, tests of veterinary medicinal products for bioequivalence.

Four pilot vaccine batches were used for preclinical and clinical tests.

Carnivac-Cov vaccine active substance is inactivated SARS-CoV virus. Aluminum hydroxide was used as an adsorbing agent. All vaccine components were thoroughly tested during incoming quality control including tests for sterility and antigen innocuity in Vero C1008 by three consecutive passages.

Preclinical tests were performed at the FGBI "ARRIAH"; 130 animals of different species (ferrets, minks, dogs, cats) were used for preclinical trials. Clinical trials were performed in 200 target animals (cats, dogs, minks, Arctic foxes, etc.) on fur farms, in veterinary clinics and animal shelters.

The animals were kept and used in accordance with the Guidelines for laboratory animal keeping and use (Directive 2010/63/EU of the European Parliament and Council of the European Union of 22 September 2012).

Serum samples from the animals were tested for specific antibodies against SARS-CoV-2 in accordance with the Methodical Guidelines for detection of anti-SARS-CoV-2 antibodies with enzyme-linked immunosorbent assay in sera of susceptible animals [16].

RESULTS AND DISCUSSION

Carnivac-Cov vaccine was examined for the following during its preclinical tests: interaction with other veterinary medicinal products, toxicity for target and laboratory animals, vaccine tolerability in healthy animals, immunity duration.

Ferrets, minks, dogs and cats were used to determine the interaction of the vaccine with other immunologicals. Animals of each species were divided into four groups isolated from each other, 5 animals per group. The animals of the first groups were immunized with Carnivac-Cov vaccine, animals of the second groups were simultaneously immunized with Carnivac-Cov vaccine and anti-rabies vaccine, animals of the third groups were vaccinated with the anti-rabies vaccine 5 days after Carnivac-Cov administration and animals of the fourth groups remained non-immunized.

Blood samples were collected from the animals 14 days after their vaccination with Carnivac-Cov for sera preparation; the sera were tested for specific antibodies against SARS-CoV-2 with enzyme-linked immunosorbent assay (ELISA). Test results showed that the antibody levels in Carnivac-Cov vaccine-immunized test animals were 1:200–1:800 [16].

No signs of depression, loss of appetite, body temperature rise or other clinical disorders were detected in test animals during observation period of 35 days that together with serological test results demonstrated Carnivac-Cov vaccine compatibility with other veterinary medicinal products.

The vaccine was tested for its toxicity in ferrets, minks, dogs, cats and white mice. Animals of each species were divided into two groups, 5 animals per group. One immunizing dose (1.0 cm^3) of the vaccine was administered intramuscularly to the animals of the first groups. The vaccine was administered three times during the day every 6 hours. The animals of the second groups remained intact (control groups). The animals of all groups were observed for clinical signs including body temperature measuring and recording the observation results during 14 days after the vaccine administration. The animals were euthanized on day 15 after the experiment had started and then subjected to post-mortem examination for possible organ and tissue lesions.

No intoxication signs were detected in animals of all test groups: general body condition, body temperature, water and feed intake, etc. were similar to that ones in control groups of animals. Obtained data showed that Carnivac-Cov vaccine was not toxic for the said animal species.

The vaccine was tested for its tolerability in healthy animals. The tests were carried out in ferrets, minks, dogs, cats injected intramuscularly by the recommended vaccine dose (1.0 cm^3) as well as by the vaccine doses that were 5, 10 and 50 times higher than the recommended dose. Each vaccine dose was administered intramuscularly twice with a 21-day interval. The animals of all groups were clinically observed for 35 days after the first vaccine administration. Afterwards, the animals were euthanized and subjected to post-mortem examination. The test findings showed that the vaccine administered at the recommended doses or at the excessive doses induced no local reactions and had

no lethal effect. Post-mortem examination showed that organs of all test animals were normal. Thus, obtained results have shown that Carnivac-Cov vaccine is safe and non-reactogenic for carnivores.

Tests for determination of the vaccine immunizing dose were carried out in 2 months-old ferrets, minks, dogs and cats (35 animals of each species). The animals were divided into isolated groups, 5 animals per group, were intramuscularly injected with the vaccine at a dose of 0.5; 1.0 and 2.0 cm^3 once and injected twice at the same doses at a 21-day interval. The animals were examined for clinical signs with daily thermometry during the whole observation period.

Blood samples were collected from all animals 14 days after the last pilot vaccine administration. The prepared sera were tested with ELISA for detection of specific anti-SARS-CoV-2 antibodies.

ELISA tests showed that average specific anti-SARS-CoV-2 antibody level in the animals vaccinated with Carnivac-Cov vaccine at a dose of 0.5 cm^3 was significantly lower than that ones in animals immunized with the vaccine at doses 1.0 and 2.0 $см^3$ (Table).

Thus, obtained results indicated that the minimal immunizing dose of the pilot vaccine was 1.0 cm^3 when the vaccine was administered intramuscularly twice at a 21-day interval.

Tests for assessment of postvaccinal immunity duration were carried out in ferrets, minks, dogs and cats (13 animals of each species) divided into groups isolated from each other. Animals of tests groups (10 animals of each species per group) were immunized with Carnivac-Cov vaccine by double intramuscular injection of 1.0 cm^3 at a 21-day interval. The animals of control groups (3 animals of each species per group) remained non-vaccinated.

Blood samples were collected from all animals 2, 4 and 6 months after immunization and the prepared sera were tested with ELISA for detection of specific anti-SARS-CoV-2 antibodies.

Sera test results showed that average specific anti-SARS-CoV-2 antibody level in samples from the immunized animals (test groups) was 1:420–1:520 during 6 months. Whereas, no specific anti-SARS-CoV-2 antibodies were detected in sera collected from non-vaccinated animals (control groups). Thus, the test results show that Carnivac-Cov vaccine induces strong immunity response against coronavirus infection (COVID-19) agent in the immunized carnivores that lasts for at least 6 months.

The vaccine contains no infectious agent or toxic substances and does not pose a potential threat to humans and environment.

Carnivac-Cov vaccine was proven safe and effective during the preclinical trials and that allowed for clinical trials of the vaccine in cats, dogs, fur animals (minks, Arctic foxes, foxes). The trials were carried out in target animals (cats, dogs, minks, Arctic foxes, foxes) of different ages on fur farms, in veterinary clinics and animal shelters. The animals were injected intramuscularly twice with Carnivac-Cov vaccine at a dose of 1.0 $см^3$ with a 21-day interval (Fig. 1–3).

No local tissue reactions to the vaccine injection, disease signs or animal deaths were detected in the animals during the observation period.

Blood samples were taken from the immunized animals (cats, dogs, fur animals) 2, 4 and 6 months after vaccination to confirm duration of the postvaccinal immunity induced by Carnivac-Cov in target animals in the field. Prepared sera were tested with ELISA for specific anti-SARS-CoV-2 antibodies.

Tests showed that average specific anti-SARS-CoV-2 antibody levels in sera collected from the immunized animals remained high throughout the whole observation period and were as follows per group: 1:485 in cats, 1:304 in dogs, 1:500 in fur animals (Fig. 4–6). Clinical trial results confirmed Carnivac-Cov vaccine safety and effectiveness.

Successful preclinical and clinical trials of Carnivac-Cov vaccine provided grounds for preparation of the

Table
Results of testing of sera from carnivores immunized with Carnivac-Cov vaccine

Таблица
Результаты исследования проб сыворотки крови плотоядных животных, иммунизированных вакциной «Карнивак-Ков»

Group No.	Animal species	Number of animals	Injected dose, volume / number of injections	Average antibody level
1	ferrets	5	0.5 cm^3/once	1:70
2		5	0.5 cm^3/twice	1:100
3		5	1.0 cm^3/once	1:140
4		5	1.0 cm^3/twice	1:440
5		5	2.0 cm^3/once	1:160
6		5	2.0 cm^3/twice	1:480
7		5	control	< 1:50
8	minks	5	0.5 cm^3/once	1:60
9		5	0.5 $см^3$/twice	1:100
10		5	1.0 cm^3/once	1:120
11		5	1.0 cm^3/twice	1:440
12		5	2.0 cm^3/once	1:120
13		5	2.0 cm^3/twice	1:340
14		5	control	< 1:50
15	dogs	5	0.5 cm^3/once	1:80
16		5	0.5 cm^3/twice	1:90
17		5	1.0 cm^3/once	1:80
18		5	1.0 cm^3/twice	1:240
19		5	2.0 cm^3/once	1:150
20		5	2.0 cm^3/twice	1:280
21		5	control	< 1:50
22	cats	5	0.5 cm^3/once	1:70
23		5	0.5 cm^3/twice	1:110
24		5	1.0 cm^3/once	1:120
25		5	1.0 cm^3/twice	1:360
26		5	2.0 cm^3/once	1:150
27		5	2.0 cm^3/twice	1:280
28		5	control	< 1:50

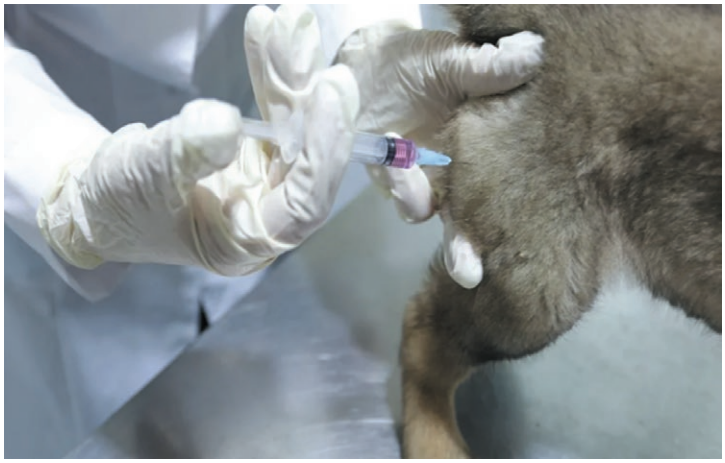

Fig. 1. Carnivac-Cov vaccine is intramuscularly administered to the puppy

Рис. 1. Внутримышечное введение вакцины «Карнивак-Ков» щенку

Fig. 2. Carnivac-Cov vaccine is intramuscularly administered to the cat

Рис. 2. Внутримышечное введение вакцины «Карнивак-Ков» кошке

Fig. 3. Carnivac-Cov vaccine is intramuscularly administered to the mink

Рис. 3. Внутримышечное введение вакцины «Карнивак-Ков» норке

registration dossier for the adsorbed inactivated vaccine against coronavirus infection (COVID-19) for carnivores and subsequent registration of the said vaccine in the Russian Federation.

CONCLUSION

Effective and safe product for specific immunization of carnivores against COVID-19 was developed based on the studies performed at the FGBI "ARRIAH". Double immunization of the animals at a dose of 1.0 cm^3 with a 21-day interval induces antibody development at the following levels: 1:485 in cats, 1:304 in dogs, 1:500 in fur animals. The vaccine has been proven safe, non-reactogenic for carnivores.

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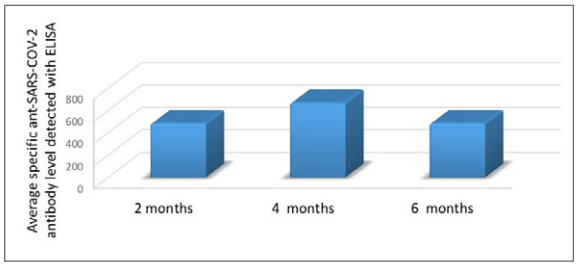

Fig. 4. Immunity duration in cats

Рис. 4. Продолжительность иммунитета у кошек

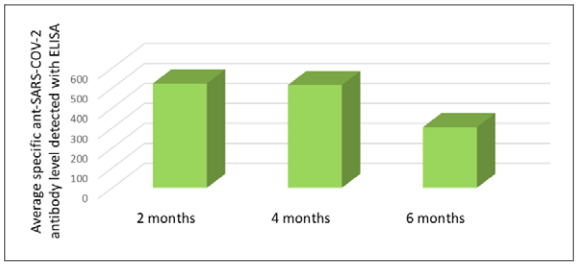

Fig. 5. Immunity duration in dogs

Рис. 5. Продолжительность иммунитета у собак

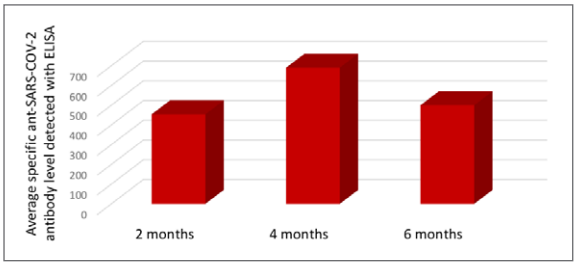

Fig. 6. Immunity duration in fur animals

Рис. 6. Продолжительность иммунитета у пушных зверей

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Tatyana S. Galkina, Candidate of Science (Veterinary Medicine), Head of Laboratory for Pet Disease Prevention, FGBI "ARRIAH", Vladimir, Russia.

Alexander A. Nesterov, Candidate of Science (Veterinary Medicine), Junior Researcher, Reference Laboratory for Bovine Diseases, FGBI "ARRIAH", Vladimir, Russia.

Alexey V. Borisov, Candidate of Science (Veterinary Medicine), Leading Researcher, Laboratory for FMD Prevention, FGBI "ARRIAH", Vladimir, Russia.

Ilya A. Chvala, Candidate of Science (Veterinary Medicine), Deputy Director for Research and Monitoring, FGBI "ARRIAH", Vladimir, Russia.

Alexander V. Kononov, Candidate of Science (Veterinary Medicine), Deputy Director for Research and Development, FGBI "ARRIAH", Vladimir, Russia.

Галкина Татьяна Сергеевна, кандидат ветеринарных наук, заведующий лабораторией профилактики болезней мелких домашних животных ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Нестеров Александр Александрович, кандидат ветеринарных наук, младший научный сотрудник референтной лаборатории болезней крупного рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Борисов Алексей Валерьевич, кандидат ветеринарных наук, ведущий научный сотрудник лаборатории профилактики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Чвала Илья Александрович, кандидат ветеринарных наук, заместитель директора по НИР и мониторингу ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Кононов Александр Владимирович, кандидат ветеринарных наук, заместитель директора по НИР и развитию ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

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SARS-CoV-2 spread in humans and animals

T. P. Akimova[1], V. P. Semakina[2], M. N. Mitrofanova[3], M. V. Zhiltsova[4], E. S. Vystavkina[5], D. G. Isakova[6], D. B. Andreychuk[7], A. K. Karaulov[8], I. A. Chvala[9], A. E. Metlin[10]

[1–5, 7–10] FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

[6] Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor), Moscow, Russia

[1] *ORCID 0000-0003-3502-1146, e-mail: akimova@arriah.ru*

[2] *ORCID 0000-0002-4078-4458, e-mail: semakina@arriah.ru*

[3] *ORCID 0000-0003-0126-9653, e-mail: mitrofanova@arriah.ru*

[4] *ORCID 0000-0002-0264-9351, e-mail: zhiltsova@arriah.ru*

[5] *e-mail: vistavkina@arriah.ru*

[6] *e-mail: dg.isakova@gmail.ru*

[7] *ORCID 0000-0002-1681-5795, e-mail: andreychuk@arriah.ru*

[8] *ORCID 0000-0002-5731-5762, e-mail: karaulov@arriah.ru*

[9] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*

[10] *ORCID 0000-0002-4283-0171, e-mail: metlin@arriah.ru*

SUMMARY

Coronaviruses are a large family of viruses and they are wide spread in animals and humans. They can cause respiratory tract illnesses of various severity. The latest recently discovered coronavirus (SARS-CoV-2) is an agent of COVID-19. The first human cases were reported in Wuhan (People's Republic of China) in December 2019. Since then, the disease infected over 153 million people and became the cause of more than 3 million deaths all over the world. Among the leaders in the confirmed cases are the USA, India, Brazil, France, Turkey and Russia. In February 2020, it was determined that some animal species, including domestic cats and dogs, can be infected with SARS-CoV-2. Reports of animal infection in zoos were submitted from the USA, Argentina, Czech Republic, Sweden, Spain, Estonia, RSA and India. Cases of SARS-CoV-2 infection in fur-farmed minks were reported by 13 countries. The most large-scale COVID-19 outbreak in minks that involved about 300 mink farms was reported in Denmark. During the COVID-19 pandemic, the agent's transmission from humans to canines (*Canidae*), felines (*Felidae*), mustelids (*Mustelidae*) and hominids (*Hominidae*) was confirmed. As of early May 2021, the disease cases in animals were reported by 33 countries. Due to COVID-19 epidemic spread and detection of animal infection cases, diagnosis tools and methods were developed in the Russian Federation, and screening tests were performed in susceptible animal populations in different regions of the country. COVID-19 monitoring results demonstrated the virus in two cats (in Moscow and Tyumen).

Keywords: Coronaviruses, COVID-19, SARS-CoV-2, epizootic situation, interspecies transmission, monitoring.

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For correspondence: Tatyana P. Akimova, Leading Veterinarian, Information Analysis Centre, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: akimova@arriah.ru.*

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Распространение коронавируса SARS-CoV-2 среди людей и животных

Т. П. Акимова[1], В. П. Семакина[2], М. Н. Митрофанова[3], М. В. Жильцова[4], Е. С. Выставкина[5], Д. Г. Исакова[6], Д. Б. Андрейчук[7], А. К. Караулов[8], И. А. Чвала[9], А. Е. Метлин[10]

[1–5, 7–10] ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

[6] Федеральная служба по ветеринарному и фитосанитарному надзору (Россельхознадзор), г. Москва, Россия

[1] *ORCID 0000-0003-3502-1146, e-mail: akimova@arriah.ru*

[2] *ORCID 0000-0002-4078-4458, e-mail: semakina@arriah.ru*

[3] *ORCID 0000-0003-0126-9653, e-mail: mitrofanova@arriah.ru*

[4] *ORCID 0000-0002-0264-9351, e-mail: zhiltsova@arriah.ru*

[5] *e-mail: vistavkina@arriah.ru*

[6] *e-mail: dg.isakova@gmail.ru*

[7] *ORCID 0000-0002-1681-5795, e-mail: andreychuk@arriah.ru*

[8] *ORCID 0000-0002-5731-5762, e-mail: karaulov@arriah.ru*
[9] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*
[10] *ORCID 0000-0002-4283-0171, e-mail: metlin@arriah.ru*

РЕЗЮМЕ

Коронавирусы составляют многочисленное семейство вирусов и широко распространены у животных и людей. Они способны вызывать у человека респираторные заболевания различной степени тяжести. Последний из недавно открытых коронавирусов (SARS-CoV-2) является возбудителем заболевания COVID-19. Первые случаи инфицирования людей SARS-CoV-2 были зарегистрированы в городе Ухань (Китайская Народная Республика) в декабре 2019 г. С тех пор данная болезнь поразила более 153 миллионов человек, став причиной более 3 миллионов смертей по всему миру. Лидерами по количеству подтвержденных случаев являются США, Индия, Бразилия, Франция, Турция и Россия. С февраля 2020 г. установлено, что некоторые виды животных, в том числе домашние кошки и собаки, могут заражаться вирусом SARS-CoV-2. Сообщения об инфицировании животных в зоопарках стали поступать из США, Аргентины, Чешской Республики, Швеции, Испании, Эстонии, ЮАР, Индии. О случаях заражения норок SARS-CoV-2 на звероводческих фермах сообщили 13 стран. Наиболее масштабная вспышка COVID-19 среди норок, охватившая около 300 норковых ферм, произошла в Дании. За время пандемии COVID-19 зафиксирована передача возбудителя от человека к представителям семейств псовых (*Canidae*), кошачьих (*Felidae*), куньих (*Mustelidae*), а также гоминид (*Hominidae*). По состоянию на начало мая 2021 г. о заболевании животных сообщили 33 страны. В связи с эпидемическим распространением COVID-19 и выявлением случаев заражения животных в Российской Федерации были разработаны средства и методы диагностики инфекции и проведены скрининговые исследования в популяции восприимчивых животных из различных регионов страны. В ходе мониторинга COVID-19 в России вирус-возбудитель был выявлен у 2 кошек – в Москве и Тюмени.

Ключевые слова: Коронавирусы, COVID-19, SARS-CoV-2, эпизоотическая ситуация, межвидовая передача, мониторинг.

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Для корреспонденции: Акимова Татьяна Петровна, ведущий ветеринарный врач информационно-аналитического центра ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: akimova@arriah.ru*.

INTRODUCTION

Coronaviruses (*Coronaviridae*) is a family of related RNA viruses of 46 species, organized in two subfamilies, that cause diseases in mammals (including humans), birds and amphibians. The name is associated with the structure of the virus, the spikes of which resemble the solar corona [1, 2].

Coronaviruses are widespread in the animal world. Viruses of the *Coronaviridae* family affect bats, cats, dogs, pigs, cattle, birds and other species [2].

The new SARS-CoV-2, which caused an outbreak of the dangerous infectious disease COVID-19, was first detected in December 2019. The World Health Organization (WHO) declared it a public health emergency of international concern on 30 January 2020, and announced it a pandemic on March 11, i.e., a disease that broke out on a global scale and covered several continents.

The detection of antibodies to the viral virion components confirms the previous disease or asymptomatic carrier state and indicates the presence of immunity. The duration and strength of the immunity to the SARS-CoV-2 in different species is currently understudied. To detect antibodies to animal coronaviruses, various methods are used: neutralization test, enzyme-linked immunosorbent assay, immunofluorescence assay [3–8].

Due to the wide spread of COVID-19 in the world and the potential interspecific transmission, the goal was to develop domestic tools and methods for diagnosing the disease in animals, as well as to conduct screening studies of biological material from susceptible animals in various regions of the Russian Federation.

One of the tasks of the work carried out was to determine the target animal populations and conduct COVID-19 primary screening in these populations using laboratory diagnostic methods.

MATERIALS AND METHODS

The data on COVID-19 animal cases were collected based on statistical data from the WAHID/WAHIS database of the World Organization for Animal Health (OIE), as well as on scientific publications of foreign and domestic authors and data from the mass media. Data on human morbidity were taken from the archived data of the Johns Hopkins University (USA) and the World Health Organization (WHO) on COVID-19 outbreaks. Mapping and analytics were performed in the FGBI "ARRIAH" using the ArcGIS ESRI services.

For screening tests, Veterinary Services of 20 Russian Federation Subjects with the participation of the Rosselkhoznadzor Territorial Administrations and inter-regional veterinary laboratories collected samples from animals (nasopharyngeal/oropharyngeal swabs, rectal swabs, fresh feces) using viscose-tipped sterile applicators. Domestic cats, dogs, and mustelids (minks, sables, and ferrets) were identified as the target population for sampling, from which 1,312 samples of biological material

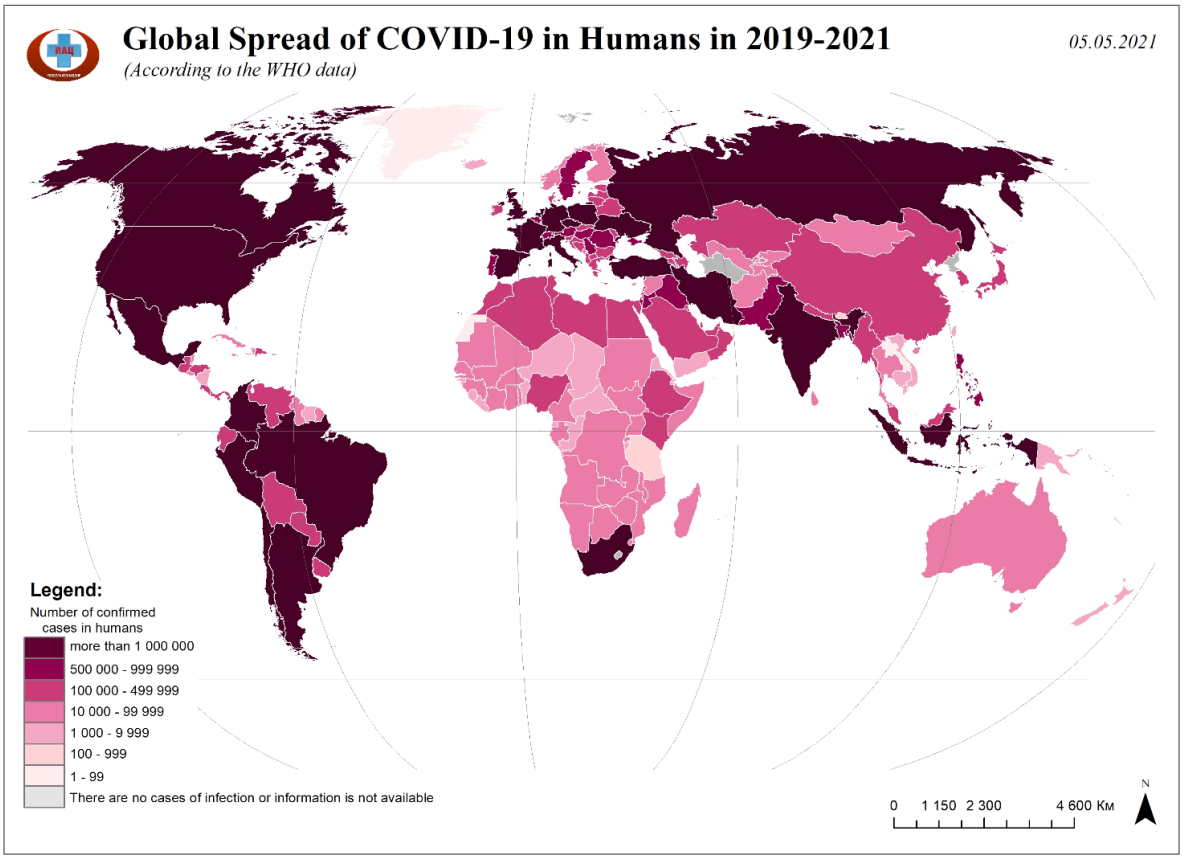

Fig. 1. Global spread of COVID-19 in humans, 2019–2021

Рис. 1. Распространение COVID-19 среди людей в мире с 2019 по 2021 г.

were collected. In addition, in order to determine the presence of infected animals in the populations of wild and domestic birds, as well as in the livestock populations (cattle, small ruminants, pigs), 122 samples of biomaterial from 26 Russian Federation regions were collected and tested. The collected samples were transported to the FGBI "ARRIAH" at a temperature of 4–8 °C. For analysis, a PCR test kit was used to detect the SARS-CoV-2 RNA (SARS-CoV-2 real-time RT-PCR, FGBI "ARRIAH") as recommended by the manufacturer.

Table 1
Number of SARS-CoV-2 infected humans across the world [11]

Таблица 1
Количество зараженных коронавирусом SARS-CoV-2 людей в мире [11]

Region	Number of cases	% of cases	Number of deaths	% of deaths
America	62,713,257	40.7	1,529,597	47.5
Europe	52,275,954	34.0	1,092,527	33.9
Southeast Asia	23,837,189	15.5	291,762	9.0
Eastern Mediterranean	9,274,240	6.0	185,875	5.8
Africa	3,330,385	2.2	83,259	2.6
Western Pacific	2,522,720	1.6	38,019	1.2
Total	153,953,745		3,221,039	

RESULTS AND DISCUSSION

SARS-CoV-2 spread in the human population

In December 2019 in China, there was an increase in the number of pneumonia cases. Investigations revealed that the disease was caused by a previously unknown virus, which was later identified as the new SARS-CoV-2 coronavirus. The infection spread quickly enough to other countries (Fig. 1) [9].

According to the WHO data, as of May 5, 2021, the number of coronavirus infected people in the world exceeded 153 million, of which more than 3.22 million died (Table 1). According to Johns Hopkins University, more than 155.2 million people were infected worldwide, and more than 3.24 million died [10]. The leader in the number of confirmed cases remains the United States, where 32.1 million infected people were recorded. In second place is India (20.7 million), in third – Brazil (14.8 million), in fourth – France (5.6 million), in fifth – Turkey (4.9 million), in sixth – Russia (4.8 million) [11].

SARS-CoV-2 spread in animal populations

The first official notification of the OIE on the transmission of the SARS-CoV-2 from humans to animals was received on February 26, 2020 from Hong Kong. The dog was quarantined after its owner was hospitalized due to the COVID-19 infection. Samples of biological material taken from the dog tested positive for SARS-CoV-2. At the same time, the animal had no clinical signs of the disease. Later, there were reports of SARS-CoV-2 detections in domestic cats and dogs in Europe, Asia, and America (Fig. 2).

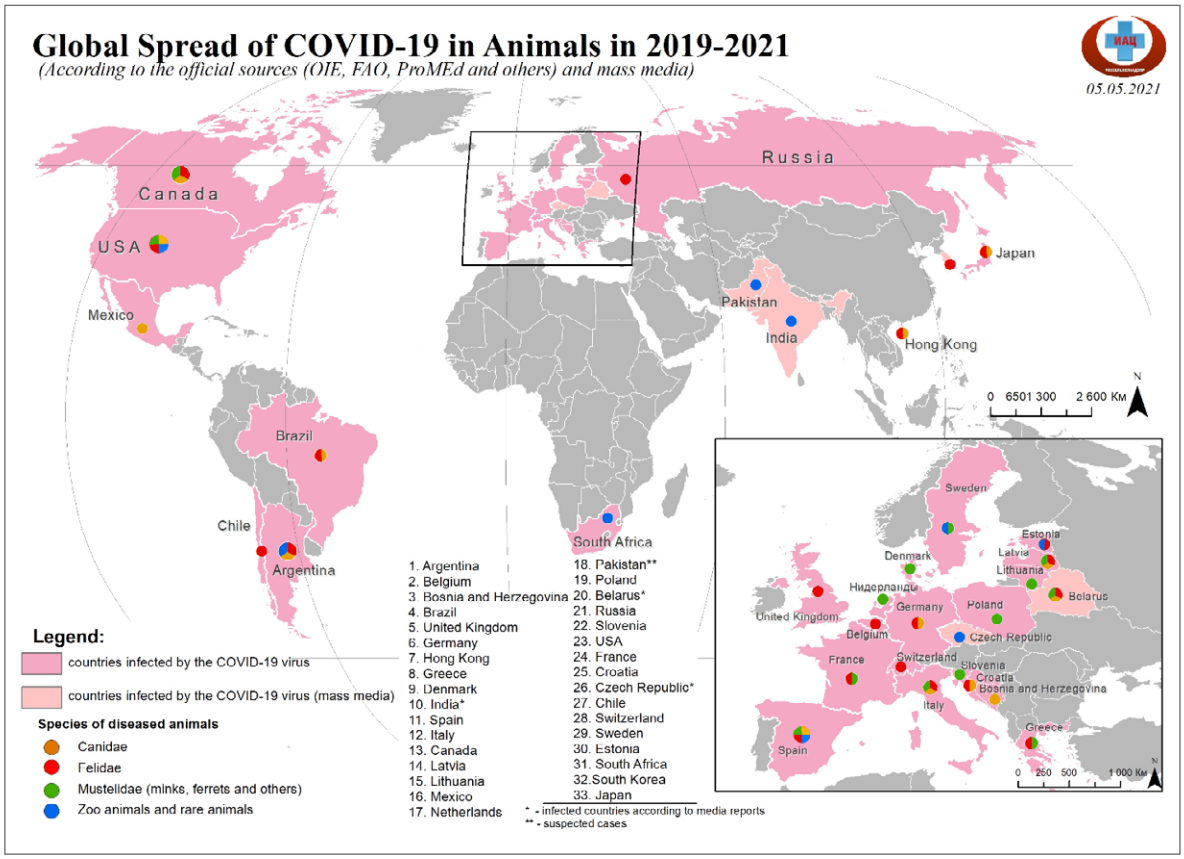

Fig. 2. Global spread of COVID-19 in animals, 2019–2021

Рис. 2. Распространение COVID-19 среди животных в мире с 2019 по 2021 г.

In Russia, the infection of animals with SARS-CoV-2 was confirmed by the FGBI "VGNKI" and the FGBI "ARRIAH". The virus was detected in two cats from Moscow and Tyumen. In the course of further COVID-19 screening tests in the populations of domestic, farm and fur animals (minks, ferrets, sables), conducted in 2020, no cases of the disease/virus carrier state were detected.

SARS-CoV-2 infection of zoo animals was first reported in March 2020 in the United States. In the Bronx Zoo (New York), a tiger with clinical signs of respiratory disease was diagnosed with SARS-CoV-2 infection during laboratory diagnostics. Five other tigers and three lions, which were kept in two enclosures at the zoo, were also infected with SARS-CoV-2. Then reports of animal infections in zoos began to arrive from other US states: tigers in Indiana, Tennessee, Virginia, Texas and Minnesota; snow leopard in Kentucky; gorilla in California; lion in Pennsylvania [12, 13]. Moreover there was information in the media that in the State of Georgia aquarium, otters got diseased with a coronavirus infection. According to the aquarium staff, the otters had symptoms of respiratory infection: sneezing, runny nose, cough and mild depression [14]. Presumably, employees of the zoos with an asymptomatic disease became a source of infection for the animals.

SARS-CoV-2 infections among zoo animals have been reported in other countries. Laboratory tests confirmed the disease in gorillas and Indian lions at the Prague Zoo (Czech Republic) [15, 16]. Representatives of the Spanish Veterinary Services reported coronavirus positives in four lions at the Barcelona Zoo [17]. In January 2021, a lion was diagnosed with COVID-19 at the Tallinn Zoo (Estonia) [18]. The Swedish National Veterinary Institute (SVA) has confirmed COVID-19 in two tigers and two lions kept at the Boras Zoo. The media reported about the suspected SARS-CoV-2 infection of two white tiger cubs in Pakistan [19]. In India, the coronavirus was found in lions at the Hyderabad Zoo [20]. The OIE also received notifications about the infection of cougars in the zoo in Johannesburg (South Africa) and in Argentina.

In April, two mink farms in the Dutch province of North Brabant in the municipalities of Larbeck and Gemert-Bakel were diagnosed with a coronavirus infection. The animals had a laboured breathing and increased mortality was recorded. Experts believe that the source of infection in minks was a human, because at the same time, some farm employees had symptoms of respiratory disease.

Sporadic cases of the SARS-CoV-2 infection have been reported in domestic ferrets in Slovenia and Spain [21, 22].

A total of 14 countries reported SARS-CoV-2 infection in mustelids (including domestic and wild). As of April 2021 COVID-19 was registered on more than 400 fur farms (Table 2).

The largest COVID-19 outbreak among minks occurred in Denmark. It covered about 300 mink farms, which were located mainly in the north of the country, in Northern Jutland. Human cases of COVID-19 were also reported, caused by new variant strains of SARS-CoV-2 detected in farm minks. To prevent the further spread of the coronavirus variants isolated from minks among people, the Danish authorities took a number of measures, including the culling of mink population on the country's farms [24]. Cases of transmission of the mutated virus from minks

Table 2
COVID-19 outbreaks on mink farms across the world [21, 23–28]

Таблица 2
Регистрация очагов COVID-19 на норковых фермах в странах мира [21, 23–28]

No.	Country	Total mink farms	Number of infected mink farms	Date of outbreak registration
1	Greece	91	23	13.11.2020 – 08.02.2021
2	Denmark	1,147	290	15.06.2020 – 07.12.2020
3	Spain	29	3	03.12.2020 – 15.03.2021
4	Italy	9	3	10.08.2020 – 18.03.2021
5	Canada	98	2	26.11.2020 – 16.12.2020
6	Latvia	9	2	04.2021
7	Lithuania	86	4	26.11.2020 – 30.03.2021
8	the Netherlands	126	69	23.04.2020 – 04.11.2020
9	Poland	350	1	27.01.2021
10	Republic of Belarus	8	1	02.2021
11	the USA	≈245	16	26.07.2020 – 25.10.2020
12	France	4	1	16.11.2020
13	Sweden	40	13	23.10.2020 – 11.11.2020
		Total	427	23.04.2020 – 04.2021

to humans were also detected in other countries (USA, Netherlands) [11, 29].

As early as in 2020, there were reports of SARS-CoV-2 registered in wild fauna. The US Department of Agriculture confirmed the first case of wild mink infection with coronavirus in Utah. The authorities of the Spanish autonomous community of Valencia also found SARS-CoV-2 in two dead wild minks [12, 25].

Fur animals that have escaped from farms can act as hosts, maintaining the virus circulation in the wild, and cause the transmission of the SARS-CoV-2 to other species of wild susceptible animals. However, there is currently no evidence of the wide spread of coronavirus in the population of wild minks living around animal farms, and the available information is insufficient to assess the likelihood of a SARS-CoV-2 reservoir occurrence in wild fauna [21].

During 18 months from the beginning of the COVID-19 pandemic, the transmission of the pathogen to canines (*Canidae*), felines (*Felidae*) (including the subfamilies of large (*Pantherinae*) and small (*Felinae*) cats), mustelids (*Mustelidae*), as well as hominids (*Hominidae*) belonging to the order Primates was recorded. The World Organization for Animal Health recommends testing of all species susceptible to the coronavirus.

Many tests have been conducted to study the effects of SARS-CoV-2 on different animal species. As shown by the results of recent experimental studies (Table 3), many mammals can be infected with the new coronavirus, including cats, dogs, mice, hamsters, bats, shrews, raccoon dogs, and deer. In laboratory conditions, intraspecific transmission of SARS-CoV-2 was observed in cats, minks, ferrets, raccoon dogs, white-tailed deer, striped skunks, some species of bats and hamsters, as well as rhesus macaques, crab-eating macaques and other Old World monkey species [29, 30]. Also, various foreign studies show that some farm animals (pigs, cattle, rabbits, ferrets, minks) are also susceptible to SARS-CoV-2 [31–34]. Data on experimental infection of cattle are scarce, and reports about pig and rabbit infections are sometimes contradictory.

An international team of biologists studied the properties of 25 amino acids of the ACE2 enzyme (angiotensin-converting enzyme 2), which serves as a receptor for SARS-CoV-2 in humans, and performed genomic and structural analyses of ACE2 in 410 vertebrate species to identify transmission routes and understand which animal species are susceptible to the virus [47, 48]. The results allowed the scientists to divide the animals into five groups.

The highest risk group included 18 species of Old World primates: representatives of the families of marmosets (*Cercopithecidae*), gibbons (*Hylobatidae*) and hominids (*Hominidae*), including humans. The second group included some rodents, marine animals (dolphins, porpoises, beluga whales, narwhals, and other cetaceans), deer, lemurs, and members of the anteater family (*Myrmecophagidae*).

A relatively large group of cloven-hoofed mammals was classified as a medium-risk group for infection. It includes domestic cattle, sheep, goats, as well as some species of cloven-hoofed animals, kept in zoos and wildlife parks (giraffes, okapis, hippos, water buffaloes, Saharan oryxes, Saharan gazelles, yaks, alpacas, bisons, etc.). In felines, the likelihood of infection was average, and in camels, horses, pigs, dogs and some other domestic animals – low. In many representatives of fish, reptiles, amphibians, monotremes, marsupials, martens, rodents and birds, the risk of infection tends to zero.

Table 3
Supposed animal hosts of SARS-CoV-2 [9, 12, 14, 26, 29–46]

Таблица 3
Предполагаемые хозяева SARS-CoV-2 среди животных [9, 12, 14, 26, 29–46]

Possible origin (natural reservoir)	Intermediate hosts (naturally susceptible animals)	Animals susceptible under experimental conditions
bats/reptiles/ pangolins	dogs, cats (domestic cats, cougars, Malay, Amur and Bengal tigers, lions, snow leopards), primates (lowland gorillas), martens (frets, European and American minks, Asian small-clawed otters)	fruit bats (Egyptian rousettes), cats, dogs, minks, ferrets, primates (rhesus macaques, crab-eating macaques, green monkeys, hamadryas, tamarins), pigs, cattle (dairy calves), white-tailed deers, raccoon dogs, tree shrews, striped skunks, North American raccoons, New Zealand rabbits, shrews, rodents (mice, bank voles, hamsters: Syrian, Chinese, deer mice, bushy-tailed woodrats, Roborovski dwarf hamsters)

The authors of the study noted that the results obtained *in silico* need to be confirmed experimentally, while they can not be considered absolutely accurate. Nevertheless, the results of the analysis significantly expanded the range of potential intermediate hosts and identified many species that may be at risk of infection with the SARS-CoV-2 through its interaction with the ACE2 receptors. In the future, these studies will help determine which animal species can infect humans with the coronavirus [47, 48].

COVID-19 monitoring studies in animals in the Russian Federation

Since June 2020, screening studies of animal populations from various RF regions have been conducted by the FGBI "ARRIAH" in order to detect the SARS-CoV-2 RNA, using the test kit of its own production. 1,312 samples of biomaterial from different animal species from 20 regions of the country were tested. During the monitoring process, a positive sample from a cat from the Tyumen Oblast was detected (Table 4).

According to the results of the study, the SARS-CoV-2 is detected sporadically in the studied animal populations (cats, dogs, minks, sables, ferrets) in the tested regions of the Russian Federation. The virus was detected in two cats – from Moscow (confirmed by FGBI "VGNKI") and Tyumen (confirmed by FGBI "ARRIAH").

In order to study the possibility of natural coronavirus infection or SARS-CoV-2 transmission to animals with unproven natural susceptibility (large and small cattle, pigs, domestic and wild birds), 122 samples of biomaterial collected on farms in various regions of the Russian Federation were tested (Table 5).

The genome of the new SARS-CoV-2 was not detected in any of the samples from wild and domestic birds, as well as from farm animals.

CONCLUSION

The number of people infected with the SARS-CoV-2 in the world has exceeded 153 million. The leaders in the number of confirmed cases are the United States (32.1 million), India (20.7 million), Brazil (14.8 million), France (5.6 million), Turkey (4.9 million), and Russia (4.8 million).

According to many virologists, humanity will be able to cope with SARS-CoV-2 only through the acquisition of immunity by the planet population. About 20 vaccines against COVID-19 have been registered in the world [11]. However, according to the WHO, as of March 1, 2021, less than 10% of the world's population has antibodies to the coronavirus.

There is currently no evidence that animals play a significant role in the spread of SARS-CoV-2 in humans. Based on the information available to date, the risk of human infection from animals is considered low. The SARS-CoV-2 is primarily transmitted with respiratory droplets from humans to humans [26, 29]. However, cases of the virus transmission from animals to humans have already been reported. For example, in Denmark, the Netherlands and the United States, there was a transmission of the mutated coronavirus from a mink to a human.

At the same time, the coronavirus can be transmitted from humans to animals. A high likelihood of infection is noted for animals that are in direct contact with humans. Thus, COVID-19 cases in companion animals (dogs, cats, ferrets) were reported in Europe, Asia and America.

Table 4
Results of testing biological samples collected from cats, dogs and mustelids for SARS-CoV-2 RNA using FGBI "ARRIAH" manufactured test-kit

Таблица 4
Результаты исследования проб биологического материала от кошек, собак и куницеобразных, полученные в ходе выявления РНК SARS-CoV-2 с использованием тест-системы производства ФГБУ «ВНИИЗЖ»

No.	Region	Species	Number of tested samples	Number of positive samples
1	Altai Krai	sables	31	0
2	Bryansk Obalst	minks, ferrets	45	0
3	Vladimir Oblast	dogs, cats	50	0
4	Kaliningrad Oblast	minks	90	0
5	Kirov Oblast	minks	30	0
6	Leningrad Oblast	sables	31	0
7	Lipetsk Oblast	minks	69	0
8	Nizhny Novgorod Oblast	cats	30	0
9	Novosibirsk Oblast	minks	35	0
10	Republic of Bashkortostan	minks, sables	70	0
11	Republic of Crimea	minks	30	0
12	Republic of Mordovia	minks	51	0
13	Republic of Sakha (Yakutia)	minks	60	0
14	Republic of Tatarstan	minks, sables	120	0
15	Republic of Udmurtia	minks	60	0
16	Sverdlovsk Oblast	cats, dogs, minks	17	0
17	Tver Oblast	minks	301	0
18	Tula Oblast	ferrets, minks, sables	95	0
19	Tyumen Oblast	cats	67	1
20	Yamalo-Nenets Autonomous Okrug	sables	30	0
		Total	1,312	1

In Russia, the infection of animals with SARS-CoV-2 was confirmed by the FGBI "VGNKI" and the FGBI "ARRIAH". The virus was detected in two cats from Moscow and Tyumen. During the COVID-19 monitoring conducted in 2020 by the FGBI "ARRIAH", no cases of the disease/carrier state were detected in other studied animal populations (dogs, minks, sables, ferrets, cattle and small cattle, pigs, domestic and wild birds).

Infection of zoo animals with SARS-CoV-2 has been reported in the United States, Argentina, Sweden, Spain, Czech Republic, Estonia, South Africa, India. Cases that have occurred on mink farms in the United States, Canada, and several European countries, including Denmark, Greece, Spain, Poland, France, Lithuania, Latvia, Belarus, Sweden, Italy, and the Netherlands, show that the virus can be transmitted from humans to minks. Sporadic cases of SARS-CoV-2 infection have been reported in domestic

Table 5
Results of testing biological samples collected from cattle, sheep and goats, pigs and birds for SARS-CoV-2 RNA using FGBI "ARRIAH" manufactured test-kit

Таблица 5
Результаты исследования проб биологического материала от КРС, МРС, свиней и птиц, полученные в ходе выявления РНК SARS-CoV-2 с использованием тест-системы производства ФГБУ «ВНИИЗЖ»

No.	Region	Species	Number of tested samples	Number of positive samples
1	Belgorod Oblast	pigs	3	0
2	Vladimir Oblast	cattle	7	0
		wild ducks, chickens	10	0
3	Voronezh Oblast	wild ducks	5	0
		pigs	9	0
4	Zabaikalsky Krai	chickens	5	0
5	Ivanovo Oblast	pigs	3	0
6	Kaluga Oblast	cattle	5	0
7	Kostroma Oblast	pigs	3	0
8	Krasnodar Oblast	ducklings	5	0
		pigs	3	0
9	Lipetsk Oblast	pigs	3	0
10	Moscow Oblast	cattle	3	0
		pigs	4	0
11	Nizhny Novgorod Oblast	cattle	3	0
12	Orel Oblast	pigs	3	0
13	Penza Oblast	turkeys	5	0
14	Perm Krai	cattle	3	0
15	Pskov Oblast	pigs	3	0
16	Republic of Bashkortostan	pigs	6	0
17	Republic of Dagestan	small ruminants	2	0
18	Republic of Crimea	swans	3	0
19	Republic of Mordovia	cattle	3	0
20	Republic of Tatarstan	cattle	4	0
21	Rostov Oblast	swans	4	0
22	Samara Oblast	cattle	3	0
23	Stavropol Krai	pigs	3	0
24	Tambov Oblast	pigs	3	0
25	Tula Oblast	pigs	3	0
26	Chelyabinsk Oblast	pigs	3	0
		Total	122	0

ferrets in Slovenia and Spain [21, 22]. Cases of coronavirus infection have also been recorded in wild mink populations in the United States and Spain, but the available information is insufficient to assess the likelihood of a SARS-CoV-2 reservoir occurrence in wild fauna.

Recent experimental studies show that many mammals, including some farm animal species, can be infected with the new coronavirus.

These findings highlight the importance of regular testing of susceptible animal species and the study of SARS-CoV-2 genetic material.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Tatyana P. Akimova, Leading Veterinarian, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Акимова Татьяна Петровна, ведущий ветеринарный врач информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Valentina P. Semakina, Leading Veterinarian, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Семакина Валентина Петровна, ведущий ветеринарный врач информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Maria N. Mitrofanova, Candidate of Science (Veterinary Medicine), Junior Researcher, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Митрофанова Мария Николаевна, кандидат ветеринарных наук, младший научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Milena V. Zhiltsova, Candidate of Science (Veterinary Medicine), Leading Researcher, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Жильцова Милена Владимировна, кандидат ветеринарных наук, ведущий научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Evgeniya S. Vystavkina, Leading Computer Programmer, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Выставкина Евгения Сергеевна, ведущий программист информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Darya G. Isakova, Head of Directorate for International Cooperation and Veterinary Control of Export/Import and Transportation, Rosselkhoznadzor, Moscow, Russia.

Исакова Дарья Георгиевна, начальник Управления ветеринарного надзора при экспортно-импортных операциях, на транспорте и международного сотрудничества, Россельхознадзор, г. Москва, Россия.

Dmitry B. Andreychuk, Candidate of Science (Biology), Head of Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Андрейчук Дмитрий Борисович, кандидат биологических наук, заведующий референтной лабораторией вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Anton K. Karaulov, Candidate of Science (Veterinary Medicine), Head of Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Караулов Антон Константинович, кандидат ветеринарных наук, руководитель информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Ilya A. Chvala, Candidate of Science (Veterinary Medicine), Deputy Director for Research and Monitoring, FGBI "ARRIAH", Vladimir, Russia.

Чвала Илья Александрович, кандидат ветеринарных наук, заместитель директора по НИР и мониторингу ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Artem Ye. Metlin, Doctor of Science (Veterinary Medicine), Deputy Director for Research and Quality, FGBI "ARRIAH", Vladimir, Russia.

Метлин Артем Евгеньевич, доктор ветеринарных наук, заместитель директора по НИР и качеству ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

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Development of the test kit for detection of SARS-CoV-2 antibodies in sera of susceptible animals

M. A. Volkova[1], N. G. Zinyakov[2], P. S. Yaroslavtseva[3], I. A. Chvala[4], T. S. Galkina[5], D. B. Andreychuk[6]
Federal State Budgetary Institution "Federal Center for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia
[1] *ORCID 0000-0002-7674-639X, e-mail: volkovama@arriah.ru*
[2] *ORCID 0000-0002-3015-5594, e-mail: zinyakov@arriah.ru*
[3] *ORCID 0000-0003-0383-9912, e-mail: yaroslavtzeva@arriah.ru*
[4] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*
[5] *ORCID 0000-0001-9494-8537, e-mail: galkina_ts@arriah.ru*
[6] *ORCID 0000-0002-1681-5795, e-mail: andreychuk@arriah.ru*

SUMMARY

The novel coronavirus infection COVID-19, caused by the SARS-CoV-2, has triggered a pandemic, and has also been reported in animal populations – in farm minks, dogs and felines: domestic cats, lions and tigers. The susceptibility of some animal species to the SARS-CoV-2 has been proven by experimental infection. Serological methods are effectively used to detect the infection in animals. Currently, methods such as neutralization test, immunofluorescence assay and enzyme-linked immunoassay are used to detect antibodies to coronaviruses. Thanks to these studies, a test kit was developed based on an indirect enzyme-linked immunoassay to detect the SARS-CoV-2 antibodies in sera of susceptible animals. The use of a purified concentrated inactivated virus as an antigen allows the detection of antibodies to various SARS-CoV-2 immunodominant proteins (S and N). The reaction conditions were optimized, and a positive-negative threshold was established by testing of 154 negative sera from animals of six species (ferrets, minks, foxes, Arctic foxes, cats and dogs). The method reproducibility analysis showed that the average value of the variation coefficient did not exceed 7%, which is an acceptable value. The specificity and sensitivity of the neutralization test, when testing 30 sera from ferrets was 100 and 92.6%, respectively. The high diagnostic sensitivity and specificity shown by testing of 50 serum samples from minks, foxes, cats and dogs with different immune status, allow us to recommend the developed test kit for screening and monitoring tests and post-vaccination immunity control.

Keywords: COVID-19, SARS-CoV-2, antibodies, enzyme-linked immunoassay.

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For correspondence: Marina A. Volkova, Candidate of Science (Biology), Leading Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: volkovama@arriah.ru.*

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Разработка тест-системы для выявления антител к вирусу SARS-CoV-2 в сыворотках крови восприимчивых животных

М. А. Волкова[1], Н. Г. Зиняков[2], П. С. Ярославцева[3], И. А. Чвала[4], Т. С. Галкина[5], Д. Б. Андрейчук[6]
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия
[1] *ORCID 0000-0002-7674-639X, e-mail: volkovama@arriah.ru*
[2] *ORCID 0000-0002-3015-5594, e-mail: zinyakov@arriah.ru*
[3] *ORCID 0000-0003-0383-9912, e-mail: yaroslavtzeva@arriah.ru*
[4] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*
[5] *ORCID 0000-0001-9494-8537, e-mail: galkina_ts@arriah.ru*
[6] *ORCID 0000-0002-1681-5795, e-mail: andreychuk@arriah.ru*

РЕЗЮМЕ

Новая коронавирусная инфекция (COVID-19), вызываемая вирусом SARS-CoV-2, стала причиной пандемии, а также была зарегистрирована в популяциях животных – у норок фермерских хозяйств, собак и представителей кошачьих: домашних кошек, львов и тигров. Доказана чувствительность некоторых видов животных к вирусу SARS-CoV-2 при экспериментальном заражении. Для выявления случаев инфицирования животных эффективно применяются серологические методы. В настоящее время для обнаружения антител к коронавирусам используют такие методы, как реакция нейтрализации, иммунофлуоресцентный метод и иммуноферментный анализ. В результате проведенных исследований была разработана тест-система на основе непрямого варианта иммуноферментного анализа для выявления антител к вирусу SARS-CoV-2 в сыворотках крови восприимчивых животных. Использование

в качестве антигена очищенного концентрированного инактивированного вируса позволяет выявлять антитела к различным иммунодоминантным белкам (S и N) SARS-CoV-2. Оптимизированы условия постановки реакции, установлен позитивно-негативный порог при исследовании 154 негативных сывороток крови от животных шести видов (хорьков, норок, лис, песцов, кошек и собак). При определении воспроизводимости метода среднее значение коэффициента вариации не превышало 7%, что является допустимым значением. Специфичность и чувствительность относительно реакции нейтрализации при исследовании 30 сывороток крови от хорьков составила 100 и 92,6% соответственно. Высокая диагностическая чувствительность и специфичность, показанные при исследовании 50 сывороток крови от норок, лис, кошек и собак с разным иммунным статусом, позволяют рекомендовать разработанную тест-систему для проведения скрининговых исследований и контроля поствакцинального иммунитета.

Ключевые слова: COVID-19, SARS-CoV-2, антитела, иммуноферментный анализ.

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Для корреспонденции: Волкова Марина Алексеевна, кандидат биологических наук, ведущий научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: volkovama@arriah.ru.*

INTRODUCTION

Coronaviruses are one of the major pathogens of mammals (including humans), amphibians and birds [1]. The novel coronavirus SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus 2) is the causative agent of COVID-19, a 2019 coronavirus disease. The SARS-CoV-2 belongs to *Nidovirales* order, *Orthocoronavirinae* subfamily, *Betacoronavirus* genus (International Committee on Taxonomy of Viruses, ICTV). Coronaviruses are enveloped RNA viruses with single-stranded unsegmented RNA [2]. The main structural proteins are nucleoprotein (N), spike protein (S), envelope protein (E), and matrix protein (M). The S-protein, consisting of two subunits S1 and S2, forms a trimer on the viral membrane. The S1 subunit contains a receptor-binding domain (RBD), which is responsible for binding of the host cell to the receptors, and the S2 subunit facilitates fusion between the viral membranes and the host cell [2–4]. The S protein is the most variable in the representatives of different genera of *Coronaviridae* family and is responsible for their transmissivity and adaptation abilities. The S2 protein is more conservative than S1 [4].

The susceptibility to the SARS-CoV-2 infection of different species is currently understudied. There are a number of reports about the SARS-CoV-2 experimental infections in ferrets, cats, dogs and pigs [5–7]. The SARS-CoV-2 was found to replicate effectively in cats and ferrets. Dogs were less susceptible to the infection, and ducks and chickens were not susceptible at all [8]. B. S. Pickering et al. showed that pigs are sensitive to nasal infection with a large dose of the SARS-CoV-2 [7]. In natural environments (in zoos), the SARS-CoV-2 genome was detected in cats, dogs, tigers, and lions, showing signs of respiratory disease. In the spring of 2020, the SARS-CoV-2 infection in minks and their deaths were reported on farms in the Netherlands [9].

The peculiarity of this viral disease is the development of antibodies to the components of viral particles, the detection of which confirms the past disease or asymptomatic carrier state and indicates the presence of immunity. The duration and strength of immunity to the SARS-CoV-2 in different species is not sufficiently studied.

Currently, various methods are used to detect antibodies to animal coronaviruses: neutralization test, enzyme-linked immunoassay, immunofluorescence assay [5, 6, 8–11]. In 2020, IDvet (France) released the ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA multi-enzyme immunoassay kit for the detection of antibodies to the SARS-CoV-2 nucleoprotein in serum, plasma and whole blood of different species. There are a number of reports on the development of other non-commercial ELISA kits for the detection of antibodies to the S1 protein receptor-binding domain (RBD) in animal blood [5, 10, 11].

Due to the COVID-19 wide spread of in the world and the infection risk for humans and animals, the goal was to develop a domestic ELISA test kit for detection of antibodies against SARS-CoV-2 antigen in sera of susceptible animals of various species to control post-vaccination immunity and conduct screening tests.

MATERIALS AND METHODS

Antigen. As an antigen in the enzyme-linked immunoassay (ELISA), the SARS-CoV-2 "variant B" strain, inactivated with β-propiolactone and cultured in Vero cells was used. Purification and concentration of the inactivated virus-containing culture liquid included low-speed centrifugation and ultracentrifugation through a 30% sucrose layer. The resulting 100-fold concentrated product was used for ELISA. The presence of the virus was confirmed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Sera. The SARS-CoV-2-free serum samples from animals (ferrets, minks, foxes, cats, dogs, and other susceptible animals) were used as negative controls), positive controls were the SARS-CoV-2-specific sera of animals (ferrets, minks, foxes, cats, dogs, and other susceptible animals) with a titer of at least 1:800.

Indirect ELISA. Antigen working dilution in a carbonate-bicarbonate buffer (pH 9.6) was added to the wells of a 96-well plastic plate (Nunc-Immuno Plates, Denmark), incubated overnight at 4 °C, blocked with a 1% skimmed milk powder solution (Carl Roth GmbH, Germany) in a tris-HCl buffer solution (0.02 M tris-HCl, 0.15 M NaCl, 0.1% twin-20)

(TBR-T), pH 7.4, for 1 hour at room temperature and then washed three times using TBR-T. The serum samples were titrated by two-fold serial dilutions for testing, starting from 1:50 dilution and using 1% skimmed milk solution in TBR-T. When testing single-diluted sera, 1:100 dilution was used as a working solution. The test and control sera were added into the plate wells in a volume of 100 µl and incubated for 45 minutes at 37 °C. The bound antibodies were detected by adding 100 µl of working dilution of protein A from *Staphylococcus aureus* / horseradish peroxidase conjugate (Sigma, USA) diluted by dilution buffer to each well of the plate. The plates were incubated for 45 minutes at 37 °C. After each stage, the wells were washed 3–4 times with a TBR-T buffer solution. To visualize the resulting complex, 100 µl of ABTS substrate solution [2,2-azino-di-(3-ethyl benzoaminosulfonate)] was added to each well and incubated for 10–15 minutes at room temperature. The reaction was stopped by adding 100 µl of a stop solution (1% sodium dodecyl sulfate) to each well. The reaction was read by spectrophotometric method at a wavelength of 405 nm. The highest serum dilution that gave an optical density (OD) of 2 or greater standard deviations above the negative control was deemed the antibody titer. S/N value (where S is the OD of the test sample, N is the OD of negative control) was determined by a single serum dilution method for each sample. To determine the positive-negative threshold, 154 sera from clinically healthy animals of different species were tested. The antibody titer, S/N value and standard deviation were calculated, the sum of the mean and two standard deviations determined the upper limit of the negative values, and the sum of the mean and three standard deviations determined the lower limit of the positive values.

Electrophoresis and immunoblotting. Protein electrophoresis was performed in 10% SDA-PAGE for 1 hour at a constant voltage of 200 V. Separated viral proteins were transferred to a nitrocellulose membrane with a pore size of 0.45 microns for 1 hour at a voltage of 100 V using Mini Trans-Blot Electrophoretic transfer cell, BioRad, USA according to the manufacturer's instructions. The membrane was incubated for 1 hour in a 1% milk powder solution in TBR-T buffer, at pH 7.4. Then they were treated with normal and the SARS-CoV-2 positive animal sera diluted 1:100 with TBR-T buffer for 1 hour while being stirred on a shaker. After soaking in a Protein A-horseradish Peroxidase Conjugate (Sigma, USA) for 1 hour, the membrane was stained with a substrate mixture including 4-chloro-1-naphthol and 0.04% hydrogen peroxide. Each step was finished by a 3–4 washings of the membrane with TBR-T buffer.

Statistical data processing. For statistical data processing, we used the program Statistica 10.0 (Stat Soft. Inc., USA).

RESULTS AND DISCUSSION

After purification and concentration of the virus-containing culture fluid, the presence of the virus in the resulting preparation was determined by SDS-PAGE electrophoresis of viral proteins and immunoblotting using specific sera (Fig. 1, 2). The polypeptide position after electrophoresis corresponded to the data published by other authors for SARS-CoV-2 [2, 4].

The testing of sera from animals vaccinated against coronavirus infection (COVID-19) by immunoblotting showed that antibodies were developed against the main immunodominant proteins: spike protein S1- and S2-subunits and N-protein, this is confirmed by the data of other researchers [10, 11].

The resulting inactivated virus material was used as an antigen in indirect ELISA to detect antibodies to SARS-CoV-2.

When developing the ELISA test kit, the reaction conditions were optimized: the antigen working dilution (1:300) and immunoperoxidase conjugate working dilution (1:10000) was determined. Analyzing 30 optical density values of tested control sera, diluted 1:100, their acceptable values were established: not higher than 0.2 for a negative control; not lower than 0.4 for a positive control.

To obtain an objective assessment of the immune response, it was necessary to establish a positive-negative threshold. 154 sera from clinically healthy animals of different species (ferrets, minks, foxes, Arctic foxes, cats and dogs),

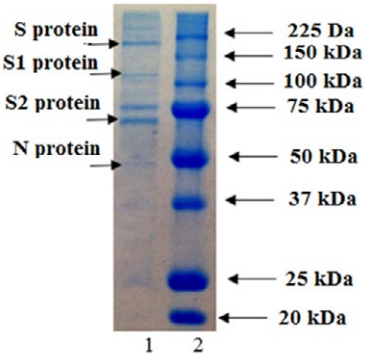

Fig. 1. SARS-CoV-2 antigen. SDS-PAGE electrophoresis, Coomassie Brilliant Blue G-250 staining:
1 – purified SARS-CoV-2 antigen protein fractions;
2 – molecular weight marker (BioRad, USA)

Рис. 1. Антиген SARS-CoV-2. Электрофорез в 10%-м ДСН-ПААГ, окрашивание Coomassie Brilliant Blue G-250:
1 – белковые фракции очищенного антигена SARS-CoV-2;
2 – маркер молекулярных весов (BioRad, США)

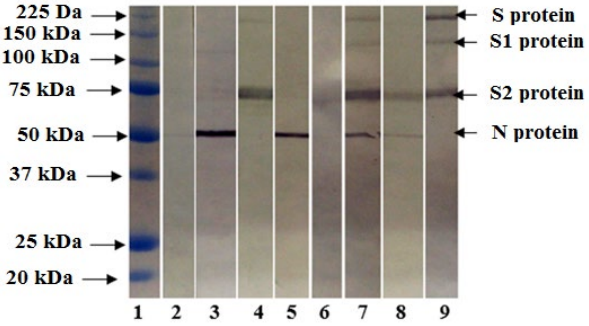

Fig. 2. SARS-CoV-2 antigen protein immunoblotting using normal and coronavirus-specific animal sera:
1 – molecular weight marker (BioRad, USA);
2, 6 – purified SARS-CoV-2 antigen protein fractions using normal ferret and cat sera;
3, 4, 5, 7, 8, 9 – purified SARS-CoV-2 antigen protein fractions using coronavirus-specific sera of ferret (3), mink (4), fox (5), cat (7, 8), dog (9)

Рис. 2. Иммуноблоттинг белков антигена SARS-CoV-2 с нормальной и специфическими к коронавирусу сыворотками крови животных:
1 – маркер молекулярных весов (BioRad, США);
2, 6 – белковые фракции очищенного антигена SARS-CoV-2 с нормальной сывороткой крови хорька и кошки;
3, 4, 5, 7, 8, 9 – белковые фракции очищенного антигена SARS-CoV-2 со специфической к коронавирусу сывороткой крови хорька (3), норки (4), лисы (5), кошки (7, 8), собаки (9)

Table 1
Results of the method reproducibility study

Таблица 1
Результаты исследования воспроизводимости метода

Serum type	Operator / day of run	Indicator			
		Mean S/N value	Standard deviation (σ)	Coefficient of variation (CV%)	Mean value CV%
Repeatability (by 6 tests)					
Weakly positive	1/1	4.44	0.205	4.62	3.70
	2/1	4.01	0.125	3.12	
	1/2	3.76	0.144	3.82	
	2/2	4.05	0.129	3.17	
Strongly positive	1/1	6.00	0.174	2.91	2.43
	2/1	6.04	0.144	2.39	
	1/2	6.05	0.285	4.71	
	2/2	6.25	0.170	2.72	
Reproducibility (by 24 tests)					
Weakly positive	2 operators / 2 days	4.07	0.280	6.99	5.12
Strongly positive	2 operators / 2 days	6.08	0.197	3.24	

not vaccinated against COVID-19, were examined in triplicate on different days. As a result, the established mean S/N value of negative sera was 1.333, and the standard deviation was 0.3769. Based on the statistical analysis, the result was considered negative if S/N was ≤ 2.1; positive if S/N was ≥ 2.5; intermediate values were considered inconclusive. When the serial dilution method was used, the antibody titer less than or equal to 1:100 was considered negative, greater than or equal to 1:200 value was considered positive.

The reproducibility of the method was evaluated by the coefficient of variation within one run and between the runs, doing retests of 2 positive serum samples from susceptible animals (ferrets) with different concentrations of antibodies (low and high). The coefficient of variation of S/N values within one run was evaluated by 6 tests conducted by one operator, the coefficient of variation between the runs – by 24 tests conducted on 4 different plates by two operators on different days (Table 1).

The results obtained by repeated ELISAs showed that the mean value of the coefficient of variation did not exceed 7%. Thus, the use of the developed test kit for the detection of the SARS-CoV-2 antibodies by indirect ELISA gave reproducible results.

To confirm the specificity of the developed method, sera specific for α-coronavirus (porcine transmissible gastroenteritis virus), β-coronavirus (bovine coronavirus), γ-coronavirus (infectious bronchitis virus) and pestivirus (bovine diarrhea virus) were used. It was shown (Table 2) that the activity of the SARS-CoV-2 antigen against heterologous sera did not exceed the background level (reaction to non-immune serum).

In order to assess the relative specificity and sensitivity of the test kit, the results obtained using the developed ELISA and neutralization test were compared using 2 × 2 contingency table. Table 3 shows the results of the testing by two assays of 30 serum samples taken from ferrets 28 days after immunization with pilot vaccines against coronavirus infection (COVID-19) of carnivorous animals (produced by the FGBI "ARRIAH"). The data obtained indicate that 3 samples were negative and 25 samples were positive in both assays. Two samples, positive in neutralization test, when showed negative results when tested by ELISA.

The ELISA specificity and sensitivity compared to these parameters of the neutralization test used for testing of postvaccinal sera was 100 and 92.6%, respectively.

Table 2
Results of SARS-CoV-2 antigen antibody detection in homologous and heterologous animal sera by ELISA

Таблица 2
Результаты выявления антител к антигену SARS-CoV-2 в гомологичных и гетерологичных сыворотках крови животных методом ИФА

No.	Sample	Antibody titer*	S/N	Qualitative assessment of the result
1	Porcine transmissible gastroenteritis virus specific serum	50	0.88	negative
2	Bovine coronavirus specific serum	50	0.78	negative
3	Bovine diarrhea virus specific serum	100	0.99	negative
4	Infectious bronchitis virus specific serum	50	0.57	negative
5	Negative control serum	100	1.00	negative
6	SARS-CoV-2 positive control serum	800	5.70	positive

* Antibody titer is the reciprocal value of the serum dilution
(Титр антител – величина, обратная разведению сыворотки).

In order to determine the diagnostic sensitivity and specificity, 50 serum samples from different species (minks – 14, foxes – 10, cats – 14 and dogs – 12) were tested before and after (after 4–6 weeks) vaccination against COVID-19 with pilot vaccines produced by the FGBI "'ARRIAH". The results of the studies are presented in Table 4. Antibodies to SARS-CoV-2 were detected in the sera of all vaccinated animals; before immunization, all serum samples were negative in ELISA.

The results of antibody detection using the developed test kit were compared with the results, obtained by the commercial IDvet kit (France). For this purpose, 44 sera from different species were tested using the FGBI "ARRIAH" test kit and the IDvet commercial kit for the detection of antibodies to the SARS-CoV-2 nucleoprotein in accordance with the manufacturer's instructions.

Antibodies to the SARS-CoV-2 were detected in all serum samples of vaccinated animals, tested using the developed test kit (Table 5).

When testing by the IDvet kit, the SARS-CoV-2-specific antibodies were detected only in the sera of vaccinated ferrets, foxes, and minks. No specific antibodies were found in the blood of vaccinated dogs and cats. Since the IDvet kit is designed to detect antibodies only to the SARS-CoV-2 nucleoprotein, it can be assumed that the level of antibodies to protein N in the sera of cats and dogs is very low, which is also confirmed by immunoblotting of these sera (Fig. 2). In serum samples from non-vaccinated pigs, virus-specific antibodies were not detected by both ELISA test kits.

Table 3
Analysis of ELISA relative sensitivity and specificity for SARS-CoV-2 antibody detection in animal sera

Таблица 3
Оценка относительной чувствительности и специфичности ИФА для выявления антител к вирусу SARS-CoV-2 в сыворотках крови животных

Neutralization test*	ELISA		
	Positive	Negative	Total
Positive samples	25/a	2/c	27/a+c
Negative samples	0/d	3/b	3/d+b
Total samples	25	5	n = 30

* Sera were tested by neutralization test at the "N. F. Gamaleya NRCEM"of the Ministry of Health of the Russian Federation (Moscow) / Исследование сывороток крови в реакции нейтрализации выполнено в ФГБУ «НИЦЭМ им. Н. Ф. Гамалеи» Минздрава России (г. Москва);
a – true-positive results (истинно положительные результаты);
b – true-negative results (истинно отрицательные результаты);
c – false-negative results (ложноотрицательные результаты);
d – false-positive results (ложноположительные результаты).

Table 4
Results of SARS-CoV-2 antibody detection in animal sera before and after vaccination against coronavirus infection (COVID-19) by ELISA

Таблица 4
Результаты выявления антител к вирусу SARS-CoV-2 в сыворотках крови животных до и после вакцинации против коронавирусной инфекции (COVID-19) животных в ИФА

No.	Serum samples	Antibody titer*	S/N**	Number of samples / positives
1	Minks before vaccination	64 ± 9	1.45 ± 0.11	7/0
2	Minks after vaccination	829 ± 216	6.03 ± 0.89	7/7
3	Foxes before vaccination	50 ± 0	1.45 ± 0.11	5/0
4	Foxes after vaccination	400 ± 0	3.61 ± 0.65	5/5
5	Cats before vaccination	50 ± 0	1.31 ± 0.08	7/0
6	Cats after vaccination	543 ± 95	5.03 ± 0.47	7/7
7	Dogs before vaccination	83 ± 11	1.69 ± 0.13	6/0
8	Dogs after vaccination	667 ± 84	4.33 ± 0.40	6/6

* Average value of the antibody titer ± standard error of the mean, where the antibody titer is the reciprocal value of the serum dilution (Среднее значение титра антител ± стандартная ошибка среднего, где титр антител – величина, обратная разведению сыворотки);
** S/N value ± standard error of the mean (значение S/N ± стандартная ошибка среднего).

CONCLUSION

The developed indirect ELISA test kit for detection of antibodies to the SARS-CoV-2 showed high specificity (100%), sensitivity (92.6%) and reproducibility and can be used for screening tests for the SARS-CoV-2 in different susceptible species and control of the immunity after vaccination against coronavirus infection (COVID-19) of animals.

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Table 5
Results of SARS-CoV-2 antibody detection in animal sera by ELISA

Таблица 5
Результаты выявления антител к вирусу SARS-CoV-2 в сыворотках крови животных в ИФА

No.	Serum samples	ELISA (FGBI "ARRIAH")		ELISA (IDvet)	
		Antibody titer*	Number of samples / positives	S/P (%)**	Number of samples / positives
1	Ferrets after vaccination	1,600 ± 0	5/5	707 ± 66	5/5
2	Minks before vaccination	50 ± 0	2/0	47 ± 9	2/0
3	Minks after vaccination	1,025 ± 225	8/8	529 ± 58	8/8
4	Foxes after vaccination	300 ± 58	4/4	208 ± 62	4/4
5	Non-vaccinated pigs	50 ± 0	4/0	3.1 ± 1.8	4/0
6	Non-vaccinated Arctic foxes	50 ± 0	2/0	2 ± 0	2/0
7	Cats before vaccination	50 ± 0	3/0	1 ± 0	3/0
8	Cats after vaccination	560 ± 264	5/5	0.80 ± 0.01	5/0
9	Dogs before vaccination	50 ± 0	4/0	8.3 ± 6.6	4/0
10	Dogs after vaccination	700 ± 205	6/6	2.40 ± 1.09	6/0

Serum samples from vaccinated animals were taken 4-5 weeks after immunization using experimental vaccines against coronavirus infection (COVID-19) produced by the FGBI "ARRIAH" (Пробы сыворотки крови от вакцинированных животных отобраны через 4–5 недель после иммунизации экспериментальными вакцинными препаратами против коронавирусной инфекции (COVID-19) производства ФГБУ «ВНИИЗЖ»);
* the average value of the antibody titer ± the standard error of the mean, where the antibody titer is the reciprocal value of the serum dilution (среднее значение титра антител ± стандартная ошибка среднего, где титр антител – величина, обратная разведению сыворотки);
** mean S/P ± standard error of the mean (среднее значение S/P ± стандартная ошибка среднего).
Interpretation of the results obtained by IDvet kit (Интерпретация результатов в наборе IDvet): S/P ≤ 50% – negative result (результат отрицательный), S/P ≥ 60% – positive result (результат положительный), 50% < S/P < 60% – inconclusive result (результат сомнительный).

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Marina A. Volkova, Candidate of Science (Biology), Leading Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Nikolay G. Zinyakov, Candidate of Science (Biology), Senior Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Polina S. Yaroslavtseva, Candidate of Science (Biology), Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Ilya A. Chvala, Candidate of Science (Veterinary Medicine), Deputy Director for Research and Monitoring, FGBI "ARRIAH", Vladimir, Russia.

Tatyana S. Galkina, Candidate of Science (Veterinary Medicine), Head of Laboratory for Pet Disease Prevention, FGBI "ARRIAH", Vladimir, Russia.

Dmitry B. Andreychuk, Candidate of Science (Biology), Head of Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Волкова Марина Алексеевна, кандидат биологических наук, ведущий научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Зиняков Николай Геннадьевич, кандидат биологических наук, старший научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Ярославцева Полина Сергеевна, кандидат биологических наук, научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Чвала Илья Александрович, кандидат ветеринарных наук, заместитель директора по НИР и мониторингу ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Галкина Татьяна Сергеевна, кандидат ветеринарных наук, заведующий лабораторией профилактики болезней мелких домашних животных ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Андрейчук Дмитрий Борисович, кандидат биологических наук, заведующий референтной лабораторией вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

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Post-mortem and histological lesions in the European minks (*Mustela lutreola*) induced by spontaneous infection with coronavirus SARS-CoV-2

I. A. Subbotina[1], I. N. Gromov[2]
Educational Establishment "Vitebsk State Awarded the "Badge of Honour" Order Veterinary Medicine Academy" (EE "VSAVM"), Vitebsk, Republic of Belarus
[1] *ORCID ID 0000-0001-8346-2988, e-mail: irin150680@mail.ru*
[2] *ORCID ID 0000-0001-8065-5661, e-mail: gromov_igor@list.ru*

SUMMARY
The paper contains data on registration of the SARS-CoV-2 virus in the European mink population. Profound and detailed studies of the virus circulation in the European mink population and the clinical manifestations of the disease, comprehensive approaches to the disease diagnosis, including epidemiological studies, clinical and post-mortem examinations, molecular genetic laboratory diagnostics (polymerase chain reaction and full-genome sequencing) contributed to better understanding of the disease features. The paper presents the data on post-mortem and histological lesions in the European minks infected with the new coronavirus SARS-CoV-2 obtained during the research. All the animals from which the pathological material was collected were infected with SARS-CoV-2, and the diagnosis was made using polymerase chain reaction (RT-PCR). The obtained and presented in the paper data reveal the features and dynamics of pathological processes in the body of infected animals (European mink), demonstrate the characteristics of the lesions in organs and tissues in case of acute and chronic disease, explain the clinical and post-mortem disease pattern and indicate the causes of animal deaths. All this together will allow veterinary specialists not only to quickly and timely diagnose the disease in the population of fur animals (European mink), but also to take necessary therapeutic and preventive measures in a timely manner, to select the most effective means for symptomatic and pathogenetic therapy as well as the most rational and effective substances and disinfection procedures.

Keywords: European mink, coronavirus, SARS-CoV-2, post-mortem lesions, histological examination.

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For correspondence: Irina A. Subbotina, Candidate of Science (Veterinary Medicine), Associate Professor, Department of Epizootology and Infectious Diseases, EE "VSAVM", 210026, Republic of Belarus, Vitebsk, 1st Dovator str., 7/11, *e-mail: irin150680@mail.ru.*

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Патологоанатомические и гистологические изменения у норки европейской (*Mustela lutreola*) при спонтанном инфицировании коронавирусом SARS-CoV-2

И. А. Субботина[1], И. Н. Громов[2]
УО «Витебская ордена «Знак Почета» государственная академия ветеринарной медицины» (УО «ВГАВМ»), г. Витебск, Республика Беларусь
[1] *ORCID ID 0000-0001-8346-2988, e-mail: irin150680@mail.ru*
[2] *ORCID ID 0000-0001-8065-5661, e-mail: gromov_igor@list.ru*

РЕЗЮМЕ
Представлены данные о регистрации вируса SARS-CoV-2 в популяции норки европейской. Проведение глубоких и детальных исследований циркуляции данного вируса в популяции норки европейской, изучение клинического проявления болезни, комплексные подходы в диагностике данной болезни, включающие эпизоотологические, клинические и патологоанатомические исследования, молекулярно-генетическую лабораторную диагностику (полимеразную цепную реакцию и полногеномное секвенирование) позволили более детально понять ряд особенностей данной болезни. В статье приведены данные собственных исследований об особенностях патологоанатомической картины и гистологических изменений у норки европейской при инфицировании новым коронавирусом SARS-CoV-2. Все животные, от которых отбирался патологический материал, были инфицированы SARS-CoV-2, диагноз ставился с использованием полимеразной цепной реакции (RT-PCR). Полученные и приведенные в исследовании данные широко раскрывают особенности и динамику патологических процессов в организме инфицированных животных (норки европейской), показывают особенности происходящих

изменений в органах и тканях как при остром, так и при хроническом течении болезни, объясняют клиническую и патологоанатомическую картину болезни и указывают на причины летальных исходов у животных. Учитывая очевидную высокую информативность результатов гистологического исследования по сравнению с неспецифичностью патологоанатомических изменений, считаем проведение его обязательным при постановке предварительного диагноза на COVID-19 у норок. Все это в совокупности позволит ветеринарным специалистам не только быстро и вовремя диагностировать данную патологию в популяции пушных животных (норки европейской), но и своевременно провести необходимые лечебно-профилактические мероприятия, подобрать наиболее эффективные средства для симптоматической и патогенетической терапии, подобрать наиболее рациональные и эффективные вещества и режимы для дезинфекции.

Ключевые слова: Норка европейская, коронавирус, SARS-CoV-2, патологоанатомические изменения, гистологическое исследование.

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Для корреспонденции: Субботина Ирина Анатольевна, кандидат ветеринарных наук, доцент кафедры эпизоотологии и инфекционных болезней, УО «ВГАВМ», 210026, Республика Беларусь, г. Витебск, ул. 1-я Доватора, 7/11, *e-mail: irin150680@mail.ru.*

INTRODUCTION

The coronavirus infection COVID-19 caused by SARS-CoV-2 virus has prevented humanity from returning to a normal active life and communication in all spheres for nearly two years already. Strict quarantine measures are still in place in many countries, in some countries lockdown have been implemented, some countries closed their borders for foreigners and imposed restrictions on leaving the country, the trade, political and social relations between countries have been impeded. And to think that at the beginning when the infection occurred and the agent was identified the humanity neglected the situation and compared it with "common seasonal influenza". But unfortunately the disease appeared to be more severe than influenza and for today more than two million people have become its victims. The coronavirus infection can, of course, be compared to the Spanish flu based on many characteristics. However, one shouldn't forget the time when the Spanish flu, having claimed about 100 million lives, occurred and healthcare level of that time. And now, take into consideration the COVID-19 impact in the century of high technologies and advanced medicine – the comparison is not in our favour. Many doctors, virologists and researchers consider that this is just the beginning of the journey of the relatively aggressive and deadly virus.

One more characteristic of the new coronavirus raises concern among scientists and epidemiologists in the world – absence of distinct species specificity. Originally it was proved that COVID-19 – is a zoonosis transmitted from an animal to a human. The sources of the virus were determined and bat is considered to be one of the basic sources. However, there is some controversy as to whether pangolins and snakes were involved in the process of mutation and agent transmission to the human. In the first days and months of the pandemics the disease was reported and studied only in humans, but currently the situation has drastically changed [1–3].

According to the official data of the World Organization for Animal Health (OIE), Food and Agricultural Organization of the United Nations (FAO), Centers for Disease Control and Prevention (CDC), World Health Organization (WHO), American Veterinary Medical Association and some other international organizations SARS-CoV-2 has currently been recovered from quite a huge number of animals. Moreover, the virus not only circulated in the body of animals but causes the disease development and death in some species. The clinical disease manifestations are similar to those in humans: common symptoms are depression, feed refusal, coughing, fever, labored breathing, dyspnea, sometimes diarrhea [4].

So far, the disease has been registered and the clinical picture in representatives of the *Felidae* family (cats, lions, leopards, tigers and pumas) as well as in fur animals (European mink, tinker) has been partially described. Multiple positive results of COVID-19 diagnostics in representatives of the *Felidae* family were obtained in the USA, some European countries (Italy, France, Belgium, Lithuania, etc), in the Russian Federation, Ukraine and China. All animals had contacts with sick people (COVID-19 positive). The infected animals had damaged respiratory organs, the major disease symptoms were nasal discharge, rapid and shallow breathing, coughing. In several cases gastrointestinal disorders were observed (diarrhea). The Chinese researches performed an experiment and proved animal-to-animal SARS-CoV-2 transmission within the cat population. Italian researchers conducted a comprehensive examination of cats and dogs in most COVID-19 affected Italian regions and detected a relatively high percent of animals with SARS-CoV-2 antibodies (dogs – more than 30%, cats – more than 40% of all tested animals), which is indicative of susceptibility of these animal species to the new virus [4, 5].

Besides data on the virus spread among pets (cats and dogs) there are data on possible virus persistence and its transmission within tinker population (a perfect biological model for studying SARS-CoV-2). The virus circulation in raccoon dogs and rabbits is also possible. There is evidence of the possibility of infecting laboratory animals (white

mice, golden Syrian hamster and guinea pig), badger, pig (with experimental infection), small ruminants. There have been published data on the possible (theoretical) infection of about 400 animal species due to the receptor protein ACE2 they have [5, 6].

Within the last months there have been reports from some zoos about infection of gorillas (San-Diego). AVMA has also provided data on profound studies of SARS-CoV-2 circulation in different animal species (more than 2000), as a result of which the virus was recovered in samples collected from civet cats, dolphins, armadillos, and anteaters. The researchers determined that 80% of the tested pets (cats and dogs) were infected. The data on SARS-CoV-2 circulation in populations of different animal species are constantly updated [4–7].

The new coronavirus circulation in the mink population and the pathological condition induced by it are worth mentioning. As the latest OIE, FAO, CDC, WHO data demonstrate minks are one of the potential (and the only confirmed as for today) source of the backward virus transmission to humans and one of the animal species in whose body the virus is likely to mutate. Denmark published information about destruction of the entire mink population (about 17 million animals) because of the possible SARS-CoV-2 virus mutation in their body and its probable transmission to humans (12 people). Later the strain with a similar mutation was detected in more than 200 people (according to official data of the National Danish Institute for Infectious diseases). However, there was no any direct proof that the infection was transmitted from minks, it was only a hypothesis. Previously, the Netherlands informed about possible infection of two humans by minks. To date, data on the infection of minks have already been published by many countries around the world (Poland, Lithuania, Denmark, the Netherlands, the USA, etc.), in a number of countries it was decided to destroy the mink population due to the threat of the virus mutation and its transmission to the human population [8–13].

The issue of the animal's role in SARS-CoV-2 spread and its persistence in their populations and the virus influence on the animal's body is considered to be practically unstudied. The research in this field is just taking shape in several countries.

Based on the above, the circulation of SARS-CoV-2, which causes COVID-19, in populations of various animal species, as well as the features of the clinical and pathological disease manifestation, histological lesions during infection with this virus are poorly understood and relevant.

The aim of the research was to identify the post-mortem and histological lesions of the European mink (*Mustela lutreola*) during SARS-CoV-2 infection.

MATERIALS AND METHODS

The carcasses of culled European minks (young animals, parent stock) kept in industrial fur farms were used as material for research. According to anamnestic data, in May – June 2020, on a number of farms, there was an increased morbidity and mortality of animals with signs of respiratory tract and cardiovascular lesions.

The PCR tests (RT-PCR) of the biological material (swabs from the mucous membranes of the oral, nasal cavities and rectum) from sick and dead animals using test systems for the detection of SARS-CoV-2 virus RNA (ArtBioTech LLC, Republic of Belarus) revealed positive results.

During autopsy of mink carcasses the nature and severity of pathomorphological lesions were taken into account, a pathological diagnosis was made [14], macro photography was performed using natural light. The autopsy was carried out in specially equipped rooms in compliance with personal and biological safety rules followed by the neutralization and disposal of biomaterial, disinfection of the room and tools, preventing contamination of rooms and equipment.

Pieces of the lungs, liver, kidneys, heart, pancreas, and spleen were taken for histological examination [15]. The resulting material was fixed in a 10% solution of neutral formalin and compressed by paraffin embedding process in accordance with the generally accepted method [16]. Dehydration and paraffinization of organ pieces was performed using an spin tissue processor with carousel system MICROM STP 120 (Germany). An automatic tissue embedding centre MICROM EC 350 was used to embed the pieces and prepare the paraffin blocks. Histological samples of the organ pieces embedded in paraffin were prepared using a rotary (pendulum) microtome MICROM HM 340 E. De-embedding and staining of histological sections with hematoxylin – eosin was performed using an automatic station MICROM HMS 70. Histological examination was performed using a light microscope "Biomed-6" (Russia). The data obtained were documented by microphotography using the DSM-510 digital reading and video input system, as well as the ScopePhoto image input and preprocessing software. Structural changes in the parenchyma and in the stroma were taken into account according to the guidelines [17, 18].

RESULTS AND DISCUSSION

The major post-mortem lesions during the acute course of the disease were characterized by the predominance of hemodynamic disorders and serious disorders of the cardiovascular and respiratory systems.

Macroscopic changes in the lungs were characterized by the simultaneous development of a number of interrelated processes, among which three main combinations were distinguished.

In the first case, severe acute venous hyperemia, serous or serous-hemorrhagic edema, alveolar emphysema were observed in the lungs (Fig. 1). Macroscopically, the lungs did not collapse, their shape was not changed, the color was dark red (almost black-red), the consistency was soft. Jell-like clots or red foam were observed in incised primary bronchi, and well – formed blood clots in dissected large arteries and incised veins. The pattern of the lobular structure is poorly visible. These processes are most likely due to the presence of fibrin in the transudate, which was further confirmed by the results of histological examination. An important sign indicating the absence of classical pneumonia is the buoyancy of the pieces in the water. On a dark red background of the lung parenchyma, the areas of emphysema were clearly visualized in the form of poorly shaped, slightly elevated foci of gray-white color. The fact of the development of this pathological process is important for the disease diagnosis, and we consider it a significant pathognomonic sign. However, when performing an autopsy of dead minks, it can be easily confused with post-mortem processes: redistribution of blood flow, cadaveric autolysis, cadaveric emphysema. In this regard, the results of histological examination are of decisive importance.

In the second case, a combination of acute serous pulmonary edema with areas of alveolar emphysema was observed. The lungs were not collapsed, with a doughy consistency, classic for edema, pink-red, light-red ("carmine" lungs), their shape was not changed. The pattern of the lobules is not pronounced, the pieces of the organ float, completely immersed in the water.

In the third case, the following combination was observed: acute serous edema, areas of alveolar emphysema, small-focal pneumonia with localization in the anterior, middle and caudal lobes of the lungs (Fig. 2–5). Against the background of the processes described above (serous edema, alveolar emphysema), the presence of small-focal pneumonia was observed. The lesions were small (up to 5–6 mm), dark red, localized subcapsularly in various parts of the lungs (caudal, middle, anterior lobes). Pieces of the affected lungs did not drown, but floated, completely submerged in the water. In this regard, this process can be easily confused with spotted hemorrhages. The small size of the inflamed areas does not allow to determine the nature of pneumonia: serous, catarrhal, fibrinous or interstitial. In this case, it is necessary to conduct a histological examination of the lungs.

When studying the heart, three variants of pathological processes were also identified, indicating the development of acute cardiac and concomitant cardiopulmonary failure.

In the first case, in our opinion the most severe and irreversible, there was an acute expansion of all cardiac cavities with the development of a classic round heart, sometimes with severe acute venous hyperemia of the myocardium (Fig. 1, 2, 4). In the second case, the blood content of the heart muscle was less pronounced, however, the characteristic signs of fatty degeneration of the myocardium with its staining in a light yellow color came to the fore (Fig. 3). Fatty degeneration of the myocardium, liver and kidneys is the morphological equivalent of acute intoxication of the body. In the third case, signs of asphyxia prevailed – acute expansion of the right ventricle and atrium, blood stagnation in the pulmonary circulation system (Fig. 5).

Considering the deep structural changes in the lungs, the development of signs of asphyxia, viremia, infectious shock, the formation of pronounced post-mortem blood coagulation looks paradoxical not only in the cavities of the heart and large arteries, but also in veins of various sizes (Fig. 6). In our opinion, this is due to a systemic imbalance in the blood coagulation and anticoagulation systems towards thrombus formation, which plays an important role in the COVID-19 pathogenesis in humans and animals.

In addition, the autopsy of mink carcasses revealed morphological signs of acute heart failure – cyanosis of the mucous membranes, especially of such organs of the oral cavity as the tongue and gums (Fig. 7), skin, skeletal muscles, acute venous hyperemia of the liver and kidneys. Most animals showed punctate hemorrhages in the renal cortex (Fig. 8), and in some specimens, serous or hemorrhagic splenitis (Fig. 9).

In some cases, these processes developed concurrently with chronic feed toxicosis, the characteristic morphological sign of which was fatty liver and interstitial hepatitis (Fig. 1). At the same time, the liver was not enlarged, the shape was not changed, the consistency was elastic, dense, yellow, the pattern of the lobular structure in the section was more distinct.

Six months after recovering from COVID-19, minks killed for diagnostic purposes showed characteristic pathological changes indicating a chronic course of the disease, long-term persistence of the virus in susceptible livestock. As in the beginning of the outbreak, the major processes were observed in the lungs, cardiovascular system and blood. Morphological signs of regeneration of the structure of previously affected organs and tissues were not recorded.

Lung examination revealed alveolar emphysema, edema, hemorrhages, small- and large-focal interstitial pneu-

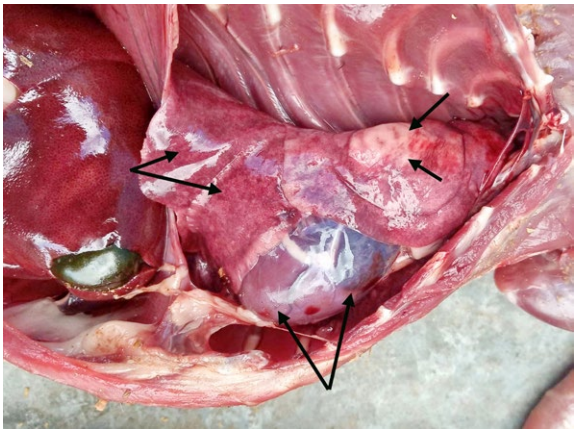

Fig. 1. Post-mortem lesions in a 6-month-old mink infected with COVID-19: acute venous hyperemia (arrows on the left), pulmonary emphysema (arrows on the right), acute cardiac dilatation (arrows below). Background processes: interstitial hepatitis, gallbladder expansion (left)

Рис. 1. Патологоанатомические изменения у 6-месячной норки при COVID-19: острая венозная гиперемия (стрелки слева), эмфизема легких (стрелки справа), острое расширение сердца (стрелки внизу). Фоновые процессы: интерстициальный гепатит, расширение желчного пузыря (слева)

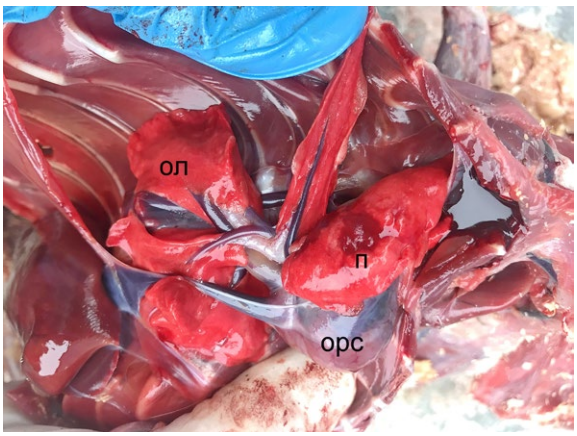

Fig. 2. Post-mortem lesions in a 6-month-old mink infected with coronavirus SARS-CoV-2: pulmonary edema (ОЛ), pneumonia sites (П), acute cardiac dilatation (ОРС)

Рис. 2. Патологоанатомические изменения у 6-месячной норки, инфицированной коронавирусом SARS-CoV-2: отек легких (ОЛ), участки пневмонии (П), острое расширение сердца (ОРС)

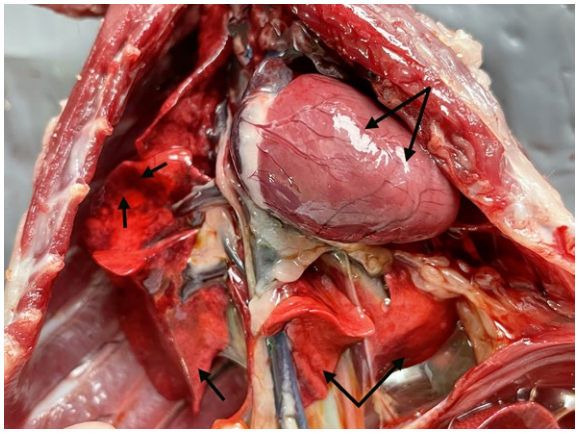

Fig. 3. Post-mortem lesions in a 6-month-old mink infected with COVID-19: pulmonary edema (arrows below), pneumonia sites (arrows on the left), acute cardiac dilatation, myocardial lipidosis (arrows on the left)

Рис. 3. Патологоанатомические изменения у 6-месячной норки при COVID-19: отек легких (стрелки внизу), участки пневмонии (стрелки слева), острое расширение сердца, жировая дистрофия миокарда (стрелки справа)

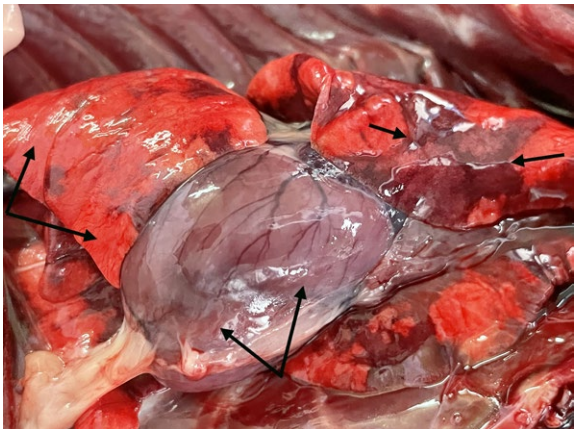

Fig. 4. Lungs of a 6-month-old mink infected with coronavirus SARS-CoV-2: pulmonary edema (arrows on the left), pneumonia and emphysema sites (arrows on the right), round heart (arrows below)

Рис. 4. Легкие 6-месячной норки, инфицированной коронавирусом SARS-CoV-2: отек легких (стрелки слева), участки пневмонии и эмфиземы (стрелки справа), круглое сердце (стрелки внизу)

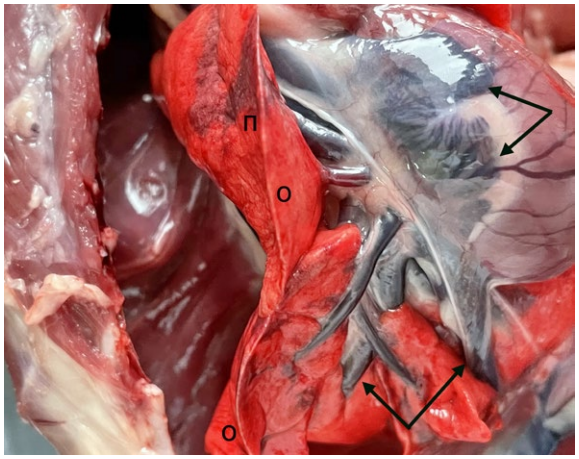

Fig. 5. Pulmonary edema (O) with pneumonia sites (П) in a 6-month-old mink. Enlargement of the left artrium and pulmonary veins (arrows)

Рис. 5. Отек легких (О) с участками пневмонии (П) у 6-месячной норки. Острое расширение левого предсердия и системы легочных вен (стрелки)

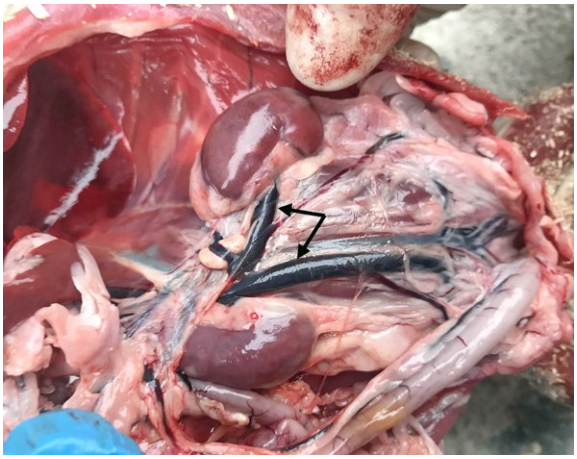

Fig. 6. Severe blood clot formation in the caudal vena cava of a 6-month-old mink infected with coronavirus SARS-CoV-2

Рис. 6. Выраженное формирование посмертных свертков крови в каудальной полой вене 6-месячной норки, инфицированной коронавирусом SARS-CoV-2

monia (Fig. 10, 11). The lungs were not collapsed, their shape was not changed, the consistency was soft with a doughy feel. The background color, as during the acute disease, was pink-red, carmine. Subcapsular, well-defined hemorrhages were common. There were also regions of classic interstitial pneumonia with compaction of the parenchyma, staining it in a "fleshy", red-brown color, as well as a more distinct pattern of the lobular structure. In all cases, the buoyancy of the affected pieces of the lungs was preserved. In some animals, an aggravating process was noted – focal chronic fibrinous pleurisy.

In the heart there were signs of acute expansion, fatty degeneration, but without venous hyperemia of the myocardium (Fig. 10, 11). In the cavities of the heart, the lumen of large arteries and veins, as in the acute course, post-mortem blood clots were detected. The renal cortex showed multiple punctate hemorrhages and brownish pigment spots, which are "old" hemorrhages (Fig. 12).

The spleen was enlarged in size, had elastic consistency, the parenchyma was red-brown in color with a steel tint. The scraping of the pulp with the back of the knife is insignificant.

Post-mortem diagnosis:

acute disease

1. Severe acute venous hyperemia, serous or serous-hemorrhagic pulmonary edema, areas of alveolar emphysema.

or

Serous pulmonary edema (carmine lungs), areas of alveolar emphysema.

Fig. 7. Acute venous hyperemia of the mucous membrane of the tongue in a 6-month-old mink

Рис. 7. Острая венозная гиперемия слизистой оболочки языка у 6-месячной норки

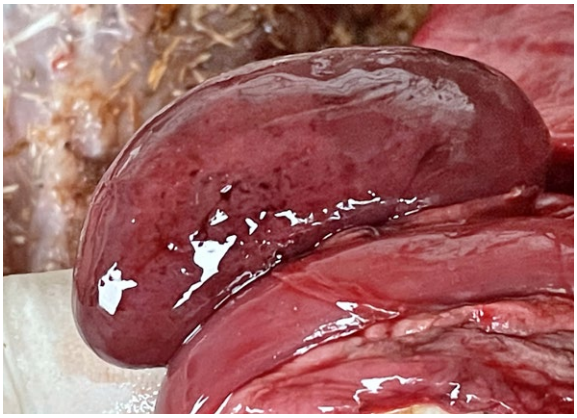

Fig. 8. Hemorrhages in the renal cortex in a 6-month-old mink infected with the coronavirus SARS-CoV-2

Рис. 8. Кровоизлияния в корковом веществе почки у 6-месячной норки, инфицированной коронавирусом SARS-CoV-2

Fig. 9. Acute serous splenitis in a 6-month-old mink

Рис. 9. Острый серозный спленит у 6-месячной норки

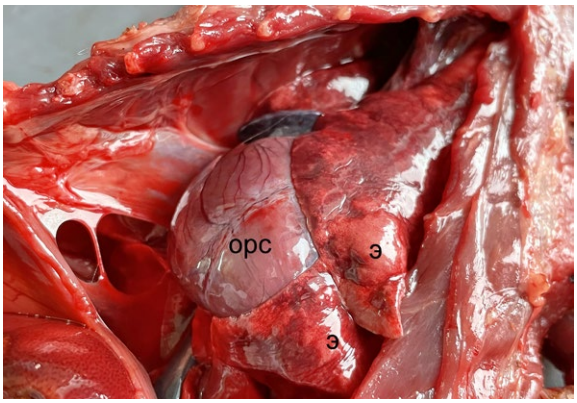

Fig. 10. Post-mortem lesions in an 18-month-old mink infected with the SARS-CoV-2 coronavirus: interstitial pneumonia, sites of emphysema (Э), acute cardiac dilatation (ОРС)

Рис. 10. Патологоанатомические изменения у 18-месячной норки, инфицированной коронавирусом SARS-CoV-2: интерстициальная пневмония, участки эмфиземы (Э), острое расширение сердца (ОРС)

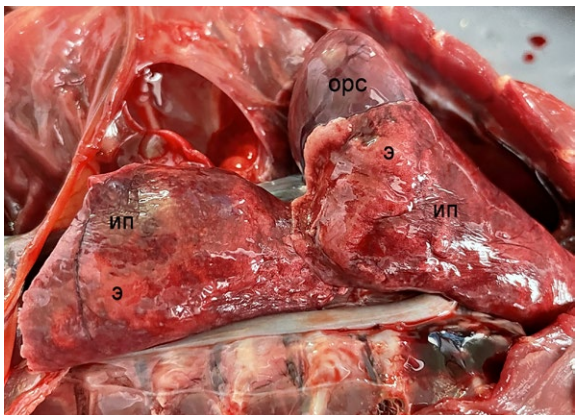

Fig. 11. Interstitial pneumonia (ИП) with emphysema sites (Э) in an 18-month-old mink. acute cardiac dilatation (ОРС), acute venous hyperemia of the myocardium

Рис. 11. Интерстициальная пневмония (ИП) с участками эмфиземы (Э) у 18-месячной норки. Острое расширение сердца (ОРС), острая венозная гиперемия миокарда

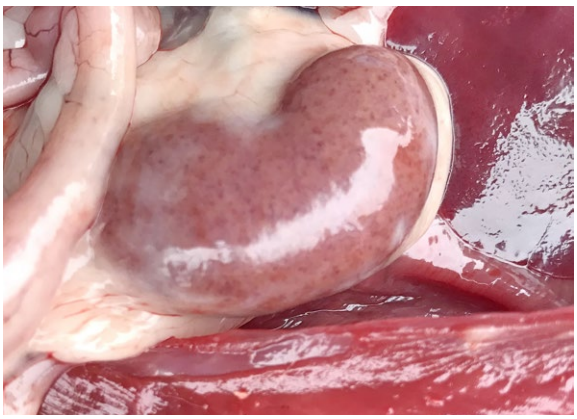

Fig. 12. Kidney of an 18-month-old mink infected with the coronavirus SARS-CoV-2. Pigmented spots in the cortex (old hemorrhages)

Рис. 12. Почка 18-месячной норки, инфицированной коронавирусом SARS-CoV-2. Пигментные пятна в корковом веществе (старые кровоизлияния)

or

Serous pulmonary edema, areas of alveolar emphysema, small focal pneumonia with localization in the anterior, middle and caudal lobes of the lungs.

2. Acute heart enlargement (round heart), acute venous myocardial hyperemia.

or

Acute heart enlargement (round heart), myocardial fatty degeneration.

or

Acute enlargement of artrium and right ventricle (cor pulmonale, asphyxia), pulmonary veins.

3. Severe post-mortem blood coagulation in the cavities of the heart, large arteries and veins.

4. Acute venous hyperemia as well as fatty liver and kidneys. Pinpoint hemorrhages in the renal cortex.

5. Acute venous hyperemia of mucous membranes, skin and skeletal muscles.

6. Acute serous or hemorrhagic splenitis (not always).

chronic disease

1. Alveolar emphysema, edema (carmine lungs) and hemorrhages in the lungs, focal interstitial pneumonia.

2. Focal fibrinous pleurisy (complication).

3. Acute heart enlargement (round heart).

4. Severe post-mortem blood coagulation in the cavities of the heart, large arteries and veins.

5. Hemorrhages and pigment spots (old hemorrhages in the kidneys).

6. Splenomegaly (hyperplasia of the spleen).

Histological diagnosis:

acute disease

• **Lungs** (Fig. 13–16) – vascular hyperemia of the microvasculature, thrombosis of arterioles, venules, interalveolar capillaries (disseminated intravascular coagulation syndrome, shock lungs), severe serous, serous-hemorrhagic edema of interstitial tissue and parenchyma, necrosis and desquamation of alveolar epithelium fibrin threads in the form of a mesh, fragments of necrotic epithelium, hemolyzed erythrocytes and eosinophilic hyaline membranes, extensive lymphoid-macrophage peribronchitis and perivasculitis, focal proliferation of fibroblasts, alveolar emphysema.

• **Liver** (Fig. 17) – acute venous hyperemia, serous edema, thrombosis of the central veins of the hepatic lobules and sinusoidal capillaries (disseminated intravascular coagulation, shock liver), multiple hemorrhages, hemosiderin deposition (hemosiderosis), total droplet fatty degeneration, areas of necrobiosis and parenchymal necrosis.

• **Pancreas –** venous hyperemia, hemostasis (especially in the area of the islets of Langerhans), vacuolar degeneration of individual acini epithelial cells.

• **Kidneys** (Fig. 18) **–** acute venous hyperemia, edema, extensive hemorrhages, large-droplet fatty degeneration of the epithelium of the urinary tubules.

• **Spleen –** focal lymphoid-macrophage infiltrates in the red pulp, hyperemia of sinusoidal capillaries.

chronic disease

• **Lungs** (Fig. 19–21) – a pronounced proliferation of interlobular and interalveolar connective tissue, lymphoid-macrophage peribronchitis and perivasculitis, the formation of nodular lymphoid tissue, chronic venous hyperemia, blood stasis in the vessels of the microvasculature, multiple hemorrhages with RBC hemolysis and hemosiderin accumulation, large regions of alveolar emphysema, atrophy or absence of alveolar epithelium.

• **Liver –** chronic venous hyperemia, hemosiderin deposition (hemosiderosis).

• **Kidneys** (Fig. 22) – venous hyperemia, serous edema of the glomeruli and intertubular connective tissue, hemosiderosis, deposition of uric acid salts in the lumen of individual urinary tubules.

• **Heart –** serous myocardial edema.

• **Spleen** (Fig. 23, 24) – ubiquitous proliferation of connective tissue (sclerotization), severe lymphoid hyperplasia of the white pulp, deposition of hemosiderin granules in the red pulp.

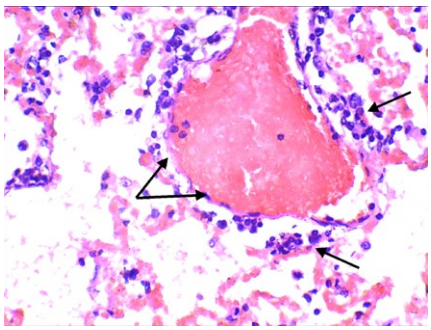

Fig. 13. Lungs of a 6-month-old mink: venous thrombosis (arrows on the left), lymphoid-macrophage perivasculitis (arrows on the right), alveolar rupture. Hematoxylin-eosin staining, magnification ×480

Рис. 13. Легкие 6-месячной норки: тромбоз венулы (стрелки слева), лимфоидно-макрофагальный периваскулит (стрелки справа), разрыв альвеол. Окраска гематоксилином и эозином, увеличение ×480

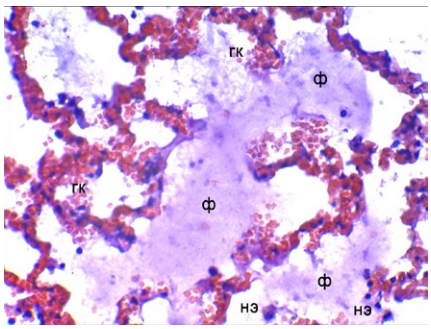

Fig. 14. Lungs of a 6-month-old mink: hyperemia of capillaries (ГК), fibrin (Ф) and necrotic epithelium (НЭ) in the alveolar lumen. Hematoxylin-eosin staining, magnification ×480

Рис. 14. Легкие 6-месячной норки: гиперемия капилляров (ГК), фибрин (Ф) и некротизированный эпителий (НЭ) в просвете альвеол. Окраска гематоксилином и эозином, увеличение ×480

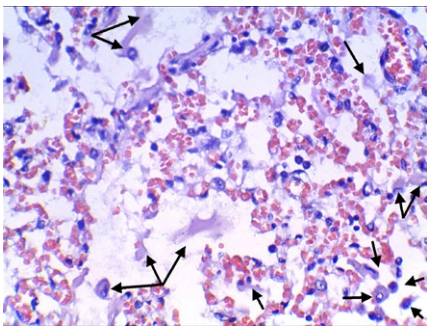

Fig. 15. Desquamated epithelium, fibrin filaments, hyaline membranes in the lumen of the lung alveoli of a 6-month-old mink. Hematoxylin-eosin staining, magnification ×480

Рис. 15. Слущенный эпителий, нити фибрина, гиалиновые мембраны в просвете альвеол легких 6-месячной норки. Окраска гематоксилином и эозином, увеличение ×480

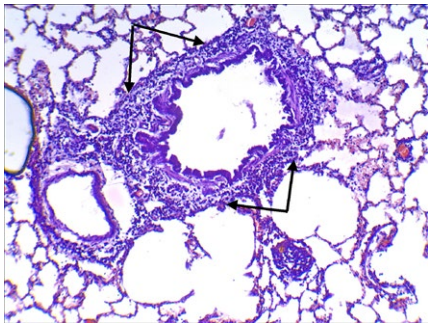

Fig. 16. Lungs of a 6-month-old mink: lymphoid-macrophage peribronchitis (arrows), emphysema. Hematoxylin-eosin staining, magnification ×120

Рис. 16. Легкие 6-месячной норки: лимфоидно-макрофагальные перибронхиты (стрелки), эмфизема. Окраска гематоксилином и эозином, увеличение ×120

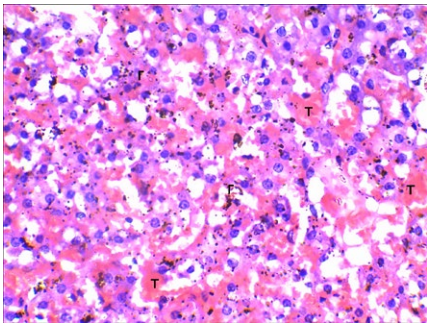

Fig. 17. Deposition of hemosiderin granules (Г) in the shock liver of a 6-month-old mink, thrombosis (Т) of sinusoidal capillaries. Hematoxylin-eosin staining, magnification ×480

Рис. 17. Отложение гранул гемосидерина (Г) в шоковой печени 6-месячной норки, тромбоз (Т) синусоидных капилляров. Окраска гематоксилином и эозином, увеличение ×480

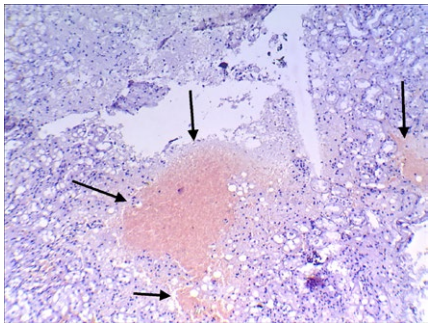

Fig. 18. Hemorrhages in the kidney of a 6-month-old mink (arrows). Hematoxylin-eosin staining, magnification ×120

Рис. 18. Кровоизлияния в почке 6-месячной норки (стрелки). Окраска гематоксилином и эозином, увеличение ×120

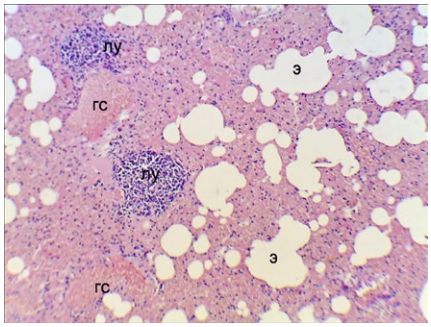

Fig. 19. Interstitial pneumonia in an 18-month-old mink. Hemostasis (ГС), lymphoid nodules (ЛУ), emphysema (Э). Hematoxylin-eosin staining, magnification ×120

Рис. 19. Интерстициальная пневмония у 18-месячной норки. Гемостаз (ГС), лимфоидные узелки (ЛУ), эмфизема (Э). Окраска гематоксилином и эозином, увеличение ×120

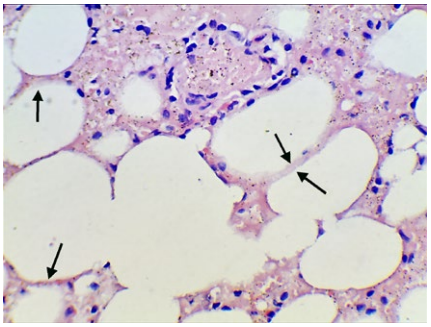

Fig. 20. Lungs of an 18-month-old mink: sclerotization, emphysema, hemosiderosis. The alveolar epithelium is atrophied or absent (arrows). Hematoxylin-eosin staining, magnification ×480

Рис. 20. Легкие 18-месячной норки: склеротизация, эмфизема, гемосидероз. Альвеолярный эпителий атрофирован или отсутствует (стрелки). Окраска гематоксилином и эозином, увеличение ×480

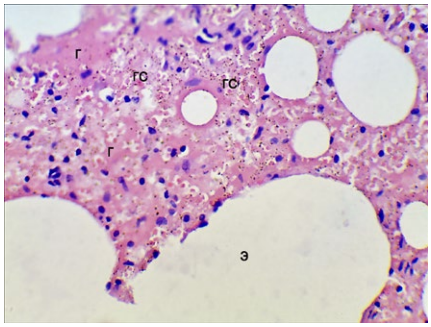

Fig. 21. Lungs of an 18-month-old mink: Sclerotization, emphysema (Э), hemorrhages with hemolysis (Г) of erythrocytes, hemosiderin granules (ГС). Hematoxylin-eosin staining, magnification ×480

Рис. 21. Легкие 18-месячной норки: Склеротизация, эмфизема (Э), кровоизлияния с гемолизом (Г) эритроцитов, гранулы гемосидерина (ГС). Окраска гематоксилином и эозином, увеличение ×480

CONCLUSION

Post-mortem lesions during the acute course of COVID-19 in minks are characterized by the predominance of hemodynamic disorders, serious disorders of the cardiovascular and respiratory systems i.e. development of acute venous hyperemia, pulmanory emphysema and microfocal pneumonia in various combinations, acute expansion of the heart or its right cavities, acute venous hyperemia of the myocardium, liver, kidneys, skin and mucous membranes, hemorrhages in the kidneys, pronounced post-mortem blood coagulation in the heart, arterial lumens and veins of various diameters. The development of serous and hemorrhagic splenitis, in our opinion, cannot be considered as a diagnostic marker of this disease. In case of chronic disease, the nature of the pathological picture remains, which is apparently associated with the long-term persistence of the pathogen in susceptible minks. Morphological signs of a long-term viral infection are focal interstitial pneumonia, lymphoid hyperplasia of the spleen (splenomegaly), as well as the formation of pigment spots at the site of hemorrhages due to the appearance of hemoglobinogenic pigments.

Histological lesions in minks during the acute course of COVID-19 are characterized by acute interstitial pneumonia and alveolitis complicated by respiratory distress syndrome and alveolar emphysema. The immediate cause of death is membranogenic pulmonary edema. Deep and irreversible changes in the vessels of the microvasculature of the internal organs (disseminated intravascular coagulation syndrome, disseminated intravascular coagulation syndrome) are a sign of shock development (infectious-toxic, septic, etc.).

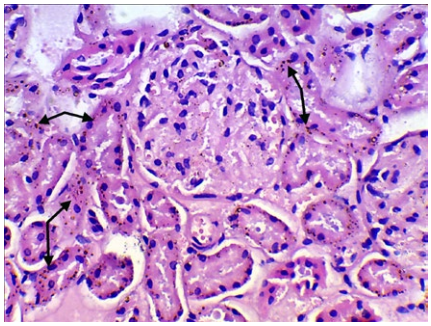

Fig. 22. Hemosiderosis of the kidney of an 18-month-old mink. Hematoxylin-eosin staining, magnification ×480

Рис. 22. Гемосидероз почки 18-месячной норки. Окраска гематоксилином и эозином, увеличение ×480

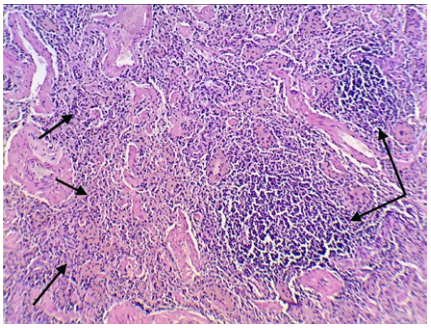

Fig. 23. Spleen of an 18-month-old mink: proliferation of a connective tissue (arrows on the left), hyperplasia of lymphoid nodules (arrows on the right). Hematoxylin-eosin staining, magnification ×120

Рис. 23. Селезенка 18-месячной норки: разрастание соединительной ткани (стрелки слева), гиперплазия лимфоидных узелков (стрелки справа). Окраска гематоксилином и эозином, увеличение ×120

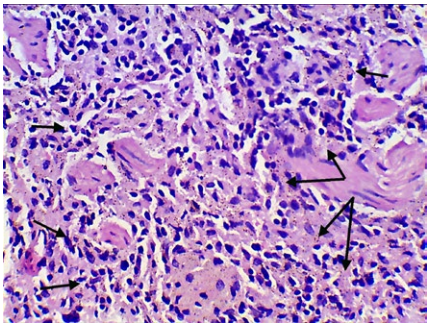

Fig. 24. Spleen of an 18-month-old mink: proliferation of a connective tissue (arrows on the left), deposition of hemosiderin granules (arrows on the right). Hematoxylin-eosin staining, magnification ×480

Рис. 24. Селезенка 18-месячной норки: разрастание соединительной ткани (стрелки слева), отложение гранул гемосидерина (стрелки справа). Окраска гематоксилином и эозином, увеличение ×480

Structural lung disorders of minks with a long course of the disease are characteristic of chronic interstitial pneumonia, complicated by alveolar emphysema, combined with profound lesions in the microvasculature (venous hyperemia, hemostasis, hemorrhages, local hemosiderosis). An aggravating process caused by prolonged pulmonary and heart failure is chronic venous hyperemia of internal organs (liver, kidneys). General hemosiderosis is a concomitant process, probably associated with prolonged intravascular hemolysis of erythrocytes. Lymphoid hyperplasia and sclerotization of the spleen are signs of a viral infection that is systemic in nature and accompanied by prolonged viremia.

Taking into account the relatively nonspecific pathological changes and the obvious high information content of the results of histological examination, we consider it's mandatory to perform it when making a presumptive diagnosis of COVID-19 in minks.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Irina A. Subbotina, Candidate of Science (Veterinary Medicine), Associate Professor, Department of Epizootology and Infectious Diseases, EE "VSAVM", Vitebsk, Republic of Belarus.

Igor N. Gromov, Doctor of Science (Veterinary Medicine), Head of the Department of Pathological Anatomy and Histology, EE "VSAVM", Vitebsk, Republic of Belarus.

Субботина Ирина Анатольевна, кандидат ветеринарных наук, доцент кафедры эпизоотологии и инфекционных болезней, УО «ВГАВМ», г. Витебск, Республика Беларусь.

Громов Игорь Николаевич, доктор ветеринарных наук, заведующий кафедрой патологической анатомии и гистологии, УО «ВГАВМ», г. Витебск, Республика Беларусь.

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Analysis of SAT-1, -2, -3 FMD outbreaks in Africa in 2017–2019

M. V. Sidorovskaya[1], S. N. Fomina[2], S. R. Kremenchugskaya[3]
FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia
[1] *ORCID 0000-0001-6218-5846, e-mail: sidorovskaya@arriah.ru*
[2] *ORCID 0000-0002-2122-9096, e-mail: fomina@arriah.ru*
[3] *ORCID 0000-0002-8634-8205, e-mail: kremenchugskaya@arriah.ru*

SUMMARY
Data on FMD spread in Africa in 2017–2019 provided by the World Animal Health Organization (OIE) and World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) were analyzed with the emphasis on the current epidemic situation in Northern Africa, and the analysis results are demonstrated as well. Brief historical information on SAT-1, -2, -3 FMDV recovery in Africa is presented. Diagnostic test results demonstrate that the abovementioned virus serotypes are circulating in the Southern, East and West African countries. However, there are reports on detection of SAT-1 FMDV in the Near East (1961–1965 and 1970) and SAT-2 FMDV in Saudi Arabia (2000), Lebanon (2003), Bahrain, Egypt and Lebanon (2012). Infection of cattle with SAT-1, -2, -3 FMDV in Southern and East Africa is associated with the contacts between the domestic livestock and wild cloven-hoofed ungulates, specifically with African buffaloes (*Syncerus caffer*). FMDV persists in buffaloes for up to 4–5 years and in buffalo herds living within the limited area of the national reserves – for up to 24 years. Buffaloes are considered to be natural reservoir of the virus. The basic disease control measure in Africa is prevention of any contacts between FMD susceptible livestock and buffaloes in the national reserves and game sanctuaries. Moreover, crucial component of FMD prevention is vaccination of bovines kept in buffer zones around the wild cloven-hoofed ungulates' habitats against the virus serotypes spread by the latter. Foot-and-mouth disease remains one of the most economically significant infections in the world and it involves losses due to the decrease of the agricultural production as well as due to the international trade restrictions.

Keywords: Foot-and-mouth disease, serotypes, topotypes, Africa, natural reservoir.

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For correspondence: Miroslava V. Sidorovskaya, Post-Graduate Student, Researcher, Reference Laboratory for FMD Diagnosis, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: sidorovskaya@arriah.ru.*

УДК 619:616.98:578.835.2:616-036.22(6)

Анализ вспышек ящура серотипов SAT-1, -2, -3 на территории Африканского континента за 2017–2019 гг.

М. В. Сидоровская[1], С. Н. Фомина[2], С. Р. Кременчугская[3]
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия
[1] *ORCID 0000-0001-6218-5846, e-mail: sidorovskaya@arriah.ru*
[2] *ORCID 0000-0002-2122-9096, e-mail: fomina@arriah.ru*
[3] *ORCID 0000-0002-8634-8205, e-mail: kremenchugskaya@arriah.ru*

РЕЗЮМЕ
Представлены результаты анализа данных Всемирной организации здравоохранения животных (МЭБ) и Всемирной референтной лаборатории по ящуру (WRLFMD) о распространении ящура на территории Африканского континента в 2017–2019 гг. с акцентом на современную эпизоотическую ситуацию в Северной Африке. Дана краткая историческая справка об открытии серотипов SAT-1, -2, -3 вируса ящура в Африке. Как показывают результаты диагностических исследований, указанные серотипы циркулируют в странах Южной, Восточной и Западной Африки. Однако имеются сообщения об обнаружении серотипа SAT-1 на Ближнем Востоке (1961–1965 и 1970 гг.) и SAT-2 в Саудовской Аравии (2000 г.), Ливии (2003 г.), Бахрейне, Египте и Ливии (2012 г.). Заболеваемость крупного рогатого скота ящуром серотипов SAT-1, -2, -3 в Южной и Восточной Африке обусловлена контактом домашнего скота с дикими парнокопытными, в частности с африканским буйволом (*Syncerus caffer*). Вирус ящура персистирует в организме буйволов до 4–5 лет, а в стадах буйволов, обитающих на ограниченных территориях национальных парков, – до 24 лет. Буйволы считаются естественным резервуаром вируса. Основной мерой борьбы с заболеванием на Африканском континенте является пресечение контактов восприимчивого поголовья скота с буйволами в национальных парках и охотничьих заповедниках. Кроме того, важным аспектом профилактики ящура является вакцинация крупного рогатого скота, находящегося

в буферных зонах вокруг ареалов обитания диких парнокопытных, против тех серотипов, которые переносятся ими. Ящур остается одной из наиболее экономически значимых инфекций животных в мире, при которой убытки складываются за счет снижения уровня производства сельскохозяйственной продукции и ограничения международной торговли.

Ключевые слова: Ящур, серотипы, топотипы, Африка, естественный резервуар.

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Для корреспонденции: Сидоровская Мирослава Владимировна, аспирант, сотрудник референтной лаборатории диагностики ящура ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: sidorovskaya@arriah.ru.*

INTRODUCTION

Foot-and-mouth disease (*Aphtae epizooticae*) was first reported in the XVI century and until now it remains one of the predominant vesicular diseases of the cloven-hoofed ungulates that causes losses to national economies and requires substantial investment in the prevention and management of disease outbreak consequences. In spite of the Progressive Control Pathway for Foot-and-Mouth Disease developed by the Food and Agriculture Organization (FAO) and European Commission for the Control of Foot-and-Mouth Disease (EuFMD), the disease is still reported in many countries of the world. The above mentioned document is the basis of the FAO/OIE Global Foot-and-Mouth Disease Control Strategy, and it allows FAO/OIE to approve national FMD control program developed by a country, which should include a number of progressive steps, *inter alia* a schedule of the susceptible animal vaccination. Continent of Africa is of particular significance as the majority of the disease outbreaks are being reported there. According to the World Animal Health Organization (OIE), from 2017 to 2019, FMD was reported in 41 African countries. The map in Figure 1 demonstrates global FMD epidemic situation as of late 2019, and it clearly illustrates that African continent leads in the number of affected countries. During the above mentioned period, there were 55 FMD affected countries in the world, of these 33 countries were African ones, 23 countries were Asian countries and only two countries were the European ones (Russian Federation, Turkey).

Sporadic cases of the infection have been reported in Africa for many decades. Figure 2 shows FMD epidemic situation in Africa in 2017–2019. Studies of the virus that caused the disease in the susceptible animal population demonstrated that serotype O virus prevailed over other FMDV serotypes (Fig. 3). The proportion of the registered SAT-1, -2, -3 viruses amounted, however, to 29% of the total number of FMD cases detected in the region. Therefore, this fact should deserve specific attention during the examination of the FMD outbreaks in Africa. Moreover, during the study period, the virus remained untyped in 15% of cases of FMD detections in cattle.

Due to the increased risk of exotic isolate introduction into the Russian Federation associated with the intensification of the trade relations with North African countries as well as due to the threat of the SAT FMD virus introduction from FMD affected African regions to the Near Eastern and West Asian countries and their further spread into the neighboring countries, the works were aimed at the detailed study of SAT FMD epidemic situation in Africa.

History of SAT-1, -2, -3 FMDV detection in Africa

The disease was continuously reported in Southern African countries back to the colonial era but only in the first half of the XX century, while studying FMDV, through the successive cross-protection tests in guinea pigs and cattle the researchers managed to determine its type difference. Development and implementation of such method as compliment fixation test allowed for further studies aimed at the FMD agent typing. As soon as such diagnostic tool as polymerase chain reaction appeared, molecular examination of the virus was made possible [1, 2].

SAT-1, -2 and -3 foot-and-mouth disease viruses were first isolated by the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD; Pirbright, Great Britain) from the samples collected from the livestock animals in Bechuanaland (Botswana) and Northern Rhodesia (Zambia) in 1948. Retrospective studies of the viruses isolated earlier in 1931 and 1937 in Southern Rhodesia demonstrated close relatedness to the isolates recovered in 1948. One more virus isolate from Southern Rhodesia appeared to be the third new serotype. The discovered serotypes were hereafter named as South Africa Territories 1, 2 and 3 (abbreviated as SAT-1, SAT-2 and SAT-3). The data on the above mentioned serotypes were published by J. B. Brooksby [1].

According to the results obtained by F. Duchatel et al. [3] during sequencing-based phylogenetic studies, SAT-1 and SAT-2 FMDV serotypes have been circulating in African wildlife for over 400 years. The scientists reconstructed the evolution of various FMDV serotypes including SAT-1 and -2 starting from the XVI century and until 2016, and they also evaluated potential influence of ecological and anthropological factors on their spread. Results of the studies were demonstrated as phylogenetic trees [3, 4].

Current FMD epidemic situation in Africa (SAT-1, -2, -3)

Spread of SAT-1 FMDV

SAT-1 virus serotype is widely spread in the countries in Sub-Saharan Africa (SSA). Singular disease outbreaks caused by the serotype were however reported on the

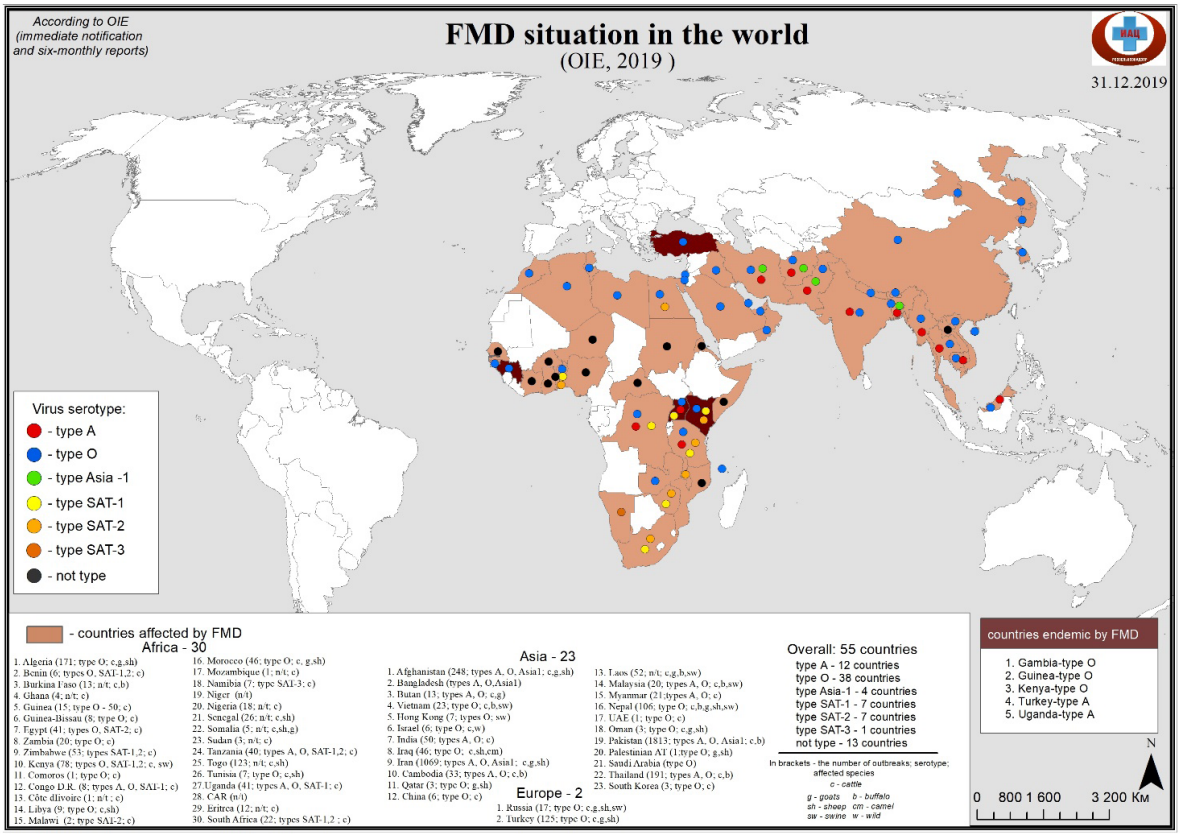

Fig. 1. Global FMD epidemic situation in 2019 (the map is made by the experts of the Information Analysis Center, FGBI "ARRIAH")

Рис. 1. Эпизоотическая ситуация в мире по ящуру в 2019 г. (карта подготовлена сотрудниками информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

Near East in 1962–1965 and 1969–1970, and then in Greece in 1962 [5]. The serotype includes 13 topotypes currently numbered with Roman numerals I–XIII. Topotype I is also known as Northwest Zimbabwe (NWZ), topotype II – Southeast Zimbabwe (SEZ), topotype III – Western Zimbabwe (WZ), topotype IV – East Africa 1 (EA-1), topotype VII – East Africa 2 (EA-2) and topotype VIII – East Africa 3 (EA-3) [6].

SAT-1 FMDV genome was detected in samples collected from cattle in Kenia in 2017. Sequencing of VP1 structural protein demonstrated that this serotype belonged to topotype I that had never occurred in the region before. FMD virus of SAT-1 serotype and topotype I was also reported in Malawi. In August and October, 2017, six SAT-1 FMD outbreaks were reported in cattle in Greater Giyani municipality, Limpopo province (RSA) [7, 8].

In early 2018, sixty-two disease outbreaks were reported in eastern and western provinces of Zimbabwe; by July the outbreaks occurred in north-east part of the country – close to the border with Mozambique. It should be noted the FMD outbreaks are rare in this region. By September 2018, there were over 100 outbreaks caused by SAT-1 FMDV. The new outbreaks were reported in Midlands and Masvingo Provinces [9, 10].

In January 2019, two FMD outbreaks (SAT-1) were again reported in cattle in Masvingo and Matabeleland Provinces, Zimbabwe [11]. During 2019, over twenty SAT-1 FMD outbreaks were reported in Masvingo Province [12]. In the third quarter, same year, SAT-1 FMD virus was isolated in Cameroon, the disease caused by this virus serotype was most recently reported in the North and Adamawa Regions of the country in August – September, 2016. The phylogenetic analysis of the recovered isolates demonstrated that they belonged to topotype X, and they were phylogenetically related to the virus isolated in Nigeria, 2015–2016 [13].

Spread of SAT-2 FMDV

This serotype includes 14 topotypes numbered with Roman numerals I–XIV [6]. Out of the three SAT serotypes SAT-2 foot-and-mouth disease virus also prevails in Sub-Saharan Africa and thus it is sufficiently researched. The outbreaks associated with this FMDV serotype were however reported in north-eastern Africa and Near East: in Yemen in 1990, in Kuwait and Saudi Arabia in 2000. In 2012, SAT-2 FMDV were reported in Egypt, Libya and Palestine. The virus of this serotype was also isolated in Bahrain [5]. Forty-three outbreaks caused by this virus serotype were reported in fourteen Egyptian provinces from February to March, 2012. Studies of VP1 gene nucleotide sequence by the WRLFMD demonstrated that the recovered isolates belonged to topotype VII and two genetic lineages Ghb-12 and Alx-12, which had been previously widely spread. SAT-2 FMDV-associated epidemic in Egypt was its first occurrence in the country from 1950. The phylogenetic analysis of the isolates that caused FMD outbreaks in Palestine (Gaza Strip) in April 2012 indicated that the virus belonged to topotype VII of Ghb-12 genetic lineage and was related to the Egyptian isolates. Again in February 2012, SAT-2 FMDV isolates belonging to topotype VII of Lib-12 genetic lineage were recovered in Libya. In March – April that year SAT-2 FMD virus of topotype IV and genetic lineage Ken-09

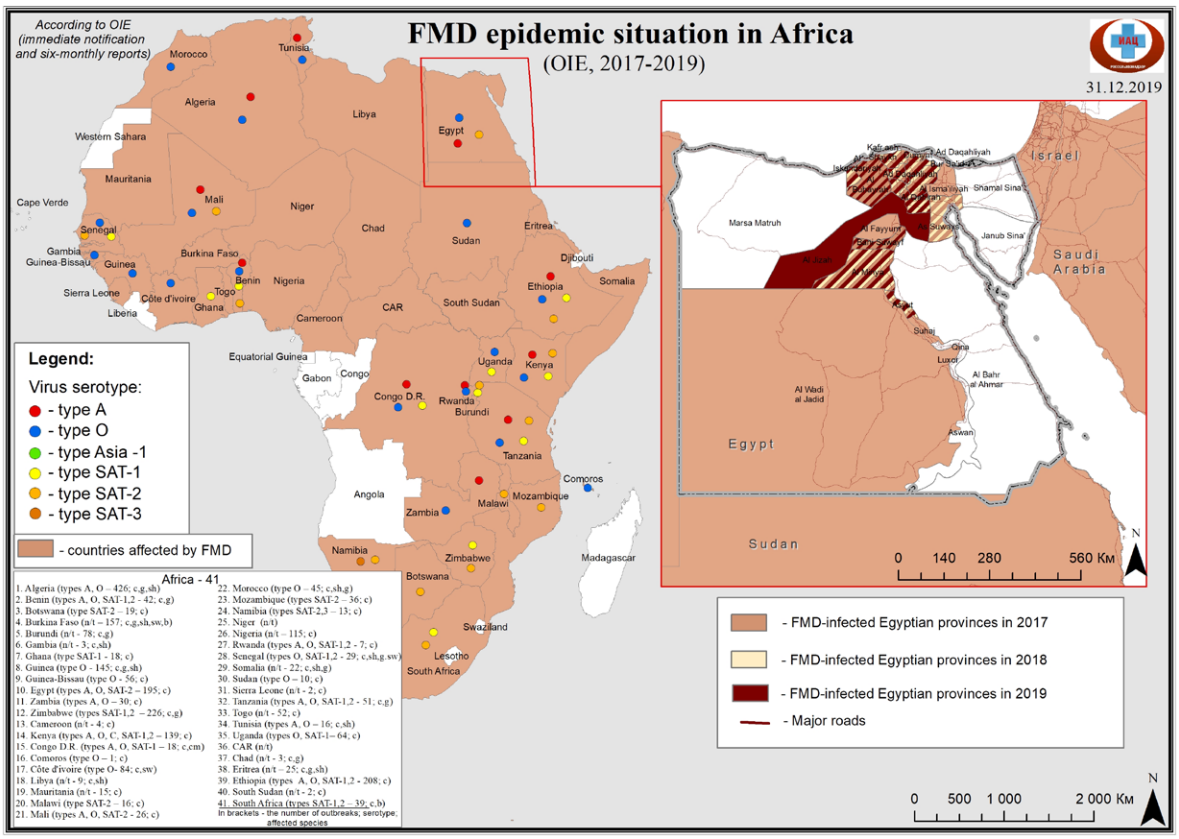

Fig. 2. FMD epidemic situation in Africa in 2017–2019 (the map is made by the experts of the Information Analysis Center, FGBI "ARRIAH")

Рис. 2. Эпизоотическая ситуация в Африке по ящуру в 2017–2019 гг. (карта подготовлена сотрудниками информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

was isolated during the disease outbreak in imported livestock in the northern part of the Kingdom of Bahrain. This was the first emergence of this serotype in the country [14].

In January 2017, SAT-2 FMDV was reported in Matabeleland North and Midlands Provinces, Zimbabwe. The epidemic lasted until March 2017, and five outbreaks were reported in cattle. FMD outbreaks were registered in the same provinces in May. No data of the virus genotyping were reported. Again in March, singular SAT-2 FMD outbreak was reported in cattle in Bushbuckridge municipality, Mpumalanga Province, RSA. It should be noted that the above mentioned outbreaks were detected near Kruger National Park [15, 16].

In February – July 2017, an FMD outbreak was reported in Namanyane settlement, Ngamiland District, Botswana. The OIE Sub-Saharan Africa Regional Reference Laboratory (SSARRL, Botswana) identified SAT-2 virus and determined genetic sequence of capsid VP1 protein of the virus. The analysis of the isolate performed by the WRLFMD demonstrated that it belonged to topotype III and was closely related to the viruses isolated from cattle in Botswana in 2015 [7].

Five outbreaks caused by SAT-2 FMDV were reported in cattle in Namibia from July to September, 2017. The outbreaks were located near Katima Mulilo Urban, Zambezi Region. Topotype VII SAT-2 FMDV was also registered in Uganda [7].

In October – December 2017, topotype III SAT-2 virus was detected in Botswana, Mozambique, Namibia, and topotype II SAT-2 virus was isolated in Zimbabwe [17].

Serotyping and genotyping results in case of FMDV isolated in Ethiopia in March 2018 demonstrated that the virus belonged to serotype SAT-2, topotype VII of the genetic lineage Ghb-12 [9].

In May 2018, singular SAT-2 FMD outbreak was reported in Thulamela settlement, Limpopo Province (RSA). No genotyping was performed [9].

From June to August 2018, topotype III SAT-2 FMDV continued its spread in cattle in Botswana. In total, seventeen outbreaks were detected in North-West District of the country [10].

In August 2018, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) published the results of the phylogenetic examination of the virus isolated from the bovine sample in Koundjourou sub-prefecture, Republic of Chad in December 2016. The published data demonstrated that the isolate belonged to serotype SAT-2, topotype VII, genetic lineage Lib-12 [10].

From May to August 2018, fourteen SAT-2 FMD outbreaks were reported in cattle in Southern (Neno) and Central (Ntcheu, Dedza and Lilongwe) [9, 10] provinces of Malawi.

In July 2018, diagnostic testing of 39 samples collected from bovines and submitted to the WRLFMD from Sudan demonstrated SAT-2 FMDV in five of them. Genotyping revealed that the virus belonged to topotype VII, genetic lineage Alx-12, while genome of topotype IV SAT-2 FMDV was isolated from the samples submitted from Kenia and collected during the period from January 2017 to June 2018 [10].

In late 2018, three new SAT-2 FMDV outbreaks were reported in cattle in the RSA (Limpopo Province). No genotyping results were published. Sub-clinical infection was detected in buffalo population in Maruleng Local Municipality, Limpopo Province [18].

Throughout 2019, SAT-2 FMDV outbreaks were still reported in Malawi, Zambia, Zimbabwe and Nigeria [11–13, 19].

In 2019, the WRLFMD tested samples collected in the Arab Republic of Egypt from January 2017 to November 2018. FMD agent of SAT-2 subtype was detected in the samples and its subsequent genotyping demonstrated that the virus belonged to topotype VII of genetic lineages Ghb-12 and Lib-12 [11].

In January 2019, five more SAT-2 FMD outbreaks occurred in cattle population in the Limpopo Province, RSA [11].

In February that year, an FMD outbreak of the mentioned serotype was reported in cattle in the Northern Region of Malawi. Phylogenetic tests demonstrated that the virus belonged to topotype I and was related to the virus previously reported in Zambia [19].

In March – April, 2019, eight SAT-2 FMD outbreaks were reported in Mashonaland East Province and fourteen outbreaks were registered in Mashonaland Central Province, Zimbabwe [11, 19].

Twelve samples collected from cattle during the period from January to April 2019 were tested by the WRLFMD, and FMD viruses of SAT-2 serotype, topotype I were identified in four of them [19]. Another SAT-2 FMD outbreak was also reported in cattle in Zambia-bordering region in Malawi in 2019 [13].

Three more FMD outbreaks were reported in the Eastern Province of Zambia in the second quarter of 2019. Testing of the recovered isolates demonstrated circulation of FMDV of serotype SAT-2, topotype I in this locality [13].

In December 2019, the Canadian Food Inspection Agency (CFIA/ACIA) in cooperation with the National Veterinary Research Institute of Nigeria (NVRI) obtained the genome sequence of the foot-and-mouth disease virus isolated from the samples collected from cattle in Plateau and Bauchi States of Nigeria in 2017–2018. SAT-2 FMDV was detected in eight samples. Sequencing of VP1 structural protein demonstrated that the virus belonged to topotype VII of genetic lineage Lib-12 [12].

In September 2019, singular SAT-2 FMD outbreak was again reported in Mashonaland Central Province, Zimbabwe. A series of fifteen SAT-2 FMD outbreaks was reported in cattle in the Limpopo Province (RSA) from November to December that year. One should emphasize that the epidemics occurred in the national buffer zone [12].

Spread of SAT-3 FMDV

Out of three SAT serotypes of the FMDV, SAT-3 has relatively small number of topotypes and the most limited prevalence. To date, five different topotypes were identified and numbered with Roman numerals I–V. The tested viruses were received from seven countries: RSA, Zimbabwe, Zambia, Namibia, Botswana, Malawi and Uganda [6, 20].

SAT-3 is one of the less studied virus serotypes. In 16 years after the last outbreak of this serotype FMDV that occurred in Kruger National Park in Limpopo Province (RSA) in 2006, the SAT-3 FMDV was isolated from clinically healthy Ankole Longhorn calf that had contact with wild buffaloes on the grasslands located in the Queen Elizabeth National Park in Uganda. According to VP1-coding nucleotide sequence, the emerged virus strain was approximately 20% different from the related isolates previously recovered from the buffaloes in Uganda in 1997. African buffaloes are considered to play an important role in the FMDV circulation maintenance in the National parks in Uganda, but no large-scale monitoring in wild animal population is carried out [21–23].

Over the study period (2017–2019), SAT-3 FMD outbreaks were reported in Zambia, Mozambique and Namibia.

Testing of a sample collected from cattle in Lukulu District (Western Province, Zambia) in May 2017 revealed topotype II SAT-3 virus and demonstrated its close phylogenetic relatedness to the isolates recovered in this area in 2015 [17].

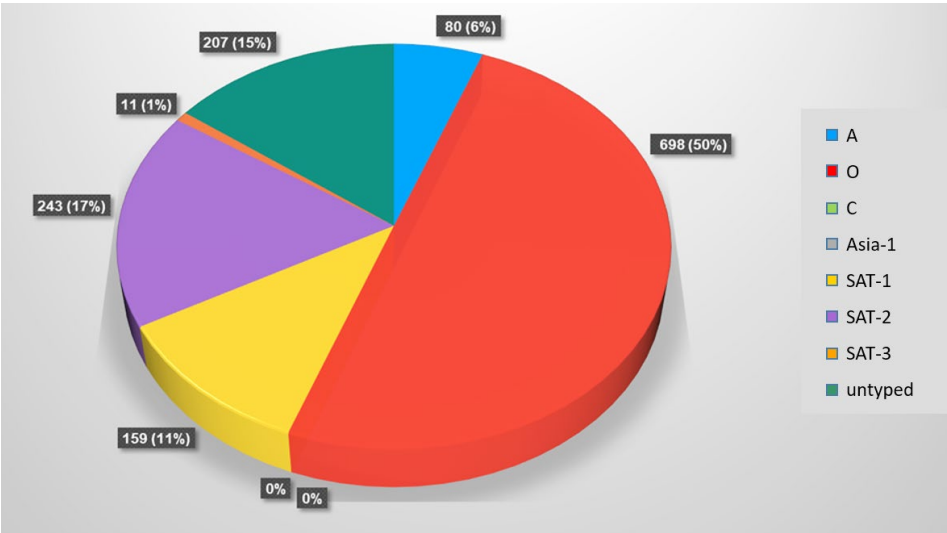

Fig. 3. Proportion of different FMDV serotypes detected in Africa in 2017–2019 (according to the OIE/FAO WRLFMD quarterly reports)

Рис. 3. Соотношение случаев обнаружения ящура разных серотипов на Африканском континенте в 2017–2019 гг. (по данным квартальных отчетов WRLFMD МЭБ/ФАО)

In December 2017, an FMD outbreak was reported in cattle in Chicualacuala District (Gaza Province, Mozambique). As the SSARRL stated (Botswana), the outbreak was caused by SAT-3 FMDV. Genotyping demonstrated that the virus belonged to topotype I and it was phylogenetically different from other viruses within this topotype [8].

In July 2019, two SAT-3 FMDV outbreaks were reported in cattle in Katima Mulilo Urban (Zambezi Region, Namibia). Data on VP1 nucleotide sequencing were received in August 2019. Further genetic tests demonstrated that the virus belonged to topotype II [13]. During the period from October to December that year, five more outbreaks of topotype II SAT-3 FMD were reported in cattle in this region [12].

WRLFMD quarterly reports show that SAT-1, -2, -3 FMD viruses of different topotypes circulate in the African countries.

The following FMDV serotypes prevail in the Eastern Africa (Ethiopia, Kenia, Sudan and Uganda): SAT-1 of topotype I, SAT-2 of topotype IV and SAT-2 of topotype VII, genetic lineages Alx-12 and Ghb-12.

The following FMDV serotypes are relevant for Western African countries (Cameroon, Republic of Chad, Nigeria): SAT-1 of topotype X, SAT-2 of topotype VII; genetic lineage Lib-12.

In the Southern African countries (RSA, Zimbabwe, Botswana, Malawi, Namibia, Mozambique, Zambia) the following FMDV serotypes are being reported: SAT-1 of topotype I; SAT-2 of topotypes I, II, III; SAT-3 of topotypes I and II.

Since 2012, topotype VII SAT-2 FMDV of genetic lineages Lib-12 and Ghb-12 have been reported in Northern Africa, specifically in Egypt and Libya.

All three serotypes of SAT FMDV circulate only in Southern Africa and infect ruminant cloven-hoofed animals. FMD outbreaks in the countries of the Southern, Eastern and Western Africa are associated with the close contacts between the domestic livestock and wild cloven-hoofed animals during grazing, in particular with African buffalo and impala (*Aepyceros melampus*) [3]. The unique trait of FMD epidemiology in Africa involves continuous maintenance of all three SAT-serotypes in African buffalo population. When crowded these animals serve as potential source of sporadic infection for livestock and other FMDV susceptible animal species. The precise mechanism of FMD transmission from buffalo to cattle is understudied, and according to F. Maree et al., it is facilitated by direct contact between these two species [23]. Natural FMD outbreak has been formed on the African continent, and buffaloes serve as a natural reservoir of the disease agent for the domestic and wild animals. This is due to the virus persistence in wild cloven-hoofed animals as in some animals the virus is maintained for up to 4–5 years and for up to 24 years in small isolated herds (30–100 animals) [24].

A. N. Burdov et al. [24] defined four factors of FMD spread in Africa associated with the animal movements:

1. Livestock animals are moved from Niger, Chad, Northern Region of Nigeria and Mali to be sold in municipal centers along the Atlantic coast and from North-Eastern Africa to be sold in the Near East.

2. Livestock movements associated with the use of community pastures, barter relations and normadism.

3. In the Western Africa, humans and livestock animals migrate in large numbers to the south during the annual drought seasons (January – February) and back to the north as soon as monsoon season starts.

4. Natural migration of wild animals in the East-African and South-African plateaus.

In spite of FMD control measures taken by a number of African countries the situation on the continent is still unstable [25].

The majority of the disease outbreaks are reported in Eastern and Western Africa, but the serotypes prevailing in the Southern Africa tend to spread deep into the continent. Data of the WRLFMD reports suggest SAT-2 FMD spread to Northern Africa. Detailed information on FMDV outbreaks in the Northern Africa in 2017–2019 are shown in Figure 4. However noteworthy is the fact that not all outbreaks are typed. This fact significantly aggravate FMD control on the continent.

According to the data published by I. McLachlan et al., many livestock owners in the African countries face annual FMD outbreaks. Grave economic losses are due to the declined production of agricultural products and loss of draft cattle. Basic activities of the private and backyard farms involve keeping and breeding of livestock for livelihood and food security purposes. In the developing countries with low and medium income the loss of profit in agriculture results in decline of health, education and meal expenses. Livestock is also an integral part of social status and cultural identity of the population in the majority of the African countries. Therefore, further measures should be taken in order to intensify FMD control in enzootic regions of Africa [26].

CONCLUSION

Current SAT-1, -2, -3 FMD epidemic situation in the African continent indicates that SAT-2 FMD continues its spread towards Northern Africa and poses real threat of introduction to the Near East and Western Asia. The disease spread is accompanied with the steadily intensified trade relations between Northern African countries, in particular, Egypt, Tunisia, Algeria, and Northern Asia as well as Russian Federation. This fact encourages the interest in thorough study of the specific features of epidemic FMDV isolates in Africa, as they are genetically different from the strains previously detected in our and neighboring countries.

SAT-1, -2, -3 FMDV demonstrate significant infectivity and they can infect different animal species in mixed populations not only in the African countries but generally outside the continent.

The fact that African buffaloes and impalas serve as natural reservoirs of all three SAT serotypes of the foot-and-mouth disease virus should be also considered. The virus can persist in wild cloven-hoofed animals for many years. This explains the existence and maintenance of the persisting FMD natural outbreak in Africa and, therefore, the threat of new outbreak occurrence is still high.

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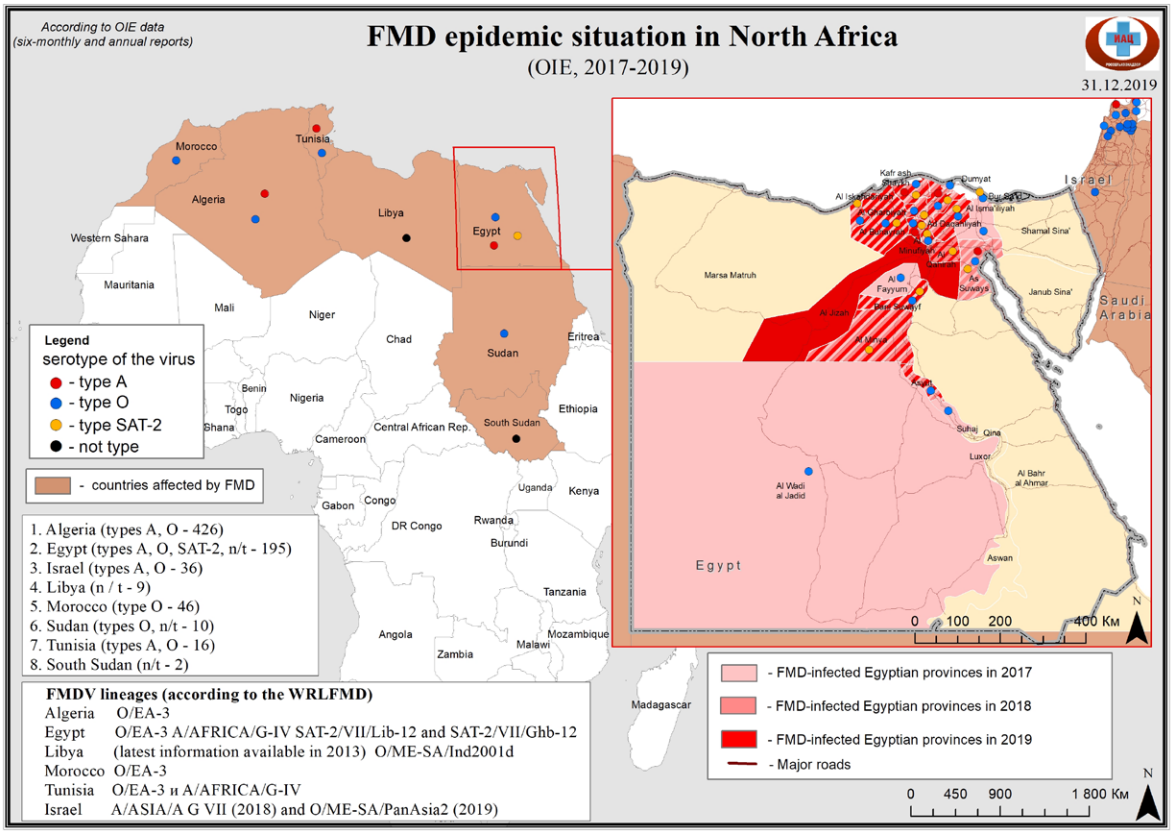

Fig. 4. FMD epidemic situation in North Africa in 2017–2019 (the map is made by the experts of the Information Analysis Center, FGBI "ARRIAH")

Рис. 4. Эпизоотическая ситуация в Северной Африке по ящуру в 2017–2019 гг. (карта подготовлена сотрудниками информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Miroslava V. Sidorovskaya, Post-Graduate Student, Researcher, Reference Laboratory for FMD Diagnosis, FGBI "ARRIAH", Vladimir, Russia.

Svetlana N. Fomina, Candidate of Science (Veterinary Medicine), Head of Reference Laboratory for FMD Diagnosis, FGBI "ARRIAH", Vladimir, Russia.

Svetlana R. Kremenchugskaya, Doctor of Science (Veterinary Medicine), Leading Researcher, Reference Laboratory for FMD Diagnosis, FGBI "ARRIAH", Vladimir, Russia.

Сидоровская Мирослава Владимировна, аспирант, сотрудник референтной лаборатории диагностики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Фомина Светлана Николаевна, кандидат ветеринарных наук, заведующий референтной лабораторией диагностики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Кременчугская Светлана Ревдитовна, доктор ветеринарных наук, ведущий научный сотрудник референтной лаборатории диагностики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

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Studying dynamic changes in body mass and mass of internal organs in laboratory rats experimentally infected with bovine leukosis virus

E. S. Krasnikova[1], R. V. Radionov[2], A. V. Krasnikov[3], A. Yu. Svetozarova[4]

[1,3,4] FSBEI HE "Michurinsk State Agrarian University" (FSBEI HE Michurinsk SAU), Michurinsk, Russia

[2] FSBEU HE "Saratov State Agrarian University named after N. I. Vavilov" (FSBEU HE Saratov SAU), Saratov, Russia

[1] *ORCID 0000-0003-4395-5862, e-mail: krasnikovaes77@yandex.ru*

[2] *ORCID 0000-0002-8586-2691, e-mail: roman5875@mail.ru*

[3] *ORCID 0000-0002-4127-8725, e-mail: krasnikov.77@mail.ru*

[4] *ORCID 0000-0002-3412-527X, e-mail: ana.svetozarova@yandex.ru*

SUMMARY

Enzootic bovine leukosis has been an urgent problem of veterinary medicine both in the Russian Federation and abroad for more than a hundred years. A number of aspects have been studied quite deeply; however, there are still areas that require additional research. These include the development of a fully adequate laboratory model for reproducing bovine leukosis virus (BLV) infection. Preliminary studies have established that BLV infection of laboratory rats is accompanied by clinical, morphological and biochemical changes in the blood, signs of immune suppression, impaired immunological reactivity of the body, and morphofunctional changes in the immunocompetent cells that correlate with bovine leukosis. In this regard, it is of interest to analyze disorders caused by these dysfunctions; the disorders are demonstrated by changed morphometric characteristics of both the body and individual organs. The aim of the research was to study dynamic changes in body mass and mass of internal organs in laboratory rats experimentally infected with BLV. There was a clear body mass increase in BLV-infected laboratory rats, then followed by a decrease down to negative numbers. The reverse trend was observed for such internal organs of the experimental animals as liver, spleen, kidneys and lungs. At first, their relative mass decreased to some extent, then increased with different dynamics in groups. The heart was the exception, as its relative mass decreased and did not increase until the end of the experiment. The data obtained correlate with those provided by a number of authors that the relative mass of various organs changes in the BLV infected animals because of proliferative, inflammatory, dystrophic and atrophic processes.

Keywords: Rats, enzootic bovine leukosis, relative mass, internal organs, average daily mass gain, relative mass gain.

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For correspondence: Ekaterina S. Krasnikova, Doctor of Science (Veterinary Medicine), Associate Professor, Professor, Department of Animal Science and Veterinary Medicine, FSBEI HE Michurinsk SAU, 393760, Russia, Tambov Oblast, Michurinsk, Internatsyonalnaya str., 101, *e-mail: krasnikovaes77@yandex.ru.*

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Изучение динамики массы тела и внутренних органов лабораторных крыс при экспериментальной инфекции вирусом лейкоза крупного рогатого скота

Е. С. Красникова[1], Р. В. Радионов[2], А. В. Красников[3], А. Ю. Светозарова[4]

[1,3,4] ФГБОУ ВО «Мичуринский государственный аграрный университет» (ФГБОУ ВО Мичуринский ГАУ), г. Мичуринск, Россия

[2] ФГБОУ ВО «Саратовский государственный аграрный университет имени Н. И. Вавилова» (ФГБОУ ВО Саратовский ГАУ), г. Саратов, Россия

[1] *ORCID 0000-0003-4395-5862, e-mail: krasnikovaes77@yandex.ru*

[2] *ORCID 0000-0002-8586-2691, e-mail: roman5875@mail.ru*

[3] *ORCID 0000-0002-4127-8725, e-mail: krasnikov.77@mail.ru*

[4] *ORCID 0000-0002-3412-527X, e-mail: ana.svetozarova@yandex.ru*

РЕЗЮМЕ

Энзоотический лейкоз крупного рогатого скота уже более ста лет является актуальной проблемой ветеринарной медицины как в Российской Федерации, так и за рубежом. Ряд аспектов изучен достаточно глубоко, но есть и такие, что требуют проведения дополнительных исследований. К их числу относится разработка адекватной во всех отношениях лабораторной модели для воспроизведения инфекции, вызванной вирусом лейкоза крупного рогатого скота (BLV-инфекция). Предварительными исследованиями было установлено, что BLV-инфекция лабораторных крыс сопровождается коррелирующими с лейкозом у крупного рогатого скота клинико-морфологическими и биохимическими изменениями в крови, признаками иммунной супрессии, нарушением иммунологической реактивности организма и морфофункциональными изменениями на уровне иммунокомпетентных клеток. В этой связи интерес представляет анализ провоцируемых данными дисфункциями нарушений, находящих свое отражение в изменении морфометрических характеристик как всего организма, так и отдельных органов. Целью исследований стало изучение динамики массы тела и внутренних органов лабораторных крыс при экспериментальной BLV-инфекции. Динамика весовых показателей тела BLV-инфицированных лабораторных крыс характеризовалась выраженной тенденцией к их увеличению с последующим снижением вплоть до отрицательных значений. Обратная тенденция была отмечена для таких внутренних органов экспериментальных животных, как печень, селезенка, почки и легкие. Сначала их относительная масса в той или иной степени снижалась, затем увеличивалась с разной динамикой по группам. Исключение составило сердце, относительная масса которого снизилась и не увеличивалась до окончания эксперимента. Полученные данные коррелируют с мнением ряда авторов, что при BLV-инфекции относительная масса различных органов изменяется в результате пролиферативных, воспалительных, дистрофических и атрофических процессов.

Ключевые слова: Крысы, энзоотический лейкоз крупного рогатого скота, относительная масса, внутренние органы, среднесуточный привес, относительный привес.

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Для корреспонденции: Красникова Екатерина Сергеевна, доктор ветеринарных наук, доцент, профессор кафедры зоотехнии и ветеринарии ФГБОУ ВО Мичуринский ГАУ, 393760, Россия, Тамбовская обл., г. Мичуринск, ул. Интернациональная, д. 101, *e-mail: krasnikovaes77@yandex.ru.*

INTRODUCTION

Enzootic bovine leukosis (EBL) – a widespread disease, especially often detected in highly productive dairy cows [1]. Cattle, sheep, and rabbits are used as models to study pathological processes in the experimentally infected animals [2]. In the previous studies, Wistar laboratory rats demonstrated susceptibility to oral and parenteral infection with EBL [3, 4]. Since it was found that laboratory animals infected with bovine leukosis virus (BLV) demonstrate clinical, morphological and biochemical changes in blood that correlate with enzootic bovine leukosis [3], signs of immune suppression and impaired immunological reactivity of the body [4], as well as morphofunctional changes at the level of immunocompetent cells [5], Wistar rats can be considered as a new laboratory model for studying BLV *in vivo*. Such conclusions require a correlation (at the level of disease pathogenesis) between laboratory and naturally susceptible animals. Therefore, it is of particular interest to analyze the disorders caused by these dysfunctions, i.e. those disorders that are demonstrated by changed morphometric characteristics of both the body and individual organs.

In this regard, the aim of the research was to study dynamic changes in body mass and mass of internal organs of laboratory rats experimentally infected with BLV.

MATERIALS AND METHODS

Wistar rats (n = 60) divided into three equal groups were used as an object of the study. The rats had an adequate diet and daily received plenty of fresh milk from the BLV-infected and diseased cows (as reported by the state veterinary service), originating from collective farm "Zarya" located in the Tamalinsky District of the Penza Oblast. Rats of Group One (I) were fed on milk from intact cows, Group Two (II) was fed on milk from BLV-infected cows and Group Three (III) was fed on milk from the cows with clinical form of bovine leukosis. The animals of each group were divided into 2 subgroups: *a* – included adult rats, *b* – included rat pups. The rat pups born during the experiment were separated from their mothers after they started self-feeding. Polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) were used to follow the dynamics of BLV infection in experimental rats 3, 6, 9 and 12 months after the experiment started.

Blood was aspirated from the lateral caudal vein into vacuum tubes with aEDTA-K3 stabilizer (ethylenediaminetetraacetic acid) and into test tubes with clot activator (PUTH, China). For PCR diagnostics the following reagent kits were used: "DNA-sorb-B", "LEUKEMIA" and "EF" (Federal State Budgetary Institution of the Central Research Institute of Epidemiology of the Rospotrebnadzor,

Table
Dynamics of BLV-infection in rats of the experimental group

Таблица
Динамика развития BLV-инфекции у крыс экспериментальных групп

Test dates	Groups and subgroups of animals											
	I*a*		I*b*		II*a*		II*b*		III*a*		III*b*	
	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
3 months after	–	–	–	–	+	+	–	+	+	–	X	X
6 months after	–	–	–	–	+	+	+	+	+	+	+	+
9 months after	–	–	–	–	+	+	+	+	+	+	+	+
12 months after	–	–	–	–	+	+	+	+	+	+	+	+

"–" – negative result (отрицательный результат);
"+" – positive result (положительный результат);
"X" – not tested (исследования не проводились).

Russia); the PCR procedure was held and the tests result were read in Bio-Rad Laboratories equipment, Inc. (USA). Serological tests were performed using a "BLV antibody ELISA kit for serum and milk (option No. 1 – screening)" produced by the FKP "Kursk Biofactory – Firm BIOK" (Russia) using Multiskan equipment (Thermo Scientific, USA). To confirm BLV infection in experimental rats, presence ("+") or absence ("–") of proviral DNA and/or antibodies were taken into account as qualitative indicators. Animals that were positive for at least one of the indicators were used in the experiment, and negatively reacting rats were culled.

Five rats from each group were subjected to euthanasia and autopsy within the established time limits. The animals were euthanized by cervical dislocation after diethyl ether anesthesia.

All the experiments on animals were carried out strictly in accordance with the Interstate guidelines for accommodation and care of laboratory animals GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22.09.2010 on the protection of animals used for scientific purposes.

Parenchymal organs of animals: kidneys, liver, spleen, heart and lungs were used as materials for morphometric studies. Electronic scales JW-1 (*e* = 0.02 g) manufactured by Acom Inc. (South Korea) were used to weigh rats and their internal organs.

RESULTS AND DISCUSSION

The results of the serological, molecular, and genetic tests presented in the table indicate positive dynamics of the infectious process in experimental animals, i.e. at least one of the diagnostic tests gave a positive result for the group. This was most likely due to biological characteristics of the infection causative agent and by the peculiarities of the disease pathogenesis. The animals of the control group remained intact throughout the whole experiment.

The data on the body mass of experimental animals indicates that the positive trend reported at the beginning of

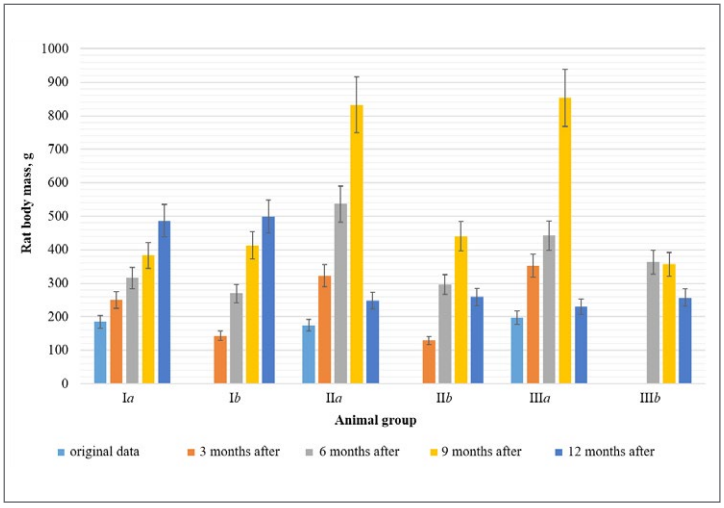

Fig. 1. Dynamic changes in animal body mass

Рис. 1. Динамика изменения массы тела животных

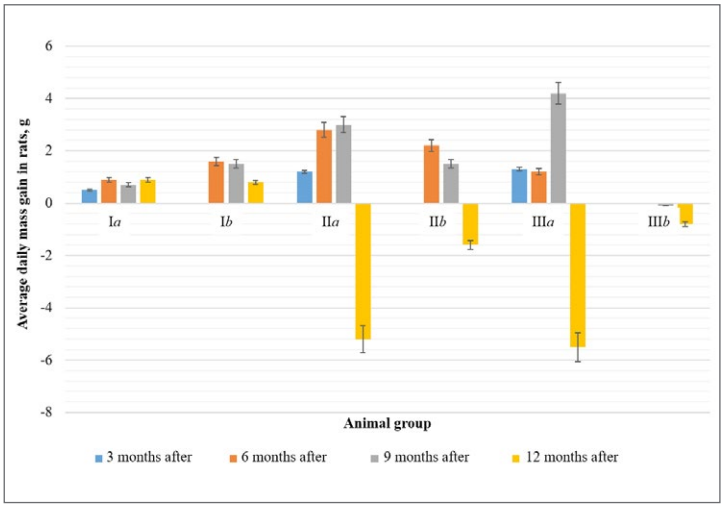

Fig. 2. Dynamic changes in average daily mass gain

Рис. 2. Динамика среднесуточного прироста массы тела

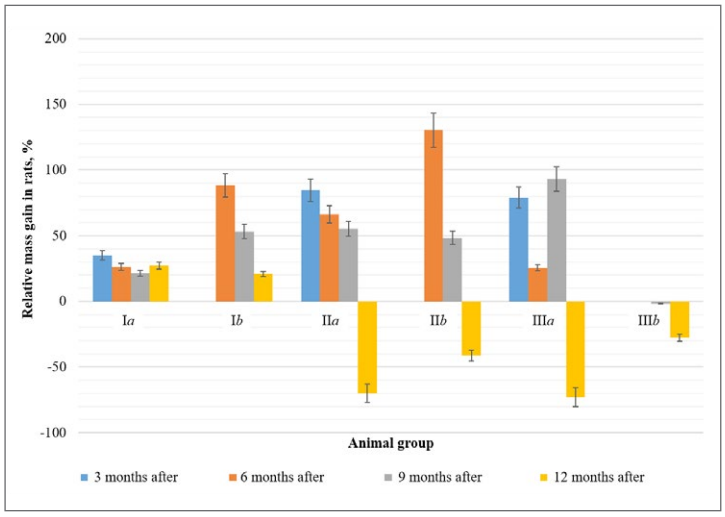

Fig. 3. Dynamics of relative body mass gain in animals

Рис. 3. Динамика относительного прироста массы тела животных

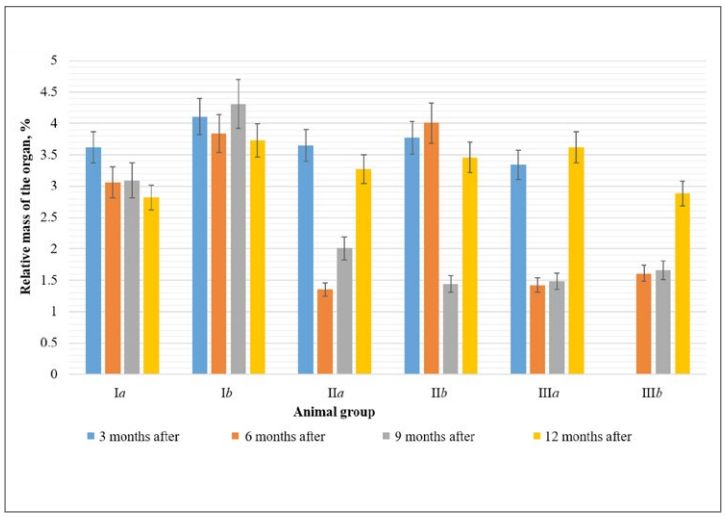

Fig. 4. Dynamic changes in relative mass of animal liver

Рис. 4. Динамика относительной массы печени животных

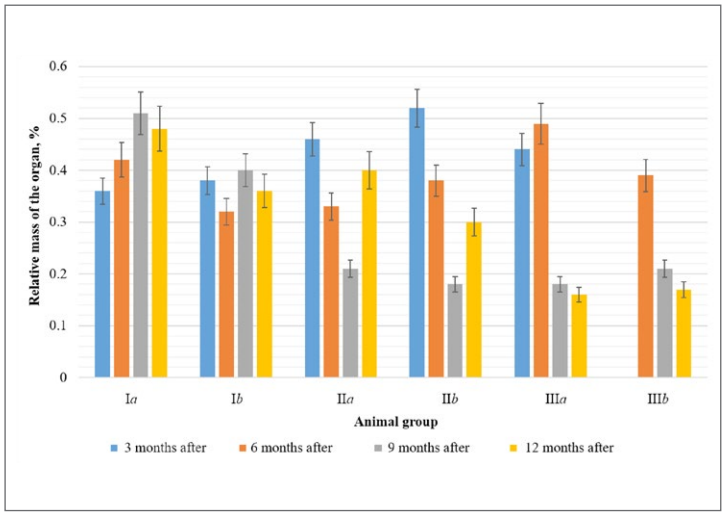

Fig. 5. Dynamic changes in relative mass of animal spleen

Рис. 5. Динамика относительной массы селезенки животных

the experiment was replaced by a negative one at the end of the observation period, and cachexia began to develop in the experimental animals following a sharp increase in the body mass. This was most pronounced in the rat pups of Group III, where absolutely no positive dynamics were reported. At the same time, the control group rats naturally gained weight. The changes in the body mass of animals and the dynamic changes in the average daily and relative mass gain of rats are shown in Figures 1–3.

As Figure 1 shows, the animals in the experimental groups had pronounced positive dynamics in the body mass change during 9 months of the experiment and sharp negative dynamics at the end of the experiment. By the third quarter of the experiment, the body mass of adult rats in the experimental groups was 2.2 times more than that in the control group, and the body mass of their pups was 1.5 times more compared to the control. The obesity in rats may be related to an increased fat content of milk from the BLV-diseased and infected cows, which is often caused by a decrease in milk volume resulting from subclinical mastitis [6]. By the end of the experiment, the body mass of rats in Groups II*a* and III*a* decreased by 3.4 and 3.7 times in comparison to the previous indicators, and the body mass of their pups decreased by 1.7 and 1.4 times, respectively, whereas the body mass of the intact animals continued to increase gradually. The steady development of cachexia may indicate changes in the metabolism of the experimental animals, since there is evidence that chronic viral infections induce metabolic disorders [7], and bovine leukosis is often accompanied by cachexia [8].

Figure 2 shows that the most pronounced positive dynamic changes in the average daily mass gain was observed in the first 9 months of the experiment in adult rats that had milk from BLV-diseased and infected cows, then the indicator became sharply negative in these animals. At the beginning of the experiment, the rat pups from Group II rats showed an increase in body mass, but by the end of the research, this indicator became negative, as well as in the parent population. However, the average daily mass gain of the rat pups from Group III first remained at zero and then slightly decreased. Dynamic changes in the average daily mass gain in animals directly correlates with the indicators of the relative mass gain, and the corresponding trends are shown in Figure 3.

The dynamic changes in the relative mass gain of rats in all groups had a negative trend (Fig. 3). The exception was observed in adult rats, whose diet included milk from cows with bovine leukosis, relative mass gain indicators in animals of this group were not constant. By the first quarter of the experiment, this indicator in Groups II*a* and III*a* was (84.6 ± 7.8)% and (78.9 ± 7.1)%, respectively, exceeding that in animals of the control group by 2.4 and 2.3 times. However, 6 and 9 months after the start of the experiment, the relative mass gain in Group II*a* animals decreased by 1.3 and 1.2 times, respectively, whereas it first sharply decreased by 3.1 times in Group III*a* rats and then increased by 3.6 times, which can be associated with metabolic changes most likely caused by the hormonal background of the animals. By the end of the experiment, this indicator was negative in all experimental groups and ranged from –(27.8 ± 2.1)% and –(41.1 ± 3.8)% in the pups from Groups III and II to –(73.0 ± 7.1)% and –(70.2 ± 6.6)% in the parent population, respectively; however, this indicator was positive in the control animals and remained at (20.9 ± 1.7)% and (27.1 ± 2.1)%.

Relative mass of the internal organs is an important criterion that characterizes the state of the body. It is known that the mass of any organ is directly related to its functional state. At the same time, changes of the organ volume and structure can be caused either by the age-related changes, or by any pathological processes [9]. Dynamic changes in the relative mass of the internal organs of the experimental animals demonstrated certain trends in different groups. The data obtained are illustrated in Figures 4–8.

Relative liver mass of the experimental rats initially showed a negative trend (Fig. 4), most likely due to a rapid increase in the body mass. By the end of the experiment, this indicator significantly increased in all experimental groups, which may be caused by cachexia, as well as by inflammatory processes and intoxication, which markers were identified during clinical, morphological and biochemical tests of blood from experimental animals [10, 11]. So, 3 and 6 months after the start of the experiment, the relative liver mass of rats of Groups II*a* and III*a* reduced by 2.3/2.2 and 1.5/2.1 times, respectively, in comparison to the control group and by the end of the experiment, this indicator already exceeded the indicators of the control group by 1.2 and 1.3 times. In the middle of the experiment relative liver mass of experiment rat pups was lower by 3.0 and 2.6 times compared to the control, and at the end – this indicator increased by 2.4 and 1.7 times compared to the previous data; whereas the control rat pups demonstrated approximately the same numbers over the entire observation period.

Relative spleen mass of animals changed during the experiment in the following way (Fig. 5). It first decreased in experimental rats by 1.4–2.1 times, varying from group to group, in connection with the body mass gain and with the exception of Group III adult animals, where the relative spleen mass first slightly increased by 1.1 times, and then sharply decreased by 2.7 times. Then, this indicator changed in Groups II*a* and II*b* and the organ increased by 1.9 and 1.6 times at the end of the experiment, which was, probably, caused either by inflammatory or proliferative processes in the spleen alongside with a decrease in the body mass gain. Group III rats and their pups demonstrated a progressive reduction in the organ volume by 3.0 and 2.1 times, respectively, compared with the control. The trend was possibly associated with atrophic processes.

Six-nine month after the start of the experiment, relative kidney mass of rats from the experimental groups (Fig. 6) decreased by 1.6/1.8/2.5 compared to the initial data for Groups II*a*/II*b*/III*a*, alongside with the body mass increase. At the end of the experiment, there was an increase in the relative mass of the organ in all the experimental groups by 1.2–1.9 times as compared to the data obtained in the third quarter. The increase may be associated with proliferative processes or kidneys hypertrophy due to intoxication. Group III*b* rats were an exception with a constant increase in the relative kidney mass, because there was not an initial increase in the relative body mass gain. It should be noted that the relative kidney mass of the experimental rat pups was 1.2 times higher than that of the rat pups from the intact animals. In adult rats from Group II this indicator was 1.3 times higher, and in rats from Group III it was 1.6 times lower than that in the intact animals.

As Figure 7 shows, relative mass of the rat lungs did not change so dynamically as in case of other organs

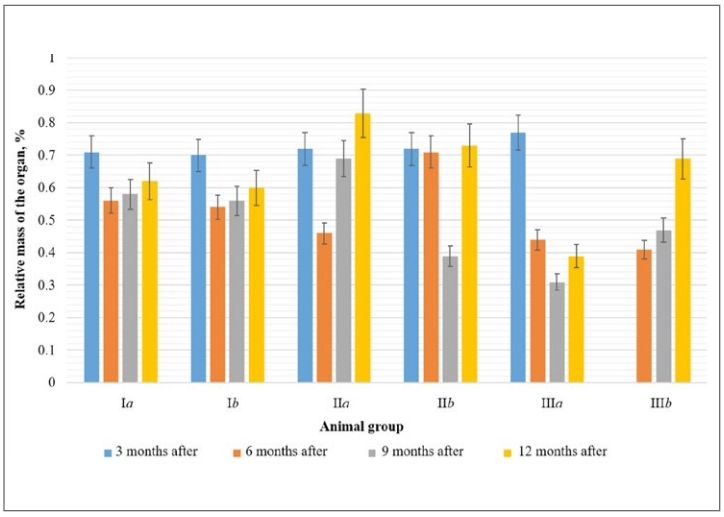

Fig. 6. Dynamic changes in relative mass of animal kidneys

Рис. 6. Динамика относительной массы почек животных

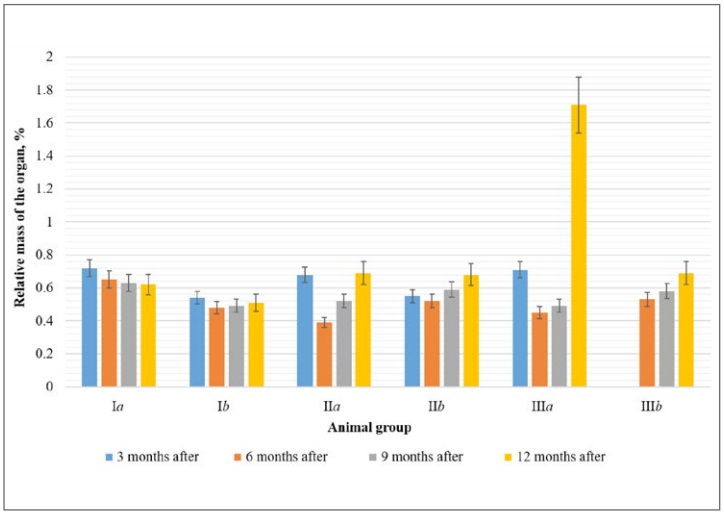

Fig. 7. Dynamic changes in relative mass of animal lungs

Рис. 7. Динамика относительной массы легких животных

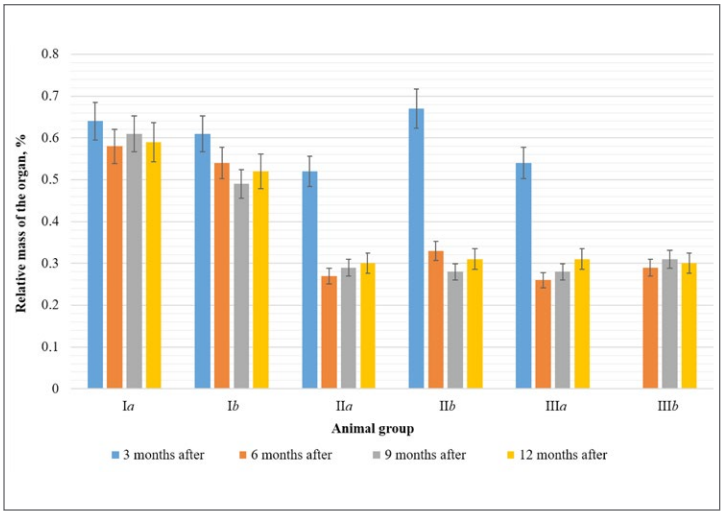

Fig. 8. Dynamic changes in relative mass of animal heart

Рис. 8. Динамика относительной массы сердца животных

described above. It increased sharply only in animals from Group III*a*. At the end of the experiment, the indicator in the given group exceeded that one in the control group by 2.8 times. This indicator increased by 1.3 times in the pups from Groups II and III, as compared to the intact ones. This was due to autopsy-confirmed single and double pneumonia in many experimental rats which could be triggered by BLV-induced immunosuppression. According to N. G. Kozyreva et al. [2], P. Dimitrov et al. [12], pneumonia often accompanies experimental BLV infection in rabbits, which confirms the research results.

Relative heart mass in animals from all the experimental groups demonstrated a dynamic decrease by 2.5–3.0 times compared to the control (Fig. 8), despite cachexia reported at the end of the experiment. This may be due to the progressive atrophy or dystrophy of the organ alongside with metabolic disorders.

CONCLUSION

Thus, the obtained results demonstrate that the experimental BLV infection is accompanied by regular changes in both absolute and relative indicators of body mass and mass of internal organs in Wistar laboratory rats. There was a clear body mass increase in BLV-infected laboratory rats, then followed by a decrease down to negative numbers. The reverse trend was observed for such internal organs of the experimental animals as liver, spleen, kidneys and lungs. At the beginning of the experiment, their relative mass decreased to some extent, then increased with different dynamics in groups. The heart was the exception, as its relative mass decreased and did not increase until the end of the experiment.

The data obtained correlate with the results provided by other authors stating that BLV infection induces disorders not only in the hematopoietic, but also in other vital organs of the animal. As a result of proliferative, inflammatory, dystrophic and atrophic processes, the relative mass of various organs changes [13], with the most pronounced changes found in the spleen, liver, kidneys and heart [14–16].

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Ekaterina S. Krasnikova, Doctor of Science (Veterinary Medicine), Associate Professor, Professor, Department of Animal Science and Veterinary Medicine, FSBEI HE Michurinsk SAU, Michurinsk, Russia.

Roman V. Radionov, Candidate of Science (Biology), Assistant, Department of Animal Diseases and Veterinary and Sanitary Expertise, FSBEU HE Saratov SAU, Saratov, Russia.

Aleksander V. Krasnikov, Doctor of Science (Veterinary Medicine), Associate Professor, Head of the Department of Animal Science and Veterinary Medicine, FSBEI HE Michurinsk SAU, Michurinsk, Russia.

Anna Yu. Svetozarova, Post-Graduate Student, Department of Animal Science and Veterinary Medicine, FSBEI HE Michurinsk SAU, Michurinsk, Russia.

Красникова Екатерина Сергеевна, доктор ветеринарных наук, доцент, профессор кафедры зоотехнии и ветеринарии ФГБОУ ВО Мичуринский ГАУ, г. Мичуринск, Россия.

Радионов Роман Владимирович, кандидат биологических наук, ассистент кафедры болезней животных и ветеринарно-санитарной экспертизы ФГБОУ ВО Саратовский ГАУ, г. Саратов, Россия.

Красников Александр Владимирович, доктор ветеринарных наук, доцент, заведующий кафедрой зоотехнии и ветеринарии ФГБОУ ВО Мичуринский ГАУ, г. Мичуринск, Россия.

Светозарова Анна Юрьевна, аспирант кафедры зоотехнии и ветеринарии ФГБОУ ВО Мичуринский ГАУ, г. Мичуринск, Россия.

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Prevalence of feline viral leukemia in Moscow and the Moscow Oblast with the analysis of hematological and biochemical blood parameters

A. P. Zenchenkova
Peoples' Friendship University of Russia (RUDN University), Moscow, Russia;
ORCID 0000-0002-8605-5103, e-mail: oshea.rorey@gmail.com

SUMMARY
Feline viral leukemia is an infectious disease that is common throughout the world. Despite the statistical records that have been successfully kept in other countries for many years, there is little data on the prevalence of the infection in the Russian Federation, and the samples are represented by a small number of animals. 11,807 blood samples collected in Moscow and the Moscow Oblast were tested for FeLV antigen and antibodies against FeLV by polymerase chain reaction, enzyme immunoassay, and immunochromatographic assay. The prevalence of feline viral leukemia was 12.8%, which correlates with the prevalence of the disease detected in developing countries, and speaks of a high disease prevalence in domestic cats in Moscow and the Moscow Oblast. Most often, feline leukemia virus was detected in free roaming domestic cats. During the diagnostic studies, the following hematological abnormalities were found: anemia, thrombocytopenia, lymphopenia, and a shift of the leukocyte formula to the left. Biochemical blood tests showed the increased levels of total protein, aspartate aminotransferase, alkaline phosphatase, and C-reactive protein. The obtained data demonstrate non-specific hematological and biochemical abnormalities in infected cats, and dictate the need for further study of the risk factors that predispose cats to the infection with this viral disease. Feline viral leukemia should be suspected in case of a non-specific clinical picture, as well as in case of abnormalities in blood biochemical and hematological parameters of free roaming cats or of those which have had a confirmed contact with their conspecifics.

Keywords: Feline viral leukemia, feline viral immunodeficiency, immunosuppression, prevalence, anemia.

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For correspondence: Anna P. Zenchenkova, Post-Graduate Student, Department of Veterinary Medicine, Agrarian Technology Institute, People's Friendship University of Russia, 117198, Russia, Moscow, Miklukho-Maklaya str., 8, building 2, *e-mail: oshea.rorey@gmail.com*.

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Превалентность вирусной лейкемии кошек в условиях г. Москвы и Московской области с анализом гематологических и биохимических параметров крови

А. П. Зенченкова
ФГАОУ ВО «Российский университет дружбы народов» (РУДН), г. Москва, Россия;
ORCID 0000-0002-8605-5103, e-mail: oshea.rorey@gmail.com

РЕЗЮМЕ
Вирусная лейкемия кошек – инфекционное заболевание, распространенное по всему миру. Несмотря на статистический учет, который успешно ведется в других странах мира на протяжении уже многих лет, данные о распространении инфекции в Российской Федерации малочисленны, а выборки представлены небольшим количеством животных. Проведено исследование 11 807 образцов крови, собранных на территории г. Москвы и Московской области, на наличие антигена вируса лейкемии кошек и/или антител против возбудителя методами полимеразной цепной реакции, иммуноферментного и иммунохроматографического анализов. Превалентность вирусной лейкемии кошек составила 12,8%, что коррелирует с превалентностью заболевания, выявленной в развивающихся странах, и характеризует высокую степень распространенности инфекции в популяции домашних кошек г. Москвы и Московской области. Наиболее часто вирус лейкемии кошек выявляли у самцов и самок, имеющих свободный доступ на улицу. При проведении диагностических исследований установлены следующие гематологические отклонения: анемия, тромбоцитопения, лимфоцитопения и сдвиг лейкоцитарной формулы

влево. Биохимический анализ крови показал повышение уровней общего белка, аспартатаминотрансферазы, щелочной фосфатазы и С-реактивного белка. Полученные данные отражают неспецифичность гематологической и биохимической картины анализа крови, представленной у инфицированных кошек, и диктуют необходимость дальнейшего изучения факторов риска, предрасполагающих кошек к заражению данным вирусным заболеванием. Ветеринарные специалисты должны подозревать заболевание вирусной лейкемией кошек в случае наличия неспецифической клинической картины, а также в случае выявления отклонений от нормы параметров гематологического и биохимического анализа крови животных, имеющих свободный доступ на улицу или имеющих подтвержденный контакт с сородичами.

Ключевые слова: Вирусная лейкемия кошек, вирусный иммунодефицит кошек, иммуносупрессия, превалентность, анемия.

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Для корреспонденции: Зенченкова Анна Петровна, аспирант департамента ветеринарной медицины Аграрно-технологического института РУДН, 117198, Россия, г. Москва, ул. Миклухо-Маклая, д. 8, корпус 2, *e-mail: oshea.rorey@gmail.com.*

INTRODUCTION

Feline viral leukemia is an infectious disease caused by feline leukemia virus (FeLV) belonging to the genus *Gammaretrovirus* of the family *Retroviridae*. This viral agent was discovered and described in 1964 by William Jarrett, and since then it has been found in blood and saliva samples from cats all over the world [1].

Feline leukemia virus is responsible for a wide range of clinical syndromes associated with immunosuppression and bone marrow disorders [2]. Thus, the clinical signs most often associated with immunosuppression are neutropenia, lymphocytopenia, and the development of such opportunistic infections as feline calicivirus infection, chlamydiosis, toxoplasmosis, and cryptococcosis [3]. Impaired bone marrow function leads to the development of non-regenerative anemia, which can be autoimmune, and thrombocytopenia. In addition, the risk of developing cancer (especially lymphoma) in infected cats is higher than in the healthy ones [4].

A large number of studies on the prevalence of feline viral leukemia in developed countries are presented in the literature sources. Thus, the prevalence of the infection in Southern Germany is 1.8% [5], in Switzerland – 3% [6], in the United States of America and Canada – 3.1% [7]. However, there is no exact data on the prevalence of feline viral leukemia in the Russian Federation due to the country's scale and the lack of necessary diagnostics. Nevertheless, in recent years, results of the studies aimed at determining the occurrence and prevalence of feline viral leukemia in certain cities of the Russian Federation have been published. Thus, the registered prevalence of feline viral leukemia in Vladivostok in 2018 was 15.9% [8].

Prevention plays a significant role in reducing the risk of virus transmission to healthy cats. The most effective measures include keeping the infected animals indoors, preventing them from contact with their conspecifics, mandatory castration of the infected animals, as well as vaccination against FeLV infection. Currently, non-adjuvanted recombinant and inactivated vaccines are available on the market, demonstrating the same effectiveness against the pathogen [9].

In connection with the above, the purpose of this research was to study FeLV prevalence in Moscow and the Moscow Oblast and to analyze abnormalities in blood biochemical and hematological parameters of the infected cats.

MATERIALS AND METHODS

Study design. 11,807 cat blood samples (both containing whole blood and serum) were tested for FeLV antigen and antibodies against FeLV. Data on animals and blood samples were collected between October 2018 and October 2019. 174 medical case histories were analyzed. The study included such criteria as gender, neuter status, access to the outdoors, and contact with conspecifics. The subject of the study was 6,529 male and 5,278 female cats.

Polymerase chain reaction. The study was based on the detection of the feline leukemia virus proviral DNA in blood of infected animals and was carried out using the Rotor-Gene Q amplifier (QIAGEN, Germany). Using a commercially available DNA extraction kit, QIAamp DNA Blood Kits (QIAGEN, Germany), proviral DNA was isolated from 200 µl of whole blood samples containing EDTA (Ethylenediamine tetraacetate, anticoagulant). Amplification was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA).

Enzyme-linked immunosorbent assay (ELISA). The study was aimed at detecting the feline leukemia virus antigen using SNAP FIV/FeLV Combo test system (IDEXX, USA) in whole blood samples containing EDTA and in the FeLV infected cat sera according to the kit instructions.

Immunochromatographic analysis (IHA). The study was based on the detection of feline leukemia virus antigen in blood serum samples using rapid test FIV Ab + FeLV Ag Combined Test (Quicking Biotech Co., Ltd., China) according to the manufacturer's recommendation.

Hematological and biochemical tests. The results of 124 general blood tests and 80 biochemical tests were assessed. Hematological testing of whole blood samples (25 µl, containing EDTA) collected from infected cats was performed using an automatic analyzer Biocode-Hycel

Celly 70 (Biocode-Hygel, France). Biochemical testing of the infected cat blood sera (up to 40 µl) was carried out using a BA-400 analyzer (BioSystems S. A., Spain).

RESULTS AND DISCUSSION

The study of 11,807 blood samples demonstrated 12.8% prevalence of FeLV (1,514 animals) in cats in Moscow and the Moscow Oblast. The obtained results correlate with the data on the disease prevalence in developing countries. For example, FeLV prevalence in Thailand is 16.5% [10], and in Brazil – 12.5% [11].

High FeLV prevalence in Moscow and the Moscow Oblast is probably due to the lack of recommendations for vaccination against this pathogen in the annual immunization schedule for healthy cats, as well as due to the large number of free roaming cats. According to the study, 89.7% of cats had their freedom to roam outdoors. Most often, feline leukemia virus was detected in male cats (61.3% – 928 animals), which may be due to their more aggressive zoo-social behavior towards their conspecifics, as well as their predisposition to live a feral-lifestyle. This observation confirms similar conclusions made in earlier studies [7]. In addition, 64.8% of cats (61 out of 94 animals) had a confirmed contact with their conspecifics at home or outdoors.

Despite the fact that some literature sources [2] provide data demonstrating that the risk of FeLV infection in non-castrated male cats is higher, the study did not prove it: 66.6% of the infected cats (618 animals) were neutered when the disease was diagnosed. Thus, the conclusion regarding the relationship between the neuter status of cats and the risk of infection with the feline leukemia virus in this study could not be made.

The summary of the results is presented in the Table.

Table
Prevalence of feline viral leukemia by sex, neuter status, and access to the outdoors

Таблица
Распространенность вирусной лейкемии кошек в зависимости от пола, статуса интактности кошек и доступа на улицу

Parameter	Number of FeLV infected cats	Prevalence, %
Sex:		
male cats	928	61.3
female cats	586	38.7
Total	1,514	12.8
Neuter status:		
neutered male cats	618	66.6
intact male cats	310	33.4
neutered female cats	411	70.2
intact female cats	175	29.8
Access to the outdoors:		
free-roaming	70	89.7
indoor keeping only	8	10.3
Total	78	–
Contact with conspecifics:		
confirmed	61	64.8
absent	33	35.2
Total	94	–

Assessment of 124 complete blood count test results revealed a decrease in red blood cell levels (RBC) in 46% of animals (57 cats), decreased hemoglobin levels (HGB) in 54.8% of animals (68 cats) and decreased hematocrit levels (HCT) in 60.5% of animals (75 cats). In addition, thrombocytopenia (PLT) was diagnosed in 58.1% of the tested cats (72 animals), an increased level of band neutrophils was found in 45.2% of cats (56 animals). Decreased lymphocytes levels were observed in 32.3% of cats (40 animals), and the increased erythrocyte sedimentation rate – in 37.9% of cats (47 animals).

Thus, the results obtained indicate that at the moment, the most common hematological abnormality occurring in infected cats in Moscow and the Moscow Oblast is anemia. Most often, it is of non-regenerative nature and can be caused by an autoimmune reaction. Therefore, diagnosis of FeLV infection should be included in the screening protocol for any cat with anemia signs.

It is worth noting that thrombocytopenia was diagnosed in more than half of the animals (58.1%), this fact can be associated with both aggregation of feline platelets induced by EDTA contained in test tubes for hematological testing, and an autoimmune reaction.

The following changes in the biochemical profile of the blood were observed during the studies: increased urea level – in 30% of cats, increased aspartate aminotransferase (AST) level – in 77.5% of cats, increased C-reactive protein level – in 87.5% of animals, hyperglycemia was observed in 56% of cats. In addition, water-electrolyte balance disorders were reported: hypokalemia and hyponatremia which may result from hypo- or anorexia were diagnosed 71.4% and 60% of cases, respectively, as well as some common disorders of the gastrointestinal tract, such as vomiting and diarrhea.

Thus, biochemical analysis of FeLV infected cat blood demonstrated non-specific results indicative of the pathologies not associated with the viral disease. It should be noted that most animals had abnormal blood biochemical parameters, typical for a non-specific inflammatory process: increased AST, C-reactive protein, and total protein levels.

CONCLUSION

Results presented in this study demonstrate a high prevalence of feline viral leukemia in Moscow and the Moscow Oblast. Based on the obtained from the medical case history of the infected animals, it can be concluded that the major risk factors associated with the disease are gender and access to the outdoors. Cats brought to a veterinary clinic had a wide range of clinical manifestations associated with viral leukemia. The most pronounced abnormality in the hematological parameters in infected cats, which may be a good reason to suspect feline viral leukemia, was anemia. We believe that further research is needed on the prevalence of feline viral leukemia in the Russian Federation, the risk factors, and the clinical signs associated with this viral disease.

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INFORMATION ABOUT THE AUTHOR / ИНФОРМАЦИЯ ОБ АВТОРЕ

Anna P. Zenchenkova, Post-Graduate Student, Department of Veterinary Medicine, Agrarian Technology Institute, People's Friendship University of Russia, Moscow, Russia.

Зенченкова Анна Петровна, аспирант департамента ветеринарной медицины Аграрно-технологического института Российского университета дружбы народов, г. Москва, Россия.

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Analysis of marker substitutions in A/chicken/Astrakhan/2171-1/2020 H5N8 isolate of avian influenza virus recovered in the Astrakhan Oblast

N. G. Zinyakov[1], A. V. Andriyasov[2], Ye. V. Ovchinnikova[3], A. A. Kozlov[4], P. D. Zhestkov[5], D. B. Andreychuk[6], I. A. Chvala[7]
FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia
[1] *ORCID 0000-0002-3015-5594, e-mail: zinyakov@arriah.ru*
[2] *ORCID 0000-0001-6314-2119, e-mail: andriyasov_av@arriah.ru*
[3] *ORCID 0000-0001-5501-4432, e-mail: ovchinnikova@arriah.ru*
[4] *ORCID 0000-0002-1466-7602, e-mail: kozlov_aa@arriah.ru*
[5] *ORCID 0000-0001-8204-280X, e-mail: zhestkov@arriah.ru*
[6] *ORCID 0000-0002-1681-5795, e-mail: andreychuk@arriah.ru*
[7] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*

SUMMARY

At the end of 2020, a large-scale bird death was registered at one of the poultry farms in the Astrakhan region, the cause of which was avian influenza. Data on detection of the marker substitutions in viral proteins of avian influenza virus A/chicken/Astrakhan/2171-1/2020 isolate are presented in the paper. Type A H5N8 avian influenza virus was identified with complex PCR-based methods in the submitted samples. Hemagglutinin gene fragment sequencing identified REKRRKR/GLF, highly pathogenic avian influenza virus isolate-characteristic amino acid sequence of the hemagglutinin cleavage site. Phylogenetic analysis of nucleotide sequences of hemagglutinin gene segment (848–1105 bp ORF) allowed A/chicken/Astrakhan/2171-1/2020 H5N8 isolate to be classified to highly pathogenic avian influenza virus genetic clade 2.3.4.4. Comparative analysis of genome segments using available databases showed that A/chicken/Astrakhan/2171-1/2020 H5N8 virus related to A/H5 avian influenza virus isolates detected in the Russian Federation in 2016–2020. Analysis of the studied virus isolate hemagglutinin amino acid identified AIV-characteristic $G_{225}QRG_{228}$ amino acids in the receptor-binding domain of the protein enabling high-affinity binding to avian epithelial cell SA*a*-2,3-gal receptors. Single mutations, 70G in NEP protein and 13P in PB1 protein, out of the list of the reported influenza virus mutations affecting successful influenza virus replication in mammals were identified. No mutations affecting virus sensitivity to anti-viral medicines, rimantadin, amantadine, oseltamivir and zanamivir, were detected. The following mutations recognized as pathogenicity determinants in mice were found: 42S in the NS1 protein and 30D protein 215A in M1 protein.

Keywords: Avian influenza, H5N8, genetic analysis, amino acid substitutions.

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For correspondence: Nikolay G. Zinyakov, Candidate of Science (Biology), Senior Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: zinyakov@arriah.ru.*

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Анализ маркерных замен изолята вируса гриппа A/chicken/Astrakhan/2171-1/2020 H5N8, выделенного на территории Астраханской области

Н. Г. Зиняков[1], А. В. Андриясов[2], Е. В. Овчинникова[3], А. А. Козлов[4], П. Д. Жестков[5], Д. Б. Андрейчук[6], И. А. Чвала[7]
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия
[1] *ORCID 0000-0002-3015-5594, e-mail: zinyakov@arriah.ru*
[2] *ORCID 0000-0001-6314-2119, e-mail: andriyasov_av@arriah.ru*
[3] *ORCID 0000-0001-5501-4432, e-mail: ovchinnikova@arriah.ru*
[4] *ORCID 0000-0002-1466-7602, e-mail: kozlov_aa@arriah.ru*
[5] *ORCID 0000-0001-8204-280X, e-mail: zhestkov@arriah.ru*

[6] *ORCID 0000-0002-1681-5795, e-mail: andreychuk@arriah.ru*
[7] *ORCID 0000-0002-1659-3256, e-mail: chvala@arriah.ru*

РЕЗЮМЕ

В конце 2020 г. на одной из птицефабрик в Астраханской области была зарегистрирована массовая гибель птиц, причиной которой стал грипп птиц. В работе представлены данные по выявлению маркерных замен вирусных белков изолята вируса гриппа птиц A/chicken/Astrakhan/2171-1/2020. В результате комплекса исследований с использованием полимеразной цепной реакции в полученных пробах был идентифицирован вирус гриппа птиц типа А подтипа H5N8. Согласно результатам секвенирования участка гена гемагглютинина установлена аминокислотная последовательность сайта расщепления гемагглютинина REKRRKR/GLF, характерная для изолятов высокопатогенного гриппа птиц. Филогенетический анализ нуклеотидных последовательностей участка гена гемагглютинина (848–1105 н. п. открытой рамки считывания) позволил установить принадлежность изолята A/chicken/Astrakhan/2171-1/2020 H5N8 к генетической кладе 2.3.4.4 высокопатогенного вируса гриппа птиц. В результате сравнительного анализа геномных сегментов с использованием доступных баз данных установлено родство вируса A/chicken/Astrakhan/2171-1/2020 H5N8 с изолятами вируса гриппа A/H5, выявленными на территории Российской Федерации в 2016–2020 гг. Анализ аминокислотной последовательности вирусного гемагглютинина анализированного изолята выявил в рецептор-связывающем центре белка аминокислоты $G_{225}QRG_{228}$, характерные для вируса гриппа птиц и обеспечивающие повышенный аффинитет к рецепторам SA*α*-2,3-gal эпителиальных клеток птиц. Из числа описанных мутаций вируса гриппа, влияющих на успешную репродукцию его в организме млекопитающих, были выявлены единичные мутации 70G в белке NEP и 13P в белке PB1. Мутаций, влияющих на чувствительность вируса к противовирусным препаратам: римантадину, амантадину, осельтамивиру и занамивиру – не обнаружено. Выявлены мутации 42S в белке NS1 и 30D, 215A в белке M1, признанные детерминантами патогенности для мышей.

Ключевые слова: Грипп птиц, H5N8, генетический анализ, аминокислотные замены.

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Для корреспонденции: Зиняков Николай Геннадьевич, кандидат биологических наук, старший научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: zinyakov@arriah.ru*.

INTRODUCTION

Avian influenza virus (AIV) is a dangerous highly contagious causative agent of respiratory illnesses in birds. Mortality in birds infected with H5 and H7 AI viruses reaches 100%.

Since 1996, highly pathogenic avian influenza (HPAI) caused by A/H5N1 AI virus has become epidemic in the South-Eastern Asian countries. The disease outbreaks occurred in 2005–2007 caused significant losses to the poultry industry of the Russian Federation. H5N8 AI virus was detected in a migratory duck in the Republic of Sakha in 2014. Then, H5N8 AI viruses have repeatedly caused outbreaks in poultry and wild bird populations in Russia as well as in Asian, African and European countries up to the present time. Epidemic HPAI situation in the Russian Federation aggravated in late 2016. In 2016–2017 H5N8 HPAI outbreaks were reported in poultry in the Rostov, Astrakhan, Samara, Moscow, Nizhny Novgorod Oblasts, Krasnodar Krai, Republic of Tatarstan, Mariy El, Kalmykia as well as in the Udmurt and Chechen Republics. The disease outbreaks caused great economic losses to Russian commercial establishments [1]. In 2018 H5N8 AI virus was detected in poultry in the Kursk, Oryol, Voronezh, Kostroma, Smolensk, Saratov, Samara, Ulyanovsk, Penza, Nizhny Novgorod, Rostov Oblasts, Udmurt Republic, Republic of Mariy-El, Chuvash Republic and Republic of Tatarstan [2].

In 2020, H5N8 AI virus widely spread across the European and Middle East countries as well as Russian Federation and Kazakhstan territories. Moreover, H5N5 AI virus was detected in the Omsk and Rostov Oblasts. In late 2020, H5N8 AI virus was detected in humans contacting to the diseased poultry on the poultry farm located in the Astrakhan Oblast (https://www.interfax.ru/russia/752017).

Human AI cases require further investigations including whole-genome sequencing followed by analysis of deduced amino acid sequence A/chicken/Astrakhan/2171-1/2020 for detection of possible markers of tropism and virulence for mammals.

MATERIALS AND METHODS

RNA extraction. RNA was extracted with RIBO-sorb kit (FBIS "Central Research Institute of Epidemiology" of the RF Federal Service for Customers' Rights Protection and Human Well-Being Surveillance, Russia; cat. No. K2-1-Et-100) according to the manufacturer's instructions.

Real time reverse transcription-polymerase chain reaction (rt RT-PCR). One-step rt RT-PCR was carried out with OneStep RT-PCR Kit (Qiagen, Netherlands; cat. No. 210212) using 25 мМ magnesium chloride solution (Promega, USA; supplied with the kit, cat. No. M8296) and a set of primers for M gene and HA, NA genes of H5N8 AIV. Reaction mix (25 µl) was prepared. The reaction mix contained 1× buffer for RT-PCR, 1.25 мМ

$MgCl_2$, 0.4 мМ dNTP, 0.4 pmol/µl of forward primer and 0.4 pmol/µl of reverse primer, 0.3 pmol/µl of fluorescent probe, 1 µl of reverse transcriptase and polymerase mix (Qiagen, Netherlands; cat. No. 210212), 5 µl of total RNA solution. Reverse transcription was performed at 50 °C for 30 min. The following temperature/time conditions were used for amplification: 95 °C – 10 min. (polymerase activation), then 40 runs, each consisting of three steps (95 °C – 10 sec., 55 °C – 35 sec., 72 °C – 10 sec.).

Reverse transcription-polymerase chain reaction (RT-PCR). Conventional RT-PCR was performed in one-step with OneStep RT-PCR Kit (Qiagen, Netherlands; cat. No. 210212) using 25 мМ magnesium chloride solution (Promega, USA; supplied with the kit, cat. No. M8296) and a set of primers for HA gene of H5 subtype AIV. Reaction mix (25 µl) was prepared. The reaction mix contained 1× buffer for RT-PCR, 1.25 мМ $MgCl_2$, 0.4 мМ dNTP, 0.4 pmol/µl of forward primer and 0.4 pmol/µl of reverse primer, 1 µl of reverse transcriptase and polymerase mix (Qiagen, Netherlands; cat. No. 210212), 5 µl of total RNA solution. Reverse transcription was performed at 50 °C for 30 min. Amplification was performed under the following temperature/time conditions: 95 °C – 10 min. (polymerase activation), then 40 runs, each consisting of three steps (95 °C – 30 sec., 58 °C – 60 sec., 68 °C – 120 sec.) and final elongation – for 7 min.

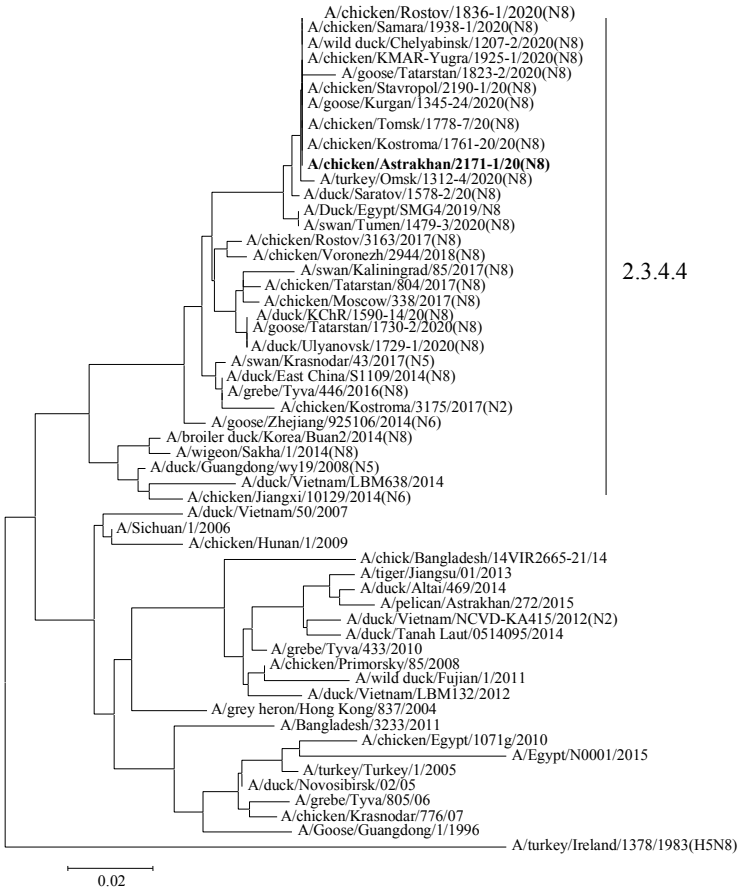

Fig. 1. Phylogenetic tree constructed using HA gene fragment sequence (848–1105 bp) of H5 HPAI virus isolates and strains

Рис. 1. Филогенетическое древо, построенное с помощью последовательностей фрагмента гена НА (848–1105 н. п.) изолятов и штаммов вируса ВПГП подтипа Н5

Sequencing. HA gene fragment nucleotide sequences were determined with automated ABI Prism 3100 sequencer using BigDye Terminator Cycle Sequencing kits (Applied Biosystems, USA) according to the manufacturer's instructions. Whole-genome sequencing was performed with MySeq analyzer (Illumina, USA) according to the manufacturer's instructions. Double stranded DNA synthesis was carried out with cDNA Synthesis System (Roche, Switzerland) according to the manufacturer's instructions. DNA libraries were prepared with commercial XT kit and Nextera XT Index Kit (Illumina, USA).

Nucleotide sequences. Nucleotide sequences of H5 subtype AIV isolates and strains published in the GenBank database, NCBI electronic source (www.ncbi.nlm.nih.gov/nucleotide), and the EpiFlu database (https://www.gisaid.org) were used.

Analysis of the nucleotide sequences and corresponding amino acid sequences was carried out with BioEdit software, version 7.0.5.3. The sequences were aligned with ClustalW multiple sequence alignment software. Phylogenetic tree was constructed with NJ algorithm using MEGA package, version 6.06.

RESULTS AND DISCUSSION

Mass mortality of poultry was reported on a poultry farm located in the Astrakhan Oblast in December 2020. Results of the tests carried out by local veterinary laboratory indicated the avian influenza virus presence in the tested samples. The samples were sent to the Reference Laboratory for Viral Avian Diseases of the FGBI "ARRIAH" for confirmation of the test results and further virus typing. Type A H5N8 AI virus was identified in the submitted samples with a complex PCR-based tests. Virus hemagglutinin cleavage site, REKRRKR/GLF, was identified based on analysis of deduced amino acid sequence. The obtained results allow detected virus to be identified as highly pathogenic avian influenza virus.

The isolated A/chicken/Astrakhan/2171-1/2020 virus was classified to genetic clade 2.3.4.4 based on phylogenetic analysis of its HA gene nucleotide sequence (Fig. 1). Diagnostic fragment of the hemagglutinin gene sequence (848–1105 bp of open reading frame – ORF) was used for the phylogenetic analysis.

Whole-genome sequencing was performed to identify marker substitutions indicative of A/chicken/Astrakhan/2171-1/2020 virus adaptation to mammals. Comparative and phylogenetic analyses showed high similarity of A/chicken/Astrakhan/2171-1/2020 virus to the vast majority of H5N8 AI virus isolates recovered in the Russian Federation in 2020 and described earlier [3]. No genomic segment reassortment events between A/chicken/Astrakhan/2171-1/2020 virus and the virus isolates of other types or H5 genetic clades were found.

HA protein amino acid sequence of A/chicken/Astrakhan/2171-1/2020 H5N8 isolate was analyzed. $G_{225}QRG_{228}$ amino acids (according to H3 subtype numbering) were detected in viral protein receptor-binding domain (Fig. 2).

According to the earlier studies, a group of $G_{225}QRG_{228}$ amino acids is characteristic of the viruses isolated from birds and targets to SA*α*-2,3-gal receptors [4].

According to the literature data, virus hemagglutinin contains marker amino acids that are located in the receptor-binding domain and conservative for the virus isolates recovered from birds but distinct from the relevant amino acids in the virus isolates recovered from mammals [4, 5].

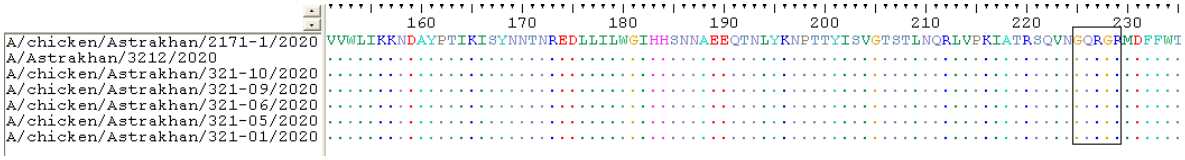

Fig. 2. Segment of deduced amino acid sequence of the virus HA receptor-binding domain

Рис. 2. Участок предсказанной аминокислотной последовательности рецептор-связывающего домена вирусного гемагглютинина

Table 1 shows HA protein amino acid residues related to receptor specificity characteristic of H5 AI viruses and human influenza virus.

For A/chicken/Astrakhan/2171-1/2020 isolate, AIV-characteristic amino acid residues are located in all above-mentioned positions (except for 159, 222, 227). Amino acid residues non-characteristic of human and avian influenza viruses are located in three positions.

H5 HPAI viruses can infect humans despite that their hemagglutinins interact predominantly with SA*α*-2,3-gal cell receptors. However, in cases of successful influenza virus replication in mammalian cells, the researchers identified mutations in other virus genes supposed to be markers of the influenza virus adaptation to mammals for its replication [6–11]. Table 2 shows amino acid residues of A/chicken/Astrakhan/2171-1/2020 isolate proteins responsible for successful influenza virus replication in birds or mammals. Amino acid substitutions facilitating virus replication in mammalian cells were identified only in NEP protein (70G substitution) and PB1 protein (13P substitution). In all other positions AIV-characteristic amino acid residues were identified.

Additionally, deduced amino acid sequences of the virus proteins mediating the virus sensitivity to medicines were analyzed. Currently adamantanes (rimantadin and amantadine) are the medicines with known mechanism of action. These medicines are blockers of ion channels formed by type A influenza virus M2 protein. Influenza virus resistance to rimantadin and amantadine could be accounted for mutations in M2 protein (L26F, 27 V27A, 30 (A→V/P), 31 (S→N/R), 34 G34E) resulting in changes in ion channel configuration. The following amino acids were identified in M2 protein of A/chicken/Astrakhan/2171-1/2020 isolate: leucine (26L), isoleucine (27I), alanine (30A), serine (31S), glycine (34G) that indicates the virus sensitivity to the adamantanes [10].

Besides adamantanes, there are also virus neuraminidase inhibitors, such as the most common oseltamivir and zanamivir. The most oseltamivir-resistant viruses have histidine-to-tyrosine substitution in position 274 (H274Y) [11]. Marker for oseltamivir resistance was not detected in A/chicken/Astrakhan/2171-1/2020 during the analysis. However, some studies showed that resistance to neuraminidase inhibitors varied depending on NA subtype of influenza virus and different NA mutations could result in different resistance levels. Thus, four marker substitutions related to complete or partial resistance to oseltamivir and zanamivir were identified for N2 subtype viruses [12].

Finally, the analysis for markers of virulence for mammals was carried out. Analysis identified serine (S) amino acid in position 42 of NS1 protein. This substitution is a marker of virulence for mice and is able to antagonize the host cell interferon induction, as well as to prevent NF-*κ*B pathway activation during immune response [7]. Moreover, 30D, 215A amino acid substitutions in M1 protein recognized as determinants of pathogenicity for mice were found [13].

Thus, despite the absence of known marker amino acid substitutions enabling effective replication in the mammals in the studied virus, identification of markers of virulence for laboratory animals indicates the need for further investigations of the biological properties of H5N8 avian influenza viruses.

Table 1
HA protein amino acid residues related to receptor specificity of influenza virus (according to H3 type)

Таблица 1
Аминокислотные остатки белка гемагглютинина, определяющие рецепторную специфичность вируса гриппа (по подтипу H3)

Position No. (according to H3 subtype)	A/chicken/ Astrakhan/ 2171-1/2020	Avian influenza virus	Human influenza virus
136	S	S	T
153	W	W	–
158	N	N/D	N
159	D	N	S
183	H	H	–
190	E	E	D
194	L	L	I
221	S	S	P
222	Q	K	–
225	G	G/N	D
226	Q	Q	L/I
227	R	S	A
228	G	G	S

CONCLUSION

Analyses of A/chicken/Astrakhan/2171-1/2020 virus have showed that it is closely related genetically to H5N8 AIV isolates recovered in 2020 during avian influenza outbreaks occurred in the Russian Federation and belongs to genetic clade 2.3.4.4 of H5 subtype. No genomic segment reassortment between the analyzed virus and both influenza viruses of other types and H5N8

Table 2
Amino acid residues determining influenza virus host range

Таблица 2
Аминокислотные остатки, определяющие спектр хозяев вируса гриппа

Protein / position No.	A/chicken/Astrakhan/ 2171-1/2020	Avian influenza virus	Mammalian influenza virus
PB1			
13	P	L	P
99	H	H	Y
368	I	I	V
PB2			
44	A	A	S
81	T	T	M
199	A	A	S
271	T	T	A
588	A	A	I
613	V	V	I
627	E	E	K
661	A	A	T
674	A	A/S	T
701	D	D	N
702	K	K	R
PA			
28	P	P	L
55	D	D	N
65	S	S	L
100	V	V	A
356	K	K	R
382	E	E	D
400	S	Q/T/S	L
409	S	S	N
552	T	T	S
NP			
33	V	V	I
61	I	I	L
100	R	R	V
109	I	I	V
136	L	L	M
214	R	R	K
283	L	L	P
293	R	R	K
313	F	F	Y
375	D	D	G/E
M1			
137	T	T	A
M2			
16	E	E	G
20	S	S/N	N
28	I	I	I/V
55	L	L	F
78	Q	Q	K
NEP			
70	G	S	G

genetic clade 2.3.4.4 viruses isolated earlier in the Russian Federation was found with whole-genome sequencing. Analysis of virus molecular markers indicates A/chicken/Astrakhan/2171-1/2020 virus adaptation to birds and absence of mutations related to adaptation to mammals, including humans. No markers of the virus resistance to anti-viral medicines of adamantane class, oseltamivir and zanamivir, were identified.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Nikolay G. Zinyakov, Candidate of Science (Biology), Senior Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Artem V. Andriyasov, Candidate of Science (Biology), Leading Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Yevgeniya V. Ovchinnikova, Candidate of Science (Biology), Senior Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Anton A. Kozlov, Candidate of Science (Biology), Junior Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Pavel D. Zhestkov, Post-Graduate Student, Leading Technologist, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Dmitry B. Andreychuk, Candidate of Science (Biology), Head of Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Ilya A. Chvala, Candidate of Science (Veterinary Medicine), Deputy Director for Research and Monitoring, FGBI "ARRIAH", Vladimir, Russia.

Зиняков Николай Геннадьевич, кандидат биологических наук, старший научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Андриясов Артем Валерьевич, кандидат биологических наук, ведущий научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Овчинникова Евгения Валерьевна, кандидат биологических наук, старший научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Козлов Антон Александрович, кандидат биологических наук, младший научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Жестков Павел Дмитриевич, аспирант, ведущий технолог референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Андрейчук Дмитрий Борисович, кандидат биологических наук, заведующий референтной лабораторией вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Чвала Илья Александрович, кандидат ветеринарных наук, заместитель директора по НИР и мониторингу ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

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Morphological characterization of spleen and bursa of Fabricius of Pekin ducks in selenium-deficient area

Ye. E. Lazareva
OOO "IBC Generium", s. Volginsky, Vladimir Oblast, Russia;
ORCID 0000-0003-2559-6851, e-mail: a-e-e@list.ru

SUMMARY

The study was aimed at examination of age-related morphology of spleen and bursa of Fabricius of Pekin ducks with a dietary selenium deficiency and its correction with an organic selenium additive. The experimental study was carried out in 85 day-old ducks divided into two groups, control group and test group, 40 ducks per group, the experiment lasted for 120 days. Control group was fed with standard mixed feed for meat-type poultry. The test group of ducks was fed with the feed supplemented with DAFS-25k organic selenium additive, 1.3 mg/kg of feed, that fully compensated selenium deficiency. The study showed that the dynamics of absolute body weights and relative weight gains in control and test groups correlated to the general biological pattern – increase in absolute parameters and decrease in relative parameters were dependent on age. Therewith, maximum relative weight gain intensity was reported at the age of 15 days and maximum increase in relative spleen and bursa of Fabricius weights was reported in at the age of 30 days. Weight gain parameter drastically decreased on day 75 and remained low up to the age of 120 days. Changes in the relative spleen and bursa of Fabricius weights were non-linear throughout the study. Therewith, in ducks of all ages tested spleen parameters remained approximately at the same level but bursa of Fabricius parameters changed – relative weight of bursa of Fabricius decreased by the age of 45 days and then increased again by the age of 75 days. Relative weight gains and relative spleen weights of ducklings in test group were higher than that ones of ducklings in control group during the examined ontogenesis periods. The opposite pattern was observed for bursa of Fabricius: the above-mentioned parameters in test group were lower than that ones in control group. It was concluded that organic selenium additive had a positive effect on development of the immune system organs and reduced the stress factor impact on duckling organism.

Keywords: Pekin ducks, spleen, bursa of Fabricius, organic selenium additive.

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For correspondence: Yelena E. Lazareva, Pharmacist-Laboratory Assistant, OOO "IBC "Generium", 601125, Russia, Vladimir Oblast, Petushinsky Raion, s. Volginsky, Zavodskaya, 273, *e-mail: a-e-e@list.ru.*

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Морфологическая оценка селезенки и клоакальной бурсы уток пекинской породы в селендефицитном регионе

Е. Э. Лазарева
ООО «МБЦ «Генериум», пгт Вольгинский, Владимирская обл., Россия;
ORCID 0000-0003-2559-6851, e-mail: a-e-e@list.ru

РЕЗЮМЕ

Целью научно-исследовательского опыта стало изучение возрастной морфологии селезенки и клоакальной бурсы уток пекинской породы при дефиците селена в рационе и его корректировке селеноргани́ческим препаратом. Экспериментальное исследование проводили в течение 120 дней на 85 утках суточного возраста, разделенных на контрольную и опытную группы по 40 голов в каждой. Контрольная группа получала стандартный комбикорм для выращивания мясной птицы, а в рацион уток опытной группы добавляли селеноргани́ческий препарат ДАФС-25к в количестве 1,3 мг/кг корма, что полностью восполняло дефицит селена. В ходе исследований установлено, что динамика абсолютной массы тела и ее относительного прироста в контрольной и опытной группах подчиняется общей биологической закономерности – повышение абсолютных показателей и снижение относительных показателей изменяются с возрастом. При этом максимальная интенсивность относительного прироста массы тела отмечается в 15-суточном возрасте, а относительная масса селезенки и клоакальной бурсы – в 30-суточном. Значение показателя прироста массы тела резко снижается на 75-е сутки и остается на низком уровне до 120-суточного возраста. Изменения относительной массы селезенки и клоакальной бурсы на всем протяжении исследования носят нелинейный характер. При этом исследуемые показатели селезенки у птиц всех возрастных групп остаются примерно на одном уровне, а показатели клоакальной бурсы претерпевают ряд изменений – к 45-суточному возрасту относительная масса органа снижается, а затем к 75-м суткам вновь возрастает. В изучаемые периоды онтогенеза показатели относительного прироста массы тела и относительной массы селезенки утят опытной группы превышали аналогичные показатели птиц контрольной группы. В отношении клоакальной бурсы наблюдается обратная картина – указанные значения

в опытной группе были меньше, чем в контрольной. Сделан вывод, что селенорганический препарат оказал положительное влияние на развитие органов иммунной системы и нивелировал воздействие стресс-фактора на организм утят.

Ключевые слова: Утки пекинский породы, селезенка, клоакальная бурса, селенорганический препарат.

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Для корреспонденции: Лазарева Елена Эдуардовна, провизор-лаборант ООО «МБЦ «Генериум», 601125, Россия, Владимирская обл., Петушинский район, пгт Вольгинский, ул. Заводская, стр. 273, *e-mail: a-e-e@list.ru*.

INTRODUCTION

Poultry farming is one of high-technology and profitable agricultural industries in the Russian Federation and supplies consumers with meat, eggs, as well as technical raw materials – down and feathers. Duck farming is a promising trend in poultry industry along with chicken farming owing to high growth rate of this poultry species [1]. Currently, special attention is paid to the meat-type duck farming, where the significant share belongs to Pekin duck farming [2]. Pekin ducks are easy to raise and can reach body weight of 3 kg at the age of 6 weeks when they are fed with a balanced diet [3].

It is impossible to realize animal and bird potential without understanding of development pattern for organs and systems thereof, where the immune system plays the key role, conferring protection from diseases of various etiologies. Several studies have been devoted to examination of the development of the poultry immune system organs [4–10], but the data on development of Pekin duck immune system organs are non-systematic and fragmented [2, 11, 12].

Feed additives fortifying poultry diet with the required microelements and vitamins as well as reducing product losses have an impact on internal organ growth and development [13, 14]. Selenium is currently recognized as one of the most important elements [15], it stimulates efficient utilization of feed metabolic energy, enhances nutrient digestibility and intake and thereby contributes to poultry performance improvement [16].

Thus, the study goal was to examine age-related morphology of spleen and bursa of Fabricius of Pekin ducks with a dietary selenium deficiency and its correction with an organic selenium additive.

MATERIALS AND METHODS

Clinically healthy Pekin ducks obtained from OOO PPK "Romashino", Moscow Oblast, were used for the study. The ducks were raised on a backyard farm located in the Gus-Khrustalny Raion, Vladimir Oblast, in accordance with requirements and standards laid down in the Methodical Guidelines for poultry establishment technological designing, RD-APK1.10.05.04-13.

All experiments were carried out in poultry in strict accordance with the International Standard, GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification as well as in accordance with Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 on protection of animals used for scientific purposes.

Experimental study was carried out in 85 day-old ducks for 120 days. The ducks were randomly divided into two groups, control group and test group, 40 birds per group. Five ducklings were subjected to diagnostic killing before the experiment to determine syntopy of immune system organs and their absolute and relative weights. Control group was fed with standard mixed feed for meat-type poultry and test group was fed with the feed supplemented with organic selenium additive, DAFS-25k, 1.3 mg/kg of the feed. The amount of feed additive was estimated based on the test of the feed for actual selenium content performed in the Kostroma Oblast Veterinary Laboratory. The poultry were provided with the free access to drinking water. The ducks were daily examined for their appearance, mobility, feed intake. Five ducks from each group were weighed and killed with generally accepted methods at a 15 day-interval.

Spleen and bursa of Fabricius were dissected and examined for their topography, colour, form, size and integrity. The ducks were weighed with a 1.0 g precision torsion balance. Spleen and bursa of Fabricius were weighed with Pocket Scale MH-200 0.01 g precision electronic balance immediately after necropsy. Relative weight was calculated in accordance with the formula proposed by G. G. Avtandilov:

$$w_0 = w_n / W \times 100\%,$$

where w_0 and W – absolute spleen (or bursa of Fabricius) weight and live body weight, respectively. Dynamics of relative weight gain in ducks was calculated in accordance with Brodi formula:

$$K = \frac{W_t - W_0}{0{,}5 \times (W_t + W_0)} \times 100\%,$$

where K – relative weight gain (%) during for a specified period of time;

W_t – weight at given age;

W_0 – initial weight.

Estimated numerical values were processed biometrically as proposed by G. F. Lakin (1990).

RESULTS AND DISCUSSION

Our studies showed that use of organic selenium feed additive at the recommended dose had no negative effect

on the Pekin ducks: birds in both groups willingly ate feed, were motile and adequately responded to external stimuli. Analysis of absolute body weight and relative body weight gain dynamics in control and test groups has indicated that it correlates to the general biological pattern – increase in absolute parameters and decrease in relative parameters depend on age (Table 1, Fig. 1). The weights of ducks in both groups changed synchronously, however, ducks of test group significantly outperformed their counterparts in control group throughout the study.

Therewith, weight gains were the highest in 15 day-old ducks and then sharply decreased at the age of 30 and 75 days that could be accounted for the onset of critical development periods – embryonic down replacement by first feathers and juvenile molting, respectively. Further, relative body weight gains in ducks remained low up to 120 days of age. Absolute weight gains in ducks of tests group were consistently higher as compared to that ones in ducks of control group owing to favourable organic selenium additive effect on body weight gain in ducks. Obtained data on dynamics of absolute and relative body weights in ducks of control and tests groups are consistent with the data provided by other authors [13], however there are insignificant differences in absolute values [2].

Table 1
Live weights and relative weight gains of Pekin ducks in control and test groups

Таблица 1
Показатели живой массы и относительного прироста уток пекинской породы контрольной и опытной групп

Age, days	Live body weight, g		Relative weight gain, %	
	Control group	Test group	Control group	Test group
1	83.60 ± 3.62		–	–
15	261.48 ± 15.59	274.50 ± 17.48*	103.09	106.62
30	458.29 ± 17.49	484.58 ± 33.56*	54.69	55.35
45	887.34 ± 32.60	971.28 ± 40.56*	63.77	66.86
60	1,512.62 ± 15.28	1,751.26 ± 28.38*	52.11	57.30
75	1,698.39 ± 16.04	2,014.49 ± 29.31*	11.57	13.98
90	2,047.27 ± 18.54	2,439.82 ± 15.49*	18.63	19.10
105	2,429.49 ± 17.74	2,950.48 ± 20.36*	17.08	18.95
120	2,670.28 ± 17.88	3,250.43 ± 14.36*	9.44	9.67

* $P \leq 0.05$ as compared to control ($P \leq 0,05$ в сравнении с контролем).

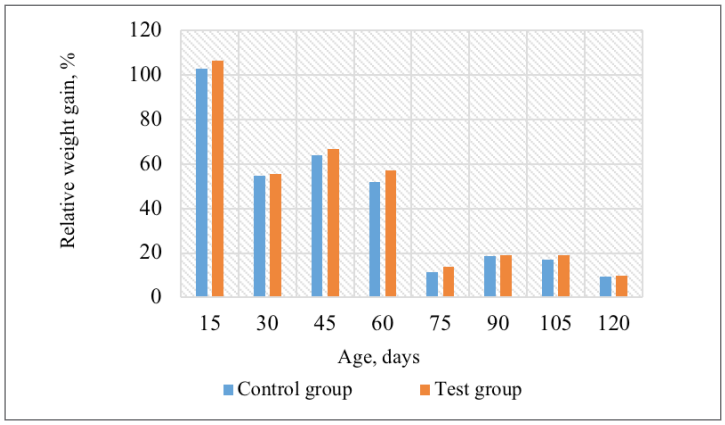

Fig. 1. Dynamics of relative weight gains of Pekin ducks in control and test groups

Рис. 1. Динамика относительного прироста массы тела уток пекинской породы контрольной и опытной групп

Examination of immune system organs of Pekin ducks showed that spleen was oval, of reddish-brown colour and located on the left side in phrenic cavity between proventriculus and gizzard (Fig. 2). Bursa of Fabricius was an elongated-oval unpaired cavity organ in the form of diverticulum of the dorsal wall of proctodeum of the cloaca and connected with it by a duct. Its dorsal surface lied close to lumbosacral bone, its ventral surface was in contact with the dorsal wall of the cloaca. Findings on syntopy and shape of the examined organs are consistent with the data obtained by other authors [9, 11].

Changes in absolute and relative spleen weights were consistent with general biological pattern: absolute spleen weight increased and relative spleen weight decreased with age. It should be noted that changers in relative spleen weights in ducks of control and test groups were non-linear (Table 2, Fig. 3).

Thus, the relative spleen weight gain was the highest at the age of 15 days and 30 days in both groups. Then, this parameter synchronously decreased in ducks by the age of 45 days that could be accounted for completion of the period of embryonic down replacement with first feathers that was stressful for birds. More drastic decrease in relative spleen weight in ducks of test group could be indicative of pronounced adaptive processes associated with selenium in the duck body. Relative spleen weights remained stable up to the age of 120 days, therewith, relative spleen weights were higher in ducks of test group. Other authors also noted that critical periods and technogenic factors had an impact on the immune system organ development [10].

Analysis of relative bursa of Fabricius weight dynamics showed that this parameter was non-linear similar to the relative spleen weight parameter (Table 3, Fig. 4).

Relative bursa of Fabricius weight drastically increased in ducks of control and test group by the age of 15 days and kept increasing and reached maximum by the age of 30 days; however this parameter decreased by the age of 45 days. Relative bursa of Fabricius weight increased in both groups at the age of 60 to 75 days probably due to the onset of the next critical period of the duck development. However, this parameter tended to decrease in ducks of both groups already from the age of 90 days. It should be noted that relative bursa of Fabricius weight in control group within the all tested age periods were higher than that one in test group that was associated with higher body weight of ducks in test group. Available literature data on bursa of Fabricius size during postnatal ontogenesis are quite contradictory. Findings on the dynamics of the relative organ weight obtained during the study are consistent with the data of some authors [17–21], but differ from the data on maximum absolute mass of the organ [17–20, 22, 23].

CONCLUSION

The maximum relative weight gain of Peking ducks was observed at the age of 15 days, then this parameter decreased with a sharp drop on day 30 and day 75. The relative weight gains in ducks of test group were higher

than in ducks of control group during all tested periods of ontogenesis.

Dynamics of changes in relative spleen weight showed that the development of the organ was almost the same both in control group and test group. Therewith, maximum values were reported in ducks at the age of 15 and 30 days and then those values just slightly and synchronously fluctuated in both groups. Relative spleen weight in ducks of test group was higher than that one in ducks of control group throughout the study.

Relative bursa of Fabricius weight in Pekin ducks intensively increased up to the age of 15 days, but reached the peak level at the age of 30 days both in control and test groups. There were more pronounced wave-like fluctuations in the relative weight of the examined organ, associated with critical periods due to diet changes and moltings in control group.

Thus, it can be concluded that the organic selenium additive had a positive effect on the development of the spleen and bursa of Fabricius, enhancing the adaptive capacity of the duck body during critical periods of its development.

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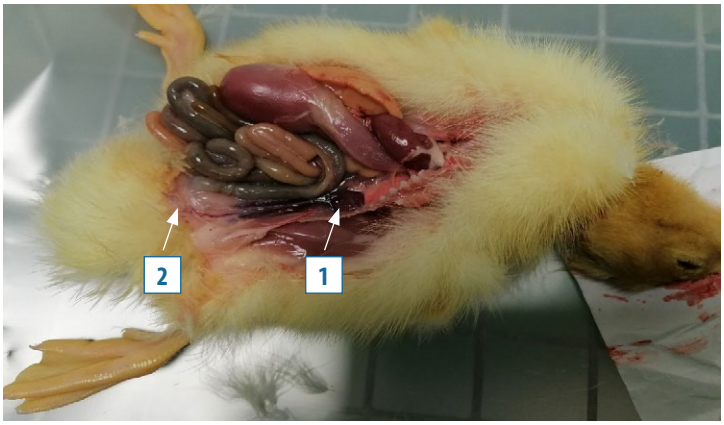

Fig. 2. Phrenic organs of day-old Pekin ducks: 1 – spleen, 2 – bursa of Fabricius

Рис. 2. Органы грудобрюшной полости суточного утенка пекинской породы: 1 – селезенка, 2 – клоакальная бурса

Table 2
Dynamics of absolute and relative spleen weight parameters of Pekin ducks in control and test groups

Таблица 2
Динамика абсолютных и относительных показателей массы селезенки уток пекинской породы контрольной и опытной групп

Age, days	Control group		Test group	
	Absolute spleen weight, g	Relative spleen weight, %	Absolute spleen weight, g	Relative spleen weight, %
1	0.13 ± 0.02	0.16	0.13 ± 0.02	0.16
15	0.62 ± 0.04	0.24	0.63 ± 0.30	0.23
30	1.15 ± 0.05	0.25	1.23 ± 0.04	0.25
45	1.73 ± 0.11	0.19	1.84 ± 0.12	0.19
60	3.00 ± 0.22	0.20	3.50 ± 0.14	0.20
75	3.23 ± 0.12	0.19	3.95 ± 0.21	0.20
90	3.99 ± 0.14	0.19	5.10 ± 0.16*	0.21
105	4.76 ± 0.17	0.20	6.40 ± 0.19*	0.22
120	5.26 ± 0.21	0.20	7.20 ± 0.18*	0.22

* $P \leq 0.05$ as compared to control ($P \leq 0,05$ в сравнении с контролем).

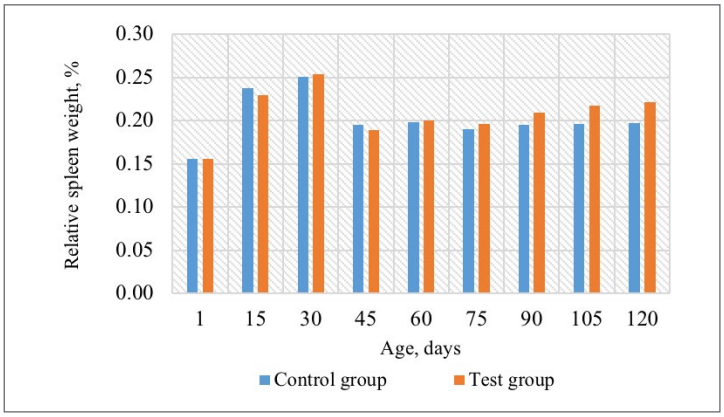

Fig. 3. Dynamics of relative spleen weight of Pekin ducks in control and test groups

Рис. 3. Динамика относительной массы селезенки уток пекинской породы контрольной и опытной групп

Table 3
Dynamics of absolute and relative bursa of Fabricius weight parameters of Pekin ducks in control and test groups

Таблица 3
Динамика абсолютных и относительных показателей массы клоакальной бурсы уток пекинской породы контрольной и опытной групп

Age, days	Control group		Test group	
	Absolute bursa of Fabricius weight, g	Relative bursa of Fabricius weight, %	Absolute bursa of Fabricius weight, g	Relative bursa of Fabricius weight, %
1	0.02 ± 0.01	0.02	0.02 ± 0.01	0.02
15	0.26 ± 0.01	0.10	0.25 ± 0.01	0.09
30	0.55 ± 0.01	0.12	0.48 ± 0.01	0.10
45	0.71 ± 0.02	0.08	0.68 ± 0.02	0.07
60	1.65 ± 0.03	0.11	1.58 ± 0.03	0.09
75	1.87 ± 0.03	0.11	2.01 ± 0.03*	0.10
90	1.64 ± 0.05	0.08	1.98 ± 0.06*	0.08
105	1.39 ± 0.05	0.06	1.35 ± 0.05	0.05
120	1.24 ± 0.06	0.05	1.11 ± 0.04	0.03

* $P \leq 0.05$ as compared to control ($P \leq 0{,}05$ в сравнении с контролем).

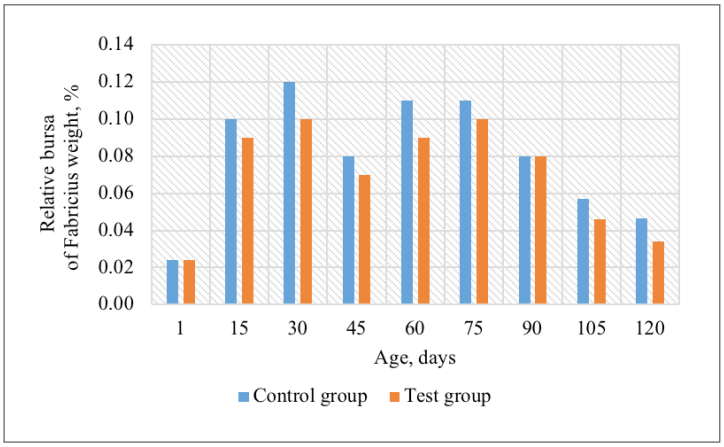

Fig. 4. Dynamics of relative bursa of Fabricius weight of Pekin ducks in control and test groups

Рис. 4. Динамика относительной массы клоакальной бурсы уток пекинской породы контрольной и опытной групп

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INFORMATION ABOUT THE AUTHOR / ИНФОРМАЦИЯ ОБ АВТОРЕ

Yelena E. Lazareva, Pharmacist-Laboratory Assistant, OOO "IBC Generium", s. Volginsky, Vladimir Oblast, Russia.

Лазарева Елена Эдуардовна, провизор-лаборант ООО «МБЦ «Генериум», пгт Вольгинский, Владимирская обл., Россия.

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Epidemic situation on enzootic bovine leukosis in public and individual farms in the Republic of Dagestan

A. R. Mustafayev
Caspian Regional Research Veterinary Institute – Branch of Dagestan Agriculture Science Center, Makhachkala, Republic of Dagestan, Russia;
ORCID 0000-0002-5142-8360, e-mail: mustafaev_arkif@mail.ru

SUMMARY
The spread of the bovine leukemia virus impedes the development of livestock production and causes considerable losses. Despite the measures implemented, the problem of bovine leukosis eradication remains relevant in different regions of Russia. The article presents data on distribution of enzootic bovine leukosis in the Republic of Dagestan. Over the past five years, the lowest level (1.02%) of leukemia virus infection in cattle in the Republic was recorded in 2020. Laboratory tests for bovine leukosis were carried out in 41 raions and 7 municipal districts: no disease was diagnosed in 12 raions and 4 municipal districts, and the animal seropositivity index in the rest of areas was less than 1%. A high level of animal infection with the leukemia virus was recorded in the following raions: Dakhadaevsky (10.3%), Shamilsky (7.9%), Tarumovsky (3.1%), Kizlyarsky (2.3%), Babayurtovsky (2.2%), as well as in the town of Yuzhno-Sukhokumsk (3.8%). In other districts, the parameter's values ranged from 1 to 2%. In total, 524,930 animal sera samples were serologically tested using the immunodiffusion method, out of which 5,362 samples were seropositive in 2020. 1,265 sera samples from animals infected with the leukemia virus were tested using the hematological method, 251 animals (19.8%) with persistent leukocytosis were identified, which is the average for the past years. Comparative analysis of the morbidity rate for bovine leukemia virus in farms of different categories showed that in public farms of the republic the percentage of infection level was higher (3.3%) than in the individual sector (0.7%). Thus, bovine leukemia infection level in the republic tends to decrease. Nevertheless, the infection and morbidity rates in cattle remain high in some raions and municipal districts, in particular, in the public sector.

Keywords: Enzootic bovine leukosis, infection level, distribution, serology, morbidity, Republic of Dagestan.

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For correspondence: Arkif R. Mustafayev, Candidate of Science (Veterinary Medicine), Leading Researcher, Laboratory of Infectious Pathology of Farm Animals, Caspian Regional Research Veterinary Institute – Branch of Dagestan Agriculture Science Center, 367000, Russia, Republic of Dagestan, Makhachkala, Dakhadaeva str., 88, *e-mail: mustafaev_arkif@mail.ru.*

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Эпизоотическая обстановка по энзоотическому лейкозу крупного рогатого скота в общественных и индивидуальных хозяйствах Республики Дагестан

А. Р. Мустафаев
Прикаспийский зональный научно-исследовательский ветеринарный институт – филиал ФГБНУ «Федеральный аграрный научный центр Республики Дагестан» (Прикаспийский зональный НИВИ – филиал ФГБНУ «ФАНЦ РД»), Республика Дагестан, г. Махачкала, Россия;
ORCID 0000-0002-5142-8360, e-mail: mustafaev_arkif@mail.ru

РЕЗЮМЕ
Распространение вируса лейкоза крупного рогатого скота препятствует развитию животноводства и наносит значительный ущерб. Несмотря на принимаемые меры, проблема ликвидации лейкоза в различных регионах России продолжает оставаться актуальной. В статье представлены данные по распространению лейкоза крупного рогатого скота в Республике Дагестан. За последние пять лет наименьший процент (1,02%) инфицированности животных вирусом лейкоза в республике отмечен в 2020 г. Лабораторные исследования на лейкоз крупного рогатого скота проводились в 41 районе и 7 городских округах, в 12 и 4 из них, соответственно, заболевание не диагностировано, в остальных показатель серопозитивности животных составил менее 1%. Высокий уровень инфицированности животных вирусом лейкоза был установлен в следующих районах: Дахадаевском (10,3%), Шамильском (7,9%), Тарумовском (3,1%), Кизлярском (2,3%), Бабаюртовском (2,2%), а также в г. Южно-Сухокумске (3,8%). В остальных районах показатели находились на уровне от 1 до 2%. Всего за 2020 г. серологическим методом с использованием реакции иммунодиффузии было исследовано 524 930 проб сыворотки крови животных, из них 5362 оказались сероположительными. От инфицированных вирусом лейкоза животных гематологическим методом

исследовали 1265 проб крови, выявлено 251 животное (19,8%) с персистентным лейкоцитозом, что является средним показателем за прошедшие годы. Сравнительный анализ распространенности вируса лейкоза крупного рогатого скота в хозяйствах разных категорий показал, что в общественных хозяйствах республики процент инфицированности выше (3,3%), чем в индивидуальном секторе (0,7%). Таким образом, лейкоз крупного рогатого скота в республике имеет тенденцию к снижению. Тем не менее в некоторых районах и городских округах инфицированность и заболеваемость животных остается высокой, особенно в общественном секторе.

Ключевые слова: Энзоотический лейкоз крупного рогатого скота, инфицированность, распространенность, серология, заболеваемость, Республика Дагестан.

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Для корреспонденции: Мустафаев Аркиф Рамазанович, кандидат ветеринарных наук, ведущий научный сотрудник лаборатории инфекционной патологии сельскохозяйственных животных, Прикаспийский зональный НИВИ – филиал ФГБНУ «ФАНЦ РД», 367000, Россия, Республика Дагестан, г. Махачкала, ул. Дахадаева, 88, *e-mail: mustafaev_arkif@mail.ru.*

INTRODUCTION

Bovine leukosis virus, or bovine leukemia virus (BLV) belongs to the genus *Deltaretrovirus* of the family *Retroviridae*. Many viruses of this family directly or indirectly (by way of malignancy or proliferation of organs with neoplastic cells) cause neoplasms or leukemias of the hematopoietic system in animals and humans. The bovine leukemia causative agent refers to exogenous viruses and causes the disease known as bovine leukosis. Due to its location-specific nature it is also called enzootic bovine leukosis.

The characteristic feature of the disease is that it mainly occurs in animals in a chronic form with no visible clinical signs. Nevertheless, bovine leukosis diagnosis is made using serological, hematological and other types of laboratory testing. The bovine leukosis is rarely clinically diagnosed in farms by veterinary specialists, and the morbidity rate in infected livestock can reach up to 3–10% depending on the epizootic tension and the animal keeping conditions.

The bovine leukemia virus is transmitted from a diseased animal to a healthy one through fluids (blood, saliva, milk, etc.) that contain cells infected with this pathogen (B-lymphocytes), and to a lesser extent – through monocytic macrophages. The transmission routes of the virus are horizontal and vertical. Cattle (cows, bulls) of different breeds and all ages (older than 5–6 months) are susceptible to the leukemia virus in natural conditions. However, in spontaneous conditions BLV can be transmitted to other species (buffalo, zebu, sheep, elk, yak, alpaca) [1–4]. As the previous study results showed, all cow breeds bred in the Republic of Dagestan (red steppe, black-and-white, Simmental, Caucasian brown, Shvitskaya, indigenous breed) of different ages are susceptible to BLV, but the highest percentage of infection was detected in animals aged 5–7 years [5].

Enzootic bovine leukosis is widely distributed in many countries (USA, Bulgaria, Uzbekistan, etc.), as well as in the Russian Federation [6–9]. The disease used to be hematologically diagnosed in the Republic of Dagestan during the Soviet times; since 1988 the serological method has been implemented in veterinary laboratories and immunodiffusion test (IDT) is used for the diagnosis. The number of infected animals detected in the Republic increased as a result of bovine leukosis serological diagnosis introduced in Dagestan. However, the failure to implement the leukosis control program, as well as the lack of health-improving and preventive measures led to increased frequency of infection in animals in the farms of the Republic [10].

In view of the above, the aim was to conduct an epizootological analysis of the enzootic bovine leukosis distribution in the Republic based on the farm status.

MATERIALS AND METHODS

The official data of the GBU RD "Republican Veterinary Laboratory", as well as the results of internal testing conducted in the Laboratory for Infectious Pathology of Livestock Animals of the Caspian Zonal NIVI within the last 10 years served the basis for the analysis of the bovine leukosis epidemic situation. A retrospective analysis was performed using hematological and serological methods. Serological diagnosis for bovine leukosis was performed using immunodiffusion test (IDT).

Diagnostic studies for enzootic bovine leukemia were conducted in accordance with the "Methodical guidelines for the diagnosis of bovine leukosis" [11], and epizootological studies were conducted in compliance with the "Methodical guidelines for bovine leukosis epizootological studies" [12].

RESULTS AND DISCUSSION

Over the past 5 years large-scale serological testing for bovine leukosis was conducted in the Republic of Dagestan using immunodiffusion test (Fig. 1). The largest number of sera samples were tested in 2019 (625,970), of which 15,578 (2.50%) were seropositive. The smallest number of studies were conducted in 2017 (7,466), when seropositive animals were detected in 577 cases (7.70%). A high level of BLV infection in cattle population was noted in 2016 – 1,433 (13.20%) sera samples out of 10,842 gave a positive result when IDT was used. Starting from 2018, the number of leukosis diagnostic tests increased after the Action Plan for Bovine Leukosis Prevention and Control in Republic of Dagestan for 2017–2020 (Order of the Government of the Republic of Dagestan No. 323-r of September 11, 2017) was adopted. Thus, 223,293 sera samples

were tested in 2018, the seropositivity of animals was 4.03% (8,998 animals). As compared to 2017, the number of serological tests for bovine leukosis in 2018 increased by almost 30 times, and the percentage of infection decreased by about 2 times. By 2020, the percentage of infection with the bovine leukemia virus was 1.02%, i.e. 5,362 out of 524,930 animals were seropositive.

As it can be seen in Figure 1, in recent years the maximum number of serological tests for bovine leukosis were performed in 2019–2020, while a high percentage of animal infection with BLV was recorded in 2016–2017. This is due to the fact that until 2018 diagnostic tests for leukosis in the Republic were carried out randomly and using small numbers of cattle kept in flat areas where transhumance is practiced.

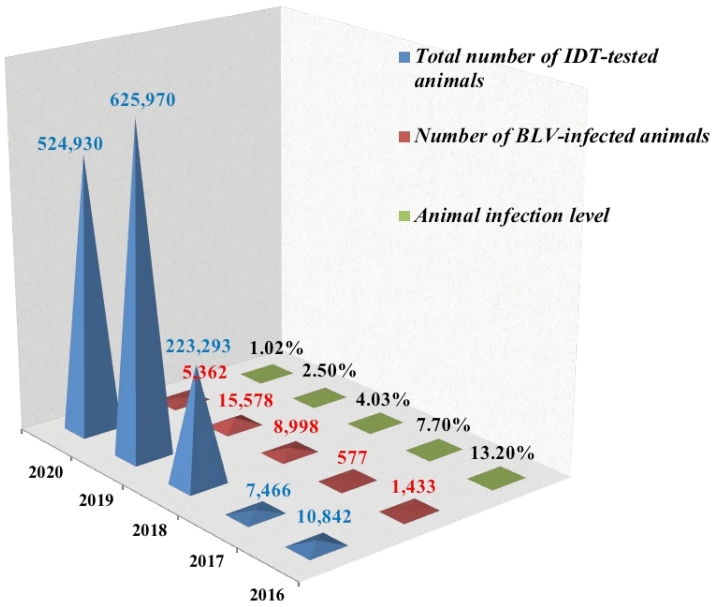

Fig. 1. Distribution of enzootic bovine leukosis in the Republic of Dagestan in 2016–2020

Рис. 1. Распространение лейкоза крупного рогатого скота в Республике Дагестан в 2016–2020 гг.

The hematological studies were conducted in the Republic to identify diseased animals among those infected with the leukemia virus over the past 10 years, which showed a high percentage of persistent leukocytosis. As it can be seen in Figure 2, the largest number of hematological tests conducted in cattle were recorded in 2019 (6,070), 2020 (1,265) and 2018 (1,202), and the lowest – in 2015 (79) and 2012 (81). A high percentage of animals with hematological disease was observed in 2011 (44.9%) and 2010 (42.8%), and a low percentage – in 2014 (16.7%) and 2015 (17.7%). Within the past 3 years the bovine leukosis morbidity was 24.3% in 2018, 24.4% – in 2019, 19.8% – in 2020. The results obtained are explained by the fact that the farms of the Republic do not carry out timely culling of infected adult livestock in case of loss of productivity, and cattle infected with the leukosis virus continue to be kept in the herd.

As it is shown in Figure 2, the bovine leukosis morbidity fluctuations over time are minimal. This is due to the fact that animals with hematological disease are not subjected to slaughter.

In the course of the epizootological analysis, the 2020 data obtained from the GBU RD "Republican Veterinary Laboratory" were analyzed (Table 1). The laboratory tests conducted in 41 raions and 7 towns of the Republic showed that bovine leukosis was detected in cattle in 29 and 3 administrative units, respectively. As it is shown in Table 1, the disease was not diagnosed in 12 raions (Akhvakhsky, Akhtynsky, Buinaksky, Dokuzparinsky, Kazbekovsky, Kaitagsky, Karabudakhkentsky, Kurakhsky, Magaramkentsky, Novolaksky, Suleiman-Stalsky and Khivsky) and in 4 towns (Makhachkala, Kaspiysk, Derbent and Dagestanskiye Ogni). A high level of BLV infection was found in the following raions: Dakhadayevsky – 10.3%, Shamilsky – 7.9%, Tarumovsky – 3.1%, Kizlyarsky – 2.3%, Babayurtovsky – 2.2%, as well as in Yuzhno-Sukhokumsk – 3.8%.

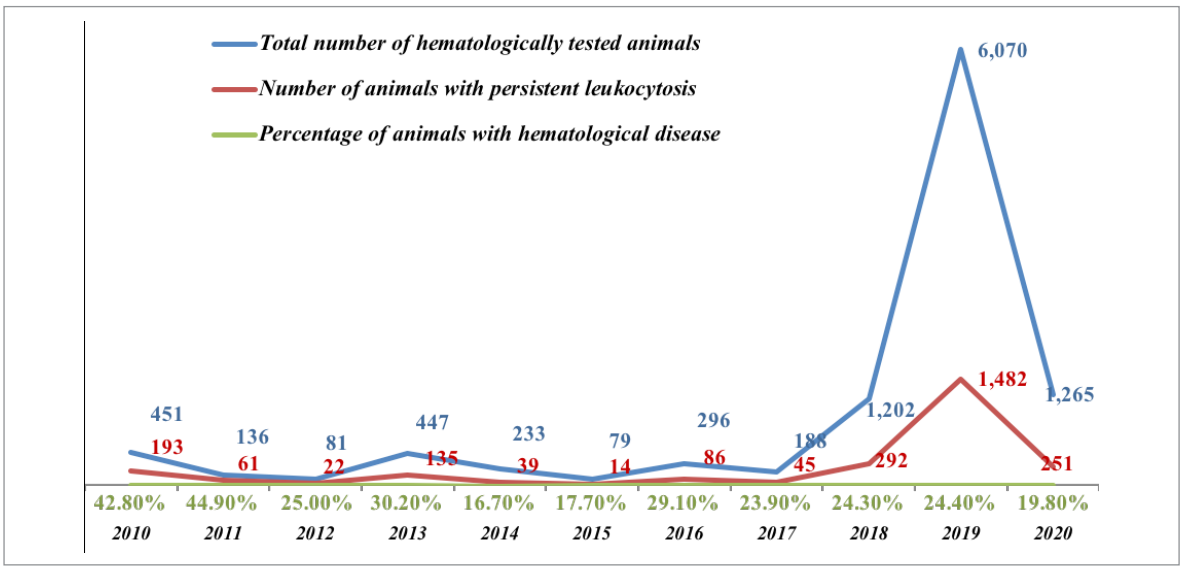

Fig. 2. Dynamics of detection of leukosis-infected animals with hematological disease in the Republic of Dagestan in 2010–2020

Рис. 2. Динамика выявления гематологически больных лейкозом животных в Республике Дагестан за 2010–2020 гг.

Table 1
Epidemic monitoring of bovine leukosis in the Republic of Dagestan in 2020 (according to the data of GBU RD "Republican Veterinary Laboratory")

Таблица 1
Эпизоотологический мониторинг лейкоза крупного рогатого скота в Республике Дагестан в 2020 г. (по данным ГБУ РД «Республиканская ветеринарная лаборатория»)

No.	Raions and municipal districts	Serological testing			Hematological testing		
		Number of animals	IDT-positive	%	Number of sera samples	Diseased animals identified	%
1	Agulsky	1,666	7	0.40	–	–	–
2	Akushinsky	35,471	131	0.37	–	–	–
3	Ahvakhsky	18,261	–	–	–	–	–
4	Akhtynsky	5,725	–	–	–	–	–
5	Babayurtovsky	12,710	284	2.20	–	–	–
6	Botlikhsky	11,378	154	1.40	–	–	–
7	Buinaksky	5,052	–	–	–	–	–
8	Gergebilsky	14,492	79	0.50	–	–	–
9	Gumbetovsky	2,834	4	0.10	–	–	–
10	Gunibsky	33,864	278	0.80	–	–	–
11	Dakhadaevsky	2,754	285	10.30	–	–	–
12	Derbentsky	13,817	31	0.20	–	–	–
13	Dokuzparinsky	2,562	–	–	–	–	–
14	Kazbekovsky	3,500	–	–	–	–	–
15	Kaitagsky	41	–	–	–	–	–
16	Kizilyurtovsky	14,159	10	0.10	–	–	–
17	Kumtorkalinsky	1,545	4	0.30	–	–	–
18	Kayakentsky	5,812	29	0.50	–	–	–
19	Karabudakhkentsky	33,310	–	–	–	–	–
20	Kizlyarsky	22,250	642	2.30	–	–	–
21	Kulinsky	18,358	135	0.70	–	–	–
22	Kurakhsky	2,855	–	–	–	–	–
23	Laksky	15,545	5	0.03	–	–	–
24	Levashinsky	16,756	145	0.90	–	–	–
25	Magaramkentsky	10,785	–	–	–	–	–
26	Novolaksky	1,494	–	–	–	–	–
27	Nogaisky	16,243	6	0.04	–	–	–
28	Rutulsky	2,776	41	1.50	–	–	–
29	Suleiman-Stalsky	3,425	–	–	–	–	–
30	Sergokalinsky	6,897	20	0.30	–	–	–
31	Tabasaransky	8,577	18	0.20	–	–	–
32	Tarumovsky	7,097	223	3.10	271	86	31.70
33	Tlyaratinsky	14,758	175	1.20	–	–	–
34	Untsukulsky	8,373	65	0.80	–	–	–
35	Khasavyurtovsky	77,467	1,368	1.80	303	59	19.50
36	Khivsky	4,893	–	–	–	–	–
37	Khunzakhsky	8,693	27	0.30	–	–	–
38	Tsumadinsky	19,622	83	0.40	–	–	–
39	Tsuntinsky	6,599	17	0.30	–	–	–
40	Charodinsky	19,280	335	1.70	685	100	14.60
41	Shamilsky	8,209	651	7.90	–	–	–
42	Kizlyar	421	4	1.00	–	–	–
43	Makhachkala	100	–	–	–	–	–
44	Kaspiysk	98	–	–	–	–	–
45	Izberbash	902	6	0.70	–	–	–
46	Yuzhno-Sukhokumsk	2,663	100	3.80	6	6	100
47	Derbent	354	–	–	–	–	–
48	Dagestankiye Ogni	487	–	–	–	–	–
	Total	**524,930**	**5,362**	**1.02**	**1,265**	**251**	**19.80**

Table 2
Distribution of bovine leukosis in individual and public farms in the Republic of Dagestan in 2020

Таблица 2
Распространение лейкоза крупного рогатого скота в индивидуальных и общественных хозяйствах Республики Дагестан в 2020 г.

No.	Raions and municipal districts	Total of animals tested for bovine leukosis					
		in individual farms			in public farms		
		Number of IDT-tested animals	IDT-positive	%	Number of IDT-tested animals	IDT-positive	%
1	Agulsky	–	–	–	1,666	7	0.40
2	Akushinsky	28,669	81	0.30	6,802	50	0.70
3	Ahvakhsky	18,261	–	–	–	–	–
4	Akhtynsky	5,725	–	–	–	–	–
5	Babayurtovsky	10,420	140	1.30	2,290	147	6.40
6	Botlikhsky	10,892	130	1.20	486	24	4.90
7	Buinaksky	5,052	–	–	–	–	–
8	Gergebilsky	14,492	79	0.50	–	–	–
9	Gumbetovsky	2,649	4	0.20	185	–	–
10	Gunibsky	33,814	278	0.80	50	–	–
11	Dakhadaevsky	–	–	–	2,754	285	10.30
12	Derbentsky	13,817	31	0.20	–	–	–
13	Dokuzparinsky	2,562	–	–	–	–	–
14	Kazbekovsky	3,500	–	–	–	–	–
15	Kaitagsky	–	–	–	41	–	–
16	Kizilyurtovsky	10,672	9		3,487	1	0.03
17	Kumtorkalinsky	1,545	4	0.30	–	–	–
18	Kayakentsky	5,812	29	0.50	–	–	–
19	Karabudakhkentsky	33,310	–	–	–	–	–
20	Kizlyarsky	22,250	642	2.30	–	–	–
21	Kulinsky	9,305	15	0.20	9,053	120	1.30
22	Kurakhsky	2,855	–	–	–	–	–
23	Laksky	15,545	5	0.03	–	–	–
24	Levashinsky	12,982	96	0.70	3,774	49	1.30
25	Magaramkentsky	10,785	–	–	–	–	–
26	Novolaksky	1,494	–	–	–	–	–
27	Nogaisky	16,243	6	0.04	–	–	–
28	Rutulsky	2,086	31	1.50	690	10	1.45
29	Suleiman-Stalsky	3,425	–	–	–	–	–
30	Sergokalinsky	6,787	20	0.30	110	–	–
31	Tabasaransky	8,577	18	0.20	–	–	–
32	Tarumovsky	–	–	–	7,097	223	3.10
33	Tlyaratinsky	13,490	151	1.10	1,268	24	1.90
34	Untsukulsky	8,253	62	0.80	93	3	3.20
35	Khasavyurtovsky	77,467	1,368	1.80			
36	Khivsky	4,771	–	–	122	–	–
37	Khunzakhsky	8,693	27	0.30	–	–	–
38	Tsumadinsky	19,460	77	0.40	159	6	3.80
39	Tsuntinsky	6,447	15	0.20	152	2	1.30
40	Charodinsky	9,686	51	0.50	9,594	280	3.00
41	Shamilsky	1,784	10	0.60	6,425	641	10.00
42	Kizlyar	421	4	1.00	–	–	–
43	Makhachkala	–	–	–	100	–	–
44	Kaspiysk	–	–	–	98	–	–
45	Izberbash	902	6	0.70	–	–	–
46	Yuzhno-Sukhokumsk	2,663	100	3.80	–	–	–
47	Derbent	354	–	–	–	–	–
48	Dagestankiye Ogni	487	–	–	–	–	–
	Total	**468,404**	**3,489**	**0.70**	**56,496**	**1,872**	**3.30**

The 1–2% morbidity rate due to leukosis was registered in 4 raions of the Republic: Khasavyurtovsky (1.8%), Charodinsky (1.7%), Botlikhsky (1.4%), Tlyaratinsky (1.2%), and in the town of Kizlyar (1.0%). In other raions and municipal districts the percentage of BLV infection was less than 1.0%. In 2020 a total of 524,930 animal sera samples were serologically tested using IDT, 5,362 (1.02%) samples among them were seropositive. In 2020 1,265 sera samples from IDT-positive animals were tested hematologically, and 251 animals (19.8%) were identified as having the hematological disease. Hematological studies of animal sera for leukosis were conducted in 3 raions (Tarumovsky, Khasavyurtovsky, Charodinsky) and in the town of Yuzhno-Sukhokumsk, which does not fully reflect the situation on bovine leukosis morbidity in the Republic.

Thus, based on the official data provided by the GBU RD "Republican Veterinary Laboratory", the number of BLV-infected animals has sharply decreased to 1.02%, being the lowest indicator in the Republic in recent years [13].

Cattle are kept in private (individual) or public (state unitary enterprise, agricultural production cooperative, small-scale farms, etc.) sectors in the Republic of Dagestan. The farm's status is also an important factor in the distribution of BLV. For example, contrary to the private sector, a large number of animals are kept together in public farms, which means that there is close contact of livestock and joint processes of cow milking, feeding, veterinary and zootechnical handling procedures. All this leads to increased level of BLV infection in animals in public farms of the Republic (Table 2).

As it is shown in Table 2, the number of cattle kept in individual farms in the Republic is larger. 468,404 samples from cattle in private farms were serologically tested in 2020, 3,489 (0.7%) samples out of them were seropositive. In total 56,496 sera samples from cattle in public farms were tested for leukosis, and the infection rate was 3.3% (1,872 animals). The number of seropositive animals in the public sector exceeds the number of seropositive animals in individual farms by more than 4 times, and the percentage of infection in livestock in some areas reaches 10% and even higher (Dakhadaevsky – 10.3%, Shamilsky – 10.0%).

Based on the above, it can be concluded that BLV is distributed in all livestock sectors in the Republic, and, in particular, the distribution is more intensive among cattle in public farms. One of the reasons for the spread of infection in the public sectors of the Republic is the entry of infected and leukosis-diseased animals from other regions back in the Soviet times [13].

CONCLUSION

The serological studies for bovine leukosis conducted in the Republic of Dagestan show that in 2020 the general level of BLV infection decreased to 1.02% as compared to previous years. The number of animals with hematological disease detected among BLV-infected animals in the farms of the Republic in 2020 remains at a high level (19.8%). Hematological studies for bovine leukosis are carried out randomly in the Republic, covering small quantities of animals and not all infected livestock population. Nevertheless, the detection rate of animals with the hematological disease is high, which indicates that animals infected with leukosis are not subjected to slaughter. A comparative analysis of the BLV frequency in farms of different categories showed that the percentage of infection in animals is higher in the public sector as compared to the private (individual) sector. Bovine leukosis is distributed in many raions and municipal districts, and that remains one of the main problems of animal husbandry in the Republic of Dagestan.

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INFORMATION ABOUT THE AUTHOR / ИНФОРМАЦИЯ ОБ АВТОРЕ

Arkiv R. Mustafayev, Candidate of Science (Veterinary Medicine), Leading Researcher, Laboratory of Infectious Pathology of Farm Animals, Caspian Regional Research Veterinary Institute – Branch of Dagestan Agriculture Science Center, Makhachkala, Russia.

Мустафаев Аркиф Рамазанович, кандидат ветеринарных наук, ведущий научный сотрудник лаборатории инфекционной патологии сельскохозяйственных животных, Прикаспийский зональный НИВИ – филиал ФГБНУ «ФАНЦ РД», г. Махачкала, Россия.

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Features of anthrax natural foci and *Bacillus anthracis* ecology

A. P. Rodionov[1], E. A. Artemeva[2], L. A. Melnikova[3], M. A. Kosarev[4], S. V. Ivanova[5]
Federal State Budgetary Scientific Institution "Federal Center for Toxicological, Radiation, and Biological Safety" (FSBSI "FCTRBS-ARRVI"), Republic of Tatarstan, Kazan, Russia
[1] *ORCID 0000-0003-0853-5678, e-mail: alexandrvetspets@gmail.com*
[2] *ORCID 0000-0002-6204-6077, e-mail: artemevaelena21@mail.ru*
[3] *ORCID 0000-0002-0159-3843, e-mail: vnivi@mail.ru*
[4] *ORCID 0000-0002-5577-486X, e-mail: kosarev@vnivi.ru*
[5] *ORCID 0000-0002-4378-8569, e-mail: 9274281396@mail.ru*

SUMMARY
Anthrax remains a global problem, both for veterinary and human medicine, due to the wide spread of its soil foci throughout the world. The ability to sporulate is the main feature of *Bacillus anthracis*, which allows the pathogen to persist in the environment for a long time. Understanding the ecology of *B. anthracis* is essential for successful control of this infection. This review analyzes the data from the global literature, reflecting the modern understanding of the vital functions of the anthrax agent in various ecological niches. As a result of the work, it was revealed that many links in the chain of *B. anthracis* lifecycle in the abiotic environment remain poorly understood. A more in-depth study is required for issues related to the mechanisms, ways of living and evolution of the anthrax causative agent outside the animal body. A separate section of the review describes the problems of anthrax foci in soil. It is shown that today there are no effective and environmentally friendly methods and means of their elimination. In addition, the question of the expediency of their use remains open. According to some researchers, the increasingly emerging initiatives for the elimination or conservation of anthrax burial sites are not only useless, but also harmful, since they exclude the possibility of further predicting the risks associated with soil foci that surround livestock burial sites and cannot be decontaminated. The study and new approaches to solution of the highlighted issues will make a significant contribution to solving the global problem of protecting animals and people from this infection.

Keywords: Anthrax, ecology, *Bacillus anthracis*, soil foci, natural focality, cattle burial site, permanently infected settlement.

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For correspondence: Alexander P. Rodionov, Junior Researcher, Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", 420075, Russia, Republic of Tatarstan, Kazan, Scientific town-2, *e-mail: alexandrvetspets@gmail.com.*

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Особенности природной очаговости сибирской язвы и экологии *Bacillus anthracis*

А. П. Родионов[1], Е. А. Артемьева[2], Л. А. Мельникова[3], М. А. Косарев[4], С. В. Иванова[5]
ФГБНУ «Федеральный центр токсикологической, радиационной и биологической безопасности» (ФГБНУ «ФЦТРБ-ВНИВИ»), Республика Татарстан, г. Казань, Россия
[1] *ORCID 0000-0003-0853-5678, e-mail: alexandrvetspets@gmail.com*
[2] *ORCID 0000-0002-6204-6077, e-mail: artemevaelena21@mail.ru*
[3] *ORCID 0000-0002-0159-3843, e-mail: vnivi@mail.ru*
[4] *ORCID 0000-0002-5577-486X, e-mail: kosarev@vnivi.ru*
[5] *ORCID 0000-0002-4378-8569, e-mail: 9274281396@mail.ru*

РЕЗЮМЕ
Сибирская язва остается глобальной проблемой как для ветеринарной, так и для гуманной медицины в связи с широким распространением ее почвенных очагов во всем мире. Способность к споруляции является главной особенностью *Bacillus anthracis*, позволяющей возбудителю сохраняться в окружающей среде в течение длительного времени. Понимание экологии *B. anthracis* необходимо для успешной борьбы с данной инфекцией. В настоящем обзоре проведен анализ данных мировой литературы, отражающих современное представление о жизнедеятельности возбудителя сибирской язвы в различных экологических нишах. В результате работы выявлено, что многие звенья в цепи жизнедеятельности *B. anthracis* в абиотической среде остаются

малоизученными. Более глубокого изучения требуют вопросы, касающиеся механизмов, способов существования и эволюции возбудителя сибирской язвы вне организма животного. Отдельным разделом обзора представлены проблемы почвенных очагов сибирской язвы. Показано, что на сегодняшний день не существует эффективных и безвредных для окружающей среды методов и средств их ликвидации. Кроме того, остается открытым вопрос о целесообразности их применения. По мнению некоторых исследователей, все чаще возникающие инициативы ликвидации или консервации сибиреязвенных скотомогильников не только бесполезны, но и вредны, так как исключают возможность в дальнейшем прогнозировать риски, связанные с почвенными очагами, которые окружают скотомогильники и не могут быть обезврежены. Изучение и новые подходы к решению освещенных вопросов внесут свой значительный вклад в решение глобальной проблемы защиты животных и людей от данной природно-очаговой инфекции.

Ключевые слова: Сибирская язва, экология, *Bacillus anthracis*, почвенные очаги, природная очаговость, скотомогильник, стационарно неблагополучный пункт.

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Для корреспонденции: Родионов Александр Павлович, младший научный сотрудник лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», 420075, Россия, Республика Татарстан, г. Казань, Научный городок-2, *e-mail: alexandrvetspets@gmail.com.*

INTRODUCTION

The main feature of the causative agent of anthrax (*Bacillus anthracis*) is the ability to form spores that persist in the environment for decades until they get the possibility to penetrate into a susceptible organism. *B. anthracis* in spore form is a perfect infectious agent. To date, there are a large number of works devoted to the processes occurring in *B. anthracis* infected macroorganism [1, 2]. However, aspects concerning *B. anthracis* relationships in soil ecosystems and the environment are still poorly understood. This review analyzes the life cycle of *B. anthracis* in various ecological niches.

Bacillus anthracis spore and sporulation

The spores are formed in the environment or in laboratory conditions when grown on nutrient media, provided there is an access of oxygen, lack of nutrients, high humidity, and temperature of 26–37 °C. One vegetative cell is capable of forming a single spore, which is located in the center or subterminally. At temperatures above 43 °C or below 12 °C, sporulation does not occur.

Sporulation is triggered by the lack of a nutrient substrate. In this case, the *spo0A* gene encoding the protein of the same name is activated. Then, by phosphorylation the Spo0A protein is activated into Spo0A~P, causing the expression of more than 200 genes. These genes are responsible for sporulation. When the endospore formation is complete, the mother cell wall is lysed, releasing the mature spore into the environment [3].

The *B. anthracis* spore consists of the core, surrounded by coats: the cortex, the coat proteins and the exosporium (Fig. 1) [4].

The spore core consists of a chromosome tightly bound to acid-soluble proteins [5]. The interaction between DNA and proteins, high levels of dipicolinic acid, calcium and other ions provide protection from a variety of adverse effects, including elevated temperatures and ultraviolet radiation.

The cortex is the inner part of the spore, surrounded by a membrane and peptidoglycan layer, which in turn are surrounded by several layers of proteins called the coat proteins.

The coat protein surface is distinguished by folds, which extend along the long axis of the spore, and allow the spore to withstand the core growth during its germination [6–10].

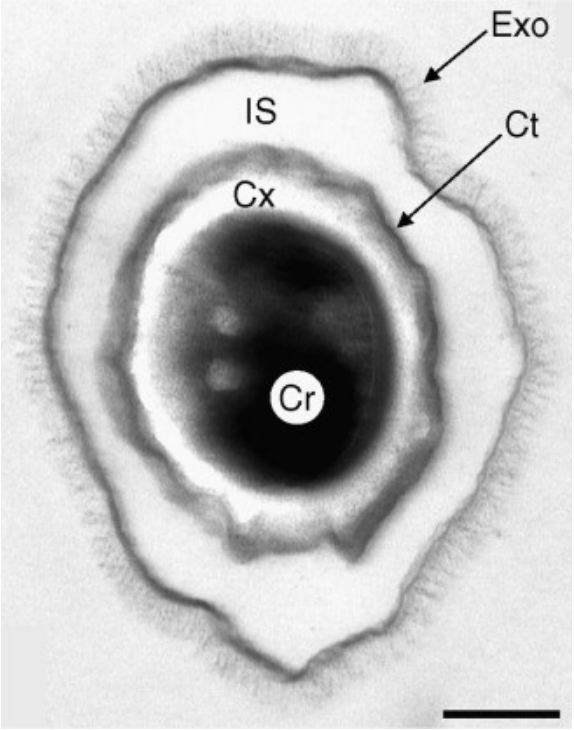

Fig. 1. Thin-section electron micrograph of a Bacillus anthracis spore (Sterne strain). Core (Cr), cortex (Cx), coat (Ct), interspace (IS) and exosporium (Exo) are indicated [4]

Рис. 1. Строение споры возбудителя сибирской язвы: Cr – ядро, Cx – кортекс, Ct – белковая оболочка, IS – промежуток, Exo – экзоспориум [4]

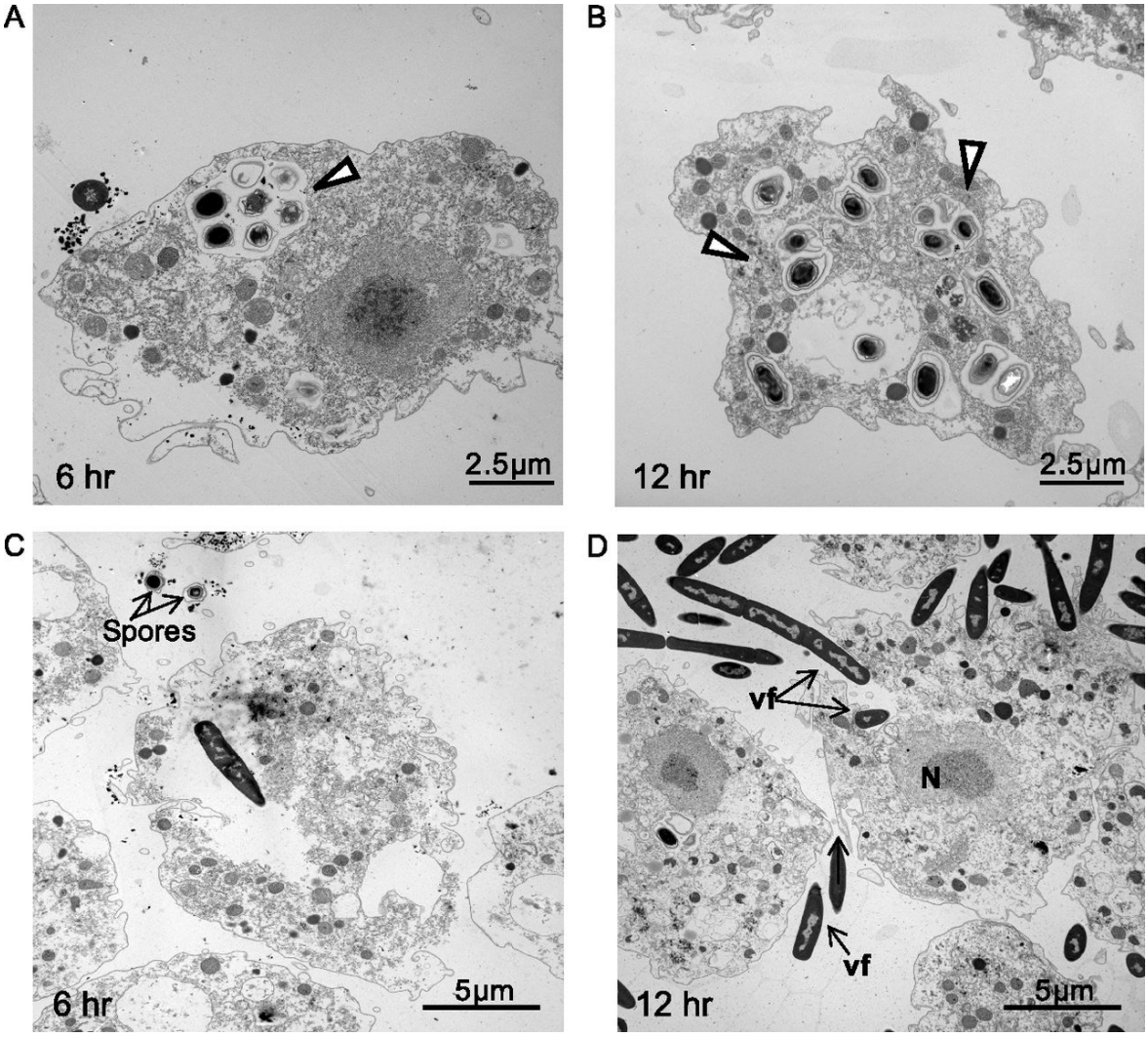

Fig. 2. Transmission electron microscopy analysis of B. anthracis-Acanthamoeba castellanii interactions [27]: *A and B – Micrographs show spores of strain 9131 contained in A. castellanii phagosomes (open arrowheads) after 6 (A) and 12 h (B) of coculture at 37 °C, respectively; C – A vegetative Sterne spore within an A. castellanii trophozoite in a phagosome after 6 h of coculture; D – Vegetative forms of Sterne inside and outside amoebas after 12 h of infection (black arrows). N – nucleus; vf – vegetative form*

Рис. 2. Просвечивающая электронная микроскопия взаимодействия B. anthracis с почвенной амебой [27]: *A и B – находящиеся внутри амебы Acanthamoeba castellanii споры штамма 9131 B. anthracis и начало их прорастания через 6 (A) и 12 ч (B) совместного культивирования при 37 °C; C – прорастание штамма Sterne B. anthracis внутри амебы; D – вегетативные формы штамма Sterne B. anthracis внутри и снаружи амебы через 12 ч после совместного культивирования (черные стрелки). Обозначения: N – ядро; vf – вегетативная форма*

These coat proteins perform a number of important functions:

1) prevent the penetration of large molecules and toxic substances;

2) protect against the aggressive action of other microorganisms [11–13].

In general, the protective functions of these structures allow spores to remain dormant for many years [14, 15].

The exosporium is the outer-most structure of the spore, in most *Bacillus* species it is separated from the underlying structure by interspace, the composition and functional purpose of which remains a mystery. The exosporium consists of a basal membrane surrounded by hair-like projections. The collagen-like glycoprotein BCLA is the main component of these projections. Thanks to the hair-like projections of the outer structure, the spores can adhere to the soil fragments, which allows them to stay on the surface and enter the body of animals during grazing. In recent years, the BCLA protein has been given special attention as a possible antigen for vaccine development [16, 17]. The BCLA protein, composing the exosporium, interacts with the phagocytes of the host organism, thereby promoting the penetration of the pathogen into the cell and its subsequent germination – the process by which the spores stop being dormant [4].

Germination is initiated by presence of essential nutrients, which is detected by receptors in the inner membrane of the spore. The binding of the receptors leads to a cascade of successive reactions, including the influx of water, the release of cations and dipicolinic acid, the pH rises to 7.7, and the glycopeptide cortex is hydrolyzed. When pH changes, intracellular enzymes are activated,

the spore coats are destroyed, and vegetative metabolism reactivates, including the production of powerful virulence factors [18].

Ecology of *B. anthracis* in soil

To date, there are several different theories of the *B. anthracis* ecology in soil. The first was put forth in 1941 [19]. According to this theory, the pathogen is able to germinate in certain «incubator areas», that is, in soils rich in organic matter, calcium, with a pH greater than 6.0 and an ambient temperature above 15.5 °C. Sporadic outbreaks of anthrax occur as a result of the pathogen germination in the surface soils under certain climatic and environmental conditions, contributing to accumulation of high concentrations, able to infect grazing animals.

A competing hypothesis suggests that these local accumulations emerge from the physical pooling of spores in rainwater depressions because of the spores hydrophobic surface character [20–22]. Furthermore, vegetative cells of *B. anthracis* were suggested to be unable to successfully compete with resident soil microbiota and have never been found in natural environments. Also the clonal genetic character of this microorganism, isolated from infected animals, argues against frequent episodes of soil proliferation. This statement is inconsistent with the fact that probe sequencing of soils contaminated with *B. anthracis* spores showed the presence of isolates that lack one or both virulence plasmids [23, 24]. The latter is indicative of the active metabolism of the pathogen in the environment, but its further fate in the soil is disputable.

Over time, an increasing number of laboratory results contradicted the established opinion that *B. anthracis* is an obligate pathogen and is able to reproduce exclusively in susceptible animals. For example, other members of the genetically homogeneous group *B. cereus sensu lato* were discovered as common inhabitants of the invertebrate gut [25], and as saprophytes in the rhizosphere of plants [26]. This gave rise to the assumption that the *B. anthracis* germination is not limited to the animal body.

After study of closely related species, similar studies were conducted in the laboratory conditions, which confirmed the capacity of *B. anthracis* to germinate in the rhizosphere of some plants [26] and inside soil amoebas (Fig. 2) [27], which significantly expanded knowledge about its life cycle and the capacity to dictribute in the environment.

In addition, domestic researchers have found that *B. anthracis* spores can persist and spread in the soil with earthworms. It was found that 50–70% of spores retain their properties and virulence in the worm gut for 30 days (the study period) [28].

No less interesting is the life cycle of *B. anthracis*, interacting with bacteriophages that mediate phenotypic changes and cause the appearance of lysogenic variants of the pathogen with a pronounced improvement in survival.

In the course of long-term studies, various *B. anthracis* phages were identified (Fig. 3) [29], including for vaccine strains with low virulence, such as Sterne, Pasteur, and Vollum [30]. As for field strains, soil isolates of *B. anthracis* often contain phage plaques when cultured [26]. In addition, studies of more than 160 natural *B. anthracis* isolates recovered from the environment and from diseased animals showed that more than 20% of them were infected with various phages. Free, infective phages for *B. anthracis* are also found in many environments, including sewage, tannery effluent, animal hair, soil and water at or near anthrax carcasses, as well as soil at non-endemic areas [29].

As an example of the bacteriophage-mediated variability of *B. anthracis*, the results of studies published in the early 21st century can be cited. The strains of bacilli isolated from wild apes in African countries: Cameroon and Côte d'Ivoire are described in the papers. The studied bacteria were characterized by the motility, resistance to penicillin and diagnostic gamma phage, the ability to form a capsule not only after induction by CO_2 and bicarbonate, the secretion of protective antigen and lethal factor. These strains had both the toxin and the capsule plasmid pBCXO1 and pBCXO2, with sizes corresponding to the *B. anthracis* viru-

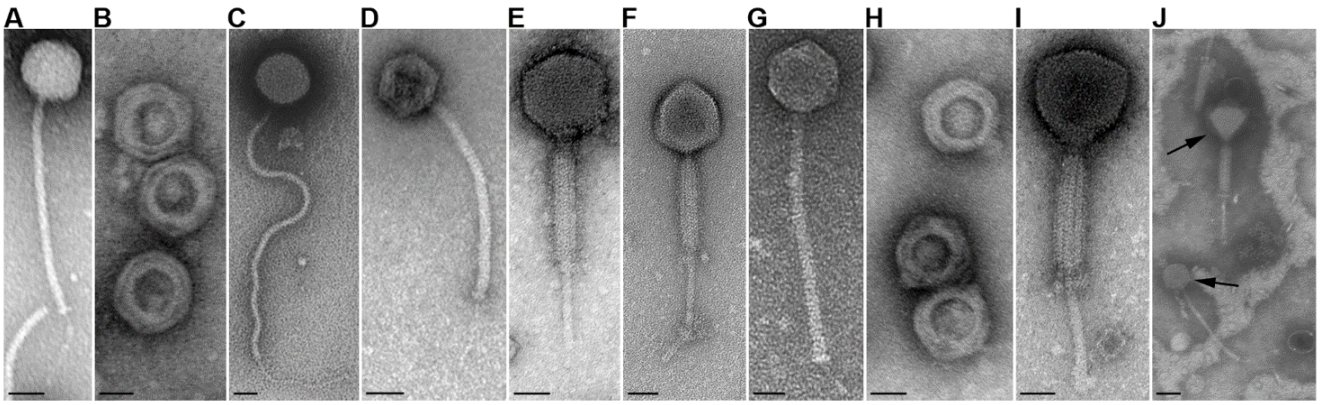

Fig. 3. Transmission electron micrographs of bacteriophages negatively stained with 2% uranyl acetate [29]. *The bacteriophages infecting B. anthracis include, (A) Wβ, (B) Wip1, (C) Wip2, (D) Wip4, (E) Wip5, (F) Frp1, (G) Frp2, (H) Htp1, and (I) Bcp1. An extract from the gut of the earthworm Eisenia fetida is shown (J) with two distinct and uncharacterized phages indicated by arrows*

Рис. 3. Просвечивающая электронная микроскопия бактериофагов, выделенных от B. anthracis, отрицательно окрашенных 2%-м раствором уранилацетата [29]. *Бактериофаги, инфицирующие B. anthracis, включают: (A) Wβ, (B) Wip1, (C) Wip2, (D) Wip4, (E) Wip5, (F) Frp1, (G) Frp2, (H) Htp1 и(I) Bcp1. J – экстракт из кишечника дождевого червя Eisenia fetida (стрелками обозначены два неидентифицированных фага)*

lence plasmids pXO1 and pXO2. Genetic analysis of these strains revealed a close relationship with both classic *B. anthracis* strains and two uncommonly virulent *B. cereus* and *B. thuringiensis* isolates. The authors of the study proposed that the newly discovered strains share a common ancestor with *B. anthracis* or that they emerged recently by transfer of the *B. anthracis* plasmids to a strain of the *B. cereus* group [31–34]. These strains were designated *B. cereus* biovar *anthracis*. These strains were as virulent for mice and guinea pigs as wild-type *B. anthracis* and remained virulent after removal of the plasmid encoding capsule synthesis. In addition to the poly-D-glutamate capsule, these strains were found to produce a hyaluronic acid capsule encoded by the pBXO1 plasmid. Such phenotypic changes enabled systemic dissemination, thus providing a clear evolutionary advantage [35]. In this regard, it is relevant to further study the life of these *B. anthracis* strains in the environment and organisms of susceptible animals as pathogens of potentially new infectious diseases.

Ecology of *B. anthracis* in the environment

More than 50 species of animals belonging to 8 orders and 23 families are susceptible to the *B. anthracis*, which explains the reason for the wide geographical spread of this infection around the world [20]. However, birds are not susceptible to this pathogen, nevertheless, they play a significant role in the epizootology and epidemiology of anthrax, contributing to the spread of spores to new territories [36].

It is known that the condition for *B. anthracis* circulation in nature is the contamination of the soil with spores after the death of a diseased animal. However, if the integrity of the carcass is preserved, the bacilli do not sporulate and die [37]. Therefore, the activity of scavenger birds has a significant impact on the circulation of the pathogen: carcass consumption contributes to spore formation, and dispersing the remains leads to widespread contamination of the soil with spores.

Experimental studies of other authors have shown that birds, consuming meat of infected animals, can secrete spores of the pathogen with excrement for a long time and transfer them in the beak and on the paws. Flying over long distances, birds can spread spores in areas where this disease has not been previously recorded [38]. In our country, scavenger birds consuming reindeer carcasses are actively involved in the spread of *B. anthracis* spores. Synanthropic birds are also dangerous. For example, studies were conducted in the UK to study the role of house sparrows *Passer domesticus* in the spread of *B. anthracis* spores. Scientists have found that 2% of these birds are carriers of *B. anthracis* spores. The researchers suggest that in countries with a high incidence of anthrax, the percentage of infected sparrows must be higher [36].

One of the indicators of anthrax prevalence in nature is the circulation of the pathogen among various rodent species. Reports on the isolation of *B. anthracis* from field rodents in the Russian Federation regions and the former Soviet Union countries evidence that anthrax is latent in naturally infected mouse-like rodents. *B. anthracis* cultures were repeatedly isolated from clinically healthy field mice showing no post-mortem changes in organs and tissues, which reflects the possibility of unhindered spore dissemination with these animal species.

The ability to transfer *B. anthracis* spores with blood-sucking insects is also very important. Flies, horseflies, ticks, and mosquitoes feed on the blood of infected animals. Then, moving and biting a healthy animal, they introduce the pathogen into a new susceptible organism. In addition, it was found that, flying into the adjacent vegetation, they secrete both spores of the pathogen and vegetative cells. The researchers noted that *B. anthracis* spores were found on the leaves of plants at a distance of 1–3 meters from the dead animal carcass [20].

Thus, in addition to susceptible animals, there are a large number of species that contribute to the maintenance and spread of the pathogen in the environment, which, in turn, makes it difficult to control this infection and requires strict measures for specific prevention of anthrax.

Problems of anthrax soil foci

One of the main reservoirs of *B. anthracis* is the soil, which is considered the second source of the disease after infected animals. Infection with *B. anthracis* spore form was reported after contacts with spore-contaminated soil in 3–14% out of total number of cases [39]. Spore-contaminated soil can remain a source of infection for many decades. To date, it has been established that anthrax bacillus spores can persist for up to 200 years. However, the exact period of possible presence in the soil and the ability to infect living organisms with *B. anthracis* spores has not yet been established [20].

A retrospective analysis of data on anthrax incidence in Russia in the 18th and 19th centuries shows that it was one of the most common diseases. During this period, more than 100,000 cases were officially recorded in the country. In the 20th century, 69,827 anthrax outbreaks occurred on the territory of our country [40]. Many carcasses were buried without prompt control, which led to a wide spread of soil foci and an increase in the number of anthrax animal burial sites on the territory of Russia.

In the Russian Federation today, there are more than 35,000 permanently anthrax infected settlements, 14,109 animal burial sites, of which 3,193 are anthrax burial sites [41]. Abandoned animal burial sites and animal burial sites with unknown geographical coordinates are particularly dangerous. Many soil foci are not marked on maps or on the terrain. Initially, these burials were under the control of local veterinary services, but over many decades, as a result of numerous reorganizations and the transfer of control functions over animal burial sites from one agency to another, the archived data on these animal burial sites were lost in most cases. As a result, on the territory of our country there are a large number of anthrax soil foci, both known and uncontrolled, which pose a great danger of potential spread and infection with this highly dangerous infection.

To date, Russian scientists have developed a number of methods for the disinfection of anthrax soil foci, but there are no effective and environmentally friendly methods among them. It should also be noted that it is impossible to accurately determine the effectiveness of disinfection of anthrax soil focus, since, according to researchers, the possibility of detecting *B. anthracis* and isolating it from the soil is no more than 1.5% [42]. In this regard, all existing anthrax burials have a potential danger to a greater or lesser extent [43].

Recently, due to the use of previously abandoned land, increase in residential development, there is a need for a detailed study of this danger, which, according to

researchers, persists because of violated conditions for burial site maintenance [44]. According to available data, on the national scale, on average, 37% of biological waste disposal sites are in poor veterinary and sanitary condition [40]. The situation related to the maintenance of burial sites in infected regions poses a potential danger and requires constant monitoring of the state of these sites.

Currently, specialists are developing methods for studying the epizootological and epidemiological danger of anthrax burials, aimed at assessing the risks of their possible impact on outbreak occurrence and the spread of infection in order to prevent it [42].

According to the researchers, the increasingly emerging initiatives for the elimination or conservation of anthrax burial sites are not only useless, but also harmful, since they exclude the possibility of further predicting the risks associated with the soil foci that surround the burial sites and cannot be decontaminated [42, 45]. In addition, local disinfection of known soil foci is not able to provide its complete elimination. Due to the fact that several dozen species of wild animals are susceptible to anthrax, which are potential carriers of it, it can be assumed that there are many other foci in the wild, and with each new animal that becomes diseased, their number increases.

CONCLUSION

One hundred and fifty years of studying the ecology of *B. anthracis* has allowed us to shed light on many aspects of the pathogen's existence in the environment, to establish its connection and interaction with various species of living organisms. However, many links in the life chain of *B. anthracis* in the abiotic environment remain poorly understood. Questions concerning the mechanisms, ways of existence and evolution of anthrax causative agent outside the animal's body also require an in-depth study, which will make a significant contribution to solving the global problem of protecting animals and people from this natural focal infection.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Alexander. P. Rodionov, Junior Researcher, Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Родионов Александр Павлович, младший научный сотрудник лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

Elena A. Artemeva, Candidate of Science (Veterinary Medicine), Head of Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Lilia A. Melnikova, Candidate of Science (Veterinary Medicine), Associate Professor, Leading Researcher, Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Maxim A. Kosarev, Candidate of Science (Biology), Head of Department of Bacteriology, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Svetlana V. Ivanova, Candidate of Science (Biology), Leading Researcher, Laboratory of Viral Anthropozoonoses, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Артемьева Елена Александровна, кандидат ветеринарных наук, заведующий лабораторией коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

Мельникова Лилия Арсентьевна, кандидат ветеринарных наук, доцент, ведущий научный сотрудник лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

Косарев Максим Аркадьевич, кандидат биологических наук, заведующий отделением бактериологии ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

Иванова Светлана Викторовна, кандидат биологических наук, ведущий научный сотрудник лаборатории вирусных антропозоонозов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

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Funding of activities of state veterinary services of Russian Federation Subjects

I. M. Klinovitskaya[1], M. A. Shibayev[2], A. K. Karaulov[3]
FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia
[1] *ORCID 0000-0001-5347-8620, e-mail: klinovitskaya@arriah.ru*
[2] *ORCID 0000-0002-9382-0109, e-mail: shibaev@arriah.ru*
[3] *ORCID 0000-0002-5731-5762, e-mail: karaulov@arriah.ru*

SUMMARY
The due performance by the veterinary service of its assigned functions depends largely on the amount of funding provided for different aspects and types of its activities. The paper presents analysis results for 13 main funded activities of veterinary services in 85 Subjects of the Russian Federation in 2019. All the funded activities were reviewed in relation to three funding sources: the federal budget, the budget of a Russian Federation Subject and extrabudgetary sources. The paper examines funding levels of the Russian Federation Subjects' veterinary services (against actual funding requirements) with respect to each object of expenditure and each funding source; besides, the share of each funding source in overall funding of the veterinary service of the country on the whole and of certain types of its activities was determined. In 2019, overall funding of the veterinary service of the country amounted to about 49.5 billion rubles which made up 96% of funding requirements for this period. The major sources of funding were the budgets of the Russian Federation Subjects (56.3%) and veterinary services' own extrabudgetary resources (43.2%). Only 0.5% of all the funds received by the state veterinary service of the Russian Federation were allocated from the federal budget. The following 4 out of 13 analyzed aspects of activities of the Subjects' veterinary services were fully funded: staff salaries, anti-epidemic activities, the purchase of reagents and test systems, the implementation of monitoring and screening programmes at the Subject level for contagious animal disease control. The funding levels for other activities of the country's veterinary service were from 9% (accreditation of veterinary laboratories and maintenance of accreditation) to 87% (the implementation of regional monitoring of food product, raw material, animal product quality and safety; animal health awareness-raising and information activities).

Keywords: Funding of veterinary service, veterinary service funding sources, funding level, federal budget, budget of Russian Federation Subject, extrabudgetary funding.

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For correspondence: Irina M. Klinovitskaya, Candidate of Science (Economics), Senior Researcher, Information and Analysis Centre, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: klinovitskaya@arriah.ru.*

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Финансовое обеспечение деятельности государственной ветеринарной службы субъектов Российской Федерации

И. М. Клиновицкая[1], М. А. Шибаев[2], А. К. Караулов[3]
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия
[1] *ORCID 0000-0001-5347-8620, e-mail: klinovitskaya@arriah.ru*
[2] *ORCID 0000-0002-9382-0109, e-mail: shibaev@arriah.ru*
[3] *ORCID 0000-0002-5731-5762, e-mail: karaulov@arriah.ru*

РЕЗЮМЕ
Выполнение ветеринарной службой на должном уровне возложенных на нее функций во многом зависит от объема финансирования различных направлений и видов деятельности. В статье представлены результаты анализа 13 основных направлений финансирования деятельности ветеринарных служб в 85 субъектах Российской Федерации в 2019 г. Все направления финансирования рассматривались в разрезе трех источников: из федерального бюджета, бюджета субъекта РФ и внебюджетных источников. В работе рассмотрены показатели обеспеченности ветслужб субъектов РФ финансированием (от реальной потребности) по каждой статье расходования средств и каждому источнику финансирования, а также определены доли каждого источника финансирования в общем объеме финансирования ветслужбы страны в целом и по конкретным направлениям ее деятельности. Общий объем финансирования ветеринарной службы страны в 2019 г. составил около 49,5 млрд рублей, что соответствует 96%-й обеспеченности от требуемого

финансирования за данный период. Основными источниками финансирования явились бюджеты субъектов РФ (их доля 56,3%) и внебюджетные средства самих ветслужб (их доля 43,2%). Из федерального бюджета было выделено только 0,5% всех денежных средств, фактически поступивших в 2019 г. в государственную ветеринарную службу РФ. Полная обеспеченность финансированием ветслужбы субъектов РФ наблюдалась по четырем (из 13 анализируемых) направлениям: на заработную плату сотрудников, на противоэпизоотические мероприятия, на закупку реагентов и тест-систем, на реализацию мониторинговых и скрининговых программ субъектового уровня по контролю заразных болезней животных. По остальным направлениям деятельности уровень обеспеченности финансированием ветслужбы страны составил от 9 (на аккредитацию и поддержание аккредитации ветеринарными лабораториями) до 87% (на проведение регионального мониторинга качества и безопасности пищевых продуктов, сырья, продукции животного происхождения; на ветеринарно-просветительскую и информационную деятельность).

Ключевые слова: Финансирование ветеринарной службы, источники финансирования ветеринарной службы, уровень финансового обеспечения, федеральный бюджет, бюджет субъекта федерации, внебюджетное финансирование.

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Для корреспонденции: Клиновицкая Ирина Михайловна, кандидат экономических наук, старший научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: klinovitskaya@arriah.ru.*

INTRODUCTION

Funding level is one of the most important characteristics of state veterinary service activities.

In order to ensure animal disease freedom in the country as a whole and in particular Subjects of the Russian Federation (hereinafter – the Subjects), preventive, diagnostic, therapeutic, veterinary and sanitary, as well as other measures are implemented that are primarily aimed at the reduction of animal production costs and prevention of significant economic losses resulting from infectious animal disease outbreaks, as well as of acute zoonotic infections in humans. The implementation of such activities requires substantial funds allocated from different sources: the federal budget, the budgets of the Subjects and extrabudgetary sources. The federal funds are provided for the control of certain highly dangerous infectious animal diseases (African swine fever, rabies, avian influenza, foot-and-mouth disease, classical swine fever, etc.) in order to ensure the country's veterinary and sanitary safety and are expended in full compliance with the Law of the Russian Federation on the federal budget for a particular period and the Budget Code of the Russian Federation [1, 2].

The consolidation of the role of the Subjects' authorities in regional economic governance increases the significance of regional budgets that are created, approved and expended at the discretion of the Subjects' authorities in accordance with the Budget Code of the Russian Federation [3, 4]. Almost all the veterinary service activities are funded from regional budgets.

The income from rendering chargeable services, bank borrowings, etc. can serve as extrabudgetary funding sources [5]. They are also regulated by the law and are necessary to provide additional funding for different activities of the Subjects' veterinary services and have proven to be an important element of sustainability of the veterinary service as a whole.

The government and its structures should ensure the availability of sufficient funding for the state veterinary service maintenance and implementation of key veterinary activities aimed at accomplishing the assigned tasks [6].

In view of the fact that available and official information on the funding of the state veterinary service of the country as a whole and of each particular Subject is very limited, the study was aimed at selection, collection and overall analysis of the most relevant indicators that allowed for assessment of funding level for different types of activities in each Region. The findings of the study aimed at the analysis of compiled quantitative data on the structure and level of funding are, for the first time, presented in this paper.

MATERIALS AND METHODS

Data for 2019 provided by the veterinary executive authorities of the Russian Federation Subjects using the primary data collection form developed by the FGBI "ARRIAH" served as a practical basis for the analysis of funding of the state veterinary service of the Russian Federation and its Subjects. The information was received from 85 Subjects. The data were collected using Assol. Express component of FGIS VetIS.

Theoretical and methodological framework for the study includes the laws of the Russian Federation and methods of analysis described in contemporary scientific articles on this issue published in the field-specific publications.

The following methods were used: analytical method, comparative analysis, descriptive statistics, compilation and grouping methods.

In this study, funding level was determined based on the ratio between actually provided and required (necessary) funding, i.e. the state veterinary services of the Subjects were given the opportunity to determine independently, based on reasonable expenses, their material and resource requirements for the execution of tasks and functions laid down in the Veterinary Law of the Russian Federation, taking into account each Region's particular

circumstances (natural climatic conditions, territorial factors, social and economic development level, specific features of economic activities, etc.).

RESULTS AND DISCUSSION

The state veterinary service of the Russian Federation is tasked with ensuring compliance with veterinary and sanitary safety requirements [7]. A certain economic foundation is required for effective functioning of the veterinary service. This means that there is a close relationship between the veterinary service's performance and funding, the level of which has an effect primarily on the organization of preventive and anti-epidemic activities, as well as on the level of staff salaries, fit-out of facilities, the availability of means of transport, the purchase of laboratory equipment, etc.

The state veterinary service of the Russian Federation is funded from three sources: the federal budget, the budgets of the Subjects and extrabudgetary funding sources. In 2019, ***the overall funding*** (from all the funding sources) of the state veterinary service amounted to about 49.5 billion rubles, of which 56.3% were allocated from the budgets of the Subjects, 43.2% – from extrabudgetary sources, and about 0.5% – from the federal budget (Fig. 1).

In the country as a whole, veterinary service funding level was 96%. Funding received from the Subjects' budgets and the federal budget was lower than requirements, as indicated by the level of funding provided from these sources – 89 and 97%, respectively. Only extrabudgetary funding of the veterinary services' activities was sufficient to cover the requirements for funding from this source.

The level of funding from the Subjects' budgets was low (not more than 50% of requirements) in 6 Regions of the Russian Federation. Full (100%) funding was provided in 54 Subjects. Funding levels for the veterinary services of the remaining 25 Subjects varied over a wide range – from 51 to 99%. Thus, the veterinary services of 31 Regions of the country were, to varying extents, underfunded as regards the implementation of their assigned tasks.

The largest part (62%) of all funds allocated to the veterinary services (from all funding sources) was used to pay ***staff salaries*** and fully covered funding requirements for such expenditures in the country as a whole. The veterinary services of 64 Subjects were 100% funded from the regional budgets for the payment of staff salaries; in other Regions, the levels of such funding varied from 40 to 99% (most of the veterinary services of these Subjects attracted additional funding from extrabudgetary sources). It should be noted that the salaries of veterinary specialists in most Regions of the Russian Federation are not high. In particular, veterinary specialist salaries, including executive salaries, were average or just above average for the Subject as a whole in 31 Subjects, and when excluding executive salaries – only in 15 Regions. Such situation requires the attention of the Subjects' authorities. Decent salaries will provide the basis for veterinary profession prestige enhancement, and this will allow for state veterinary service staffing with qualified personnel for appropriate implementation of the tasks assigned [8].

In order ***to attract and retain*** qualified personnel, many veterinary services implemented social support measures for veterinary specialists that are to be funded from local budgets and extrabudgetary sources only. The level of funding for these expenditures in the country as a whole (from all the funding sources) was 18%, from the budgets of the Subjects – only 16%, from extrabudgetary sources – 50%. No funding from any sources was envisaged for these expenditures in 54 (out of 85) Subjects. The veterinary service funding requirements for these expenditures were fully covered through local budgets in 16 Regions only. Underfunding of this aspect of veterinary service activities may result in lower level of competence among veterinary specialists. To improve the situation, more active attraction of additional budgetary and extrabudgetary funds is required, in particular in order to retain veterinarians in rural locations, to provide material incentives and advanced training for them.

One of important characteristics of the veterinary services of the Subjects is the level of veterinary specialists' qualification. Over the past 5 years, about 67% of the veterinary specialists of the country's state veterinary service received ***advanced training***. According to the veterinary services of the Subjects, in 2019, more than 91 million rubles were spent on advanced training; three quarters of these funds were provided from extrabudgetary sources, funding level was 99%. Funding from the Subjects' budgets covered 43% of requirements in the country as a whole. The amount of funding allocated for advanced training of veterinary specialists from regional budgets

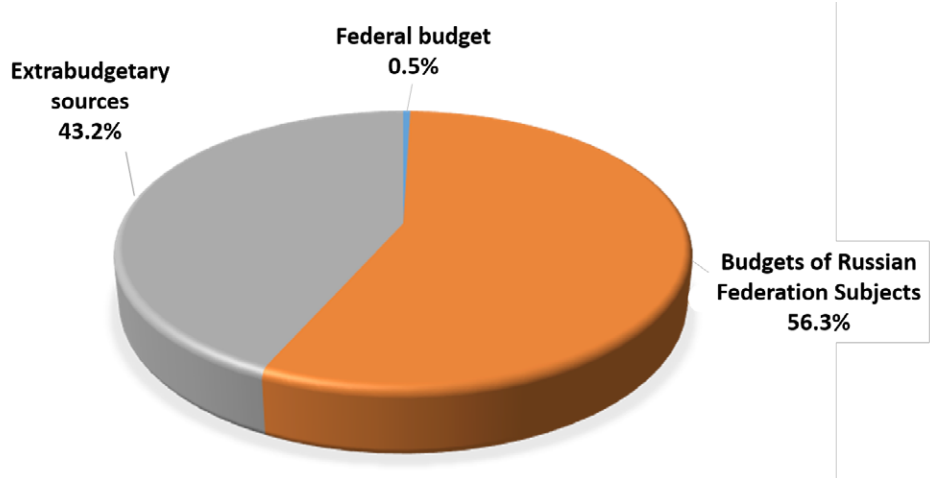

Fig. 1. Funding structure of the state veterinary service of the Russian Federation in 2019

Рис. 1. Структура финансирования государственной ветеринарной службы РФ в 2019 г.

over the year varied from 7 thousand to 8 million rubles. Extrabudgetary funding amount varied from 80 thousand to 7 million rubles for a Subject. Notably, funding allocated for such expenditures from local budgets was higher than that from extrabudgetary sources in 7 Subjects only; no funding was provided from any sources in 12 Subjects. In the country as a whole, funding level for advanced training of veterinary specialists was 72%.

One of the main tasks of the veterinary service is ***the implementation of anti-epidemic activities*** aimed at prevention and control of infectious animal diseases. In the country as a whole, funding requirements for the implementation of anti-epidemic activities were fully covered.

The budgets of the Subjects were the key source of funding for such activities; their share of the overall funding for such expenditures was 93%. Funds attracted from the federal budget accounted for 2% of the total funds spent on anti-epidemic activities in the country, funds attracted from extrabudgetary sources – for 5%. Funding from the federal budget was allocated to 17 Subjects that needed and applied for federal budget funding for 2019. According to the Subjects' veterinary executive authorities, 60 Subjects had no need for federal budget funding, but in 2018 the outbreaks of highly dangerous and quarantinable diseases, such as rabies, brucellosis, African swine fever, avian influenza, lumpy skin disease, sheep pox, etc. were reported there (one to six infections in a Subject), and that could have served as a ground for applying for federal budget funding for anti-epidemic activities for 2019. Many of these Subjects may have intended to solve this issue through local budget funding only; these include 5 Subjects where 1 to 3 diseases (African swine fever, rabies, brucellosis) were reported, however they did not plan to receive and received no funding from any source in 2019.

Most of the Subjects (58 out of 85) were fully funded for the implementation of anti-epidemic activities from regional budgets and thus were able to purchase disinfectants, vaccines, diagnostic kits, test systems, expendables, etc. in sufficient quantities. The level of funding was very low (only 8% of requirements) in one Oblast. Funding situation can be considered as close to critical in 2 Subjects where funding levels were 20 and 28%. Besides, 8 Regions of the country received no funding from this source. In the remaining 16 Subjects, 43 to 99% of requirements for anti-epidemic activity funding from local budgets were covered.

Thus, the analysis results show that there is an obvious connection between funding and infectious animal disease freedom (including with respect to quarantinable and highly dangerous diseases) of a Subject. Consequently, underfunding of such activities has negative impact on the epidemic situation in the Regions of the country.

Many indicators, in particular the level of funding for ***the purchase and fit-out of facilities*** for the veterinary service activities, show the extent to which the Subjects' authorities are interested in veterinary service development. In the country as a whole, the level of such funding was as low as 37% of requirements. The level of funding provided from the Subjects' budgets was 25%. The problem was solved mainly through extrabudgetary funding that covered 93% of requirements. No federal budget funding was provided for. The level of funding for these expenditures was very low (3, 5 and 12%) in 3 Subjects. The veterinary services in 41 Regions received no funding for these purposes from local budgets. At the same time, the veterinary services in other 34 Subjects were 100% funded from the Subjects' budgets for the purchase and fit-out of facilities required for their activities. Funding levels varied within the range of 25–96% in the remaining 7 Regions. Appropriate implementation of duties and responsibilities assigned to the veterinary service, in particular the number and scope of veterinary activities conducted, depend on the condition and fit-out of its facilities.

Funding for ***the purchase of laboratory instruments and equipment*** (including those required for veterinary and sanitary examination) was provided from the Subjects' budgets and extrabudgetary sources (only one Oblast received funding for these expenditures from the federal budget) and covered 67% of required funding for the veterinary service of the country as a whole. The overall level of veterinary service funding provided from the Subjects' budgets was 65%, of that provided from extrabudgetary sources – 73%. The level of funding was influenced by the situation in 9 Subjects, which, according to the submitted data, had the need for funding but in fact no funds were allocated to them. The veterinary services of 3 Oblasts were significantly underfunded (with their funding levels being 8, 12 and 15%) from the Subjects' budgets. The veterinary services in 46 Subjects received full (100%) funding from this source. Due to underfunding, veterinary laboratories were provided with less diagnostic tools than required, and that naturally affected the technical capabilities and modernization of laboratories.

The situation is much better as regards the funding of veterinary laboratories for ***the purchase of reagents and test systems*** in the country as a whole. Funds for these expenditures were provided from three sources, and 100% of funding requirements were covered. Most of the funds for these purposes were allocated from extrabudgetary sources being 2.4 times higher than funds provided from the Subjects' budgets and 11 times higher than those received from the federal budget. The federal budget funds were allocated to 7 Subjects only, and that allowed to fully cover the requirements for funding from this source.

The level of funding provided from local budgets for these expenditures was 100% in 46 Subjects. No funding from this source was envisaged for many veterinary services (in 30 Subjects). The level of funding from the Subject's budget was the lowest (5%) in one Oblast; in the other 8 Subjects, funding levels were 45–96%. Underfunding of laboratories can affect their technical capabilities, reduce the range of veterinary services provided, and this, in turn, will have impact on epidemic situation control in the Region.

As for veterinary laboratories' activities related to ***accreditation and maintenance of accreditation***, 73% of funds received for these purposes were attracted from extrabudgetary sources and only 27% – from the budgets of the Subjects. Extrabudgetary funding almost fully (97%) covered the requirements, whereas funds allocated from the Subjects' budgets covered only 3% of required funding from this source. This suggests that 60 Subjects received no funding from local budgets; in order to make up a shortfall of funding, funds from extrabudgetary sources were attracted. The situation is quite the opposite in 18 Subjects the authorities of which are interested in accreditation of the veterinary laboratories of the Region and, consequently, in the maintenance of

their status as evidenced by 100% funding provided for these activities. In the remaining 7 Subjects, the levels of funding provided by the Subjects were 9 to 59%. It is known that the accreditation of laboratories in a certain field serves as an evidence of their competence upon which the reliability of test results and the validity of managerial and organizational decisions made on their basis depend.

Funding for the purchase of various ***means of transport*** (in particular, specialized ones) was provided from the local budgets of the Subjects, as well as from extrabudgetary sources and covered 39 and 85% of requirements, respectively, in the country as a whole. In absolute terms, funds provided from the Subjects' budgets were 1.7 times more than those provided from extrabudgetary sources. Full (100%) funding from the Subjects' budgets was provided in 40 Regions of the country. However, no such funding was provided in 34 Subjects. The situation was close to critical in 5 Oblasts, where funding levels were 3 to 36%. Underfunding for the purchase of means of transport, especially of specialized ones, can result in undue replacement and maintenance of vehicles and, consequently, affect the mobility of veterinary service specialists and implementation of the full range of disinfection activities, and this can be critical for providing prompt veterinary assistance, especially in remote areas.

The purchase of ***Komarov's disinfection units (DUKs) and other disinfection units*** was 93% funded from the local budgets, and only 7% of funds were attracted from extrabudgetary sources. However, data show that funding allocated from the budgets was not sufficient, since it covered only 23% of required funding from the Subjects. This was due to the situation when the veterinary services of 13 Subjects had the need for funding for the purchase of such disinfection units, but received none. Altogether, 56 veterinary services received no funding from local budgets. At the same time, the veterinary services in 22 Regions were fully provided with funding from this source. For the remaining 7 veterinary services, this indicator varied over a wide range of 16–88%. In the country as a whole, the level of funding from both sources was 24%.

Funding for such essential activities as implementation of ***monitoring and screening programmes*** at the Subject level aimed at contagious animal disease control, the tasks and objectives of which are determined by the Subjects' veterinary services, was to be provided from the local budgets. Indeed, 96% of funds allocated for these purposes were provided from the Subjects' budgets and 4% – from extrabudgetary sources. Funding from the Subjects' budgets was allocated in 25 Regions of the country (full funding was provided). Regional funding was not provided for in 60 Subjects, despite the fact that the veterinary services of 6 Regions had the need for such funding during this period. In some Subjects, certain funds for implementation of such programmes were allocated from the local budgets, though there was no planned requirement for such funding. In the country as a whole, funding requirements for monitoring and screening programme implementation were fully covered.

Regional ***monitoring of quality and safety*** of food products, raw materials, animal products, feed and biological materials, the tasks and objectives of which are also determined by the Subject's veterinary service, was funded from regional budgets (89%) and extrabudgetary sources (11%). The situation was most favourable in one third of the Subjects (26 Subjects) where funding requirements for these expenditures were fully covered. The complete absence of funding was reported by 55 Regions of the country, 5 of which had the need for such funding. As for the remaining 3 Subjects, their funding levels were 25, 60 and 70%. In the country as a whole, the level of funding from both sources was 87%; in particular, the level of funding provided from the Subjects' budgets was 86%, from extrabudgetary sources – 89%. Increased funding will allow expanding the scope of tests for quality and safety parameters for food products, raw materials, animal products, feed, etc.

One of important tasks in the work of veterinary services is carrying out ***animal health awareness-raising and information activities*** (organization of citizens' meetings, production of leaflets, presentations, lectures, media publications, website maintenance, etc.) [9]. In total, about 40 million rubles were spent for their implementation in the country, which covered 87% of funding required for these purposes. Funding was provided from two sources: regional budgets and extrabudgetary sources (40 and 60%, respectively.) On average, across the country, the level of funding from the Subjects' budgets was 74%, from extrabudgetary sources – 99%. Funding from local budgets was provided in 27 out of 85 Regions of the country, 25 of which were 100% funded for these activities (in 2 Subjects, the funding levels were 0.4 and 11%). No funding from the local budgets was provided for in other Regions of the Russian Federation. Besides, the veterinary services of 37 Subjects received no funding from any source.

The complete absence of funding or low funding of such activities is likely to be indicative of the lack of the Subject authorities' interest in animal health awareness-raising and information activities among the people of the Region, which are primarily aimed at preventing animal diseases and enabling people to act independently in various situations. N. I. Pirogov, a great Russian surgeon, wrote, "Medicine of the future is preventive medicine" [10].

Funding levels for various activities of the state veterinary service described above are more visibly shown in Figure 2.

CONCLUSION

The analysis of information provided by the veterinary executive authorities of the Russian Federation Subjects allowed for objective assessment of funding level of the veterinary services of the country as a whole and of individual Subjects in relation to their main activities.

In the country as a whole, funding level was 96%. The budgets of the Subjects were the major source of funding which covered over half of total actual expenditures over the year. The largest part (62%) of all the funds allocated to the veterinary services was used to pay staff salaries, the rest of the funds were distributed among other activities.

Annual funding was fully provided from local budgets in 54 Regions of the country. The funding requirements for the following activities were fully covered through funding from the Subjects' budgets:

– staff salaries – in 64 Subjects;

– the attraction and retaining of qualified personnel – in 16 Subjects;

– advanced training – in 35 Subjects;

– the implementation of anti-epidemic activities – in 58 Subjects;

– the purchase and fit-out of facilities for the veterinary service activities – in 34 Subjects;
– the purchase of laboratory instruments and equipment – in 46 Subjects;
– the purchase of reagents and test systems – in 46 Subjects;
– the accreditation of veterinary laboratories and its maintenance – in 18 Subjects;
– the purchase of means of transport – in 40 Subjects;
– the purchase of DUKs and other disinfection units – in 22 Subjects;
– the implementation of monitoring and screening programmes at the Subject level – in 25 Subjects;
– the implementation of regional quality and safety monitoring for food products, raw materials, etc. – in 26 Subjects;
– animal health awareness-raising activities among the public – in 25 Subjects.

The effectiveness in veterinary activities depends on many factors, such as veterinary specialists' qualification level, the availability of specialized means of transport and equipment, the availability of facilities, public awareness level, etc. It is therefore essential that all the state veterinary service activities be fully funded from the budgets of various levels.

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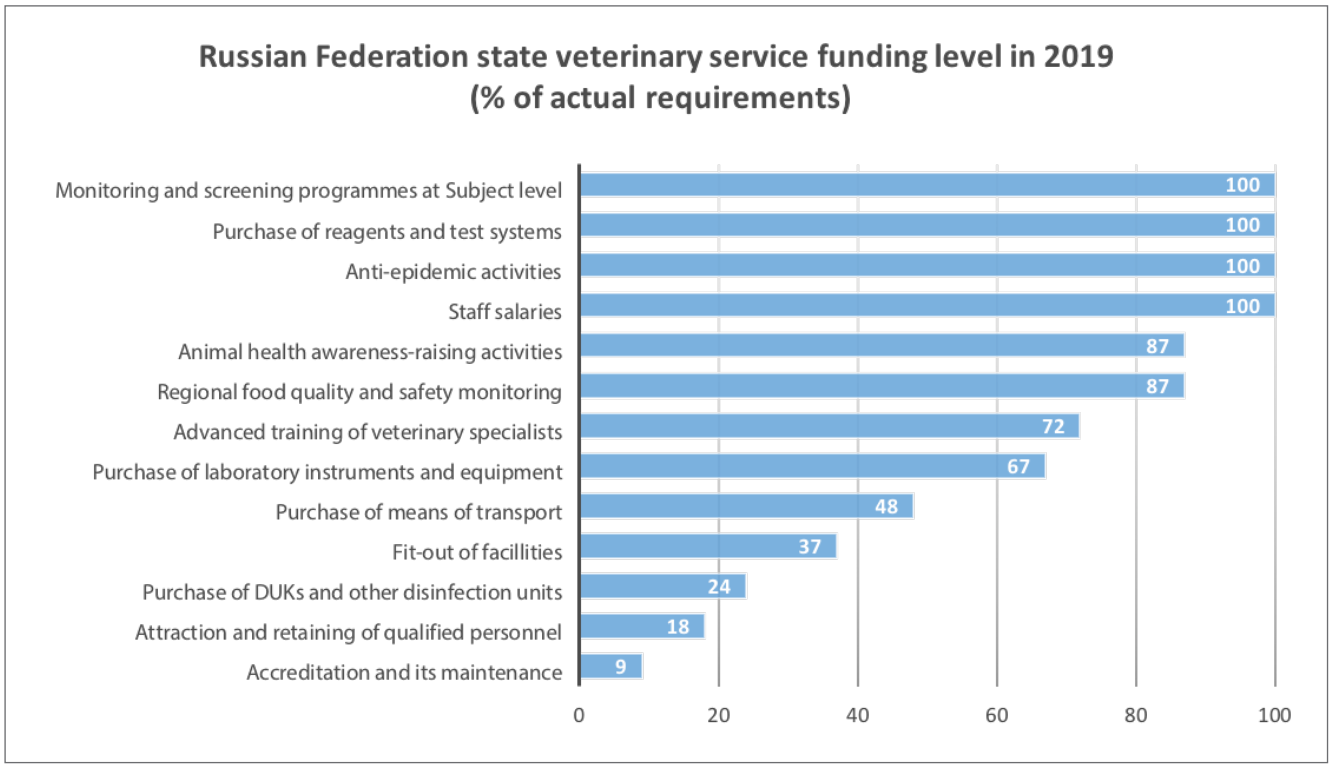

Fig. 2. Funding of the state veterinary service of the Russian Federation in 2019

Рис. 2. Финансирование государственной ветеринарной службы РФ в 2019 г.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Irina M. Klinovitskaya, Candidate of Science (Economy), Senior Researcher, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Mikhail A. Shibayev, Candidate of Science (Veterinary Medicine), Head of Sector, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Anton K. Karaulov, Candidate of Science (Veterinary Medicine), Head of Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia.

Клиновицкая Ирина Михайловна, кандидат экономических наук, старший научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Шибаев Михаил Александрович, кандидат ветеринарных наук, заведующий сектором информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Караулов Антон Константинович, кандидат ветеринарных наук, руководитель информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

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Investigation of the effects of Afyonkarahisar Region hot springs water on blood calcium and some hormon levels in experimentally-created osteoporosis in rats

Bülent Elitok[1], Tolgahan Saygin[2], Yavuz Ulusoy[3], Bahadir Kilinç[4]

[1,2] Afyon Kocatepe University, Afyonkarahisar, Turkey

[3,4] Veterinary Control Central Research Institute, Ministry of Agriculture and Forestry, Ankara, Turkey

[1] *ORCID: 0000-0003-3336-4479, e-mail: elitok1969@hotmail.com*

[3] *e-mail: yavuz.ulusoy@tarim.gov.tr*

[4] *e-mail: bahadir.kilinc@tarim.gov.tr*

SUMMARY

Today, one of the most common non-communicable diseases, which, according the World Health Organization experts, dominates the structure of human morbidity and mortality is osteoporosis. The aim of this study was to determine the effect of water from the hot springs of the Afyonkarahisar region on the calcium content and the levels of certain hormones in the blood of rats with experimentally induced osteoporosis. 25 female albino rats of the same age were used in the experiment. Ovaries were removed from all animals under anesthesia with ketamine (200 mg/kg) and xylazine (10 mg/kg), after which they were divided into two groups: control (10 animals) and experimental (15 animals). The animals of the control group were given tap water twice a day through an orogastric tube and they were bathed in it for 15 minutes at the same time, the water temperature was (35 ± 2) °C. The animals of the experimental group were given fresh water from the Süreyya I hot spring using the same method. Blood clinical, hematological and biochemical parameters were measured prior to the study, as well as on day 1, 7, 14, and 21 after the ovariectomy operation. The ovariectomy demonstrated inconsistence of the tested blood parameters with the standard ones. In the course of the treatment, by day 21 of the experiment, the parameters normalized, and the most noticeable changes were observed in the rats of the experimental group ($p < 0.05$). The results of the work performed showed that Süreyya I hot spring water contributed to a significant improvement in the clinical, hematological and biochemical blood parameters in rats with osteoporosis, therefore, it can be used for prevention and treatment of this disease in combination with other types of treatment.

Keywords: Afyonkarahisar, balneotherapy, osteoporosis, rat.

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For correspondence: Bülent Elitok, Doctor of Science, Associate Professor, Department of Internal Medicine, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200, Turkey, Afyonkarahisar, *e-mail: elitok1969@hotmail.com*.

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Исследование воздействия термальной воды из источника региона Афьонкарахисар на содержание кальция и уровни некоторых гормонов в крови крыс с экспериментально воспроизведенным остеопорозом

Bülent Elitok[1], Tolgahan Saygin[2], Yavuz Ulusoy[3], Bahadir Kilinç[4]

[1,2] Университет Афьон Коджатепе, г. Афьонкарахисар, Турция

[3,4] Центральный научно-исследовательский институт ветеринарного контроля, Министерство сельского и лесного хозяйства, г. Анкара, Турция

[1] *ORCID 0000-0003-3336-4479, e-mail: elitok1969@hotmail.com*

[3] *e-mail: yavuz.ulusoy@tarim.gov.tr*

[4] *e-mail: bahadir.kilinc@tarim.gov.tr*

РЕЗЮМЕ

На сегодняшний день одним из наиболее распространенных неинфекционных заболеваний, которое, по данным экспертов Всемирной организации здравоохранения, занимает ведущее место в структуре заболеваемости и смертности населения, является остеопороз. Целью настоящего исследования было определение влияния воды из термальных источников региона Афьонкарахисар на содержание кальция и уровни некоторых гормонов в крови крыс с воспроизведенным остеопорозом. В эксперименте использовали 25 самок крыс-альбиносов одного возраста. У всех животных под анестезией с использованием кетамина (200 мг/кг) и ксилазина (10 мг/кг) удалили яичники, после чего их разделили на две группы: контрольную (10 особей) и опытную (15 особей). Животных контрольной группы дважды в день через орогастральный зонд выпаивали водопроводной водой и в течение 15 минут в одно и то же время купали в ней, температура воды составляла (35 ± 2) °С. Животные опытной группы в том же режиме получали свежую воду из горячего источника Süreyya I. Клинические, гематологические и биохимические параметры крови измеряли до начала исследования, а также на 1, 7, 14 и 21-е сут после овариэктомии. Показано, что после овариэктомии отмечались отклонения от нормы величин исследуемых показателей крови. В процессе лечения к 21-м сут эксперимента наблюдался процесс нормализации показателей, наиболее заметные изменения произошли у крыс опытной группы ($p < 0,05$). По итогам работы было установлено, что вода из термального источника Süreyya I способствовала значительному улучшению клинических, гематологических и биохимических показателей крови у крыс с остеопорозом, следовательно, ее можно использовать для профилактики и лечения данного заболевания в комплексе с другими видами лечения.

Ключевые слова: Афьонкарахисар, бальнеотерапия, остеопороз, крыса.

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Для корреспонденции: Elitok Bülent, доктор наук, доцент, кафедра терапии факультета ветеринарной медицины, Университет Афьон Коджатепе, 03200, Турция, г. Афьонкарахисар, *e-mail: elitok1969@hotmail.com.*

INTRODUCTION

Osteoporosis is a disease characterized by low bone density and increased brittleness in the bones and is one of the common syndromes of the menopause period [1]. It was reported that osteoporosis was responsible for 1,700 bone fractures per day in Europe, and this number will increase with rising in the elderly population and put a great burden on the health system [2]. As a matter of fact, not only the resorption in the bone accelerates in the menopause, but also some changes in many blood parameters, especially hormones, such as estrogen [3].

Various substances in hot spring waters have been shown to provide significant benefits in reducing the symptoms of osteoporosis by reducing the osteoclastic effect [4]. In addition, it is stated that the calcium, which is found in the hot water and fast absorbed in the intestine, and increases the ionized calcium level in the blood and eliminates the calcium deficit. Therefore, the oral intake of the spa waters will provide great benefits in closing the calcium deficiencies [5]. It has been reported that treatment with hot spring waters can also benefit ovaries to work more regularly, actively and for a longer period of time, by helping the circulatory system to work more actively, to help nutrition and regulate tissues [6].

In this study, it was aimed to reveal the effectiveness of Süreyya I hot spring water, which has rich mineral content, especially calcium, in the treatment of osteoporosis.

MATERIALS AND METHODS

The experimental part of this study was carried out in Afyon Kocatepe University Experimental Animals Application and Research Center, in accordance with the Directive of Afyon Kocatepe University Experimental Animals Ethics Committee (AKUHADYEK) with the report number 137-18, and supported by Afyon Kocatepe University Scientific Research Projects Board (AKÜBAPK) as Master's Thesis Project with No. 19.SAĞ.BİL.04.

Animal Material. In this study, 25 female Albino rats of the same age were used. Ten of 25 rats served as control group (CG), while 15 rats assigned as study group (SG). All the rats was kept in plastic cages in Afyon Kocatepe University Experimental Animals Application and Research Center in a stable environment where the same humidity (50–60%) and heat (17–22 °C) conditions were created for 12 hours night and 12 hours day. During the study, the rats were provided to get *ad libitum* rat food.

Creating Experimental Osteoporosis. In this study, ovariectomy operation was performed according to the method reported by M. Berkoz et al. [7]. According to this method ketamine (200 mg/kg) and xylazine (10 mg/kg) anesthesia was administered intraperitoneally, following disinfection and shaving of the operation area in female rats. Then, the abdominal cavities were opened, the tubers were ligated using '0 chrome caTRIGut, and ovaries was removed from uterus. Finally, peritoneum, connective tissue, muscle tissue and skin were sutured. Operation site was disinfected with Batikon.

Groups and Treatment Procedure. A total of 25 rats, 10 from the control group and 15 from the study group, whose ovaries were removed, were taken into the 21-day treatment period according to the following method:

1. Control Group (CG). Totally 10 ovariectomized rats received tap water twice a day with an orogastric tube at the same time every day with the calculation of 1 L/33 kg body

weight. In addition, all rats in this group were bathed at (35 ± 2) °C in tap water for 15 minutes at the same time every day. After bathing, the rats were placed in their cages.

2. Study Group (SG). In order to treat 15 ovariectomized rats in this group, Süreyya I hot spring water was given twice a day with an orogastric tube at the same time every day with the calculation of 1 L/33 kg bw. In addition, all rats in this group were bathed at (35 ± 2) °C in Süreyya I hot spring water for 15 minutes at the same time every day, and following the bath, the rats were placed in their cages.

Süreyya I Spa Spring water, whose terrapeutic effectiveness on osteoporosis was investigated in this study, is a volcanic origin mineral water, containing carbon dioxide and rich in minerals such as calcium, magnesium, and it has a mineral content of more than 4 g (4,046.8 µg/L) per liter.

Blood samples were collected according to the method previously described by H. B. Waynforth and P. A. Flecknell [8] before the study, after the ovariectomy operation, and on days of 1st, 7th, 14th and 21st following ovariectomy operation. Blood samples were collected under ketamine (200 mg/kg) and xylazine (10 mg/kg) anaesthesia acoording to the method described by M. A. Suckow et al. [9].

Clinical Examinations. Body temperatures (T), respiration rates (R) and heart frequencies (P) were measured and recorded in all the rats during the study.

Hematological Examinations. For hematological examinations, blood samples collected into blood tubes containing EDTA, and measured in Chemray Brand blood count commercial test kits (Rayto Life and Analytical Sciences Co., China). Total leukocyte (WBC), erythrocyte (RBC), hematocrit (HCT), hemoglobin (HB), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), lymphocytes (LYM), neutrophils (NEUT), eosinophils (EOS), monocytes (MON) and basophils (BAS) were measured hematologically.

Blood Biochemical Examinations. Gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB) and glucose (GLU), triglyceride (TRIG), total cholesterol (TCHOL), high-density lipoprotein (HDL), low density lipoprotein (LDL) was measured in Cobas Integra 400 Plus (Roche Diagnostics GmbH, Germany) analyzer using commerical kits. In addition, estradiol (E2), calcitonin (CT) and calcium (Ca) measurements were made with ChemWell, Chromate 4300 Elisa Reader device (Awareness Technology, Inc., USA) using commercial Elisa kits (Sunred Biological Technology Co., Ltd, China).

Statistical Analyses. Analysis of variance (ANOVA) were used for statistical analyses. Intra-group differences were revealed by Duncan test. For statistical analyses, Windows-compatible SPSS Statistics 18.1 (IBM, USA) package program was used, and $p < 0.05$ was determined as an important value.

Table 1
Statistical comparison of body temperature, pulse frequency and respiratory rate

Таблица 1
Статистическое сравнение температуры тела, частоты пульса и дыхания у животных

Time of indicator measurement by groups		Parameters (X ± SD)		
		T (°C)	P (frequence/min)	R (rate/min)
BS ($n = 25$)		37.30 ± 0.30	313.16 ± 45.22^{d}	104.23 ± 32.23^{d}
AOF ($n = 25$)		37.30 ± 0.20	309.18 ± 43.24^{d}	103.12 ± 30.20^{d}
AT 1st day	CG ($n = 10$)	37.20 ± 0.30	312.26 ± 41.14^{d}	104.18 ± 26.34^{d}
	SG ($n = 15$)	37.30 ± 0.20	333.27 ± 32.21^{cd}	109.16 ± 24.12^{c}
AT 7th day	CG ($n = 10$)	37.30 ± 0.20	313.12 ± 22.18^{d}	105.13 ± 18.24^{d}
	SG ($n = 15$)	37.30 ± 0.20	346.12 ± 21.12^{c}	116.20 ± 12.22^{b}
AT 14th day	CG ($n = 10$)	37.30 ± 0.20	314.11 ± 10.25^{d}	106.04 ± 10.10^{d}
	SG ($n = 15$)	37.20 ± 0.20	357.22 ± 11.28^{b}	118.45 ± 8.10^{a}
AT 21st day	CG ($n = 10$)	37.20 ± 0.20	313.13 ± 6.13^{d}	105.16 ± 4.24^{d}
	SG ($n = 15$)	37.30 ± 0.20	464.31 ± 7.16^{a}	119.21 ± 3.16^{a}
Normal physiological parameters		37.00–38.00	360.00 ± 3.30	100.90 ± 4.40

$^{a–d}$ The values in the column are statistically significant ($p < 0.05$).
$^{a–d}$ Значения в столбце являются статистически значимыми ($p < 0,05$).
BS – before study (до исследования), AOF – after ovariectomy (после овариэктомии), AT – after treatment (после лечения), CG – control group (контрольная группа), SG – study group (экспериментальная группа).

TEST RESULTS

Although no age difference was detected between the groups ($p > 0.05$), the mean bw before starting the study (310.2 g) was found to be statistically significantly higher ($p > 0.05$) than the mean bw after ovariectomy operation (286.4 g). The most important loss of body weight was mesured in SG rats (283.2 g) on 21st day of treatment.

Clinical Findings

The clinical findings determined for both groups are presented in Table 1. According to this Table, while no significant difference was observed in periods measured in terms of T ($p > 0.05$), it was determined that P and R statistically significant differences ($p < 0.05$) occurred, and the highest levels were formed on day 21 in SG animals.

Hematological Findings

The results of the hematological measurements shown in Table 2. According to this Table; it was determined that mean WBC, NEUT and MCV increased significantly ($p < 0.05$) following ovariectomy operation. Conversely, mean RBC, HB, HCT, LYM and MCHC decreased significantly ($p < 0.05$). With the start of the treatment period, mean RBC, HB, HCT, LYM, and MCHC levels increased in both groups, but these increases were statistically more significant ($p < 0.05$) in SG animals. However, it was observed that the biggest heamtological changes in the last day of study (21th day) in SG animals.

Blood Biochemical Findings

The blood biochemical measurements shown in Table 3. According to this Table; it was seen that mean GGT, AST, LDL, TRIG, TCHOL and GLU increased significantly ($p < 0.05$), whereas mean ALB, TP, CT, E2, HDL and Ca decreased significantly ($p < 0.05$) following ovariectomy operation. On the other hand, it was determined that a reverse course was shaped in terms of the mentioned parameters, and the most important changes occurred in SG rats and on the 21st day of the study ($p < 0.05$) during treatment period.

DISCUSSION AND CONCLUSION

In our study, it was observed that the mean bw decreased after the ovariectomy operation, and the lowest

Table 2
Results of hematology blood tests

Таблица 2
Результаты гематологических исследований крови животных

Time of indicator measurement by groups		Parameters (X ± SD)											
		WBC (10^3/mm^3)	RBC (10^6/mm^3)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	LYM (%)	NEUT (%)	EOS (%)	MON (%)	BAS (%)
BS ($n = 25$)		13.56 ± 3.12^{c}	8.57 ± 4.11^{a}	13.26 ± 3.22^{a}	42.23 ± 5.12^{a}	49.26 ± 5.32^{g}	15.48 ± 3.21	31.40 ± 3.23^{ab}	70.14 ± 12.22^{a}	27.14 ± 5.18^{f}	3.30 ±1.10^{a}	0.45 ± 0.10^{b}	NS
AOF ($n = 25$)		18.29 ± 4.16^{a}	6.48 ± 5.22^{c}	10.37 ± 4.12^{b}	37.46 ± 7.22^{c}	57.20 ± 7.18^{ab}	16.01 ± 3.43	27.71 ± 5.18^{c}	56.43 ± 7.44^{d}	43.10 ±6.34^{a}	1.20 ± 1.30^{d}	0.30 ± 0.20^{c}	NS
AT 1st day	CG ($n = 10$)	18.47 ± 4.08^{a}	6.23 ± 3.43^{c}	10.28 ± 4.32^{b}	37.12 ± 6.44^{c}	58.97 ± 6.20^{a}	16.52 ± 3.28	27.70 ± 4.22^{c}	55.28 ± 6.47^{d}	43.20 ± 3.20^{a}	1.24 ± 0.50^{d}	0.27 ± 0.10^{c}	NS
	SG ($n = 15$)	17.34 ± 4.22^{ab}	6.78 ± 3.27^{c}	10.79 ± 3.37^{b}	38.12 ± 5.32^{bc}	56.28 ± 5.44^{b}	15.90 ± 2.09	28.29 ± 4.19^{bc}	55.14 ± 6.13^{d}	43.30 ± 3.10^{a}	1.45 ± 0.40^{c}	0.20 ± 0.20^{d}	NS
AT 7th day	CG ($n = 10$)	17.16 ± 3.44^{ab}	6.96 ± 2.23^{bc}	11.04 ± 2.23^{bc}	38.14 ± 3.32^{a}	54.81 ± 4.18^{c}	15.89 ± 2.53	26.43 ± 3.23^{c}	56.18 ± 4.36^{e}	43.10 ± 1.40^{a}	1.14 ± 0.30^{ab}	0.30 ± 0.20^{c}	NS
	SG ($n = 15$)	15.45 ± 3.14^{b}	7.48 ± 2.31^{b}	12.34 ± 2.43^{ab}	40.12 ± 2.23^{ab}	53.60 ± 4.24^{bc}	16.59 ± 2.16	30.78 ± 3.41^{b}	60.11 ± 4.21^{c}	38.10 ± 1.10^{c}	1.50 ± 0.40^{c}	0.40 ± 0.28^{b}	NS
AT 14th day	CG ($n = 10$)	16.01 ± 2.26^{b}	7.04 ± 1.12^{b}	11.19 ± 1.36^{bc}	39.16 ± 2.09^{a}	55.61 ± 4.12^{d}	15.91 ± 2.06	28.58 ± 2.48^{bc}	58.48 ± 3.26^{d}	40.40 ± 1.40^{b}	1.70 ± 0.30^{bc}	0.30± 0.20^{c}	NS
	SG ($n = 15$)	14.05 ± 2.16^{bc}	8.03 ± 1.06^{ab}	13.24 ± 1.34^{a}	41.48 ± 1.16^{ab}	51.66 ± 3.14^{e}	16.50 ± 1.18	31.90±2.78^{ab}	61.24 ± 1.13^{b}	36.27 ± 1.30^{d}	2.10 ± 0.30^{b}	0.40 ± 0.40^{b}	NS
AT 21st day	CG ($n = 10$)	15.87 ± 1.08^{b}	7.34 ± 0.36^{bc}	11.45 ± 0.34^{bc}	39.15 ± 0.37^{b}	53.34 ± 3.17^{bc}	15.63 ± 1.46	29.27 ± 1.46^{b}	57.28 ± 0.57^{d}	39.60 ± 0.60^{b}	2.00 ± 0.40^{b}	0.50 ± 0.20^{ab}	NS
	SG ($n = 15$)	13.08 ± 1.22^{c}	8.69 ± 0.27^{a}	13.75 ± 0.28^{a}	42.65 ± 0.32^{a}	49.09 ± 2.16^{gf}	15.83 ± 1.43	32.24 ± 1.39^{a}	63.24± 0.66^{b}	34.20 ± 0.50^{e}	3.10 ± 0.30^{a}	0.60 ± 0.30^{a}	NS

$^{a–f}$ The values in the column are statistically significant ($p < 0.05$).

$^{a–f}$ Значения в столбце являются статистически значимыми ($p < 0,05$).

BS – before study (до исследования), AOF – after ovariectomy (после овариэктомии), AT – after treatment (после лечения),
CG – control group (контрольная группа), SG – study group (опытная группа).
WBC – white blood cells (лейкоциты), RBC – red blood cells (эритроциты), HB – hemoglobin (гемоглобин),
HCT – hematocrit (гематокрит), MCV – mean corpusculare volume (средний объем эритроцитов),
MCH – mean corpuscular hemoglobin (среднее содержание гемоглобина в эритроците),
MCHC – mean corpuscular hemoglobin concentration (средняя концентрация гемоглобина в эритроците),
LYM –lymphocyte (лимфоциты), NEUT – neutrophils (нейтрофилы), EOS – eosinophils (эозинофилы),
MON – monocyte (моноциты), BAS – basophils (базофилы).

Table 3
Blood biochemical findings of the animals

Таблица 3
Результаты биохимических исследований крови животных

Time of indicator measurement by groups		Parameters (X ± SD)											
		AST (IU/L)	GGT (IU/L)	ALB (g/dl)	TP (g/dl)	GLU (g/dl)	E2 (pg/L)	CT (pg/ml)	Ca (mg/dl)	TCHOL (mg/dL)	HDL (mg/dl)	LDL (mg/dl)	TRIG (mg/dl)
BS ($n = 25$)		153.25 ± 43.20^{c}	13.24 ± 3.12^{c}	40.20 ± 12.23^{ab}	66.62 ± 21.44^{a}	120.14 ± 32.18^{f}	14.48 ± 4.24^{a}	5.27 ± 2.22^{a}	6.28 ± 2.18^{a}	91.82 ± 9.27^{f}	45.12 ± 10.25^{a}	67.48 ± 14.17^{f}	97.23 ± 23.14^{e}
AOF ($n = 25$)		175.68 ± 52.30^{a}	17.48 ± 6.24^{a}	34.48 ± 18.10^{d}	54.48 ± 32.26^{d}	156.16 ± 27.28^{a}	1.03 ± 0.36^{d}	2.16 ± 1.43^{e}	3.46 ± 1.24^{d}	139.34 ± 25.23^{a}	26.17 ± 12.32^{c}	138.44 ± 19.56^{a}	213.48 ± 37.23^{a}
AT 1st day	CG ($n = 10$)	176.48 ± 51.13^{a}	17.56 ± 6.44^{a}	34.10 ± 17.30^{d}	55.14 ± 31.23^{d}	147.13 ± 25.12^{b}	0.56 ± 0.13^{de}	2.28 ± 1.34^{e}	3.53 ± 1.14^{d}	138.31 ± 22.16^{a}	26.40 ± 10.03^{c}	138.41 ± 17.24^{a}	214.44 ± 38.56^{a}
	SG ($n = 15$)	172.14 ± 50.21^{a}	17.04 ± 5.16^{a}	35.14 ± 16.40^{d}	56.34 ± 27.33^{d}	138.13 ± 13.48^{c}	1.54 ± 0.10^{d}	2.33 ± 1.25^{e}	3.67 ± 1.10^{d}	133.17 ± 21.34^{b}	27.14 ± 10.43^{c}	132.45 ± 15.12^{b}	207.21 ± 35.23^{a}
AT 7th day	CG ($n = 10$)	172.14 ± 50.21^{a}	17.04 ± 5.16^{a}	35.14 ± 16.40^{d}	56.34 ± 27.33^{d}	138.13 ± 13.48^{c}	1.54 ± 0.10^{d}	2.33 ± 1.25^{e}	3.67 ± 1.10^{d}	137.22 ± 18.48^{a}	26.13 ± 9.20^{c}	137.36 ± 12.44^{a}	213.44 ± 31.09^{a}
	SG ($n = 15$)	174.18 ± 37.40^{a}	16.87 ± 3.32^{a}	35.34 ± 11.18^{d}	56.45 ± 28.22^{a}	139.34 ± 11.13^{d}	0.32 ± 0.10^{e}	2.88 ± 1.32^{d}	3.41 ± 1.12^{d}	124.24 ± 15.67^{c}	36.21 ± 7.24^{b}	114.11 ± 12.32^{c}	176.14 ± 24.45^{b}
AT 14th day	CG ($n = 10$)	171.16 ± 22.10^{a}	16.48 ± 2.26^{a}	36.09 ± 10.11^{a}	57.14 ± 22.12^{c}	132.13 ± 11.12^{d}	1.30 ± 0.00^{a}	2.85 ± 1.48^{d}	3.64 ± 0.46^{d}	135.56 ± 14.12^{b}	27.02 ± 7.33^{c}	136.47 ± 9.13^{b}	211.07 ± 25.32^{a}
	SG ($n = 15$)	156.10 ± 18.10^{c}	13.08 ± 2.34^{c}	40.30 ± 7.32^{ab}	61.44 ± 17.08^{b}	124.22 ± 10.22^{e}	3.30 ± 0.00^{b}	4.13 ± 0.32^{b}	4.76 ± 0.58^{bc}	116.26 ± 10.33^{d}	37.28 ± 5.18^{b}	103.46 ± 8.26^{d}	154.21 ± 22.21^{c}
AT 21st day	CG ($n = 10$)	167.20 ± 11.10^{ab}	16.07 ± 1.28^{a}	37.03 ± 5.12^{c}	58.14 ± 12.18^{c}	131.16 ± 8.24^{de}	1.20 ± 0.00^{a}	3.04 ± 0.56^{c}	3.71 ± 0.43^{d}	134.20 ± 9.27^{b}	27.24 ± 5.43^{c}	134.12 ± 6.14^{b}	209.03 ± 14.27^{a}
	SG ($n = 15$)	151.48 ± 9.10^{c}	12.98 ± 1.14^{c}	41.10 ± 4.14^{a}	67.03 ± 9.16^{a}	118.33 ± 7.12^{f}	3.20 ± 0.00^{b}	4.27 ± 0.48^{b}	5.45 ± 0.37^{b}	105.48 ± 6.25^{e}	37.24 ± 4.11^{b}	83.29 ± 5.47^{e}	121.18 ± 13.14^{d}

$^{a–f}$The values in the column are statistically significant ($p < 0.05$).

$^{a–f}$Значения в столбце являются статистически значимыми ($p < 0,05$).

BS – before study (до исследования), AOF – after ovariectomy (после овариэктомии), AT – after treatment (после лечения),
CG – control group (контрольная группа), SG – study group (экспериментальная группа).
AST – aspartate aminotransferase (аспартатаминотрансфераза),
GGT – gamma-glutamyl transferase (гамма-глутамилтрансфераза), ALB – albumin (альбумин),
TP – total protein (общий белок), GLU – glucose (глюкоза), E2 – estradiol (эстрадиол), CT – calcitonin (кальцитонин),
Ca – calcium (кальций), TCHOL – total cholesterol (общий холестерин),
HDL – high-density lipoprotein (липопротеины высокой плотности),
LDL – low density lipoprotein (липопротеины низкой плотности), TRIG – triglyceride (триглицериды).

mean bw was obtained in SG rats treated with Süreyya I hot spring water. This finding was found to be consistent with findings reported by researchers [10] who found that treatment with mineral waters reduced lipid absorption, but increased burning.

In our current study, P frequencies and R rates in SG rats which were treated with hot spring water were higher. Y. Agishi claimed that hot spring water caused an increase in circulating flow, resulting in vasodilation in peripheral vessels and step-up in P frequency and R rate [11].

D. L. Millis et al. reported that leukocytosis occurred in the hemogram after the experimental ovariectomy operation in the dogs, while neutrophilia, lymphopenia and eosinopenia were noticeable in the differential blood picture [12]. In our study, similarly measurements were determined following the ovariectomy operation. Moreover, it was found that the percentages of NEUT increased, while the percentages of LYM and EOS decreased. With the transition to the treatment period, it was determined that WBC and NEUT levels decreased in SG rats treated with Süreyya I hot spring water. This finding is consistent with some researchers [13] who reported that hot mineral waters suppress the immune system and that adrenocorticotropic hormone and cortisol release in baths made with these waters increase and lead to a decrease in T-lymphocytes. In addition, it has been reported that Mg deficiency has an encouraging effect on the occurrence of inflammatory symptoms, therefore it may have an effect on leukocytosis and macrophage activation [14]. In our current study, the detection of lower mean WBC acount in SG rats treated with Mg rich Süreyya I hot spring water was found to be compatible with findings found out by these above researchers.

As in humans, the increase in turnover due to rapid bone loss in the early period of the absence of estrogen is observed in rats [15]. Although there are 3 types of estrogen, E1 (estrone), E2 (estradiol), E3 (estriol), E2 measurements are used in the determination of estrogen levels, since estradiol (E2) is the strongest and most produced type of estrogen [16]. Indeed, estrogen has a direct effect on bone structure, acting on calcium metabolism, bilateral effect on calcium absorption and excretion [17]. In this study, determining that E2 and Ca levels decreased after ovariectomy operation supports the findings of these researcher. However, an increase of E2 and Ca levels in SG rats can be accepted as an evidence that the spa treatment we applied increased E2 and Ca levels.

In our study, it was also determined that AST, GGT and GLU levels measured after ovariectomy operation were high, whereas E2, CT, Ca, TP and ALB levels were low. After treatment period, E2, CT, Ca, TP and ALB levels were higher in SG rats which treated with Süreyya I hot spring water when compare to CG rats which treated with tap water. Additionally, AST and GGT levels were still higher in CG animals, unlike SG rats.

It has been reported that LDL, TRIG and TCHOL levels increase significantly in the case of menopause, whereas HDL levels decrease [18]. Similar findings were determined following ovariectomy operation. With the start of treatment, complete improvement in lipid profile was observed; LDL, TRIG and TCHOL levels decreased, while HDL levels increased in SG rats which treated with Süreyya I hot spring water. These data confirm the findings of some researchers [19] who reported that mineral waters were highly effective in normalizing the degraded blood lipid levels. Abnormal lipid profile was still observed in CG rats even on day of 21 of the treatment.

Calcitonin (CT) is a hormone produced by thyroid gland C cells that increases calcium and phosphate accumulation in the bone and slows the osteolysis process by decreasing osteoclast activity [20]. In our study, although CT levels decreased after ovariectomy operation, it was found that by the onset of the treatment period, CT levels significantly increased in SG animals, while there was an opposite situation in CG animals. Our findings was in line with the findings obtained by M. Cecchettin et al. who reported that spa treatments had positive effects on bone density and increased CT levels in women with osteoporosis in the postmenopausal period undergoing spa treatment [21].

Some researchers reported that bone-derived osteocalcin hormone regulates the insulin secretion of the pancreas, while estrogen has a direct effect on it [22]. It has also been reported that blood GLU levels have a direct relationship with CT levels so that glucagon infusions decrease CT levels, while Ca infusions decrease glucagon levels and lead to an increase in CT levels [23]. Higher GLU levels following ovariectomy in this study was in full agreement with results of another study [24]. On the other hand, increased GLU levels were observed in CG animals even at the and of the study, despite normalized in SG rats.

Consequently, it has been determined that Süreyya I hot spring water was very successful in treatment of osteoporosis in the rats with osteoporosis. Hence, it can be used safely in removing unwanted symptoms of osteoporosis itself or when combined with other medical treatments.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Bülent Elitok, Associate Professor, Doctor of Science, Department of Internal Medicine, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey.

Tolgahan Saygin, Afyon Kocatepe University, Afyonkarahisar, Turkey.

Yavuz Ulusoy, Doctor of Science, Head of Pathology Laboratory, Veterinary Control Central Research Institute, Ministry of Agriculture and Forestry, Ankara, Turkey.

Bahadir Kilinç, Veterinarian, Pathology Laboratory, Veterinary Control Central Research Institute, Ministry of Agriculture and Forestry, Ankara, Turkey.

Bülent Elitok, доцент, доктор наук, кафедра терапии, факультет ветеринарной медицины, Университет Афьон Коджатепе, г. Афьонкарахисар, Турция.

Tolgahan Saygin, Университет Афьон Коджатепе, г. Афьонкарахисар, Турция.

Yavuz Ulusoy, доктор наук, заведующий лабораторией патологии, Центральный научно-исследовательский институт ветеринарного контроля, Министерство сельского и лесного хозяйства, г. Анкара, Турция.

Bahadir Kilinç, ветеринарный врач лаборатории патологии, Центральный научно-исследовательский институт ветеринарного контроля, Министерство сельского и лесного хозяйства, г. Анкара, Турция.

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Contact information:

tel. +7(4922) 26-15-12 (ext. 25-20), *e-mail: romenskaya@arriah.ru*

tel. +7(4922) 52-99-62, *e-mail: elnikova@arriah.ru*

tel.+7 (4922) 26-15-12 (ext. 23-10), *e-mail: komova@arriah.ru*

tel: +7 (4922) 26-15-12 (ext. 22-27), *e-mail: nikeshina@arriah.ru*

tel: +7 (4922) 26-15-12 (ext. 25-01), *e-mail: guseva_ey@arriah.ru*