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DECEMBER | ДЕКАБРЬ №4 [35] 2020

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CONTENTS

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Analysis of Asia-1 foot-and-mouth disease global spread in 1999–2019

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SUMMARY
In spite of current foot-and-mouth disease (FMD) preventive measures, the disease outbreaks are annually reported in different countries of the world. FMD tends to extensive spread and growing into epidemics. While being a transboundary infection according to the OIE/FAO classification, FMD severely affects the economy and international trade. The paper describes the analysis of the data on global spread of type Asia-1 virus-induced FMD in 1999–2019. The virus of this type is most often reported in such Asian countries as Afghanistan, Pakistan, China, Nepal, Iran, Myanmar, from where it can spread to FMD free countries. In China, Asia-1 FMD outbreaks were reported from 2001 to 2009. Previously exotic for our country, the virus of this type was first reported in the Primorsky, Khabarovsk, Zabaikalsky Krais and Amur Oblast in 2005–2006. The results of the phylogenetic analysis of the recovered isolates demonstrated that FMD emergence in the Subjects of the Russian Federation was attributed to the virus introduction from the neighboring territories. Possible virus introduction from China resulted in significant economic expenditures on FMD containment and eradication. In view of the close trade and economic relations between the Russian Federation and such Asia-Pacific countries as China, India, Japan and Republic of Korea, one should place greater focus on the risk of FMDV introduction into the Russian Federation from these countries. Of key importance is intensification of the international cooperation with the Asian countries in the area of joint activities aimed at FMD freedom maintenance.

Key words: foot-and-mouth disease virus (FMDV), type Asia-1, susceptible animals, outbreak, virus introduction, disease spread.

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Анализ распространения вируса ящура типа Азия-1 в мире с 1999 по 2019 год

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РЕЗЮМЕ
Несмотря на принимаемые меры профилактики, направленные на предупреждение возникновения ящура, вспышки заболевания ежегодно регистрируют в различных странах мира. Ящур имеет тенденцию к широкому распространению и приобретению размаха эпизоотий. Являясь по современной классификации МЭБ/ФАО трансграничной инфекцией, ящур оказывает крайне негативное влияние на экономику и международную торговлю. В статье представлен анализ данных по распространению в мире в 1999–2019 гг. ящура, вызванного вирусом типа Азия-1. Наиболее часто вирус данного типа регистрируется на территории таких азиатских стран, как Афганистан, Пакистан, Китай, Непал, Иран, Мьянма, откуда он может распространяться в свободные от ящура страны. В Китае вспышки заболевания ящуром типа Азия-1 регистрировались с 2001 по 2009 г. Ранее считавшийся экзотическим для нашей страны, вирус этого типа был впервые зарегистрирован в 2005–2006 гг. на территории Приморского, Хабаровского, Забайкальского краев и Амурской области. Как показали результаты филогенетического анализа полученных изолятов, возникновение ящура в субъектах Российской Федерации было обусловлено заносом вируса из сопредельных территорий. Вероятный занос вируса из Китая привел к значительным экономическим затратам на ликвидацию ящура и недопущение дальнейшего распространения заболевания. Учитывая тесные торгово-экономические связи Российской Федерации с государствами Азиатско-Тихоокеанского региона, где основными партнерами являются Китай, Индия, Япония и Республика Корея, следует уделять повышенное внимание риску заноса вируса ящура из этих стран на территорию нашей страны. Важную роль играет укрепление международного сотрудничества со странами Азии с целью принятия совместных мер по обеспечению благополучия по ящуру.

Ключевые слова: вирус ящура, тип Азия-1, восприимчивые животные, вспышка инфекции, занос вируса, распространение болезни.

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, which severely affects the economies of the countries worldwide. The key method for the disease prevention involves immunization of the susceptible animals in the areas of high risk of FMD introduction and spread [1, 2]. The disease control is complicated by the diversity of the virus serotypes (types A, O, C, Asia-1, SAT-1, SAT-2, SAT-3), its genetic variability and restricted specificity of the animal immunity within one serotype. There are currently a number of FMD endemic countries. Global FMD situation is extremely sensitive and in spite of all preventive measures taken, the disease outbreaks are annually reported in different countries [3].

Due to the threat of Asia-1 FMD occurrence in Russia, especially along the borders with China, Mongolia and Middle Asian and Transcaucasian countries, the work was aimed at the analysis of the domestic and foreign published reports on global spread of this virus type in 1999–2019.

MATERIALS AND METHODS

Globally reported FMD outbreaks were analyzed with reference to the open access publications in the databases of the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD, Pirbright, Great Britain) and World Animal Health Organization (OIE) [4–6].

Comparative historical and comparative geographical tools were used for the analysis of Asia-1 FMD epidemic situation.

RESULTS AND DISCUSSION

Serotype Asia-1 foot-and-mouth disease virus (FMDV) is endemic in the Asian region and it was first isolated from the pathological material submitted to the World Reference Laboratory for Foot-and-Mouth Disease in Pirbright from Pakistan in 1954. Retrospective studies of some atypical isolates delivered from Indian town Izzatnagar in 1951–1952 demonstrated that they belonged to serotype Asia-1 and were the earliest documented FMDV isolates of this type. In 1957, Asia-1 FMD was introduced into the Near East, where the first outbreaks were reported in Israel. Later, the serotype became globally widespread [7–9].

In September 1999, Asia-1 FMD became widely spread in Iran, and in October that year, it was reported in Turkey. Then in 2000–2001, it was introduced into Armenia, Georgia, Greece and Azerbaijan, and in 2003 – in Tajikistan. In 2001–2005, the outbreaks of this FMDV type were also reported in Afghanistan, Bhutan, India, China, Laos, Mongolia, Myanmar, Nepal, Pakistan and Thailand. In Vietnam, the FMD outbreak occurred in Ha Giang Province in 2006. In January 2007, FMD clinical signs were reported in calves imported from Liaoning province (China) to the North Korean P'yŏngyang-si Provinces (Ryongkok-Ri, Sangwŏn-gun). In China, the first notifications of Asia-1 FMD were made on March 9, 2005 by Hong Kong, where the disease clinical signs were reported in cattle. Hereafter, the disease was reported in seven more provinces. In 2006, sixteen FMD outbreaks of this type were reported in the country; in 2008 – eight outbreaks and in 2008 – three outbreaks. Of particular note is the fact that in 2007 Asia-1 FMDV carriers were found among cattle in the Xinjiang Uyghur Autonomous Region located in the northwestern part of China along the border with Kazakhstan, Tajikistan, Mongolia and Russian Federation. The disease outbreaks were also reported in this autonomous region in February 2008 and January 2009. FMDV of the same type was detected in other Chinese provinces: Sichuan, Hunan, Guizhou and Shaanxi [10, 11]. In 2009–2011, Asia-1 FMDV of novel genetic lineage Sindh-08 became widespread in Pakistan, Bahrain, Iran, Afghanistan and it was also detected in Eastern Antalya, Turkey in 2011 [8]. In May 2011, the virus of this genetic lineage was officially confirmed in Tajikistan [12]. In 2013, Asia-1 FMDV of the novel genetic lineage BD-18 (G-IX) was reported in Bangladesh [8].

According to the OIE data for 2017, outbreaks of Asia-1 G-VIII FMDV originally detected in Kingdom Bahrain in 2009 were reported in Nepal, Afghanistan and Myanmar.

In 2018, Asia-1 FMD was detected in the following countries: Nepal, Afghanistan, Iran and Bangladesh. Iran and Afghanistan were determined to belong to the genetic lineage Sindh-08.

In 2019, Afghanistan, Bangladesh and Pakistan notified FMDV outbreaks (Fig. 1).

According to the diagram below (Fig. 2), Asia-1 FMD outbreaks were most often notified by Afghanistan (2001–2005, 2009–2011, 2017–2019) and Pakistan (2001–2005, 2009–2011, 2019). In China, FMD of this type was reported in 2001–2009. Asia-1 FMDV circulation is most likely to occur due to uncontrolled migration of cloven-hoofed animals and illegal importation and exportation of animal and plant products [13].

The Russian Federation had been free from Asia-1 FMD until 2005, when fifteen FMD outbreaks were detected in the Amur Oblast, Khabarovsk and Primorsky Krais (Fig. 3). Even though these regions were within the zone where susceptible animals were subjected to preventive immunization against FMD, vaccination against Asia-1 FMD was not carried out. On 9 June 2005, the first outbreak was

confirmed in Busse village (Svobodnensky Raion, Amur Oblast) located on the left bank of the Amur River that separates the settlement from China. In the second half of August 2005, new Asia-1 FMD outbreaks were reported in the Khabarovsk and Primorsky Krais. Around the same time, FMD occurrence was notified in China-bordering Dornod aimag, Mongolia.

In the Khabarovsk Krai FMD was detected in cattle in four settlements in the Bikinsky and Vyazemsky Raions located near the border with China. On 21 August 2005, FMD was confirmed in cattle owned by KGUSP "Lermontovskoye", Bikinsky Raion. The cattle pasturelands were located near Dobrolyubovo settlement on the Ussury River floodplain that borders China. On 23 August 2005, FMD was reported in cattle on the grassland belonging to KGUSP "Lonchakovskoye", Bikinsky Raion. In Vyazemsky Raion FMD was diagnosed in cattle in Vidnoe settlement. On 24 August, in the same raion one more FMD outbreak was reported in Sheremetyevo unit of KGUSO "Kotikovo", where 56 cattle were kept.

Asia-1 FMD became widespread in the Primorsky Krai. The first FMD suspicion was identified in cattle on 26 August 2005, in the backyard in Krasny Kut settlement (Spassky Raion) located near the border with China. From 27 August to 2 September, FMD was detected in seven more settlements in six raions of the Primorsky Krai, four of which bordered China. Thus, in Pavlo-Fedorovka settlement, Kirovsky Raion, and in Abramovka settlement, Mikhailovsky Raion, FMD was diagnosed in cattle on 27 August; and on 28 August FMD was reported in Ignatevka settlement, Pozharsky Raion; on 31 August – on "Primorsky Ris" farm in Sivakovka settlement, Khorolsky Raion; on 2 September FMD clinical signs were reported in cows and in a pig in the backyards in Luchki settlement of the same raion. The disease was confirmed in cows in the backyards in Pervomaiskoe settlement, Khankaisky Raion, on August 30, and in Slavyanka settlement, Khasansky Raion, on September 2. In the Primorsky Krai the last outbreaks were reported in the Lesozavodsky Raion, namely in Nevskoe settlement bordering China [14].

Asia-1 FMD outbreaks continued in 2006. FMD was reported in cattle in the settlements located near the Russian-Chinese border: in Srednyaya Borzya (Kalgansky Raion, Chita Oblast) – on 23 January, and in Kuropatino settlement (Tambovsky Raion, Amur Oblast) – on 22 February [15].

Phylogenetic analysis demonstrated that the Russian isolates were genetically closely related to the virus that had caused large-scale Asia-1 FMD epidemics in China in 2005–2006 [16].

FMD buffer zone was established in Russia and it comprised all the southern border of the country [1, 17]. After the FMD outbreaks caused by Asia-1 virus, trivalent vaccine against type A, O and Asia-1 FMD is used for preventive immunization in the buffer zone. Until 2015, routine preventive vaccination of FMD susceptible animals was also carried out in the Moscow and Vladimir Oblasts, as FMD vaccine manufacturing facilities are located here. Nowadays, these facilities are not part

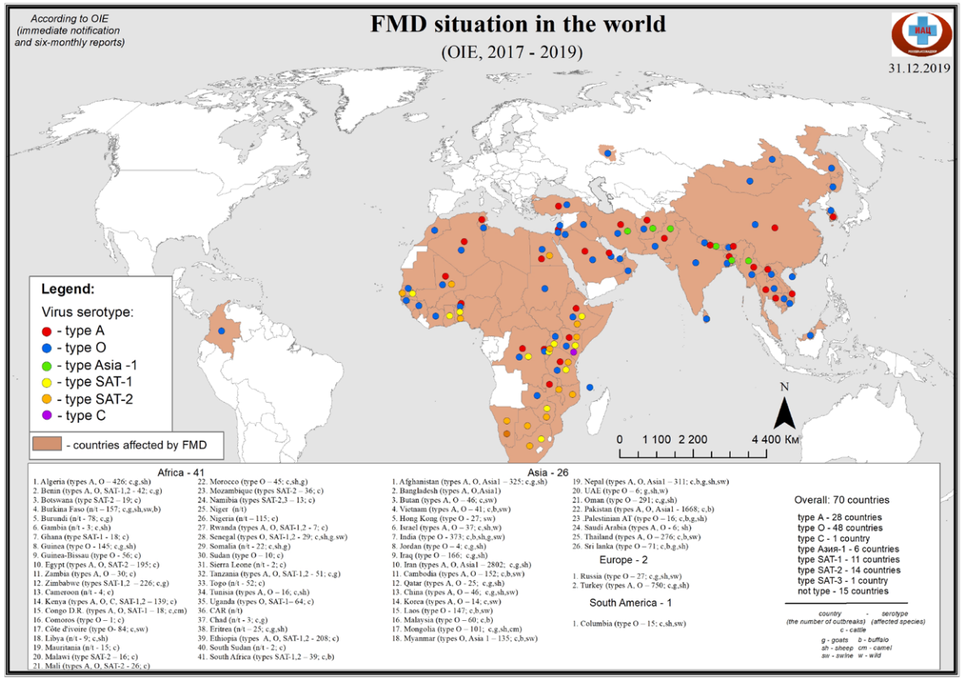

Fig. 1. Global FMD epidemic situation in 2017–2019 (the map has been prepared by the officials of the Information Analysis Centre, FGBI "ARRIAH")

Рис. 1. Эпизоотическая ситуация в мире по ящуру в 2017–2019 гг. (данная карта подготовлена сотрудниками Информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

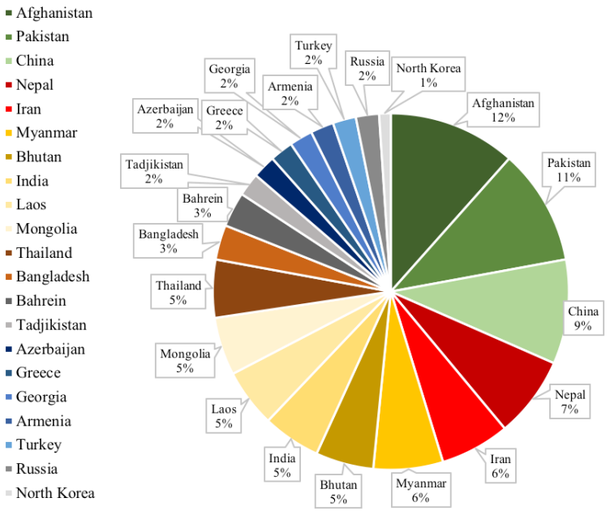

Fig. 2. Frequency of Asia-1 FMD reports in different countries

Рис. 2. Частота регистрации ящура типа Азия-1 в различных странах

of the buffer zone, since they are strictly compliant with the relevant safety requirements aimed at the agent escape prevention (Fig. 4).

On 18 October 2016, Asia-1 FMD outbreak was reported in cattle on one of the farms in Vyshmanovo settlement (Sobinsky Raion, Vladimir Oblast). The disease was eradicated in the primary outbreak area by seizure and destruction of all susceptible animals located in the infected settlement. Epidemic investigation failed to conclusively identify the source of the agent [18].

Of topical significance today is maintenance of FMD freedom in the Russian Federation through the implementation of such measures as monitoring of the global disease situation, preventive vaccination of susceptible animals in the buffer zone using relevant production virus strains and diagnostic tests aimed at the FMDV detection [17, 19, 20].

CONCLUSION

Summary of the data obtained during the analysis suggest that actual situation on global Asia-1 FMDV spread is different from the officially reported one. This is particularly true for Asian and Near Eastern countries, where strict reporting is not carried out due to a number of reasons and in spite of the continuous virus circulation in the regions. Uncontrolled migrations of the wild cloven-hoofed animals (saigas, dzerens, buffaloes) and insufficient financing of the veterinary services in these countries mean that in many outbreaks the viruses remain untyped thus extremely distorting the actual situation with global FMD spread.

Asia-1 FMD inflicted significant damage to Russian livestock production and economy in 2005–2006, when possible virus introduction from China resulted in multiple infection outbreaks in the country as well as in heavy expenditures on their eradication.

In order to minimize the risk of FMDV introduction into our country, special consideration should be given to monitoring of the epidemic situation in Asian countries and to the strengthening of the safety control of the products imported to the Russian Federation. Intensification of the international cooperation with APAC countries in implementation of joint measures for FMD prevention is of major importance.

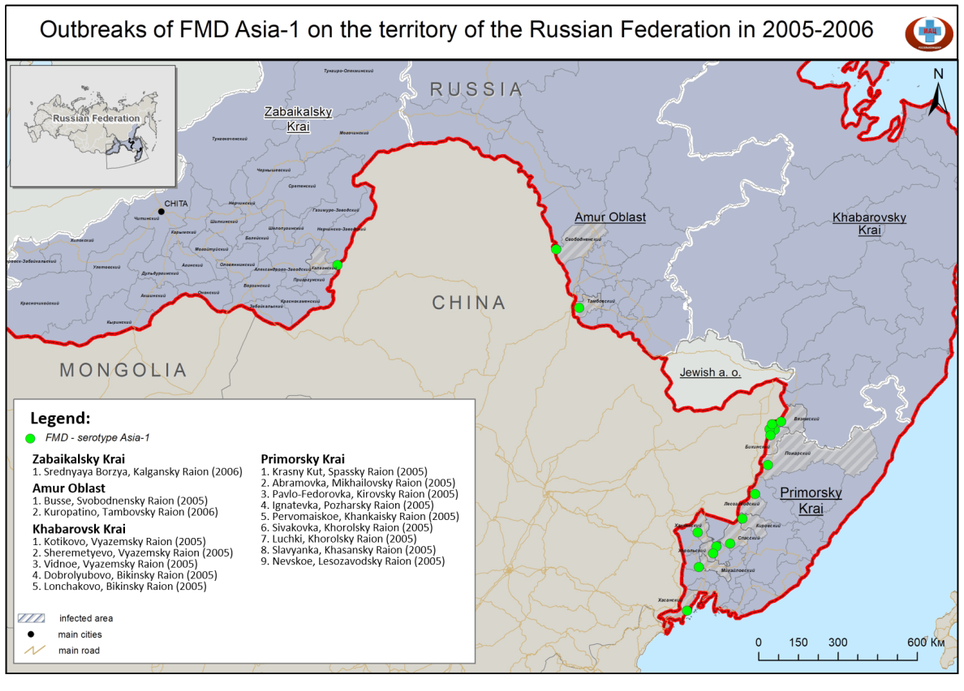

Fig. 3. Asia-1 FMD spread in the Russian Federation in 2005–2006 (the map has been prepared by the officials of the Information Analysis Centre, FGBI "ARRIAH")

Рис. 3. Распространение ящура типа Азия-1 на территории Российской Федерации в 2005–2006 гг. (данная карта подготовлена сотрудниками Информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

Fig. 4. Zone of preventive vaccination against FMD (A, O, Asia-1) in the Russian Federation (the map has been prepared by the officials of the Information Analysis Centre, FGBI "ARRIAH")

Рис. 4. Зона профилактической иммунизации против ящура (A, O, Азия-1) на территории Российской Федерации (данная карта подготовлена сотрудниками Информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

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Quality profile of milk from high producing dairy cows vaccinated against mastitis

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SUMMARY

One of the raw milk quality criteria is the count of somatic cells, produced by the cow's immune system to fight infectious diseases of the mammary gland. The paper presents the analysis of somatic cell count and total bacteria count of milk from cows, vaccinated against mastitis using Startvac vaccine. Tests were performed as a comparison between a dairy unit and a farm under different management conditions and using different milking techniques. Six months after the start of the vaccine application the somatic cell count at the dairy unit decreased by 60 thousand/ml, at the farm by 182 thousand/ml. The agent profile was represented by the following bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus* spp., *Pseudomonas aeruginosa*. *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Bacillus*, *Lactobacillus* were also isolated from udder secretion. After a year of immunization somatic cell count both at the unit and on the farm decreased by 245 and 216 thousand/ml respectively; it is noteworthy that 43.75% of microflora isolated from mammary gland secretion was represented by *Streptococcus* spp. After two years of the vaccine use the somatic cell count was equal to 371 and 725 thousand/ml at the unit and on the farm respectively. Tests of mammary gland secretions revealed *Streptococcus* spp. in 27.27% of cases, *Staphylococcus aureus* and *Enterococcus faecium* were isolated in 18.18% of tested samples. It was established that after three years of the vaccine use the major cause of mastitis in cows was *Streptococcus* spp. (55.00%). During four years of tests, a downward trend in somatic cell count of bulk milk from high producing dairy cows as well as in the number of agents responsible for inflammation in a mammary gland was detected. Somatic cell count of milk from vaccinated animals decreased by 286 and 432 thousand/ml at the unit and on the farm respectively. During the test period *Staphylococcus aureus* isolation rate declined by 19.41%.

Key words: mastitis, Startvac mastitis vaccine, somatic cells, mastitis agents, milk quality.

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Показатели качества молока высокопродуктивных коров на фоне применения противомаститной вакцины

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РЕЗЮМЕ

Одним из качественных показателей сырого молока является содержание в нем соматических клеток, играющих защитную роль против инфекционных заболеваний молочной железы коров. В статье приведен анализ уровня соматических клеток и бактериальной обсемененности молока на фоне применения противомаститной вакцины Startvac. Исследования проводились в сравнении: на базе комплекса и фермы, различающихся условиями содержания и технологией доения. Через 6 месяцев с начала применения вакцины уровень соматических клеток в комплексе снизился на 60 тыс./мл, на ферме – на 182 тыс./мл. Структура возбудителей была представлена такими бактериями, как *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus* spp., *Pseudomonas aeruginosa*. В секрете вымени также были выделены *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Bacillus*, *Lactobacillus*. После года иммунизации животных количество соматических клеток в комплексе и на ферме снизился на 245 и 216 тыс./мл соответственно, при этом выделенная из секрета молочной железы микрофлора в 43,75% случаев была представлена *Streptococcus* spp. Спустя два года с начала применения вакцины показатель соматических клеток в сборном молоке в комплексе и на ферме составил 371 и 725 тыс./мл соответственно. Исследование секрета молочной железы показало наличие в 27,27% случаев *Streptococcus* spp.; *Staphylococcus aureus* и *Enterococcus faecium* выделены в 18,18% исследуемых проб. Установлено, что спустя три года иммунизации основной причиной мастита у коров было наличие *Streptococcus* spp. (55,00%). За четырехлетний период исследований выявлена тенденция к снижению показателя соматических клеток в сборном молоке высокопродуктивных коров, а также спектра возбудителей, вызывающих воспаление в молочной железе. Количество соматических клеток в сборном молоке на фоне иммунизации животных снизилось в условиях фермы и комплекса на 286 и 432 тыс./мл соответственно. За период исследования наблюдается снижение высеваемости *Staphylococcus aureus* на 19,41%.

Ключевые слова: мастит, противомаститная вакцина Startvac, соматические клетки, возбудители мастита, качество молока.

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INTRODUCTION

One of the most essential prerequisites for the increase in milk yields and dairy farming performance is the improvement of existing breeds and their genetic potential [1]. Herewith, milk quality in dairy production is prioritized.

A quality parameter of raw milk is the number of somatic cells, which are in fact cells of different tissues and organs, playing a protective role against udder infectious diseases. They include white blood cells (WBCs), red blood cells (RBCs), columnar, squamous and cuboidal epithelial cells [2–5]. Numerous genetic and environmental factors influence the number and types of WBCs, which compose the majority of somatic cells in milk from healthy animals. Quality of milk from high producing dairy cows is adversely affected by infection-induced mammary gland inflammation, which is manifested by increased levels of somatic cells. The overwhelming majority of mammary gland somatic cells is represented by neutrophils [6, 7]. A low somatic cell count is a reliable indicator of high quality milk, free from pathogenic organisms.

Antimicrobial therapy, notwithstanding the presence of sensitive pathogens, is often unsuccessful; moreover, the drug components are secreted with milk for a long time. Search for different approaches that allow producing milk with a low somatic cell count and maintaining this count at the optimal level is a vital task in modern veterinary practice. One of the measures to prevent mastitis, i.e. to maintain low somatic cell counts, is the vaccination of cows [2, 8].

The study objective was to evaluate the effect of mastitis vaccine Startvac (Laboratorios Hipra, Spain) on somatic cell count and total bacterial count of milk from high-producing dairy cows.

MATERIALS AND METHODS

The study was conducted in 2016–2020 in the Laboratory for Reproductive Pathology and Young Animal Diseases and Department of Veterinary and Laboratory Diagnosis under Testing Laboratory of the FSBSI UrFASRC UrB of RAS.

Experimental studies were performed at the breeding farm, located in the Sysertsky Raion of the Sverdlovsk Oblast. 1,400 Holstein-Friesian cows with annual milk production rate of 9,299 kg are kept on the farm. The average period of the cow's use for milk production is 3.8 lactations. The tests were conducted as a comparison between the dairy unit (free stall housing and milking in parallel system milking parlors) and on the farm (tie stall housing and milking using pipeline milking machines).

Mastitis vaccine Startvac for cows (Laboratorios Hipra, Spain) was registered in the Russian Federation in 2010. Externally the vaccine is a homogenous white-yellow emulsion. It is filled in glass bottles 1, 5 and 25 doses per each; the bottles are sealed using rubber stoppers and aluminum caps. The vaccine is produced from inactivated cells of *Escherichia coli* (J5) and *Staphylococcus aureus* (CP8), containing slime associated antigenic complex (SAAC), with the following excipients added: liquid paraffin 9.5 mg/ml and benzyl alcohol 10.5 mg/ml. One inoculation dose (2 ml) contains at least 50 effective immunogenic doses of *Escherichia coli*, strain J5 and at least 50 effective immunogenic doses of *Staphylococcus aureus* (CP8) strain SP 140. The vaccine is administered intramuscularly in the amount of 2 ml, its temperature shall be +15 to +25 °C. The solution shall be shaken before use.

The first immunization using Startvac vaccine of all cows on the breeding farm was conducted in December 2016. Three weeks later, all animals were revaccinated and then boostered every three months. By the time when the paper was ready, 14 vaccinations had been performed with the most recent one to occur in April 2020.

Bulk raw milk was tested every month for somatic cell count using DCC cell counter (GMU Tumba DeLaval International AB, Sweden).

During the test period milk samples were collected for further molecular, genetic and microbiological tests and evaluation of vaccination effect on the presence of agents, responsible for inflammation in the mammary gland. The samples collected were tested by real-time polymerase chain reaction using Rotor-Gene 3000 (Corbett Research, Australia) and the following test-kits "Vetscreen. STREPTOPOL-V", "Vetscreen. STAPHYPOL", "Vetscreen. COLYPOL", "Vetscreen. STREPTOPOL" (OOO "IDS", Moscow). For the purposes of bacteriological and microbiological testing milk samples were seeded onto liquid and solid nutrient media, in particular beef extract broth, beef extract agar, Endo agar, Sabouraud agar, mannitol salt agar, enterococci agar, Hiss serum sugars. The recovered isolates were identified using Bergey's Manual of Systematic Bacteriology and manual of pathogenic and opportunistic fungi. During the study of mastitis vaccine effectiveness, 125 milk samples from high producing cows were tested.

RESULTS AND DISCUSSION

The previous studies, conducted on the breeding farm, showed numerous mastitis cases in cows during the year. The morbidity in 2015 was 12.2% out of the total number of tested cows; in 2016, the number of udder inflammations grew up to 22.1%, which is 1.8 times higher as compared with the previous year. In 2015, clinical mastitis was detected in 6.8% and subclinical mastitis was found in 5.4% of animals. In 2016 there was a change in this ratio: more animals suffered from subclinical mastitis (17.4%) and clinical mastitis was identified in 4.8% of animals [9]. Before vaccination somatic cell count in bulk milk from high producing cows, kept at the dairy unit, was 695 thousand/ml, and on the farm this value was equal to 916 thousand/ml. The most commonly detected microorganisms were *Staphylococcus aureus* (29.42%) and *Streptococcus* spp. (23.53%). *Enterococcus* bacteria were recovered from 11.76% of samples. 17.65% of tested samples contained *Aspergillus* mold. Gram-negative bacteria of *Klebsiella* spp. (5.88%), *Pseudomonas* (5.88%), *Enterobacter* (5.88%) were also isolated. Moreover, the microorganisms were detected as monocultures (27.3%), and as mixed cultures of bacteria (55.6%), fungi and yeasts (17.1%). These tests demonstrated that mastitis in animals, and as a result increased somatic cell counts in milk, were caused by a rather wide range of agents.

Due to a high incidence of subclinical mastitis in lactating cows, responsible for increased somatic cell counts in milk, and a large number of animals suffering from udder inflammations, caused by *Staphylococcus aureus*, it was decided to vaccinate animals with Startvac mastitis vaccine.

When the vaccination started, the somatic cell count in milk samples from cows, kept at the dairy unit and milked using special milking equipment, was 559 thousand/ml. The somatic cell count in bulk milk from cows, kept on the farm and milked using a stationary pipeline equipment, was 822 thousand/ml.

After vaccination, the somatic cell count started decreasing gradually, and then grew insignificantly, but the general declining trend continued. In six months after vaccination had started the somatic cell count at the dairy unit and on the farm decreased by 60 and 182 thousand/ml correspondingly. At that moment, the mastitis in cows was caused predominantly by *Enterococcus faecium* (20.00%), *Staphylococcus aureus* (17.33%), *Streptococcus* spp. (17.33%), *Pseudomonas aeruginosa* (14.67%), *Streptococcus agalactiae* (8.02%). *Staphylococcus saprophyticus* (5.33%), *Staphylococcus epidermidis* (5.33%), *Enterococcus faecalis* (5.33%), *Escherichia coli* (1.33%), *Bacillus* (1.33%), *Lactobacillus* (1.33%) were also isolated from milk. In 2.67% of tested milk samples, no pathogenic and opportunistic microorganism growth was observed.

After one year of mastitis vaccine application, tests of bulk milk showed stable decrease in somatic cell counts at the dairy unit with free stall housing and milking in parallel system milking parlors, SCC was equal to 450 thousand/ml, which is 245 thousand/ml less as compared to the value, obtained before vaccination. On the farm with a tie stall housing and milking using pipeline milking machines no steady tendency in decline of somatic cell counts was observed. After one year of Startvac vaccine use the somatic cell count decreased by 261 thousand/ml, but in the following month a rise in this value by 227 thousand/ml was observed (Fig. 1). The microflora isolated from milk in 43.75% cases was represented by *Streptococcus* spp., and by *Streptococcus agalactiae* in 31.25% of tested samples. The proportion of *Staphylococcus aureus* and *Escherichia coli* positive samples, responsible for mastitis, was the same (12.50% each).

After two years of vaccination against mastitis somatic cell counts in bulk milk at the dairy unit and on the farm were 371 and 725 thousand/ml correspondingly (Fig. 2). Thus, a gradual decrease of this indicator was observed at the dairy unit, whereas on the farm this decrease was intermittent. The major part of the mastitis agents was represented by *Streptococcus* spp. (27.27%). The numbers of *Staphylococcus aureus* (18.18%) and *Enterococcus faecium* (18.18%) were equal in the tested samples. The percentage of *Escherichia coli* among the pathogens isolated from milk was 9.10%. The number of samples, in which no microorganism growth was detected, was 27.27%.

After three year-immunization of high-producing cows, the somatic cell count in bulk milk was at the level of 630 thousand/ml on the farm and 263 thousand/ml at the dairy unit, which is 286 and 432 thousand/ml lower than this value before the vaccination program was applied. Results of molecular genetic and microbiological studies showed that most cases of mastitis in cows were caused by *Streptococcus* spp. (55.00%). *Staphylococcus aureus* was

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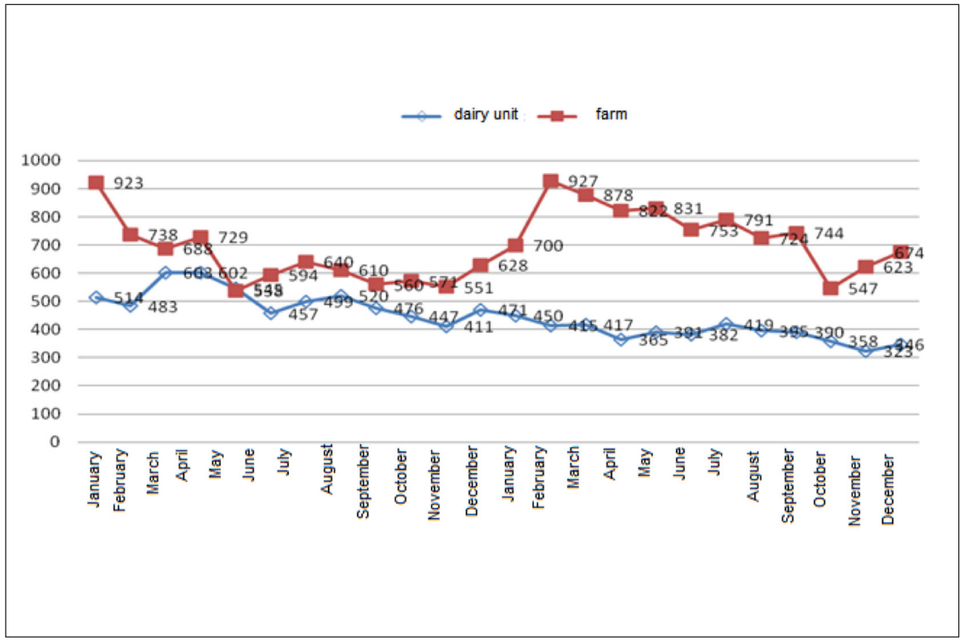

Fig. 1. Dynamics in somatic cell counts in 2017–2018

Рис. 1. Динамика уровня соматических клеток в период с 2017 по 2018 г.

isolated in 10.00% of the tested samples, *Escherichia coli* and *Streptococcus agalactiae* were detected at the same levels (5.00%). In 25.00% of the studied samples, there was no growth of pathogenic and opportunistic microorganisms.

The results of molecular genetic and microbiological tests performed during vaccination of highly producing cows with Startvac mastitis vaccine showed a significant reduction in the range of pathogens, causing inflammation in the mammary gland. There was a 19.41% decrease in the isolation rate of *Staphylococcus aureus*, which is explained by an increase in antibodies levels in vaccinated animals. White blood cells, contained in milk, perform one of the main protective functions against mastitis agents. For the normal functioning of the immunity protective properties, antibodies must induce the production of white blood cells and be targeted against certain bacterial agents. Earlier studies on the immunobiological status of cows established that inflammatory reactions occurring in the mammary gland are accompanied by changes in the overall resistance of the animal body, including immunological blood parameters. When studying the body's response to the vaccine administration, evaluated by overall resistance rates, an increase in the number of T-lymphocytes by 12.1% and B-lymphocytes by 7.0% was noted. The opsonophagocytic assay showed an increase in the phagocytic activity of neutrophils by 14.3%. Animals showed a persistent decrease in the level of circulating immune complexes in the blood to (106.8 ± 3.4) c. u., which is explained by suppression of inflammatory response in the mammary gland due to the activation of humoral immunity factors [10].

CONCLUSION

Application of the mastitis vaccine Startvac and use of the major mastitis therapy regime used by the breeding establishment for high producing cows result in a positive downward trend in somatic cells counts in bulk milk. Somatic cell counts in milk of vaccinated cows from the dairy unit with free stall housing and milking in parallel system milking parlors decreased by 432 thousand/ml, while in milk of high-producing cows from the farm with a tie stall housing and milking using pipeline milking machines, the level of somatic cells decreased by only 286 thousand/ml. The difference in somatic cells counts in bulk milk of high-producing cows kept in the dairy unit and farm conditions can be explained by differences in the husbandry practices and milking technologies, which, in turn, can affect the formation and number of pathogenic and opportunistic microorganisms responsible for mastitis. This assumption will serve as the target for further research in this direction. Vaccination of animals facilitated the reduction of milk bacterial contamination caused by *Staphylococcus aureus* by 19.41%. Thus, the obtained results showed promising use of the vaccine in future and its introduction into the mastitis control and milk quality improvement program.

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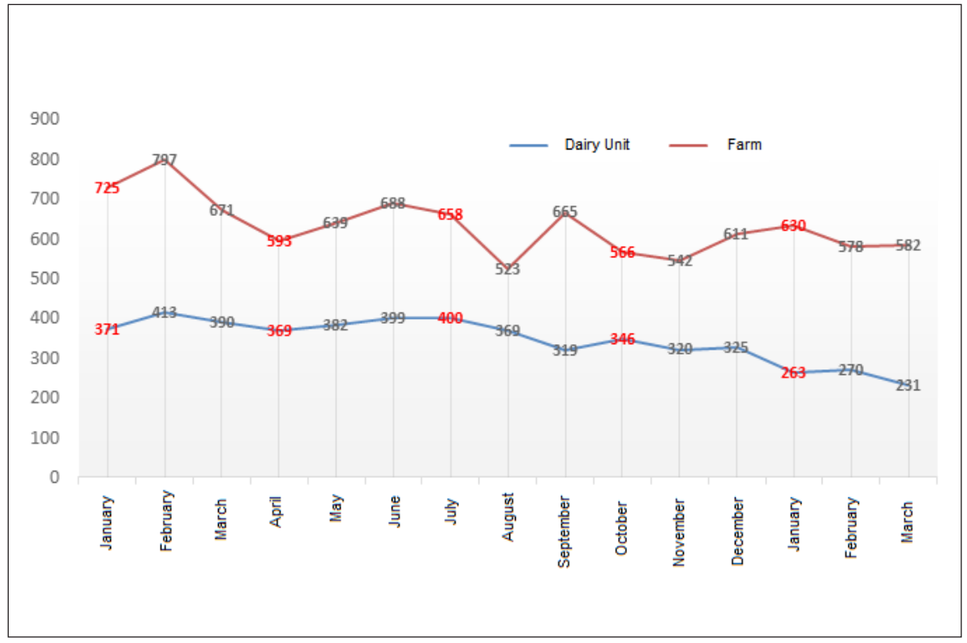

Fig. 2. Dynamics in somatic cells counts in 2019–2020

Рис. 2. Динамика уровня соматических клеток в период с 2019 по 2020 г.

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Improvement of bovine tuberculosis diagnosis

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SUMMARY

Detection of animals with non-specific reactions to tuberculin is one of the major problems in bovine tuberculosis (TB) diagnosis. There is a need to find and improve methods for detection of the sensitization causes. This paper presents the results of comparative studies of different ways to stabilize red blood cells in order to obtain diagnosticums for indirect hemagglutination (IHA) test. The article describes the stages of red blood cells stabilization and sensitization and demonstrates the diagnostic significance of Fili stabilization method using formaldehyde as a fixative. The highest antibody titers (1:3000 and 1:4000) were received in hyper-immune sera of rabbits immunized with *Mycobacterium bovis* using a homologous diagnosticum. Practical importance of the sensitins homologous to the infection is shown during testing of 1,911 serum samples collected from animals of different categories (diseased; healthy and reacting to tuberculin; healthy and not reacting to tuberculin) with IHA test using diagnosticums produced from *Mycobacterium bovis* and *Mycobacterium fortuitum*. Based on the positive results of the IHA test, TB was diagnosed in 87.5% of animals originating from an infected farm during post-mortem examination. The results of the IHA test agreed with those of the intradermal tuberculin test in 37.7% of cases. Diagnostic antibody titers were found in 206 TB infected animals with no reaction to the intradermal test. However, the post-mortem examination revealed TB changes in internal organs. The obtained data suggest a possibility to use the IHA test to detect TB infected animals with non-specific reactions to tuberculin.

Key words: tuberculosis, indirect hemagglutination test, differentiation, cattle, standardization, antibodies, red blood cells, sensitization.

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К совершенствованию диагностики туберкулеза крупного рогатого скота

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РЕЗЮМЕ

Выявление животных с неспецифическими реакциями на туберкулин – одна из наиболее актуальных проблем в диагностике туберкулеза. Очевидна необходимость поиска и совершенствования методов для выявления причин сенсибилизации. В работе представлены результаты сравнительного изучения различных способов стабилизации эритроцитов с целью получения диагностикума для проведения реакции непрямой гемагглютинации. Отражены этапы стабилизации и сенсибилизации эритроцитов. Показана диагностическая значимость метода стабилизации Фили с использованием формальдегида в качестве фиксатора. Наиболее высокие титры антител (1:3000 и 1:4000) получены в гипериммунных сыворотках крови кроликов, иммунизированных *Mycobacterium bovis*, с гомологичным диагностикумом. Практическая значимость гомологичных заражению сенситинов показана при исследовании 1911 проб сывороток крови животных из хозяйств различных категорий (больные; здоровые, реагирующие на туберкулин; здоровые, не реагирующие на туберкулин) в реакции непрямой гемагглютинации с диагностикумами, изготовленными из *Mycobacterium bovis* и *Mycobacterium fortuitum*. В неблагополучном по заболеванию хозяйстве, на основании полученных в реакции непрямой гемагглютинации позитивных результатов, при проведении патологоанатомического вскрытия диагноз на туберкулез установили у 87,5% животных. Отмечено совпадение результатов реакции непрямой гемагглютинации с показаниями внутрикожной туберкулиновой пробы в 37,7% случаев. У 206 больных туберкулезом животных обнаружены диагностические титры антител при отсутствии реакции на внутрикожную пробу. Однако при проведении патологоанатомического исследования были выявлены изменения туберкулезного характера внутренних органов. Полученные данные указывают на возможность использования реакции непрямой гемагглютинации при выявлении больных туберкулезом животных с неспецифическими реакциями на туберкулин.

Ключевые слова: туберкулез, реакция непрямой гемагглютинации, дифференциация, крупный рогатый скот, стандартизация, антитела, эритроциты, сенсибилизация.

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INTRODUCTION

Often, in TB-infected cattle herds there is a certain number of undetected diseased animals even after a comprehensive diagnosis. There is data (although scattered) on the possibility to detect more TB-infected animals using serodiagnostic methods.

Various serological tests were used to detect specific antibodies in animals: agglutination, precipitation, complement fixation, indirect and direct hemagglutination tests, enzyme immunoassay and others. However, not all of them have found wide practical application due to their low effectiveness and frequent inconsistency with the intradermal tuberculin test results.

Indirect hemagglutination (IHA) test was first used for TB diagnosis by F. I. Awad [1], who used a polysaccharide extract from *Mycobacterium tuberculosis* strain H37Rv as an antigen. Polysaccharide antigen is adsorbed on sheep red blood cells (RBCs), which agglutinate with homologous antibodies of the test serum. The following can be used as an antigen: heated and dialyzed tuberculin [2–8]; tuberculin obtained by ultrasound exposure to mycobacteria [9, 10]; phosphatide fraction of lipids extracted from mycobacteria [11], etc.

Since animal blood serum contains heterogeneous hemagglutinins, various methods of their adsorption by depletion of test sera [12, 13] or by treatment of RBCs with a tannin [14], trypsin or papain [12] solution have been proposed.

There are several ways to stabilize red blood cells, different in the chemical nature of the fixative, and RBC treatment methods. Aldehydes (for example, formaldehyde, glutaraldehyde, acetaldehyde), hydroxal, osmium tetrachloride, and others can be used for RBC fixation. Most often, formaldehyde is used in concentrations from 0.5 to 20%. In some cases, its concentration in RBC suspension is increased gradually by dialysis or stepwise. The duration of RBC fixation with formalin ranges from 1.5–2.0 to 48 hours.

The opinions of researchers regarding the interpretation of diagnostic antibody titres detected in healthy and TB-infected cattle are contradictory. Thus, J. Komura et al. considers blood serum dilution 1: 40 [15] a diagnostic titre, while O. V. Martma – 1:160 [16], G. V. Dunaev – 1:32 [6], E. I. Buryak – 1:64 [17], N. P. Ovdienko – 1:16 [18], etc.

There are various techniques, which, to some extent, can also influence the results.

Thus, the use of different antigens, heterogeneous hemagglutinin adsorption techniques, RBC membrane stabilization and sensitization methods and test techniques leads to contradictory results, which can be clearly seen by different levels of antibody titers considered as diagnostic to distinguish between the diseased and healthy animals. Therefore, the diagnostic value of IHA test for TB diagnosis is debatable.

The aim of this work was to study various RBC membrane stabilization methods used for sensitization with polysaccharide antigen, to determine the practical value of IHA test using different diagnosticums and to compare it with other test methods used on farms with different TB epidemic situations.

MATERIALS AND METHODS

For the purpose of this study, a polysaccharide from *Mycobacterium bovis* and *Mycobacterium fortuitum* was obtained following the procedure: a weight of dry cells of each strain was ground to powder in a grinding mill. Polysaccharide fractions were extracted from the bacterial mass of *Mycobacterium* using an aqueous beta-naphthol alcohol solution with further cooling with cold (0 ... –2 °C) ethyl alcohol. Then the antigen was separated by centrifugation at 3–5 thousand rpm, washed twice with alcohol and ether, dried in a thermostat, rubbed and transferred to vials.

Hyperimmune sera were obtained from rabbits immunized with *M. bovis*, *M. bovis* bacillus Calmette – Guérin, *M. avium*, *M. scrofulaceum* using Freud method.

Chess-border antigen titration was performed using the hyperimmune sera.

1,911 serum samples were collected from healthy and TB-infected cattle, as well as from animals with non-specific reactions to tuberculin, and were tested with IHA test.

The test was performed according to the generally accepted method on Titertek plates, which allowed to reduce the component consumption significantly and to observe their exact dosage.

All the tests were conducted in strict accordance with the interstate standards for the care and keeping 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 European Union Council as of 22.09.2010 on the protection of animals used for scientific purposes.

RESULTS AND DISCUSSION

Comparative studies of RBC membrane stabilization methods for IHA test is an important element in improving the technology of preparing antigenic erythrocyte diagnosticums. There are several ways to stabilize red blood cells using mainly aldehydes as a fixative. There are no fundamental differences between them, since in all the cases the main criteria determining the quality of stabilization are the concentration of the fixative during treatment, fixative – RBCs ratio, the duration of treatment, and the temperature [19–22]. For the purposes of comparative testing, four methods were used, the details are provided in Table 1.

Stabilized under the conditions specified in Table 1, sheep RBCs were tested for spontaneous agglutination in inactivated serum and used for polysaccharide sensitization. RBC sensitization was performed by mixing various dilutions (from 1:500 to 1:4000) of polysaccharides with a 10% suspension of stabilized RBCs in 1:4 ratio and incubated at 38 °C for 12 hours with constant shaking in Vibrotherm shaking water bath. RBCs were washed twice and a 2% suspension was prepared in a phosphate-buffered saline solution with the addition of 1% normal inactivated adsorbed bovine serum. Depending on the dilution of polysaccharides intended for RBC sensitization (from 1:500 to 1:4000, at 1:500 interval), 32 variants of diagnosticums were prepared.

For antigen titration, 2 positive and 2 negative sera were used. They were diluted with phosphate-buffered saline solution in 1:4 ratio and inactivated in a water bath at 64 °C for 30 min. To adsorb heterogeneous hemagglutinins, a 5% suspension of sheep RBCs was added to each serum, kept in a water bath at 37–38 °C for one hour and shaken periodically. Then the RBCs were separated from the sera by centrifugation.

The test was performed on ELISA plates in accordance with the generally accepted procedure. Thus, the volume of the components was reduced tenfold. Each diagnosticum sample was tested with sera dilutions of up to 1:2048. Tests were performed both with negative and positive sera. Also, hyperimmune sera were used (from rabbits immunized with *M. avium* and *M. scrofulaceum*, one serum from each).

0.02 ml of a 2% suspension of sensitized red blood cells was added to each plate well containing 0.05 ml of the diluted sera. Serum in a dilution of 1:8 and 0.02 ml of diagnosticum (control of serum adsorption) was added to well 11.

0.05 ml of buffer + 0.02 ml of diagnosticum was added to well 12 (control of spontaneous agglutination of the erythrocyte diagnosticum). An antigen titer was considered a dilution, in which "+++" agglutination was observed with positive serum and "++++" with hyperimmune antiserum.

Testing of the sera collected from control and healthy animals originating from TB-free farms revealed no hemagglutinins reacting with the diagnosticum produced from *M. fortuitum* in any of the antigen dilutions. Hemagglutinins at 1:500 and 1:1000 titers were detected in 1:32 sera dilutions during RBC membrane stabilization by VIEV method (All-Russian Research Institute of Experimental Veterinary Medicine) using a diagnosticum produced from *M. bovis*. In 193 samples of sera obtained from animals known to be healthy, hemagglutinins in higher titers could not be detected. The highest antibody titers were obtained in hyperimmune sera of rabbits immunized with *M. bovis* using a homologous diagnosticum in dilutions of 1:3000 and 1:4000, with RBC membrane stabilization by Fili method. Antibodies were detected in sera dilutions of 1:4096. Hemagglutinins were detected in 1:1024 sera dilutions using diagnosticums prepared by three other methods of RBC membrane stabilization. At the same time, the difference between antibody titers in hyperimmune sera homologous to the *M. bovis* antigen and in heterologous sera was the highest when RBCs were stabilized using Fili method. Thus, when using a diagnosticum produced from *M. bovis* with a homologous serum, hemagglutination titer was 1:4096, while with the sera from rabbits immunized with *M. avium,* – 1:1024, *M. scrofulaceum* – 1:256 and *M. bovis* bacillus Calmette – Guérin – 1:1024.

The situation was opposite in IHA test with a diagnosticum produced from *M. fortuitum*. The highest antibody titers (1:2048) in this case were received in sera of rabbits immunized with *M. scrofulaceim*. Based on these results, a dilution of 1:1500 (double titer) was taken as an antigen titer, when stabilizing RBCs with Fili method.

IHA test using sera with known antibody titers was performed to standardize diagnosticums. Then the diagnosticums were filled in 5 ml ampoules and used for IHA testing, as needed.

The testing was performed in animals originating from four farms with different TB epidemic situation. By the time of the study 75.6% of animals coming from the first farm had demonstrated a positive reaction to the intradermal tuberculin test (tuberculosis isolation unit). On the second farm (which had been infected for a long time), health status was improved based on the systemic study results by isolation and further slaughter of positively reacting animals. 3.9% of animals from that farm resulted positive to the tuberculin test. On the third farm, positive animals were detected during the routine testing (6.5% in this study), but the autopsy and the laboratory testing of the materials obtained from those animals did not confirm the diagnosis. The fourth farm was free from the disease, with no animals reacting to tuberculin.

Table 1
Different methods and procedures for red blood cells stabilization

Таблица 1
Различные способы и режимы стабилизации эритроцитов

No.	Method	Fixative	Concentration of the fixative during treatment, %	Ratio of the fixative to red blood cells	Treatment duration, hours	Temperature, °C
1	Fili	Formaldehyde	5.7	0.32:1	2	37
2	Weinbach	Formaldehyde	1.5	0.375:1	18–20	37
3	Ling	Formaldehyde	4.4	0.75:1	24	4
4	VIEV	Glutaric aldehyde	2.5	0.5:1	18–20	37

1,911 blood samples were taken from animals on those farms to obtain sera, which was tested with IHA using diagnosticums produced from *M. bovis* and *M. fortuitum*. The results are presented in Table 2.

The results show an expressed difference between the number of positive animals detected with IHA test using a diagnosticum produced from *M. bovis* and the number of animals tested using a diagnosticum produced from *M. fortuitum* on the TB infected farms. On the first two farms, more serum samples with a diagnostic antibody titer were detected using a *M. bovis*-based diagnosticum than using a *M. fortuitum*-based diagnosticum. On the contrary, when testing sera from the third farm, a greater number of detections were obtained with a diagnosticum produced from *M. fortuitum*. At the same time, the IHA test conducted using a diagnosticum produced from *M. bovis* was positive only for 15% of the tested animals kept in the isolation unit.

On all the four farms, animals with positive and negative IHA results were euthanized for diagnostic purposes. TB was diagnosed in 87.5% of IHA positive animals from the disease-free farms. During the post-mortem examination and the laboratory testing of the biological material collected from animals with non-specific reactions to tuberculin, no changes in internal organs typical for tuberculosis were detected, and the disease was not diagnosed. In 22.1% of cases, cultures of atypical mycobacteria were isolated from the materials.

When comparing the results of studies obtained with the intradermal tuberculin test and IHA test, it was found that the consistency of the results was 37.7%. At the same time, diagnostic antibody titers were detected in 5 animals in the isolation unit and in 201 animals with no reaction to the intradermal tuberculin test on the infected farm. Post-mortem examination of the internal organs of 20 euthanized animals from the infected farm and of 5 animals from the isolation unit, revealed TB in 75 and 100% of cases, respectively. Consequently, IHA test enhances the ability of TB diagnosis.

CONCLUSION

Experimental studies and practical experience in the use of serological methods for TB diagnosis provide additional information on animal immunity. The data available on serological techniques (complement fixation test, indirect hemagglutination test, etc.) with the use of various antigens for the detection of TB-infected animals are contradictory and scattered. The results of this study show that the effectiveness of a serological technique depends on the antigen quality and the method of its use (in this case RBCs sensitization), and are consistent with the data obtained by other researchers. Studies have shown that the best way to sensitize RBCs is Fili method, which allowed higher antibody titers to be obtained with homologous sera as compared to the heterologous ones. It is logical to assume that IHA testing using various mycobacterial diagnosticums will reveal the cause of the macroorganism's sensitization to tuberculin. Testing of sera of animals sensitized with atypical mycobacteria showed that the number of animals positive in IHA test based on *M. fortuitum* diagnosticum was significantly higher than the number of animals positive in IHA test based on *M. bovis* diagnosticum. The opposite was observed in TB-infected farms – a relatively low effectiveness of IHA test of blood sera from TB-infected animals using a diagnosticum prepared from heterologous mycobacteria, which is probably associated with the developed TB process suppressing the immunity.

Thus, the presented results show that diagnosticums prepared from mycobacteria homologous to the infection are effective for the differentiation of non-specific reactions to tuberculin, and allow to expand the understanding of mechanisms for animal TB diagnosis improvement.

Table 2
Results of IHA tests of serum samples collected from animals originating from different farm

Таблица 2
Результаты исследования проб сывороток крови от животных из различных хозяйств в реакции непрямой гемагглютинации

Farm No.	Number of serum samples	Antibodies were detected using diagnosticums produced from:			
		M. bovis		*M. fortuitum*	
		quantity	%	quantity	%
1	180	27	15.0	2	1.1
2	1,016	206	20.3	73	7.2
3	522	43	8.2	79	15.1
4	193	–	–	–	–

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Detection of *Helicobacter suis* bacteria in pigs of different age groups

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SUMMARY

Currently, the pathogenesis of gastric ulcer in pigs remains largely unexplored. The origin of this pathology is most often associated with the type and the technologies of feeding, stresses and disorders of homeostasis of the animal body. The possible involvement of bacteria of the genus *Helicobacter* in the development of chronic gastritis and gastric ulcer disease in pigs was suggested by the researchers relatively recently. The article comprises the results of investigations aimed at detection of *Helicobacter suis* bacteria and the contamination degree of porcine gastric mucosa in pigs of different age groups. The stomachs, obtained from suckling pigs, fattening pigs and sows in the slaughterhouse of the Mari El Republic, were examined. The study determined the dependence of pathomorphological changes in the gastric mucosa of pigs on the detection of *H. suis* in microscopic and biochemical tests as well as in PCR. Thus, no pathomorphological changes in the gastric mucosa of suckling pigs were detected. Severe hyperkeratosis, erosions, and ulcers were found on the stomach mucosa of fattening pigs and sows that were infected with *H. suis* bacteria. Sows also had ulcerative lesions in the non-glandular region of esophagus. In the biomaterial of suckling piglets the DNA of *H. suis* bacteria was found only in the pyloric region of the stomach, while in fattening pigs, the DNA of these bacteria was most often isolated from the fundal region, and in sows – from the fundal and cardial regions. This indicates a shift in colonization by helicobacters of the mucous membrane of the stomach from the pyloric to the cardiac section increased with animal age. The obtained research data provide the additional evidence of the etiological role of *H. suis* in the pathogenesis of gastric ulcer in pigs.

Key words: *Helicobacter suis,* gastric mucosa, pigs, laboratory diagnostics.

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Выявление бактерий *Helicobacter suis* у свиней разных возрастных групп

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РЕЗЮМЕ

В настоящее время патогенез язвенной болезни желудка свиней остается в значительной степени неизученным. Возникновение данной патологии наиболее часто связывают с типом и технологией кормления, стрессами и нарушениями гомеостаза организма животных. Сравнительно недавно исследователями было выдвинуто предположение о возможном участии бактерий рода *Helicobacter* в развитии хронического гастрита и язвенной болезни свиней. В статье приводятся результаты исследований по выявлению *Helicobacter suis* и степени обсемененности слизистой оболочки желудка свиней разных возрастных групп. Материалом для исследования служили желудки, полученные от молочных поросят, откормочных свиней и свиноматок в убойном пункте Республики Марий Эл. Определена зависимость выявленных патоморфологических изменений слизистой оболочки желудка свиней от наличия *H. suis*, обнаруженной в микроскопических и биохимических тестах, а также с помощью полимеразной цепной реакции. Так, выраженных патоморфологических изменений на слизистой желудка молочных поросят выявлено не было. У откормочных свиней и свиноматок, у которых было установлено инфицирование бактериями *H. suis*, на слизистой оболочке желудка обнаруживали выраженный гиперкератоз, эрозии и язвы. Также у свиноматок наблюдали язвенные поражения нежелезистой части желудка в области пищеводного отверстия. У молочных поросят ДНК бактерий *H. suis* была выделена только в биоматериале из пилорического отдела желудка, тогда как у откормочных свиней наиболее часто ДНК обнаруживали в фундальном отделе, а у свиноматок – в фундальном и кардиальном отделах. Это указывает на сдвиг колонизации хеликобактериями слизистой оболочки желудка

от пилорического к кардиальному отделу с увеличением возраста животного. Полученные данные представляют дополнительные доказательства этиологической роли *H. suis* в патогенезе язвенной болезни желудка свиней.

Ключевые слова: *Helicobacter suis,* слизистая оболочка желудка, свиньи, лабораторная диагностика.

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INTRODUCTION

Helicobacter suis – is a spiral-shaped gram-negative bacterium characterized by 0.6–0.8 μm in diameter and 2.3–6.7 μm in length, forming 4–8 closely spaced spirals. Periplasmic fibrils are not observed. Such type bacteria are characterized by active motility and amphitrichous flagellation type, with 4 to 10 flagella seen at both ends of the cells. The flagella are blunt-ended or have spherical ends that are twice the mean diameter of the flagella body. *H. suis* is a non-capsulating and non-sporulating bacterium [1, 2].

For *H. suis* cultivation the following media are used: brucella agar, meat-liver peptone agar (MLPA), meat peptone agar (MPA), supplemented with 20% fetal calf serum or 10% defibrinated horse blood. The seeds are cultivated under microaerophilic and capnophilic conditions at 37 °C for 5–7 days. Slight growth of bacteria is observed under the anaerobic incubation conditions. *Spirillum*-like forms are transformed into coccoid forms in the course of cultivation in growth media [1, 3].

The possible involvement of *H. suis* in the development of chronic gastritis of the pyloric region of porcine stomach was proposed at the end of the XX century when M. M. Queiroz et al. in 1990 described in their paper a spiral organism detected in the course of microscopic examination of swabs taken from the pig stomachs [4]. Then ensued the studies performed by E. N. Mendes et al. [5], M. M. Queiroz et al. [6], C. De Witte et al. [7] and other authors who examined the correlation between the bacteria presence in the stomach mucosa and gastric ulcer disease in pigs. However, G. M. Grasso et al. [8], S. I. Melnichouk et al. [9] detected no such correlation.

It is should also be stated that currently *H. suis* – is the most prevalent (after *H. pylori*) type of *Helicobacter* in humans capable to induce diseases of gastrointestinal tract [10–12]. Nowadays the most acceptable point of view is that *H. pylori* represents an important risk factor of chronic gastritis development in humans. The study of such bacteria type led to a fundamental change in treatment of human ulcer disease. In particular, implementation of preventive measures in Moscow in 2016 led to reduction in the burden of ulcer disease by 77% and by 64% in its spread compared to 1994 [10]. *H. pylori* eradication as a strategy of stomach cancer prevention in humans in type B atrophic gastritis generates a considerable interest. Largely thanks to it, a significant reduction of stomach cancer is observed in Western Europe, USA and Japan [10, 13].

However, the pathogenesis of gastric ulcer in pigs remains largely unexplored [7]. The origin of this disease is most often associated with such factors as: ration, size of feed particles, permanent stress and homeostasis disorders in animal body. Multiple ulcer processes are observed in animal body due to metabolism disturbance, alimentary dystrophy, poisonings, infectious diseases (foot-and-mouth disease, necrobacillosis, tuberculosis). Sulfate or copper carbonate feeding, swill feeding (as well as toxic and excessive sour feed), protein and selenium deficiency, imbalance of the main and sour equivalents and hypovitaminosis are considered as the predisposing causes. Some authors claim that *H. suis* bacteria causing hyperkeratosis, erosions and ulcers play a certain role in ulcer disease pathogenesis in pigs. Various data on the spread of such type bacteria in pigs are given in papers. For example, E. N. Mendes et al. [5] detected *H. suis* in 10.8% cases, M. M. Queiroz et al. [6] – in 90%, A. Hellemans et al. [14] – in 80%. Currently it can be concluded that *H. suis* morbidity in pigs vary from 10.8 to 90.0%, but most studies report on 60% [2, 7, 11].

Influence on animal health, economic losses in pig breeding industry and zoonotic value of *H. suis* bacteria prove the necessity of a detailed study of the microorganism itself and the epidemiology of lesions which it may cause [2, 3, 15, 16].

The aim of this study is to detect *H. suis* bacteria, to determine the contamination degree of the stomach mucosa of pigs belonging to different age groups and to compare the pathomorphological changes in the mucosa with the degree of its colonization with *Helicobacter* bacteria.

MATERIALS AND METHODS

Stomachs obtained from 5 suckling pigs (1–2 months), 20 fattening pigs (8–10 months) and 4 sows (3 years) of Large White breed were used as a research material. All pigs came to a specialized slaughterhouse from one and the same holding of the Republic of Marij El during one work shift.

After the pre-mortem inspection of animals, conducted by the state veterinarian only healthy animals were allowed for slaughter. The removed stomachs were opened

by incising along the greater curvature and washed with sterile water in the slaughterhouse. The pathomorphological properties of the stomach mucosa were examined according to the method of M. J. Hessing et al. [17], who proposed to use the scale from 0 to 5, where 0 stands for the intact mucosa; 1 – moderate hyperkeratosis, covering less than 50% of the surface; 2 – severe hyperkeratosis covering more than 50% of the surface; 3 – hyperkeratosis and several small erosions (less than 5 single lesions, 2.5 cm^2 in area); 4 – hyperkeratosis and extensive erosions (more than 5 lesions and/or more than 2.5 cm^2); 5 – hyperkeratosis, large erosions (more than 10 lesions or more than 5 cm^2) and/or ulcers.

In order to evaluate the level of microorganism contamination of the stomach mucosa of cardial, fundal and pyloric stomach regions of pigs the imprint smears were prepared and subject to Gram-staining. Contamination level was determined using a semi-quantitative method by three degrees: I – to 10, II – from 10 to 20, III – more than 20 microbial bodies per light microscope with ×1000 magnification [18].

The results of microscopic investigations were proved by CLO-test, which principle is based on the property of *Helicobacter* bacteria to recover urease, an enzyme that catalyzes the hydrolysis of urea, forming ammonia and carbon dioxide. As a result of the reaction which takes place in the course of the test, the pH of the media shifts to alkaline which is identified by the indicator. Speed of the indicator colour change depends on the urease activity, which in its turn depends on the bacterial count. Thus, the colonization degree of the stomach mucosa with *H. suis* can be indirectly assessed – the higher the contamination degree the faster the speed of the indicator colour change. In order to conduct the test, biopsy samples (5 × 5 mm) were aseptically taken from the mentioned areas of the stomach mucosa in the first 10 minutes after the slaughter and were put into tubes with reagents. CLO-test results were considered after 1, 3 and 24 hours, evaluating the speed of solution colour change.

Specific detection of *H. suis* bacteria was performed by polymerase chain reaction (PCR) according to D. De Groote et al. [19]. For this purpose, the samples of mucosa from cardial, fundal and pyloric regions of the stomach were collected into Eppendorf tubes. Genomic DNA from the biomaterial was extracted using "DNA-SORBENT" reagent kit (OOO NPF "Lytech", Moscow) in accordance with manufacturer's instruction. Homologous regions of *H. suis* 16S rDNA gene 433 bp in size represented the identifiable DNA fragments. Species-specific PCR was performed using V832f TTGGGAGGCTTTGTCTTTCCA and V1261r GATTAGCTCTGCCTCGCGGCT primers, proposed by D. De Groote et al. [19], according to the following programme: 94 °C – 4 minutes; 94 °C – 30 seconds; 60 °C – 60 seconds; 72 °C – 10 seconds during 40 cycles. Detection of amplification products was performed using horizontal electrophoresis by their electrophoretic separation in 2% agarose gel with ethidium bromide added and fluorescent visualization in UV-transilluminator.

RESULTS AND DISCUSSION

Macroscopic evaluation of suckling pig stomach mucosa condition demonstrated that in 3 samples the mucosa was not damaged (0 points according to J. J. C. Hessing et al. method), in 2 samples moderate hyperkeratosis, covering less than 50% (1 point) of the surface was noticed. While testing fattening pig stomach mucosa condition the following data was obtained: in 1 sample the mucosa was undamaged (0 points), 5 samples demonstrated moderate hyperkeratosis (1 point), and 6 samples – severe hyperkeratosis (2 points), hyperkeratosis and several small erosions (3 points) were detected in 4 samples, 3 samples revealed hyperkeratosis and extensive erosions (4 points), 1 sample – hyperkeratosis and large erosions (5 points). Stomach mucosa condition of sows was characterized by severe hyperkeratosis (2 points) in 1 sample, 2 samples demonstrated hyperkeratosis and several small erosions (3 points) and 1 sample – hyperkeratosis and extensive erosions (4 points).

Imprint smears of the stomach mucosa were prepared for primary microscopy from all the stomachs obtained under the conditions of the slaughter point. The stomach mucosa fragments of intact stomachs were taken from the cardial and fundal regions incising along the greater and the lesser curvature; from the pyloric region – along the vertexes of the imaginary equilateral triangle with pylorus in the middle (at 2–4 cm distance from it). In case of stomachs with detected pathomorphological changes – from the affected sites. The obtained biomaterial was put in tubes with thioglycollate medium and transported within 2–3 hours in thermal bag with cooling agent.

While investigating the imprint smears of suckling pig stomach mucosa no microorganisms with forms typical for *Helicobacter* were detected.

In microscope examination of imprint smears obtained from fattening pig stomach mucosa curved, spiral-shaped gram-negative bacilli (0.2–0.8 μm in diameter and 2–5 μm in length) were detected in 8 of 20 stomachs in presence of stained mucus. Microorganisms with forms characteristic of *H. suis* were found in 8 samples from the fundal region of the stomachs and in 4 samples from the pyloric region, with no similar bacteria detected in the cardial region. The II degree of contamination with microorganisms was determined in 5 samples taken from the fundal region mucosa, and the III degree – in 3 samples. In imprint smears from the pyloric region of the stomach mucosa the II degree of contamination was registered in 4 samples.

Microorganisms, bearing the forms characteristic of *H. suis* were detected in 1 of 4 stomachs in the course of examination of imprint smears, taken from sow stomach mucosa. The II contamination degree of mucosa was determined in the cardial and fundal regions of the stomach. No characteristic microorganisms were detected in the imprint smear taken from the pyloric region.

Urease activity of tested samples was determined by the CLO-test. *H. suis* contamination degree of the stomach mucosa was evaluated in the test by the speed of the indicator colour change during 24 hours. If the solution colouring changed within 1 hour it indicated significant contamination of stomach with the microorganism, from 1 to 3 hours – moderate contamination, from 3 to 24 hours – insignificant *H. suis* presence in the biopsy sample (Table 1). In case of low contamination degree of the stomach mucosa, the urease activity may be insignificant and the possibility of false-negative result achievement exists. It should be noted that CLO-test represents the indirect method because it does not detect *Helicobacter* but only the urease activity which may be demonstrated by other bacteria, for example *Proteus*.

The investigation data represented in Table 1 demonstrate high urease activity detected in stomach samples obtained from fattening pigs. In biopsy samples from suckling pig stomachs no urease activity was detected.

The biomaterial taken from different pig stomach regions was tested by PCR with *H. suis* species-specific primers – V832f and V1261r. The investigation results for *H. suis* DNA presence in the mucosa of cardial, fundal and pyloric regions of the stomach are shown in Table 2.

As Table 2 shows, *H. suis* DNA was detected in the biomaterial taken from the pyloric region of the stomach of one suckling pig. *H. suis* genome was recovered from the mucosa of 17 out of 20 fattening pig stomachs, with that in 4 cases – in the cardial region, in 15 cases – in the pyloric region and in 17 cases – in the fundal region of the stomach mucosa (36 positive samples in total). While investigating the material obtained from sows, *H. suis* DNA was detected in stomachs of all 4 animals, whilst 2 samples of the stomach mucosa from the cardial region, 4 samples – from the fundal and 1 sample from the pyloric regions were found positive (7 positive samples in total).

The results of the tests performed in order to determine the presence or absence of the pathomorphological changes in porcine stomach mucosa were compared with the results of *H. suis* identification in microscopic and biochemical tests and bacterium genome detection in PCR. For this purpose, the biomaterial obtained from pigs of each age group was divided into two groups: *H. suis*-positive (*H. suis* "+") and *H. suis*-negative (*H. suis* "–") in PCR. The data on microscopic evaluation of pig stomach mucosa, microscopy and urease test were put into the corresponding groups. The results are represented in Table 3.

Table 3 shows that moderate hyperkeratosis of the stomach mucosa covering less than 50% of the surface was observed in the pyloric region of the stomach of one suckling pig from which the *H. suis* DNA was extracted. No relevant bacteria were detected by microscopic and biochemical methods. In group representing *H. suis*-negative suckling pigs moderate hyperkeratosis of stomach mucosa was detected once, however it was impossible to detect *H. suis* by any of the test methods used.

Fattening pigs demonstrated marked hyperkeratosis and erosions of the stomach mucosa in group of *H. suis*-positive animals, whereas no marked inflammatory reactions were detected in group of *H. suis*-negative animals. In microscopy of the imprint smears of the stomach mucosa gram-negative bacilli with *Helicobacter*-characteristic forms were detected in 8 samples from *H. suis*-positive group in presence of stained mucus. The samples were urease-positive in all cases. The III degree of *H. suis* contamination of fundal region stomach mucosa was determined in 2 of 3 stomachs (which macroscopic lesions were given a 4-point mark), and the II degree – in one stomach.

H. suis DNA was detected in all tested samples in PCR of sow stomach mucosa. Hyperkeratosis of cardial, fundal and pyloric regions was observed in macroscopic evaluation of stomach mucosa condition. The II degree of *H. suis* contamination was estimated in the cardial and fundal region of the stomach affected by ulcers (with a 4-point macroscopic evaluation mark) in the area of esophageal opening (its non-glandular part). All biomaterial samples gave a positive response in CLO-test.

Table 1
Contamination degree of samples from the pyloric region of pig stomachs determined by the urease activity in CLO-test

Таблица 1
Степень обсемененности образцов из пилорического отдела желудков свиней, установленная по наличию уреазной активности в CLO-тесте

Animal age group	Number of tested stomachs	Number of positive samples during		
		1 hour	3 hours	24 hours
suckling pigs	5	0	0	0
fattening pigs	20	8	14	17
sows	4	1	2	4

Table 2
***H. suis* DNA detection in pig stomach samples by PCR**

Таблица 2
Обнаружение ДНК *H. suis* в образцах желудков свиней посредством ПЦР

Animal group (number of tested stomachs)	Number of positive samples taken from the stomach mucosa			Total number of positive samples	Number of animals with *H. suis* DNA detected in stomachs
	cardial region	fundal region	pyloric region		
suckling pigs (5)	0	0	1	1	1
fattening pigs (20)	4	17	15	36	17
sows (4)	2	4	1	7	4

CONCLUSION

The epizootological investigation data on the spread of *H. suis* bacterium in pigs given in different publications differ greatly. The number of detected cases vary from 10.8 to 90.0%. It can be explained by the following reasons: firstly, ulcer disease has a multi-factor etiology; secondly, *H. suis* represents a microorganism which is difficult to extract and it was considered to be uncultivated *in vitro* till 2008; thirdly, the material for investigations was taken from animals of different age; fourthly, the mucosa samples were obtained from different regions of animal stomachs. Nevertheless, the majority of researchers report on high (60% and more) prevalence of *H. suis* among pigs.

The conducted tests demonstrated that the results of macroscopic evaluation of the stomach mucosa lesions

Table 3
Determination of dependence between the pathomorphological changes in porcine gastric mucosa and *H. suis* detection in microscopic and biochemical tests and its genome detection in PCR

Таблица 3
Определение зависимости патоморфологических изменений СОЖ свиней от обнаружения *H. suis* в микроскопических и биохимических тестах и выявления его генома в ПЦР

Animal group (number of animals)	Macroscopic evaluation of stomach mucosa lesions, points						Microscopy +	CLO-test +
	0	1	2	3	4	5		
Group 1: *H. suis* "+" suckling pigs (1)	0	1	0	0	0	0	–	–
Group 2: *H. suis* "–" suckling pigs (4)	3	1	0	0	0	0	–	–
Group 1: *H. suis* "+" fattening pigs (17)	0	4	5	4	3	1	8	17
Group 2: *H. suis* "–" fattening pigs (3)	1	1	1	0	0	0	12	0
Group 1: *H. suis* "+" sows (4)	0	0	1	2	1	0	2	4

in each age group of pigs differed. The highest level of lesions was detected in the group of fattening pigs. In *H. suis*-positive (according the PCR data) group of fattening pigs severe hyperkeratosis and erosions in stomach mucosa were mostly observed. According to the results of microscopic investigations and urease test, high level of *Helicobacter* contamination was detected in the biomaterial taken from animals belonging to this age group. In all fattening pigs *H. suis* DNA was extracted in the fundal region of the stomach. Pathomorphological changes in the mucosa of suckling pigs were insignificant or totally absent, *H. suis* DNA was extracted in one out of five stomachs tested. Stomach mucosa condition of sows was characterized by moderate lesions, however, *Helicobacter* were detected in all mucosa samples in the course of microscopic investigations, biochemical tests and PCR.

In suckling pigs *H. suis* was found in the pyloric region of the stomach, thus, in fattening pigs – it was more often detected in the fundal region, and in sows – in fundal and cardial regions. This may indicate a shift in *Helicobacter* colonization of stomach mucosa from the pyloric to cardial region with the increase of the animal age.

The results obtained show that *H. suis* may be one of the factors playing a certain role in the development of pig stomach ulcer pathogenesis.

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Efficacy of a complex plant-based preparation for poultry mycotoxicosis

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SUMMARY
The article presents results of efficacy studies for a complex plant-based preparation for poultry mycotoxicosis. Feed additive fibralin contains polysaccharides (dried sugar beet pulp) and phospholipids (rapeseed lecithin) in the proportion 4:1. Eighteen-day-old "Ross-308" broiler chickens with average weight of (665.10 ± 4.28) g were tested, since such mycotoxins as T-2 toxin, zearalenone and aflatoxin B_1 were detected in their feeds. Maximum admissible level of each toxin was not exceeded, however, their cumulative effect on poultry resulted in mycotoxicosis. Use of fibralin in the feed (3 kg per one ton) for 10 days reduced clinical signs of intoxication, increased flock survival by 13.5% and stimulated body weight gain by 15.8%. Pharmacological effect of fibralin was demonstrated by improvement of blood morphobiochemical parameters in poultry, i.e. reduction of leukocytes by 19.3% and cholesterol by 13.6%; and an increase in the number of erythrocytes by 19.4%, hemoglobin by 8.1% and calcium by 9.5%. Antitoxin therapy had a positive effect on liver structure and functions and that fact was confirmed by a decrease in aminotransferase level in serum and normal levels of total protein. The data obtained may justify the use of this natural bio-preparation as a product with antitoxic and hepatoprotective properties and the use of fibralin for mycotoxicosis treatment of poultry.

Key words: mycotoxicoses, poultry, fibralin, beet pulp, lecithin.

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Эффективность комплексного препарата на основе вторичных растительных ресурсов при микотоксикозе сельскохозяйственной птицы

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РЕЗЮМЕ
Представлены результаты изучения эффективности комплексного препарата на основе вторичных растительных ресурсов при микотоксикозе сельскохозяйственной птицы. Препарат фибралин содержит комплекс веществ полисахаридной (сухой свекловичный жом) и фосфолипидной (рапсовый лецитин) природы в соотношении 4:1. Исследования проведены на 18-суточных цыплятах-бройлерах кросса «Росс-308» со средней массой тела (665,10 ± 4,28) г, в кормовых рационах которых были обнаружены микотоксины: Т-2 токсин, зеараленон и афлатоксин B_1. Концентрация токсинов по отдельности не превышала максимально допустимого уровня, но их сочетанное воздействие на организм птицы обуславливало развитие микотоксикоза. Применение фибралина в дозе 3 кг на тонну корма в течение 10 дней привело к снижению клинических признаков интоксикации, повышению сохранности поголовья на 13,5% и интенсивности приростов массы тела на 15,8%. Фармакологический эффект фибралина проявился улучшением морфобиохимических параметров крови птицы за счет снижения концентрации лейкоцитов на 19,3% и холестерина на 13,6% при увеличении содержания эритроцитов на 19,4%, гемоглобина – на 8,1% и кальция – на 9,5%. Антитоксическая терапия оказала положительное действие на структурное и функциональное состояние печени, что подтвердилось снижением уровня аминотрансфераз в сыворотке крови и нормализацией концентрации общего белка. Полученные данные могут служить основанием для применения данного биологического комплекса природного происхождения в качестве препарата с антитоксическими и гепатопротекторными свойствами и использования фибралина при терапии микотоксикозов у птицы.

Ключевые слова: микотоксикозы, птица, фибралин, свекловичный жом, лецитин.

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INTRODUCTION
An increase in the number of mycotoxicoses cases in animals and poultry is reported now and this situation poses high economic and environmental risks. There are hundreds of various mycotoxins; they differ in their chemical structure and in their impact on animals. Most widespread mycotoxins are aflatoxin, zearalenone, T-toxin, fumonisin and ochratoxin. Many mycotoxins cause severe irreversible changes in animals that frequently result in death. This problem is aggravated by the fact that mycotoxins (transmitted to animals via feeds) can accumulate in food products of animal origin, thus posing a significant risk for human health [1, 2].

Taking into account the above, we believe that veterinary science faces now a crucial task to develop effective complex antitoxic preparations. A wide range of preparations that have antitoxic, hematoprotective, antioxidant properties includes fibralin based on such plant by-products as beet pulp and rapeseed lecithin.

Beet pulp fibers included into preparation normalize motor functions of biliary tract, i.e. stimulate bile excretion and prevent stases in hepatobiliary system. Beetroot contains many microelements and vitamins, and beet pectin found in its fibres has physicochemical properties (i.e. low etherification degree with a great number of free carboxyl groups) of the best natural adsorbent and complexing agent for different xenobiotics [3–5].

Lecithin, the second component of fibralin, is a complex of essential phospholipids with a range of different functions. It is a component of cellular membranes (in the form of phosphatidylcholine); an emulsifier and regulator of cholesterol crystallization; it contains omega-6 polyunsaturated fatty acids that normalize lipid transport in blood and improve intestinal lipid absorption; it plays an important role in immune protection and has an antioxidant effect etc. [6–8].

The purpose of the research is to analyze fibralin efficacy for mycotoxicosis in broiler chickens.

MATERIALS AND METHODS
Fibralin contains polysaccharides (dried beet pulp) and phospholipids (rapeseed lecithin) in the proportion 4:1.

Fibralin efficacy was tested in backyard "IP I. V. Remesnik" (the Dinsky Raion, the Krasnodar Krai). 440 eighteen-day-old "Ross-308" cross broilers with average weight of (665.10 ± 4.28) g were used. Deaths were reported in the backyard during 5 days before the experiment, when starter diet was being replaced by grower diet.

Mixed feed "Rost" was tested in ELISA for mycotoxins. For this purposes we used Stat Fax® 2600 (USA) analyzer and a test kit for indirect competitive ELISA (ZAO "Farmatekh", Russia). The test revealed mycotoxins in the feeds: T-2 toxin – 0.016 mg/kg; zearalenone – 0.018 mg/kg; aflatoxin B_1 – 0.002 mg/kg. Maximum admissible level of each toxin was not exceeded, however, their cumulative effect on poultry resulted in mycotoxicosis.

In order to assess fibralin efficacy we divided the chickens into 3 groups: experimental group No. 1 (200 chickens) received 3 kg of fibralin per ton of feed; experimental group No. 2 (200 chickens) received 1.5 kg of another preparation for comparison "AtoxBio Plus" (OOO "TekhnoFeed", Russia) per ton of feed; control group No. 3 (40 chickens) received no treatment. Thus, the preparations were given for 10 days.

Clinical observations were performed during the experiment; chickens were weighed at the beginning and at the end and dead birds were subject to post-mortem examination. On day 1 and day 10 of the experiment, we took 10 chickens from each group for general and biochemical blood tests. Biochemical blood tests were performed in automatic chemistry analyzer Vitalab Selectra Junior (the Netherlands) with chemical reagents from ELITech Clinical System (France) and Analyticon Biotechnologies AG (Germany), general blood test was performed in automatic analyzer Mythic 18 Vet (Switzerland).

Table 1
Body weight gain of broiler chickens treated for mycotoxicosis

Таблица 1
Динамика массы тела цыплят-бройлеров при лечении микотоксикоза

Groups	Body weight, g	
	Day 1 of the experiment	Day 10 of the experiment
Experimental No. 1	664.90 ± 4.26	1204.90 ± 11.3
Experimental No. 2	665.40 ± 4.41	1192.50 ± 12.9
Control No. 3	664.80 ± 4.18	1040.70 ± 10.7

The following benchmarks were used to evaluate treatment effectiveness: survival rate, clinical status, appetite, locomotor activity, results of general and biochemical blood tests, body weight gain.

All the animal tests were performed in compliance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 "On protection of animals used for scientific purposes".

Statistical analysis was performed using Statistica 6.0 software. Validation criterion was determined by Student's table.

RESULTS AND DISCUSSION

During the whole experiment 3 chickens were found dead (1.5%) in group No. 1 (received fibralin) on the first two days of the experiment; 5 chickens died in group No. 2 (2.5%) and 6 birds died in control group (15%).

Gravimetric measurements showed weight gain in all the three groups, however, weight gain rate was obviously lower in chickens from control group (received no treatment) than that in birds from experimental groups. Thus, on day 10 of the experiment the difference from experimental group No. 1 was 15.8% and from group No. 2 – 14.6% (Table 1).

Assessment of hematological status revealed that white blood cell count in chickens from all groups was close to upper normal level, on average $(37.10 \pm 1.27) \times 10^9$/L. At the end of the experiment white blood cell (WBC) count in chickens from group No. 3 increased by 9.1% and accounted for $(39.80 \pm 0.96) \times 10^9$/L, whereas WBC concentration in groups No. 1 and No. 2 decreased by 19.3 and 16.2%, respectively. Besides, the group that received no treatment demonstrated a progressive decrease in red blood cell (RBC) count by 12.1% – $(2.90 \pm 0.22) \times 10^{12}$/L and in hemoglobin level by 6.7% – (84.10 ± 2.08) g/L as compared to their original levels. Broiler chickens from experimental groups No. 1 and No. 2 demonstrated positive dynamics, i.e. an increase in RBC count by 19.4 and 12.5%; and hemoglobin by 8.1 and 11.6%, respectively, as compared to the original data.

Laboratory blood tests revealed that treatment for mycotoxicosis with antitoxic preparations was accompanied by positive changes in biochemical profile of chickens, with a number of better values reported in experimental group No. 1 (Table 2).

At the beginning of the experiment, the chickens demonstrated higher levels of liver enzymes, i.e. alanine aminotransferase (ALT) was higher than the reference value and aspartate aminotransferase (AST) activity was reported at the upper normal level. These data suggest there was a mild cytolytic process in liver caused by the given combination and concentration of mycotoxins. Antitoxin therapy in experimental groups had a positive effect on liver structure and functions as it was confirmed by a drop in aminotransferases level in chicken serum: in group No. 1 – ALT decreased by 30.5% ($p \le 0.01$) and AST by 15.1%; in group No. 2 – ALT decreased by 15.1% ($p \le 0.05$) and AST by 6.1%. On day 10, the enzyme activity in the control group increased as compared to the original values: ALT increased by 19.7% ($p \le 0.05$) and AST by 12.6% ($p \le 0.05$). These changes indicate an acceleration in hepatocyte cytolysis accompanied by mycotoxicosis that pushes the enzymes out into the intracellular space and increases their level in blood.

A baseline study revealed hypercholesterolemia that demonstrated early signs of liver damage in chickens. Hypercholesterolemia is a sign of acute liver dysfunction in the early stages of the disease. It is well-known that cholesterol concentration drops lower than the normal value, when an acute condition becomes a chronic one. These changes in cholesterol profile were as well observed during the experiment: those broiler chickens that had mycotoxins in their diet and received no treatment had cholesterol below lower limit of normal at the end of the experiment, i.e. (2.46 ± 0.09) mmol/L with the significant difference from the original data of 33.7% ($p \le 0.01$). Due to the treatment provided, cholesterol concentration was optimal in group No. 1 by 13.6% and in group No. 2 by 7.3% as compared to the background data.

Hepatic protein synthesis in chickens from experimental groups improved as it was indicated by normalization of total protein concentration that increased by 16.7% ($p \le 0.05$) in group No. 1 and by 15.8% in group No. 2 on day 10 of the experiment as compared to the original values. At the end of the experiment, hypoproteinemia became more evident in control chickens, with a decrease in total protein by 5.9%.

There was also a 10.1% drop in creatinine concentration in group No. 3 as compared to the original values; however, this parameter did not significantly change in experimental groups.

Pharmacological effect that fibralin has on protein metabolism is explained not only by improved hepatic protein synthesis influenced by a hepatoprotective component of the preparation, but also by the intake of beet pulp proteins represented by such amino acids as lysin, arginine, leucine, phenylalanine, threonine, valine, methionine and cystine.

In addition, it is the mineral content of beet pulp (a lot of calcium, potassium, sodium, magnesium, cuprum, cobalt) that leads to changes in calcium and phosphorus metabolism in chickens treated for mycotoxicosis. Total calcium in chicken blood reported in baseline studies was at the lower limit of normal – (2.10 ± 0.09) mmol/L on average. At the end of the experiment, the chickens from experimental group No. 1 (received fibralin) demonstrated an increase in calcium level up to (2.30 ± 0.06) mmol/L, which complies with the normal values. No significant changes were reported in mineral metabolism in the other groups.

Table 2
Blood chemistry values for broiler chickens treated for mycotoxicosis ($M \pm m$; $n = 10$)

Таблица 2
Биохимические показатели крови цыплят-бройлеров при лечении микотоксикоза ($M \pm m$; $n = 10$)

Values	Groups		
	Experimental No. 1	Experimental No. 2	Control No. 3
	Day 1 of the experiment		
ALT, u/L	22.60 ± 1.77	21.30 ± 1.67	23.90 ± 1.32
AST, u/L	296.50 ± 5.60	284.50 ± 3.50	291.70 ± 4.40
Cholesterol, mmol/L	3.74 ± 0.18	3.69 ± 0.11	3.71 ± 0.12
Glucose, mmol/L	8.70 ± 0.55	8.90 ± 0.46	9.10 ± 0.41
Creatinine, μmol/L	29.10 ± 1.32	28.50 ± 1.15	29.80 ± 0.79
Total protein, g/L	31.20 ± 0.85	29.80 ± 1.22	30.40 ± 0.43
Calcium, mmol/L	2.10 ± 0.12	2.10 ± 0.09	2.00 ± 0.05
Phosphorus, mmol/L	1.28 ± 0.16	1.34 ± 0.12	1.36 ± 0.18
	Day 10 of the experiment		
ALT, u/L	15.70 ± 0.39**	18.10 ± 0.48*	28.60 ± 0.54*
AST, u/L	251.50 ± 5.60	267.30 ± 3.40	328.90 ± 4.70*
Cholesterol, mmol/L	3.23 ± 0.07*	3.42 ± 0.13	2.46 ± 0.09**
Glucose, mmol/L	10.90 ± 0.63	9.40 ± 0.36	8.20 ± 0.27
Creatinine, μmol/L	32.30 ± 0.71	29.10 ± 0.62	26.80 ± 0.36
Total protein, g/L	36.40 ± 0.43*	34.50 ± 1.30	28.60 ± 0.74
Calcium, mmol/L	2.30 ± 0.06	2.20 ± 0.11	1.90 ± 0.13
Phosphorus, mmol/L	1.35 ± 0.15	1.39 ± 0.24	1.42 ± 0.17

* $p \le 0.05$;
** $p \le 0.01$ – differences are verified in relation to background data (различия достоверны по отношению к фоновым данным).

CONCLUSION

Consequently, the carried out research demonstrated that a complex use of substances with adsorptive, hepatoprotective, antioxidant and metabolism-stabilizing properties improves survival rate and productivity of poultry fed with mycotoxin-contaminated feeds. Use of fibralin against combined mycotoxicosis in broiler chickens (at a dose of 3 kg per ton of feed) reduces clinical signs of intoxication, normalizes morphological and biochemical parameters of blood, increases survival and weight gain rates in the flock.

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Prospects of betulin application in broiler farming

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SUMMARY

It is an urgent task today to seek and implement nature-like technologies in broiler production and obtain biologically complete and safe poultry products, thus refusing from antibiotic use in feed. Feed additives based on natural components can be an effective tool for implementation of preventive and therapeutic veterinary measures. The paper presents research study results of application of betulin-based phytobiotics in broiler farming. The tests were carried out on Ross-308 cross-breed broiler chickens within the production cycle at one of the poultry farms of the Sverdlovsk Oblast. Birds of the experimental group received compound feed supplemented with dry betulin at 2.5 mg/kg of live weight from day 21 to day 35 of growing. The introduction of betulin-based feed additive into the diet contributed to increase in live weight gain and 7.6% pectoralis muscle output as compared with the control group. It was established that the phytobiotic consumption resulted in reduced deposition of subcutaneous and abdominal fat, higher biological value of meat by increasing the ash content, improved technological properties of meat due to increasing water-holding capacity of muscle fiber and intensity of formation and maturation of muscle fiber. Histological studies of pancreatic tissue samples from broiler chickens showed increase in the mass of islets of Langerhans and insulin-producing cell complexes. The pancreas was activated due to effects of the betulin-based feed additive. The results obtained indicate that the use of betulin in broiler production is a promising trend.

Key words: broiler chicken, feed additive, betulin, phytobiotics, live weight, muscle fibre, fat, pancreas, islets of Langerhans.

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Перспективы применения бетулина в бройлерном птицеводстве

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РЕЗЮМЕ

Поиск и внедрение природоподобных технологий при выращивании цыплят-бройлеров и получении биологически полноценной и безопасной продукции птицеводства в условиях отказа от кормовых антибиотиков на сегодняшний день является актуальной задачей. При проведении профилактических и терапевтических ветеринарных мероприятий эффективным средством могут быть кормовые добавки на основе природных компонентов. В работе представлены результаты научного исследования по применению фитобиотика на основе бетулина в бройлерном птицеводстве. Испытания проведены в условиях технологического цикла на одной из птицефабрик Свердловской области на цыплятах-бройлерах кросса «Росс-308».

Птице в опытной группе бетулин в сухом виде вводили в комбикорм с 21-го по 35-й день выращивания из расчета 2,5 мг/кг живой массы. Введение в рацион кормовой добавки на основе бетулина способствовало повышению прироста живой массы и выхода грудных мышц на 7,6% по отношению к контрольной группе. Установлено, что потребление цыплятами фитобиотика приводило к снижению отложения подкожного и абдоминального жира, повышало биологическую полноценность мяса за счет увеличения содержания зольных элементов, улучшало технологические свойства мяса за счет повышения влагоудерживающей способности мышечного волокна, а также за счет интенсивности формирования и созревания мышечного волокна. При проведении гистологических исследований образцов тканей поджелудочной железы цыплят-бройлеров выявлено увеличение площади островков Лангерганса, инсулин-продуцирующих клеточных комплексов. Под воздействием кормовой добавки на основе бетулина происходила активация работы поджелудочной железы. Полученные результаты свидетельствуют о том, что применение бетулина в бройлерном производстве является перспективным направлением.

Ключевые слова: цыплята-бройлеры, кормовая добавка, бетулин, фитобиотик, живая масса, мышечное волокно, жир, поджелудочная железа, островки Лангерганса.

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INTRODUCTION

The trend of implementing nature-like technologies in broiler poultry farming is determined by the European Union requirements and is related to the production of biologically complete and safe products. By 2025 Russian poultry establishments will have to completely refuse from feed antibiotics in broiler chicken production, therefore, the search for and introduction of alternative biological additives into production seem to be extremely urgent today [1, 2]. Phytobiotics based on natural components and used for preventive and therapeutic veterinary measures fit well into the technological cycle and demonstrate pronounced biological and economic effects [3–6]. The betulin-based phytobiotic is currently one of the promising modern developments.

Betulin is derived from birch bark (from Latin *Bétula*). It belongs to triterpene alcohols and has a high physiological activity. The substance has antiseptic, wound-healing and anti-inflammatory properties, as well as anti-ulcer, choleretic and hepatoprotective activity. It can be used for prevention of liver diseases, which often occur in meat-producing poultry and are associated with alimentary factors [7]. The hypolipidemic and hypocholesterolemic activity of betulin is effective for normalizing lipid metabolism [8]. It also has antibacterial effect on some streptococcus bacteria [9, 10].

Betulin has been used in medicine for a long time, yet its unique properties have been reviewed in veterinary medicine and agriculture just recently. This phytobiotic has aroused increased interest of both scientists and practitioners; the possibility of its use in the technological cycle is being looked into, and its positive zootechnical, as well as physiological and biochemical effects on chicken are being studied. Betulin enhances the vaccination efficacy in chicks, which is economically beneficial for a poultry establishment [3, 9].

Betulin used in poultry improves glycemic indicators. It was proved to reduce sera lipid concentration, which is shown in a statistically significant decrease in the concentration of total cholesterol and triglycerides, and is also accompanied by an improvement in the liver function parameters (a pronounced decrease in the activity of alanine aminotransferase and aspartate aminotransferase). At the same time the antioxidant and anti-inflammatory effect of betulin was established (indicators of lipid peroxidation, catalase activity, superoxide dismutase, concentration of anti-inflammatory cytokines). The normalization of the ratio of immunoregulatory subpopulations of lymphocytes (CD4+ and CB8+), a decrease in the blood concentration of inflammatory cytokines (IL-6, IL-8, IL-12, IL-18, TNFα, IFNg) were shown. Due to its antiseptic, wound-healing and anti-inflammatory properties betulin can be used for wound aseptic care and treatment of gastrointestinal inflammation (antiulcer activity) [3, 8].

The aim of this paper is to study effects of the betulin-based feed additive on growth performance and biological parameters of Ross-308 cross-breed broilers.

MATERIALS AND METHODS

Animals. Two groups of 80 Ross-308 cross broiler roosters in each group were formed to conduct research in production conditions using the analogy principle. Chickens of the experimental group received feed supplemented with dry betulin at 2.5 mg per kg of body weight at days 21–35 of growing, the second group was control.

All experiments were carried out in poultry in accordance with the requirements of Directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes of September 22, 2010.

Histological examination. The tissue samples were taken for histological analysis from five broiler chickens selected by random sampling in each group (in accordance with the recommendations of the All-Russian Scientific Research and Technology Institute for Poultry, 2010) and were subjected to control slaughter. Histological samples of pectoral muscles and pancreas were fixed in 10% neutral formalin solution. The general study was conducted and structural changes were examined in paraffin sections, using hematoxylin and eosin staining according to the generally accepted method. Histological findings were recorded using Leica DM2500 light microscope with a Leica camera.

Biochemical blood tests were carried out using Chem Well 2910 Combi automatic biochemical analyzer (Awareness Technology Inc., USA) and standard Vital Diagnostics SPb (Russia) and Diasys (Germany) test kits.

The digital data were processed using standard statistical methods of Microsoft Excel 2007 and Statistica 6.0 software. The reliability was calculated using the Student's *t*-test.

Table
Broiler chicken growth parameters before and after the experiment ($n = 160$)

Таблица
Технологические показатели выращивания цыплят-бройлеров до и после опыта ($n = 160$)

Parameter	Control Group	Experimental Group
Body weight at day 21, g	520.00 ± 18.56	522.80 ± 26.31
Body weight at day 35, g	1 924.80 ± 44.93	1 942.80 ± 57.70
Body weight gain, g	1 404.80	1 420.00
Homogeneity, %	85	90
Livability, %	100	100

RESULTS AND DISCUSSION

Ross-308 cross hybrid was breed by specialists of the Aviagen company (U.K.) and is the result of a complex cross-breeding scheme of five breeds in four generations. This is a fast growing broiler with efficient feed conversion and high meat performance. Increased body weight indicated a positive effect on body of broiler chickens when various additives were introduced into the diet during the feeding period. Moreover, it is important to take into account whether the increase occurred due to muscle growth or fat deposition. Therefore, the birds were weighed at the beginning and at the end of the experiment. The results are presented in the Table.

The data presented in the Table indicate an insignificant increase in body weight of the birds in the experimental group. However, it should be noted that the anatomical cutting of carcasses revealed 36% less subcutaneous and abdominal fat deposition on the internal organs and mesentery of the intestine in broilers of the experimental group, and the performance of the pectoral and leg muscles thereof was 7.6% higher in relation to the control group. This confirms the hypolipidemic properties of betulin.

The flock livability indicators were at 100% in both groups. The homogeneity of the flock shows the uniformity of broiler body weight gain during the rearing period, it was 5% higher in the experimental group as compared with the control, indicating a positive effect of betulin on the growth and development parameters of broilers.

The histological examination was conducted for a more detailed study of muscle fiber characteristics. The correlation of meat quantity and quality with the size of muscle fibers was established. A higher number of muscle fibers indicated greater and better meat quality, as well as lower fat content. The muscle fiber thickness decreases with an increase in the mass of individual muscles due to the appearance of thinner new ones. The structure of the muscle tissue and interstitial connective tissue of chickens in the experimental groups (Fig. 1 and 4), as compared with the control ones, was represented by a compact muscle tissue, with nearly completed maturation process and fine

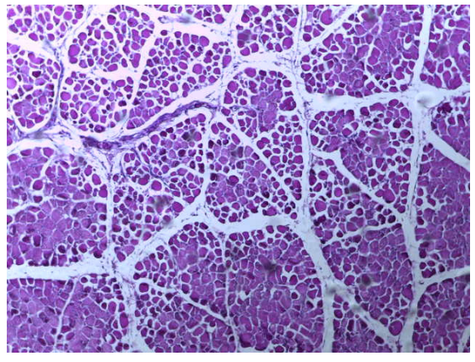

Fig. 1. The structure of femoral muscle fiber bundles in birds of the experimental group. Hematoxylin and eosin staining (100× magnification)

Рис. 1. Структура пучков мышечных волокон бедренной группы мышц птиц опытной группы. Окраска гематоксилином и эозином (увеличение ×100)

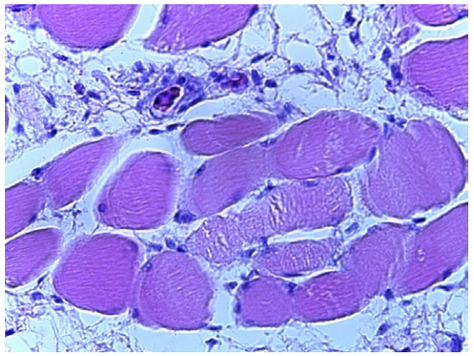

Fig. 2. Immature muscle fibers in the bundle (control group). Hematoxylin and eosin staining (400× magnification)

Рис. 2. Незрелые мышечные волокна в пучке (контрольная группа). Окраска гематоксилином и эозином (увеличение ×400)

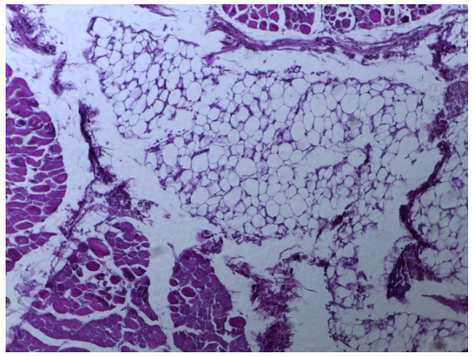

Fig. 3. Muscle fiber bundles with a fat cell layer (control group). Hematoxylin and eosin staining (200× magnification)

Рис. 3. Пучки мышечных волокон с прослойкой жировых клеток (контрольная группа). Окраска гематоксилином и эозином (увеличение ×200)

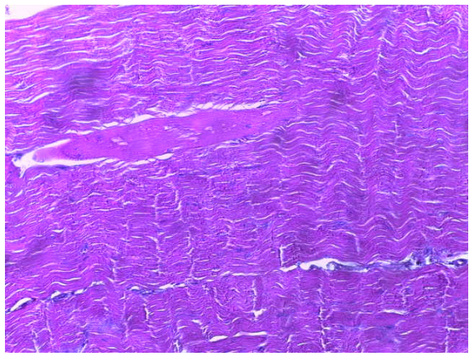

Fig. 4. The breast muscle of the birds in the experimental group. The striated pattern is clearly expressed indicating the maturity of this muscle fiber. Hematoxylin and eosin staining (200× magnification)

Рис. 4. Грудная мышца цыплят опытной группы. Поперечнополосатая исчерченность четко выражена, что свидетельствует о созревании данного мышечного волокна. Окраска гематоксилином и эозином (увеличение ×200)

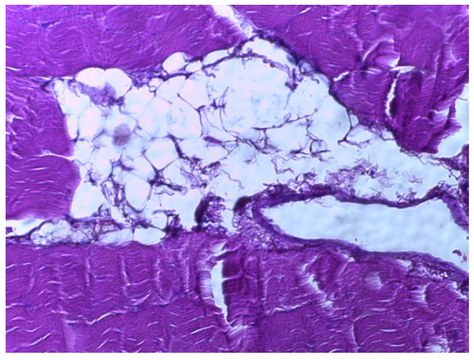

Fig. 5. Perivascular deposition of large fat droplets in the muscles of control broilers. Hematoxylin and eosin staining (200× magnification)

Рис. 5. Периваскулярное отложение крупных капель жира в мышцах контрольных бройлеров. Окраска гематоксилином и эозином (увеличение ×200)

droplets of fat located perivascularly. Larger blood vessels appear inside the muscle, which contributes to better tissue nutrition.

In the samples obtained from birds of the control group, the process of formation and maturation of muscle tissue in both the thoracic and femoral muscle groups had a tendency towards incomplete maturation (Fig. 2) with formation of coarse adipose connective tissue. Adipose tissue, which is located along the intermediate connective tissue, contains a significant amount of fat. Adipocytes are larger, in some areas they are located tightly to each other, forming a continuous layer. Fatty layers with connective tissue sometimes penetrate between small muscle bundles (Fig. 3 and 5).

It should be noted that introduction of betulin-based phytobiotics into the diet promoted the formation of maturer muscle fiber. This kind of meat is the safest for humans and has good nutritional properties.

Betulin had a positive effect on the water-holding capacity of the pectoral muscle fibers, which is an important technological parameter for cooling, freezing or storage processes, as well as for manufacture of sausages and smoked products from broiler meat. The loss of meat juice during heat treatment causes dehydration of tissues, decrease in juiciness, deterioration in the consistency, structure and taste of the finished product. Studies have shown that betulin increased the water-holding capacity of breast muscles in broiler chickens by 5.1% as compared with the control group.

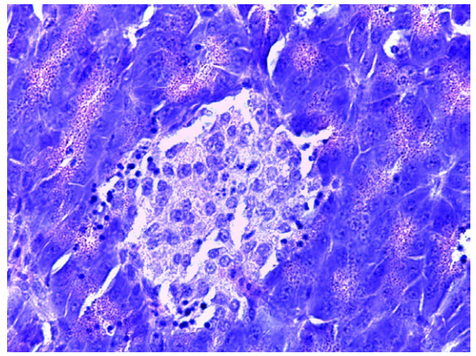

Fig. 6. Pancreas of chickens in the control group. The islets of Langerhans are clearly defined, beta cells are in active secretion. Hematoxylin and eosin staining (400× magnification)

Рис. 6. Поджелудочная железа цыплят контрольной группы. Островки Лангерганса четко очерчены, бета-клетки в состоянии активной секреции. Окраска гематоксилином и эозином (увеличение ×400)

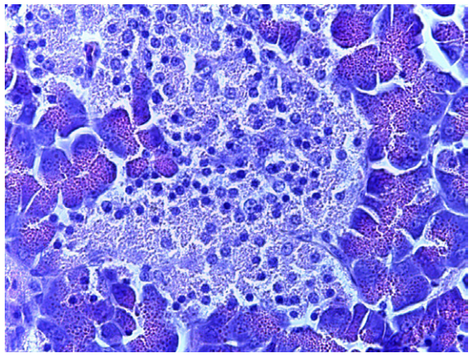

Fig. 7. Pancreas of chickens in the experimental group. The size of the islets of Langerhans has sharply increased. Hematoxylin and eosin staining (400× magnification)

Рис. 7. Поджелудочная железа цыплят опытной группы. Островки Лангерганса резко увеличены в размерах. Окраска гематоксилином и эозином (увеличение ×400)

Additional tests of muscle tissue chemical composition showed increased accumulation of ash elements in the muscle fiber of chickens by 41.9% in the experimental group (1.08 ± 0.16 in the control group, 1.86 ± 0.25 in the experimental group), which also indicates increase in biological value of meat from broiler chicken that received betulin.

Biochemical blood tests showed significant changes in the concentration of uric acid in the control (450.07 ± 35.97 μmol/L) and experimental (230.17 ± 26.78 μmol/L) groups (at $p \leq 0.01$). This is due to blocking the breakdown of purine bases in broilers. The processes of protein synthesis and the formation of muscle fiber proceeded more intensively in chickens in the experimental group.

It was found that betulin had impact on the development and condition of internal organs. Histological studies of the broiler chicken pancreas in the control group showed that the islets of Langerhans were clearly delineated, the beta cells were in active secretion (Fig. 6).

The pancreas of broilers in the experimental group that received the betulin-based feed additive secreted normally, but the islets of Langerhans sharply increased in size (Fig. 7). Active insulin production was recorded. Thus, the increased area of islets of Langerhans (insulin-producing cells) was revealed in the pancreas of broiler chickens. The pancreatic function was activated due to betulin effect.

CONCLUSION

The study showed that the betulin-based feed additive decreased the deposition of subcutaneous and abdominal fat with a slight difference in body weight of broiler chickens; increased the meat biological value due to raised content of ash elements; improved the meat technological properties by increasing the water-holding capacity of muscle fibers; promoted the accumulation of proteins and minerals in meat and the intensity of the formation and maturation of muscle fibers. The increased area of islets of Langerhans (insulin-producing cells) was recorded in the pancreas of birds indicating the pancreatic activation.

The betulin-based feed additive fits perfectly into the broiler production technology and is one of the promising areas for implementation of environmentally friendly technologies in poultry farming.

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Canine parvovirus enteritis: epidemic situation analysis and perspectives

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SUMMARY

Parvovirus enteritis is one of canine dangerous diseases which poses a particular concern for practitioners and dog owners around the world. Parvovirus type 2 (CPV-2) can affect dogs at any age, but puppies between 6 weeks and 6 months old are most susceptible to infection. One of the main biological properties of parvovirus is its continuous genetic evolution, which led to the replacement of the original virus type by new antigenic variants – CPV-2a, CPV-2b and CPV-2c. According to the literature data, all three variants of the virus are currently circulating in the domestic dog population worldwide. The paper presents analysis of the epidemic situation and seasonal occurrence of canine parvovirus enteritis in certain regions of the Russian Federation in 2017–2019. It was shown that parvovirus enteritis was ranked first among the registered infectious diseases of dogs and accounted for 37% during the study period. It has been established that the disease is registered all year round, but the frequency of disease cases depends on the season. Canine parvovirus infection mainly occurs in spring, late autumn and early winter, which is probably associated with changes in daily temperature during these periods and decreased animal resistance. Despite extensive vaccination, the main reason for the wide spread of the virus is either interference with maternal antibodies in vaccinated puppies or low level of immune protection in adult dogs. It has been concluded that it is necessary to monitor the parvovirus circulation and spread in order to study the genetic and antigenic properties of newly identified isolates for the timely update of vaccine strains used for development of specific means of prevention.

Key words: parvovirus (CPV), canine parvovirus enteritis, canine diseases, epidemic situation.

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Парвовирусный энтерит собак: анализ эпизоотической ситуации и перспективы

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РЕЗЮМЕ

Парвовирусный энтерит является одним из опасных заболеваний собак и вызывает определенную обеспокоенность у практикующих врачей и владельцев собак по всему миру. Парвовирус 2-го типа (CPV-2) может поражать собак в любом возрасте, но наиболее подвержены риску заражения щенки в возрасте от 6 недель до 6 месяцев. Одним из основных биологических свойств парвовируса является его непрерывная генетическая эволюция, которая привела к тому, что исходный тип вируса был заменен новыми антигенными вариантами – CPV-2a, CPV-2b и CPV-2c. Согласно литературным данным, в настоящее время все три варианта вируса циркулируют в популяции домашних собак по всему миру. В работе представлен анализ эпизоотической ситуации и сезонности заболеваемости собак парвовирусным энтеритом в 2017–2019 гг. в отдельных округах Российской Федерации. Показано, что среди регистрируемых инфекционных болезней собак парвовирусный энтерит находится на первом месте, его доля за исследуемый период составила 37%. Установлено, что заболевание фиксируется круглогодично, но частота случаев зависит от сезона. Заражение собак парвовирусом в основном происходит весной, поздней осенью и ранней зимой, что, вероятно, связано с перепадами суточной температуры в эти периоды и снижением резистентности организма животных. Несмотря на обширную вакцинацию, основной причиной широкого распространения вируса является либо вмешательство материнских антител у вакцинированных щенков, либо низкая эффективность иммунной защиты у взрослых собак. Сделан вывод о необходимости проведения мониторинга циркуляции и распространения парвовируса с целью изучения генетических и антигенных свойств вновь выявляемых изолятов для своевременного обновления вакцинных штаммов, используемых при создании средств специфической профилактики.

Ключевые слова: парвовирус (CPV), парвовирусный энтерит собак, болезни собак, эпизоотическая ситуация.

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INTRODUCTION

The causative agent of canine parvovirus enteritis is parvovirus type 2 (Canine parvovirus type 2, CPV-2), which belongs to the genus *Protoparvovirus* in the family *Parvoviridae*. It is one of the most dangerous canine intestinal pathogens. CPV-2 is closely related to feline panleukopenia virus and mink enteritis virus. There is also canine parvovirus type 1 (CPV-1, or MVC – minute virus of canines) that was first isolated from dog faeces in 1967. It significantly differs from CPV-2 in its molecular biological and antigenic properties and does not play an important role in canine infectious pathology. Canine parvovirus type 2 was identified in the late 1970s and caused serious outbreaks in dog breeding kennels and shelters worldwide. This virus, like all parvoviruses, replicates in rapidly dividing cells and is very resistant to environmental conditions: it can survive for more than six months in faeces, on contaminated surfaces of objects used for pet care, and bedding. The virus is resistant to fat solvents, such as ether and chloroform, but is inactivated by many detergents and disinfectants (sodium hypochlorite, etc.) [1].

Soon after its emergence CPV-2 underwent genetic evolution giving rise to successively two antigenic variants (CPV-2a and CPV-2b), which gradually replaced the original type. In the late 90s a new antigenic variant of the virus, CPV-2c, was detected in Europe and America. In 1996 a number of additional mutations were described, some of which led to antigenic changes in the virus. The studies of CPV-2 strains carried out in Italy revealed emergence of a virus mutant with an amino acid substitution at position Glu-426 in the VP2 capsid protein. The CPV-2c type quickly spread to other countries and is currently circulating together with CPV-2a and CPV-2b. Since its detection, CPV-2c has also been found in Asia, Europe, North and South America and Africa. Canine parvovirus binds to cells using the cell transferrin receptor (TfR); mutations in the capsid protein VP2 gene contribute to broadening the range of potential virus hosts. Thus, the original CPV-2 strains cause intestinal infection only in dogs, as compared with the CPV-2a/2b isolates, which, under experimental and natural conditions, can infect animals belonging to the Felidae family, and the CPV-2c variants, originally isolated from leopards, affect dogs and cats. The experimental infection of ferrets, minks and cats suggested minor manifestations of parvovirus enteritis clinical signs [2–4]. Despite the antigenic differences in the VP2 protein, all parvovirus serovariants give cross-reactions in hemagglutination assays and neutralization tests using polyclonal sera [1, 5]. Canine parvovirus is undergoing continuous evolution, and new genetic variants of the virus, differing in antigenic characteristics, can affect the susceptibility of young animals to infection during the period when the level of protective maternal antibodies in them lowers to minimum values. This variability in the virus genome can negatively impact the effectiveness of vaccination, although it has recently been shown that dogs immunized with a single live attenuated vaccine based on the original CPV-2 strain were protected from infection with the field virus strain type 2c [2, 3].

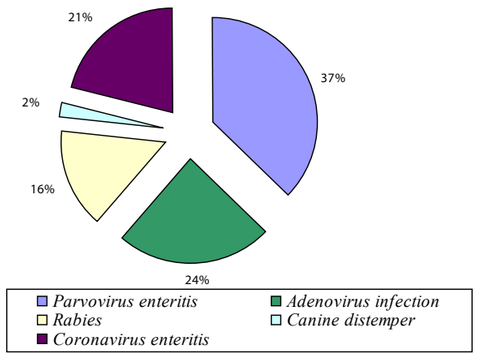

Fig. 1. Morbidity rates for canine infectious diseases in the Russian Federation in 2017–2019

Рис. 1. Заболеваемость собак инфекционными болезнями в Российской Федерации в 2017–2019 гг.

Parvovirus enteritis is a highly contagious viral disease of dogs characterized by vomiting, hemorrhagic gastroenteritis, diarrhea, myocarditis, leukopenia, dehydration, which can cause mortality in animals. The disease is generally acute, death occurs within 2–3 days after onset of clinical signs. The incubation period for natural infection lasts up to 10 days and depends on the organism's resistance and the amount of viral particles entering the gastrointestinal tract of the animal [1, 5, 6]. Parvovirus can affect dogs at any age, but the disease is most severe in puppies between 6 weeks and 6 months old. All dogs are susceptible to this disease, although mixed-breed animals are believed to be less susceptible to infection than purebreds. Dog breeds such as Rottweilers, Doberman Pinschers, English Springer Spaniels, American Pit Bull Terriers and German Shepherds are at risk of parvovirus infection [1, 7, 8].

CPV-2 infection occurs by the alimentary route through oral mucosa after contact with the faeces of infected dogs or contaminated surfaces. The virus enters the body by infecting mainly lymphoid tissues, intestinal epithelium and bone marrow [7]. In newborn puppies the virus causes severe myocarditis which becomes clinically apparent several weeks after infection. Canine parvovirus is also manifested by decreased numbers of leukocytes as a result of inflammation in the bone marrow and lymphoid tissues. After vaccination or inoculation with the field virus strains nearly all adult dogs develop intense immunity to the agent during their lifetime. Infected adult animals have abortive, asymptomatic, persistent and mild clinical forms of the disease. Mortality due to parvovirus enteritis ranges from 30–50%, but in some cases it can reach 100% [3, 4, 8, 9].

Vaccination is the main control method for this disease in domestic animal population. Inactivated and modified live virus vaccines were developed in 1979 being the first products for CPV-2 control. Vaccines provide protective immunity which allow controlling parvovirus infection in dogs. Nevertheless, the virus is still widespread in nature and circulates worldwide. Its genetic evolution and the emergence of new antigenic variants CPV-2a, CPV-2b and CPV-2c cause interest and determine research relevance for many scientists from different countries. This calls for continuous epidemiological surveillance to identify new CPV-2 variants that will help to assess the need to update the currently available canine parvovirus enteritis vaccines.

MATERIALS AND METHODS

The official data of the FGBI "Center for Veterinary Medicine" of the Ministry of Agriculture of the Russian Federation on morbidity rates of canine parvovirus enteritis in some Subjects of the Russian Federation for 2017–2019 were used in the study. The retrospective analysis of the epidemic situation was carried out, the data obtained were provided graphically and cartographically. The data on CPV-2 global distribution used in the study were taken from such bibliographic and reference databases as PubMed, Web of Science, Scopus.

RESULTS AND DISCUSSION

The canine virus disease situation in some regions of the Russian Federation is presented in Figure 1. It shows that parvovirus enteritis was most often registered in 2017–2019 (37%), the morbidity rate for adenovirus infection was 24%, for coronavirus enteritis – 21%, for rabies – 16%, for canine distemper – 2% and for infectious hepatitis – less than 1%.

Analyzing the data obtained over the past three years, it can be noted that parvovirus enteritis is annually recorded in dogs in various regions of the country (Fig. 2–4).

The program of basic comprehensive vaccination in dogs is aimed at formation of immunity to parvovirus, as well as decreased mortality level in the population and a reduction in the risk of the virus spread. However, the circulating field antigenic variants of the virus have completely replaced the original type CPV-2 [3, 4, 7, 10], which is still used in the production of most commercial vaccines,

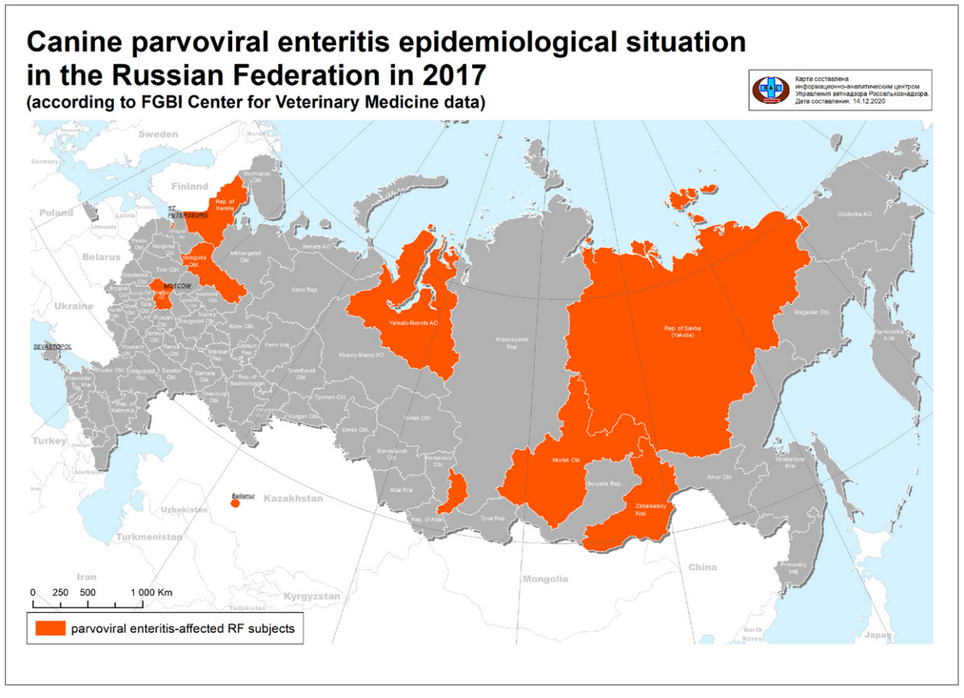

Fig. 2. Distribution of parvovirus enteritis in RF Subjects in 2017

Рис. 2. Распространенность парвовирусного энтерита в субъектах РФ в 2017 г.

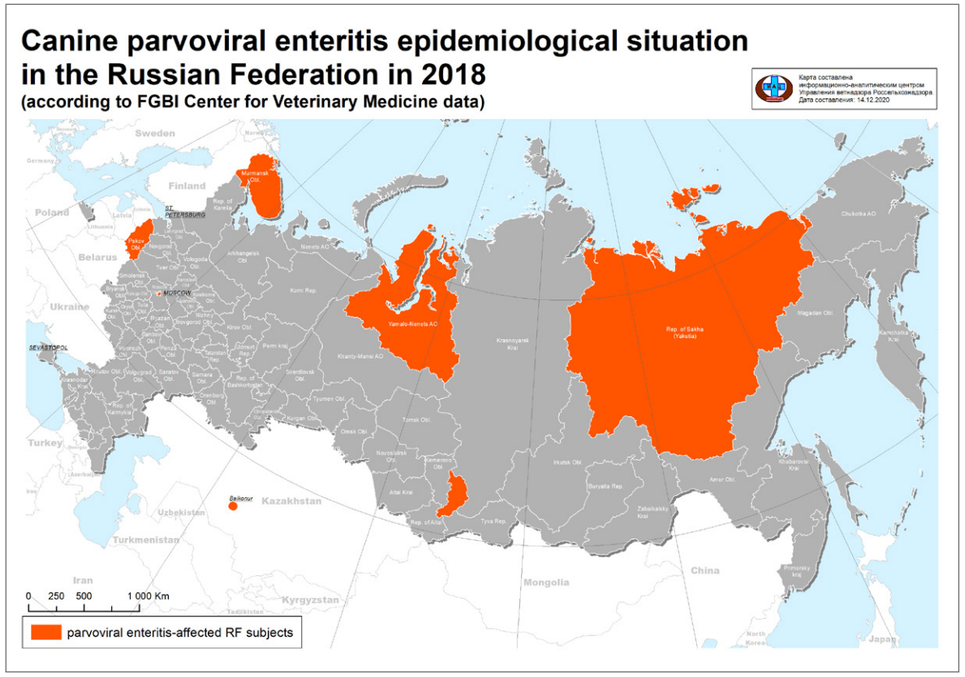

Fig. 3. Distribution of parvovirus enteritis in RF Subjects in 2018

Рис. 3. Распространенность парвовирусного энтерита в субъектах РФ в 2018 г.

Fig. 4. Distribution of parvovirus enteritis in RF Subjects in 2019

Рис. 4. Распространенность парвовирусного энтерита в субъектах РФ в 2019 г.

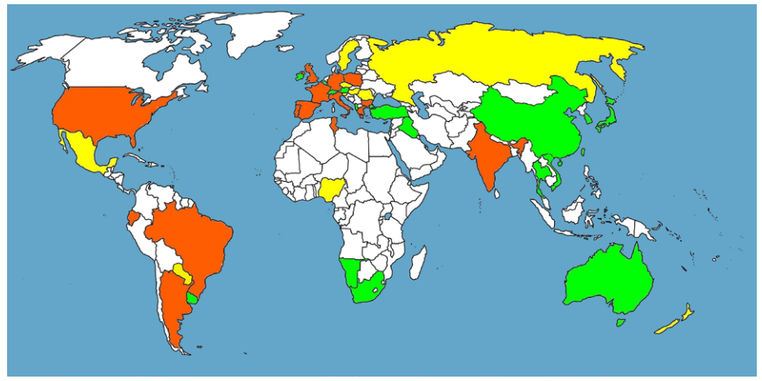

Fig. 5. Global geographic distribution of three CPV-2 variants.
Orange areas – presence of three virus variants; green areas – presence of two out of three virus variants; yellow areas – presence of one out of three virus variants [4] (https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.000540)

Рис. 5. Географическое распределение в мире трех вариантов CPV-2.
Оранжевый – наличие трех вариантов вируса; зеленый – наличие двух из трех вариантов вируса; желтый – наличие одного из трех вариантов вируса [4] (https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.000540)

therefore the issue of efficacy of the presently used vaccines remains open.

According to the literature data on the epidemic situation regarding canine parvovirus infection in the world, CPV-2 (CPV-2a, CPV-2b, CPV-2c) variants have been registered in 42 countries on five continents (Fig. 5).

At present the CPV-2a, CPV-2b and CPV-2c types are unevenly distributed in the world. The co-circulating virus variants are recorded in Continental European countries. Thus, CPV-2a and CPV-2b prevail in France and Belgium, types CPV-2a and CPV-2c – in Italy, type CPV-2a – in Eastern Europe, type CPV-2c – on the Iberian Peninsula, all these three variants of the virus are common in Germany. The circulation of CPV-2b/2c and CPV-2a/2c is recorded in North and South America. The CPV-2a and CPV-2b types prevail in Asia, Great Britain, Australia and Japan. All three types of CPV-2 co-circulate in the north of Africa, while CPV-2a and CPV-2b are more common in the south [4, 10].

Our early studies showed that parvovirus infection rates in dogs were higher in spring and autumn. Perhaps this is due to the increase in the number of stray animals during this period. Another equally important factor is the high resistance of the virus to physical and chemical treatment, as well as the possibility of its preservation in the external environment for up to several months [1, 9, 11].

Analysis of the parvovirus morbidity dynamics in dogs in the Central Federal District of the Russian Federation in 2017–2019 revealed the largest number of diseased animals in the spring and autumn periods (Fig. 6). An increase in the number of dogs with parvovirus enteritis was noted from April to June and from October to December in 2018 and 2019. Figure 6 shows that the peak of animal morbidity in 2019 was registered in late autumn and the first winter month. It has been established that over the past three years the number of reported cases of canine parvovirus enteritis in this region has increased.

Parvovirus enteritis in dogs was recorded in the Northwestern Federal District throughout year 2017. The number of diseased animals increased from March to December, the peak morbidity was recorded in the period

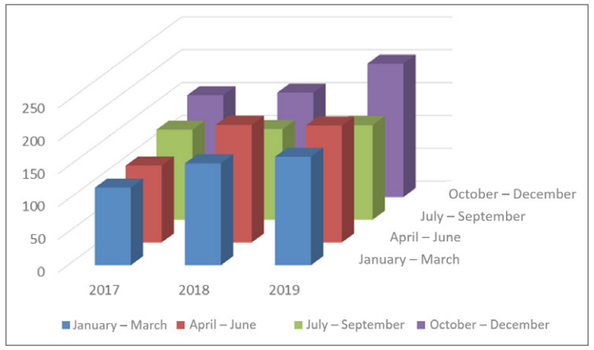

Fig. 6. Morbidity dynamics of canine parvovirus enteritis in the Central Federal District in 2017–2019

Рис. 6. Динамика заболеваемости собак парвовирусным энтеритом в Центральном федеральном округе в 2017–2019 гг.

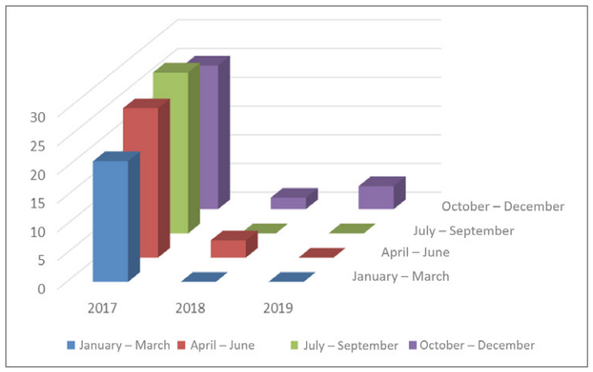

Fig. 7. Morbidity dynamics of canine parvovirus enteritis in the Northwestern Federal District in 2017–2019

Рис. 7. Динамика заболеваемости собак парвовирусным энтеритом в Северо-Западном федеральном округе в 2017–2019 гг.

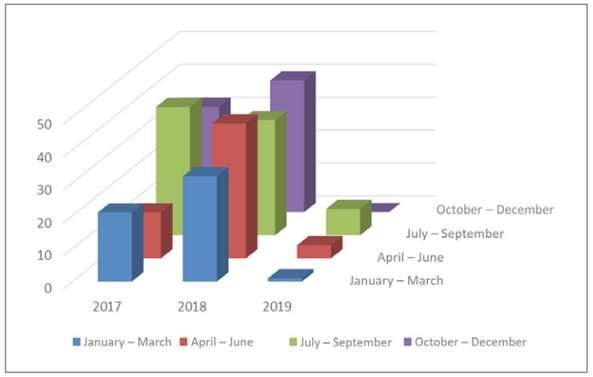

Fig. 8. Morbidity dynamics of canine parvovirus enteritis in the Urals Federal District in 2017–2019

Рис. 8. Динамика заболеваемости собак парвовирусным энтеритом в Уральском федеральном округе в 2017–2019 гг.

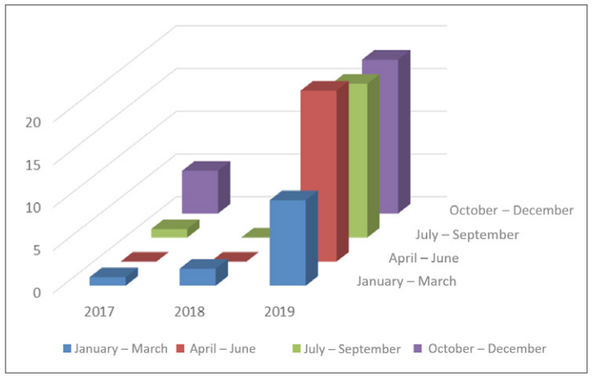

Fig. 9. Morbidity dynamics of canine parvovirus enteritis in the Siberian Federal District in 2017–2019

Рис. 9. Динамика заболеваемости собак парвовирусным энтеритом в Сибирском федеральном округе в 2017–2019 гг.

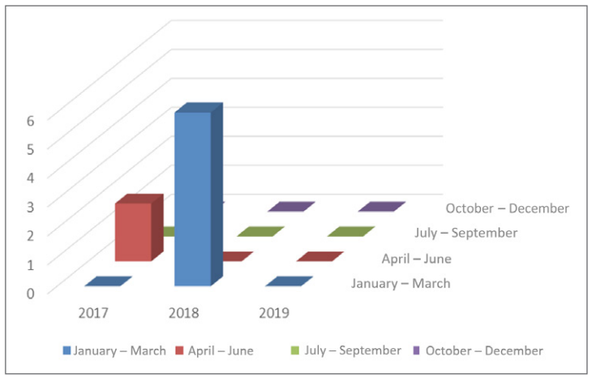

Fig. 10. Morbidity dynamics of canine parvovirus enteritis in the Far Eastern Federal District in 2017–2019

Рис. 10. Динамика заболеваемости собак парвовирусным энтеритом в Дальневосточном федеральном округе в 2017–2019 гг.

of July – September. However, in 2018–2019 there was a decrease in the number of cases of canine parvovirus infection, and single cases were recorded in spring and late autumn (Fig. 7).

Figure 8 shows that the number of dogs with parvovirus enteritis increased from July to December in the Ural Federal District in 2017. The morbidity rate in dogs was high throughout the year 2018, with a peak in spring and autumn. In 2019 single disease cases were reported from April to September.

Single cases of canine parvovirus enteritis occurred in the Siberian Federal District in 2017–2018; and in 2019 the disease was recorded throughout the year, the number of diseased animals increased from March to December (Fig. 9).

Analysis of the parvovirus morbidity dynamics in dogs in the Far Eastern Federal District in 2017 revealed the largest number of diseased animals in the period of April – June, and the peak of morbidity was recorded in January – March 2018. In 2019 canine parvovirus infection was not reported. The analysis showed that for the three years there was a decrease in the number of registered cases of canine parvovirus enteritis in the Far Eastern Federal District (Fig. 10).

The data analysis for Baikonur city over the past three years suggests increase in registered cases of canine parvovirus enteritis. The seasonality of disease cases is clearly demonstrated in Figure 11 showing that its peak was in late autumn 2017 and in summer-autumn 2018. However, an increase in the number of canine parvovirus infection cases was noted in the spring – summer period 2019.

Thus, the analysis of the epidemic situation of canine parvovirus enteritis in some Subjects of the Russian Federation in 2017–2019 shows that the disease was recorded all year round but the frequency of reported cases depended on the season. Canine parvovirus infection occurred more often in spring, late autumn and early winter. This may be associated with significant daily temperature fluctuations and climatic differences during these seasons in some federal districts of the country and, probably, with the decreased level of animals' body resistance thus increasing the risk of infection.

Despite extensive vaccination, the main reasons for the wide spread of the virus remain either interference due to maternal antibodies in vaccinated puppies (the so-called 'window of susceptibility') or low level of immune protection efficacy in adult dogs. Another reason is that the circulating CPV-2 field strains have completely replaced the original type of the virus used for production of most commercial vaccines, which may fail to provide effective cross-protection.

CONCLUSION

The analysis of the scientific study results showed that canine parvovirus type 2 is globally widespread and circulates in the Russian Federation. The estimated data on the epidemic situation with regard to canine parvovirus enteritis over the past three years in some Subjects of Russia indicate a correlation between seasonality and frequency of reported disease cases.

It should be noted that it is necessary to monitor the circulation and spread of parvovirus in order to study the genetic and antigenic properties of newly detected isolates for the timely renewal of vaccine strains used for development of specific prevention means.

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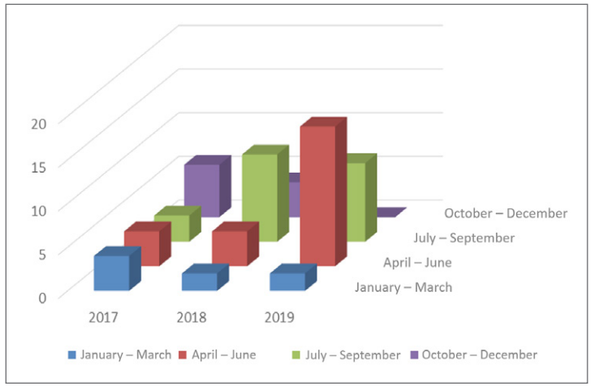

Fig. 11. Morbidity dynamics of canine parvovirus enteritis in Baikonur city in 2017–2019

Рис. 11. Динамика заболеваемости собак парвовирусным энтеритом в г. Байконуре в 2017–2019 гг.

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Determination of fungal genera composition and total toxicity of feed produced in the Republic of Crimea

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SUMMARY

Animal mycotoxicoses caused by ingestion of toxicogenic micromycete-contaminated feed are of major concern for agricultural industry and of great importance for production of agricultural products. In 2017–2019, feed and raw feed materials produced in the Republic of Crimea were tested for mold fungi and genera composition thereof; the feedstuffs were tested for total toxicity by bioassay in rabbits. A total of 252 samples including 124 grain feed samples, 70 forage samples, 58 mixed feed samples were selected for testing. Tests showed that the major detected contaminants were members of the following genera: *Mucor* (67.9%), *Penicillium* (26.6%), *Aspergillus* (13.1%), *Fusarium* (9.1%), *Alternaria* (8.7%), *Stachybotrys* (3.6%) and *Rhizopus* (2.0%). It was revealed that feed were exposed to mold fungi contamination during vegetation and harvesting as well as during transportation and storage. Thus, in spring the feed were more often contaminated with micromycetes of *Penicillium* genus (37.8%) and *Stachybotrys* genus (6.7%); feed collected and tested in autumn were more often contaminated with toxicogenic mold fungi of *Fusarium* genus (14.9%), *Alternaria* genus (13.9%) and *Rhizopus* genus (3.0%); in winter members of *Mucor* genus (78.0%) and *Aspergillus* genus (22.0%) were most often detected in feed. Tests for determination of total toxicity showed that 9 (7.3%) and 10 (8.1%) samples out of 124 tested grain feed samples were low toxic and evidently toxic, respectively. Tests of mixed feed samples for toxicity showed that 5 samples (8.6%) and 2 (3.4%) samples out of 58 mixed feed samples were low toxic and evidently toxic, respectively. It was shown that the proportion of contaminated feed was the highest in spring (25.0%) as compared to proportion of the contaminated feed in winter (18.2%), in autumn (13.7%) and in summer (4.5%).

Key words: fungi, mycological analysis, micromycetes, contamination, total toxicity.

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УДК 619:615.9:636.085

Определение родового состава грибов и общей токсичности кормов, произведенных на территории Республики Крым

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РЕЗЮМЕ

Микотоксикозы животных, причиной которых является употребление контаминированных токсинообразующими микромицетами кормов, представляют серьезную проблему для сельского хозяйства и имеют большое значение при производстве сельскохозяйственной продукции. В период с 2017 по 2019 г. проведен анализ кормов и кормового сырья, произведенных на территории Республики Крым, на содержание и родовой состав плесневых грибов, изучена общая токсичность кормовой продукции методом постановки биопробы на кроликах. Для проведения исследований было отобрано 252 образца, из которых бóльшую часть составили зерновые корма – 124 пробы; 70 проб были представлены грубыми кормами, 58 – комбикормами. В результате проведенных исследований установлено, что основными контаминантами были представители таких родов, как: *Mucor* (67,9%), *Penicillium* (26,6%), *Aspergillus* (13,1%), *Fusarium* (9,1%), *Alternaria* (8,7%), *Stachybotrys* (3,6%) и *Rhizopus* (2,0%). Выявили, что корма подвержены контаминации плесневыми грибами как в период вегетации и уборки, так и во время перевозки и хранения. Так, микромицетами родов *Penicillium* (37,8%) и *Stachybotrys* (6,7%) чаще были контаминированы корма весной; токсинообразующими плесневыми грибами родов *Fusarium* (14,9%), *Alternaria* (13,9%) и *Rhizopus* (3,0%) – корма, отобранные и исследованные осенью; представителей родов *Mucor* (78,0%) и *Aspergillus* (22,0%) наиболее часто выявляли в зимний период. При определении общей токсичности кормов и кормовой продукции установили, что из 124 исследуемых образцов зерновых кормов слаботоксичными были 9 (7,3%), токсичными – 10 проб (8,1%). Из 58 испытуемых образцов комбикорма слабую токсичность проявили 5 проб (8,6%), а выраженную токсичность – 2 образца (3,4%). Показано, что самый высокий процент токсичного корма выявляли в весенний период (25,0%), зимой данный показатель составил 18,2%, осенью – 13,7%, летом – 4,5%.

Ключевые слова: плесневые грибы, микологический анализ, микромицеты, контаминация, общая токсичность.

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INTRODUCTION

Quality of feed becomes very important in the context of animal and poultry farming intensification. Contamination of feed with mold fungi being producers of mycotoxins, low-molecular-weight secondary metabolites of micromycetes, are of particular concern [1, 2]. Mycotoxin diversity, high toxicity and various mycotoxicosis manifestations make the said issue highly significant.

There are currently more than 100,000 various fungi species including more than 250 toxicogenic fungi species [2–4]. There are following mycotoxins: aflatoxins, trichothecene mycotoxins, ochratoxins, fumonisin, zearalenone and its derivatives, ergot alkaloids, cyclopiazonic acid, patulin, citrinine, etc. Ochratoxin A, deoxynivalenol, T-2 toxin, zearalenone and aflatoxin are the most widespread and dangerous [5, 6].

Contamination of feed and feed products with mycotoxins reduces feed nutritional value and contributes to development of various non-infectious diseases – foodborne mycotoxicoses that can be acute or chronic. Disease clinical signs and course depend on the level of feed contamination, animal physiological status and pathogenic characteristics of the fungi contaminating feed products. Acute mycotoxicosis is characterized with neurotoxic symptoms: agitation or depression, fatigue, incoordination, gastrointestinal and cardiovascular disorders, hyporeflexia, convulsions. Chronic mycotoxicosis is characterized with depression, anemia, decrease in weight and reduced performance, abortions [7, 8].

Contamination of feed with mycotoxins at low concentrations also poses a serious threat: after the ingestion by farmed animals they can partially accumulate in tissues and organs and after being metabolized they can enter the products derived from these animals (meat, milk, eggs). Thus, aflatoxin B_1, ingested with the feed transforms in aflatoxin M_1 and is excreted with milk. Therefore, products derived from the animals that have been fed with mycotoxin-contaminated feed are potentially toxic for humans [1, 9].

It is important to note that mycotoxins are characterized by uneven distribution - their concentration in different points of the same batch of feed varies significantly that affects the test results [1].

Failure to observe the requirements for raw feed materials and finished feed harvesting and storage is one of the main causes of the mold fungi growth and, therefore, results in changes in the fungal microflora species and quantitative composition. The mycotoxin contamination level in feed depends on the level of its exposure to toxicogenic mold fungi [10].

According to the Food and Agricultural Organization of the United Nations (FAO), annually, mycotoxins contaminate about 25% of the world grain crop. The majority of countries in the world regulates mycotoxin content in feed and food. In the Russian Federation, maximum allowable levels for mycotoxins are laid down by Technical Regulations of the Customs Union 021/2011 on food safety and 015/2011 on grain safety, GOSTs and TUs for different types of products and feed. According to the literature data, the following mycotoxins are the most often detected in the Russian Federation: deoxynivalenol, T-2 toxin, zearalenone and aflatoxin. There are no documents regulating allowable levels of mold fundi species contamination [11].

Moreover, mycological control for mold fungi exposure allows for detection of toxicogenic micromycete contamination of feed and feed products at early stages. Toxicogenic micromycetes include fungi of the following genera: *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Fusarium*, *Alternaria*, *Helminthosporium*, *Ustilago*, *Tilletia*, *Nigrospora* etc., that can produce mycotoxins under favorable conditions (relative humidity of 85–95%, temperature of 4–30 °C) [3]. Therewith, the mycological analysis is an important step of the feed quality control.

The work was aimed at determination of generic composition of mold fungi contaminating the feed produced in the territory of the Republic of Crimea, examination of dependence of mold fungi exposure level on the season of the year and environmental conditions (temperature, humidity), and determination of total toxicity of feed by bioassay.

MATERIALS AND METHODS

Test materials: samples of feed and mixed feed taken on farms and backyards located in the Republic of Crimea. Samples were collected in accordance with GOST ISO 6497-2014 "Feeding stuffs. Sampling", GOST 13586.3-2015 "Grain. Acceptance rules and sampling methods", GOST 13496.0-80 "Mixed feeding-stuffs. Rules of sampling of average sample".

Reagents and nutrient media: distilled water (GOST 6709-72 "Distilled water. Specifications"), Czapek-Dox and Sabouraud media (OOO "SPC Biokompas-S", Russia), filter paper, formaldehyde, OP-7 surfactant, ammonia, pure acetone for analysis (OOO "KhlorenKhima", Russia), refined sunflower oil (GOST 1129-2013 "Sunflower oil. Specifications").

Laboratory animals: rabbits, live weight: 2.0 to 2.5 kg, having integral non-pigmented skin.

All experiments in animals were carried out in strict accordance with the international standards on laboratory animal keeping and handling laid down in GOST 33216-2014 "Guidelines for accommodation and care of animals. Species-specific provisions for laboratory rodents and rabbits" and GOST 33215-2014 "Guidelines for accommodation and care of animals. Environment, housing and management", adopted by the Interstate Council for Standardization, Metrology and Certification as well as in accordance with Directive of the European Parliament and the Council 2010/63/EU of 22 September 2010 on protection of animals used for scientific purposes.

Sample preparation methods: mycological analysis was performed in accordance with GOST 13496.6-71 "Mixed feed. Method of detachment of fungi" and in accordance with the "Methodical guidelines for sanitary and mycological evaluation of feed and for feed quality improvement" (approved by the USSR Ministry of Agriculture on February 25, 1985). The test materials were seeded in agar media-containing Petri dishes for mold fungi cultivation. Tests were carried out in quintuplicate. The seeds in Petri dishes were kept in thermostat at 25 °C for 10 days. The Petri dishes were examined at day 3, 5, 7 and 10 of incubation [12, 13].

The thermostat and isolation room were tested for cleaning quality and sterility, respectively, in accordance with the "Methodical guidelines for disinfection, pre-sterilization cleaning and sterilization of medical devices" [14]. Before test material seeding, two Petri dishes with the agar medium were placed on the laboratory bench and two Petri dishes with the agar medium were placed in thermostat. The said Petri dishes were opened and left for 15 minutes, then, they were placed in thermostat and kept similarly to the seeded material.

Grain feed was tested for external and internal spore contamination. To test grains for external spore contamination, the test materials were seeded onto Sabouraud medium in Petri dishes, 10 grain kernels per dish, so as the kernels did not touch each other.

To test grain for internal spore contamination the grain kernels were treated with 3% formaldehyde solution (treatment period – 2 minutes) and washed once with 0.2% ammonium solution and then with distilled water after their treatment; the treated kernels were placed onto Sabouraud agar in Petri dishes, 10 grain kernels per dish. Five Petri dishes were used for each test.

Forage feed was chopped into 2 cm-long pieces in a sterile Petri dish. Then, the pieces were transferred with a sterile forceps onto Czapek-Dox agar in Petri dishes (10 forage pieces per dish, 5 dishes) [13].

Granulated mixed feed was ground with LZM-1 laboratory mill; 10 g of the feed were taken and poured with 100 ml of 0.1% aqueous OP-7 surfactant solution to prepare primary suspension. The following suspension dilutions were prepared using serial dilution method: 1:1 000 dilution – for good-quality feed and 1:10 000 dilution – for the feed with spoilage signs (macroscopic signs of mold fungi contamination, characteristic odour, etc.). The 1:1 000 dilution and 1:10 000 dilution were seeded onto 5 and 8 Petri dishes containing agar medium, respectively [12, 13].

Generic classification of fungi was performed based on morphological characteristics of the colonies, mycelia and sporangium structure [15].

Determination of total toxicity of feed. Test feed samples were ground and sifted through a 1 mm-pore-size sieve for tests for total toxicity. Ground feed sample (50 g) was transferred to 500 cm^3 conical flask with tight stopper and 150 cm^3 of acetone were added. The flask was placed on orbital shaker and the extraction was performed for 3 hours. Resulting extract was filtered through paper filter and transferred to the cup for evaporation. It was concentrated up to complete solvent odour removal and to oily residual mass. Vegetable oil was added to make the sample volume to 1 cm^3 when amount of the extract after evaporation was not sufficient for testing [13].

Bioassay was performed in rabbits that have integral non-pigmented non-scaly skin. Vegetable oil used for the extract dissolution was preliminary tested for its toxicity in rabbits by applying it twice on the sheared skin area at an 24-hour interval. Skin reactions were recorded on the next day after second application and then for 3 days. The vegetable oil that had not induce any redness of the rabbit epidermis was considered suitable for use.

Then, one half of the extract was applied to 6 × 6 cm preliminary sheared skin area of rabbit thigh or shoulder with glass spatula by gentle rubbing. The second half of the extract was applied on the next day. The oily extract of the test feed was kept in refrigerating chamber before the second application. The skin reactions were recorded on the next day after the second extract application and the rabbits were observed for the next 3 days.

One 6 × 6 cm sheared skin area was used as a control one. No extract was applied to this area. One rabbit was used for maximum four simultaneous bioassays.

Results of tests of feed for their toxicity were evaluated based on inflammatory reaction presence or absence. The feed sample was considered toxic when apparent hyperemia, soreness, swelling, manifested by a strong thickening of the skin as well as ulcers or solid scab were observed on the skin area in rabbits [13].

RESULTS AND DISCUSSION

At first stage, the level of feed contamination with micromycetes and generic composition of mold fungi contaminating feedstuffs harvested on the territory of the Republic of Crimea were determined.

Table 1
Characterization of tested feed samples

Таблица 1
Характеристика исследуемых образцов корма

Sampling period	Average weight of collected sample	Number of collected samples	Sample type
Winter	2.0 kg	23	grain feed (barley, wheat, oat)
		17	forage (hay, straw)
		10	mixed feed
Spring	2.0 kg	22	grain feed (barley, wheat, oat)
		13	forage (hay, straw)
		10	mixed feed
Summer	2.0 kg	30	grain feed (barley, wheat, oat)
		12	forage (hay, straw)
		14	mixed feed
Autumn	2.0 kg	49	grain feed (barley, wheat, oat)
		28	forage (hay, straw)
		24	mixed feed

Tests were performed in the FGBI "ARRIAH" Branch located in the Republic of Crimea in 2017–2019.

Feed and feed product samples were collected in backyards and on farms located in the Belogorsky, Krasnogvardeysky, Leninsky and Saksky Raions of the Republic of Crimea. Average sample weight was 2.0 kg (for each sample taken separately). Numbers of collected samples and sampling periods are given in Table 1.

A total of 252 feed samples including 124 grain feed samples, 70 forage samples and 58 mixed feed samples were collected for tests of the feedstuffs harvested in the Republic of Crimea for toxicogenic mold fungi and for fungal generic composition. Feed samples were collected and tested in different seasons of the year to determine dependence of the level of feed contamination with mold fungi on environmental conditions (air temperature, relative humidity).

Mold fungi were differentiated and classified to genera based on the grown colonies morphology; mycelium and sporangium structures were examined by light microscopy.

Results of tests of mold fungi contaminating the feedstuffs harvested in the territory of the Republic of Crimea are shown in Table 2.

Micromycetes of the following genera were detected during feed sample tests for determination of contaminating fungi generic composition: *Mucor* – in 171 samples (67.9%), *Penicillium* – in 67 samples (26.6%), *Aspergillus* – in 33 samples (13.1%), *Fusarium* – in 23 samples (9.1%), *Alternaria* – in 22 samples (8.7%), *Stachybotrys* – in 9 samples (3.6%), *Rhizopus* – in 5 samples (2.0%).

A total of 50 feed samples were collected in winter period. Mold fungi of the following genera were detected during mycological tests of the said samples: mold fungi of *Mucor* genus were detected in 39 samples (78.0%), *Aspergillus* – in 11 samples (22.0%), *Penicillium* – in 7 samples (14.0%), *Fusarium* – in 6 samples (12.0%), *Alternaria* – in 5 samples (10.0%), *Stachybotrys* – in 2 samples (4.0%), *Rhizopus* – in one sample (2.0%).

In spring 45 feed samples were collected and tested, mold fungi of the following genera were detected: mold fungi of *Mucor* genus were detected in 33 samples (73.3%), *Penicillium* – in 17 samples (37.8%), *Aspergillus* – in 9 samples (20.0%). Micromycetes of *Alternaria* genus and *Stachybotrys* genus were detected in 3 (6.7%) and 3 samples (6.7%), respectively. Mycromycetes of *Rhizopus* genus were detected in 1 sample (2.2%) out of the tested feed samples.

In summer, 56 feed samples were collected for testing. The feed samples were found to be contaminated with the mold fungi of the following genera: mold fungi of *Mucor* genus were detected in 43 samples (76.8%), *Penicillium* – in 20 samples (35.7%), *Aspergillus* and *Fusarium* genera – in 2 samples (3.6%) and 2 samples (3.6%), respectively. Mold fungi of *Stachybotrys* genus were detected in one sample (1.8%).

In autumn, 101 feed samples were collected and tested, 56 (55.4%) out of them were found to be contaminated

Table 2
Generic diversity of the fungi contaminating tested feed

Таблица 2
Родовое разнообразие грибов, контаминирующих исследуемые корма

Genus of detected micromycete	Sample type	Number of contaminated feed samples collected in different seasons of the year				
		winter	spring	summer	autumn	total
Mucor	grain feed	18	16	22	23	79
	forage	13	10	9	17	49
	mixed feed	8	7	12	16	43
Penicillium	grain feed	4	9	14	12	39
	forage	1	2	2	6	11
	mixed feed	2	6	4	5	17
Alternaria	grain feed	2	3	0	5	10
	forage	0	0	0	4	4
	mixed feed	3	0	0	5	8
Aspergillus	grain feed	4	6	2	5	17
	forage	4	3	0	3	10
	mixed feed	3	0	0	3	6
Stachybotrys	grain feed	1	1	1	0	3
	forage	1	2	0	3	6
	mixed feed	0	0	0	0	0
Fusarium	grain feed	6	0	2	10	18
	forage	0	0	0	2	2
	mixed feed	0	0	0	3	3
Rhizopus	grain feed	0	0	0	0	0
	forage	1	0	0	1	2
	mixed feed	0	1	0	2	3

with fungi of *Mucor* genus, 23 samples (22.8%) – with *Penicillium,* 15 samples (14.9%) – *Fusarium*. Fungi of *Alternaria* genus were detected in 14 samples (13.9%), *Aspergillus* genus – in 11 samples (10.9%); micromycetes of *Stachybotrys* genus and *Rhizopus* genus were detected in 3 (3.0%) and 3 (3.0%) samples, respectively.

According to the literature data, fungi of *Rhizopus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Aspergillus*, *Fusarium*, *Mucor*, *Trichoderma*, *Stachybotrys* genera and others also play dominating role in contamination of plant feed in the Russian Federation and in European countries; their genera and species diversity depend on the environmental climatic conditions [3, 16, 17].

The tests have shown that feedstuffs are exposed to mold fungi both in vegetation and harvesting periods as well as during their transportation and storage. Some toxicogenic micromycetes rapidly grow and multiply at temperatures below 20 °C, the other ones – at 30–50 °C. Favorable conditions for phytopathogenic micromycetes propagation at the stage of feed crop growing and failure

Table 3
Results of tests of feed for total toxicity

Таблица 3
Результаты исследований общей токсичности кормов

Season	Toxicity of feed samples					
	grain feed			mixed feed		
	non-toxic	low toxic	toxic	non-toxic	low toxic	toxic
Winter	18	2	3	9	1	0
Spring	16	2	4	8	1	1
Summer	29	1	0	13	1	0
Autumn	42	4	3	21	2	1

to comply with the feed harvesting technology can result in mycotoxin accumulation in the products. Degree of feed exposure to toxicogenic micromycetes directly influences the level of feed contamination with mycotoxins and, as a result, agricultural product safety and probability of mycotoxicoses in animals.

Analysis of the test results showed that fungal generic composition in feed changes depending on the season of the year. Thus, in spring the feed was more often contaminated with micromycetes of *Penicillium* genus (37.8%) and *Stachybotrys* genus (6.7%); in autumn, the feed was more often contaminated with toxicogenic mold fungi of *Fusarium* genus (14.9%), *Alternaria* genus (13.9%) and *Rhizopus* genus (3.0%); members of *Mucor* genus (78.0%) and *Aspergillus* genus (22.0%) were the most often detected in winter.

Taking into account the direct influence of the degree of exposure of the feed to toxicogenic mold fungi on the level of contamination of feed with mycotoxins, the next stage of the work was to determine the total toxicity of samples of micromycete-contaminated feed and feed products.

A total of 124 grain feed samples and 58 mixed feed samples were tested for toxicity by bioassay in rabbits. Feed toxicity was assessed based on presence or absence of inflammatory reaction in rabbits and the tested feed samples were classified into three categories: toxic, low toxic and non-toxic [13]. The feed sample was considered toxic when apparent hyperemia, soreness, swelling manifested by a strong thickening of the skin as well as ulcers or solid scab were observed on the rabbit skin area pre-treated with the said feed extract. The extract of low toxic feed caused hyperemia that lasted for 2–3 days, skin exfoliation, soreness and swelling manifested by slight thickening of the skin followed by formation of discrete crusts. The feed sample was considered non-toxic when inflammatory reaction was absent or hyperemia was observed for maximum 2 days and no skin exfoliation was observed after treatment with the feed extract.

Results of tests of feed for total toxicity are given in Table 3.

Tests of 124 grain feed samples for their toxicity showed that 9 samples (7.3%) out of them were low toxic, 10 samples (8.1%) were toxic. Tests of 58 mixed feed samples showed that 5 samples (8.6%) out of them were low toxic, 2 samples (3.4%) were evidently toxic.

In winter 33 samples were tested: 3 grain feed samples (9.1%) were considered toxic and 2 grain feed samples (6.1%) and 1 mixed feed sample (3.0%) were considered low toxic.

In spring 32 samples were tested, 4 grain feed samples (12.5%) and 1 mixed feed sample (3.1%), were found toxic and 2 grain feed samples (6.3%) and 1 mixed feed sample (3.1%) were found low toxic.

In summer 44 samples were tested: 1 grain feed sample (2.3%) and 1 mixed feed sample (2.3%) were found low toxic.

In autumn 73 samples were tested: 3 grain feed samples (4.1%) and 1 mixed feed sample (1.4%) were considered toxic, 4 grain feed samples (5.5%) and 2 mixed feed samples (2.7%) were considered low toxic.

The figure shows that the proportion of the feed found contaminated was the highest in spring (15.6%).

Feed and feed products collected and tested in all seasons were found toxic to various degree. Thus, 4 samples (5.5%) were found toxic and 6 samples (8.2%) were found low toxic out of 73 samples collected and tested in autumn. Three samples (9.1%) were found toxic and 3 samples (9.1%) were found low toxic out of 33 samples collected and tested in winter. Five samples (15.6%) were found toxic and 3 samples (9.4%) were found low toxic out of 32 samples collected and tested in spring. No toxic samples were found and only 2 samples (4.5%) were found low toxic out of 44 samples collected and tested in summer.

Feed and feed products considered low toxic and toxic based on the laboratory test results are not allowed for feeding livestock animals [18].

CONCLUSION

Tests showed that fungal genera composition changes depending on the season of the year. Thus, feedstuffs were more often contaminated with micromycetes of *Penicillium* genus (37.8%) and *Stachybotrys* genus (6.7%) in spring; with toxicogenic mold fungi of *Fusarium* genus (14.9%), *Alternaria* genus (13.9%) and *Rhizopus* genus (3.0%)

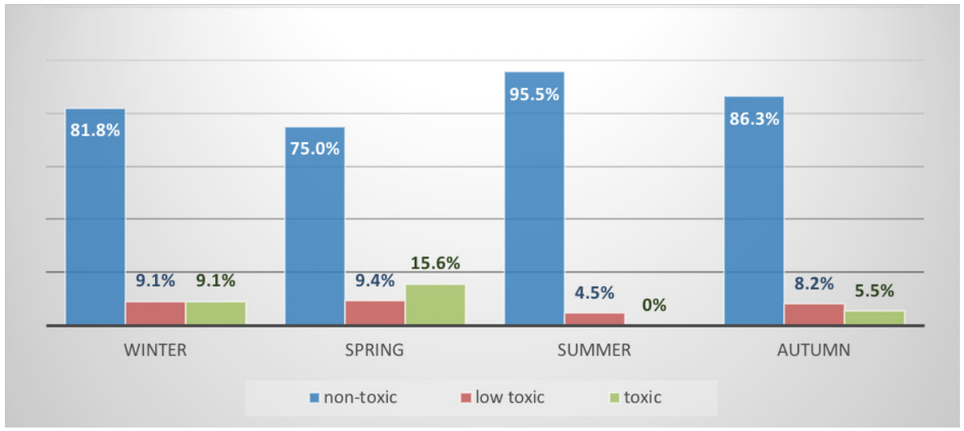

Fig. Total toxicity of feed and feed products in different seasons of the year

Рис. Общая токсичность кормов и кормовой продукции в зависимости от сезона года

in autumn; with fungi of *Mucor* genus (78.0%) and *Aspergillus* genus (22.0%) in winter.

Tests of feed for total toxicity showed that 9 samples (7.3%) were low toxic and 10 samples (8.1%) were low toxic out of tested 124 grain feed samples. Five samples (8.6%) were considered low toxic and 2 samples (3.4%) were considered toxic out of tested 58 mixed feed samples.

Low toxic feed samples were detected during all four seasons: in summer (4.5%), autumn (8.2%), winter (9.1%) and spring (9.4%). Toxic feed samples were detected in autumn (5.5%), winter (9.1%) and spring (15.6%). No toxic feed samples were detected in 44 feed samples collected and tested in summer.

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Preparation of rabbit kidney immortalized cell culture

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SUMMARY
Preparation of immortalized cell lines obtained from organs and tissues of farm animals is an essential area of biotechnology. The paper presents results of continuous (immortalized) cell line preparation from a primary trypsinized cell culture of an adult rabbit kidney. Cytomorphologic analysis and karyotyping were performed during the process of subcultivation in the cell culture at passages 1, 3, 24, 31, 38, 56, 66, 75, 86, 101. Dynamics of spontaneous continuous cell line formation during long-term serial passaging was examined using standard nutrient media and fetal serum. Contrary to the known cell lines of rabbit origin (*Oryctolagus cuniculus* L.), immortalization was not accompanied with enhanced cell production and cell size reduction. The prepared continuous cell line in its adhesive phase was up to 200 μm in size and its productivity was about 7,000 cells/cm^2. Significant differences (compared to the known cell lines) in the karyotype were detected during passaging. The formed genotype was found to be near-tetraploid when the CCL cultural properties were stabilized at passages 66–101. The known cell lines – rabbit kidney (RK-13) and rabbit cornea (SIRC) – can be characterized as pseudotriploid basing on their karyotype. This culture demonstrated low sensitivity to viruses – causative agents of rabbit diseases and sensitivity to heterologous porcine and bovine viruses.

Key words: continuous cell line, primary trypsynized cell culture, immortalization, apoptosis, telomere.

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Получение иммортализованной культуры клеток почки кролика

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РЕЗЮМЕ
Получение иммортализованных клеточных линий, происходящих из органов и тканей сельскохозяйственных животных, является актуальным направлением биотехнологии. В статье представлены результаты получения постоянной (иммортализованной) клеточной линии из первично трипсинизированной культуры клеток почки взрослого кролика. В процессе субкультивирования проводили цитоморфологические и кариологические исследования на 1, 3, 24, 31, 38, 56, 66, 75, 86, 101-м пассажах. При проведении длительных последовательных пассажей была прослежена динамика спонтанного формирования постоянной клеточной линии с использованием стандартных питательных сред и эмбриональной сыворотки. В отличие от известных клеточных линий, произошедших от обыкновенного домашнего кролика (*Oryctolagus cuniculus* L.), было отмечено, что иммортализация не сопровождалась увеличением продуктивности клеток и уменьшением их размеров. Размеры клеток полученной перевиваемой линии достигали в адгезированном состоянии 200 микрометров, продуктивность составляла 7000 клеток на квадратный сантиметр. Значительные отличия (в сравнении с известными линиями) в процессе пассирования обнаруживались и в кариотипе. При стабилизации культуральных свойств постоянной линии клеток на 66–101-м пассажах генотип сформировался как околотетраплоидный. Известные клеточные линии – почка кролика (RK-13) и роговица глаза кролика (SIRC) – по кариотипу оказались псевдотриплоидные. Данная культура оказалась малочувствительной к вирусам – возбудителям болезней кроликов и чувствительной к гетерологичным вирусам свиней и крупного рогатого скота.

Ключевые слова: постоянная линия клеток, первично трипсинизированная культура, иммортализация, апоптоз, теломеры.

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INTRODUCTION
Immortalization of subcultured cells of mammalian organs can be considered as a shift away from terminal proliferation arrest which is eventually observed in all normal somatic cells. In early 60s of the last century L. Hayflick and P. S. Moorhead discovered that normal diploid human fibroblasts proliferate *in vitro* only a certain number of times and subsequently they stop dividing and start aging [1–3]. Given that the process of aging clearly differs from any form of a cell's death, for instance, apoptosis, it is essential to distinguish these statuses during successive passages. Studies, performed by researchers in 1990s demonstrated that immortalization is associated with activation of telomere maintenance mechanism via telomerase activity or other unknown process [2, 4]. Immortalization is a complex process associated with many genetic modifications a part of which may not be related to telomere maintenance [4]. There is evidence for existence of three basic compensatory mechanisms of maintaining viability and stimulating cell line proliferation in case of the genetic material (telomere) loss: polyploidization of the initial cell clone, oncogene amplification, and amplification of whole autosomes or their fragments.

Cell culture specialists know only a small amount of stable and high performance continuous cell lines (CCL) of rabbit origin (*Oryctolagus cuniculus* L.). The most widespread lines are: RK-13 (rabbit kidney, obtained in 1963), SIRC (rabbit cornea, obtained in 1965), RSK (rabbit skin). Methods of karyotyping used for identification of the RSC continuous cell line obtained from the VIEV revealed that it belonged to porcine cell lines [5]. The rest cell lines from the Catalogue of the Russian Cell Culture Collection (RCCC), split ratio 1:2, have the status of subcultures [6]. Preparation of non-malignant continuous (immortalized) mammalian cell lines, including those from rabbit organs, is not performed by stereotypical manipulations. Stable CCL develop spontaneously: through long-term passaging in stable conditions or through changing cultivation conditions – change of media, sera and other components.

Organs of newborn animals or their embryos are used for long-term passaging of mammalian cells. The status of these cells is indicative of their longer mitotic activity *in vitro*.

The aim of the research was preparation of the primary cell culture, sensitive to rabbit viruses, from a kidney of an adult animal, maintaining of the subculture during long-term successive passages, and studying the degree of the prepared cell line transformation.

MATERIALS AND METHODS
Kidneys of a 4–5 month old rabbit weighing 2.0–2.5 kg were taken for the test. The primary cell culture was prepared using a modified method of fractional trypsinization [7–9]. Following testing of primary and subcultured cells for sensitivity to rabbit viruses, the cells were subcultured on DMEM/F-12 (pH 7.1–7.2) with 0.1% lactalbumin hydrolysate, containing 5–10% bovine fetal serum [10]. Subsequently the subculture was adapted to the MEM-like medium.

Rabbit kidney cells were cultured in a thermostat according to the generally accepted method in glass and plastic vessels with different growth areas at (38.0 ± 0.5) °C.

Cell morphology tests. Morphology of native cells from the primary trypsinized culture was studied using a phase-contrast microscopy technique.

For cell culture identification P. S. Moorhead's method for formed metaphase plate karyotyping (1960) was used.

Lifetime cytochemical detection of mycoplasma contamination and cell morphology studies were performed using 0.001% acridine orange dye.

Comparative studies of two stable widespread cell lines of rabbit origin: RK-13 – rabbit kidney and SIRC – rabbit cornea cells, were performed.

RESULTS AND DISCUSSION
Any process of primary cell subcultivation includes long-term continuous passaging of the prepared populations [11]. In this respect monitoring of cytogenetic and cultural characteristics of cells derived from an adult rabbit kidney was performed. For this purpose subculturing was performed twice a week starting with passage 4, split ratio 1:2, 1:3. Herewith, the monolayer confluence was 100%, cells remained large – up to 50 μm in diameter [10]. Besides DMEM/F-12 MEM-like (1/3) was added to the culture medium starting with passage 4, and starting with passage 45 the culture was grown on MEM-like medium with 10% bovine fetal serum (Germany).

Cytomorphologic analysis and karyotyping were performed during the process of subcultivation in the cell culture at passages 1, 3, 24, 31, 38, 56, 66, 75, 86, 101. The cells were subject to cryopreservation at approximately the same passages and afterwards their ability to revive was observed. Vials with cells derived from an adult rabbit kidney (RK) concentrated to the density of (2.0–5.0) × 10^6 cells/ml in the fresh growth medium with 10% dimethylsulphoxide, were frozen using a programmable freezer and stored in liquid nitrogen. The cells were thawed by intense agitation in a water bath at 37–38 °C. The survival rate was 80–90%. The optimal level of the cell culture proliferative activity observed prior to freezing was achieved already at passage 2 after thawing (Fig. 1).

The level and dynamics of proliferation have become stable by passage 38. By the same passage population

polymorphism decline was observed. In contrast to the primary subcultured populations epithelioid and spindle-shaped cells began to prevail (Fig. 2). In case the culture was kept over three days pseudo-syncytia (Fig. 3) were formed which hindered examination and studying of cytoplasmic structure. Cells grown in culture vessels of different volume had an equal morphological status.

Micrometry of the RK confluent monolayer was performed at passage 101. Figure 4 shows a micrometer grid used for cell size measuring (minimal cell size – 10 μm). The average size of epithelioid cells was 30–50 μm. The size of spindle-shaped cells reached 100 μm. During pseudosyncytium formation the number of cells did not increase, the cells remained alive for up to ten days at 37 °C, pH did not demonstrate any critical change and was about 6.9–7.0.

As seen from the Table the productivity of the subculture and the forming continuous cell line was not very high. Growth rate on the flat surfaces (300 cm^2) reached 4.1 within four days and 1.7 – in rotating vessels (800 cm^2) within three days. When in 4 hours the seeding concentration was 40,000 cells/ml adherent cells covered the entire surface of the culture vessel. In 72 hours the monolayer became dense and the productivity increased up to 100,000 cells/ml.

Cell micrometry data correlated with the productivity of the prepared cell line which did not exceed 130,000/cm^2.

The prepared cell culture was used in different FGBI "ARRIAH" units for virological testing. It turned out to be sensitive to infectious bovine rhinotracheitis, classical swine fever, and Aujeszky's disease viruses. Cytopathic effect was detected in the RK cell culture after the first passage of the myxomatosis virus. The decrease of the virus cytopathic effect (CPE) was observed during subsequent passaging. CPE of the rabbit haemorrhagic disease virus was not observed in RK cell culture.

The main objective of the research was not achieved as the prepared cell line had low sensitivity to the viruses – agents of rabbit diseases. Although many heterologous viruses reproduced effectively with titer increase at passages. That is why studies of cytological and karyotypic changes in the new continuous cell line were continued.

Cytokaryotypic transformation. The studies of cytokaryotypic signs of the most common CCLs derived from rabbit organs and tissues – RK-13 (rabbit kidney) and SIRC (rabbit cornea) – revealed that both of these cultures have a near-triploid karyotype (Fig. 5, 6) and a distinct cell morphology with a predominance of epithelial-like cells in RK-13 (Fig. 7) and spindle-shaped cells in SIRC (Fig. 8).

In appropriate cultivation conditions the SIRC cell line produces extracellular matrix (presumably, of protein origin) which aggregates cells during trypsinization and monolayer formation. In this case one of the reasons for continuous cell line formation derived from different rabbit organs is gene amplification by 40–47% due to autosome extracopying. Probably this is the reason for telomerase activity increase and formation of stable cell line immortalization.

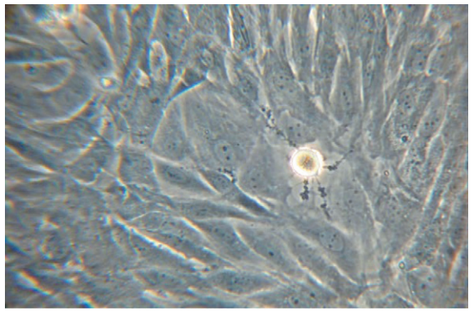

Fig. 1. Morphology of RK cells, passage 2
Рис. 1. Морфология клеток ПКр, 2-й пассаж

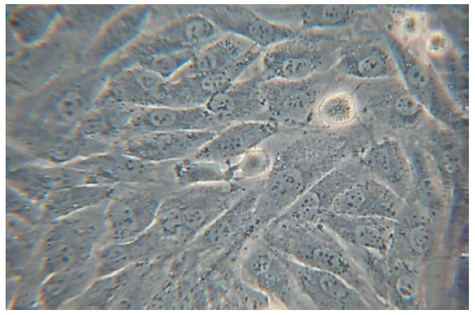

Fig. 2. Morphology of RK cells, passage 38–56
Рис. 2. Морфология клеток ПКр, 38–56-й пассаж

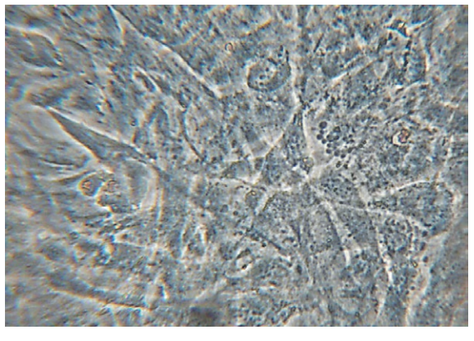

Fig. 3. RK pseudosyncytium
Рис. 3. Псевдосинцитий ПКр

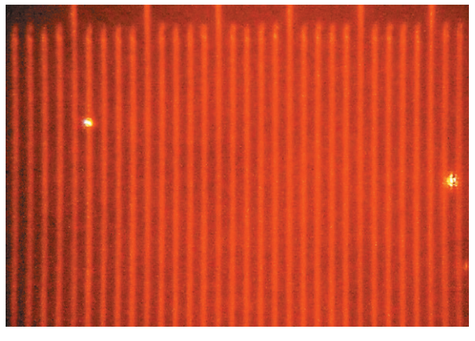

Fig. 4. Micrometer grid, cell size – 10 μm
Рис. 4. Микрометрическая сетка, размер одного деления 10 мкм

Table
Dynamics of the RK continuous cell line proliferation in different culture media

Таблица
Динамика пролиферации постоянной линии клеток ПКр в разных культуральных сосудах

Conditions for cell growth on a monolayer	Seeding concentration, mln/cm^3	Cultivation time, days	Cell productivity, mln	Cell productivity, 1 cm^2	Growth rate
Glass culture vessel (cultivation flask), volume 1,500 ml (300 cm^2)	0.02	4	25.0 ± 0.2	83,000	4.1
	0.04	3	30.0 ± 0.2	100,000	2.5
	0.05	3	40.0 ± 0.1	130,000	2.6
Rotating vessel, volume 3,000 ml (800 cm^2)	0.07	3	50.0 ± 0.2	62,000	1.7
	0.10	3	70.0 ± 0.2	87,000	1.7

Near-triploid chromosome set in comparison with normal, diploid karyotype is a consistent and specific sign of a long-term passaging of RK-13 and SIRC continuous cell lines. Split ratio of these "old" CCLs reaches 1:4, 1:8.

Immortalization of cell lines considerably changes cytomorphological and physiological properties of tissue-derived primary cells. This particularly affects the CCL sensitivity to viruses. Very often continuous cell lines become non-sensitive to homologous viruses and sensitive to heterologous viruses.

Thus, RK-13 cells are sensitive to African horse sickness, swine vesicular disease, cowpox and at the same time to myxomatosis and rabbit pox viruses. SIRC CCL is used for roseola virus reproduction.

As previously noted the prepared RK cell line exhibited immortalization signs starting from passage 30. Performed karyotyping analysis demonstrated the tendency for RK cell hyperdiploidization starting from passage 38 (Fig. 9). Starting from passage 56 near-tetraploid population prevalence was observed (Fig. 10). The performed karyotyping

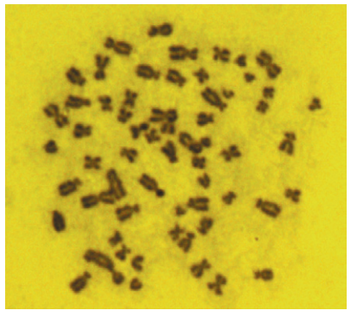

Fig. 5. RK-13 metaphase (62 chromosomes)
Рис. 5. Метафаза RK-13 (62 хромосомы)

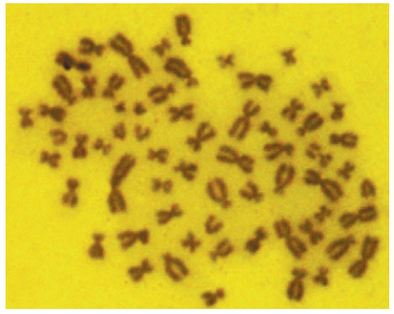

Fig. 6. SIRC metaphase (65 chromosomes)
Рис. 6. Метафаза SIRC (65 хромосом)

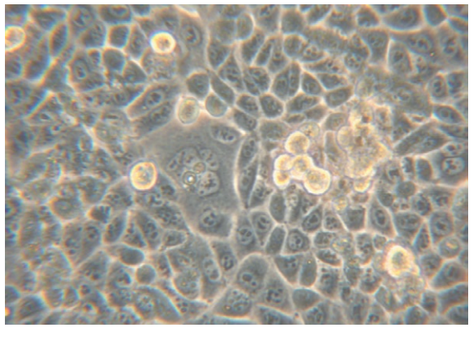

Fig. 7. RK-13 CCL morphology
Рис. 7. Морфология ПЛК RK-13

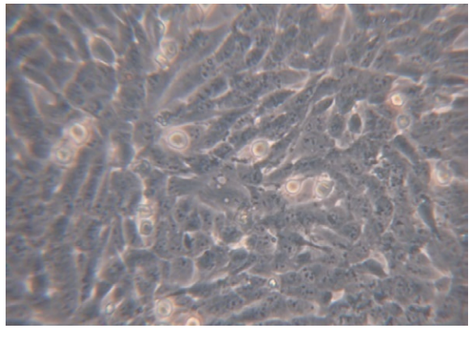

Fig. 8. SIRC CCL morphology
Рис. 8. Морфология ПЛК SIRC

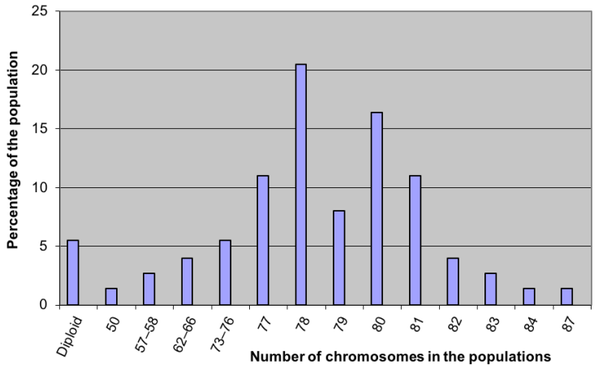

Fig. 9. RK cell line ideogram, passage 38

Рис. 9. Кариограмма клеточной линии ПКр, 38-й пассаж

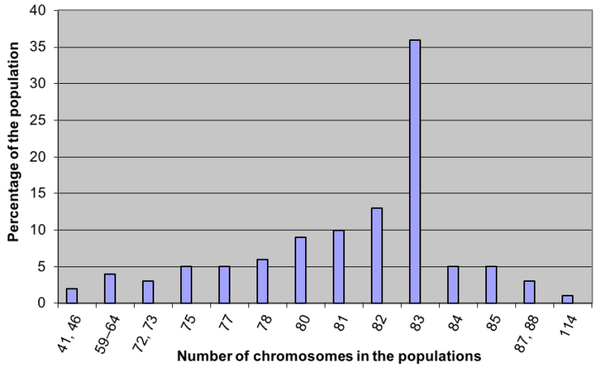

Fig. 10. RK cell line ideogram, passage 56

Рис. 10. Кариограмма клеточной линии ПКр, 56-й пассаж

at passages 66, 75, 86 and 101 demonstrated stability of near-tetraploid population (77–83 chromosomes) in the karyotype (Fig. 11).

Variability of the RK cell line populations basing on the amount of chromosomes was consistent with the variability of the immortalized cell line with stable cytomorphological and cultural characteristics. It should be noted that the number of polyploids at long-term passaging in standard optimal conditions and keeping for up to 10 days at 37 °C was 1%.

The cytochemical analysis of the CCL derived from an adult rabbit kidney demonstrated polymorphism both of cell and nucli (Fig. 12). This method did not detect mycoplasmas in the intercellular space and on the cell membranes. Absence of latent contamination allows performing long-term passaging of the CCL without changing split ratio, culture conditions, and without a cryo stage.

This culture turned out to be sensitive to viruses of infectious bovine rhinotracheitis, classical swine fever, and Aujeszky's disease viruses. Cytopathic changes detected in the RK culture were caused by the rabbit myxomatosis virus and the titer increase was not observed during passaging.

CONCLUSION

Immortalization of cell cultures derived from organs of farmed animals has several patterns which are the reason for rather a small amount of permanent cultures. There are, for instance, only five cell lines derived from a European rabbit (*Oryctolagus cuniculus* L.). It is well known that the probability of spontaneous formation of continuous cell lines from organs and embryos and newly-borns is higher than from organs of adult animals and such cell lines are more preferable as they contain a large amount of stem cells with an increased proliferation activity [3]. As demonstrated by E. L. Duncan and R. R. Reddel spontaneous immortalization of non-tumorigenic origin can have unpredictable ways of cell transformation *in vitro* [2, 11]. The results of the research for rabbit kidney CCL stabilization at the tetraploidy level bring new perspectives to use of adult animal organs. It was established that the prepared subculture was not highly productive but it can be used as a substrate for cultivation of porcine and bovine heterologous viruses.

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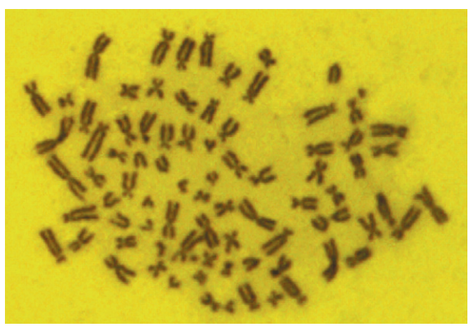

Fig. 11. Metaphase plate of the RK cell line, passage 56, 83 chromosomes

Рис. 11. Метафазная пластинка клеточной линии ПКр, 56-й пассаж, 83 хромосомы

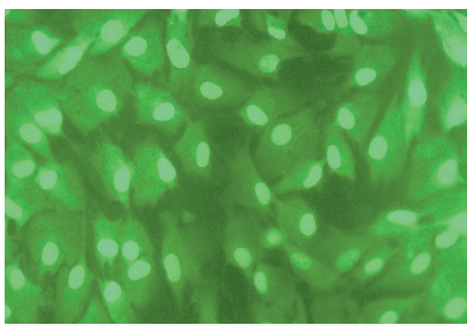

Fig. 12. RK cytochemical preparation. Staining with acridine orange, lens ×40

Рис. 12. Цитохимический препарат ПКр. Окраска акридиновым оранжевым, объектив ×40

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Determination of FMDV 146S particle concentration by spectrometric method during viral RNA quantification

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SUMMARY

During FMD vaccine production, special attention is paid to the concentration of 146S particles bearing the critical biological features of FMDV and being the main components that have an effect on vaccine immunogenicity. For this reason, each batch of vaccine raw material is tested for 146S component concentration. The paper presents the results of the use of a spectrometric method for whole particle concentration determination during quantification of FMDV RNA extracted after immune capture. It is an inexpensive, easy-to-perform method allowing for determination of FMDV 146S particle concentration in the non-inactivated culture suspension. 146S particle concentration was found to depend on the number of RNA molecules extracted from virions after their strain-specific immune capture and quantitatively detected by the spectrometric method. The presented method allows for determination of 146S component concentration in the non-inactivated vaccine raw material using the proposed linear model. The spectrometric method showed 94.5–99.5% correlation with real-time reverse transcription polymerase chain reaction and complement fixation test based on the results of tests of 360 non-inactivated suspensions of FMDV of all types. Tests of the positive control demonstrated 99.0–99.6% compatibility of actual and expected results. FMDV genome and 146S particles were not detected in the negative control, and that was in line with expectations.

Key words: FMDV RNA, 146S component concentration, spectrometric analysis, immune capture.

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Определение концентрации 146S частиц вируса ящура спектрометрическим способом при оценке количества вирусной РНК

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РЕЗЮМЕ

В процессе промышленного производства противоящурных вакцин особое внимание уделяют концентрации 146S частиц, которые наделены важнейшими биологическими свойствами вируса ящура и являются основными компонентами, влияющими на иммуногенную активность вакцинных препаратов.

По этой причине каждую серию вакцинного сырья исследуют на определение концентрации 146S компонента. В статье представлены результаты изучения применения спектрометрического метода для определения концентрации полных частиц при оценке количества РНК вируса ящура, выделенной после иммунного захвата. Данный способ является дешевым, простым в исполнении, позволяет определять концентрацию 146S частиц вируса ящура в неинактивированной культуральной суспензии. Выявлено существование зависимости между концентрацией 146S частиц и количеством молекул РНК, выделенных из вирионов после их штаммоспецифического иммунного захвата и количественно детектированных спектрометрическим методом. Представленный метод позволяет определять значение концентрации 146S компонента вируса ящура в неинактивированном сырье для вакцины с применением предложенной линейной модели. Корреляция спектрометрического способа с полимеразной цепной реакцией с обратной транскрипцией в режиме реального времени и реакцией связывания комплемента при тестировании 360 неинактивированных суспензий вируса ящура всех типов составила 94,5–99,5%. Для положительного контроля совпадение фактических и ожидаемых результатов соответствовало 99,0–99,6%. В отрицательном контрольном образце геном и 146S частицы вируса ящура не обнаружены, что соответствовало ожиданиям.

Ключевые слова: РНК вируса ящура, концентрация 146S компонента, спектрометрический анализ, иммунный захват.

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INTRODUCTION

Foot-and-mouth disease is a highly contagious viral disease characterized by fever that affects wild and domestic cloven-hoofed animals [1]. The disease agent is an RNA-virus of the order *Picornavirales*, family *Picornaviridae*, genus *Aphthovirus*. Foot-and-mouth disease virus (FMDV) is highly antigenically variable due to mutations in the capsid protein genes and encompasses A, O, C, Asia-1, SAT-1, SAT-2, SAT-3 types and multiple genetic lineages [2].

FMDV virion is about 23–25 nm in diameter. The genome is a single-stranded positive-sense RNA consisting of approximately 8,500 nucleotide bases. The virion has a molecular weight of about 8,080,000 Da [3]. When reproduced in susceptible cell lines, FMDV forms 4 variants of components: 146S particles (virions) containing one viral RNA molecule and 60 polypeptide copies, each is a complex of VP_1-VP_2-VP_3-VP_4 proteins; 75S component lacking RNA and comprising 60 copies of VP_1-VP_3-VP_0 polypeptide; 12S particles represented by VP_1, VP_2, VP_3 proteins; 3.8S subunits consisting of non-structural VP_9 protein [4].

The system of measures for FMD control and prevention includes mass immunization of cattle and small ruminants, as well as post-vaccination immunity level monitoring [1, 5]. During FMD vaccine production, special attention is paid to the concentration of 146S particles bearing the critical biological features of FMDV and being the major components that have an effect on vaccine immunogenicity [4]. Quantitative complement fixation test (CFT) and real-time reverse transcription polymerase chain reaction (rtRT-PCR) are used to determine 146S component concentration in the vaccine raw material used for every batch. The first technique has several disadvantages: it is labour-intensive and time-consuming (the test lasts up to 3 days) and does not allow for simultaneous testing of a large number of samples, its cost is rather high [6]. Real-time RT-PCR reduces the test duration to 4 hours, but requires expensive equipment and reagents [7]. Besides, when a sample contains an excessive amount of ballast components, reliability of accurate determination of 146S immunogenic component concentration may be decreased due to anticomplementarity in CFT and high ballast protein content in the sorbent at the RNA extraction stage.

It is, therefore, reasonable to search for an alternative method for FMDV 146S component quantification in the non-inactivated vaccine raw material.

The aim of the study is to assess spectrometric method potential for determination of 146S particle concentration during FMDV RNA quantification.

MATERIALS AND METHODS

Virus. Non-inactivated suspensions of culture FMDV A/Turkey/2006, A/ARRIAH/2015, O/Primorsky/2012, O/Primorsky/2014, Asia-1/Tajikistan/2011, C_1/Zakarpatsky/1972, SAT-1/Akhalkalaksky/62, SAT-2/Saudi Arabia 7/2000, SAT-3/Bechuanaland 1/65 vaccine strains deposited in the FGBI "ARRIAH" Collection of Microorganism Strains were used. The virus was reproduced in the continuous suspension culture of baby hamster kidney cells (BHK-21).

Immunoassay plate coating. A 24-well plate with DNA/RNA/DNAase/RNAase-free surface was coated with strain-specific anti-FMDV polyclonal antibodies in a volume of 1.0 cm^3 with IgG concentration in suspension of 5.0 μg/cm^3 at (4 ± 2) °C during 18 hours. Open binding sites were blocked with 0.5% bovine serum albumin (BSA) suspension at (37 ± 1) °C for 1 hour. This is a preparatory process that should be carried out beforehand, prior to analysis.

Immune capture of FMDV virions. The virus suspension was added to the wells coated with antibodies to the virions of a particular FMDV strain, 3.0 cm^3 of the suspension per well, and incubated at 37 °C for 1 hour. Immune complexes were formed, with some of them being 146S particle – strain-specific antibody complexes. The suspension was removed from the wells, but the film on the bottom

was left intact. Then 1/15 M phosphate buffered saline (PBS) was added to the wells, 1.0 cm^3 of PBS per well, with subsequent resuspension.

RNA extraction from FMDV 146S particles. 10 ml of a solution containing 50% of phenol (pH < 7.0) and 50% of 4 M guanidine isothiocyanate (GITC) were added to 1.0 cm^3 of 146S particle – strain-specific antibody immune complex suspension and incubated for 20 minutes at 23–25 °C. The prepared lysate was centrifuged at 14,000 rpm for 8 minutes. The supernatant was transferred into a centrifuge tube, 2.5 cm^3 of chloroform were added, and the content was incubated for 4 minutes with periodic mixing. After exposure, the mixture was centrifuged at 14,000 rpm for 12 minutes at 4–5 °C. As a result of precipitation, the tube content was fractionated into three phases: 1) the straw-coloured lower phase containing the complex of phenol and chloroform with bound lipids and polypeptides; 2) the white interphase comprising peptide components and DNA; 3) the upper phase representing a clear RNA extract [8, 9]. The entire upper phase was collected into an empty tube, while other fractions were left intact, and 4.5 cm^3 of 100% isopropyl alcohol were added. The prepared mixture was incubated for 8 minutes at 23–25 °C; after that the tube content was centrifuged at 14,000 rpm for 8 minutes at 23–25 °C. The supernatant was removed, and FMDV RNA pellet remained in the tube. 2.0 cm^3 of 80% ethanol were added to the RNA pellet. The content was mixed and pelleted at 14,000 rpm for 6 minutes at 23–25 °C. The supernatant was removed, and the RNA pellet was dried with an air stream at ambient temperature for 5 minutes. Then 0.1 cm^3 of TE buffer (10 mM tris(oxymethil)aminomethane, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0–7.2) free from RNAases and Mg^{2+} cations was added to RNA; the content was heated at 55–60 °C for 2–3 minutes to maximally dissolve FMDV RNA. Thirty-fold eluates of FMDV RNA standard dilutions and the negative control were prepared.

Assessment of FMDV RNA eluate purity using spectral analysis. FMDV RNA eluate spectral absorbance was measured at wavelengths of 205–325 nm. Using the said spectral range, the preparation was tested for residual phospholipids, polysaccharides and guanidine isothiocyanate, phenol, polypeptides, large conglomerates by optical density (OD) determination at 205, 235, 270, 280 and 320 nm, respectively [10, 11]. RNA eluate was considered to be free from protein and phenol contamination when the extinction coefficient R_1 (OD_{262}/OD_{280}) was 1.8–2.2 and its value was close to 2.0. FMDV RNA extract was considered to be uncontaminated with polysaccharides when the extinction coefficient R_2 (OD_{262}/OD_{235}) was 2.00–2.02 [12]. When 1% of RNA is replaced with polysaccharide components, the R_2 value decreases by 0.002. $R_2 > 2.02$ was indicative of nucleic acid dissociation and the presence of nucleotides in the eluate. OD_{320} tending to zero reflected the absence of large suspended particles in the extract [8].

Complement fixation test (CFT). Quantitative CFT was used for FMDV 146S particle concentration determination; the test was carried out in compliance with requirements [6].

Real-time reverse transcription polymerase chain reaction (rtRT-PCR). Real-time RT-PCR was used for FMDV genome detection and quantification in the non-inactivated vaccine raw material. The amount of components for the reaction, as well as time and temperature parameters for thermal cycling are specified in the previously described requirements [7].

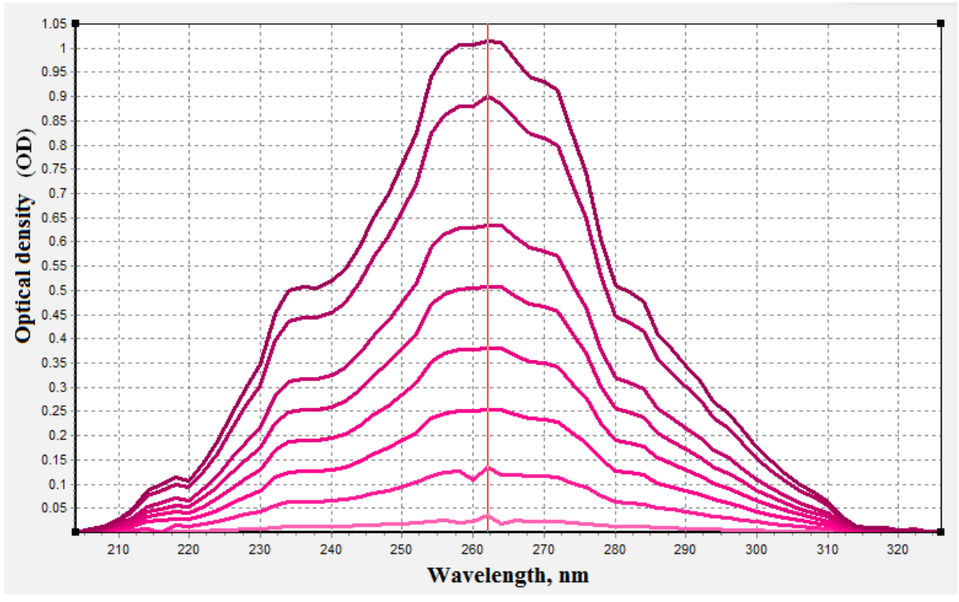

Fig. 1. Spectrograms of dilutions of standard FMDV Asia-1/Tajikistan/2011 strain RNA eluate. The figure shows (from top downwards) graphs for some dilutions of eluates corresponding to the following 146S particle concentrations: 4.0; 3.5; 3.0; 2.5; 2.0; 1.5; 1.0; 0.5; 0.1 μg/cm^3 (n = 3)

Рис. 1. Спектрограммы разведений стандартного элюата РНК вируса ящура штамма Азия-1/Таджикистан/2011. Сверху вниз отражены графики для некоторых разведений элюатов, соответствующих концентрациям 146S частиц: 4,0; 3,5; 3,0; 2,5; 2,0; 1,5; 1,0; 0,5; 0,1 мкг/$см^3$ (n = 3)

RESULTS AND DISCUSSION

During the first stage of the study, a non-inactivated suspension of culture FMDV Asia-1/Tajikistan/2011 strain with 146S particle concentration of (4.00 ± 0.11) μg/cm^3 (based on CFT and rtRT-PCR data) used as a positive standard was tested. Immune capture of FMDV virions was carried out using an immunoassay plate coated with polyclonal antibodies specific to the whole virus particles of the specified strain. BHK-21 cell suspension with a concentration of cells of (3.00 ± 0.10) mln/cm^3 not infected with FMDV served as a negative control. As a result of serological reaction, a suspension of FMDV 146S particles bound with strain-specific immunoglobulins G was prepared. The suspension was used for RNA extraction from the whole virus particles, and a 30-fold extract of FMDV Asia-1/Tajikistan/2011 strain RNA in a volume of 0.1 cm^3 was prepared.

During the next stage of the study, the purity of FMDV Asia-1/Tajikistan/2011 strain (positive standard) RNA eluate was assessed by spectral analysis in the ultraviolet region. Extinction values were registered for every 2 nm in the range from 205 to 325 nm, and the entire absorption spectrum of RNA was recorded using Spectrum software version 5.0 (Fig. 1).

The test results for 30-fold preparation showed that $OD_{205\text{-}259}$ and $OD_{263\text{-}325}$ values did not exceed $OD_{260\text{-}262}$ values (0.001–1.006 < 1.007–1.014 and 1.013–0.004 < 1.007–1.014), thus indicating the high purity of the prepared RNA eluate. During spectral analysis, no pronounced peaks were observed on the graph at wavelengths of 205, 235, 270, 280 and 320 nm, and that was indicative of the almost total absence of contamination with phospholipids, polysaccharides and residual GITC, phenol, polypeptides, large conglomerates, respectively. The extinction coefficient R_1 was 1.988 (OD_{262}/OD_{280} = 1.014/0.510), i.e. close to the normal value of 2.000, and that showed the absence of DNA and the presence of trace amounts of protein impurities. Nucleic acid degradation and free nucleotides were not observed in the solution, since R_1 did not exceed 2.000. The extinction coefficient R_2 (OD_{262}/OD_{235} = 1.014/0.506) of 2.004 showed that the eluate of FMDV RNA (positive standard) was not contaminated with polysaccharides and GITC. Given that the R_2 value decreases by 0.002 when 1% of RNA is replaced with polysaccharide components, no carbohydrate impurities were detected in the prepared eluate.

During the following stage of the study, quantification of RNA molecules extracted from FMDV 146S particles was performed. One-stranded RNAs have their absorption maxima at 252–271 nm due to high spectral absorbance of ribonucleoside-5′-triphosphates in this range, in particular: adenosine-5′-triphosphate (λ_{ATP}) – 259 nm, uridine-5′-triphosphate (λ_{UTP}) – 262 nm, guanosine-5′-triphosphate (λ_{GTP}) – 252 nm, cytidine-5′-triphosphate (λ_{CTP}) – 271 nm [13]. Spectral studies demonstrate that maximum absorption wavelengths of whole RNA monomers are within the same range; thus, extinction is the highest in the specified wavelength range [14]. In the course of the study, nucleotide analysis of RNAs of a wide variety of isolates and strains of seven types of FMDV available in the GenBank database was carried out [3]. Based on the test results, average percentages of ribonucleoside-5′-triphosphates (W_{ATP}, W_{UTP}, W_{GTP}, W_{CTP}) in the genome were determined. The test results are presented in Table 1.

Based on the data obtained, average maximum absorption wavelengths (λ_{max}) of RNAs were calculated for each FMDV type using the following formula: $\lambda_{max} = \lambda_{ATP} \times W_{ATP} + \lambda_{UTP} \times W_{UTP} + \lambda_{GTP} \times W_{GTP} + \lambda_{CTP} \times W_{CTP}$. It was determined that λ_{max} for the genome of FMDV strains of type A was 261.64 nm, type O – 261.51 nm, type C – 261.68 nm, type Asia-1 – 261.63 nm, type SAT-1 – 261.74 nm, type SAT-2 – 261.70 nm, type SAT-3 – 261.77 nm. λ_{max} values were experimentally determined by biospectrometry of RNA eluates of all FMDV types and found to be close to empirical values – 262 nm (Table 1).

The number of one-stranded RNA molecules in the pure preparation was calculated on the basis of the Bouguer – Lambert – Beer law which states that the intensity of a parallel beam of monochromatic light decreases when it passes through the absorbing medium [10]. Using this law, it is possible to relate the amount of light absorbed to the concentration of absorbing particles. At λ = 260–262 nm, average extinction coefficient for one-stranded RNA is 0.024 $(μg/cm^3)^{-1}$ cm^{-1}. Therefore, the optical density of a solution with nucleic acid concentration of 1.00 μg/cm^3 exposed to ultraviolet light at a wavelength of 260 nm (OD_{260}) is 24.000, and, consequently, optical density of 1.000 corresponds to an eluate with RNA concentration of 41.67 μg/cm^3 [13]. Measurements showed that FMDV RNA has its absorption maximum at λ = 262 nm; the OD_{262} value for a highly purified extract with RNA concentration of 41.67 μg/cm^3 is 1.000.

The use of Bouguer – Lambert – Beer law is relevant for viral RNA molecule quantification in diluted eluates. However, at high analyte concentrations (> 1.00 μg/cm^3), the distance between the molecules of an ultraviolet absorbing material is significantly reduced. As a result, the effect of each particle on surface charge distribution between the neighboring molecules grows, and this may lead to a change in RNA absorbance at the specified wavelength. Thus, for FMDV RNA molecule quantification in high concentration preparations, analyte dilutions should be made using TE buffer. Prior to

Table 1
Ribonucleoside-5′-triphosphate percentages and maximum absorption wavelengths for RNAs of different FMDV types

Таблица 1
Процентное содержание рибонуклеозид-5′-трифосфатов и длины волн с максимальным поглощением РНК вируса ящура разных типов

FMDV type	Average percentages of ribonucleoside-5′-triphosphates in FMDV genome (*W*), %				Maximum absorption wavelengths for RNAs, nm	
	ATP	UTP	GTP	CTP	calculated	empirical
A	25.1	20.9	25.7	28.3	261.64	262
O	25.6	21.4	25.9	27.1	261.51	262
C	25.1	21.2	25.4	28.3	261.68	262
Asia-1	24.7	21.2	25.9	28.2	261.63	262
SAT-1	25.2	20.9	25.1	28.7	261.74	262
SAT-2	25.4	21.0	25.3	28.6	261.70	262
SAT-3	25.3	20.9	24.9	28.9	261.77	262

Table 2
Relationship between 146S particle concentration and 30-fold number of RNA molecules extracted from whole particles of FMDV ($n = 3$, $p < 0.005$)

Таблица 2
Зависимость между концентрацией 146S частиц и 30-кратным количеством молекул РНК, выделенных из полных частиц вируса ящура ($n = 3$, $p < 0,005$)

Type of control sample	146S particle concentration, μg/ml	Average number of RNA molecules extracted from FMDV 146S particles in 30-fold eluate		
		theoretical value	based on spectrometric method data	reliability, %
positive standard	0.1	7,457,680,118	7,315,278,733	98.05
	0.2	14,915,360,236	14,349,200,591	96.05
	0.3	22,373,040,353	21,383,122,450	95.37
	0.4	29,830,720,471	28,698,401,183	96.05
	0.5	37,288,400,589	36,013,679,916	96.46
	0.6	44,746,080,707	43,328,958,649	96.73
	0.7	52,203,760,825	49,800,166,759	95.17
	0.8	59,661,440,943	57,115,445,492	95.54
	0.9	67,119,121,060	66,118,865,471	98.49
	1.0	74,576,801,178	73,715,501,078	98.83
	1.1	82,034,481,296	79,061,281,690	96.24
	1.2	89,492,161,414	88,346,058,544	98.70
	1.3	96,949,841,532	95,379,980,402	98.35
	1.4	104,407,521,649	100,163,047,266	95.76
	1.5	111,865,201,767	109,166,467,245	97.53
	1.6	119,322,881,885	114,512,247,858	95.80
	1.7	126,780,562,003	122,952,954,088	96.89
	1.8	134,238,242,121	130,830,946,570	97.40
	1.9	141,695,922,239	137,302,154,679	96.80
	2.0	149,153,602,356	147,712,359,030	99.02
	2.1	156,611,282,474	153,620,853,391	98.05
	2.2	164,068,962,592	158,403,920,255	96.42
	2.3	171,526,642,710	164,875,128,365	95.97
	2.4	178,984,322,828	170,502,265,852	95.03
	2.5	186,442,002,946	180,349,756,454	96.62
	2.6	193,899,683,063	190,197,247,056	98.05
	2.7	201,357,363,181	196,949,812,040	97.76
	2.8	208,815,043,299	203,421,020,150	97.35
	2.9	216,272,723,417	211,580,369,506	97.78
	3.0	223,730,403,535	221,146,503,234	98.83
	3.1	231,188,083,652	224,522,785,726	97.03
	3.2	238,645,763,770	228,743,138,841	95.67
	3.3	246,103,443,888	237,465,201,946	96.36
	3.4	253,561,124,006	245,061,837,553	96.53
	3.5	261,018,804,124	255,190,685,029	97.72
	3.6	268,476,484,242	261,943,250,013	97.51
	3.7	275,934,164,359	265,038,175,631	95.89
	3.8	283,391,844,477	271,790,740,615	95.73
	3.9	290,849,524,595	284,451,799,961	97.75
	4.0	298,307,204,713	287,546,725,579	96.26
negative control	0.0	0	not detected	–

measuring the absorbance of an eluate dilution, TE buffer background values should be automatically subtracted. When calculating 146S RNA concentration in the preparation, conversion factor for FMDV nucleic acid ($F_{\text{FMDV RNA}} = 41.67$) and dilution factor (*DF*) should be taken into account; besides, background values for samples (OD_{320}) and the negative control ($OD_{260\,C-}$) should be subtracted from OD_{262}. The following should be included when calculating the number of FMDV RNA molecules ($N_{\text{FMDV RNA}}$): Avogadro's number (6.022045×10^{23} mole^{-1}), the average molecular weight of a ribonucleoside ($Mw_{\text{ribonucleoside}} = 340.5$ Da), FMDV RNA length ($L = 8{,}500$ nucleobases) [13]; besides, nucleic acid weight should be converted from micrograms to grams according to the International System of Units (Le Système International d'Unités) [11] (the conversion factor is $1/10^6$), and the number of molecules for 30-fold eluate should be converted into that for 1-fold dilution (the conversion factor is $1/10^{1.48}$) (the total conversion factor is $1/10^{7.48}$).

When FMDV is reproduced in susceptible cell lines, 146S particles (virions) are formed, with each of them comprising one RNA molecule. It should be noted that FMDV suspensions contain RNA as part of virions (98–99%), and also a small amount (1–2%) of RNA in the free state [4, 15]. In other words, when calculating the number of RNA molecules extracted from the whole particles, a coefficient of 0.98 should be included.

The number of RNA molecules extracted from FMDV 146S particles ($N_{\text{RNA 146S}}$) should be calculated using the following formula taking into account the above mentioned parameters:

$$N_{\text{RNA 146S}} = 0.98 \times \frac{41.67 \times DF \times (OD_{262} - OD_{320} - OD_{260\,C-}) \times N_A}{10^{7.48} \times Mw_{\text{ribonucleoside}} \times L},$$

where *DF* is the dilution factor for the eluate of RNA extracted from FMDV 146S particles;

OD_{262} is the optical density value for the eluate of RNA extracted from FMDV 146S particles at a wavelength of 262 nm;

OD_{320} is the optical density value for the extract of RNA extracted from FMDV 146S particles at a wavelength of 320 nm;

$OD_{260\,C-}$ is the optical density value for the negative control at a wavelength of 260 nm;

N_A is the Avogadro constant (6.022045×10^{23} mole^{-1});

$Mw_{\text{ribonucleoside}}$ is the average molecular weight of a ribonucleoside (340.5 Da);

L is FMDV genome length (8,500 nucleobases);

41.67 is the factor for FMDV RNA ($F_{\text{FMDV RNA}}$);

$1/10^{7.48}$ is the total factor for conversion of weight from micrograms to grams and for conversion of FMDV RNA molecule number for 30-fold eluate into that for 1-fold dilution.

The next stage of the study was to search for a model for FMDV 146S particle concentration determination based on the number of molecules of the extracted viral RNA. To identify the relationship between FMDV 146S particle concentration and the number of viral RNA molecules, serial dilutions of 30-fold standard of RNA extracted from FMDV Asia-1/Tajikistan/2011 strain suspension with whole particle concentration of 4.0 μg/cm^3 were made; thus, the standard samples of nucleic acid corresponding to 146S particle concentrations of 0.1 to 4.0 μg/cm^3 (in increments of 0.1 μg/cm^3) were prepared. Prior to measuring the absorbance of the prepared standard samples, TE buffer background values were automatically subtracted. Spectral analysis of the prepared positive standards was performed, and extinction values were determined at wavelengths of 262 nm and 320 nm. The optical density of the negative control was measured to detect the presence of non-specific RNA molecules that were found to have their absorption maxima at $\lambda = 260$ nm. Spectral analysis of some of the specified standards is presented in Figure 1. The results of measurements and calculations of the number of RNA molecules extracted from 146S particles are presented in Table 2 demonstrating that the $N_{\text{RNA 146S}}$ values for all dilutions of 30-fold standard with 146S particle concentrations of 0.1 to 4.0 μg/cm^3 were between 7,315,278,733 and 287,546,725,579, respectively.

It is known that whole, and free RNA content in the virus suspensions is low [4]; hence, there is a direct relationship FMDV particles comprise one RNA molecule between the number of 146S particles and the number of FMDV RNA molecules quantified in the same volume of non-inactivated suspension after the virus reproduction. It is known that the molecular weight of a whole FMDV virion (Mw_{146S}) is 8,080,000–8,167,500 Da on average [3, 4, 15]. Given that the virus genome length is 8,500 nucleobases [3, 4] and the average molecular weight of a ribonucleside is 340.5 Da, the molecular weight of the viral RNA ($Mw_{\text{FMDV RNA}}$) is about 2,894,250 Da, and this is on average 2.79–2.82 times less than Mw_{146S}. Put differently, theoretically 146S particle concentration (μg/cm^3) in FMDV suspensions is on average 2.79–2.82 times higher than viral RNA concentration (μg/cm^3); hence,

$$\frac{m_{146S}}{m_{\text{RNA 146S}}} = 2.79 \div 2.82, \text{ or } m_{\text{RNA146S}} = \frac{m_{146S}}{2{,}79 \div 2{,}82}.$$

This expression is substituted into the equation

$$\frac{m_{146S}}{N_{\text{RNA 146S}}}$$

instead of $m_{\text{RNA 146S}}$, taking into account that

$$N_{\text{RNA 146S}} = \frac{m_{\text{RNA}} \times N_A}{10^6 \times L \times Mw_{\text{ribonucleoside}}},$$

where m_{RNA} is FMDV RNA weight;

N_A is the Avogadro constant (6.022045×10^{23} mole^{-1});

L is FMDV genome length (8,500 nucleobases);

$Mw_{\text{ribonucleoside}}$ is the average molecular weight of a ribonucleoside (340.5 Da);

$1/10^6$ is the factor for weight conversion from micrograms to grams.

By rearrangements, we obtain

$$\frac{N_{\text{RNA 146S}}}{m_{146S}} = 7.38 \times 10^{10} - 7.46 \times 10^{10}.$$

This means that theoretically the number of RNA_{146S} molecules is on average $7.38 \times 10^{10} - 7.46 \times 10^{10}$ times higher than 146S particle concentration, as was experimentally confirmed by tests of dilutions of standards with known 146S particle concentrations using the proposed method. The test results are presented in Tables 2 and 3.

The data presented in Table 2 show that reliability of quantification of RNA molecules extracted from FMDV suspensions with 146S particle concentrations of 0.1 to 4.0 μg/cm^3 was between 95.03 and 99.02%.

Based on the data obtained on the number of viral RNA molecules in the dilutions of the standard

Table 3
Relationship between 146S particle concentration and the number of RNA molecules extracted from whole particles of FMDV Asia-1/Tajikistan/2011 strain ($n = 3, p < 0.005$)

Таблица 3
Зависимость между концентрацией 146S частиц и количеством молекул РНК, выделенных из полных частиц вируса ящура штамма Азия-1/Таджикистан/2011 ($n = 3, p < 0,005$)

146S particle concentration in a standard, µg/ml	Average optical density (OD) values			Calculated number of RNA_{146S} molecules
	standard samples		negative control	
	OD_{262}	OD_{320}*	$OD_{260\,C-}$*	
0.1	0.026 ± 0.001	0.002	0.006	7,315,278,733
0.2	0.051 ± 0.001	0.000	0.006	14,349,200,591
0.3	0.076 ± 0.001	0.001	0.006	21,383,122,450
0.4	0.102 ± 0.001	0.002	0.006	28,698,401,183
0.5	0.128 ± 0.001	0.001	0.006	36,013,679,916
0.6	0.154	0.002	0.006	43,328,958,649
0.7	0.177 ± 0.001	0.001	0.006	49,800,166,759
0.8	0.203	0.003	0.006	57,115,445,492
0.9	0.235 ± 0.001	0.002	0.006	66,118,865,471
1.0	0.262 ± 0.001	0.003	0.006	73,715,501,078
1.1	0.281 ± 0.001	0.004	0.006	79,061,281,690
1.2	0.314 ± 0.001	0.003	0.006	88,346,058,544
1.3	0.339 ± 0.001	0.003	0.006	95,379,980,402
1.4	0.356 ± 0.001	0.005	0.006	100,163,047,266
1.5	0.388	0.004	0.006	109,166,467,245
1.6	0.407	0.005	0.006	114,512,247,858
1.7	0.437 ± 0.001	0.006	0.006	122,952,954,088
1.8	0.465 ± 0.001	0.006	0.006	130,830,946,570
1.9	0.488 ± 0.001	0.004	0.006	137,302,154,679
2.0	0.525 ± 0.001	0.007	0.006	147,712,359,030
2.1	0.546 ± 0.001	0.005	0.006	153,620,853,391
2.2	0.563	0.005	0.006	158,403,920,255
2.3	0.586 ± 0.001	0.007	0.006	164,875,128,365
2.4	0.606 ± 0.001	0.005	0.006	170,502,265,852
2.5	0.641 ± 0.001	0.008	0.006	180,349,756,454
2.6	0.676 ± 0.001	0.006	0.006	190,197,247,056
2.7	0.700	0.002	0.006	196,949,812,040
2.8	0.723 ± 0.001	0.002	0.006	203,421,020,150
2.9	0.752 ± 0.002	0.003	0.006	211,580,369,506
3.0	0.786 ± 0.001	0.004	0.006	221,146,503,234
3.1	0.798 ± 0.002	0.001	0.006	224,522,785,726
3.2	0.813 ± 0.001	0.005	0.006	228,743,138,841
3.3	0.844 ± 0.001	0.001	0.006	237,465,201,946
3.4	0.871 ± 0.002	0.003	0.006	245,061,837,553
3.5	0.907 ± 0.001	0.001	0.006	255,190,685,029
3.6	0.931 ± 0.002	0.002	0.006	261,943,250,013
3.7	0.942 ± 0.001	0.002	0.006	265,038,175,631
3.8	0.966 ± 0.001	0.002	0.006	271,790,740,615
3.9	1.011 ± 0.002	0.003	0.006	284,451,799,961
4.0	1.024 ± 0.002	0.002	0.006	287,546,725,579

OD_{262} – extinction value at 262 nm; OD_{320} – extinction value at 320 nm;
$OD_{260\,C-}$ – extinction value for the negative control at 260 nm;
* values remained unchanged when measured.
OD_{262} – значение экстинкции при 262 нм; OD_{320} – значение экстинкции при 320 нм;
$OD_{260\,К-}$ – значение экстинкции для отрицательного контроля при 260 нм;
* значения при измерениях не менялись.

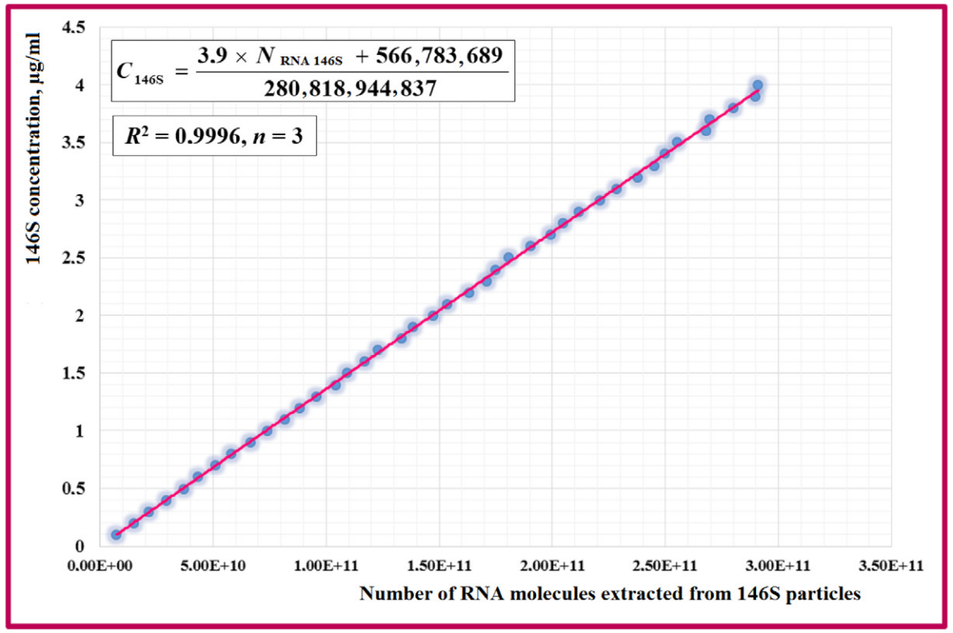

Fig. 2. Relationship between FMDV 146S particle concentration and the number of viral RNA molecules in standard solutions. The number of molecules is expressed in exponential notation (E = 10)

Рис. 2. Зависимость концентрации 146S частиц вируса ящура от количества молекул вирусной РНК в стандартных растворах. Количество молекул выражено в экспоненциальном формате (E = 10)

and corresponding 146S particle concentrations, a calibration graph of the relationship between C_{146S} and $N_{RNA\ 146S}$ was plotted represented as the model

$$C_{146S} = \frac{3.9 \times N_{RNA\ 146S} + 566,783,689}{280,818,944,837}$$

with the coefficient of determination R^2 of 0.9996 (Fig. 2). No FMDV was detected in the negative control.

The obtained model based on the number of FMDV RNA molecules extracted from 146S particles after immune capture of virions allows for determination of whole particle concentration in the non-inactivated culture virus suspensions.

During the final stage of the study, the presented method for 146S particle concentration determination was tested using 360 non-inactivated suspensions of the following FMDV vaccine strains: A/Turkey/2006 (40 samples), A/ARRIAH/2015 (40 samples), O/Primorsky/2012 (40 samples), O/Primorsky/2014 (40 samples), Asia-1/Tajikistan/2011 (40 samples), C_1/Zakarpatsky/1972 (40 samples), SAT-1/Akhalkalaksky/62 (40 samples), SAT-2/Saudi Arabia 7/2000 (40 samples), SAT-3/Bechuanaland 1/65 (40 samples). The samples were parallely tested with rtRT-PCR and CFT in triplicate (Table 4).

The compatibility of actual and expected (based on rtRT-PCR and CFT data) results of culture FMDV 146S component concentration determination was 96.2–99.4% for A/Turkey/2006 strain, 94.9–99.3% for A/ARRIAH/2015, 97.2–99.2% for O/Primorsky/2012, 97.0–99.3% for O/Primorsky/2014, 97.6–99.5% for Asia-1/Tajikistan/2011, 95.2–97.7% for C_1/Zakarpatsky/1972, 94.8–97.3% for SAT-1/Akhalkalaksky/62, 96.5–99.1% for SAT-2/Saudi Arabia 7/2000, 94.5–98.5% for SAT-3/Bechuanaland 1/65. Tests of the positive control demonstrated 99.0–99.6% compatibility of actual and expected results. FMDV genome and 146S particles were not detected in the negative control, and that was in line with expectations.

Thus, the spectrometric method for determination of culture FMDV 146S component concentration showed 94.5–99.5% correlation with rtRT-PCR and CFT based on the test results.

CONCLUSION

A method for spectrometric determination of 146S particle concentration during quantification of FMDV RNA extracted after immune capture is proposed. This is an inexpensive, easy-to-perform method for determination of FMDV 146S particle concentration in the non-inactivated vaccine raw material with high ballast component content.

146S particle concentration was found to depend on the number of RNA molecules extracted from virions after their strain-specific immune capture and quantitatively detected using the spectrometric method. The presented spectrometric method allows for FMDV 146S particle concentration determination in the non-inactivated vaccine raw material using the following linear model

$$C_{146S} = \frac{3.9 \times N_{RNA\ 146S} + 566,783,689}{280,818,944,837}.$$

The proposed method showed 94.5–99.5% correlation with rtRT-PCR and CFT. Tests of the positive control

Table 4
Compatibility of actual and expected results of 146S particle concentration determination by spectrometric method with rtRT-PCR and CFT ($n_{tests} = 3$)

Таблица 4
Степень совпадения фактических и ожидаемых результатов спектрометрического способа определения концентрации 146S частиц с ОТ-ПЦР-РВ и РСК ($n_{иссл.} = 3$)

Sample status	FMDV strain	Number of samples	Compatibility of spectrometric method results with other methods, %	
			rtRT-PCR [7]	CFT
test samples	A/Turkey/2006	40	97.0–99.4	96.2–97.1
	A/ARRIAH/2015	40	96.8–99.3	94.9–97.0
	O/Primorsky/2012	40	98.3–99.2	97.2–98.5
	O/Primorsky/2014	40	98.0–99.3	97.0–98.1
	Asia-1/Tajikistan/2011	40	98.4–99.5	97.6–98.5
	C_1/Zakarpatsky/1972	40	97.0–97.7	95.2–97.1
	SAT-1/Akhalkalaksky/62	40	96.1–97.3	94.8–96.0
	SAT-2/Saudi Arabia 7/2000	40	98.0–99.1	96.5–98.2
	SAT-3/Bechuanaland 1/65	40	97.9–98.5	94.5–97.9
positive control	Asia-1/Tajikistan/2011	40	99.2–99.6	99.0–99.3
negative control	–	40	100	100

demonstrated 99.0–99.6% compatibility of actual and expected results. FMDV genome and 146S particles were not detected in the negative control, and that was in line with expectations.

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Investigation of healing effects of Afyonkarahisar Region thermal spring water on experimentally-induced nephritis in mice

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SUMMARY

The aim of this study was to determine the efficacy of Afyokarahisar termomineral water in the treatment of nephritis. For this purpose, 40 Albino mouse at the same daily age were used. Nephritis was induced by adding adenine to their feed at a rate of 0.2% for 6 weeks. After nephritis was induced, a 21-day treatment period was started, and the mice were equally divided into two groups as control and study. While control group mice were received tap water daily and bathing in tap water, study group animals were given fresh Süreyya I hot spring water daily and bathing in this water. Clinical, hematological, blood biochemical and histopathological examinations were performed before the study, after nephritis formation, and on days of 1st, 7th, 14th and 21st of treatment period. Results of this study showed that WBC, NEUT and MCV levels increased significantly ($p < 0.05$) following nephritis formation, while RBC, HB, HCT, LYM, MCH and MCHC levels decreased significantly ($p < 0.05$). It was also determined that AST, GGT, GLU, BUN and IgG levels of blood biochemical parameters were significantly increased ($p < 0.05$) and TP, ALB levels decreased significantly ($p < 0.05$) after nephritis formation. At the end of the study, it was seen that all the measured parameters turned to the normal range in the study group animals, whereas problems still continuing with control group animals. Consequently, it was concluded that Süreyya I hot spring water was very successful in the treatment of nephritis and considered as an option in the treatment of nephritis.

Key words: Afyonkarahisar, balneotherapy, biochemistry, mouse, nephritis, treatment.

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Исследование лечебного действия термальной воды региона Афьонкарахисар на экспериментально индуцированный нефрит у мышей

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РЕЗЮМЕ

Целью настоящего исследования является определение эффективности применения термоминеральной воды региона Афьонкарахисар в лечении нефрита. Для этой цели использовали 40 мышей-альбиносов одного возраста. Нефрит воспроизвели путем добавления аденина в их корм из расчета 0,2% на протяжении 6 недель. Перед началом 21-дневного курса лечения минеральной водой индуцированного нефрита мышей разделили на две равные группы: контрольную и экспериментальную. Животных контрольной группы ежедневно выпаивали водопроводной водой и купали в ней, животным экспериментальной группы ежедневно давали свежую воду из горячего источника Süreyya I, а также устраивали ежедневные ванны с использованием этой воды. Клинические, гематологические, биохимические и гистопатологические исследования проводили до исследования, после развития нефрита, а также на 1, 7, 14 и 21-й день эксперимента. Полученные результаты показали, что после развития нефрита уровни лейкоцитов (WBC), нейтрофилов (NEUT) и среднего объема эритроцитов (MCV) в крови значительно увеличились ($p < 0,05$), в то время как уровни эритроцитов (RBC), гемоглобина (HB), гематокрита (HCT), лимфоцитов (LYM), среднее содержание гемоглобина в эритроците (MCH) и средняя концентрация гемоглобина в эритроците (MCHC) значительно снизились ($p < 0,05$). Также было установлено, что после развития нефрита уровни аспартатаминотрансферазы (AST), гамма-глутамилтрансферазы (GGT), глюкозы (GLU), содержание азота мочевины в крови (BUN) и уровень IgG значительно повысились ($p < 0,05$), а уровни общего белка (TP) и альбумина (ALB) значительно снизились ($p < 0,05$). В конце исследования у животных из экспериментальной группы все параметры крови нормализовались, тогда как у мышей контрольной группы такого эффекта не наблюдалось. Это позволило сделать вывод, что термальная вода из источника Süreyya I эффективна в лечении нефрита и может рассматриваться как один из вариантов терапии.

Ключевые слова: Афьонкарахисар, бальнеотерапия, биохимия, мышь, нефрит, лечение.

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INTRODUCTION

Kidneys are the organs that play an important role in metabolic activities such as absorption of minerals and water, the acid-base balance and the functionality of the buffer systems [1]. Nephritis is a condition in which nephrons, which are functional units of the kidneys, become inflamed which can adversely affect the kidney function [2].

It has been shown that people who use hot springs as balneotherapy have important benefits in endemic nephropathic cases [3]. So, P. B. Nocco reported that balneotherapeutic cures, including inpatient and bath therapies, were very important for regaining health, especially in the treatment of chronic cases such as nephritis [4]. In addition, alkaline mineral waters were used for the purpose of detoxification [5, 6]. A. L. Rodgers claimed that the calcium oxalate stones formed in the kidneys over time had achieved very successful results from the treatment with waters containing calcium and magnesium, and these waters had have effects not only for therapeutic purposes but also for prophylactic purposes [7]. R. Siener et al. reported that consuming water containing magnesium and bicarbonate similarly regulates urinary pH, magnesium and citrate excretion, prevents calcium oxalate stone formation and prevents increased calcium excretion [8].

In this study, it is aimed to reveal the importance of treatment with Süreyya I hot spring water with rich content in the borders of Afyonkarahisar Province in the treatment of experimental nephritis.

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 Ethical Committee (AKUHADYEK) and was referred to with the report numbered 59-18 of the board and supported by Afyon Kocatepe University Scientific Research Projects Board (AKÜBAPK) as the Master's Thesis with Project number 18.SAĞ.BİL.12.

Animal Material. In this study, 40 Albino mice of the same age were used. Mice were kept in a stable environment under equal heat and humidity conditions, 12 hours day and 12 hours night, in Afyon Kocatepe University Experimental Animals Application and Research Center. Throughout the experiment, all the animals were fed *ad libitum* food.

Creating Experimental Nephritis. Four of the 40 mice were exempted to take blood samples before performing the nephritis procedure in all animals. Nephritis was created in remaining mice by adding 0.2% adenine to their food for 6 weeks [9]. After the nephritis was created, 4 out of 36 mice were ex for collecting blood and histopathological examinations, and the remaining 32 mice with nephritis were divided equally and randomly into 2 groups as below:

1. Control Group (CG). Tap water was added to the drinkers of 16 mice with nephritis, and they were provided with access to refreshed water and food *ad libitum* every day. All the mice in CG were bathed with tap water at (35 ± 2) °C for 15 minutes at the same time once a day up to end of this study, which was 21 days.

2. Study Group (SG). Fresh hot spring water was added to the drinkers of 16 mice with nephritis, and it was provided to reach the refreshed daily hot spring water and food *ad libitum*. All the mice in SG were bathed with fresh hot spring water at (35 ± 2) °C for 15 minutes at the same time once a day.

Süreyya I hot spring water, used for treatment in this study, has a total mineral content of 4046.8 mg/L, and it is in the thermomineral water group with sodium bicarbonate, carbon dioxide, magnesium, calcium, fluoride and silicon.

Clinical Examinations. Respiratory rates (R), body temperatures (T) and heart frequencies (P) were measured in all of the mice used in the study, and the results were recorded for statistical comparisons.

Collecting Blood and Tissue Samples. Blood and kidney tissue samples were exhausted under ketamine/xylazine (100/10 mg/kg) anesthesia [10] before the study, after nephritis formation (NF), and on 1st, 7th, 14th and 21st days following the treatment period.

Hematological Examinations. In blood samples; total leukocytes (WBC), lymphocytes (LYM), hemoglobin (HB), mean corpuscular volume (MCV), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), neutrophil (NEUT), eosinophil (EOS), erythrocytes (RBC), monocytes (MON) and basophils (BAS) were measured using Chemray Brand blood count commercial test kits (Rayto Life and Analytical Sciences Co., China).

Blood Biochemical Examinations. Blood urea nitrogen (BUN), serum aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), total protein (TP), glucose (GLU) were measured in Cobas Integra 400 Plus (Roche Brand (Roche Diagnostics GmbH, Germany) analyzer. Immunoglobulin G (IgG) levels were determined with ChemWell, Chromate 4300 Elisa Reader device (Awareness Technology, Inc., USA) using commerical Elisa kits (Sunred Biological Technology Co., Ltd, China).

Blood Gases Examinations. After taking blood samples to 500 IU liquid heparin plastic syringes for 1 ml of blood, which were prepared simultaneously with histopathological sampling, the tip of the sterile syringe was closed immediately, and measurements were made before < 3 hours. In blood samples partial carbon dioxide pressure (pCO_2), pH, total carbon dioxide concentration (TCO_2), bicarbonate (HCO_3^-), base deficit (BE), potassium (K^+), sodium (Na^+), chlorine (Cl^-), calcium (Ca^{2+}) values were made using a portable blood gas analyzer EDAN i15 Vet (EDAN Instruments, Inc., China) using commercial cartridges.

Histopathological Examinations. Obtained kidney samples by method mentioned above were sent to the Veterinary Control Central Research Institute, Pathology Laboratory to perform histopathological examinations in 10% formol, where 5 micron thick sections were taken, stained with hematoxylin-eosin and examined in a light microscope.

Statistical Analyses. Statistical calculations for CG and SG were made in accordance with the variance analysis (ANOVA) method. The Duncan's test was used to determine the significance of the difference in the groups. Statistical analyzes were provided through the Windows-compatible SPSS Statistics 18.1 (IBM, USA) package program. The data levels obtained are presented as mean ± standard error, and $p < 0.05$ is considered to be important.

Table 1
Statistical comparison of body temperature, pulse frequency and respiratory rate

Таблица 1
Статистическое сравнение температуры тела, частоты пульса и частоты дыхания

Time of indicator measurement by groups		Parameters (X ± SD)		
		T (°C)	P (frequence/min)	R (rate/min)
BS ($n = 40$)		37.10 ± 0.16^b	326.30 ± 44.22^e	117.04 ± 18.36^f
ANF ($n = 36$)		38.50 ± 0.14^a	327.31 ± 45.10^e	131.24 ± 25.43^e
AT 1st day	CG ($n = 16$)	38.50 ± 0.20^a	331.28 ± 33.40^d	132.54 ± 21.45^e
	SG ($n = 16$)	38.30 ± 0.18^a	363.14 ± 32.20^b	141.14 ± 22.25^c
AT 7th day	CG ($n = 12$)	38.30 ± 0.20^a	334.26 ± 27.20^e	135.30 ± 17.12^d
	SG ($n = 12$)	38.20 ± 0.10^a	378.23 ± 25.44^a	148.10 ± 15.23^b
AT 14th day	CG ($n = 8$)	38.20 ± 0.10^a	342.16 ± 22.23^c	137.10 ± 8.05^d
	SG ($n = 8$)	38.10 ± 0.12^a	374.25 ± 21.14^a	157.30 ± 7.20^a
AT 21st day	CG ($n = 4$)	38.10 ± 0.16^a	345.13 ± 9.21^c	142.18 ± 6.33^c
	SG ($n = 4$)	38.00 ± 0.10^a	376.44 ± 6.12^a	159.23 ± 4.22^a

[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), ANF – after nephritis formation (после развития нефрита),
AT – after treatment (после лечения), CG – control group (контрольная группа), SG – study group (экспериментальная группа).

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 ($n = 40$)		8.38 ± 2.32^{e}	7.80 ± 2.32^{b}	13.53 ± 2.48^{b}	43.36 ± 5.32^{a}	55.04 ± 5.12^{bc}	17.19 ± 2.16^{b}	32.08 ± 2.43^{a}	60.50 ± 2.10^{a}	34.10 ± 4.14^{f}	2.30 ± 0.50	3.50 ± 0.40	NS
ANF ($n = 36$)		16.14 ± 4.10^{a}	5.34 ± 1.27^{d}	7.67 ± 3.34^{e}	31.37 ± 4.16^{f}	58.69 ± 5.24^{a}	14.38 ± 2.21^{d}	24.47 ± 3.06^{d}	47.84 ± 4.20^{e}	48.14 ± 5.28^{a}	2.56 ± 0.30	3.40 ± 1.50	NS
AT 1st day	CG ($n = 16$)	16.21 ± 4.20^{a}	5.31 ± 1.56^{d}	7.65 ± 3.16^{e}	31.43 ± 4.13^{f}	59.20 ± 5.32^{a}	14.43 ± 2.08^{d}	24.34 ± 3.32^{d}	47.34 ± 4.20^{e}	48.21 ± 4.16^{a}	2.68 ± 0.56	3.50 ± 0.40	NS
	SG ($n = 16$)	15.23 ± 3.04^{ab}	6.04 ± 1.47^{cd}	8.07 ± 3.14^{de}	32.78 ± 3.47^{e}	54.28 ± 5.16^{c}	13.35 ± 2.13^{c}	24.83 ± 3.02^{d}	47.30 ± 4.10^{e}	46.68 ± 4.25^{b}	2.34 ± 0.50	3.40 ± 0.30	NS
AT 7th day	CG ($n = 12$)	16.10 ± 2.42^{a}	5.48 ± 1.08^{d}	7.81 ± 2.06^{e}	32.14 ± 2.15^{e}	58.66 ± 3.27^{a}	14.28 ± 1.46^{c}	24.28 ± 2.36^{d}	47.32 ± 2.30^{e}	48.03 ± 2.38^{a}	2.54 ± 0.30	3.20 ± 0.30	NS
	SG ($n = 12$)	14.03 ± 2.05^{b}	6.78 ± 1.04^{c}	9.47 ± 2.03^{c}	38.22 ± 2.23^{c}	56.38 ± 3.41^{b}	13.94 ± 1.35^{c}	24.80 ± 2.48^{d}	53.38 ± 2.14^{c}	42.38 ± 2.43^{c}	2.10 ± 0.20	3.10 ± 0.30	NS
AT 14th day	CG ($n = 8$)	15.86 ± 1.23^{ab}	5.81 ± 0.68^{c}	8.02 ± 1.17^{d}	34.18 ± 1.54^{e}	58.82 ± 3.05^{a}	13.81 ± 0.43^{c}	23.44 ± 1.37^{e}	48.16 ± 1.30^{e}	47.30 ± 1.54^{ab}	2.42 ± 0.40	3.60 ± 0.30	NS
	SG ($n = 8$)	12.13 ± 1.17^{c}	7.74 ± 0.51^{b}	11.34 ± 1.09^{b}	41.45 ± 1.56^{b}	53.54 ± 3.20^{d}	14.65 ± 0.52^{c}	27.39 ± 1.27^{c}	56.24 ± 1.14^{b}	39.34 ± 1.20^{d}	2.05 ± 0.20	3.00 ± 0.20	NS
AT 21st day	CG ($n = 4$)	14.86 ± 0.67^{b}	5.97 ± 0.44^{b}	8.57 ± 0.68^{d}	35.13 ± 0.67^{d}	58.83 ± 2.12^{a}	14.36 ± 0.34^{c}	24.40 ± 0.44^{d}	50.36 ± 0.68^{d}	45.23 ± 0.46^{b}	2.32 ± 0.10	3.80 ± 0.20	NS
	SG ($n = 4$)	9.23 ± 0.48^{d}	8.46 ± 0.37^{a}	13.01 ± 0.53^{a}	44.02 ± 0.54^{a}	52.03 ± 2.09^{d}	15.37 ± 0.41^{b}	29.56 ± 0.39^{b}	60.10 ± 0.57^{a}	36.03 ± 0.57^{e}	2.00 ± 0.10	2.60 ± 0.10	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), ANF – after nephritis formation (после развития нефрита), AT – after treatment (после лечения),
CG – control group (контрольная группа), SG – study group (экспериментальная группа), NS – non-significant (не значимо),
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).

TEST RESULTS

Since the mice had the same date of birth, there was no age difference in the mice ($p > 0.05$). The mean body weight (bw) before the study was calculated as 29.7 g, whereas it was calculated as 28.7 g after the NF, and a significant ($p < 0.05$) reduction was observed in terms of bw averages. On the 21st day of treatment, it was observed that mean bw of CG mice was 28.1 g, while that of SG mice was 29.1 g, and there was a statistically significant differences ($p < 0.05$) between CG and SG animals.

Clinical Findings

The clinical findings obtained during the study are shown in Table 1.

When Table 1 was examined, by nephritis formation, significant ($p < 0.05$) changes were seen in terms of body temperature, respiration and heart frequencies. So, mean R rates and P frequencies increased with the start of the treatment period, but this increase was more significant ($p < 0.05$) in the study group mice.

Hematological Findings

Hematological examination findings are shown in Table 2.

According to this table; NEUT, MCV, WBC levels were increased statistically significant ($p < 0.05$) following NF, whereas HCT, HB, RBC, LYM, MCHC and MCH levels significantly decreased ($p < 0.05$). On the 21st day of the study, it was found that LYM, HCT, RBC, HB, MCH and MCHC levels increased in both groups, but NEUT, MCV, WBC levels decreased. These changes were statistically more significant ($p < 0.05$) in SG animals.

Blood Biochemical Findings

The blood biochemical analysis were shown in Table 3.

According to this Table; it was observed that GGT, AST, BUN, GLU and IgG levels increased significantly ($p < 0.05$) after NF, while ALB, TP and levels decreased significantly ($p < 0.05$). On the contrary, in the following days, TP, ALB levels were increased in both groups, whereas GGT, AST, BUN, GLU and IgG levels were decreased ($p < 0.05$). It was observed that the most significant differences occurred in SG mice in the last week of the study, and this difference was statistically significant ($p < 0.05$).

Blood Gases Findings

Blood gases analyses results were shown in Table 4.

When this table is examined, it was observed that pCO_2, pH, HCO_3^-, BE, TCO_2, Ca^{2+} and K^+ levels decreased

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)	TP (g/dl)	ALB (g/dl)	GLU (g/dl)	BUN (mmol/L)	IgG (mg/ml)
BS ($n = 40$)		96.72 ± 12.38^{h}	3.63 ± 0.56^{e}	53.96 ± 5.47^{b}	33.78 ± 2.34^{a}	172.15 ± 0.69^{d}	21.74 ± 3.25^{f}	3.17 ± 1.45^{e}
ANF ($n = 36$)		269.17 ± 32.45^{a}	8.96 ± 3.43^{a}	34.06 ± 6.45^{f}	21.08 ± 3.44^{e}	275.27 ± 0.56^{a}	36.48 ± 5.23^{a}	8.16 ± 3.57^{a}
AT 1st day	CG ($n = 16$)	272.13 ± 42.34^{a}	8.97 ± 3.71^{a}	34.04 ± 6.48^{f}	21.01 ± 3.66^{e}	208.13 ± 0.30^{a}	37.04 ± 6.18^{a}	8.45 ± 3.44^{a}
	SG ($n = 16$)	263.12 ± 38.32^{ab}	8.78 ± 3.58^{a}	34.69 ± 5.17^{f}	22.23 ± 3.32^{d}	203.21 ± 0.44^{b}	36.91 ± 6.53^{a}	7.79 ± 3.08^{b}
AT 7th day	CG ($n = 12$)	253.25 ± 23.21^{b}	8.61 ± 2.45^{a}	34.23 ± 4.31^{f}	22.16 ± 2.54^{d}	196.11 ± 0.48^{b}	36.76 ± 4.21^{a}	8.17 ± 2.14^{a}
	SG ($n = 12$)	231.32 ± 19.34^{c}	7.41 ± 2.32^{b}	42.14 ± 4.32^{d}	26.34 ± 2.56^{c}	178.14 ± 0.53^{c}	28.64 ± 4.06^{d}	6.10 ± 2.03
AT 14th day	CG ($n = 8$)	234.07 ± 14.54^{c}	8.57 ± 1.37^{ab}	36.12 ± 3.21^{ef}	23.02 ± 1.21^{cd}	187.35 ± 0.48^{c}	34.17 ± 2.34^{b}	7.78 ± 1.34^{b}
	SG ($n = 8$)	162.44 ± 15.16^{f}	5.73 ± 1.44^{c}	48.16 ± 3.09^{c}	30.94 ± 1.41^{b}	173.43 ± 0.53^{d}	24.13 ± 2.11^{e}	4.32 ± 1.27^{d}
AT 21st day	CG ($n = 4$)	213.47 ± 10.21^{d}	7.32 ± 0.65^{b}	38.03 ± 1.53^{e}	25.21 ± 0.58^{c}	183.22 ± 0.10^{c}	30.14 ± 1.08^{c}	6.48 ± 0.65^{c}
	SG ($n = 4$)	103.34 ± 8.11^{g}	3.98 ± 0.59^{d}	56.05 ± 1.48^{a}	33.35 ± 0.49^{a}	166.17 ± 0.10^{e}	20.83 ± 0.53^{f}	3.15 ± 0.51^{e}

[a–h] The values in the column are statistically significant ($p < 0,05$).
[a–h] Значения в столбце являются статистически значимыми ($p < 0,05$).
BS – before study (до исследования), ANF – after nephritis formation (после развития нефрита),
AT – after treatment (после лечения), CG – control group (контрольная группа), SG – study group (опытная группа).
AST – aspartate aminotransferase (аспартатаминотрансфераза),
GGT – gamma-glutamyl transferase (гамма-глутамилтрансфераза), TP – total protein (общий белок),
ALB – albumin (альбумин), GLU – glucose (глюкоза), BUN – blood urea nitrogen (содержание азота мочевины в крови),
IgG – immunoglobulin G (уровень иммуноглобулина G).

Table 4
Blood gases findings of the animals
Таблица 4
Результаты исследования газового состава крови

Time of indicator measurement by groups			Parameters (X ± SD)							
			pH	pCO_2 (mmHg)	HCO_3^- (mmol/L)	BE (mEq/L)	TCO_2 (mmol/L)	LACT (mmol/L)	K^+ (mmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Ca^{2+} (mmol/L)
BS (n = 40)			7.32 ± 0.03^{b}	44.03 ± 0.42^{a}	21.91 ± 0.57^{b}	–3.3 ± 0.28^{b}	15.41 ± 1.58^{b}	4.68 ± 1.47rd	8.78 ± 2.36^{a}	163.28 ± 14.18^{f}	117.34 ± 12.43^{g}	10.68 ± 2.09^{a}
ANF (n = 36)			7.22 ± 0.02^{d}	32.78 ± 2.14^{e}	13.02 ± 1.63^{de}	–13.1 ± 0.45^{f}	11.14 ± 2.27^{d}	7.03 ± 2.05^{b}	5.69 ± 2.21^{d}	264.32 ± 17.23^{a}	196.48 ± 13.44^{a}	6.47 ± 2.12^{c}
AT 1st day	CG (n = 16)		7.22 ± 0.02^{d}	32.68 ± 2.32^{e}	12.91 ± 1.43^{e}	–13.2 ± 0.44^{f}	12.05 ± 2.29^{c}	7.15 ± 2.28^{a}	5.58 ± 2.43^{d}	267.31 ± 13.21^{a}	193.57 ± 14.44^{a}	6.51 ± 2.25^{c}
	SG (n = 16)		7.23 ± 0.03^{d}	33.07 ± 2.48^{d}	13.64 ± 1.44^{e}	–12.4 ± 0.35^{e}	11.07 ± 2.33^{d}	6.87 ± 2.13^{ab}	5.31 ± 1.47^{d}	253.36 ± 18.25^{ab}	182.37 ± 12.24^{b}	6.89 ± 2.38^{c}
AT 7th day	CG (n = 12)		7.24 ± 0.02^{d}	33.78 ± 2.23^{d}	14.02 ± 0.68^{de}	–11.9 ± 0.34^{de}	11.70 ± 1.35^{a}	6.49 ± 1.68^{b}	5.37 ± 1.13^{d}	248.22 ± 15.12^{b}	181.38 ± 11.47^{b}	6.96 ± 1.43^{c}
	SG (n = 12)		7.30 ± 0.02^{bc}	37.48 ± 2.15^{bc}	17.82 ± 0.49^{c}	–7.5 ± 0.21^{c}	11.56 ± 1.47rd	4.78 ± 1.37^{c}	6.22 ± 1.37^{c}	214.25 ± 13.27^{d}	158.34 ± 9.33^{d}	8.02 ± 1.35^{b}
AT 14th day	CG (n = 8)		7.27 ± 0.02^{c}	35.12 ± 1.54^{c}	15.61 ± 0.43^{d}	–10.1 ± 0.17^{d}	12.11 ± 0.66^{c}	6.54 ± 0.67^{b}	5.91 ± 0.68rd	225.34 ± 11.11^{c}	164.33 ± 8.13^{c}	7.17 ± 0.76^{bc}
	SG (n = 8)		7.34 ± 0.03^{b}	42.02 ± 1.13^{b}	21.89 ± 0.31^{b}	–3.2 ± 0.14^{b}	14.80 ± 0.47rd	4.22 ± 0.53^{c}	7.53 ± 0.67^{b}	178.35 ± 10.28^{d}	121.31 ± 7.35^{f}	9.49 ± 0.77^{ab}
AT 21st day	CG (n = 4)		7.30 ± 0.02^{bc}	36.87 ± 0.56^{bc}	17.52 ± 0.23^{d}	–7.7 ± 0.10^{c}	12.83 ± 0.39^{c}	6.04 ± 0.38^{b}	6.18 ± 0.43^{c}	203.44 ± 6.16^{e}	147.34 ± 5.14^{e}	8.06 ± 0.45^{b}
	SG (n = 4)		7.39 ± 0.02^{a}	44.13 ± 0.47^{a}	25.79 ± 0.19^{a}	–1.3 ± 0.10^{a}	15.82 ± 0.28^{a}	3.89 ± 0.39^{d}	8.99 ± 0.33^{a}	147.34 ± 5.22^{g}	103.42 ± 3.28^{h}	10.84 ± 0.38^{a}

$^{a-h}$ The values in the column are statistically significant (p < 0,05).
$^{a-h}$ Значения в столбце являются статистически значимыми (p < 0,05).
BS – before study (до исследования), ANF – after nephritis formation (после развития нефрита), AT – after treatment (после лечения),
CG – control group (контрольная группа), SG – study group (опытная группа).
pH – hydrogen ion concentration (концентрация ионов водорода), pCO_2 – CO_2 partial pressure (парциальное давление углекислого газа),
HCO_3^- – bicarbonate (бикарбонат), BE – base excess (сдвиг буферных оснований), TCO_2 – total CO_2 (общая концентрация углекислого газа),
LACT – lactate (лактат), K^+ – potassium (калий), Na^+ – sodium (натрий), Cl^- – chlorine (хлор), Ca^{2+} – calcium (кальций).

following NF, whereas LACT, Na^+ and Cl^- levels increased. However, with the onset of treatment period, a reverse course has taken shape in these parameters. On the other hand, it was observed that the most important changes in terms of these parameters occurred in SG animals in the last week of the study, and the difference was statistically significant (p < 0.05).

Histopathological Findings

In our current study, severe glomerulonephritis was detected in tissue sections taken from nephritic mice in which infiltration detected in the vascular lumen (Fig. 1).

It was found that there was almost a complete improvement in SG animals which treated with hot spring water at the end of the treatment period (Fig. 2), while CG animals which treated with tap water continued to show histopathological symptoms of nephritis in their kidney tissue sections (Fig. 3).

DISCUSSION AND CONCLUSION

In this study, it was found that the mean of bw decreased with the NF, and this difference was found to be statistically significant (p < 0.05). On day 21st of the experiment the average bw of the CG mice was reported to be lower as compared to that of the SG mice (p < 0,05). These findings have been found to be compatible with study U. Lange et al., which reported that treatment with mineral waters supports fat breakdown and decreases the absorption of fat from the intestines, leading to a decrease in live weight [11].

It was found that the body temperatures increased in mice with nephritis, when compared to the pre-study, and this rising was statistically significant (p < 0.05). However, there was no significant (p > 0.05) changes in terms of R and P. With the initiation of treatment; it was observed that the levels for R and P increased in both groups, but the highest levels were shaped in SG animals. These findings support the findings of the researchers P. Greco-Otto et al., who reported that spa treatment increased heart rate, improvement in peripheral vessels, and an increase in heart and indirectly respiration rate [12]. Moreover, hot baths have been reported to stimulate the sympathetic nervous system, leading to an increase in blood pressure, heart and respiratory frequency [13].

It was observed that mean NEUT, WBC and MCV counts were continuing in high levels in CG animals, while SG mice which received Süreyya I hot spring water and bathed have a decreased. So, it has been reported that hyperthermal waters have an immunosuppressive effect and T-lymphocyte and EOS numbers decrease significantly in hyperthermal baths in both healthy individuals and those with chronic inflammatory disease [14].

It was found that mean RBC, HB, HCT, MCH and MCHC counts decreased statistically (p < 0.05) in the period following NF. It has been previously reported that these parameters decrease, the anemia table is shaped after nephritis, and this is due to a decrease in the production of erythropoietin produced in kidneys in rats with kidney damage caused by adenine [15]. The improvement in the red blood cell index after treatment in SG animals proves that Süreyya I hot spring water is extremely effective in terms of healing of kidney damage and anemic parameters. Furthermore, Süreyya I hot spring water is rich in Mg, and the inflammatory markers were low levels in SG animals by starting treatment period.

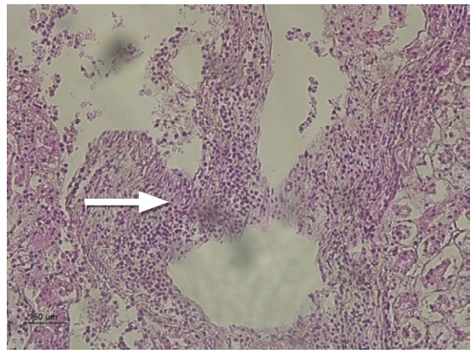

Fig. 1. Severe glomerulonephritis (white arrow), 10×–40×

Рис. 1. Выраженный гломерулонефрит (белая стрелка), увеличение 10×–40×

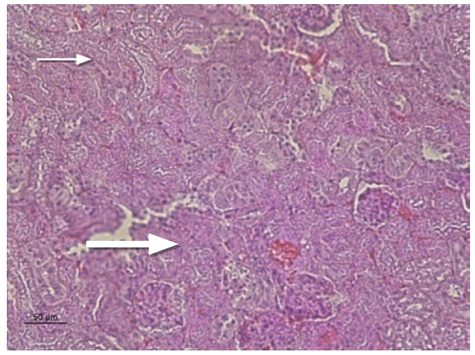

Fig. 2. Completely healed tubule and glomerul structure at the end of day 21 in study group animals (tubule – thin arrow, glomerul – thick arrow), 10×–20×

Рис. 2. Восстановление структуры канальцев и клубочков на 21-е сут у животных экспериментальной группы (канальцы – тонкая стрелка, клубочки – толстая стрелка), увеличение 10×–20×

As known, magnesium deficiency is one of the reasons specific clinical inflammatory syndrome, because its deficiency causes dyslipidemia, hypertension, insulin resistance, endothelial activation and prothrombic differences with the regulation of inflammation and oxidative stress markers [16].

In the current study, it was observed that GGT, AST, BUN and IgG levels increased significantly (p < 0.05) following NF, whereas ALB, TP and GLU levels decreased (p < 0.05). On the contrary, it was found that ALB, TP and GLU levels increased, whereas GGT, AST, BUN and IgG levels decreased by starting treatment period. On the other hand, the most important changes were seen in SG animals, and the difference was statistically significant (p < 0.05). Our these findings has been supported by the findings of researchers C. Pereira et al., who reported that UREA, ALT, AST, CREA levels decreased and TP and ALB levels increased as a result of consuming mineral

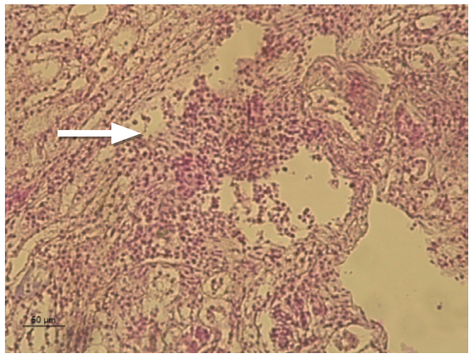

Fig. 3. Continuation of the glomerulonephritis structure at the end of day 21 in control group animals (white arrow), 10×–20×

Рис. 3. Гистопатологические признаки гломерулонефрита на 21-е сут в срезах почечной ткани животных контрольной группы (белая стрелка), увеличение 10×–20×

water in the experimental metabolic syndrome experimentally created by fructose [16]. Even though albumin levels decreased significantly following nephritis, it was thought to be related to liver damages, but it also indicated that there was no reabsorption from kidney tubules [17].

It has been explained that balneotherapy can be effectively applied to lower blood sugar levels. It has been reported that these beneficial effects of the balneotherapy might be related to its ability to control high blood sugar that causes glucose tolerance [18]. In the current study, GLU levels were high in nephritis-induced mice, but with the onset of treatment, the gradually decrease in GLU levels, the most important decreases were detected in SG mice, and these findings were consistent with these researchers reported above.

Unfortunately, we did not find a study measuring blood gases in the treatment of nephritis with hot spring waters in widely literature searches. In our study, it was observed that LACT, Cl^- and Na^+ levels increased significantly ($p < 0.05$) following NF, whereas Ca^{2+} and K^+ levels decreased. After the treatment was initiated, the opposite results were obtained, and it was determined that LACT, Cl^- and Na^+ levels decreased, while Ca^{2+} and K^+ levels increased. Compared with CG, these changes were seen to be faster and statistically more significant ($p < 0.05$) in SG animals. Hypercalcemic and hyperchloremic metabolic acidosis occurs after nephritis. Blood pH, HCO_3^- and pCO_2 levels decrease, Cl^-, Na^+ and LACT levels increase [19]. In addition to kidneys and lungs, mineral waters of suitable nature have been reported to provide acid-base balance in the liver and regulate the metabolism of LACT and some amino acids [20]. In the study, it was determined that the parameters mentioned in the 21st day of SG animals which treated with Süreyya I hot spring water, and reached physiological levels, and the metabolic acidosis case improved. So, L. Xu et al. previously reported that LACT levels significantly improved after 21 days of spa treatment in studies conducted in individuals living under bad conditions [19].

In our current study, severe glomerulonephritis was detected in the tissue sections of the kidneys of the mice with nephritis along with inflammatory infiltration in the vascular lumen (Fig. 1). Although a progressive improvement was observed in SG which treated with Süreyya I hot spring water at the end of this study (Fig. 2), it was observed that the nephritis picture continues in CG animals treated with tap water. These histopathological findings prove that the Süreyya I hot spring water provides significant improvements in the nephritis case histopathologically as well as clinical, hematological, blood biochemical and blood gasses findings.

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Рецензия на монографию «Африканская чума свиней в условиях особо охраняемых природных территорий (опыт Воронежского заповедника)». Б. В. Ромашов, Н. Б. Ромашова, Е. А. Стародубцева, А. С. Мишин.

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African swine fever (ASF) epidemic situation in the Russian Federation as well as its tendencies for the whole infection period are persistently specified by "positive" dynamics both in the domestic pigs and in wild boars being at a relatively close ratio of 1.5:1. This clearly suggests the need of urgent change of the attitude to the issue as well as its comprehensive solutions. Clearly prescribed measures compliant with the previous instruction of 1980 and rules approved in 2016 are used for ASF control in the domestic pigs. As for the wildlife, there is a kind of collision and up to confrontation between the biologists, game managers, ecologists, animal advocates and national services involved in the implementation of the anti-epidemic measures. Herewith, the first ones demonstrate total ignorance of epizootology and parasitology canons; they absolutely disregard everything coming from the veterinary science, accuse the veterinary services of all troubles and resort to empty rhetoric lacking any line of reasoning; and all that is echoed by ratings-hungry mass media and even some research journals.

Unfortunately, among the domestic publications only three or four authoritative research papers consider epizootological investigation of "boar – ASF" issue that is significantly lower in number as compared to the ambitious printed materials of the opposition. In this regard, publication of the monograph by B. V. Romashov et al. is just a gift, and extremely well-timed one. Without going into details of the monograph, general and specific ideas should be mentioned that are deemed the most notable from the reviewer's point of view.

As usual in such cases, general statistic data and specific features of the tested object (i.e. large isolated group of boars generally having the properties of the ideal (panmictic) population) were used as the primary, benchmark data for the epizootological analysis. The authors demonstrate actual scientific and practical experience of ASF experts' field operations, which significance (not scale) can, say the least of it, be compared with the disease eradication activities in three regions of the USSR in 1977.

This is due to the unique properties of this epizootic event – i.e. the fact of the development of the complete natural cycle of the large-scale epizootic wave without any outside intervention (it is commonly known that there is no vaccination and the final diagnosis is made nearly *post factum*). The point is that for five months in 2016 (March – July) total mortality of the boars occurred on 32 ths ha area of the Voronezh natural reserve due to ASF introduction and spread. The initial boar population amounted to 532 animals and the average population density amounted to 16.6 animals/1000 ha. The boars actively used the feeding stations and accumulated mostly in the bottomland area of the reserve. The situation development was specified by the typical epizootic curve. The results obtained during this unique and objectively documented and published research can serve as an example of the description of the natural ASF epidemic.

Even the first glance through the publication provides much food for thoughts, questions and objections in the best sense of the term. Here are just some of them.

As a discussion, one cannot agree with the authors' conclusion on the impossibility of stable natural ASF circulation in boars due to high virulence of the agent and the boars' minor role in ASF epizootology (pp. 4, 16, 17 et seq.). Indeed, the incidence of the natural infection reported in the Russian Federation is mostly sporadic and lacks evident temporal or spatial continuity; although, the veterinarians are not inclined to consider the data submitted by game management authorities to be reliable. However, the situation is quite opposite in the ASF-infected countries in Central Europe, where the boar population density is relatively high: the endemicity is associated with the boar morbidity with rare, even sporadic, index-cases in domestic pigs occurring on 9:1 ratio as well as with described hallmarks of the evolution to at least the disease chronicity and moderate virulence of genotype II virus (see, for example: https://doi.org/10.1186/s40813-018-0109-2).

According to multiple foreign analytical publications, the evidence of the boar being the only reservoir of the infection is not even discussed. Rare cases of domestic pigs' involvement in the epidemic process are also not considered to be a problem. One can hardly imagine the human factor to play any role with such a pattern of the epidemic process. Moreover, nearly complete boar depopulation (97.5%) in Lithuania allowed prevention of ASF spread in the country in 2014–2017 as compared to Latvia and specifically Estonia, who ignored the boar depopulation at proper time.

This is also true of the early conclusion stating "there are no biological and ecological prerequisites for ASFV hotspots" (p. 99). How can then be explained the fact that clusters of natural infection occurred in the Novgorod, Nizhny Novgorod and Oryol Oblasts of the Russian Federation in 2019, and they were specified by natural nodality, chronological and spatial distinctness, minimum one year endemic stability and sporadic index-outbreaks in the backyards?

It is the first time when the publication on ASF describes possible fate of the diseased boars and specifically emphasizes the loss of migration instinct. While observing such mortality and in order to control the natural infection, it would be important to have reliable information about dominating natural environment chosen by the infection victims for their departure (there are already some data on this issue in the foreign publications).

The vector "domestic outbreak → wild fauna" is not covered even hypothetically, except for singular reference to the fact that boars are infected from the dumped pig carcasses. In the conclusion the authors use the concept "anthropopressure" for that, i.e. abstract parasitological term, which appears to be of virtual character. Until this vector is considered, all arguments about epizootological safety of boars are just scholastic.

Widespread and quite obvious euphemism stating that "boar is one of the key components of fauna and biodiversity" remains unclear. By the way, before the mid-XXth century there were no boars at all in the USSR with minor exceptions of the southern part of the country. What is specific advantage of the further spread of the boars all over the country? And how one should understand this if in the Russian Federation boar is considered to be an invasive, i.e. environmentally harmful species, but in such authoritative document as Agroecological atlas of Russia and bordering countries it is mentioned as game and pest animal (for illustration see: https://www.youtube.com/watch?v=1iCePTe178w).

There are several editorial shortcomings. For example, in the description of ASF virus: DNA is located not inside the supercapsid envelope, but inside the capsid. The term "virus volatility" is not correct, as ASF cannot be transmitted by air. The list of references is extremely limited and mostly includes domestic authors, while in the Baltic States and Poland there are many published reports about ASF epizootology researches with authoritative conclusions.

All the above mentioned should be considered as the reviewer's opinion, who meanwhile highly appreciates multiple and often critical references to his own publications in the monograph.

As a treatise, the book by B. V. Romashov et al. as a whole complies with the current standards, efficiently compiled and written in good language. Detailed illustrations are also an upside of the monograph.

The monograph is actually unique and rich in facts novel for science and practice both in general and specific epizootological context. The unique experience deserves attention of everybody engaged in veterinary epidemiology, natural nodality, infection emergence and veterinary education.

Publications of the FGBI "ARRIAH" researchers in foreign journals included in the abstract and citation databases, 2018–2020.

Subject: "Lumpy skin disease"

Публикации сотрудников ФГБУ «ВНИИЗЖ» в зарубежных научных журналах за 2018–2020 гг., включенных в международные реферативные базы данных и системы цитирования.

Тема: «Заразный узелковый дерматит крупного рогатого скота»

1. Pestova Y., Byadovskaya O., Kononov A., Sprygin A. A real-time high-resolution melting PCR assay for detection and differentiation among sheep pox virus, goat pox virus, field and vaccine strains of lumpy skin disease virus. *Mol. Cell. Probes*. 2018; 41: 57–60. DOI: 10.1016/j.mcp.2018.08.003.

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3. Sprygin A., Pestova Y., Prutnikov P., Kononov A. Detection of vaccine-like lumpy skin disease virus in cattle and *Musca domestica* L. flies in an outbreak of lumpy skin disease in Russia in 2017. *Transbound. Emerg. Dis*. 2018; 65 (5): 1137–1144. DOI: 10.1111/tbed.12897.

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Торговое наименование лекарственного препарата: Вакцина против пастереллеза крупного рогатого скота инактивированная эмульсионная

Международное непатентованное, или группировочное, или химическое наименование лекарственного препарата: Вакцина против пастереллеза крупного рогатого скота инактивированная эмульсионная

Лекарственная форма: эмульсия для инъекций

Дозировка: 10 см³ (10 доз), 50 см³ (50 доз) и 100 см³ (100 доз)

Регистрационное удостоверение выдано бессрочно, со сроком действия 5 лет
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М.П.

The vaccine is produced from inactivated suspension of bacterial strains *Mannheimia haemolytica* serotype A:1, *Pasteurella multocida* serogroup A and anatoxin *Mannheimia haemolytica* with oil-based adjuvant Montanide (SEPPIC, France). Components of the vaccine, which is unrivalled at the Russian market of veterinary preparations, were chosen to match bovine pasteurellosis serovariants circulating in the Russian Federation. The vaccine also includes anatoxin that plays two key roles in the process of disease progression. First, it protects bacterial cells by leukolysis and, second, it releases neutrophil substances causing lung tissue damage.

Clinical and preclinical studies of the inactivated emulsion vaccine against bovine pasteurellosis (carried out in accordance with Federal Law No. 61-FZ "On Circulation of Medicines" and in compliance with the OIE recommendations) confirmed its innocuity and efficacy. High level of protection from pasteurellosis in immunized population was demonstrated on livestock farms of the Russian Federation.

Nowadays, respiratory problems in young cattle are still among the most urgent and economically significant problems of the veterinary medicine. Pasteurellosis is a widespread infectious disease, its treatment is difficult, expensive and it is not always successful. It is explained by wide circulation of antibiotic resistant strains of *Mannheimia haemolytica* and *Pasteurella multocida* and by difficulties in reaching the required inhibition concentration of the preparation in lungs; therefore, vaccination is the key element in prevention and control of pasteurellosis.

Calves on affected farms should be vaccinated after they reach 10 days of age; twice and with a 3–4 week interval. On farms at risk of pasteurellosis and in case the disease may occur during transportation and when animals are introduced into a new herd, or into different animal facilities, the animals shall be vaccinated once, 3 weeks before the anticipated risk. The vaccine is administered at the dose of 1.0 cm^3 via the intramuscular route into the middle third of the neck.

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