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AND PHYTOSANITARY SURVEILLANCE
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Monkeypox and other orthopoxvirus zoonoses

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SUMMARY

The paper highlights the current knowledge on infection biology, epidemiology and evolution of monkeypox virus (MPXV), cowpox virus (CPXV), buffalopox virus (BPXV), camelpox virus (CMLPV), as well as addresses some factors that modulate dynamics of orthopoxvirus transmission, manifestation of orthopoxvirus infections and their preservation in nature. Despite the elimination of the historically infamous smallpox, orthopoxviruses remain a serious veterinary and health problem. Their role is currently increasing while the number of persons not immune to smallpox grows. Along with this, there is a genetic transformation of pathogens. In this regard, the risks of human infection with orthopoxviruses of zoonotic nature are increasing. The problem of monkeypox, cowpox, buffalopox and camelpox and the respective agents included in the genus of zoonotic orthopoxviruses presents the greatest interest. Along with the increased number of human monkeypox cases in 2020–2022, a retrospective analysis of the last 20 years shows that the activity of monkeypox outbreaks in the XXI century intensified in Central African countries. Cowpox outbreaks in Europe and camelpox outbreaks in Southwestern and Central Asia have also become more active. In 2011, in India, the camelpox virus overcame the interspecies barrier and caused a clinical pox-like disease in humans. Scientists are alarmed by these facts as the camelpox virus genome is 99% homologous to the genome of the smallpox virus. This requires strengthening the epizootological and epidemiological monitoring of orthopoxvirus zoonotic pathogens.

Keywords: review, monkeypox, cowpox, buffalopox, camelpox, orthopoxviruses, zoonoses

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Оспа обезьян и другие ортопоксвирусные зоонозы

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РЕЗЮМЕ

В представленной работе освещено текущее состояние знаний, касающихся биологии инфекции, эпидемиологии и эволюции вируса оспы обезьян (MPXV), оспы коров (CPXV), оспы буйволов (BPXV), оспы верблюдов (CMLPV), а также некоторые факторы, которые модулируют динамику передачи ортопоксвируса, проявление ортопоксвирусных инфекций и их сохранение в природе. Несмотря на ликвидацию исторически печально известной натуральной оспы, ортопоксвирусы остаются серьезной проблемой ветеринарии и здравоохранения. Их роль в настоящее время возрастает на фоне увеличения количества людей, которые не имеют иммунитета против натуральной оспы. Наряду с этим наблюдается генетическая трансформация возбудителей, что становится причиной роста рисков поражения человека ортопоксвирусами зоонозной природы. Наибольший интерес представляет проблема оспы обезьян, оспы коров, оспы буйволов и оспы верблюдов, возбудители которых входят в род зоонозных ортопоксвирусов. На фоне учащения проявления случаев заболевания человека оспой обезьян в 2020–2022 гг. ретроспективный анализ последних 20 лет показывает, что активность очагов оспы обезьян в XXI в. возрастала в государствах Центральной Африки. Также активизировались очаги оспы коров в Европе, оспы верблюдов в Юго-Западной и Центральной Азии. В 2011 г. в Индии вирус оспы верблюдов преодолел межвидовой барьер и вызвал клиническую оспоподобную форму заболевания у человека. Подобные факты тревожат ученых, так как геном вируса оспы верблюдов на 99% гомологичен геному вируса натуральной оспы. Это требует усиления эпизоотологического и эпидемиологического мониторинга за возбудителями ортопоксвирусных зоонозов.

Ключевые слова: обзор, оспа обезьян, оспа коров, оспа буйволов, оспа верблюдов, ортопоксвирусы, зоонозы

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INTRODUCTION

Why do poxviruses rank high as potential viral threats? This family includes many pathogens that affect both vertebrate (including humans) and invertebrate representatives of the animal kingdom. Despite eradication of the infamously known smallpox, the poxviruses belonging to the genus *Orthopoxvirus* and causing serious zoonotic diseases remain a challenge in veterinary medicine and healthcare. This review summarizes general characteristics of orthopoxviruses, as well as addresses current and future threats posed by these viruses to humans, domestic and wild animals. In-depth studies of this genus' representatives allow expanding fundamental biological knowledge and understanding the methodological approaches to prevention and control of other infectious diseases of zoonotic nature [1].

Currently, there is an increase in the number of humans worldwide that are not immune to smallpox, along with genetic transformation of zoonotic pathogens of orthopoxvirus nature. This increases the risk of infection in humans. Another risk factor is the ability of poxviruses to overcome the species barrier, as it occurred in case of monkeypox virus [2–7]. A retrospective analysis carried out within the last 20 years shows that the activity of smallpox outbreaks in monkeys increased in the XXI century in African countries [8, 9]. Outbreaks of cowpox in Europe [2, 6, 7], buffalopox [4, 10–12, 15] and camelpox in Southwest and Central Asia [13, 14] also intensified. In 2011, in India, the camelpox virus overcame the interspecies barrier and caused a clinical pox-like form of the disease in humans [16–19]. Scientists are alarmed by these facts [20–22], as the camelpox virus genome is 99% homologous to that of the smallpox virus [23]. Multiple mutations were identified in some genes, including the C18L gene responsible for the host species' gene [24].

GENERAL CHARACTERISTICS OF ORTHOPOXVIRUSES

The family *Poxviridae* consists of two subfamilies: *Chordopoxvirinae* (poxviruses of vertebrates) and *Entomopoxvirinae* (poxviruses of insects). The subfamily *Chordopoxvirinae* is represented by large DNA-viruses of a brick-like or ovoid shape. They are grouped into genera and infect mammals, with the exception of *Avipoxvirus* (infect specifically birds) and *Crocodylidpoxvirus* (crocodiles serve as natural hosts). The genus *Parapoxvirus* is isolated and its members possess a unique spiral envelope that distinguishes them from other poxviruses (Orf virus, bovine papular stomatitis virus and sealpox virus). Agents of molluscum contagiosum and currently eradicated smallpox are the only poxviruses that have humans as their host and reservoir [25].

The best-known is the genus *Orthopoxvirus*, which includes vaccinia virus, as well as monkeypox, cowpox, buffalopox, camelpox viruses and some other orthopoxviruses [25–28].

According to the IX International Committee on Taxonomy of Viruses (ICTV) Report (2012), the genus *Orthopoxvirus* included 11 virus species (see the Table).

The taxonomy of the genus *Orthopoxvirus* is constantly updated. In the XXI century the new representatives of orthopoxviruses were detected in North America – *Skunkpox virus* (SKPV), and in Africa – *Uasin Gishu disease virus* (UGDV), which was named after the Kenyan province. In 2010, 2015 and 2017 three more new members of the genus *Orthopoxvirus* were identified in Georgia (Akhmeta and Van regions), USA (Alaska) and Italy: *Akhmeta virus*, *Alaskapox virus*, and feline poxvirus, respectively [29–32]. The emergence of mutated animal orthopoxviruses that are similar to the smallpox virus cannot be excluded [33].

In 2018, foreign researchers developed the first complete synthesis of a horsepox virus, the work results were published in the *PLoS ONE* [34].

To date, complete nucleotide sequences of orthopoxviruses genome have been determined – they are deposited in the GenBank international database. It should be noted that the first full-genome sequencing of the smallpox virus isolated in India in 1967 was carried out by researchers of the State Scientific Centre of Virology and Biotechnology "VEKTOR" [35–40]. Babkin I. V. determined the nucleotide sequence of hemagglutinin and fusion protein genes for various strains of the genus *Orthopoxvirus*. To develop molecular methods for the diagnosis and differentiation of orthopoxviruses, they were offered to use the sequence of the A27L gene encoding a conserved virion protein [26].

The divergence of poxviruses from the original virus and separation into modern genera began about 500 thousand years ago. The *Orthopoxvirus* progenitor might emerge about 300 thousand years ago. Gradually, various species began to emerge within the genus (Fig. 1) [23, 24]. Calculations showed that species evolutionarily close to the smallpox virus – camelpox virus and taterapoxvirus – separated from a common ancestor (apparently rodent virus) about (3.4 ± 0.8) thousand years ago. In the process of evolution the genus *Orthopoxvirus* split into two main branches. At the same time, the genetic picture of evolution is very diverse and differs significantly for individual orthopoxviruses [23, 24, 33, 41, 42].

Orthopoxviruses multiply in cell cytoplasm, and there are several stages of replication in the cells of infected animals that are described in detail and are practically the same for different representatives of the genus [43–45].

These viruses are sensitive to various disinfectants, including solutions of 1% sodium hypochlorite, 1% sodium

Table
Classification of orthopoxviruses [29]

Members of the genus *Orthopoxvirus* based on taxonomy of viruses			
1991			
Variola virus (VARV); *Monkeypox virus* (MPXV); *Cowpox virus* (CPXV); *Camelpox virus* (CMLPV); *Ectromelia virus* (ECTV); *Vaccinia virus* (VACV) (subspecies: *Buffalopox virus*, *Rabbitpox virus*); *Raccoonpox virus* (RCN); *Taterapox virus* (TATV)			
	1995		
	Volepox virus (VPXV)		
		2000	
		Uasin Gishu disease virus (UGDV) – named after the Kenyan province, affects horses*	
			2010
			Skunkpox virus (SKPXV)
Total 8 species	Total 9 species	Total 10 species	Total 11 species

* not been approved as species.

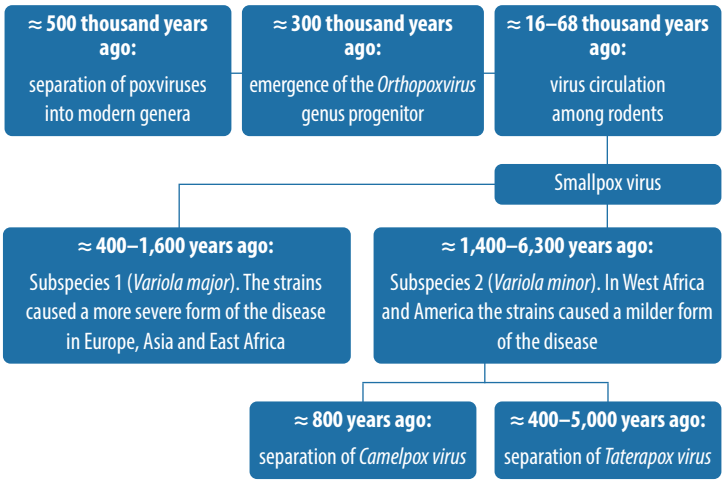

Fig. 1. Evolution of poxviruses (based on [33])

hydroxide, 1% peracetic acid, formaldehyde, 0.5–1% formalin and 0.5% quaternary ammonium compounds. They are destroyed by autoclaving or boiling for 10 minutes, as well as if exposed to ultraviolet light [39, 46].

It is believed that the monkeypox, cowpox, buffalopox and camelpox viruses are genetically similar, might infect humans and induce cross-immunity [26–28].

MONKEYPOX

Some infectious diseases occurring in monkeys pose a danger to humans and other animals [47–51].

Monkeypox is a zoonotic disease caused by the monkeypox virus (MPXV) (Fig. 2), genome of which is represented by double-stranded DNA. MPXV belongs to the family *Poxviridae*, the genus *Orthopoxvirus* [48].

This disease is endemic in some Central and West African countries. The circulation of the virus among wild animals was established, monkeypox cases were recorded in humans in Africa and countries outside the African continent [52–53].

The natural MPXV reservoir and transmission mechanism have not been definitively identified. Infection occurs aerogenically (by airborne droplets), orally, via damaged skin. Diseased primates (12 species), virus-infected humans, rodents are the sources of infection [52]. The disease is characterized by intoxication syndrome, vesicular-pustular rash on skin and mucous membranes [48]. Unfortunately, the collected data on this disease in monkeys are limited and scattered.

The pathogen was first isolated in 1958 at the State Serum Institute in Copenhagen from a crab-eating macaque with pustular rashes on the skin, and designated as a monkeypox virus [54, 55].

The virus is contagious, causes disease in almost all simian species and can infect other animals, such as ground squirrels (*Spermophilus tridecemlineatus*), black-tailed prairie dogs (*Cynomys ludovicianus*), Kellen's dormouse (*Graphiurus kelleni*), mice, steppe marmots (*Marmota bobak*). In Africa, MPXV is found in many animal species, such as striped squirrels, tree squirrels, Gambian rats, striped mice. The pathogen immunologically cross-reacts with other orthopoxviruses, but has specific antigens that are detected using monoclonal antibodies. The monkeypox clinical picture varies in different simian species. The disease is most severe in orangutans. Green monkeys develop a disease of moderate severity; rhesus macaque, hamadryas baboons and chimpanzees have a mild form of infection. The incubation period in animals experimentally infected parenterally ranged from 3 to 8 days [54].

In natural conditions the incubation period lasts 10 days. The disease begins acutely: the temperature rises, cough develops, lethargy and loss of appetite are observed. Generalized lymphadenopathy often develops by the end of the first week, lasting up to 3 weeks. At day 3–14 papular rashes are found on the skin and mucous membranes, undergoing ulceration with spread to the lips, eyelids, mucous membranes of the mouth and pharynx. Later, a papule turns into a pustule. The crusts are formed and disappear on day 21, scars appear. Mortality ranges from 3 to 40% (in orangutans) [27, 50, 55].

Laboratory diagnosis is based on molecular biological (PCR), immunochemical (various modifications of ELISA), virological (virus isolation in cell culture, using the chorioallantoic membrane of chicken embryonated eggs and laboratory animals) and serological test methods. It is advisable that personnel working with monkeys, especially those animals arriving during the quarantine period, should be vaccinated against monkeypox [48, 50, 55].

The monkeypox virus is not an evolutionary progenitor of the smallpox virus, but it is also considered dangerous for humans [48, 56]. At the end of the XX century monkeypox rarely occurred in humans, but in the 2020s the frequency and geographical distribution of infection cases in humans increased [57, 58]. Monkeypox was first diagnosed in a traveler from Nigeria in the USA in 2001 [59], and some more cases were identified in 2003. A prairie dog was identified as the source of infection [60].

In September 2017 a major outbreak of human monkeypox occurred in Bayelsa State (Nigeria) [61]. During the examination of 21 diseased people the following clinical signs were recorded: skin rash – 100% of cases, fever – 80.1%, itching – 66.7%, malaise – 61.9%, lymphadenopathy – 61.9%, chills and sweating – 61.9%, headache – 57.1%, oral sores – 52.4%, genital ulcers – 41.6%, sore throat – 42.8%, myalgia – 23.8%, pain – 23.8%, cough – 19.0%, conjunctivitis – 19.0%, nausea and vomiting – 14.3%, sensitivity to light – 14.3%, hepatomegaly – 9.5%, dehydration – 9.5%, vulvar swelling – 9.5%, poor appetite – 9.5%, tongue sores – 9.5%, scrotal swelling – 9.5%, diarrhea – 4.8%. This outbreak and the subsequent export of the virus with travelers from Nigeria to other parts of the world in 2018–2020 caused serious concerns of scientists who assumed that MPXV could occupy the ecologic and immunological niche left vacant by the smallpox virus [61, 62].

The clinical picture caused by MPXV in humans is similar to that of smallpox, but they differ epidemiologically [56]. Vaccination against smallpox protects people from monkeypox. Human-to-human transmission of the virus was reported [52].

Skin lesions of a monkeypox affected patient are shown in Figures 3, 4.

According to the WHO, by mid 2022 the number of people infected with monkeypox virus exceeded 3.4 thousand people in 50 countries of the world. More than 86% of all infected individuals were residents of European countries [63].

The MPXV biological properties were studied using non-human primates, prairie dogs, African squirrels, ground squirrels and immunodeficient mice [64]. Virus titration in prairie dogs showed that Congo Basin clade MPXV isolates are more virulent via intranasal route than West African MPXV isolates [65]. According to A. A. Sergeev et al., steppe marmot are the most sensitive to the monkeypox virus, while rabbits and guinea pigs intranasally challenged with suspended Congo Basin MPXV V79-1-005 strain did not demonstrate any observable signs of the disease. The results of this work suggested that steppe marmots could be used as model animals for studying the properties of this virus [64, 66].

Thus, MPXV is the causative agent of monkeypox. The disease is similar to smallpox as regards its clinical manifestations. The virus is mainly detected using laboratory diagnosis methods. Current epizootological monitoring of monkeys and other susceptible animals is necessary due to the fact that monkeypox is recognized as the most important orthopoxvirus infection in humans in the era following smallpox eradication [54, 66, 67].

COWPOX

Until the 1970s it was believed that cowpox virus (CPXV) causes outbreaks only in cattle population, demonstrating clinical signs that are more often manifested in the form of local (lesions on the skin of the udder and on the nipples), more rarely systemic infection (which is more typical for calves). Later it was found out that a much wider range of animals are susceptible to the virus; moreover, CPXV is pathogenic to humans and can cause systemic infection in people with weakened immune status [21, 22, 24, 44, 68].

The causative agent of cowpox is a DNA poxvirus with complex symmetry, belonging to the *Poxviridae* family, the genus *Orthopoxvirus*, the *Cowpox virus* species (clades Brighton Red – CPXV-BR, GRI-90-CPXV-GRI) [68].

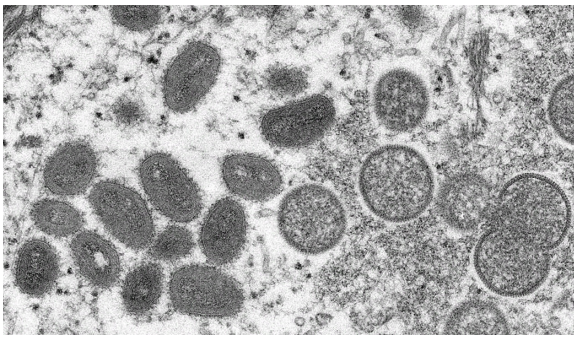

Fig. 2. Monkeypox virus (https://nashpoz.ru/wp-content/uploads/2022/05/2022-05-19T105043Z_1152629469_RC2T9U9TZXD0_RTRMADP_3_HEALTH-MONKEYPOX-PORTUGAL-SPAIN.JPG.jpg)

The virus propagates well in the chorioallantoic membrane of chicken embryonated eggs, forming plaques, and in certain cell cultures (Vero, MRC-5, RK13, etc.), inducing a cytopathic effect [69].

CPXV replication in cutaneous cells of infected animals goes through a number of stages described in detail and practically indistinguishable from other orthopoxviruses [70–73].

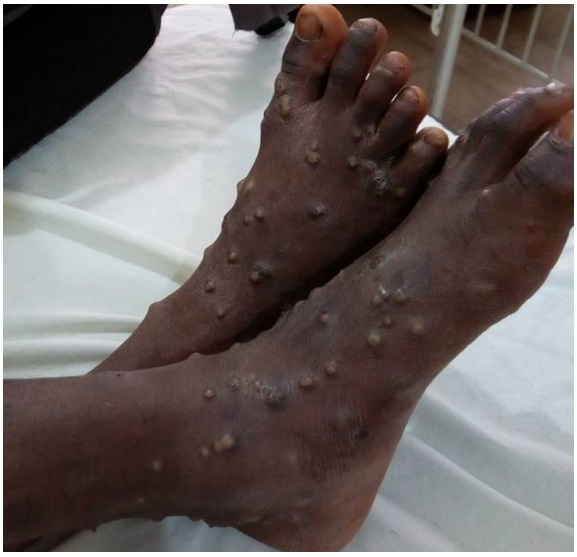

Fig. 3. Vesicular-pustular lesions on feet of monkeypox affected patient [61]

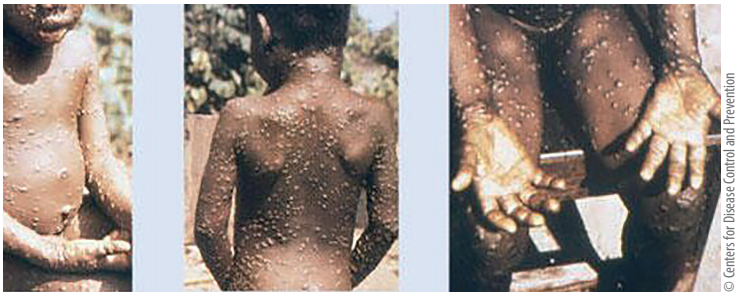

Fig. 4. Child with monkeypox virus infection

The clinical signs of cowpox in different animals are quite similar, regardless of the infected species, and are mainly manifested as skin damage. The virus is epitheliotropic, the disease often starts with the appearance of vesicular lesions, that later develop into a pustule with a depressed center.

CPXV lesions in humans are usually localized and self-limiting, but can lead to mortality in patients with immunosuppression [74, 75].

The study of the ecology of cowpox virus revealed that this pathogen spread among laboratory and wild rodents in natural biocenoses [70, 76–79]. High antibody titers detected in felines (family *Felidae*) and some other carnivora indicate a high infectivity of this virus. Outbreaks caused by CPXV were reported in lions, leopards, cheetahs, snow leopard, bush dog, banded mongoose (*Mungos mungo*) and jaguarundi (*Herpailurus yagouaroundi*), as well as elephants, rhinoceroses, camels in zoos and circuses [80–83]. Mortality among exotic animals and felines is high, although no exact data are available. Exotic zoo animals can get infected if they are kept in close proximity to other animals that come into contact with wild rodents [75].

Numerous data on CPXV and its antibodies detected in wild rodents allowed D. K. Lvov [68] to make an assumption about the leading role of these animals as the main reservoir of cowpox. Wild rats can be either a primary reservoir or a reinforcing host [75, 79, 84].

Cowpox virus shows environmental stability. It survives at low temperatures, in crusted scabs, in glycerin. The virus-containing material subjected to heat treatment is inactivated within a few minutes and it is relatively resistant to disinfectants [85].

Diseased animals and virus carriers are the source of the pathogen. Cowpox virus enters the body via aerogenic or alimentary route during contact of diseased and healthy animals via damaged skin and mucous membranes (udder, nipples, scrotum, head, neck, thighs).

The clinical signs of the disease have been specified in detail for cattle (Fig. 5). The disease is most commonly manifested as mastitis associated with reduced milk yield and lactation [85].

Animals recover in 3–4 weeks if no complications occur, and the disease is delayed for 1.5–2.0 months in case of complications. Adult animals generally have a mild form of disease. Calves develop bronchopneumonia and gastroenteritis.

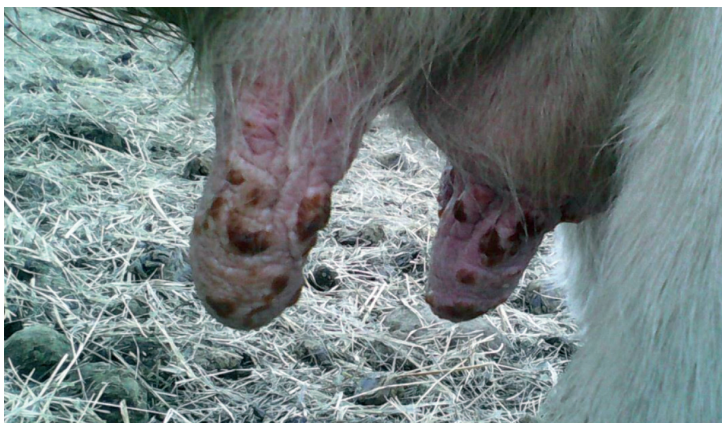

Fig. 5. Affected udder nipples [86]

The virus penetrates blood, lymph nodes and internal organs. The viremia is accompanied by increased body temperature, depression. Convalescent cows develop lifelong immunity [85].

Reports on human cowpox cases appeared in the XVIII century. Cowpox was considered an occupational disease of milkers. The disease in humans is generally benign, but complications may occur in unvaccinated persons and individuals with weakened immunity. In rare cases systemic infection or death are observed [40]. Natural CPXV isolates that are poorly studied or not studied at all are potentially dangerous [66, 70, 87]. In the future, the frequency of human infection may increase [75].

In 2008, four individuals were infected with cowpox virus in Krefeld (Germany). The CPXV HumKre08/1 virus was isolated. CPXV-infected rats from a pet store were a source of infection. All animals died. In the same year, another infection case was diagnosed – cowpox virus was isolated from an employee of a private reptile zoo in Landau (Fig. 6), it was named CPXV HumLan08/1.

The HA gene sequence of both virus strains turned out to be different. Figure 7 shows the evolutionary relationships of orthopoxvirus isolates recovered during the specified outbreaks and reference orthopoxvirus strains [75].

Cowpox virus played a certain role in the specific prevention of smallpox in humans. In 1796 E. Jenner developed a method of vaccination against this disease by inoculating CPXV to humans [88].

At present, CPXV should be considered as a rodent virus – zoonosis with natural nidality. Human infection is possible through contact not only with diseased cows, but also with any infected animal. The risk of cowpox outbreaks is high. Epizootological and epidemiological monitoring is required [68, 75, 79, 84].

BUFFALOPOX

Buffalopox is a contagious viral disease affecting buffaloes (*Bubalus bubalis*) and, less often, cows. Information on this infection is systematized in the review by S. V. Borisevich et al. [15]. The disease is zoonotic. Humans (mainly nursing staff and milkers) get infected from animals.

The causative agent is the vaccinia virus.

Till 5 to 80% of buffaloes get diseased during outbreaks. The incubation period lasts 2–4 days. The disease clinical manifestations include pox sores on the udder, nipples, eyelids, in the groin area, on the scalp. Severe forms occur in association with systemic rash. In 10 days the sores resolve with scab formation. There might be complications: eye swelling and bulging, corneal ulcer, ear discharge. Recovery occurs within 1–2 months. Transmission of the virus from animal to animal occurs through milkers, however, there is no confirmed data on human-to-human infection yet [89–91]. The disease does not cause a high mortality among animals, but results in decreased productivity, milk yield reduction and trade restrictions. Outbreaks are reported in countries where buffaloes are bred as dairy cattle [89, 90].

For the diagnosis and differentiation of the buffalopox virus, methods of viral isolation, modern test systems for the virus detection, including a polymerase chain reaction based on buffalopox virus (C18L) specific gene, and methods for determination of specific antibodies were developed [92].

The incubation period in humans infected with buffalopox virus lasts 3–19 days. Lesions occur on the fingers or forearms and, as a rule, are accompanied by a mild fever, which begins on day 1–4 and lasts 4–5 days. Recovery occurs within 2 weeks [90]. There is an assumption that the buffalopox virus became pathogenic for animals and humans due to adaptive evolution [15, 89, 93].

The possibility of VACV interspecies transmission, including cows, buffaloes and humans, implies potential reoccurrence of the virus and emergence of new outbreaks. Epizootological and epidemiological monitoring is necessary [92, 94].

CAMELPOX

Camelpox is a zoonotic contagious disease that occurs with the formation of typical cutaneous and mucosal nodular-pustular smallpox lesions. Camelpox virus (CMLPV) belongs to the family *Poxviridae*, the genus *Orthopoxvirus* [95].

The disease is recorded on almost all continents where camel husbandry is practiced except Australia (where the dromedary camel was introduced in the XIX–XX centuries) and South America (where lamas and related species are considered livestock animals) [95–97]. Serological tests showed a high prevalence of CMLPV antibodies [98].

The nucleotide sequence analysis showed that CMLPV is most related to the smallpox virus. Vaccination of camels with vaccinia strains showed good results.

Camelpox virus multiplies in cell cultures (Vero, MA-104, MS, BHK, camel skin) and in primary cell cultures (lamb testicles, lamb kidneys, camel embryo kidney, calf kidneys, chicken embryo fibroblasts) [99, 100], hemagglutinates cockerel erythrocytes [99], is stable at pH of 5–8.5.

The incubation period lasts 9–15 days. Camelpox clinical manifestations vary from mild smallpox lesions localized on skin to moderate and severe lesions with systemic infection. It might depend on the CMLPV strain or the immune status of animals [98]. Skin lesions appear on day 1–3 after the onset of fever: first erythematous spots, papules and vesicles, and then pustules that turn into crusts, localizing on eyelids, nostrils and ear edges. They can spread to neck, limbs, genitals, mammary glands and perineum. Lymph nodes get enlarged. In case of systemic disease, lesions are found on the mucous membranes of the mouth and respiratory tract, sometimes blindness is observed [98, 101–103].

Recovery occurs in 4–6 weeks. Pregnant cows might abort. Mortality is usually associated with secondary infections and septicemia [30, 96, 98].

Transmission of the pathogen occurs via contact with infected animals in a contaminated environment. The route of infection is aerogenic or through abrasions on the skin. The virus is shedded with milk, saliva, and discharge from the eyes and nose. Dried scabs formed during smallpox infection may contain a live virus for at least 4 months and contaminate the environment [97].

Immunity against camelpox is both antibody- and cell-mediated. It is considered that circulating antibodies are not indicative of the animal's immune status [98]. Lifelong immunity is acquired after natural infection. A live attenuated vaccine gives protection against the disease for 6 years [104].

The camelpox virus is species-specific and does not infect other animals, including cattle, sheep and goats [18, 102].

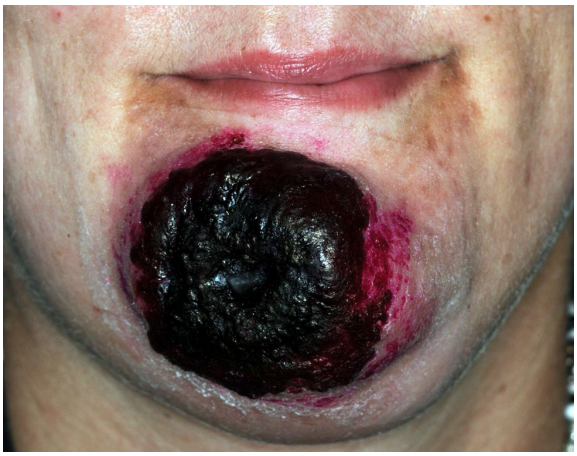

Fig. 6. Patient affected by cowpox virus strain CPXV HumLan08/1 [75]

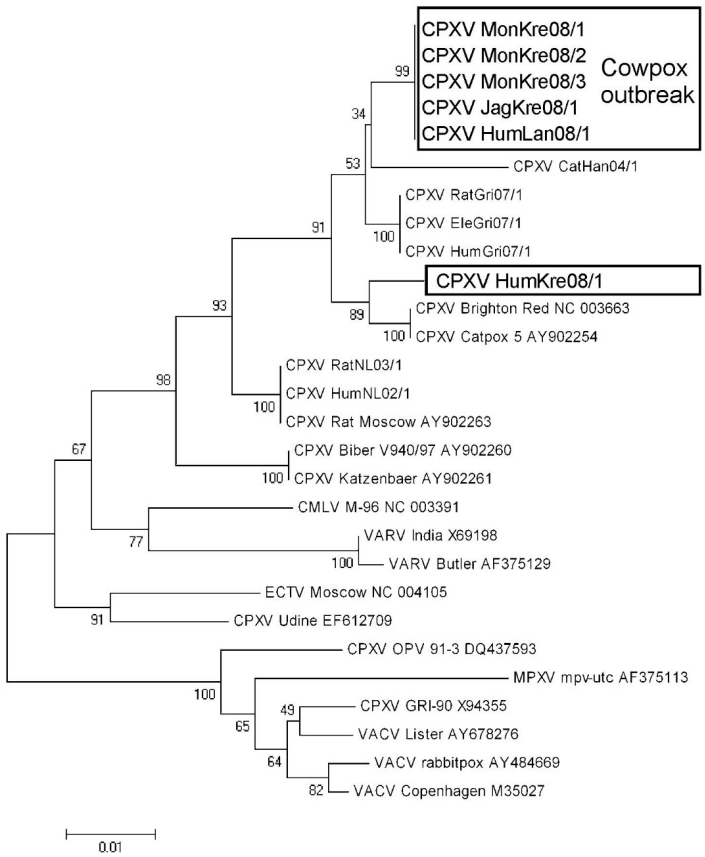

Fig. 7. Evolutionary relationships of orthopoxvirus recovered isolates and reference strains [75]

CONCLUSION

Along with the increased number of human monkeypox cases in 2020–2022, measures are required to strengthen epizootological and epidemiological monitoring of zoonotic orthopoxviruses.

Outbreaks of orthopoxvirus infections make it urgent to develop reliable and species-specific rapid methods for detecting relevant pathogens, to expand the panel of DNA samples of orthopoxviruses and include fowlpox virus, rabbit myxoma virus, chickenpox virus, herpes simplex virus type I and II.

Epizootological and epidemiological issues, molecular and biological mechanisms of virus replication and virus-host interaction should be considered prospective research developments within orthopoxvirus studies.

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Respiratory diseases in young cattle

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SUMMARY

The following review considers modern scientific data on respiratory diseases in young cattle. The problem of respiratory diseases in calves does not lose its relevance, since these pathologies rank second in frequency after diseases of the digestive system. In order to compile the data, the works of domestic and foreign researchers and collectives available in the collections of scientific conferences, seminars, symposiums, as well as in peer-reviewed periodicals, materials of dissertations and abstracts were reviewed. The group of pathologies under consideration is sufficiently diverse and can be caused by high animal density in the premises, overheating, hypothermia, unbalanced feeding, micronutrient deficiency, decreased body resistance, unfavorable epidemic situation and many other factors. However, out of the major calf diseases, particular mention should be made of pneumonia, which is most often caused by viruses. In this case agents can induce bacterial infection which aggravates and complicates the course of viral diseases. Microorganisms, such as *Salmonella*, *Pasteurella* and others, contribute to secondary infection and cause mixed forms of pneumonia. Bronchopneumonia is another disease covered in the article. It is a respiratory pathology characterized by inflammation of both the bronchi and lungs. As a rule, such disease types are most common in industrial animal husbandry, they are widespread and cause significant economic damage to the dairy and beef cattle breeding industries. The article pays great attention to these pathologies, justifies the importance of comprehensive preventive measures and timely diagnosis for livestock industries. To reduce the incidence of respiratory diseases in young cattle, it is necessary to strictly follow technological and hygienic standards for animal keeping and feeding. The use of combined medicines and preparations containing microelements increases treatment effectiveness.

Keywords: review, young cattle, respiratory organs, respiratory diseases, pneumonia, bronchopneumonia

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Респираторные заболевания молодняка крупного рогатого скота

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РЕЗЮМЕ

В обзорной статье рассматриваются современные научные сведения о заболеваниях органов дыхания у молодняка крупного рогатого скота. Проблема респираторных заболеваний у телят не теряет актуальности, поскольку данные патологии занимают второе по распространенности место после болезней органов пищеварения. С целью систематизации данных проведен обзор работ отечественных и зарубежных исследователей и коллективов, доступных в сборниках научных конференций, семинаров, симпозиумов, а также в рецензируемых периодических научных изданиях, материалах диссертаций и авторефератов. Рассматриваемая группа патологий в достаточной степени разнообразна, может провоцироваться скученным содержанием животных, перегревом, переохлаждением, несбалансированным кормлением, дефицитом микроэлементов, снижением резистентности организма, неблагоприятной эпизоотической ситуацией и многими другими факторами. Однако среди основных заболеваний телят следует особо выделить пневмонию, которую чаще всего вызывают вирусы. При этом возбудители способны инициировать деятельность ряда бактерий, усугубляющих и осложняющих

течение вирусных заболеваний. Такие микроорганизмы, как сальмонеллы, пастереллы и другие, становятся участниками вторичного инфекционного процесса и создают смешанные формы пневмоний. Другое освещаемое в работе заболевание – бронхопневмония. Это респираторная патология, характеризующаяся воспалением одновременно бронхов и легких. В промышленном животноводстве такие формы заболеваний, как правило, встречаются наиболее часто, имеют повсеместное распространение и наносят значительный экономический ущерб отраслям молочного и мясного скотоводства. Названным патологиям в статье уделяется наибольшее внимание, обосновывается важность комплексных мер профилактики и своевременной диагностики в условиях промышленных животноводческих предприятий. Для снижения частоты возникновения респираторных заболеваний среди молодняка крупного рогатого скота необходимо четко следовать технологическим и гигиеническим нормам содержания и кормления животных. Использование комбинированных лекарственных средств и препаратов, содержащих микроэлементы, повышает эффективность лечения.

Ключевые слова: обзор, молодняк крупного рогатого скота, органы дыхания, респираторные заболевания, пневмония, бронхопневмония

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INTRODUCTION

Respiratory system pathologies in young cattle are one of the major causes of livestock losses in dairy farming. Among calves in the first month of life, they are recorded in 17.2–23.6% of cases [1]. Respiratory diseases in calves are mostly caused by the following pathogens: parainfluenza-3 viruses, infectious rhinotracheitis, and parvo- and adenoviruses, as well as diarrhea, influenza and immunodeficiency viruses. In this regard, a comprehensive approach to the prevention and treatment of respiratory diseases in animals is required [2–4]. Thus, timely diagnosis, prevention and treatment of non-infectious respiratory diseases, among which pneumonia and bronchopneumonia causing the inflammation of the bronchi and lungs, is of crucial importance for cattle preservation and increase in livestock product manufacturing [5–7]. The pathology begins with serous exudation in the lung parenchyma, which is typical for catarrhal pneumonia. When first the bronchi are affected and then the process spreads rapidly through the bronchial tree, such a disease, occurring mainly in calves, is called bronchopneumonia [8, 9]. One of the predominant reasons triggering the disease in winter is hypothermia, in summer – overheating. Poor-quality feeding, lack of nutrients during intrauterine development and after birth has a negative impact on calves' body resistance [10, 11].

The purpose of this article is to analyze modern reported data on respiratory diseases in young cattle, their causes, as well as on methods of their prevention and therapy.

MATERIALS AND METHODS

Methodological approaches are substantiated by the study of works of domestic and foreign researchers presented in the collections of scientific conferences, seminars, symposiums, peer-reviewed periodicals, materials of dissertations and abstracts. The obtained data as well as the research findings have been analyzed. The review presents data on the study of young cattle, conducted on the basis of farms in the Saratov, Voronezh, Nizhny Novgorod, Novosibirsk, and the south of the Tyumen Oblasts.

The respiratory system of cattle is comprised of the upper (nasal cavity, paranasal sinuses, part of the oral cavity, pharynx) and the lower (larynx, trachea, bronchi, lung alveoli) airways. The upper respiratory tract, trachea and bronchi make up the conducting zone of the respiratory system. The main function of the respiratory system is gas exchange – the delivery of oxygen to the body and the removal of carbon dioxide from it. The air entering the lungs is warmed up and disinfected in the upper respiratory tract, while gas exchange occurs in the lower respiratory tract, in the alveoli [12].

Animals can be affected by respiratory diseases of different etiologies up to several times a year. In food-producing animals, respiratory diseases account for about 35% of the total number of diseases of non–infectious etiology, while in non-food producing animals – 13–15% [13, 14]. Respiratory diseases in calves can be caused by non-infectious and infectious factors: viruses and secondary bacterial infection caused by pathogenic microflora.

Upper respiratory tract infections include pharyngitis (inflammation of the mucous membranes and lymphoid tissue of the pharynx), rhinitis (inflammation of the nasal mucosa), frontal and maxillary sinusitis (inflammation of the mucous membrane lining the sinuses), sore throat and tonsillitis (inflammation of the tonsils). Lower respiratory tract infections include bronchitis (inflammation of the bronchi), pneumonia (inflammation of the alveoli), bronchopneumonia (inflammatory process in the bronchi and alveoli, accompanied by the accumulation of exudate in the alveoli), laryngitis (inflammation of the larynx),

tracheitis (inflammation of the mucous membranes of the trachea), pleurisy (inflammation of the pleural linings), alveolitis (inflammation of the pulmonary alveoli) [15, 16].

Despite different etiology and ways of transmission, respiratory diseases share a number of common features in their pathogenesis. These features include mucus secretion in the bronchi; superficial peeling of the mucosa, the elements of which enter the bronchial lumen together with blood; decreased functional pulmonary volume; impairment of lung diffusion capacity, leading to changes in blood composition; bronchial and lung walls become less elastic, which leads to the increased energy expenditure for breathing. Severe forms of respiratory diseases may lead to animal death or emergency slaughter [17, 18].

Non-infectious factors contributing to the development of respiratory diseases include embryo developmental disorders, hypoxia, gestosis, provoking changes in the functional activity of the fetoplacental complex, adaptation disorders in the postnatal period. They may also include the increased density of animals in the premises and gas contamination [19, 20]. Exceeded levels of carbon dioxide, ammonia, methane, hydrogen sulfide cause irritation of the mucous membranes and lead to the inflammation of the respiratory tract. Violation of sanitary and hygienic standards in the premises where animals are kept leads to high contamination of the mucous membranes by opportunistic and pathogenic microorganisms. Respiratory diseases in animals may occur in case of decreased activity of antioxidant defense enzymes, insufficient intake of vitamins that act as antioxidants, since increasing free radical oxidation is a key factor in the inflammatory process [20, 21]. In addition to the lack of vitamins, non-specific resistance of calves is negatively affected by the deficiency of trace elements involved in adequate functioning of the immune system and the synthesis of antioxidant enzymes, for example, selenium, iodine and zinc.

Viruses and bacteria infect endothelial cells of the bronchi, penetrate into respiratory tract tissue, and then spread throughout the body with the blood flow. Temperature changes, high humidity in the premises, inadequate diet as well as vitamin and mineral imbalance also contribute to the disease occurrence. Viral infections, especially in case of contributing factors, spread rapidly and cause inflammatory reactions. Secondary bacterial infections cause purulent processes in the lungs, which leads to the death of animals [22].

Viral infections include infectious rhinotracheitis (herpesvirus infection), which damages the mucous membrane of the respiratory tract in young animals, and when complicated by bacterial agents, the disease proceeds in the form of purulent pneumonia. Some of the main clinical signs include: rhinitis, serous-catarrhal discharge from nasal cavity, conjunctivitis. The disease mainly affects calves of 1.5–4.0 months of age.

Lung damages are observed in calves of 3–6 months of age in case of parainfluenza-3. Clinical signs include cough and purulent sputum discharge from the nasopharynx, increased body temperature. The disease is often associated with other viral and bacterial agents, for example, *Mycoplasma bovis*, *Pasteurella multocida*, *Mannheimia haemolytica*. There is a high probability of the immunosuppressive effect of the virus [23].

Respiratory syncytial viral infection, occurring in the form of latent or acute respiratory illness, is manifested by high fever, catarrh of the upper respiratory tract, serous rhinitis. The prognosis of the disease is most often favorable.

Viral diarrhea is a disease of the mucous membranes of cattle which most often occurs as a result of intrauterine infection. The infection affects the animal's immune system and is characterized by the development of both intestinal and respiratory syndromes, formation of ulcers on the mucous membranes is observed.

Secondary bacterial infections include pasteurellosis (the causative agent *Mannheimia haemolytica* and *Pasteurella multocida*, serotype A), which cause pneumonia in the presence of contributing factors, or complicate the course of the primary viral infection.

The most common causative agent of mycoplasmosis in cattle is *Mycoplasma bovis*, which is becoming the main cause of pneumonia in young animals. The disease is often heralded by viral diarrhea, rhinotracheitis, coronavirus infection, often at an early age. Calves demonstrate decreased appetite, depression, nasal discharge, wet cough, wheezing. Other bacteria that cause secondary pneumonia in cattle are *Haemophilus*, *Klebsiella*, *Streptococcus* [22].

Pneumonia in calves occurs due to the extension of the infection from the upper respiratory tract. Pathogenic microorganisms located on the mucous membrane of the nasal cavity, paranasal sinuses, middle ear and larynx can be the cause of infection of the lower respiratory system. Rhinitis and inflammation of the paranasal sinuses are common in one-month-old calves and herald pneumonia [24]. In stressful conditions, when the body's defense mechanisms are compromised, pathogenic microflora causes the development of inflammation [25]. The inflammatory process can be localized in the alveoli, bronchi, individual lung lobes or in the entire lung. By the nature of inflammatory exudate, pneumonias can be catarrhal, fibrinous, serous, putrefactive, hemorrhagic, purulent and mixed. Catarrhal and fibrinous are the most common ones. Clinical signs of pneumonia include: increased respiratory rate with wheezing (more than 60 breaths/min); nasal (less often ocular) discharge; cough, sometimes with purulent sputum; temperature of 41–42 °C; diarrhea; dull demeanour; absence of rumination. In 4–5 months old calves pneumonia can become chronic with no distinct symptoms, while reduction in liveweight gain is observed. If the disease is caused by contributing factors and secondary bacterial infections, treatment with antibiotics is required. In case of viral and secondary bacterial infections, specific prevention is effective [22, 26].

Bronchopneumonia is manifested by inflammation of the bronchi and lung lobes with the accumulation of exudate and desquamated epithelial cells in the alveoli [27, 28]. Opportunistic microorganisms cause inflammation of the bronchi, bronchioles and alveoli. In addition to significant pathological changes in the lungs, malfunction of the central nervous, cardiovascular and other body systems is observed [29].

According to a number of authors, bronchopneumonia occupies a leading position in terms of morbidity and transmission rates among other pathologies of non–infectious etiology in 1.5–3.5-month-old calves. According to some data, in the Voronezh, Nizhny Novgorod,

Novosibirsk Oblasts, in different years, pathologies of the respiratory system affect 29.10–59.36% of calves per year, with the mortality rate of 6–35%. Animals that have suffered from the disease demonstrate developmental and growth delays, which makes them unsuitable for further use [30]. Clinical examination of calves with this pathology reveals dull demeanour: lowered ears, decreased appetite, standing on their own away from the herd; then respiratory signs appear – increased temperature (up to 40 °C), nasal discharge, cough, dyspnea, wheezing [31]. There are three main steps in bronchopneumonia treatment: stopping bacterial growth and reproduction, removal of accumulated exudate from the bronchi and detoxification of the animal's body [30]. To prevent bronchopneumonia in calves, it is necessary to control hygiene and temperature in the premises, as well as to provide a balanced diet for animals [3].

The mineral status of the animal, which depends on its mother's diet and the feed given to young animals, has a significant impact on the occurrence of respiratory diseases. Fetal intrauterine growth restriction due to the deficiency of such trace elements as copper, selenium, zinc, cobalt, manganese leads to 2.08-fold increase in respiratory disease cases in young animals in the neonatal period, 7.14-fold increase in bronchopneumonia cases, as compared to animals from cows with the physiological course of pregnancy; this, together with other factors or complicated course of pregnancy, leads to the weakening of the antioxidant defense system and, hence, to oxidative stress [32, 33].

Results of studies by D. Shukla et al. [34] proved the role of cobalt in the antioxidant protection of the lungs; its deficiency, along with the deficiency of copper, zinc, manganese and selenium, regulating the activity of superoxide dismutase, catalase and glutathione peroxidase, is a risk factor for the development of bronchopneumonia in calves.

Intrauterine disorders due to lack of trace elements in pregnant cows contribute to the development of respiratory diseases in calves. Maternal health determines fetal growth and wellbeing, the quality of colostrum and milk [35]. Shaposhnikov I. T. et al. [36] found that in case of protein and carbohydrate metabolism disorders and calcium deficiency, antioxidant deficiency is developed in down calvers, which leads to the development of respiratory and gastrointestinal diseases in their offsprings. Other researchers [33, 37] proved the role of oxidative stress in the pathogenesis of respiratory diseases in young cattle. The authors found that antioxidant deficiency, caused, in part, by the lack of trace elements, disrupts the regulation of free radical oxidation processes and contributes to the excessive amount of toxic protein and lipid peroxidation byproducts in the bronchoalveolar fluid and blood of sick animals, which not only damages cell membranes but also inhibits the immune system.

Pneumonia and bronchopneumonia morbidity rates in calves depend on chemical, physical, biological and environmental factors. This has been confirmed by studies conducted by V. M. Aksenova et al. [27]. Failure of the lung and bronchi defense mechanisms affected by pathogenic microflora, and other factors compromising the immunity, influence the occurrence and the course of respiratory infections [38, 39]. It has been proved that hypothermia of young animals leads to reductions in total immunoglobulin levels in blood serum and to the development of respiratory system pathologies in 59–69% of calves. Imbalance in "organism – environment" system may also play a certain role in the development of lung diseases in calves. There is a correlation between emissions of carbon monoxide, hydrocarbons into the atmosphere and the incidence of respiratory diseases in animals. Increase in the mortality rate from respiratory system pathologies in young animals was recorded in cities with the increased level of air pollution [40].

Calves under one year of age are most susceptible to respiratory diseases, and it should be noted that recovered animals can get infected again [3]. Since these pathologies are widespread, the incidence rate is quite high, and there is also a risk of disease reccurence, it is necessary to develop effective treatment regimens using modern drugs [31].

In case of calves recovering from bronchopneumonia, in addition to conventional therapy, including etiotropic treatment and novocaine blockade with 0.6% hydrogen peroxide in 0.9% sodium chloride at a dose of 0.4 mL/kg, it is recommended to administer 4 mL of "Antimiopathic" on the first day, which helps to correct antioxidant deficiency and acid-base balance. This product is based on vitamins and trace elements, it successfully replenishes deficient elements and helps to normalize trace element homeostasis.

To prevent the development of respiratory diseases in newborn calves, calvers should be injected with "Antimiopathic" in a single dose of 10 mL 60, 40 and 20 days before calving; this will enhance antioxidant protection in the newborn calves and reduce their oxidative stress, compensate for postnatal hypoxia, acidosis and will lead to the early formation of colostral immunity [19].

Respiratory diseases cause important economic losses in industrial animal husbandry. To increase the effectiveness of treatment of pneumonia in livestock, it is necessary to optimize young animal nutrition as well as to use combination medicines and preparations containing trace elements. Animal keeping standards must comply with the existing requirements [41]. A comprehensive approach is needed in the treatment of pneumonia and bronchopneumonia, since their etiology is multifactorial. Therefore, in modern production, veterinarians use vitamin and mineral complexes, drugs and broad-spectrum antibiotics to treat respiratory diseases [42].

CONCLUSION

Nowadays, respiratory diseases are widespread in young cattle, they are characterized by a variety of pathoetiology, but the most common factor in the development of pathology is decreased body resistance. Therefore, it is necessary to take measures to prevent and promptly detect pneumonia, bronchopneumonia and other respiratory system diseases, which include clinical examination of animals and blood tests, as well as to introduce effective treatment and prevention regimens.

To reduce the incidence of respiratory diseases in young cattle, it is necessary to strictly follow technological and hygienic standards for animal keeping and feeding. Timely vaccination of calves and the use of vitamin and mineral premixes have a high preventive effect.

Such preventive measures contribute to the reduction of morbidity and, consequently, reduce the costs of treatment and the likelihood of early death in young animals, thus, preventing economic losses.

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Assessment of the epizootic situation by invasive diseases in reindeer farms in the Murmansk Oblast

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SUMMARY
Reindeer invasive diseases cause significant damage to reindeer farms due to reduction in animal productivity and deterioration in quality of reindeer products. Helminthiases take a special place among them. Knowledge of the epizootic situation will contribute to the successful organization of a system of therapeutic and preventive measures to protect the domestic reindeer stock from helminthiases. The article presents assessment results for the invasive disease epizootic situation in reindeer farms in the Murmansk Oblast in 2018–2021. The research was carried out in two large reindeer farms – APC "Tundra" and APC HFE SEN "Olenevod" during the planned slaughter of reindeer. A total of 4,048 deer carcasses of all ages were examined and 199 samples of faeces were tested. A retrospective analysis of the veterinary service's data showed that, among helminthiases, mainly cysticercosis is recorded in reindeer herds of the Murmansk Oblast. The prevalence of cestodes infection varies from 0.16 to 0.83% depending on the year, however the extensiveness of cysticercosis invasion of reindeer is decreasing. The prevalence of oedemagenosis varied in different age and sex groups from 25 to 100%. It was found that reindeer of all ages were infested with paramphistomiasis (12.50–15.15%), setariasis (5.36–6.06%), nematodiasis (3.0–6.0%), dictyocaulosis (3.03–3.57%), protostrongylosis (3,0%) and, to the least extent, echinococcosis (0.04%). Helminths of the genus *Taenia*, class *Cestoda*, that cause cysticercosis, mainly infest young animals – extensiveness of invasion (EI) is 0.50–0.81%. Thus, oedemagenosis and paramphistomiasis prevail in the structure of helminth infections; they are recorded in all reindeer herds. It was established that invasive diseases occur in the form of mixed invasions. Mixed invasions most often occur in the following associations: oedemagenosis + protostrongylosis, oedemagenosis + paramphistomiasis + setariasis, oedemagenosis + paramphistomiasis + cysticercosis (finnosis), oedemagenosis + dictyocaulosis + protostrongylosis.

Keywords: domestic reindeer, epizootic situation, invasive diseases, helminthiases

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Оценка эпизоотической ситуации по инвазионным заболеваниям в оленеводческих хозяйствах Мурманской области

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РЕЗЮМЕ
Инвазионные заболевания наносят значительный ущерб оленеводческим хозяйствам за счет снижения продуктивности животных и ухудшения качества оленеводческой продукции. Особое место среди них занимают гельминтозы. Знание эпизоотической обстановки будет способствовать успешной организации системы терапевтических и профилактических мероприятий по защите поголовья домашних северных оленей от паразитарных заболеваний. В статье представлены результаты оценки эпизоотической ситуации по инвазионным заболеваниям в оленеводческих хозяйствах Мурманской области за период с 2018 по 2021 г. Исследования были проведены в двух крупных оленеводческих хозяйствах – СХПК «Тундра» и СХПК ОПХ МНС «Оленевод»

во время планового убоя северных оленей. Всего было исследовано 4048 туш оленей всех возрастов и 199 проб фекалий. Ретроспективный анализ данных ветеринарной службы показал, что из гельминтозов в оленеводческих стадах Мурманской области в основном регистрируется цистицеркоз (*Cysticercosis*). Процент зараженности животных цестодами варьирует по годам от 0,16 до 0,83%, при этом наблюдается уменьшение экстенсивности инвазии северного оленя. Распространенность эдемагеноза по разным половозрастным группам составляла от 25 до 100%. Показано, что олени всех возрастов болеют парамфистоматозом (12,50–15,15%), сетариозом (5,36–6,06%), нематодиреллезом (3,0–6,0%), диктиокаулезом (3,03–3,57%), протостронгилезом (3,0%) и в меньшей степени эхинококкозом (0,04%). Гельминтами рода *Taenia* класса *Cestoda*, вызывающими цистицеркоз, заражается преимущественно молодняк, экстенсивность инвазии составляет 0,50–0,81%. Таким образом, в структуре заболеваемости гельминтозами доминирующее положение занимают эдемагеноз и парамфистоматоз, регистрируемые во всех оленеводческих стадах. Установлено, что инвазионные болезни протекают в форме микст-инвазий, чаще всего в следующих ассоциациях: эдемагеноз + протостронгилез, эдемагеноз + парамфистоматоз + сетариоз, эдемагеноз + парамфистоматоз + цистицеркоз (финноз), эдемагеноз + диктиокаулез + протостронгилез.

Ключевые слова: домашний северный олень, эпизоотическая ситуация, инвазионные болезни, гельминтозы

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INTRODUCTION

Reindeer husbandry is a traditional ancient occupation of most peoples of the Far North. Deer is a major component of the ecosystem of the Northern regions and the welfare, level of economic and social development of indigenous peoples largely depend on its rational use. Preservation and further development of domestic reindeer husbandry, increasing its productivity and profitability are impossible without proper organization and implementation of effective measures against infectious and invasive animal diseases that can cause significant damage to reindeer farms.

The main diseases causing significant economic damage to reindeer husbandry are necrobacteriosis, oedemagenosis, cefenomyosis and helminthiases. These infections are often registered in reindeer herds, reduce the animals' productive qualities [1–8] and often cause mortality [9–11]. Helminthiases should be particularly noted.

Reindeer are ruminants, but due to scarce food supply in their habitats, they developed food preferences untypical of ruminants. They can eat fallen horns of their relatives and moose, brackish soil, chicks, bird eggs and droppings, as well as lemmings. Cases of deer drinking from puddles and simultaneously urinating and defecating were also recorded. These and other factors increase the likelihood of helminth invasion of deer [12].

Currently, there is only general information on diversity of parasites in reindeer [10, 13–21], and detailed information on parasitic infections is limited. Complete data on parasitic prevalence and diversity will contribute to a more targeted fight against infestations.

Reindeer diseases caused by helminths are widespread in reindeer herds both in the Russian Federation [15–17, 22, 23] and in the Scandinavian countries (Norway, Finland) [24, 25].

Thus, in the Republic of Sakha (Yakutia) the infection rate of deer with *Setaria cervi* was 32.8% [15], with *Cysticercus tarandi* – 13.3%, *Cysticercus parenchimatosa* – 10%, *Paramphistomum cervi* – 10%, *Echinococcus granulosus* – 10% among all tested animals. Larvae of nasopharyngeal gadflies (*Cephenomyia trompe*) affected 36.6% of the deer population, infection with larvae of hyperdermic gadfly was established at 100% (*Oedemagena tarandi*, oedemagenosis) [16].

The invasiveness of *Dictyocaulus eckerti* (dictyocaulosis) in wild reindeer was 100%, in domestic reindeer it was 45.5% for adult animals and 100% for calves of the current year of birth [22]. For comparison: in Norway, the prevalence of dictyocaulosis in wild reindeer varied from 28 to 80% depending on the period [24].

The level of prevalence of *Oedemagena tarandi* in deer grazing on the territory of the Khanty-Mansi Autonomous Okrug – Yugra, is much lower than in the Republic of Sakha (Yakutia). The extensiveness of oedemagenosis invasion varied from 0.71% in 2012 to 10.37% in 2015 [17].

The helminthiasis epizootic situation with regard to animal husbandry in the Murmansk Oblast remains practically unstudied. To date, there is little data and research works in the field of epizootology, epizootic process studies, as well as scarce information on clinical signs, treatment and prevention of reindeer invasive diseases in the region.

Timely epizootological monitoring, development and implementation of preventive, quarantine and health improvement measures in reindeer breeding farms are necessary conditions for the control of diseases caused by helminths [14].

For successful organization of a system of therapeutic and preventive measures to protect domestic reindeer population from parasitic diseases, knowledge of the biology of pathogens and their pathogenic effect on the host

organism, as well as information on the epizootic situation are necessary [26].

Therefore, the task was set to assess the epizootic situation of invasive diseases in reindeer breeding farms of the Murmansk Oblast in order to develop an effective strategy and tactics for the control, prevention and eradication of helminthiases.

MATERIALS AND METHODS

Studies on the spread of invasive diseases and helminth infestation of reindeer were conducted in 2018–2021 in two large reindeer breeding farms of the Murmansk Oblast ("Tundra" and "Olenevod" farms) during the planned slaughter of animals at the slaughter sites in the Lovozero settlement. In total, 4,048 carcasses from deer of all ages (2,812 from the "Tundra" farm and 1,236 from the "Olenevod" farm), as well as 199 fecal samples were examined.

Meat inspection included examination of animal's rumen, reticulum and abomasum. The helminthiases distribution was studied based on antemortem and postmortem diagnosis and taking into account the epizootological data. At the same time, coproscopic (ovoscopy, larvoscopy, helminthoscopy), flotation (according to Fulleborn) and sedimentation (sequential washing) tests were performed, incomplete helminthological autopsy was carried out and individual organs were examined according to K. I. Scriabin's method [27].

The species diversity of helminth fauna was determined morphologically by microscopy of macro- and micro-preparations using deer helminth identification guide [28].

Table 1
Extensiveness of helminthiasis infestation of domestic reindeer in reindeer farms of the Murmansk Oblast

Holding	Number of tested carcasses	Helminthiasis	Number of infested carcasses	Extensiveness of invasion, %
"Tundra" farm	2,812	cysticercosis	14	0.50
		echinococcosis	1	0.04
	56	paramphistomiasis	7	12.50
		setariasis	3	5.36
		dictyocaulosis	2	3.57
"Olenevod" farm	1,236	cysticercosis	10	0.81
		echinococcosis	–	–
	33	paramphistomiasis	5	15.15
		setariasis	2	6.06
		dictyocaulosis	1	3.03

Table 2
Extensiveness and intensity of helminthic invasion in reindeer of different sex and age groups

Holding	Sex and age group	Number of samples	II, eggs/g faeces	EI, %		
				Nematodiasis	Protostrongylosis	Paramphistomiasis
"Tundra" farm	button bucks under 1 year old	14	3–4	3	–	–
	bucks	2	4–5	3	–	–
	does	11	3–4	6	–	–
	females under 1 year old	104	–	–	–	–
"Olenevod" farm	button bucks under 1 year old	50	3–5	–	3	–
	does	6	–	–	–	–
	females under 1 year old	5	–	–	–	–

RESULTS AND DISCUSSION

Based on meat inspection results of parenchymal organs and gastrointestinal tract of 2,812 deer carcasses belonging to the "Tundra" farm, and 1,236 carcasses submitted to the slaughtering site from the "Olenevod" farm, infection with helminths of the genus *Taenia* (*Taenia hydatigena*), class *Cestoda* (mainly young animals) was detected, the extensiveness of invasion (EI) was 0.5 and 0.81%, respectively. Infection with echinococcosis agent (*Echinococcus canadensis*) was detected only in the "Tundra" farm, while the EI was insignificant and amounted to 0.04% (Table 1).

Of the total number of deer carcasses tested, 56 carcasses from the "Tundra" farm and 33 carcasses from the "Olenevod" farm demonstrated helminths of the genus *Paramphistomum* (*Paramphistomum cervi*) belonging to the digenetic trematodes (deer of all ages were infested) – with EI 12.50 and 15.15%, respectively, causative agents of setariasis (*Setaria tundra* of a genus of parasitic roundworms phylum *Nematoda*) – EI 5.36 and 6.06%, and dictyocaulosis (*Dictyocaulus eckerti*, phylum *Nematoda*) – EI 3.57 and 3.03%, respectively.

Thus, in the study of slaughter products, it was found that the extensiveness of domestic reindeer invasion in reindeer farms in the Murmansk Oblast varies from 0.04 to 15.15%. The most common parasitic diseases are paramphistomiasis (15.15%), setariasis (6.06%), dictyocaulosis (3.57%); less common are cysticercosis (0.81%) and echinococcosis (0.04%).

Based on results of coproscopy (ovoscopy, larvoscopy, helminthoscopy), combined sedimentation-flotation studies and sequential washings, the extensiveness (EI) and intensity (II) of helminthic invasion in reindeer of different age and sex groups were determined (Table 2). It was revealed that the EI of domestic reindeer with nematodiasis was from 3 to 6%, with protostrongylosis – 3%, the intensity of invasion was from 3 to 5 eggs/g of feces.

Retrospective analysis conducted based on reports of the veterinary service of the Lovozersk Animal Disease Control Station for 2018–2021 showed that of all helminthiases predominantly cysticercosis (or finnosis) is registered in reindeer of the Murmansk Oblast (Table 3).

Analyzing the results of meat inspection on farms, it can be noted that in 2018 the highest EI for finnosis (0.39%) was in the "Tundra" farm. In the "Olenevod" farm the maximum invasion rate (1.79%) was observed in 2020. In

Table 3
Retrospective analysis of cysticercosis distribution in reindeer in the Murmansk Oblast

Year	"Tundra" farm			"Olenevod" farm			Average, by farms		
	Total number of slaughtered animals	Animals with finnosis detected	EI, %	Total number of slaughtered animals	Finnosis detected	EI, %	Total number of slaughtered animals	Animals with finnosis detected	EI, %
2018	3,883	15	0.39	1,520	1	0.07	5,403	16	0.30
2019	2,995	7	0.23	1,902	1	0.05	4,897	8	0.16
2020	2,816	7	0.25	1,735	31	1.79	4,551	38	0.83
2021	3,381	5	0.15	1,338	8	0.60	4,719	13	0.28

2018–2021, the percentage of cestodes infestation for all farms, on average, was relatively low and varied from 0.16 to 0.83%. In 2021, there was a decrease in EI, which is associated with the use of ivermectin-based drugs and increased effectiveness of reindeer anti-parasitic treatment and animal health control measures.

At the next stage, the prevalence of hypodermic gadfly larvae infestation in reindeer of the Murmansk Oblast was studied by sex and age groups (Table 4).

Analysis of the obtained data shows that the highest invasiveness of domestic reindeer is observed in the "Olenevod" farm, where the EI was 100% for all sex and age groups. In the "Tundra" farm, the EI was significantly lower, but nevertheless quite high, especially in the groups of breeding bucks (71.4%) and calves (50.7%). On average, the extensiveness of edemagenous invasion in the entire studied reindeer population of both farms was 70.3%.

Such a high level of invasion with *Oedemagena tarandi* larvae is due to the fact that not all livestock (under 50%) of domestic reindeer are subjected to treatment against hypodermic gadfly, and the treatment terms are incompliant.

Analysis of the meat inspection results and studies conducted using a combined method showed that the invasions identified in reindeer of the Murmansk Oblast, as a rule, occur in the form of mixed invasions in various associations, the most frequent being: oedemagenosis + protostrongylosis, oedemagenosis + paramphistomiasis + setariasis, oedemagenosis + paramphistomiasis + cysticercosis (finnosis), oedemagenosis + dictyocaulosis + protostrongylosis. Similar data were obtained by researchers from Finland – more than half (53.3%) of the surveyed deer population had mixed parasitic infections [25].

Table 4
Infestation of reindeer by hypodermic gadfly larvae in reindeer farms in the Murmansk Oblast

Sex and age group	"Olenevod" farm					"Tundra" farm				
	Hides examined, pcs.	Infested by larvae, pcs.	Larvae recorded, pcs.	Medium II per animal, pcs.	EI, %	Hides examined, pcs.	Infested by larvae, pcs.	Larvae recorded, pcs.	Medium II per animal, pcs.	EI, %
Bucks	43	43	3,736	86.9	100	7	5	451	90.2	71.4
Does	6	6	475	79.2	100	4	1	87	87.0	25.0
Deer calves	5	5	397	79.4	100	73	37	3,005	81.2	50.7
Total	54	54	4,608	85.3	100	84	43	3,543	82.4	51.2

CONCLUSION

The results of the conducted studies have shown that invasive diseases of reindeer caused by helminths – representatives of three classes: trematodes, nematodes and cestodes – are registered in all surveyed farms of the Murmansk Oblast.

The most common parasitic diseases are paramphistomiasis (12.50–15.15%), setariasis (5.36–6.06%), nematodiasis (3.0–6.0%), dictyocaulosis (3.03–3.57%), protostrongylosis (3.0%), echinococcosis (0.04%) is less common. Helminths of the genus *Taenia,* class *Cestoda*, causing cysticercosis, infect mainly young animals, EI is 0.50–0.81%. The prevalence of oedemagenosis varied by different age and sex groups of animals from 25 to 100%. Oedemagenosis and paramphistomiasis occupy a dominant position and are recorded in all herds.

A retrospective analysis carried out during the meat inspection of venison on the basis of veterinary reporting documents within planned animal slaughter showed that among all helminthiases, mainly cysticercosis (finnosis) is registered in reindeer herds of the Murmansk Oblast.

It was established that invasive diseases occur in the form of mixed invasions. The following associations are most often registered: oedemagenosis + protostrongylosis, oedemagenosis + paramphistomiasis + setariasis, oedemagenosis + paramphistomiasis + cysticercosis (finnosis), oedemagenosis + dictyocaulosis + protostrongylosis.

The conducted studies made it possible to assess the epizootic situation of invasive diseases in reindeer breeding farms of the Murmansk Oblast. The data obtained will contribute to the successful organization of a system of therapeutic and preventive measures to ensure protection of domestic reindeer population from helminthiases.

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Bovine leukosis incidence in Republic of Dagestan in 2021

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SUMMARY

Results of epizootological analysis of data on bovine leucosis (BL) incidence in the Republic of Dagestan in 2021 are reported. Bovine blood was diagnostically tested for leukosis in 32 veterinary laboratories and diagnostic offices including SBI RD Republican Veterinary Laboratory. 720,489 sera were serologically tested and 7,188 (1.0%) samples were serologically positive for bovine leukaemia virus (BLV). Among the infected animals, 527 ones were subjected to haematologic testing. Persistent leukocytosis was reported in 153 (29.03%) blood samples of haematologically tested BL diseased cattle. Statistical analysis of BLV prevalence in the republic was performed for 41 Raions and 8 municipalities. High percentage of BLV infection in the animal population was reported in fourteen Raions: Kumtorkalinsky (5.8%), Gunibsky (5.3%), Tarumovsky (3.3%), Karabudakhentsky (2.9%), Akhvakhsky (2.0%), Kizlyarsky (1.8%), Charodinsky (1.7%), Kazbekovsky (1.6%), Babayurtovsky (1.5%), Tlyaratinsky (1.1%), Dakhadayevsky (1.04%), Sergokalinsky (1.02%), Novolaksky (1.0%), Shamilsky (1.0%), and in four municipalities: Makhachkala (2.0%), Izberbash (1.14%), Khasavyurt (1.1%) and Yuzhno-Sukhokumsk (1.0%). In 21 Raions and two municipalities, BLV seropositivity was below 1.0%. No BLV infected animals were detected in the Agulsky, Akhtynsky, Dokuzparinsky, Magaramkentsky, Khivsky, Suleiman-Stalsky Raions and in Derbent and Dagestanskiye Ogni municipalities. Studies of BLV prevalence in 2015–2021 demonstrated that the highest level of the animal infection was reported in 2015 (13.9%) and the lowest – in 2021 (1.0%). However, the number of animals serologically tested in 2021 exceeded the number of animals tested over the whole study period. Therefore, the Republic of Dagestan remains BL infected region.

Keywords: bovine leukosis, prevalence, epizootological analysis, incidence, Republic of Dagestan

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Конъюнктура заболеваемости лейкозом крупного рогатого скота на территории Республики Дагестан за 2021 год

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РЕЗЮМЕ

Представлены результаты эпизоотологического анализа данных по заболеваемости лейкозом крупного рогатого скота на территории Республики Дагестан в 2021 г. Диагностические исследования крови крупного рогатого скота на лейкоз были проведены в 32 ветеринарных лабораториях и диагностических кабинетах, в том числе ГБУ РД «Республиканская ветеринарная лаборатория». Серологическим методом было исследовано 720 489 проб сыворотки крови, из них 7188 (1,0%) образцов оказались серопозитивными к вирусу лейкоза крупного рогатого скота. Из числа инфицированных животных гематологическому исследованию подвергнуто 527 голов. Персистентный лейкоцитоз выявлен в 153 (29,03%) пробах крови гематологически больного лейкозом крупного рогатого скота. Проведен статистический анализ распространения вируса лейкоза крупного рогатого скота в республике в разрезе 41 района и 8 городских округов. Высокий процент инфицированности поголовья вирусом лейкоза крупного рогатого скота выявлен в 14 районах: Кумторкалинском (5,8%), Гунибском (5,3%), Тарумовском (3,3%), Карабудахкентском (2,9%), Ахвахском (2,0%), Кизлярском (1,8%), Чародинском (1,7%), Казбековском (1,6%), Бабаюртовском (1,5%), Тляратинском (1,1%), Дахадаевском (1,04%), Сергокалинском (1,02%), Новолакском (1,0%), Шамильском (1,0%); 4 городах: Махачкале (2,0%), Избербаше (1,14%), Хасавюрте (1,1%), Южно-Сухокумске (1,0%). В 21 районе и в 2 городских округах показатель серопозитивности к вирусу лейкоза крупного рогатого скота составил менее 1,0%. В Агульском, Ахтынском, Докузпаринском, Магарамкентском, Хивском, Сулейман-Стальском районах, городах Дербент и Дагестанские Огни инфицированные вирусом лейкоза крупного рогатого скота животные не выявлены. При изучении

динамики распространения вируса лейкоза крупного рогатого скота в 2015–2021 гг. установлено, что наибольший уровень инфицированности животных был в 2015 г. (13,9%), а наименьший – в 2021 г. (1,0%), но численность поголовья, подвергшегося серологическому исследованию, в 2021 г. превосходила таковую за весь анализируемый период. Таким образом, Республика Дагестан остается регионом, неблагополучным по лейкозу крупного рогатого скота.

Ключевые слова: лейкоз крупного рогатого скота, распространенность, эпизоотологический анализ, заболеваемость, Республика Дагестан

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INTRODUCTION

Bovine leukosis (enzootic bovine leukosis, EBL) is induced by bovine leukemia virus (BLV), which causes neoplastic growth of hemoblasts in blood through malignant degeneration and proliferation of immune cells, more specifically through the growth of the number of leukocytes (B- leukocytes) [1–5].

This bovine viral disease is widely spread in the USA, Canada, China, Japan and Russian Federation. West European countries (Norway, Finland, Sweden, Denmark, etc.) and New Zealand are considered BLV-free. In such countries as Italy, Portugal, Latvia, Greece, Romania, Bulgaria and Belarus, BL cases are sporadic [6–12]. According to the animal health recommendations specified in the Terrestrial Animal Health Code of the World Organization for Animal Health[1], any premises should be considered leukosis free in case 99.8% of the animals in the herd were BLV free for the last three years [13, 14].

Over the recent years, a number of programs aimed at BLV spread prevention and disease eradication on the livestock farms were approved in the Republic of Dagestan. They included "Plan for bovine leukosis prevention and control in the Republic of Dagestan in 2017–2020"[2] (approved by the order of the Republic of Dagestan government of 11 September 2017 No. 323-p), "Bovine leukosis prevention and eradication on the farms in the Republic of Dagestan" (approved by order of the Republic of Dagestan government of 28 June 2018 No. 76)[3].

Hematological and serological diagnostic tests of blood of nearly total bovine animal population are performed in the Republic for the detection of BL infected and diseased animals. Bovine leukosis, however, remains the pressing challenge in the region. One of the reasons of such situation is absence of targeted activities for the disease control in Dagestan as well as lack of full-scale preventive measures stipulated by the Veterinary Law[4].

[1] https://fsvps.gov.ru/fsvps-docs/ru/oie/oie_terrestrial_code_g_t1.pdf.
[2] https://docs.cntd.ru/document/450340001.
[3] https://docs.cntd.ru/document/550147549.
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Therefore, the goal was to study the BLV spread in the region and to perform an epizootological analysis of the data on BL incidence in the Republic of Dagestan in 2021.

MATERIALS AND METHODS

The basic materials used for the investigation of enzootic bovine leukosis in the Republic of Dagestan included reports of the Republican, Interraion and Raion veterinary laboratories for 2021 as well as data notified by the Raion diagnostic offices. Bovine (native) blood sera delivered from different settlements and livestock farms of various types of ownership were used in the studies.

Serological and hematological tests were performed in accordance with "Methodical instructions for diagnosis of bovine leukosis" using immunodiffusion assay (AGID) and hematology analyzer. The epizootological studies were performed in compliance with "Methodical instructions for epidemiological study of bovine leukosis" [15, 16].

RESULTS AND DISCUSSION

Bovine leukosis diagnostic tests were performed in 32 Interraion, Zonal veterinary laboratories including SBI RD Republican Veterinary Laboratory as well as in the diagnostic offices located in the Raions. Biological materials (blood, sera) collected from the animals were delivered to the veterinary laboratories according to their service area. However, there are distant pastures, where the animals are mostly moved in autumn and winter. All diagnostic tests of the animals kept on the premises located in such areas are performed in the zonal veterinary laboratories [17, 18]. In 2021, officers of the veterinary laboratories of the Republic of Dagestan subjected 720,489 bovine serum samples to serological tests for BLV, and 7,188 (1%) of the samples were reported seropositive. During the same period blood collected from 527 animals was hematologically tested for persistent leukocytosis, and 153 (29.03%) blood samples from hematologically diseased cattle were identified.

Table 1 demonstrates the results of serological and hematological tests of bovine sera and whole blood samples for EBL. Therefore, the highest amount of serological tests was performed in the laboratories located in the lowland area of Dagestan, where the major part of the animal

Table 1
BL epizootological monitoring in the Republic of Dagestan, 2021

No.	Veterinary laboratory	Serological tests			Hematological tests of AGID-positive animals		
		Number of animals	AGID-positive	%	Number of blood samples	Diseased animals detected	%
1	Republican	47,538	2,377	5.0	103	22	21.4
2	Agulskaya	4,444	–	–	–	–	–
3	Akushinskaya	24,974	–	–	–	–	–
4	Babayurt	44,456	789	1.8	312	90	28.8
5	Botlikh	51,768	–	–	–	–	
6	Buynaksk	28,242	81	0.3	–	–	–
7	Gumbetovsky	–	–	–	–	–	–
8	Gunib	16,236	48	0.3	–	–	–
9	Dakhadayevskaya	16,565	–	–	–	–	–
10	Derbent	18,075	25	0.14	–	–	–
11	Dokuzparinskaya	22,193	–	–	–	–	–
12	Izberbash	15,608	134	0.9	–	–	–
13	Kasumkent	21,764	–	–	2	–	–
14	Kizilyurt	38,339	188	0.5	–	–	–
15	Kizlyarsk	41,244	680	1.6	–	–	–
16	Kochubeyskaya	42,574	973	2.3	90	32	35.6
17	Kulinskaya	11,847	4	0.03	–	–	–
18	Kurakh	8,257	–	–	–	–	–
19	Lakskaya	17,866	4	0.02	–	–	–
20	Levashy	19,775	48	0.2	–	–	–
21	Maydanovskaya	24,124	–	–	–	–	–
22	Nogayskaya	16,051	11	0.07	–	–	–
23	Rutulskaya	6,402	–	–	–	–	–
24	Tabasarany	12,208	29	0.2	–	–	–
25	Tarumovskaya	26,313	803	3.1	–	–	–
26	Tlyaratinskaya	3,631	–	–	–	–	–
27	Khasavyurt	89,979	969	1.08	20	9	45.0
28	Khivskaya	6,437	–	–	–	–	–
29	Khunzakh	4,217	–	–	–	–	–
30	Tsuntinskaya	10,253	–	–	–	–	–
31	Charodinskaya	12,640	25	0.2	–	–	–
32	Shamilskaya	16,469	–	–	–	–	–
	Total	**720,489**	**7,188**	**1.0**	**527**	**153**	**29.03**

population is accumulated: in Khasavyurt Zonal Veterinary Laboratory – 89,979 bovine serum samples; in the Republican Veterinary Laboratory – 47,538 bovine serum samples; in Babayurt Veterinary Laboratory – 44,456 bovine serum samples; in Kochubeyskaya Veterinary Laboratory – 42,574 bovine serum samples, in Kizlyarsk Veterinary Laboratory – 41,244 bovine serum samples; in Kizilyurt Veterinary Laboratory – 38,339 bovine serum samples; in Tarumovskaya Veterinary Laboratory – 26,313 bovine serum samples, in Izberbash Veterinary Laboratory – 15,608 bovine serum samples. BLV seropositivity rate amounted to 1.08% (969 animals); 5.0% (2,377 animals); 1.8% (789 animals); 2.3% (973 animals); 1.6% (680 animals); 0.5% (188 animals); 3.1% (803 animals); 0.9% (134 animals), respectively. Analysis of the data submitted by the nine veterinary laboratories demonstrated that the level of the BLV infected animals was below 0.5%: in Derbent – 0.14% (25 animals), in Buynaksk – 0.3% (81 animals), in Gunib – 0.3% (48 animals), in Levashy – 0.2% (48 animals), in Tabasarany – 0.2% (29 animals), in Charodinskaya – 0.2% (25 animals), in Nogaysk – 0.07% (11 animals), in Kulinsk – 0.03% (4 animals), in Laksk – 0.02% (4 animals). In other 14 veterinary laboratories and diagnostic offices, located mostly in the mountainous regions, no bovine leukosis was diagnosed. No animal sera were tested for specific precipitating antibodies against BLV in Gumbetovsky Diagnostic Office.

The resulted data demonstrate that there is large-scale serological testing for BLV antibodies performed in the Republic, but hematological tests of blood sera are random, and the complete picture of EBL prevalence cannot be, therefore, made. Nevertheless, those little if any hematological analysis data obtained in 2021 and earlier indicate that the incidence level is high and hematologically diseased animals are not removed from the herd [19].

On the next stage, epizootological data on bovine leukosis in Raions and towns of the Republic in 2021 were analyzed. Dagestan consists of 41 Raions and 10 municipalities. Bovine sera were serologically tested for leukosis nearly in each administrative unit (Table 2). Total of 720,489 blood samples were tested and 7,188 (1.0%) of them turned seropositive. High percentage of BLV infected animals was reported in 14 Raions and 4 municipalities. The highest number of the seropositive animals is housed on the lowland farms and on remote pasture premises located in the foothill and mountainous regions. BLV infection in these Raions and municipalities ranged from 1.0 to 5.8%: in Kumtorkalinsky Raion – 5.8%, in Gunibsky Raion – 5.3%, in Tarumovsky Raion – 3.3%, in Karabudakhkentsky Raion – 2.9%, in Akhvakhsky Raion – 2.0%, in Kizlyarsky Raion – 1.8%, in Charodinsky Raion – 1.7%, in Kazbekovsky Raion – 1.6%, in Babayurtovsky Raion – 1.5%, in Tlyaratinsky Raion – 1.1%, in Dakhadayevsky Raion – 1.04%, in Sergokalinsky Raion – 1.02%, in Novolaksky and Shamilsky Raions – 1.0% in each, as well as in municipalities of Makhachkala – 2.0%, Izberbash – 1.14%, Khasavyurt – 1.1% and Yuzhno-Sukhokumsk – 1.0%. In 21 Raions and 2 municipalities BLV seropositivity level was below 1.0%. In other 6 Raions (Agulsky, Akhtynsky, Dokuzparinsky, Magaramkentsky, Khivsky, Suleiman-Stalsky) and two municipalities (Derbent, Dagestanskiye Ogni) no BLV infected animals were detected.

Table 2
BL diagnostic tests in the Raions and municipalities of the Republic of Dagestan, 2021 (according to SBI RD Republican Veterinary Laboratory)

No.	Raions and municipalities	Serological tests		
		Number of animals	AGID-positive	Infected animals detected, %
1	Agulsky	4,552	–	–
2	Akushinsky	45,590	180	0.4
3	Akhvakhsky	14,887	299	2.0
4	Akhtynsky	10,391	–	–
5	Babayurtovsky	11,372	174	1.5
6	Botlikhsky	32,405	130	0.4
7	Buynaksky	28,213	81	0.3
8	Gergebilsky	24,863	193	0.8
9	Gumbetovsky	11,356	35	0.31
10	Gunibsky	24,432	1,300	5.3
11	Dakhadayevsky	13,605	141	1.04
12	Derbentsky	14,754	22	0.15
13	Dokuzparinsky	9,077	–	–
14	Kazbekovsky	14,897	243	1.6
15	Kaytagsky	5,477	2	0.04
16	Kizilyurtovsky	14,397	45	0.3
17	Kumtorkalinsky	5,256	305	5.8
18	Kayakentsky	7,840	56	0.7
19	Karabudakhkentsky	9,228	263	2.9
20	Kizlyarsky	25,874	467	1.8
21	Kulinsky	22,206	111	0.5
22	Kurakhsky	9,090	2	0.02
23	Laksky	22,863	170	0.7
24	Levashinsky	21,965	77	0.4
25	Magaramkentsky	15,192	–	–
26	Novolaksky	10,674	106	1.0
27	Nogaysky	15,139	10	0.07
28	Rutulsky	9,201	36	0.4
29	Suleiman-Stalsky	9,297	–	–
30	Sergokalinsky	6,353	65	1.02
31	Tabasaransky	12,453	29	0.2
32	Tarumovsky	30,225	983	3.3
33	Tlyaratinsky	13,536	151	1.1
34	Untsukulsky	13,493	93	0.7
35	Khasavyurtovsky	59,650	305	0.5
36	Khivsky	7,196	–	–
37	Khunzakhsky	12,880	54	0.4
38	Tsumadinsky	16,568	88	0.5
39	Tsuntinsky, Bezhtinsky District*	6,528 3,215	11 –	0.2 –
40	Charodinsky	20,298	349	1.7
41	Shamilsky	23,306	224	1.0
42	Kizlyar m.	7,086	20	0.3
43	Makhachkala m.	14,785	290	2.0
44	Kaspiysk m.	706	4	0.6
45	Izberbash m.	702	8	1.14
46	Yuzhno-Sukhokumsk m.	3,895	39	1.0
47	Derbent m.	560	–	–
48	Dagestanskiye Ogni m.	456	–	–
49	Khasavyurt m.	2,505	27	1.1
	Total	**720,489**	**7,188**	**1.0**

*Bezhtinsky District – administrative region within Tsuntinsky Raion.

It was established that the level of BLV infection of the animals in the Republic did not decrease in 2021 (1.0%) as compared to 2020 (1.02%), and the number of the animals diagnostically tested for bovine leukosis increased [20].

Bovine leukosis epizootic data for 2015–2021 were reviewed for the examination of BLV spread dynamics.

The Figure demonstrates that the highest amount of the serological tests for bovine leukosis was performed in 2021, and the number of the infected animals was the lowest in this period. In 2015, only 7,310 bovine blood samples were tested and the seropositivity amounted to 13.9% (1,016 animals). High percentage of the infected animals was associated with the random testing for leukosis and lack of any program for the disease prevention and control in the Republic of Dagestan. Large-scale diagnostic testing for bovine leukosis was started in the region in 2018 and it was performed according to the "Plan of measures for bovine leukosis prevention and control to be implemented in the Republic of Dagestan in 2017–2020", but no disease control and preventive measures were implemented. As soon as the Plan was approved, the number of animals subjected to diagnostic tests increased dramatically: 223,293 animals in 2018; 625,970 animals in 2019; 524,930 in 2020; and 720,489 animals in 2021. The percentage of the BLV infected animals decreased from 4.03% (2018) to 1.0% (2021).

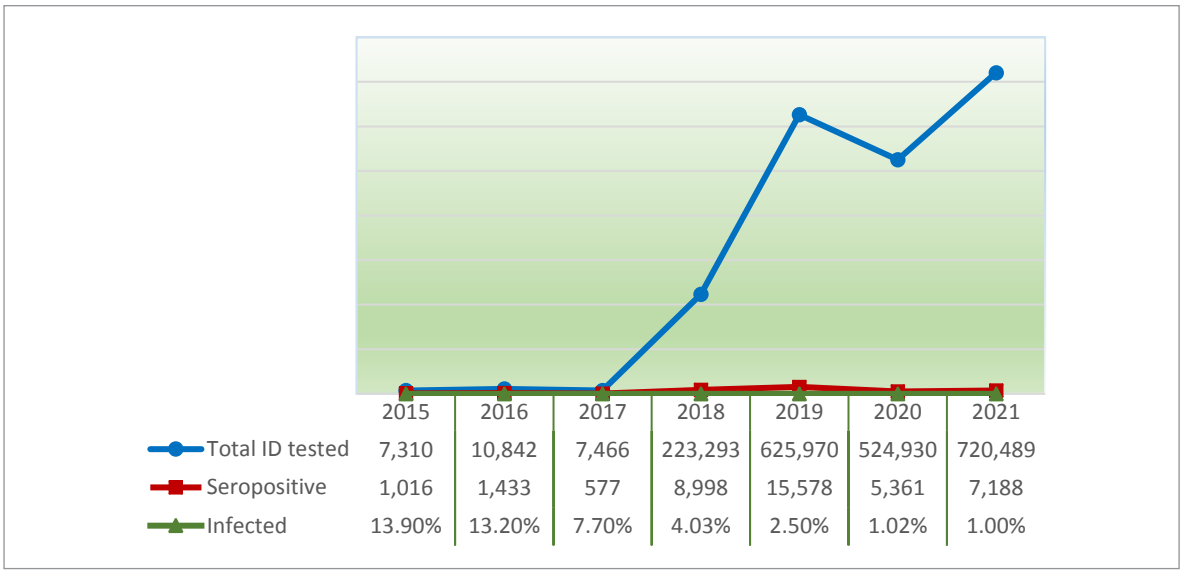

Fig. BL spread dynamics in the Republic of Dagestan, 2015–2021

Therefore, it can be concluded that the percentage of the BLV-infected animals and level of the leukosis incidence remain still high. This situation can be explained by the fact that systemic activities on the on-farm prevention and control of the disease have been started just recently and are carried out not in full extent.

CONCLUSION

In 2021, diagnostic tests for bovine leukosis were performed in all veterinary laboratories in the Republic of Dagestan, excluding Gumbetovsky diagnostic office.

720,489 bovine serum samples were subjected to serological tests, out of which 7,188 (1.0%) were seropositive. High levels of animal infection were identified in 14 Raions and 4 municipalities. In 2 municipalities and 21 Raions BLV seropositivity level was below 1.0%. No BLV infected animals were detected in Agulsky, Akhtynsky, Dokuzparinsky, Magaramkentsky, Khivsky, Suleiman-Stalsky Raions and in Derbent and Dagestanskiye Ogni municipalities. Over the period from 2015 to 2021 the lowest percentage of BLV infected animals was reported in 2021 – 1.0% of tested animals.

Proceeding from the above the conclusion can be made that bovine leukosis is diagnosed almost in all veterinary laboratories of the Republic, the incidence level is high and targeted control and preventive measures are incomplete.

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Actual bovine tuberculosis situation in the Republic of Dagestan

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SUMMARY

Lack of statistical data and inconsistences in science and practice make it impossible to give at least approximate tuberculosis prevalence rates in the Republic of Dagestan. Every year the number of tuberculin reacting animals is increasing. For example out of 2,944 tested heifers of breeding age in 2014–2019, up to 30% of animals had positive reactions in tests. During this period out of 1,166 emergency slaughtered animals, tuberculosis was confirmed in 326 animals (28%). Bacteriological tests revealed 291 mycobacterium cultures, 107 out of them were *Mycobacterium bovis*, the other 184 cultures were identified as atypical ones. Based on the species differentiation of 58 cultures, 22 Group II cultures (according to Runyon classification) were isolated; 18 out of them belonged to *Mycobacterium gordonae*, 2 to *Mycobacterium flavescens*, and species of two cultures could not be identified. Four cultures of Group III were species of *Mycobacterium intracellulare*. Out of 32 cultures of Group IV, two belonged to *Mycobacterium smegmatis*, seven to *Mycobacterium fortuitum* and one to *Mycobacterium phlei*, 22 cultures were not identified. To elucidate the role of milk in tuberculosis epidemiology 82 samples of milk from reactors from two farms were tested. In the farm, where reactors were awaiting their removal for a long time, mycobacteria were detected in 20% of milk samples, whereas in the recently infected farm the detection rate was 4%, which suggests that long awaiting periods present high risks. Microscopic, conventional phenotypic and targeted biochemical tests indicate that pseudo-allergic reactions, revealed by tests, result from the atypical mycobacteria of the mentioned groups and species, which present in the animal organism, and seem to be responsible for the tuberculin sensibilization. Timely and comprehensive diagnostic and animal health measures will improve the situation.

Keywords: tuberculosis, cattle, mycobacteria, atypical mycobacteria, infected farms, tuberculin, allergy tests, differentiation, identification, pseudo-allergic reactions

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Актуализированная эпизоотическая ситуация по туберкулезу крупного рогатого скота в Республике Дагестан

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РЕЗЮМЕ

Из-за несовершенства статистических данных и несоответствия расчетных и практических показателей не представляется возможным привести хотя бы приблизительные цифры о заболеваемости животных туберкулезом в Республике Дагестан. С каждым годом число реагирующих на туберкулин животных растет. Так, из 2944 исследованных телок случного возраста в 2014–2019 гг. выявлено до 30% особей, реагирующих на введение туберкулина. За этот период из 1166 подвергнутых вынужденному убою животных диагноз на туберкулез подтвержден у 326 (28%). При проведении бактериологических исследований удалось изолировать 291 культуру микобактерий, из них к *Mycobacterium bovis* отнесено 107 культур, остальные 184 идентифицированы как атипичные. Во многих хозяйствах одновременно с *Mycobacterium bovis* выделялись и нетуберкулезные кислотоустойчивые микобактерии. При видовой

дифференциации 58 культур изолировано 22 культуры второй группы (по Раньону), 18 из которых отнесены к *Mycobacterium gordonae*, 2 – к *Mycobacterium flavescens*, у двух видовую принадлежность установить не удалось. Четыре культуры третьей группы являются представителями вида *Mycobacterium intracellulare*. Из 32 культур четвертой группы 2 отнесены к *Mycobacterium smegmatis*, 7 – к *Mycobacterium fortuitum* и 1 – к *Mycobacterium phlei*, у 22 культур вид не установлен. Для выяснения роли молока в эпизоотологии туберкулеза исследовано 82 пробы от реагирующих на туберкулин животных двух хозяйств. В одном, где реагирующие животные передерживались длительный период, микобактерии в молоке выявлялись в 20% случаев, в другом, где туберкулез выявлен недавно, доля обнаружения составляла 4%, что говорит о большой опасности длительной передержки животных с положительной аллергической реакцией. Проведенные микроскопические, традиционно фенотипические и узкие биохимические исследования свидетельствуют, что выявляемые в процессе диагностики парааллергические реакции обусловлены наличием в организме животных атипичных микобактерий отмеченных групп и видов, которые, по-видимому, обуславливают сенсибилизацию организма к туберкулину. Своевременное и полное выполнение диагностических и ветеринарно-санитарных мероприятий позволит улучшить ситуацию в республике.

Ключевые слова: туберкулез, крупный рогатый скот, микобактерии, атипичные микобактерии, неблагополучные пункты, туберкулин, аллергические исследования, дифференциация, идентификация, парааллергические реакции

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INTRODUCTION

In recent years, certain success has been achieved in many districts of the Republic of Dagestan due to comprehensive organizational, economic, veterinary and sanitary measures aimed at eradication and prevention of bovine tuberculosis (BT). There has been a noticeable improvement in hygiene practices employed across animal production and increase in the number and quality of diagnostic tests. Diseased cattle are timely isolated and slaughtered. At the same time, bovine tuberculosis in some regions of the Republic still poses a serious threat to animal production and human health [1, 2].

Comprehensive research conducted earlier on the BT epidemic situation in all natural and climatic zones of the Republic showed the highest prevalence of the disease in the lowland farms and a steady increase in the incidence due to fundamentally different factors that compromise the immunobiological status of animals. Out of 26 infected farms, identified in the Republic in recent years, only three were located in the mountainous area. This is explained not by the resistance to tuberculosis of local cattle, but by the fact that in mountainous areas, due to the small size of farms, the delivery of feed and contacts between animals, including in the private sector, are restricted, and abundant vegetation of alpine and subalpine meadows, significant solar aeration, and large pastures, etc. are available [1–5].

The results of the study suggest that farms become infected due to various reasons: the introduction of infected young replacement animals, feeding with raw milk, pooling and regrouping of young replacement animals and cows from farms with different animal health statuses, long awaiting periods for diseased animals, inappropriate veterinary, preventive and organizational and management measures, etc. [5–12].

Along with the study of various pathogen introduction ways, indicators of the incidence rate and the influence of various factors on the spread of tuberculosis are of considerable interest. These data are directly related to the organization of preventive measures, investigation of the likely timing of the pathogen introduction into the farm, as well as evaluating of the performance and reliability of diagnostic tests [13–17].

The incidence rate of cattle re-infection in the infected areas depends mainly on the quality of feeding and management conditions. Severe violations of hygienic conditions, micro- and macroclimate, unbalanced diet lead to a decrease in individual resistance of the macroorganism, contribute to the transmission of the pathogen from one animal to another and reduce the incubation period [18–23].

In this regard, a large group of transient nontuberculous acid-fast mycobacteria and mycobacteria-like microorganisms related to mycobacteria, which are widespread in nature and are not fastidious to environmental conditions and resistant, is of particular interest. Under these conditions, it is acceptable that infection of cattle with non-tuberculosis microorganisms occurs with a frequency no less than infection with tuberculosis-causing pathogens, which ultimately results in sensitization of the body to mammalian PPD-tuberculin and false positive results due to the low specificity of the diagnostic tests used [1, 2, 24–27].

In general, the problem of diagnosing tuberculosis, including in Dagestan, involves nonspecific reactions to tuberculin. Reacting animals are often identified among livestock purchased outside the Republic [1, 2, 7].

In this regard, the aim of the work was to obtain additional data on bovine tuberculosis situation in the Republic of Dagestan and the causes of nonspecific sensitization to mammalian PPD-tuberculin.

MATERIALS AND METHODS

In total, from 2014 to 2019, 2,944 heifers of breeding age were subjected to skin test. For the post-mortem examination, 1,166 animals were slaughtered using a humane method. 291 cultures of mycobacteria were isolated from pathological material obtained from 67 farms. Identification and differentiation of 104 cultures was carried out based on the Runyon classification.

The intradermal tuberculin skin test, post-mortem examinations of animals slaughtered for diagnostic purposes and laboratory tests of pathological material were performed in accordance with the "Manual on the Diagnosis of Animal Tuberculosis" (2002)[1]. The mammalian tuberculin produced by the FKP "Kursk Biofactory" (Russia) was injected intradermally using a needle-free BI-7 injector (JSC MIZ-Vorsma, Russia). The reactions were recorded and evaluated 72 hours after injection by measuring the thickness of the skin using TB caliper. Animals with increased skin thickness by three or more millimeters in comparison with a healthy area were considered reactors [3, 5, 11].

When conducting a simultaneous test, a complex allergen from atypical mycobacteria (CAM) was used together with mammalian PPD-tuberculin. The results were evaluated by the intensity of responses to tuberculin and CAM. A more intense reaction to tuberculin indicated homologous infection.

During the post-mortem examination, attention was paid to the site and size of granulomas (tubercles), the nature of inflammation in the lymph nodes, vessels and capsules surrounding the tubercles. The necrosis color, the density of the junction with the surrounding capsule, the capsule inner surface as well as, the consistency of the nodule contents when incised were analyzed. Post-mortem focal catarrhal and catarrhal-purulent inflammations were detected in the lungs of cattle from farms infected with tuberculosis for a long period. Extensive lesions in the lungs, caused by lobular and lobar pneumonia with multiple necrotic foci, along with lymphadenitis of the bronchial and portal lymph nodes, became a visible confirmation of advanced tuberculosis.

Isolated mycobacteria were identified in accordance with GOST 26072-89 (ST SEV 3457-81) "Agricultural animals and poultry. Methods of laboratory diagnostics of tuberculosis"[2] and GOST 27318-87 (ST CMEA 5627-86) "Agricultural animals. Methods of identification of non-typical microbacteria"[3].

The lymph nodes (parotid, submandibular, pre-scapular, bronchial, portal, supramental) served as the material for laboratory testing. The seed was pre-treated using the Sumiyoshi-Löwenstein and Hohn method by acid exposure for 30 minutes.

Primary identification was performed taking into account cultural characteristics: colony growth rate on dense nutrient media, colony color, pigmentation and colony morphology.

The material was seeded on blood agar by spreading 2–4 drops of the suspensions from the Löwenstein – Jensen medium. Next, the plates with blood agar were placed in an incubator at a temperature of 37 °C with oxygen. The results were analyzed visually after 24–48 hours of incubation.

The relation of the isolated culture to *Mycobacterium tuberculosis complex* or to non-tuberculous acid-fast mycobacteria was confirmed by specific laboratory and conventional phenotypic, microscopic and targeted biochemical methods. Among the biochemical methods, niacin, nitrate reduction and heat stable catalase tests were used [28].

RESULTS

TB skin tests of 2,944 heifers of breeding age, supplied in 2014–2019, showed up to 30% reactors in some groups. Reactions in individual animals persisted for up to a year, tuberculin allergic reactions disappeared and occurred from time to time.

In one of the farms, 29 reactors were identified out of the purchased 136 animals by the end of the quarantine. These animals were tested simultaneously after 40 days, with 20 reacting and 13 of them repeatedly reacting. The control slaughter of three animals did not reveal any post-mortem lesions consistent with tuberculosis. The results of bacteriological tests of animals slaughtered for diagnostic purposes were negative. After 45 days, the animals were re-tested and only five animals re-reacted. The next testing was performed after 6 months, all previously reacting animals showed no reactions, on the contrary, positive reactions to tuberculin were registered in 43 animals, who had not reacted before.

A similar situation was observed in other farms that purchased genetically improved or breeding heifers.

In 2014, 28 of the imported animals were slaughtered for diagnostic purposes, tuberculosis was not detected in any case, but the introduced animals continued to react to tuberculin.

To clarify the results of skin tests in 2014–2019, 1,166 animals were subjected to control slaughter. At the same time, tuberculosis lesions were found in the lymph nodes (pharyngeal, bronchial, mediastinal, submandibular), as well as generalization involving parenchymal organs in 326 animals, representing 28.0% (Table).

The test results showed a decreasing coincidence of the skin test results with post-mortem results. Thus, during control tests of reactors in 2014, tuberculosis lesions were detected in 77.0% of animals. In 2019, thanks to targeted actions, including keeping of livestock in isolators, on-farm processing, introduction of healthy animals and the implementation of veterinary and sanitary measures pursuant to regulations, the proportion of diseased animals with tuberculosis lesions decreased to 9.2%.

291 cultures of mycobacteria were isolated during laboratory tests of pathological material of slaughtered animals from 67 farms. Their differentiation enabled to reveal that 107 cultures in 31 farms were *Mycobacterium bovis*, 184 isolated cultures in 36 farms belonged to atypical mycobacteria. Atypical mycobacteria were isolated simultaneously with *Mycobacterium bovis* in 15 farms.

[1] https://files.stroyinf.ru/Data2/1/4293744/4293744181.pdf.
[2] https://docs.cntd.ru/document/1200025492.
[3] https://base.garant.ru/5917269.

Table
Results of post-mortem and bacteriological tests for tuberculosis

Year	Slaughtered	Detected	%	Samples tested	Cultures isolated			Tested among atypical ones	Group by Runyon			
					in total	including			I	II	III	IV
						Mycobacterium bovis	atypical					
2014	122	94	77.0	15	10	10	–	–	–	–	–	–
2015	115	47	40.9	11	7	3	4	–	–	–	–	–
2016	165	59	35.8	167	105	24	81	55	–	22	1	32
2017	348	91	26.1	195	105	50	55	26	–	17	1	8
2018	243	19	7.8	116	48	11	37	16	–	12	1	3
2019	173	16	9.2	150	16	9	7	7	–	1	1	5
Total	1,166	326	28.0	654	291	107	184	104	–	52	4	48

In a number of farms, despite a significant number of reactors, tuberculosis was not established by post-mortem examinations and bacteriological tests. In most cases, atypical mycobacteria were isolated from the pathological material of these animals.

Of 104 cultures of atypical mycobacteria being differentiated according to the Runyon classification, 52 were classified as Group II (scotochromogens), 4 as Group III (nonchromogens) and 48 as Group IV (rapid growers), Fig. 1.

Detections of reactors not showing any visible pathological lesions in internal organs are frequent in Dagestan. Isolation of such animals does not stop the detection of new reactors. Therefore, the observed phenomenon is of interest and is the reason for its comprehensive study.

According to various publications, laboratory tests of biological materials often reveal atypical mycobacteria belonging to Ranyon IV organisms – *Mycobacterium fortuitum* and *Mycobacterium chelonae,* which can be potentially pathogenic for both animals and humans.

At the same time, the sensitizing and pathogenetic roles of rapid growers for cattle remain understudied and controversial. In this regard, when assessing the tuberculosis situation, special attention should be paid to identification of isolated mycobacteria, including atypical ones, since knowing the species has a great practical and theoretical importance for successful prevention and eradication of tuberculosis on farms.

Nontuberculous acid-fast mycobacteria have a number of common features with true tuberculosis agents (morphology, tinctorial properties, acid-, alcohol- and alkali-resistance), but at the same time they have a number of properties similar to saprophytic mycobacteria (colony shape, growth rate, enzymatic activity, drug resistance).

In order to differentiate species within groups, 58 cultures were subjected to a more detailed testing.

As a result, out of 22 cultures of Group II, 18 were identified as *Mycobacterium gordonae*, 2 as *Mycobacterium flavescens*, two species could not be identified.

It was found that all 4 cultures of Group III are representatives of *Mycobacterium intracellulare* species.

Of 32 cultures of Group IV, 2 were classified as *Mycobacterium smegmatis*, 7 as *Mycobacterium fortuitum* and 1 as *Mycobacterium phlei*, 22 cultures were not identified (Fig. 2).

The data obtained reflect the high species heterogeneity of atypical mycobacteria in cattle reactors.

Of all the tested cultures, 34 different species were established in 34, which is 58.6%.

The results of quantitative distribution show that the largest number of identified species was classified as Group II. They can probably play a significant role in the sensitization of cattle to mammalian PPD-tuberculin, but further studies using a larger number of strains are needed to confirm the correlation between the species and sensitization.

The distribution analysis of the identified species showed that the highest number of different species of atypical mycobacteria isolated from reactors' pathological material was observed in Group IV, which is an experimental confirmation of numerous published data. At the same time, considering that only 31.3% of cultures

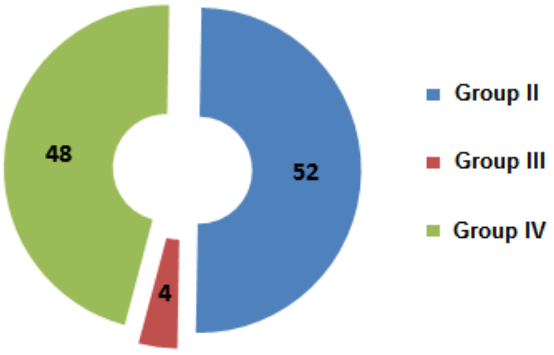

Fig. 1. Grouping of isolated atypical mycobacteria based on Runyon classification

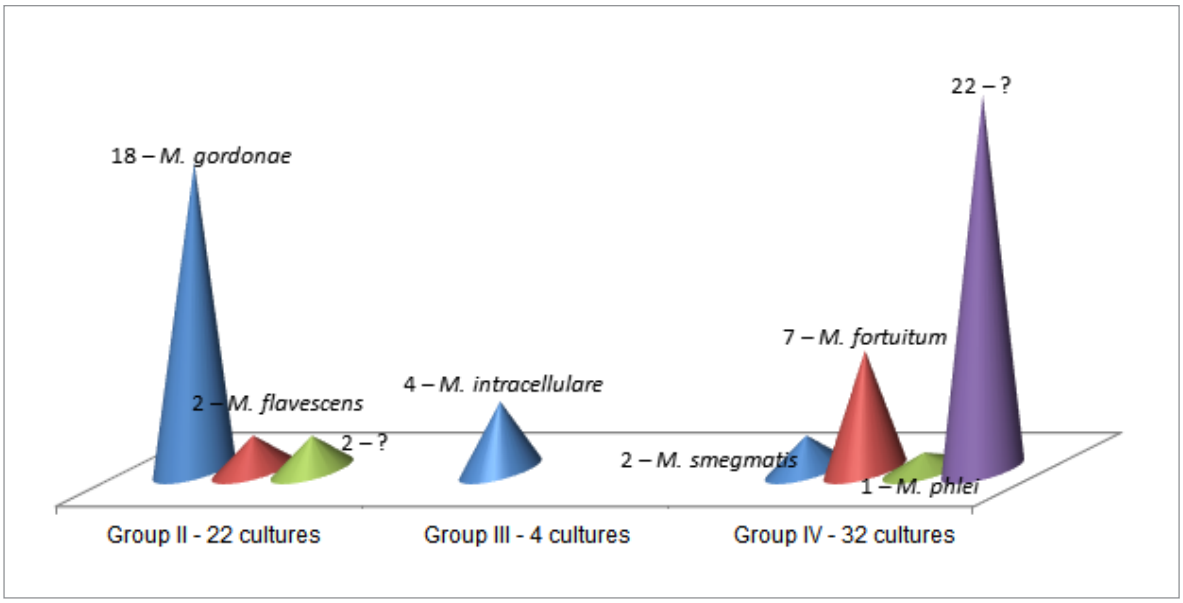

Fig. 2. Species differentiation of atypical mycobacteria

(10 out of 32) were identified in this group, it becomes obvious that representatives of this group play a particularly important role in the sensitization of the macroorganism to tuberculin.

In general, the data obtained became the basis for further dynamic monitoring of the circulation of nontuberculous acid-fast microorganisms in biological material in order to optimize differential diagnosis of bovine tuberculosis.

To clarify the role of milk in the epizootology of tuberculosis, 82 samples from reactors from two farms were tested. One of the farm had been infected for a long period; the other had been infected recently.

In the first farm, where reactors awaited their removal for long periods, mycobacteria in milk were detected in 20% of cases; in the second farm, the detection rate was 4%, which suggests the great danger of prolonged awaiting periods.

CONCLUSION

The data obtained give grounds to believe that the pseudoallergic reactions in skin tests are caused by the presence of atypical mycobacteria of the above mentioned groups and species in animals, which apparently cause sensitization of the organism to tuberculin.

The results of species differentiation did not allow identification of a certain group of atypical mycobacteria species causing increased sensitization to tuberculin.

Isolation of pure mycobacterium cultures from pathological material and their identification should be carried out in close connection with the detection of allergic reactions to tuberculin.

Due to the complex contradictory BT situation in the Republic of Dagestan, a comprehensive plan of anti-tuberculosis measures was drawn up. At the same time, the main attention was paid to the protection of BT free farms from the introduction of tuberculosis, timely and complete identification and removal of diseased animals and reactors from farms, measures to destroy the pathogen in the environment and raising healthy young animals to replace diseased livestock. Diagnostic activities were strengthened in free farms and farms where the disease is eradicated under veterinary control.

Timely and comprehensive anti-tuberculosis measures will allow achieving positive results in the disease control.

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Classical swine fever: a retrospective analysis of the epizootic situation in the Russian Federation (2007–2021) and forecast for 2022

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SUMMARY
The paper presents trends in the epizootic situation on classical swine fever (CSF) in the Russian Federation, for 2007–2021. Most likely, a drop in the number of CSF outbreaks throughout the country results from two factors: a geographical shift of the disease outbreaks from the European part of Russia to the eastern regions bordering on China (into the wild boar population), as documented between 2015 and 2021, and a large-scale vaccination of domestic pigs practiced in the recent years. The introduction and spread of CSF in the Russian Federation are, most likely, associated with the internal risk factors (i.e. quality of anti-epizootic measures, mainly vaccination) and with the territories, where the virus circulates in wild boars. Expansion of vaccination coverage since 2011 is one of the factors contributing to a decrease in the number of clinical CSF cases registered in domestic pigs of the Russian Federation. The infection spread in domestic pigs is still on a downward trend. For purposes of analysis, current trends of CSF spread in domestic pigs and wild boars in the Russian Federation, as well as the volume of the vaccine used, were visualized in relative numbers (taking into account total number of pigs in the country) used to build a regression model. Currently, vaccination against classical swine fever in the Russian Federation (and its good quality) is an essential prerequisite to contain the infection spread in the country.

Keywords: classical swine fever, the Russian Federation, epizootic situation, retrospective analysis, vaccination

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Классическая чума свиней: ретроспективный анализ эпизоотической ситуации в Российской Федерации (2007–2021 гг.) и прогноз на 2022 г.

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РЕЗЮМЕ

Представлена тенденция развития эпизоотической ситуации по классической чуме свиней в Российской Федерации в 2007–2021 гг. Констатируется факт территориального смещения в 2015–2021 гг. очагов инфекции из европейской части России в восточные, приграничные с Китаем регионы в популяцию дикого кабана и усиления вакцинопрофилактики в домашней популяции в последние годы, что, вероятнее всего, было определяющим в снижении количества очагов классической чумы свиней на всей территории страны. Основные особенности заноса и распространения инфекции в Российской Федерации с большей вероятностью связаны с внутренними факторами риска (качество исполнения противоэпизоотических мероприятий, главным образом вакцинации) и территориями циркуляции вируса среди диких кабанов. К числу факторов, способствующих снижению числа регистрируемых клинических случаев классической чумы свиней в популяции домашних свиней Российской Федерации, можно отнести прирост с 2011 г. объемов вакцинации. Тренд неблагополучия в популяции домашних свиней остается ниспадающим. Тенденции развития эпизоотической ситуации по классической чуме свиней на территории Российской Федерации в популяциях домашних и диких свиней и объемы применения вакцин для целей анализа были визуализированы в относительных величинах, учитывающих общую численность поголовья свиней в стране, которые использовали для построения регрессионной модели. На основе анализа дан прогноз на 2022 г. в условиях сохранения выбранной в стране стратегии борьбы с заболеванием. Вакцинация против классической чумы свиней в Российской Федерации и ее качество на данный момент остается предопределяющим фактором сдерживания распространения эпизоотии на территории страны.

Ключевые слова: классическая чума свиней, Российская Федерация, эпизоотическая ситуация, ретроспективный анализ, вакцинация

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INTRODUCTION

Classical swine fever (CSF) is a transboundary viral disease, which remains one of the most serious swine diseases in the world. It is caused by an RNA virus belonging to *Pestivirus* genus of *Flaviviridae* family [1]. There are three CSF virus genotypes with three to four subgenotypes, that do not directly correlate with the virulence. It is important to note that genetic diversity does not make it possible to obtain true serotypes and it has no impact on the vaccine effectiveness [2, 3]. A wide range of CSF clinical signs (including the ones observed after vaccination) requires a laboratory confirmation of the disease. In most countries with well-developed pig industry CSF occurs, at least, sporadically [4, 5].

Wild boars are a reservoir of the virus, which is able to circulate for a long time in the infected population, thus, posing a threat to poorly protected pig farms and backyards. It can be assumed that the disease is also endemic in certain countries of South and Central America and in some parts of Eastern Europe and Asia [2, 6, 7]. Little is known about the situation in Africa [4, 5].

CSF control strategy in the disease-free countries mainly includes conventional measures to control an outbreak of a highly dangerous disease: quarantine, destruction of all animals in the infected herd, contact tracing, timely and reliable diagnosis, creation of restriction zones, tracing commodities associated with the risk of the virus spread. Once such strategy is in place, vaccination is assumed as a possible, however, an emergency option, although it was practiced at the first stage of the disease control in the currently disease-free countries [2, 8]. Vaccines are used as a routine option to contain the disease in CSF endemic regions of Asia, Eastern Europe, America and some African countries [4, 8].

In Russia, the situation on CSF in domestic pigs is getting less tense [9] in most parts of the country, which is, probably, explained by mass vaccination and strong anti-epizootic measures (passive surveillance, compartmentalization, regionalization). At the same time, taking into account CSF potential for rapid territorial and transboundary spread, it seems appropriate to retrospectively analyze the epizootic situation in the Russian Federation and to discuss the expected course of events, assessing infection status of the wild boar population and the use of vaccination.

MATERIALS AND METHODS

For this research, we used data from the World Animal Health Organization information system (WAHIS) together with the official veterinary reports from the FGBI "Veterinary Center" [5].

The information was processed with the help of descriptive statistics, correlation and regression analysis using STATISTICA 10 software (StatSoft, Inc., 2011).

For the purposes of analysis, CSF trends in domestic pigs and wild boars in the Russian Federation, as well as the volume of the vaccine used, were visualized in relative numbers K1 and K2 for the period from 2007 to 2020. Total pig population in the country was taken into account; K1 is the ratio between the number of outbreaks per year to the total pig population (million animals), K2 is the ratio between the number of animals vaccinated against CSF (million animals) and the total pig population (million animals). K1 and K2 values for 2011–2018 were used to build a regression model [10].

Prognostic values for CSF outbreaks in 2022 were calculated based on "Poisson random walk" model using the Poisson distribution. Calculations were done in the @RISK program using Monte Carlo simulation in 10,000 iterations. The time interval from 2010 to 2021 was chosen for analysis.

The analysis results and the forecast are given in discussion and conclusion.

RESULTS AND DISCUSSION

Epizootic situation on classical swine fever in different countries. According to the official data of the World Organization for Animal Health (WOAH) for 2021, only 38 countries on different continents are officially free from CSF, and 3 countries have separate disease-free zones. A difficult situation is registered in Asian and South American countries. Thus, from 2017 to 2019 more than 300 CSF outbreaks were recorded in Indonesia; more than 150 outbreaks were reported in Vietnam within the same period; more than 50 outbreaks – in China and India (each); 18 outbreaks – in Nepal; 11 outbreaks – in Thailand; more than 230 outbreaks – in Cuba; 150 outbreaks – in Peru; 115 outbreaks – in Ecuador; 25 outbreaks – in Colombia; 11 outbreaks – in the Dominican Republic and Haiti (each) [5].

More than a quarter-century Japan remained free from CSF without vaccination. However, in 2018, the virus was introduced into the wild boar population and the infection spread widely. Next year, compared to the previous year, Japan reported a decrease in the number of outbreaks from 1,633 to 972 due to vaccination of wild boars [5, 11, 12].

In South Korea, since 2017, there has been a rapid increase in the number of seropositive wild boars caught near the demilitarized zone bordering on North Korea. CSF spread in South Korean wild boars was reported from west to southeast, due to such external factors as small-scale hunting, geographical features and road network development. The virus introduction was associated with infection circulating in wild boars in China, where the disease is endemic [13].

In Colombia, 134 CSF outbreaks were reported in the Atlantic coast region within six years (from 2013 to 2018). The first outbreak in 2013 was associated with the import of infected pigs from Venezuela, where, under the current socio-economic circumstances, pig prices were lower from those in Colombia. The role of the illegal trade in pork and animals between the countries is still unknown, but the fact that the Colombian police confiscated 48.8 tons of pork and 778 smuggled live pigs in the departments of Guajira, North Santander, Arauca, Cesar and the metropolitan area of Bogota (from 2013 to 2018) confirms that such trade shall not be underestimated. Most outbreaks (95%) were reported in the backyards. CSF introduction and spread mainly resulted from import of infected pigs (38%) and movement of people (37%) [14].

Brazil remained CSF-free for ten years. In 2018, CSF virus was re-introduced to the country and 38 outbreaks were registered in domestic pigs. In 2019, 30 more outbreaks were recorded in domestic pigs; for 10 months of 2020, 2 outbreaks were also reported in domestic pigs. However, due to the lack of accurate data, it is difficult to judge what exactly caused the situation [15].

Owing to the absence of CSF outbreaks, some countries (mainly African) have declared freedom of their territories from CSF without any official recognition from the WOAH [5].

The WOAH classifies CSF as one of those six diseases, which require official recognition for a country to get the freedom status [5]. As of 2021, 38 countries in the world were recognized CSF-free. In 2021, disease-free status was reinstated in Colombia and Brazil for those zones, which are key pig industry centres in Latin America; and Romania lost its status in 2020 based on the findings of the WOAH mission aimed to check compliance with the provisions of the Terrestrial Animal Health Code. Infection eradication programs were earlier implemented in most officially recognized disease-free countries. Those programmes were implemented step by step, often included vaccination at the first stage to reduce the number of clinical cases and contain the disease spread, however, the vaccination was totally excluded at the final stages, regardless of the large economic costs [2, 16].

The list of the countries where vaccination against CSF is currently practiced (according to WAHIS) includes: Armenia, Azerbaijan, Belarus, Bosnia and Herzegovina, Bulgaria (only wild boars), People's Republic of China, Taiwan, Colombia (in some zones), Cuba, Dominican Republic, Ecuador, Macedonia, Haiti, Hong Kong, India, Indonesia, Korea, Madagascar, Moldova, Montenegro, Myanmar, Nepal, Peru, Philippines, Serbia, Timor, Ukraine, Vietnam, Thailand, Russia [5].

Thus, the global epizootic situation on classical swine fever (in Eurasia, Central and South America) remains tense. The main CSF control measures include: vaccination and prevention of involvement of wild boars and domestic pigs (from a poorly protected population) into the epizootic process.

Epizootic situation on classical swine fever in the Russian Federation in 2007–2021. CSF cases are annually registered in Russia. From 2007 to November 2021 (as of 25 November 2021), 42 CSF outbreaks were reported in domestic pigs and 51 in wild boars.

Figure 1 shows that infection trends have different directions for populations of CSF susceptible animals. So, a downward trend is shown for domestic pigs (with an average number of outbreaks per year – 2.47 ± 2.48). On the contrary, an upward trend is shown for wild boars (with an average number of outbreaks per year – 3.00 ± 2.50). Thus, CSF situation tends to improve in the population of domestic pigs; however, it tends to worsen for wild boars.

Retrospective data obtained after examination of CSF outbreaks show that, within the analyzed time period, the outbreaks in domestic pigs were reported on the farms

with low biosecurity levels, where animals were often fed food wastes or where failures in vaccination against CSF were observed. A serious problem arouse from the impossibility to find accurately the pathogen source and its transmission paths on such farms. Virtually all the options were plausible: introduction with feed; feeding food wastes to the animals; because of pig handlers; import of live animals; contacts between free-ranging domestic pigs and wild boars, etc. Taking into account multiple ways of the disease introduction, there is a permanent risk of further virus spread in the pig population on poorly protected farms. Therefore, it was difficult to detect the ways of further spread while searching for connections between suspicious/disease-related farms.

The pathogen introduction into the wild boar population poses a significant risk of CSF spread. Alongside with it, an increase in the number of CSF cases registered in the recent years in the Far East is alarming.

The analysis of CSF territorial distribution in the Russian Federation from 2016 to 2020 (no cases detected in 2021) showed that the disease was registered in wild boars in the Primorsky Krai and the Amur Oblast, and in domestic pigs it was only reported in the Primorsky Krai and the Moscow Oblast (Fig. 2).

Since 2013, there has been a geographical shift of CSF-affected area to the western and eastern borders of the Russian Federation. In addition to it, since 2015, CSF outbreaks were reported exclusively along the eastern border of the country: in the Amur Oblast and the Primorsky Krai with one backyard incident in the Zibrovo village, the Serpukhov District of the Moscow Oblast being an exception. On 7 July 2018, CSF was diagnosed there with the help of polymerase chain reaction carried out by GBUV MO "Moscow Regional Veterinary Laboratory". The fact that no data are available on other confirmatory laboratory tests (i.e. differentiation of the detected genetic material: an epizootic or vaccine strain) raises doubts about reliability of the made diagnosis.

The FGBI "ARRIAH" regularly receives materials for CSF laboratory tests. In 2020, pathological material was received from the Primorsky Krai, taken from wild boars shot in three districts of the region: Shkotovsky (FGBI "GOOH "Orlinoye"), Pogranichny (near the settlement of Sofye-Alekseyevskoe), Spassky (near the settlement of Novovladimirovka).

Initially, the received material was tested in the FGBI "Primorsky Interoblast Veterinary Laboratory" using real-time reverse transcription polymerase chain reaction (RT-PCR). The test resulted in detection of CSF RNA. Later on, the FGBI "ARRIAH" tests confirmed CSF agent in the samples, and helped to type the detected virus. For this purpose, E2 gene fragment of the virus was amplified, sequenced and subjected to a comparative analysis.

Phylogenetic analysis showed that the Primorsky isolates of 2020 belong to subgenotype 2.1 of CSF virus (Fig. 3). At the same time, isolates from the Shkotovsky and Spassky raions belong to Cluster 2.1d and are genetically very close to Primorsky isolates recovered in 2015–2019.

The CSF virus isolate from the border region belongs to a different cluster and is genetically closely related to the virus isolated from a wild boar in the Amur Oblast in 2019.

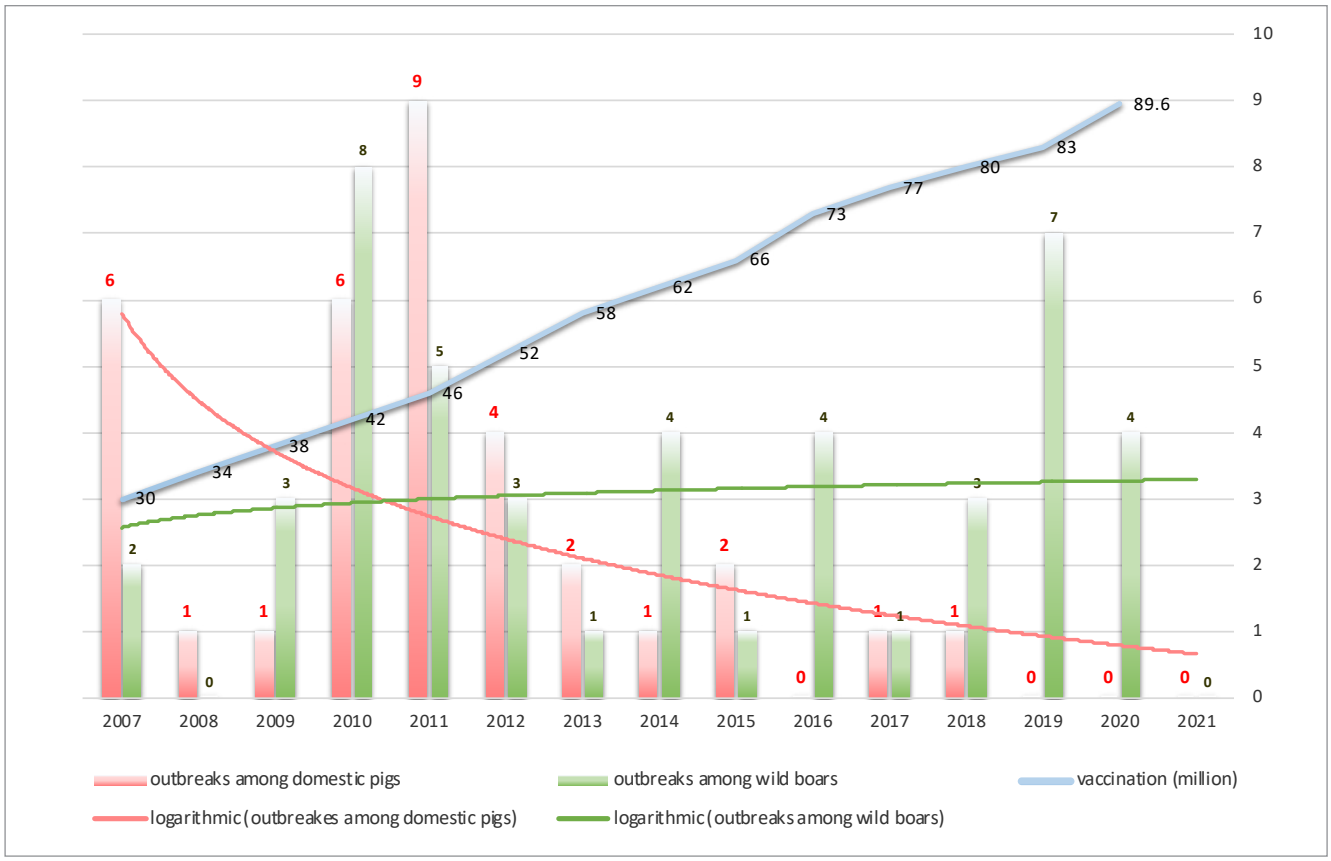

Fig. 1. Number of CSF outbreaks in the Russian Federation among domestic pigs and wild boars in 2007–2021

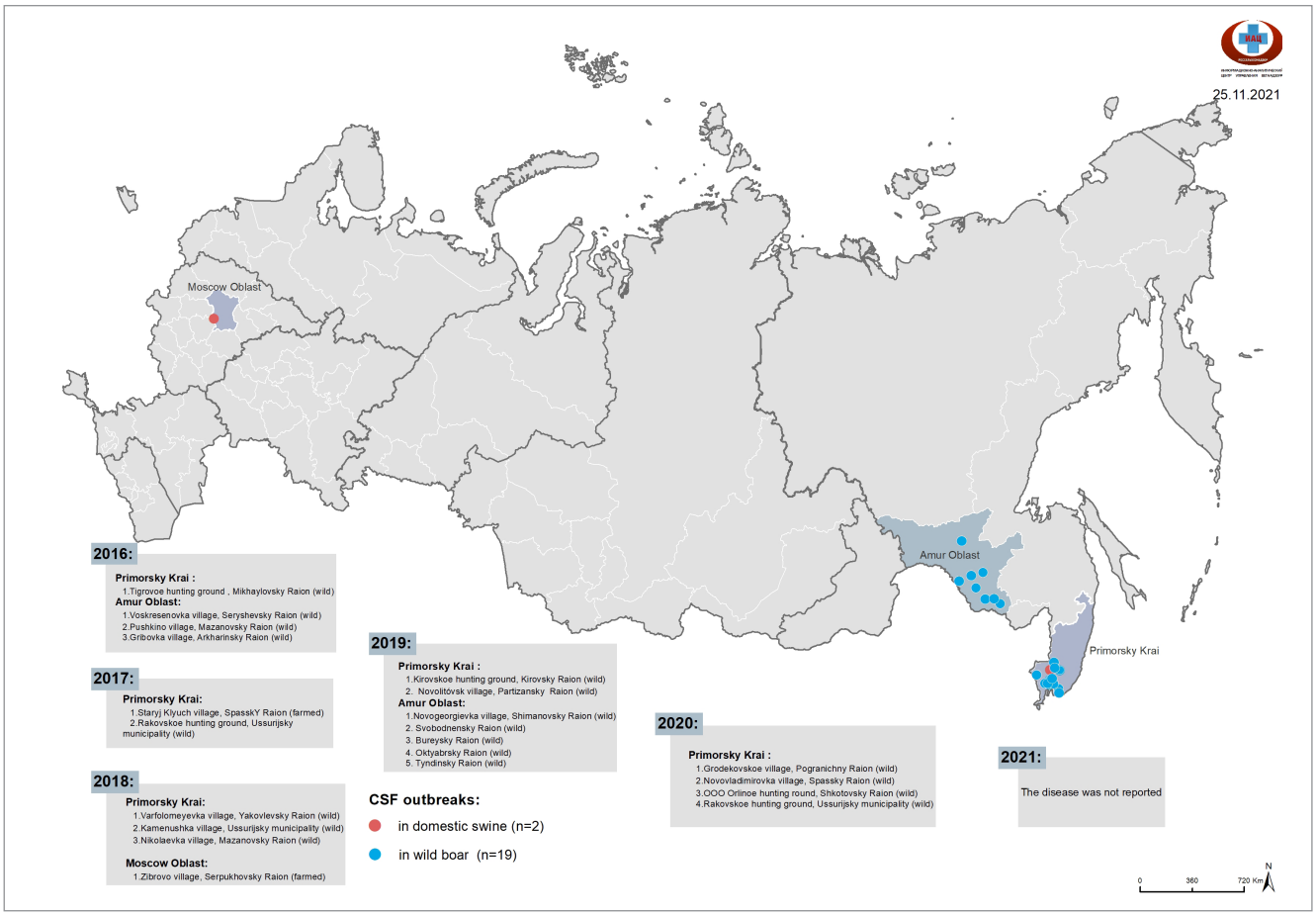

Fig. 2. Territorial distribution of CSF outbreaks in the Russian Federation in 2016–2021

CSF virus subgenotype 2.1 is endemic in China and Southeast Asia, and it can be assumed that it was originally introduced into the Far Eastern Federal District from the PRC. Earlier, the disease outbreaks were repeatedly recorded in the regions of the Russian Federation bordering on China. The isolates that caused these outbreaks (Primorsky/2007, EAO/2012, Amur/2014 and Primorsky/2015) are genetically close to the Chinese isolates of CSF virus, thus suggesting that the disease was imported. It is worth noting that CSF virus in China is very diverse: subgenotypes 1.1, 2.1, 2.2 and 2.3 circulate in the country [3, 9, 17]. The situation that so far only subgenotype 2.1 of CFS virus has been detected in the Far Eastern Federal District can be explained by the fact that this particular genotype is predominant in the PRC [18]. There are 4 clusters today in China (2.1a, 2.1b, 2.1c and 2.1d) within the subgenotype 2.1 [9].

It shall be also noted that, in 2017, CSF was registered in wild boars in the north of South Korea (in province Gyeonggi-do), and then in 2018–2019 (in Gangwon-do province). 16 CSF virus strains, isolated in 2017–2019 from wild boars, were identical to YC16CS strain (subgenotype 2.1d) isolated in 2016 during CSF outbreak in breeding pigs not far from the border on North Korea [13].

As mentioned above, all the Primorsky isolates of 2015–2020 belong to subgenotype 2.1 of CSF virus, which is predominant in China, therefore, it can be assumed that it has been circulating in wild boars in the Primorsky

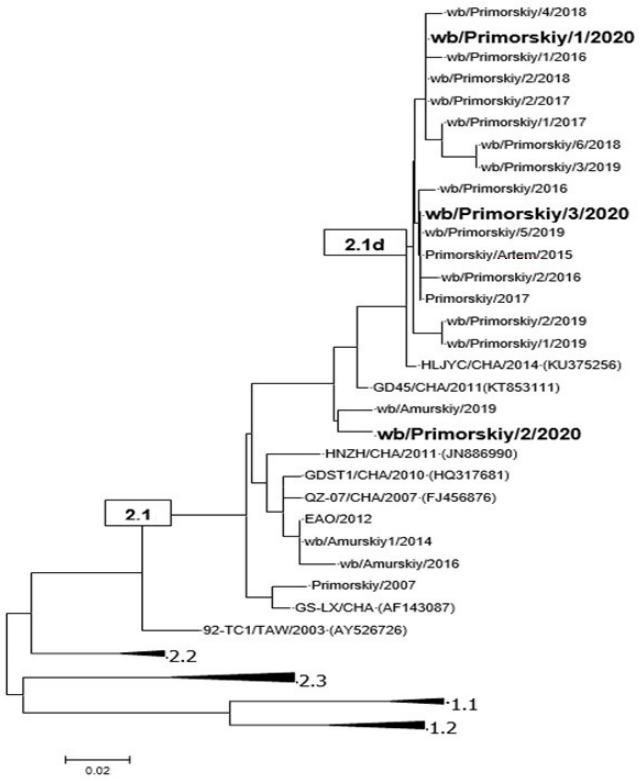

Fig. 3. Position of CSF virus Primorsky isolates (2020) in the phylogenetic tree (comparison of E2 gene nucleotide sequences; wb – wild boar)

Krai since 2015. Thus, S. V. Terebova et al. [19–21] noted that in 2015–2019 a natural and anthropurgic focus was very likely formed in this region. As shown in the available reports, risks of CSF spread in the Primorsky Krai remained high due to the facts that individual populations in some backyards were not vaccinated for a long time or vaccination coverage was insufficient; animals were purchased from unreliable sources without veterinary accompanying documents; and CSF-affected free-range pig farms are located close to the forest edges.

According to the Chinese researchers, C-strain-based vaccines (Chinese vaccine strain – C-strain) are able to induce protection against all the identified CSF virus subgroups [3, 18, 22, 23]. Mass vaccination of domestic pigs in the Russian Federation with domestically produced live vaccines also induces protection in most susceptible animals, regardless of the circulating virus isolates [2, 24]. It helps to reduce significantly the risk of pathogen introduction from infected wild boars, especially in areas located far from the affected ones. However, such a risk shall be also taken into account, when situation with the vaccination refusal changes. Taking into account the difficult situation on CSF in China and close economic ties between our countries (including the option of illegal cross-border transportation of products contaminated with CSF pathogen), one shall not exclude the re-introduction of new CSF subgenotypes into the Far Eastern Federal District and the formation of natural and anthropurgic foci.

Vaccination. The mass vaccination of animals supposedly resulted in a decrease in the number of reported CSF cases in Russia [9, 24, 25]. The use of live attenuated vaccines in the country makes it impossible to differentiate vaccinated animals from the infected ones, although these vaccines are believed to induce a more effective and long immune response [2, 22, 26].

Those factors that help to reduce the number of CSF outbreaks in domestic pigs include: routine vaccination, regionalization of the Russian territory, strong anti-epizootic measures, a decrease in the number of poorly protected farms [9, 24, 25]. Thus, in 2007–2018, population of domestic pigs on small farms with low biosecurity level (backyards, small-scale farms, the farm of an individual entrepreneur, etc.) decreased from 7.6 to 2.9 million. However, the number of pigs on large-scale farms, on the contrary, increased from 8.7 to 20.8 million over the same period. However, implementation of all the above-mentioned measures (especially vaccination refusal) is not sufficient to radically change the epizootic situation.

To assess the overall vaccination against CSF in the Russian Federation, veterinary reports (forms 1-vet and 1-vet-A) were analyzed, taking into account statistical data on the number of domestic pigs. For 27 years (from 1991 to 2017), a correlation was established (r = -0.49769 at $p < 0.05$) between an increase in the vaccination coverage and a decrease in the number of registered CSF outbreaks in the domestic pigs (Fig. 4). The K1 value tended to reach 2.5 only from 2011 (at K2 = 0.13 ± 0.15 ($M \pm m$) from 2011), at the same time from 1991 to 2011 K1 did not exceed 3.0 and averaged 1.84 ± 0.35 ($M \pm m$), while K2 was 10.06 ± 11.65 ($M \pm m$) (at min = 0.45; max = 39.00). Therefore, we chose for analysis the period starting from 2011.

Using a regression analysis, we assessed dependence of the number of registered CSF outbreaks (using K2 coefficient) and vaccination (K1 coefficient) from 2011 to 2018 (Table).

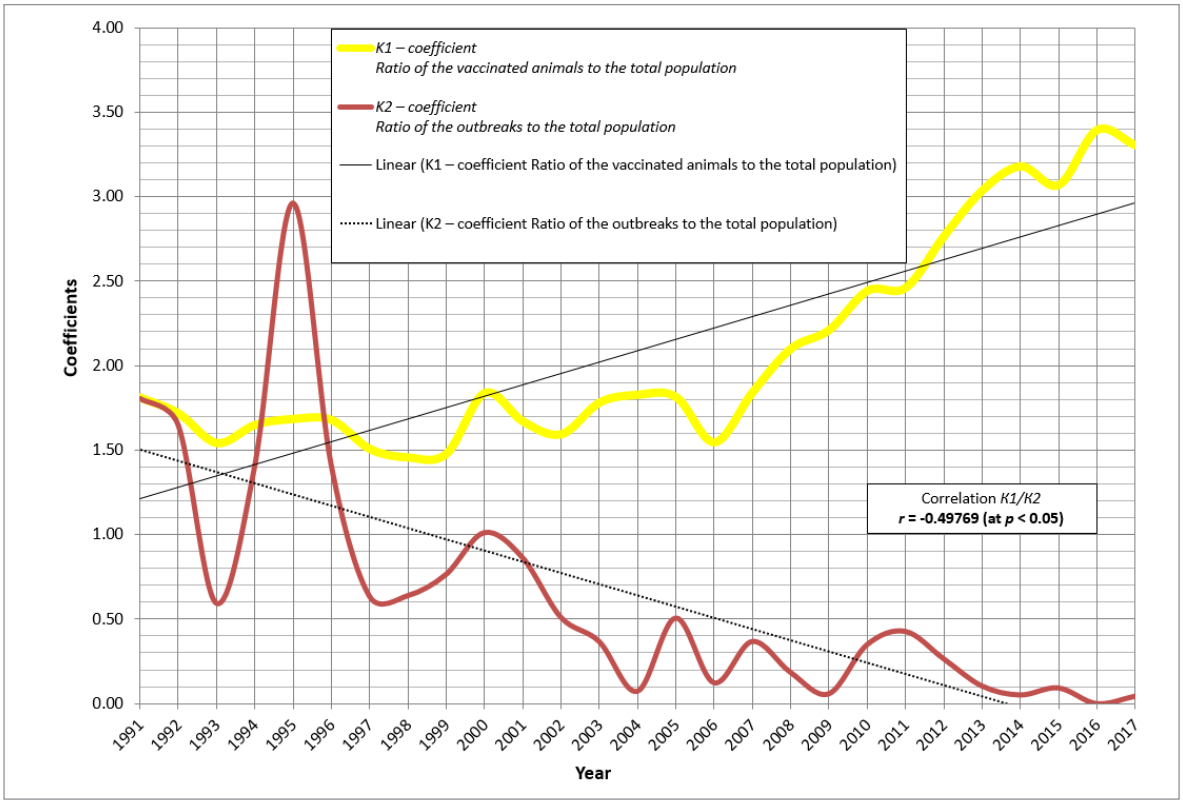

Fig. 4. Coefficient K1 (number of vaccinated animals) and K2 (number of outbreaks) in 1991–2017

The determination coefficient (*R*-square) in the presented model (0.948 > 0.8) indicates that the correlation between analyzed K1 and K2 parameters can be well explained, given that the strong inverse correlation between K1 and K2 (r = -0.97) is significant ($p < 0.05$). The analysis of variance indicates the significance of differences in mean values ($F < F_0$; 0.0000444645), the coefficient of "*x*" variable is negative (*t*-statistics indicate its significance; $p < 0.000045$) and remains within the negative limits of the 95% confidence interval. Coefficient "Y" also maintains its sign within the 95% confidence interval and is significant ($p < 0.00002$). The absolute error of approximation (MAPE = 39%) is in the range of 20% < A < 50%, so we can say that the model-fitting accuracy is satisfactory.

Based on the data of the obtained regression model, vaccination of livestock with K1 value = 3.372 will allow to reach K2 < 0.0001 (which means 1 outbreak per 10,000 years, which is evaluated as a "minor risk").

Thus, expansion of the CSF vaccination coverage from 2011 can be among the factors that contribute to a decrease in the number of CSF clinical cases in domestic pigs of the Russian Federation.

Immunization of pigs against CSF is mostly known as a forced measure, unable to stop virus-carriage in the previously infected herd [7, 27, 28]. In the pig herds, even among the vaccinated ones, there are always unprotected animals with a weak immune system due to the low age-associated immunoreactivity of piglets and suppression of the post-vaccination immunity with colostral antibodies. Latently infected sows transplacentally transmit the pathogen to the young, who become virus-carriers [1, 27–30]. It has been reported that the mass use of live vaccines affects the adaptive evolution of CSF virus, including recombination of epizootic and vaccine strains [23]. These disadvantages of the vaccination are viewed as a potential risk of the long-term virus circulation on the affected farms. Therefore, when country's status of freedom from CSF is determined, the international recommendations (Chapter 1.6 of the Terrestrial Animal Health Code) take this into account and prescribe not to immunize either domestic pigs or captive wild boars against CSF in the last 12 months; or, if vaccination was carried out, it is recommended to differentiate immune pigs from the infected ones [5, 7].

On the other hand, in many countries, where CSF eradication programs were successfully implemented without any vaccination, flaws in epizootic surveillance and infection control systems were fraught with the mass disease spread. Thus, in 1997–1998, 429 CSF outbreaks were registered in the Netherlands; in 2000, 16 outbreaks were reported in the UK, and 49 outbreaks were reported in Spain in 2001–2002. In 2006–2007, 1,597 CSF outbreaks were reported in domestic and wild pigs in Romania. To stabilize the epizootic situation in the country, a decision was made to temporarily return to immunization of domestic pigs. In 2006–2009, CSF was registered in Croatia (129 outbreaks among domestic pigs), Hungary (225 outbreaks in wild boars), Bulgaria (12 outbreaks in domestic and 4 in wild pigs), Slovakia (more than 10 outbreaks in wild pigs) [5, 9, 24]. After 10 years of CSF freedom, in 2018, 38 outbreaks were reported in Brazil in domestic pigs, and after 11 months of 2019 there were more than 30 outbreaks. Measures taken to stabilize the situation in the country were mainly aimed at strengthening the system of CSF epizootological surveillance and control in the disease-affected areas [15]. In Japan, where vaccination was not practiced for more than 26 years, CSF was reported in 2018 in wild boars and pigs. Spatiotemporal analysis conducted in Gunma and Saitama prefectures revealed anthropogenic factors in the disease spread. In response to the outbreaks, from March to May 2019, wild boars from certain areas of Aichi and Gifu prefectures were twice subjected to peroral immunization using commercial Pestiporc Oral vaccine (IDT Biologika GmbH, Germany), which failed to prevent CSF spread, although a decrease

Table
Regression analysis of CSF registered outbreaks (using coefficient K2) and vaccination (using coefficient K1), from 2011 to 2018

Year	K2 (Y)	K1 (*x*)	(Y) Calculated	Regression residuals (0)
2011	0.427807487	2.459893048	0.396101668	0.031705819
2012	0.265957447	2.765957447	0.263219277	0.00273817
2013	0.104712042	3.036649215	0.145694436	-0.040982394
2014	0.051282051	3.179487179	0.083679218	-0.032397167
2015	0.093023256	3.069767442	0.131315667	-0.038292411
2016	0	3.395348837	-0.010040318	0.010040318
2017	0.043478261	3.334782609	0.016255407	0.027222854
2018	0.042194093	3.367088608	0.00222928	0.039964813

MAPE ($\sum([0]/Y) / n \times 100\%$) = 39%
Mean absolute percentage error (MAPE) is satisfactory (20% < A <50%)

Regression statistics	
Multiple *R*	0.973724123
R-square	0.948138669
Adjusted *R*-square	0.939495113
Standard error	0.035700785
Observations	8

Analysis of variance					
	df	*SS*	*MS*	*F*	Significance level of *F*
Regression	1	0.139808953	0.139808953	109.6931347	4.44645E-05
Residual	6	0.007647276	0.001274546		
Total	7	0.147456229			

Data on coefficients of the regression equation						
	Coefficient	Standard error	*t*-statistics	*P*-value	Low 95%	High 95%
Y-intersection	1.464100669	0.12814027	11.42576543	2.6961E-05	1.1505527	1.7776486
Variable *x*	-0.434164811	0.041453852	-10.47344904	4.44645E-05	-0.5355987	-0.3327309

in the number of cases in the severely affected areas was noticeable [11]. Similar results of wild boar vaccination in the Primorsky Krai in 2004–2016 also demonstrated that the mass mortality of wild boars was reduced, however, the virus circulation was not prevented [19, 20].

The negative aspects associated with no-vaccination CSF eradication policy (stamping out, logistical and technological costs for biosafety and control, ethical issues) suggest that immunization is the key measure to control future outbreaks, taking into account serious concern about the global threat posed by re-emergence of a population immunologically naive to CSF. Therefore, the most urgent task today is to continue research with the purpose to create more effective vaccines against CSF [2, 22, 26].

Regarding DIVA strategy, it is worth noting that the first generation of marker vaccines (commercial subunit E2 vaccines), despite their safety, did not demonstrate the same high effectiveness as live attenuated vaccines did [2, 22]. In some countries, work is ongoing to create vaccines that could be used as part of the DIVA strategy. However, their use for CSF control and eradication must be combined with well-organized and implemented epizootological surveillance, quarantine measures at the borders and biosafety of the pig industry [22].

We believe that today CSF zoning of the Russian Federation with creation of disease-free zones without vaccination and preservation of vaccination in disease-affected (risk) zones is the most progressive way as demonstrated by the already implemented FMD zoning of the country's territory [5]. In terms of general veterinary and sanitary and quarantine measures, FMD and CSF zoning are similar, but CSF-focused surveillance shall be strengthened separately, as well as CSF-related quarantine measures in the existing zones. In our opinion, the CSF control with the use of live attenuated vaccines practiced for decades in the Russian Federation paved the way for vaccination refusal in future and for successful eradication of this infection, as demonstrated by officially CSF-free countries or countries with CSF-free zones [2, 14, 15].

CSF outbreaks expected in 2022 in the Russian Federation. Modeling for the possible number of CSF cases in 2022 showed that an average of 3 (95% confidence interval: 0–5) outbreaks can be expected in the population of domestic pigs, and 3 (0–6) outbreaks in the wild boar population (Fig. 5).

CONCLUSION

Over the past decade, there has been a geographical shift of CSF-affected area from the central to the eastern regions of the Russian Federation along the borders. However, unlike the previous years 2019–2020, the CSF trend showed an upward direction in the wild boar population (a growth), which is associated with the disease spread in the Primorsky Krai and the Amur Oblast. If the existing level of specific prevention is in place, the long-term forecasts suggest that the situation with a low number of outbreaks in domestic pigs will persist (sporadic CSF outbreaks).

The short-term forecasts for 2022 suggest that there will be 0–5 CSF outbreaks in the population of domestic pigs (with the expected average of 3) and 0–6 outbreaks in the population of wild boars (with the expected average of 3). These are domestic pigs on small-scale pig farms with low biosafety (biosecurity) level (where violations (non-compliances) are reported during vaccination), who are expected to become a CSF target population in 2022.

The main peculiarities of the infection spread in the Russian Federation are more likely associated with the internal risk factors (i.e. quality of antiepizootic measures, mainly vaccination) and the territories of the virus circulation among wild boars. In terms of wild boars, the susceptible animals from the Primorsky Krai bordering on the PRC and the DPRK will be the main CSF target in 2022, as a high probability was earlier reported in the area (since 2015) to form a natural and anthropurgic foci of the infection and CSF virus was widespread in the wild boar populations of the region (China, South Korea, the DPRK and the Primorsky Krai of the Russian Federation). At the same time, one shall not exclude potential anthropogenic impact which may result in penetration of the infection into any regions of the Russian Federation.

Thus, expansion of the CSF vaccination coverage from 2011 can be among the factors that contribute to a decrease in the number of CSF clinical cases in domestic pigs of the Russian Federation. Currently, vaccination

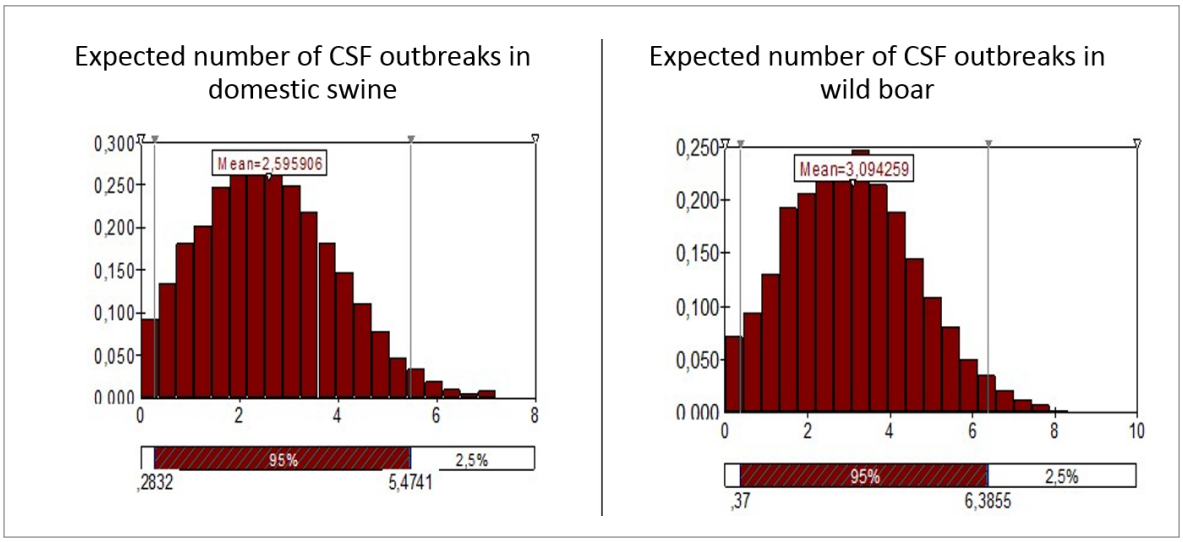

Fig. 5. The projected number of CSF outbreaks in the population of domestic pigs and wild boars for 2022

against CSF (and its good quality) in the Russian Federation is an essential prerequisite to contain the infection spread in the country. Infection spread in domestic pig population is still on a downward trend.

To make a reasonable choice of a future CSF control strategy, it is required to introduce an effective system of epizootological surveillance that will be able to confirm freedom from the infection or to determine the exact area of infection (including latent carriers) in domestic pigs and wild boars in different regions of the country. It is unacceptable to confirm freedom from the disease, simply relying on the fact that there are no notified CSF outbreaks in the population, where vaccination is widely practiced.

If a decision is made to refuse vaccination, new measures shall be efficiently implemented step by step (being tried and tested on farms with a high level of biosecurity). First, they shall be implemented in the areas of the lowest CSF risk occurrence, and only after that, if successful, throughout the whole country.

We believe that CSF zoning of the Russian Federation with creation of disease-free zones without vaccination and preservation of vaccination in disease-affected (risk) zones is the most progressive way, as demonstrated by the already implemented FMD zoning of the country's territory.

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African swine fever in the Republic of Crimea in 2015–2018

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SUMMARY

During the analyzed period of 2015–2018 number of pigs kept on backyards and in agricultural organizations as well as number of wild boars living in the Crimean lands declined by 25.34% and 26.09%, respectively, due to African swine fever introduction. In 2016–2018, ASF epizootic situation in domestic pigs and wild boars was the most complicated in the Sovetsky, Razdolnensky, Belogorsky Raions and in Sudak municipality, respectively. The unauthorized burials of dead domestic pigs, which could have caused the dangerous disease agent introduction into the wildlife were detected. Number of tests for ASF carried out within both passive and active monitoring increased during the said period: 527 tests were carried out in 2015 and 7,754 tests were carried out in 2018. In 2018, ASF virus was detected with polymerase chain reaction (PCR) in 8 samples of pathological materials from wild boars, that was 0.1% out of total number of the samples tested in the FGBI "ARRIAH" Branch in the Republic of Crimea and FGI RC "Regional State Veterinary Laboratory of the Republic of Crimea". Large-scale diagnostic tests performed in domestic pigs and wild boars contributed to rapid diagnosis of outbreaks and disease eradication. It should be noted that in case of ASF occurrence in domestic pigs only, the disease could be eradicated with a complex of anti-epizootic measures in initial outbreak areas. Absence of the disease in the Crimean Peninsula during the last years proves the effectiveness of the measures taken for ASF spread prevention.

Keywords: African swine fever, wild boar, domestic pig, Republic of Crimea, epizootological monitoring

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Африканская чума свиней на территории Республики Крым в 2015–2018 гг.

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РЕЗЮМЕ

За анализируемый период с 2015 по 2018 г. на территории Республики Крым вследствие заноса африканской чумы свиней численность поголовья домашних свиней в личных подсобных хозяйствах и сельскохозяйственных организациях сократилась на 25,34%, популяция дикого кабана, обитающего в крымских угодьях, уменьшилось на 26,09%. В 2016–2018 гг. наиболее напряженная эпизоотическая ситуация по африканской чуме среди домашних

свиней сложилась в Советском, Раздольненском, Белогорском районах, а среди диких кабанов – в городском округе Судак. Выявлены факты несанкционированных захоронений трупов домашних свиней, которые, возможно, явились причиной заноса возбудителя опасной болезни в дикую фауну. За данный период времени количество проводимых лабораторных исследований на африканскую чуму свиней в рамках как пассивного, так и активного мониторинга увеличилось: в 2015 г. было проведено всего 527 исследований, а в 2018 г. выполнено 7754 исследования. В 2018 г. в 8 образцах патологического материала от диких кабанов с использованием полимеразной цепной реакции был выявлен вирус африканской чумы свиней, что составило 0,1% от общего количества исследований, проведенных в Филиале ФГБУ «ВНИИЗЖ» и ГБУ РК «Региональная государственная ветеринарная лаборатория Республики Крым». Реализация широкомасштабных диагностических исследований среди поголовья домашних свиней и диких кабанов способствовала быстрому выявлению вспышек и ликвидации заболевания. Следует отметить, что при возникновении африканской чумы свиней только среди домашних свиней болезнь удается искоренить с помощью комплекса общепринятых противоэпизоотических мероприятий в первичных очагах. Отсутствие заболевания на территории Крымского полуострова в последние годы доказывает эффективность проводимых профилактических мероприятий по недопущению распространения африканской чумы свиней.

Ключевые слова: африканская чума свиней, дикий кабан, домашняя свинья, Республика Крым, эпизоотологический мониторинг

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INTRODUCTION

The Russian Federation is perceived in the world as an unpredictably endemic territory for contagious animal diseases posing a threat to global livestock sector. In this regard, epizootological surveillance of dangerous infectious diseases becomes extremely important [1–6].

Therewith, epizootological surveillance refers to the process of continuous animal health monitoring including recording data on the disease spread and the analysis of the collected data. The surveillance results are the basis for predicting the epizootic situation development and used for targeting the infectious animal disease agent containment measures. The surveilance is also required for determination of the epizootic status of a particular territory for international trade and transportation [4, 6, 7].

The surveillance is divided into passive and active depending on the used data collection methodology and tools. Passive surveillance is mainly aimed at early detection of infected animals (incident diagnosis). Active surveillance provides for broader population-based studies to estimate the disease prevalence or to prove absence of the disease.

Historically, measures attributed to passive surveillance were predominantly carried out in Russia while active surveillance measures were carried out rarely.

In 2011, the Russian Federation joined the World Trade Organization (WTO). Annual official laboratory epizootological surveillance for highly dangerous animal diseases is one of the requirements of the Agreement on Russia's accession to the WTO. Accordingly, the first Official Laboratory Epizootological Monitoring Plan[1] was approved by Rosselkhoznadzor Order No. 120 of 8 April 2011. Tests carried out under this Order were of active surveillance character, but, probably, in order not to confuse epizootological surveillance with the veterinary surveillance, term used in Russia, a similar term is used for its designation – epizootological monitoring [6, 8, 9].

Official laboratory epizootological monitoring covering extensive list of infectious animal diseases including African swine fever (ASF) has been carried out annually in the Russian Federation for the Agreement implementation since 2011 [8].

MATERIALS AND METHODS

Data on ASF situation, numbers of domestic pigs and wild boars in the Republic of Crimea in 2015–2018 were obtained from the Federal State Statistics Service Department for the Republic of Crimea and city of Sevastopol, Rosselkhoznadzor Territorial Administration for the Republic of Crimea and city of Sevastopol, State Veterinary Committee of the Republic of Crimea, Ministry of Agriculture of the Republic of Crimea, State Committee for Forestry and Hunting of the Republic of Crimea. The methodology for wild boar number estimation was not specified.

Pathological material samples collected from domestic pigs and wild boars for testing were provided by the Raion

[1] Laboratory tests to be performed within the Rosselkhoznadzor measures taken for compliance with the WTO SPS Agreement requirements at the Russian Federation accession to the WTO for 2011: approved by Rosselkhoznadzor Order No. 120 of 8 April 2011. Accessed at: https://base.garant.ru/2175474.

Veterinary Prophylactic Centers and hunting farms of the Republic of Crimea and city of Sevastopol as agreed by the Rosselkhoznadzor Territorial Administration for the Republic of Crimea and city of Sevastopol. Pathological material sampling methods were not indicated in the accompanying documents.

Results of laboratory tests for ASF were provided by GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" and supplemented by the data obtained in the framework of official epizootological monitoring for ASF carried out by the Laboratory for Molecular Diagnostics of the Laboratory and Diagnostic Centre of the FGBI "ARRIAH" Brach in the Republic of Crimea.

ASF virus DNA was extracted from tested samples using sorbent method in accordance with the instruction for use of "Ampli Prime DNA-sorb-V" reagent kit for DNA extraction from clinical samples ("NextBio" LLC, Russia).

Real-time polymerase chain reaction (rtPCR) was performed with "ASF" test system for detection of African swine fever virus (FBSI "Central Research Institute of Epidemiology" of the Federal Service for Consumer Rights Protection and Human Welfare, Russia) and Rotor-Gene Q amplifier (QIAGEN GmbH, Germany).

Analysis of the ASF outbreaks reported in the Republic of Crimea was carried out based on open publications of the Rosselkhoznadzor and World Organization for Animal Health (WOAH) databases [8, 10].

Maps were provided by the Information Analysis Centre of the FGBI "ARRIAH" (Vladimir).

The purpose of this work was to provide a general overview of the ASF situation in the Republic of Crimea in 2015–2018, to study of wild boar and domestic pig population dynamics as well as to analyze passive and active monitoring data for determination of the disease introduction risk and disease spread in the region.

RESULTS AND DISCUSSION

Despite the fact that Republic of Crimea was ASF free, disease prevention centre was established in 2015 in view of expansion of the epizooty in Ukraine. According to the State Veterinary Committee of the Republic of Crimea, the centre staff-members monitored the health status of wild boars living in the eastern part of the peninsula, and together with Rosselkhoznadzor specialists participated in inspections of foodstuffs supplied to Crimea from Ukraine [11, 12].

There were 2,300 wild boars and 167,151 domestic pigs including 77,340 domestic pigs kept in backyards and 89,811 domestic pigs kept in agricultural holdings in the Republic of Crimea in 2015 [13–15].

A total of 527 pathological and biological material samples from domestic pigs kept in backyards and agricultural holdings as well as from wild boars were tested with PCR at the GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" in 2015 in the framework of passive surveillance for determination of ASF introduction risk and spread in the region (Table 1).

A total of 158 and 145 pathological (biological) material samples were collected from domestic pigs kept in agricultural holdings and backyards, respectively, that accounted for 0.18 and 0.19% of the total population; 224 pathological material samples collected from wild boars were tested that accounted for 9.74% of total wild boar population. No ASF virus genome was detected in the tested samples.

In 2015, the Republic of Crimea was considered ASF free based on the test results for but the disease outbreaks were reported in domestic pigs and wild boars in bordering countries and in the Krasnodar Krai [11, 16, 17].

In 2016, the wild boar population decreased by 23.22% (1,766 animals), and domestic pig population decreased by 13.47% (79,578 animals in backyards and 65,062 animals in agricultural holdings) [11, 15, 18].

According to the State Veterinary Committee of the Republic of Crimea and the Rosselkhoznadzor Territorial Administration, the first ASF outbreak was reported in late January 2016 on the farm (s. Novoselovskoe, Razdolnensky Raion) and adjacent territories where unauthorized burials of domestic pigs were found. Notice on imposed quarantine was published on the Rosselkhoznadzor Territorial Administration site. Later, the disease outbreaks were registered in the Belogorsky (s. Aromatnoe) and Leninsky Raions [8, 11, 15].

Multiple unauthorized burials of domestic pigs and dead domestic pigs were found in the Republic of Crimea that facilitated the said infectious disease spillover

Table 1
Laboratory tests for ASF carried out by the FGI RC "Regional State Veterinary Laboratory of the Republic of Crimea" in 2015

Raion in the Republic of Crimea	Pathological (biological) materials			
	from domestic pigs		from wild boars	
	number of samples	PCR results	number of samples	PCR results
Bakhchisaraysky	16	–	132	–
Belogorsky	16	–	25	–
Dzhankoi	15	–	–	–
Kirovsky	2	–	2	–
Krasnogvardeysky	158	–	–	–
Krasnoperekopsky	15	–	–	–
Leninsky	10	–	1	–
Nizhnegorsky	10	–	–	–
Pervomaysky	15	–	–	–
Razdolnensky	10	–	–	–
Saksky	15	–	–	–
Simferopolsky	5	–	22	–
Sovetsky	5	–	–	–
Chernomorsky	10	–	–	–
Alushta/Yalta	–	–	22	–
Simferopol	1	–	–	–
Sudak	–	–	20	–
Total	303	0	224	0

Table 2
Laboratory tests for ASF carried out by the FGI RC "Regional State Veterinary Laboratory of the Republic of Crimea" in 2016

Raion in the Republic of Crimea	Pathological (biological) materials			
	from domestic pigs		from wild boars	
	number of samples	PCR results	number of samples	PCR results
Bakhchisaraysky	146	–	41	–
Belogorsky	80	–	21	–
Dzhankoi	81	–	–	–
Kirovsky	45	–	–	–
Krasnogvardeysky	936	–	–	–
Krasnoperekopsky	135	–	–	–
Leninsky	103	–	–	–
Nizhnegorsky	30	–	–	–
Pervomaysky	92	3	–	–
Razdolnensky	220	26	–	–
Saksky	137	–	–	–
Simferopolsky	240	–	11	–
Sovetsky	35	–	–	–
Chernomorsky	127	–	–	–
Alushta/Yalta	1	–	1	–
Kerch	4	–	–	–
Simferopol	4	–	2	–
Sudak	10	–	–	–
Feodosia	4	–	11	–
Total	2,430	29	87	0

to wildlife. Thus, a dead wild boar was found on Yevpatoria city beach in January 2016. ASF virus genetic material was detected in pathological material samples collected from the said animal and simultaneously tested with PCR at the FGBSI "Federal Research Centre for Virology and Microbiology" and at the ASF Reference Laboratory of the FGBI "ARRIAH".

State of natural emergency had been introduced in the Republic of Crimea since February 8, 2016 due to ASF outbreak.

The disease outbreaks were registered on the pig farm located in the Prevomaysky Raion and in the Leninsky Raion (s. Pesochnoe) in November 2016.

According to the Rosselkhoznadzor Territorial Administration, the quarantine zone covered three settlements: Pesochnoe, Ostanino and Zeleny Yar. Therewith, 180 pigs (0.23% of the total domestic pig population) were found to be infected and destroyed in the backyard of the outbreak [8, 11].

In 2016, 2,517 PCR tests of pathological and biological material samples taken from domestic pigs kept in backyards, agricultural holdings and from wild boars were carried out at the GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" in the framework of passive monitoring for ASF in the region (Table 2).

A total of 1,176 and 1,254 pathological and biological material samples were collected from domestic pigs raised in agricultural holdings and in backyards, respectively, that accounted for 1.81 and 1.58% of the total domestic pig population, and tested. Eighty-seven pathological material samples were collected from wild boars (4.93% of the wild boar population recorded in the peninsular) and tested.

It should be noted that ASF virus genome was detected in 29 pathological material samples from backyard domestic pigs that accounted for 1.15% of total number of tests.

According to the Rosselkhoznadzor Territorial Administration data, seven ASF outbreaks in domestic pigs (s. Aromatnoe, s. Pesochnoe, s. Dmitrovka, s. Chapayevo, s. Novoselovskoe, s. Berezovka, s. Razdolnoe) were detected and officially confirmed in the Belogorsky, Leninsky, Sovetsky, Pervomaysky and Razdolnensky Raions. A total of 268 infected backyard pigs (0.34% of total number of pigs in the said backyards) were destroyed in the infected settlements (Figure 1).

In the beginning of 2017 the number of domestic pigs in the peninsular decreased by 9.15% (66,489 backyard pigs and 64,913 pigs kept in agricultural holdings), and number of wild boars decreased by 11.10% (1,570 animals) as compared to that ones in 2016 [15, 18].

In 2017 the first ASF outbreaks were reported in January in the backyards in s. Dmitrovka, Sovetsky Raion. Hence, state of local emergency was introduced and quarantine was imposed (Figure 2).

In March 2017, ASF outbreaks were detected in s. Karasevka (Belogorsky Raion) as well as in the Sovetsky Raion (near s. Zavetnoe and s. Dmitrovka), where two sites of unauthorized burials of dead pigs were found. The results of laboratory tests carried out at the FGBI "ARRIAH" revealed ASFV genome in some samples taken on the burial sites [8, 11, 12].

In May 2017, dead wild boars were found 2.5–3 km from s. Gromovka (Sudak municipality), 1.5 km from Sudak city, and 1.3 km from s. Fersmanovo (Dobrovskoe rural community, Simferopolsky Raion). ASFV genome was detected in some pathological material samples collected from the dead wild boars and tested at the FGBI "ARRIAH" [8, 11].

Earlier, the Rosselkhoznadzor Territorial Administration for the Republic of Crimea informed on detection of unauthorized dead animal dumping sites in the Belogorsky and Sovetsky Raions, ASFV genome was detected in all biomaterial samples taken from the said dead animals. The zone of the imposed quarantine covered areas adjacent to s. Mezhgorye (Zelenogorskoe rural community, Belogorsky Raion) where dead wild boars were found and three sites near s. Kurortnoe (Aromatnovskoe rural community, Belogorsky Raion) where wild boar remains were found. Three areas of Sudak municipality (2.5 and 3 km north-west from s. Gromovka and 1.5 km north-west from

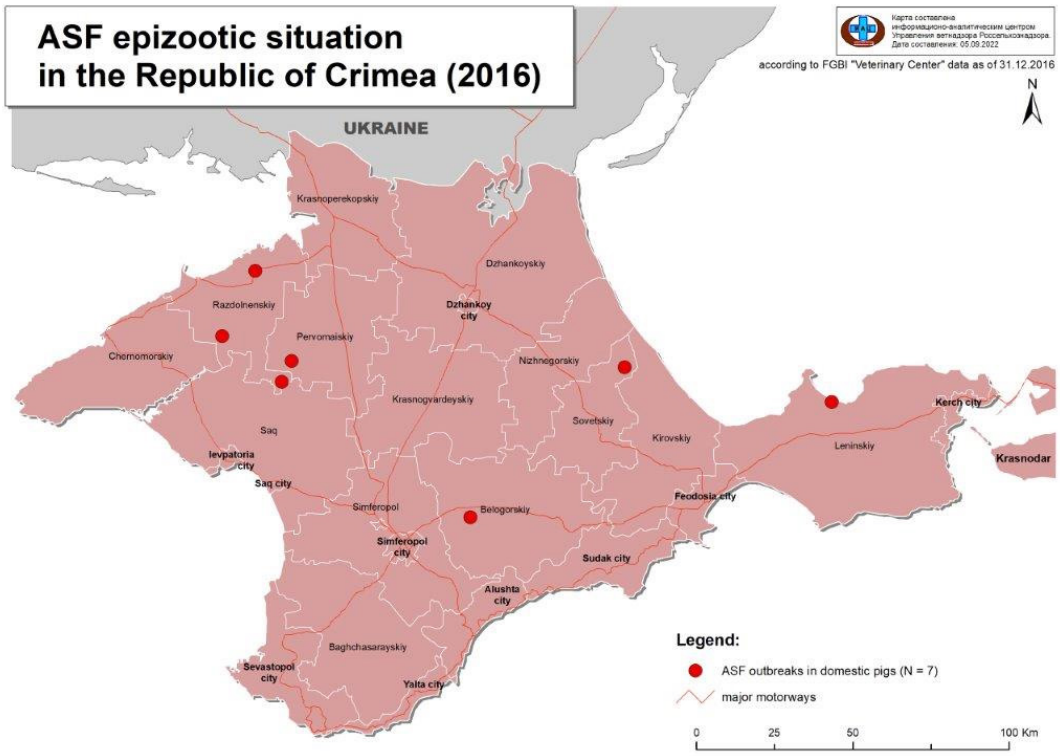

Fig. 1. ASF epizootic situation in the Republic of Crimea in 2016

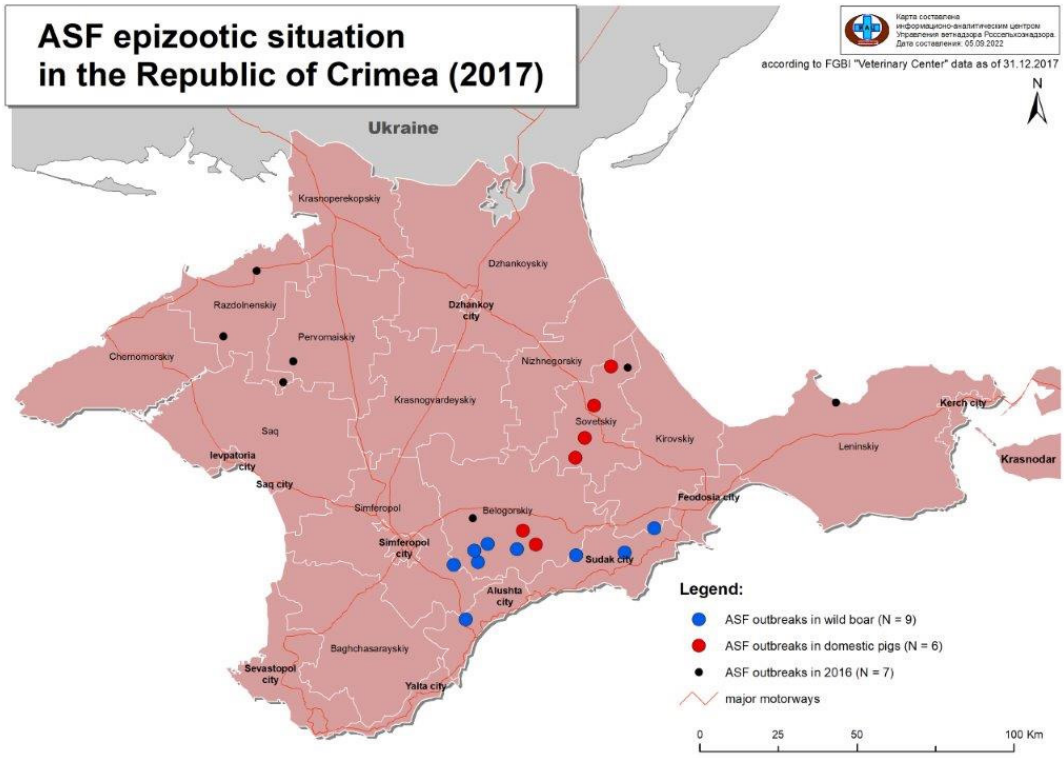

Fig. 2. ASF epizootic situation in the Republic of Crimea in 2017

Sudak city) were also included in the quarantine zone. The Rosselkhoznadzor experts noted that ASF outbreaks were reported in wildlife [8, 11, 12].

Despite the fact that no new ASF outbreaks had been registered in the territory of the Republic of Crimea since June 2017, the State Veterinary Service of the region continued to carry out preventive measures aimed at the disease occurrence and spread prevention.

In October 2017, the burial of eight dead pigs was found 1.47 km to the north from s. Sovetsky. ASFV genome was detected in pathological material samples collected from all dead and subjected to laboratory testing [8, 11, 12].

Table 3
Laboratory tests for ASF in the Republic of Crimea in 2017

Raion in the Republic of Crimea	GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" (passive monitoring)				FGBI "ARRIAH" Branch in the Republic of Crimea (active monitoring)			
	pathological (biological) materials							
	from domestic pigs		from wild boars		from domestic pigs		from wild boars	
	number of samples	PCR results	number of samples	PCR results	number of samples	PCR results	number of samples	PCR results
Bakhchisaraysky	324	–	45	–	–	–	–	–
Belogorsky	110	–	19	–	–	–	5	–
Dzhankoi	330	–	–	–	–	–	–	–
Kirovsky	75	–	4	1	–	–	–	–
Krasnogvardeysky	1,600	–	–	–	200	–	–	–
Krasnoperekopsky	107	–	–	–	–	–	–	–
Leninsky	100	–	–	–	–	–	–	–
Nizhnegorsky	100	–	–	–	–	–	–	–
Pervomaysky	256	–	–	–	–	–	–	–
Razdolnensky	225	–	–	–	–	–	–	–
Saksky	230	–	–	–	–	–	–	–
Simferopolsky	435	–	8	–	200	–	13	–
Sovetsky	25	–	–	–	38	13	–	–
Chernomorsky	190	–	–	–	–	–	–	–
Sevastopol	–	–	–	–	–	–	53	–
Alushta/Yalta	5	–	–	–	–	–	7	–
Yevpatoria	5	–	–	–	–	–	–	–
Kerch	11	–	–	–	–	–	–	–
Simferopol	20	–	13	5	–	–	–	–
Sudak	10	–	–	–	–	–	–	–
Feodosia	15	–	–	–	–	–	–	–
Total	4,173	0	89	6	438	13	78	0

In 2017, 516 PCR tests of pathological and biological materials from backyard pigs and pigs kept in agricultural holdings as well as from wild boars were carried out by the FGBI "ARRIAH" Branch in the Republic of Crimea in the framework of active monitoring for determination of ASF introduction risk and spread in the region. The GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" performed 4,262 tests for the same period (Table 3).

Specialists of the Laboratory for Molecular Diagnostics of the FGBI "ARRIAH" Branch in the Republic of Crimea examined pathological and biological material from domestic pigs kept in agricultural holdings (200 samples) and in backyards (238 samples), which amounted to 0.31% and 0.36% of the total number of animals kept in holdings of different ownership types, respectively.

Seventy-eight pathological material samples (4.97% of total wild boar population) were collected from wild boars for testing.

ASF virus genome was detected in 13 pathological material samples collected from backyard pigs that accounted for 2.52% of total number of PCR tests performed in the FGBI "ARRIAH" Branch in 2017.

The GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" tested 2,254 pathological and biological material samples (3.47% of total population) taken from pigs kept in agricultural holdings and 1,919 pathological and biological material samples (2.89% of total population) taken from backyard pigs. Eighty-nine pathological material samples taken from wild boars (5.67% of the recorded population) were tested.

ASFV genome was detected in 6 pathological material samples from wild boars that accounted for 0.14% of total PCR tests carried out in the laboratory for reporting period.

According to the Rosselkhoznadzor data, 15 ASF outbreaks were reported in the Republic of Crimea in 2017 including 6 outbreaks in pigs kept in backyards and agricultural holdings and 9 outbreaks in wild boar population. Six settlements located in the Sovetsky and Belogorsky Raions were recognized as ASF infected: s. Luchevoe, s. Khlebnoe, s. Dmitrovka, s. Rovenka, s. Alexeyevka, s. Karasevka.

ASF outbreaks in wild boar population were reported near s. Gromovka (Sudak municipality), s. Fersmanovo, s. Druzhnoe (Simferopolsky Raion), near s. Mezhgorye, s. Kurortnoe and s. Zemlyanichnoe (Belogorsky Raion) as well as s. Lavanda (Alushta municipality) (Figure 2).

At the beginning of 2018 the wild boar population increased by 7.65% and was 1,700 animals, whereas the domestic pig population decreased by 5.02% (59,900 backyard pigs and 64,900 pigs kept in agricultural holdings) [8, 11, 12, 15].

In 2018, 1,000 PCR tests of pathological and biological materials from pigs kept in backyards and agricultural holdings as well as from wild boars were carried out by the FGBI "ARRIAH" Branch in the Republic of Crimea in the framework of active monitoring for determination of ASF introduction risk and spread. The GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" performed 6,754 tests (Table 4).

In February 2018, ASFV genome was identified in the pathological materials collected from dead wild boars detected to the south of Asret district (Sudak municipality) and tested by specialists of the FGBI "ARRIAH" Branch in the Republic of Crimea. The said results were confirmed by the Reference Laboratory for African swine fever of the FGBI "ARRIAH" (Vladimir).

Table 4
Laboratory tests for ASF in the Republic of Crimea in 2018

Raion in the Republic of Crimea	GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" (passive monitoring)				FGBI "ARRIAH" Branch in the Republic of Crimea (active monitoring)			
	pathological (biological) materials							
	from domestic pigs		from wild boars		from domestic pigs		from wild boars	
	number of samples	PCR results	number of samples	PCR results	number of samples	PCR results	number of samples	PCR results
Bakhchisaraysky	219	–	294	–	60	–	–	–
Belogorsky	168	–	38	3	26	–	–	–
Dzhankoi	463	–	–	–	45	–	5	–
Kirovsky	270	–	3	–	–	–	–	–
Krasnogvardeysky	1882	–	–	–	–	–	–	–
Krasnoperekopsky	314	–	–	–	–	–	–	–
Leninsky	156	–	–	–	–	–	–	–
Nizhnegorsky	190	–	–	–	65	–	–	–
Pervomaysky	300	–	–	–	150	–	–	–
Razdolnensky	191	–	–	–	50	–	–	–
Saksky	590	–	–	–	–	–	–	–
Simferopolsky	1023	–	60	–	400	–	7	–
Sovetsky	146	–	–	–	–	–	–	–
Chernomorsky	199	–	–	–	56	–	2	–
Sevastopol	148	–	–	–	–	–	127	–
Alushta/Yalta	5	–	–	–	–	–	2	–
Kerch	12	–	–	–	–	–	–	–
Simferopol	20	–	3	–	–	–	–	–
Sudak	10	–	–	–	–	–	5	5
Feodosia	20	–	30	–	–	–	–	–
Total	6,326	0	428	3	852	0	148	5

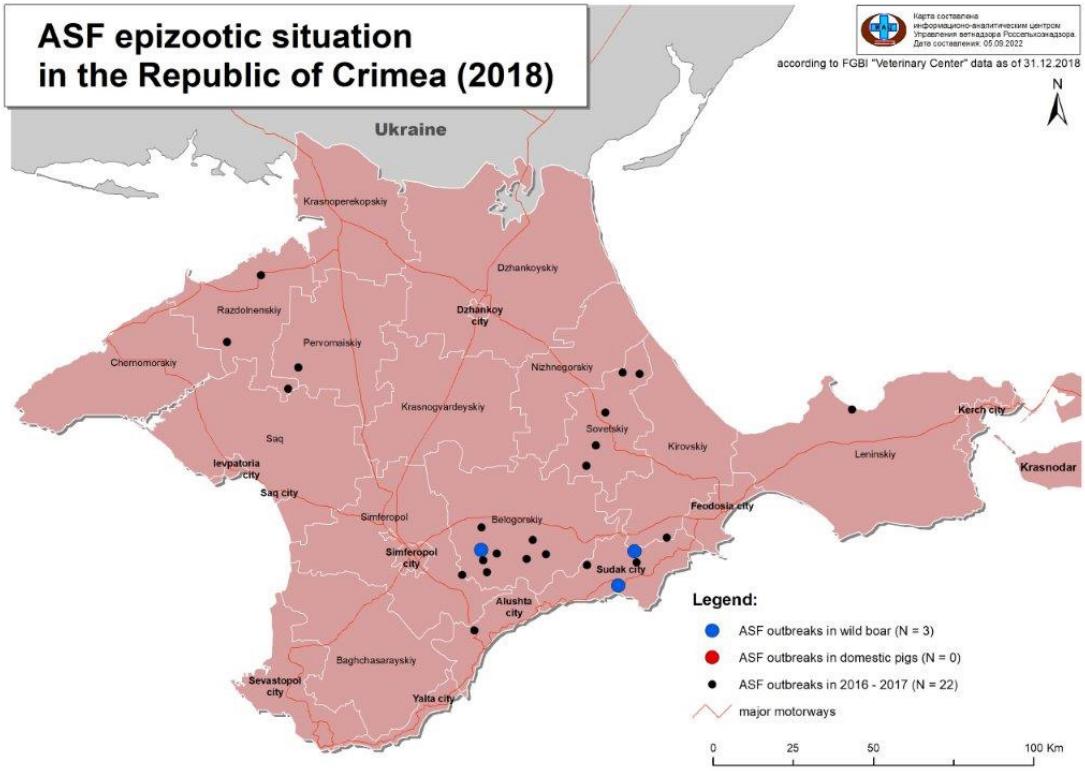

Fig. 3. ASF epizootic situation in the Republic of Crimea in 2018

In April 2018, ASFV genome was identified in the pathological materials collected from wild boars (s. Mezhgorye, Belogorsky Raion) and tested by specialists of the GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea" (Figure 3).

A total of 3,445 and 3,723 pathological and biological material samples from pigs kept in agricultural holdings and from backyard pigs that accounted for 5.32 and 6.22% of total pig population kept in holdings of different ownership type, respectively, were tested in 2018 [8, 11, 18]. A total of 576 pathological material samples from wild boars (33.88% of the total population) were tested. ASFV genome was detected in 8 pathological material samples that accounted for 0.1% of total PCR tests carried out in the FGBI "ARRIAH" Branch in the Republic of Crimea and the GBI RC "Regional State Veterinary Laboratory of the Republic of Crimea".

CONCLUSION

African swine fever has not been reported in the Republic of Crimea since 2016. Since logistics in the Republic is arranged in such a way that products come not only from the Subjects of the Russian Federation, the ASF virus can be introduced by any route: starting from unauthorized movements of domestic animals that could be ASFV infected, and products derived from them, ending with animal buyers, feed sellers, etc.

Unfortunately, it is the human factor that most often plays a role in the ASF virus spread, as evidenced by illegal burials of dead domestic pigs, which could have resulted in infection of wild animals.

Thousands of pigs had been destroyed in 2016–2018 due to ASF introduction to the Crimean peninsula. ASF epizootic situation in domestic pigs and wild boars was the most complicated in the Sovetsky, Razdolnensky, Belogorsky Raions and in Sudak municipality, respectively. As a result, the population of pigs kept in backyards and agricultural holdings and population of wild boars decreased by 25.34% and 26.09%, respectively.

Number of laboratory tests performed in the framework of passive and active monitoring aimed at determination of ASF introduction risk and spread had increased by ten-folds from 2015 to 2018. Thus, 527 pathological and biological material samples from domestic pigs and wild boars were tested in 2015 whereas in 2018 a total of 7,754 of such samples were tested. Large-scale diagnostic tests performed in domestic pigs and wild boars contributed to rapid diagnosis of outbreaks and timely anti-epizootic measures implementation.

The disease has been eradicated owing to timely measures for ASF spread prevention that is evidenced by results of monitoring tests carried out in the Republic of Crimea in following years.

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Bovine leukosis control measures

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SUMMARY
Bovine leukosis is one of the most common infectious diseases of farm animals, causing significant economic damage due to a decrease in production of livestock products, premature culling and slaughter of cows and servicing bulls. The disease needs special attention and control on behalf of the on-farm veterinarians and zootechnicians. The article briefly describes epizootic situation on bovine leukosis in the Russian Federation in 2004–2020. It also includes a report on the disease situation in "Sibirskaya Niva" LLC (the Irkutsk Oblast) for 2015–2021 and assesses effectiveness of health support and disease prevention measures taken on the farm. The paper gives a brief description of the agricultural establishment: its zoosanitary status as well as zootechnical, veterinary, therapeutic and preventive measures (disinsection, deratization, vaccination). Based on the data obtained, we found the ultimate cause of bovine leukosis on the farm: presumably, these were crossbred animals brought into the farm. In order to eradicate the disease, "Sibirskaya Niva" LLC has developed a plan on health support and disease prevention, which includes veterinary, zootechnical and economic measures. Thus, due to the actions taken from 2015 to 2019, the number of infected cows and heifers reduced by 6.42 and 2.78 times, correspondingly. At the same time, the overall number of infected animals decreased by 9.9%. Task-oriented measures taken by the state veterinary services made it possible to steadily reduce the number of infected animals by 2020. The comprehensive approach embracing the farm peculiarities has proven to be effective to quickly eliminate bovine leukosis, as the above-mentioned agricultural establishment exemplified it.

Keywords: leukosis, bovine leukosis virus, hematologic tests, serological tests, health support and preventive measures

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Комплекс мероприятий по борьбе с лейкозом крупного рогатого скота

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РЕЗЮМЕ
Лейкоз крупного рогатого скота – одно из наиболее распространенных инфекционных заболеваний сельскохозяйственных животных, наносящих значительный экономический ущерб вследствие недополучения продуктов животноводства, преждевременной выбраковки и убоя коров и быков-производителей. Болезнь требует особого внимания и контроля со стороны специалистов ветеринарной и зоотехнической служб хозяйств. В статье кратко охарактеризована эпизоотическая обстановка по лейкозу крупного рогатого скота на территории Российской Федерации в 2004–2020 гг., а также представлены результаты изучения ситуации по заболеванию за 2015–2021 гг. в ООО «Сибирская Нива» Иркутской области и оценена эффективность оздоровительно-профилактических мероприятий, проведенных в хозяйстве. Приведена краткая характеристика сельскохозяйственного предприятия: зоогигиенические показатели, зоотехнические, ветеринарные и лечебно-профилактические (дезинсекция, дератизация, вакцинация) мероприятия. На основании полученных данных была установлена первопричина появления лейкоза крупного рогатого скота в хозяйстве: предположительно, источником вируса явились ввезенные помесные животные. Для ликвидации заболевания в ООО «Сибирская Нива» разработан план оздоровительно-профилактических мероприятий, включающий ветеринарные, зоотехнические и организационно-хозяйственные процедуры. В результате проведенной работы

с 2015 по 2019 г. инфицированность среди коров и телок снизилась в 6,42 и 2,78 раза соответственно. При этом общий уровень инфицированности животных уменьшился на 9,9%. Благодаря целенаправленной работе, проводимой государственной ветеринарной службой, к 2020 г. удалось добиться стабильного снижения уровня инфицированности животных. На примере данного сельхозпредприятия доказана эффективность спланированного с учетом особенностей хозяйства комплексного подхода, позволившего в короткие сроки провести оздоровление хозяйства от лейкоза крупного рогатого скота.

Ключевые слова: лейкоз, вирус лейкоза крупного рогатого скота, гематологические исследования, серологические исследования, оздоровительно-профилактические мероприятия

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INTRODUCTION

Bovine leukosis is a chronic infectious disease caused by bovine leukemia virus (BLV). The damage caused by the disease on farms includes a drop in production of milk, slaughter products and offspring; premature culling and slaughter of cows and servicing bulls [1].

Leukosis is registered in most countries with well-developed livestock industry. The disease is mostly spread in the USA and in some countries of Central Europe, as well as in Denmark and Sweden. Based on the report of the Rosselkhoznadzor Information and Analysis Center on the epizootic situation in the Russian Federation, 18,636 animal cases were registered in the country in 2020 and 442 sites were affected with bovine leukosis [1–6]. Figure 1 depicts an upward trend in the year-on-year dynamic changes in the number of the affected sites.

In 2020, new outbreaks of bovine leukosis were registered in the Altai, Krasnodar, Perm, Primorsky, Khabarovsk Krais, in the Republics of Dagestan, Ingushetia, Kalmykia, Karelia, Crimea, North Ossetia, Tatarstan, in the Khanty-Mansi Autonomous Okrug, in the Amurskaya, Astrakhan, Vladimir, Voronezh, Irkutsk, Kaliningrad, Kaluga, Kirov, Kurgan, Kursk, Novgorod, Tyumen and Chelyabinsk Oblasts [5, 6].

The increase in the number of leukosis cases and lack of effective prevention tools and methods add urgency to the problem which needs to be solved not only for the purposes of veterinary medicine, but also for general biology [7, 8].

The purpose of the research is to assess measures taken to control bovine leukosis in "Sibirskaya Niva" LLC.

To achieve the purpose, the following tasks were set:

– to study epizootic situation on bovine leukosis in "Sibirskaya Niva" LLC;

– to analyze measures taken to control bovine leukosis in "Sibirskaya Niva" LLC;

– to assess effectiveness of the measures taken over the past 7 years.

MATERIALS AND METHODS

Primary veterinary records and veterinary reports were used as a practical basis to study the epizootic situation on bovine leukosis in "Sibirskaya Niva" LLC (the Irkutsk Oblast), as well as to assess the disease control measures.

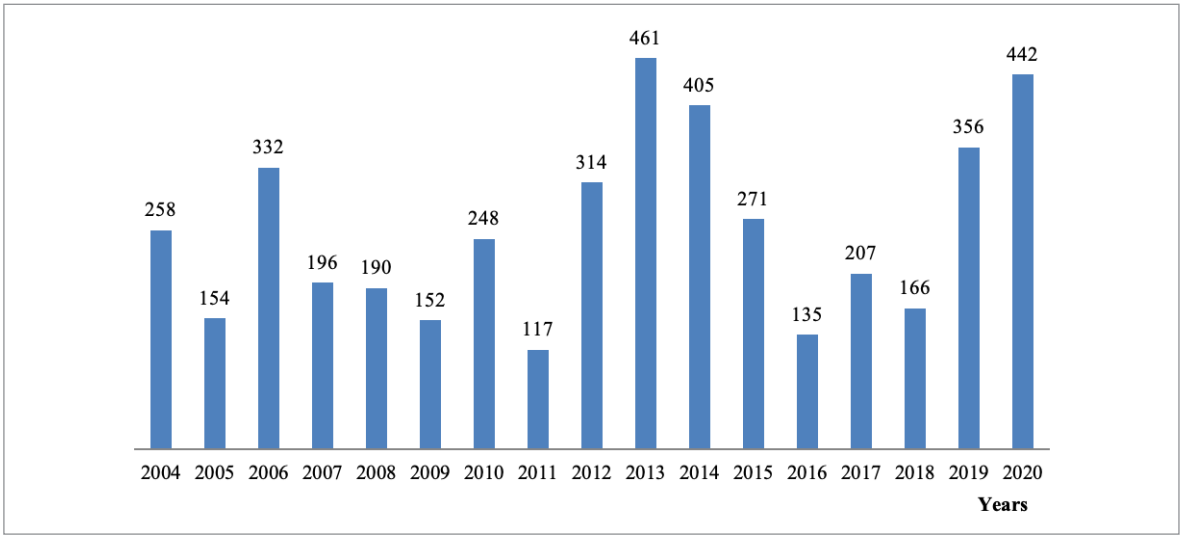

Fig. 1. Number of bovine leukosis-affected sites in the Russian Federation in 2004–2020 [6]

RESULTS AND DISCUSSION

"Sibirskaya Niva" LLC keeps one animal species, i.e. black-and-white dairy cattle. There are no driveways or cow tracks next to the farm. The livestock farms are located at least 500 m away from residential areas and household buildings. The animals are kept in standard cow barns designed for 100 animals. These are brick-built barns complying with the relevant zoosanitary requirements and having artificial lighting, central heating and combined supply and exhaust ventilation.

Cow watering site is equipped with individual water bowls with water supplied from artesian wells. Animal feeds are stored in a specially designated storage facility protected from birds and rodents and silage is stored in a sealed trench. Feeds are regularly tested for bacterial contamination, mycotoxins and heavy metals.

The feed preparation room, isolation pen and veterinary pharmacy properly comply with the sanitary and hygiene requirements.

The manure is removed out of the cow barns twice a day with a scraper conveyor, then transported to the manure storage site located 600 m away from the farm for further biothermal decontamination. On-farm dead livestock and slaughter wastes are buried in burial sites in the settlement of Orinsk or burned in a trench.

Formaldehyde, caustic soda, chloramine and bleach are used on the farm for disinfection. Required amounts of disinfectants are stored in a pharmacy warehouse in sealed original containers with labels on. The use of disinfectants is registered in a special log. A mobile disinfection station (UDP) and a spray nozzle SAG-1 are used for disinfection of cattle barns. For preventive purposes cattle barns are disinfected after the grazing season begins and before the housing season starts. The calving area is sanitized, as soon as the animals leave calving cubicles. Calve nurseries are sanitized every time after the calves are moved to calf barns.

Disinfection of the farm production facilities includes 4 stages:

1. Spray treatment of the interior surfaces in the production facilities and in ventilation shafts with a hot 2% caustic soda solution, exposure time – 30 minutes.

2. Mechanical cleaning: removal of left-over feed, manure, mechanical impurities using pressure washing unit from the mobile disinfection station.

3. Wet disinfection with a hot 5% caustic soda solution, exposure time – 1 hour (concentration of the working solution is 1 L/m^2).

4. Indoor air aerosol disinfection with a 40% formaldehyde solution (15 mL/m^3), exposure time – 2 hours. Then the room is ventilated, the remaining formaldehyde is neutralized by sprayed 20% hydrous ammonia. Walls (to a height of 1.5 m), feeders and cubicle divisions are treated with 15% freshly slaked lime. Disinfection quality control is ensured by the Irkutsk Interregional Veterinary Laboratory which tests farm swabs for coliform bacteria.

For disinsection purposes, a 1% aqueous solution of chlorophos is used to destroy insect breeding sites. Before blood-sucking insect (horseflies) season starts, the skin of animals is treated with a 2% solution of chlorophos. Deratization is carried out regularly after rodents are detected; more often in autumn and winter when the rodents move indoors. For these purposes, baits with zinc phosphide are used. Special attention is given to preventive measures: floors are repaired, doors and gates are sealed, grain feeds scattered on the floor are removed, etc.

Farm workers are provided with overalls and personal protective equipment in accordance with the regulations. The overalls are washed once a month in the laundry room. The clothes are pre-soaked for 30 minutes in a 2% formaldehyde solution. Employees can enter the farm only through the shower and changing facilities, where they take off their casual clothes, take a shower and put on overalls. Industrial and sewage wastewater goes to the settlement sewage system and pass through a sewage treatment plant. Awareness-raising campaign aimed at educating farm workers is in place. The farm workers are monthly lectured on veterinary and sanitary topics.

The dairy herd is restored from the young replacements reared on the farm. The cows showing signs of estrous behavior are artificially inseminated (manocervical method) in compliance with veterinary and sanitary rules (the external genitalia are washed with a 1:5000 solution of furacilin, disposable pipettes and gloves are used). Semen from serving bulls of "Irkutskgosplem" LLC is used for insemination of cows and heifers.

Until 2014, no cases of bovine leukosis were registered in "Sibirskaya Niva" LLC. This is primarily explained by the fact that routine serological diagnosis of leukosis using agar gel immunodiffusion (AGID) test in the Irkutsk Oblast began in 2014. It can be assumed that before some leukosis-infected animals were culled due to weight losses and a decrease in milk production. Probably, the infection was introduced into the farm in 2014, following delivery of mixed-bred heifers. Large gatherings of cattle during blood collection, vaccination, therapeutic manipulations, etc. play a key role in transmission of bovine leukosis to healthy susceptible animals.

In 2015, serological tests of blood sera samples from the entire herd [9] revealed that the number of leukosis infected cows and heifers was 32.1 and 13.9% (on average 19.5%), respectively (Fig. 2, 3, Table). Following the tests, the farm was declared leukosis affected.

In order to eradicate the disease the following health support action plan was elaborated by the Institute of Experimental Veterinary Medicine of Siberia and the Far East of the SFSCA RAS together with the Veterinary Services of "Sibirskaya Niva" LLC.

Veterinary measures:

1. Pursuant to results of serological tests of animal blood sera, the dairy herd shall be divided into leukosis infected and healthy cattle.

2. Leukosis infected cows shall be segregated in one of the yards and tested for leukosis 2 times a year using a hematological method. Blood sera from animals of this group shall not be serologically tested [4, 9].

3. The sick animals shall be culled, sent to meat processing plant or emergently slaughtered on the farm.

4. Calves from the sick cows shall be reared in isolation, shall be fed colostrum from mother cows for 10 days and then milk from healthy animals, bulk pasteurized milk or calf milk replacer [9].

5. Blood sera samples from healthy cows shall be serologically tested every quarter. Reactors shall be transferred to the infected group [2, 10].

6. The main dairy herd shall be restored from seronegative heifers [11, 12].

7. Sera samples from the young cattle shall be serologically tested at the age of 6, 12, 18 months and before they join the main herd. Reactors shall be moved to the fattening group with subsequent slaughter for meat. It is also possible to subject these animals to immunostimulating therapy in combination with the use of immunomodulators [13, 14].

Veterinary and zootechnical measures:

1. Ensure clear identification numbering of cattle of all sex and age groups.

2. Prohibit the use of non-sterile tools and other materials during animal handling.

3. Prevent contacts between infected and intact animals.

4. Any on-farm animal regrouping shall be authorized by the chief veterinarian.

5. AGID-positives shall be culled.

Economic measures:

1. Employees shall be timely assigned to immobilize animals during mass blood sampling for leukosis tests, and vehicles shall be provided for transportation of animals to the meat processing plant.

2. Key implementers of the on-farm health support measures shall get bonus payments.

3. Quarterly reports from livestock specialists on implementation of the action plan aimed at ensuring farm freedom from bovine leukosis shall be reviewed.

4. The action plan shall be annually corrected following its implementation assessment.

The use of such a detailed approach [5, 15, 16] during implementation of the health support plan in 2015–2021 made it possible to achieve a stable decrease in the number of leukosis infected animals (Fig. 2, 3, Table).

The dairy herd animals, whose blood sera contained BLV-specific antibodies, as revealed by serological tests, were subjected to hematological tests. It resulted in detection of cows with hematologic stage which helped to reduce their number in the herd over time [5, 6, 11–14, 17].

As a cost-saving measure for the farm, no BLV genotyping was carried out. Leukosis causative agent is known to include 10 genetic groups and several subgroups.

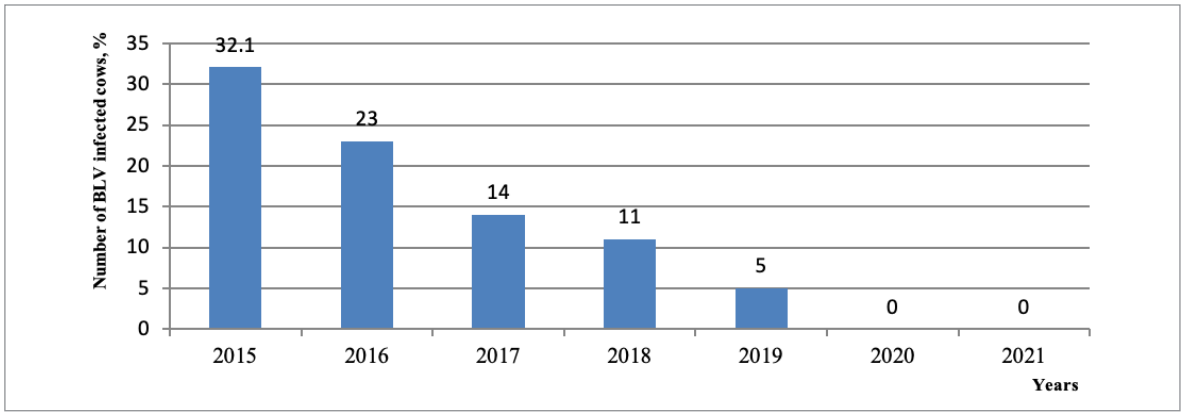

Fig. 2. Dynamic changes in the number of BLV infected cows, 2015–2021

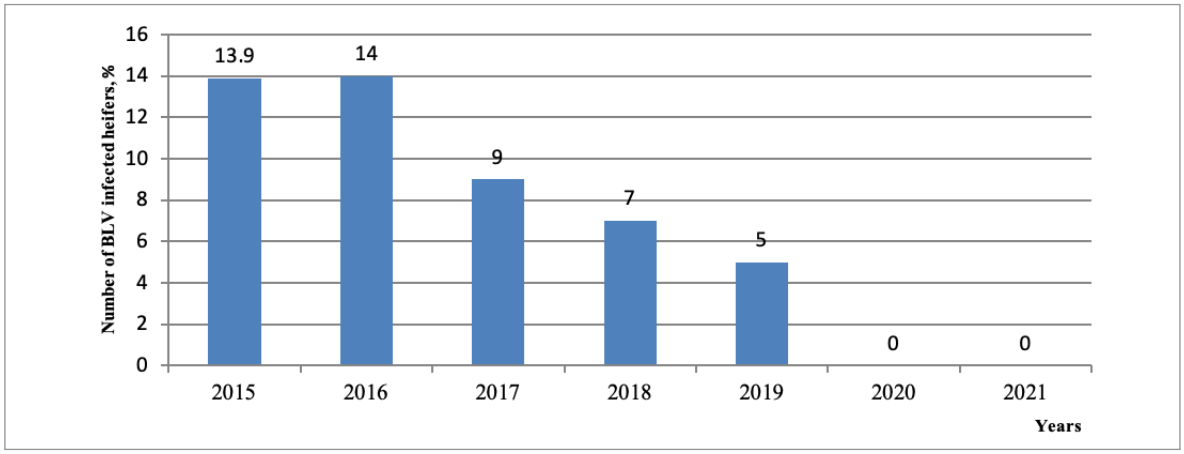

Fig. 3. Dynamic changes in the number of BLV infected heifers, 2015–2021

Table
Changes in the overall number of the infected livestock, 2015–2021

Year	2015	2016	2017	2018	2019	2020	2021
Number of infected animals, %	19.5	19.1	11.5	11.0	9.6	–	–

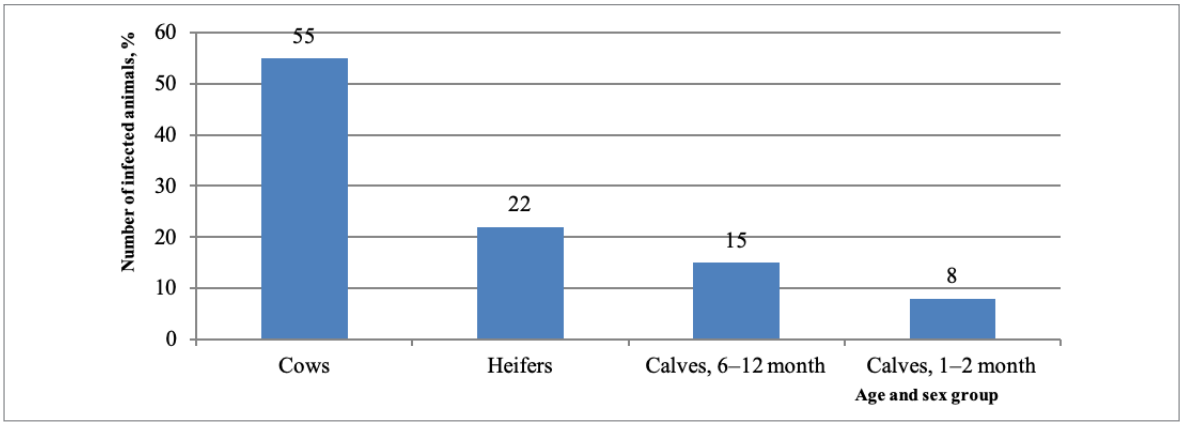

Fig. 4. Assessing the number of BLV-infected animals in 2019

BLV isolates recovered in the Russian Federation belong to genetic groups 4, 7 and 8 [5, 18, 19]. In the Irkutsk Oblast BLV belonging to the Belgian and Australian subgroups circulates in the cattle population.

Calves from infected and sick cows were healthy and received milk only from healthy animals, since BLV can be transmitted through colostrum or milk [5, 16, 19, 20].

From 2015 to 2019, the number of infected cows and heifers decreased by 6.42 and 2.78 times, respectively. At the same time, the overall number of infected animals decreased by 9.9%.

Figure 4 depicts data on BLV spread in different age and sex groups in 2019. The highest percentage of infected animals was reported in cows and heifers.

In 2020, AGID revealed no specific precipitating antibodies to BLV antigens in blood sera from the herd.

Thus, thanks to the purposeful work done by the state veterinary services from 2015 to 2019, the number of BLV-infected animals decreased to 9.6%, and since 2020 no infected animals have been detected on the farm. Lethality and mortality rates were not taken into account, since the disease was chronic and no deaths were recorded. The animals were culled at the very first clinical signs and when changes in blood leukogram were reported. As of 1 October 2020, the disease was completely eradicated on "Sibirskaya Niva" LLC farm.

CONCLUSION

Bovine leukosis was diagnosed on "Sibirskaya Niva" LLC farm following positive results of serological and hematological tests. Epizooty of bovine leukosis on the farm broke out in 2015 and was presumably caused by imported crossbreeds.

In order to eradicate the disease, a plan of health support and preventive measures (veterinary, zootechnical and organizational and economic) was developed.

The measures taken to prevent and eliminate bovine leukosis based on the integrated plan (taking into account peculiarities of the farm as well as timely diagnosis) made it possible to eradicate the disease and improve health status of the herd.

The research was conducted before the Order of the Ministry of Agriculture of the Russian Federation No. 156 of 24 March 2021 was published ("Validating veterinary rules for preventive, diagnostic, restrictive and other measures, the establishment and cancellation of quarantine and other restrictions aimed at preventing the spread and elimination of outbreaks of bovine leukosis").

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Determination of indicators for tests of polysept (polyhexamethylene guanidine hydrochloride) for flocculation properties

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SUMMARY

In vaccine production, it is particularly important to purify the virus-containing suspension in order to remove ballast proteins and fats, which, when present in high concentrations, are responsible for depression or allergic reactions in animals. Polyguanidine and its derivatives have long been used for such purposes. At present, the market offers polyhexamethylene guanidine hydrochloride, a cationic polyelectrolyte with a unique combination of physico-chemical and biocidal properties allowing for it to be used in nearly all spheres of economy. Flocculation properties of polysept (polyhexamethylene guanidine hydrochloride) vary from batch to batch, and this has necessitated the development of a test system for determination of the incoming material quality, which has a significant impact on virus antigen concentration during vaccine production. Seven batches of polyhexamethylene guanidine were tested for flocculation properties, changes in FMDV immunogenic component concentration in the virus-containing suspension, osmolality of solutions at different percentage concentrations. Indicators of incoming material suitability for FMD vaccine production were determined. The batches of polysept should be tested for flocculation properties at different concentrations of the polymer (0.007, 0.0105 and 0.01575%) in dynamics during 24 hours. After this period, the turbidity of solutions should not exceed 30 FNU (formazin turbidity) at concentrations of 0.0105 and 0.01575%. It is also necessary to determine the osmolality of polysept solutions at different percentage concentrations (6, 8, 10, 12, 14%). Osmolality values should be within the following ranges: 260 ± 20 mOsm for a 6% solution; 330 ± 25 mOsm for an 8% solution; 400 ± 25 mOsm for a 10% solution; 460 ± 30 mOsm for a 12% solution; 520 ± 20 mOsm for a 14% solution.

Keywords: polyhexamethylene guanidine hydrochloride, foot-and-mouth disease virus antigen, immunogenic components, flocculation, turbidity, osmolality

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Определение критериев для исследования флокулирующих свойств полисепта (полигексаметиленгуанидин гидрохлорида)

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РЕЗЮМЕ

При производстве вакцин важную роль играет очистка вирусной суспензии от балластных белков и жиров, высокая концентрация которых вызывает угнетение организма животных или аллергические реакции. На протяжении длительного времени для этих целей применяли полигуанидин и его производные. В настоящее время на рынке предлагают катионный полиэлектролит полигексаметиленгуанидин гидрохлорид, обладающий уникальным сочетанием физико-химических и биоцидных свойств, которые позволяют использовать его практически во всех сферах народного хозяйства. Партии полисепта (полигексаметиленгуанидин гидрохлорида) отличаются друг от друга по флокулирующим свойствам, поэтому возникла необходимость разработать тест-систему для определения качества поступающей продукции, существенно влияющего на потерю антигена вируса при производстве вакцин. Были изучены как флокулирующие свойства, так и потеря иммуногенных компонентов вируса ящура из вируссодержащей суспензии, а также осмоляльность растворов разной процентной концентрации семи серий полигексаметиленгуанидин гидрохлорида. Установлены критерии пригодности поступающей продукции для производства противоящурных вакцин: проверка в динамике флокулирующих качеств партий полимера при разных его концентрациях (0,007; 0,0105; 0,01575%) на протяжении 24 ч. Через указанное время мутность раствора должна быть не более 30 FNU (формазиновая степень мутности) при концентрациях 0,0105 и 0,01575%. Также необходимо определять осмоляльность растворов полисепта разной процентной концентрации (6, 8, 10, 12, 14%). Значение осмоляльности должно укладываться в следующие границы: 6%-й раствор – 260 ± 20 mOsm; 8%-й – 330 ± 25 mOsm; 10%-й – 400 ± 25 mOsm; 12%-й – 460 ± 30 mOsm; 14%-й – 520 ± 20 mOsm.

Ключевые слова: полигексаметиленгуанидин гидрохлорид, антиген вируса ящура, иммуногенные компоненты, флокуляция, мутность, осмоляльность

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INTRODUCTION

In vaccine production, the purification of the virus-containing suspension is of particular importance. Polyguanidine and its derivatives have long been used for such purposes. At present, the market offers polyhexamethylene guanidine hydrochloride (PHMG hydrochloride), a cationic polyelectrolyte with a unique combination of physico-chemical and biocidal properties allowing for it to be used in nearly all spheres of economy [1–8].

Polyhexamethylene guanidine hydrochloride is a water-soluble chlorine-containing polymer with a molecular mass of 10,000 Da, represented by formula $[–NH–C=(NH\times HCl)–NH–(CH_2)_6–]_n$. Chlorine included in its composition is a complex salt of hydrogen chloride with a strong basic nitrogen of the compound. The preparation with the empirical formula $(C_7H_{16}N_3Cl)_n$ manufactured under the trademark "Polysept" (OOO "Pharma-Pokrov", Russia) is a water-soluble polymer product, which has the characteristics of both a cationic polyelectrolyte and a polymer, contains polar guanidine and nonpolar hexamethylene groups imparting adhesive and surfactant properties to it and can therefore be broadly applied in economy. PHMG hydrochloride has a high bactericidal and fungicidal effect. Its 0.05% solutions kill both gram-positive and gram-negative microorganisms within 5–25 minutes. The product is safe for humans, animals and the environment [8–10].

Physico-chemical properties of PHMG hydrochloride: it is colourless and odourless (some low quality product samples may smell like ammonia), fireproof, explosion-safe, fully soluble in water, alcohol-soluble, does not lose its properties at subzero temperatures, does not decompose and retains its physico-chemical and biocidal properties when heated to 120 ± 5 °C. The shelf life is at least 5 years for a 20% aqueous solution and at least 7 years for a 100% concentrate.

Biocidal properties of PHMG hydrochloride: it is a biocide with a broad-spectrum antimicrobial activity against gram-negative and gram-positive bacteria (in particular, mycobacteria causing tuberculosis and legionellosis), viruses (including enteric and post-transfusion hepatitis viruses, human immunodeficiency virus, poliomyelitis virus, influenza virus, herpesviruses, etc.), fungi, in particular mold, yeast and yeast-like fungi, fungi of the genus *Candida*, dermatophytes.

Product form: lumps (pellets) containing at least 95–98% of PHMG hydrochloride or an aqueous solution containing 20% of PHMG hydrochloride. Where necessary, aqueous solutions containing up to 50% of the active ingredient can be prepared [4].

PHMG hydrochloride is produced by the interaction of hexamethylene diamine and guanidine hydrochloride [11, 12].

Table 1
Formazin turbidity reference values

Turbidity, FNU				
< 0.10	0.1–15	16–100	101–750	> 750
ultra-low	low	medium	high	ultra-high

Polysept has been found to have flocculation properties. It is applied as a 9% solution (1.5–2.0 mg of dry matter per 1 L of waste water) [13].

In the production of inactivated vaccines against foot-and-mouth disease, polysept is added in the form of a 5 or 10% aqueous solution to reach the final concentration of 0.005–0.03% (pH 7.6–8.0). Flocculated ballast proteins are removed by centrifugation, separation or sedimentation. The use of PHMG hydrochloride concentrations greater than 0.03% results in a significant reduction of the virus concentration; a decrease in FMDV infectivity titre and 146S component concentration are observed [10, 14].

Unfortunately, flocculation properties and other characteristics of polysept may vary from batch to batch, leading to a decrease in the concentration of the virus protein used for vaccine production. It is therefore important to develop a test system for tests of PHMG hydrochloride batches for flocculation properties.

The aim of the study is the selection of a test system for tests of polysept (PHMG hydrochloride) for its flocculation properties.

MATERIALS AND METHODS

Cell line. BHK-21/SUSP/ARRIAH, a continuous suspension culture of neonatal Syrian hamster kidney cells, was used for the study [15]. The cells were grown in metal fermenters with a working capacity of up to 1,800 dm^3 in accordance with the Master formula record for production of the vaccine against FMD of various types.

PHMG hydrochloride was supplied by OOO "Pharma-Pokrov" (Russia), TU 9392-001-32963622-99, batches: No. 343 of 09 July 2020; No. 522 of 20 November 2020; No. 48 of 21 February 2021; No. 57 of 26 February 2021; No. 71 of 26 March 2021; No. 219 of 27 August 2019; No. 168 of 27 August 2021.

To perform the study, 10 and 20% solutions were prepared in an enameled container using demineralized water. The mixture was heated to 90–100 °C with constant stirring until complete dissolution of the polymer, cooled at 18–25 °C and then placed in the cold chamber (4–8 °C).

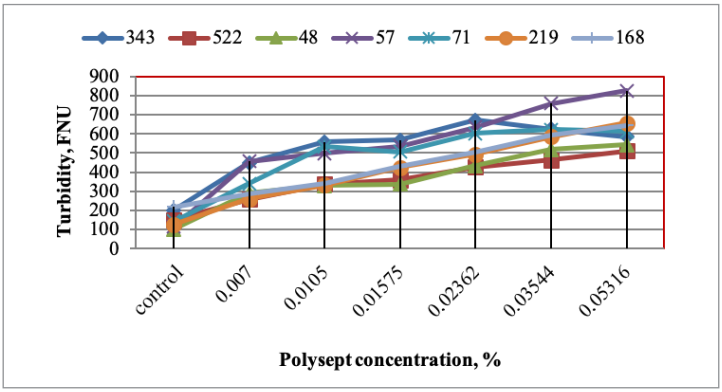

Fig. 1. Turbidity dynamics in the zero-hour sample at different polysept concentrations

The turbidity of the prepared PHMG hydrochloride solution was measured using a portable HI 98713 turbidity meter equipped with an IR-diode (Hanna Instruments, Germany) according to the manufacturer's instruction. Turbidity was reported in FNU (a formazin turbidity unit).

The values and corresponding degrees of turbidity are presented in Table 1 [16].

To measure the osmolality of the tested solutions at different concentrations (6, 8, 10, 12, 14%), an OSKR-1M cryoscopic medical osmometre (Russia) was used.

The concentration of FMDV total viral protein and its components was determined according to the "Methodical guidelines for determination of concentration of 146S, 75S, 12S components of vaccine strains of culture FMD virus with complement fixation test (CFT)" [17].

Tests for flocculation properties. The tests were carried out as follows: the inactivated FMD virus suspension was transferred into 0.5 dm^3 bottles (0.4 dm^3 of the suspension per bottle), then a 10% polysept solution was added to reach the final concentrations of 0.007, 0.0105, 0.01575, 0.02362, 0.03544, 0.05316% (1:5). Samples for turbidity tests were collected after 0 (a zero-hour sample), 2, 4, 6, 8 and 24 hours. The concentrations of total viral protein and its components were determined 24 hours after addition of polysept.

Statistical processing of data. Numerical data were statistically processed by generally accepted methods of variation statistics using a personal computer and Microsoft Excel software.

RESULTS AND DISCUSSION

During the first stages of the study, different batches of polysept were tested for their flocculation properties. For this purpose, a 10% polysept solution was added to the inactivated FMD virus suspension at different concentrations in the total volume. Samples were collected at different time intervals. It was found that the higher the flocculant percentage, the greater were turbidity values in the zero-hour samples (Fig. 1, Table 2). Turbidity in the controls varied from 101.5 ± 19.9 to 219.3 ± 10.8 FNU; in the polymer-supplemented suspension, turbidity varied from 258.0 ± 32.9 to 826.0 ± 61.6 FNU depending on the batch and concentration of polysept (the differences were significant, $p < 0.005$).

According to literature data, the adsorption of a flocculant onto dispersed phase particles can occur as a result of electrostatic, chemical interactions, ion exchange, under the effect of Van der Waals forces. Flocculation effectiveness, floc size and density depend largely on the intensity and duration of mixing, as well as on flocculant amount. The addition of a small amount of the flocculant leads to a sharp increase in floc hardness.

The formation of aggregates of particles, i.e. the binding of particles through the formation of bridges with the polymer, is a result of interaction between macromolecules adsorbed onto dispersed phase particles and loose particles. The adsorption of ionogenic flocculants onto oppositely charged dispersed phase particles occurs mainly due to electrostatic attraction. Flocculation rate is the highest when the concentration of flocculant-coated particles and that of uncoated particles are the same [18–20].

Table 2
Turbidity dynamics at different polymer concentrations ($n = 3$)

No.	PHMG batch No.	Time (hours)	Turbidity (FNU)						
			0.007%	0.0105%	0.01575%	0.02362%	0.03544%	0.05316%	Control
1	343	0	448.2 ± 12.0	557.8 ± 41.3	568.8 ± 8.6	672.8 ± 17.7	625.4 ± 34.6	585.4 ± 9.5	200.6 ± 69.5
		2	354.2 ± 9.2	238.8 ± 24.6	109.0 ± 15.0	85.6 ± 11.7	88.7 ± 3.0	69.3 ± 4.7	180.0 ± 60.0
		4	307.6 ± 12.6	117.5 ± 29.5	71.2 ± 11.4	53.3 ± 8.2	59.4 ± 5.7	49.4 ± 3.8	173.6 ± 51.3
		6	304.6 ± 11.2	85.5 ± 10.4	51.2 ± 12.8	38.2 ± 8.8	30.6 ± 8.3	19.0 ± 1.4	169.2 ± 52.7
		8	294.4 ± 14.0	70.0 ± 20.5	35.3 ± 10.6	25.8 ± 3.7	24.9 ± 2.3	19.3 ± 3.1	170.4 ± 51.8
		24	54.4 ± 22.4	47.2 ± 11.3	19.6 ± 3.5	13.1 ± 1.5	15.1 ± 2.3	12.5 ± 3.1	142.8 ± 30.1
2	522	0	258.0 ± 32.9	337.3 ± 38.8	361.0 ± 27.6	426.3 ± 29.5	464.0 ± 26.2	511.3 ± 22.3	150.7 ± 27.0
		2	223.3 ± 25.2	216.7 ± 20.8	340.7 ± 24.0	111.3 ± 3.2	85.7 ± 4.5	74.2 ± 3.3	163.0 ± 37.5
		4	184.7 ± 32.3	243.7 ± 40.5	108.7 ± 9.9	70.4 ± 5.1	58.0 ± 2.4	56.6 ± 3.6	163.7 ± 42.7
		6	183.0 ± 28.2	222.0 ± 23.6	70.7 ± 12.1	47.6 ± 0.4	38.4 ± 1.4	34.7 ± 0.29	160.3 ± 39.0
		8	181.0 ± 27.5	147.3 ± 29.7	68.5 ± 18.7	38.1 ± 1.1	29.3 ± 2.6	23.0 ± 3.0	127.0 ± 26.9
		24	155.0 ± 24.5	99.9 ± 0.2	34.9 ± 4.5	23.4 ± 5.2	18.7 ± 1.8	12.5 ± 1.5	124.3 ± 29.9
3	48	0	290.7 ± 37.4	330.0 ± 35.8	337.7 ± 33.3	435.3 ± 55.5	517.7 ± 58.5	543.7 ± 40.9	101.5 ± 19.9
		2	236.67 ± 16.7	88.6 ± 11.3	90.9 ± 19.1	79.3 ± 34.4	102.9 ± 11.5	85.2 ± 13.9	110.7 ± 10.1
		4	159.7 ± 25.9	62.0 ± 8.3	47.8 ± 14.3	49.4 ± 4.0	47.5 ± 8.0	49.7 ± 9.3	101.3 ± 1.5
		6	74.5 ± 21.1	44.4 ± 8.4	40.4 ± 2.7	30.0 ± 3.7	46.0 ± 13.4	35.4 ± 1.5	104.8 ± 9.9
		8	49.1 ± 16.4	29.1 ± 9.9	26.3 ± 8.0	25.0 ± 1.7	32.3 ± 8.8	30.0 ± 3.0	102.4 ± 5.6
		24	41.3 ± 1.6	19.8 ± 5.8	12.7 ± 2.2	15.9 ± 6.7	17.2 ± 4.4	18.6 ± 10.8	101.0 ± 4.6
4	57	0	457.7 ± 40.2	498.3 ± 34.3	536.7 ± 55.9	634.7 ± 37.2	760.0 ± 23.9	826.0 ± 61.6	114.0 ± 16.6
		2	136.0 ± 7.9	105.8 ± 11.6	99.4 ± 5.5	97.0 ± 15.1	90.3 ± 9.5	90.6 ± 2.5	118.7 ± 3.2
		4	81.0 ± 7.9	66.5 ± 3.3	75.2 ± 13.1	69.3 ± 11.8	79.7 ± 13.8	85.8 ± 2.3	101.7 ± 7.6
		6	59.4 ± 0.6	58.2 ± 3.3	46.8 ± 2.2	47.1 ± 1.8	43.3 ± 2.9	38.3 ± 2.9	110.0 ± 10.0
		8	48.5 ± 2.0	37.0 ± 4.0	30.9 ± 0.9	35.2 ± 4.6	31.3 ± 5.5	31.0 ± 1.7	106.7 ± 5.8
		24	23.3 ± 7.5	21.9 ± 1.9	18.8 ± 1.2	15.8 ± 2.7	18.2 ± 1.7	19.6 ± 2.5	76.2 ± 7.7
5	71	0	339.7 ± 10.0	533.3 ± 41.6	506.3 ± 11.85	602.0 ± 13.1	623.3 ± 25.2	613.3 ± 18.6	144.0 ± 30.4
		2	263.7 ± 23.7	110.3 ± 11.9	79.2 ± 4.6	90.3 ± 3.2	102.2 ± 6.9	126.0 ± 4.6	119.0 ± 14.5
		4	83.7 ± 3.3	65.1 ± 3.2	47.9 ± 4.6	62.6 ± 6.2	59.0 ± 8.1	55.0 ± 8.7	105.7 ± 12.5
		6	84.3 ± 4.0	44.6 ± 5.9	35.4 ± 2.4	44.5 ± 4.0	45.6 ± 13.2	57.8 ± 0.4	123.0 ± 21.1
		8	91.7 ± 7.6	41.8 ± 2.2	29.8 ± 6.9	36.0 ± 4.2	40.0 ± 7.9	45.4 ± 11.3	116.7 ± 14.6
		24	39.6 ± 15.9	19.6 ± 2.1	12.9 ± 1.5	12.9 ± 1.5	14.7 ± 1.3	15.1 ± 3.1	111.7 ± 15.5
6	219	0	260.3 ± 11.7	331.7 ± 17.2	423.3 ± 25.2	495.0 ± 59.1	585.0 ± 43.9	657.3 ± 30.6	128.0 ± 19.2
		2	214.3 ± 16.2	101.9 ± 15.0	81.7 ± 11.7	77.4 ± 9.8	82.2 ± 8.1	96.0 ± 3.0	155.3 ± 34.0
		4	82.7 ± 8.3	66.5 ± 19.2	50.5 ± 8.1	47.6 ± 1.9	57.9 ± 6.4	74.0 ± 4.9	114.6 ± 21.6
		6	124.7 ± 70.5	61.8 ± 5.5	44.0 ± 2.6	40.4 ± 7.8	43.7 ± 7.0	59.5 ± 13.0	112.5 ± 17.7
		8	69.8 ± 10.4	46.5 ± 6.0	29.0 ± 3.4	31.0 ± 1.7	32.2 ± 2.8	44.1 ± 4.4	107.5 ± 21.0
		24	34.9 ± 10.9	19.2 ± 5.5	12.0 ± 2.2	10.2 ± 3.6	13.9 ± 1.0	12.6 ± 3.7	117.0 ± 20.7
7	168	0	289.3 ± 7.0	339.3 ± 22.5	429.7 ± 20.0	505.3 ± 26.8	595.7 ± 6.7	645.3 ± 41.3	219.3 ± 10.8
		2	210.0 ± 13.1	99.55 ± 8.0	82.4 ± 10.8	83.1 ± 16.4	86.5 ± 13.8	82.5 ± 2.3	115.7 ± 9.6
		4	76.2 ± 18.1	65.8 ± 14.7	53.2 ± 11.9	54.3 ± 12.8	60.1 ± 4.8	57.4 ± 10.9	110.3 ± 6.4
		6	69.8 ± 7.6	44.7 ± 2.5	42.7 ± 9.1	42.8 ± 8.3	46.4 ± 4.3	54.3 ± 16.3	101.3 ± 4.2
		8	58.0 ± 13.9	34.8 ± 0.8	32.2 ± 5.4	28.7 ± 2.2	34.4 ± 9.6	36.0 ± 13.5	111.0 ± 15.8
		24	30.6 ± 12.5	17.0 ± 0.8	17.5 ± 3.5	16.7 ± 1.6	14.9 ± 1.5	15.8 ± 3.8	99.0 ± 22.3

Based on perceptions of the chemical nature of flocculation processes, it is logical that the higher the content of the flocculant, the more intensive flocculation is, and this was observed during the tests.

Tests of polysept of different batches (at the concentration of 0.007%) for its ability to precipitate cell debris showed the following: after 4 hours, four of the tested batches demonstrated a 68.3–82.0% (by 3.1–5.7 times) decrease in turbidity, which declined to medium values (the differences are significant, $p < 0.005$); batch No. 48 demonstrated a 74% (by 3.9 times) decrease in turbidity after 6 hours; batch No. 343 demonstrated an 88% (by 8.2 times) decrease in turbidity after 24 hours; batch No. 522 demonstrated no cell debris precipitation even after 24 hours (Fig. 2A, Table 2).

When the amount of the added flocculant was increased by 1.5 times (0.0105%), six of the tested batches demonstrated a 57–81% (by 2.3–5.3 times) decrease in turbidity of the polymer-supplemented suspension; however, the turbidity still remained rather high (Fig. 2B, Table 2). After 4 hours, five PHMG hydrochloride batches demonstrated an 80–87% decrease in turbidity, which declined to medium values (16–100 FNU). Batch No. 343 demonstrated a decrease in turbidity after 6 hours, and batch No. 522 did not demonstrate satisfactory cell debris precipitation even after 24 hours.

In further tests, when polysept concentration was increased to 0.05316% (by 7.5 times), rapid precipitation of debris was observed as soon as after 2 hours. After 24 hours (according to FMD vaccine production technology), the turbidity of the suspension was 10–20 FNU, and this was indicative of satisfactory flocculation (Fig. 2C–F, Table 2).

Debris sedimentation was observed in the control samples containing no flocculant (Fig. 2G, Table 2); however, turbidity decreased only by 1.1–2.2 times as a result of natural sedimentation, and, after 24 hours, the turbidity still exceeded 100 FNU, being unsatisfactory in terms of the vaccine production technology.

At the following stages of the study, the concentrations of total viral protein and FMDV antigen composition were determined.

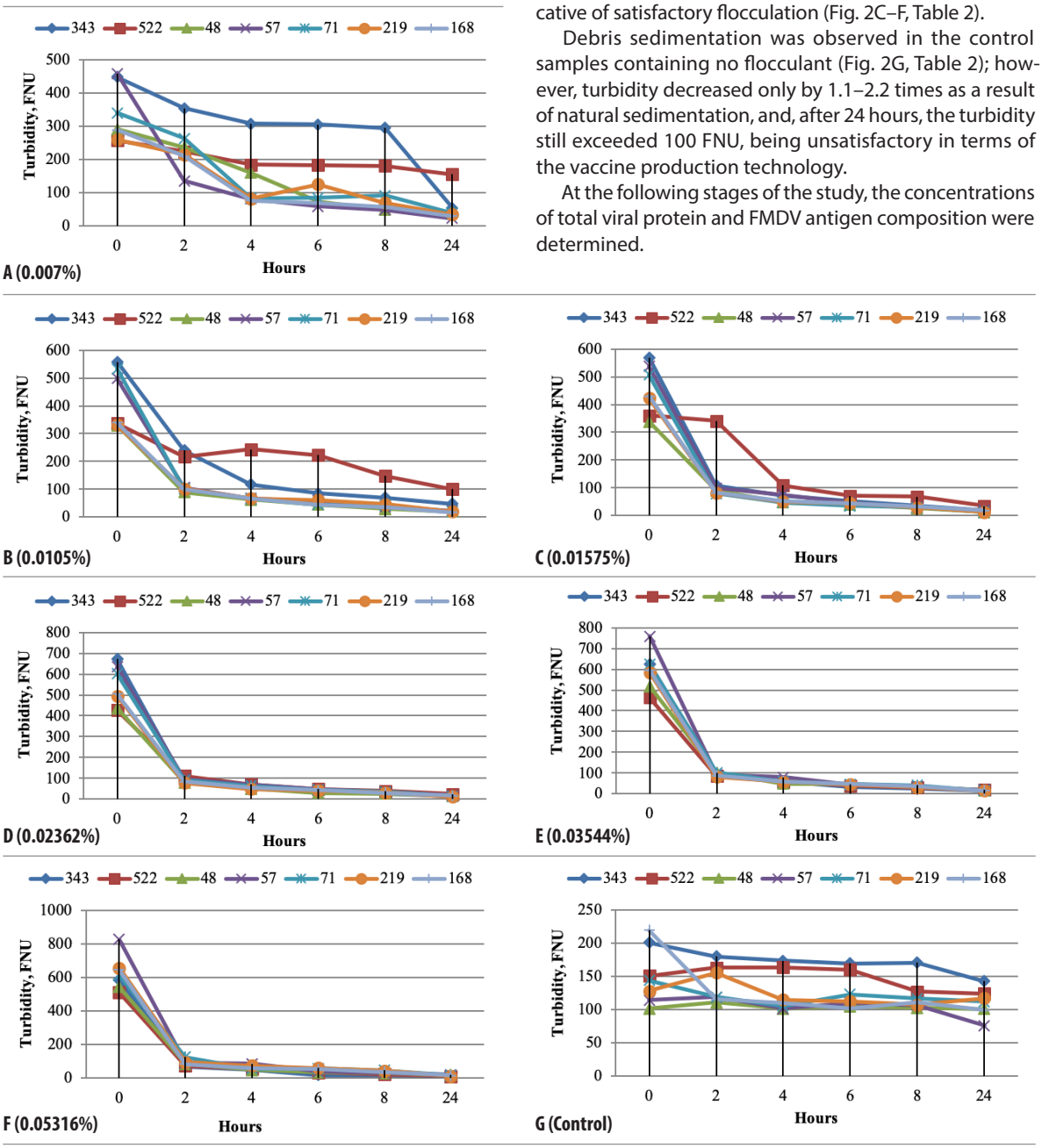

Fig. 2. Dynamics of flocculation properties of polysept batches at different polymer concentrations

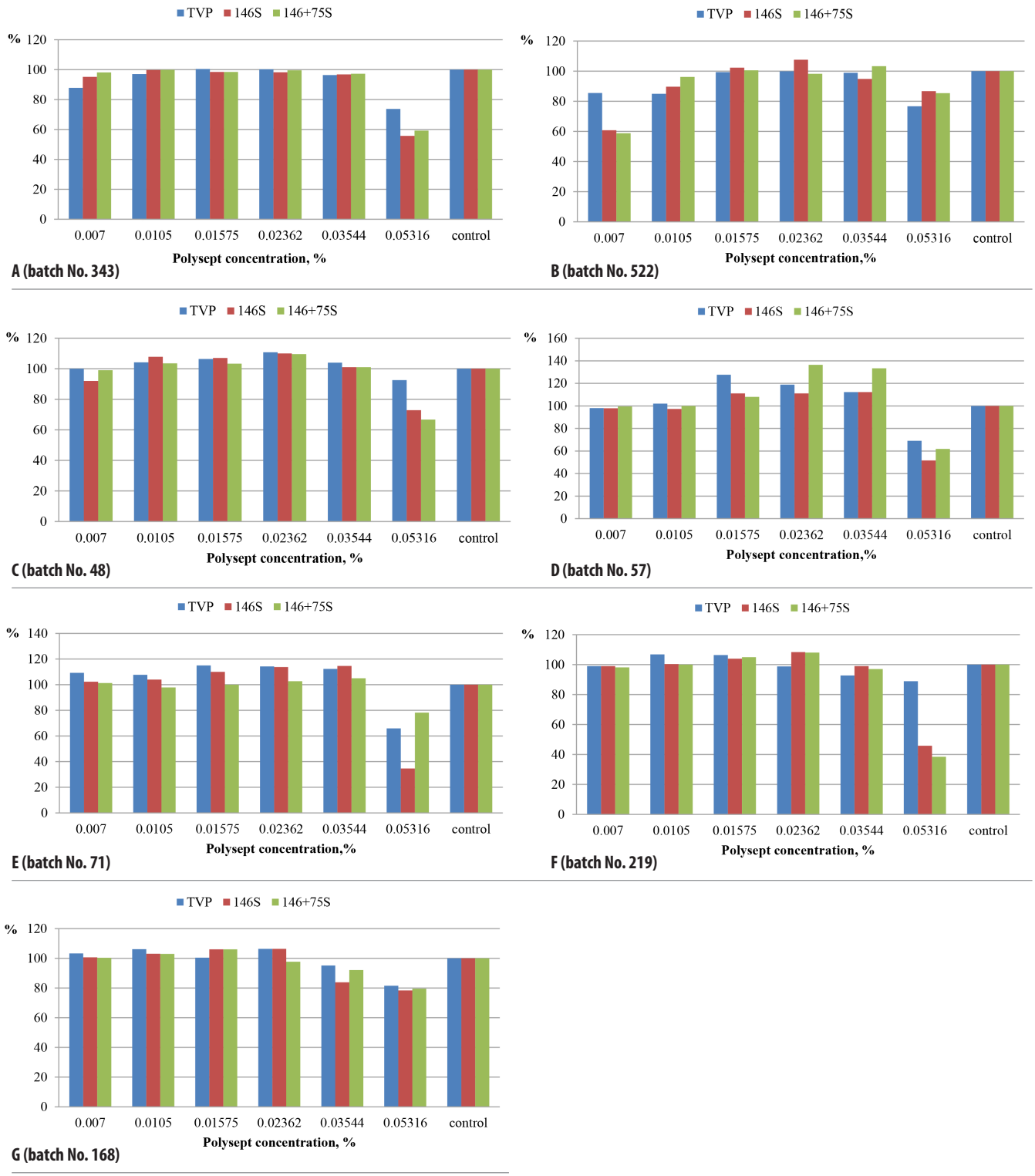

Fig. 3. Relative changes in concentrations of total viral protein (TVP) and immunogenic components of FMD virus supplemented with polysept of different batches

The study involved the use of antigens of different FMDV strains, and it was therefore incorrect to apply absolute measures when calculating losses at different PHMG hydrochloride concentrations. In view of this, the losses were reported using relative measures, with measures in the control production sample (0.01% of polysept) taken as 100%. The test results are presented in Figure 3.

Since the use of higher flocculant concentrations resulted in better antigen purification and lower anticomplementary activity of viral antigen preparations as determined with CFT, this could probably explain a slight rise in the content of total viral protein and immunogenic components of FMD virus when polysept concentration was increased from 0.007 to 0.03544%.

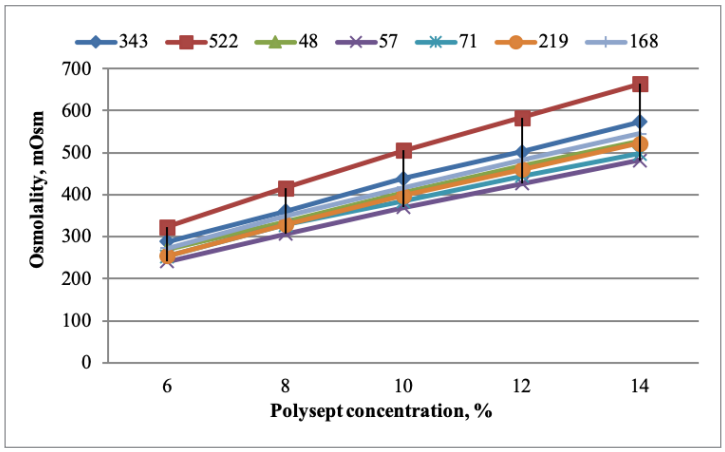

Fig. 4. Osmolality dynamics at different polysept concentrations

Table 3
Osmolality reference values for determination of polyhexamethylene guanidine hydrochloride suitability for FMD vaccine production

PHMG hydrochloride concentration, %	Osmolality, mOsm
6	260 ± 20
8	330 ± 25
10	400 ± 25
12	460 ± 30
14	520 ± 20

All the batches of polysept at the concentration of 0.05316% demonstrated an 8.0–65.4% decrease in FMDV total viral protein and immunogenic component content. It was found that, when the turbidity of the suspension was very high, CFT tests yielded false (erroneously low) results, and the immunogenic component content was 14.5–39.3% lower as compared with the control (Fig. 3).

At the final stage, the osmolality of solutions of polysept of all the tested batches was measured at different concentrations of the flocculant (Fig. 4). Batch No. 522 polysept osmolality was considerably different from that of other batches at all the tested concentrations (the differences were significant, $p < 0.001$). In particular, at PHMG hydrochloride concentration of 6%, the osmolality of this batch was 324 ± 4 mOsm, whereas in other batches it varied from 241 ± 3 to 288 ± 4 mOsm. As for 14% polymer solutions, the differences were even higher: 664 ± 8 mOsm in batch No. 522, and 482 ± 5 and 573 ± 10 mOsm in the rest of the batches.

Thus, to determine the suitability of polysept batches for FMD vaccine production, it is necessary to test the polymer for its flocculation properties at different concentrations (0.007, 0.0105, 0.01575%) over the period of 24 hours. The application of higher PHMG hydrochloride concentrations is economically disadvantageous.

The production of FMD vaccines involves the use of a 10% polysept solution. At this PHMG hydrochloride concentration, all the batches found suitable for production demonstrated the osmolality values that ranged from 370 mOsm to 440 mOsm. Batch No. 522 demonstrated a higher osmolality, namely 504 ± 5 mOsm.

CONCLUSION

As a result of the tests performed, the following indicators of PHMG hydrochloride suitability for FMD vaccine production were determined:

– flocculation properties of a batch of polysept at different concentrations (0.0105 and 0.01575%) evaluated in dynamics over 24 hours. After this period, the turbidity of 0.0105 and 0.01575% polysept solutions should not exceed 30 FNU (Table 2);

– osmolality of PHMG hydrochloride solutions measured at different concentrations (6, 8, 10, 12, 14%). The osmolality should be within the reference values specified in Table 3.

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Optimization of medium composition and study of growth stages of *Mycoplasma bovis* "Kaluga 2020" isolate

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SUMMARY

Mycoplasma bovis is considered one of bovine mycoplasmosis pathogens responsible for respiratory diseases, mastitis, arthritis and keratoconjunctivitis. The paper presents results of the study on optimizing the component composition of the culture medium for *Mycoplasma bovis* "Kaluga 2020" isolate, as well as the study of this pathogen's growth stages. The color-changing units assay and the culture method combined with colony-forming unit quantification were used for determination of *Mycoplasma* activity. It was found that when cultured in an optimized nutrient medium based on modified Hayflick broth, the microorganism enters a logarithmic growth phase after first 24 hours of growth, in 72 hours the *Mycoplasma* culture enters a stability phase, and a decline phase is recorded in 84 hours. The effect of percentage content of glucose, fresh yeast extract and horse serum in the nutrient medium on accumulation of *Mycoplasma bovis* "Kaluga 2020" isolate was evaluated using the one-factor-at-a-time approach. It was found that the greatest effect on *Mycoplasma* accumulation was exerted by such growth factors as fresh yeast extract and horse serum in the nutrient medium ($p < 0.05$), while changes in the amount of glucose did not stimulate *Mycoplasma bovis* growth. Based on results of the conducted studies, the appropriate composition was determined and the optimal content of growth factors in the medium for culturing *Mycoplasma bovis* "Kaluga 2020" isolate was selected: 12.5% of fresh yeast extract and 25% of horse serum. The use of the optimized nutrient medium based on modified Hayflick broth allowed 5-fold increase in accumulation of *Mycoplasma* biomass (3.98×10^9 CFU/ml) compared to the standard medium (0.79×10^9 CFU/ml).

Keywords: *Mycoplasma bovis*, cattle, "Kaluga 2020" isolate, optimization, nutrient media, colony-forming units (CFU), color-changing unit (CCU), biological activity

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Оптимизация состава питательной среды и изучение стадий роста изолята «Калуга 2020» *Mycoplasma bovis*

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РЕЗЮМЕ

Mycoplasma bovis является одним из возбудителей микоплазмозов крупного рогатого скота, вызывающим респираторные болезни, маститы, артрит и кератоконъюнктивит. В статье представлены результаты исследования по оптимизации компонентного состава питательной среды для культивирования изолята «Калуга 2020» *Mycoplasma bovis*, а также изучения стадий роста возбудителя. Для определения активности микоплазм использовали метод измерения цветоизменяющих единиц и культуральный метод с подсчетом колониеобразующих единиц. Установлено, что при культивировании в оптимизированной питательной среде на основе модифицированного бульона Хейфлика микроорганизм вступает в фазу логарифмического роста по истечении первых 24 ч роста, через 72 ч культура микоплазм переходит в стабильный период, а через 84 ч регистрируется фаза спада. Влияние процентного содержания глюкозы, свежего дрожжевого экстракта и сыворотки крови лошади в питательной среде на накопление изолята «Калуга 2020» *Mycoplasma bovis* оценивали с использованием метода «один фактор за раз». Было установлено, что наибольшее влияние на накопление микоплазм оказывало содержание в питательной среде таких факторов роста, как свежий дрожжевой экстракт и сыворотка крови лошади ($p < 0,05$), в то время как изменение количества глюкозы не стимулировало рост *Mycoplasma bovis*. В результате проведенных исследований определен подходящий состав и подобрано оптимальное содержание факторов роста в среде для культивирования изолята «Калуга 2020» *Mycoplasma bovis*: 12,5% свежего дрожжевого экстракта и 25% сыворотки крови лошади. Применение оптимизированной питательной среды на основе модифицированного бульона Хейфлика позволило увеличить накопление биомассы микоплазм в 5 раз ($3,98 \times 10^9$ КОЕ/мл) по сравнению со стандартной средой ($0,79 \times 10^9$ КОЕ/мл).

Ключевые слова: *Mycoplasma bovis*, крупный рогатый скот, изолят «Калуга 2020», оптимизация, питательные среды, колониеобразующие единицы (КОЕ), цветоизменяющая единица (ЦИЕ), биологическая активность

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INTRODUCTION

Mycoplasma bovis (*M. bovis*) is a causative agent of bovine mycoplasmoses, which is widespread all over the world and is common in the Russian Federation as well [1, 2]. This pathogen is one of the etiological agents of bovine respiratory diseases, also causing mastitis, arthritis and keratoconjunctivitis [3, 4].

M. bovis was first isolated from cattle with severe mastitis in the USA in 1961 [5, 6]. *M. bovis* is considered responsible for a quarter to a third of all economic losses in cattle industry due to respiratory diseases [7].

Laboratory diagnosis of bovine mycoplasmoses includes cultural, serological and molecular test methods [8, 9]. At the same time, the pathogen isolation by culturing in nutrient media is one of the most reliable methods of the disease diagnosis. Currently, various types of nutrient media are widely used for *M. bovis* cultivation, including Hayflick medium [10], modified PPLO medium [11], Eaton's medium [12] and others.

Optimization of nutrient medium composition is one of the most important aspects for improving the mycoplasma culture technique, as well as for diagnostic studies using isolation method [13]. At the same time, the complexity of the component composition of nutrient media and the long period of mycoplasma growth require multistage studies [14].

Mycoplasma growth rate and activity are estimated using several methods: color-changing unit (CCU) assay, colony-forming unit (CFU) count, measurement of turbidity, reduction of tetrazolium salts to formazane, determination of adenosine triphosphate (ATP) cell concentrations using luciferin-luciferase luminometry, etc. [15].

The aim of the paper is to study *M. bovis* growth dynamics during *in vitro* cultivation and to optimize the component composition of Hayflick nutrient medium.

MATERIALS AND METHODS

Isolate. M. bovis "Kaluga 2020" isolate recovered from biological material samples of calves demonstrating clinical signs of respiratory disease in 2020 was used for the study. The *M. bovis* isolate was identified using real-time polymerase chain reaction.

Nutrient media. Modified Hayflick broth was used as a standard liquid nutrient medium [16]. BBL™ Mycoplasma Broth Base (BD, USA) was used for its preparation. BBL™ Mycoplasma broth base in an amount of 20 g was dissolved in 1 L of distilled water, thoroughly mixed and sterilized by autoclaving at a temperature of (121 ± 0.5) °C for 15 minutes, then it was cooled to (50 ± 2) °C. 20 mL of non-heated horse serum, 0.5 mL of 40% glucose solution, 10 mL of fresh 25% yeast extract solution, 1.5 mL of 0.5% phenolic red solution, 1 mL of penicillin solution (200,000 units/mL) and 0.04 mL of 10% thallium acetate solution were added to 100 mL of the medium. The pH of the finished broth was adjusted to 7.8 by adding 1.0 M NaOH solution.

To prepare a solid nutrient medium, 6.7 g of Bacto™ Agar (BD, USA) was added to the modified Hayflick broth. After autoclaving, the semi-product was cooled to a temperature of (50 ± 2) °C, and medium supplements were

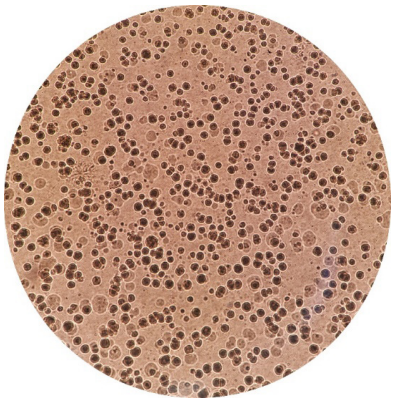


Fig. 1. 3-day-old M. bovis culture grown in solid nutrient medium (magnification 20×)

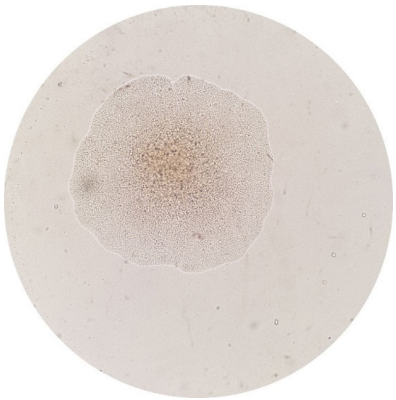


Fig. 2. 4-day-old M. bovis culture grown in solid nutrient medium (magnification 40×)

added according to the above-specified procedure. The prepared nutrient medium was poured into Petri dishes, cooled until completely solidified and stored at a temperature of (4 ± 2) °C.

Determination of M. bovis "Kaluga 2020" isolate activity. To determine the mycoplasma activity, the CCU assay and culture method with CFU count were used. A series of 10-fold successive dilutions of the suspension containing *M. bovis* "Kaluga 2020" isolate (10^{-1}–10^{-5}) were prepared in Hanks' saline solution. 10 µL of microbial suspension of each dilution were inoculated in solid nutrient medium in three duplicates. The inoculations were cultivated in a thermostat at a temperature of (37 ± 0.5) °C in 5% CO_2-enriched atmosphere for 9 days. Reading of titration results was conducted by counting single colonies and calculating the average CFU number in 10 µL of the highest suspension dilution, in which the growth of *M. bovis* colonies was observed. The obtained value was used to calculate the CFU number in 1 mL of the initial suspension of the test material.

The activity of *M. bovis* "Kaluga 2020" isolate was evaluated in 96-well culture plates using the color-changing units [17, 18] method. 20 µL of initial *M. bovis* suspension were mixed with 180 µL of modified Hayflick broth with phenolic red in the first wells of the plate, then serial 10-fold dilutions of the test suspension were prepared (10^{-1}–10^{-10}). Wells without *M. bovis* were used as a nutrient medium control. The plates were incubated at a temperature of (37 ± 0.5) °C in 5% CO_2-enriched atmosphere for 14 days. Accumulation of *M. bovis* metabolic products results in a pH shift to acidity, which causes a color change of the indicator from red to yellow. The color change of the nutrient medium and *M. bovis* activity in the culture suspension were recorded every 24 hours.

The concentration (titer) was determined in CCU/mL as the maximum dilution of *M. bovis*-containing suspension, in which a color change was observed [19].

The pH of the medium was monitored using a pH meter according to the operating instructions of the device.

Statistical analysis. The Minitab/Statistics program (version 19.1, USA) was used for experimental data analysis. The results obtained were found to be reliable ($p < 0.05$).

Table 1
Correlation between *M. bovis* biological activity and CCU value

Duration of cultivation, h	Medium color change	Mycoplasma biological activity		pH
		lg CFU/mL	lg CCU/mL	
24	from red to red-orange	7.0	5.0	7.4
72	from red-orange to orange	8.9	10.0	7.2
96	from orange to orange-yellow	8.0	9.0	7.1
168	from orange-yellow to yellow	6.4	4.0	6.8

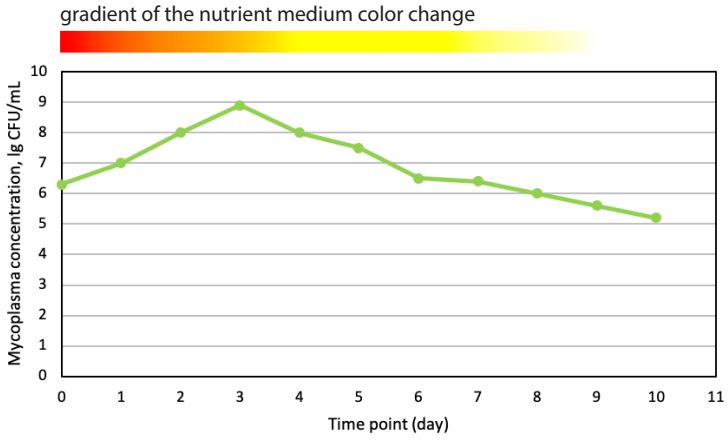


Fig. 3. Growth phases and accumulation of M. bovis "Kaluga 2020" isolate when cultivated in modified Hayflick broth

TEST RESULTS

During cultivation of *M. bovis* on solid modified Hayflick medium the colonies with irregular edges and a knobby protuberance resembling a fried egg were observed (Fig. 1, 2).

Determination of growth phases and maximum accumulation time of M. bovis "Kaluga 2020" isolate when cultivated in modified Hayflick broth. *M. bovis* "Kaluga 2020" isolate was cultivated at a temperature of (37 ± 0.5) °C for 10 days, while samples of culture suspension were taken every 24 hours and the *M. bovis* activity was estimated by titration methods using CFU count (culture method) and color-changing units assay. The study results showed that the "Kaluga 2020" isolate culture entered the logarithmic growth phase after 24 hours of cultivation and the maximum level of mycoplasma biomass

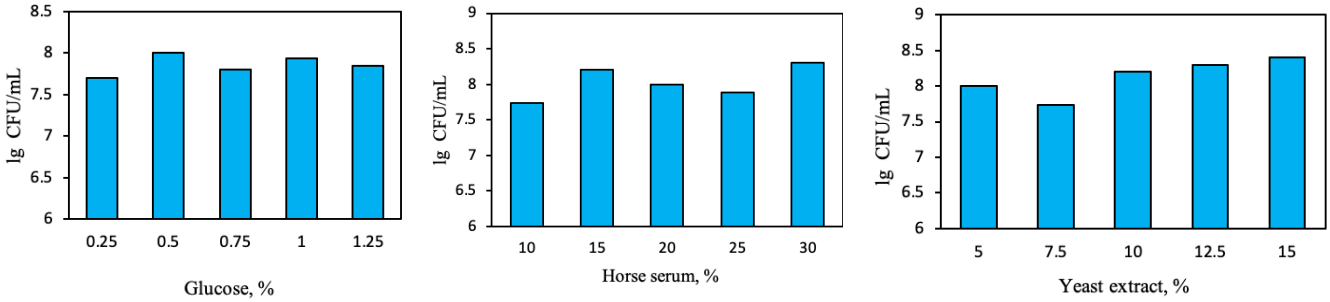


Fig. 4. Effect of various components of nutrient medium on accumulation of M. bovis "Kaluga 2020" isolate

accumulation was recorded in 72 hours, after which a decline phase occurred with a decrease in the biological activity of the cultivated material (Fig. 3).

Correlation of biological activity values of mycoplasmas identified by color-changing units assay and cultural method. Analysis of the obtained results showed that the maximum isolate accumulation was observed in 72 hours of cultivation (8.9 lg CFU/mL). The indicated mycoplasma concentration correlated with the change in the color of the nutrient medium (10 lg CCU/mL), which corresponded to a pH of 7.2. With longer cultivation, the biological activity of the cultured material was recorded to decrease to 6.4 lg CFU/mL, which was comparable to the concentration of microorganisms equal to 4 lg CCU/mL (Table 1).

Determination of the optimal nutrient medium composition for M. bovis "Kaluga 2020" isolate cultivation. At the first stage of the work, the modified Hayflick medium components that most significantly affected the accumulation of *M. bovis* "Kaluga 2020" isolate were determined. The studies were carried out by modifying the standard composition of the Hayflick medium with respect to the three components: glucose, fresh yeast extract and horse serum. At the same time, the percentage quantity of only one of the three components was changed in each series of experiments, while the standard parameters of other growth factors remained the same.

To determine the effect of glucose on *M. bovis* growth, a nutrient medium containing this component at 0.25; 0.50; 0.75; 1.00; 1.25% was used, fresh yeast extract – at 5.0; 7.5; 10.0; 12.5; 15.0%, horse serum – at 10, 15, 20, 25, 30%. Changes in the biological activity of mycoplasmas were monitored by CFU count using titration method.

The obtained results showed that the content of two growth factors in the nutrient medium is of the highest importance for *M. bovis* accumulation: fresh yeast extract and horse serum, while glucose does not have a significant effect (Fig. 4).

After identification of the most significant growth factors of the nutrient medium, it was necessary to determine their optimal ratio. For this purpose, testing of 25 experimental nutrient media with different amounts of these components was conducted. *M. bovis* accumulation was determined by titration and expressed in lg CFU/mL.

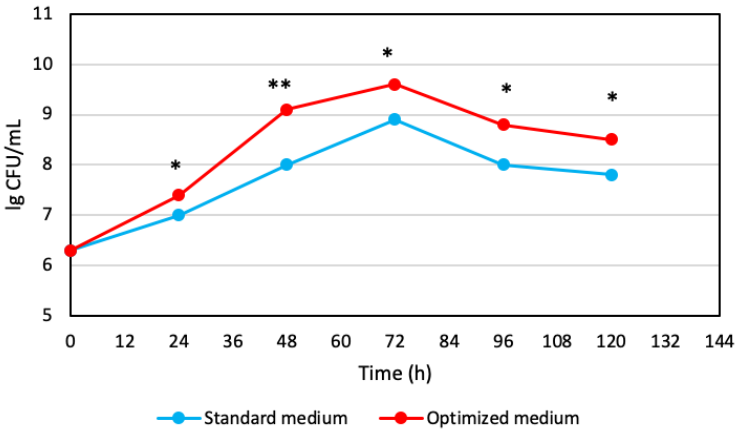
According to Table 2, the *M. bovis* maximum activity (9.60 lg CFU/mL) is observed when cultivated in a nutrient medium containing 12.5% fresh yeast extract and 25% horse serum.

The obtained results are confirmed by the data on changes in the biological activity of *M. bovis* "Kaluga 2020" isolate when cultured in a standard and optimized Hayflick nutrient medium (Fig. 5).

Table 2
Effect of nutrient medium composition on accumulation of *M. bovis* "Kaluga 2020" isolate

Experiment number	Contents of the studied components		lg CFU/mL
	fresh yeast extract, %	horse serum, %	
1	5.0	10	7.80
2	7.5	10	7.90
3	10.0	10	8.00
4	12.5	10	8.20
5	15.0	10	7.90
6	5.0	15	7.70
7	7.5	15	7.90
8	10.0	15	8.00
9	12.5	15	8.20
10	15.0	15	7.80
11	5.0	20	7.70
12	7.5	20	7.90
13	10.0	20	8.10
14	12.5	20	8.00
15	15.0	20	7.80
16	5.0	25	8.10
17	7.5	25	7.90
18	10.0	25	8.00
19	**12.5**	**25**	**9.60**
20	15.0	25	7.79
21	5.0	30	7.50
22	7.5	30	7.85
23	10.0	30	8.00
24	12.5	30	7.80
25	15.0	30	7.60

When cultured in an optimized medium the biological activity of *M. bovis* was on average 3.98×10^9 CFU/mL, which is 5 times higher than that of *M. bovis* when cultured in a standard Hayflick nutrient medium (0.79×10^9 CFU/mL).



** p < 0.05; ** p < 0.001*

Fig. 5. Activity of M. bovis "Kaluga 2020" isolate when cultivated in standard and optimized Hayflick medium

DISCUSSION

Based on the results of the conducted studies, it can be concluded that there is a correlation between the growth phase, the accumulation of mycoplasmas, the CFU indicator and the nutrient medium color (CCU parameter).

The transition from red to orange corresponded to the phase of exponential growth of mycoplasmas, and when the growth peak was reached, a change in the color of the medium from red-orange to orange was observed. During further cultivation the medium colour changed successively to orange-yellow and yellow, which corresponded to the decline phase as a result of cell lysis and intracellular ATP depletion. Similar results are confirmed by the foreign colleagues' data [15, 20].

Mycoplasma cultivation is considered a laborious technique [18], and the search for optimal media for obtaining high-quality biological material of these microorganisms, as well as for production of specific means of mycoplasmosis immunoprophylaxis is of urgent importance [21].

It was established that glucose had a minor effect on *M. bovis* growth and accumulation, which is consistent with the literature data [22, 23].

The use of optimized nutrient medium based on modified Hayflick broth containing 12.5% fresh yeast extract and 25% horse serum showed a 5-fold increase in accumulation of *M. bovis* "Kaluga 2020" isolate (3.98×10^9 CFU/mL) as compared to the standard medium (0.79×10^9 CFU/mL). The results obtained in similar studies on optimization of the nutrient medium composition for *M. hyopneumoniae* cultivation indicate only a 3-fold increase in mycoplasma accumulation as compared to the standard medium [13, 24].

Studying *M. bovis* growth, as well as determining the log phase, can play an important role in future investigations on isolation of this species mycoplasmas, cultivation and vaccine development.

CONCLUSION

The study results showed that after first 24 hours of cultivation in a liquid medium *M. bovis* "Kaluga 2020" isolate entered the phase of logarithmic growth and reached the maximum accumulation level in 72 hours. A decline phase occurred and the biological activity of the resulting material decreased with longer cultivation in 84 hours.

It was found that the growth factors – fresh yeast extract and horse serum – had the greatest effect on accumulation of *M. bovis* "Kaluga 2020" isolate when cultivated in Hayflick medium. The optimal content of these components in the culture medium was selected – 12.5 and 25%, respectively. An optimized nutrient medium based on modified Hayflick broth can be used to obtain bacterial material for the development of diagnostic products and means of specific prevention of *M. bovis*-induced diseases.

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Long-term storage of C-141 reference strain of melioidosis agent (*Burkholderia pseudomallei*)

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SUMMARY

Melioidosis is a highly dangerous infectious disease caused by Hazard Group II bacteria *Burkholderia pseudomallei*, against which specific prevention and treatment tools have not been developed yet. Both humans and animals suffer from the disease. Previously the disease was prevalent in Southeast Asia regions, but currently is reported almost in all continents of the globe. Potential possibility of the agent introduction to the Russian Federation as well as the risk of malevolent use of this agent as a tool of bioterrorism dictates the need for storage of this pathogen in the microorganism collections to study its properties, develop and test diagnostic, detection and identification means. Microorganism Collection Laboratory of the FSBSI "FCTRBS-ARRVI" is responsible for storage and preservation of *Burkholderia pseudomallei* C-141 reference strain, submitted by Federal State Scientific Institution "Russian Research Anti-Plague Institute "Microbe" under the Rospotrebnadzor (Saratov city) for research purposes in 1983 and as a back-up strain in case of its loss by other collections. The purpose of the work was to study the preservation of biological properties of freeze-dried *Burkholderia pseudomallei* C-141 strain after 11 years of storage. It was established that under optimal storage conditions (temperature of 4–8 °C, skimmed milk as a cryoprotectant) the strain remained viable and retained its biological properties during the whole observation period. C-141 strain showed saccharolytic, oxidase, catalase and proteolytic activities, did not generate hydrogen sulphide, which is consistent with the melioidosis agent biochemical features. The strain was refreshed by passaging in golden hamsters and Burkholderia culture was isolated and freeze-dried. *Burkholderia pseudomallei* C-141 freeze-dried strain was tested for quality parameters, records were made and the strain was deposited.

Keywords: particularly dangerous diseases, melioidosis, strain, storage, passage, lyofilyzation

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УДК 619:616.98:579.8

Опыт длительного хранения референтного штамма С-141 возбудителя мелиоидоза (*Burkholderia pseudomallei*)

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РЕЗЮМЕ

Мелиоидоз – особо опасное инфекционное заболевание, вызываемое бактериями *Burkholderia pseudomallei*, относящимися к II группе патогенности, против которого не разработаны специфические средства профилактики и лечения. Мелиоидозом болеют как люди, так и животные. Ранее заболевание было распространено в районах Юго-Восточной Азии, в настоящее время регистрируется почти на всех континентах земного шара. Потенциальная возможность завоза возбудителя мелиоидоза на территорию Российской Федерации, а также опасность преднамеренного применения его в качестве средства биологического терроризма диктует необходимость содержания данного патогена в коллекциях микроорганизмов для проведения исследований по изучению его основных свойств, разработке и испытанию средств диагностики, индикации и идентификации. Лаборатория коллекции штаммов

микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ» осуществляет хранение и поддержание референтного штамма С-141 *Burkholderia pseudomallei*, полученного из ФКУН Российский научно-исследовательский противочумный институт «Микроб» Роспотребнадзора (г. Саратов) в 1983 г., для проведения научно-исследовательских работ, а также в качестве дублирующего штамма в случае утраты его в других коллекциях. Целью работы являлось изучение сохранности биологических свойств штамма С-141 *Burkholderia pseudomallei* после 11 лет хранения в лиофилизированном виде. Установлено, что при оптимальных условиях хранения (температура от 4 до 8 °С, криопротектор – обезжиренное молоко) штамм сохранял свою жизнеспособность и биологические свойства в течение всего срока наблюдения. Штамм С-141 обладал сахаролитической, оксидазной, каталазной и протеолитической активностью, сероводород не образовывал, что соответствует биохимическим признакам возбудителя мелиоидоза. Проведено освежение штамма путем пассажа через организм золотистых хомячков, выделена культура буркхольдерий, которая была лиофилизирована. Лиофилизированный штамм С-141 *Burkholderia pseudomallei* был проверен по показателям качества, на него оформлен паспорт, штамм заложен на хранение.

Ключевые слова: особо опасные болезни, мелиоидоз, штамм, хранение, пассаж, лиофилизация

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Для цитирования: Артемьева Е. А., Мельникова Л. А., Родионов А. П. Опыт длительного хранения референтного штамма С-141 возбудителя мелиоидоза (*Burkholderia pseudomallei*). *Ветеринария сегодня*. 2022; 11 (3): 268–272. DOI: 10.29326/2304-196X-2022-11-3-268-272.

Конфликт интересов: Авторы заявляют об отсутствии конфликта финансовых/нефинансовых интересов, связанных с написанием статьи.

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INTRODUCTION

The strains of microorganisms are stored and maintained in public and national collections existing on the premises of research institutions. The collections include from hundreds to thousands or more of storage units. They are formed by the microbial strains submitted by biosecurity organizations, veterinary, medical, phytosanitary and other institutions, conducting microbiological tests [1, 2]. The main task of collections of microorganisms is to maintain the cultures in conditions that exclude their loss, change or degradation of morphological, biochemical, serological and toxic properties, as well as sensitivity to antibiotics during long-term storage [3, 4]. The collections include back-up, archival, field, reference and production strains and are the national heritage of the country, which ensure its biological and food security [5]. The microbial strains stored in the collections are needed for fundamental and applied scientific research, the development of therapeutic, diagnostic and preventive drugs, as well as for the development of modern test-kits, immunobiological products and therapeutic drugs against infectious diseases reported on the territory of the Russian Federation (anthrax, brucellosis, etc.), as well as against diseases, posing a potential risk of being introduced into the country from infected regions [6]. One of such infections is the highly dangerous disease melioidosis, which is endemic throughout Southeast Asia region [7]. In this regard, it is impossible to exclude the threat of introduction of this zoonosis into the territory of our country, as well as the risk of acts of sabotage using *Burkholderia pseudomallei* as an agent of biological terrorism. This creates the need to store strains of this microorganism in collections, to maintain and study the biological properties of the pathogen [8]. The laboratory of the microorganism strain collection of the FSBSI "FCTRBS-ARRVI" is responsible for maintaining the collection of *B. pseudomallei* strains, being a potentially dangerous biological agent.

The purpose of this study was to study the preservation of the biological properties of freeze-dried *B. pseudomallei* strain C-141 after 11 years of storage.

MATERIALS AND METHODS

Biological safety. The work was conducted in the Microorganism Collection Laboratory of the FSBSI "FCTRBS-ARRVI" in accordance with SanPiN 3.3686-21[1].

Strains. Freeze-dried *B. pseudomallei* reference strain C-141 (isolated from the patient's blood in 1948 in Saigon), received in accordance with the established procedure from the FSSI "Russian Research Anti-Plague Institute "Microbe" under the Rospotrebnadzor (Saratov city) in 1983 was used in this work.

Nutrient media. To revive the freeze-dried culture of the strain under study and to study the preservation of its morphological properties, meat peptone glycerol agar (MPGA) and meat peptone glycerol broth (MPGB) were used.

To detect the formation of hydrogen sulfide, MPGB with 4% glycerol was used; MPGA with 4% glycerol was used to study catalase activity. The above-mentioned nutrient media are produced by the FSBSI "FCTRBS-ARRVI".

Biochemical properties were analyzed using Hiss' media (NPO Microgen, Russia).

Laboratory animals. Five golden hamsters were used to revive the strain. All animal experiments were conducted in strict accordance with the interstate standards for

[1] SanPiN 3.3686-21 Sanitary and epidemiological requirements to prevent infectious diseases: approved by Resolution of the Chief Medical Officer of the Russian Federation No. 4 dated 28.01.2121. Available at: http://vnipchi.rospotrebnadzor.ru/s/203/files/ND/safety/95493_64.pdf.

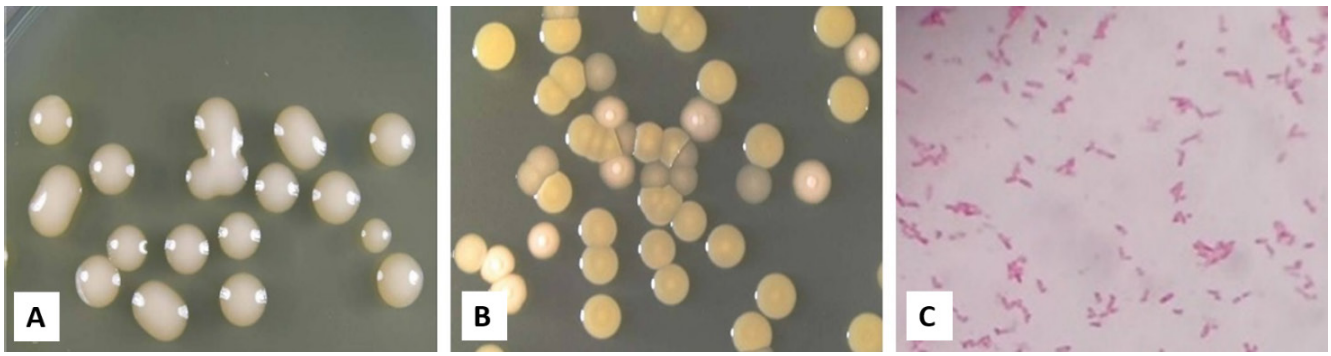

Fig. Cultural and morphological properties of B. pseudomallei C-141 strain: A – day-old culture, grown on meat peptone agar; B – dissociated culture in 48–72 hours of incubation; C – Gram stained smear of the tested culture

laboratory animal handling adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, 18.03.1986).

Equipment. The culture was freeze-dried using LZ-9.2 freeze-dryer (Frigera, Czech Republic).

Test methods. After the viability of the strain was established and second generation culture was obtained by passaging, its biological properties were studied for compliance with specifications according to MU 4.2.2787-10 "Laboratory Diagnostics of Melioidosis" [9]. To study the cultural characteristics, the strain was inoculated on MPGA and MPGB. Tinctorial and morphological properties were determined by microscopy of the preparations based on a 2-day culture fixed in a Nikiforov mixture and stained according to Gram. The mobility of bacterial cells was established by microscopic examination of native preparations made by the hanging drop technique. The formation of saccharolytic enzymes was determined using Hiss' media. The proteolytic properties were tested by seeding the culture using inoculating loop in 12% gelatin, and then 2–3 drops of culture in saline solution were added to skimmed milk. The results were recorded during 3 days. The formation of hydrogen sulfide was determined in MPGB using an indicator paper soaked in lead acetate and inserted into a test tube. The oxidase activity was analyzed by applying a 1% solution of hydrogen peroxide to the surface of a culture grown on MPGA. The final reading of the results was performed on 7–10th day.

After testing the basic properties of *B. pseudomallei* C-141 strain, it was passaged in golden hamsters by subcutaneous injection of a 2-day agar culture suspension. The animals were monitored for 5–6 days. The dead animals were autopsied and samples from liver, spleen, lungs, injection site, heart blood were inoculated onto MPGA and MPGB, which were cultivated at 37 °C for 3–4 days [10].

A pure culture of the *B. pseudomallei* C-141 strain was lyophilized using skimmed milk as a cryoprotector. The freeze-drying conditions were designed for this type of pathogen.

The lyophilized culture was tested for its viability, its basic properties were studied using the data obtained, a data sheet of the strain was issued, reports were filled out, and then ampoules with the strain were deposited.

RESULTS AND DISCUSSION

Replenishment of the laboratory's collection of microbial strains of the FSBSI "FCTRBS-ARRVI" with the *B. pseudomallei* C-141 strain was dictated by the need to develop methods for differential diagnosis of glanders and melioidosis, since the causative agents of these particularly dangerous infectious diseases are antigenically closely related. In addition, this strain is deposited in the collection as a back-up strain in case it is lost in other collections. For 38 years, the strain has been stored in native state on MPGA (working cultures) and in lyophilized form and has been periodically subjected to testing for its viability and biological properties. The culture of the strain stored in its native state is re-inoculated onto MPGA every 3 months and tested for its basic properties. Lyophilized cultures are tested once every 5 years.

Currently, the storage of the *B. pseudomallei* C-141 strain in the collection and the relevant works are dictated by the ongoing globalization in all spheres of human activity, the development of tourism, especially in tropical climate areas, international relations in the field of trade, sports, etc., creating a risk of introducing exotic infections on the territory of our country, including melioidosis. Being a biological agent, *B. pseudomallei* creates a potential threat of its deliberate use as a means of biological terrorism. Melioidosis was included in the list of socially significant hazardous diseases by Decree of the Government of the Russian Federation No. 715 of December 1, 2004 concerning approval of the list of socially significant diseases and the list of hazardous diseases[2].

At the first stage, the cultural properties of the *B. pseudomallei* C-141 strain stored for 11 years in freeze-dried form, were studied. After 24 hours of cultivation at a temperature of 37 °C, small, translucent, convex colonies of grayish color with smooth edges and a smooth surface grew on the MPGA (Fig. A). After 48–72 hours of incubation, signs of culture dissociation appeared: some colonies were transparent, others had a folded surface (Fig. B). Growth manifested by light turbidity was recorded on the MPGB after a day; in the following days the turbidity increased, a precipitate and a surface film formed, which after 4–5 days became folded, changing its color from gray-yellow to brown.

[2] Concerning the approval of the list of socially significant diseases and the list of hazardous diseases: approved by Decree of the Government of the Russian Federation No. 715, dated 01.12.2004. Available at: https://docs.cntd.ru/document/901916651.

Microscopy of smears prepared from a 2-day old agar culture of the strain, fixed in the Nikiforov mixture and Gram stained demonstrated small, gram-negative cells located singly, in pairs and in short chains with characteristic bipolar staining (Fig. C). When viewed under a microscope of the "hanging drop" preparation made from *Burkholderia* culture, the rectilinear movement of bacteria was recorded.

Testing of the biochemical properties of the *B. pseudomallei* C-141 strain showed that it had a saccharolytic activity: it oxidized glucose and changed the color of the indicator and the nutrient medium. The inoculations on MPGB did not generate hydrogen sulfide: the indicator paper impregnated with lead acetic acid did not turn black. The strain had oxidase, catalase (when 1 mL of 1% hydrogen peroxide solution was applied to the *Burkholderia* culture grown on MPGA, gas bubbles appeared) and proteolytic (it coagulated and peptonized milk, liquefied 12% gelatin) activity (Table).

The results of the test showed that the biochemical properties of the C-141 strain are consistent with the species characteristics of *B. pseudomallei* provided for by the Bergey's manual [11]. This fact suggests that the strain optimal storage conditions and the work carried out for 38 years contributed to the preservation of its viability without loss of its biological properties.

To maintain the basic properties of strains during their long-term storage, it is necessary to revive them in susceptible animal models [12]. For melioidosis agent, such models are golden hamsters, white mice and guinea pigs. Golden hamsters were used in this work. The disease in these animals proceeds in an acute form, death occurs within 3–5 days, depending on the strain and the dose, besides these animals are convenient for maintenance and care. *B. pseudomallei* C-141 strain was passaged by subcutaneous injection of live culture to golden hamsters at a dose of 10^9 live microbial cells per 1 cm^3 suspended in saline solution. Then the hamsters were monitored. Depression, lethargy, anorexia, death on day 4–6 were registered in animals. During the autopsy, necrotic nodules, 2–3 mm in diameter, were found in the liver, spleen and lungs. Bacteriological testing of samples from internal organs and heart blood revealed a culture with *B. pseudomallei*-consistent biological properties.

The primary task of microbial collections is to preserve the strains in an unchanged state for a long time. For this purpose freeze-drying is most often used [13]. The isolated culture of the *B. pseudomallei* C-141 strain was lyophilized to preserve the basic properties for a long time. Skimmed milk was used as a cryoprotector, which is effectively used for freeze-drying of glander pathogen strains [14].

Lyophilization was carried out according to the previously optimized conditions:

1. Freezing of the material for 18 hours to minus 40 °C in the freezer.
2. Transfer of the material to the freeze-dryer, cooling the plate to minus 52 °C.
3. Vacuumization.
4. Lyophilization in automatic mode for 12 hours.
5. Turning on heating (p) after 17 hours from loading of the material with the following parameters: plate temperature 10 °C, medium temperature 0 °C.

Table
C-141 *B. pseudomallei* biochemical properties

No.	Parameter	of tested strain	in Bergey's manual
1	Glucose oxidation	+	+
2	Hydrogen sulfide formation	–	–
3	Oxidase activity	+	+
4	Milk coagulation	+	+
5	12% gelatin liquefaction	+	+

6. Turning on heating (p + 1) after 18 hours with the following parameters: plate temperature 20 °C, medium temperature 5 °C, vacuum 0.5 trr.
7. Next 24 hours: plate temperature 32 °C, medium temperature 25 °C, vacuum 0.05 trr.

The freeze-drying process was completed at a relative humidity of the material in the range from 2 to 3.5% [15]. Testing of the *B. pseudomallei* C-141 strain after lyophilization showed its viability and preserved basic properties.

After refreshing the strain by passaging in susceptible animal models, lyophilization and quality control in accordance with SanPiN 3.3686-21, a data sheet was issued and corresponding entries were made in the registration form No. 517/u "Card of individual registration of a collection pathogenic biological agent", after which the strain was deposited.

CONCLUSION

The results of the study showed that under optimal storage conditions (temperature from 4 to 8 °C, skimmed milk as a cryoprotector), the *B. pseudomallei* lyophilized C-141 strain retained its viability and biological properties for 11 years of storage (observation period). The strain had saccharolytic, oxidase, catalase and proteolytic activity, did not generate hydrogen sulfide, which is consistent with biochemical features of the *B. pseudomallei* species.

Refreshing of the strain by passaging in susceptible animal models (golden hamsters) and preservation by lyophilization will ensure its maintenance in a viable state and preservation of its basic properties for a long period of time.

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Isolation and study of biological properties of *Bordetella bronchiseptica*-specific bacteriophages

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SUMMARY

The paper presents results of the studies on isolation of bacteriophages active against *Bordetella bronchiseptica*. Three new bacteriophages were recovered from clinical samples from animals with respiratory signs:*vB_BbrS_2/200220.7.2*, *vB_BbrS_4/200220.7.1*, *vB_BbrM_5/200220.7.2*. The bacteriophage isolation method and basic biological properties thereof have been specified in detail. The lytic activity of isolated bacteriophages was determined by the agar layer method varied from $(2.3 \pm 1.4) \times 10^8$ to $(9.0 \pm 0.2) \times 10^8$ PFU/mL, and the lytic spectrum ranged from 61.5 to 76.9%. The bacteriophage titer stability was shown during 8-month storage of phage lysate with no preservative added. The morphology of bacteriophage plaques was tested in various nutrient media and analyzed based on two parameters: size and transparency. Dissociation of plaques into clear colonies, turbid colonies, and clear colonies with turbid halos was observed in the media. Plaques also were varied in size from 0.6 ± 0.2 to 2.6 ± 0.1 mm. Great thermal stability was noted during exposure of bacteriophages to high temperatures ranging from +40 to +95 °C with 5 °C increment. The specificity study showed that the isolated bacteriophages lyse closely-related bacteria. The electron microscopy of each bacteriophage revealed such parameters as the average diameter of the head and the average length of the tail. In accordance with the international classification of viruses by morphological characteristics the *vB_BbrS_2/200220.7.2* and *vB_BbrS_4/200220.7.1* phages have been assigned to the family *Siphoviridae*, *vB_BbrM_5/200220.7.2* bacteriophage has been assigned to the family *Myoviridae*. The obtained results of *in vitro* studies have shown that the isolated bacteriophages can be promising for phage therapy of *Bordetella bronchiseptica*-induced diseases in veterinary medicine.

Keywords: bacteriophages, *Bordetella bronchiseptica*, phage therapy, phage prophylaxis

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Выделение и изучение биологических свойств бактериофагов, специфичных к *Bordetella bronchiseptica*

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РЕЗЮМЕ

Представлены результаты собственных исследований по выделению бактериофагов, активных в отношении *Bordetella bronchiseptica*. Из клинического материала от животных с признаками респираторных заболеваний выделено три новых бактериофага: *vB_BbrS_2/200220.7.2*, *vB_BbrS_4/200220.7.1*, *vB_BbrM_5/200220.7.2*. Подробно описана методика выделения бактериофагов и их основные биологические свойства. Литическая активность выделенных бактериофагов, определяемая методом агаровых слоев, варьировала от $(2,3 \pm 1,4) \times 10^8$ до $(9,0 \pm 0,2) \times 10^8$ БОЕ/мл, а спектр литического действия составил от 61,5 до 76,9%. Показана стабильность титра бактериофагов при хранении фаголизата в течение 8 месяцев без добавления консерванта. Морфологию негативных колоний бактериофагов изучали на различных питательных средах и анализировали по двум признакам: размер и прозрачность. На средах наблюдалась диссоциация негативных колоний на прозрачные, мутные, и прозрачные с мутными ореолами. Бляшки также

различались по размеру: от 0,6 ± 0,2 до 2,6 ± 0,1 мм. Отмечены высокие показатели температурной устойчивости при воздействии на бактериофаги высокой температуры от +40 до +95 °С с шаговым интервалом 5 °С. Изучение специфичности показало, что выделенные бактериофаги лизируют близкородственные бактерии. В ходе электронно-микроскопических исследований для каждого бактериофага были определены такие параметры, как среднее значение диаметра головки и среднее значение длины хвоста. В соответствии с международной номенклатурой вирусов по морфологическим параметрам фаги *vB_BbrS_2/200220.7.2* и *vB_BbrS_4/200220.7.1* отнесены к семейству *Siphoviridae*, бактериофаг *vB_BbrM_5/200220.7.2* – к семейству *Myoviridae*. Полученные результаты исследований *in vitro* показали, что выделенные бактериофаги могут быть перспективными для применения в ветеринарной медицине в фаготерапии заболеваний, вызванных бактерией *Bordetella bronchiseptica*.

Ключевые слова: бактериофаги, *Bordetella bronchiseptica*, фаготерапия, фагопрофилактика

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INTRODUCTION

Bordetella bronchiseptica is an aerobic gram-negative motile bacterium. It causes respiratory diseases in most domestic animals (pigs, rabbits, cats, dogs) and wild animals of all ages, however, young individuals under one year of age and animals with chronic diseases are at risk of infection most of all [1–3]. The bacteria multiply in the epithelial cilia of the respiratory tract; infection is airborne, the incubation period lasts from 5 to 20 days [2, 4].

Respiratory infection (bordetellosis) caused by *B. bronchiseptica* develops quite quickly, causing infectious tracheobronchitis in dogs, commonly known as "kennel cough", pneumonia, rhinitis in cats and rabbits [3, 5, 6]. In some cases, the disease can actively progress to bronchopneumonia and result in death [1, 7, 8]. *B. bronchiseptica* is the cause of atrophic rhinitis and bronchopneumonia in pigs. Atrophy of the nasal septum and nasal conch can occur [3, 4, 9, 10]. It has been reported cases of human infections with *B. bronchiseptica* bacteria [2, 3, 5, 6, 11–14]. The disease mainly affects children, people with chronic diseases and weakened immunity. People get infected by direct contacts with sick animals, the possibility of cross-infection between humans is not excluded [5, 6, 11–14].

To date, the main means of bordetellosis therapy in pets and livestock are antibiotics, but their effectiveness is reduced due to the spread of *B. bronchiseptica* antimicrobial resistant strains [15, 16]. This entails an active increase in morbidity among animals, a reduced productivity of farm animals, and, therefore, economic losses in animal production. There is a direct threat to human health, since there is a risk of infection with antimicrobial-resistant strains of *B. bronchiseptica* from carrier animals.

The development of new tools and methods of prevention and treatment of bordetellosis is an urgent task in veterinary medicine. One of the promising and safe solutions to this issue is the use of products based on bacteriophages. The prospects for the use of phages active against *B. bronchiseptica* are conditioned by their safety, and the lack of a negative impact on the normal flora of the body [4, 17].

Bacteriophages can be used not only as an alternative to antibiotics, but also in combination with all types of traditional antimicrobial therapy [17]. In a study conducted by G. Y. Park et al. [10], it was found that bacteriophages have therapeutic potential against respiratory diseases caused by *B. bronchiseptica* and can participate in the suppression of bacterial inflammation. However, the mechanism of this process remains under-studied.

The purpose of this study was to isolate bacteriophages active against *B. bronchiseptica*, to study their biological properties for further evaluation of potential practical application in veterinary medicine.

MATERIALS AND METHODS

Strains of *B. bronchiseptica* bacteria were isolated from clinical material from rabbits, dogs and cats (mucosal swabs, faecal samples, water from drinkers). Samples were submitted from animal shelters, veterinary clinics and veterinary institutes of Moscow and the Moscow Region. The *B. bronchiseptica* pathogenic isolates were selected based on the results of identification of microorganisms by microscopic, biochemical and mass spectrometric methods to create a working collection of strains of RPC "MikroMir". The strains were certified and deposited into the collection of microorganisms of RPC "MikroMir". All bacterial strains under study were preliminarily tested for the absence of profages in the culture by S. Luria and D. Darnell method [18], as well as using induction by ultraviolet radiation [19].

B. bronchiseptica bacteria were cultured at a temperature of (37 ± 0.5) °C for 24 hours on BHI agar (HiMedia Laboratories Pvt. Limited, India) with 5% sterile defibrinated sheep blood added.

The isolation of bacteriophages and the study of their biological properties were carried out by methods proposed by M. Adams [20] and D. M. Goldfarb [21]. Bacteriophages were isolated from samples of biomaterial of animals from which strains of *B. bronchiseptica* had been previously isolated. The samples were resuspended in 20 mL of isotonic saline solution. Large particles and bacteria were removed from the resulting suspension by low-speed centrifugation (5,000 rpm, 20 min) using Avanti J-E centrifuge (Beckman Coulter, Inc., USA) [22]. Pathological materials of the liquid phase were centrifuged without preliminary resuspending at the same parameters.

The supernatant was separated from the precipitate and centrifuged on an Optima L-90K ultracentrifuge (Beckman Coulter, Inc., USA) at a high speed (27,000 rpm, for 120 min). The precipitate was resuspended in 0.05 M Tris-HCl buffer (pH 7.0–7.2) and filtered through membrane filters (pore sizes 1.2; 0.45; 0.22 microns) of Sartorius, Germany. The presence of phages in the filtrate was detected by the Gratia method [20, 21]. The detection of various types of plaques on the bacterial lawn of the test culture suggested the presence of several types of phages in the tested material [22]. Pure bacteriophage lines were obtained from morphologically homogeneous plaques. For this purpose, 0.1 mL of an 18-hour culture of the test strain was inoculated into flasks with 20 mL of BHI broth and incubated in a growth chamber (Binder, Germany) at 37 °C. A fragment of an agar plate with a single phage plaque was introduced into the log-phase culture. A flask without a plaque fragment served as a control. The contents of the flasks were cultured in a growth chamber (Binder, Germany) at 37 °C for 24 hours, after this, clearing was observed in the flasks with the test strain, and pronounced turbidity of the medium was observed in the control flask. Then the contents of the flasks were centrifuged for 20 min at 5,000 rpm on an "Avanti J-E" centrifuge (Beckman Coulter, Inc., USA) [22]. The collected supernatant was successively filtered through different membranes (pore size 1.2; 0.45; 0.22 microns). The obtained phagolysate was tested again by the agar layer method [20, 21]. The procedure was repeated until homogeneous plaques were obtained.

To obtain a sufficient amount of phagolysate with a consistently high titer, bacteriophages were cultured according to the following method: 0.3 mL of a phage-sensitive culture of *B. bronchiseptica* was added to flasks with 50 mL of BHI broth. The suspension was incubated in an orbital shaker-incubator (BioSan ES-20/60, Latvia) at 140 rpm at 37 °C for 2.5 hours. Then 3.0 mL of a pure bacteriophage line was introduced into the flasks and cultured at 37 °C for 18–24 hours at 140 rpm. After this time, the contents of the flasks were centrifuged at a low speed for 20 min at 5,000 rpm, then the phage particles were re-precipitated at 27,000 rpm for 120 min on an "Optima L-90K" centrifuge (Beckman Coulter, Inc., USA). The precipitate was resuspended in 0.05 M Tris-HCl buffer (pH 7.0–7.2) and filtered through membrane filters (pore sizes 1.2; 0.45; 0.22 microns). To determine the titer, the phagolysate was titrated according to generally accepted methods on dense nutrient media (Gratia method) [20, 21], after which the filtrate was placed in sterile test tubes for storage at 4 °C.

The lytic activity of the isolated phages was determined by the Gratia method [20, 21].

The spectrum of lytic activity was studied on 13 test cultures of *B. bronchiseptica* by spot testing [22].

The bacteriophage plaque morphology was studied on various dense nutrient media: 1.5% BHI-agar (HiMedia Laboratories Pvt. Limited, India) with the addition of 5% sterile defibrinated sheep blood, 1.5% BHI-agar (HiMedia Laboratories Pvt. Limited, India), 1.5% FPH agar (FBSI SSC PMB, Russia), 1.5% Bordetelagar (FBSI SSC PMB, Russia) [23]. 1.0 mL of dilutions of the titrated bacteriophage and 0.1 mL of bacterial suspension were added to tubes with 0.8 or 0.4% BHI-agar (HiMedia Laboratories Pvt. Limited, India), then were inoculated on pre-prepared plates with solid agar (1.5%). The morphology of plaques was studied after incubation for 18–20 hours at 37 °C [22].

Electron microscopy of the obtained bacteriophages, counterstained with 1% uranyl acetate, was performed on a transmission electron microscope JEM-1011 (JEOL, Japan). The figures were taken using a side-mounted camera Erlangshen ES500W (Gatan, USA). The bacteriophage parameters were measured using the ImageJ program.

The specificity of the phage was studied by spot testing of phagolysate on the lawn of closely related bacteria: *Bordetella parapertussis* C-95, *Bordetella parapertussis* C-94, *Bordetella pertussis* C-99. The strains were obtained from the RPC "MikroMir" collection of microorganisms.

To study the thermal stability of isolated bacteriophages, phagolysates were heated in a dry block heater "Termit" ("DNA Technology" LLC, Russia) with a temperature increase from 40 to 95 °C. A sample was taken from the test tube every 20 min, while increasing the temperature by 5 °C [24]. The control tubes were not heated. The activity of the tested phages was determined by the Gratia method [20, 21].

The titer stability of the phage in the sealed tube was studied using the Gratia method and stored at 4–8 °C without the addition of a preservative for 8 months [20, 21].

RESULTS AND DISCUSSION

Three new virulent bacteriophages were isolated from samples of animal biomaterial (Fig. 1), which were designated as: *vB_BbrS_2/200220.7.2*; *vB_BbrS_4/200220.7.1*; *vB_BbrM_5/200220.7.2*.

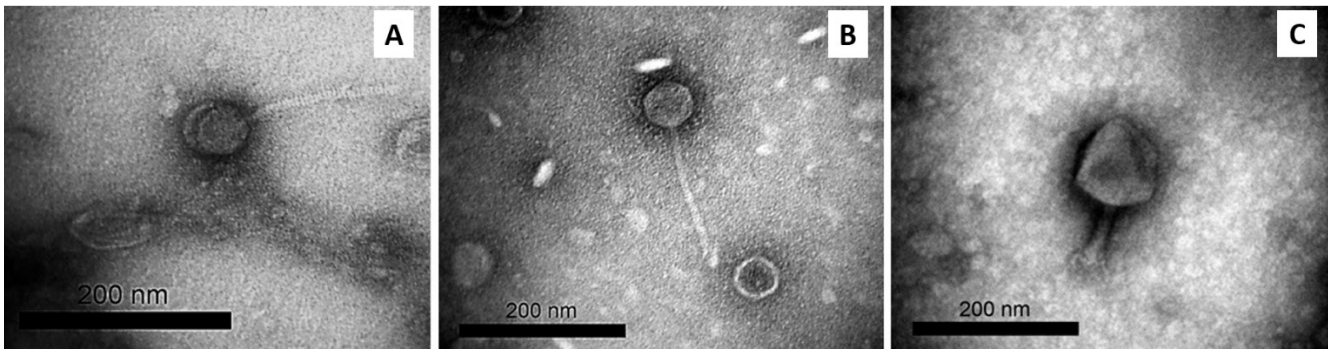

Fig. 1. Electron micrographs of bacteriophages active against B. bronchiceptica: A – vB_BbrS_2/200220.7.2; B – vB_BbrS_4/200220.7.1; C – vB_BbrM_5/200220.7.2
(1% uranyl acetate solution was used as a counterstain, 250,000× magnification)

Table 1
Morphology of bacteriophage plaques

Nutrient medium	Plaque morphology		
	vB_BbrS_2/200220.7.2	*vB_BbrS_4/200220.7.1*	*vB_BbrM_5/200220.7.2*
1.5% BHI-agar with blood	– clear, Ø 1.8 ± 0.3 mm; – turbid, Ø 0.9 ± 0.1 mm; – clear with turbid halo, Ø 2.3 ± 0.3 mm	– clear, Ø 1.2 ± 0.3 mm; – turbid, Ø 1.0 ± 0.1 mm; – clear with turbid halo, Ø 2.4 ± 0.1 mm	– clear, Ø 0.6 ± 0.2 mm; – turbid, Ø 1.9 ± 0.1 mm
1.5% BHI-agar	– turbid, Ø 1.1 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm
1.5% FPH-agar	– turbid, Ø 1.0 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm	– turbid, Ø 1.0 ± 0.1 mm
1.5% Bordetelagar	– clear, Ø 2.2 ± 0.3 mm; – turbid, Ø 0.9 ± 0.1 mm; – clear with turbid halo, Ø 2.4 ± 0.2 mm	– clear, Ø 1.6 ± 0.2 mm; – turbid, Ø 1.0 ± 0.1 mm; – clear with turbid halo, Ø 2.6 ± 0.1 mm	– clear, Ø 1.5 ± 0.2 mm; – turbid, Ø 1.0 ± 0.1 mm; – clear with turbid halo, Ø 2.1 ± 0.1 mm

The plaque morphology was studied on various nutrient media and analyzed by two parameters: size and transparency. The manifestation of these signs depended on the composition of the nutrient medium and the concentration of agar in the upper layer. Table 1 presents the results of the analysis of the morphology of plaques obtained in *B. bronchiseptica* strains. Dissociation of plaques into clear, turbid and clear with turbid halos was observed on various media. Plaques also varied in size, the smallest ones had a diameter of 0.6 ± 0.2 mm, and the largest – 2.6 ± 0.1 mm. It is worth noting that this plaque morphology was observed when 0.8% agar was used in the upper layer. At the same time, when using 0.4% agar in the upper layer, mainly large (2.5 ± 0.1 mm) clear plaques were formed on BHI- and FPH-agar nutrient media, i.e. agar dilution promotes the formation of more clear plaques and an increase in their size. This means, the composition of the nutrient medium has a significant effect on the morphology of plaques, which corresponds to the conclusions made by N. Ramesh et al. [23]. In addition, in M. Adams' publication [20] it was also noted that the plaque count will not give the absolute number of phage particles

Table 2
Lytic spectrum of isolated bacteriophages active against *B. bronchiseptica*

No.	Strains	Bacteriophages		
		vB_BbrS_2/200220.7.2	*vB_BbrS_4/200220.7.1*	*vB_BbrM_5/200220.7.2*
1	*B. bronchiseptica* 200220.7.1	++++	++++	++++
2	*B. bronchiseptica* 200220.7.2	++++	++++	++++
3	*B. bronchiseptica* 200220.6.1	–	+	–
4	*B. bronchiseptica* 1	+	–	++
5	*B. bronchiseptica* 2	++++	++++	–
6	*B. bronchiseptica* 3	++	++	++
7	*B. bronchiseptica* 4	–	+	–
8	*B. bronchiseptica* 200220.4.4	+++	–	–
9	*B. bronchiseptica* C-93	–	+	–
10	*B. bronchiseptica* C-97	+	+++	+
11	*B. bronchiseptica* C-98	+	+	+
12	*B. bronchiseptica* 43	–	+	+
13	*B. bronchiseptica* 44	–	–	++

"–": no plaque; "+": a plaque with multiple secondary bacterial colonies; "++": a plaque with few secondary bacterial colonies; "+++": a plaque with sporadic secondary bacterial colonies; "++++": clear plaques without any secondary colonies of bacteria grown [25].

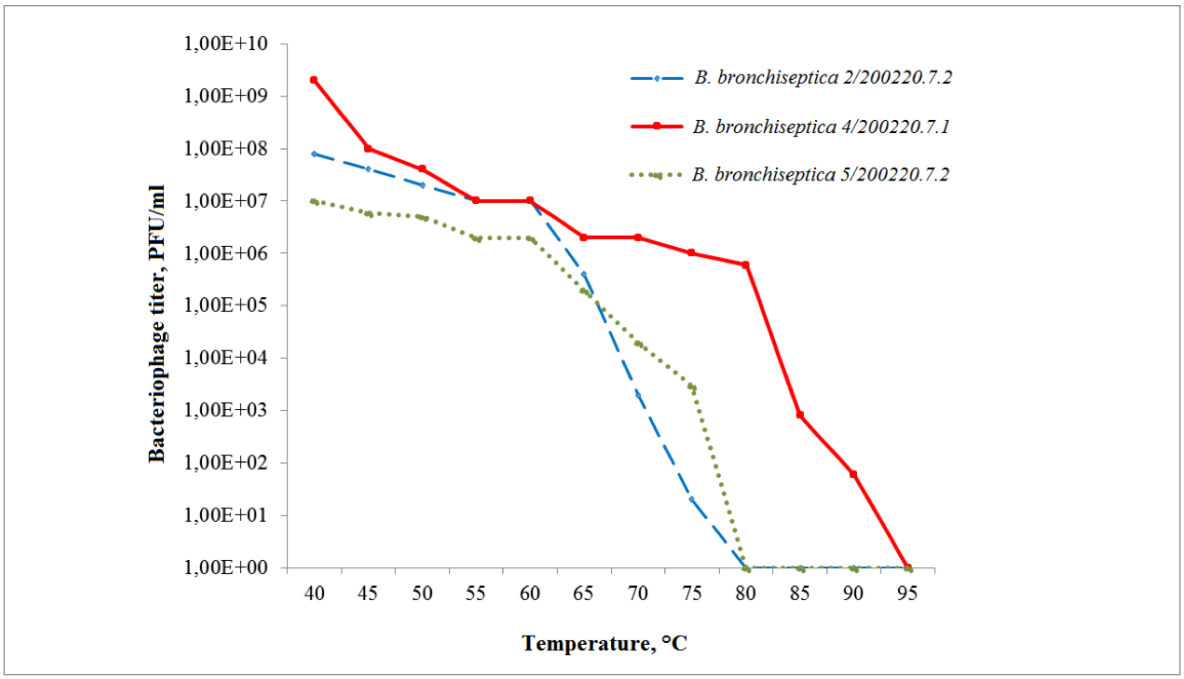

Fig. 2. Heat resistance of isolated bacteriophages

Table 3
Temporal variations in lytic activity of bacteriophages active against *B. bronchiseptica*

Bacteriophage	Titer before storage, PFU/mL	Titer after 8 months of storage, PFU/mL
vB_BbrS_2/200220.7.2	$(2.3 \pm 1.4) \times 10^8$	$(3.1 \pm 0.4) \times 10^8$
vB_BbrS_4/200220.7.1	$(9.0 \pm 0.2) \times 10^8$	$(8.6 \pm 0.2) \times 10^8$
vB_BbrM_5/200220.7.2	$(6.1 \pm 1.2) \times 10^8$	$(4.1 \pm 1.1) \times 10^8$

present in the inoculum; this number depends on the nutrient medium and the strain of sensitive bacteria.

Titers of *vB_BbrS_2/200220.7.2*; *vB_BbrM_5/200220.7.2* bacteriophages were determined on a test strain of *B. bronchiseptica* 200220.7.2 and were $(2.3 \pm 1.4) \times 10^8$; $(6.1 \pm 1.2) \times 10^8$ PFU/mL, respectively. The bacteriophage *vB_BbrS_4/200220.7.1* had the highest titer – $(9.0 \pm 0.2) \times 10^8$ PFU/mL. Its titer was determined on a test strain of *B. bronchiseptica* 200220.7.1.

The results of tests of lytic activity spectrum of isolated bacteriophages are presented in Table 2. The tests were performed in triplicate. Tests demonstrated that the isolated bacteriophages had different spectrum of lytic action. The maximum spectrum of lytic action was shown by phage *vB_BbrS_4/200220.7.1*, which lysed 10 out of 13 bacterial strains (76.9%). Bacteriophages *vB_BbrS_2/200220.7.2* and *vB_BbrM_5/200220.7.2* 61.5% of *B. bronchiseptica* strains were lysed.

For each isolated bacteriophage, parameters such as the average diameter of the head (from vertex to vertex) and the average length of the tail were determined. Virions of the phage *vB_BbrS_2/200220.7.2* consist of 49 ± 2.67 nm icosahedral head and a flexible non-contractile tail, 171 ± 2.26 nm long. Virions of the bacteriophage *vB_BbrS_4/200220.7.1* have morphology, which is similar to the *VB_BbrS_2/200220.7.2* phage: 61 ± 2.88 nm icosahedral head and a flexible non-contractile tail, 158 ± 6.17 nm long. Virions of the phage *vB_BbrM_5/200220.7.2* consist of 108 ± 3.49 nm icosahedral head and a straight contractile tail, 69 ± 5.37 nm long. In accordance with the international classification of viruses by morphological parameters phages *vB_BbrS_2/200220.7.2* and *vB_BbrS_4/200220.7.1* are assigned to the family *Siphoviridae*, bacteriophage *vB_BbrM_5/200220.7.2* to the family *Myoviridae*.

The specificity test showed that the isolated bacteriophages lyse not only the strains of *B. bronchiseptica*, but also active against the strains of *Bordetella parapertussis* C-95 and *Bordetella parapertussis* C-94.

The testing of bacteriophage thermal stability demonstrated that heating of phages for 20 min at more than 40 °C results in a decrease in their lytic activity (Fig. 2). Bacteriophages *vB_BbrS_2/200220.7.2* and *vB_BbrM_5/200220.7.2* are completely inactivated at 80 °C, bacteriophage *vB_BbrS_4/200220.7.1* is inactivated at 95 °C. The tests were performed in triplicate.

To use the isolated phages in production, it is necessary to study the change in their lytic activity over time [26, 27]. Bacteriophage filtrates were stored in vials at 4–8 °C without any preservative added. Lytic activity was determined after 8 months. It was found that during this period of time, the titer of bacteriophages did not decrease. The results are presented in Table 3.

CONCLUSION

Thus, bacteriophages *vB_BbrS_2/200220.7.2*, *vB_BbrS_4/200220.7.1*, *vB_BbrM_5/200220.7.2*, according to the results of *in vitro* studies, are promising for further scientific research in the context of phage therapy of diseases caused by the bacterium *B. bronchiseptica*. The use of drugs based on virulent bacteriophages in veterinary medicine will safely and effectively eliminate the infection without affecting the normal flora, minimize the risk of transmission of *B. bronchiseptica* antimicrobial-resistant strains to humans, improve the livestock productivity and economic performance in the animal production.

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Electroporation of mouse embryonic stem cells with Neon device

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SUMMARY

Mouse embryonic stem cells are widely used as a promising material for producing of new cellular systems with desired properties in cellular and molecular biology, pharmacology, virology, medicine, veterinary medicine and biotechnology. Each type of cells requires different electroporation conditions that are determined experimentally. Therefore, the main goal was to optimize conditions of electroporation with Neon® Transfection System, a new-generation device, by selecting and changing of various parameters (voltage, impulse width and number of impulses) to maximize efficiency of D3 embryonic stem cell line transfection and to maintain cell viability. The following parameters were found to be the most optimal for the said cells: impulse voltage – 1200 V, impulse width – 10 ms, number of impulses – 3. Under given conditions, viability of the cells after electroporation was 91%, and transient transfection efficiency (24 hours after electroporation) assessed based on bacterial *β*-galactosidase production was 88%. It was shown that with higher cell density any electroporation condition tested yielded higher transfection efficiency ranging between 34 and 88%. It was demonstrated that only 5 out of 12 tested protocols with different parameters could be successfully used for insertion of DNA plasmid carrying *lacZ Escherichia coli* gene into D3 cell line. Thus, the experiment results show the more optimal conditions can be selected experimentally taking into account available information on electroporation protocols for similar cell types recommended by the device manufacturer. Electroporation of mouse embryonic stem cells with the new-generation device can be an effective method for *in vitro* insertion of nucleic acids into the cells of interest to the researcher.

Keywords: embryonic stem cells, insertion of exogenous DNA plasmid, Neon electroporation device, *lacZ Escherichia coli* gene, transfection efficiency, viability

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Электропорация эмбриональных стволовых клеток мыши с помощью прибора Neon

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РЕЗЮМЕ

Эмбриональные стволовые клетки мыши широко используются в качестве перспективного материала для создания новых клеточных систем с заданными свойствами в клеточной и молекулярной биологии, фармакологии, вирусологии, медицине, ветеринарии и биотехнологии. Для каждого типа клеток требуются разные условия электропорации, которые должны быть определены экспериментально, поэтому была поставлена цель путем подбора и из-

менения различных параметров (напряжения, ширины импульса и количества импульсов) оптимизировать условия электропорации при использовании электропоратора нового поколения Neon® Transfection System, обеспечивающие высокую эффективность трансфекции эмбриональных стволовых клеток линии D3 и их жизнеспособность. Установлено, что наиболее подходящими параметрами для данных клеток являются: импульсное напряжение – 1200 V, ширина импульса – 10 ms, количество импульсов – 3. При данных условиях жизнеспособность клеток после электропорации составила 91%, а эффективность временной трансфекции (24 ч после электропорации), оцениваемая по продукции бактериальной *β*-галактозидазы, достигала 88%. Показано, что при более высокой клеточной концентрации любые испытанные режимы электропорации обеспечивают более высокую эффективность трансфекции в диапазоне от 34 до 88%. Продемонстрировано, что для введения ДНК плазмиды с геном *lacZ Escherichia coli* в клетки линии D3 из 12 изученных протоколов с разными параметрами можно успешно использовать 5. Таким образом, полученные в эксперименте результаты показывают, что, имея предварительную информацию о режимах электропорации аналогичного типа клеток, которую рекомендует производитель прибора, можно подобрать экспериментальным путем более оптимальные условия. Электропорация эмбриональных стволовых клеток мыши с использованием электропоратора нового поколения может быть эффективным методом введения нуклеиновых кислот в представляющие интерес для исследователя клетки *in vitro*.

Ключевые слова: эмбриональные стволовые клетки, введение экзогенной ДНК плазмиды, электропоратор Neon, ген *lacZ Escherichia coli*, эффективность трансфекции, жизнеспособность

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INTRODUCTION

Since their discovery in 1981 [1, 2] mouse embryonic stem cells (ESCs) have been widely used as a promising material for the creation of new cellular systems with desired properties in cellular and molecular biology, pharmacology, virology, medicine, veterinary medicine and biotechnology. These cells have following features: high rate of doubling, specific marker gene expression confirming their origin, ability to respond to growth factors, causing morphological and biochemical changes leading to the ESCs differentiation into cells with a phenotype similar to more than 225 cell types in culture, and retention of early pre-implantation embryo properties both *in vitro* and *in vivo*.

The introduction of exogenous DNA into mouse ESCs enables genetic modification of their genomes, but also use of such cells for gene-edited animal creation [3]. Methods of recombinant molecule introduction into ESCs require careful selection due to their morphological features: small cells with a large nucleus and a narrow cytoplasm rim that grow in dense colonies, usually on a fibroblast monolayer. Electroporation is considered as the most acceptable way of highly efficient introduction of exogenous DNA into these cells without cellular changes and with retained differentiation capacity [4]. During such transfection, cells are exposed to a high-voltage impulse in the presence of exogenous nucleic acid. High voltage causes short-term permeability of the cell membrane, which allows foreign nucleic acids to enter the cell [5, 6]. Each type of cells requires different electroporation conditions that are determined experimentally. Intensity of the electric field and the impulse duration shall be taken into account, as they are key parameters for achieving maximum transfection efficiency and maintaining cell viability after the procedure. The impulse applied to the cells can be generated in two different waveforms: rectangular and exponential decay. Rectangular waveforms rely on a constant charge being applied to the cells for a set time. The use of rectangular signals allows applying multiple impulses. During the exponential decay waveform, the initial voltage is set, and the attenuation duration (time constant) is the product of capacitance setting and resistance of the circuit including the sample. Since the sample resistance mainly depends on the ionic strength of the electroporation buffer, while the resistance is constant, effect of capacitance setting changing on the impulse can be determined empirically [7]. Buffer solution components are also have an impact on transfection efficiency and cell viability. Earlier, buffer solution with high ionic strength (low resistance), such as phosphate-buffered saline (PBS) or serum-free growth medium, was adapted to for mouse ESC-D3 electroporation with high capacitance [8].

The goal was to optimize the electroporation conditions providing high efficiency of mouse ESCs transfection and viability due to purchasing of Neon® Transfer System (Invitrogen, Thermo Fisher Scientific, USA), new generation electroporator. Unlike previously systems used for electroporation, for example Gene Pulser (Bio-Rad), unique 100 or 10 µL tips are used as an electroporation chamber for electrical impulse exposure instead of cuvettes. It was demonstrated that the above-said device could be used for successful transfection of the foreign

DNA incorporation-incompetent cells, primary and immortalized hematopoietic cells, as well as stem cells and cells of various tissues [9–11].

MATERIALS AND METHODS

Mouse embryonic stem cells (D3 line) were tested. The mouse ESCs were cultivated in DMEM containing 4.5 g/L of glucose, 10% fetal bovine serum (HyClone, USA), essential amino acid solution, 2 мМ *α*-glutamine, 0,1 мМ *β*-mercaptoethanol and antibiotics: penicillin and streptomycin at final concentration of 50 U/mL and 50 μg/mL, respectively (NPP "PanEco", Russia). The ESCs were cultivated in monolayer of mouse diploid embryonic fibroblasts pre-treated with mitomycin C (final concentration: 30 μg/mL for 3 hours) to block mitosis.

pcDNATM3.1/His/lacZ plasmid comprising nucleotide sequence of *lacZ Escherichia coli* marker gene is used for transfection. Foreign DNA was re-precipitated with ethanol (70%) and dissolved in sterile buffer (0.1 мМ EDTA, 1 мМ Tris-HCl, pH 8.0) before transfection.

Transfection was performed by electroporation using Neon® Transfection System device and starter kit of reagents according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, USA). The day before transfection the ESCs were passaged to separate them from feeder layer be taking an advantage of differences in cell adhesion and seeded in Petri dishes (60 mm in diameter) without fibroblasts 24 hours before transfection so that they were in an exponential growth phase on the day of electroporation.

The cells were washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions (PBS-2); 1 mL of trypsin (NPP "PanEco", Russia) was added and the cells were incubated at 37 °C for 2 minutes before electroporation. The cells were resuspended after adding 9 mL of supplemented DMEM and then transferred to sterile 15 mL polypropylene tube (Sarstedt, Germany) and centrifuged at 200 g for 5 minutes. The precipitate was resuspended in 10 mL of PBS and the cells were calculated in Goryaev's chamber. The cell were precipitated again and buffer solution R from the kit for electroporation was added to the precipitate: up to final cell concentration of 1.0×10^7 cells/mL in the first case and two times less concentration (0.5×10^7 cells/mL) in the second case, as well as DNA plasmids, pcDNATM3.1/His/lacZ (final concentration of 1 μg/mL). DNAs and ESCs were mixed in the buffer and 10 μL of the mixture were collected by a pipette with tips for electroporation. The pipette is fixed in the holder for electroporation and exposed to electric current using preliminary set or manually entered protocols in Neon device. The transfected cells were transferred (10 μL) to the well of 24-well tissue culture plate containing 500 μL of suitable growth medium without antibiotics and the procedure was repeated 11 times for each cell concentration only changing the parameters for the electroporator. The plate with transfected mouse ESCs and control (non-transfected ESCs) was incubated at 37 °C in humidified 5% CO_2 atmosphere for 24 hours.

The cells were tested for their viability after electroporation through their staining with trypan blue (0.02% solution). Percentage of viable cells was calculated as the ratio of the number of unstained cells to the total number of cells multiplied by 100.

Transfection efficiency was assessed based on presence of *β*-galactosidase, *lacZ E.* coli gene expression product in the cells. For this purpose, X-gal (Sigma-Aldrich, USA) was used as a substrate. The cells were fixed with cold methanol (–20 °C) on ice for 15 minutes before staining. Proportion of blue-green-stained cells (*β*-galactosidase-producing cells) was calculated providing that at least 1,000 cells were analyzed. Non-transfected ESCs served as negative control.

Table
Optimization of conditions for electroporation of D3 mouse embryonic cell line in 10 μL tip (Neon)

Protocol No.	Impulse voltage, V	Impulse width, ms	Number of impulses	Transfection efficiency/viability, % (m ± SEM)	
				cell concentration: 0.5×10^5	cell concentration: $1,0 \times 10^5$
1	1,000	10	3	29±0.1/70±0.04	39±0.01/83±0.5
2	1,000	20	2	37±0.6/77±0.05	39±0.5/79±0.1
3	1,000	40	1	37±0.7/64±0.3	37±0.01/68±0.7
4	1,200	10	3	74±0.02/86±0.8	88±0.7/91±0.04
5	1,200	20	2	66±0.03/87±0.01	70±0.6/90±0.3
6	1,200	20	3	65±0.04/85±0.1	76±0.5/89±0.7
7	1,200	40	1	44±0.5/58±.001	57±0.4/60±0.3
8	1,400	10	3	71±0.05/70±0.3	78±0.1/88±0.1
9	1,400	20	2	68±0.2/70±0.5	81±0.1/90±0.5
10	1,400	20	3	65±0.4/66±0.6	70±0.7/72±0.6
11	1,400	30	3	33±0.7/59±0.02	57±0.4/66±0.1
11	1,500	10	3	26±0.6/46±0.3	35±0.1/48±0.01
12	1,500	40	1	22±0.07/34±0.4	34±0.2/41±0.2

The cells were visualized with inverted phase-contrast microscope (Carl Zeiss, Germany) using AxioVision Rel. 4.8. software. The experiments were performed in triplicate. Arithmetic mean (m) and standard error of the mean (SEM) were calculated.

RESULTS AND DISCUSSION

Several experiments for introduction of foreign DNA plasmid containing *lacZ E. coli* marker gene in mouse ESCs with electroporation using Neon® Transfection System device designed for mammalian cells and starter reagent kit. Two sets of electroporation parameters recommended by the device manufacturer were used for mouse ESCs transfection (protocols No. 5 and 8 in the Table). Several combinations of voltage (1,000; 1,300; 1,400 or 1,500 V), impulse duration (10, 20, 30, 40 ms) and number of impulses (1–3), as indicated in the Table, were additionally tested. Different cell concentrations, 0.5×10^5 or 1.0×10^5 cells in 10 µl, were used to optimize conditions.

Data given in the Table show that the best results were obtained when protocols No. 4 and 9 were used: transfection efficiency was the highest and proportion of stained cells was higher than that one when the other electroporation parameters were used.

The method produces reproducible results that has been determined by comparing its effectiveness in three repeated experiments. Parameters recommended by the device manufacturer for mouse ESCs (protocols No. 5 and 8 given in the Table) were also efficient but were inferior to the expected parameters. Thus, the manual states that 79% and 88% efficiency of the mouse ESCs transfection with *EGFP* gene-containing plasmid at 99% and 96% cell viability can be achieved after 48 hours using the proposed parameters in accordance with protocols No. 5 and 8, respectively.

Data analysis showed that cell concentration in the suspension was one of the most important variables having an impact on efficiency of the transfection performed with Neon device. Obtained results allows us to conclude that at a higher cellular concentration, in our case twofold concentration, any tested electroporation protocols provided higher transfection efficiency in the range of 34 to 88%. The said values varied from 22 to 74% when lower cell concentration was used.

Unlike standard cuvette-based electroporation methods, the Neon system uses unique reaction chambers – Neon tips that generate higher electric field for biological samples. The tips maximizes the gap size between two electrodes while minimizing the surface area of each electrode. This results in minimal pH change, less ion formation, and negligible heat generation. This design enhances transfection efficiency and cell viability as well as maintains ergonomic workflow. It would be of interest to assess cell viability after electroporation since the transfection occurred in the mixture microvolume (buffer solution, DNA and cells). Cells subjected to electroporation and not subjected to electroporation (not exposed to foreign DNA and electric impulse) were handled in a similar way. The results showed that ESCs viability (1.0×10^5 cell concentration) after transfection according to protocol No. 4, 5, 6, 8, 9 parameters (Table) was comparable with that one of control ESCs 95 ± 0.2% (ESCs not subjected to electroporation) was as follows: 91, 90, 89, 88 and 90%, respectively. The cell viability was the highest (91%) when the following parameters were used: impulse voltage – 1,200 V, impulse width – 10 ms, number of impulses – 3.

Mouse ESCs, promising for engineering a laboratory model for viral infection studies [12] and being immortalized cells, i.e. immortal in culture, were selected for the experiments. *lacZ E. coli* gene being a part of pCMV-lacZ plasmid was successfully introduced in the said cells with Gene Pulser device (Bio-Red, USA) at transfection efficiency of 35% during previous experiments [8]. Therewith, maximum proportion of the cells survived after electroporation was 82%. Comparative examinations of mouse D3 embryonic stem cell electroporation with Neon device (from 34 to 88%) for electroporation efficiency have shown the advantage of the new-generation electroporator where transfection occurs in tips. It should be noted that the Neon system allows the use of very small volumes for transfection, is miniaturized for the use of tips for electroporation and 10 or 100 µL volumes for transfection.

During the experiment foreign DNA plasmid, pcDNATM3.1/His/lacZ, was successfully introduced into mouse ESCs at transfection efficiency of 88% by changing electroporation parameters. Data on the efficiency of foreign RNA [13–15], DNA and proteins introduction into cells non-competent to take up foreign material using Neon electroporator are currently accumulated [16–18]. The experiment data are consistent with these reports, however, cells shall be tested for their quality and the parameters shall be optimized for each cell culture before transfection. Differences noted in the electroporation protocols published in scientific literature [9, 10, 16] suggest that even minor changes in cell growth conditions could have a significant impact on foreign DNA introduction into them.

CONCLUSION

Thus, the results obtained during the experiment show that more optimal conditions can be selected experimentally, when preliminary information on electroporation modes for similar cell type recommended by the manufacturer is available. Electroporation is shown to be a very effective method for nucleic acid introduction into the cells of interest, including those that are often considered difficult for transfection. The high transfection efficiency for the cells described in this paper is achieved by optimization or determination of electroporation parameters for Neon device. The following parameters are found the most suitable parameters for mouse D3 embryonic stem cells: impulse voltage – 1,200 V, impulse width – 10 ms, number of impulses – 3, at which cell viability after electroporation is 91%, and the temporary transfection efficiency (24 hours after electroporation) assessed based on bacterial protein *β*-galactosidase production is 88%.

The analysis of the obtained results indicates the need to select optimal conditions for electroporation of mouse D3 ESCs due to the peculiarities of their behavior in culture. The concentration of cells in the suspension is shown to be one of the most important variables affecting transfection efficiency under optimized electroporation conditions. Obtained results allows us to conclude that at a higher cellular concentration, two-fold concentration in our case, any tested electroporation protocols provide higher transfection efficiency in the range of 34 to 88%.

The best electroporation conditions for any cell type can be obtained by using a system that allows adjustment of parameters including waveform, voltage, as well as cell density, which can significantly affect both the cell viability after electric current exposure and transfection efficiency. Proportion of viable cells (comparable to that one in control, i.e. non-transfected cells) or transfection efficiency can be increased by changing these parameters. It is demonstrated that only 5 out of 12 tested protocols with different parameters can be successfully used for introduction of DNA plasmid carrying *lacZ Escherichia coli* gene into ESCs-D3. Thus, Neon® Transfection System electroporator (Invitrogen, Thermo Fisher Scientific, USA), allows selection of conditions that are the most suitable for creating cells with the specified properties.

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OIE REGIONAL REFERENCE LABORATORY FOR FOOT AND MOUTH DISEASE

РЕГИОНАЛЬНАЯ РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ЯЩУРУ

OIE REFERENCE LABORATORY FOR HIGHLY PATHOGENIC AVIAN INFLUENZA AND LOW PATHOGENIC AVIAN INFLUENZA (POULTRY) AND NEWCASTLE DISEASE

РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ВЫСОКОПАТОГЕННОМУ И НИЗКОПАТОГЕННОМУ ГРИППУ ПТИЦ И НЬЮКАСЛСКОЙ БОЛЕЗНИ