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CONTENTS

СОДЕРЖАНИЕ

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Heart and skeletal muscle inflammation – novel dangerous disease of farmed *Salmonidae*

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SUMMARY
Heart and skeletal muscle inflammation (HSMI) is one of the most widespread economically relevant diseases of farmed Atlantic salmon (*Salmo salar*), and it poses serious danger to its aquaculture. The disease was first reported in Norway in 1999. In 2006, the Norwegian researchers demonstrated its viral etiology. Heart and skeletal muscle inflammation is a novel and understudied highly contagious transboundary disease of *Salmonidae* characterized by erythrocyte damage, blood circulation failure, jaundice and aggregated signs of heart and skeletal muscle inflammation. The disease associated economic damage to aquaculture is enormous. Total cumulative mortality can reach 30% and morbidity can amount to 100%. Loss of quality of the commercial fish products due to melanised foci in the salmons' muscles further increases the disease-associated economic losses. Aquacultured Atlantic salmon is the most susceptible to HSMI. Rainbow trout, chub salmon and bull trout are also susceptible species and the list is still being continued. The disease is caused by the virus belonging to genus *Orthoreovirus* in the family *Reoviridae*. Currently Piscine orthoreovirus demonstrates the tendency towards its global spread. The virus-induced disease is reported in Norway, Scotland, Ireland, Iceland, France, Germany, Italy, Denmark, the Faroe Islands, Chile, Canada, Atlantic coast of the USA and Alaska. The majority of the outbreaks are registered in Central and Northern parts of Norway, which borders the Murmansk Oblast. The vicinity of the affected areas to Russia, the Gulf Stream passing the Norwegian shore while moving towards the Murmansk Oblast as well as wild *Salmonidae* migration to the Barents Sea, White Sea and Pechora Sea through the Norwegian territorial waters coupled with high stability of the virus compose high threat of *Piscine orthoreovirus* introduction to the Russian Federation from the adjacent countries.

Key words: *Piscine orthoreoviruses*, heart and skeletal muscle inflammation (HSMI), epidemic situation.

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Воспаление сердечных и скелетных мышц – новое опасное заболевание культивируемых лососевых рыб

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РЕЗЮМЕ
Воспаление сердечных и скелетных мышц в настоящее время является одним из самых распространенных, экономически значимых заболеваний культивируемого атлантического лосося и представляет серьезную угрозу его аквакультуре. Впервые болезнь выявлена в 1999 г. в Норвегии. В 2006 г. норвежские ученые доказали ее вирусную этиологию. Воспаление сердечных и скелетных мышц является трансграничной, высококонтагиозной, новой, еще недостаточно изученной вирусной болезнью лососевых рыб, характеризующейся поражением эритроцитов, симптомокомплексом воспаления сердечных и скелетных мышц, нарушением кровообращения и желтухой. Экономический ущерб, наносимый этим заболеванием аквакультуре, чрезвычайно велик. Общая кумулятивная смертность может достигать 30%, а уровень заболеваемости составлять 100%. Снижение качества товарной рыбной продукции из-за меланизированных участков в мышцах лососевых еще больше повышает экономические потери от этого заболевания. Наиболее чувствителен к воспалению сердечных и скелетных мышц выращиваемый в аквакультуре атлантический лосось. Восприимчивыми видами также являются радужная форель, чавыча и кумжа, и этот список продолжает пополняться. Возбудителем заболевания является вирус, относящийся к роду *Orthoreovirus* семейства *Reoviridae*. На сегодняшний день ортореовирус рыб имеет тенденцию к глобальному распространению. Заболевание, вызванное этим вирусом, регистрируют в Норвегии, Шотландии, Ирландии, Исландии, Франции, Германии, Италии, Дании, на Фарерских островах, в Чили, Канаде,

на Атлантическом побережье США и на Аляске. Наибольшее количество очагов отмечают в Средней и Северной Норвегии, пограничной с Мурманской областью. Близость неблагополучных территорий к России, протекающий мимо норвежских берегов в сторону Мурманской области Гольфстрим, а также миграция диких лососевых через норвежские территориальные воды в Баренцево, Белое и Печорское моря в совокупности с высокой стабильностью вируса создают высокий уровень угрозы заноса ортореовируса рыб с территории сопредельных стран на территорию Российской Федерации.

Ключевые слова: ортореовирусы рыб, воспаление сердечных и скелетных мышц, болезни рыб, эпизоотическая ситуация.

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DISEASE BACKGROUND

Heart and skeletal muscle inflammation (HSMI) was first reported in the Atlantic salmon (*Salmo salar*) grown on an aquaculture farm in Norway in 1999 [1]. For a long time the disease cause remained unknown. The virus etiology was supposed only in 2004, and later the infectious nature of the disease was confirmed by the Norwegian researchers [1, 2]. In 2006, the virus was observed during electronic microscopy [3]. In 2010, the virus was identified as reovirus using molecular and biological methods [4], and it was later attributed to genus *Piscine orthoreovirus* (PRV) [5]. In 2014, O. W. Finstad et al. demonstrated that PRV replicated in erythrocytes [6]. In 2016, Japanese scientists proved that erythrocytic inclusion body syndrome (EIBS) known since 1970-s was caused by PRV genetic variant in a coho salmon in Japan [7], and the orthoreoviruses were subdivided into three types. The latest evidence of the orthoreovirus being the HSMI agent was demonstrated by O. Wessel et al. in 2017 [8].

CLASSIFICATION

Heart and skeletal muscle inflammation is caused by *Piscine orthoreovirus*. PRV belongs to genus *Orthoreovirus*. PRV greatly differs from aquareoviruses. M. J. T. Kibenge et al. performed the phylogenetic analysis of S1 gene segment of many available virus isolates that allowed for grouping the Norwegian PRV strains in one genotype (PRV-1) including subgenotypes Ia and Ib. The Canadian PRV strains belonged to genotype Ia, and the Chilean PRV strains isolated from the Atlantic salmon belonged to subgenotype Ib [5].

Two more PRV genetic variants were described over the past few years that are adapted not to the Atlantic salmon but to other salmonids. One of such variants designated as PRV-2 is an EIBS agent on coho salmon aquaculture farms in Japan [7].

Another genetic variant designated as PRV-3 (also called Y virus or PRV-*Om*) induces HSMI-like disease in rainbow trout [9]. The same genotype includes PRV strains isolated from coho salmon in Chile [10]. PRV-3 was demonstrated to be closer related to PRV-1 than to PRV-2 [11]. PRV-3 also replicates in the Atlantic salmon but pathogenicity of this virus genotype for this fish species is lower as compared to the rainbow trout [12].

VIRUS STABILITY

Orthoreoviruses are stable against disinfecting agents. The virions are stable at pH 2–9 and up to 55 °C; they are also stable against fat-dissolving agents and detergents. UV irradiation reduces the virus infectivity.

EPIDEMIC DATA

Heart and skeletal muscle inflammation is a severe disease characterized by high morbidity and duration. Progressive increase of the severity of the lesions during the months before the clinical signs' onset is indicative of the fact that the subclinical infection can be present on the farm for a very long time [2]. In Norway, the HSMI morbidity of the aquacultured Atlantic salmon amounts up to 100% with 20% mortality. In Chile, the mortality rate does not usually exceed 5% during freshwater culture. During seawater culture two mortality peaks are reported in fish: the first one – in two months after transfer from the freshwater to the seawater and it amounts to 2–10%; the second peak starts in six months after the first one. During the second peak the mortality reaches 30% and it is associated with secondary infections. HSMI outbreaks in the Atlantic salmon are reported both in winter and in summer. This suggests that seasonality and water temperature have no impact on the disease development. HSMI was long considered to occur only in fish in seawater, usually 5–9 months after the Atlantic salmon is transferred to seawater cages from the freshwater cages. Over the recent years (2016–2019) the disease has been also reported on the freshwater farms [13]. In freshwater pools PRV infects young fish before smoltification [14]. According to the latest data PRV-1 is present in nearly every batch of cultivated Atlantic salmon during the seawater phase [8]. Adult fish is more often infected as compared to young fish; males are more often diseased than females. The disease outbreaks associated with high mortality last for several weeks. The amount of the virus detected using real-time reverse-transcription polymerase chain reaction (PCR) directly correlates with the disease development [15]. The virus is isolated from the wild fish, but no clinical signs are reported. This is due to the fact that in natural environment the diseased fish quickly dies or it is captured by the predators. PRV is preserved in tissues of convalescent fish for over one year [16]. A. B. Kristoffersen et al. demonstrated that HSMI risk increases with the increase of the fish lifespan, growth of the virus concentration in the environment and increase of the fish stocking density [17].

Geographic distribution and susceptible species. In spite of the vast PRV spread in the cultured Norwegian salmon [18] and in wild Atlantic salmon [4], HSMI is reported

only in cultured fish. In addition to Norway, PRV is widely spread in aquacultured Atlantic salmon and coho salmon in Chile [5, 10], Scotland, Ireland, Iceland and on the Faroe Islands [19], in cultured and wild Atlantic salmon in Denmark [20], Atlantic salmon, cutthroat trout, steelhead trout, and chum salmon in Canada [5], cultured chinook salmon and coho salmon along the US Atlantic coast [21], aquacultured chinook salmon and wild coho salmon on Alaska [22]. K. A. Garver et al. experimentally infected coho salmon, chinook salmon and sockeye salmon with PRV [16], but they identified no heart lesions or immune response pathology. Orthoreovirus was also isolated from 3% of andromous sea-trout tested, but it was not detected in Arctic char [23]. L. Bigarre et al. described increased PRV-induced mortality in brown trout population in France [24]. The virus was isolated from a farmed Atlantic salmon escaped from the cage that was considered as one of the routes of the disease spread [25]. During PCR-screening of the samples collected from the marine fish captured along the Norwegian seacoast, C. R. Wiik-Nielsen et al. detected low PRV concentrations in Atlantic capelin, European horse mackerel and great silver smelt . The authors suggested the interactions to be more complex and involving several virus carriers and reservoirs as no positive samples were collected from fish captured near the aquaculture farms, where HSMI outbreaks were reported [26].

For Norway HSMI is a serious problem due to a great number of epidemic outbreaks; in 2014 the disease was reported on 181 farms [27]. Chile reported 44 PRV cases in the Atlantic salmon in the first half of 2015 [28]. In British Columbia (Canada) 75% of tested Atlantic salmon were PRV-positive [29]. Retrospective molecular and genetic tests of archived clinical samples collected in 1974-2013 on Alaska and in British Columbia demonstrated the virus presence in the samples collected from different salmonid species in 1970-s, i.e. before the establishment of Atlantic salmon aquaculture.

The anemia-accompanied disease designated as EIBS was first reported in rainbow trout in 1977 [30] and in 1987 in chinook salmon from the Pacific Northwest of the USA [31]. In 2016, orthoreovirus was isolated from EIBS-infected coho salmon in Japan and it was classified as PRV-2 [7]. Nevertheless, while the cases occurred in 1977 and 1987 precede the evidence of the etiological role played in EIBS, it is still unknown, what etiological agent (PRV-1 or PRV-2) is the cause of the above mentioned erythrocyte lesions. Spread of PRV-2 outside of Japan has not been evidenced so far.

A new HSMI-like disease of freshwater rainbow trout was reported in Norway in 2013. Its agent was designated as PRV-3 [9]. PRV-3 was reported in salmonids with cardiac signs in Chile [10] and in clinically healthy adult rainbow trout in Norway [32]. Over the recent years, in several European countries including Scotland, German, Italy and Denmark, during the disease outbreaks the virus has been detected in rainbow trout both with and without clinical manifestations of the disease [11]. In 2017, H. Hauge et al. published results of the experiments on contact transmission of PRV-3 and demonstrated that the virus of this genotype extensively replicates in the blood of the rainbow trout and it is easily transmitted to a naïve host, while its replication capacity in the Atlantic salmon is limited [12]. PRV-3 is also supposed to be associated with proliferative darkening syndrome (PDS) reported in brown trout in the European pre-alpine countries [33].

Transmission. Horizontal virus transmission was confirmed during the laboratory experiment on infected and contact fish [2]. Based on these experiments the researchers concluded that the virus is excreted and transmitted with water. H. Hauge et al. isolated the virus from the feces of the experimentally infected fish [34]. The maximal amount of the virus was excreted two-three weeks after the experimental infection that agrees with the peak levels of the virus accumulation in blood [6]. Vertical virus transmission is less studied but, though it is unlikely to be the basic one, it should be still taken into account until proven otherwise [26]. The route of the virus introduction into the susceptible organism is not yet known. The possibility of the PRV transmission with the marine species cannot be excluded. In 2012, C. R. Wiik-Nielsen et al. detected PRV genome in greater argentine (*Argentina silus*), capelin (*Mallotus villosus*), Atlantic horse mackerel (*Trachurus trachurus*), and Atlantic herring (*Clupea harengus*) [26]. Therefore, PRV poses a potential threat to the survival of the wild Atlantic salmon.

PATHOGENESIS

PRV target cells include myocytes, erythrocytes and macrophages. At the early stage of the infection the virus affects the salmon's erythrocytes, herewith at the acute phase of the infection the affection may reach 50% over a short period of time [6]. Inclusion bodies similar to those observed at EIBS were detected in the erythrocytes. Unlike the mammalian erythrocytes the fish ones are nucleated cells capable of protein production [35], and therefore they are able to maintain the virus replication. D. Morera and S. A. MacKenzie demonstrated that fish erythrocytes are involved in the immune response [36].

PRV infection of the erythrocytes results in their dysfunction, decrease of the hemoglobin levels and anemia. The fish becomes more sensible to stress factors and hypoxic conditions [37]. Cardiac workload increases and in combination with the virus replication in the cardiac muscle cells it results in the damage and dysfunction of the organ. Heart failure leads to circulation deficiency (stagnation). Post-mortem examination of dead salmon most often reveals the circulation deficiency. The liver fails to perform its function that results in its lesions. PRV replication in the erythrocytes leads to the infectious hemolytic anemia and subsequent jaundice [10]

According to the published data, PRV is the cause of black (melanised) foci in red and white skeletal muscles of cultivated Atlantic salmon in Norway [38]. Poikilothermal animals including fish have a population of melanin-producing leukocytes being a strong anti-oxidant. Focal discoloration of muscles is associated with melanin-producing macrophages (melanomacrophages), which are normal for the lymphoid organs of fish [39]. Their function is protection against oxidative damage. Pathogenesis of the melanised foci is the following: cytotoxic immune cells employ oxidation for the inactivation of pathogens. The virus replication in myocytes (chronic antigen stimulation) results in chronic inflammatory responses. Melanomacrophages concentrate in those inflammation sites where chronic inflammation occurs. Therefore, melanised foci in muscle tissue appear to be the result of autoimmune reactions.

PRV is ubiquitous; however HSMI does not always occur. This suggests that the key triggers of the disease are stress and environmental factors.

CLINICAL SIGNS

HSMI usually occurs in 5–9 months after the Atlantic salmon is transferred from the freshwater cages to the seawater ones. By appearance the diseased fish does not differ from the healthy ones. Ruffled scales, pale gills and exophthalmos are reported in case of acute disease. Behavioral changes are also observed. The diseased fish are lethargic, reduce feed intake and become poor swimmers. They usually swim near the cage net facing the water current that is indicative of the lack of oxygen; mortality also increases [1].

In natural environment HSMI is complicated with co-infection with other viral pathogens (salmonid alphavirus – agent of pancreas disease (PD), infectious hematopoietic necrosis virus (IHNV), piscine myocarditis virus (PMCV) etc.) as well as with bacterial and fungal diseases (*Piscirickettsia salmonis*, *Flavobacterium psychrophilum*, *Saprolegnia* sp. etc.) thus making the clinical presentation unclear [40].

POST-MORTEM LESIONS

Post-mortem examination of the Atlantic salmon demonstrates signs of anemia (pale gills and heart, jaundice), hemodynamic abnormalities and cardiac failure (ascite, enlarged and deformed heart, hemorrhages in the pericardium, swim bladder and visceral fat), liver lesions (hepatomegaly, discoloration and hemorrhages), swollen spleen and kidneys (Fig. 1). One of the most frequent post-mortem lesions is hemopericardium (hemorrhages in pericardial cavity) that induces cardiac tamponade and can result in internal bleeding and death [1, 3, 10, 19]. Pancreas is not damaged in case of HSMI. HSMI post-mortem lesions are indicative of circulatory deficiency (stagnation) due to cardiac failure.

Main post-mortem HSMI-like lesions in Chilean farmed Atlantic coho salmon include pale gills, hemopericardium, pale heart and yellow liver [10].

Muscle lesions are often reported at later stages of the disease. Spread of melanised foci in the rainbow

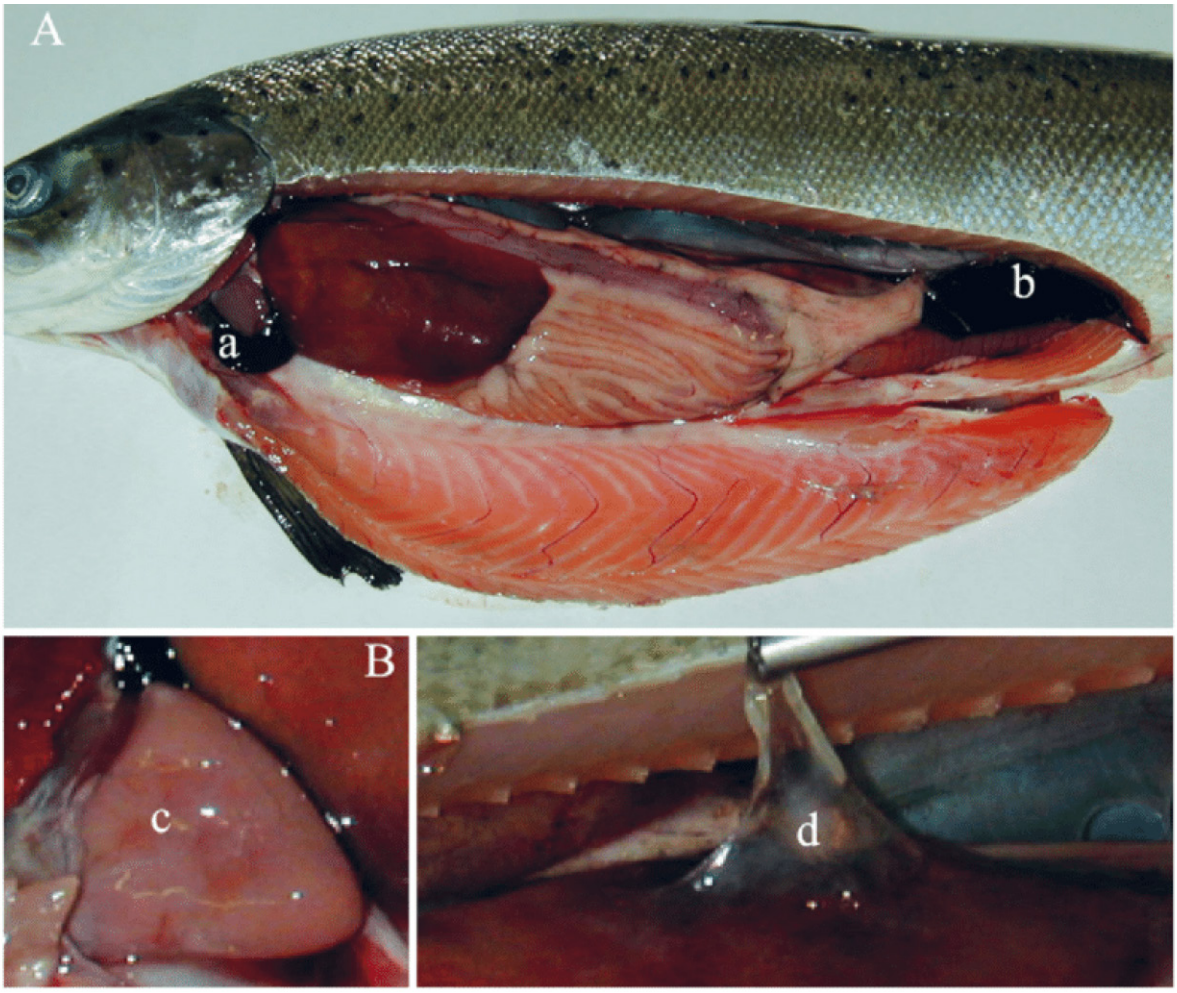

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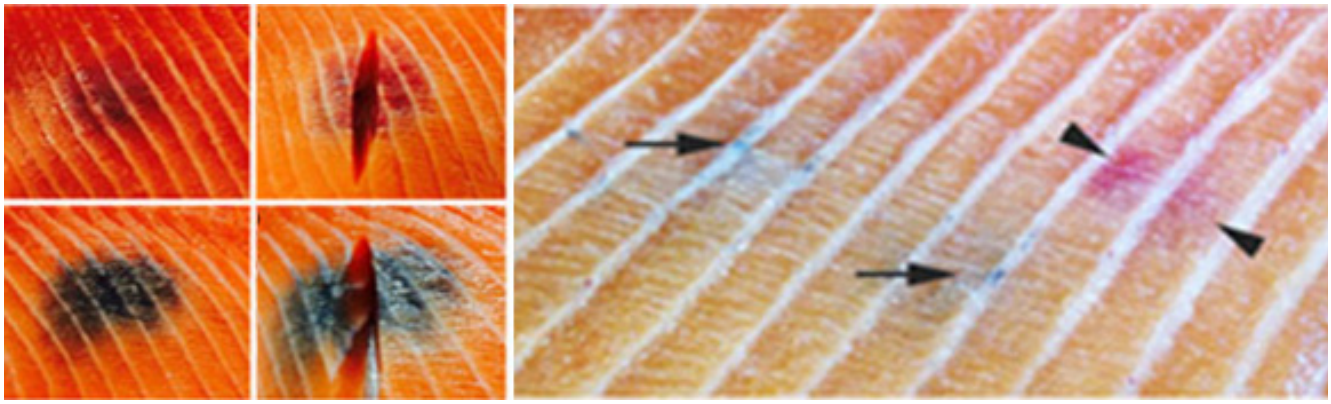
Fig. 1. Salmo salar. Macroscopic lesions in heart and skeletal muscle inflammation
A – haemopericardium (a), swollen spleen (b);
B – pale heart (c), fibrinous layer on the liver (d).

Рис. 1. Макроскопические изменения при воспалении сердечных и скелетных мышц (HSMI) у атлантического лосося
A – гемоперикард (a), увеличение селезенки (b);
B – бледное сердце (c), фибриновая пленка на печени (d).



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(https://veterinaryresearch.biomedcentral.com/track/pdf/10.1186/s13567-015-0244-6)

Fig. 2. Muscle lesions in case of aggregated signs of heart and skeletal muscle inflammation (HSMI)

Рис. 2. Изменения в мышцах при симптомокомплексе воспаления сердечных и скелетных мышц (HSMI)

trout muscles is insignificant in spite of aquaculture environment similar to the one of the Atlantic salmon (Fig. 2). There are no reports of similar lesions in wild salmonids [38].

One more HSMI sign is jaundice syndrome in chinook salmon and coho salmon. The jaundice syndrome is associated with the infectious hemolytic anemia due to PRV replication in erythrocytes [10].

HISTOPATHOLOGY

Histopathological HSMI lesions are reported in the heart and red skeletal muscle (myocarditis and red skeletal muscle necrosis), Fig. 3. During the degeneration and necrosis of cardiac muscle cells both compact and spongy myocardiums are involved in the extensive inflammation. Infiltrates are composed of monocytes. Extensive epicarditis is reported that is generally closely related to myocarditis. Myodegeneration and necrosis are observed at later stages of the disease. Red muscle inflammation occurs similarly to the one in the heart: degeneration signs, loss of cross striation, eosinophilia, vacuolization and karyorrhexis appear in the affected myocytes. Focal hepatic necrosis, circulatory deficiency, edema and erythrocyte accumulation are observed in some organs [1, 2, 3, 6, 27]. Histopathological lesions in coho salmon with PRV-3-associated disease were different from those reported in orthoreovirus-infected Atlantic salmon and rainbow trout in Norway. In the Chilean coho salmon the heart inflammation was diffuse and as a rule involved the spongy myocardium; red muscle lesions were absent or insignificant [10].

The key histological signs of jaundice include hemorrhages in all organs and severe hemosiderosis in combination with erythrophagocytosis in kidneys and spleen [10].

IMMUNITY

PRV infection activates both cellular and antibody immune responses [41]. As it was mentioned above, cardiac tissues are the site of the virus replication. The immune response to the virus replication involves emergence of

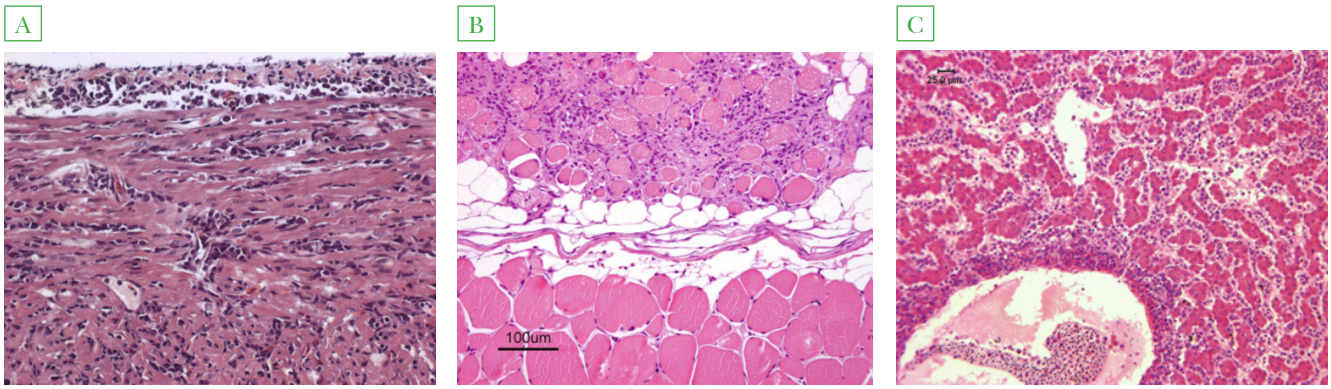

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Fig. 3. HSMI histopathological lesions
A – heart inflammation;
B – red muscle degeneration and inflammation (top);
C – liver inflammation.

Рис. 3. Гистопатологические поражения при ВССМ
А – воспаление в сердце;
В – дегенерация и воспаление красных мышц (вверху);
С – воспаление в печени.

cytotoxic T-lymphocytes (CD8+) in the heart that is indicative of this immune response to be directed to the virus-infected cells. O. W. Finstad et al. noted that cytotoxic cellular responses to the infected cells play an important role in HSMI immunity. However, such responses are directly accountable for the development of lesions in the heart and skeletal muscles. Atlantic salmon erythrocytes are the target cells for PRV replication [6], and they can play an immediate role in the immune response [36]. The erythrocytes react to the infection by means of the interferon-mediated immune response [42], which is capable of further protection against IHNV, salmonid alphavirus and, probably, other infectious agents [40, 43].

DIAGNOSTIC TOOLS

HSMI diagnosis is based on the presence of histologically recovered signs of myocardium inflammation extending to epicardium and endocardium, myocardium necrosis and red skeletal muscle myositis and necrosis [1] in combination with reverse transcription quantitative PCR (RT-qPCR) results. The target tissue for diagnosis usually involves the heart as the virus presence is to be related to the cardiac muscle pathology. HSMI should be differentiated from pancreas disease and cardiomyopathy syndrome (CMS) [45].

PREVENTION AND CONTROL

Heart and skeletal muscle inflammation (HSMI) is not subject to notification to the World Organization for Animal Health (OIE). This PRV-associated disease was officially reported only in Norway [4, 46] and Chile [10]. HSMI was excluded from National List-3 and in Norway it is currently registered as a non-notifiable disease [27].

In spite of specific antibodies produced after the PRV infection [41], the virus is ubiquitous in the populations of the virus-susceptible fish. This is indicative of the immune system's failure to eliminate the virus completely, and this poses the challenge of the effective vaccine development. Some production companies and research institutions have officially targeted on the development of HSMI vaccines using various strategies (whole-virion vaccines, recombinant subunit vaccines and DNA-vaccines). No vaccines have been developed so far.

The diseased fish is extremely sensitive to stress factors and hypoxic conditions. PRV diagnostic testing of the fish on aquaculture farms is needed. In case of PRV-positive fish detection and in order to reduce HSMI-associated losses one should abandon from immunosuppressive and stressful treatments, limit the fish transportation and take maximal care of the fish. All these will decrease the cardiac workload and result in the reduction of mortality.

Use of disinfectants reducing the amount of the viruses and opportunistic bacteria in the environment is appropriate for the disease prevention. Anti-inflammatory agents also demonstrated benefits during HSMI control. There are reports on functional feeds having antioxidant and anti-inflammatory actions that aid to the reduction of HSMI-associated losses and share of the fish discarded due to melanised spots in the muscle [47].

One more effective HSMI control tool involves breeding and culture of the disease-resistant fish.

CONCLUSION

Heart and skeletal muscle inflammation is an infectious fish disease with high morbidity and long disease course. During fish farming intensification of the culture process is inevitable and it involves increased stocking thus aiding to the spread of the pathogen, increase of stress and reduction of the disease resistance. Currently, there is a tendency towards the wide spread of the disease (European countries, North and South Americas); new virus variants are detected and the range of the disease susceptible fish species is expanded. The PRV-induced disease is the third significant disease of cultured Atlantic salmon in Norway. Heart and skeletal muscle inflammation is ubiquitous in cultured and wild Atlantic salmon in Norway. Since 2010, from 100 to 200 HSMI affected sites have been annually reported in Norway [13]. The highest number of the disease outbreaks is reported in the middle and northern parts of Norway that are bordering on the Murmansk Oblast. Up to 20% of the Atlantic salmon fillet produced at the Norwegian fish processing plants demonstrate melanised foci [48] thus impairing the product quality and increasing the economic losses born by the aquaculture sector due to the disease. All that is indicative of the fact that PRV-infection consequences can be graver as it was previously supposed.

In spite of the above mentioned HSMI data, in 2014 the Norwegian Food Safety Authority delisted the disease from National List-3 of notifiable diseases. Therefore, the decrease of the disease-affected sites being reported since 2015 is unlikely to fit the reality. Furthermore, there is no any national HSMI control program is Norway, and no positive impact on the situation can be envisaged.

Piscine orthoreovirus is reported in migratory wild Atlantic salmon [38] that is indicative of natural long-distance spread of the virus. This fact is supported by high genetic similarity of the PRV variants isolated on different continents [10].

The above mentioned data reflect a major threat of PRV introduction to the Russian Federation from the neighboring countries.

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Polymerase chain reaction for detection of some highly dangerous viral fish disease agents

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SUMMARY
Today viral fish diseases cause major losses in the world aquaculture. Pathogen spread often occurs during the transportation of fish from infected farms to the disease-free ones. Therefore, the import of fish stocking material to Russia from countries with a different epidemic situation requires risk-based monitoring and forecasting. Diagnostics is of primary importance in the complex of measures to prevent the spread of viral infections in fish. To date, laboratory diagnostics of viral fish diseases is based on pathogen isolation and its identification using serological methods which require a lot of time and are performed only in large research institutes with specialized laboratories. Molecular diagnostic methods are more sensitive and high-performance. The article presents the results of using reverse transcription polymerase chain reaction to detect a number of highly dangerous viral diseases of fish (*Salmonidae*). As a result of this work, primers were selected and the temperature and time conditions of the reaction were optimized for the identification of infectious hematopoietic necrosis, viral hemorrhagic septicemia and infectious salmon anemia. The results obtained during the research allowed us to establish that this diagnostic method is highly specific with analytical sensitivity to infectious salmon anemia virus of 2.5 lg TCD_{50}/cm^3, to infectious hematopoietic necrosis – 2.9 lg TCD_{50}/cm^3 and to viral hemorrhagic septicemia – 4.2 lg TCD_{50}/cm^3. The described method was used to identify reference and field strains available at the FGBI ARRIAH Reference Laboratory for Aquaculture Diseases and isolated in different years in fish farms in the territory of the Russian Federation. The research data correlated with the results obtained from virus neutralization in cell culture and ELISA performed using commercial kits. The proposed method of RT-PCR allows to detect pathogens both in fish with pronounced clinical signs and in latent virus carriers.

Key words: fish diseases, infectious salmon anemia, viral hemorrhagic septicemia, infectious hematopoietic necrosis, reverse transcription polymerase chain reaction, specificity, analytical sensitivity.

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Применение полимеразной цепной реакции для выявления возбудителей некоторых особо опасных вирусных болезней рыб

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РЕЗЮМЕ
В настоящее время основной ущерб мировой аквакультуре наносят вирусные болезни рыб. Возбудители болезней часто проникают из одних рыбоводных хозяйств в другие при перевозках инфицированных рыб из неблагополучных хозяйств в благополучные. Поэтому завоз рыбопосадочного материала в Россию из стран с различной эпизоотологической ситуацией требует мониторинга и прогнозирования на основе возможных рисков. Ведущее место в комплексе мероприятий по предотвращению распространения вирусных инфекций рыб занимает диагностика. На сегодняшний день лабораторная диагностика болезней рыб вирусной природы основана на выделении возбудителя и его идентификации серологическими методами, которые требуют больших затрат времени и выполняются только в крупных научно-исследовательских институтах, имеющих профильные лаборатории. Более чувствительными и высокопроизводительными являются молекулярные методы диагностики. В статье представлены результаты применения полимеразной

цепной реакции с обратной транскрипцией по выявлению ряда особо опасных вирусных болезней рыб семейства лососевых. В результате проделанной работы были подобраны праймеры и оптимизированы температурно-временные условия проведения реакции для идентификации инфекционного некроза гемопоэтической ткани, вирусной геморрагической септицемии и инфекционной анемии лососевых. Результаты, полученные в ходе исследований, позволили установить, что данный диагностический метод является высокоспецифичным с аналитической чувствительностью для вируса инфекционной анемии лососевых – 2,5 lg $ТЦД_{50}/см^3$, инфекционного некроза гемопоэтической ткани – 2,9 lg $ТЦД_{50}/см^3$ и вирусной геморрагической септицемии – 4,2 lg $ТЦД_{50}/см^3$. С помощью описанного метода была проведена идентификация референтных и полевых штаммов, имеющихся в референтной лаборатории по болезням аквакультуры ФГБУ «ВНИИЗЖ» и выделенных в разные годы в рыбоводческих хозяйствах на территории Российской Федерации. Данные, полученные в ходе исследований, коррелировали с результатами вирусовыделения на культуре клеток и иммуноферментного анализа с использованием коммерческих наборов. Предложенная методика проведения полимеразной цепной реакции с обратной транскрипцией позволяет обнаружить возбудителей болезней у рыб как с явными клиническими признаками, так и со скрытым вирусоносительством.

Ключевые слова: болезни рыб, инфекционная анемия лососевых, вирусная геморрагическая септицемия, инфекционный некроз гемопоэтической ткани, полимеразная цепная реакция с обратной транскрипцией, специфичность, аналитическая чувствительность.

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INTRODUCTION

Today viral fish diseases have a world-wide distribution. About a quarter of the detected viruses cause diseases that result in serious losses in aquaculture. Among the most dangerous diseases are infectious hematopoietic necrosis, infectious salmon anemia, viral hemorrhagic septicemia, etc.[1] Despite the fact that most infections do not pose an immediate risk to human health, they have a negative impact on the fish growth rate, its marketable condition, the quality of fish products, and are accompanied by high mortality rates [1].

Disease prevention, i.e. prevention of pathogen introduction into disease-free regions, is the major tool for controlling infectious fish diseases all over the world. Therefore, the import of fish stocking material to Russia from countries with different epidemic situations requires risk-based monitoring and forecasting [2, 3, 4].

Diagnostics is of primary importance in preventing the spread of viral fish infections. To date, laboratory diagnosis of viral fish diseases is based on pathogen isolation and its identification using serological methods which require a lot of time and are performed only in large research institutes with specialized laboratories [1, 5].

Molecular diagnostic methods are more sensitive and require less time than culture and serological methods traditionally used to identify pathogens of viral fish diseases. Over the past decades, molecular diagnosis of diseases (including fish diseases) has advanced significantly. Such methods include polymerase chain reaction (PCR) which allows to detect the virus both in fish with pronounced clinical signs and in latent virus carriers[2].

In Russia there aren't any methods that are low cost, easy to use and, most importantly, high sensitive. Only imported kits and test systems for viral fish disease diagnosis are available on the domestic market.

The purpose of this study was to assess the possibility of using reverse transcription polymerase chain reaction (RT-PCR) for the detection and identification of highly dangerous viral diseases of fish (*Salmonidae*), such as viral hemorrhagic septicemia, infectious hematopoietic necrosis and infectious salmon anemia.

MATERIALS AND METHODS

The following strains were used in the study: Orenburg/14 strain of spring viraemia of carp virus (SVCV), Arkus 32/87 strain of infectious hematopoietic necrosis virus (IHNV), FLD/2004 strain of infectious pancreatic necrosis virus (INPV) and Aland strain of viral haemorrhagic septicaemia virus (VHSV), which are stored in the collection of microbial strains at the FGBI "ARRIAH", as well as CCBB strain of infectious salmon anemia virus (ISAV), obtained from the American Type Culture Collection (ATCC) in 2019.

The total RNA of the studied viruses was isolated using RNA-Extran commercial kit (Syntol, Russia) according to the recommendations of the manufacturer. The synthesis of cDNA from an RNA template was performed using OT-1 Test kit (Syntol, Russia), according to the recommendations of the manufacturer. The PCR was performed in a 25 µl reaction mixture using Encyclo Plus PCR kit (ZAO "Evrogen", Russia) according to the package insert. PCR was performed in a PTC-200 DNA Engine Cycler (Bio-Rad, USA). Previously published PCR primers were used [6, 7, 8]. The primers were synthesized by the Syntol company (Russia).

Amplicons obtained during PCR were analyzed by electrophoresis using 2% agarose gel and 10 mg/ml ethidium bromide in 1× trisborate buffer at 10 V/cm for one hour. PCR product (15 µl) was added to each well.

[1] Vasilkov G. V., Grishchenko L. I., Engashev V. G., Kanaev A. I., Larkova Z. I., Osetrov V. S. Fish Diseases: Reference book. Ed. by V. S. Osetrov. 2nd ed., revised. M.: Agropromizdat; 1989. 288 p.

[2] Voronin V. N., Kuznetsova E. V., Strelkov Y. A., Chernyshova N. B. Fish Diseases in Aquaculture in Russia: Practical guidance. SPb.: FSBSI "VNIRO"; 2011. 264 p.

M100 (5 µl; Syntol, Russia) was used as a molecular weight marker (100 bp increment). The gel was documentedusing Gel Doc XR+ Documentation System (Bio-Rad, USA).

The reaction sensitivity was analyzed by using ten-fold serial dilutions of viral cDNA. The analytical sensitivity limit was considered to be the highest dilution at which a positive result was registered.

RESULTS AND DISCUSSION

Based on the analysis of the studied viruses' genome sequences represented in the GenBank database of the U.S. National Center for Biotechnology Information (NCBI), and the recommendations of the World Organization for Animal Health (OIE), primers specific for conserved regions of the eighth segment of the ISA virus, of nucleocapsid (N) gene of VHSV and of G gene of IHNV, allowing to identify the maximum number of known strains and isolates of these viruses, were selected. The primer sequences are presented in Table 1.

During a series of experiments, temperature and time conditions for PCR were optimized. When amplifying a fragment of the IHN virus genome, the number of cycles was increased from 30 to 35, which allowed a larger number of amplicons to be produced, thereby increasing the sensitivity of the reaction. When amplifying a fragment of the ISA virus genome, the annealing time was reduced from 45 to 30 seconds, and the synthesis time was reduced from 90 to 60 seconds, which allowed to reduce the reaction time without losing its effectiveness. When amplifying a fragment of the VHS virus genome, the annealing temperature was increased from 52 to 55 °C, which increased the specificity of the reaction.

The optimized amplification parameters for the studied infections are presented in Table 2.

The specificity of the selected primers was tested experimentally during the study of cultures inoculated with SVC, VHS, IPN, IHN and ISA viruses using RT-PCR. The virus genome fragments obtained after amplification were analyzed using agarose gel. The gel was examined under UV-light (wavelength 312 nm) and the reaction results were interpreted based on the presence or absence of luminous bands.

Figure 1 shows the electrophoregrams of the analysis.

From the data presented in Figure 1, it can be seen that for wells No. 2 containing IHN, ISA and VHS virus strains, clear fragments are observed at 693 bp (*a*), 211 bp (*b*) and 811 bp (*c*), respectively. As for the other wells, including the ones with negative control, these fragments are not observed, which indicates the high specificity of the selected primers for detecting the pathogens causing the above-mentioned infections and the absence of contamination of the used reagents of the tested or foreign RNA.

Analytical sensitivity was estimated on ten-fold serial dilutions of cDNA obtained from reverse transcription with the RNA extracted from ISA, VHS and IHN virus cultures which had initial infectivity titres of 5.5; 7.2 and 6.9 lg TCD_{50}/cm^3, respectively. The sensitivity limit was considered to be the highest dilution at which a positive result was registered. The calculated values of the analytical sensitivity of the optimized PCR were 2.5 lg TCD_{50}/cm^3 for the ISAV, 2.9 lg TCD_{50}/cm^3 for the IHNV, and 4.2 lg $TCD_{50}/см^3$ for the VHSV.

The results of analytical sensitivity testing are provided in Figure 2 and Table 3.

Table 1
Design of primers for detection of pathogens causing infectious salmon anemia, viral hemorrhagic septicemia and infectious hematopoietic necrosis

Таблица 1
Структура праймеров для выявления возбудителей инфекционной анемии лососевых, вирусной геморрагической септицемии и инфекционного некроза гемопоэтической ткани

Primer sequence (5′–3′)	Size of PCR-product, bp	Reference
ISAV		
F-GAAGAGTCAGGATGCCAAGACG	211	M. Devold et al. [6]
R-GAAGTCGATGAACTGCAGCGA		
VHSV		
F – GGGGACCCCAGACTGT	811	OIE. Manual of Diagnostic Tests for Aquatic Animals [8]
R-TCTCTGTCACCTTGATCC		
IHNV		
F – AGAGATCCCTACACCAGAGAC	693	E. J. Emmenegger et al. [7]
R – GGTGGTGTTGTTTCCGTGCAA		

F – forward primer;
R – reverse primer.

Table 2
Amplification parameters

Таблица 2
Параметры амплификации

PCR stage	Number of cycles	Temperature, °C	Stage duration, min
Pre-denaturation	1	94	3
Denaturation	35	94	1/2
Annealing of primers		59 (ISA) 50 (IHN) 55 (VHS)	1/2
Synthesis		72	1
Final synthesis	1	72	7
Storage	1	4	20

Thus, the proposed RT-PCR method allows to detect ISA, IHN and VHS viruses at their minimum infectivity titres of 2.5; 2.9 and 4.2 lg $TCD_{50}/см^3$, respectively. The specificity of the selected primers was tested using the BLAST program, as well as experimentally. The PCR results presented in Figure 1 show that the selected primers hybridize only with the fragments complementary to the target viral RNA and do not interact with the RNA of nontargets.

The described method was used to identify reference and field strains isolated on fish farms in the territory of the Russian Federation in different years and available at the FGBI "ARRIAH" Reference Laboratory for Aquaculture

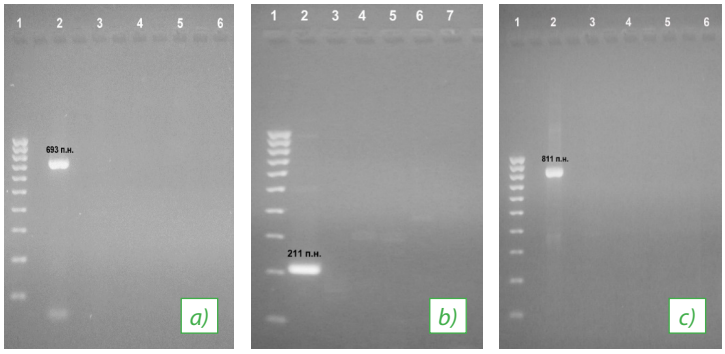

Fig. 1. Electrophoregrams of RT-PCR specificity testing results for detection of infectious hematopoietic necrosis (IHN) (a), infectious salmon anemia (b) and viral hemorrhagic septicemia (c) viruses

a) 1 – molecular weight markers "M100";
2 – Arcus 32/87 strain of the IHN virus;
3 – Aland strain of the viral hemorrhagic septicemia (VHS) virus;
4 – FLD/2004 strain of infectious pancreatic necrosis (IPN) virus;
5 – Orenburg/14 strain of the spring veremia of carp (SVC) virus;
6 – negative control (sterile water free of RNase and DNase);
b) 1 – molecular weight markers "M100";
2 – CCBB strain of the infectious salmon anemia (ISA) virus;
3 – Orenburg/14 strain of the spring veremia of carp (SVC) virus;
4 – Aland strain of the VHS virus;
5 – Arcus 32/87 strain of the IHN virus;
6 – FLD/2004 strain of infectious pancreatic necrosis (IPN) virus;
7 – negative control (sterile water free of RNase and DNase);
c) 1 – molecular weight markers "M100";
2 – Aland strain of the VHS virus;
3 – Arcus 32/87 strain of the IHN virus;
4 – FLD/2004 strain of infectious pancreatic necrosis (IPN) virus;
5 – Orenburg/14 strain of the spring veremia of carp (SVC) virus;
6 – negative control (sterile water free of RNase and DNase).

Рис. 1. Электрофореграммы результатов тестирования специфичности метода ОТ-ПЦР для выявления вирусов ИНГТ (a), ИАЛ (b) и ВГС (c)

a) 1 – маркер молекулярных весов «M100»;
2 – штамм «Аркус 32/87» вируса ИНГТ;
3 – штамм «Аланд» вируса ВГС;
4 – штамм «FLD/2004» вируса ИНПЖ;
5 – штамм «Оренбург/14» вируса ВВК;
6 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);
b) 1 – маркер молекулярных весов «M100»;
2 – штамм «ССВВ» вируса ИАЛ;
3 – штамм «Оренбург/14» вируса ВВК;
4 – штамм «Аланд» вируса ВГС;
5 – штамм «Аркус 32/87» вируса ИНГТ;
6 – штамм «FLD/2004» вируса ИНПЖ;
7 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);
c) 1 – маркер молекулярных весов «M100»;
2 – штамм «Аланд» вируса ВГС;
3 – штамм «Аркус 32/87» вируса ИНГТ;
4 – штамм «FLD/2004» вируса ИНПЖ;
5 – штамм «Оренбург/14» вируса ВВК;
6 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз).

Table 3
Determination of optimized RT-PCR analytical sensitivity

Таблица 3
Результаты определения аналитической чувствительности оптимизированной ОТ-ПЦР

No.	Dilution	Virus infectivity titre, Lg TCD_{50}/см^3	RT-PCR result
		ISAV	
1	original sample	5.5	+
2	10^{-1}	4.5	+
3	10^{-2}	3.5	+
4	10^{-3}	2.5	+
5	10^{-4}	1.5	–
6	10^{-5}	0.5	–
		IHNV	
1	original sample	6.9	+
2	10^{-1}	5.9	+
3	10^{-2}	4.9	+
4	10^{-3}	3.9	+
5	10^{-4}	2.9	+
6	10^{-5}	1.9	–
		VHSV	
1	original sample	7.2	+
2	10^{-1}	6.2	+
3	10^{-2}	5.2	+
4	10^{-3}	4.2	+
5	10^{-4}	3.2	–
6	10^{-5}	2.2	–

"+" – positive result;
"–" – negative result.

Diseases. The data obtained in the studies using RT-PCR correlated with the results obtained by virus isolation in cell culture and by ELISA using commercial kits produced by TestLine Clinical Diagnostics Ltd. (Czech Republic) and Bio-X Diagnostics S.A. (Belgium).

The results presented in the article on the use of conventional gel-based RT-PCR show that despite the high effectiveness of this method, it is still inferior to more modern molecular genetic diagnostic methods, such as real-time RT-PCR and microchip-based RT-PCR. The main advantages of the latter two methods: they are more sensitive, less time consuming and there is reduced contamination due to the absence of the gel electrophoresis. Despite a number of advantages, real time RT-PCR is not widely used in our country for viral fish disease diagnosis and was used only by M. I. Doronin et al. together with the employees of the company OOO "Lumex-marketing" (Saint Petersburg) for IHNV and VHSV detection [9].

It should be mentioned that in Russia data on the use of RT-PCR for diagnosis of such viral fish diseases as IHN, VHS and ISA is scarce. There is a limited number of publications on the development and use of this molecular genetic method [2, 9, 10, 11], while it has become commonly used abroad. In many countries, selective amplification of viral genome fragments using PCR with subsequent sequencing is used not only for the diagnosis of IHN, VHS and ISA viruses, but also for the development of methods for their typing [12, 13, 14].

CONCLUSION

As a result of the research, primers were selected and the temperature and time conditions of PCR were optimized to detect such viral fish diseases as viral hemorrhagic septicemia, infectious hematopoietic necrosis and infectious salmon anemia.

The results demonstrate high specificity of this diagnostic method and its analytical sensitivity of 2.5 lgTCD_{50}/см^3 when detecting infectious salmon anemia virus, 2.9 lg TCD_{50}/см^3 – infectious hematopoietic necrosis virus and 4.2 lg TCD_{50}/см^3 – viral hemorrhagic septicemia virus. The RT-PCR allowed to reduce testing time to 5 hours, while the "gold" standard – virus isolation – takes several weeks.

The proposed method can be used to detect ISA, IHN and VHS viruses in monitoring studies of pathological material samples collected from salmonid fishes. The peculiarity of this method is that it allows to detect infected fish before the onset of clinical signs, in latent virus carries, which is especially important for cross-border transportation of hydrobionts.

Upon the results of the studies, guidelines for ISA, IHN and VHS virus identification using gel-based RT-PCR were prepared and approved by the Scientific Council of the FGBI "ARRIAH".

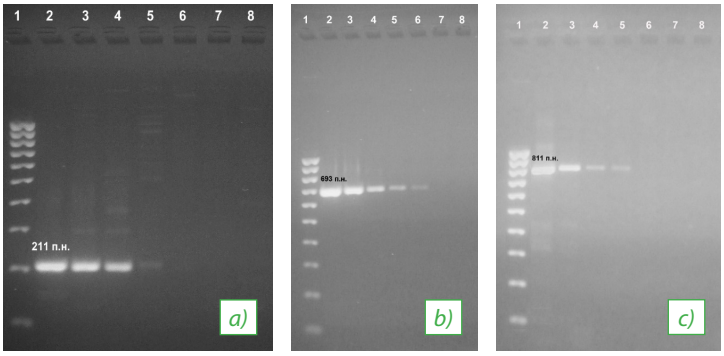

Fig. 2. Electrophoregrams showing PCR analytical sensitivity testing results for detection of ISA (a), IHN (b) and VHS (b) viruses

a) *1 – molecular weight markers "M100";*
2 – original sample (CCBB strain of the ISA virus);
3–7 – a series of ten-fold dilutions of CCBB strain of the ISA virus (10^{-1}–10^{-5});
8 – negative control (sterile water free of RNase and DNase);

b) 1 – molecular weight markers "M100";
2 – original sample (Arcus 32/87 strain of the IHN virus);
3–7 – a series of ten-fold dilutions of Arcus 32/87 strain of the IHNV (10^{-1}–10^{-5});
8 – negative control (sterile water free of RNase and DNase);

c) 1 – molecular weight markers "M100";
2 – original sample (Aland strain of the VHS virus);
3–7 – a series of ten-fold dilutions of Aland strain of the VHS virus (10^{-1}–10^{-5});
8 – negative control (sterile water free of RNase and DNase).

Рис. 2. Электрофореграммы результатов тестирования аналитической чувствительности ПЦР для выявления вирусов ИАЛ (a), ИНГТ (b) и ВГС (c)

a) 1 – molecular weight markers «M100»;
2 – исходный образец (штамм «CCBB» вируса ИАЛ);
3–7 – серия десятикратных разведений штамма «CCBB» вируса ИАЛ (10^{-1}–10^{-5});
8 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);

b) 1 – molecular weight markers «M100»;
2 – исходный образец (штамм «Аркус 32/87» вируса ИНГТ);
3–7 – серия десятикратных разведений штамма «Аркус 32/87» вируса ИНГТ (10^{-1}–10^{-5});
8 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз);

c) 1 - molecular weight markers «M100»;
2 – исходный образец (штамм «Аланд» вируса ВГС);
3–7 – серия десятикратных разведений штамма «Аланд» вируса ВГС (10^{-1}–10^{-5});
8 – отрицательный контроль (стерильная вода, свободная от РНКаз и ДНКаз).

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Miramistin effect on BHK-21 and PSGK-30 cell line proliferation and FMD virus reproduction in these cells

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SUMMARY
At present, Miramistin drug (benzyldimethyl [3-(myristoylamino) propyl] ammonium chloride monohydrate), which has a broad bactericidal effect, is used in veterinary practice. This antiseptic is active against most viruses, mycoplasmas, bacteria, fungi and protozoa. According to toxicometric parameters, Miramistin is classified as a low-hazard substance. Use of cell structures as test systems for assessing the toxicity of pharmacological substances instead of conventional tests on experimental animals allows us to better clarify the possible mechanism of the effect of the investigated substance. Since many types of mycoplasmas affect the genitourinary system organs, to assess the cytotoxicity of Miramistin kidney mammalian cells of various mammals can be used as test systems, in particular, the newborn Syrian hamster (VNK-21), Siberian mountain ibex (PSGK-30), and others. The possibility of using BHK-21 and PSGK-30 cell monolayer as test systems for assessing the baseline cytotoxicity of Miramistin was shown. When studying toxicity of the drug, the effect of its various concentrations on the cell morphology was studied, the number of viable cells and the total protein content were determined as an indicator of the cell mass increase. The results of a cytomorphological study indicate that the Miramistin maximum permissible concentration of for BHK-21 and PSGK-30 cell monolayer is 25 μg/cm^3. The use of this antibacterial drug in higher concentrations caused the appearance and increased signs of endogenous intoxication and degeneration. When evaluating the proliferative activity of cells under the influence of the Miramistin antibiotic in different concentrations, it was found that increasing the dose to 50, 75, 100, 125, 150 μg/cm^3 leads to decrease in the rate of cell growth compared to the control. The Miramistin content in the growth medium in an amount of up to 25 μg/cm^3 did not affect the intensity of protein synthesis. Presence of Miramistin in the culture medium at the maximum permissible concentration causes a slight decrease in the reproduction of foot and mouth disease virus in BHK-21 and PSGK-30 cell cultures by 4.5 and 4.0%, respectively, compared with the control without antibiotic. Since mycoplasmas are the most common contaminants of cell cultures, further studies will be aimed at exploring the possibility of using Miramistin for decontamination of BHK-21 and PSGK-30 cell monolayers.

Key words: Miramistin, BHK-21 and PSGK-30 cell monolayers, Mycoplasma.

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Влияние мирамистина на пролиферацию клеток линий ВНК-21 и ПСГК-30 и репродукцию вируса ящура в них

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РЕЗЮМЕ

В настоящее время в ветеринарной практике применяется препарат мирамистин (бензилдиметил[3-(миристоиламино)пропил] аммоний хлорид моногидрат), обладающий широким бактерицидным действием. Данный антисептик активен против большинства вирусов, микоплазм, бактерий, грибов и простейших. По параметрам токсикометрии мирамистин классифицируется как малоопасное вещество. Использование клеточных структур в качестве тест-систем для оценки токсичности фармакологических веществ вместо классических тестов на экспериментальных животных позволяет лучше прояснить возможный механизм действия исследуемого вещества. Поскольку многие виды микоплазм поражают органы мочеполовой системы, для оценки цитотоксичности мирамистина в качестве тест-систем можно использовать клетки почек разных млекопитающих, в частности новорожденного сирийского хомячка (ВНК-21), сибирского горного козерога (ПСГК-30) и другие. Показана возможность применения монослойных клеточных ВНК-21 и ПСГК-30 в качестве тест-систем для оценки базовой цитотоксичности мирамистина. В процессе исследования токсичности препарата изучали влияние его различных концентраций на морфологию клеток, определяли количество жизнеспособных клеток и содержание общего белка как показателя прироста клеточной массы. Результаты цитоморфологического исследования свидетельствуют о том, что предельной допустимой концентрацией мирамистина для монослойных клеточных линий ВНК-21 и ПСГК-30 является 25 мкг/см^3. Применение данного антибактериального препарата в бóльших концентрациях вызывало появление и нарастание признаков эндогенной интоксикации и дегенерации. При оценке пролиферативной активности клеток под влиянием антибиотика мирамистина в разных концентрациях выявлено, что увеличение дозы препарата до 50, 75, 100, 125, 150 мкг/см^3 приводит к снижению кратности прироста клеток по сравнению с контролем. Содержание в ростовой среде мирамистина в количестве до 25 мкг/см^3 не влияло на интенсивность синтеза протеинов. Наличие в культуральной среде мирамистина в предельно допустимой концентрации вызывает незначительное снижение репродукции вируса ящура в культурах клеток ВНК-21 и ПСГК-30 на 4,5 и 4,0% соответственно по сравнению с контролем без антибиотика. Поскольку микоплазмы являются наиболее распространенными контаминантами клеточных культур, дальнейшие исследования будут направлены на изучение возможности применения мирамистина для деконтаминации монослойных клеточных линий ВНК-21 и ПСГК-30.

Ключевые слова: мирамистин, монослойные клеточные линии ВНК-21, ПСГК-30, микоплазма.

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INTRODUCTION

Mycoplasmosis of animals is a group of infectious diseases caused by microorganisms such as *Tenericutes*, *Mollicutes* class, *Mycoplasmataceae* family, *Mycoplasma* genus. Mycoplasmas are the most common cell culture contaminants. These prokaryotic microorganisms tightly adhere to the cell membrane, receive various nutrients and growth factors from it. Mycoplasma metabolites, including hydrogen peroxide and ammonia, accumulate in the culture medium and can have a pronounced cytopathic effect [1, 2, 3]. Mycoplasmas cause significant metabolic disorder in the cells, the consequence of which may be changes in the level of RNA and protein synthesis, the composition of the cell membrane, the appearance of chromosomal aberrations, induction or activation of cytokine expression, and a sharp decrease in proliferative activity [4]. Irreversible changes in the cells caused by the influence of mycoplasmas and their metabolic products lead to a distortion of the results of laboratory studies in which cell lines are used as test systems.

Mycoplasmas are resistant to sulfonamides, penicillins, streptomycin, but are sensitive to tetracyclines, macrolides, and fluoroquinolones [1, 3]. At present, Miramistin (benzyldimethyl[3-(myristoylamino)propyl] ammonium chloride monohydrate), which has a broad bactericidal effect, is used in veterinary practice. The drug belongs to the group of cationic surfactants, namely to quaternary ammonium compounds. According to the toxicometric parameters, Miramistin is classified as a low-hazard substance[1]. The drug is active against most viruses, bacteria, fungi and protozoa. The mechanism of Miramistin action is manifested in the process of active interaction of this compound with lipids and polysaccharides of the cytoplasmic membrane of mycoplasmas, leading to their death [2, 5].

Analysis of the microorganism ability to multiply and grow on media containing a decreasing concentration of a drug substance allows us to determine the minimum inhibitory concentration (MIC) of an antibiotic[2] that inhibits the life of mycoplasma *in vitro*. According to A. M. Dunaevsky et al., the MIC for Miramistin is 10–100 μg/cm^3 [6].

A study of the biological activity of antibiotics involves assessment of their cytotoxicity, which manifests itself in the occurrence of pathological changes in cells [1]. Depending on the concentration of the active substance and the sensitivity of cell receptors, a wide range of changes can be observed in the cell, which can manifest themselves in the form of a cytostatic effect or in the form of a cytocidal effect that causes its death [4].

To assess the toxicity of pharmacological substances, currently test systems using monolayer cell cultures are

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widely used instead of classical tests on experimental animals [5, 7, 8]. Since a large number of mycoplasma species affect the organs of the genitourinary system, cells obtained from the kidneys of various mammals, in particular, the newborn Syrian hamster (BHK-21), Siberian mountain capricorn (PSGK-30), and others, are used as test systems to assess the cytotoxicity of Miramistin. A wide range of methods are used to study the cytotoxicity of antibiotics in cell culture, including determining the number of viable cells, colony formation, measuring the concentration of total cellular protein, assessing morphological changes in cells, and others [8].

The purpose of this study was to evaluate the effect of Miramistin on proliferation of BHK-21 and PSGK-30 cells, as well as on the reproduction of foot and mouth disease virus in them.

MATERIALS AND METHODS

Antibiotic. Miramistin antibiotic (LLC Scandia Eco, Russia) with concentrations of 5, 10, 25, 50, 75, 100, 125 and 150 μg/cm^3 in the culture medium was used for analysis. Four controls were used in the study: No. 1 – tetracycline (5 μg/cm^3); No. 2 – spiramycin (10 μg/cm^3); No. 3 – ciprofloxacin (18 μg/cm^3) [9]; No. 4 is a control, which is a cell suspension in a growth medium without antibiotics.

Test systems. To assess the baseline cytotoxicity of Miramistin, BHK-21 and PSGK-30 monolayer cell cultures, free of mycoplasma, were used as a test system.

A quantitative method for evaluating the effects of Miramistin on cell cultures. The effect of Miramistin on BHK-21 and PSGK-30 cell monolayer was evaluated by cell viability during three consecutive passages. The analysis consisted of counting the number of living and dead cells after dispersing the monolayer with a mixture of trypsin and versene (1:1) at a temperature of 37 °C. Proliferation intensity was estimated by the Proliferation Index (PI), using the following formula:

$$PI = a/b,$$

where a – live cell concentration in 48 hours after inoculation;

b – inoculation concentration.

When PI > 1, cell growth is observed, when PI = 1, no cell proliferation is observed, when PI < 1, cell death is observed.

Spectrophotometric method for assessing cell proliferation using a gentian violet. BHK-21 and PSGK-30 cells were seeded in 96-well culture plates in the amount of 250–300 cells per well. Miramistin in predetermined concentrations was added to the growth medium during the cell seeding. The cells without antibiotics as well as cells with tetracycline, spiramycin and ciprofloxacin in the specified above concentrations were used as the controls. After 48 hours of cultivation, the cells were fixed with a 70% ethanol solution and stained with a 0.1% gentian violet solution. To extract the dye from the cells, a 7% acetic acid solution in a volume of 200 μl per well was used. The proliferative activity of cells was determined by the optical density for a suspension of gentian violet associated with cellular proteins at a 570 nm wavelength.

Qualitative assessment of cell viability consisted in intravital visual observation under an inverted microscope of the cell morphology after 2 days of cultivation using an isotonic 0.4% aqueous solution of trypan blue.

Virus. The FMD Master seed virus (A/Zabaykalsky/2013 strain), obtained in the PSGK-30 cell culture at passage 5, was used as seed for infection of the specified cell monolayer at a dose of 0.05 TCD_{50}/cell. Virus reproduction was carried out for 15–17 hours until the appearance of a 95–100% cytopathic effect.

Determination of the FMDV 146S component. The amount of 146S particles of foot and mouth disease virus in inactivated material was determined using a quantitative variant of the complement fixation test [10].

Statistical data processing. The test was performed in triplicate. The data obtained were statistically processed, calculating the arithmetic mean values, the degree of reliability of the statistical difference between the average values determined by the Fisher's difference method. The differences were considered statistically significant at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

At the first stage of the study the Miromistin baseline cytotoxity in BHK-21 и PSGK-30 monolayers widely used as diagnostic test-systems in veterinary practice was determined. The choice of the antibiotic concentration was based on the data of its clinical tests [2, 3], as well as on the results of the Miramistin MIC analysis for mycoplasma, which falls within the range of 10–100 μg/cm^3 [2, 3, 6]. On that basis, the following tested Miramistin concentrations were selected: 5, 10, 25, 50, 75, 100, 125, 150 μg/cm^3. Cultivation of cells with the specified amount of the drug was performed during three consecutive passages.

Cytomorphological differences in the control and experimental samples. Cells without antibiotics, as well as grown in media with tetracycline (5 μg/m^3), spiramycin (10 μg/cm^3), ciprofloxacin (18 μg/cm^3) had a distinct plasma membrane, a clear nuclear membrane, and transparent homogeneous cytoplasm (Fig. 1). In 48 hours after each passage in the control samples cell clones were dense and formed by more than 100 cells. Morphological characters of BHK-21 and PSGK-30 cells with Miramistin contents of 5, 10, and 25 μg/cm^3 were most similar to the control. For these variants the clones were dense and were formed by 85–100 cells. Clones of the BHK-21 и PSGK-30 cell lines as a result of cultivation in the miramestin supplemented medium at concentrations 50, 75, 100, 125 μg/cm^3 in comparison with the control without the antibiotic had a loose structure and contained 65–75, 45–60, 25–40, 15–25 cells respectively.

When using 150 μg/cm^3 of Miramistin, clones were formed from single cells characterized by significant granularity and an increased number of lysosomes. The presented morphological changes were the result of local cytoplasmic degradation that develops in the cell under the influence of a toxic factor such as excessive Miramistin. In other words, the results of a cytomorphological study indicate that maximum permissible concentration of Miramistin for BHK-21 and PSGK-30 cell monolayer is 25 μg/cm^3. The use of this antibacterial drug in higher concentrations resulted in the appearance and increase in signs of endogenous intoxication and degeneration.

A study was made of the effect of Miramistin in test concentrations on the proliferation index of BHK-21 and PSGK-30 cell lines basing on the results of three consecutive passages in comparison with the controls. The results are shown in the table and in Figure 2.

From the data presented in the table, it follows that the Proliferation Index of the BHK-21 cell line in the controls without antibiotic was 2.18 ± 0.16, and in the controls

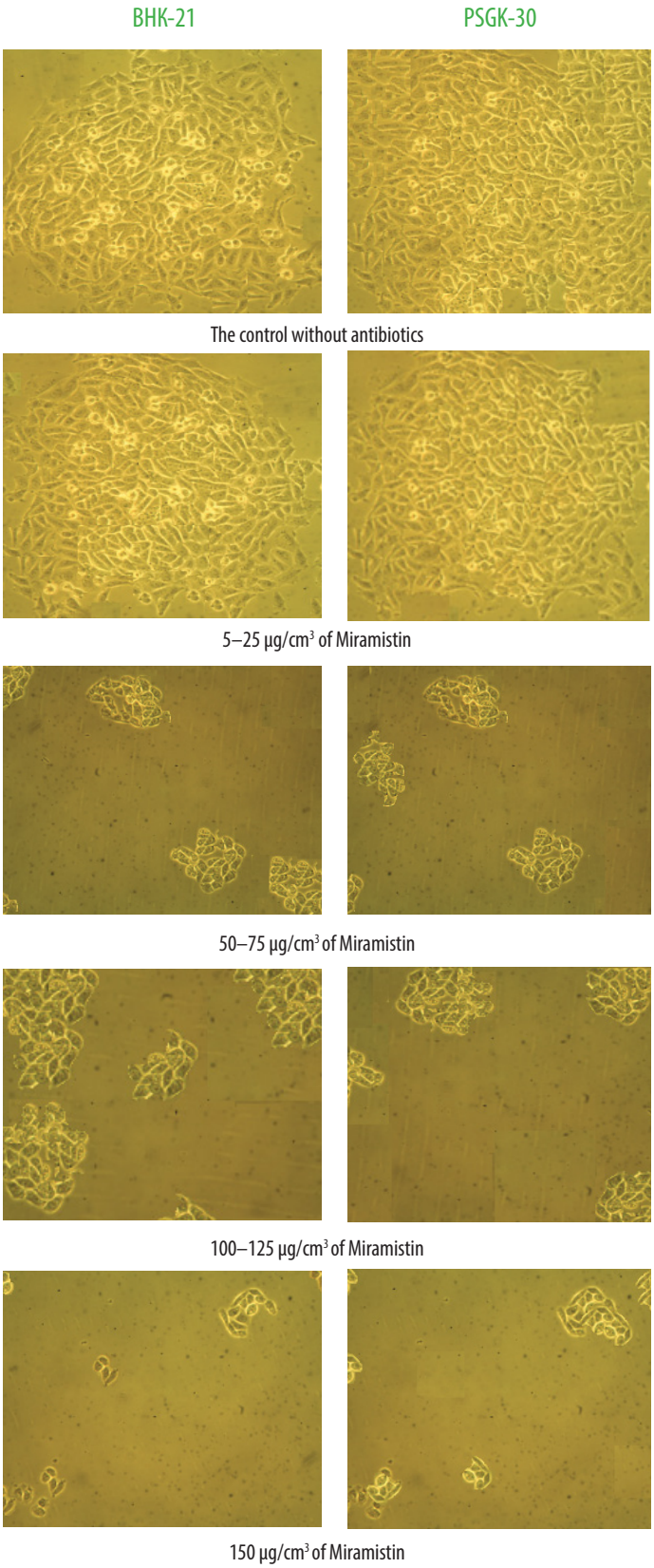

Fig. 1. Morphology of BHK-21 and PSGK-30 cell lines in clones, formed in 48 hours of cultivation in the medium supplemented with different Miramistin concentrations

Fig 1. Морфология клеток линий BHK-21 и ПСГК-30 в клонах, образовавшихся через 48 ч культивирования в среде с мирамистином в различных концентрациях

with antibiotics – 2.17 ± 0.19 – 2.18 ± 0.17. When using Miramistin in amounts of 5–25 μg/cm³, this parameter corresponded to 2.17 ± 0.19 – 2.18 ± 0.16. An increase in the dose of the drug to 50, 75, 100, 125, 150 μg/cm³ led to a decrease in the cell proliferation index compared to the control by 4.6; 5.4; 7.5; 10.9 and 21.7 times, respectively. If the amount of antibiotic in the growth medium was 150 μg/cm³, a visible cell death was observed. Thus, as a result of studying the proliferation of BHK-21 cells, it was determined that Miramistin in concentrations up to 25 μg/cm³ does not induce the cytotoxic effect.

For PSGK-30 cells, the proliferation index in the control without antibiotic was 2.00 ± 0.15, in the medium with control antibiotics – 1.88 ± 0.15 – 1.97 ± 0.14. The proliferative potential of PSGK-30 cells when using Miramistin at concentrations of 5–25 μg/cm³ was at the control level and was equal to 1.93 ± 0.18 – 1.97 ± 0.15. When the content of this antibiotic in the medium was 50, 75, 100, 125, 150 μg/cm³, the cell proliferation index, compared to the control, decreased by 4.9; 5.3; 6.8; 10.0 and 21.1 times, respectively. When 150 μg/cm³ of Miramistin was added into the growth medium, cell death was observed. Thus, when assessing the proliferation index in comparison with the control parameters, it was revealed that the growth of the PSGK-30 cell monolayer in a medium containing up to 25 μg/cm³ of Miramistin did not have a cytotoxic effect.

Cell proliferation was also evaluated by the relative determination of the total cellular protein using spectrophotometry. The results of the study are presented in Figure 3, from which it follows that the protein content of BHK-21 cells grown in a medium with 5, 10, 25 μg/cm³ of Miramistin corresponded to control parameters of optical density and amounted to 0.57–0.59. When the specified antibiotic was added into the medium at concentrations of 50, 75, 100, 125, 150 μg/cm³, the amount of cellular proteins decreased by 4.3; 5.1; 8.0; 11.2 and 18.7 times, respectively, compared to the control (optical density was 0.13; 0.11; 0.07; 0.05; 0.03). Thus, results of total cellular protein determination compared to the control, revealed that cultivation of the BHK-21 cell monolayer in the medium with up to 25 μg/cm³ of Miramistin did not reduce the translation intensity.

The amount of synthesized total protein of PSGK-30 cells grown in the medium with 5, 10, 25 μg/cm³ of Miramistin was at the level of control parameters (optical density was 0.58–0.60). In the presence of the indicated antibiotic in the medium at concentrations of 50, 75, 100, 125, 150 μg/cm³, the amount of cellular protein decreased by 4.8; 5.3; 7.3; 9.7 and 19.3 times, respectively, compared to the control (optical density was 0.12; 0.11; 0.08; 0.06; 0.03). In other words, results of total cellular protein determination compared to the control, revealed that that cultivation of the PSGK-30 cell monolayer in a medium containing up to 25 μg/cm³ of Miramistin did not reduce the translation intensity.

According to the results of Miramistin cytotoxic potential assessmen, it was revealed that this antibacterial drug does not have any destructive effect on BHK-21 and PSGK-30 monolayers at a concentration of up to 25 μg/cm³. At the same time, basing on the literature sources [2, 3, 6] it is know that the antibiotic concentrations presented are included in the MIC range for representatives of the *Mycoplasma* genus, and therefore, Miramistin in the indicated amounts allows the prevention of mycoplasma contamination of BHK-21 and PSGK-30 cell lines.

Table
Assessment of BHK-21 and PSGK-30 cell line proliferative activity influenced by different Miramistin concentrations ($n = 3, p < 0.05$)

Таблица
Оценка пролиферативной активности клеток линий BHK-21 и ПСГК-30 под влиянием антибиотика мирамистина в разных концентрациях ($n = 3, p < 0,05$)

Cell line	Passage number	Cell Proliferation index											
		In the medium supplemented with different Miramistin concentrations (μg/cm³)								Control			
										With antibiotics			Without antibiotics
		5	10	25	50	75	100	125	150	No. 1	No. 2	No. 3	No. 4
BHK-21	1	2.21 ± 0.15	2.17 ± 0.17	2.22 ± 0.14	0.45 ± 0.15	0.40 ± 0.14	0.31 ± 0.13	0.22 ± 0.13	0.10 ± 0.15	2.22 ± 0.16	2.20 ± 0.17	2.22 ± 0.20	2.23 ± 0.19
	2	2.18 ± 0.15	2.20 ± 0.20	2.10 ± 0.15	0.47 ± 0.17	0.40 ± 0.14	0.29 ± 0.14	0.20 ± 0.12	0.09 ± 0.14	2.20 ± 0.20	2.22 ± 0.20	2.10 ± 0.13	2.10 ± 0.15
	3	2.15 ± 0.18	2.15 ± 0.21	2.20 ± 0.14	0.49 ± 0.11	0.40 ± 0.11	0.29 ± 0.11	0.18 ± 0.11	0.11 ± 0.11	2.12 ± 0.19	2.10 ± 0.19	2.20 ± 0.20	2.20 ± 0.14
	M ± m	2.18 ± 0.16	2.17 ± 0.19	2.17 ± 0.14	0.47 ± 0.14	0.40 ± 0.14	0.29 ± 0.13	0.20 ± 0.13	0.10 ± 0.12	2.18 ± 0.17	2.17 ± 0.19	2.17 ± 0.17	2.18 ± 0.16
PSGK-30	1	1.90 ± 0.14	2.01 ± 0.15	1.95 ± 0.12	0.37 ± 0.18	0.35 ± 0.18	0.30 ± 0.18	0.18 ± 0.18	0.10 ± 0.16	1.80 ± 0.17	2.03 ± 0.15	1.95 ± 0.14	1.95 ± 0.14
	2	1.98 ± 0.17	1.90 ± 0.21	2.00 ± 0.15	0.39 ± 0.21	0.34 ± 0.17	0.28 ± 0.16	0.19 ± 0.12	0.09 ± 0.17	2.00 ± 0.15	1.81 ± 0.20	2.06 ± 0.16	2.05 ± 0.15
	3	2.02 ± 0.17	1.88 ± 0.17	1.90 ± 0.16	0.40 ± 0.17	0.39 ± 0.17	0.27 ± 0.17	0.20 ± 0.12	0.09 ± 0.13	1.92 ± 0.14	1.80 ± 0.17	1.90 ± 0.14	2.00 ± 0.15
	M ± m	1.97 ± 0.15	1.93 ± 0.18	1.95 ± 0.15	0.39 ± 0.19	0.36 ± 0.16	0.28 ± 0.13	0.19 ± 0.11	0.09 ± 0.13	1.91 ± 0.15	1.88 ± 0.15	1.97 ± 0.14	2.00 ± 0.15

No. 1 – tetracycline (5 μg/cm³), No. 2 – spiramycin (10 μg/cm³), No. 3 – ciprofloxacin (18 μg/cm³), No. 4 – without antibiotic (w/a).

At the next stage, Miramistin influence on the sensitivity of BHK-21 PSGK-30 cell monolayers to the reproduction of foot and mouth disease virus was studied using the example of A/Zabaykalsky/2013 strain. Cells grown in a nutrient medium with 25 μg/cm³ of Miramistin were used for infection. The virus culture medium contained the same amounts of antibiotic that were added to the growth medium. The results of the test are shown in Figure 4.

From the data presented in Figure 4, it follows that as a result of the FMDV, A/Zabaykalsky/2013 strain cul-

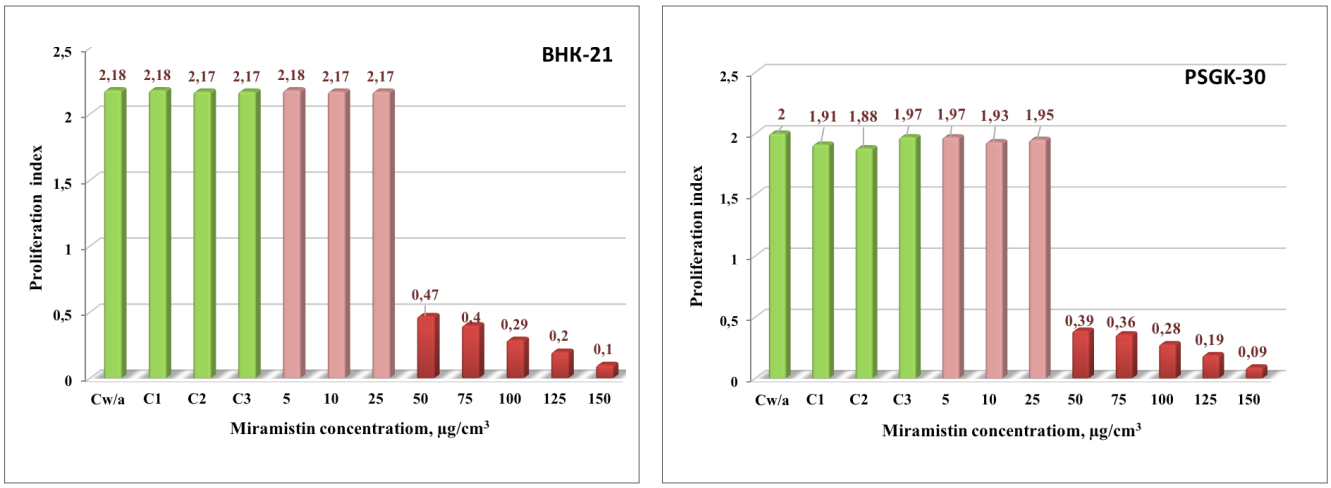

Fig. 2. Assessment of proliferation index of BHK-21 and PSGK-30 cell lines cultivated in the medium with different Miramistin concentrations (5, 10, 25, 50, 75, 100, 125, 150 μg/cm³)
Cw/a – control without antibiotics;
C1 – tetracycline-supplemented medium (5 μg/cm³);
C2 – spiramycin-supplemented medium (10 μg/cm³);
C3 – ciprofloxacin-supplemented medium (18 μg/cm³).

Рис. 2. Оценка кратности прироста клеток линий BHK-21 и ПСГК-30, выращенных в среде с разным количеством мирамистина (5, 10, 25, 50, 75, 100, 125, 150 мкг/см³)
Cw/a – контроль без антибиотика;
C1 – среда с тетрациклином (5 мкг/см³);
C2 – среда со спирамицином (10 мкг/см³);
C3 – среда с ципрофлоксацином (18 мкг/см³).

tivation in BHK-21 cells grown with the addition of Miramistin, the concentration of the 146S component in the suspension was 0.64 ± 0.05 μg/cm³. The specified content of complete FMDV particles correlates with data for the control without antibiotics (0.67 ± 0.05 μg/cm³) and for the control with tetracycline (0.62 ± 0.05 μg/cm³), spiramycin (0.63 ± 0,06 μg/cm³) and ciprofloxacin (0.63 ± 0.05 μg/cm³). Therefore, the use of Miramistin at a concentration of 25 μg/cm³ does not reduce the sensitivity of the BHK-21 cell monolayer to infection and reproduction of foot and mouth disease virus.

During the reproduction of FMDV A/Zabaikalsky/2013 strain in PSGK-30 cells cultured in Miramistin-supplemented medium, the amount of 146S particles was 0.48 ± 0.05 μg/cm³. The obtained values correlate with the data for the control without antibiotic (0.50 ± 0.06 μg/cm³) and for the controls with tetracycline (0.46 ± 0.07 μg/cm³), spiramycin (0.48 ± 0.05 μg/cm³) and ciprofloxacin (0.46 ± 0.08 μg/cm³). Thus, Miramistin in an amount of 25 μg/cm³ does not reduce the sensitivity of the PSGK-30 monolayer to infection and reproduction of foot and mouth disease virus.

The results of the tests performed confirm that the presence of Miramistin antibiotic in the specified above maximum permissible concentrations in the growth and maintenance media does not cause a decrease in the concentration of the 146S component of the FMD virus compared to other drugs against mycoplasmas and the control without antibiotics.

CONCLUSION

The performed study showed the possibility of using BHK-21 and PSGK-30 cell monolayers as test systems for assessing Miramistin baseline cytotoxicity.

According to the results of assessing the cytotoxic potential of this antibacterial drug, when analyzing the morphological state and the cell proliferation index, as well as the amount of synthesized cell proteins, it was found that Miramistin did not have a toxic effect on BHK-21 and PSGK-30 cell monolayers at a maximum dose of 25 μg/cm³. The use of Miramistin in higher concentrations caused the appearance and growth of signs of endogenous intoxication, which manifested itself in a changed cell morphology, appearance of granularity and an increase in the number of lysosomes, as well as in a decrease in the cell proliferation index and proteins synthesized by them.

It was found that addition of 25 μg/cm³ of Miramistin in the growth and maintenance medium did not cause a decrease in the concentration of the 146S component during the reproduction of foot-and-mouth disease virus unlike addition of other antibiotics against mycoplasmas or no antibiotics at all. As the result of FMDV A/Zabaikalsky/2013 strain cultivation in BHK-21 cell line grown in a medium with 25 μg/cm³ of Miramistin, the concentration of the 146S component was 0.64 ± 0.05 μg/cm³, which is 4.5% lower than the control without an antibiotic; 3.2; 1.6 and 1.6% higher in comparison with tetracycline, spiramycin and ciprofloxacin. When foot-and-mouth disease virus was reproduced in PSGK-30 cells, which were also cultured in a medium with 25 μg/cm³ of Miramistin, the amount of 146S particles was 0.48 ± 0.05 μg/cm³, which is 4.0% lower compared to the control without antibiotic, 4.3% higher compared to tetracycline and spiramycin and equal for the medium with spiramycin.

Further research will be aimed at studying the possibility of using Miramistin for decontamination of BHK-21 and PSGK-30 cell monolayers from mycoplasmas.

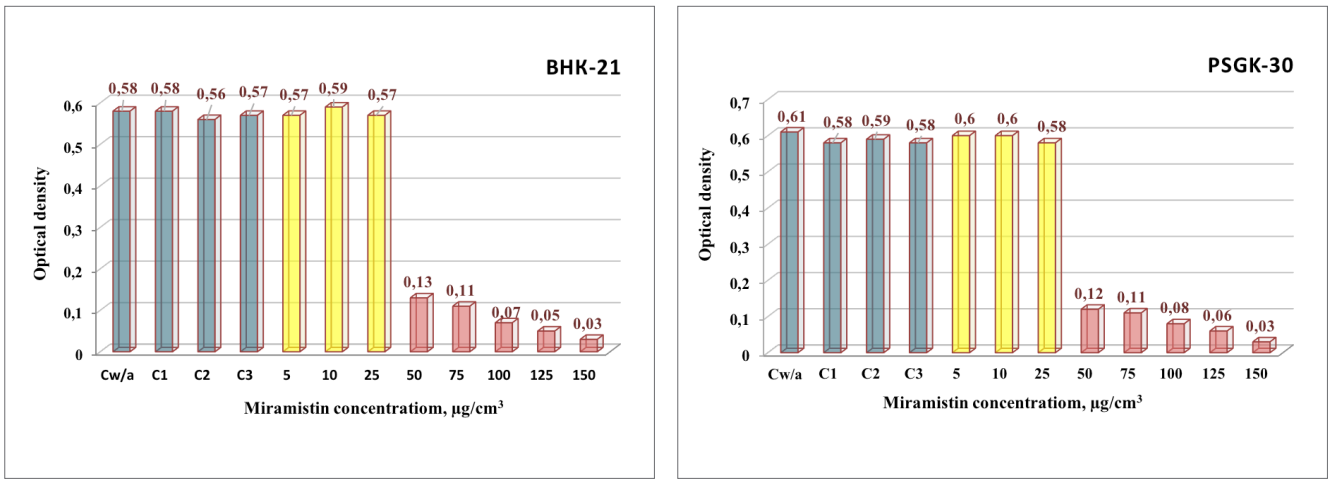

Fig. 3. Assessment of BHK-21 and PSGK-30 cell line proteins in 48 hours cultivation in the medium with different Miramistin concentrations (5, 10, 25, 50, 75, 100, 125, 150 μg/cm³)
Cw/a – control without antibiotics;
C1 – tetracycline-supplemented medium (5 μg/cm³);
C2 – spiramycin-supplemented medium (10 μg/cm³);
C3 – ciprofloxacin-supplemented medium (18 μg/cm³).

Рис. 3. Гистограммы оценки протеинов клеток линий BHK-21 и ПСГК-30 через 48 ч культивирования в среде с разным количеством мирамистина (5, 10, 25, 50, 75, 100, 125, 150 мкг/см³)
Cw/a – контроль без антибиотика;
C1 – среда с тетрациклином (5 мкг/см³);
C2 – среда со спирамицином (10 мкг/см³);
C3 – среда с ципрофлоксацином (18 мкг/см³).

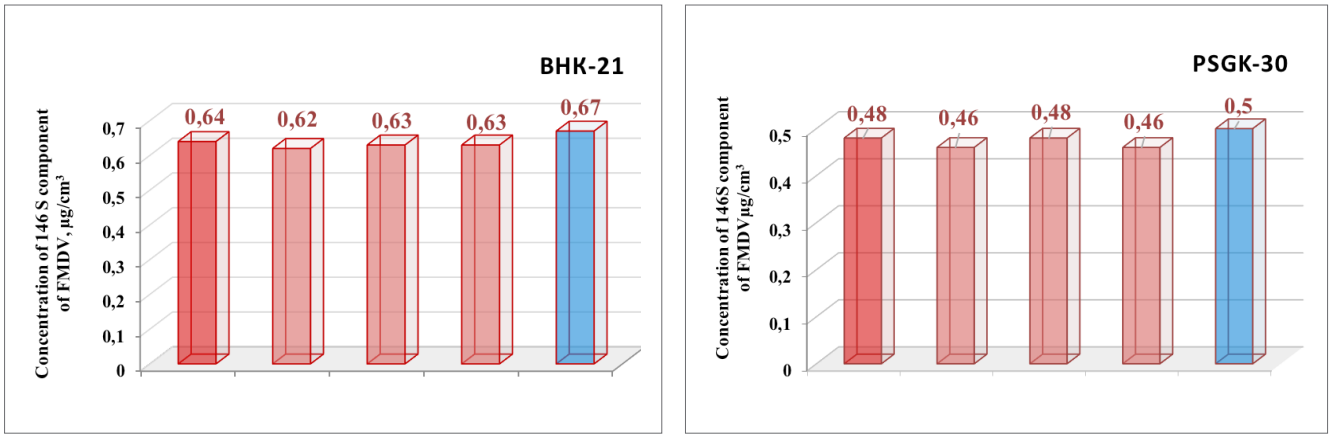

Fig. 4. Concentration of 146 S component of FMDV strain A/Zabaikalsky/2013 in inactivated suspensions after reproduction in BHK-21 and PSGK-30 cell lines cultivated in the Miramistin-supplemented medium compared with the controls
1 – Miramistin-supplemented medium;
2 – tetracycline-supplemented medium (5 μg/cm³);
3 – spiramycin-supplemented medium (10 μg/cm³);
4 – ciprofloxacin-supplemented medium (18 μg/cm³);
5 – control without antibiotics.

Рис. 4. Концентрация 146S компонента вируса ящура штамма A/Забайкальский/2013 в инактивированных суспензиях после репродукции в клетках линий BHK-21 и ПСГК-30, культивируемых в среде с мирамистином, в сравнении с контролями
1 – среда с мирамистином;
2 – среда с тетрациклином (5 мкг/см³);
3 – среда со спирамицином (10 мкг/см³);
4 – среда с ципрофлоксацином (18 мкг/см³);
5 – контроль без антибиотика.

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Studies on humoral immunity in dogs after use of rabies inactivated vaccines formulated with Montanide ISA 70 VG and GEL 01 adjuvants

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SUMMARY
Despite all the efforts made, the issue of rabies in the world does not lose its relevance. As an acute endemic infection, it poses a considerable danger to both animals and humans. The leading role in the transmission of rabies to humans belongs to diseased dogs; stray animals can be potential sources of rabies agent, which increases the risk of transmitting a deadly virus to humans. Rabies prevention in dogs around the world is carried out by counting the number of these animals, their mandatory routine immunization and evaluating the effectiveness of vaccination against the accumulation of rabies virus-neutralizing antibodies. Inactivated vaccines based on different viral strains formulated with a wide range of adjuvants are used to induce a protective level of humoral immunity against rabies in dogs (≥ 0.5 IU/cm^3), which allows vaccines with high safety and effectiveness for the target animal species to be obtained. The article presents the results of the study of humoral immunity in 20 non-pedigree dogs 21 days after the administration of rabies inactivated vaccines based on culture rabies virus from ARRIAH strain formulated with various adjuvants. The presented rabies vaccines formulated with oil adjuvant Montanide ISA 70 VG and gel adjuvant Montanide GEL 01 were innocuous and safe and induced strong immunity in all vaccinated animals. The vaccine formulated with Montanide ISA 70 VG adjuvant in case of a single administration in the dose of 1.0 cm^3 induces formation of rabies virus-neutralizing antibodies in the level of 2.4 times higher than the vaccine formulated with Montanide GEL 01 adjuvant. The highest levels of rabies antibodies in dogs were 48.1 ± 3.7 and 28.3 ± 1.5 IU/cm^3 and were observed with the use of rabies inactivated emulsion vaccine in the doses of 3.0 and 1.0 cm^3 respectively.

Key words: rabies, inactivated vaccine against rabies, humoral immunity, rabies virus-neutralizing antibodies, dogs.

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Изучение гуморального иммунитета собак при использовании антирабических инактивированных вакцин, изготовленных с применением адъювантов Montanide ISA 70 VG и GEL 01

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РЕЗЮМЕ

Несмотря на все прилагаемые усилия, проблема бешенства в мире не теряет своей актуальности. Являясь острой природно-очаговой инфекцией, представляет огромную опасность как для животных, так и для человека. Ведущая роль в процессе передачи вируса бешенства человеку принадлежит заболевшим собакам, безнадзорные животные могут быть потенциальными источниками возбудителя бешенства, что повышает риск передачи смертельно опасного вируса человеку. Профилактика бешенства среди собак во всем мире осуществляется путем учета численности этого вида животных, их обязательной регулярной иммунизации и оценки эффективности вакцинации по уровню накопления антирабических вируснейтрализующих антител. Для формирования защитного уровня гуморального иммунитета у собак против бешенства (≥ 0,5 МЕ/см^3) применяют инактивированные вакцины, полученные на основе различных штаммов вируса с использованием широкого спектра адъювантов, что позволяет получать вакцины с высокими показателями безопасности и эффективности для целевых видов животных. В статье представлены результаты исследования гуморального иммунитета у 20 беспородных собак на 21 сут после введения антирабических инактивированных вакцин из культурального вируса бешенства штамма «ВНИИЗЖ» с применением различных адъювантов. Представленные вакцины против бешенства, изготовленные с использованием масляного адъюванта Montanide ISA 70 VG и гелевого адъюванта Montanide GEL 01, были авирулентными, безвредными и индуцировали напряженный иммунитет у всех привитых животных. Вакцина на основе адъюванта Montanide ISA 70 VG при однократном введении в дозе 1,0 см^3 способствует выработке вируснейтрализующих антирабических антител в 2,4 раза выше по сравнению с препаратом, полученным с использованием адъюванта Montanide GEL 01. Наиболее высокие титры антител против бешенства у собак составляли 48,1 ± 3,7 и 28,3 ± 1,5 МЕ/см^3 и были отмечены при использовании антирабической инактивированной эмульсионной вакцины в дозах 3,0 и 1,0 см^3 соответственно.

Ключевые слова: бешенство, инактивированная вакцина против бешенства, гуморальный иммунитет, антирабические вируснейтрализующие антитела, собаки.

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INTRODUCTION

Rabies is a major viral disease of humans and animals and also one of the most dangerous zoonoses, causing lesions of the central nervous system, encephalomyelitis, and paralysis with inevitable lethal outcome. The agent belongs to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus,* species *Rabies lyssavirus* [1].

Rabies is a world-wide problem, to which international organizations (WHO, OIE, FAO, GARC) and veterinary services of many countries pay special attention [2, 3]. The disease leads to significant costs associated with animal mortality, management of outbreak consequences, preventive and quarantine measures, management of wild animal population, catching of stray cats and dogs and diagnostic testing [4, 5]. It is estimated that the annual global economic loss from rabies is more than $8.6 billion [6].

The virus spreads easily, so the disease can become an epizootic. A large number of natural foci of rabies is mainly maintained due to wild animals, though the pathogen is also transmitted to domestic animals, and this fact requires increased attention because of their close proximity to humans [7]. It is considered that the leading role in the process of rabies virus transmission to humans belongs to diseased dogs because of their high sensitivity to the virus as well as a number of biological and ecological features peculiar to them (tendency to form packs, ability to move considerable distances, etc.). Most cases of rabies transmission from dogs to humans are associated with viral saliva getting into wounds inflicted by bites [4, 8]. Currently, more than half of the population of the Russian Federation has companion animals. At the same time, the problem of free-roaming dogs is acute, and their packs have become common in cities, towns and settlements [7, 9]. They may be potential sources of the rabies agent, which increases the risk of transmitting the deadly virus to humans.

Rabies prevention in dogs worldwide is carried out by taking into account the number of this animal species, its mandatory routine immunization, and by assessing the effectiveness of vaccination against the level of accumulation of rabies virus-neutralizing antibodies (VNA). To induce a protective level of humoral immunity in dogs against rabies (≥ 0.5 ME/cm^3) [2], inactivated vaccines obtained from different rabies virus strains and formulated with a wide range of adjuvants are used, which allows to obtain vaccines with high safety and efficiency indicators for target animal species [2, 5, 9, 10, 11].

The aim of this work was to study the possibility of protecting dogs from rabies through the use of rabies inactivated vaccines based on "ARRIAH" strain of rabies virus and formulated with Montanide ISA 70 VG and GEL 01 adjuvants.

MATERIALS AND METHODS

Vaccines. Experimental batches of rabies inactivated vaccines were made based on "ARRIAH" rabies virus strain, reproduced in suspension cell line from the Syrian baby hamster kidney (BHK-21). Rabies virus was inactivated with aminoethylethylenimine (AEEI) solution. The suspension of the inactivated antigen was purified from ballast proteins by simple sedimentation. To enhance immune response, vaccines were formulated with Montanide ISA 70 VG oil adjuvant, consisting of mineral oil and non-ionic emulsifier, and Montanide GEL 01 gel adjuvant, based on highly purified finely dispersed sodium polyacrylate (SEPPIC, France) (Table 1).

Laboratory animals. We used thirty non-pedigree dogs weighing 10-15 kg, and twenty white mice weighing 10-12 g.

All tests on animals were conducted in strict accordance with the Interstate Standards for the keeping and care of laboratory animals GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

Immunization of animals. For the purposes of the research, all dogs were divided into 6 groups per 5 animals, the vaccines were administered by intramuscular route. The following animals were immunized with rabies inactivated adsorbed vaccine: animals of group 1 (No. 1–5) were immunized with a single undiluted dose of 1.0 cm^3, animals of group 2 (No. 6–10) – at a dose of 3.0 cm^3, animals of group 3 (No. 11–15) – twice at a dose of 1.0 cm^3 with inoculation interval of 7 days. Rabies emulsion vaccine was administered to dogs of group 4 (No. 16–20) a single time at a dose of 1.0 cm^3, group 5 (No. 21–25) – at a dose of 3.0 cm^3, group 6 (No. 26–30) – twice at a dose of 1.0 cm^3 with an interval between inoculations of 7 days (Table 2).

Evaluation of innocuity of rabies virus antigen in cell culture. The innocuity of the inactivated rabies virus suspension was controlled by inoculation into the monolayer continuous BHK-21 cell line during three consecutive passages using rabies immunoglobulin G labeled with FITC. As a positive control, we used a freeze-dried suspension of non-inactivated rabies virus of "ARRIAH" strain with infectivity titer of 7.00 lg TCID50/cm^3. A freeze-dried suspension of inactivated rabies virus served as negative control. The material was considered avirulent if no fluorescent glow specific to rabies virus was detected in any of the three consecutive passages [2].

Evaluation of safety of rabies inactivated vaccines on white mice. Before administration to white mice, the emulsion vaccine against rabies was destroyed up to the isolation of the antigenic phase. For this purpose, the preparation was triply frozen and thawed, and then centrifuged for 30 min at 7000 g (rotor diameter – 6 cm, speed – 10,000 rpm). The process resulted in the sedimentation of the antigen at the bottom of the tube, which was used for testing by intracerebral injection of 0.03 cm^3 to ten white mice.

The adsorbed vaccine was diluted 4-fold by the saline solution and the resulting suspension was injected intracerebrally per 0.03 cm^3 to ten mice [2].

The animals were observed for 21 days. The vaccine was considered safe if all vaccinated animals remained clinically healthy during the whole period of observation, without rabies signs [2].

Testing of safety of the vaccine on dogs. For the analysis of the vaccines for safety, ten animals from groups 2 and 5 (No. 6–10 – for adsorbed vaccine, No. 21–25 – for emulsion vaccine) were administered a triple dose of the vaccine into neck muscles (3.0 cm^3 each). The safety of the re-administration of a single dose of these vaccines into neck muscles at a 7-day interval was also assessed in 10 dogs of groups 3 and 6 (No. 11–15 – for adsorbed vaccine, No. 26–30 – for emulsion vaccine). The clinical condition of the animals was observed for 14 days. The vaccine was considered safe on condition that all dogs remained clinically healthy at the end of the observation period, without necrosis in the inoculation area.

Table 1
Rabies inactivated vaccines for dogs made of ARRIAH strain of rabies virus formulated with different adjuvants

Таблица 1
Антирабические инактивированные вакцины для собак, изготовленные на основе штамма «ВНИИЗЖ» вируса бешенства с применением разных адъювантов

No.	Type of vaccine	Adjuvant	Adjuvant/ antigen ratio	Type of emulsion
1	inactivated adsorbed	Montanide GEL 01	10/90	–
2	inactivated emulsion	Montanide ISA 70	70/30	W/O

W/O – water-in-oil (inverse emulsion).

Determination of the number of rabies virus-neutralizing antibodies (VNA). Evaluation of strength of post-vaccination humoral rabies immunity in dogs 21 days after vaccination was carried out by analysis of sera taken from animals No. 1–5, 16–30 in neutralization reaction (FAVN modification) with the use of monolayer cell line BHK-21 according to the OIE recommendations on rabies [2]. Each serum was tested in triplicate.

Statistical data processing consisted in determination of arithmetic mean values of rabies antibody titer and reliability of statistical difference between the mean values according to Student-Fisher method [12].

RESULTS AND DISCUSSION

At the first stage of the study the innocuity of the obtained rabies virus antigen of "ARRIAH" strain in the monolayer cell line BHK-21 was evaluated. As a result of the analysis, no fluorescent glow specific to rabies virus was detected in any of the three consecutive passages, which indicated the complete inactivation of the virus.

At the second stage, two proposed vaccines were tested for innocuity in 20 white mice and safety in 20 dogs (Nos. 6–15, 21–30). According to the results of the analysis, the produced vaccines were found to be innocuous and safe, since all immunized animals remained clinically healthy

Table 2
Immunization of dogs with rabies inactivated vaccines based on ARRIAH strain of rabies virus formulated with different adjuvants

Таблица 2
Иммунизация собак антирабическими инактивированными вакцинами на основе штамма «ВНИИЗЖ» вируса бешенства и разных адъювантов

Group No.	Animal No.	Vaccine characteristics		Inoculation dose, cm^3
		type	adjuvant	
1	1–5	inactivated adsorbed	Montanide GEL 01	1.0
2	6–10			3.0
3	11–15			1.0 + 1.0
4	16–20	inactivated emulsion	Montanide ISA 70 VG	1.0
5	21–25			3.0
6	26–30			1.0 + 1.0

Table 3
Evaluation of level of post-vaccination humoral immunity against rabies in dogs by FAVN after administration of rabies inactivated vaccines formulated with different adjuvants ($n_{of\ tests} = 3, p < 0.005$)

Таблица 3
Оценка степени поствакцинального гуморального иммунитета у собак против бешенства в FAVN после введения антирабических инактивированных вакцин с применением разных адъювантов ($n_{исследований} = 3, p < 0,005$)

Group No.	Vaccine characteristics		Inoculation dose, cm^3	Animal No.	Titer of rabies VNA 21 days after vaccination, IU/cm^3
	type	adjuvant			
1	inactivated adsorbed	Montanide GEL 01	1.0	1	13.4 ± 1.2
				2	13.5 ± 0.8
				3	10.3 ± 0.9
				4	10.9 ± 1.5
				5	10.9 ± 1.7
				M ± m	**11.8 ± 1.5**
4	inactivated emulsion	Montanide ISA 70 VG	1.0	16	29.6 ± 1.7
				17	28.6 ± 1.4
				18	27.4 ± 1.5
				19	29.8 ± 1.5
				20	26.3 ± 1.6
				M ± m	**28.3 ± 1.5**
5	inactivated emulsion	Montanide ISA 70 VG	3.0	21	43.4 ± 2.8
				22	52.3 ± 3.9
				23	50.8 ± 3.6
				24	48.5 ± 3.7
				25	45.6 ± 3.9
				M ± m	**48.1 ± 3.7**
6	inactivated emulsion	Montanide ISA 70 VG	1.0 + 1.0	26	17.5 ± 0.8
				27	15.5 ± 1.5
				28	14.9 ± 1.4
				29	17.2 ± 0.8
				30	16.9 ± 0.8
				M ± m	**16.4 ± 1.1**

during the whole period of observation, without signs of rabies and without tissue necrosis at the injection site.

The next stage of the study was devoted to studying post-vaccination humoral immunity in 20 dogs after administration of rabies vaccines based on "ARRIAH" strain of rabies virus and formulated with Montanide ISA 70 VG and Montanide GEL 01 adjuvants. The animals of four groups were immunized according to the scheme presented in Table 2. Before and 21 days after vaccination, blood was taken from dogs and serums were tested by neutralization test (FAVN modification) [2]. It was found that prior to vaccination, the serum did not contain antibodies against rabies virus.

Data in Table 3 and Figures 1–4 show that VNA titers in the animals of group 1 vaccinated with rabies inactivated adsorbed vaccine formulated with Montanide GEL 01 adjuvant once at a dose of 1.0 cm^3, averaged 11.8 ± 1.5 ME/cm^3. This value is 2.4 times lower than that of group 4 where dogs were immunized once at a dose of 1.0 cm^3 with Montanide ISA 70 VG adjuvant and the antibodies against rabies virus were 28.3 ± 1.5 IU/cm^3. Given that the level of humoral immunity in dogs vaccinated with the emulsion vaccine is higher than in the case of the adsorbed vaccine, further studies were conducted with a vaccine formulated with Montanide ISA 70 VG adjuvant, and the dose was increased three times, and the animals were vaccinated twice at a dose of 1.0 cm^3 at 7-day intervals. Thus, in animals of group 5 which were administered the emulsion vaccine once at a dose of 3.0 cm^3, VNA titers stood at 48.1 ± 3.7 ME/cm^3, which was 4.1 and 2.4 times higher than in animals of groups 1 and 4 respectively. VNA titers in dogs of group 6 which were vaccinated with this preparation twice per 1.0 cm^3, were on average 16.4 ± 1.1 IU/cm^3, which was 1.4 times higher than in animals of group 1.

As a result of the comparative analysis of the obtained data, it was established that the rabies inactivated emulsion vaccine formulated with Montanide ISA 70 VG adjuvant stimulated the formation of a stronger humoral immunity in comparison with the adsorbed vaccine formulated with Montanide GEL 01 adjuvant. Thus, in the group of animals immunized with the emulsion vaccine a single time at a dose of 1.0 cm^3, the VNA titer was 1.7 times higher compared to the data for adsorbed vaccine. The level of rabies antibodies in dogs immunized with emulsion vaccine once at a dose of 3.0 cm^3 and twice at a dose of 1.0 cm^3 was 4.1 and 1.4 times higher respectively compared to animals vaccinated with the adsorbed vaccine.

Comparing average values of rabies antibodies titers in dogs 21 days after vaccination with emulsion vaccine at different doses, it was found that the highest level of VNA was achieved with a single administration of the preparation at a dose of 3.0 cm^3 (48.1 ± 3.7 IU/cm^3).

A single administration of the rabies vaccine to dogs at a dose of 1.0 cm^3 and a double administration at a dose of 1.0 cm^3 21 days after immunization induced accumulation of VNA in titers of 28.3 ± 1.5 and 16.4 ± 1.1 IU/cm^3, which is 2.4 and 2.9 times lower in comparison with a single administration of the above-mentioned vaccine at a dose of 3.0 cm^3. It should be noted that during the whole period of observation (21 days) the condition of all animals vaccinated with the presented vaccines at different doses was satisfactory.

As a result of the conducted studies, it was found that the developed rabies inactivated vaccines based on "ARRIAH" strain of rabies virus formulated with Montanide ISA 70 VG and Montanide GEL 01 adjuvants 21 days after the administration induced protective level of antibodies in dogs (above 0.5 IU/cm^3) and thus met the OIE requirements for immunogenicity [2]. At the same time the highest levels of humoral immunity in dogs against rabies of 48.1 ± 3.7 and 28.3 ± 1.5 IU/cm^3 were observed when using rabies inactivated emulsion vaccine at doses of 3.0 and 1.0 cm^3 respectively.

CONCLUSION

The immunobiological properties of two rabies inactivated vaccines based on "ARRIAH" strain of rabies

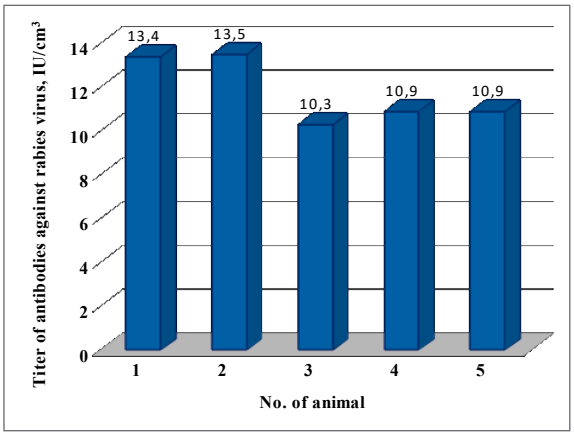

Fig. 1. Humoral immunity in dogs 21 days after single administration of anti-rabies inactivated adsorbed vaccine based on Montanide GEL 01 adjuvant at a dose of 1.0 cm³ (according to the FAVN data)

Рис. 1. Гуморальный иммунитет у собак на 21 сут после однократного введения антирабической инактивированной сорбированной вакцины на основе адъюванта Montanide GEL 01 в дозе 1,0 см³ (по данным FAVN)

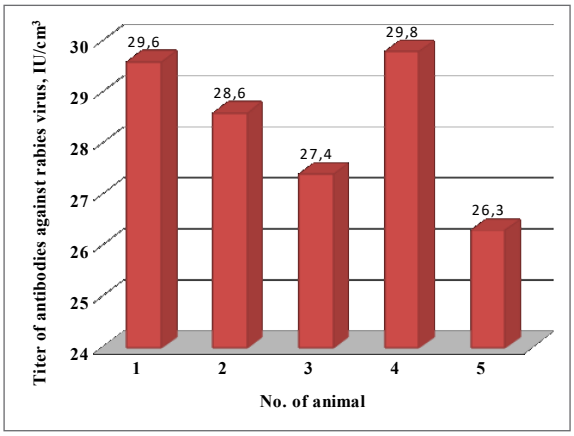

Fig. 2. Humoral immunity in dogs 21 days after single administration of anti-rabies inactivated emulsion vaccine based on Montanide ISA 70 VG adjuvant at a dose of 1.0 cm³ (according to the FAVN data)

Рис. 2. Гуморальный иммунитет у собак на 21 сут после однократного введения антирабической инактивированной эмульсионной вакцины на основе адъюванта Montanide ISA 70 VG в дозе 1,0 см³ (по данным FAVN)

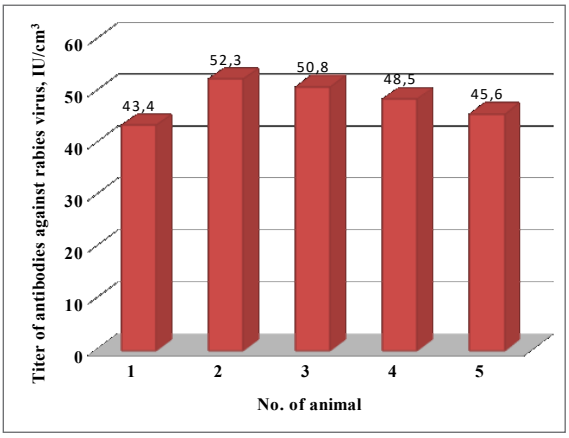

Fig. 3. Humoral immunity in dogs 21 days after single administration of anti-rabies inactivated emulsion vaccine based on Montanide ISA 70 VG adjuvant at a dose of 3.0 cm³ (according to the FAVN data)

Рис. 3. Гуморальный иммунитет у собак на 21 сут после однократного введения антирабической инактивированной эмульсионной вакцины на основе адъюванта Montanide ISA 70 VG в дозе 3,0 см³ (по данным FAVN)

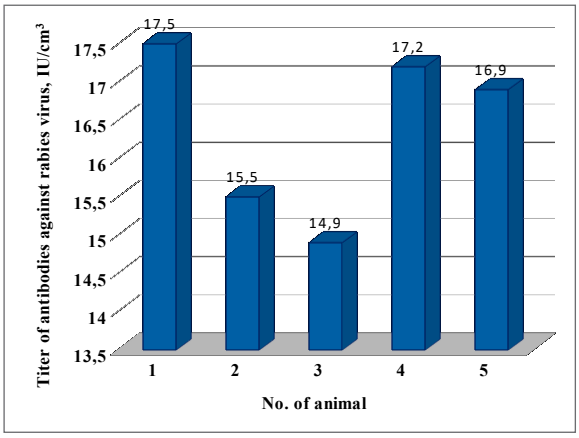

Fig. 4. Humoral immunity in dogs 21 days after double administration of anti-rabies inactivated emulsion vaccine based on Montanide ISA 70 VG adjuvant at a dose of 1.0 cm³ at 7-day interval (according to the FAVN data)

Рис. 4. Гуморальный иммунитет у собак на 21 сут после двукратного введения антирабической инактивированной эмульсионной вакцины с применением адъюванта Montanide ISA 70 VG по 1,0 см³ с интервалом 7 сут (по данным FAVN)

virus were studied using Montanide ISA 70 VG and Montanide GEL 01 adjuvants.

When tested on white mice and dogs, it was found that the obtained vaccines were safe.

Humoral immunity was assessed in 20 non-pedigree dogs 21 days after the administration of the developed vaccines at different doses. It was found that the developed rabies inactivated vaccines stimulated the formation of protective level of antibodies in dogs and met the OIE requirements for immunogenicity.

It was found that the highest levels of humoral immunity in dogs against rabies 21 days post-vaccination were 48.1 ± 3.7 and 28.3 ± 1.5 IU/cm³ and were observed when using rabies inactivated emulsion vaccine at doses of 3.0 and 1.0 cm³ respectively. The vaccine formulated with Montanide ISA 70 VG oil adjuvant at a single administration of a dose of 1.0 cm³ contributed to the formation of rabies VNA at the level 2.4 times higher compared to the vaccine formulated with Montanide GEL 01 adjuvant.

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Testing of Ferarabivac anti-rabies live vaccine for wild carnivores for its immunogenicity and protectivity

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SUMMARY

Rabies is one of the most important human and animal viral diseases, being one of the most dangerous zoonoses, causing damage to the central nervous system with an inevitable fatal outcome. This disease is of global concern, and it attracts special attention of international organizations (WHO, OIE, FAO, GARC) and of veterinary services in many countries around the world. A variety of anti-rabies vaccines have been used for specific rabies prevention in wild carnivores, however, the safety and effectiveness of some of them is doubtful. New, more advanced products are being developed, one of which is Ferarabivac, a live oral vaccine. The vaccine was tested for its immunogenicity and protectivity in wild carnivores. The optimal immunizing dose was 2.0 cm^3, with the infectivity titre of RV-97 strain of at least 6.00 lg $KKID_{50}/cm^3$. Anti-rabies antibody titres detected in the blood sera of foxes and raccoon dogs 14 days post vaccination, were 0.70 ± 0.18 and 0.73 ± 0.19 IU/cm^3, respectively, which provided protection against rabies virus infection (≥ 0.50 IU/cm^3). Rabies virus neutralizing antibodies in foxes reached their maximum level of 4.30 ± 0.32 IU/cm^3 50 days post vaccination. Antibody titres in vaccinated raccoon dogs also reached their maximum level of 4.53 ± 0.27 IU/cm^3 50 days post vaccination. The minimum protective threshold levels of serum neutralizing antibodies was determined 12 months after the vaccination, and it was 0.62 ± 0.28 and 0.71 ± 0.17 IU/cm^3 in foxes and raccoon dogs, respectively, which proves the necessity to perform booster vaccination one year later. No animals vaccinated against rabies with Ferarabivac live vaccine showed any clinical signs of the disease during the entire observation period following the challenge test carried out 30 days post vaccination.

Key words: rabies of wild carnivorous animals, Ferarabivac anti-rabies live oral vaccine, rabies virus neutralizing antibodies.

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Исследование иммуногенных и протективных свойств антирабической живой вакцины «Ферарабивак» для диких плотоядных животных

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РЕЗЮМЕ

Бешенство имеет первоочередное значение в ряду вирусных болезней человека и животных, являясь одним из самых опасных зоонозов, вызывая поражение центральной нервной системы с неизбежным летальным исходом. Заболевание представляет собой мировую проблему, которой уделяют особое внимание международные организации (ВОЗ, МЭБ, ФАО, GARC) и ветеринарные службы многих стран мира. Для специфической профилактики бешенства среди диких плотоядных животных применяют разнообразные антирабические вакцины, однако существуют сомнения в безопасности и эффективности некоторых из них. Ведутся разработки новых, более совершенных препаратов, одним из которых является антирабическая живая оральная вакцина «Ферарабивак». Проведены исследования по изучению ее иммуногенных и протективных свойств для диких плотоядных животных. Оптимальная иммунизирующая доза препарата составляет 2,0 $см^3$ с титром инфекционной активности вируса бешенства штамма РВ-97 не менее 6,00 lg $ККИД_{50}/см^3$. Через 14 сут после оральной иммунизации данной вакциной антирабические антитела обнаружены в сыворотке крови лисиц и енотовидных собак в титрах

0,70 ± 0,18 и 0,73 ± 0,19 МЕ/см^3 соответственно, что обеспечивало защиту от заражения вирусом бешенства (≥ 0,50 МЕ/см^3). Спустя 50 сут уровень антирабических вируснейтрализующих антител у лисиц достигал максимальных значений и составлял 4,30 ± 0,32 МЕ/см^3. Титр антител у вакцинированных енотовидных собак достигал максимальных значений также спустя 50 сут и был равен 4,53 ± 0,27 МЕ/см^3. Минимальный пороговый уровень вируснейтрализующих антител определяли через 12 месяцев после иммунизации, он составлял у лисиц и енотовидных собак 0,62 ± 0,28 и 0,71 ± 0,17 МЕ/см^3 соответственно, что доказывает необходимость проведения повторной вакцинации животных против бешенства через год. В результате контрольного заражения через 30 сут после вакцинации все животные, иммунизированные антирабической живой вакциной «Ферарабивак», в течение всего срока наблюдения не проявляли клинических признаков бешенства.

Ключевые слова: бешенство диких плотоядных животных, антирабическая живая оральная вакцина «Ферарабивак», антирабические вируснейтрализующие антитела.

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INTRODUCTION

According to the International Committee on Taxonomy of Viruses, rabies is caused by viruses belonging to the *Lyssavirus* genus *Rhabdoviridae* family of the *Mononegavirales* order [1]. The *Lyssavirus* genus includes the classic rabies virus (RABV), which belongs to phylogenetic group 1 and is widely spread among various animal species around the world [1, 2].

Rabies is of primary importance among human and animal viral diseases, being one of the most dangerous zoonoses, causing damage to the central nervous system with an inevitable fatal outcome [3, 4]. Thus, rabies is a global challenge in the field of infectious pathology, epidemiology, epizootology and ecology [5].

To date, the developed countries, including Russia, where vaccine manufacturing technologies meet modern requirements, have replaced the production of tissue-based anti-rabies vaccines with live and inactivated culture-based rabies vaccines for parenteral and oral use [4].

A variety of anti-rabies vaccines are used for specific rabies prevention in wild carnivores, however, the safety and effectiveness of some of them is doubtful. Currently, new and more advanced vaccines are being developed [4, 6, 7]. WHO, OIE, FAO and GARC experts and specialists constantly highlight the necessity to improve existing anti-rabies vaccines and to develop the newer and safer ones [4].

Anti-epidemic measures implemented in the territory of the Russian Federation in recent years do not result in significant containment of rabies virus spread in animals [2]. In Russia, domestic oral anti-rabies vaccines are used in wild carnivores. The vaccines are based on the attenuated rabies virus strain, which ensures protection against any rabies virus variant belonging to phylogenetic group 1 [8, 9, 10].

The effectiveness of preventive vaccination depends on the route of administration, quality of the produced vaccines, and number of immunized wild carnivores [2, 10]. For each type of target animals, the vaccine efficacy should be demonstrated by statistically reliable studies that include oral vaccination, subsequent challenge test and assessment of vaccine protective efficacy. The most susceptible young animals, for which the vaccine is recommended, were used in the study. Rabies virus neutralizing antibody titers (VNA) should confirm the vaccine efficacy for each target animal species. For this purpose we studied the protective antibody production using VN assay (RFFIT) [8] and the duration of immunity in wild carnivores [6, 7].

The production of anti-rabies vaccines for animals is regulated by the requirements of the World Organization for Animal Health (OIE), according to which the vaccine should provide strong immunity in target animal species (immunogenicity index ≥ 1.0) and induce VNA titer (at least 0.5 IU/cm^3) [4].

The aim of this work was to study immunogenic and protective properties of FGBI "ARRIAH"-manufactured live oral vaccine Ferarabivac in wild carnivores.

MATERIALS AND METHODS

Rabies virus. Rabies virus vaccine strain RV-97 deposited in the Collection of strains of microorganisms of the FGBI "ARRIAH" was used to produce Ferarabivac anti-rabies live attenuated oral vaccine (FGBI "ARRIAH"). Standard control virus strain (CVS-27) was used for challenge.

Vaccine. Ferarabivac anti-rabies live attenuated oral vaccine for wild carnivores was used in the study. The vaccine contained rabies virus vaccine strain RV-97 (infectivity titre ≥ 6.0 lg $KKID_{50}$/cm^3) at the dose of 1.0, 2.0 and 5.0 cm^3.

Animals. Red foxes aged 9–12 months (115 animals) and raccoon dogs aged 9–12 months (85 animals) were used to study the vaccine immunogenicity. The animals were purchased from animal farms in the Moscow region. According to the VN assay results, none of the animals had rabies virus VNA.

All the tests were conducted in strict accordance with the Interstate Guide for Care and Use of Laboratory Animals, GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22.09.2012 on the protection of animals used for scientific purposes.

Determination of the rabies virus infectivity titre. Virus infectivity was determined using a monolayer cell line of the newborn Syrian hamster kidneys (BHK 21/2-17), followed by staining with fluorescein isothiocyanate

(FITC)-labeled anti-rabies immunoglobulin G [11]. Virus titers were calculated using Spearman-Kerber method [12] and expressed in lg $KKID_{50}/cm^3$.

Brain and blood sampling. Sampling was performed in compliance with the FGBI "ARRIAH"s Guidelines for brain, blood serum and bone tissue sampling and shipment for rabies diagnosis and assessment of the efficacy of oral vaccination [13].

Fluorescent antibody test (FAT) Smears prepared from a composite sample of brain tissues of target animals were examined by direct FAT, which consists in binding FITC-labeled anti-rabies antibodies to the specific antigen, and further examination for fluorescent antibody-antigen complexes using fluorescence microscopy [14].

Study of vaccine immunogenicity. The duration of the protective immunity in the target animals at the end of the stated protection period was assessed by FAVN using a monolayer BHK-21/2-17 cell line and FITC-immunoglobulin in accordance with the OIE recommendations for rabies [4]. Positive OIE Standard Serum of dog origin (6.7 IU/cm^3) and negative OIE Standard Serum of dog origin were used (ANSES, Nancy, France). Each blood serum was tested in triplicates.

Study of vaccine protectivity. To study the efficacy of vaccination, 25 vaccinated and 10 control animals were used. Challenge was performed on day 30 post vaccination at a dose of 25,000 LD_{50}/cm^3. After the challenge, the animals were observed daily for 90 days. As soon as the animals began to show clinical signs of the disease, they were euthanized and the presence of the virus was confirmed by FAT. At the end of the observation period, all survived animals were euthanized and the brain smears were studied using FAT.

Statistical data processing. The obtained data were statistically processed by calculating arithmetic mean values and the confidence interval of the difference between means computed using the Student's-Fischer equation [12]. The differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Ferarabivac anti-rabies live oral vaccine is designed to prevent rabies in wild carnivores, and is the only effective immunization route in this case is feeding the vaccine to the animals.

At the first stage, the immunizing dose was determined for 30 red foxes. The animals were divided into 3 groups (10 animals per group). Animals in the first group were fed 1 bait containing 1.0 cm^3 of the vaccine, in the second group – 1 bait containing 2.0 cm^3 of the vaccine, in the third group – 1 bait containing 5.0 cm^3 of the vaccine. Blood sera were collected and tested for rabies VNA on days 14, 21, 30 and 60 post vaccination (dpv). The results are shown in Table 1.

The data in Table 1 demonstrate that when foxes ate the bait containing 1.0 cm^3 of the vaccine the anti-rabies VNA titre amounted to 0.30 ± 0.29; 0.90 ± 0.25; 1.25 ± 0.25; 2.00 ± 0.21 IU/cm^3 respectively. Eating of the vaccine at a dose of 2.0 cm^3 resulted in anti-rabies VNA level at 0.69 ± 0.18; 2.20 ± 0.23; 3.20 ± 0.34; 4.20 ± 0.38 IU/cm^3 respectively. Eating of 5.0 cm^3 dose resulted in anti-rabies VNA titres at 0.78 ± 0.25; 2.60 ± 0.35; 4.00 ± 0.28; 4.96 ± 0.30 IU/cm^3 on 14, 21, 30, 60 dpv respectively. According to the obtained data and the OIE requirements as well as in the context of cost-effectiveness the dose of 2.0 cm^3 with infectivity titre being at least 6.00 lg $KKID_{50}/cm^3$ is an effective immunizing dose of the vaccine and it ensures the animal protection against rabies infection in 14 days post immunization. When animals were fed the baits containing 5.0 cm^3 of the vaccine, the comparable anti-rabies VNA levels were induced but it required considerable amount of the vaccine raw material. Administration of the immunizing dose of 1.0 cm^3 on 14 dpv resulted in accumulation of anti-rabies VNA at the amount insufficient for the animal protection against rabies (< 0.50 IU/cm^3).

During the next stage of the research, we arranged an experiment aimed at the examination of the Ferarabivac vaccine immunogenicity in 25 foxes and 25 raccoon dogs. One vaccine dose containing 2.0 cm^3 of the rabies virus with titre 6.00 lg $KKID_{50}/cm^3$ was orally administered to each animal. In 14, 30, 50, 60, 70, 80 and 90 dpv blood samples were collected from the animals and sera were tested for the virus-specific antibody titres. The test results are shown in Table 2 and Figure 1.

The data shown in Table 2 demonstrate that Ferarabivac anti-rabies live oral vaccine induced anti-rabies

Table 1
Determination of immunizing dose of Ferarabivac anti-rabies live oral vaccine for wild carnivores ($n = 10, p < 0,05$)

Таблица 1
Определение иммунизирующей дозы антирабической живой оральной вакцины «Ферарабивак» для диких плотоядных животных ($n = 10, p < 0.05$)

Days post vaccination	Anti-rabies VNA titre (IU/cm^3)* after vaccine feeding at various doses		
	1.0 cm^3	2.0 cm^3	5.0 cm^3
14	0.30 ± 0.29	0.69 ± 0.18	0.78 ± 0.25
21	0.90 ± 0.25	2.20 ± 0.23	2.60 ± 0.35
30	1.25 ± 0.25	3.20 ± 0.34	4.00 ± 0.28
60	2.00 ± 0.21	4.20 ± 0.38	4.96 ± 0.30

* VN assay data (FAVN modification).

Table 2
Assessment of the immunogenicity of Ferarabivac anti-rabies live oral vaccine in foxes and raccoon dogs ($n = 25, p < 0.05$)

Таблица 2
Оценка иммуногенной активности антирабической живой оральной вакцины «Ферарабивак» на лисицах и енотовидных собаках ($n = 25, p < 0,05$)

Days post vaccination	Anti-rabies VNA titre, IU/cm^{3*}	
	foxes	raccoon dogs
14	0.70 ± 0.18	0.73 ± 0.19
30	3.20 ± 0.22	3.50 ± 0.22
50	4.30 ± 0.32	4.53 ± 0.27
60	4.20 ± 0.40	4.30 ± 0.30
70	4.00 ± 0.29	4.09 ± 0.32
80	3.81 ± 0.42	3.90 ± 0.23
90	3.70 ± 0.35	3.80 ± 0.41

* VN assay data (FAVN modification).

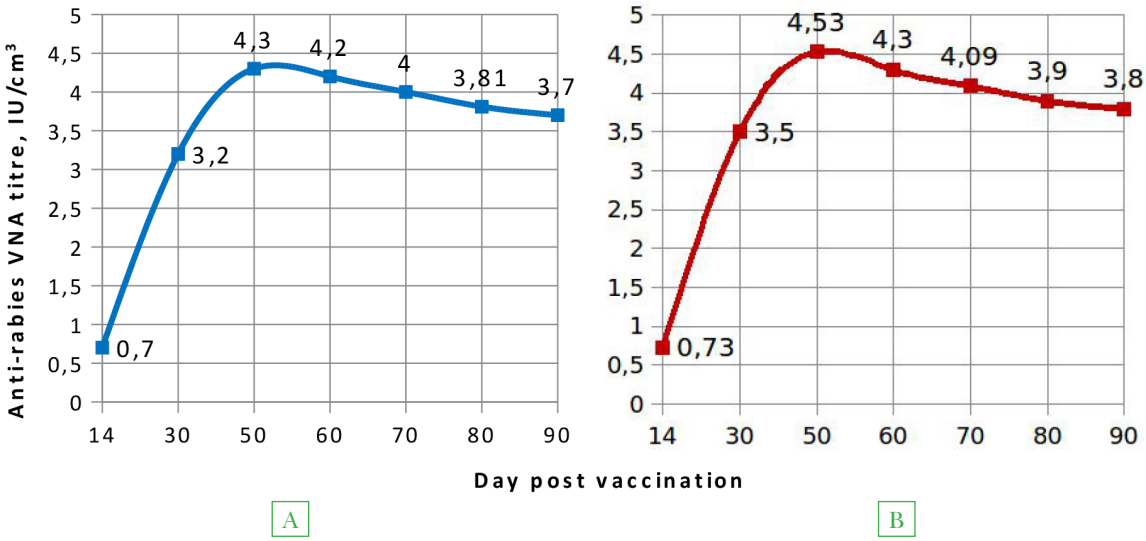

Fig. 1. Immunogenicity of Ferarabivac anti-rabies live oral vaccine in foxes (A) and raccoon dogs (B)

Рис. 1. Иммуногенная активность антирабической живой оральной вакцины «Ферарабивак» при исследовании на лисицах (А) и енотовидных собаках (Б)

VNA production in the animals. Thus in 14 days post oral vaccination the anti-rabies antibodies were detected in the sera collected from foxes and raccoon dogs at titres 0.70 ± 0.18 and 0.73 ± 0.19 IU/cm³ respectively, that ensured protection against the rabies virus infection (≥ 0,50 МЕ/см³), and in 30 dpv the titres amounted to 3.20 ± 0.22 and 3.50 ± 0.22 IU/cm³ respectively. On 50 dpv the anti-rabies VNA level in foxes reached its peak and amounted to 4.30 ± 0.32 IU/cm³. VNA titre subsequently decreased but on 90 dpv it was still high and amounted to 3.70 ± 0.35 IU/cm³ that ensured protection against rabies virus infection (≥ 0.50 IU/cm³). VNA titres in Ferarabivac vaccinated raccoon dogs reached their peak level in 50 dpv and amounted to 4.53 ± 0.27 IU/cm³, and hereafter they gradually decreased and on 90 dpv they amounted to 3.80 ± 0.41 IU/cm³.

Ferarabivac anti-rabies live oral vaccine potency in wild carnivores was tested in 35 foxes, which were subdivided in two groups: experimental group (No. 1) – 25 foxes, and control group (No. 2) – 10 foxes; and in 35 raccoon dogs, which were also subdivided into experimental group (No. 3) – 25 animals, and control group (No. 4) – 10 animals. The animals in groups No. 1 and 3 were immunized with the vaccine at a dose of 2.0 cm³. Groups No. 2 and 4 included control non-vaccinated animals. Blood samples were collected from the animals before the infection, and they were tested for anti-rabies VNA titre. In 30 days post the vaccine consumption the animals were challenged with control rabies virus strain CVS-27 at a dose of 25,000 LD_{50}/cm³. The animals' clinical condition was examined daily for 90 days. The results of the experiment are shown in Tables 3 and 4.

After challenge all animals immunized with Ferarabivac anti-rabies live oral vaccine demonstrated no rabies clinical signs. In a month post immunization the mean titres of anti-rabies virus antibodies in foxes and raccoon dogs amounted to 3.24 ± 0.08 and 3.26 ± 0.22 IU/cm³ respectively. The control animals died on day 12–20 post infection. Disease-specific death was confirmed using FAT. The remaining animals were observed for 90 more days after the death of the last control animal. Upon the observation period termination the animals were euthanized and their brains were tested using FAT. No rabies virus was detected in the tested brain samples.

At the final stage of the research, the immunity duration was tested in 25 foxes and 25 raccoon dogs, which were vaccinated with Ferarabivac at a dose of 2.0 cm³ with infectivity titre of the attenuated rabies virus strain RV-97 being at least 6.0 lg $KKID_{50}$/cm³. The animals were observed for a year and blood samples were collected at a regular basis for anti-rabies VNA testing. The test results are shown in Table 5 and Figure 2.

The results demonstrated in Table 5 and Figure 2 indicate that after foxes' immunization with Ferarabivac vaccine the VNA were detected in 0.5 month after single vaccination, and their titres amounted to 0.70 ± 0.31 IU/cm³. Anti-rabies antibody titres reached their peak by month 2 and amounted to 4.30 ± 0.32 IU/cm³. Hereafter, they gradually decreased and amounted to 3.00 ± 0.31 IU/cm³ in six months and to 0.62 ± 0.28 IU/cm³ in 12 months.

After the raccoon dogs' immunization with Ferarabivac vaccine the anti-rabies VNA were detected in 0.5 month after singular feeding with the vaccine and their titres amounted to 0.70 ± 0.29 IU/cm³. Anti-rabies antibody levels reached their peak by month 2 and amounted to 4.51 ± 0.27 IU/cm³. Hereafter, they gradually decreased and reached the value of 3.10 ± 0.43 IU/cm³ in six months and 0.71 ± 0.17 IU/cm³ in 12 months. Otherwise stated, Ferarabivac anti-rabies live oral vaccine provided 12-month protection of wild carnivores against rabies as the titre of the anti-rabies VNA amounted to ≥ 0.5 IU/cm³ that is compliant with the OIE requirements for anti-rabies vaccines [4]. The minimal VNA threshold level was determined in 12 months post immunization that indicates that the animals should be vaccinated against rabies in a year.

CONCLUSION

Immunogenicity and protectivity of the Ferarabivac anti-rabies live oral vaccine for wild carnivores were tested.

The optimal immunization dose of the vaccine was determined to be 2.0 cm³ with infectivity titre of attenuated rabies virus strain RV-97 being at least 6.00 lg $KKID_{50}$/cm³.

Table 3
Challenge of foxes with CVS 27 strain one month post vaccination with Ferarabivac ($n = 3, p < 0,05$)

Таблица 3
Результаты заражения контрольным штаммом вируса бешенства CVS-27 лисиц через месяц после иммунизации вакциной «Ферарабивак» ($n_{иссл.} = 3, p < 0,05$)

Group No.	Animal No.	Anti-rabies VNA titre determined using virus neutralization assay, IU/cm³	Challenge results	
			presence/ absence of rabies clinical signs	brain testing using FAT
1 (experimental)	1	3.20 ± 0.21	–	neg.
	2	3.32 ± 0.20	–	neg.
	3	3.18 ± 0.24	–	neg.
	4	3.25 ± 0.19	–	neg.
	5	3.31 ± 0.20	–	neg.
	6	3.24 ± 0.21	–	neg.
	7	3.42 ± 0.25	–	neg.
	8	3.14 ± 0.18	–	neg.
	9	3.24 ± 0.27	–	neg.
	10	3.20 ± 0.25	–	neg.
	11	3.28 ± 0.21	–	neg.
	12	3.15 ± 0.24	–	neg.
	13	3.25 ± 0.21	–	neg.
	14	3.38 ± 0.25	–	neg.
	15	3.39 ± 0.20	–	neg.
	16	3.18 ± 0.24	–	neg.
	17	3.05 ± 0.19	–	neg.
	18	3.38 ± 0.20	–	neg.
	19	3.08 ± 0.21	–	neg.
	20	3.42 ± 0.27	–	neg.
	21	3.40 ± 0.19	–	neg.
	22	3.24 ± 0.27	–	neg.
	23	3.20 ± 0.27	–	neg.
	24	3.36 ± 0.21	–	neg.
	25	3.19 ± 0.24	–	neg.
	M ± *m*	3.26 ± 0.22		
2 (control)	26	0.00	+	pos.
	27	0.00	+	pos.
	28	0.00	+	pos.
	29	0.00	+	pos.
	30	0.00	+	pos.
	31	0.00	+	pos.
	32	0.00	+	pos.
	33	0.00	+	pos.
	34	0.00	+	pos.
	35	0.00	+	pos.
	M ± *m*	0.00		

«+» – presence of rabies clinical signs and death of the animal;
«–» – absence of rabies clinical signs.

Table 4
Challenge of racoon dogs with CVS 27 strain one month post vaccination with Ferarabivac ($n = 3, p < 0.05$)

Таблица 4
Результаты заражения контрольным штаммом вируса бешенства CVS-27 енотовидных собак через месяц после иммунизации вакциной «Ферарабивак» ($n_{иссл.} = 3, p < 0,05$)

Group No.	Animal No.	Anti-rabies VNA titre determined using virus neutralization assay, IU/cm³	Challenge results	
			presence/absence of rabies clinical signs	brain testing using FAT
1 (experimental)	1	3.45 ± 0.24	–	neg.
	2	3.41 ± 0.21	–	neg.
	3	3.58 ± 0.19	–	neg.
	4	3.35 ± 0.19	–	neg.
	5	3.39 ± 0.22	–	neg.
	6	3.47 ± 0.21	–	neg.
	7	3.61 ± 0.20	–	neg.
	8	3.74 ± 0.26	–	neg.
	9	3.50 ± 0.27	–	neg.
	10	3.40 ± 0.25	–	neg.
	11	3.58 ± 0.21	–	neg.
	12	3.51 ± 0.24	–	neg.
	13	3.39 ± 0.21	–	neg.
	14	3.58 ± 0.25	–	neg.
	15	3.60 ± 0.20	–	neg.
	16	3.48 ± 0.24	–	neg.
	17	3.55 ± 0.28	–	neg.
	18	3.64 ± 0.20	–	neg.
	19	3.48 ± 0.22	–	neg.
	20	3.49 ± 0.27	–	neg.
	21	3.51 ± 0.19	–	neg.
	22	3.49 ± 0.27	–	neg.
	23	3.70 ± 0.27	–	neg.
	24	3.76 ± 0.21	–	neg.
	25	3.69 ± 0.26	–	neg.
	M ± *m*	3.78 ± 0.23		
2 (control)	26	0.00	+	pos.
	27	0.00	+	pos.
	28	0.00	+	pos.
	29	0.00	+	pos.
	30	0.00	+	pos.
	31	0.00	+	pos.
	32	0.00	+	pos.
	33	0.00	+	pos.
	34	0.00	+	pos.
	35	0.00	+	pos.
	M ± *m*	0.00		

«+» – presence of rabies clinical signs and death of the animal;
«–» – absence of rabies clinical signs.

Table 5
A 12-months' study of immunity duration in wild animals vaccinated with Ferarabivac ($n = 3, p < 0.05$)

Таблица 5
Исследование продолжительности иммунитета в течение 12 месяцев у диких животных, иммунизированных вакциной «Ферарабивак» ($n_{иссл.} = 3, p < 0,05$)

Period post vaccination, month	Anti-rabies VNA titre determined using VN assay, IU/cm³	
	foxes	raccoon dogs
0.5	0.70 ± 0.31	0.70 ± 0.29
1	3.20 ± 0.22	3.50 ± 0.22
2	4.30 ± 0.32	4.51 ± 0.27
3	3.71 ± 0.35	3.80 ± 0.41
4	3.55 ± 0.41	3.62 ± 0.25
5	3.46 ± 0.23	3.46 ± 0.35
6	3.00 ± 0.31	3.10 ± 0.43
8	2.30 ± 0.19	2.63 ± 0.23
9	1.89 ± 0.37	2.01 ± 0.19
10	1.21 ± 0.15	1.53 ± 0.25
12	0.62 ± 0.28	0.71 ± 0.17

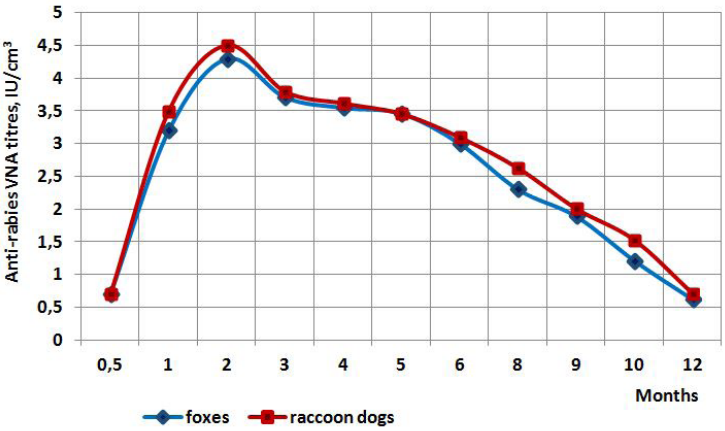

Fig. 2. Immunity duration in foxes and raccoon dogs vaccinated with Ferarabivac anti-rabies live oral vaccine

Рис. 2. Длительность иммунитета против бешенства у лисиц и енотовидных собак, вакцинированных антирабической живой оральной вакциной «Ферарабивак»

It was demonstrated that in 14 days post oral immunization with the vaccine the anti-rabies virus antibodies were detected in sera of foxes and raccoon dogs and their titres amounted to 0.70 ± 0.18 and 0.73 ± 0.19 IU/cm³ respectively, thus providing protection against rabies virus infection (≥ 0.50 IU/cm³). The anti-rabies VNA levels in foxes reached their peak in 50 days, and they amounted to 4.30 ± 0.32 IU/cm³. VNA titres subsequently decreased but, nevertheless, in 90 days they still remained high and amounted to 3.70 ± 0.35 IU/cm³. In the Frearabivac-vaccinated raccoon dogs the VNA titres reached their peak levels in 50 days and amounted to 4.53 ± 0.27 IU/cm³. Hereafter, they gradually decreased, and in 90 days post immunization they amounted to 3.80 ± 0.41 IU/cm³. Minimal VNA threshold level was reported in 12 months post immunization and it amounted to 0.62 ± 0.28 and 0.71 ± 0.17 IU/cm³ in foxes and raccoon dogs respectively, that is indicative of the need of the rabies vaccination of the animals to be repeated in a year.

It was found that in 30 days post challenge all animals immunized with Ferarabivac anti-rabies live oral vaccine demonstrated no rabies clinical signs during the whole observation period. In a month post immunization the mean titres of anti-rabies virus antibodies in foxes and raccoon dogs amounted to 3.24 ± 0.08 and 3.26 ± 0.22 IU/cm³ respectively. The control animals died on day 12–20 post infection. Disease-specific death was confirmed using FAT. The remaining animals were observed for 90 more days after the death of the last control animal. Upon the observation period termination the animals were euthanized and absence of the rabies virus was confirmed by testing their brain tissues using FAT.

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Experience in African swine fever control in the Russian Federation and its value for the other countries

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SUMMARY
Basic characteristics of African swine fever, being one of the most significant transboundary infections with a devastating potential for pig production, are presented in the paper. It occurred in Georgia for the first time in 2007 and spread among domestic pigs and wild boar in the European part of the Russian Federation. After that, ASF virus was detected in Belarus, Ukraine and since 2014, the disease has been reported in the European countries. Having spread in Europe, African swine fever became an epizooty, caused by ASFV genotype II and exhibiting a deteriorating trend. Modern ASF infected areas in the Russian Federation, Europe, China and Asia are described. Currently the disease is the most serious epidemiological problem due to huge losses (high mortality among susceptible animals); ability to occur and spread in the most unexpected parts of the world and absence of specific prevention tools and means. Russia has developed a high level of expertise in the disease prevention, control and eradication. A scientifically justified set of measures to prevent and eradicate ASF, which has proved its effectiveness in the disease control, was developed and introduced. The core of this set is formed by biosafety assurance along the whole production chain at the establishments of any type of ownership involved into breeding, keeping, slaughter, processing, storage, movement and marketing of live pigs and pig products; by wild boar population control and improvement of waste management approaches. Other ASF infected countries can benefit from the Russian Federation's experience in ASF control by adapting it to the concrete region with due regard to local social and economic conditions.

Key words: African swine fever (ASF), biological safety, ASF control measures, experience in ASF control.

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Опыт борьбы с африканской чумой свиней в Российской Федерации и его значение для других стран

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РЕЗЮМЕ
Представлены основные характеристики африканской чумы свиней как наиболее важной трансграничной инфекции с катастрофическим разрушительным потенциалом для свиноводства. Появившись в Грузии в 2007 г., АЧС распространилась среди домашних свиней и кабанов на территории европейской части Российской Федерации. Затем вирус АЧС был обнаружен в Беларуси, на Украине, а с 2014 г. заболевание стали регистрировать в странах Евросоюза. Распространившись на территории Европы, африканская чума свиней приняла характер широкой эпизоотии, вызванной вирусом АЧС II генотипа, с трендом на ухудшение ситуации. Описан современный ареал распространения АЧС в Российской Федерации, странах Европы, Китае и Азии. В настоящее время болезнь является самой серьезной проблемой эпизоотологии ввиду: чрезвычайно большого прямого ущерба (высокой летальности восприимчивых животных); способности к возникновению и эпизоотическому распространению в самых неожиданных регионах мира; отсутствия средств специфической профилактики и лечения. В России накоплен богатый опыт предотвращения возникновения, распространения и ликвидации этой болезни. Разработан и внедрен научно обоснованный комплекс мер по профилактике и ликвидации АЧС, доказавший свою эффективность в условиях борьбы с данным заболеванием свиней. В основу комплекса мер положены: обеспечение биобезопасности по всей производственной цепочке предприятий, связанных с разведением, содержанием, убоем, переработкой, хранением, перемещением и реализацией живых свиней и свиноводческой продукции, независимо

от форм собственности; регуляция численности популяции кабана; совершенствование проблемных вопросов по обращению с биологическими отходами. Опыт борьбы с АЧС в Российской Федерации можно рекомендовать другим неблагополучным по данному заболеванию странам, но с адаптацией его к конкретному региону с учетом местного социально-экономического состояния.

Ключевые слова: африканская чума свиней (АЧС), биологическая безопасность, комплекс мер по противодействию АЧС, опыт борьбы с АЧС.

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INTRODUCTION

African swine fever (ASF) (lat. *Pestis africana suum*; Montgomery's disease) is a contagious disease of pigs, it can occur in acute, subacute, chronic or asymptomatic form and is characterized by fever, hemorrhagic diathesis, inflammatory and parenchymal dystrophies and necrosis. ASF is caused by DNA virus of *Asfarviridae* family, *Asfivirus* genus. Under natural conditions domestic pigs and wild boar of all ages and breeds are susceptible to the disease at any season.

This disease, exotic for the European and Asian countries, is enzootic in many African countries, where 24 genotypes are circulating, and in the island of Sardinia. In 2007 ASF was firstly reported in Georgia in domestic pigs and wild boar; after penetrating wildlife, the disease spread in the territory of bordering countries, including in the Russian Federation. After that, ASF was detected in Ukraine, Belarus and since 2014, the disease has been reported in European countries. Having spread in Europe, African swine fever became an epizooty, caused by ASFV genotype II and exhibiting a deteriorating trend. Since that time, ASF has been demonstrating a rampant diffuse spread [1].

Ten European countries are currently infected with ASF. Within less than five years after ASF occurred in Lithuania, the disease has infected a huge territory, including Latvia, Estonia, Poland, Czech Republic, Hungary, Romania, Bulgaria, Slovakia, Serbia.

China, the largest pork producer in the world, firstly reported ASF in 2018. Up to 2020 ASF has been registered in many South Asian countries. The forecast is unfavourable, that is why the list of ASF infected countries, will likely be extended.

Currently African swine fever is the most important transboundary disease with a devastating potential; one of the most serious challenges in epizootology due to huge direct losses (high mortality among susceptible animals), ability to occur and spread in the most unexpected parts of the world and absence of specific prevention tools and means [2]. ASF epidemic process analysis showed that the biggest number of outbreaks in pigs occurs in backyards, peasant farms and smallholder farms with the least number reported by large commercial pig holdings [3].

Experience has shown that the best tool to control ASF is to ensure biological security of a pig farm. Only a large farm can afford full funding of biological security measures, because it requires considerable investments.

A wild boar is an equal participant of ASF epidemic process. The population of wild boar shall be strictly regulated, otherwise biological security is compromised. Evident transboundary nature of the disease and an important role of a wild boar was demonstrated in Russia, Europe and other countries.

A set of ASF control measures, which has proved its effectiveness in addressing the problem, was developed and introduced in Russia. Constant monitoring, analysis and modelling of the epidemic situation as well as annual forecasts are performed to control the disease. In case of ASF unfavorable scenario, the need in expanded international cooperation in ASF control, experience sharing and ASF awareness campaigns arises.

MATERIALS AND METHODS

Data of the World Organization for Animal Health (OIE), FGBI "Veterinary Centre" under the RF MoA, FGBI "ARRIAH" Information and Analysis Centre on ASF epidemic situation in the Russian Federation and in the world since 2007 as well data from the public sources on ASF control measures in infected countries were used.

Retrospective analysis of epidemic trend in the Russian Federation and other countries was carried out. General tendencies in ASF epidemic were shown in graphs and maps.

RESULTS AND DISCUSSION

African swine fever after occurrence and spread in Georgia in 2007, entered other neighboring countries: Armenia, Azerbaijan, Iran and Russia (Fig. 1).

Up to 2020, the disease has infected many Russian regions, East European and Baltic countries, struck pig production in China, North and South Koreas and South East countries. The humanity has never faced an epizooty of such a magnitude.

According to experts, ASF can be especially detrimental to Asian countries, where 80% of global pig population is concentrated. More than 500 million pigs are kept in China. Losses, inflicted by ASF up to 2020, amount to a trillion CNY [4].

ASF control in China, South Korea, Vietnam, Laos, Cambodia and the Philippines is complicated by a specific pig management, i.e. most of animals are kept at smallholder farms and in backyards with inadequate biological security; poor registration, insufficiently regulated and sometimes not regulated (even illegal) long-distance movements of potentially dangerous goods (pigs, pork products, pig feeds); poor infrastructure of slaughter, processing, storage and marketing of pork products; feeding with food wastes.

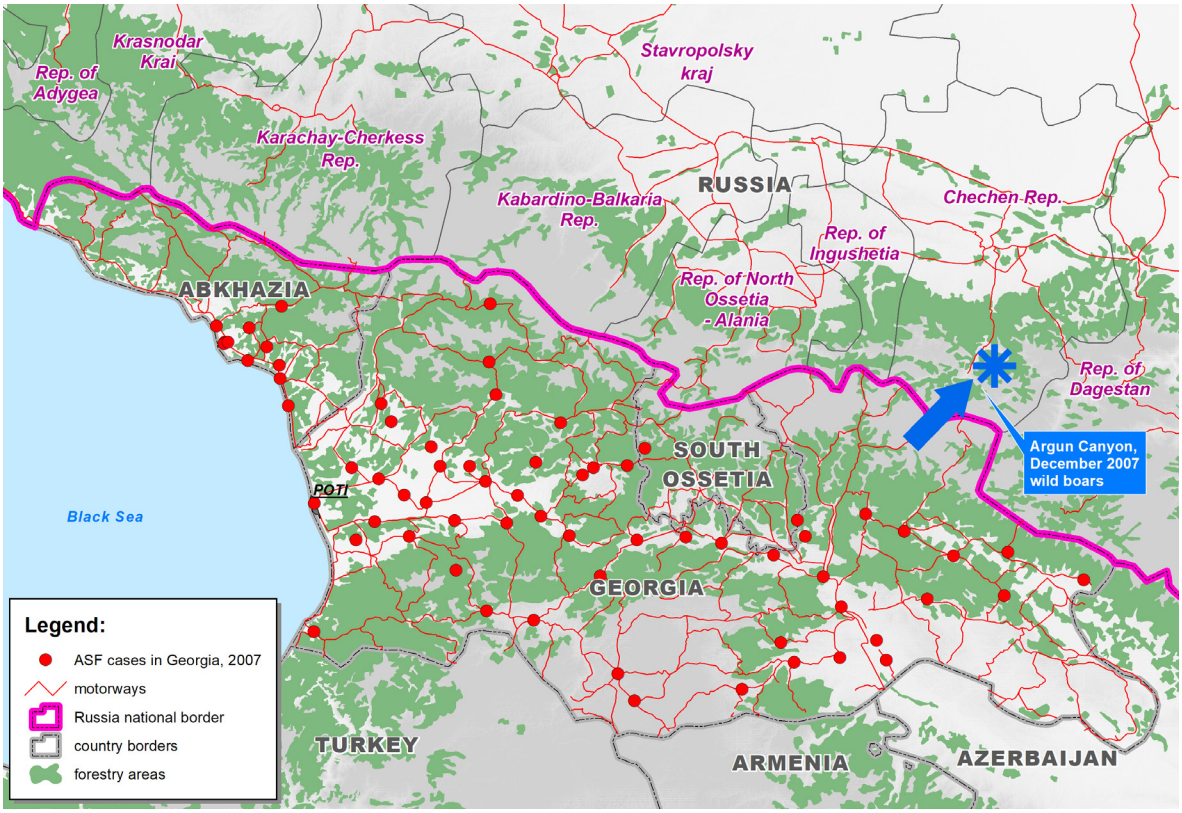

Fig. 1. ASF introduction into the Russian territory in 2007 (https://www.fsvps.ru/fsvps-docs/ru/iac/asf/2015/asf_chronology_15.pdf)

Рис. 1. Проникновение АЧС на территорию России в 2007 г.

To control and eradicate ASF, analysis of global experience in the disease combating is needed. At the meeting of the Standing Group of Experts on ASF in Ho Chi Minh the experts discussed the ways to prevent rapid spread of the disease in Asia. Taking into account the basic causes of ASF spread in Asian region, the participants concluded that the major tool in the disease control must be the improvement of biological security along the whole production chain of pig holdings (farms, slaughterhouses, processing plants, cold storages, etc.) [5].

Average speed of ASF epidemic development in 2007–2017 in Eurasia was 1.273 countries per year [3]. The calculation of ASF spread speed in China and South East Asia due to explosive nature of ASF infection in different regions poses certain challenges (Fig. 2.)

From our perspective, countries, which face ASF for the first time, often make systemic mistakes, i.e. no decisive measures are taken to prevent and eradicate the disease (rapid diagnostics, restrictions, destruction of infected pigs and pig products, biological waste management) with due regard to ASFV high resistance to external factors and its persistence in wild nature; insufficient attention is paid to wild boar population regulation (being one of the key factors in ASF spread and persistence in wild life); poor awareness campaigns and inadequate knowledge about ASF danger not only among the farmers, but also among the veterinarians.

Our country has developed a high level of expertise in this disease prevention, control and eradication [3]. The experience shows that it is practically impossible to reach high biosecurity levels in backyards. That is why under the current epidemic conditions it is necessary to transfer stepwise the pig production to middle-sized and large commercial holdings, located in ASF free regions and using compartmentalization system (animal health status assessment at pig farms). Smallholder farms and backyards should shift to alternative livestock productions, like poultry or rabbit productions, and if sufficient land is available, sheep and goat production or meat and milk production. A comprehensive approach of the Russian Veterinary Service to upgrading of biological security at all facilities, involved into the production cycle; the regionalization of the country territory and use of e-certification for transported products has proved its effectiveness. Currently this approach is well known in the international community (OIE and FAO). Now China and Vietnam, which are in a dismal state, make use of the lessons, learned by Russia.

The retrospective analysis of ASF situation in the Russian Federation during the past three years shows a declining trend in the number of outbreaks among both domestic pigs and wild boar (Fig. 3).

There are three underlying postulates:

1. Biological security along the whole production chain at establishments, involved into breeding, keeping, slaughter, processing, storage, movements and marketing of live pigs and pig products, regardless of their ownership type.
2. Wild boar population regulation.
3. Solution of biological waste management issues.

Regional programmes on ASF control have been developed as well as the mechanisms for their implementation

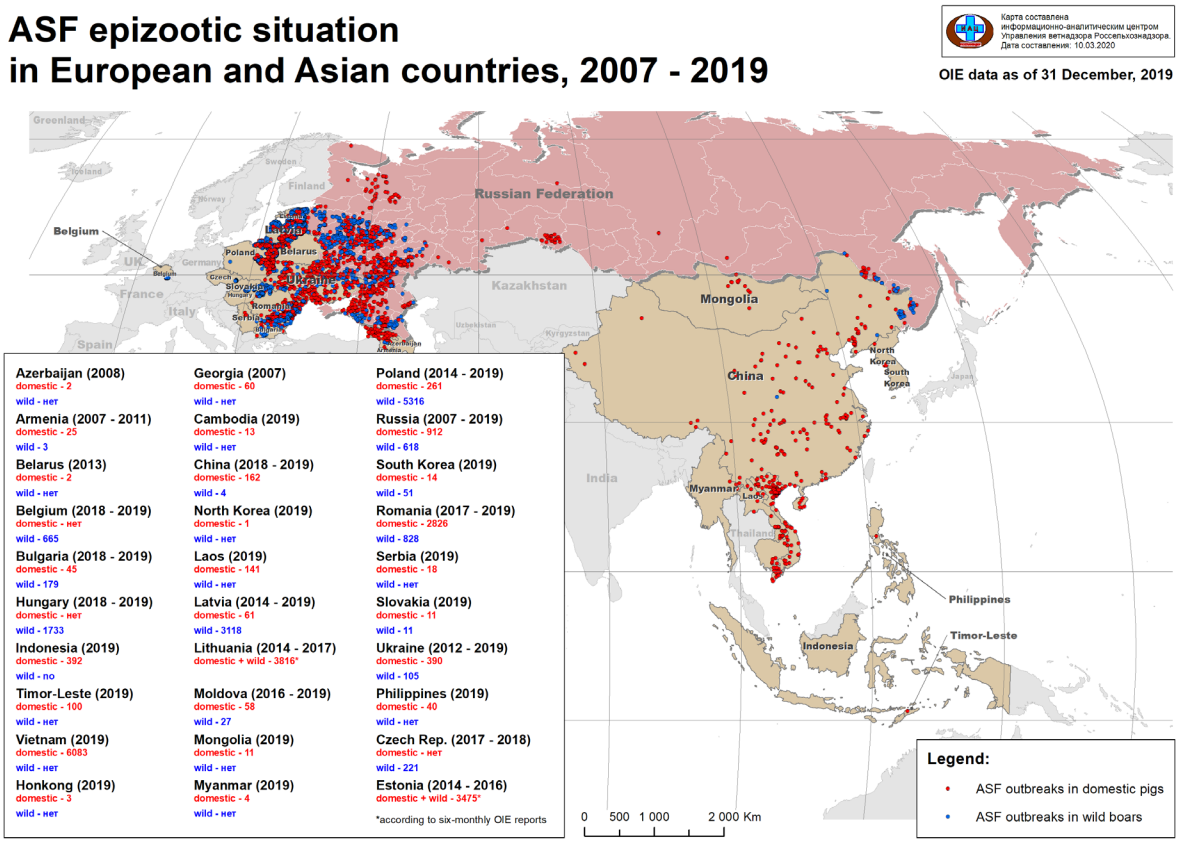

Fig. 2. ASF outbreaks in Europe and Asia in 2007–2019 (https://www.fsvps.ru/fsvps-docs/ru/iac/asf/2019/12-31/05.pdf)

Рис. 2. Вспышки АЧС в странах Европы и Азии в 2007–2019 гг.

and financing have been sought in the Russian Federation. At the same time at the federal level coordinating bodies (commissions, headquarters), first under the Ministry of Agriculture, then under the Russian Government, have been established. Scientifically justified federal laws of a great strategic potential, having a real impact on ASF outbreak prevention and eradication, have been adopted. Here are just a few to mention: the Veterinary Law and the Federal Law on Hunting and Preservation of Hunting Resources have been amended 12 times. Numerous orders have been issued by the Russian Ministry of Agriculture and Russian Ministry of Natural Resources, including those registered by the Ministry of Justice of the Russian Federation:

– Order of the RF Ministry of Agriculture No. 258 of 23.07.2010 (amended on 15.10.2013) "On Approval of Rules to Determine Animal Health Status of Pig Holdings and Organizations, Involved into Pig Slaughter, Processing and Storage of Pig Products" (Registered by the RF Ministry of Justice on 12.11.2010, No. 18944);

– Order of the RF Ministry of Agriculture No. 114 of 29.03.2016 "On Approval of Veterinary Rules of Pig Keeping for the Purposes of Reproduction, Growing and Marketing" (Registered by the RF Ministry of Justice on 04.07.2016, No. 42749);

– Order of the RF Ministry of Agriculture No. 635 of 14.12.2015 "On Approval of Veterinary Rules of the RF Territory Regionalization" (Registered by the RF Ministry of Justice on 23.03.2016, No. 1508);

– Order of the RF Ministry of Agriculture No. 161 of 22.04.2016 "On Approval of the List of Animal Species Subject to Identification and Registration";

– Agro-industrial Regulatory Document (RD-APK) 3.10.07.05-17. Veterinary and Sanitary Requirements for Design, Construction, Reconstruction and Operation of Animal Production Facilities (Moscow, 2017);

– Order of the RF Ministry of Agriculture No. 213 of 31.05.2016 "On Approval of Veterinary Rules of Preventive, Diagnostic, Restrictive and other Measures, Quarantine Imposition and Removal and other Actions, Taken to Prevent the Spread and Eradicate African Swine Fever Outbreaks" (Registered by the RF Ministry of Justice on 24.08.2016, No. 43379);

– Order of the RF Ministry of Agriculture No. 329 of 06.07.2017 "On Approval of Veterinary Rules of Pig, Pig Product and Feed Transportation by Motor Vehicles" (Registered by the RF Ministry of Justice on 03.08.2017, No. 47649);

– Order of Rosselkhoznadzor No. 235 of 19.03.2018 "On Approval of Check-lists, used by Officials of the Federal Service for Veterinary and Phytosanitary Surveillance Territorial Offices for Scheduled Audits Within the Federal State Veterinary Monitoring";

– Decree of the Russian Federation Government No. 2048-p of 30.09.2016 "On Approval of the Action Plan to Prevent Introduction and Spread of African Swine Fever in the Russian Federation";

and some other acts, which are regularly amended and supplemented.

To restore the order it is also needed to enhance responsibility of animal owners for violations of veterinary rules and legal requirements. Physical persons and business operators have already experienced the consequences of the judicial practice.

The major achievement of Russia, as we believe, is the understanding by all social strata that ASF is an awful disaster! It affects every person, authorities of all levels and business society.

There shall be no exclusions from adopted rules to control ASF. As there are no means of specific prophylaxis and treatment it is important to strengthen the requirements for biological security at pig holdings of different compartments, especially of industrial holdings, and to implement the policy of alternative livestock production.

Russia's experience in ASF control is invaluable for the modern world. The developed set of measures made it possible to preserve and even develop pig production as an industry; every year the pig population is increasing, pig performance and pork production are growing. According to the National Union of Pork Producers total annual pork production increased by 2.4 times from 2005 to 2018 [6]. The international expert society acknowledged that Russia is becoming a pork exporter [7].

This is facilitated by promising developments, conducted by the Rosselkhoznadzor and the FGBI "ARRIAH" subordinate to it, i.e. regionalization of the country, animal registration, e-certification (FGIS MERCURY), etc.

Implementation of the RF Presidential Decree No. 204 of 07.05.2018 envisages expanding of agro-industrial product exports equal to 45 billion USD up to 2024, including increase in pork production. That is why everybody will have to work hard and implement the set of ASF control measures.

CONCLUSIONS

1. The Russian Federation has developed a set of practical measures to prevent and eradicate ASF, covering regulatory framework on federal and regional levels as well as fieldwork, which has proved its effectiveness in ASF control.

2. Intensive industrial pork production allows systematic control of ASF and has unquestionable advantages over small-scale pork production, including backyards.

3. Industrial pork production, compliant with all developed and scientifically justified requirements for biological security along the whole production chain at establishments, involved into breeding, processing, storage, transportation and marketing of live pigs and pork products, regardless of their ownership type; regionalization of the Russian Federation territory; electronic certification can contribute to solution of the issues, related to the national food security and meat product export in an optimal manner.

4. Other ASF infected countries can benefit from the Russian Federation's experience in ASF control by adapting it to the concrete region with due regard to local social and economic conditions.

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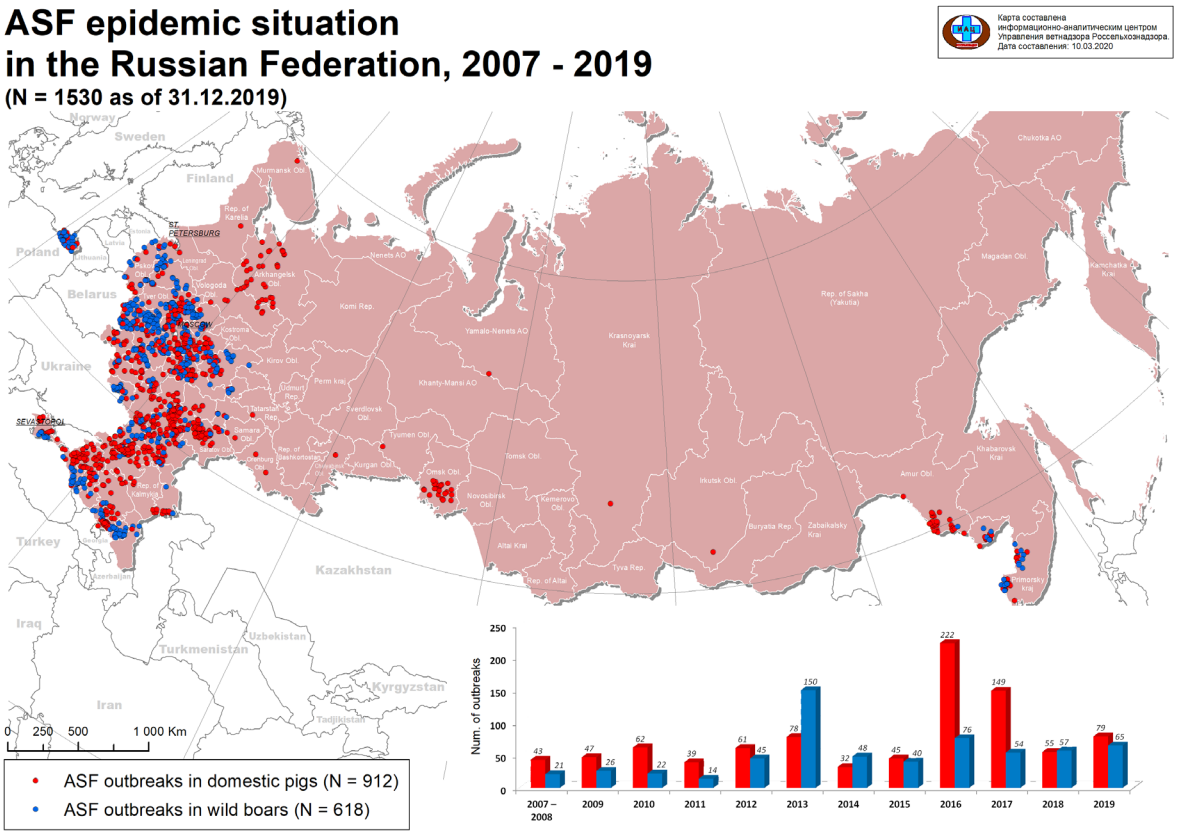

Fig. 3. Declining trend in the number of ASF outbreaks in Russia since 2016 (https://www.fsvps.ru/fsvps-docs/ru/iac/asf/2019/12-31/03.pdf)

Рис. 3. Тренд снижения количества вспышек АЧС в России с 2016 г.

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Heterogeneity of avian infectious bronchitis virus population

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SUMMARY
Avian infectious bronchitis is one of the most common viral infections causing enormous economic losses in the global poultry industry. Due to the lack of mechanisms to correct errors during genome replication, the virus can quickly mutate and generate new strains. This is facilitated by widespread use of live vaccines, simultaneous circulation of field viruses belonging to different serotypes in one flock and rapid spread of the virus. Previous studies of avian infectious bronchitis virus strains and isolates identified in the Russian Federation poultry farms showed that 50% of samples tested positive for the 4-91, D274, H-120, Ma5 vaccine strains, and the other half of samples tested positive for the field viruses belonging to eight GI genetic lineages, while the G1-19 (QX) lineage was dominant. The paper presents identification and genotyping results of the avian infectious bronchitis virus in one of the poultry farms in the Saratov Oblast (the Russian Federation) in 2018–2019. The samples of internal organs and blood, as well as oropharyngeal and cloacal swabs were taken from chicks and layers of different ages in the parent and replacement flocks. The vaccine strain, GI-19 field isolates and variant isolates that do not belong to any of the known genetic lineages were detected. Analysis of test results within a two-year period showed that it is important to study samples taken from birds of different ages. The virus undergoes modification and adaptation inducing new genetic forms by infecting several poultry generations, due to which the heterogeneity of the virus population is observed not only in the poultry farm as a whole or in a separate department, but also within one organism. The identified isolates showed tropism for the tissues of intestine, reproductive organs, and, in rare cases, trachea and lungs. The data obtained indicate that, despite the vaccination used, a genetically diverse population of the infectious bronchitis virus circulates in the poultry farm, while the infection may not manifest itself at an early age, but may affect the flock productivity in the future due to pathological changes in the reproductive organs of laying chickens.

Key words: avian infectious bronchitis virus, genetic analysis, genetic lineage, virus population heterogeneity.

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Гетерогенность вирусной популяции при инфекционном бронхите кур

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РЕЗЮМЕ

Инфекционный бронхит кур является одной из наиболее распространенных вирусных инфекций, наносящих огромный экономический ущерб птицеводству во всем мире. По причине отсутствия механизмов коррекции во время репликации генома вирус может быстро мутировать и генерировать новые штаммы. Этому способствует широкое использование живых вакцин, одновременная циркуляция полевых вирусов, относящихся к разным серотипам в одном стаде, и быстрое распространение вируса. Проведенные ранее исследования выявленных на птицефабриках Российской Федерации штаммов и изолятов вируса инфекционного бронхита кур показали, что 50% положительных проб относятся к вакцинным штаммам 4-91, D274, H-120, Ma5, вторая половина положительных проб представлена полевыми вирусами, которые относятся к 8 генетическим линиям генотипа GI, при этом доминирующей является линия G1-19 (QX). В данной работе представлены результаты по выявлению и генотипированию вируса инфекционного бронхита кур на одной из птицефабрик Саратовской области Российской Федерации в 2018–2019 гг. Внутренние органы, ротоглоточные и клоакальные смывы, кровь отбирали от цыплят и кур-несушек разных возрастов из родительского стада и стада ремонтного молодняка. Выявлены: вакцинный штамм, полевые изоляты генетической линии GI-19 и вариантные изоляты, не относящиеся ни к одной из известных генетических линий. Анализ результатов исследований за двухлетний период показал, что важно исследовать пробы, взятые от птиц разного возраста. Инфицируя несколько поколений птиц, вирус изменяется и приспосабливается, порождая новые генетические формы, благодаря чему наблюдается гетерогенность вирусной популяции не только на птицефабрике в целом или в отдельном цехе, но и в одном организме. Выявленные изоляты обладали тропизмом к тканям кишечника, репродуктивных органов и, в единичных случаях, трахеи и легких. Полученные данные свидетельствуют о том, что на птицефабрике, несмотря на применяемую вакцинацию, циркулирует генетически разнородная популяция вируса инфекционного бронхита, при этом инфекция может не проявляться в раннем возрасте, но может повлиять на продуктивность стада в дальнейшем за счет патологических изменений органов репродукции кур-несушек.

Ключевые слова: вирус инфекционного бронхита кур, генетический анализ, генетическая линия, гетерогенность вирусной популяции.

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INTRODUCTION

Avian infectious bronchitis (IB) is one of the most important viral infections causing significant economic losses in the global poultry industry. The causative agent of the disease is an RNA-containing virus belonging to order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*, which can quickly mutate due to the lack of correction mechanisms during genome replication, i.e. it is able to generate new viral strains.

IB prevention, along with appropriate biosafety measures, is based on routine vaccination. However, this approach is hindered by the high genetic diversity of the virus, leading to the constant emergence of new variants against which cross-protection may be absent. The protection level against a particular variant can be achieved by using either one vaccine based on a strain of the same virus genotype (homologous vaccination), or several vaccines based on different lineages to expand the scope of protection (heterologous vaccination) [1, 2]. For this purpose, various vaccines and immunization schedules are implemented. However, there are still difficulties in selecting attenuated heterologous vaccine virus strains that would provide effective ways to protect poultry from the disease. Despite its importance for disease control, the widespread use of IB vaccines has some significant drawbacks. Live attenuated vaccine strains can enter non-vaccinated herds, restore virulence and also participate in natural recombination. In addition, their application complicates the IB diagnosis, since many detectable field viruses are closely related to vaccine strains.

The analysis of IB virus strains and isolates identified in poultry farms of the Russian Federation showed that approximately 50% of positive samples belong to vaccine strains 4-91, D274, H-120, and Ma5. The second half of the positive samples is represented by the IB field viruses, which belong to 8 genetic lineages of the GI genotype: GI-1 (Mass), GI-12 (D274), GI-13 (793B), GI-14 (B1648), GI-16 (Q1), GI-19 (QX), GI-22, GI-23 (Variant-2). In addition, isolates that are natural recombinants and variant virus isolates that do not belong to any of the known genotypes were detected in poultry farms of the Russian Federation. The dominant group is the genetic lineage GI-19 (QX) [3].

The IB clinical manifestations depend on a number of factors, including virulence and tropism of the virus. The port of the IB virus entry is the respiratory tract, then it spreads systemically, affecting epithelial cells in many tissues. The severity of clinical signs depends on the virus strain and the poultry keeping conditions, such as the microclimate in the poultry house, dust, stocking density, age and type of birds, its immune status (vaccination, immune suppression, presence of maternal antibodies), presence of concomitant infections that are also important factors.

The IB mortality is usually very low but it may increase after secondary bacterial infections [4].

Field isolates of the GI-19 (QX) genetic lineage have tropism for epithelial cells of almost all organ systems, thereby inducing all possible syndromes of avian infectious bronchitis. Researchers describe viruses of this genetic lineage as respiratory, nephropathogenic, affecting reproductive organs, and there are also data indicating that some field strains damage the intestinal tract [5, 6]. Most of the IB virus field isolates were isolated and described during outbreaks with the acute course of the disease. However, there are known cases of the detection of the IB GI-19 genetic lineage virus with asymptomatic infection or with mild respiratory disorders [7].

The aim of this research was to study biological material from poultry of various ages kept in different units of one individual poultry farm for the presence of the IB virus genome, followed by phylogenetic analysis of the obtained nucleotide sequences of the S1 gene fragment.

MATERIALS AND METHODS

Samples of poultry biological material were tested using reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR according to methodical instructions [8, 9].

Comparative analysis of the S1 gene fragment of approximately 500 nucleotide bases (position 112–653 n.b. of S gene of H120 strain) was performed for IB virus genotyping. Nucleotide sequences of prototype strains proposed by V. Valastro et al. were used for the analysis [10].

The nucleotide sequences were determined according to Sanger method using fluorescence labeled chain-terminating nucleotides involving ABI Prism 3130 automated sequencer (Applied Biosystems, USA) according to the manufacturer's instructions.

The obtained nucleotide sequences were compared with IB virus sequences deposited in the international database NCBI (http://www.ncbi.nlm.nih.gov) using BioEdit software, version 7.0.5.3.

IB specific antibodies were detected in blood sera using the FGBI "ARRIAH" ELISA test kit for determining antibodies to avian infectious bronchitis virus when testing sera in single dilution in accordance with the instructions. The test results were recorded using Tecan spectrophotometer plate reader (Austria) at a wavelength of 405 nm using the SINKO-IFA software. The test was considered positive if the antibody titer was 725 or higher.

RESULTS AND DISCUSSION

Live poultry (9 replacement chickens and 12 layers of the parent stock) from one of the poultry farms in the Saratov Oblast were received and tested for the presence of IB virus genetic material in the FGBI "ARRIAH" (Table 1).

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

No abnormalities were detected in live chickens at 19–290 days of age, received in December 2018, which were visually examined and subjected to autopsy. Multiple cysts of reproductive organs with a watery liquid of more than 100 ml were observed during autopsy of chickens at 361 days of age, received in October 2019.

Oropharyngeal and cloacal swabs, as well as samples of tissues of internal organs (trachea, lungs, kidneys, intestines, reproductive organs) were collected from each chicken for PCR testing. At the first stage the tissue samples of the internal organs, pooled within each group (i.e. 8 samples) were tested (Table 1). Pooled sample No. 1 from 19-day-old chicks from Unit 4 was negative in RT-PCR and real-time RT-PCR, despite the fact that all chicks were vaccinated with a bivalent vaccine based on strains H120 and D274 at two days of age and with the vaccine based on strain 4–91 at 12–14-days of age according to the manufacturer's instructions. The IB virus genome was detected in other pooled samples (No. 2–8) (Table 2). Similar results were obtained inblood sera tests using enzyme-linked immunosorbent assay (ELISA), when IB virus-specific antibodies were detected in poultry of all groups except for Group 1 (Table 3).

The 4-91 IBV45-18 vaccine derivative was detected in sample No. 4 (the nucleotide sequence of the S1 gene fragment was 99% homologous with the vaccine strain and differed from it by 4 bp). The data in the scientific literature indicate that the passaging of vaccine virus strains in live poultry and chicken embryonated eggs sometimes leads to single nucleotide and amino acid substitutions. It is interesting to note that one and the same vaccine virus

Table 1
Characteristics of poultry received for testing

Таблица 1
Характеристика поступившей для исследования птицы

Date of Submission	Group No.	Poultry	Unit No.	Age, days
12.2018	1	Replacement chickens (3 birds from each unit)	4	19
	2		7	60
	3		1	124
	4	Laying chickens of the Ross-PM3-cross parental stock (2 birds from each unit)	21	192
	5		7	228
	6		15	290
10.2019	7	Laying chickens of the Ross-PM3-cross parental stock (3 birds from each unit)	1	361
	8		18	361

Table 2
Genotyping results of avian infectious bronchitis virus

Таблица 2
Результаты генотипирования вируса инфекционного бронхита кур

Date	Group No.	Poultry age, days	Genetic relation	Isolate name
12.2018	2	60	GI-19	IBV42-18
	3	124	GI-19	IBV42-18 IBV43-18
	4	192	GI-13, 4-91 vaccine strain derivative	IBV45-18
	5	228	Variant isolate	IBV44-18
	6	290	GI-19	IBV43-18
10.2019	7	361	Variant isolate	IBV43-19
	8	361	GI-19	IBV44-19

strain used for production of different vaccines batches by one or more manufacturers mutates differently after passaging. These differences may indicate different sources of the original virus strain obtained for the production of this vaccine, as well as the methods and number of the virus passages carried out for its attenuation [11].

The following field viruses were detected in the other remaining positive samples: three isolates of the S1 gene fragment of the GI-19 genetic lineage (IBV42-18, IBV43-18, IBV44-19) and two variant isolates with a unique primary structure (IBV44-18, IBV43-19), not related to any genetic lineage (Table 2, Fig. 1).

The phylogenetic analysis showed that IBV44-19 isolate of GI-19 lineage, identified in 2019, formed a separate branch and differed in amino acid composition from isolates IBV42-18 and IBV43-18 identified in 2018, by 7.5 and 10.3% respectively. Whereas the isolates IBV42-18 and IBV43-18 differed only by 2.7% (Fig. 2) and were found in birds at 60, 124 and 290 days of age, and a mixture of these two isolates was detected in Group 3 (at 124 days of age).

The IBV variant isolates IBV44-18 and IBV43-19 having a unique structure and not related to any genetic lineage were identified in Samples No. 5 and 7. These isolates had 13.7% differences in amino acid composition. Earlier, in March 2018, the other two similar variant isolates (IBV08-18 and IBV09-18) were identified in the material from laying hens at the age of 359 and 285 days kept in different units on this poultry farm. These isolates differed from isolates IBV44-18 and IBV43-19 by 10.3% in amino acid composition, which indicates their different origin (Fig. 1, 3). Variant isolates result from the accumulation of mutations (point substitutions, deletions, insertions, and recombinations) that occur due to IB virus replication errors. A variant isolate may appear non-viable, or it may circulate for a long time on the farm and continue changing.

At the next stage of research process the virus tropism was determined. For this purpose, oropharyngeal, cloacal swabs and samples of tissues of internal organs were taken from each bird and then individually tested using real-time RT-PCR (Table 3). The IB virus isolate of the genetic lineage GI-19 (QX) IBV42-18 was recovered in the pooled sample from three 60-day-old chickens using RT-PCR and sequencing analysis. The samples of intestines and testes from two chicks were positive in real-time RT-PCR.

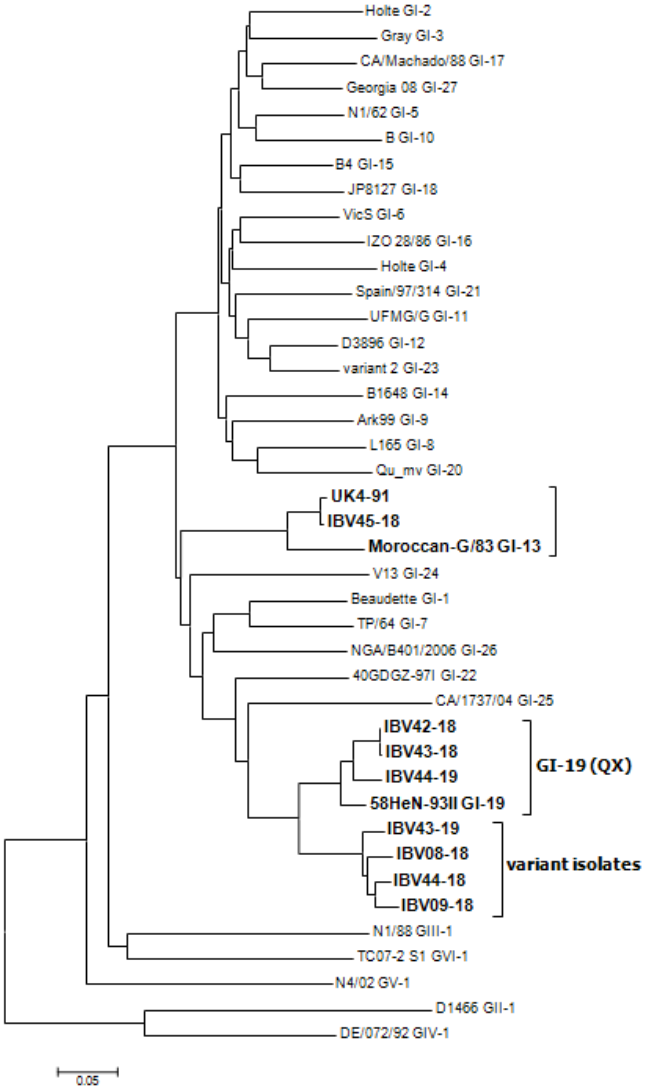

Fig. 1. Phylogenetic relationships based on analysis of the S1 gene fragment of IB virus isolates identified in a poultry farm in Saratov Oblast in 2018–2019

Рис. 1. Филогенетические связи на основе анализа фрагмента гена S1 изолятов вируса ИБК, выявленных на птицефабрике Саратовской области в 2018–2019 гг.

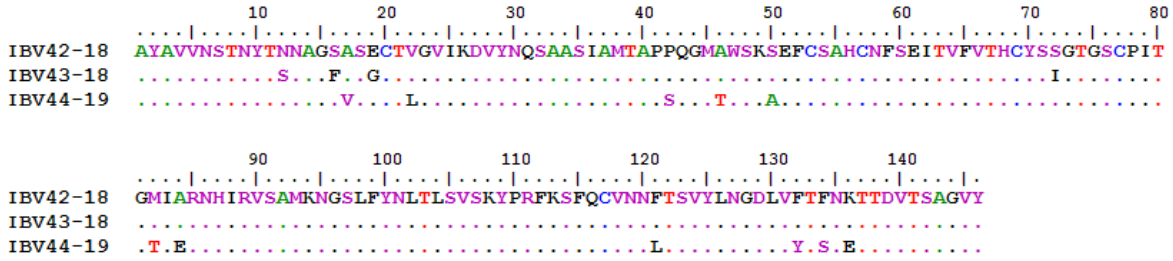

Fig. 2. Comparison of amino acid sequences of identified GI-19 isolates

Рис. 2. Сравнение аминокислотных последовательностей выявленных изолятов генетической линии GI-19

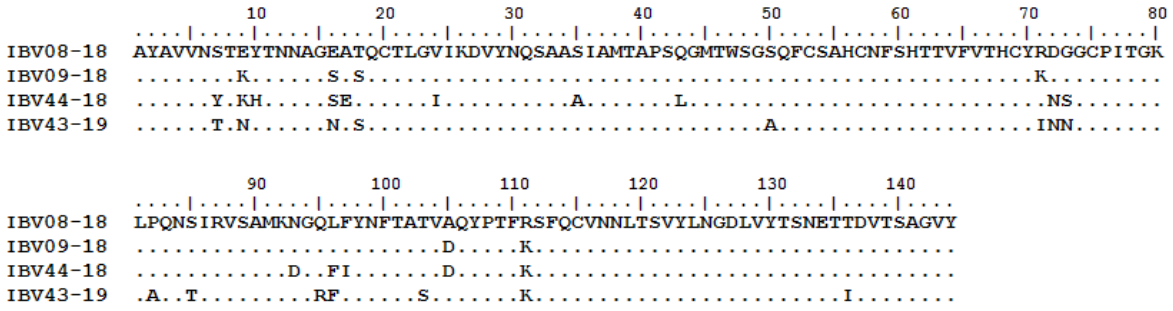

Fig. 3. Comparison of amino acid sequences of identified variant isolates

Рис. 3. Сравнение аминокислотных последовательностей выявленных вариантных изолятов

A combination of field viruses IBV42-18 and IBV43-18 of the IB virus genetic lineage GI-19 (QX) was recovered in Group 3 with replacement chicks (124 days of age) using RT-PCR and sequencing analysis. The samples taken from each organ and the pooled tissue sample from one of the three chicks tested negative in real-time RT-PCR. The IB virus genome was detected in the biological material of trachea and lungs in two other chicks of this group, as compared with other age groups, where only samples of intestinal tissues and reproductive organs were positive. Sequencing of the fragment of the S1 gene of the virus detected in samples obtained from the tissues of the trachea and intestines of chick No. 8 was carried out. It was found that the field virus IBV42-18 persisted in the intestine, and the vaccine strain 4-91 was detected in the trachea of the same chick.

Vaccine strain 4-91 (IBV45-18), two variant isolates (IBV44-18, IBV43-19) and two field virus (IBV43-18, IBV44-19) of the IB virus genetic lineage GI-19 (QX) were recovered in samples of tissues of internal organs from poultry of the parent flock of the ages of 192, 228, 290 and 361 days using RT-PCR and sequencing methods. The samples of intestinal tissues, reproductive organs, and one cloacal swab were positive for IB virus in real-time RT-PCR (Table 3).

An analysis of study results obtained at one poultry farm over a two-year period showed that the detection of IB virus using molecular methods depended on the type of sampling, the number of samples, the poultry age, tested organs etc. While infectious bronchitis causes reproductive and nephropathogenic syndromes, the clinical manifestations are often observed later, many months after infection, and the virus itself may no longer be found in the tissues of the bird, so it is important to examine samples from birds of different ages. On the other hand, the IB virus can persist for a long time in the environment. By infecting several poultry generations, the virus changes and adapts, causing new genetic forms, due to which the heterogeneity of the viral population is observed not only in the poultry farm as a whole or in a separate unit, but also in one organism. The virus can be represented via a set of virions containing both slightly modified but closely related genomes and genomes belonging to different genetic lineages even within one bird, contributing to the rapid adaptation and survival of the virus in the host organism. In addition, the diversity of the virus in one poultry farm, in particular, the appearance of new isolates with a high percentage of amino acid differences (8–10% and higher), indicates an external route of the virus introduction.

CONCLUSION

Phylogenetic analysis of the nucleotide sequences of the avian infectious bronchitis virus isolates recovered in one of the poultry farms in the Saratov Oblast of the Russian Federation in 2018–2019 was carried out. The heterogeneity of the viral population was shown in the layer parent flock and the replacement flock, where, besides the vaccine strain used, three field viruses of the GI-19 genetic lineage and two variant isolates not belonging to any of the known genetic lineages were identified. The data obtained indicate that, despite the vaccination implemented, a genetically heterogeneous IB virus population is circulating in the poultry farm, and it may not manifest itself at an early age, but may affect flock productivity due to pathological changes in layer reproductive organs.

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Table 3
Test results for tissue samples of internal organs from birds of different ages for presence of avian infectious bronchitis virus

Таблица 3
Результаты исследований проб тканей внутренних органов птиц разных возрастов на наличие вируса ИБК

Poultry number and age			Antibody titer	Real-time RT-PCR results, Ct							
				Organ pooled sample	Trachea	Lungs	Kidney	Intestine	Oviduct/ testes*	Oropharyngeal swabs	Cloacal swabs
1	Replacement stock	19	negative	negative	not tested	not tested	not tested	not tested	not tested	negative	negative
2			negative	negative	not tested	not tested	not tested	not tested	not tested	negative	negative
3			negative	negative	not tested	not tested	not tested	not tested	not tested	negative	negative
4		60	**3,220**	**33.9**	negative	negative	negative	**32.0**	negative	negative	negative
5			equivocal	negative	negative	negative	negative	negative	negative	negative	negative
6			**2,474**	**35.7**	negative	negative	negative	**36.6**	**35.0***	negative	negative
7		124	**3,600**	**33.7**	**35.6**	**39.8**	negative	**30.3**	negative	negative	negative
8			**5,012**	**34.7**	**38.5**	negative	negative	**34.7**	negative	negative	negative
9			**3,373**	negative	negative	negative	negative	negative	negative	negative	negative
10	Parent stock	192	not tested	**34.5**	negative	negative	negative	**29.9**	negative	negative	negative
11			**7,542**	**34.4**	negative	negative	negative	**31.8**	**38.5**	negative	negative
12		228	**4,925**	**34.4**	negative	negative	negative	**32.2**	negative	negative	negative
13			**5,857**	**35.2**	negative	negative	negative	**32.3**	**36.7**	negative	**38.4**
14		290	**3,341**	**33.8**	negative	negative	negative	**30.5**	**39.7**	negative	negative
15			**2,602**	negative	negative	negative	negative	negative	negative	negative	negative
16		361	**2,894**	not tested	negative	negative	negative	negative	negative	negative	negative
17			**5,454**	**37.9**	negative	negative	negative	**37.6**	negative	negative	negative
18			**3,828**	not tested	negative	negative	negative	negative	negative	negative	negative
19			**7,916**	**36.2**	negative	negative	negative	**34.1**	negative	negative	negative
20			**4,609**	not tested	negative	negative	negative	negative	negative	negative	negative
21			**2,931**	not tested	negative	negative	negative	negative	negative	negative	negative

negative – negative result;
equivocal – equivocal result;
not tested – not subjected to testing.

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Characteristics of microbial contamination of animal raw materials and products in the Russian Federation from 2015 to 2018

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SUMMARY

To date, there is a whole system of legal documents, regulating food security issues in the Russian Federation. Monitoring of food quality and safety is performed on the federal level, on the level of the Russian Federation Subjects and on the municipal level based on the developed and adopted regulatory and methodical documents. The paper presents the analysis of ASSOL information system data related to microbial contamination of animal raw materials and products, collected within the following official activities: "Laboratory Testing within Official Monitoring of Food Safety and Quality" and "Laboratory Testing of Animal Raw Materials and Products, Feed and Biological Materials for the Purposes of Food Safety and Quality Assurance". Microbiological test data were obtained from 37 Russian laboratories, subordinate to the Rosselkhoznadzor, within 2015–2018. The analysis performed showed that the maximum number of tests was performed for the following pathogenic microorganisms: *Salmonella* bacteria (29.5% within official monitoring and 26.8% within official programme). The highest number of non-compliances within monitoring was revealed when testing for total viable count (total mesophilic anaerobic and facultative anaerobic microorganisms) – 14.8% and Coliforms – 8.98%; within the official programme most violations were related to yeast (18.8%), yeasts and molds (18.5%) and TVC (12.4%). The parameters, showing less than 1% of positives within official programme testing, were identified. They include *Vibrio parahaemolyticus*, *Proteus* bacteria and sulphite-reducing clostridia. The necessity in further tests for safety and quality of animal raw materials and products in the Russian Federation was justified.

Key words: animal raw materials and products, microbiological parameters of safety, official monitoring, risk-oriented approach.

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Характеристика микробной контаминации сырья и продукции животного происхождения в Российской Федерации за период с 2015 по 2018 год

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РЕЗЮМЕ

На сегодняшний день в Российской Федерации сложилась целая система нормативно-правовых документов, регулирующих вопросы обеспечения продовольственной безопасности. Мониторинг качества и безопасности пищевых продуктов осуществляется на федеральном уровне, уровне субъектов Российской Федерации, уровне муниципальных образований на основе разработанных и утвержденных в установленном порядке нормативных и методических документов. В статье представлен анализ данных информационной системы «Ассоль» по микробной контаминации сырья и продукции животного происхождения, полученных при выполнении государственных работ «Проведение лабораторных исследований в рамках плана государственного мониторинга качества и безопасности пищевых продуктов» и «Проведение лабораторных исследований сырья, продукции животного происхождения, кормов и биологического материала в целях обеспечения качества и безопасности пищевых продуктов». Данные по микробиологическим показателям были получены в 37 подведомственных Россельхознадзору лабораториях Российской Федерации за период с 2015 по 2018 г. На основании выполненного анализа установлено, что максимальное количество исследований приходилось на обнаружение патогенных микроорганизмов: бактерий рода *Salmonella* (29,5% при проведении государственного мониторинга и 26,8% при выполнении государственного задания) и *Listeria monocytogenes* (22 и 21% соответственно). Наибольшее количество несоответствий при проведении мониторинга установлено по показателям КМАФАнМ (количество мезофильных анаэробных и факультативно анаэробных микроорганизмов – 14,8%) и БГКП (бактерии группы кишечной палочки – 8,98%); при выполнении государственного задания – по показателям дрожжи (18,8%), дрожжи и плесневые грибы (18,5%) и КМАФАнМ (12,4%). Определены показатели, процент выявления которых при выполнении государственных работ составил менее 1. Это парагемолитический вибрион, бактерии рода *Proteus* и сульфитредуцирующие клостридии. Обоснована необходимость дальнейших исследований по оценке безопасности и качества сырья и продукции животного происхождения на территории Российской Федерации.

Ключевые слова: сырье и продукция животного происхождения, микробиологические показатели безопасности, государственный мониторинг, риск-ориентированный подход.

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INTRODUCTION

To date, there is a legal and regulatory system, regulating safety of animal raw materials and products in the Russian Federation. To ensure animal and public health safety several legal acts have been developed, including RF Veterinary Law No. 4979-1, Federal Laws on food safety and quality No. 29-FZ and technical regulation No. 184-FZ [1, 2].

The basic documents regulating processes of production (manufacturing), storage, transportation (movement), marketing and rendering of food products are Customs Union Regulations Nos. 021/2011, 033/2013, 034/2013 and Eurasian Economic Union Regulation No. 040/2016 [1, 2].

Pursuant to CU TR 021/2011 on food safety, the food business operator shall develop, introduce and maintain procedures, based on HACCP principles [2].

The major provisions of HACCP concept are given in the Directives of the European Council. HACCP principles are recommended for practical use by Codex Alimentarius Commission and are obligatory for the European Union (EU) countries [3, 4].

The importance of HACCP system is acknowledged by all countries in the world, but the degrees of this system implementation vary a lot in different countries. If maintenance of HACCP principles is mandatory for the EU, the adherence to these principles in the Russian Federation is far from being strict [3, 4, 5, 6].

Pursuant to Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products laboratory tests shall be performed for two major groups of substances: group A – substances having anabolic effect and unauthorized substances and group B – veterinary drugs and contaminants. Microbiological criteria are not included into animal product monitoring scope due to a well developed HACCP system in the EU countries and a great share of food business operators' responsibility for the food safety [6].

As stated above the level of HACCP system implementation in the Russian Federation is not adequate yet [3, 4, 5]. In this context to ensure the compliance with the requirements of the WTO Agreement on Application of Sanitary and Phytosanitary Measures the microbiological criteria were included into the Official Food Quality and Safety Monitoring Plan by the Order of the Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor).

Starting from 2007 the Food Monitoring Plan in the Russian Federation has been implemented by the Rosselkhoznadzor.

The target of this study is to analyze the data of ASSOL Information System on microbial contamination of animal raw materials and products, collected within the following public works: "Laboratory Testing within Official

Monitoring of Food Safety and Quality" (hereinafter "Food Quality and Safety Monitoring") and "Laboratory Testing of Animal Raw Materials and Products, Feed and Biological Materials for the Purposes of Food Safety and Quality Assurance" (hereinafter "Food Safety") during 2015–2018.

MATERIALS AND METHODS

Pursuant to Regulation on Monitoring of Food Quality and Safety and Public Health, approved by Ordinance of the RF Government No. 883 on November 22, 2000, food safety and quality monitoring shall be performed on the federal level, on the level of the Russian Federation Subjects and on the municipal level in accordance with developed and adopted regulatory and methodical documents [2].

ASSOL Information System data for 2015–2018 related to the public works implementation ("Food Safety and Quality Monitoring" and "Food Safety") from 37 laboratories subordinate to the Rosselkhoznadzor were analyzed. Microbiological criteria of safety and quality were focused on.

Microsoft Excel program was used for diagram constructions.

RESULTS AND DISCUSSION

Public works "Food Safety and Quality Monitoring" and Official Epidemiological Monitoring with respect to microbiological tests in 2015–2018.

Pursuant to Orders of the Rosselkhoznadzor No. 831 of 31.12.2014, No. 993 of 31.12.2015, No. 995 of 30.12.2016, No. 1304 of 28.12.2017 on laboratory tests within the implementation of the Rosselkhoznadzor activities aimed at fulfillment of WTO SPS Agreement commitments, taken under accession of the Russian Federation to the WTO the Official Food Quality and Safety Monitoring Plan was approved.

131,545 microbiological tests were conducted in 2015–2018. Data are shown in Table 1.

As shown in Table 1 the highest number of tests was performed for pathogenic *Salmonella* (29.5 % out of total number) and *L. monocytogenes* (22%). Further, in descending order of quantity, tests for total viable count (20.1%) and coliforms (15.6%) are listed. Tests for *Staphylococcus aureus* account for 4% out of total number of tests. Insignificant numbers of tests were performed for *Clostridium* sulphite-reducing bacteria (0.6%), *Proteus* bacteria (0.5%) and *Vibrio parahaemolyticus* (0.4%).

It should be noted that food spoilage indicators (yeasts and molds) were not included into the Official Food Quality and Safety Monitoring Plan.

Table 1 also shows the number of positive results for the above-mentioned microorganisms. The biggest number of positives accounts for TVC (14.8%), somatic cells (9.2%) and coliforms (9.0%), which suggests the unsatisfactory conditions at animal raw material and product manufacturing plants in the Russian Federation.

L. monocytogenes were detected in 4.2% of samples tested, *Salmonella* bacteria in 1.8%.

The detection rate for *Staphylococcus aureus* was 0.9%, for *Clostridium* sulphite-reducing bacteria – 0.6%,

Table 1
Characteristics of microbial contamination of animal raw materials and products, detected within official monitoring of food quality and safety in 2015–2018

Таблица 1
Характеристика микробной контаминации сырья и продукции животного происхождения, выявленной при реализации государственного мониторинга качества и безопасности пищевых продуктов в 2015–2018 гг.

Criteria	Number of tests according to Monitoring Plan	Share in microbiological tests, %	Number of positive tests	Detection rate, %
Total viable count	26,411	20.1	3,901	14.8
Somatic cells	4,146	3.2	383	9.2
Coliforms	20,520	15.6	1,843	9.0
Enteropathogenic *Escherichia coli*	3,351	2.5	191	5.7
Listeria monocytogenes	28,958	22.0	1,229	4.2
Total bacterial count	2,129	1.6	87	4.1
Salmonella	38,762	29.5	712	1.8
Staphylococcus aureus	5,234	4.0	45	0.9
Clostridium sulphite-reducing bacteria	798	0.6	5	0.6
Proteus bacteria	731	0.5	4	0.6
Vibrio parahaemolyticus	505	0.4	0	0
Total	**131,545**	**100**	**8,400**	**6.4**

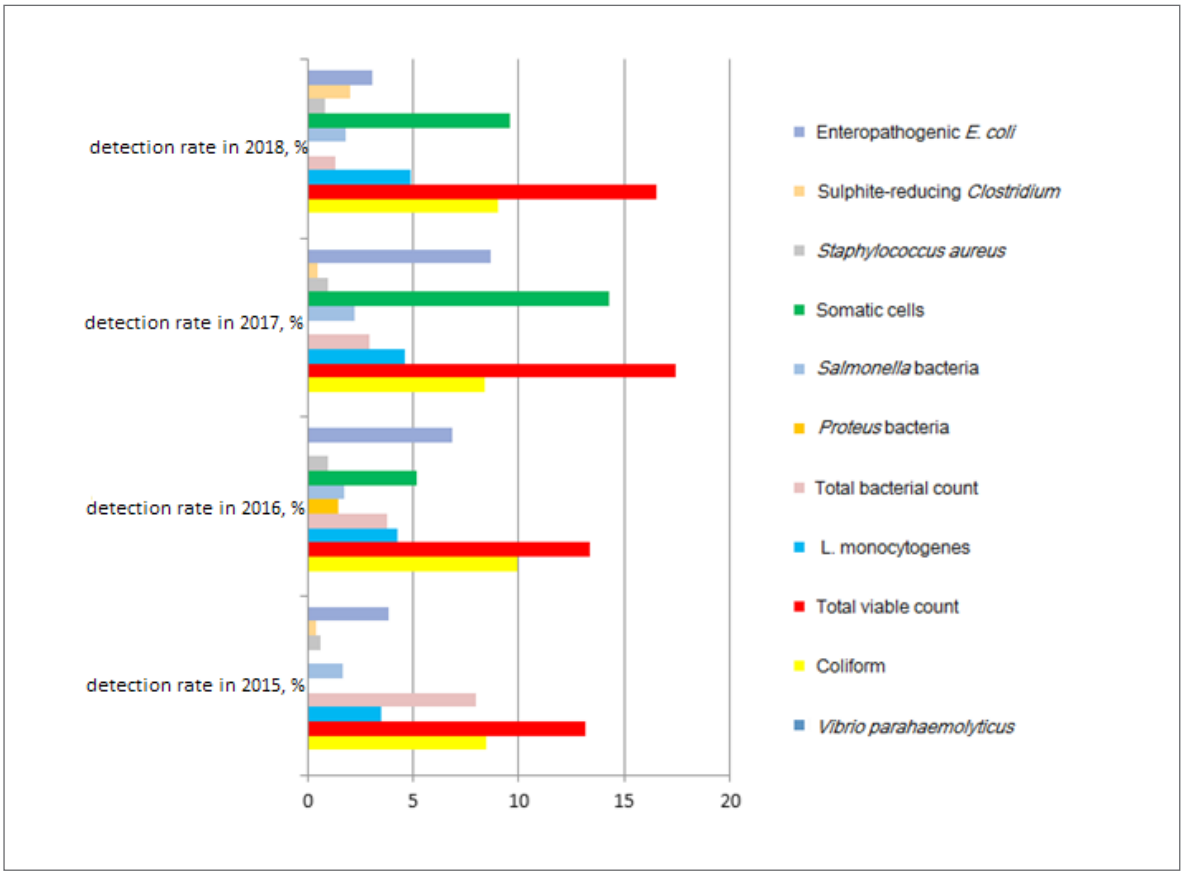

Fig. 1. Microbiological contaminant detection dynamics within official monitoring of food quality and safety in 2015–2018

Рис. 1. Динамика выявления микробиологических контаминантов при выполнении государственного мониторинга качества и безопасности пищевых продуктов за период с 2015 по 2018 г.

Proteus bacteria – 0.6%. *Vibrio parahaemolyticus* was not detected.

Percentage of non-compliances related to enteropathogenic *Escherichia coli* and total bacterial count in animal feeding stuffs were 5.7 и 4.1% respectively, which is indicative of technological violations in the production and storage processes.

Figure 1 presents detection rates for different microorganisms by years.

The detection rate for *Staphylococcus aureus* is within 0.6–1.0%, for *Salmonella* bacteria is 1.7–2.3% and for coliforms is 8.5–9.1%. Sulphite-reducing *Clostridium* were detected in 2015, 2017 and 2018, exceeded levels of somatic cells in raw milk products were reported in 2016, 2017 and 2018; *Proteus* bacteria were isolated only in 2016. Positive trend towards decreasing proportion of non-compliances related to total bacterial count in feeding stuffs of animal origin from 8% in 2015 to 1.3% in 2018 should be noted. The percentage of non-compliances, associated with total viable count in animal products was within 13.2–13.3 in 2015–2016, but it grew up to 17.4 in 2017. In 2018 the number of TVC violations decreased by 0.9% and was equal to 16.5%.

The analysis of monitoring data showed an upward trend in the number of detections of pathogenic *L. monocytogenes* from 3.5% in 2015 to 4.9% in 2018. We believe that this case can be explained by the biofilm-forming capability of *L. monocytogenes*, thus increasing the risk of animal product secondary contamination.

In 2015–2018 pursuant to the Orders of the Rosselkhoznadzor laboratory tests within the Official Epidemiological Monitoring were performed.

Data on detections of animal salmonellosis, listeriosis and campylobacteriosis agents are given in Table 2.

According to the data, presented in Table 2, 36,160 tests were performed for *Salmonella*. The detection rate was 1.5%.

Within the period under review *L. monocytogenes* were isolated from animal pathological material only in two cases (0.03%). It should be noted that the detection rate for this microorganism is 4.2% (see Table 1). We believe, that this difference can be explained by several reasons.

First of all, due to the differences in methodical approaches to listeria testing. To date *L. monocytogenes* isolation from food products is performed pursuant to GOST 32031-2012 "Food Products. Methods of *Listeria monocytogenes* detection", which envisages two-step enrichment of the sample. Pathological material shall be tested pursuant to "Methodical recommendations on laboratory diagnostics of human and animal listeriosis" (approved by USSR Ministry of Health on September 4, 1986 and USSR State Agriculture Committee on February 13, 1987). According to the methodical recommendations for the bacteriological test saline solution should be added to the suspension of brain and parenchymal organs (ratio 5:1); then this mixture should be inoculated into meat-peptone or Hottinger broth following by re-inoculation into liver or blood agar or potassium tellurite agar.

Secondly, the secondary contamination, caused by violation of production and processing technologies could be the reason of the product contamination.

The third possible reason, impeding bacteriological diagnostics of listeriosis in pathological material, can be concomitant microflora, able to inhibit vital functions of listerias.

The detection rate for *Campylobacter* in tests performed in 2015–2018 was 1.1%.

According to the World Health Organization *Campylobacter* bacteria are one of the most common reasons of acute diarrheal diseases [7, 8, 9, 10]. Nevertheless, tests for *Campylobacter* in animal products are currently not included into the public works plan in the Russian Federation.

Characteristics of microbial contamination of animal products detected in the course of the "Food Safety" official programme implementation in 2016–2018.

The official programme on food safety testing in 2015–2018 was implemented based on the following Orders of the Rosselkhoznadzor: No. 790 of 26.12.2014, No. 915 of 17.12.2015, No. 081-00037-16-00 of 29.12.2015, No. 081-00032-17-00 of 30.12.2016, No. 081-00015-18-00 of 29.12.2017. Data on microbiological testing results have been introduced into the ASSOL system since 2016.

The results of the official programme on microbiological testing for food safety in 2016–2018 are given in Table 3.

Table 2
Number of tests for pathogenic microorganisms, performed in 2015–2018 within epidemic monitoring

Таблица 2
Количество исследований на патогенные микроорганизмы при проведении эпизоотологического мониторинга в 2015–2018 гг.

Bacteria	Number of tests	Number of positives	Detection rate, %
Salmonella	36,160	546	1.5
Campylobacter	1,269	14	1.1
L. monocytogenes	7,831	2	0.03

117,001 tests for microbiological parameters were performed during three years in total, 6,686 non-compliances were revealed with the detection rate equal to 5.7%.

Distribution of tests by criteria is the following: the biggest number comes from pathogenic microorganisms: *Salmonella* (26.8%) and *L. monocytogenes* bacteria (21.0%). They are followed by tests for TVC (19.7%) and coliforms (19.1%). The share of tests for *Staphylococcus aureus* is 7.0%, and this is almost twice higher than the monitoring test rate. The official programme envisages testing for food

Table 3
Microbial contamination of animal products, detected within the official programme in 2016–2018

Таблица 3
Микробная контаминация продукции животного происхождения, выявленная при реализации государственного задания за период с 2016 по 2018 г.

Criteria	Tests in total	Share in microbiological tests, %	Number of positive tests	Detection rate, %
Yeasts	968	0.8	182	18.8
Yeasts and molds	1,079	1.0	200	18.5
Lactic acid bacteria	306	0.3	39	12.8
Total viable count	23,016	19.7	2,842	12.4
Molds	594	0.5	51	8.6
Coliforms	22,391	19.1	1,911	8.5
Enteropathogenic *Escherichia coli*	2,124	1.8	137	6.5
L. monocytogenes	24,562	21.0	766	3.1
Pseudomonas aeruginosa	173	0.1	5	2.9
Total bacterial count	564	0.5	10	1.8
Staphylococcus aureus	8,170	7.0	130	1.6
Salmonella	31,392	26.8	404	1.3
Sulphite-reducing *Clostridium*	1,074	0.9	7	0.7
Proteus	588	0.5	2	0.3
Total	**117,001**	**100**	**6,686**	**5.7%**

spoilage indicators like yeasts and molds. Their combined share was 2.3%. Small number of tests accounts for tests for sulphite-reducing *Clostridium* (0.9%), *Proteus* (0.5%), *Pseudomonas aeruginosa* (0.1%). 2,688 tests (2.3%) were carried out to check feeding stuffs of animal origin (total bacterial count and enteropahogenic *E. coli*) .

The highest number of non-compliances was detected in tests for yeasts (18.8%), yeasts and molds (18.5%). They are followed by tests for lactic acid bacteria (12.8%) and TVC (12.4%), molds (8.6%), coliforms (8.5%), enteropathogenic *E. coli* (6.5%), *Pseudomonas aeruginosa* (2.9%), total bacterial count (1.8%) and *Staphylococcus aureus* (1.6%).

Among pathogenic microorganisms the highest detection rate was shown by *L. monocytogenes* (3.1%). The number of non-compliances in testing for *Salmonella* was 1.3%.

The detection rates for sulphite-reducing *Clostridium* and *Proteus* bacteria were 0.7 and 0.3% respectively.

The analysis of data, shown in Figure 2, proves a high number of non-compliances in tests for coliforms (the detection rate was within 8.2–8.9%) and total viable count (10.7–13.4%) and small number of detections in tests for sulphite-reducing clostridia (0.7% in average for the period under review).

Non-compliances in tests for different criteria were not revealed every year. *Pseudomonas aeruginosa* positives were reported in 2016 (3% detection rate) and 2017 (2.7%), *Proteus* bacteria were detected only 2016 (0.6%). Positives for molds were found in 2016 (9.6% detection rate) and in 2017 (7.3%). Exceeded levels of total bacterial counts were reported in 2017 (2.4%) and 2018 (0.5%).

Up to 2018 an increasing trend in the yeast detection rate was noted (from 14.6% in 2016 up to 23.6% in 2018). In 2016 no tests for lactic acid bacteria were performed. From 2017 to 2018 the detection rate in tests for this criterium increased from 4 to 15.6%.

The detection rate for *L. monocytogenes* was 2.8% in average in 2016 and 2017, but up to 2018 it reached 3.7%. The detection rate for *Salmonella* grew from 0.9% in 2016 to 1.5% in 2017 and 2018.

The presented data suggest the trend of decreasing in detection of *Staphylococcus aureus* in food products and enteropathogenic *E. coli* in feeding stuffs.

The analysis of microbiological test results, obtained during public works

Pursuant to Programme "Reform of Controlling and Supervising Activities", approved in December 2016, the Rosselkhoznadzor developed the institutional dossier of the project implementation in this area. The dossier envisages the implementation of several types of official veterinary surveillance and control at the border crossing points of the Russian Federation and (or) at the customs clearance facilities. It also ensures the application of a risk-oriented approach during control and surveillance activities within the established types of the control (surveillance).

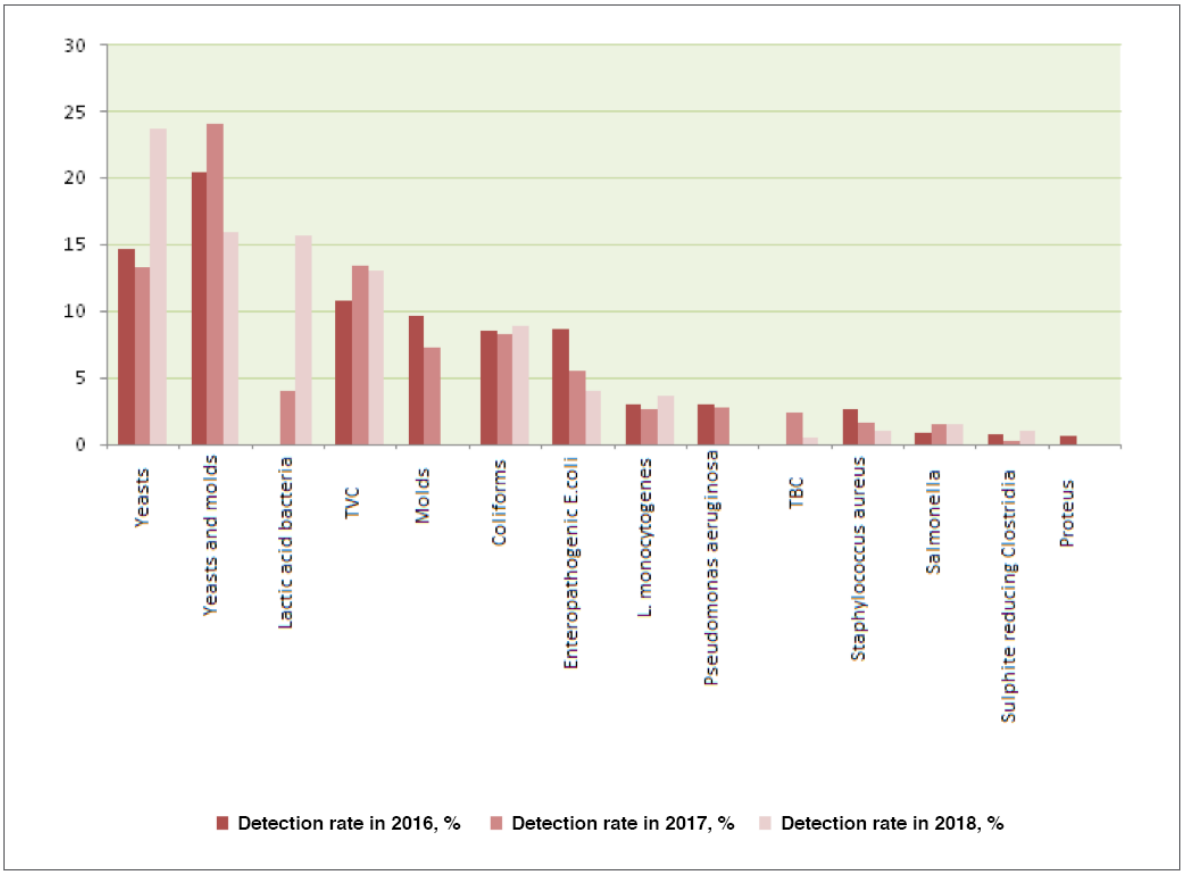

Fig. 2. Dynamics of non-compliances detection in microbiological tests within the official programme "Food Safety" in 2016–2018

Рис. 2. Динамика выявления несоответствий по микробиологическим показателям при выполнении государственного задания «Безопасность пищевой продукции» в 2016–2018 гг.

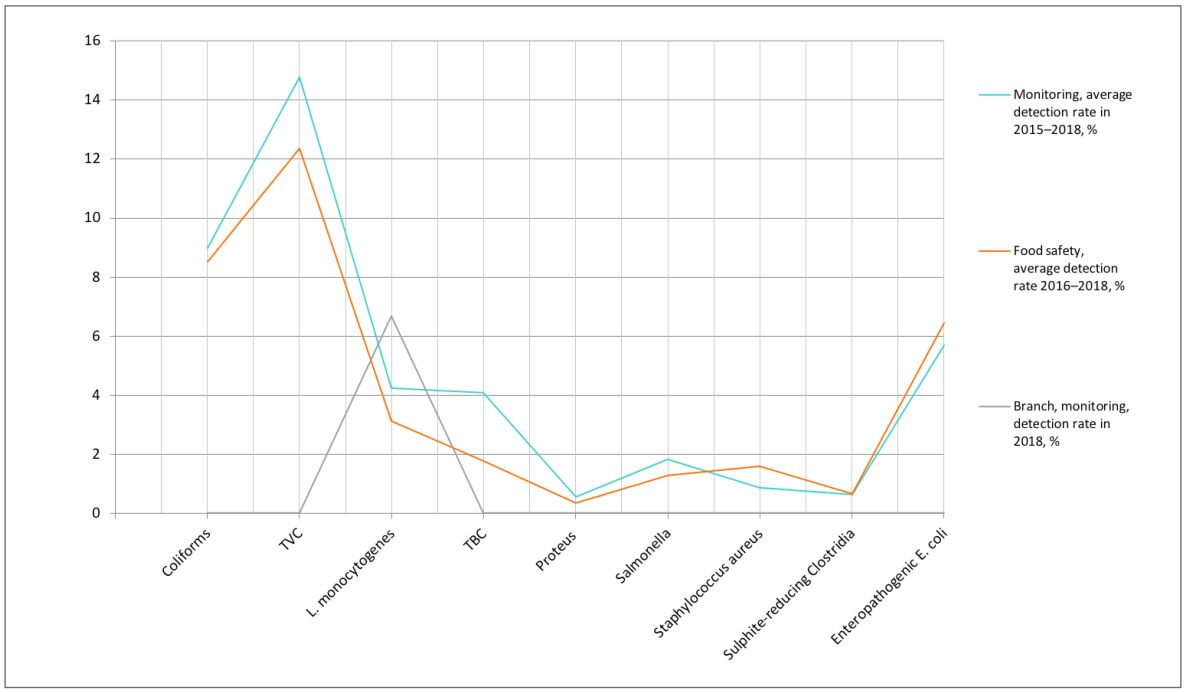

Fig. 3. Results of official microbiological testing

Рис. 3. Результаты выполнения государственных работ по микробиологическим показателям

Table 4
Total tests and total positives in tests for major microbiological parameters within official activities in 2016–2018

Таблица 4
Общее количество исследований и общее количество положительных исследований по основным микробиологическим показателям при проведении государственных работ за период с 2016 по 2018 г.

Criteria	2016		2017		2018	
	Total tests (OP+MP)	Total positives (OP+MP)	Total tests (OP+MP)	Total positives (OP+MP)	Total tests (OP+MP)	Total positives (OP+MP)
Salmonella	22,338	297	18,013	333	18,497	300
L. monocytogenes	15,984	584	14,500	508	14,450	601
TVC	15,078	1,806	13,235	1,998	12,613	1,820
Coliforms	12,927	1,175	11,916	989	12,845	1,148
Staphylococcus aureus	4,263	89	3,470	38	4,248	39
Sulphite-reducing *Clostridium*	595	3	561	2	462	6
Vibrio parahaemolyticus	411	0	392	0	343	0

OP – Official Programme "Food Safety";
MP – "Food Safety and Quality Monitoring Programme".

The comparison of total results obtained in the course of the "Food Safety and Quality Monitoring" and "Food Safety" programmes for the period under review, given in Figure 3, reflects the risk-oriented approach to planning of the parameters and lists of microbiological criteria by different activities. It should be noted that the detection rates of major microbial contaminants in animal raw materials and products are similar with the

analogous ones, shown by the official programme implementation.

Starting from 2018 the FGBI "ARRIAH" Branch in the Republic of Crimea has been participating in the implementation of the State Monitoring Plan. The Food Safety Laboratory of the Laboratory and Diagnostic Centre performed tests, including those for *Salmonella* and *L. monocytogenes*, the detection rate of the latter was 6.7%, which is 1.5–2.0 times higher than the analogous rate for Russia. No *Salmonella* were detected.

Data on total number of tests performed and noncompliances detected within public works are presented in Table 4 by years.

These data suggest a successful application of a risk-oriented approach during public works planning, but it does not eliminate the need for further research in this area.

Previously we analyzed the microbial contamination of animal products in the EU countries using RASFF data. The major microbial contaminants of animal products reported were *Salmonella* bacteria (2,719 notifications out of 3,769), *E. coli* (548 notifications) and *L. monocytogenes* (440 notifications) [11].

L. monocytogenes detection rate, revealed during public works implementation in the Russian Federation, is 2.5 times higher than *Salmonella* bacteria, which is different from the European data, where *Salmonella* were detected 6 times oftener than *L. monocytogenes* [11].

It should be noted that EU Commission Regulation No. 1441/2007 of 05.12.2007 on microbiological criteria for foodstuffs, does not contain coliforms and TVC criteria. Unlike the EU countries presence of these hygiene product indicators is checked in the Russian Federation during the implementation of the public works on food safety. The detection rates for these criteria are the highest.

CONCLUSIONS

Total 205,750 tests for microbiological safety were performed within public works in 37 laboratories subordinate to the Rosselkhoznadzor in 2015–2018.

The highest number of tests were carried out for pathogenic *Salmonella* (29.5% under official monitoring and 26.8% under official programme) and *L. monocytogenes* (22 and 21% respectively). The detection rate for this microorganisms in animal raw materials and food products was equal to 1.8% for official monitoring and 1.3% for official programme; the rates for *L. monocytogenes* were 4.2 and 3.1% respectively.

The parameters, showing less than 1% of positives within public works testing, were identified. They include *Vibrio parahaemolyticus*, *Proteus* bacteria and sulphite-reducing clostridia. The lowest detection rate of these microorganisms during the whole period under review is the reason to reduce the number of such tests.

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Mycotoxicological monitoring. Part 1. Complete mixed feed for pigs and poultry (2009–2018)

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SUMMARY
Results of the ten-year annual mycotoxicological testing of complete mixed feeds for pigs and poultry submitted by holdings and processing establishments located in the Northwestern, Central, Southern, Volga and Ural Federal Districts are presented. Competitive ELISA tests showed that the occurrence of T-2 toxin, deoxynivalenol, zearalenone, fumonisins of group B, alternariol, ochratoxin A, citrinin, mycophenolic acid, ergot alkaloids and emodin was about 5% and quantities thereof varied within one or three orders; quantities of T-2 toxin, deoxynivalenol, zearalenone, fumonisins, and ochratoxin A might exceed maximum admissible levels for feed grains. Diacetoxyscirpenol, aflatoxin B_1, sterigmatocystin and cyclopiazonic acid belonged to group of rare contaminants. Level of feed contamination with T-2 toxin and emodin was found to be consistently high during the said period; in some of the years occurrence of deoxynivalenol, fumonisins as well as ochratoxin A, citrinin, mycophenolic acid and ergot alkaloids increased. In 2016–2018, mixed feed contamination with alternariol increased whereas contamination with fumonisins steadily decreased and level of zearalenone occurrence remained consistently low. Evidence for a wide occurrence of emodin known as "diarrheic factor" as well as for sporadic increase in mixed feed contamination with alternariol, citrinin, mycophenolic acid, mycotoxins having the highly dangerous toxic impact and long-term adverse effects, was detected for the first time. These data confirmed the need for their inclusion into the regulated group of substances significant for public health. General features of pig and poultry feed contamination as well as usefulness of regional surveys for intoxication risk prediction are described. Special attention is paid to the importance of the projects for creation of common information resources that could become a unique scientific basis for innovations in feed poisoning prevention. Original monitoring data systematized and summarized in the paper are given in electronic format in section Additional materials.

Key words: complete mixed feed, mycotoxins, monitoring, enzyme-linked immunosorbent assay (ELISA).

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Микотоксикологический мониторинг. Сообщение 1. Полнорационные комбикорма для свиней и птицы (2009–2018 гг.)

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РЕЗЮМЕ
Представлены результаты 10-летнего ежегодного микотоксикологического обследования полнорационных комбикормов для свиней и сельскохозяйственной птицы, предоставленных хозяйствами и перерабатывающими предприятиями Северо-Западного, Центрального, Южного, Приволжского и Уральского федеральных округов. Методом конкурентного иммуноферментного анализа показано, что Т-2 токсин, дезоксиниваленол, зеараленон, фумонизины группы В, альтернариол, охратоксин А, цитринин, микофеноловая кислота, эргоалкалоиды и эмодин встречаются с частотой более 5%, их количества варьируют в пределах одного-трех порядков, и количества Т-2 токсина, дезоксиниваленола, зеараленона, фумонизинов и охратоксина А могут

превышать регламенты допустимого содержания в зерне, предназначенном на кормовые цели. Диацетоксисирпенол, афлатоксин B_1, стеригматоцистин и циклопиазоновая кислота относятся к группе редких контаминантов. Установлено, что высокая загрязненность комбикормов Т-2 токсином и эмодином сохраняет по годам устойчивый характер, в отдельные годы возрастает встречаемость дезоксиниваленола, фумонизинов, а также охратоксина А, цитринина, микофеноловой кислоты и эргоалкалоидов. В 2016–2018 гг. отмечено обострение ситуации по загрязненности комбикормов альтернариолом при положительной тенденции снижения контаминации фумонизинами и сохранения стабильно низкой встречаемости зеараленона. Факт обширной распространенности эмодина, известного как «диарейный фактор», а также спорадического, в отдельные периоды наблюдений, возрастания контаминации комбикормов альтернариолом, цитрининои и микофеноловой кислотой – микотоксинами с особо опасными формами токсического действия и негативными отдаленными последствиями – выявлен впервые. Эта информация подтверждает необходимость их введения в группу нормируемых санитарно-значимых показателей. Обсуждаются общие черты контаминации комбикормов для свиней и птицы, а также целесообразность региональных обследований для прогнозирования рисков развития интоксикаций. Особое внимание уделяется актуальности выполнения проектов, направленных на формирование единых информационных ресурсов, которые могут стать уникальной научной базой для инноваций в сфере профилактики кормовых отравлений. Исходные данные мониторинга, систематизированные и обобщенные в данной работе, представлены в электронном виде в разделе «Дополнительные материалы».

Ключевые слова: полнорационные комбикорма, микотоксины, мониторинг, иммуноферментный анализ.

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INTRODUCTION

Feed safety plays a crucial role in successful animal farming and processing industry development. Various responses of livestock sex/age groups and species to natural and anthropogenic factors as well as various diet compositions make feed material control extremely difficult. Completeness of the data on expected toxicants for each type of feed (their occurrence and levels) is a prerequisite for solving the said problem.

In our country, there is a long overdue need for the projects aimed at creation of common information resources that could become a unique scientific basis for innovations in feed poisoning prevention as well as top-requested reference manual for specialists engaged in feed production and animal feeding. Implementation of such projects requires establishment of principles that will be mandatory for the project participants as well as selection of optimal data entry system convenient for data storage, replenishment and multi-purpose use.

Complete mixed feed is a basis for modern pig and poultry industries. Summarizing and updating data on feed contamination with mycotoxins is one of the key problems remained unsolved. Creation of common information resource containing data on mycotoxicological tests of feed was firstly discussed at the 3rd Congress of Russian Toxicologists in 2008[1]. Feed and agricultural product monitoring is arranged and carried out by the Rosselkhoznadzor but access to the monitoring results is limited and data published in scientific-practical and scientific-production periodicals, Congress and Conference Proceedings are associated with small samplings often without any indications of tested sample number and origin or presented after being processed in accordance with conditional criteria [1, 2]. This impedes or makes data summarizing and free use impossible.

Recently, scientific periodicals have actively applied the experience gained in creation of replenishable electronic databases, for example, microorganism genotype collections [3]. A series of publications summarizing data of the analytical studies performed based on common approach that allows users to process the said data in accordance with their objectives could be an initial stage of the project for creation of mycotoxicological monitoring information resource in our country.

The goal of the paper is to summarize results of tests of complete mixed feed for pigs and poultry for mycotoxin contamination for 2009–2018 and provide original data contained in the electronic database.

MATERIALS AND METHODS

Average samples (1,338 samples) from complete mixed feed batches intended for various sex/age animal groups including SK-type feed for pigs (1,075 samples) and PK-type feed for poultry (263 samples), submitted to the laboratory by specialists of feed mills and veterinarians of pig and poultry establishments located in the Northwestern, Central, Southern, Volga and Ural Federal Districts, predominantly from the areas where such industries were intensively developed for the purpose of routine control, incoming control and diagnosis of mycotoxicoses in 2009–2018.

Group of target mycotoxins included T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins of B group (FUM), alternariol (AOL), ochratoxin A (OA), citrinin (CIT), aflatoxin B_1 (AB_1), cyclopiazonic acid (CPA), mycophenolic acid (MPA), ergot alkaloids (EA) and emodin (EMO).

Samples preparation was carried out using unified method based on liquid extraction and indirect

[1] Burkin A. A., Kononenko G. P. Monitoring test methods for risk assessment of acute and chronic mycotoxicoses occurrence. *3rd Congress of Russian Toxicologists: Abstracts (December 2–5, 2008).* Ed. By G. G. Onishenko, B. A. Kurlyandsky. M.; 2008; 71–73. (in Russian)

Table 1
Mycotoxins in complete mixed feed for pigs (summarized data for 2009–2018)

Таблица 1
Микотоксины в полнорационных комбикормах для свиней (обобщенные данные 2009–2018 гг.)

Toxin	Occurrence n^+/n (%)	Level, µg/kg				
		Range		mean	median	90% percentile
		min	max			
T-2	949/1,075 (88.3)	4	500	31	20	66
DON	411/1,075 (38.2)	39	1,580	198	100	400
DAS	5/1,075 (0.5)	32	250	89	51	175.2
ZEN	125/1,075 (11.6)	18	151	36	26	64.6
FUM	277/1,043 (26.6)	40	6,300	421	190	975
AOL	377/1,043 (36.2)	20	998	61	38	106.6
OA	294/1,051 (30.0)	4	105	10	6	20
CIT	120/1,050 (11.4)	20	250	43	32	71
AB_1	0/1,051 (–)	–	–	–	–	–
STE	14/1,042 (1.3)	4	177	22	7	27.2
CPA	5/1,042 (0.5)	59	148	89	74	128.8
MPA	88/1,042 (8.5)	10	5,400	99	26	64.3
EA	135/1,043 (12.9)	5	3,970	92	14	65.8
EMO	760/915 (83.1)	20	1,255	78	51	151.3

n – number of tested samples;
n^+ – number of mycotoxin-containing samples.

competitive ELISA, validated in 1995–2008 and then included in official control methods[2]. Detection limits determined based on 85% antibody binding were as follows: 2 µg/kg (AB_1), 3 µg/kg (EA), 4 µg/kg (T-2, OA, STE), 20 µg/kg (ZEN, AOL, CIT, MPA, EMO) and 50 µg/kg (DAS, DON, FUM, CPA). The following coding pattern was used for filling-in record form in the database: mixed feed type (PK, SK), recorded year (1–10), detected mycotoxins.

Microsoft Excel 2016 and Statistica, Version 6 programmes were used for statistical processing including calculation of percentage of occurrence based on n^+/n ratio and following three values for positive samples – the arithmetical mean, the median and the 90% percentile.

RESULTS AND DISCUSSION

Thirteen out of fourteen target mycotoxins (except for AB_1) were detected in feed for pigs (Table 1). T-2 and EMO were detected more often than others, DAS, STE and CPA were detected in less than 5% of samples; detection rate for other mycotoxins varied from 8.5 to 38.2%. The quantity of toxins generally varied within one-two orders and in case of EA – within three orders. Median displacement to the smaller values as compared to the mean value was demonstrated for all contaminants that was indicative of unified dissymmetric distribution of contents where a half of accumulation levels was less than other ones. Moreover, the largest quantities were always higher than threshold concentrations for 90% of values (90% percentile), and the most strongly pronounced for MPA and EA. All this was indicative of possible abnormally high toxin accumulation levels in typical situations. The highest T-2, DON, ZEN, FUM and OA levels detected in mixed feed were higher than admissible levels for feed grains [4].

In poultry feed, AB_1 was detected in 1.1% of samples, DAS was not detected and therewith there was a clear similarity with pig feed in the T-2 and EMO prevalence, rare detection of STE and CPA, similar occurrence for other 10 mycotoxins – 9.5–47.9%, degree of variation in their levels and character of the median displacement to the mean values and 90% percentile to the highest levels (Table 2). Such similarity can be accounted for the common raw feed materials used for the feed formulas (wheat, barley, corn grains, sunflower meal and cake, soybean meal) as well as similar their proportions in the formulas despite of variations related to the animal category. The rate of fusariotoxins (T-2, DON, ZEN, FUM), AOL as well as OA, CIT, MPA, EA and EMO occurrence was more than 5% in complete mixed feed intended both for pigs and poultry.

Selective tests of mixed feed initiated by the ARRIVSHE in 1997 were described in the earlier publications as far as the data were gained. In 1997–2004 mycotoxin contamination level in 766 feed consignments used in commercial poultry industry was 34.6–79.5%, T-2 was the most prevalent in feed (38.5% of samples) at the level of 30–59 µg/kg,

[2] GOST 31653-2012 Feed. Immunoenzyme method for mycotoxin detection. M.: Standardinform; 2012. 11 p. (in Russian)

Table 2
Mycotoxins in complete mixed feed for poultry (summarized data for 2009–2018)

Таблица 2
Микотоксины в полнорационных комбикормах для сельскохозяйственной птицы (обобщенные данные 2009–2018 гг.)

Toxin	Occurrence n^+/n (%)	Level, μg/kg				
		range		mean value	median	90% percentile
		min	max			
T-2	208/263 (79.1)	4	280	25	12	63.3
DON	85/263 (32.3)	50	757	181	112	462.4
DAS	0/263 (–)	–	–	–	–	–
ZEN	45/263 (17.1)	20	334	37	25	54.6
FUM	95/263 (36.1)	50	5,000	350.5	165	585
AOL	126/263 (47.9)	20	595	85	47	200
OA	87/263 (33.1)	4	107	9	5	14.6
CIT	25/263 (9.5)	20	194	55	33	132
AB_1	3/263 (1.1)	2	12	6	4	10.4
STE	4/263 (1.5)	8	11	10	10.5	11
CPA	2/263 (0.8)	50	123	86.5	86.5	115.7
MPA	32/263 (12.2)	20	158	43	31.5	80.5
EA	35/263 (13.3)	3	5,000	311	15	159.6
EMO	149/201 (74.1)	14	536	76	50	162

n – number of tested samples;
n^+ – number of mycotoxin-containing samples.

in some samples – 550 μg/kg, DON (13.7%), ZEN (10.3%) and FUM (9.9%) were detected less frequently, rate of OA detection at the level of 10–33 μg/kg varied from 15.2 to 42.2%, AB1 was detected rarely (1.6%) and no CTE was detected[3]. Results for 2005–2009 supported conclusions made at the previous stage and provided new information that DAS occurence was far lower as compared to other fusariotoxins, CIT took an active part in contamination and CPA was absent[4,5], and allowed the first specific proposals on mycotoxicological control improvement to be formulated [5]. Later, the following brief reports were published: on frequent occurrence of EMO, antrachinonic toxin known as "diarrheic factor", within the range of dozens to thousands μg/kg in the set of 29 samples[6]; EA occurrence in 2008–2013 [6]; AOL occurrence in 2009–2014 and MPA occurrence in 2007–2014[7] including reporting of abnormal accumulation of this toxin (5,400 μg/kg) in dense caked fraction of mixed feed for piglets due to heavy infestation by highly toxicogenic *Penicillium* spp. fungi[8]. Detailed analysis of the whole data body allowing assessment of feed contamination with mycotoxins is presented for the first time.

Dynamics of annual variation in occurrence of 10 major contaminants is given in Figure. Level of T-2 and EMO contamination remained high that was indicative of wide occurrence of their producers. Significant variations in DON, FUM and AOL occurrence with multi-fold increase in particular years appeared to be associated with specific contamination of the grain supplied to regional feed mills since level of *Fusarium* spp. and *Alternaria* spp. infestation was different in various territories and years. In 2016–2018 situation on AOL contamination became aggravated. In general, decrease in feed contamination with FUM and consistently low feed contamination with ZEN can be noted as a positive trend for the last four years (Fig.).

Drastic increase in OA, CIT, MPA and EA detection in particular years could be accounted for known occurrence in endemic areas as well as combination of adverse factors

[3] Kononenko G. P. System of mycotoxicological control of products subjected to veterinary/sanitary and ecological surveillance: Author's Abstract, Thesis for Doctor Degree (Biology). M.; 2005. 49 p. (in Russian)

[4] Kononenko G. P., Burkin A. A. Mycotoxicological control of raw feed materials and mixed feed. *Current aspects of veterinary pharmacology, toxicology and pharmacy: Proceedings of the Congress of Russian Pharmacologists and Toxicologists.* St-P.; 2011; 242–244. (in Russian)

[5] Kononenko G. P., Burkin A. A. Achievements and challenges of creation of data base of human health-significant mycotoxins in feed. *Modern Mycology in Russia.* M.: National Academy of Mycology; 2012; 428–429. (in Russian)

[6] Kononenko G. P., Burkin A. A. Emodin: contamination of grain feed. *Advances in Medical Mycology.* 2007; 9: 88–89. (in Russian)

[7] Burkin A. A., Kononenko G. P. Alternariol occurrence in biological objects. *Modern Mycology in Russia: Proceeding of the III International Mycological Forum (April 14-15, 2015).* Ed. by Yu. T. Dyakov, Yu. V. Sergeyev. M.: National Academy of Mycology; 2015; 5: 223–225. (in Russian)

[8] Kononenko G. P., Burkin A. A. Mycophenolic acid: occurrence in biological objects. *Modern Mycology in Russia: Proceeding of the III International Mycological Forum (April 14-15, 2015).* Ed. By Yu. T. Dyakov, Yu. V. Sergeyev. M.: National Academy of Mycology; 2015; 4: 201–203.

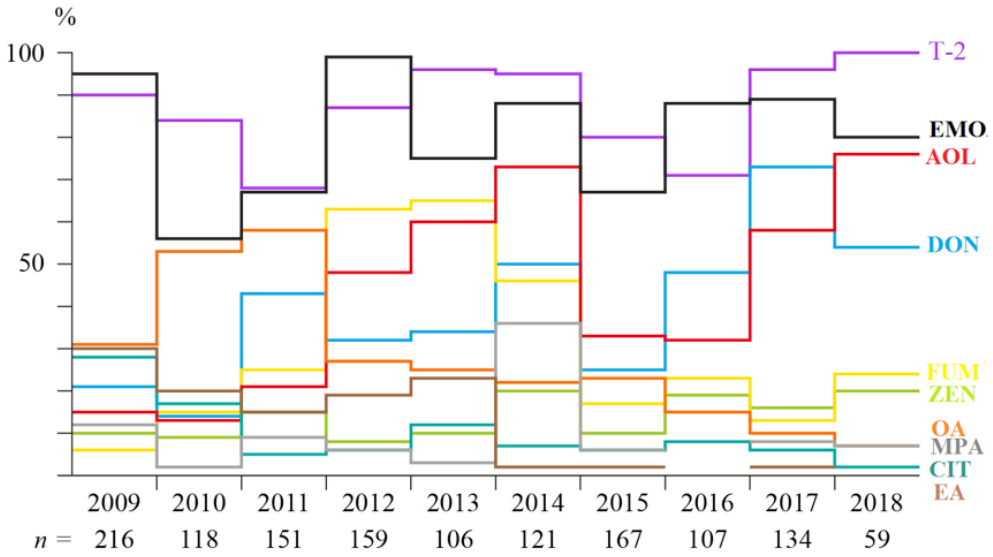

Fig. Dynamics of annual occurrence of T-2 toxin (T-2) deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), ergot alkaloids (EA) and emodin (EMO) in mixed feed for pigs and poultry (summarized data)

Рис. Динамика ежегодной встречаемости Т-2 токсина (Т-2), дезоксиниваленола (ДОН), зеараленона (ЗЕН), фумонизинов (ФУМ), альтернариола (АОЛ), охратоксина А (ОА), цитринина (ЦИТ), микофеноловой кислоты (МФК), эргоалкалоидов (ЭА) и эмодина (ЭМО) в комбикормах для свиней и птицы (обобщенные данные)

during agricultural product harvesting, transportation and pre- and post-processing storage.

Original data of the 10-year monitoring of complete mixed feed for pigs and poultry systematized and summarized in the paper are given in electronic format in section Additional materials are given at: http://doi.org/10.29326/2304-196X-2020-1-32-60-65. Access to the entire database allows any other variants of the data processing, for example data on single and concomitant contamination, joint OA and CIT occurrence that is rather frequent [7, 8] and can enhance their nephrotoxic effect [9], correlation between T-2 and DAS amounts that are similar in toxicity level as well as calculation of excessive contamination cases when regulations on maximum admissible levels for mycotoxins are put into effect at the country level. It should be noted, that wide geographic area where samples used for the said study have been collected does not allow regional aspects to be taken into account. Nevertheless, data obtained for particular territories are very valuable since they provide an unique opportunity to gain understanding of feed grain contamination. Feed grain testing for risk prediction is very difficult due to large-scale harvesting and various soil and climate factors that can influence the mycotoxicological situation. Under these circumstances, feed mixes with large proportion of grain ingredients can be used as ecological markers. In some European countries tracing of mycotoxin contamination in food grains based on results of tests of bakery products sold through retail chains was tested, recognized to be cost-effective and has been put in international practice [10, 11, 12]. Unfortunately, there have been yet no specific regional projects on mixed feed assessment in our country and sample testing results are presented in aggregated form sometimes without indication of type of tested mixed feed in theses covering a wide range of related aspects [13, 14].

CONCLUSION

General features and peculiarities of mycotoxin contamination of complete mixed feed intended for pigs and poultry have been established during extensive monitoring carried out for the last 10 years with annual data collection. Obtained results confirmed importance of regular testing of such feed for their contamination with fusariotoxins, ochratoxin A, regulated in feed grains as well as ergot alkaloids. For mixed feed, it is recommended to include emodin, antrachinonic toxin known as diarrheic factor as well as alternariol, citrin and mycophenolic acid having highly dangerous toxic impacts and long-tern adverse effects into the list of substances significant for public health. Obtained data could be useful for assessment of the situation in feed producing industry and providing rationale for mycotoxin regulation in mixed feed and product control system improvement at processing establishments.

Additional materials to the paper (records forms with database) can be found at: http://doi.org/10.29326/2304-196X-2020-1-32-60-65.

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Evaluation of veterinary laboratory proficiency based on results of interlaboratory comparisons organized by FGBI "ARRIAH" in 2018–2019

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SUMMARY
Laboratory diagnosis is a crucial component in implementation of the set of anti-epidemic measures aimed at contagious animal disease control. The need for unswerving trust in the quality of laboratory performance is a matter of importance not only for service providers and customers, but also for inspecting organizations, accreditation bodies, etc. that establish performance requirements for diagnostic laboratories. Incorrect laboratory test results can lead to a misdiagnosis and, therefore, to grave consequences. One of the forms of experimental verification of a laboratory's performance with a view to determine the laboratory's competence and to verify its compliance with accreditation criteria as part of inspection control of the laboratory's activities is interlaboratory comparison. The laboratory can prove its competence at a particular time, as well as clearly demonstrate how stable the quality of its test results is by summarizing and analyzing the results of its participation in interlaboratory comparisons. The analysis of the results of the interlaboratory comparisons (programmes for detection of causative agents or antibodies to the causative agents of avian influenza, Newcastle disease, rabies, classical swine fever, African swine fever, bluetongue, lumpy skin disease) organized by the FGBI "ARRIAH" for the veterinary laboratories of the Russian Federation in 2018–2019 is presented. The results showed that most of the laboratories had passed the tests successfully. The results submitted by participants were unsatisfactory in some interlaboratory comparison programmes (rabies virus detection using fluorescent antibody technique; detection of avian influenza, classical swine fever and lumpy skin disease viruses using polymerase chain reaction). That highlights the need for those participants who failed the tests to improve their laboratory testing quality.

Key words: interlaboratory comparisons, infectious animal diseases, polymerase chain reaction, enzyme-linked immunosorbent assay, fluorescent antibody technique.

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Оценка квалификации ветеринарных лабораторий по результатам межлабораторных сличительных испытаний, организованных ФГБУ «ВНИИЗЖ» в 2018–2019 гг.

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РЕЗЮМЕ

Важной задачей при проведении комплекса противоэпизоотических мероприятий, направленных на борьбу с заразными болезнями животных, является лабораторная диагностика. Необходимость в постоянном доверии к качеству работы лаборатории важна не только для исполнителей и заказчиков, но и инспектирующих организаций, органов по аккредитации и др., которые устанавливают требования к деятельности диагностических лабораторий. Недостоверные результаты лабораторных исследований могут привести к постановке неправильного диагноза, а значит, и к серьезным последствиям. Одной из форм экспериментальной проверки деятельности лаборатории с целью определения ее компетентности и подтверждения соответствия критериям аккредитации при инспекционном контроле за ее деятельностью являются межлабораторные сличительные (сравнительные) испытания. Обобщая и анализируя результаты участия в межлабораторных сличительных испытаниях, лаборатория может не только подтвердить свою компетентность в конкретный момент, но и наглядно продемонстрировать, насколько стабильно качество результатов ее анализов. Представлен анализ результатов межлабораторных сличительных испытаний, организованных в 2018–2019 гг. ФГБУ «ВНИИЗЖ» для ветеринарных лабораторий России, по программам выявления возбудителей или антител к возбудителям гриппа птиц, ньюкаслской болезни, бешенства, классической чумы свиней, африканской чумы свиней, блютанга, заразного узелкового дерматита крупного рогатого скота. Результаты показали, что большинство лабораторий успешно справились с испытаниями. Неудовлетворительный результат был получен участниками по отдельным программам межлабораторных сличительных испытаний (выявление вируса бешенства методом флуоресцирующих антител; выявление вирусов гриппа птиц, классической чумы свиней и заразного узелкового дерматита крупного рогатого скота методом полимеразной цепной реакции). Это указывает на необходимость повышения качества лабораторных исследований не справившихся с заданием участников.

Ключевые слова: межлабораторные сличительные испытания, инфекционные болезни животных, полимеразная цепная реакция, иммуноферментный анализ, метод флуоресцирующих антител.

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INTRODUCTION

Several regions of the Russian Federation retain the status of infected with respect to some infectious animal diseases. Effective control of contagious diseases requires a completes set of anti-epidemic measures with laboratory diagnosis being one of its crucial components. Incorrect laboratory test results can lead to grave consequences. The need for unswerving confidence in laboratory performance quality is a matter of importance not only for service providers and customers, but also for inspecting organizations, accreditation bodies, etc. that establish performance requirements for diagnostic laboratories [1].

One of the ways to evaluate laboratory performance is interlaboratory comparison (ILC). This form of laboratory proficiency testing is widely used, includingin international practice [2]. In addition to the monitoring of test result reliability, the objectives of interlaboratory comparisons are the identification of problems in the work of the laboratory and differences between laboratories, the determination of effectiveness and comparability of test or measurement methods, and a number of others. Successful participation in interlaboratory comparisons helps the laboratory gain more trust from customers. Besides, participation in interlaboratory comparisons is mandatory for the laboratories accredited in the national laboratory accreditation system [3, 4].

The Rosselkhoznadzor subordinate FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH") is accredited in the national accreditation system as an ILC provider according to GOST ISO/IEC 17043-2013 (Certificate of Accreditation No. RA.RU.430258, date of entry into the registry of accredited persons: March 16, 2018). In 2019, the competence of the FGBI "ARRIAH" as an ILC provider was confirmed following the results of the on-site audit (Order of the Federal Service for Accreditation (RusAccreditation) No. PK1-1180 dated June 21, 2019).

Interlaboratory comparison programmes developed by the ILC provider in accordance with the approved scope of accreditation allow for verification of laboratory proficiency in the diagnosis of highly dangerous infectious animal diseases using such methods as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and fluorescent antibody (FA) technique.

The aim of the paper is to analyze the results of the ILC rounds organized by the FGBI "ARRIAH" in 2018–2019.

MATERIALS AND METHODS

The results of the interlaboratory comparisons organized by the FGBI "ARRIAH" in 2018–2019 under the following 10 programmes were used for the paper:

– detection of avian influenza type A virus RNA;

– detection of antibodies to avian influenza type A virus using ELISA;

– detection of antibodies to Newcastle disease virus using ELISA;

– detection of rabies virus antigen using fluorescent antibody technique;

– detection of antibodies to African swine fever virus using ELISA;

– detection of African swine fever virus genome;

– detection of antibodies to classical swine fever virus using ELISA;

– detection of classical swine fever virus genome;

– detection of antibodies to bluetongue virus using ELISA;

– detection of lumpy skin disease virus genome.

Pursuant to the approved interlaboratory comparison plan, one round of interlaboratory comparisons was carried out for each programme annually. The Split-Sample Scheme was applied: identical control samples prepared from the same material or a specially prepared material split into two or more parts were used, so that each ILC participant could test the control samples from this set of samples [1]. All control samples are validated in terms of assigned values, homogeneity and stability in strict compliance with the procedure approved by the ILC provider.

Data obtained for each ILC programme are provided in detail in the reports that are available in the Interlaboratory Comparisons section on the web-site of the ILC provider (FGBI "ARRIAH") (http://www.arriah.ru/main/lprovedenie-msi). Information about participants is presented in anonymized form.

RESULTS AND DISCUSSION

The proficiency of 46 veterinary laboratories located in 37 regions of the Russian Federation with respect to diagnosis of seven dangerous animal diseases (avian influenza, Newcastle disease (ND), rabies, classical swine fever (CSF), African swine fever (ASF), lumpy skin disease (LSD), bluetongue) using PCR, ELISA and fluorescent antibody technique was evaluated based on the results obtained within the ILC programmes. The consistency of submitted results with the assigned values for the coded control samples served as a criterion for evaluation. The ILC result was considered satisfactory when all the control samples in the panel were correctly identified. All the ILC programmes were based on the qualitative analysis of the assigned control sample. When at least one control sample did not agree with the assigned value, the ILC result was recognized unsatisfactory.

Table 1 shows the total number of participating laboratories for each ILC programme of 2018–2019 and the number of participants that made mistakes and failed the tests.

Data presented in Table 1 show that inconsistent results were identified amongthe ILC results for 4 out of 10 implemented programmes. These programmes required the use of fluorescent antibody technique or PCR. At the same time, the participants of all the ILC programmes for the detection of infectious animal disease agents with ELISA passed the tests successfully using commercial test kits produced by domestic and foreign manufacturers.

The greatest number of mistakes were found to have been made by the participants of the proficiency tests related to the detection of rabies virus antigen using fluorescent antibody technique which is the main tool for rabies diagnosis (90.7% of laboratories passed the test). The unsatisfactory ILC results with respect to the diagnosis of this deadly animal and human disease are particularly troubling in the light of unfavorable rabies epidemic situation across much of Russia's territory. It is important to note that fluorescent antibody technique requires the high qualifications and experience of personnel, as well as the appropriate maintenance of fluorescence microscopes (timely replacement of lamps, etc.). Besides, at least two specialists should be involved in the interpretation of fluorescent antibody test results.

As for the ILC programmes for the detection of avian influenza, CSF and LCD viruses using PCR, the percentages of the laboratories that passed the tests were 96.2, 94.1 and 93.9%, respectively, out of the total number of participants. PCR procedure comprises several stages (sample preparation, nucleic acid isolation, PCR as such, the interpretation and analysis of results), and this increases the likelihood of making a mistake at any of its stages and producing an unsatisfactory final result. This technique

Table 1
Results of interlaboratory comparison programmes carried out in 2018–2019

Таблица 1
Результаты реализации программ МСИ за 2018–2019 гг.

No.	Parameter according to the scope of accreditation (corresponds to a particular ILC programme)	Test method	Number of participants*	Participants that passed the ILCs successfully	
				number	percentage of the total number
1	AI type A virus RNA	PCR	26	25	96.2
2	Antibodies to AI type A virus	ELISA	10	10	100
3	Anti-NDV antibodies	ELISA	6	6	100
4	Rabies virus antigen	MA	43	39	90.7
5	Anti-ASFV antibodies	ELISA	10	10	100
6	ASFV genome	PCR	17	17	100
7	Anti-CSFV antibodies	ELISA	34	34	100
8	CSFV genome	PCR	51	48	94.1
9	Antibodies to bluetongue virus	ELISA	6	6	100
10	LSDV genome	PCR	49	46	93.9

* In a case where a laboratory participated in the interlaboratory comparisons twice (in 2018 and 2019), it was recorded as two participants.

Table 2
Mistakes made by participants of interlaboratory comparison rounds in 2018–2019

Таблица 2
Ошибки участников раундов МСИ в 2018–2019 гг.

No.	Parameter according to the scope of accreditation (corresponds to a particular ILC programme)	Test method	Number of participants that failed the ILC	Type of mistake	
				false positive result	false negative result
1	AI type A virus RNA	PCR	1	0	1
2	Rabies virus antigen	FA technique	4	1	3
3	CSFV genome	PCR	3	0	3
4	LSDV genome	PCR	3	0	3

requires a particularly high qualification and the availability of specialized rooms.

When the result is unsatisfactory, the participating laboratory is encouraged to review the adopted test procedure, to identify the possible causes of mistakes, and to develop adequate corrective actions (further training of the staff; the enhanced control of the tests performed; the repair or replacement of equipment and measuring tools; diagnostic kit replacement, etc.). The effectiveness of corrective actions should be confirmed by the laboratory's repeated participation in the interlaboratory comparisons at the earliest possible time.

Table 2 shows mistakes made by the participants of ILC rounds in 2018–2019.

Data in Table 2 show that most of mistakes were related to false negatives, i.e. the control samples that contained a disease agent or antibodies to it were characterized as negative ones. Such mistakes in laboratory diagnosis can have grave consequences such as late diagnosis, underestimation of the disease spread risk and, consequently, reduce the effectiveness of anti-epidemic measures.

Even a well-managed laboratory comprising experienced personnel can sometimes produce unsatisfactory test results. Monitoring is of particular importance for evaluation of test result reliability; it can be carried out not only through interlaboratory comparisons, but also by means of intralaboratory controls (repeated tests of samples under standard conditions, the use of alternative equipment, performance of the same test by a different staff member, the use of the diagnostic test kit from a different manufacture for testing, etc.). However, even with existence of internal controls, the laboratory is obliged to monitor its performance by means of comparison with other laboratories' results, i.e. through participation in interlaboratory comparisons [3]. The laboratory's proficiency shall be evaluated taking into account the results of the previous rounds of tests, the frequency of its participation in interlaboratory comparisons, the level of coverage of the laboratory's scope of activities by proficiency testing programmes, the ability of the personnel to use the results of participation in interlaboratory comparisons to improve the laboratory's performance, etc. [5]. Continuous satisfactory results of the laboratory's participation in interlaboratory comparisons can be indicative of its high competence in performing particular types of tests.

After having eliminated existing mistakes, all the participants of the ILC rounds organized by the FGBI "ARRIAH" that had failed the 2018 tests participated in the comparisons again in 2019 and produced satisfactory results.

CONCLUSION

The analysis of the results of the veterinary laboratory proficiency tests organized by the FGBI "ARRIAH" in 2018–2019 demonstrated that most of the participants had passed the tests successfully. The proficiency of some laboratories was recognized unsatisfactory mainly because of the false negative results produced by them. The greatest number of mistakes was made by the ILC participants within the programmes for rabies virus antigen detection using fluorescent antibody technique: 90.7% of laboratories passed the test successfully. Besides, unsatisfactory results were presented by the participants of the ILC programmes for detection of avian influenza, CSF and LSD viruses using PCR (96.2, 94.1 and 93.9% of laboratories, respectively, passed the tests). The data obtained indicate the existence of mistakes in the laboratory diagnosis of infectious animal diseases as regards some participants that failed the interlaboratory comparisons, as well as highlight the need for actions to improve their laboratory test quality. Diagnostic laboratories should pay more attention to the internal control of their testing quality (preferably, using reference samples) and the control of the diagnostic test kits they use, carry out verification of the diagnostic techniques employed and ensure the adequate level of knowledge and skills of the personnel involved.

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The Monograph "Animal rabies" is devoted to a challenging issue of continued rabies cases which even in the XXI century raises concern of the WTO, OIE and FAO.

Rabies is one of the oldest and dangerous diseases of animals and humans. According to the WTO assessment it is among five zoonoses posing the greatest economic damage and constant threat for the life of humans and animals. Rabies is included in the OIE list of notifiable diseases.

One million of different animal species die of rabies each year. 110 countries of the world report a complicated rabies situation. Animal rabies especially in wild carnivores is one of the most important international criteria (WTO, FAO and OIE) for assessing biological and ecological safety of the human environment.

Analysis of the data characterizing rabies epidemic situation in the Russian Federation 1991–2019 demonstrates that it remains complicated despite measures taken. Rabies natural foci have become more active and the number of cases in wild carnivores has increased. Domestic carnivores, cats and dogs, have become more involved in the epidemic process, which makes specific prevention more relevant.

The analytical and own investigations of the authors are aimed at studying rabies epidemic data: rabies spread in the Russian Federation and neighboring countries, epidemic monitoring data, risk assessment in case of rabies occurrence in animals, rabies manifestations in different animal species. A large section is devoted to the rabies diagnosis: developed schemes and methods of brain sampling, safety measures during handling the rabies virus contaminated material are presented. Conventional methods as well as methods of laboratory diagnosis using florescence test, different variants of enzyme-immunoassay, polymerase chain reaction, latex agglutination test, immunochromatographic analysis, rabies virus isolation in cell culture, and rabies virus detection in saliva, developed and improved by the authors, are described.

A comprehensive review and results of the investigations of the rabies virus field isolates and vaccine strains using nucleotide sequencing and phylogenetic analysis performed by the authors are presented.

Section "Rabies prevention and control measures" revealing the concept of rabies prevention in carnivores in the natural environment is important when it comes to practical use. Experience in oral vaccination against rabies in the neighboring countries is comprehensively analyzed and vaccines produced in different countries are characterized.

Publication of the monograph "Animal rabies" by K. N. Gruzdev and A. Ye. Metlin was a significant event contributing to understanding rabies spread, diagnosis, and prevention. When performing analytical studies of the literary sources and own experience the authors, not without a reason, raise a question of the necessity of intergovernmental and interregional interaction between veterinary services for control of such zoonosis as rabies.

The reviewed monograph – is a tangible contribution of Russia to the achievement of the goal of rabies eradication in humans all over the world which was initiated by the WTO, OIE, FAO, GARC and outlined in the new programmes (2015) for solving fundamental and veterinary and epidemiological applied tasks aimed at the infection surveillance and control.

The monograph contains a great many of own scientific, fundamental and applied data, analytical generalizations and conclusions. The book has a proper design and is well illustrated. We consider that it will be useful for specialists in the field of veterinary as well sanitary and epidemiological surveillance, researchers, students of different levels – post-graduate students, advanced training course participants as well as specialists dealing with infectious pathology and epidemiology.

Food and Agriculture Organization
of the United Nations (FAO)

PRESS RELEASE

Oleg Kobiakov appointed Director of FAO Liaison Office with the Russian Federation

28 February 2020,
Moscow, Russian Federation

FAO Director-General Qu Dongyu appointed Oleg J. Kobiakov the Director of Liaison Office with the Russian Federation effective from 28 February 2020.

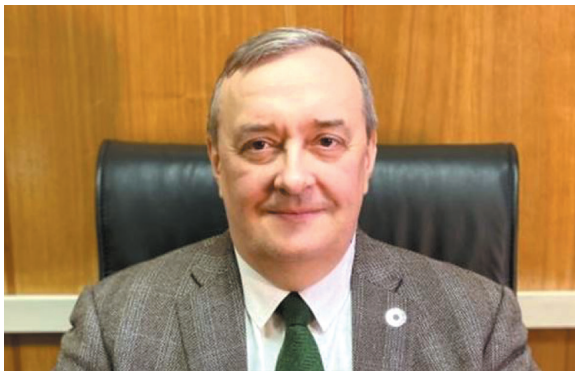
Oleg Kobiakov, a Russian national, is a seasoned diplomat with 35 years of experience at Russia's Ministry of Foreign Affairs (MFA). He graduated from the Moscow State Institute of International Relations majoring in history, economics and law (1985), and completed the PhD programme in diplomacy and international relations at the Centre d'études diplomatiques et stratégiques in Paris, France (2000).

His diplomatic career encompasses postings to the Russian embassy in Prague, permanent missions to the European Union (EU) in Brussels, to UNESCO in Paris, and to FAO in Rome, as well as service at the Department of International Organizations of MFA and the Minister's Cabinet.

In 2011–2016, he was an alternate representative, then Deputy Permanent Representative of Russia, to Rome-based UN agencies. Prior to joining FAO, he was Head of Division for the UN Economic and Social Council and UN Regional Commissions at the Russian MFA.

Kobiakov has devoted over 12 years of his professional career to promoting and supporting relations between FAO and the Russian Federation. He has a broad knowledge of topics related to FAO's areas of work and extensive practical experience in international negotiations on issues of agriculture, forestry, and fisheries and their adaptation to modern challenges, in particular food insecurity, healthy nutrition, and sustainable development.

He possesses a proven track record of administering Russian contributions to UN agencies, especially FAO, the World Food Programme, United Nations Economic Commission for Europe, and the Economic and Social Commission for Asia and the Pacific, in addition to directing respective technical cooperation projects.



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Oleg Kobiakov played a key role in the establishment of the Permanent Mission of the Russian Federation to Rome-based UN agencies in 2011 and in the opening of the FAO Liaison Office in Moscow four years later. He served as rapporteur for FAO's European Regional Conferences in Baku, Azerbaijan (2012) and Voronezh, Russia (2018), as well as a member of the Executive Committee of the FAO European Commission on Agriculture from 2015–2017.

Oleg Kobiakov is fluent in English, Czech, and Russian (mother tongue), and speaks French and Italian.

LINKS:

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– scientific and technical activities aimed at ensuring biological safety and freedom of the Russian Federation from dangerous and economically important infectious aquaculture diseases; epidemiological and diagnostic tests for the said diseases;

– study of aquatic animal disease agent occurrence in the Russian Federation for Russian product export support;

– study of virus isolates for diagnostic tool improvement according to epidemic situation;

– research activities for improvement and implementation of methods for effective diagnosis and prevention of dangerous and economically important aquaculture diseases;

– scientific and practical assistance to field veterinarians in viral fish disease diagnosis and prevention;

– independent tests of food products derived from aquaculture as well as fish feed for quality and safety to determine their compliance with standards and requirements laid down in current regulations;

– prediction and assessment of risks associated with aquatic animal disease occurrence and introduction in the Russian Federation;

– participation in the development of programmes and regulations on infectious aquaculture disease prevention and eradication, scientific and methodical support for the Rosselkhoznadzor and its Territorial Administrations and subordinated organizations in their activities focused on viral fish diseases.

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