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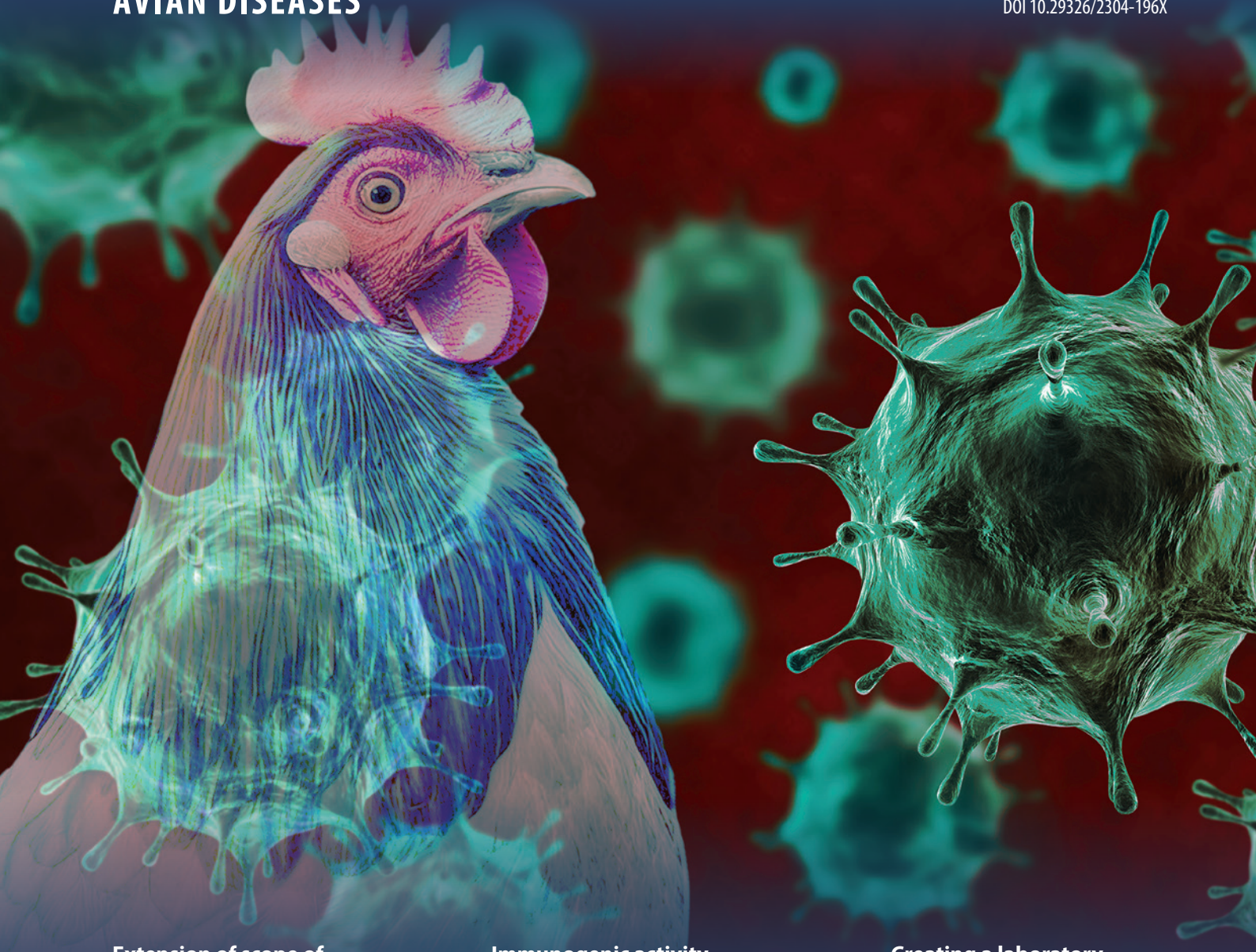

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Extension of scope of susceptible mammalian species as avian influenza global situation developed in 2023–2024

Milena V. Zhiltsova, Tatiana P. Akimova, Mariya N. Mitrofanova, Valentina P. Semakina, Evgeniya S. Vystavkina
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ABSTRACT

Introduction. Highly pathogenic avian influenza currently requires the close attention of the international community. Determining the factors affecting transmission and replication of avian influenza virus in mammals and analysing the evolutionary processes involved will suggest which virus lineages will have the spillover potential and infect non-typical hosts, including humans.

Objective. The paper is aimed at studying the avian influenza epidemic situation in mammals, description of the features of the avian influenza epizootic process, retrospective analysis of influenza outbreaks in non-typical hosts.

Materials and methods. The study was carried out in the Information and Analysis Centre of the Veterinary Surveillance Department of the Federal Centre for Animal Health (Vladimir). The data obtained was based on statistical data from the database of the World Organisation for Animal Health WAHIS and scientific publications of foreign and domestic authors. Cartographic analysis was carried out using ArcGIS geographic information system (ESRI, USA).

Results. The avian influenza virus H5N1 epizootic process in 2022–2024 involved mammalians of various families (*Bovidae, Mustelidae, Ursidae* etc.) in which the disease had not been previously recorded. Strict biosecurity measures and updated alert systems are of crucial importance to effectively prevent the spread of the disease. In a limited number of countries (Bangladesh, Dominican Republic, China, Egypt, Indonesia, Laos, Vietnam, EU countries, etc.), vaccination has been used as a preventive and emergency measure to protect birds from influenza.

Conclusion. Transmission of highly pathogenic avian influenza virus to mammals of different species, including livestock, may be the start of a future pandemic. The recently recorded virus spillover indicates emergence of adaptive mutations and poses a threat to animal health, public health, food security and biodiversity.

Keywords: review, avian influenza, mammals, livestock, cattle, epizootic situation, unusual hosts, extension of host scope, control strategies

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Расширение спектра восприимчивых видов млекопитающих в ходе развития эпизоотической ситуации в мире по гриппу птиц за 2023–2024 гг.

М. В. Жильцова, Т. П. Акимова, М. Н. Митрофанова, В. П. Семакина, Е. С. Выставкина
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РЕЗЮМЕ

Введение. Высокопатогенный грипп птиц в настоящее время требует самого пристального внимания всего международного сообщества. Определение факторов, влияющих на передачу и репликацию вируса гриппа птиц у млекопитающих, а также анализ происходящих эволюционных процессов позволит предположить, какие вирусные линии будут иметь потенциал к преодолению видового барьера и инфицированию нетипичных хозяев, в том числе людей.

Цель исследования. Изучение эпизоотической ситуации по гриппу птиц среди млекопитающих, описание особенностей эпизоотического процесса при гриппе птиц, ретроспективный анализ вспышек гриппа у нетипичных хозяев.

Материалы и методы. Работу выполняли в информационно-аналитическом центре Управления ветнадзора при ФГБУ «Федеральный центр охраны здоровья животных» (г. Владимир). Сбор сведений осуществляли на основе статистического материала базы данных Всемирной организации здравоохранения животных WAHIS и научных публикаций зарубежных и отечественных авторов. Картографический анализ проводили с помощью географической информационной системы ArcGIS (ESRI, США).

Результаты. С 2022 по 2024 г. в эпизоотический процесс, вызванный вирусом гриппа подтипа H5N1, были вовлечены млекопитающие различных семейств, у представителей которых ранее болезнь не регистрировали: полорогие, куницеобразные, медвежьи и др. Для эффективного предотвращения распространения заболевания важны строгие меры биобезопасности и актуализация систем оповещения. В ограниченном числе стран (Бангладеш, Доминиканская Республика, Китай, Египет, Индонезия, Лаос, Вьетнам, страны Евросоюза и др.) в качестве профилактической экстренной меры для защиты птиц от гриппа использовали вакцинацию.

Заключение. Передача вируса высокопатогенного гриппа птиц млекопитающим разных видов, в том числе сельскохозяйственным животным, может дать старт будущей пандемии. Межвидовая передача вируса, регистрируемая в последнее время, указывает на возникновение адаптивных мутаций и представляет собой угрозу здоровью животных, общественному здравоохранению, продовольственной безопасности и биоразнообразию.

Ключевые слова: обзор, грипп птиц, млекопитающие, сельскохозяйственные животные, крупный рогатый скот, эпизоотическая ситуация, расширение спектра хозяев, стратегии контроля

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INTRODUCTION

Avian influenza virus continues to pose a threat to animal and human health. H5 and H7 subtypes have caused numerous outbreaks in wild and domestic birds and resulted in mortality of at least 600 million poultry since 2005. Many countries are now concerned about the development and application of different strategies aimed at avian influenza control.

Unlike H5N2, H5N3, H5N4, H5N5 and H5N6 subtypes detected in a rather limited area or within the continent, the H5N1 subtype started a large-scale intercontinental spread [1].

H5N1 subtype has caused a significant number of outbreaks in many countries in Europe, Africa, Asia and the Americas [2].

The virus interspecies spillover usually results in a dead-end infection. The probability that a complete set of adaptive mutations is acquired in a single immunocompetent host and transmitted onwards to other hosts is extremely low. Adaptive mutations occurring during an epizooty could enhance adaptiveness of the virus through increased polymerase activity to allow transmission to less susceptible hosts. This is demonstrated by the results of both experimental infections and isolation of the virus from atypical wild and livestock hosts during outbreaks: spread of high pathogenicity avian influenza (HPAI) H5N1 to farmed pigs in Indonesia, and transmission to cattle and goats in the USA [3].

Evident changes in the epidemiology and ecology of the virus now pose a threat to animal health, public health, food security and biodiversity. Conventional control measures such as biosecurity, stamping-out and movement restrictions, although important, may not be sufficient. Most countries have mechanisms in place to facilitate the regular exchange of information and best practices to coordinate disease control policies and develop evidence-based national strategies [4, 5].

The aim of the study was to investigate the avian influenza epizootic situation in mammals, to characterize the avian influenza epizootic process and to retrospectively analyze influenza outbreaks in atypical hosts.

MATERIALS AND METHODS

The study was carried out in the Information Analysis Centre of the Veterinary Surveillance Department, Federal Centre for Animal Health (Vladimir). The data were collected using statistical material from the WAHIS database of the World Organisation for Animal Health (WOAH) and scientific publications of foreign and domestic authors. Cartographic analysis was carried out using ArcGIS geographic information system (ESRI, USA).

DYNAMICS OF HPAI OUTBREAKS AMONG ATYPICAL HOSTS, INCLUDING LIVESTOCK

During the ongoing global outbreak of avian influenza caused by the HPAI A/H5N1 virus, both birds and many mammalian species were found to be infected. In 2022–2024 there was a marked change in the scope and ratio of atypical hosts naturally infected with HPAI. Mammalian species, including cattle and small ruminants, were involved in the process.

The significant increase in the number of detected mammalian infections (Fig. 1) from 139 in 2022 to 275 in 2023 is linked to the spread of infection and implementation of expanded avian influenza monitoring programmes [6]. It cannot be excluded that some atypical hosts may be

important reservoirs of infection. HPAI A/H5N1 virus has recently demonstrated easy spillover into wildlife and agriculture, and has the potential to trigger a global pandemic.

The virus has currently impacted a variety of mammalian species worldwide, including those classified as endangered and threatened, potentially exacerbating their conservation status. The most likely source of mammalian infection appears to be close contact with infected birds, with some evidence suggesting potential mammal-to-mammal transmission [7].

Previously we described the HPAI global situation in mammals in 2022 [8]. The virus was said to have a high ability to spillover from birds to mammals such as mustelids (minks, otters, ferrets, badgers), felines (domestic cats, cougars, leopards, lynxes), pinnipeds (common seals, grey seals), bears (brown, grizzly, American black), bottlenose dolphins, skunks, foxes, opossums, raccoons. HPAI manifestations in mammals range from asymptomatic to severe forms.

HPAI virus strains that have already adapted to various mammalian species currently continue to circulate.

HPAI EPIZOOTIC SITUATION IN ATYPICAL HOSTS IN 2023–2024

In addition to the increased number of HPAI cases reported in mammals, there has been a change in the ratio of diseased animals belonging to various families over the last 2 years (Fig. 2, Table).

Thus, in 2022 more than 50% of infected animals belonged to the *Canidae* family (red foxes). In 2023, there was an increase in the number of cases among pinnipeds, felines, farmed mustelids, as well as among new species: forest polecat, coatis. In 2024 a significant number of outbreaks were recorded in bovids (cattle, goats).

Deaths of seals due to HPAI A/H5N1 infection in 2022 have been confirmed in Quebec (Canada) and along the US coast [9]. HPAI outbreak that began in November 2022 in Peruvian pelicans along the Peruvian coast and adjacent islands spread to marine mammals, particularly to South American sea lions, causing mass mortality by early 2023. Researchers have confirmed the virus entry into Peru from North America, presumably due to wild bird migration [10, 11, 12, 13].

There have been several cases of HPAI H5 transmission among other domestic and wild birds, as well as zoo animals and wild predators.

Extension of the species range and increase in the number of reported cases are not only linked to the disease spread, but also to the implementation of HPAI monitoring programmes in various countries [6].

In 2024, infection cases of influenza A/H5N1 began to be reported in cattle. Reports coming from the USA on HPAI positive tests in dairy cattle and goats, as well as probable transmission of the H5N1 subtype virus between cattle in dairy herds, are of concern because of the possibility of rapid adaptation and further interspecies spillover. All animals demonstrated similar clinical signs [14, 15]. The likely source of infection was cow feed or water that were accessible to wild birds. Cases of HPAI transmission from cattle to humans were recorded [16, 17].

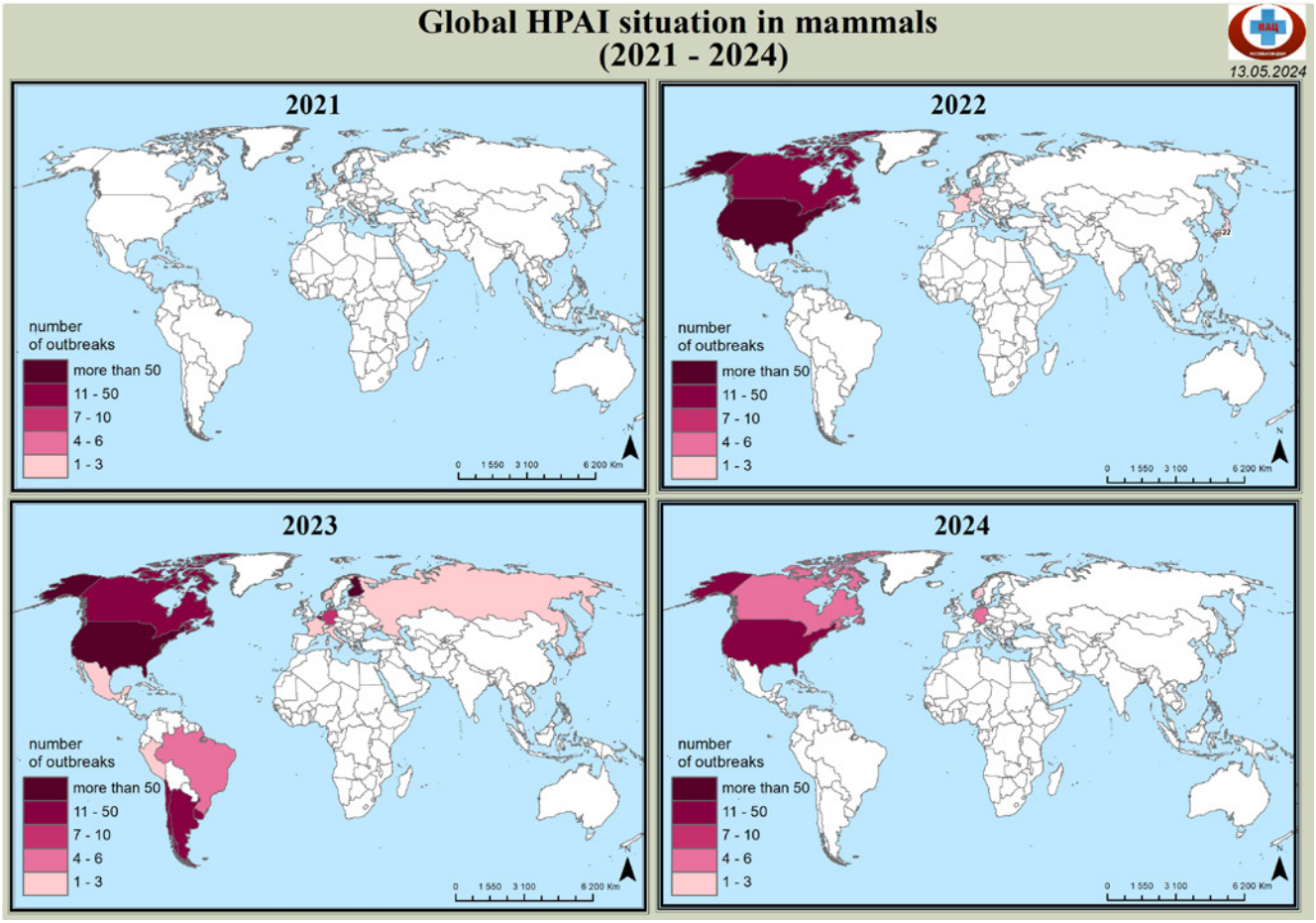

Fig. 1. Avian influenza epizootic situation in mammals in 2021–2024

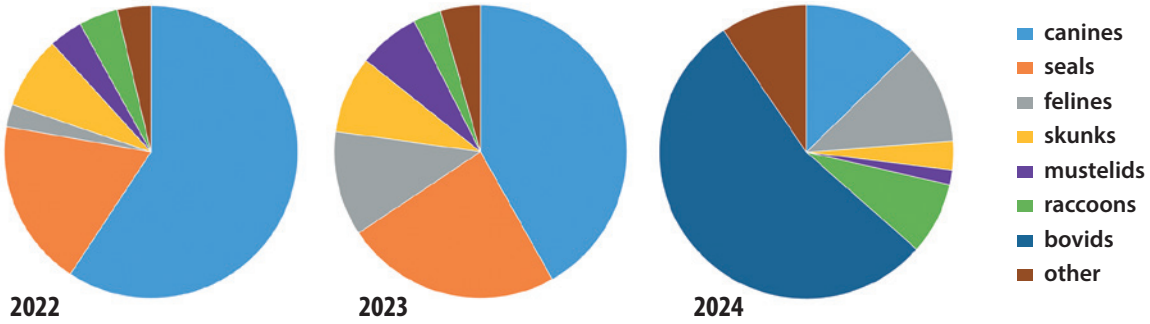

Fig. 2. Global distribution of avian influenza outbreaks by mammalian families in 2022–2024

Recombination of the North American viruses probably occurred shortly before the outbreak emerged in cattle. All isolates recovered from cattle were reassortants of the Eurasian and North American genotypes first detected in late 2023. The outbreak in goats was not linked to the outbreak in cattle. The HPAI H5N1 outbreak in cattle likely went undetected for an extended period. Researchers assume that the onset of the event occurred between 13 November 2023 and 18 January 2024 [18].

In light of recent HPAI situation in the USA the American Association of Bovine Practitioners (AABP) has taken steps to redefine the disease syndrome caused by the avian influenza virus and observed in cattle and designate it as bovine influenza A virus (BIAV), which requires further studies [19, 20, 21].

Experts at the Centers for Disease Control and Prevention (CDC) believe that the current HPAI-associated risk to humans is low, but people who come in contact with infected birds or animals are at greater risk of contracting HPAI A/H5N1 [22].

Infected cattle showed a non-specific course of infection, reduced feed intake and a sharp drop in milk yield, but severe systemic influenza infection was demonstrated in domestic cats receiving raw (unpasteurised) milk from diseased cows. In addition, cases of cow-to-cow transmission were reported [23].

In response to this situation, the GF-TADs (Global Framework for the Progressive Control of Transboundary Animal Diseases) meeting was held on 4 April 2024 to address identified cases of high pathogenicity influenza in dairy cattle and goats in the United States of America and the detection of the virus in humans. The importance of early detection and transparency of notifications, as well as cooperation between different national agencies, was emphasized [24].

The WOAH continues to monitor the situation to determine the risks to animal and human health. Timely reporting is crucial to objectively assess the disease situation and prevent any type of misinformation. Based on the data available the WOAH points out that restrictions on the movement of healthy cattle and products thereof are not recommended unless justified by an import risk analysis conducted in accordance with Chapter 2.1 of the WOAH Terrestrial Animal Health Code [25, 26].

GLOBAL CONTROL STRATEGIES

The first protective measure against HPAI spread is early detection of outbreaks. Establishing accurate warning systems is essential for effective prevention and control of the disease. Strict biosecurity and hygiene measures are also necessary to prevent outbreaks. When infection is detected in poultry, a culling policy is usually applied [27].

Vaccination of poultry may be recommended under certain conditions. Vaccines used should comply with the standards specified in the WOAH guidelines [28]. In early 2023 it was allowed to conduct emergency vaccination of wild birds against HPAI as an immediate response to an outbreak or when there was an increased risk of infection entry.

Concerns about international trade restrictions hamper use of vaccination, although its inclusion as a control tool has been endorsed by international standards adopted by the World Assembly of WOAH National Delegates. Unjustified trade restrictions on poultry and poultry products from vaccinated flocks have a huge impact on a sector that contributes significantly to global food security and the economy [29].

To date, vaccination has only been used in a limited number of countries as a preventive or emergency measure to protect birds against HPAI [30, 31]. According to various sources (including the WOAH), more than 30 countries have resorted to the use of vaccination against avian influenza since 2005 [32, 33, 34]. The countries that have officially declared HPAI vaccination are: Armenia, Belarus, Bangladesh, Dominican Republic, China (including Hong Kong), Egypt, El Salvador, Germany, Indonesia, Jordan, Kazakhstan, Democratic People's Republic of Korea, Kuwait, Laos, Mongolia, Mexico, Niger, Pakistan, Peru, Singapore, Sudan, Turkmenistan, Vietnam, Ecuador, Uruguay and others. In some European countries (Ireland, Great Britain) vaccination is allowed only in zoos [35, 36, 37]. In Russia, preventive vaccination against HPAI is practiced in farms (except poultry farms) according to the "Veterinary rules for the implementation of preventive, diagnostic, restrictive and other measures, establishment and lifting of quarantine and other restrictions aimed at preventing spread and eradication of high pathogenicity avian influenza", approved by Order of the Ministry of Agriculture of Russia No. 158 of 24 March 2021.

In May 2023 the US Animal and Plant Health Inspection Service (APHIS) announced that it had approved the emergency use of avian influenza vaccine to prevent additional deaths among California condors. Prior to this outbreak, the US authorities had stated that strict biosecurity protocols, including enhanced disinfection procedures as well as destruction of infected birds, were sufficient to mitigate the HPAI effects. Work is currently underway to develop a vaccine against HPAI for cattle, as it is believed that vaccination will help reduce the risk of the disease spreading to new animal species and lower potential losses to dairy production facilities. Vaccination of farm poultry has long

Table
Highly pathogenic avian influenza H5 in mammals in 2023–2024 (according to the WOAH data)

Country	Animal species	Outbreaks	Virus type
Argentina	South American fur seal	2	H5
	South American sea lion	14	H5
	Southern elephant seal	2	H5
Belgium	Red fox	16	not typed
	Forest polecat	2	not typed
Brazil	South American fur seal	5	H5N1
	South American sea lion		
Germany	Grey seal	1	H5N1
	Pine marten	1	H5N1
	Red fox	6	H5N1
	Raccoon	1	H5N1
Italy	Domestic cat	1	H5N1
	Domestic dog	1	H5N1
	Red fox	2	H5N1
Canada	American mink	2	H5N1
	Raccoon	3	H5N1
	Raccoon	3	H5N5
	Domestic cat	2	H5N1
	Red fox	1	H5N5
	Red fox	7	H5N1
	Skunk	1	H5N5
	Skunk	9	H5N1
	Domestic dog	1	H5N1
Latvia	Red fox	1	H5N1
Norway	Red fox	1	H5N1
	Red fox	2	H5N5
Peru	South American sea lion	2	H5
	Lion	1	H5
Russia	Northern fur seal	1	H5N1
USA	American mink	2	H5N1
	American black bear	1	H5N1
USA	Abert's squirrel	1	H5N1
	Polar bear	1	H5N1
	Dolphin [13]	1	H5N1
	Raccoon	6	H5N1
	Fisher	2	H5N1
	Domestic cat	11	H5N1
	Common seal	1	H5N1
	Opossum	1	H5N1
	Cougar	17	H5N1
	Red fox	20	H5N1
	Lynx	6	H5N1
	Skunk	17	H5N1
	Goat	1	H5N1
	Cattle	33	H5N1
Uruguay	Coatis	1	H5N1
	South American sea lion	8	H5N1
	South American fur seal	3	H5N1
Finland	American mink	6	H5N1
	Otter	2	H5N1
	Raccoon dog	9	H5N1
	Red fox	13	H5N1
	Arctic fox	48	H5N1
	Lynx	1	H5N1
	Sable	1	H5N1
France	Red fox	1	H5N1
Chile	Marine otter	2	H5
	Eurasian river otter	1	H5
	South American sea lion	31	H5
South Korea	Domestic cat	2	H5N1
Japan	Red fox	2	H5N1

been controversial among researchers and farmers in the US. Poultry producers are concerned about the cost and complexity of vaccinating millions of birds, as well as trade restrictions [38, 39].

In May 2023, the 27 Member states of the European Union agreed to implement a vaccination strategy against avian influenza. The tasks were shared between the states: France developed a vaccine for ducks, the Netherlands – for laying hens, Italy – for turkeys and Hungary – for Peking ducks. Preliminary results were promising: in Hungary, the mortality of vaccinated geese (HVT-H5 manufactured by Ceva Sante Animale) after challenge was 2.93% compared to 76.23% in the control group, and there was also a reduction in the viral shedding. In Italy, a high level of clinical protection of turkeys was achieved using HVT-H5 vaccine with a subunit or DNA vaccine administered as a booster. Homologous vaccination gave unsatisfactory results (25 to 40% protection) [40].

Study results of Dutch researchers showed that both HVT-H5 vaccines by Ceva Animal Health and Boehringer

Ingelheim effectively protected poultry in 8 weeks after vaccination [41].

France was the first European country to introduce mandatory vaccination of ducks from October 2023, despite the risk of trade restrictions introduced by third countries (USA, Japan) [42, 43, 44]. No outbreaks were reported among vaccinated poultry in the south of the country thereafter. In the period from 2 December 2023 to 15 March 2024 the diseased birds were predominantly found in unvaccinated poultry populations. The number of HPAI cases detected in wild birds and commercial poultry was lower than in the same period last year [45]. According to the European Food Safety Authority report, no outbreaks were reported in poultry in France from 16 March to 14 June 2024 [46].

Positive results of HPAI vaccination are also recorded in Bangladesh, where poultry have been vaccinated since 2012. The number of outbreaks before vaccination was 18 times higher than after it. The latest avian influenza outbreaks in the country were reported in 2019. The studies conducted in Bangladesh suggest that poultry vaccination can be part of a holistic strategy to mitigate HPAI consequences if accompanied by monitoring to avoid latent spread [47, 48].

Preventive vaccination has also been used successfully in Hong Kong since 2003, where no HPAI outbreaks in poultry population have been reported since 2018 [49].

Emergency vaccination with Nobilis Influenza H5N2 vaccine conducted in the Czech Republic in 2021 helped to preserve the national breed of geese. Poultry breeders and the public perceive the possibility of vaccination very positively [40].

CONCLUSION

Influenza A virus, including the H5N1 subtype, can infect many animal species. In recent years, some HPAI virus strains have adapted to new mammalian species, which demonstrates likelihood that the virus will acquire a set of additional adaptive mutations. Transmission of avian influenza A virus to mammals, including humans, could be the first step towards a future pandemic. Identification of factors affecting transmission and replication of the virus in mammals will make it possible to predict which viral lineages are more likely to spillover and cause disease in atypical hosts, including humans [50].

Although infection with HPAI mammalian strains is rare, a growing number of publications indicate an increasing prevalence of the disease, emphasizing the need for preventive measures to limit transmission, thereby preventing a potential future epidemic in humans.

Environmental and epizootological changes that occurred due to avian influenza outbreaks in recent years have raised doubts regarding the exclusive use of stamping-out programmes. International organisations (WOAH, European Food Safety Authority, European Commission) suggest that preventive vaccination can minimise the number of outbreaks and the duration of the epizootic. The use of vaccines may reduce the risk of avian influenza spillover to new animal species and lower potential losses. However, vaccination should complement, not replace, other preventive and control measures such as avian infection monitoring, early detection and biosecurity, and is recommended as part of a comprehensive approach to control of avian influenza outbreaks.

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Role of rotavirus, coronavirus and *Escherichia coli* in disease etiology in young cattle (review)

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ABSTRACT

Introduction. One of the most prevalent groups of pathologies detected in young cattle involves gastrointestinal diseases. They are often caused by infectious agents, among which rotavirus, coronavirus and pathogenic *Escherichia coli* are predominant.

Objective. Analysis and systematization of up-to-date information on the role of rotavirus, coronavirus and pathogenic *Escherichia coli* strains in the etiology of diseases of cattle, including young animals, data on the incidence of these infections in the Russian Federation and other countries of the world as well as relevance of vaccination against the above-mentioned pathogens.

Results. The paper provides information on the structure of rotavirus, coronavirus and *Escherichia coli*, on the biological properties of the pathogens, and factors affecting the disease form and severity. Based on the analysis of domestic and foreign scientific publications, data on the prevalence of colibacillosis, rotavirus and coronavirus infections are presented, and the main methods of their control are described. The significance of the vaccines for the prevention of these diseases is confirmed, the factors influencing the vaccine prevention effectiveness are listed, and measures to increase it are given.

Conclusion. The average global incidence of rotavirus infection is 32.7%, coronavirus infection is 18.4%, and colibacillosis is 39.1%. In Russia, the prevalence rate of the above-mentioned diseases is 41.4, 33.1 and 30.2%, respectively. Thus, in the Russian Federation, the incidence of bovine rotavirus and coronavirus infections exceeds the global average by 8.7 and 14.7%, respectively. The colibacillosis situation in Russia is better than in most countries: the disease is reported by 8.9% less frequently than the global average. High genetic diversity and prevalence of the above-mentioned pathogens require an integrated approach to their control. One of the most effective methods is vaccination, which makes the development of effective and safe vaccines against rotavirus, coronavirus and *Escherichia coli* infections an urgent task.

Keywords: review, rotavirus, coronavirus, *Escherichia coli*, young cattle, respiratory and intestinal pathologies

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Значение ротавируса, коронавируса и *Escherichia coli* в этиологии болезней молодняка крупного рогатого скота (обзор)

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РЕЗЮМЕ

Введение. Одной из самых распространенных групп патологий, встречающихся у молодняка крупного рогатого скота, являются болезни желудочно-кишечного тракта. Частой их причиной являются возбудители инфекций, среди которых преобладающее значение имеют ротавирус, коронавирус и патогенная форма кишечной палочки.

Цель исследования. Анализ и систематизация актуальной информации о роли рота-, коронавируса и патогенных штаммов *Escherichia coli* в этиологии болезней крупного рогатого скота, в том числе молодняка, сведений о заболеваемости этими инфекциями на территории Российской Федерации и других стран мира, а также актуальности вакцинопрофилактики против вышеназванных патогенов.

Результаты. В статье представлена информация о строении ротавируса, коронавируса и *Escherichia coli*, биологических свойствах возбудителей, факторах, влияющих на форму и тяжесть течения болезней. На основании анализа научной литературы отечественных и зарубежных авторов представлены данные о распространенности колибактериоза, ротавирусной и коронавирусной инфекций, а также описаны основные методы их контроля.

Подтверждена важность вакцин для профилактики указанных болезней, перечислены факторы, влияющие на эффективность вакцинопрофилактики, и приведены меры ее повышения.

Заключение. Средний уровень заболеваемости ротавирусной инфекцией в мире составляет 32,7%, коронавирусной инфекцией – 18,4%, колибактериозом – 39,1%. В России показатель превалентности вышеупомянутых болезней равен 41,4; 33,1 и 30,2% соответственно. Таким образом, в Российской Федерации уровень заболеваемости рота- и коронавирусной инфекциями крупного рогатого скота превышает средний показатель в мире на 8,7 и 14,7% соответственно. Эпизоотическая ситуация по колибактериозу в России благополучнее, чем в большинстве стран: болезнь регистрируется реже среднего мирового значения на 8,9%. Большое генетическое разнообразие и распространенность вышеупомянутых возбудителей требуют комплексного подхода для борьбы с ними. Одним из наиболее эффективных способов является вакцинопрофилактика, что делает разработку эффективных и безопасных вакцинных препаратов против ротавирусной, коронавирусной инфекций и эшерихиоза актуальной задачей.

Ключевые слова: обзор, ротавирус, коронавирус, *Escherichia coli*, молодняк крупного рогатого скота, респираторные и кишечные патологии

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INTRODUCTION

Gastrointestinal (GI) diseases are one of the most prevalent groups of pathologies in young cattle. The GI disorders are most often associated with the lack of a complex of necessary measures to prevent infectious diseases in young animals: ineffective or untimely vaccination, untimely feeding with colostrum, non-compliance with hygienic standards in the maintenance of production facilities, faulty diet formulation and non-compliances with the feeding technics. The combination of these factors creates conditions for the development of enteritis of infectious etiology in cattle [1, 2].

One of the most common causes of enteritis in young cattle is the effect of rotaviruses, coronaviruses and *Escherichia coli*. Therefore, they make the most significant impact on the health of calves as compared to other infectious agents that cause GI disorders, thus requiring appropriate preventive measures [3, 4].

The novelty of the analytical study lies in the generalization of scientific information on the bovine disease situation caused by rotaviruses, coronaviruses and pathogenic *E. coli* strains in the Russian Federation and other countries of the world.

The purpose of this review is to analyze and systematize up-to-date information on the role of rotavirus, coronavirus and pathogenic *E. coli* strains in the etiology of bovine diseases, *inter alia* in young animals, data on the incidence of these infections in the Russian Federation and other countries of the world, as well as relevance of vaccine prevention of the above-mentioned pathogens.

ROLE OF ROTAVIRUSES IN THE DEVELOPMENT OF BOVINE PATHOLOGIES

The taxonomic group *Reoviridae* includes non-enveloped viruses containing double-stranded, segmented RNA represented by 11 segments. Rotaviruses belong to the family *Sedoreoviridae*. The rotavirus capsid consists of 3 layers. The outer layer is represented by VP4 and VP7 proteins, the middle one – by VP6, the inner one – by VP1, VP2 and VP3, and the virion size is 70 nm. The segmented nature of the genome is the reason for the rotavirus reassortment [2].

Since the rotaviruses are widespread, young cattle can be infected with them from their first days of life. The rotavirus infection in calves is clinically manifested by depression, diarrhea, and dehydration (Fig. 1). During the milk feeding, the feces of the diseased calves are of yellow or white color and of varied consistency (from watery to thick), however, the presence of blood in the feces is not typical for rotavirus infection. In case of secondary bacterial infection, the mortality rate of newborn calves can reach 60%. During autopsy, catarrhal or catarrhal-hemorrhagic enteritis is reported in dead animals [2].

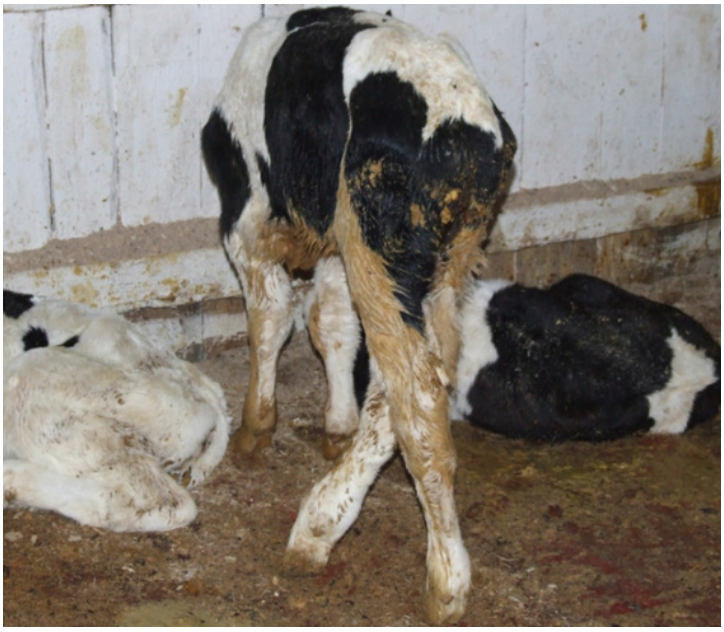

Fig. 1. Clinical manifestation of rotavirus infection, characterized by diarrhea and depression (photo from the personal archive of A. V. Kononov)

Rotavirus infection is most acute during the cold season, and the severity of the disease depends upon the decrease in the indoor temperature. The risk of severe disease is also increased by feeding with colostrum from cows that lack rotavirus antibodies as well as by the presence of other enteropathogenic infectious agents [2].

In addition to young animals, the adult animals can also be infected with rotavirus, but their infection is asymptomatic. The number of asymptomatic carriers on the infected farms can reach 44%. The infected adult animals play a significant role in the virus spread: within a few weeks, one such animal can shed up to 10^{10} viral particles per 1 g of feces. Since this virus has a high resistance to environmental factors, the pathogen can circulate on the farm for a long time and infect a large number of susceptible animals, including calves [2].

Enteritides of rotavirus etiology are reported in calves more often than other infectious GI diseases. On the infected farms, they can infect up to 100% of calves, and vaccination of cattle may be ineffective due to the rotavirus reassortment and emergence of new recombinant virus variants [5]. The incidence of the rotavirus infection in cattle in the world and in the Russian Federation can reach 70% or higher (Table 1 and 2).

Based on the systematized data, it can be concluded that the average prevalence of rotavirus infection in cattle in the countries of the world was 32.7% in 1981–2021.

Table 1
Bovine rotavirus infection prevalence in the countries of the world

Country	Region	Estimated prevalence, %	Source
Australia (2011)	Australia	79.90	[6]
Australia (2004–2005)		26.00	[7]
China (1984–2021)	Asia	35.70	[8]
Iran (2000)		34.00	[9]
Iran (1981)		31.74	[10]
Iran (2001)		28.80	[10]
Iran (2010)		27.90	[10]
Norway (2004–2007)	Europe	67.70	[11]
Switzerland (2005–2006)		58.70	[12]
Spain (2000)		43.50	[10]
Spain (1998)		42.70	[10]
Turkey (2007)		41.17	[10]
Belarus (2020–2021)		39.60	[13]
Ukraine (2012)		28.60	[14]
Sweden (2003)		13.00	[15]
Sweden (1987–1988)		5.40	[16]
Argentina (1994–2003)	South and North America	42.00	[17]
Brazil (2007)		33.00	[10]
USA (2010)		12.20	[18]
Brazil (2007)		11.00	[19, 20]
Costa Rica (1981)		10.00	[10]
Costa Rica (1998)		7.00	[10]

Table 2
Bovine rotavirus prevalence in the Russian Federation

Region of the Russian Federation	Federal district	Estimated prevalence, %	Source
Republic of Dagestan (2001–2005)	North Caucasian	77.90	[21]
Irkutsk Oblast (2020)	Siberian	44.40	[22]
Irkutsk Oblast (2004–2017)		17.60	[5]
Central Black Earth Region (2017–2018)	Central	22.30	[23]
12 Oblasts (2007–2011)	Central, Volga and Far Eastern	44.55	[24]

As for the Russian Federation, the issue of the rotavirus infection prevalence in cattle also remains relevant. The data in Table 2 show that over the past 20 years, the incidence on farms in the Russian Federation has averaged 41.4%, which exceeds the same indicator in other countries by 8.7%. Such a high prevalence may be due to the non-compliances with calf housing conditions and lack of appropriate preventive measures targeted to all age groups of cattle on the farms of the Russian Federation.

ROLE OF CORONAVIRUSES IN THE DEVELOPMENT OF BOVINE PATHOLOGIES

Bovine coronavirus belongs to the *Coronaviridae* family, genus *Betacoronavirus*, and species *Betacoronavirus gravedinis*. The genome is represented by a single-stranded (+) RNA and it is the longest among the RNA viruses. The virion is 65–210 nm in diameter and contains a supercapsid.

Coronavirus infection is ubiquitous. During their lifetime, up to 90% of animals become infected with coronavirus. Based on the clinical picture, there are three disease forms: intestinal, respiratory, and so-called winter dysentery. The development of one disease form or another depends not on the serotype of the pathogen, but on the age of the recipient [2].

The intestinal form is most typical for young animals from the first days of life to the age of five months. It is characterized by inflammatory lesions of the large and small intestines, which lead to severe diarrhea (often with blood), as well as to high mortality rate, which can reach 20% [2, 5].

The respiratory infection is typical for calves from two to six months of age, which is characterized by rhinitis, cough, fever, loss of appetite, and often by concurrent diarrhea (Fig. 2). In severe cases, dyspnea, bronchopneumonia, and weight loss up to exhaustion and death are reported [2].

Fig. 2. Clinical manifestation of coronavirus infection, characterized by depression (photo from the personal archive of A. V. Kononov)

In adult animals, the disease occurs in the form of winter dysentery, which is characterized by the following clinical signs: profuse diarrhea (up to 100% of cases), often with blood, cough, seromucous nasal discharge, harsh rapid breathing. The disease leads to the decrease in milk yield from 25 to 90%, while restoring the previous milk yield can take from 2.5 to 4 months [25].

The adult animals can be asymptomatic coronavirus carriers, while being a source of infection transmission with feces (96%) and nasal mucus (84%). Over 70% of adult animals can shed the virus despite the presence of antibodies. This is due to the fact that a long period of persistence and shedding by the recovered animals are typical for the coronavirus [2].

Low temperatures and lesser exposure to UV rays in winter not only contribute to the pathogen persistence,

Table 3
Prevalence of bovine coronavirus infection in the countries of the world

Country	Region	Estimated prevalence, %	Source
Australia (2011)	Australia	21.60	[6]
Japan (1995–1997)	Asia	57.00	[27]
Iran (2010)		3.10	[10]
Norway (2004–2007)	Europe	39.30	[11]
Belarus (2020–2021)		28.70	[13]
Ukraine (2012–2019)		22.40	[14]
Switzerland (2005–2006)		7.80	[12]
Spain (1998)		7.30	[10]
Turkey (2007)		1.96	[10]
Sweden (2003)		1.00	[15]
Brazil (2007)	South and North America	22.00	[10]
Brazil (2007)		16.00	[20]
Costa Rica (1998)		9.00	[10]
USA (2010–2011)		20.90	[18]

Table 4
Prevalence of bovine coronavirus infection in the Russian Federation

Region of the Russian Federation	Federal district	Estimated prevalence, %	Source
Republic of Dagestan (2001–2005)	North Caucasian	62.60	[21]
Siberia (2010)	Siberian	71.30	[28]
Siberia (2022)		11.80	[29]
Irkutsk Oblast (2020)		11.10	[22]
Irkutsk Oblast (2004–2017)		2.20	[5]
Central Black Earth Region (2017–2018)	Central	26.70	[23]
14 oblasts (2007–2011)	Central, Volga, Southern and Far Eastern	45.90	[24]

but also reduce the overall level of animal resistance, which leads to 50–60% increase in the amount of virus shed into the environment, which, in turn, results in the increase in the number of cases of coronavirus infection. In addition to the time of year, the bovine infection with the coronavirus is also affected by the physiological condition of the animal, for example, during the calving period and the first two weeks after it, the number of the virions shed by the infected animal increases [2].

The virus can enter the body of calves not only by alimentary route (through contaminated surfaces or contaminated udder and perineum of cows), but also by airborne route, thus resulting in a high risk of infection [2, 26].

The analysis of publications from 1995 to 2022 showed that the prevalence of bovine coronavirus in the world was 18.4% (Table 3), in Russia – 33.1% (Table 4), which is 14.7% more than the global average.

DEPENDENCE OF THE DISEASE FORM IN YOUNG CATTLE ON *E. COLI* PATHOGENIC PROPERTIES

Among the bacterial diseases of young cattle, colibacillosis (escherichiosis) caused by various *E. coli* serovariants is the most prevalent.

E. coli bacteria have a complex antigenic structure comprising three types of antigens: somatic O-antigen (contains 164 variants); capsular K-antigen (90 variants) and flagellar H-antigen (55 variants). These antigens in various combinations make more than 9,000 serovariants, 170 of which demonstrate pathogenic properties [2, 30].

E. coli strains that cause animal diseases have various pathogenicity factors, which include polysaccharides, adhesins, enterotoxins, etc. Their functions include the following: hindering and weakening the immune response (capsular polysaccharides), destruction of body cells (enterotoxins), attachment of bacteria to the surface of susceptible cells (adhesins), etc. [2].

The escherichiosis causative agents are subdivided into two groups: diarrheagenic (DEC – diarrheagenic *E. coli*) and extraintestinal pathogenic (ExPEC – extraintestinal pathogenic *E. coli*). Five main diarrheagenic groups are relevant for cattle: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), Shiga toxin-producing (STEC) and necrotoxigenic (NTEC) *E. coli* [31].

Enterotoxigenic *E. coli* attach to the surface of enterocytes using fimbrial adhesins. The distinctive feature of the representatives of this pathogroup is presence of thermostable (stI and stII) and/or thermolabile (ltI and ltII) toxins that induce the secretion of electrolytes and water. This leads to diarrhea in infected animals and, as a result, to dehydration and death [2, 32, 33].

Despite the fact that EPEC less frequently cause GI disorders in calves than EHEC and ETEC, they require monitoring by the veterinarians due to their continuous on-farm circulation. The analysis of the frequency of occurrence of different *E. coli* pathogens in calves demonstrated that EPEC circulates almost twice as frequently in the healthy animals (14.6%) than in the diseased ones (7.5%) [4].

Enteropathogenic *E. coli* is characterized by the presence of the *eae* gene encoding the adhesive pathogenicity factor intimin and by the lack of ability to produce Shiga toxin (stx). Due to intimin, the bacterium attaches to enterocytes, after which they are rejected that further leads to diarrhea [4].

Based on the presence of the *eae* gene, Shiga toxic *E. coli* are divided into 2 groups: EHEC (STEC LEE+), which have the specified gene in their genome, and STEC (STEC LEE-), which lack it. The common features of the both groups include long-term persistence in the host, localization in the small intestine, and presence of genes encoding the ability to produce stx [2, 32].

Based on the studies on the identification of various *E. coli* pathogens in calves conducted in 18 countries, it was found that STEC LEE+ is less common than STEC LEE–: in healthy calves, the frequency of their occurrence is 10.7 and 19.4%, respectively, and in the diseased ones – 6.0 and 18.2% [4].

Necrotoxigenic *E. coli* has a specific set of genes encoding cytotoxic necrotizing factor (CNF) and cytolethal distending toxin (CDT). This pathogroup has many properties of *E. coli* that cause diseases with extra-intestinal symptoms, such as presence of different fimbrial and afimbrial adhesins and the ability to resist the complement system [4, 33].

Currently, there are two known types of cytotoxic necrotizing factors: CNF1 and CNF2. Presence of genes encoding the cytotoxic necrotizing factor 1 (CNF1) is more common in strains that cause diarrhea. The genes encoding the cytotoxic necrotizing factor 2 (CNF2) are found in *E. coli*, causing sepsis [33, 34].

It should be noted that commensal *E. coli*, when interacting with pathogenic species, can acquire new genetic determinants encoding not only cell protective mechanisms, but also pathogenicity factors. Therefore, it is a mistake to classify a strain as pathogenic only on the basis

Table 5
Prevalence of bovine colibacillosis in the countries of the world

Country	Region	Estimated prevalence, %	Source
Australia (2011)	Australia	17.40	[6]
Iran (2013)	Asia	86.70	[9]
Iran (2010)		76.45	[34]
India (2009)		75.00	[9]
Pakistan (1997)		54.00	[9]
India (1993)		23.00	[9]
Germany (1997)	Europe	42.00	[9]
Spain (2008)		35.90	[9]
Ukraine (2012–2019)		31.68	[14]
France (1999)		20.30	[9]
Sweden (1987–1988)		11.50	[16]
Sweden (1993)		11.50	[9]
Switzerland (2005–2006)		5.50	[12]
Brazil (2007)	South and North America	69.00	[20]
Mexico (2000)		63.70	[9]
USA (2010–2011)		1.80	[18]

Table 6
Prevalence of bovine colibacillosis in the Russian Federation

Region of the Russian Federation	Federal District	Estimated prevalence, %	Source
Amur Oblast (2003–2005)	Far Eastern	33.00	[36]
Amur Oblast (2016–2019)		28.50	[37]
Republic of Bashkortostan (2014–2016)	Volga	30.00	[38]
Perm Krai (2010–2020)		14.40	[38]
Irkutsk Oblast (2004-2017)	Siberian	18.50	[39]
Irkutsk Oblast (2001–2010)		10.35	[38]
Rostov Oblast (2021)	Southern	74.20	[40]
Krasnodar Krai (1996–2015)		43.55	[38]
Rostov Oblast (2017)		19.26	[41]

of a serovariant, since there are *E. coli* that are included in the same serovar, but belong to different pathogroups and, as a result, cause different pathological processes. Such a tendency to variability may even lead to the acquisition of pathogenicity factors of different pathogens by one microorganism. For example, one of the publications mentions a hybrid strain containing EHEC and NTEC genes [31, 35].

Infection of young animals with the colibacillosis causative agent occurs by the alimentary route. Infected adult animals play an important role in the *E. coli* spread, contaminating water, various indoor surfaces and bedding with bacteria, as a result of contact with which the infectious agent can get on the udder and later be transmitted to the calf [2].

In the beginning of the postnatal period, colibacillosis in calves takes more often an enteritic form and less often a septic form. The severe enteritic form is manifested by heavy diarrhea, rapid dehydration of the animal, sunken eyes, depression and exhaustion, dry and grayish skin. This disease form often ends with the death of the animal. When calves are housed in good sanitary conditions and the animal has colostral antibodies, the enteritis form may be mild [2].

Septic escherichiosis is caused by non-diarrheagenic *E. coli* (ExPEC). This form of colibacillosis develops due to untimely feeding with colostrum (primary) or in the presence of viral diseases (secondary). The septic disease clinical signs are expressed by ataxia, lameness, anorexia, hard breathing. The animal dies in 24–48 hours after their onset [2].

According to the scientific publications for the period from 1987 to 2021, the most escherichiosis-infected countries are Mexico, Brazil, India and Iran. The average

global disease prevalence is 39.1% (Table 5). In Russia, the incidence rate is at the level of 30.2% (Table 6).

It should be noted that *E. coli* causes diseases not only in calves, but also in adult animals, which, depending on the pathogen properties, can develop such infections as mastitis, metritis and endometritis. Tests of milk from mastitis-diseased animals demonstrated that the level of *E. coli* was four times higher than in the milk of healthy cows, which confirms the role of *E. coli* as one of the causative agents of bovine mastitis [42].

Currently, it has not been fully clarified whether any pathogenic *E. coli* strains cause mastitis, or whether the cause of infection are *E. coli* of some individual pathogroup. There is evidence that the mastitis causative agent is an individual pathogenic *E. coli* group – MPEC (mammary pathogenic *E. coli*), which includes many extra-intestinal pathogenic *E. coli* strains: when studying the genome of *E. coli* isolated from mastitis-diseased cows, the ExPEC-typical pathogenicity factors were identified. However, there is evidence of isolation of *E. coli* of STEC group from the diseased animals, which indicates its possible involvement in the disease onset [42, 43].

Metritis and endometritis are also common *E. coli*-induced pathologies on the farms, which should not be underestimated, since under certain conditions they can lead to infertility and further culling of cows. Despite the fact that there is currently no clear opinion about the presence of a separate *E. coli* pathogroup that causes metritis and endometritis, some scientists identify six virulence genes, on the basis of which the *E. coli* strain that causes these pathologies can be presumably identified. kpsMTII gene is emphasized among them, which is probably responsible for the disease severity. Some publications note that presence of this gene carrying *E. coli* in the uterine microflora 9.2-fold reduces the probability of successful insemination [44].

MEASURES FOR PREVENTION OF VIRAL AND BACTERIAL DISEASES IN YOUNG CATTLE

All the above-mentioned data demonstrate that the issue of escherichiosis, rotavirus and coronavirus infections is quite acute for the Russian Federation and foreign countries and requires effective measures to solve it.

Vaccine prevention remains one of the most effective ways to control infectious diseases, including enteritis of viral and bacterial etiology, which is confirmed by publications of domestic and foreign authors.

For example, the study conducted in Canada in 2022 showed a two-fold decrease in the disease incidence in calves immunized with a live vaccine against coronavirus infection [45].

The results of immunization of pregnant cows with an inactivated vaccine against rotavirus and coronavirus infections on the infected farms of the Russian Federation, Belarus and Ukraine demonstrated that feeding newborn calves with colostrum and milk derived from vaccinated animals reduced the disease incidence in young animals 7 times and their mortality 6.4 times [46].

In Estonia, when studying the effect of the duration of feeding newborn calves from cows immunized with various inactivated vaccines against rotavirus, coronavirus infections and colibacillosis with colostrum and transitional milk, it was demonstrated that vaccination of pregnant animals reduced the mortality of young animals in comparison with the control groups. Feeding calves with colostrum and milk from vaccinated animals during the first 14 days after birth resulted in four-fold decrease in their mortality due to diarrhea. However, reduced period of colostrum and transitional milk feeding resulted in the decrease of the colostral immunity level in young animals [47].

In addition to preventing GI diseases, the vaccines with an optimal set of rotavirus, coronavirus and *E. coli* antigens can potentially reduce the number of pneumonia, mastitis and metritis cases in older animals on the farms.

When developing new products, it should be borne in mind that the effectiveness of specific preventive measures is influenced by such factors as the vaccine composition and the vaccination program implementation.

The first group of factors includes use of ineffective adjuvants or low-quality virus-containing source materials, as well as use of the active vaccine ingredient comprising strains with a low degree of antigenic matching with the field isolates circulating in a particular region. In this regard, monitoring of field isolates of rotavirus, coronavirus and pathogenic *E. coli* circulating in the Russian Federation and tracing changes in their genome will make it possible to timely adjust preventive measures and determine the most relevant vaccine composition when choosing from existing products or when developing new ones.

The effect of antigenic affinity of the pathogen vaccine strains and field isolates on the quality of vaccine prevention may indicate a greater effectiveness of the use of domestic products, since they are developed basing on the strains isolated in the Russian Federation.

The decrease in the effectiveness of immunization depends on the incorrect use of specific preventive measures: non-compliance with the dose, frequency and deadlines of vaccination; insufficient susceptible animal immunization coverage; non-compliance with the conditions of the vaccine storage and preparation for use; immunization of the diseased animals, etc.

CONCLUSION

Rotavirus-, coronavirus- and *E. coli*-induced infections are of great importance for animal husbandry, as they affect not only newborn calves (GI disorders, shortage of the replacement population, mortality of calves, treatment costs, etc.), but also adult cattle (mastitis, metritis, source of infection, etc.) thus resulting in significant economic losses.

The pathogens are widespread in many countries of the world. Herewith, the average incidence of rotavirus infection is 32.7%, coronavirus infection is 18.4%, and colibacilliosis is 39.1%.

The issue of the aforementioned infections does not lose its relevance for our country either. Thus, in Russia, the incidence of rotavirus and coronavirus infections exceeds the global average by 8.7 (41.4%) and 14.7% (33.1%), respectively. Of these diseases, escherichiosis is the least prevalent (30.2%), which may be due to significant seasonal temperature fluctuations in our country throughout the year and the active use of antibiotics on the Russian farms.

The wide variety and prevalence of the above-mentioned pathogens require an integrated approach to prevent infection of cattle (both young and adult animals);

an adequate diet, practicing good hygiene, housing and feeding, implementation of animal quarantine measures, etc. are essential. When speaking of measures to prevent rotavirus, coronavirus infections and colibacilliosis, it should be mentioned that vaccination is one of the most effective ways to control them. Using a vaccine with an optimal set of antigens will not only protect calves from developing GI disorders and reduce their mortality, but also potentially reduce the number of pneumonia, mastitis and metritis cases in older animals on the farms, which makes the development of effective and safe vaccines against rotavirus, coronavirus infections and escherichiosis an urgent task.

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Lumpy skin disease in the Middle East – historical and statistical data

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ABSTRACT

Introduction. Lumpy skin disease (LSD) is currently a matter of veterinary concern due to the significant economic losses of the livestock industry. The risk of LSD spread and penetration into the disease-free countries is increasing every year. Therefore, timely monitoring of the infection spread for the development a strategy for this disease control becomes of current importance. Description of the disease manifestations and course, evaluation of historical and statistical data on LSD spread in the Middle Eastern countries as well as the further joint actions at the international level are presented in this review.

Objective. Analysis of historical and statistical data on clinical lumpy skin disease in the Middle Eastern countries.

Materials and methods. The following electronic databases were used for relevant data searching and collection: PubMed, Web of Science, eLIBRARY.RU, mdpi.com, frontiersin.org, researchgate.net, etc. English literature data for 10 years were analyzed.

Results. In 1988 LSD spread outside the African continent then LSD stayed for a short time within the Middle Eastern countries and two years later spread further to the west and east. Despite the further spread, recurrent LSD outbreaks were reported in the Middle Eastern countries over the next few years. Many countries in the Middle East still face the problem of uncontrolled livestock movement, lack of high-quality laboratory diagnostics, and irregular contacts with international health and surveillance organizations aggravated by the unstable political situation in the region. These problems indicate the importance of LSD control at the international level, the significance of regional and international cooperation and effective biosafety policies.

Conclusion. Role of the Middle East region in LSD virus spread, probable causes of LSD infection in the region, trends for further actions for LSD control were determined.

Keywords: review, lumpy skin disease, cattle, Middle East, historical outbreak, transboundary animal diseases

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Заразный узелковый дерматит крупного рогатого скота на Ближнем Востоке – исторические и статистические данные

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РЕЗЮМЕ

Введение. Заразный узелковый дерматит крупного рогатого скота, также известный как нодулярный дерматит, на данный момент представляет собой актуальную проблему ветеринарии вследствие значительного экономического ущерба, причиняемого животноводческой отрасли. Риск распространения заразного узелкового дерматита крупного рогатого скота и проникновения его в благополучные по заболеванию страны с каждым годом увеличивается. В связи с этим актуальным вопросом становится своевременное отслеживание распространения инфекции для выработки стратегии борьбы с ней. В представленном обзоре рассмотрены особенности проявления и течения заболевания, оцениваются исторические и статистические данные по распространению заразного узелкового дерматита крупного рогатого скота в странах Ближнего Востока и будущие направления совместных действий на международном уровне.

Цель исследования. Анализ исторических и статистических данных проявления заразного узелкового дерматита у крупного рогатого скота в странах Ближнего Востока.

Материалы и методы. Сбор теоретического материала проводился в электронных библиотеках с использованием ресурсов: PubMed, Web of Science, eLIBRARY.RU, mdpi.com, frontiersin.org, researchgate.net и др. Проанализированы англоязычные литературные данные за последние 10 лет.

Результаты. После выхода вируса заразного узелкового дерматита крупного рогатого скота за границы Африканского континента в 1988 г. он ненадолго задержался в пределах стран Ближнего Востока, уже через два года проникнув далее на запад и восток. Несмотря на дальнейшее распространение, в ближневосточных странах еще в течение нескольких последующих лет отмечались повторные вспышки заболевания. Многие страны Ближнего Востока все еще сталкиваются с проблемой бесконтрольного перемещения скота, отсутствием возможностей для проведения качественной лабораторной диагностики, нерегулярностью контактов с международными организациями в сфере здравоохранения и надзора, усугубляемыми нестабильностью политической ситуации в регионе. Данные проблемы подчеркивают важность борьбы с заразным узелковым дерматитом крупного рогатого скота на международном уровне, значение регионального и международного сотрудничества и проведения эффективной политики биобезопасности.

Заключение. Определена роль Ближневосточного региона в распространении возбудителя болезни, названы вероятные причины неблагополучия региона по данному заболеванию, сформулировано направление дальнейших действий в рамках борьбы с заразным узелковым дерматитом крупного рогатого скота.

Ключевые слова: обзор, заразный узелковый дерматит, крупный рогатый скот, Ближний Восток, историческая вспышка, трансграничные болезни животных

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INTRODUCTION

Lumpy skin disease is currently a matter of veterinary concern due to the significant economic losses to the livestock industry caused by this disease. Lumpy skin disease is caused by a DNA virus genus *Capripoxvirus*, family *Poxviridae*, and affects cattle and water buffaloes and characterized by skin nodules in infected animals. This virus is antigenically closely related to sheep poxvirus and goat poxvirus, therefore it can infect small ruminants without any clinical disease manifestations [1, 2, 3].

The disease is enzootic, rapidly explorative and sometimes fatal infection, despite the fact that, as often reported, LSD is characterized by high incidence rate (more often about 20%, but it can vary from 3 to 85%) and rather low mortality rate (less than 5%) [4, 5, 6, 7]. LSD affects food-producing animals that results in reduced milk yields in dairy animals and decreased weight gains in meat animals that has a negative effect on food security due to limited availability of high-quality animal products on the world market. Reproductive performance in infected animals as well as quality of hides derived from slaughtered animals significantly reduce [2, 5, 6, 8, 9, 10]. In addition, the disease affects trade links and requires financial costs for anti-epizootic measures. Taking into account its ability to spread rapidly across the borders of countries and cause significant damage, the World Organization for Animal Health has included LSD in the list of the most economically significant and notifiable transboundary viral animal diseases.

Lumpy skin disease was first reported in 1929 in Northern Rhodesia (now Zambia) and since that time the disease outbreaks have occurred in various parts of Africa. But the disease was contained to the African continent. In 1988–1989 the disease cases were registered in Egypt and Israel, and then LSD began to progressively spread to the countries of the Middle East, Eastern Europe, Russia and the Balkan Peninsula [4, 11, 12].

In 2019 LSD has already been reported in South and East Asia. This has put livestock industry at risk in such countries as Afghanistan, Pakistan, India, etc. LSD is also detected in cattle in China, Cambodia, Singapore, and Indonesia, and there is a threat of LSD spreading to the LSD-free countries with significant livestock populations (Australia, etc.) [4, 6]. There are also the first reports LSD in humans [10], although, according to other data, the virus is not transmitted to humans [2].

The risk of further LSD spread and penetration into the disease-free countries is increasing every year. Therefore, timely monitoring of the infection spread for the development a strategy for this disease control becomes of current importance.

Description of the disease manifestations and course, evaluation of historical and statistical data on LSD spread in the Middle Eastern countries as well as the further joint actions at the international level are presented in this review.

The work was aimed at analysis of historical and statistical data on clinical lumpy skin disease in the Middle Eastern countries.

MATERIALS AND METHODS

English literature data on LSD in the Middle East for the last 10 years were analysed. The following electronic databases were used for relevant data searching and collection: PubMed, Web of Science, eLIBRARY.RU, mdpi.com, frontiersin.org, researchgate.net, etc.

STUDY RESULTS

Lumpy skin disease is transmitted by biting insects. The virus is transmitted by blood-sucking mosquitoes

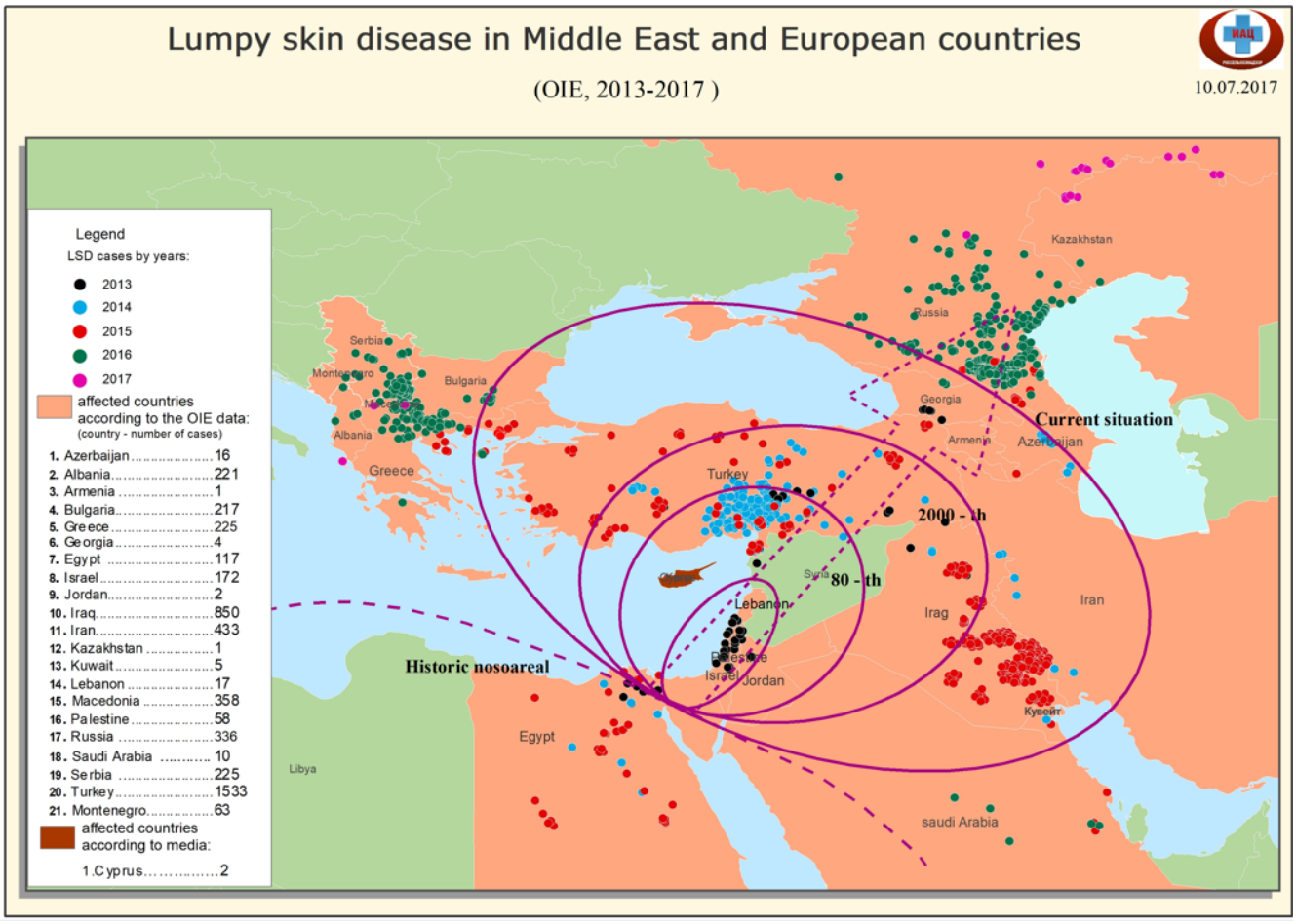

Fig. 1. LSD outbreaks reported from 2013 to 2017 in the Middle East region and neighboring countries [18]

(*Aedes aegypti*), biting flies (*Stomoxys calcitrans*, *Haematobia irritans*, *Musca domestica*) and some tick species (*Rhipicephalus appendiculatus*, *Rhipicephalus decoratus*, *Amblyomma hebraeum*) [13]. LSD can also be transmitted by non-vector route, through contact of animals with fomites (feeders, waterers), through milk, semen, or in utero [6, 10]. Hot and humid climate, rainy seasons, and low-lying, swampy terrain are factors contributing to multiplication of LSD vectors [1, 4, 9].

Clinical LSD takes the following forms: acute to subclinical or chronic form. The main symptoms include fever, lack of appetite, oedema, generalized nodular skin lesions, enlarged lymph nodes, emaciation, decreased lactation and abortions in pregnant animals. Severe LSD form is characterized with ulcerative mucosal lesions in oral cavity, larynx, trachea and esophagus. LSD virus localized in the reproductive system can cause temporary or permanent infertility in cows and bulls [7, 8, 9].

The virus is excreted by various routes: via lachrymal and nasal secretions, blood, saliva, milk, and semen of the infected animals. Nodules appearing on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly and then ulcerating can be attributed to the priority routes of virus shedding [9].

Strict quarantine of animals entering the farm, control of disease vectors, and preventive livestock vaccination are the most important measures for LSD eradication and prevention. Measures for LSD control at the international level, regional and international cooperation, and an effective biosafety policy are also of great importance [14].

The first LSD case in Northern Rhodesia in 1929 was considered to be hypersensitive response of animals to insect bites and was designated by the term "pseudocraniavaria". The infectious nature of the disease was discovered in the period from 1943 to 1945, when LSD was already recorded in Southern Rhodesia (now Zimbabwe), Botswana and South Africa. By 1946, the disease had spread to Mozambique, in 1950 it was detected in Angola and Zaire, in 1954 – on Madagascar, in 1956 – in Namibia, Tanzania and Uganda. Over the next three decades, LSD were reported in different African countries (Kenya, Sudan, Chad, Niger, Ethiopia, etc.), and therefore the virus has long been considered endemic to African countries [4, 10].

After the disease was reported in Egypt in 1988–1989, LSD spread outside the African continent and was detected in Israel. Earlier, in 1984, LSD outbreak was reported in Oman, but disease cases were not confirmed, although the disease cases were repeatedly reported in 2009. In 1986, LSD was detected in Kuwait, and the second LSD outbreak occurred in 1991. The disease was reported in Lebanon in 1993, in Yemen in 1995, in the UAE in 2000, in Bahrain in 1993 and again in 2002, and in Saudi Arabia and Iraq in 2013 [4, 15].

In 2014 LSD outbreak was reported in Azerbaijan. In 2015 LSD was registered in such European countries as Greece, Albania and Russia (another extensive outbreak occurred in Russia in 2017). Large-scale vaccination against

LSD was initiated in European countries for prevention of the wide spread of infection, but disease cases nevertheless occurred [16]. In 2016 and 2018 LSD was reported in Georgia, and in 2018 it was detected in such countries of the Balkan Peninsula as Greece, Bulgaria, North Macedonia, Serbia, Kosovo and Albania [4, 17].

Figure 1 shows map of LSD outbreaks reported in 2013–2017 in the Middle East and neighbouring countries [18].

Since 2019 LSD has become a serious problem for livestock industry in Asian countries. The disease caused devastating outbreaks in Bangladesh (2019–2020), then in India and China (2020), spread further to Nepal, Bhutan, Sri Lanka, Vietnam and Malaysia (2020). In 2021 LSD was reported in Thailand, Laos NDR, Pakistan and detected in Indonesia and Singapore in 2022 [4].

Morbidity and mortality reported during the Eurasian epizootics are approximately 10% and 1%, respectively, which, according to some authors, depends on the genetic predisposition of cattle to LSD.

As for Middle East region, LSD stayed for a short time within the region after spread outside the African continent and two years later spread further to the west and east. In the Middle East region, recurrent disease outbreaks were reported over the next few years. Some of them reported in the scientific literature are described below.

In 2012–2013, LSD virus was detected in Syria and Iraq. The unstable political situation, civil conflicts and wars had a negative impact on the veterinary services activities, contributing to the further pathogen spread to neighbouring countries of Southwest Asia [1, 6]. No specific treatment of infected animals was performed, and conservative intervention was limited to symptomatic therapy [6].

In 2013, LSD was detected in Turkey near the borders to Syria and Iraq [19, 20, 21, 22, 23]. The infected animals were not vaccinated against the infection. Mass immunization of cattle had been started in Turkey since 2014, but outbreaks had been still recorded until 2019 [6, 24].

In 2013 LSD was found in cattle in Jordan. Infected herds were treated with broad-spectrum antibiotics and anti-inflammatory drugs [6].

In 2014 LSD was still recorded in Iraq (9 disease outbreaks were reported in 2014) [9, 25].

In 2014–2016, the disease outbreaks were reported in Iran, where the disease presumably spread from Iraq due to the uncontrolled movements of infected animals across the common border [26, 27, 28]. According to P. Sameea Yousefi et al. [7] 683 cattle were examined for LSD clinical signs (fever, lack of appetite, decreased milk yield, detection of typical skin nodules and enlarged lymph nodes) during two-year observation period and the above-mentioned signs were detected in 122 cattle (prevalence – 17.9%). Therewith, the highest disease incidence rate was observed in animals older than 5 years, and the lowest in animals younger than 6 months. In vaccinated cattle, clinical signs were observed in 40.8% of animals, in non-vaccinated cattle – in 71.3% of animals. At the disease prevalence of 17.9%, the total mortality and lethality in the four examined Iranian provinces were 3.5 and 19.7%, respectively, that was non-consistent with earlier data from Turkey (12.3% morbidity and 6.4% mortality) [29], Oman (13.6–29.7% morbidity and 15.4–26.3% mortality) [30], Jordan (26.0% morbidity and 1.9% mortality) [31],

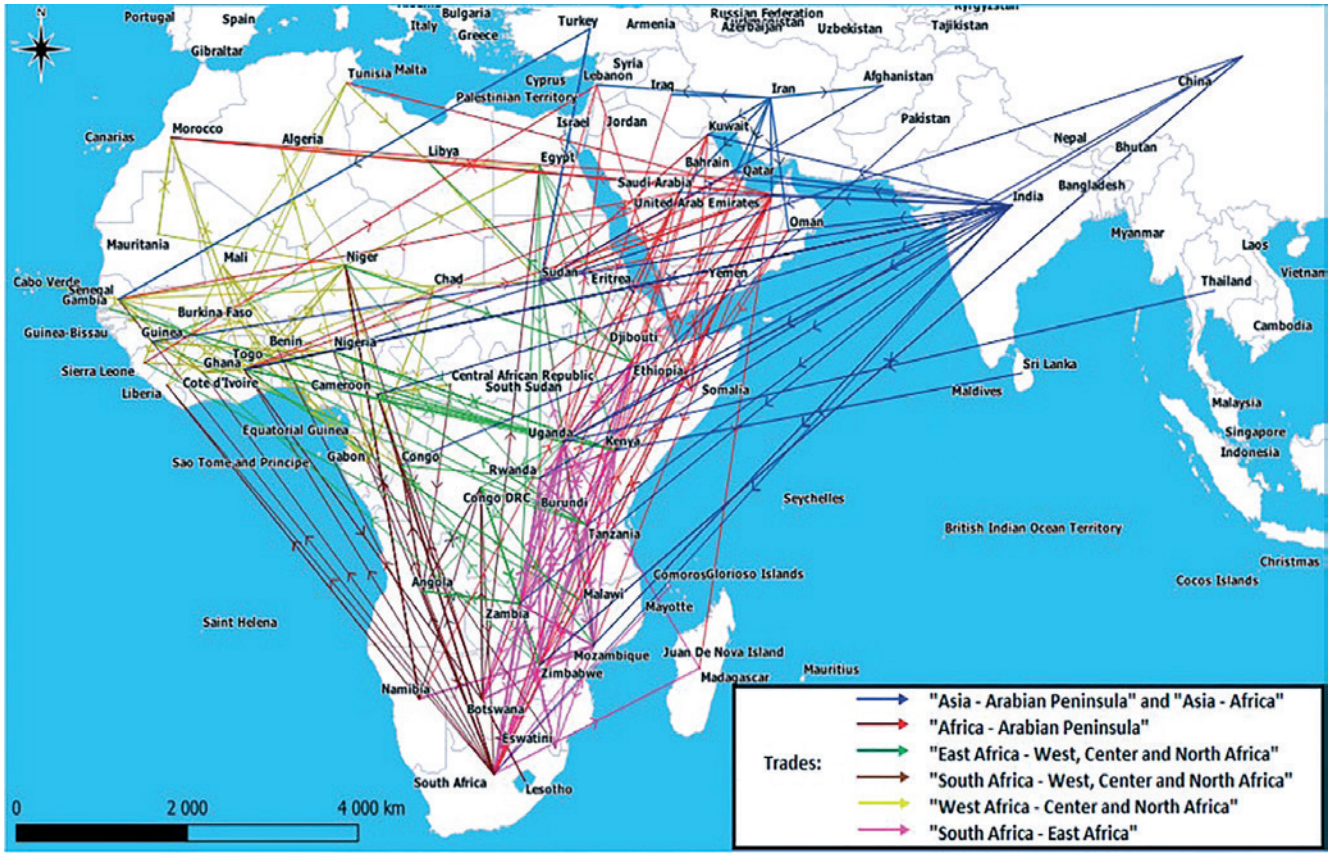

Fig. 2. Mapping of irregular flows of domestic ruminants in Africa and their links to Asia [34]

Table
LSD outbreaks sequentially reported in different world regions

Year	Country	Virus origin	Possible ways of further spreading	Time of LSD virus staying within one region before further spreading
1929	Zambia	unclear	neighbouring African countries	59 years within the African continent
1943	Botswana	Zambia	Zimbabwe and South Africa	
1944–1945	Zimbabwe, South Africa	Zambia, Botswana	Sudan and Ethiopia	
1946–1956	Mozambique, Angola, Madagascar, Tanzania, Uganda	Zambia, Botswana	Border countries where cattle was imported	
1957	Kenya	unclear	African countries – cattle importers	
1971	Sudan	insufficient data	insufficient data	
1973–1974	Chad, Niger, Nigeria	Cameroon via Gongola State in Nigeria	insufficient data	
1981–1983	Ethiopia	Sudan	insufficient data	
1983	Somalia	insufficient data	insufficient data	
1988–1989	Egypt	African countries	European countries and Israel	2 years within the Middle East countries
1989	Israel	Egypt	insufficient data	
1990–2010	Mediterranean countries	Egypt, Israel	All neighbouring countries	20 years – spread to the Mediterranean countries
2012–2014	Mediterranean countries	Syria, Iraq	Greece, Bulgaria	6–7 years – Eurasian LSD epizootic (spread of the virus in the South Eastern European, Middle Eastern and Eurasian countries)
2013	Turkey, Iran	Syria, Iraq	insufficient data	
2015–2017	Russia	Turkey, Azerbaijan, Iran, Kazakhstan	Northern regions of Europe	
	Countries located in the Balkan Peninsula	Turkey	Central and South Asian countries	
2019	Bangladesh	Perhaps neighbouring countries	India, Myanmar	5 years – spread in Southeast Asian Countries
	India		Nepal, Bhutan	
2019	China	Kazakhstan, Russia	Taiwan	
2020	Nepal	India, China	unknown	
	Vietnam	China	unknown	
2021	Thailand	unknown	insufficient data	
	Pakistan	India	not clear	
	Mongolia	Russia, China	China, India	
	Cambodia	not clear	not clear	
2022	Afghanistan	unknown	insufficient data	
	Korea	China, Nepal	insufficient data	
	Indonesia	India	Indonesia, threat to the northern regions of Australia	
2023	Libya	unknown	Tunisia and other Northwest African countries (Maghreb)	

Saudi Arabia (6.0% morbidity and 0.99% mortality) [32]. This inconsistency of the data on the disease prevalence and mortality is explained by the authors of the above study by variability of factors contributing to LSD (climate humidity, ambient temperature, etc.).

In 2018 and 2019, outbreaks continued in Iraq, namely in Basra province. The overall disease prevalence in all age groups of cattle was 18.7% (112 infected animals were detected out of 600 examined animals). LSD was detected in 92.8% of diseased animals, arthritis in 17.8% of diseased animals, and lymphadenopathy was detected in 2.5% of diseased animals [8].

The organization and quality of animal movement control and specific LSD prevention in the Middle Eastern countries depend on the well-coordinated activities of veterinary services. Stamping out of infected animals carried out in Egypt and Israel enabled LSD eradication in these countries in 2006. However, other Middle Eastern countries still face the problem of uncontrolled livestock movements, lack of high-quality laboratory diagnostics, and irregular contacts with international organizations in the field of animal health and surveillance, exacerbated by the unstable political situation in the region [1].

DISCUSSION AND CONCLUSION

Lumpy skin disease is a serious problem for livestock industry in many countries due to its rapid spread and resulting economic losses. The disease originated from African countries has gradually affected Middle Eastern and Asian countries and now threatens Western Europe and Australia. Joint efforts aimed at livestock movement control, reducing the risk factor, destruction of contaminated objects (dead animals and infected materials) are required for prevention of the further LSD spread and for LSD eradication in the countries already affected by this disease.

Currently, infection spread control is of particular importance due to the significant number of outbreaks reported in the Middle East, and risk of LSD transmission out of this region both to the west, towards the Balkan Peninsula and Europe [33], and to the east and southeast, towards Asian countries and further to Australia and New Zealand, having large cattle populations and remaining free from the disease. The further LSD spread could lead to significant economic losses in the global livestock sector.

Therefore, the Middle East can be considered as a port of entry for the infection spreading outside Africa to Europe and Asia. Thus, Figure 1 clearly shows the spread of the disease from Egypt and Israel to other countries in the Middle East, then westward to the Balkan Peninsula, northward to the southern regions of Russia, and eastward to Asian countries.

The rapid LSD virus spread in different countries can be accounted for cattle movements across international borders and for migration of blood-sucking insects regarded as disease vectors.

Figure 2 shows the directions of domestic ruminant movements in Africa and Middle East and links of livestock migration flows in these regions with Asian countries [34]. The map shows that the Middle East has close links with African and other Asian countries as for livestock purchase and sale. This significantly increases the risk of transmission of infectious large and small ruminant diseases through the territories of Middle Eastern countries to new territories.

Time intervals of LSD appearance on each of the continents were examined.

Aggregated table on the reported disease outbreaks shows that since its first occurrence in Zambia, from 1929 to 1988, i.e. for 59 years, the disease had remained within the borders of the African continent, and therefore LSD was considered endemic to African countries. There is no information about the country from which LSD virus was introduced into the Middle East, in Egypt, in 1988, although there are data on its further spread: the virus was introduced from Egypt to Israel, and in the next year both of these countries became the port of the pathogen entry to the Mediterranean countries, as well as other Middle Eastern countries. That was the way of LSD virus spread from the African continent to the Middle Eastern countries and further to the western (Mediterranean countries) and eastern (South, East and Southeast Asian countries) regions [35].

It should be noted that LSD virus "stayed" in the Middle East for the short period before its further spread to the Balkan Peninsula and further to the east to other Asian countries. This can be accounted for historically highly intensive trade relations in this region, climate suitable for virus vectors and insufficient control by veterinary surveillance authorities.

Specific preventive vaccination is currently the only effective method for LSD outbreak prevention, therefore, mass vaccination appears to be an important component of the general disease control strategy. In addition to cattle vaccination, strict quarantine measures for incoming animals, as well as slaughter of the diseased animals are required for successful eradication of LSD. Veterinary supervisory authorities should ensure strict control over LSD susceptible animal movements between neighbouring countries. Thus, the disease control should be carried out in animal farming industry at all levels: from farm to international supervisory agencies.

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Prevention of respiratory diseases of pigs of viral-bacterial etiology in conditions of import substitution

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ABSTRACT

Introduction. Pig farming, as a fast-growing branch of animal husbandry, is capable of prompt achieving a significant increase in the production of pork with high nutritional properties and biological value. One of the acute problems of pig farming is respiratory diseases of viral and bacterial etiology. In the current economic conditions, reducing the dependence of the Russian pig farming on technological imports is of particular significance. Production of domestically manufactured feeds and veterinary drugs should be considered as the most important condition for achieving the technological sovereignty of the Russian Federation.

Objective. To analyze the provision of pig farming with domestic vaccines against such significant porcine respiratory diseases as swine influenza, porcine enzootic (mycoplasmal pneumonia), porcine reproductive and respiratory syndrome and circovirus infection as well as to identify factors that hinder the development of immunobiological drugs against these diseases.

Materials and methods. The information base of the research included data from pig-breeding organizations of the Russian Federation, the Rosselkhoznadzor's state register of veterinary medicinal products, reference and special literature, publications of research institutions.

Results. Agents of swine influenza, porcine enzootic (mycoplasmal) pneumonia, porcine reproductive and respiratory syndrome, porcine circovirus infection are the most prevalent pathogens that cause respiratory diseases in pigs on the pig farms. Over the past few years, Russian biofactories have been developing import substitution programs for the necessary immunobiological drugs. By the end of 2023, the domestic establishments manufactured 19.3 billion doses of veterinary vaccines, which is 3 billion doses more than in 2022.

Conclusion. Vaccination is the most efficient and cost-effective way to prevent viral infections. However, domestic immunological drugs against swine influenza have not yet been developed in our country, and vaccines against porcine enzootic (mycoplasmal) pneumonia, porcine reproductive and respiratory syndrome, porcine circovirus infection require modification due to high variability of the agents.

Keywords: review, vaccines, import substitution, pig farming, porcine respiratory diseases, national security

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Профилактика респираторных болезней свиней вирусно-бактериальной этиологии в условиях импортозамещения

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РЕЗЮМЕ

Введение. Свиноводство, как скороспелая отрасль животноводства, способно в короткие сроки добиться существенного увеличения производства свинины с высокими пищевыми свойствами и биологической полноценностью. Одной из острых проблем отрасли являются респираторные болезни вирусно-бактериальной этиологии. В сложившихся экономических условиях особое значение приобретает снижение технологической импортозависимости

российского свиноводства, поэтому выпуск кормовых и ветеринарных препаратов отечественного производства необходимо рассматривать как важнейшее условие достижения технологического суверенитета Российской Федерации.

Цель исследования. Анализ обеспеченности свиноводства отечественными вакцинами против таких значимых респираторных болезней свиней, как грипп, энзоотическая (микоплазменная) пневмония, репродуктивно-респираторный синдром, цирковирусная инфекция, а также выявление факторов, которые препятствуют разработке иммунобиологических лекарственных препаратов против указанных заболеваний.

Материалы и методы. Информационной базой исследований являлись данные свиноводческих организаций Российской Федерации, государственный реестр лекарственных средств для ветеринарного применения Россельхознадзора, нормативно-справочная и специальная литература, публикации научно-исследовательских учреждений.

Результаты. Возбудители гриппа, энзоотической (микоплазменной) пневмонии, репродуктивно-респираторного синдрома, цирковирусной инфекции являются наиболее распространенными патогенами, которые вызывают респираторные болезни свиней на свиноводческих комплексах. На протяжении последних лет российские биофабрики разрабатывают программы импортозамещения необходимых иммунобиологических лекарственных препаратов. По итогам 2023 г. отечественные предприятия выпустили 19,3 млрд доз вакцин для ветеринарного применения, что на 3 млрд доз больше по сравнению с 2022 г.

Заключение. Вакцинация является наиболее эффективным и экономичным способом профилактики вирусных инфекций. Однако отечественные иммунологические лекарственные препараты против гриппа свиней еще не разработаны в нашей стране, а вакцины против энзоотической (микоплазменной) пневмонии, репродуктивно-респираторного синдрома, цирковирусной инфекции свиней требуют доработки в связи с высокой изменчивостью возбудителей.

Ключевые слова: обзор, вакцины, импортозамещение, свиноводство, респираторные болезни свиней, национальная безопасность

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INTRODUCTION

According to the Food Security Doctrine approved by the President of the Russian Federation in 2010, the food security of the Russian Federation is a state of the economy that ensures the country's food independence and guarantees physical and economic availability of food products compliant with the legislative requirements of the Russian Federation.

Pork, along with poultry meat, is the most affordable type of meat for the population and the key raw material for the meat processing industry, whereas pig farming, as a fast-growing branch of animal husbandry, is capable of prompt achieving a significant increase in production of pork, characterized by high nutritional properties and biological value [1]. According to the National Union of Pig Breeders, the total volume of industrial pork production in 2023 amounted to 5,627.2 thousand tons of live weight, which is 352.1 thousand tons more than in 2022, and in the past decade this value has been annually increasing by at least 3–4%.

In the current economic environment, reduction of the Russian pig industry dependence on the imports is of particular importance. The predominance of foreign-made technological equipment, feed additives and veterinary drugs in the domestic market may make the industry dependent on the international situation. Therefore, an increase in the level of technical equipment of facilities, modernization of manufacturing equipment, domestic manufacture of feed and veterinary drugs should be considered as the most essential for improving the industry's effectiveness and sustainable development [2].

The subprogram "Development of technologies for the production of veterinary medicinal products" will be one of the priorities of the "Federal Research and Technology Program for Agriculture Development in 2017–2030", approved by Decree of the Government of the Russian Federation of 25 August 2017 No. 996. The comprehensive research plan of the subprogram includes, *inter alia*, development of scientific foundations for the production of new domestic vaccines for the prevention of porcine infectious diseases, thus contributing to animal health protection, as well as animal livability and improvement of their performance for the purpose of import substitution.

One of the acute problems of pig farming that slow down the pork production growth rate are respiratory diseases of viral and bacterial etiology, such as swine influenza, porcine enzootic (mycoplasmal) pneumonia, porcine reproductive and respiratory syndrome (PRRS), and porcine circovirus infection, which cause significant economic damage to pig farms in the Russian Federation [3].

The purpose of the study is to analyze the availability of domestic vaccines against viral and bacterial respiratory diseases of pigs in the Russian Federation, as well as to identify factors that hinder the development of immunobiological drugs against these diseases.

MATERIALS AND METHODS

The information base of the research included data from pig-breeding organizations of the Russian Federation, the Rosselkhoznadzor's state register of veterinary medicinal products, reference and special literature, publications of research institutions. The range of vaccines was

analyzed basing on the data from domestic biofactories and manufacturers of diagnostic, preventive and therapeutic products against infectious animal diseases.

RESULTS AND DISCUSSION

Over the past few years, Russian biofactories and specialized research institutions have been developing programs targeted at the substitution of the imports of the relevant and promising immunobiological medicinal products. Guided by the goal of achieving technological sovereignty of the Russian Federation, the key players are filling scarcity niches and investing in the development of new medicinal products.

According to the National Veterinary Association (NVA), which includes such leading manufacturers of veterinary pharmaceutical and immunological products as VIC Group of Companies, AVZ, NITA-FARM, Apicenna, Vetbiochem LLC, Avivac, etc., in 2023, the total veterinary medicinal product market in Russia amounted to 97.7 billion rubles, VAT included. A significant contribution to the growth was made by domestic manufacturers – NVA members, who collectively increased production by more than 25% in 2023. By the end of the year, domestic establishments produced 19.3 billion doses of vaccines for veterinary use, which is 3 billion doses more than in 2022. Positive dynamics is also observed in the extension of the product range by the domestic establishments: in 2023, about 100 new medicinal products were developed and authorized, which is 70% more than in 2022.

Porcine respiratory disease complex (PRDC) is a serious challenge for pig farming as it causes devastating economic losses due to lower growth rates of young animals, increased mortality and high cost of treatment. PRDC is a multifactorial disease, the development of which depends on the combination of infectious pathogens, exposure to environmental stressors and defects in the animal breeding system [4]. The diseases included in this complex, together with swine influenza, are present in all major countries – global pork manufacturers, and *Mycoplasma hyopneumoniae*, PRRS virus and porcine circovirus type 2 (PCV-2) triad are the most common pathogens causing PRDC in Asian countries. These etiological agents suppress the host's immune system and amplify the replication of each other and other pathogens. This results in the weakening of the animal population, high mortality of young animals, degradation of the boar semen performance, as well as additional costs associated with diagnostic, quarantine and therapeutic measures.

Swine influenza is an acute respiratory disease of pigs caused by type A influenza virus belonging to the family *Orthomyxoviridae*. The disease is accompanied by high morbidity (up to 100%) and low mortality (10–15%). Fever, apathy, anorexia, serous nasal discharge and upper respiratory tract lesions are typical for it [5, 6].

The swine influenza transmission patterns vary between and within the countries due to such factors as climate, pig population and farming methods. The main vehicles of the virus transmission are airborne and contact ones, as well as with personnel and care products. Humans and pigs have the same set of receptors in the respiratory cells, so interspecific transmission of influenza A viruses occurs in both directions. Introduction of effective measures for influenza control and prevention will, therefore, help to maintain the health of not only pigs, but also humans [7].

Upon contact with the respiratory mucous membrane, which is, as a rule, the portal of the infection entry, the influenza virus starts its replication thus leading to the necrosis of the affected tracheal and bronchial cells, impaired blood circulation, damage to the vascular system and further, in complex cases, to hemorrhages on the skin and mucous membranes as well as hemorrhages in internal organs.

Currently, at least three different subtypes of influenza A virus (H1N1, H1N2 and H3N2) are jointly circulating all over the world, *inter alia* in the Russian Federation. Herewith, pigs can act as a "mixing vessel" in which influenza viruses of various origins can reassort (including with avian and human influenza pathogens), creating new progeny viruses capable of replicating and spreading in humans [7].

Vaccination is the main tool for swine influenza control. However, despite the large number of vaccines, the disease still cannot be effectively controlled, as the pathogen strains are very diverse and prone to mutations. Therefore, development of the vaccines capable of providing broad heterologous protection against antigenically diverse virus strains is crucial for the effective disease control [8, 9, 10].

The majority of globally used modern vaccines against swine influenza contain inactivated whole viruses with an adjuvant for intramuscular injection and are used either in sows for protection during pregnancy and in piglets during suckling, or in repair young animals to make their clinical signs milder. These products are targeted at the induction of the serum antibodies that neutralize the influenza virus on the respiratory mucous membranes. In the foreign countries, the inactivated vaccines are manufactured locally and contain various antigenic and genetic virus strains circulating in the relevant region, which is indicative of high evolutionary capacities of the virus [7]. For example, about half of the vaccines used in the United States are customized and herd-specific. One of the solutions to this problem could be an approach to constructing vaccines involving cocktail of numerous immunologically promising amino acid sequences of various strains of swine influenza virus [11].

Only foreign immunological medicinal products for swine influenza prevention are currently listed in the Rosselkhoznadzor's register of the veterinary medicinal products. All vaccines are inactivated and contain type A swine influenza virus of subtypes H1N1 and H3N2 (Italy, Spain) and subtypes H1N1, H1N2 and H3N2 (Germany), Table 1. Thus, despite a fairly wide range of products available on the Russian market, it is necessary to develop a domestic inactivated vaccine against swine influenza comprising those virus strains that circulate in the Russian Federation.

Porcine enzootic (mycoplasmal) pneumonia is a chronic infectious disease caused by the bacterium *Mycoplasma hyopneumoniae*, which is accompanied by cough, catarrhal bronchopneumonia and a decrease in such performance parameters such as survival rate, average daily weight gain, feed conversion rate [12]. The infection is mainly spread via airborne route, indirect transmission and infection through contact with wild boars are also possible [13].

The disease pathogenesis is very complex and has not been fully examined. The agent attaches to the ciliated epithelium of the trachea, bronchi, and bronchioles, causing damage to the mucosal mucociliary clearance system (ciliostasis), which interferes with the normal functioning of the cilia, leads to a delayed and ineffective immune

response, and contributes to a higher susceptibility of animals to other respiratory infections [14].

Mycoplasma hyopneumoniae is able to enhance replication of PRRS virus and PCV-2, increasing severity of pneumonia in pigs. Practicing pig breeders most often use vaccination rather than antibiotic treatment and prefer combined vaccines to effectively control the entire range of porcine respiratory diseases. Immunization against *M. hyopneumoniae* is usually carried out at the age of 21 days. If the pathogen is detected in swabs from the larynx of suckling piglets, vaccination can be carried out at 7 days of age to avoid *M. hyopneumoniae* transmission in weaning piglets and to control enzootic pneumonia during fattening on the farms [15, 16]. As for breeding sows, in some herds pigs are immunized against *M. hyopneumoniae* during the quarantine, before they are delivered to the breeding sow premises. Such practice allows to avoid immunity upset in breeding population by reducing the bacterial load and severity of clinical signs in vaccinated pigs in *M. hyopneumoniae*-positive herds [17].

The majority of the commercially available bacterin vaccines are adjuvanted whole-cell preparations of the inactivated cultured *M. hyopneumoniae* [18]. For the prevention of porcine enzootic pneumonia, a domestic VERRES-M.hyo (Vetbiochem LLC, Moscow) inactivated vaccine has been enlisted in the Rosselkhoznadzor register of veterinary medicinal products, as well as a number of foreign-made vaccines that can be used in the disease-affected breeding and commercial pig holdings (Table 2).

The main advantages of the vaccination involve an increase in the daily weight gain of piglets (2–8%) and feed conversion rate (2–5%), as well as a reduction in animal mortality. Moreover, the period of reaching the slaughter weight is shortened, the clinical signs of lung lesions and their treatment costs are reduced [19]. However, the disadvantage of these vaccines is that protection against the onset of *M. hyopneumoniae*-caused clinical signs and lesions is often incomplete, and vaccination leads to only a slight decrease in the transmission rate. There is, therefore, a need to develop new vaccines capable of providing more efficient protection. New vaccines are currently being actively tested, including aerosol and feed-based vaccines, as well as subunit and DNA vaccines. Feed-based vaccines or aerosol vaccines could significantly facilitate the operational procedure of the mass immunization of pigs, and would also create immunity at the infection entrance gate, i.e. in the respiratory tract. However, as a result of the experiments, it was found that even triple aerosol immunization was less effective than intramuscular administration, so this method still needs to be improved [12, 14, 19].

Porcine reproductive and respiratory syndrome (PRRS) is a quarantinable contagious viral disease that is manifested in reproductive dysfunction in sows and boars and severe pneumonia in newborn piglets and weaned piglets during fattening.

The PRRS etiological agent is an RNA-genome arterivirus (genus *Betaarterivirus*, family *Arteriviridae*) capable of replication in the pig macrophages, thus resulting in the increased animal susceptibility to primary and secondary infections, decreased animal growth as well as morbidity and mortality development and increase [20].

The virus transmission can be both horizontal and vertical. The infection mostly occurs through the contact with the diseased animals, as well as through vehicles, clothing and footwear of the personnel, through blood-sucking insects and birds. In addition, the infection is possible through boar semen, where the pathogen remains active for up to 2 weeks. The virus can cross the placental barrier in the second half of pregnancy and infect the fetus, and the surviving piglets become the virus carriers. There is also some evidence of the infectious agent airborne (aerosol) transmission [21].

Table 1
Register of main vaccines against swine influenza registered in the Russian Federation

Vaccine	Vaccine type	Influenza virus strain used	Manufacturer
Bayovac® Influ	inactivated	X53a (H1N1), MRC 11 (H3N2)	Fatro S.p.A., Italy
GRIPORK	inactivated	A(H1N1)OLL, A(H3N2)GHA	Laboratorios Hipra, S.A., Spain
Respiporc FLU 3	inactivated	Haselünne/IDT2617/2003 (H1N1), Bakum/1832/2000 (H1N2), Bakum/IDTI769/2003 (H3N2)	IDT Biologika GmbH, Germany

Table 2
Register of main vaccines against porcine enzootic (mycoplasmal) pneumonia registered in the Russian Federation

Vaccine	Vaccine type	Influenza virus strain	Manufacturer
VERRES-M.hyo	inactivated	*Mycoplasma hyopneumoniae*	Vetbiochem LLC, Russia
Ingelvac MycoFLEX®	inactivated	*Mycoplasma hyopneumoniae* (strain J)	Boehringer Ingelheim Vetmedica GmbH, Germany
Porcilis® M Hyo ID Once	inactivated	*Mycoplasma hyopneumoniae* (strain 11)	Intervet International B.V., Netherlands
Suvaccin MN-One	inactivated	*Mycoplasma hyopneumoniae* (strain P-5722-3)	Zoetis Inc., USA
Hyogen	inactivated	*Mycoplasma hyopneumoniae* (strain 2940)	Ceva Sante Animale, Hungary

The disease economic damage involves losses due to reproductive dysfunction of sows (abortions, stillbirth, death of 80–100% of newborn piglets) and the cost of diagnostic and quarantine measures, especially during acute and massive outbreaks, when 1–3% of adult breeding stock may die [22, 23, 24, 25].

Vaccination is the main tool for PRRS prevention, however, genetic studies demonstrated that the virus genome has one of the highest mutation rates among the RNA viruses, which contributes to its extensive antigenic and genetic variability [26]. There are at least three subtypes of the PRRS type 1 virus, differentiated based on ORF-5 gene analysis [27]. The reported genetic diversity of the field virus isolates is the main obstacle to the disease control [28].

A number of both domestic and foreign manufactured vaccines have been currently authorized in the Russian Federation, which can be subdivided into two large groups: live attenuated vaccines and inactivated vaccines. The range of the key Russian vaccines against PRRS is presented in Table 3.

Table 3
Register of main vaccines against porcine reproductive and respiratory syndrome registered in the Russian Federation

Vaccine	Vaccine type	PRRS virus strain used	Manufacturer
VERRES-PRRS	inactivated	domestic author's strain OB	Vetbiochem LLC, Russia
ARRIAH-PRRS inact	inactivated	production strain KPR-96, European genotype	Federal Centre for Animal Health, Russia
ARRIAH-RePovac	inactivated combined	production strain KPR-96, European genotype	Federal Centre for Animal Health, Russia
ARRIAH-Aujeszky+PRRS	inactivated combined	production strain KPR-96, European genotype	Federal Centre for Animal Health, Russia
Resvac	live dry	strain PRRS-1SBC, genotype 1	Shchelkovo biocombinat, Russia

Taking into account both safety aspects and wide variety of PRRS virus strains, the inactivated vaccines are preferable to attenuated ones, but despite these advantages, they are not effective enough. The inactivated products induce a lower immune response than the attenuated live ones, since the vaccine virus strains do not replicate in the vaccinated animals. The inactivated vaccines are not recommended to be used for immunization of seronegative animals. Vaccination of seropositive animals (due to natural infection or immunization with live vaccines) with inactivated products, nevertheless, causes a pronounced secondary humoral and cellular immune response, which allows them to be used in combined vaccination programs [29].

The effectiveness of the attenuated live vaccines is due to the fact that they ensure the development of not only humoral, but also cellular immune response against the PRRS virus. However, live vaccines have significant disadvantages. The protective immune response caused by the attenuated PRRS vaccines depends on the genetic diversity of the field virus strains circulating in a given region. The greatest effect of immunization is assumed to be achieved when the vaccine virus is antigenically similar to the field one. Moreover, there are serious concerns about the safety of the attenuated vaccines, since viremia develops after immunization of pigs with live products and a vaccine virus is shed during a few weeks, which can be directly or indirectly transmitted to the unvaccinated susceptible animals [30].

Circovirus infection in pigs is a viral disease, mainly of weaning piglets [31]. The causative agent is PCV-2, which belongs to the *Circovirus* genus of the *Circoviridae* family, which includes small single-stranded non-enveloped DNA viruses with an unsegmented circular genome [32]. The mechanisms of PCV-2 recognition, attachment and penetration into the body are currently not fully understood. The virus is believed to use a relatively common cellular receptor, since the virus replication and PCV-2 antigen were reported in many different cell types [33]. After entering the host's body and completing the 2–4-week incubation period, PCV-2 replicates in the lymph nodes, infects B cells and spreads throughout the body through the lymphatic system. Viremia in pigs is detected between days 7 and 14 after the virus inoculation. PCV-2 is capable of causing long-term infection and the viral DNA is detected in pigs for up to 125 days after experimental infection [34].

Porcine circovirus type 2 can be transmitted by several routes. The main route is with infected excretions (including urine, saliva, semen) or through the direct contact with the infected pigs. The virus can also be transmitted placentally, although this transmission route is less frequent [35, 36]. Experiments in piglets also demonstrated that some slaughter products (lymphoid tissue, skeletal muscles, and bone marrow) can be a source of infection in pigs and, when fed to experimental animals for 3 days they can lead to viremia and seroconversion in all animals [37, 38].

The causative agent of circovirus infection in pigs is widespread in many countries of the world with developed industrial pig breeding and it causes significant economic damage due to high morbidity and mortality, decreased performance and reproductive capacities of the animals [38].

There are currently four types of porcine circoviruses globally identified: PCV-1, PCV-2, PCV-3 and PCV-4 [39]. PCV-2 plays an important role in the pathologies of piglets of 6–16 weeks of age. It causes damage to various systems, but the disease clinical signs develop only in young animals with compromised immune system. When replicating in the cells of the piglets' immune system, PCV-2 causes immunodeficiency disorders, which increase the susceptibility to other infectious agents, reduce the immune response to vaccination, and result in the animal death [40].

Specific prevention of circovirus infection is successfully carried out with inactivated and recombinant subunit vaccines, which significantly reduce the piglets' morbidity and mortality during finishing and fattening [38].

The viral subunit vaccine is formulated with the components of the main viral immunogen by means of genetic engineering. The commercial subunit vaccines against porcine circovirus infection have been developed and manufactured primarily based on the expression of the recombinant capsid protein ORF-2 in the baculovirus expression system.

To prepare an inactivated vaccine, the PCV-2-infected cells are inactivated by a physical or chemical method, as a result of which the virus loses its infectious capacities, but at the same time retains its immunogenicity [40]. To date, several domestic vaccines against circovirus infection have been registered and certified in Russia, which contain the recombinant capsid protein ORF-2 of porcine circovirus type 2 (Table 4).

The technology of manufacturing vaccines against porcine circovirus infection is constantly being updated due to high frequency of mutations in the HCV-2 genome and emergence of new virus subtypes. Currently, nine PCV-2 genotypes are known (from PCV-2a to PCV-2i). Circovirus genotypes 2a, 2b, and 2d are widespread worldwide, while other genotypes are detected sporadically [41]. The emergence of new virus genotypes leads to ineffective vaccination, which dramatically increases the spread of circovirus infection outbreaks. To date, PCV-2d is the most common and dominant genotype, it has a higher virulence, causes more serious clinical signs and pathological lesions as compared to classical genotypes 2a and 2b. The majority of

the commercially available vaccines against porcine circovirus infection are based on capsid proteins of PCV-2a and PCV-2b viruses and they are often ineffective against PCV-2d. In this regard, there is a need to develop new effective vaccines to protect against the most clinically significant PCV-2 genotypes [42].

CONCLUSION

Porcine respiratory diseases are a serious problem causing devastating economic losses in the pig industry due to decreased animal growth rate, as well as increased livestock mortality and cost of treatment. Among the multiple etiological agents such pathogens as *Mycoplasma hyopneumoniae*, porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 remain the most prevalent PRDC-causing ones in the Russian Federation. Swine influenza also causes great economic damage to production, being at the same time a potentially dangerous agent for humans.

Vaccination is one of the most efficient and cost-effective means to prevent the viral infections. However, domestic immunological medicinal products against swine influenza have not yet been developed, and vaccines against enzootic (mycoplasmal) pneumonia, porcine reproductive and respiratory syndrome, and porcine circovirus infection require further development due to high variability of the pathogens, which hinders the development of a universal vaccine product.

In the current economic conditions, it is of particular importance to reduce the use of foreign immunobiological drugs and to accelerate domestic vaccine production in order to achieve technological sovereignty of the Russian Federation. In this regard, manufacturers of veterinary medicinal products are actively developing new vaccines and improving existing ones, and they are working to expand the collection of pathogens that can later become the basis for the development of new medicinal products. However, the full cycle of the development of a single vaccine takes from three to five years, and the domestic manufacturers cannot fully cover the needs of the industry so far.

Government support provided to the manufacturers of the veterinary medicinal products as regards to the accelerated registration of the medicinal products contributes to the introduction of new capital-intensive projects, and creation of new production facilities will allow for the significant increase in the volume of the manufactured products in the coming years.

Table 4
Register of main vaccines against porcine circovirus infection registered in the Russian Federation

Vaccine	Vaccine type	PCV-2 strain used	Manufacturer
VERRES-CIRCO	recombinant	recombinant viral capsid protein ORF-2	Vetbiochem LLC, Russia
ReCircoVac	recombinant	recombinant viral capsid protein ORF-2 (PCV2b)	Armavir Biofactory, Russia
Circostop	inactivated	strain PCV2/SHBC	Shchelkovo biocombinat, Russia

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Development of test-system for detection of H5 and H7 avian influenza virus RNA by multiplex real-time RT-PCR assay using internal control

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ABSTRACT

Introduction. High pathogenicity avian influenza is a dangerous highly contagious viral infection of domestic and wild birds that recently has become widespread in Europe, Asia, Africa and Americas. The causative agent of the disease is type A influenza virus of subtypes H5 and H7. Real-time RT-PCR is one of the most rapid and effective techniques for avian influenza virus identification and typing, so development of the test system based on this technique with internal control to be used for control of the reaction main stages is of current importance. At the same time, the multiplex format of RT-PCR allows for simultaneous identification of several targets that reduces the consumption of reagents and the reaction time.

Objective. Development of test-system for detection of H5 and H7 avian influenza virus RNA with multiplex real-time RT-PCR in biological samples and its characterization.

Materials and methods. H5, H7, H3, H4, H10, H16 avian influenza virus, Newcastle disease virus, infectious bursal disease virus, infectious bronchitis virus, Marek's disease virus, avian adenovirus isolates were used. MS2 bacteriophage was used as internal control.

Results. Optimal primer-probe combinations were selected, test-system characteristics were determined: specificity for homologous and heterologous avian disease viruses, analytical sensitivity, reaction amplification efficiency, repeatability and reproducibility.

Conclusion. Determination of the developed test system validation parameters has shown that it is specific only for H5 and H7 avian influenza virus, its analytical sensitivity for each subtype was 1.5 lg EID_{50}/cm^3, and the amplification efficiency was 92 and 97%, respectively. The test system was validated through its use for testing biological samples submitted to the laboratory, the test results were consistent with the results of tests with standard diagnostic methods used in the Reference Laboratory for Avian Viral Diseases of the Federal Centre for Animal Health.

Keywords: high pathogenicity avian influenza, H5 avian influenza virus, H7 avian influenza virus, real-time RT-PCR, test-system

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Разработка тест-системы для выявления РНК вируса гриппа птиц подтипов H5 и H7 методом мультиплексной ОТ-ПЦР в режиме реального времени с использованием внутреннего контрольного образца

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РЕЗЮМЕ

Введение. Высокопатогенный грипп птиц является особо опасной высококонтагиозной вирусной инфекцией домашних и диких птиц, в последние годы получившей широкое распространение на территории стран Европы, Азии, Африки и Америки. Возбудитель заболевания – вирус гриппа типа А подтипов H5 и H7. Одним из наиболее быстрых и эффективных способов идентификации и типирования вируса гриппа птиц является ОТ-ПЦР в режиме реального

времени, в связи с чем представляется актуальной разработка тест-системы на основе данного метода с использованием внутреннего контрольного образца для возможности контроля основных этапов проведения реакции. При этом постановка реакции в мультиплексном формате позволяет одновременно идентифицировать несколько целевых мишеней, что уменьшает расход реагентов и время постановки реакции.

Цель исследования. Разработка тест-системы для выявления в пробах биологического материала РНК вируса гриппа птиц подтипов H5 и H7 методом мультиплексной ОТ-ПЦР в режиме реального времени и определение ее основных характеристик.

Материалы и методы. Использовали изоляты вируса гриппа птиц подтипов H5, H7, H3, H4, H10, H16, вирусы ньюкаслской болезни, инфекционной бурсальной болезни, инфекционного бронхита кур, болезни Марека и аденовирус птиц. В качестве внутреннего контрольного образца служил бактериофаг MS2.

Результаты. Подобраны оптимальные сочетания систем праймеров и зондов, определены характеристики тест-системы: специфичность в отношении гомологичных и гетерологичных вирусов болезней птиц, аналитическая чувствительность, эффективность реакции амплификации, повторяемость и воспроизводимость.

Заключение. При определении валидационных характеристик разработанной тест-системы установлена ее специфичность в отношении только вируса гриппа птиц подтипов H5 и H7, аналитическая чувствительность для каждого подтипа составила 1,5 lg $ЭИД_{50}/см^3$, эффективность амплификации – 92 и 97% соответственно. Проведена апробация тест-системы при исследовании поступающих в лабораторию проб биологического материала, результаты соответствовали таковым для стандартных диагностических методов, используемых в референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ».

Ключевые слова: высокопатогенный грипп птиц, вирус гриппа птиц подтипа H5, вирус гриппа птиц подтипа H7, ОТ-ПЦР-РВ, тест-система

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INTRODUCTION

Avian influenza is one of the most dangerous viral diseases of poultry and wild birds, that affects primarily respiratory and digestive systems. The disease is caused by virus of genus *Alphainfluenzavirus*, *Orthomyxoviridae* family. The virus genome is a single-stranded (–)RNA composed of the 8 segments allowing high-rate virus evolution owing to reassortment [1]. Avian influenza virus (AIV) is classified into 16 subtypes by hemagglutinin (HA) and 9 subtypes by neuraminidase based on antigenic differences in surface proteins [2].

The natural reservoir of the AIV is wild waterfowl. The infected wild waterfowl are asymptomatic or demonstrate mild clinical signs. The virus naturally spread along the migration routes of wild migratory birds and at the same time is transmitted to poultry [3, 4]. High pathogenicity AIV is the most dangerous for poultry industry, since it can cause severe, rapidly developing disease with 100% mortality. High pathogenicity AIVs are believed to evolve under natural conditions from low-pathogenic H5 and H7 viruses through point mutations in HA gene causing accumulation of multiple basic amino acid at the HA cleavage site [4, 5, 6, 7, 8]. High pathogenicity avian influenza is to be notified to the World Organization for Animal Health (WOAH), regardless of the causative virus subtype.

Outbreaks of the disease caused by H5 high pathogenicity AIV were regularly reported in poultry and wild birds in the Russian Federation in 2021 – early 2024 (H5N1, H5N5 and H5N8 virus isolates were recovered) [9]. In 2024, high pathogenicity avian influenza outbreak caused by H7N3 AIV was reported in poultry kept at a poultry establishment in Australia [10]. Previously, disease cases, including cases in humans, caused by H7 AIV were reported in the North and South American, European, African and Asian countries [11, 12].

Current high pathogenicity avian influenza situation in the Russian Federation requires ongoing monitoring. Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) is the most rapid and accurate technique for detection of AIV RNA in biological materials from various poultry and wild bird species that enables simultaneous virus typing. This technique has high sensitivity, specificity, and is relatively rapid allowing high throughput.

Highly effective test system for detection of H5 and H7 AIV RNA by real-time RT-PCR is proposed in this paper. The developed test system contains exogenous internal control, that allows control of the main reaction stages (extraction of nucleic acids, reverse transcription and PCR) and elimination of false negative results.

The study was aimed at the development of real-time RT-PCR-based test system enabling simultaneous detection H5 and H7 virus RNAs in biological materials and control of the reaction procedure at all stages, starting with the nucleic acid extraction.

MATERIALS AND METHODS

Viruses. Isolates of AIV of various subtypes, Newcastle disease, infectious bursal disease, infectious bronchitis, Marek's disease viruses and avian adenovirus obtained from the working collection of the Reference Laboratory for Avian Viral Diseases of the Federal Centre for Animal Health were used (Table 1). MS2 bacteriophage with an infectious activity titre of 10^6 PFU/cm^3 served as internal control [13].

RNA extraction was performed using a "RIBO-prep" reagent kit for RNA/DNA extraction from clinical samples

(AmpliSens®, Russia) according to the manufacturer instructions. At the extraction stage, internal control was added to each sample (including negative extraction control), 0.01 mL of internal control per sample.

Primers and probes. Several sets of primers and probes for amplification of H5 and H7 AIV HA gene fragments were selected and tested based on the analysis of publications on development of test systems and methods for detection of H5/H7 AIV in biological samples with real-time RT-PCR [14, 15, 16, 17]. Since real-time RT-PCR was performed in a multiplex format, the dyes included in the TaqMan probes were selected in such a way as to generate a stable fluorescent signal and not inhibit the signals in other detection channels (Green/H5, Orange/H7, Crimson/internal control). The selected primers and probes were synthesized by the Syntol company (Russia), and the specific primers and probe for internal control [13] were synthesized by the Alkor Bio Company Ltd. (Russia).

Real-time RT-PCR was performed in one step using amplification reagents manufactured by the Syntol company (Russia) in Rotor-Gene 6000 programmable amplifier (Corbett Research Pty Ltd, Australia). The reaction mix (20 µL per sample) contained: deionized (bidistilled) water – 5.35 µL; 10× PCR buffer – 2.5 µL; 25 mM $MgCl_2$ solution – 4 µL; 25 mM deoxynucleoside triphosphate (dNTP) solution – 0.4 µL; forward and reverse primer solutions for AIV/H5, 10 pmol/µL – 1 µL of each primer; fluorescent probe for AIV/H5, 10 pmol/µL – 0.75 µL; forward and reverse primer solutions for AIV/H7, 10 pmol/µL – 1 µL of each primer; fluorescent probe for AIV/H7, 10 pmol/µL – 0.75 µL; forward and reverse primer solutions, fluorescent probe solution for MS2, 10 pmol/µL – 0.5 µL of each primer; SynTaq DNA polymerase – 0.25 µL; MMLV-revertase – 0.5 µL. The reaction was carried out according to the following procedure: reverse transcription – 20 min at 40 °C; polymerase activation – 8 min at 95 °C; 40 PCR cycles – 10 s at 95 °C; 35 s at 55 °C; 15 s at 72 °C. The fluorescence signal was detected at the stage of primer annealing using the Green/H5, Orange/H7, and Crimson/internal control channels.

Table 1
Avian virus isolates used for the study

Virus	Strain/isolate
H5N2 avian influenza virus	A/duck/Italy/5952/2015
H5N2 avian influenza virus	A/avian/Italy/6558/2015
H5N2 avian influenza virus	A/duck/Italy/6926/2017
H5N1 avian influenza virus	A/duck/Altai/469/2014
H5N1 avian influenza virus	A/dalmatian pelican/Astrakhan/485-1/2022
H5N5 avian influenza virus	A/shelduck/Kalmykia/1814-1/2021
H5N8 avian influenza virus	A/duck/KChR/1590-20/2020
H7N2 avian influenza virus	A/chicken/Italy/1670/2015
H7N3 avian influenza virus	A/turkey/Italy/9289/02
H7N7 avian influenza virus	A/duck/Italy/4932/2018
H3N8 avian influenza virus	A/wild duck/Primorsky/1872-13/21
H4N6 avian influenza virus	A/wild duck/Primorsky/1872-11/21
H9N2 avian influenza virus	A/chicken/Udmurtya/2008-1/21
H9N2 avian influenza virus	A/gull/Tyva/767-113/21
H10N7 avian influenza virus	A/wild duck/Primorsky/1872-13/21
H16N3 avian influenza virus	A/mallard/Khabarovsk/12/14
Infectious bronchitis virus	H-120
Infectious bursal disease virus	Winterfield 2512
Newcastle disease virus	LaSota (genotype II)
Avian adenovirus	KR95 (type C)
Marek's disease virus	3004

The test system was examined for its *specificity* by performing real-time RT-PCR using RNAs extracted from homologous and heterologous viruses (Table 1).

The test system was assessed for its *analytical sensitivity* by performing real-time RT-PCR with extracted RNA of serial 10-fold dilutions (10^{-8}–10^{-3}) of a virus-containing suspension (AIV strains: A/duck/KChR/1590-20/2020 H5N8 and A/turkey/Italy/9289/02 H7N3, initial infectivity titre was 8.5 lg EID_{50}/cm^3) with internal control in triplicate for each dilution. The reaction sensitivity for each sample was estimated as the virus amount (measuring units – lg EID_{50}/cm^3) corresponding to the last dilution, at which at least 95% of positive results were obtained (in 20 repeats) [18].

For *repeatability* assessment, positive samples were tested 3 times in 5 repeats during three days. Mean threshold cycle (Ct), standard deviation, and coefficient of variation for the obtained results were determined within one real-time RT-PCR run and between runs.

To determine the *reaction efficiency (E)*, the results obtained during the reaction runs for analytical sensitivity testing were used. The reaction efficiency was estimated after plotting a linear regression graph (in the coordinates "virus dilution" / "threshold amplification cycle Ct") according to the following formula:

$$E = (10^{(-1/m)} - 1) \times 100\%,$$

where m is the slope coefficient of the straight line [19, 20, 21].

RESULTS AND DISCUSSION

Optimal combinations of primers and probes were determined based on the results of testing of primer and probe combinations for amplification of H5 and H7 AIV HA gene fragments. Nucleotide sequences are shown in Table 2.

Selected sets were tested in real-time RT-PCR with AIV strains of H5N2, H5N1, H5N5, H5N8, H7N2, H7N3 and H7N7 subtypes and internal control in mono- and multiplex reaction format. Threshold cycle value, above which the reaction results should be considered negative, was set at 36.00 for the Green/H5 and Orange/H7 channels. In all tests, H5 or H7 AIV RNAs were confirmed to be present only in the samples containing the relevant virus subtype.

Optimal internal control concentration was determined by performing real-time RT-PCR using several 10-fold dilutions of the MS2 virus-containing suspension (10^5–10^7 PFU/cm^3) and simultaneous identification of one or two specific targets of the test system (AIV/H5 and AIV/H7). Based on the test results, concentration of 10^6 PFU/cm^3 was selected that enabled a stable

fluorescent signal increase in detection channel for internal control without inhibiting the signal in other channels for AIV/H5 and AIV/H7 (Fig. 1) and did not exceed sensitivity of the primers-probe system for internal control while simultaneously identifying AIV/H5 and AIV/H7 at high concentrations. When the Ct value is > 35 for Crimson/internal control detection channel, the results of the entire study are considered unreliable, i.e. errors were made at the stage of nucleic acid extraction, reverse transcription, or PCR, or the test sample contains impurities capable of inhibiting the reaction.

Test of real-time RT-PCR test-system for its specificity using extracted RNAs of H3, H4, H9, H10, H16 AIV and other RNA- and DNA-containing viruses (Newcastle disease,

Table 2
Primers and probes used for amplification of H5 and H7 AIV HA gene fragments

Name	Oligonucleotide structure
H5LH1	ACATATGACTACCCACARTATTCAG
H5RH1	AGACCAGCTAYCATGATTGC
H5Zond	(FAM)TCWACAGTGGCGAGTTCCCTAGCA(RTQ1)
LH6H7	GGCCAGTATTAGAAACAACACCTATGA
RH4H7	GCCCCGAAGCTAAACCAAAGTAT
H7Zond	(ROX)CCGCTGCTTAGTTTGACTGGGTCAATCT(BHQ2)

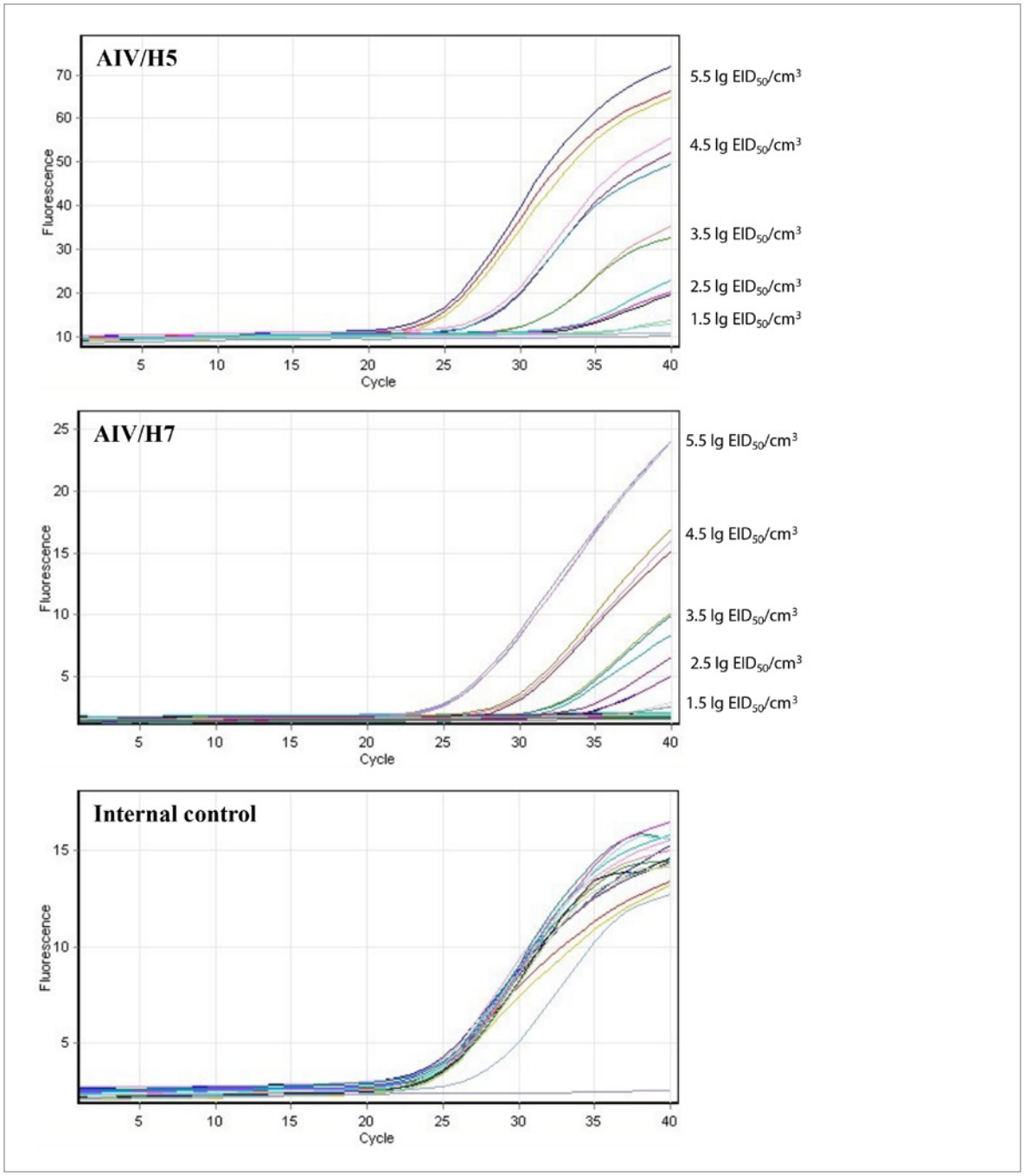

Fig. 1. Graphs of fluorescence intensity increase during real-time RT-PCR on Green (10-fold AIV/H5 dilutions), Orange (10-fold AIV/H7 dilutions) and Crimson (internal control) channels

Table 3
Real-time RT-PCR Ct values for 10-fold AIV/H5 and AIV/H7 dilutions

Detection channel / AIV subtype (virus titre in the initial suspension was 8.5 lg ED_{50}/cm^3)	The average Ct value for dilution, $n = 3$					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Green/H5	19.74 ± 0.13	23.13 ± 0.02	26.76 ± 0.53	30.37 ± 0.44	33.80 ± 0.07	–
Orange/H7	21.20 ± 0.11	25.17 ± 0.08	28.23 ± 0.03	31.39 ± 0.39	35.08 ± 0.52	–

"–" – negative result.

infectious bursal disease, infectious bronchitis, Marek's disease viruses, adenovirus) showed the absence of cross-reactions with the above-listed pathogens.

Analytical sensitivity of the test-system used for testing 10-fold dilutions of the virus with an infectious titre of 8.5 lg EID_{50}/cm^3 (Fig. 1, Table 3) for AIV/H5 the sensitivity limit corresponded to 10^{-7} dilution of the virus (in 20 repeats a positive result was obtained in 95% of cases) at the average Ct value of 34.16 ± 0.54 and a coefficient of variation of 1.59%; for AIV/H7 the sensitivity limit corresponded to a virus dilution of 10^{-7} (in 20 repeats, positive result was obtained in 100% of cases) with average Ct value of 35.17 ± 0.65 and a coefficient of variation of 1.84%.

Accordingly, the minimum virus amount that can be detected by the developed test system is 1.5 lg EID_{50}/cm^3 for AIV/H5 and AIV/H7.

Linear regression graphs were plotted for reactions with AIV/H5 and AIV/H7 to determine the efficiency parameters (Fig. 2). The following parameters should be taken into account for evaluation of the reaction efficiency:

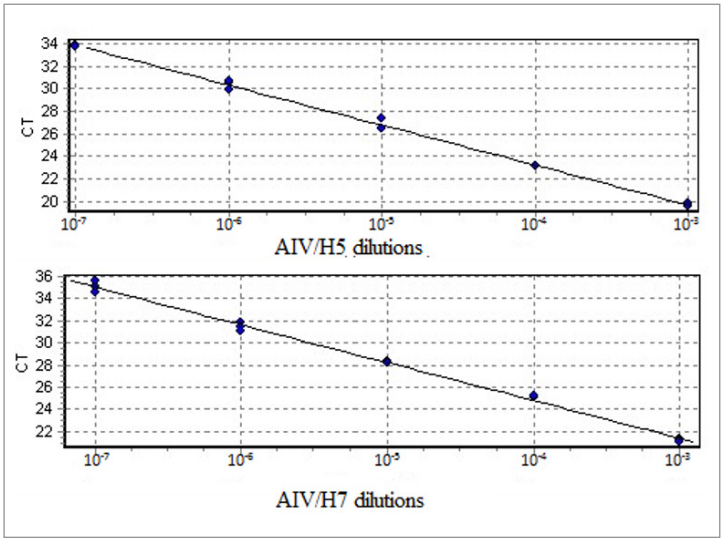

Fig. 2. Graphs of standard straight lines based on real-time RT-PCR results when 10-fold H5 and H7 AIV dilutions were used

Table 4
Reaction efficiency parameters for AIV/H5 and AIV/H7

Detection channel / AIV subtype	Correlation coefficient (R^2)	Straight line slope (m)	Reaction efficiency (E), %
Green/H5	0.997	–3.537	91.74
Orange/H7	0.996	–3.398	96.92

straight line slope (m) and correlation coefficient (R^2). Ideally (at 100% efficiency), m is –3.32, but values in the range from –3.2 to –3.5 are considered optimal. Values greater than 0.98 are optimal for R^2 [20, 22]. Determined reaction efficiency parameters for the developed test system are presented in Table 4.

The reaction efficiency for AIV/H5 (Green channel) was 91.74%, for AIV/H7 (Orange channel) was 96.92%. Parameters such as straight line slope coefficient and the coefficient of determination for AIV of both subtypes correspond to optimal values [20, 22].

The reproducibility of the test system was assessed based on standard deviation (SD) for each series of 10-fold dilutions (10^{-7}–10^{-3}, $n = 3$). For AIV/H5 standard deviations varied from 0.02 to 0.53; for AIV/H7 standard deviations varied from 0.03 to 0.52.

For repeatability assessment, the same viruses were used at a 10^{-4} dilution, each sample was tested in 5 repeats. For AIV/H5, the average Ct value within the runs varied from 22.89 to 23.36; SD was 0.22–0.33; the coefficient of variation was from 0.92 to 1.17%. For AIV/H7, the average Ct value ranged from 24.53 to 25.06; the SD – from 0.18 to 0.23, and the coefficient of variation – from 0.71 to 0.94%. Repeatability values between runs for AIV/H5 were as follows: average Ct – 23.09 ± 0.32, coefficient of variation – 1.41%; for AIV/H7: average Ct – 24.53 ± 0.31, coefficient of variation – 1.25%.

A total of 434 biological samples were tested for H5 and H7 AIV RNA with the developed test system, AIV/H5 RNA was detected in 268 samples. No AIV/H7 RNA was detected in tested samples. The results obtained using the developed test system correspond to those obtained during testing of the same samples by standard molecular diagnostic methods used by the Federal Centre for Animal Health Reference Laboratory for Avian Viral Diseases [23].

CONCLUSION

The test system for detection of H5 and H7 AIV RNA by real-time RT-PCR was developed. The proposed test system parameters were determined: the specificity was 100% (AIV/H5 and AIV/H7), analytical sensitivity limit was 1.5 lg EID_{50}/cm^3 (AIV/H5 and AIV/H7), the reaction efficiency was 92% (AIV/H5) and 97% (AIV/H7). The developed test system can be used for qualitative analysis of H5 and H7 AIV RNA in biological samples from birds and other animals.

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Immunogenic activity of "ARRIAH-AviFluVac" vaccine against high-pathogenicity H5N1 avian influenza virus relevant for Russia in 2023

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ABSTRACT

Introduction. Vaccination against high-pathogenicity avian influenza (HPAI) is a well-proven way to control the disease. Inactivated whole-virion products are the most popular among the influenza vaccines. It is important to study immunogenicity of "ARRIAH-AviFluVac" vaccine against currently circulating HPAI viruses.

Objective. To assess immunogenic activity of "ARRIAH-AviFluVac" inactivated vaccine against high-pathogenicity avian influenza virus (H5N1 subtype) which was relevant for Russia in 2023.

Materials and methods. For testing purposes 4 vaccine dilutions were prepared containing whole and diluted H5 avian influenza virus antigen (1/25, 1/50 and 1/100). Each diluted sample was used to vaccinate a separate group of 4-week-old chickens. On day 28 post vaccination, the chickens were challenged with avian influenza virus A/gull/Kirov/998-1/2023 H5N1, which was isolated during an outbreak in the Russian Federation and was phylogenetically defined as high-pathogenicity agent belonging to the Asian genetic lineage of HPAI subtype H5 (clade 2.3.4.4b). Dead and sick chickens were reported in the infected groups for 6 days.

Results. The chickens vaccinated with a whole antigen dose were found to be completely protected from the clinical signs after the challenge. A decrease in the antigen concentration in the vaccine volume decreased the vaccine-induced protection. The mortality rate after the challenge of control (intact) chickens was 10/10. An analysis of the dependence of the vaccine protectivity on the volume of the antigen immunizing dose showed that one inoculation dose contained 97 PD_{50}. An analysis of the link between protection and strength of the post-vaccination humoral immunity allowed to calculate that the expected mean antibody titer in the group, which corresponds to 90% protection in the vaccinated birds, was 5.7 $\log_2$, or ≈ 1:52.

Conclusion. "ARRIAH-AviFluVac" vaccine demonstrates high immunogenicity against high-pathogenicity avian influenza virus (H5N1) which was relevant for Russia in 2023.

Keywords: high-pathogenicity avian influenza, inactivated vaccines, antigen dose in the vaccine, vaccine protective effect

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Иммуногенная активность вакцины «ВНИИЗЖ-АвиФлуВак» против актуального для России в 2023 году вируса высокопатогенного гриппа птиц H5N1

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РЕЗЮМЕ

Введение. Вакцинопрофилактика высокопатогенного гриппа птиц является надежным способом борьбы с болезнью. Среди антигриппозных вакцин наиболее широкое распространение имеют инактивированные цельновирионные препараты. Изучение иммуногенной активности вакцины «ВНИИЗЖ-АвиФлуВак» против актуальных вирусов высокопатогенного гриппа птиц является важной задачей.

Цель исследования. Оценка иммуногенной активности инактивированной вакцины «ВНИИЗЖ-АвиФлуВак» против актуального для России в 2023 г. высокопатогенного вируса гриппа птиц подтипа H5N1.

Материалы и методы. Для испытаний готовили 4 вакцинных образца, содержащих цельный и разведенный 1/25, 1/50 и 1/100 антиген вируса гриппа птиц H5 в прививном объеме. Каждым препаратом была привита отдельная группа птиц 4-недельного возраста. Через 28 сут куры были заражены вирусом гриппа птиц A/gull/Kirov/998-1/2023 H5N1, который был выделен во время вспышки заболевания на территории Российской Федерации и филогенетически определен как высокопатогенный возбудитель, принадлежащий к азиатской генетической линии вируса высокопатогенного гриппа птиц подтипа H5 (клада 2.3.4.4b). В группах зараженных птиц в течение 6 дней регистрировали погибших и больных особей.

Результаты. Установили, что птицы, привитые цельной дозой антигена, были полностью защищены от клинического проявления болезни после контрольного заражения. Уменьшение концентрации антигена в прививном объеме обусловило снижение протективной защиты вакцины. Показатель смертности после заражения контрольных (интактных) цыплят составил 10/10. Анализ зависимости протективной активности вакцины от величины иммунизирующей дозы антигена показал, что одна прививная доза содержала 97 $ПД_{50}$. Исследование связи протективной защиты и напряженности поствакцинального гуморального иммунитета позволило определить, что ожидаемый среднегрупповой титр антител, который соответствует защите 90% вакцинированных птиц, составил 5,7 $\log_2$, или ≈ 1:52.

Заключение. Вакцина «ВНИИЗЖ-АвиФлуВак» обладает высокой иммуногенной активностью против актуального для России в 2023 г. вируса высокопатогенного гриппа птиц подтипа H5N1.

Ключевые слова: высокопатогенный вирус гриппа птиц, инактивированные вакцины, доза антигена в вакцине, протективный эффект вакцины

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INTRODUCTION

High-pathogenicity avian influenza (HPAI) is now a matter of concern for poultry farming all over the world. HPAI (H5N1) virus is the cause of devastating epizooties that cause significant economic damage. For example, 11 million birds had been detroyed in France by March 2022 due to HPAI (H5N1) spread; and, by September 2022, losses in the United States had exceeded 20 million chickens [1, 2]. Totally, 67 countries on five continents reported HPAI (H5N1) outbreaks in 2022, resulting in loss of more than 131 million poultry [3]. From April to June 2023, HPAI (H5N1) outbreaks were reported in 25 European countries in domestic and wild birds, with a total of 98 and 634 episodes, respectively [4].

As of 17 October 2023, the following HPAI (H5N1) outbreaks were registered in the Russian Federation, as the Rosselkhoznadzor reported: 57 settlements – in wild birds; 6 settlements – on poultry farms; 8 settlements – in backyard poultry [5]. It was noted that the disease affected atypical wild avian species that year, namely seagulls. For example, there was an outbreak at the Borisovskiye Prudy in Moscow with dead seagulls detected. H5N1 subtype virus genome was isolated from the remains found there [6]. In the central regions of Russia, HPAI-infected poultry farms are all located in the immediate vicinity of the settlements where HPAI (H5N1) outbreaks were recorded in wild birds [5], which clearly indicates the source of the virus spread.

The influenza large-scale spread is primarily explained by the pathogen characteristics. At the synthesis stage in an infected cell, viral RNA does not have a repair mechanism and retains all possible "errors" in the structure, which with a probability of at least $1/10^6$ determine changes in the virus phenotype [7]. Compared to DNA-viruses with the maximum probability of error during genome replication of $1/10^9$, this is a three times difference. Each round of RNA-virus replication generates a mixed population with many variants, most of which are not viable, but some of them contain mutations that can become dominant under appropriate conditions [8, 9]. At the phenotypic level, these may be changes in antigenic properties and/or changes in the pathogen tropism. In the first case, the modified agent can evade the immune response of the macroorganism, in the second case, it can increase virulence.

We emphasize that the influenza virus genome is represented by independent RNA fragments (8 fragments). If one cell is infected with various virus variants, recombination may occur, i.e. the exchange of genome fragments, which will lead to qualitative changes in the pathogen properties, including a change of the host specificity [1]. For example, in June 2023, influenza A (H5N1) virus was detected in 24 domestic cats in Poland. Infected animals showed neurological and respiratory signs, and in some cases, death was reported. In July 2023, two human cases of influenza A virus subtype H5N1 were reported in the UK, and in two cases influenza A virus subtype H9N2 was isolated [4].

Thus, the scheme depicting known avian influenza virus ecological niches (Fig. 1) only partially reflects the natural habitat of the pathogen [10].

Along with restrictive measures, specific prevention is a well-proven way to control HPAI. Inactivated whole-virion vaccines are the most popular among anti-influenza vaccines [11, 12]. Protectivity of such vaccines depends on two related factors: the antigen concentration in the vaccine and the structural correspondence between the vaccine antigens and the field isolate [12, 13].

At the same time, for biosafety reasons it is recommended to use low-pathogenicity virus variants to obtain antigens [14]. "ARRIAH-AviFluVac" is an example of an inactivated vaccine for specific prevention of HPAI based on a low-pathogenicity virus variant.

The objective of the research was to assess effectiveness of "ARRIAH-AviFluVac", the inactivated vaccine against HPAI H5N1 which caused local outbreaks in several regions of Russia in 2023.

To achieve the objective the following tasks were set:

– to determine phylogenetic type of the HPAI isolate recovered in the outbreak in the Russian Federation, which will be used to test the vaccine protective effect;

– to assess 50% protective dose contained in the inoculation volume;

– to calculate post-vaccination antibody titer that protects 90% of the vaccinated birds.

MATERIALS AND METHODS

The research object: "ARRIAH-AviFluVac" inactivated emulsion vaccine against avian influenza (H5). The antigen concentrations (D) in the vaccine inoculation dose (represented by "Yamal" production strain of low pathogenicity avian influenza H5 virus) were regulated by diluting it with saline solution in the ratios of 1/25, 1/50 and 1/100. When preparing vaccine samples, the active component (antigen) was combined with an oil adjuvant in a ratio of 30:70 (by weight) and emulsified on a high-speed Silverson laboratory mixer (Great Britain) at a speed of 6,000 rpm for 5 minutes. The emulsion stability after mixing was evaluated after centrifugation at 1,000 g for 10 min. The emulsion was considered stable, if creaming of the light (oil) fraction did not exceed 5% by volume, and no creaming of the heavy (water) fraction was observed.

Thus, vaccine samples were prepared containing a whole antigen (D = 1) and antigens diluted at 1/25, 1/50 and 1/100 (D = 25, D = 50 and D = 100) from the initial concentration.

Poultry. For the experiment, avian influenza virus seronegative 4-week-old Lohmann Brown cross-breds were used. The chickens were handled in accordance with GOST 33215-2014 and in accordance with Directive 2010/63/EU (dated 22.09.2010) on protection of animals used for scientific purposes.

Vaccination. Each vaccine sample was tested in a separate group of 10 chickens. The vaccine was injected intramuscularly into the chest area at a dose of 0.5 cm^3. Additionally, a virus control group was formed (10 chickens), where no vaccination was performed (intact chickens). Chicken groups were kept in isolated rooms with autonomous ventilation, water and feed supply.

Chicken embryos. SPF chicken embryos (9–11-day-old) were used for the research (VALO BioMedia GmbH, Germany).

Isolation of avian influenza virus. The pathological material obtained from the seagulls that died of HPAI was used. A 10% tissue suspension was prepared on a phosphate buffer (pH 7.2–7.4), which was centrifuged for 15 min at 1000 g. Antibiotics were added to the supernatant (100 U/mL of benzylpenicillin sodium salt, 100 µg/mL of streptomycin sulfate and 50 U/mL of nystatin). The resulting material was injected into the allantoic cavity of chicken embryos in a volume of 0.2 cm^3. Embryos were incubated at a temperature of 37 °C and a relative humidity of 60–70% with ovoscopy done daily. The embryos that died 24 hours after incubation or more were used for harvesting extraembryonic fluid. The death specificity was confirmed by hemagglutinating activity in the hemagglutination test and by identification of hemagglutination inhibition with a specific serum [15].

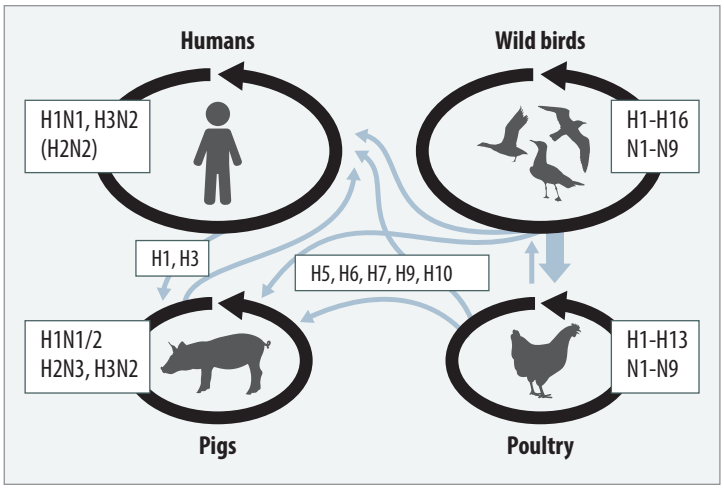

Fig. 1. Ecology of influenza A virus ([10] with changes). Hemagglutinin (H) and neuraminidase (N) variants are indicated. Black arrows show the pathogen circulation in host species, gray arrows show the virus interspecies spread

Calculating virus titer in chicken embryos. Method of limiting dilutions was used. Serial tenfold dilutions of the virus material in a phosphate buffer (pH 7.2–7.4) were prepared. Each dilution was tested in a group of embryos ($n \geq 5$). The material was inoculated into the allantoic cavity in a volume of 0.2 cm^3. The virus presence, which means i.e. a positive reaction, was confirmed, if the embryo death was observed after more than 24 hours of incubation. The titer was calculated according to Karber and expressed as EID_{50}/cm^3.

Reverse transcription polymerase chain reaction (RT-PCR). The total RNA was isolated using RNeasy Mini Kit (QIAGEN, the Netherlands, cat. No. 74106) in accordance with the manufacturer's instructions. One RT-PCR stage was performed using OneStep RT-PCR Kit (QIAGEN, the Netherlands, cat. No. 210212) with appropriate primer systems for detecting avian influenza virus genome and identifying H5N1 subtype.

Sequencing the virus genome. Nucleotide sequences of the gene fragments were determined using ABI Prism 3130 automatic sequencer (Applied Biosystems, USA). BioEdit application software package, Version 7.0.5.3, was used to analyse and compare nucleotide and corresponding amino acid sequences. Sequences of isolates and strains of A/H5 avian influenza virus previously published in the international GenBank database were also used for comparative analysis (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database). The phylogenetic tree was constructed and edited using NJ algorithm in MEGA package, Version 7.

Hemagglutination assay (HA assay). Samples of antigen-containing materials were examined in HA assay according to the procedure described in instructions for a hemagglutination inhibition test kit used to detection of antibodies to avian influenza virus subtype H5. The titer of hemagglutinating units was calculated.

Hemagglutination inhibition test (HI test). Avian blood serum samples were tested in HI test in accordance with the instructions for the hemagglutination inhibition test kit used for detection of antibodies to avian influenza virus subtype H5 (Federal Centre for Animal Health, Russia) [16]. The antibody titer was calculated. The result was considered positive, if the titer was 1:16 and more, that is 4 $\log_2$.

Challenge. Immunized and intact birds were challenged on day 28 post vaccination. HPAI strain A/gull/Kirov/998-1/2023 H5N1 was used for the challenge at a dose of 6.0 lg EID_{50}. The viral material was injected intramuscularly into the thigh area in a volume of 0.5 cm^3. Clinical status of the challenged birds was monitored for 10 days.

Processing experimental data. Conventional methods were used to process the set of variables (mean values, standard deviations, and standard errors of the mean were calculated). Elements of regression analysis were used. Special statistical methods are described in the text. Calculations were done and the graphs were drawn using Excel application.

RESULTS AND DISCUSSION

Virus isolation, virulence assessment and phylogenetic analysis. It was found that the tested biological material contained the infectious virus that was lethal to the embryos (specific mortality was 23/30). Samples of extraembryonic fluid tested positive in HA assay (from 1:64 to 1:256) and RT-PCR revealed high concentraiton of avian influenza virus genome in them (mean Ct value = 18).

The hemagglutinin cleavage site of the isolated avian influenza virus had a structure -REKRRKR-, which made it possible to characterize is as potentially highly virulent.

Virus-containing extraembryonic fluid intravenously injected to ten 5-week-old chickens, seronegative to the avian influenza virus, resulted in death of 9 chickens (90%) during the following 10 days after injection (i.e. 10-fold dilution in phosphate buffer was used and each chicken received 0.1 cm^3). The dead chickens showed typical clinical signs of HPAI (diarrhea, nasal discharge, cyanosis of unfeathered skin areas). The death specificity was confirmed by RT-PCR, which revealed HPAI virus genome in the biological material. The results obtained corresponded to the clinical signs of HPAI [14].

Comparative genetic analysis of hemagglutinin fragment nucleotide sequences revealed that the virus belongs to the Asian genetic lineage of HPAI virus subtype H5 (clade 2.3.4.4.b), which earlier became epizootic for Asia, Europe, Africa, North and South America. The isolated virus was identified as avian influenza virus strain A/gull/Kirov/998-1/2023 H5N1. The strain position in the phylogenetic tree is shown in Figure 2.

Fig. 2. A phylogenetic tree based on the full-length hemagglutinin gene sequences

Table
Indicators of vaccine immunogenicity against H5N1 avian influenza virus

Indicators according to the tested antigen concentrations			
Antigen concentration, D*	HI titre	Clinical indicator	Protective activity (P), %
	$\log_2 T$**	$\sum c/n$***	$P = (1 - \sum c/n) \times 100$
1	6.67	0/10	100
1:25	5.33	1/10	90
1:50	4.33	4/10	70
1:100	2.00	6/10	50
control	0.47	10/10	0

* antigen concentration in the inoculation volume;
** mean log antibody titer in the group of the vaccinated chickens, $n = 3$;
*** $\sum c$ – number of clinically diseased and dead birds after the challenge [indicator assessed during the experiment];
n – number of chickens in the group before the challenge.

According to GenBank and GISAID (EpiFlu) databases, the most genetically related to A/gull/Kirov/998-1/2023 H5N1 are H5N1 subtype viruses detected in 2023 in several European countries. Taking into account the time of detection in the European countries (i.e. February – May 2023) and comparing the data to the GISAID database (EpiFlu), identical isolates had been actively circulating for several months, at least, since the beginning of 2023.

Thus, taking into account spread of H5N1 influenza virus in the region, as well as introduction and spread of infection in a number of RF regions, A/gull/Kirov/998-1/2023 H5N1 virus strain was used in further work.

Assessing vaccine immunogenicity. All vaccine samples containing certain antigen concentrations were tested in birds in parallel. On day 28 post vaccination, mean $\log_2$ antibody titer against avian influenza virus was calculated in HI test ($\log_2 T$).

Further, all experimental groups were infected with the A/gull/Kirov/998-1/2023 H5N1 strain. For 10 days, current clinical indicators were examined daily in each group ($c = a + b$, where a and b are the number of clinically diseased and dead birds, respectively). At the end of the observation, the accumulated clinical indicators in the groups were assessed ($\sum c/n$, where n is the number of birds in the group before infection) and the protective activity of the vaccine type $P = (1 - \sum c/n) \times 100$ was calculated.

Indicators of the vaccine immunogenicity established in experimental groups are shown in the Table.

The relationship between the antigen concentration in the inoculation volume (D) and the vaccine protective activity (P) was studied. Regression analysis was used to construct the most probable model of the relationship between the antigen concentration and the vaccine protective activity [17]. The following linear regression equation was drawn up: $P = (-0.5184)\ D + 100.31$ ($R^2 = 0.98$), where P is the predicted index value corresponding to the given D.

Correlation regression analysis was used to graphically represent the regression of P and D. The results are shown in Figure 3.

Using the regression equation, it was calculated that the antigen concentration providing protection for 50% (PD_{50}) of the vaccinated poultry is 97, which corresponds to initial antigen concentration of 1:97. Thus, the vaccine protectivity is 97 PD_{50}, which is consistent with the requirements of the World Organization for Animal Health, i.e. $PD_{50} \geq 50$.

Studying relationship between vaccine protectivity and humoral antibody titers. The relationship between humoral antibody titers (T, $\log_2$) and vaccine protectivity was analysed (P, %), using the data obtained for the tested antigen concentrations. The results are shown in Figure 4.

Regression line P by $\log_2 T$ is shown, where 'P is the index predicted by equation 'P = (11.093) $\log_2 T$ + 26.667, with an adequacy score of $R^2 = 0.97$.

The resulting equation allowed us to calculate the expected antibody titer (T_{90}), which corresponds to 90% protection of the vaccinated poultry. The desired estimate was $\log_2 T_{90} = 5.7$.

It is known that HPAI outbreaks on poultry farms (for example, in the USA) mostly coincide geographically with the migration routes of wild waterfowl [1]. Given the role of avifauna in the disease spread in some regions of the Russian Federation, A/gull/Kirov/998-1/2023 H5N1 strain should be considered epizootically dangerous. Therefore, it is justified to use the strain for challenge purposes to assess the protectivity of the vaccine against H5N1.

The antigen concentration in the inoculation volume of the inactivated vaccine is the most important characteristic of the vaccine. The major antigen (hemagglutinin) can be measured in absolute units, such as weight units [11], or in units of action, such as PD_{50}. The inoculation volume of an effective avian influenza vaccine is considered to contain 50 PD_{50} [12, 14], which corresponds to 0.3–7.8 [12] or 3 μg of hemagglutinin [14].

The challenge procedure fully corresponded to the generally accepted method [14]. The results obtained demonstrate that the inoculation volume of "ARRIAH-AviFluVac" contained 97 PD_{50}, which ensured protection of 100% of the immunized poultry who received one inoculation volume, thus, proving the possibility to practically use it in accordance with the attached instructions. This means that the antigenic potential of "ARRIAH-AviFluVac"

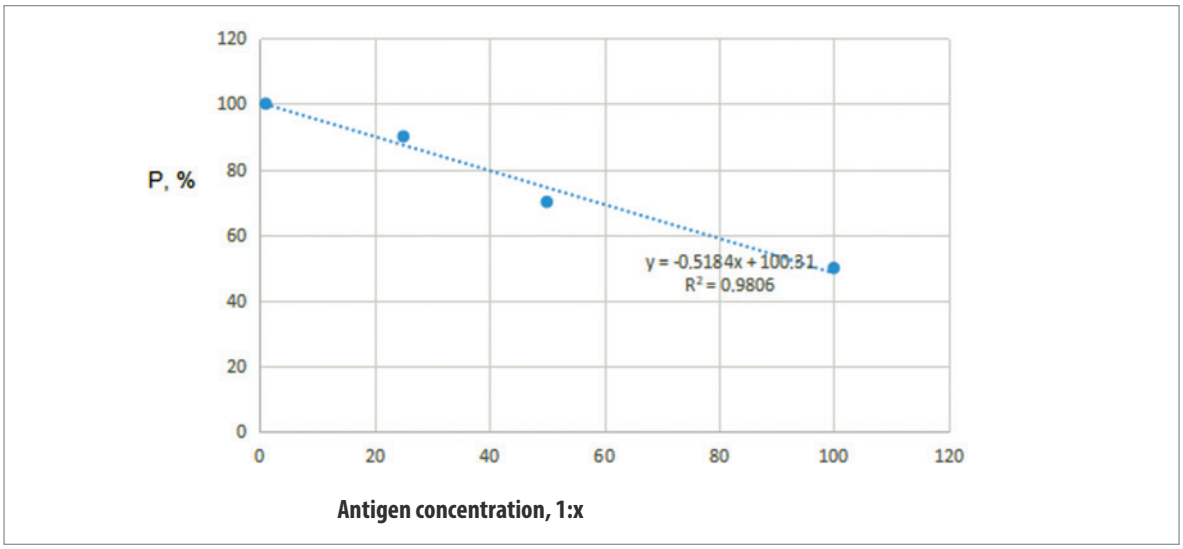

Fig. 3. Relationship between the tested antigen concentrations and protectivity of the vaccine against HPAI H5N1

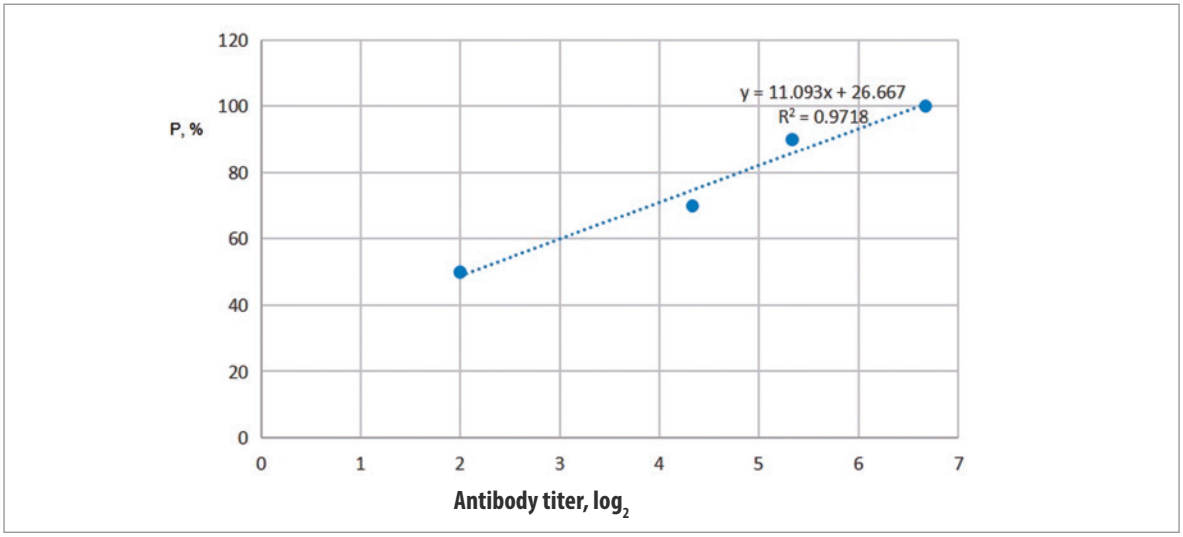

Fig. 4. Relationship between H5 virus antibody titer and level of the vaccine protectivity

vaccine against A/gull/Kirov/998-1/2023 H5N1 strain is 1.9 times higher than the recommended protective activity [14].

Previously, protective properties of a "Yamal" strain-based vaccine [18] were analysed and the vaccine induced effective protection against heterologous HPAI H5N8 subtype (A/duck/KChR/1590-20/2 strain). A/duck/KChR/1590-20/2 virus also belongs to genetic clade 2.3.4.4b. The results of the phylogenetic analysis given in this work indicate an active antigenic drift of influenza A virus subtype H5N1 in Eurasia in 2016–2023 (Fig. 1). Those avian influenza virus isolates against which the "Yamal" strain-based vaccine has proved to be effective, are shown in Figure 2 in a black triangle.

Since poultry are protected from generalized avian influenza mainly by antibodies developed against viral hemagglutinin [19], HI test data are important to indicate post-vaccination humoral antibody level. HI antibody titer of 4 $\log_2$ is known to protect the poultry from infection and death [14, 19], and antibody titer of 6.5 $\log_2$ prevents, inter alia, local replication of the virus [20]. At the same time, it is required that the antibody titer should be more than 4 $\log_2$ in 80% of the poultry population [21]. Within this research, 90% protection of the vaccinated birds corresponded to the expected titers of 5.7 $\log_2$ for "ARRIAH-AviFluVac" vaccine.

CONCLUSIONS

The following conclusions can be made based on the obtained results.

1. The virus isolated from the pathological material was identified as H5N1 A/gull/Kirov/998-1/2023 strain, which belongs to the Asian genetic lineage of H5 subtype (clade 2.3.4.4b). The strain is characterized as epizootically dangerous for the Russian Federation.

2. It has been established that "ARRIAH-AviFluVac" inactivated vaccine ensures protection from HPAI virus strain A/gull/Kirov/998-1/2023 (H5N1) and, on day 28 after vaccination, it completely prevents clinical manifestation of the disease during challenge. The protective potential of the vaccine was 97 PD_{50} in one inoculation dose.

3. It is shown that "ARRIAH-AviFluVac" vaccine induces intense humoral immunity against influenza in poultry on day 28 after administration. The post-vaccination

HI antibody titer to HPAI, corresponding to protection of 90% of the vaccinated poultry, was as predicted, i.e. 5.7 $\log_2$.

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Creating a laboratory model of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* associated infection

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ABSTRACT

Introduction. Respiratory mycoplasmosis and infectious synovitis are economically significant and notifiable avian diseases, therefore, the issue of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* control on poultry farms is of great importance. Vaccination is one of the ways to ensure specific prevention, however, when a vaccine is developed, its protective properties are assessed with special focus. Challenge does not always lead to the disease manifestation due to its predominantly chronic and factor-dependant nature.

Objective. Laboratory simulation of the factors that contribute to the disease manifestation and a histological analysis of pathological changes in the infected and vaccinated poultry.

Materials and methods. Seronegative and vaccinated 67-day-old Haysex white cross chickens were selected for the experimental purposes. We used S6 strain of *Mycoplasma gallisepticum*, WVU 1853 strain of *Mycoplasma synoviae* and A/chicken/Amursky/03/12/H9N2 strain of low-pathogenicity avian influenza virus.

Results. The associated infection of mycoplasmoses and low-pathogenicity avian influenza is manifested as a disease with pathohistological changes that include mild respiratory and joint disorders. Histological tests of the infected non-vaccinated poultry revealed damaged tracheal ciliated epithelium with desquamation. The poultry vaccinated against mycoplasmosis and experimentally infected showed no signs of epithelial separation, however, local submucosal edema was observed in the trachea. Non-vaccinated poultry infected with low-pathogenicity avian influenza virus H9N2, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* demonstrated dystrophic changes and lymphocyte infiltration in the third eyelid gland which suggested an inflammation. Lymphocytic lung tissue infiltration was detected both in the vaccinated and non-vaccinated experimentally infected poultry. All groups of chickens, except for the control one, demonstrated lymphocyte depopulation in the cortical substance of the fabricium sac.

Conclusion. The study resulted in developing a challenge procedure for poultry using *Mycoplasma gallisepticum* and *Mycoplasma synoviae* agents, in defining conditions for clinical manifestation of mycoplasmoses, in detecting infection-caused pathological changes at the cellular level.

Keywords: *Mycoplasma gallisepticum, Mycoplasma synoviae,* histology, challenge

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Воспроизведение ассоциированной инфекции, обусловленной *Mycoplasma gallisepticum* и *Mycoplasma synoviae*, в лабораторных условиях

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РЕЗЮМЕ

Введение. Респираторный микоплазмоз и инфекционный синовит птиц являются экономически значимыми и нотифицируемыми болезнями, поэтому вопрос борьбы с *Mycoplasma gallisepticum* и *Mycoplasma synoviae* на птицеводческих предприятиях является актуальным. Применение вакцин – один из способов специфической профилактики, однако при разработке препаратов особое внимание уделяется оценке их протективных свойств. Контрольное заражение не всегда приводит к проявлению болезни ввиду ее преимущественно хронического течения и факторности.

Цель исследования. Воссоздание факторов, способствующих проявлению болезни в лабораторных условиях, и выявление патологических изменений в организме зараженных и иммунизированных птиц на гистологическом уровне.

Материалы и методы. В качестве подопытных животных были отобраны серонегативные и вакцинированные куры кросса Хайсекс белый в возрасте 67 сут. В ходе опыта использовали штамм S6 *Mycoplasma gallisepticum*, штамм WVU 1853 *Mycoplasma synoviae* и штамм A/chicken/Amursky/03/12/H9N2 вируса низкопатогенного гриппа птиц.

Результаты. Ассоциированное течение микоплазмозов с низкопатогенным гриппом птиц проявляется заболеванием и патогистологическими изменениями, среди которых легкие респираторные расстройства и суставной синдром. При гистологическом исследовании у зараженных невакцинированных птиц выявили нарушение целостности реснитчатого эпителия трахеи с очагами десквамации. У вакцинированной против микоплазмоза и экспериментально инфицированной птицы признаков отслаивания эпителия не наблюдалось, однако выявляли локальный отек подслизистого слоя трахеи. В железе третьего века у невакцинированных птиц, зараженных вирусом низкопатогенного гриппа птиц H9N2, *Mycoplasma gallisepticum* и *Mycoplasma synoviae*, отмечали дистрофические изменения и инфильтрацию лимфоцитами, что свидетельствовало о наличии воспаления. В группе как вакцинированных, так и невакцинированных экспериментально инфицированных птиц в тканях легких выявляли лимфоцитарную инфильтрацию. Во всех группах птиц, кроме контрольной, наблюдали картину депопуляции лимфоцитов в корковом веществе фабрициевой сумки.

Заключение. Результатом данного исследования является создание метода проведения контрольного заражения птиц *Mycoplasma gallisepticum* и *Mycoplasma synoviae*, а также выявление условий для клинического проявления микоплазмозов, установление патологических изменений на клеточном уровне вследствие инфицирования.

Ключевые слова: *Mycoplasma gallisepticum, Mycoplasma synoviae*, гистология, контрольное заражение

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INTRODUCTION

Respiratory mycoplasmosis and infectious synovitis are economically important diseases caused by *Mycoplasma gallisepticum* and *Mycoplasma synoviae* [1]. The peculiarity of these mycoplasmoses is their chronic nature with exacerbation resulting from reduced natural resistance or caused by stress factors of various origin [2]. Vaccination is one of the ways to prevent these diseases [3]. When vaccines are developed, special attention is paid to their protective properties demonstrated in the challenge tests. Taking into account that avian mycoplasmoses are factor-dependent, it is rather difficult to reproduce the infectious process in the laboratory. Thus, the issue of testing protective properties of vaccines against respiratory mycoplasmosis and infectious synovitis has become especially important in the antimicrobial resistance control time.

Mycoplasma gallisepticum causes respiratory mycoplasmosis in chickens and turkeys, with the following signs: rales, coughing, rhinorrhea, aerosacculitis. This disease is predominantly chronic, spreads slowly in the flock, mycoplasma-carriers frequently occur. The disease can be aggravated by such stress factors as vaccination, poor feeding, draughts, high concentration of ammonia in the air, etc. Post-mortem lesions resulting from respiratory mycoplasmosis include serous, serous-fibrinous or fibrinous exudate in the nasal cavity and suborbital sinuses. Tracheal mucosa is hyperemic, lungs are full of blood, pneumonia may occur. Pathognomonic sign consists in serous, serous-fibrinous or fibrinous aerosacculitis of thoracic or abdominal air sacs: with thickened and opaque walls and exudate accumulated in the cavity. The non-complicated form is characterized by no lesions of parenchymatous organs [4, 5].

Mycoplasma synoviae is the pathological agent causing infectious avian synovitis, characterized by arthritis, tendovaginitis, synovitis and anemia. The disease clinical signs may include lameness, pale comb, stunting and swelling in the metatarsal and tibiotarsal joints, plantar surface of the paw, and thoracic bursa [6]. During subacute and chronic disease, the surface of the affected joints is macerated, covered with exudate crusts and necrotic masses [7, 8, 9]. Periarticular tissues and tendon sheaths in the affected joints are edematous, transparent exudate accumulates in the joint cavity; the chronic course is characterized by a significant amount of fibrinous masses [10]. The disease may also manifest itself as a respiratory syndrome indistinguishable from respiratory mycoplasmosis. Egg Apical Abnormalities (EAA) is believed to be a pathognomonic sign of infectious synovitis, which causes significant economic losses due to the need to cull table and hatching eggs [4, 11].

Despite the extensive list of avian mycoplasmosis characteristics, such characteristics as chronic nature and factor-dependence create certain difficulties in creating infections in the laboratory.

It is difficult to assess protective properties of the vaccine developed for specific prophylaxis of chronic diseases. For acute infections, such as high-pathogenicity avian influenza and Newcastle disease, it is easy to assess vaccine protective properties in challenge tests; however, this

method has significant limitations for chronic infections, including mycoplasmosis, because it is not always possible to reproduce well-pronounced clinical signs in the laboratory [12, 13, 14]. In addition, mycoplasmosis pathogens can persist in the body for a long time and remain undetected by the immune system (biological mimicry) [4, 9, 15].

Thus, creating a laboratory model of mycoplasmosis to assess protective properties of newly developed vaccines is assumed a timely and relevant task. Given that infections of mycoplasma origin belong to factor-dependent diseases, it was necessary to select a trigger for clinical manifestation [16, 17]. For this purpose, a reproduction model of co-infection caused by *M. gallisepticum*, *M. synoviae* and low pathogenicity avian influenza virus (subtype H9N2) with zero intravenous pathogenicity index (IVPI = 0) was tested in the experiment. According to some foreign authors, co-infection with low pathogenicity avian influenza virus (H3N8) significantly affects *M. gallisepticum* pathogenesis [18]. Infection of clinically healthy birds with this pathogen in the laboratory does not lead to clinical signs. However, on poultry farms, the co-infection of low pathogenicity avian influenza and mycoplasmosis causes respiratory infection [10, 19].

A laboratory-created model of mycoplasma and low pathogenicity avian influenza co-infection may be used both for assessing vaccine protective properties and for analyzing the role of each pathogen in the pathogenesis of mixt-infection.

MATERIALS AND METHODS

Strains. Strains S6 of *M. gallisepticum* and WVU 1853 of *M. synoviae* were used during the experiment. Strain of low-pathogenicity avian influenza virus A/chicken/Amursky/03/12/H9N2 (hereinafter – H9N2) was used as a co-infecting agent (trigger).

Inactivated emulsion combined *vaccine* against respiratory mycoplasmosis and infectious synovitis, produced by the Federal Centre for Animal Health (pilot batch).

Infectious doses. M. gallisepticum culture with activity of 6.0 $\log_2$ hemagglutinating units and *M. synoviae* with activity of 3.0 $\log_2$ agglutinating units were used for infection. The infectious doses of low pathogenicity avian influenza virus was 10^6 lg EID/0.5 cm^3.

Poultry. Seronegative and vaccinated 67 day-old Hisex white egg cross chickens were used for the experimental purposes. The chickens were kept in the animal facilities of the Federal Centre for Animal Health, the keeping and feeding conditions corresponded to zoo-hygiene requirements.

The dose and method of infection. The infection pattern and methods are given in Table 1.

Clinical observation and post-mortem examination. During the whole experiment (35 days post vaccination), the experimental chickens were monitored to assess their general condition (mobility, body condition score, reaction to external stimuli, crowding, depression, feed and water refusal, etc.).

On day 14 post infection, the chickens were euthanized and post-mortem examination was performed to describe lesions in organs and tissues. Pieces of organs and tissues were sampled for histological tests.

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

Histological tests were conducted in the Center for preclinical tests located in the Federal Centre for Animal Health. Organ sections were stained with hematoxylin and eosin, examined microscopically and followed by taking photographs.

Serological tests. Levels of specific antibodies to *M. gallisepticum* and *M. synoviae* were measured in avian sera using ELISA test kits manufactured by the Federal Centre for Animal Health; antibodies to the avian influenza virus H9N2 subtype were detected using hemagglutination inhibition (HI) test kits also manufactured by the Federal Centre for Animal Health.

RESULTS AND DISCUSSION

On day 3 after infection of non-vaccinated chickens with a combination of H9N2 + *M. gallisepticum* + *M. synoviae* pathogens (group No. 2), mild respiratory symptoms were observed, in particular, the chickens were passive, had watery eyes, conjunctival injection (reported in 6 out of 10 chickens). Between days 5–10, the following signs were noted: persistent skin hyperemia spotted in featherless patches on the heads, watery eyes and rhinorrhea. Nasal exudate dried into scabs, which easily fall off by themselves (in 9 out of 10 chickens). At the same time, 5 chickens from the group showed a change in behavior consisting in hypodynamia. Post-mortem examination of chickens in this group revealed signs of catarrhal laryngotracheitis with petechial hemorrhages (Fig. 1).

On day 7 post infection, some chickens from group No. 2 demonstrated lameness, in addition the birds were apathetic. Pronounced swelling, skin cracks and exudation were observed on the plantar surface of the paws (Fig. 2). Chickens spend a very high proportion of their daylight hours in recumbency. Opening of the affected sole parts showed severe swelling of the soft tissues with serous exudate; the soft tissue was swollen and had a gel-like consistency. These signs may suggest an articular inflammation caused by infectious synovitis.

Table 1
Infection procedure (scheme and methods)

Group number	Number of birds in the group	Vaccine	Infection
1	10	Inactivated emulsion combined vaccine against respiratory mycoplasmosis and infectious synovitis (pilot batch)	H9N2 (intranasally, ocularly); *M. gallisepticum*, *M. synoviae* (intranasally, ocularly, intramuscularly)
2	10	Not vaccinated	H9N2 (intranasally, ocularly); *M. gallisepticum*, *M. synoviae* (intranasally, ocularly, intramuscularly)
3	10	Not vaccinated	H9N2 (intranasally, ocularly)
4	10	Not vaccinated	*M. gallisepticum*, *M. synoviae* (intranasally, ocularly, intramuscularly)
5 (control)	5	Not vaccinated	Was not carried out

Fig. 1. Petechial and striped hemorrhages on the tracheal mucosa in the non-vaccinated poultry after infection with H9N2 + M. gallisepticum + M. synoviae (photo by D. A. Kozlov)

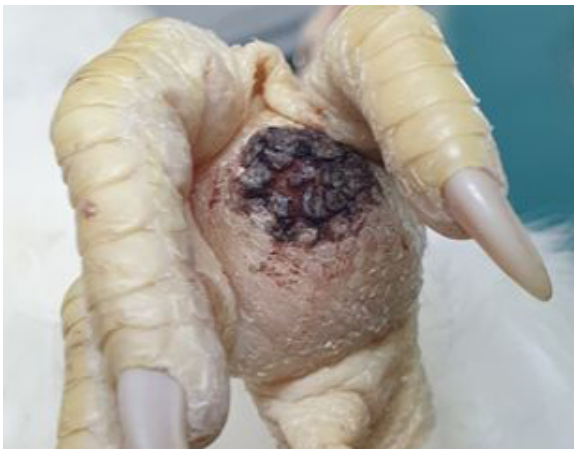

Fig. 2. Inflammation of the plantar surface of the foot in the non-vaccinated chicken on day 7 after infection with H9N2 + M. gallisepticum + M. synoviae: edema, exudation (photo by D. A. Kozlov)

In groups No. 1 (vaccinated against mycoplasmosis), No. 3 (infected with H9N2), No. 4 (infected with *M. gallisepticum* and *M. synoviae*) and No. 5 (negative control), no visible clinical abnormalities were observed.

Morphological examination of the respiratory tract revealed that non-vaccinated poultry infected with H9N2 influenza virus, *M. gallisepticum* and *M. synoviae* (group No. 2) had disrupted ciliated epithelium in trachea with desquamation. Chickens vaccinated against mycoplasmosis infected with H9N2 + *M. gallisepticum* + *M. synoviae* (group No. 1), showed no signs of desquamation, however, local swelling of the submucosal layer was detected. Morphological structure of the trachea in control chickens was preserved, all layers were clearly visible (Fig. 3). At the same time, lymphocytic infiltration of lung tissue was detected in both groups of vaccinated and non-vaccinated chickens. All chicken groups, except for the control one, demonstrated a drop in lymphocytes in the cortical layer in the bursa of Fabricius, which proves activation of the immune system and the redistribution of lymphocytes after infection.

After infecting the non-vaccinated chickens using intranasal and ocular methods (H9N2 + *M. gallisepticum* + *M. synoviae*), the third eyelid gland demonstrated dystrophic changes and lymphocytic infiltration (Fig. 4), which suggested inflammation [16, 20]. The group of the challenged vaccinated chickens, on the contrary, demonstrated a drop in lymphocyte in the third eyelid gland tissue.

All the experimental chickens, except for the control ones, showed signs of accidental thymus involution. White pulp of the spleen predominated over the red one in the non-vaccinated infected chickens. The experimental non-vaccinated chickens infected with H9N2 virus (group No. 3) had lymphocytic infiltration of kidneys and focal hemorrhages.

Histological tests of the intestines in all groups revealed that the duodenum serous and muscular membranes were preserved, autolysis was observed in the apical region of the intestinal villi and intestinal crypts were well expressed. Chyme was detected in the intestinal lumen. Cecal lymphoid follicles were identified on the border between the small and large intestine. They were structured, oval-shaped, without a pronounced reactive center.

The observed hyperemia of the vessels in various organs probably results from insufficient exsanguination.

The disease specificity after infection was confirmed by testing samples of chicken sera in ELISA and HI test. Table 2 shows mean antibody titers in vaccinated and non-vaccinated chickens before and after the challenge.

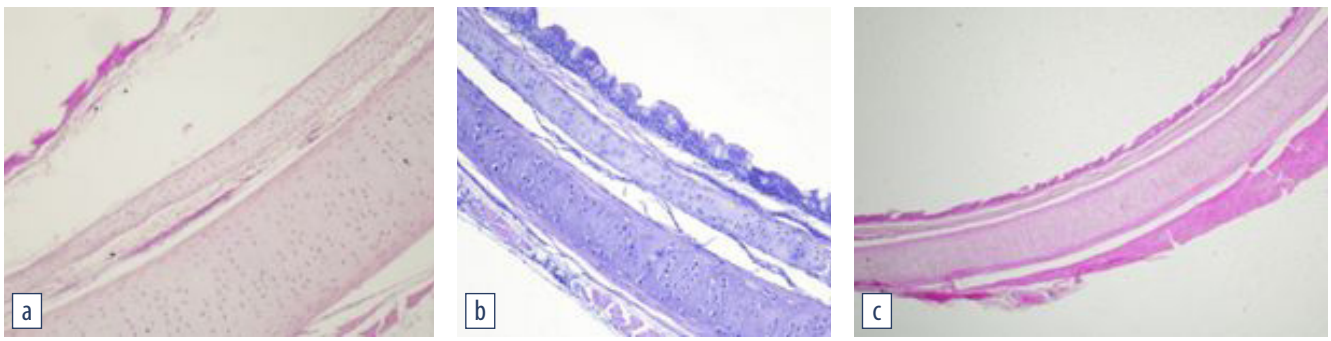

Fig. 3. Morphological structure of the trachea:
a – vaccinated and infected chicken; desquamation of the ciliated epithelium and submucosal swelling;
b – vaccinated and infected chicken; preserved tracheal structure;
c – control group; normal tracheal structure (hematoxylin and eosin staining, magnification 100×; photo by O. A. Chupina, V. V. Pronin)

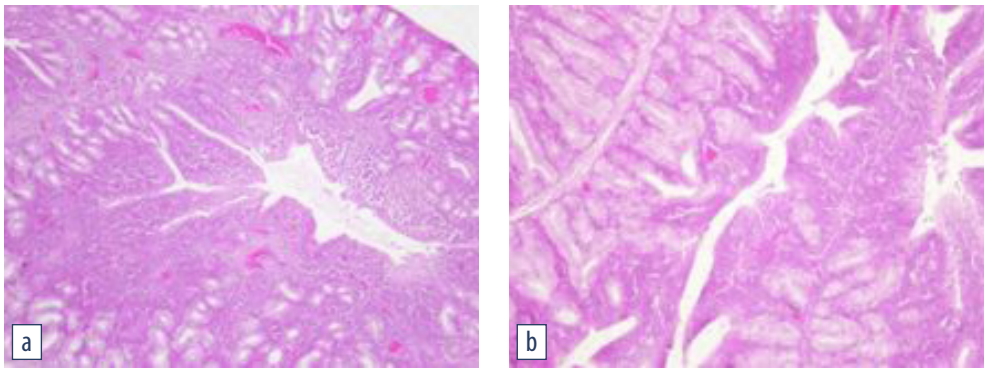

Fig. 4. Histological examination of the third eyelid gland:
a – non-vaccinated infected chicken; lymphocytic infiltration and dystrophic changes;
b – control group; normal structure of the third eyelid gland (hematoxylin and eosin staining, magnification 100×; photo by O. A. Chupina, V. V. Pronin)

The data obtained demonstrate that the immune system of the non-vaccinated chickens reacted to infection with each pathogen. At the same time, mycoplasmosis antibody titer in groups No. 2 and 4 significantly increased after infection (age-related), indicating mycoplasmas reproduction in chickens and stimulation of the immune response. Similarly, for groups 2 and 3, an age-related increase in the titer of anti-hemagglutinins to the H9N2 influenza virus indicated its replication in the body. In group 1 (vaccinated poultry), titers of antibodies to *M. gallisepticum*, *M. synoviae*, and H9N2 influenza virus also increased after infection depending on the age. However, it should be noted that on day 21 after vaccination, mycoplasmosis pathogens were also administered intramuscularly, which increased the immune response (a booster effect) and was accompanied by an increase in the titer of specific antibodies. At the same time, the absence of a clinically pronounced disease and histopathological changes in immunized birds indicated the vaccine effectiveness.

CONCLUSIONS

1. Low-pathogenicity avian influenza virus (H9N2 subtype) with a zero intravenous pathogenicity index can be used as a co-infecting agent to assess the protective activity of mycoplasmosis vaccines during a laboratory challenge test.

2. The co-infection of mycoplasmosis and low-pathogenicity avian influenza is manifested by a clinically pronounced disease and histopathological lesions.

3. The clinically associated form of respiratory mycoplasmosis and infectious synovitis is reproduced in the laboratory after preliminary infection of poultry with the low-pathogenicity avian influenza H9N2 virus and is accompanied by mild respiratory disorders and joint failure. Histological examination of infected non-vaccinated birds revealed damaged tracheal ciliated epithelium with desquamation. A chicken vaccinated against mycoplasmosis and challenged with H9N2 influenza virus, *M. gallisepticum* and *M. synoviae*, showed no desquamation

Table 2
Antibody titre before and after vaccination and infection

Group	Mean antibody titer in group			
	Before challenge / vaccinations	Post vaccination (day 21)	Post Challenge	
			day 7	day 14
1	Mg = 102 ± 64 Ms = 34 ± 12 H9N2 = 0	Mg = 2,688 ± 902 Ms = 2,830 ± 803 H9N2 = 0	Mg = 4,013 ± 1,012 Ms = 3,590 ± 899 H9N2 = 3.4 ± 0.33	Mg = 7,105 ± 1,812 Ms = 7,200 ± 1,679 H9N2 = 4.3 ± 0.15
2	Mg = 84 ± 53 Ms = 12 ± 8 H9N2 = 0	Not vaccinated	Mg = 1,002 ± 402 Ms = 948 ± 899 H9N2 = 3.3 ± 0.4	Mg = 3,013 ± 914 Ms = 2,590 ± 688 H9N2 = 4.4 ± 0.3
3	H9N2 = 0	Not vaccinated	H9N2 = 4.0 ± 0.44	H9N2 = 4.5 ± 0.3
4	Mg = 66 ± 43 Ms = 24 ± 12	Not vaccinated	Mg = 1,383 ± 212 Ms = 907 ± 64	Mg = 2,080 ± 765 Ms = 1,648 ± 966
5	Mg = 16 ± 9 Ms = 28 ± 12 H9N2 = 0	Not vaccinated	Not challenged	
			Mg = 206 ± 82 Ms = 118 ± 89 H9N2 = 0.6 ± 0.3	Mg = 304 ± 102 Ms = 194 ± 90 H9N2 = 0.5 ± 0.2

Mg – *Mycoplasma gallisepticum*; Ms – *Mycoplasma synoviae*. For Mg and Ms geometric mean ELISA titers were calculated in the group, for H9N2 the titers were expressed as HI $\log_2$.

signs, however, local swelling of the submucosal layer was detected. Trachea morphological structure in control chickens was preserved, all layers were clearly visible.

4. A significant increase in antibody titers after infection of chickens non-vaccinated against mycoplasmosis and low-pathogenicity avian influenza suggested pathogen reproduction in chickens.

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Functional and metabolic activity of neutrophils in young cattle sensitized with a non-agglutinogenic strain of *Brucella*

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ABSTRACT

Introduction. Brucellosis remains one of the most common highly dangerous zoonotic infections. Resistance to the pathogenic microorganisms of the genus *Brucella* depends on the appropriate cell-mediated immunity, which includes the activation of the bactericidal mechanisms of phagocytes. Despite the repeatedly proven role of neutrophils in the fight against many bacterial pathogens, the functions of these immunocompetent cells in the setting of brucellosis have long remained unstudied.

Objective. The study aimed to examine the functional and metabolic activity of neutrophils in young cattle sensitized with a non-agglutinogenic strain of *Brucella*.

Materials and methods. The functional and metabolic state of neutrophils in young cattle immunized against brucellosis with a vaccine produced from the non-agglutinogenic RB-51 strain of *Brucella abortus* was assessed on days 7, 14, 21, 28, 35 after immunization using nitroblue tetrazolium (NBT) test, as well as based on the level of the enzymatic activity of myeloperoxidase and the content of non-enzymatic cationic proteins. The measurements were made photometrically in the spontaneous and stimulated variants of the test, with subsequent calculation of stimulation coefficients. Disintegrated and corpuscular antigens prepared from *Brucella* vaccine strains with different antigen structures were used as reaction stimulants.

Results. It was found that the functional and metabolic status of neutrophils in young cattle immunized with the non-agglutinogenic strain of *Brucella* is characterized by increased neutrophil activity in the NBT test on days 7 and 35 of the experiment, by the absence of significant changes in the enzymatic activity of myeloperoxidase and a decrease in the content of non-enzymatic cationic proteins on days 7–14 after vaccination.

Conclusion. The most pronounced increase in stimulation coefficients was observed when using disintegrated *Brucella* antigens as a reaction stimulant. The highest stimulation coefficients were registered on day 28 after vaccination during the assessment of the oxygen-dependent metabolism of neutrophils with the NBT test and on day 14 during the assessment of the oxygen-independent metabolism.

Keywords: neutrophils, antigens, cattle, vaccination, *Brucella*

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Функционально-метаболическая активность нейтрофилов у молодняка крупного рогатого скота, сенсибилизированного неагглютиногенным штаммом бруцелл

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РЕЗЮМЕ

Введение. Бруцеллез остается одной из наиболее распространенных инфекций в группе особо опасных зоонозов. Устойчивость к патогенным микроорганизмам рода *Brucella* зависит от полноценного клеточно-опосредованного иммунитета, включающего в себя активацию бактерицидных механизмов

фагоцитов. Несмотря на неоднократно доказанную роль нейтрофилов в борьбе со многими бактериальными патогенами, функции этих иммунокомпетентных клеток при бруцеллезе оставались неизученными в течение продолжительного времени.

Цель исследования. Изучение функционально-метаболической активности нейтрофилов у молодняка крупного рогатого скота, сенсибилизированного неагглютиногенным штаммом бруцелл.

Материалы и методы. У молодняка крупного рогатого скота, иммунизированного против бруцеллеза вакциной из неагглютиногенного штамма *Brucella abortus* RB-51, оценивали функционально-метаболическое состояние нейтрофилов на 7, 14, 21, 28, 35-е сут после иммунизации в тесте с нитросиним тетразолием, а также по уровню ферментной активности миелопероксидазы и содержанию неферментных катионных белков. Измерения показателей проводили фотометрическим способом в спонтанном и стимулированном вариантах постановки с последующим расчетом коэффициентов стимуляции. В качестве стимуляторов реакции применяли дезинтеграты бруцелл и корпускулярные антигены, изготовленные из вакцинных штаммов бруцелл с разной антигенной структурой.

Результаты. Было установлено, что при иммунизации молодняка крупного рогатого скота неагглютиногенным штаммом бруцелл функционально-метаболический статус нейтрофилов характеризуется усилением активности нейтрофилов в тесте с нитросиним тетразолием на 7-е и 35-е сут исследования, отсутствием выраженных изменений в показателях ферментной активности миелопероксидазы, а также снижением количества неферментных катионных белков на 7–14-е сут после вакцинации.

Заключение. Наиболее выраженное увеличение коэффициентов стимуляции отмечается при применении в качестве стимулятора реакции дезинтегратов бруцелл. При оценке кислородзависимого метаболизма нейтрофилов в тесте с нитросиним тетразолием максимальные значения коэффициентов стимуляции отмечали на 28-е сут после вакцинации, при оценке кислороднезависимого метаболизма – на 14-е сут.

Ключевые слова: нейтрофилы, антигены, крупный рогатый скот, вакцинация, бруцеллы

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INTRODUCTION

Despite the scientifically based system of brucellosis control measures in place in animal farming, bovine brucellosis remains endemic in most territories of the Russian Federation and poses a risk to livestock farms [1, 2].

One of the main components of the said system is now specific prevention [3, 4, 5, 6] mainly aimed at the reproduction of asymptomatic or latent infection in farm animals in combination with non-sterile immunity turning into post-infection sterile one [7, 8, 9].

The resistance of a macroorganism to the pathogenic microorganisms of the genus *Brucella* at the first stages of infectious process development depends on the activity of cellular protection factors, namely the activation of the bactericidal mechanisms of phagocytes [10, 11, 12]. Polymorphonuclear neutrophils are the main phagocytic cells responsible for protection against brucellosis. The functional and metabolic status of neutrophils determines the severity of the inflammatory reaction that develops in response to the entry of infectious pathogens into the body [13, 14, 15]. The bactericidal properties of neutrophils are provided by hydrolytic enzymes, cationic proteins and reactive oxygen species [16, 17, 18].

The examination of the enzymatic and non-enzymatic systems of neutrophils makes it possible to detect changes in the body at the early stages of infectious process development, prior to the occurrence of more profound changes in the organs and systems, which are detected with conventional test methods. Scientific literature describes the specific features of neutrophil system functioning revealed by tests in laboratory and other animals [19, 20, 21].

Cell-mediated immunity to brucellosis in food producing animals is an issue of particular scientific interest today. *In vitro* tests for cellular response to stimulation with *Brucella* antigens prepared at the Omsk Agrarian Scientific Center can be considered an informative and objective approach to analyzing the immunological restructuring of the body at the early post-vaccination stages, which is very important when evaluating the effectiveness of immunobiological products.

The study aimed to examine the functional and metabolic activity of neutrophils in young cattle sensitized with a non-agglutinogenic strain of *Brucella*.

MATERIALS AND METHODS

The work was performed at the Department of Veterinary Medicine of the Omsk Agrarian Scientific Center.

The test material was heparinized bovine peripheral blood. Sampling was carried out before vaccination and on days 7, 14, 21, 28, 35 after vaccination.

Bacterial strains. The antigens were prepared using the following *Brucella* strains from the bioresource collection of the Department of Veterinary Medicine of the

Omsk Agrarian Scientific Center: *Brucella abortus* 16/4 in the stable R-form and *Brucella abortus* 19 in the S-form.

Young animals were immunized with bovine brucellosis vaccine based on the non-agglutinogenic RB-51 strain of *B. abortus* (the USA).

Animals. The experiment was carried out in 4–5-month-old Red Steppe heifers (n = 50). The animals were loose housed and received a balanced diet.

The antigens were prepared at the scientific laboratory using the modified methods of N. P. Ivanov [22].

C_S is a corpuscular antigen prepared from *B. abortus* 19 strain.

C_R is a corpuscular antigen prepared from *B. abortus* 16/4 strain.

D_S is a disintegrated antigen prepared from *B. abortus* 19 strain by ultrasonic disintegration.

D_R is a disintegrated antigen prepared from *B. abortus* 16/4 strain by ultrasonic disintegration.

The functional and metabolic state of neutrophils was assessed with nitroblue tetrazolium (NBT) test using a modified method [23], as well as based on the content of cationic proteins and myeloperoxidase using a modified method described by N. M. Khitrik [24]. The measurements were made photometrically in the spontaneous (without antigen treatment) and stimulated (with antigen treatment) variants of the test. The test results were read using a Fluorofot STD-Less-486-M multichannel immunochemistry analyzer (Russia) and expressed in relative optical density units, with subsequent calculation of the stimulation coefficient according to the following formula:

$$\text{Stimulation coefficient} = \frac{\text{value for stimulated sample}}{\text{value for spontaneous sample}}.$$

The disintegrated (D_R and D_S) and corpuscular (C_R and C_S) *Brucella* antigens were used as reaction stimulants.

The mathematical processing of the obtained numerical data was carried out using the standard methods of variation statistics involving the determination of arithmetic mean (M) values and the calculation of arithmetic mean errors (m). Student's t-test was used to assess the significance of differences (p). We also applied the normalized deviation method involving automatic determination with a special computer software [25] using the following formula:

$$t = \frac{M_2 - M_1}{S_{d_1}},$$

where t is the normalized deviation;

M is the mean for the test (M_2) and control (M_1) groups;

S_d is the standard deviation for the control group.

RESULTS AND DISCUSSION

The study was conducted on a brucellosis-free commercial farm where bovine brucellosis vaccine based on the non-agglutinogenic *B. abortus* RB-51 strain is used on a regular basis.

The initial stage of the study included the assessment of the functional and metabolic state of neutrophils with NBT test, tests for the enzymatic activity of myeloperoxidase and non-enzymatic cationic protein content carried out at different time points after the sensitization of the animals with the non-agglutinogenic strain of *Brucella*.

It was found that after an increase by day 7 from the start of the experiment, the spontaneous tetrazolium activity of neutrophils showed a slight downward trend and reached a minimum by day 28, then it increased again, but did not reach a significant difference as compared with the baseline values.

When the corpuscular (C_S, C_R) and disintegrated (D_S, D_R) antigens were added to the phagocyte cell suspension, the increased generation of oxygen radicals in neutrophil granulocytes was also observed by day 7; then it returned to the initial level (that before the administration of the vaccine) on days 14–28 from the start of the experiment depending on the antigen used. It should be noted that on day 35, there was again an increase in the induced NBT activity. In particular, a statistically significant 2.1-fold ($p < 0.05$) and 1.9-fold ($p < 0.05$) increase was registered after stimulation with the C_S and D_S antigens, respectively, as compared with the relevant values before vaccination.

From day 14 after vaccination, an increase in the NBT stimulation coefficient was observed when using the corpuscular C_S, C_R and disintegrated D_S antigens as inducers, whereas the stimulating effect of the D_R antigen was observed starting from day 21. It should also be noted that the stimulation coefficient reached its maximum values on day 28 from the start of the experiment, especially when the phagocytes interacted with the disintegrated D_S and D_R antigens (the coefficient increased 1.6- and 2.3-fold, respectively, as compared with the values before sensitization with *Brucella*). Subsequently, a decrease in the NBT stimulation coefficients was observed; however, the coefficient remained at the same level when the C_S antigen was used (Table 1).

The data from the tests confirmed our previous findings. In particular, the highest stimulation coefficients in the tests for the tetrazolium activity of neutrophils in guinea pigs immunized with a non-agglutinogenic strain of *Brucella* had been observed on day 28 after immunization [26].

The spontaneous and stimulated enzymatic activity of myeloperoxidase, which also characterizes the oxygen-production ability of neutrophils, did not show any statistically significant changes during the dynamic tests. The stimulation coefficients reached their maximum values on day 21 from the start of the experiment, except when the disintegrated D_S antigen was added to the blood samples (Table 2).

Neutrophil cationic proteins are another indicator, which, unlike the previous two, characterizes the anaerobic metabolism of phagocytes. The dynamic tests revealed certain specific features of its changes that were not observed during the tests for the tetrazolium and enzymatic activity of neutrophils. In particular, the spontaneous activity of antimicrobial peptides (2.15 ± 0.48) at the beginning of the experiment) reached a minimum (1.03 ± 0.03) on day 14 after the sensitization of the animals with *Brucella* and then, after a short-term slight increase (up to 1.23 ± 0.19) on day 28, decreased again (to 1.07 ± 0.11) on day 35.

When the blood samples were treated with the corpuscular antigen prepared from the S-strain, the activity of cationic proteins decreased 1.3-fold ($p < 0.05$) by day 14 from the start of the experiment, then 1.78-fold ($p < 0.05$)

Table 1
Stimulation coefficient dynamics in tests for tetrazolium activity of neutrophil granulocytes in young cattle at different time points after vaccination, $M \pm m$

Antigen	Days after vaccination					
	Before vaccination	7	14	21	28	35
C_S	0.63 ± 0.17	0.65 ± 0.02	1.00 ± 0.28	1.05 ± 0.06	1.00 ± 0.08	1.03 ± 0.24
C_R	0.72 ± 0.31	0.75 ± 0.13	0.89 ± 0.10	0.85 ± 0.10	1.04 ± 0.14	0.80 ± 0.11
D_S	0.82 ± 0.44	0.71 ± 0.09	0.85 ± 0.22	0.87 ± 0.09	1.33 ± 0.19	0.80 ± 0.16
D_R	0.78 ± 0.25	0.70 ± 0.07	0.72 ± 0.14	1.18 ± 0.33	1.80 ± 0.32	0.73 ± 0.13

Table 2
Stimulation coefficient dynamics in tests for enzymatic activity of myeloperoxidase of neutrophil granulocytes in young cattle at different time points after vaccination, $M \pm m$

Antigen	Days after vaccination					
	Before vaccination	7	14	21	28	35
C_S	0.87 ± 0.13	0.99 ± 0.02	0.99 ± 0.01	1.02 ± 0.01	0.99 ± 0.01	1.00 ± 0.01
C_R	1.00 ± 0.02	0.99 ± 0.02	0.97 ± 0.01	1.03 ± 0.01	0.95 ± 0.01	0.97 ± 0.01
D_S	1.01 ± 0.03	1.00 ± 0.01	1.00 ± 0.004	0.98 ± 0.02	0.85 ± 0.10	0.99 ± 0.01
D_R	0.96 ± 0.02	0.98 ± 0.02	0.97 ± 0.01	1.03 ± 0.01	0.94 ± 0.01	0.96 ± 0.01

on day 28 and 1.67-fold ($p < 0.05$) on day 35 as compared with the baseline values. When the antigen prepared from *Brucella* R-strain was used, a significant decrease in the oxygen-independent metabolism of neutrophils was registered from day 21 after the administration of the non-agglutinogenic *Brucella* strain to young cattle.

When the disintegrated D_S- and D_R-antigens were used, a decrease in the activity of neutrophil cationic proteins was observed at a later time point. In particular, the antimicrobial activity of phagocytes decreased 1.73-fold ($p < 0.05$) and 2.24-fold ($p < 0.05$) on day 28 from the start of the experiment and 2.46-fold ($p < 0.05$) and 3.4-fold ($p < 0.05$) on day 35, respectively, as compared with the initial values.

On day 14 after the sensitization of the animals with *Brucella*, the stimulation coefficient reached its maximum value, with the most pronounced 1.54-fold ($p < 0.05$) and 1.68-fold ($p < 0.05$) increase, as compared with the baseline values, having been observed when using the disintegrated D_S and D_R antigens, respectively. At the subsequent time points of the study, the coefficient decreased (Table 3).

The stimulation coefficient dynamics in the tests for non-enzymatic cationic proteins of neutrophils in young cattle confirmed our previous findings from the experiment in guinea pigs immunized with a non-agglutinogenic strain of *Brucella*, during which the most pronounced activity of non-enzymatic cationic proteins of neutrophils was observed on day 14 after the administration of the immunobiological [27].

At the next stage of the study, a special computer software was applied to statistically process the stimulation coefficients of the NBT test, myeloperoxidase and cationic proteins, using the normalized deviation method to determine the degree of their transformation as compared with the mean values at different time points of the experiment. When the deviation from the mean exceeded +1.0 sigma, the difference was considered statistically significant, which was indicative of a pronounced specific sensitization of neutrophils to *Brucella*. The results are presented in Table 4.

The data show that in the NBT test, a pronounced specific sensitization was registered on days 14–35 from the start of the experiment when using the corpuscular

Table 3
Stimulation coefficient dynamics in tests for non-enzymatic cationic proteins of neutrophils in young cattle at different time points after vaccination, $M \pm m$

Antigen	Days after vaccination					
	Before vaccination	7	14	21	28	35
C_S	0.89 ± 0.18	1.22 ± 0.21	1.30 ± 0.10	1.49 ± 0.31	0.86 ± 0.12	0.96 ± 0.13
C_R	1.24 ± 0.14	1.25 ± 0.27	1.80 ± 0.26	1.03 ± 0.03[a]	1.10 ± 0.26	1.38 ± 0.16
D_S	1.43 ± 0.18	1.45 ± 0.35	2.20 ± 0.19[a]	1.41 ± 0.24	1.40 ± 0.20	1.16 ± 0.19
D_R	1.28 ± 0.18	1.31 ± 0.28	2.15 ± 0.11[a]	1.52 ± 0.29	0.96 ± 0.12	0.74 ± 0.06[a]

[a] $p < 0.05$.

Table 4
Determination of specific sensitization of neutrophils to *Brucella* by analysis of stimulation coefficients of NBT test, myeloperoxidase and cationic proteins

Antigen	Days after vaccination				
	7	14	21	28	35
	NBT test				
C_S	+0.05	+1.21	+1.39	+1.21	+1.30
C_R	+0.52	+0.30	+0.24	+0.60	+0.13
D_S	–0.14	+0.41	+0.61	+0.67	–0.20
D_R	–0.19	–0.14	+0.93	+2.38	–0.10
	myeloperoxidase				
C_S	+0.50	+0.53	+0.67	+0.50	+0.54
C_R	–0.30	–0.72	+0.96	–1.47	–0.90
D_S	–0.17	–0.29	–0.53	–2.78	–0.44
D_R	+0.40	+0.22	+1.69	–0.66	–0.07
	cationic proteins				
C_S	+1.06	+1.32	+1.93	–0.10	+0.21
C_R	+0.05	+2.23	–1.47	–0.56	+0.55
D_S	+0.06	+2.42	–0.07	–0.09	–0.85
D_R	+0.09	+2.76	+0.76	–1.01	–1.69

C_S antigen and on day 28 when using the disintegrated D_R antigen as a stimulant, whereas in the tests for the enzymatic activity of myeloperoxidase, it was registered only on day 21 when using the disintegrated D_R antigen as an inducer (t = +1.69).

In the tests for cationic proteins, which perform their function under anaerobic conditions, specific sensitization was registered at earlier time points as compared with the indicators of oxygen-dependent metabolism. In particular, when the C_S antigen was used, a deviation from the mean that exceeded +1.0 sigma was observed from day 7 to day 21, whereas when the C_R-, D_S- and D_R-antigens were used, it was observed only on day 14. It should be noted that the disintegrated antigens induced a more pronounced specific sensitization (from +2.42 and above).

CONCLUSION

The results of the tests performed show that the functional and metabolic activity of neutrophils in young cattle immunized with *B. abortus* RB-51 strain is characterized by a 1.1–2.4-fold increase in the spontaneous and stimulated tetrazolium activity of neutrophils on days 7 and 35 after vaccination irrespective of the antigen used, a 1.2–2.1-fold decrease in the concentration of cationic proteins from days 7–14 from the start of the experiment and the absence of any pronounced changes in the content of myeloperoxidase.

A pronounced (1.5–2.3-fold) increase in the stimulation coefficients was observed when the disintegrated antigens were used. Based on the results of the mathematical processing involving the use of the normalized deviation method, the highest stimulation coefficients were observed during the assessment of the aerobic metabolism of neutrophils (NBT test) on day 28 after the inoculation of the vaccine strain when using the D_R antigen (t = +2.38) and during the assessment of anaerobic metabolism (cationic proteins) on day 14 when using the D_R and D_S antigens (t = +2.42 and +2.76, respectively), which was indicative of a pronounced specific sensitization of neutrophils to *Brucella*.

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Preparation of recombinant SARS-CoV-2 nucleocapsid protein

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ABSTRACT

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

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

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

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

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

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

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Получение рекомбинантного нуклеокапсидного белка SARS-CoV-2

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РЕЗЮМЕ

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

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

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

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

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

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

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

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INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

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

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

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

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

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

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

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

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

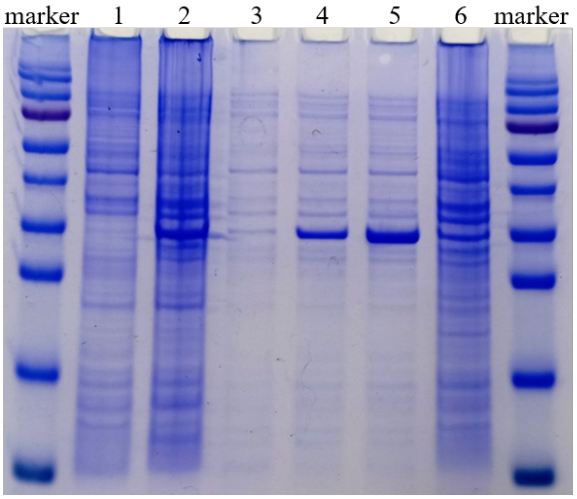

Fig. 1. Determination of optimal period of recombinant SARS-CoV-2 nucleocapsid protein expression:
protein molecular weight marker (170, 130, 95, 72, 55, 43, 34, 26, 17, 10 kDa);
1 – E. coli JM109 cell lysate;
2 – cell lysate of SARS-CoV-2 N-gene-containing E. coli clone, 18 hours after incubation;
3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

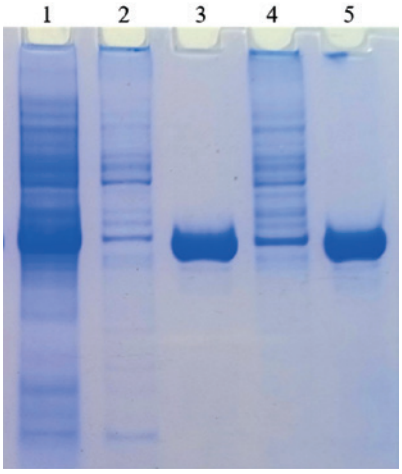

Fig. 2. Effect of lysing buffer composition on purified recombinant SARS-CoV-2 nucleocapsid protein solubility and yield:
1 – cell lysate of recombinant E. coli clone, 4 hours after induction;
2 – sediment of recombinant protein-expressing cells after treatment with lysis buffer No. 1 containing 8 M urea;
3 – purified recombinant protein when denaturing buffer containing 8 M urea was used;
4 – sediment of recombinant protein-expressing cells after treatment with lysis buffer No. 2 containing 6 M Gu-HCl;
5 – purified recombinant protein when denaturing buffer containing 6 M Gu-HCl was used

hydrochloride (Gu-HCl) – for lysis buffer No. 2. Under denaturing conditions, most of the protein was cleared from cellular debris by centrifugation. The protein yield was approximately the same when both 8 M urea and 6 M GuHCl were used (Fig. 2). It was decided to use 8 M urea as a denaturing agent.

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

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

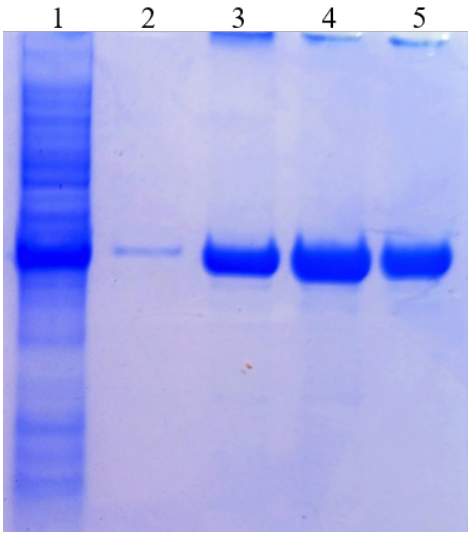

Fig. 3. Effect of elution buffer composition on purified recombinant SARS-CoV-2 nucleocapsid protein yield:
1 – cell lysate of recombinant E. coli clone, 4 hours after induction;
2 – purified recombinant protein when buffer A was used;
3 – purified recombinant protein when buffer B was used;
4 – purified recombinant protein when buffer C was used;
5 – purified recombinant protein when buffer D was used

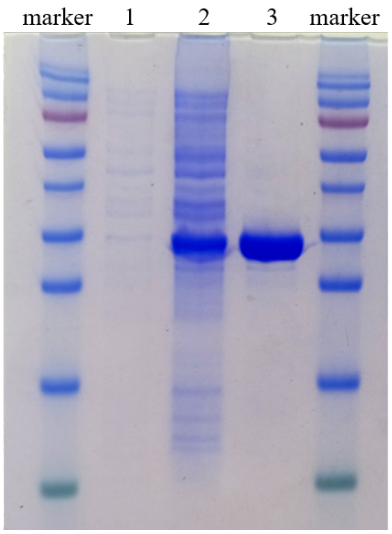

Fig. 4. Assessment of recombinant SARS-CoV-2 nucleocapsid protein size and purification level:
protein molecular weight marker (170, 130, 95, 72, 55, 43, 34, 26, 17, 10 kDa);
1 – cell lysate of recombinant E. coli clone before induction;
2 – cell lysate of recombinant E. coli clone, 4 hours after induction;
3 – purified recombinant protein

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

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

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

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

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

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

CONCLUSION

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

E. coli clones expressing recombinant nucleocapsid protein were prepared.

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

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

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Polymorphisms in *TLR4* gene associated with risks of bovine mastitis development

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ABSTRACT

Introduction. Inflammatory mammary diseases in cows remain the most common challenge in dairy industry, notwithstanding the improved preventive measures and treatment schemes. One of the methods to prevent mastitis in dairy cows is the genetic selection of the most disease-resistant individuals. Toll-like receptor 4 (*TLR4*) plays a central role in the innate immune response. There are publications about *TLR4* significance for mastitis development, its genetic polymorphisms associated with somatic cell counts.

Objective. Determination of genetic diversity and association with the development of clinical mastitis for three polymorphic loci of *TLR4*.

Materials and methods. To achieve the objective cattle health history ($n = 421$) was used, subclinical mastitis was diagnosed using rapid test for somatic cell counting in milk, TaqMan real-time polymerase chain reaction was used for genotyping of cattle for rs8193046, rs8193060, rs29017188 polymorphisms.

Results. Association studies established that rs8193046 and rs29017188 polymorphisms are the most promising candidates to be used in selection programs aimed at mastitis risk mitigation in the Ural populations. For rs8193060 no reliable results of association tests are obtained, though risk of mastitis in GCG haplotype-animals (for SNP rs8193046, rs8193060, rs29017188 alleles) is statistically lower.

Conclusion. It is noted that the abovementioned polymorphisms can be used for marker-assisted selection of cattle to prevent risks of mastitis in the populations in the Ural.

Keywords: *TLR4*, mastitis, cattle, risk of disease development, association tests

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Ассоциации полиморфизмов гена *TLR4* с риском развития мастита крупного рогатого скота

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РЕЗЮМЕ

Введение. Воспалительные заболевания молочной железы коров остаются наиболее распространенной проблемой молочного скотоводства, несмотря на оптимизируемые профилактические меры и схемы лечения. Одним из способов предупреждения развития мастита у коров молочного направления продуктивности является генетическая селекция наиболее устойчивых к заболеванию особей. Толл-подобный рецептор 4 (*TLR4*) играет ключевую роль во врожденном иммунитете, в литературе имеются данные о его значимом влиянии на развитие мастита, описаны ассоциации генетических полиморфизмов гена *TLR4* со значениями индекса соматических клеток.

Цель исследования. Определение генетического разнообразия и степени ассоциации с развитием клинического мастита для 3 полиморфных локусов, расположенных в гене *TLR4*.

Материалы и методы. Для достижения поставленной цели использованы данные анамнеза крупного рогатого скота ($n = 421$), проведена диагностика субклинического мастита при помощи экспресс-теста для определения количества соматических клеток в молоке, при генотипировании крупного рогатого скота по полиморфизмам rs8193046, rs8193060, rs29017188 применена полимеразная цепная реакция в реальном времени по технологии TaqMan.

Результаты. При проведении ассоциативных тестов установлено, что полиморфизмы rs8193046 и rs29017188 являются наиболее перспективными кандидатами для использования в селекционных программах для снижения риска заболеваемости маститом в популяциях Уральского региона. Для rs8193060 отдельно достоверных результатов ассоциативных тестов не выявлено, однако животные с гаплотипом GCG (для аллелей SNP rs8193046, rs8193060, rs29017188) имеют статистически значимый более низкий риск развития мастита.

Заключение. Отмечено, что данные полиморфизмы можно использовать для маркер-ориентированной селекции крупного рогатого скота для профилактики риска развития мастита в популяциях Уральского региона.

Ключевые слова: *TLR4*, мастит, крупный рогатый скот, риск развития заболевания, ассоциативные тесты

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INTRODUCTION

Inflammation of a mammary gland (mastitis) is one of the costliest diseases in cattle. Preventive measures and treatment regimens are being optimized, including in order to save labor and expenses. Such measures include preventive vaccination and administration of various antimicrobial drugs [1, 2, 3]. An alternative to reduce mastitis incidence in farms is genetic selection. Long-term selection of dairy cattle for high milk flow, preferred due to machine milking, resulted in weakening of the mammary streak canal sphincter that represents a physical barrier for pathogen entry [4]. Susceptibility to mastitis is based on a number of factors, both external (nutrition, keeping practices, stress factors, milking techniques) and internal (immune mechanisms, important to be understood in order to increase the resistance of animals) [5].

The immune response plays a key role in the disease pathogenesis. Toll-like receptor 4 (*TLR4*), as an intrinsic immune receptor, exhibits widespread *in vivo* expression and its dysregulation significantly contributes to the onset of various diseases, encompassing cardiovascular disorders, neoplastic conditions, and inflammatory ailments [6]. The search for associations with colibacillosis risks revealed that *TLR4* (rs8193046) gene polymorphism *G** allele frequency was higher in diarrheic calves than in control animals [7]. In a study of the association of single nucleotide polymorphism (SNP) with the risk of paratuberculosis caused by *Mycobacterium avium*, it was shown that *A/G* heterozygotes produced a higher risk of this infectious disease [8]. An experiment aimed to find polymorphism haplotypes in *TLR4* gene and conducted in different cattle populations revealed that the *A** allele is present in all haplotypes and might negatively effect on milk somatic cells. The *C** allele also has a negative effect on this value and the *G** allele might positively effect on milk somatic cells [9]. It is worth noting that in this study, no corrections for multiple comparisons were made in the search for statistically significant haplotypes. At the same time, in a study of the some SNP associations with risks of subclinical mastitis, it was shown that individuals with *G/G* genotype had higher average somatic cell counts [10].

In the case of rs8193060, indications were obtained for the association with reproductive traits: incidence of cystic ovaries, early reproductive disorders, calving ease, and production longevity [11]. There is evidence of the genetic association of polymorphisms with paratuberculosis infection, moreover it was established that the *C/T* genotype might be beneficial [12]. The study aimed to find polymorphism haplotypes in the *TLR4* gene for rs8193060 produced ambiguous results: the *C** allele might confer both positive and negative effects [9]. The analysis of rs8193060 associations with somatic cell counts showed that *T/T* genotype is not beneficial [10].

The rs29017188 polymorphism has the highest pleiotropic effect based on full-genome studies. There is evidence of its effect on the calving interval [13], lactation persistence [14] and milk composition [15].

Unfortunately, to date, no mechanisms have been identified for how exactly *TLR4* gene polymorphisms affect the body's immune functions. Bhat R. R. et al. described the supposed mechanism of SNPs falling in *TLR4* promoter and 5′ untranslated region. Researchers have found absence of heterozygous condition in these loci in individuals with susceptibility to mastitis, which is most likely due to transcriptional factor binding profile, which ultimately changes the expression of this gene [16].

Based on the above, the aim of the study was to analyze the genetic diversity and to search for associations of *TRL4* polymorphisms with the risks of mastitis in cattle.

MATERIALS AND METHODS

For genotyping of 421 cattle for rs8193046, rs8193060, rs29017188, the protocol previously described by A. Q. de Mesquita et al. [10] with a change in oligonucleotides for rs8193046 was used (Table 1). Herewith all animals were genotyped for rs8193060 and rs29017188, and 387 out of them for rs8193046. Animals from five farms of the Ural region were used.

The criterion for inclusion an animal into a risk group was clinical and subclinical mastitis in the disease history. Rapid tests measuring somatic cell counts were used to diagnose subclinical mastitis. If no mastitis had been recorded in the animal during three lactation periods, and the rapid test showed a negative result, the animal was considered resistant to mastitis. Blood was collected from the tail vein of all animals into vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid) as an anticoagulant.

Table 1
Oligonucleotide sequences

SNP	Oligonucleotide sequence	Amplicon length, bp
rs8193046	F, GAGAGGAGAGTTGCTTGGAAGTCT	107
	R,GCTCCATGCACTGGTAACTAATGT	
	P1, [HEX]CAGGAAGACACCGCA[BHQ1]	
	P2, [ROX]CAGGAAGACACCACA[BHQ2]	
rs8193060	F, CCACTCGCTCCGGATCCT	79
	R,CCTTGGCAAATTCTGTAGTTCTTG	
	P1, [HEX]ACTGCAGTTTCAACCGTATC[BHQ1]	
	P2, [ROX]ACTGCAGCTTCAACCGTA[BHQ2]	
rs29017188	F, CCAGCTTCCTCTTGTTGTTACTTCA	150
	R,CGGGAGGAGAGGAAGTGAGA	
	P1, [HEX]TATTTATCTCCTCTGCCACCGGA[BHQ1]	
	P2, [ROX]TTATCTCCTCTGCCACCCGAG[BHQ2]	

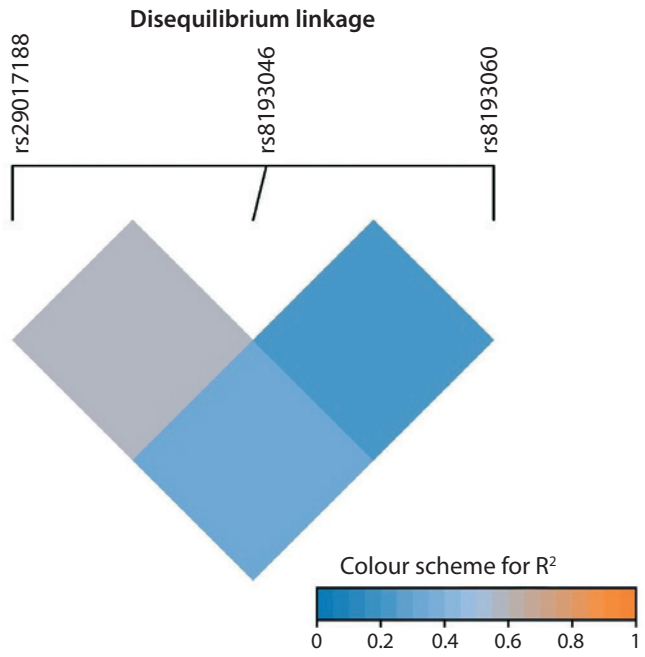

Fig. 1. Linkage disequilibrium (R^2) plot of the studied SNPs

The genotype distribution was analyzed for compliance with Hardy – Weinberg principle; linkage disequilibrium and the Shannon diversity index were calculated using GenAlEx package (version 6.5) for Microsoft Excel [17]. Linkage disequilibrium graphs were constructed using SRplot web tool [18].

Association tests for each SNP individually, the search for the most common haplotypes and their associations with mastitis risks were performed by SNPassoc R package (version 2.1.0) [19].

RESULTS AND DISCUSSION

The allelic diversity and genotype distribution are shown in Table 2. When calculating the Hardy – Weinberg proportion, 1 degree of freedom was used. Based on the analysis results, a statistically significant deviation from equilibrium allele distribution was revealed for rs8193046 polymorphism. Such deviations can occur for a number of reasons: selective pressure, genotyping errors, inbreeding. The most likely explanation is the pressure of artificial selection.

Based on the linkage disequilibrium analysis, it can be concluded that the pairs of rs8193046 and rs8193060 alleles, as well as rs29017188 and rs8193060 demonstrate linkage disequilibrium: $R^2 = 0.2$ and $R^2 = 0.4$, respectively (Fig. 1).

The genetic diversity of the studied cattle populations was also evaluated by *TLR4* gene polymorphisms. The Shannon diversity index approaching 1 reflects a high diversity level. Thanks to the analysis, it was found that the diversity between populations is low (D′ = 0.015). However, on average, a higher diversity index value (D′ = 0.403) can be observed within populations, from which it can be concluded that they are genetically stable (Fig. 2). Since there are no differences in the diversity for these polymorphisms between the studied populations, further tests were performed jointly.

Association tests were performed individually for each of the *TLR4* gene polymorphisms to identify associations with mastitis risks. A summary of the results is presented in Table 3: rs29017188 SNP showed the largest number of statistically significant inheritance models, including taking into account the Bonferroni correction, while the recessive inheritance of mastitis risk was significant for rs8193046 and rs29017188. The recessive inheritance for rs8193046 also has the lowest Akaike information criterion (AIC) value, and the odds ratio (OR) suggests that animals with *A/A* genotype have a higher risk of mastitis.

Table 2
Genotype distribution, allele frequency, and *p*-value of Hardy – Weinberg equilibrium

Locus	Genotype	Number of animals	Genotypic frequency, %	Alleles	Allelic frequency	X^2 (*p*-value)
rs8193046 *G > A*	*A/A* *A/G* *G/G*	89 145 153	23.0 37.5 39.5	*A** *G**	323 451	20.397 (< 0.0001)
rs8193060 *T > C*	*C/C* *C/T* *T/T*	184 191 46	43.7 45.4 10.9	*C** *T**	559 283	0.116 (0.734)
rs29017188 *G > C*	*C/C* *C/G* *G/G*	75 212 134	17.8 50.4 31.8	*C** *G**	362 480	0.314 (0.575)

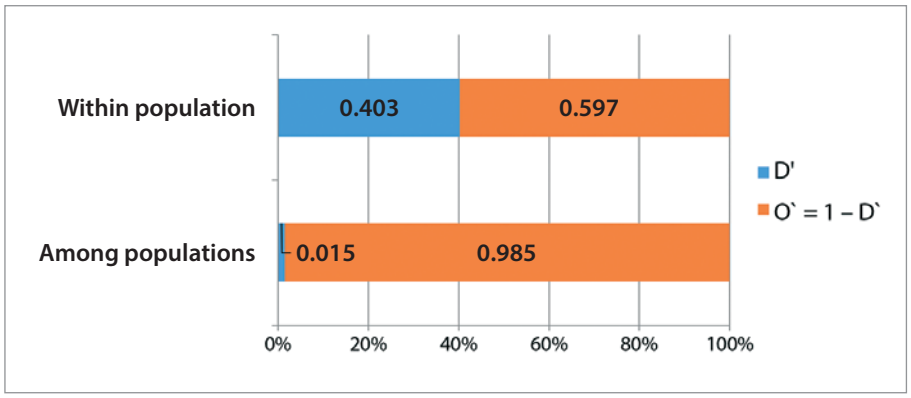

Fig. 2. Shannon diversity index for intra- and interpopulation assessment: D' – diversity index; O' – overlap index

For rs29017188, the recessive inheritance model also had the lowest AIC value, OR for the *C/C* genotype was 2.30, this means the risk of mastitis is presumably more than 2 times higher in individuals of the studied populations.

The results of the haplotype search showed that ACC, GCG, and GTG (for the rs8193046, rs8193060, and rs29017188 SNP alleles, respectively) are the most common, accounting for more than 85% of the total sample (Table 4).

Animals with the GCG haplotype have a statistically significant lower risk of mastitis (for SNPs rs8193046, rs8193060, rs29017188). We assume that an increase in the proportion of individuals with this haplotype in farms where mastitis in dairy cows is challenging may have a positive effect on the disease occurrence. The results of the search for associations of haplotypes with phenotypes coincide with the results of the identification of individual polymorphism associations and are more consistent with the data obtained by P. Wang et al. [9].

CONCLUSION

In the course of the study, the genetic diversity of the Ural dairy cattle populations was analyzed. The rs8193046 polymorphism of the *TRL4* gene revealed deviation from the Hardy – Weinberg equilibrium, which is most likely due to the influence of artificial selection pressure.

Based on the results of association tests, it was assumed that SNP rs8193046 and rs29017188 are the most promising candidates for use in breeding programs to reduce the risk of mastitis in the studied populations. It is worth noting the low effectiveness of genomic estimate extrapolation even among populations of the same breed; however, the results obtained during the study coincide with the previously published data [9]. The GCG haplotype for rs8193046, rs8193060, and rs29017188 was found to be statistically significant based on the association tests. This haplotype can be probably used for positive selection to reduce the risk of clinical mastitis in dairy cattle populations.

Table 3
Results of association tests for each of the SNPs for five inheritance models

SNP	Codominant	Dominant	Recessive	Overdominant	log-additive
rs8193046	0.04887*	0.17175	**0.01478***	0.47568	0.02935*
rs8193060	0.51985	0.34612	0.73568	0.25287	0.58147
rs29017188	**0.00671***	0.06545	**0.00248***	0.57736	**0.00368***

* *p*-value ≤ 0.05; in bold – *p*-value ≤ 0.016 (Bonferroni correction).

Table 4
Haplotypes and their associations with mastitis risks

rs8193046, rs8193060, rs29017188	Frequency	OR	95% confidence interval	*p*-value
ACC	0.3491	1.00	reference haplotype	–
GCC	0.0643	0.86	0.45–1.64	0.6515
GCG	0.2145	0.53	0.36–0.80	**0.0022***
GTG	0.3020	0.74	0.52–1.05	0.0894
Other rare haplotypes	0.0701	0.60	0.33–1.12	0.1083

* *p*-value ≤ 0.05; in bold – *p*-value ≤ 0.016 (Bonferroni correction).

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Optimization of freeze-drying process for anti-*Chlamydia* hyperimmune serum

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ABSTRACT

Introduction. The distribution area of *Chlamydia* infection in livestock and wild animals currently extends across almost all continents. At present, initial diagnosis, screening tests and certain stages of epizootiological investigations aimed at *Chlamydia* carrier detection are conducted in the Russian Federation using the "Antigen and Serum Kit for Serological Diagnosis of Chlamydiosis in Livestock". It is important in the production of diagnostics to ensure stability of different test kit components during their storage and transportation. One way of addressing this issue is to stabilize diagnosticum components by freeze-drying.

Objective. The study was aimed at optimization of freeze-drying process for specific anti-*Chlamydia* serum, the serum assessment for compliance with characteristics laid down in the technical specifications for the test kit control and testing of the serum for its stability.

Materials and methods. The serum was prepared using blood from sheep immunized with emulsion vaccine based on *Chlamydia psittaci* AMK-16 strain. Prior to freeze-drying, the hyperimmune sera were subjected to freezing to a temperature of minus 60 °C. The sera were freeze-dried using a Scientz 30F freeze-dryer (China). Two freeze-drying procedures with different temperature conditions and chamber pressures were applied. The resulting sera were tested for compliance with the technical specifications for the diagnostic test kit. The freeze-dried sera were put into storage for 24 months and tested with complement fixation test during this period.

Results. Based on the test results, the freeze-drying procedure employing a lower pressure and the highest heating temperature was found to be the most effective for the specific sera. The serum tests for compliance with characteristics laid down in the technical specifications for the test kit showed that the serum quality met all relevant requirements. The stability test results demonstrated that the hyperimmune serum freeze-dried using the improved procedure remains specific during 24 months.

Conclusion. The work performed allowed for optimization of freeze-drying process for specific anti-*Chlamydia* serum intended for the diagnostic test kit. The resulting serum is fully compliant with characteristics laid down in the technical specifications for the diagnosticum. It was established that the freeze-dried serum shelf life is at least two years, during this period the serum retains its activity and physico-chemical properties.

Keywords: chlamydiosis, *Chlamydia psittaci*, sublimation, freeze-drying (lyophilization), serum, test kit

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Отработка режима лиофилизации гипериммунной хламидийной сыворотки

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РЕЗЮМЕ

Введение. Современный ареал хламидийной инфекции сельскохозяйственных и диких животных охватывает почти все континенты. В настоящее время для постановки первичного диагноза, проведения скрининговых исследований и отдельных этапов эпизоотологических обследований с целью выявления хламидионосителей в нашей стране применяют «Набор антигенов и сывороток для серологической диагностики хламидиозов сельскохозяйственных животных». При производстве средств диагностики важной задачей является обеспечение стабильности различных компонентов тест-систем в процессе их хранения и транспортировки. Одним из путей решения этой проблемы является стабилизация различных компонентов диагностических препаратов посредством лиофилизации.

Цель исследования. Отработка режима лиофилизации специфической хламидийной сыворотки, оценка ее соответствия характеристикам, заявленным в технических условиях на контроль тест-системы, и испытание стабильности этого компонента.

Материалы и методы. Сыворотку получали из крови овец, иммунизированных эмульсионным вакцинным препаратом штамма «АМК-16» *Chlamydia psittaci*. До проведения процедуры сублимации гипериммунные сыворотки замораживали до температуры минус 60 °С. Лиофилизацию сывороток проводили на аппарате Scientz 30F (Китай) двумя способами, различающимися температурными режимами и давлением в камере. Готовые препараты сывороток крови оценивали на соответствие техническим условиям диагностического набора. Полученные сублиматы закладывали на хранение на срок 24 мес. и исследовали в реакции связывания комплемента на протяжении этого периода.

Результаты. В ходе проведенных исследований было установлено, что наиболее эффективным оказался способ лиофилизации специфических сывороток, при котором процесс сублимации проходил при более низком давлении и наиболее высокой температуре нагрева. Оценка соответствия полученного препарата характеристикам, заявленным в технических условиях на тест-систему, показала, что качество сыворотки отвечало всем требованиям. Результаты изучения стабильности гипериммунной сыворотки продемонстрировали, что высушенный усовершенствованным способом препарат не теряет своей специфичности на протяжении 24 мес.

Заключение. В результате проведенной работы был отработан оптимальный режим лиофилизации специфической хламидийной сыворотки для диагностической тест-системы. Полученный препарат полностью соответствует характеристикам, заявленным в технических условиях на диагностикум. Установлено, что длительность хранения лиофилизированной сыворотки составляет не менее двух лет, в течение данного периода ее активность и физико-химические свойства не снижаются.

Ключевые слова: хламидиоз, *Chlamydia psittaci*, сублимация, лиофилизация, сыворотка крови, тест-система

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INTRODUCTION

Chlamydiosis is an infectious disease common for animals and humans. It is caused by gram–negative bacteria of the family *Chlamydiaceae*, genus *Chlamydia* [1, 2, 3, 4]. Once in the body of an animal (cattle, sheep, pigs, horses, rodents, birds, cats, dogs and many others), *Chlamydia* affects various body systems [5] including the immune system, which, in turn, facilitates subsequent co-infection with other pathogens [1]. The concurrent clinical signs of chlamydiosis and other infections in the animals hamper the accurate and timely diagnosis [6]. *Chlamydia* infection more frequently takes a chronic course, which also hinders the timely detection of infected animals after the introduction of the pathogen to the herd [7].

The distribution area of *Chlamydia* infection in livestock and wild animals currently extends across almost all continents and therefore its diagnosis is an urgent issue [8, 9].

At present, initial diagnosis, screening tests and certain stages of epizootiological investigations aimed at *Chlamydia* carrier detection are conducted in the Russian Federation using the "Antigen and Serum Kit for Serological Diagnosis of Chlamydiosis in Livestock" (ROSS RU D-RU.RA01.V.19342/23) [10, 11].

It is important in the production of diagnostica to ensure stability of biological properties of various test kit components during their manufacture, storage and transportation [12, 13]. One way of addressing this issue is to stabilize the protein molecules of different diagnosticum components by freeze-drying [14, 15, 16].

The "Antigen and Serum Kit for Serological Diagnosis of Chlamydiosis in Livestock" manufactured by the Federal Center for Toxicological, Radiation and Biological Safety (Russia) contains two antigens (specific and control) and two animal sera (positive and negative to *Chlamydia* antigen). All components of the kit are freeze-dried [10].

During the test kit development, specific freeze-drying conditions were selected for each component of the kit. It should be noted that the freeze-drying procedure should be developed for each particular freeze-dryer depending on its design and characteristics, and freeze-drying parameters may vary for each component.

Scientz 30F freeze-dryer (China) was purchased as part of renovation of the technical facilities of the diagnostic test kit production site. It has more powerful vacuum pump and refrigeration unit. This has allowed for drying time reduction with the batch size remaining the same. Moreover, owing to the electronic control unit for drying parameters available in the freeze-dryer, the temperature and vacuum control ranges have become more accurate that makes it possible to set more process control points. Therefore, the drying procedure used previously for the old less powerful machine lacking precise control of the process parameters became unacceptable. New parameters and criteria for freeze-drying process control had to be developed and optimized. The scientific novelty of the work lies in the development of new parameters for freeze-drying of specific anti-*Chlamydia* serum using new equipment while maintaining the serum characteristics laid down in the technical specifications.

The study was aimed at optimization of freeze-drying process for specific anti-*Chlamydia* serum, the serum assessment for compliance with characteristics laid down in the technical specifications for the test kit control and the serum testing for its stability.

MATERIALS AND METHODS

The work was carried out at the Laboratory for Animal Viral Diseases, Federal Center for Toxicological, Radiation and Biological Safety.

Strain. Chlamydia psittaci AMK-16 strain with infectious titre of $10^{-5.4}$ LD_{50}/0.3 mL deposited to the Microorganism Strain Collection of the Federal Center for Toxicological, Radiation and Biological Safety (reference No. 11 of 5 September 2017) was used for the inactivated antigen preparation.

Biological models. To prepare the antigen, *Chlamydia* was cultivated in 6–7-day-old embryonated chicken eggs. Romanov sheep aged 1.5 years with a live weight of 45–50 kg were used to prepare hyperimmune sera.

During the experiment, the animals were handled in compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Test kits. The complement fixation test (CFT) was carried out using the "Antigen and Serum Kit for the serological diagnosis of *Chlamydia* in farm animals" (Federal Center for Toxicological, Radiation and Biological Safety, Kazan).

Equipment. The hyperimmune sera were freeze-dried using a Scientz 30F freeze dryer (China).

Methods. The antigen for immunization of the sheep was prepared from infected yolk membranes of embryonated chicken eggs that died on days 4–7 after infection by homogenizing them in phosphate buffered saline (pH 7.2) at a ratio of 1:9 with subsequent differential centrifugation to remove ballast substances, inactivation with formalin and concentration [17].

The serum was prepared from the blood collected from the sheep immunized with specific *Chlamydia* antigen emulsified in the original oil-lanolin adjuvant. The sera were tested for their antigenic activity with CFT using specific *Chlamydia* antigen in accordance with TU 9388-020-00492374-2007.

The sheep were bled under anesthesia. The blood was taken from the jugular vein. The blood was collected in sterile glass vessels, the inner surface of which was moistened with saline solution. To separate the serum, the vessels with blood were placed in a thermostat for 40–60 minutes, then blood clots were separated from the walls of the vessels, and the vessels were placed in a refrigerator at a temperature of 4 °C for 24 hours. The separated serum was decanted to remove the clots, centrifuged at 3.5 thousand rpm for 20 minutes to remove red blood cells and then preserved with boric acid.

The resulting sera were filled into paired glass ampoules (OST 64-2-485-85, Russia), 1.0 cm^3 per ampoule, using a BioHit single-channel pipette (Finland). In total, three batches of hyperimmune ovine sera were prepared for the study.

All sera were divided into two equal parts and freeze-dried using two different freeze-drying procedures.

Before freeze-drying, the sera in ampoules were frozen in a freeze dryer chamber at a temperature of minus 60 °C for 14 hours.

The freeze-dried sera were put for storage for 24 months. at a temperature of 18–22 °C. The freeze-dried hyperimmune sera were tested with CFT every month to assess its activity and to determine the shelf life.

The sera were tested for their antigenic activity with CFT before and after freeze-drying in accordance with the "Instruction for the use of the "Antigen and Serum Kit for Serological Diagnosis of Chlamydiosis in Livestock" approved by the Director of the Federal Center for Toxicological, Radiation and Biological Safety on 19 May 2016 (ROSS RU D-RU.RA01.V.19342/23).

The test was carried out in a volume of 1.0 cm^3 in Florinsky test tubes. The working dose of the antigen was used for the test. The sera were inactivated during 30 minutes and titrated by preparing 2-fold dilutions (starting from 1:5). Before test, the complement was titrated in the hemolytic system using its doubled dose; immune anti-*Chlamydia* and known negative serum as well as control antigen were used to control the test specificity. The hemolytic system was prepared using a 2.5% mixture of washed ram red blood cells and standard hemolytic serum at doubled titer. The test was carried out on water bath at a temperature of 37 °C. A 1:10 serum dilution was taken as the diagnostic titre, and a 1:5 dilution was considered inconclusive [18].

The resulting serum was to comply with the characteristics laid down in TU 9388-020-00492374-2007.

The prepared serum was tested for the following parameters: appearance, colour, presence of extraneous matter and mold, moisture content, solubility, CFT activity and specificity as well as shelf life.

The serum was visually examined for its appearance, colour and presence of extraneous matter and mold.

To test the freeze-dried serum for its solubility, saline solution was added to the ampoules with the serum, 1.0 cm^3 per ampoule, the ampoules were shaken and the time until the freeze-dried serum completely dissolved was recorded.

The moisture content in the freeze-dried serum was determined in accordance with the rules laid down in GOST 24061-2012. Freeze-dried sera weighing 0.1 g were ground into powder. The prepared samples were evenly distributed over the bottom of a preliminarily weighed weighing vessel. After weighing, the weighing vessels containing the serum samples were placed in a drying cabinet and kept at a temperature of 105 °C for 60 minutes. Then, they were cooled and weighed.

Moisture content was calculated according the following formula [19]:

$$X = \frac{M_1 - M_2}{M_1 - M_0} \times 100,$$

where X is moisture content, %;

M_1 is the weight of the weighing vessel containing the serum sample before drying, g;

M_2 is the weight of the weighing vessel containing the serum sample after drying, g;

M_0 is the weight of the weighing vessel without the serum sample, g.

STUDY RESULTS

During the first stage of the study, the prepared sheep sera were tested for their antigenic activity. The sera of all the three batches reacted with *Chlamydia* antigen at a titre of 1:160 when tested with CFT. Then the sera filled in ampoules, 1.0 cm^3 per ampoule, and frozen to a temperature of minus 60 °C, were freeze-dried using two different procedures. Temperature and vacuum pressure values during hyperimmune sheep serum freeze-drying according to procedure 1 are shown in Figure 1.

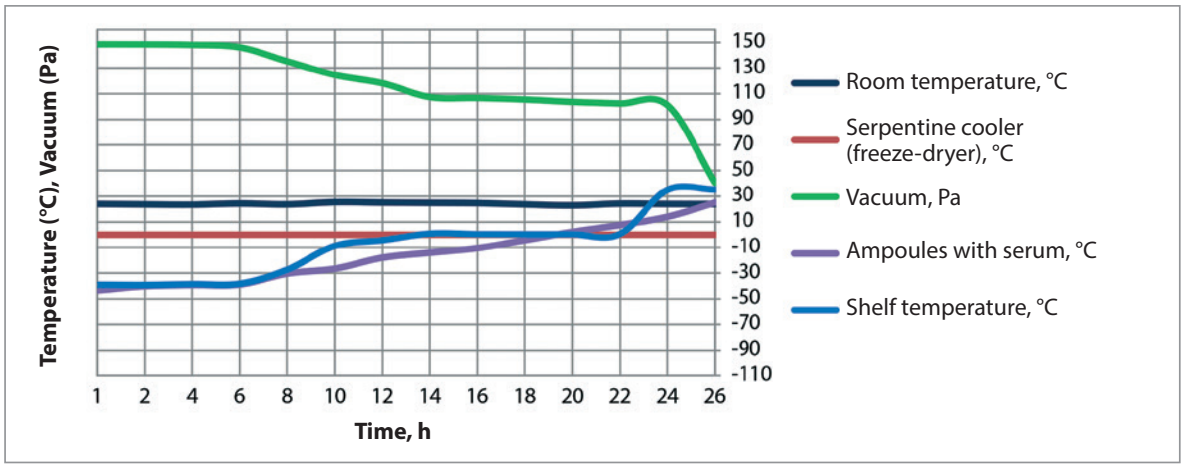

Fig. 1. Temperature conditions and vacuum pressure during hyperimmune serum freeze-drying using procedure 1

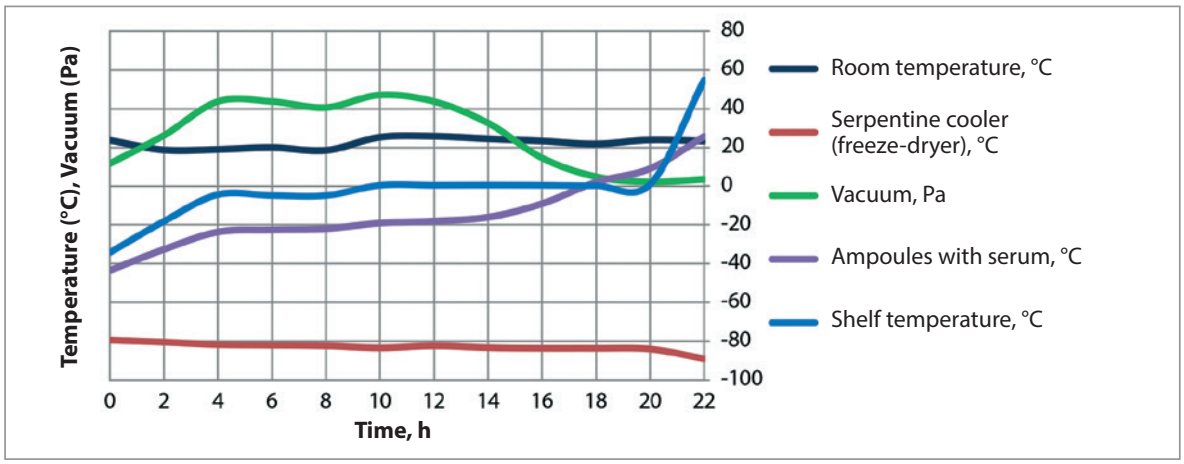

Fig. 2. Temperature conditions and vacuum pressure during hyperimmune serum freeze-drying using procedure 2

This freeze-drying procedure included keeping sera under vacuum for 26 hours with a gradual pressure decrease from 148 to 100 Pa within the first 25 hours and to 40 Pa within the subsequent one hour. The shelves were cooled during the first 7 hours. Then, during the next 6 hours (hour 8 to 13 after the freeze-drying start), the shelves were heated to a temperature of 0.2–0.8 °C, and the temperature of the shelves was kept within these limits for hour 14 to 21 after the freeze-drying start. The shelves of the freeze-dryer were switched to heating mode (up to a temperature of 35 °C) on hour 22 of the freeze-drying. The serum was dried under such conditions for another 4 hours until its temperature in the ampoules reached 25 °C.

Temperature and vacuum pressure values during hyperimmune sheep serum freeze-drying using procedure 2 are shown in Figure 2.

The difference between the procedures was that under procedure 2 the vacuum pressure was maintained at 11 Pa during the 1st hour after placing the sera into the freeze dryer. During the next 3 hours, the pressure in the freeze dryer chamber gradually increased to 43 Pa and was maintained at this level for the next 5 hours. From hour 9 to hour 11 after the freeze-drying start the pressure in the chamber was increased to 47 Pa. From hour 13 to hour 20 of the freeze-drying process, the pressure in the chamber was decreased to 2.2 Pa. The freeze dryer shelves were cooled for 1 hour. From hour 2 to hour 9 the shelves were gradually heated to a temperature of 0.6 °C. Temperature of the shelves was kept at this level for the next 10 hours (starting from hour 10 to hour 20 after the freeze-drying start). During the last 2 hours of the serum freeze-drying, the freeze dryer shelves were heated from 1 to 54 °C until the serum temperature in the ampoules reached 25 °C.

Figure 3 presents the photographs of hyperimmune sera freeze-dried using the two procedures described above.

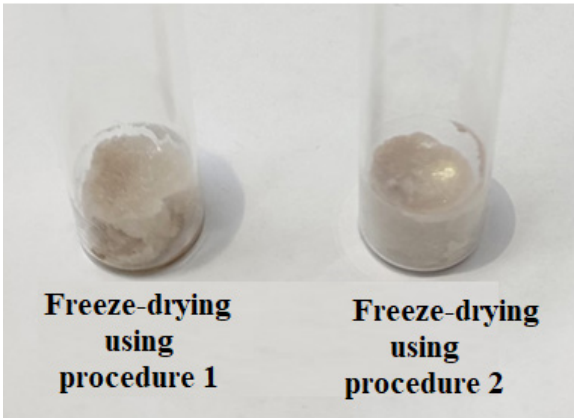

Fig. 3. Appearance of sera from the same batch freeze-dried using two different procedures

Table
Physico-chemical and biological parameters of freeze-dried anti-*Chlamydia* hyperimmune sera and their compliance with characteristics laid down in technical specifications

Parameter	Serum characteristics in accordance with TU 9388-020-00492374-2007	Serum freeze-dried using procedure 1	Serum freeze-dried using procedure 2
Appearance	Dry homogeneous amorphous mass in the form of cake	–	+
Colour	Light cream	+	+
Extraneous matter, mold	Not allowed	+	+
Moisture content, %, maximum	4	+	+
Solubility	The contents of the ampoules shall dissolve in saline solution within 2–5 minutes and be a homogeneous suspension	– (undissolved fragments are observed after 10 minutes)	+
Activity: CFT titre, at least	1:80	+	+
Specificity in CFT	Shall react only with specific *Chlamydia* antigen	+	+

The table shows the results of the freeze-dried serum tests for compliance with characteristics laid down in technical specifications.

It was found that the serum freeze-dried using procedure 1 did not comply with the specified characteristics. No cake-like homogeneous mass formed in the ampoules. The resulting serum did not dissolve properly in the water. The serum freeze-dried using procedure 2 fully complied with the specified characteristics.

The serum samples freeze-dried using procedure 2 were used for subsequent tests of hyperimmune serum for their activity during the long-term storage.

The results of tests of the freeze-dried *Chlamydia* hyperimmune serum for its antigenic activity carried out during 24 months after its freeze-drying are shown in Figure 4.

It was found that the activity of hyperimmune sheep sera did not decrease below the antibody titre specified in the technical specifications (1:40) during two years after its freeze-drying. On month 4, 12 and 21 of storage, one serum out of three reacted at a titre of 1:80, so the mean titre at this time of the study was 1:133. During the following months, all sera reacted at a titre of 1:160. A decrease in the titer of anti-*Chlamydia* antibodies detected at some time points of the study could be associated with errors in CFT procedure.

DISCUSSION

As practice shows freeze-drying is the optimal preservation method for the sera intended for long-term storage [20]. Previously, optimal freeze-drying procedure was determined for the equipment available at that time for freeze-drying of the specific anti-*Chlamydia* serum being a component of the manufactured diagnostic kit. The sera freeze-dried using that procedure complied with the technical specifications for the manufactured test-kit. New modern freeze-dryer with more powerful characteristics, larger drying chamber and electronic control unit enabling temperature and vacuum monitoring and regulating, was purchased as part of technical renovation of the production site.

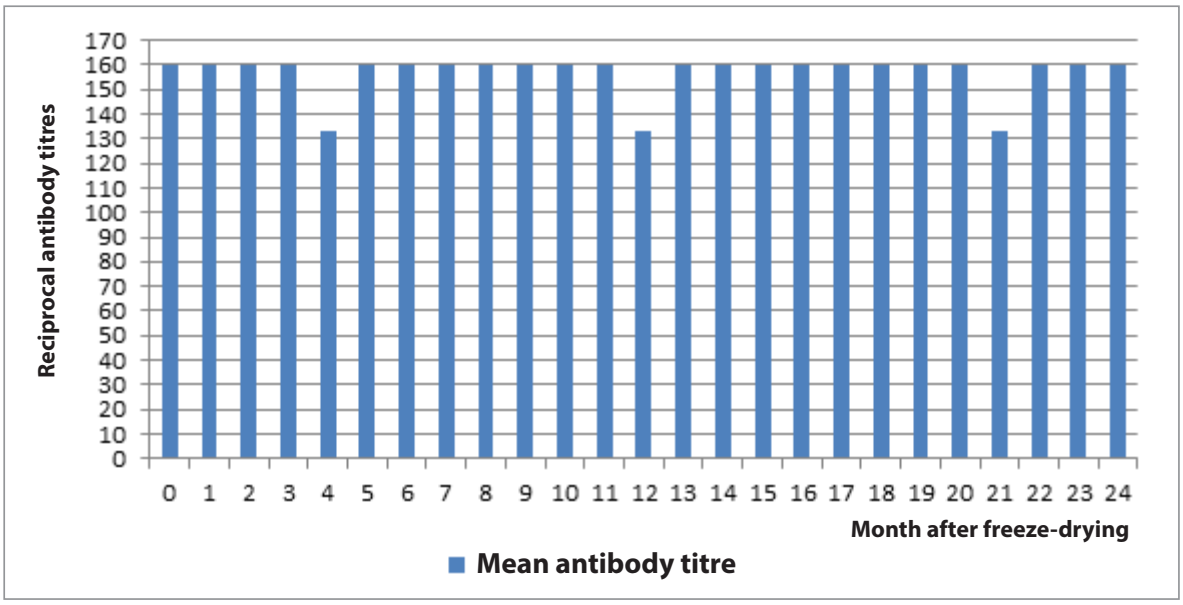

Fig. 4. Anti-Chlamydia hyperimmune ovine serum activity during its long-term storage (24 months)

Two procedures for specific anti-*Chlamydia* serum freeze-drying are described in the paper. The first procedure was as close as possible to the freeze-drying procedure used for old equipment. That freeze-drying procedure was found unsuitable for the new equipment. Therewith, a new freeze-drying procedures was developed (the second procedure).

Information on the development of freeze-drying procedures for specific sera and immunoglobulins used for diagnostic test systems is available in the scientific literature. The freeze-drying period for these products ranges from 24 to 30 hours [14, 16, 21, 22]. The first freeze-drying procedure used during the study took 26 hours. The freeze-drying procedure developed for the new equipment (the second freeze-drying procedure) made it possible to reduce the drying time to 22 hours, which was the first difference between the two methods.

The second difference was the temperature conditions for heating of freeze-dryer shelves. With the first procedure for the specific serum freeze-drying, the temperature of the freeze-dryer shelves ranged from minus 39 to minus 38 °C for the first 6 hours. Whereas with the second procedure for specific serum freeze-drying, the serum was loaded into freeze-dryer chamber cooled to minus 35 °C and starting from the first hour the shelves were heated first to minus 4 °C (for the first 8 hours), then to plus 0.5 °C (for the next 10 hours). The sublimation process at the specified parameters in the first case lasted for 22 hours, in the second case – for 20 hours. In the first case, the sublimation process was too slow during the first 6 hours after the freeze-drying start due to very low temperature and relatively low vacuum. Then, during heating of the shelves, the vacuum levels throughout the drying process also remained low (from 135 to 101 Pa at the sublimation stage). All of this together prevented complete moisture evaporation from the serum during set time period. The residual moisture condensed during the final drying process and dissolved part of the freeze-dried serum cake in the ampoule (Fig. 3). The optimal temperature and vacuum enabling preparation of freeze-dried specific serum of appropriate quality were selected for the second freeze-drying procedure.

In the paper of A. V. Komissarov et al. data on the correlation of the serum freeze-drying time with the amount of the serum to be freeze-dried are presented. The data indicate that the freeze-drying time reduces with a decrease in the amount of the serum loaded into the freeze-dryer [14]. In our study, not all moisture evaporated and condensed on serpentine cooler during the set time interval when the first freeze-drying procedure was used, although this freeze-drying procedure was optimal for the previous freeze-dryer. This was accounted for increase in the amount of the serum subjected to freeze-drying in the new freeze-dryer having larger drying chamber. Considering this, in order to optimize the freeze-drying process, the time for cooling freeze-dryer shelves could be increased, which would improve the drying process, but would significantly extend the sublimation time period. However, the period of freeze-drying of various products can be reduced by selecting the optimal mode of accelerated heating of the freeze-dryer shelves within subzero temperatures at the stage of sublimation [23]. This principle was used for the second procedure for specific anti-*Chlamydia* serum freeze-drying. The new procedure for serum freeze-drying has enabled preparation of a high-quality component for the diagnostic kit in large volumes.

CONCLUSION

Freeze-drying process for specific anti-*Chlamydia* serum intended for the diagnostic test kit has been optimized during the study. The resulting serum is fully compliant with characteristics laid down in the technical specifications for the diagnosticum, TU 9388-020-00492374-2007 "Antigen and Serum Kit for Serological Diagnosis of Chlamydiosis in Livestock".

The optimized freeze-dried procedure differed from the previously used procedure in the reduced heating time of the freeze-dryer shelves at subzero temperatures and increased vacuum, which, in turn, enabled shortening of the serum freeze-drying time and preparation of high-quality component used for the test-kit manufacturing.

It has been established that the freeze-dried serum shelf life is at least two years, during this period the serum retains its activity and physico-chemical properties.

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Metal nanoparticles, silver nanoparticles and their impact on human and animal health (review)

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ABSTRACT

Introduction. Due to increased prevalence of different diseases and antimicrobial resistance development in recent year, such advancements of the humankind as nanomaterials have gained the significance. A relatively small amount of data (lack of data) on biological distribution, pharmacokinetics and potential toxicity of nanometals for the organism hinders the development of safer and more effective drugs.

Objective. Analysis and summary of data published in modern scientific literature on studies of metal nanoparticles and silver nanoparticles, their distribution and impact on human and animal health, as well as their use in biomedicine and veterinary medicine.

Materials and methods. Publications were searched for in eLIBRARY.RU, cyberleninka.ru, scholar.google.ru, www.mdpi.com, www.researchgate.net, www.sciencedirect.com, PubMed database. The literature published during last six years and more recent publications have been used.

Results. Nanostructures can be organic, inorganic and hybrid. One of the most studied inorganic materials are metal nanoparticles. They are widely used both in engineering and biomedicine, in particular in veterinary medicine, as bactericidal and virucidal agents, anti-cancer drugs and diagnostic tools. In the CIS members, silver nanoparticles are most commonly used. It is known that shape, size and surface electric charge affect the antibacterial activity of nanostructures. Several types of silver-based drugs are available at the market now: colloidal, silver cluster and zerovalent silver. Zerovalent silver-based drugs are least toxic. Nanoparticle-based drugs can reach target tissues through local administration such as oral, inhalation, subcutaneous administration, and directly into blood flow by intraperitoneal or intravenous injection. Biodistribution of metal nanostructures depends on particle type, their size, surface, interaction with proteins as well as routes of exposure, doses and hydrophobic properties. Pharmacokinetics of silver nanoparticles does not differ from that of metal nanoparticles, furthermore nanosilver does not accumulate in spleen, liver, kidneys and lungs which is potentially toxic.

Conclusions. Further in-depth studies of nanoparticle biodistribution, compatibility and potential toxicity are needed to facilitate the development of more effective and safe therapeutic drugs.

Keywords: review, nanoparticle types, metal nanoparticles, nanosilver, biodistribution, nanosilver drugs, bactericidal activity, toxicity

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Наночастицы металлов, наночастицы серебра и их влияние на организм человека и животных (обзор литературы)

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РЕЗЮМЕ

Введение. В связи с ростом числа заболеваний различной этиологии и развитием антибиотикорезистентности в последние несколько лет возросла значимость такого достижения человечества, как наноматериалы. Сравнительно небольшое количество данных (недостаток данных) о биораспределении, фармакокинетике, а также потенциальной токсичности нанометаллов для организма замедляет разработку более безопасных и эффективных лекарственных средств.

Цель исследования. Анализ и обобщение данных современной научной литературы, посвященной изучению наночастиц металлов и наночастиц серебра, их распределения и влияния на организм человека и животных, а также по применению в сфере биомедицины и ветеринарии.

Материалы и методы. Поиск источников производился в системах eLIBRARY.RU, cyberleninka.ru, scholar.google.ru, www.mdpi.com, www.researchgate.net, www.sciencedirect.com, базе данных PubMed. Использовалась литература, опубликованная за последние 6 лет, и более ранние исследования.

Результаты. Наноэлементы делят на органические, неорганические и гибридные. Одной из наиболее изученных неорганических наноструктур являются наночастицы металлов. Они находят широкое применение как в инженерии, так и в биомедицине (ветеринарии) в качестве бактерицидного и вирулицидного агента, средств для борьбы с раком, а также в сфере диагностики. На территории СНГ популярными нанометаллами являются наночастицы

серебра. Известно, что на антибактериальную активность нанообъектов влияют их форма, размер и поверхностный заряд. Сейчас на фармацевтическом рынке существует несколько видов препаратов серебра, представленные в различных формах: коллоидное (катионное), кластерное и нульвалентное (металлическое) серебро. Препараты нульвалентного серебра наименее токсичные по сравнению с остальными. Лекарства на основе наноразмерных частиц можно вводить оральным, ингаляционным и дермальным способами, а также непосредственно в системный кровоток посредством внутрибрюшинной или внутривенной инъекции. Биораспределение металлических наноструктур зависит от типа частиц, их размера, поверхностного заряда, поверхностного покрытия, связи с белками, а также от путей воздействия, дозы и гидрофобности. Фармакокинетика наночастиц серебра не отличается от распределения наночастиц металлов, при этом наноразмерное серебро способно накапливаться в селезенке, печени, почках и легких, что может вызывать потенциальный токсический эффект.

Заключение. Необходимы дальнейшие углубленные исследования биораспределения, совместимости и потенциальной токсичности наночастиц, которые помогут разработать более эффективные и безопасные лекарственные препараты.

Ключевые слова: обзор, виды наночастиц, наночастицы металлов, наносеребро, биораспределение, препараты наносеребра, антибактериальная активность, токсичность

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INTRODUCTION

Overuse of antimicrobials inhibits the symbiotic microflora and contributes to the development of drug-resistant pathogens, thus hindering the drug therapeutic action and causing side effects and complications. New resistant mechanisms of some pathogens threaten the scope of treatment for many infectious diseases. Successful treatment of even common illnesses such as pneumonia, sepsis, and foodborne diseases is hindered and sometimes impossible due to the reduced effectiveness of antimicrobials[1]. The growing global antimicrobial resistance (AMR) concern has created an urgent need to reduce the use of antimicrobials and search for the most effective drugs to replace them [1].

The field of nanotechnology is growing rapidly. The use of nanomaterials to face biomedical and veterinary challenges, such as the diagnosis and treatment of various diseases, is currently one of the priority scientific trends. Metal nanoparticles (MNPs) proposed for use in public and animal health possessing unique chemical and biological properties that make them versatile in their functions are of particular interest among the wide range of nanoparticles (NPs) [2].

Currently, there are three main groups of action of nanostructures on biological objects:

1) modification (iron and copper NPs);

2) toxicity (NPs of copper, aluminum oxide, silver, iron, iron hydroxide);

3) mutagenicity (NPs of silicon, nickel hydroxide, iron oxide, titanium dioxide, gold, zinc oxide, copper oxide and silver) [2].

The most commonly used metal NPs are silver, gold, iron oxide, copper and zinc [3]. In veterinary medicine, these nanomaterials are mainly used as antiviral and antimicrobial agents [4].

Silver nanoparticles (AgNPs) are of particular interest among MNPs. They are mainly used for antimicrobial and anticancer therapy. AgNPs are also applied in the promotion of wound repair and bone healing, or as the vaccine adjuvants and anti-diabetic agents [5].

While nanotechnology is regarded as one of the foremost technologies already applied in diverse subjects, its application in veterinary science is still in its infancy stages when compared to other sister disciplines. Herewith, it already has revealed new opportunities in molecular biology, biotechnology, and has revolutionized virtually all veterinary medicine and animal science disciplines these days by offering new, small-scale devices and materials that are beneficial to living organisms [6]. NPs increasingly invade animal therapeutics, diagnostics, production of veterinary vaccines, used as farm disinfectants, for animal breeding and reproduction, and even in the field of animal nutrition. Their replacement of commonly used antibiotics directly reflects on the public health, as they minimize the problem of drug resistance in both human and veterinary medicine, and the problem of drug residues in milk and meat [7].

Nanometals-based products, particularly AgNP-based products, are actively studied and used as antimicrobial, antiviral, antifungal [8] and antitumor agents, as well as analgesic drugs [9] and dietary supplements to increase animal performance, improve their immunity, and even as a part of a synergetic anti-AMR bacteria system [10, 11, 12, 13].

Despite the fact that nanoobjects are already being used to solve various biomedical and veterinary problems, there is currently insufficient data on the biodistribution of nanoelements in the body. At the same time, understanding the patterns of NPs distribution in the body, taking into account their different composition and structure, is of paramount importance for identification of the prospects of their further biological and medical use [14].

[1] The World Health Organization. Antibiotic resistance. https://www.who.int/ru/news-room/fact-sheets/detail/antibiotic-resistance

The paper reviews the achievements in nanomaterial use over the past 20 years. This review is intended to provide valuable information for researchers interested in the medical and veterinary applications of MNPs, namely of AgNPs.

The purpose of the work is to analyze and summarize the data of modern scientific literature on the study of MNPs and AgNPs, in particular in the field of biomedicine and veterinary medicine, as well as on the study of their distribution and impacts on the human and animal body.

MATERIALS AND METHODS

The research data concerning the study of MNPs, namely of AgNPs, over the past 20 years have been used for this review. The published data on the properties of MNPs and AgNPs, their impact on the human and animal body, and their application in the field of veterinary medicine and biomedicine have been analyzed.

The literature was searched and analyzed using the following online resources: eLIBRARY.RU, cyberleninka.ru, scholar.google.ru, www.mdpi.com, www.researchgate.net, www.sciencedirect.com, PubMed databases.

Foreign and domestic reviews (57%) on NPs, their types, synthesis, distribution and impacts on the body, application in various fields; and research papers (43%), presenting the results of the studies on the use of MNPs and AgNPs as diagnostic agents, therapeutic drugs, dietary supplements and others have been selected for the analysis. 66% of the publications analyzed have been published over the past 6 years (11.5% of them were published in 2023, 7.7% in 2024), and 34% are earlier studies.

TYPES OF NANOPARTICLES USED IN MEDICINE AND DIAGNOSTICS

To date, many types of NPs have been developed that are used in the biomedical and veterinary fields. NPs are divided into organic, inorganic and hybrid. Most organic nanostructures are biocompatible, biodegradable, and non-toxic, while most inorganic NPs are smaller in size, exhibit better penetration capability, drug loading capacity, excellent stability, tunable degradation rates and release profile [15, 16, 17, 18, 19].

Inorganic NPs include particles of metals or their oxides, semiconductor NPs (silicon oxide), which include quantum dots, as well as carbon derivatives (graphene, fullerenes, carbon nanotubes). Organic nanoobjects are represented by structures based on lipids and their derivatives (liposomes, lipid NPs, micelles), as well as synthetic compounds of a polymeric nature: linear (classical) and branched (dendrimers, dendrons) [15].

In this review, inorganic NPs, namely MNPs, are focused on. They can be produced in the form of spheres, nanocapsules, rods and other shapes that are highly stable and effective in various conditions with easily controlled physicochemical properties. Unfortunately, MNPs have some drawback: the complexity of manufacturing (uniformly sized, homogeneous in shape and surface charge) and the difficulty of eliminating them from the body [20].

The most commonly used MNPs in biomedicine are gold, silver, copper oxide, zinc oxide, magnesium oxide, iron oxide, titanium dioxide, and aluminum NPs [21, 22, 23, 24, 25].

Silver nanoparticles have long been widely studied to be used in various fields of biomedicine and veterinary medicine due to their antimicrobial properties and antioxidant activity [26]. They are effective both against gram-negative and gram-positive bacteria and are incorporated into fabric wound dressings [27].

Gold nanoparticles are another group of MNPs that are being extensively explored and have shown promise in medicine and diagnostics, for example, antibacterial, in anticancer therapy for targeted drug delivery and reducing the tumor growth. In addition, AgNPs are used in spectroscopy and to enhance optical imaging [28, 29].

In addition, the last frequently explored group of MNPs are *metal oxides*. The use of zinc oxide, ZnO; copper (II) oxide, CuO; magnesium oxide, MgO; titanium (IV) oxide, TiO_2; aluminum oxide, Al_2O_3; iron (II, III) oxide, Fe_3O_4 has been studied for a long time [30, 31, 32, 33, 34, 35, 36, 37].

Iron oxide is currently gaining popularity due to its magnetic properties. Iron oxide NPs are used as drug delivery vehicles, for magnetic resonance imaging, cancer diagnosis, and tissue engineering [30]. Tin oxide has unique electrical properties that depend on the size of its NPs [31]. Tungsten trioxide is often used as sensing material for chemiresistive gas sensors [32]. Titanium dioxide conducts electricity, therefore, it has found application in optical and solar energy, as well as in the medical, food and microbiological industries for the photocatalytic sterilization [33, 34]. Magnesium oxide NPs are utilized to reduce air pollution and as catalysts for organic reactions [35]. Copper oxide NPs have found applications in various catalytic fields, including oxidation and photothermy [36]. Magnesium, copper, aluminum, and zinc oxides have also proven to be potential antibacterial and antifungal agents [37].

SCIENTIFIC INTEREST IN SILVER AND GOLD NANOPARTICLES AND THEIR ANTIBACTERIAL ACTIVITY

Over the past 20 years, the number of publications found for "gold nanoparticles in medicine" search query has changed annually on Google Scholar[2] web search engine from 1,360 links in 2003 to 61,900 in 2023, with the largest number found in 2022 (67,800). For "silver nanoparticles in medicine" search query there were 904 publications found in 2003, and in 2023, their number reached 51,500; the largest number (56,600) was also available in 2022 (Fig. 1).

The number of publications for "gold nanoparticles in veterinary medicine" and "silver nanoparticles in veterinary medicine" search queries is almost 9 times less (Fig. 2). The lowest number of links in English on these topics was observed in 2003 (127 and 103, respectively). The largest number of publications for "gold nanoparticles in veterinary medicine" query was available in 2023 (7,570). The number of links for "silver nanoparticles in veterinary medicine" in the same year was the largest in the last 20 years (7,910).

For "наночастицы золота в медицине" search query in Russian, there were only 12 publications on the same web search engine in 2003 and 225 in 2023 (Fig. 3). For "наночастицы серебра в медицине" search query in Russian, 395 publications were found in 2023, whereas in 2003

[2] https://scholar.google.com

there were only 20. The largest number of publications on "наночастицы золота в медицине" was in 2018 (438), and on "наночастицы серебра в медицине" – in 2016 (636).

The number of publications on "наночастицы золота в ветеринарии" and "наночастицы серебра в ветеринарии" in Russian was 5–8 times less than the search results for NPs in the medicine. At the same time, in 2005 absolutely no links were found for each of the search queries (Fig. 4).

The largest number of publications on "наночастицы серебра в ветеринарии" was in 2020 (136), and on "наночастицы золота в ветеринарии" – in 2018 (57).

Further analyzing the interest in the use of nanostructures in medicine, 569 queries on "наночастицы золота в медицине" were recorded in the Yandex search engine from January 2018 to December 2023 (Fig. 5). The largest number of queries was in 2022 (140). The number of queries on "наночастицы серебра в медицине" on yandex.ru[3] during the same period was 749. The peak of popularity of search queries on this topic occurred in 2023 (202 requests).

At the same time, there have been no search queries for "наночастицы золота в ветеринарии" and "наночастицы серебра в ветеринарии" on this engine over the past 5 years.

The graphs show (Fig. 1–4) that links in English on nanostructures in medicine and veterinary medicine exceed the number of links in Russian by more than 100 times. Also, based on the number of publications appearing during the year, it is clear that foreign colleagues' interest in MNPs in medicine decreased only a year ago, in contrast to the interest of our colleagues, which has tended to decrease over the past five years. At the same time, data from Yandex Wordstat suggest an annual increase in the interest of scientists from Russia and the CIS countries in AgNPs in the field of biomedicine. Gold nanoparticles have not aroused stable interest among researchers over the past six years (Fig. 5).

It has been established that the number of foreign studies in the field of veterinary medicine devoted to MNPs is increasing every year (Fig. 2). Russian-speaking veterinary scientists were not consistently interested in investigation of gold and silver NPs since 2015–2016 (Fig. 4).

A difference of 8–9 times between the number of links, both in English and in Russian, on the use of NPs in medicine and in veterinary medicine may be due to the fact that NPs in veterinary practice have not yet found such wide application as in public health. However, research on the use of NPs to treat and diagnose diseases of pets and livestock is also conducted. Vaccines are being developed against a number of significant bacterial and viral diseases, such as equine influenza, bovine viral diarrhea, and Newcastle disease, as well as NPs-based carriers for the delivery of imaging tools, antibiotics, vitamins, and drugs, including those targeting neoplastic diseases [38].

Thus, despite the declining interest of Russian scientists in investigation of nanomaterials use in medicine and veterinary medicine, judging by the number of publications on this topic, our compatriots are more interested in AgNPs and their use as a base for therapeutic drugs. And this is not surprising, because NPs have long been the most widely used antibacterial nanoagents due to their broad spectrum of action against a variety of bacteria, viruses and fungi [39].

[3] https://wordstat.yandex.ru

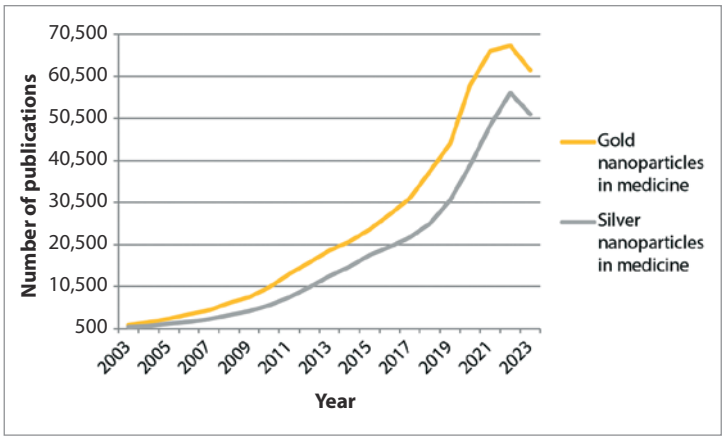

Fig. 1. Publications in English for "gold nanoparticles in medicine" and "silver nanoparticles in medicine" search queries in Google Scholar from 2003 to 2023

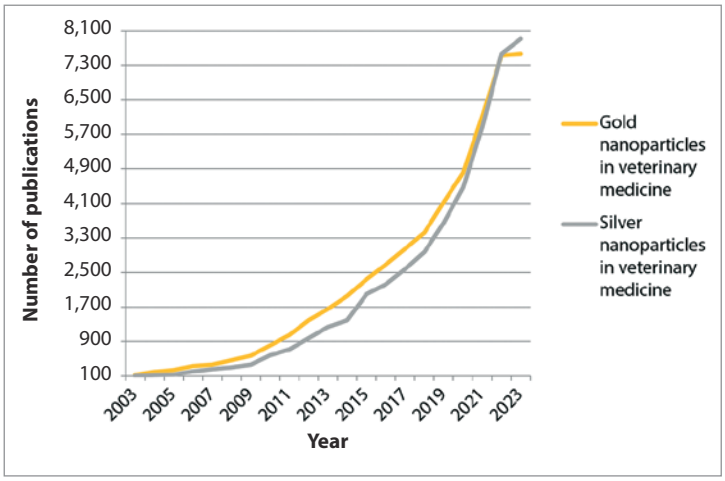

Fig. 2. Publications in English for "gold nanoparticles in veterinary medicine" and "silver nanoparticles in veterinary medicine" search queries in Google Scholar from 2003 to 2023

The earliest known reference to the use of silver in medicine goes back to the 19th century, when it was used to prevent gonococcal conjunctivitis in newborns, and later, in the 20th century, silver was used by surgeons for local treatment of burn wounds and as internal antiseptics [40, 41, 42].

Colloidal silver has been produced in a wide variety of forms for over 100 years. Currently, there are many ways to synthesize more effective forms of colloidal AgNPs.

The methods of synthesis of nanoparticles can be conditionally divided into two groups: reduction of silver ions (Ag^+) and dispersal to nanoscale sizes. The first group involves chemical methods, and the second group involves physical methods. At the same time, nanoscale silver can have various geometric shapes: spherical, pyramids, rods, cubes, etc. [43]. The bactericidal effect of NPs depends on different parameters including size, shape, and the surface charge of the particles [39].

The shape of nanoparticles. As shown by the results of studies conducted in 2016 and 2019 aimed to explore the effect of NPs shapes and facets on antibacterial activity, crystalline particles with a high-atom-density and a higher

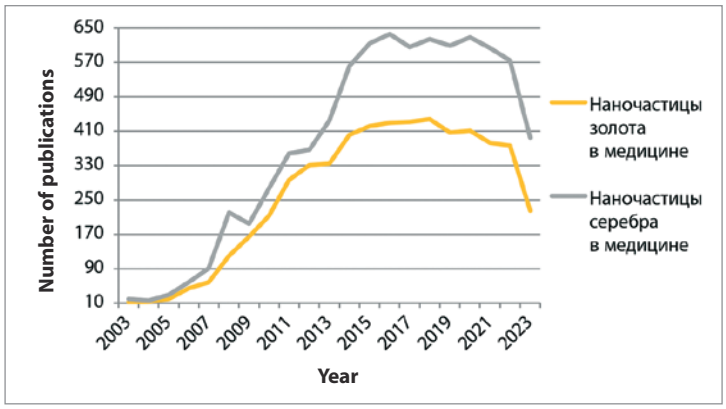

Fig. 3. Publications in Russian for "наночастицы золота в медицине" and "наночастицы серебра в медицине" search queries in Google Scholar from 2003 to 2023

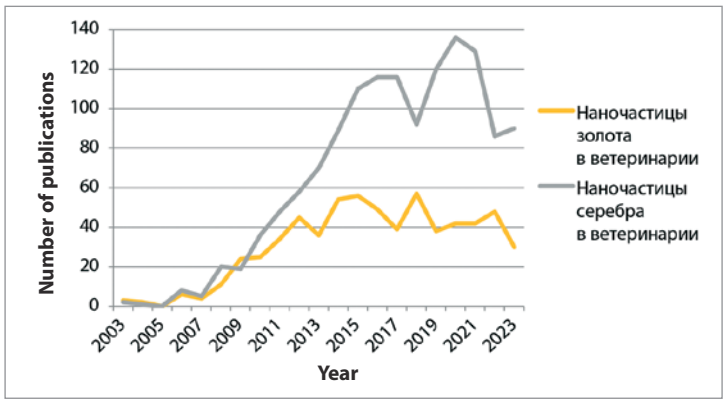

Fig. 4. Publications in Russian for "наночастицы золота в ветеринарии" and "наночастицы серебра в ветеринарии" search queries in Google Scholar from 2003 to 2023

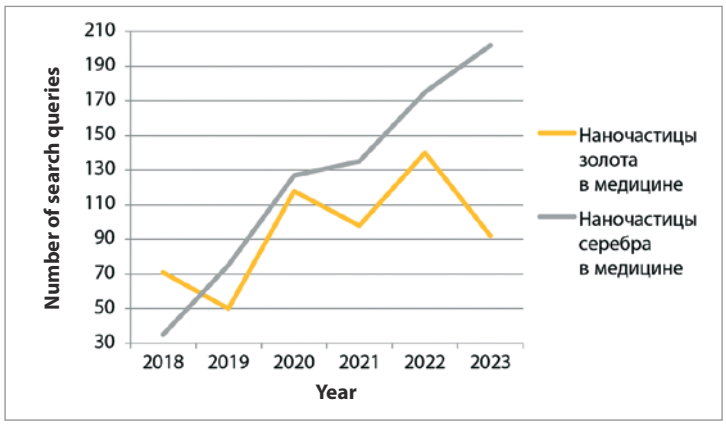

Fig. 5. Publications for "наночастицы золота в медицине" and "наночастицы серебра в медицине" search queries in Yandex from 2003 to 2023

number of facets have better activity against bacteria. For example, triangular silver nanoprisms with 111 facets have a higher atom density and, accordingly, exhibited better antibacterial efficiency than that of the spherical and rod-shaped silver particles with 100 and 110 facets [44, 45]. For example, S. Pal et al. synthesized spherical, rod-shaped NPs and truncated triangular nanoplates, and then evaluated their antibacterial activities against *E. coli* in solution and on agar plates. The researchers concluded that truncated triangular nanosilver exhibited the highest biocidal activity followed by silver nanospheres and nanorods. Scanning transmission electron microscopy revealed that all nanostructures can bind to membrane surface, alter the cell membrane permeability and subsequently cause the cell death. However, truncated nanotriangles present the highest percentage of exposed facets, which favor the direct interaction with the main components of the cell membrane, lead to the enhanced surface binding, cell uptake, and efficiently killing of bacteria [39].

Helmlinger J. et al. studied the effect of NPs shape on *Staphylococcus aureus*. They concluded that nanoplatelets exhibited the highest toxicity, followed by nanospheres, nanorods, and finally nanocubes [46].

The size of the nanoparticles. Experimental studies observed that the antibacterial activity was directly proportional to the decrease in NPs size: efficiency decreased as NP size increased. For example, AgNPs with a size of 1 to 10 nm are more effective in inhibiting bacterial growth [46, 47, 48]. This is probably due to the concentrated accumulation of NPs in the cell membrane and cytoplasma of microorganisms [49, 50]. It is also suggested that the increased antibacterial activity may be because of the fact that smaller nanoelements are able to release their toxic components at a higher rate due to higher surface-to-volume ratio as the size of the NPs decreases [47, 51]. In addition, recent studies showed that small and medium sized AgNPs strongly affect mitochondrial electron transport, autophagy and phagocytosis, and the integrity and organization of organelles [52].

The surface charge of nanoparticles. The antimicrobial activity of NPs can be changed by modifying their surface charge. Hu C. et al. demonstrated that AgNPs with a positive surface charge have increased antibacterial activity [53]. Antimicrobial activity is also mediated by released Ag^+ ions from NPs surface. This occurs because of oxidative dissolution: first, metallic silver is oxidized in the presence of dissolved oxygen, and then the formed basic oxide is dissolved in acidic conditions. Silver ions also possess high affinity to electron-donating groups that are extensively present on membrane or proteins. Ag^+ ions can readily coordinate with DNA, RNA, peptides forming the insoluble compounds and thus hindering the cell division and reproduction [39].

SILVER NANOPARTICLE-BASED DRUGS

It can be said that rapidly developing AMR brings silver-based drugs to the forefront again.

Currently, there are several types of silver-based drugs of different dosage forms available on the pharmaceutical market.

The most well known drugs are based on colloidal (cationic) silver (Ag^+): these are silver oxide, silver salts (nitrates, sulfates, phosphates), silver complexes (citrates or lactates), and free silver aqueous cations. Colloidal silver products available on the market are "Tinosan SDC" (BASF, Germany), "Argolife" (Art Life, Russia), silver sulfate (Aurat, Russia) [54].

There are also metallic micro-dispersed and nano-dispersed forms of silver products (cluster silver), in which the main amount of silver is in the low-toxic metallic form Ag^0. Cluster silver products are highly effective and less toxic than products containing a higher amount of cationic silver [55]. Such products include: "AgBion-2" (Concern

"Nanoindustria", Russia), "Argovit" (Vector-Vita, Russia), "Poviargol" (Institute of macromolecular compounds, Russia), "Argonica" (VectorPro, Russia).

Nulvalent (metallic) silver is a separate category of products, namely colloidal ion-free silver (Ag^0), for example, of the trademark "KND" (Sentosa Factoring NP, Russia): colloidal silver concentrate "KND-S", colloidal silver and copper concentrate "KND-SM", colloidal silver concentrate "KND-S-K", cosmetic raw materials and supplements "AREGONA (KND-SP)" [54].

As noted above, preparations containing silver in a finely dispersed form are significantly less toxic than products based on silver salts. Nulvalent silver products are much less toxic than those based on cluster silver. This is due to the almost complete absence of cationic Ag in nulvalent silver.

Cationic silver is also reduced in its composition and incompatible with many components of practical systems (for example, with saline solutions), unlike cluster and nulvalent silver, which are more compatible and stable [55].

Nanosilver-based drugs are very promising for use in veterinary medicine and zootechnics. AgNPs can be used for biosafety purposes on farms, for hatchery fumigation, sterilization of poultry houses and cages. It has been found that AgNPs can improve the adaptive immune system of birds [56] and hatching rates [57]. In 2023, physiologically stable, bio-compatible AgNPs were produced which may be used for targeted drug delivery in veterinary medicine that could offer enhanced therapeutic efficacy with minimal side effects [58]. In the same year, it was found that the addition of nanosilver to milk fed to calves has a positive effect on their metabolism. Therefore, nanosilver can be used to prevent infectious diseases of calves during the first month after their birth, which will mitigate the risks of AMR development and improve livestock production performance [59].

DISTRIBUTION OF NANOPARTICLES IN THE BODY

The distribution of a pharmaceutical substance containing nanoelements in organs and tissues changes significantly, affecting the pharmacodynamics of the drug. In this regard, the study of NPs biodistribution is the most important stage in the studies [60]. However, currently most NPs are still in the preclinical evaluation phase with few approved for clinical use. Most articles are devoted to *in vitro* studies of nanomaterials and there are relatively few publications on *in vivo* biodistribution studies. At the same time, the lack of concrete data on the distribution and accumulation of NPs in organs and tissues limits their application [61, 62].

Pharmacokinetic studies are needed to assess the distribution of NPs and their toxicity. Absorption, distribution, metabolism and elimination are the four processes that make up pharmacokinetics [63]. Few pharmacokinetic studies of nanoforms have been conducted, and only nanomaterials are controlled[4,5], but there are no standards and regulations regarding NPs biodistribution, which makes the evaluation of this parameter difficult.

The biodistribution of MNPs depends on NPs' type, size, surface charge, protein binding, effects, dose and/or hydrophobicity [62, 63].

The rate and degree of absorption are influenced by the physiological environment and the NPs' characteristics. Nanoformulations pass across physiological and physical barriers that selectively block the transport of molecules, reducing NPs bioavailability. Cellular uptake is heavily influenced by size, surface charge, and shape [64, 65]. The route of administration and the characteristics of the NPs affect absorption [62].

The MNPs with a negative surface charge has a higher absorption rate at the gastrointestinal membrane in the oral route, and it is related to the size of the small intestine. The pulmonary route has a larger contact area, which makes absorption easier [62].

The major routes of MNPs-based drug administration are oral, inhalation, dermal and directly into the blood stream by intraperitoneal or intravenous injection [63].

Generally, blood half-life is shorter in rodents than in larger laboratory animals (e.g., rabbits or monkeys) and differs between intravenous and oral exposures. Oral, dermal, or inhalational absorption is low (≤ 5%), but may increase with smaller sizes, negative charge, and appropriate coatings [63].

Metallic NPs can be distributed throughout the body, primarily accumulating in the liver, spleen, and lymph node due to nonspecific uptake by reticuloendothelial cells, and could remain in the body for ≥ 6 months. Metallic NPs (≤ 100 nm) can cross the blood-brain barrier (BBB), favored by coating with BBB-permeable neuropeptides. Placental transfer depends on the stage of embryonic/placental maturation and surface composition of NP, and may be enhanced by coating with biocompatible molecules (e.g., ferritin or polyethylene glycol). Renal and biliary excretion is generally low due to persistent accumulation in tissues, but renal elimination could be substantially increased with smaller sizes and specific coatings (e.g., glutathione) [66].

DISTRIBUTION AND TOXICITY OF SILVER NANOPARTICLES

The absorbed AgNPs are dispersed throughout many systems, including the dermis, respiratory, spleen, digestive, urinary, nervous, immune, and reproductive systems. The primary distribution sites are the spleen, liver, kidneys, and lungs. Little AgNP deposition seen in the teeth and bones [63].

In addition to directly exposed tissues, NPs are also delivered to various organs with blood circulation. Nanosilver particles easily penetrate the body and cross biological barriers (BBB and blood-testis barrier) and can potentially produce a cytotoxic effect. Thus, non-specific distribution of AgNPs may produce cytotoxicities such as dermal toxicity, ocular toxicity, respiratory toxicity, hepatobiliary toxicity, neurotoxicity and reproductive toxicity, which limit the applications of AgNPs. The potential cytotoxicity of AgNPs depends on the routes of administration and the properties or characteristics of the AgNPs, such as the size, shape, and concentration (Fig. 6) [63].

However, the specific mechanisms of MNPs and AgNPs distribution and accumulation in various tissues and organs, as well as their potential toxicity, have not yet been sufficiently studied [5, 62].

[4] On supervision of nanotechnology products and nanomaterial-based drugs: Regulation of the Chief Medical Officer of the Russian Federation 23.07.2007 No. 54. https://docs.cntd.ru/document/902056894

[5] Procedure and organization of control over nanomaterials: Guidelines of 17.10.2011 MI 1.2.2966-11, htpps://docs.cntd.ru/document/1200095623

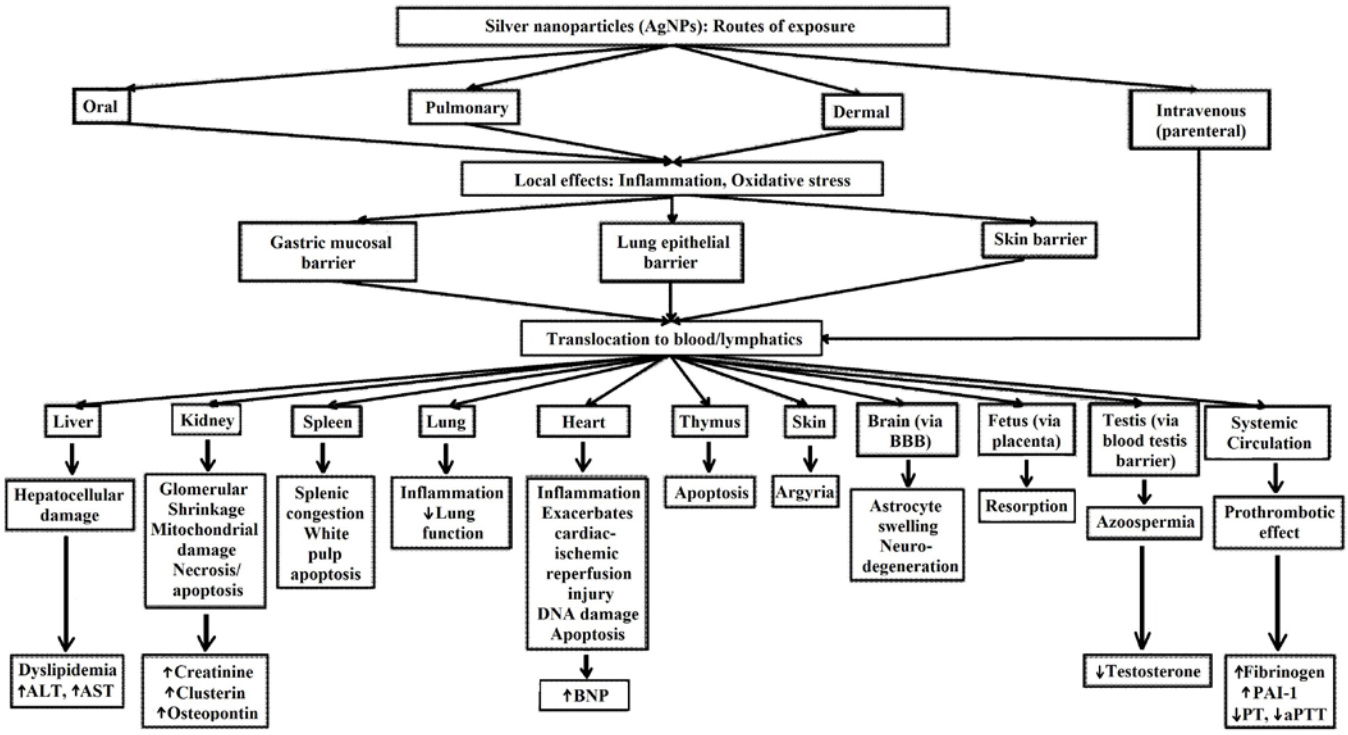

Fig. 6. Biodistribution and toxicity of silver nanoparticles for different exposure routes [63]

IMPACT OF SILVER NANOPARTICLES ON ANIMAL BODY

Silver nanoparticles may have different effects on the physiological parameters of animals, depending on the duration of use and doses of silver-based drugs. At the same time, both healthy and diseased animals always demonstrate changes in the biochemical and morphological blood parameters. Nanosilver mainly affects red blood cells and platelets, to a lesser extent affecting monocytes and leukocytes. Previously, O. A. Zeinalov et al. noted a moderate increase in platelet count and a decrease in white blood cell counts in healthy mice treated with high doses of highly dispersed metallic AgNPs [67]. Also E. M. Tsygankov et al. detected an increase in red blood cells and platelet counts in replacement flocks after using a cluster silver-based drug [68]. Study of 2021 found a significant decrease in white blood cell levels after using nanodispersed silver to treat cows with serous mastitis, and a slight decrease in monocyte counts and an increase in hemoglobin levels were also observed [11]. When using highly dispersed nanosilver in mice with Newcastle disease, the monocyte count decreases, the mean corpuscular hemoglobin concentration decreases, and red blood cells, hemoglobin, and hematocrit levels increase [69].

Silver nanoparticles are particularly attractive for veterinary medicine as dietary supplements used to increase animal performance and immunity [10, 11, 12, 13]. There are publications describing the promising use of virocidal agents based on nano- and organic silver to prevent Newcastle and Aujeszky's diseases [69, 70, 71]. It is also well known that NPs in drinking water or dietary supplements exert anabolic effect; they can increase body weight and muscle mass [72, 73, 74, 75].

However, as mentioned above, AgNPs mainly accumulate in the "filter organs" of the body, and can cross biological barriers. Toxic effects and cognitive impairment are noted after prolonged use of silver-containing drugs in animals, presumably due to the accumulation of AgNPs in the brain; and the use of silver-based drugs during mating, pregnancy and lactation of animals leads to a significant accumulation of AgNPs in tissues and organs not only of parents, but also of their offspring [76, 77].

Thus, AgNPs administered in doses not exceeding 10 mg per 1 kg of body weight per day have biotic effects: they stimulate the respiratory function of the blood, increase red blood cells and hemoglobin levels; they stimulate the body defenses by increasing white blood cell count in the bloodstream [67, 68, 78]. Low doses and administration of nanosilver for maximum 30 days exert no significant effect on the gut microbiota and increase the animal performance [72, 73, 74, 75]. The use of silver-containing drugs in high concentrations, as well as their prolonged use, negatively affects the mammalian body, and even can cause death [76, 77, 79].

Therefore, further in-depth studies of the biodistribution, compatibility and potential toxicity of NPs are still needed to facilitate the development of effective dietary supplements and safe drugs [5, 60, 62, 63, 66, 79].

CONCLUSIONS

Based on the analysis of publications it can be concluded that.

1. Over the past 20 years, a wide range of nanomaterials have been introduced in biomedicine, veterinary medicine and diagnostics. They are divided into organic and inorganic NPs. The latter include NPs of gold, silver, copper oxide, zinc oxide, magnesium oxide, iron oxide, titanium dioxide and aluminum.

2. The use of NPs in veterinary practice has not yet found such widespread use as in public health, but it keeps expanding every year.

3. AgNPs are of the greatest interest to Russian scientists, as they have long proven themselves as an antibacterial nanoagent.

4. There are physical and chemical methods for NPs synthesis. They include reduction of silver ions (Ag^+) and dispersal to nanoscale sizes.

5. The antibacterial activity of AgNPs is influenced by their shape, size and surface charge.

6. Currently, there are three types of silver-based drugs: colloidal (cationic), cluster and nulvalent (metallic).

7. The biodistribution of MNPs is affected by the type of particles, their size, surface charge and coating, protein binding, as well as the exposure route and dose.

8. The distribution of AgNPs does not differ from the MNPs pharmacokinetics, while nanoscale silver most often accumulates in the spleen, liver, kidneys and lungs, which potentially can be cytotoxic.

9. Nanosilver administration in low doses and maximum for 30 days strengthens the immunity and improves the performance of animals, and prolonged use of AgNPs and/or administration in high concentrations contributes to the accumulation of silver in mammalian organs and tissues, exerting a toxic effect.

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PCR-RFLP analysis of insecticide resistance to pyrethroids, organophosphates and carbamates in *Musca domestica* L.

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ABSTRACT

Introduction. Zoophilic flies play a significant role in animal disease transmission, and insecticide resistance being a relevant veterinary issue globally is an obstacle to effective fly population control. Molecular methods are more commonly used to monitor and diagnose insecticide resistance in insect populations.

Objective. The study aims to assess distribution of the main mutations associated with resistance to pyrethroids, organophosphorus compounds and carbamates in three field populations of *Musca domestica* L. collected in 2021–2023 in livestock facilities of the Tyumen Oblast.

Materials and methods. Genotyping of *CYP*, *vssc* and *ace-2* genes was performed using polymerase chain reaction and restriction fragment length polymorphism analysis.

Results. One mutation in the *vssc* gene (L1014F) associated with resistance to pyrethroids and two mutations in the *ace-2* gene (G342A, G342V) conferring resistance to organophosphorus compounds and carbamates were found. The resistant allele L1014F was present in 40–70% of the tested insects of all three populations with 30–55% frequency. The G342A allele was found in 10 and 60% of insects from two populations with frequencies of 5 and 30%, respectively. The G342V allele was detected in 40% insects of only one population with a frequency of 25%.

Conclusion. The results obtained indicate the potential for conferring resistance to pyrethroids, organophosphorus compounds and carbamates in the studied populations of *Musca domestica*, which should be taken into account when selecting disinsectants for livestock-keeping facilities and protecting animals from insects. Further molecular tests of *Musca domestica* flies from the regions bordering the Tyumen Oblast will be useful for developing a strategy to contain spread of resistant alleles in local populations.

Keywords: house flies, insecticides, insecticide resistance, resistance markers, molecular diagnosis

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Анализ инсектицидной устойчивости к пиретроидам, фосфорорганическим соединениям и карбаматам у *Musca domestica* L. методом ПЦР-ПДРФ

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РЕЗЮМЕ

Введение. Значимым фактором в распространении заболеваний животных являются зоофильные мухи, контроль численности которых осложняется проблемой инсектицидной резистентности, актуальной для ветеринарии и медицины во всем мире. Для мониторинга и диагностики устойчивости к инсектицидам в популяциях насекомых все большее применение находят молекулярные методы.

Цель исследования. Оценка распространения основных мутаций, ассоциированных с резистентностью к пиретроидам, фосфорорганическим соединениям и карбаматам, в трех природных популяциях *Musca domestica* L., собранных в 2021–2023 гг. в животноводческих помещениях Тюменской области.

Материалы и методы. Методом полимеразной цепной реакции с анализом полиморфизма длин рестрикционных фрагментов выполнено генотипирование генов *CYP*, *vssc* и *ace-2*.

Результаты. Выявлена одна мутация в гене *vssc* (L1014F), связанная с устойчивостью к пиретроидам, и две мутации в гене *ace-2* (G342A, G342V), обеспечивающие резистентность к фосфорорганическим соединениям и карбаматам. Резистентный аллель L1014F присутствовал у 40–70% исследованных особей всех трех популяций с частотой 30–55%. Аллель G342A обнаружен у 10 и 60% особей двух популяций с частотой 5 и 30% соответственно. Аллель G342V выявлен у 40% особей только одной популяции с частотой 25%.

Заключение. Полученные результаты свидетельствуют о потенциале формирования устойчивости к пиретроидам, фосфорорганическим соединениям и карбаматам в исследованных популяциях *Musca domestica*, что необходимо учитывать при выборе средств для дезинсекции животноводческих помещений и защиты животных от насекомых. Дальнейшие молекулярные исследования *Musca domestica* из граничащих с Тюменской областью регионов будут полезны для выработки стратегии по сдерживанию распространения резистентных аллелей в локальных популяциях.

Ключевые слова: комнатная муха, инсектициды, инсектицидная резистентность, маркеры устойчивости, молекулярная диагностика

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INTRODUCTION

Insects are a significant factor in the spread of various human and animal diseases [1, 2], including synanthropic and zoophilic flies, in particular *Musca domestica* L. house fly (*Diptera*: *Muscidae*) [3, 4]. The ability of adult *M. domestica* to be a mechanical vector of such pathogens as helminth eggs, protozoa, viruses and bacteria, including antibiotic-resistant strains, has been demonstrated in a number of studies [4, 5, 6, 7]. Thus, *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* causing bovine respiratory diseases were recovered from *M. domestica* collected at feedlots from animals suffering from bovine respiratory disease symptoms [5]. When homogenates prepared from house flies from US dairy and livestock farms were tested, tetracycline and florphenicol resistance genes with prevalence ranging from 5 to 95.8% were identified in recovered bacteria [6]. The ability of Newcastle disease virus to persist in an infectious dose in the gut of flies for four days after feeding with infected milk and for one day in chicken droppings has been shown under laboratory conditions [7], which increases the risk of disease spread via flies present in poultry farms. Given the veterinary importance of zoophilic flies, it is necessary to control their numbers.

Despite the great interest in pest control biological methods, the chemical method based on the use of synthetic insecticidal agents remains widely used. Synthetic pyrethroids, neonicotinoids, organophosphorus compounds (OPCs), and carbamates are most often used for protecting animals from insects and disinsecting livestock premises both in Russia and abroad [4, 8]. *M. domestica* quite rapidly develop resistance against insecticides when used intensively: for example, more than 20-fold increase of resistance to permethrin [9] and alpha-cypermethrin [10] was revealed under laboratory conditions over 10–20 generations. According to a number of studies, resistance to pyrethroids (deltamethrin, permethrin, beta-cyfluthrin, cypermethrin) was observed in house fly field populations in China [11, 12], Pakistan [9], Iran [13], USA [14], Saudi Arabia [10, 15], the Moscow and Kaluga Oblasts of the Russian Federation [8]. In the Tyumen Oblast, tolerant and exceptionally highly pyrethroid-resistant field populations were also recorded [16, 17]. OPC-resistant house fly populations were found, for instance, in China [12], Iran [18], and Saudi Arabia [15, 19]. Insecticide resistance of *M. domestica* field populations makes it difficult to control their numbers.

The molecular target of pyrethroids is voltage-sensitive sodium channels (*vssc*), and the presence of mutations in the genes encoding this protein, i.e. knock-down resistance (*kdr*), is recognised as a marker of resistance to pyrethroids [14, 20]. Of the five known alleles associated with target insensitivity and, consequently, pyrethroid resistance of insects, the *kdr* (L1014F) and *kdr-his* (L1014H) are the most frequently investigated [13, 14, 20]. Target insensitivity is often combined with another major mechanism of pyrethroid resistance, namely enhanced detoxification of insecticides via cytochrome P450-dependent monooxygenases (*CYP*). A confirmed molecular marker of this type of resistance is the presence of a 15-base pair (bp) insertion in the *CYP6D1* gene [21, 22]. Acetylcholinesterase (AChE), encoded by the *ace* gene, is a key enzyme of the cholinergic system and a major target of OPC and carbamate insecticides, which block the transmission of nerve impulses at cholinergic synapses. Resistance to OPC and carbamates may result from insensitivity of AChE due to mutations in

the *ace* gene or due to mutations in the carboxylesterase gene, leading to an increase in the hydrolytic activity of the enzyme with respect to OPC [20, 23]. *M. domestica* is known to have only one AChE-encoding gene, *ace-2* [24], and six major mutations associated with resistance to OPC and carbamates have been described in detail: V260L, A316S, G342A, G342V, F407Y, and G445A [25, 26, 27].

Analysis of insecticide resistance in *M. domestica* field populations in Russia is traditionally carried out using toxicological methods [8, 17, 28], which allow establishing the presence of a stable phenotype and the level of resistance and do not explain the mechanisms underlying insecticide resistance [29]. The resistance mechanisms are defined and the potential for its formation is assessed using biochemical and molecular methods [30], and these steps are critical for rationalized selection of insecticidal agents and development of insecticide application schemes. As compared to traditional toxicological methods, molecular tests provide more complete information on the population structure, and the combination of toxicological and molecular methods allows objective assessment of the level of adaptation of the population to insecticide load [31]. Among molecular methods for detecting mutations associated with insecticide resistance, PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) is used [32]. The PCR-RFLP method is cost-effective, easy to implement and requires only basic molecular genetic equipment; it is widely available and is a good alternative to sequencing.

The aim of the study was to test *Musca domestica* flies collected from three field populations in the Tyumen Oblast for the presence of mutations in *CYP*, *vssc* and *ace-2* genes associated with resistance to pyrethroids, OPC and carbamates by PCR-RFLP.

MATERIALS AND METHODS

The study was aimed at *M. domestica* flies of three field populations: Nov (56.53700°, 65.24238°), Cha (56.781583°, 65.96014°), Nik (55.55352°, 70.62864°) collected in livestock facilities of the Tyumen Oblast in 2021–2023. The first generation (F1) was obtained from the collected insects of each population under insectarium conditions, 3–5 day old adult flies were frozen and stored at –80 °C before they were used for testing.

DNA was isolated from adult flies (5 females and males of each population) using alkaline lysis [33]. The amplification process was performed with GeneExplorer GE-96G (Bioer, China) using an individual primer pair for each gene. P1, P2, P3, P4 primers were used for genotyping mutations in the *vssc* gene, and AceF and AceR primers taken from the study of X. Qiu et al. [32] were used for the *ace-2* gene. For genotyping of mutation in the *CYP6D1* gene, the S35 and AS2 primers and restrictase were used according to F. D. Rinkevich et al. [34]. The amplification conditions were identical except for the temperature of primer annealing (Table 1): at 95 °C for 5 min, further at 95 °C for 20 s, at 62–53 °C for 30 s, at 72 °C for 30 s (5 cycles), at 95 °C for 20 s, at 60–51 °C for 30 s, at 72°C for 30 s (35 cycles), at 72 °C for 10 min. The PCR reaction mixture included: 1 µL of total DNA; 4 µL of 5X ScreenMix-HS PCR prepared mix (Eurogen, Russia); 0.3 µL of each primer (25 µM); 14.4 µL of purified sterile water (18.2 µS/cm). The restriction enzymes and test conditions are indicated in Table 1. Visualization of restriction results was performed through electrophoresis with 2% agarose gel containing ethidium bromide.

RESULTS AND DISCUSSION

The prevalence and frequency of mutations associated with resistance to pyrethroids and OPCs have been investigated in *M. domestica* field populations in Denmark [35], Turkey [36], Iran [26, 37], USA [14, 34], Kazakhstan [22], United Arab Emirates (UAE) [38] and other countries. Regarding *M. domestica* populations in the Russian Federation, resistance to pyrethroids and other insecticides was previously assessed using mainly toxicological methods [8, 17, 28]. Data on molecular test results of the house fly field populations and the genetic potential for insecticide resistance in local populations of the Russian Federation have not been published in the open access.

Sse9I and Fat I restrictases are used *for* ***vssc genotyping*** with PCR-RFLP. The Sse9I restrictase cuts the amplicon into 2 fragments of 96 and 60 bp, respectively, in the presence of the L1014F mutation. The L1014H mutation is detected using the Fat I enzyme, which, in the presence of the mutation, cuts the 220 bp amplicon into fragments of 170 and 50 bp long, respectively [22]. Combining the both test results, we identified the following genotypes (Fig. 1): 1014 (L/L), 1014 (L/F), 1014 (F/F). The L1014F mutation was detected in 70% of the tested flies of the Nov and Cha populations and in 40% of the flies of the Nik population (Table 2).

Table 1
PCR-RFLP assay conditions

Gene	Primers (5′–3′)	Annealing temperature, °C	Amplicon length, bp	Restrictase	Mutation	Restriction conditions
vssc	P1. GTGCTGTGCGGAGAGTGG P2. GAAGCCTCCATCCTGGGAG	60	156	Sse9I	L1014F	3 h – 55 °C; 20 min – 65 °C
	P3. AGCTGTATACCCTTCTTCT P4. CGAAGTTGGACAAAAGCAAA	51	220	Fat I	L1014H	
CYP6D1	S35. AGCTGACGAAATTGATCAATCAGT AS2. CATTGGATCATTTTTCTCATC	59	732–711	Hpy 188III	CYP6D1v	1 h – 37 °C; 20 min – 65 °C
ace-2	AceF. CGGTGCATTTGGGTTTCTAC AceR. CGTAACCGCTAAGATCTGCTG	57	609	Mh1 I	G342	3 h – 37 °C; 20 min – 80 °C
				Aco I	G342A	3 h – 37 °C; 20 min – 65 °C

Table 2
Distribution of detected mutations associated with insecticide resistance in three populations of *M. domestica* in the Tyumen Oblast

Population	Number of flies	Proportion of flies with L1014F mutation, %	Number of flies with the genotype			Allele frequency, %	Proportion of flies with mutation, %		Number of flies with the genotype			Allele frequency, %	
			L/L	L/F	F/F	F	G342A	G342V	G/G	G/A	G/V	A	V
Nov	10	70	3	3	4	55	0	0	10	0	0	0	0
Cha	10	70	3	4	3	50	60	0	4	6	0	30	0
Nik	10	40	6	2	2	30	10	40	5	1	4	5	25

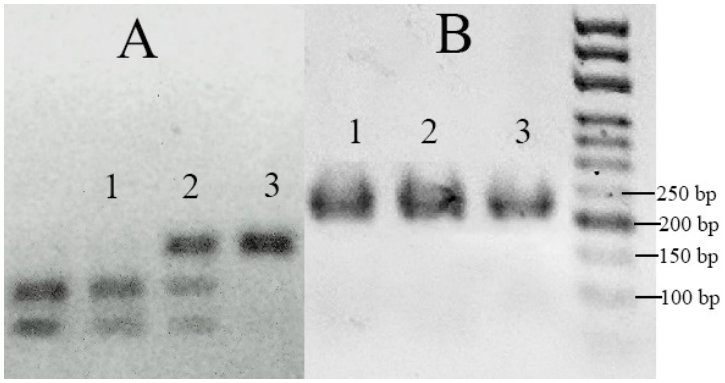

Fig. 1. Electrophoregram for PCR-RFLP amplification products of the vssc gene region: A – using Sse9I restrictase; B – using Fat I restrictase; 1 –1014 (F/F), 2 – 1014 (L/F), 3 – 1014 (L/L)

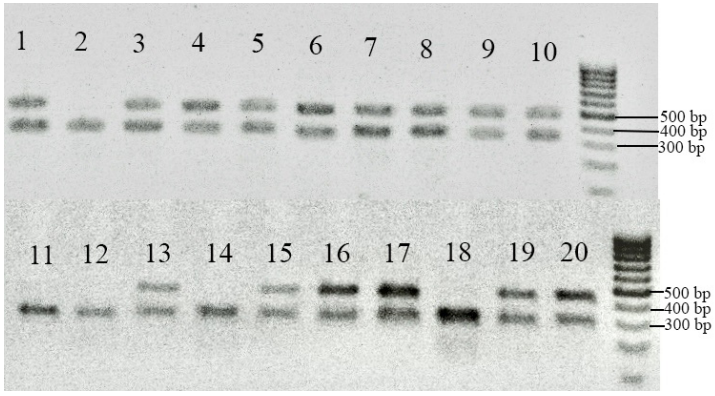

Fig. 2. Electrophoregram for PCR-RFLP amplification products of CYP6D1 gene region using Hpy 188III restrictase: 1–20 – different M. domestica species

Hpy 188III restrictase is used ***for CYP genotyping*** with PCR-RFLP. The resistant allele *CYP6D1v1* is characterised by a 15 bp insertion that disrupts the recognition sequence of the Hpy 188III enzyme. As a result, after restriction, fragments of 432 and 279 bp will be characteristic of the wild-type genotype, and 732 bp will be characteristic of the genotype carrying the mutation [34]. No resistant allele of *CYP6D1v1* was detected during the study, but Figure 2 shows that in some flies the 432 bp band is additionally cut by the Hpy 188III enzyme.

PCR-RFLP assay of the ace-2 gene was performed using Mh1 I and Aco I enzymes. The Mh1 I restrictase has a restriction site (GGC) that is characteristic of the wild-type genotype, 342G. After restriction, the two fragments of 361 and 248 bp detected in the electrophoregram are indicative of a wild type genotype, and a 609 bp fragment is indicative of the G342A or G342V mutation. The Aco I restrictase identifies the G342A mutation and cuts the amplicon into 2 fragments of 361 and 248 bp long. Thus, combining the two assays allows the detection of 6 different genotypes [32]. In our study we managed to detect 3 different genotypes (Fig. 3). G342A or G342V mutations were found in the Nov population. In the Nik population, the proportion of flies with G342A and G342V mutations was 10 and 40%, respectively. In the Cha population, only G342A mutation was detected in 60% of flies (Table 2).

In total, 3 (L1014F, G342A, G342V) out of 5 tested mutations were identified using the PCR-RFLP. The distribution frequencies of the resistant alleles in the three populations are presented in Table 2. The *kdr* mutation (L1014F) was found in the hetero- and homozygous state in 7 out of 10 flies of the Nov and Cha populations and in 4 out of 10 flies of the Nik population. The *kdr-his* mutation (L1014H) was not detected in any of the three populations. Test results for field populations of *M. domestica* in Turkey showed that the frequency of *kdr* and *kdr-his* alleles was 8 and 20%, respectively [36]. A survey of six field populations of the house fly in Kazakhstan showed the presence of the *kdr* allele in one of the populations with a frequency of 5% and the *kdr-his* allele in another population with a frequency of 14.3% in the heterozygous state [22]. Interestingly, the L1014F mutation was not reported in the Iranian population of *M. domestica*, and the percentage of *kdr-his* polymorphism (L1014H) was low at 4.7% [37]. On the contrary, in the USA, the *kdr* (L1014F) mutation was present in all six studied populations of house flies found in poultry and livestock farms, and *kdr-his* (L1014H) mutation was present in five populations. The frequency of *kdr-his* and *kdr* alleles varied widely in the populations, ranging from 12.5–28.1% and 7.1–76.6%, respectively [14]. A recent paper reported the detection of the *kdr* allele in *M. domestica* flies from the United Arab Emirates with the frequencies ranging from 9.4 to 46.9% [38]. The frequency of the resistant *kdr* allele (30–55%) in house fly populations in the Tyumen Oblast is comparable to that of populations from the USA and the UAE.

According to literature data, the knockdown resistance was first reported in house flies in the 1950s as insensitivity of sodium channels to the action of dichlorodiphenyltrichloroethane (DDT). It was later found that such resistance was associated with a nucleotide substitution (cytosine for thymine) in the *vssc* gene, resulting in the replacement of leucine with phenylalanine at position 1014 (L1014F) of the sodium channel alpha subunit [39]. As a result, structural changes in the protein molecule occur, affecting the interaction of the insecticide with the target. This

mutation also leads to the formation of resistance to pyrethroids, as they have a similar mechanism of action to DDT. The L1014F mutation, in addition to *M. domestica*, has been found in other two-winged insects (e.g., *Culex* and *Anopheles* mosquitoes, *Haematobia* fatheads), red cockroach (*Blattella germanica*), cat flea (*Ctenocephalides felis*), rat flea (*Xenopsylla cheopis*), triatomine bugs (e.g., *Triatoma infestans*), and other arthropods [39, 40].

One of the sufficiently described mechanisms of resistance to pyrethroids in insects is the enhancement of detoxification mediated by cytochrome P450-dependent monooxygenases (*CYP*) [41]. This type of insecticide resistance in *M. domestica* is associated with increased expression of the *CYP6D1* gene in the presence of 15 bp insertion (*CYP6D1v1* allele) [34]. In the USA, the resistant *CYP6D1v1* allele was detected with a frequency of > 75% in 5 studied populations of *M. domestica* [14]. According to V. Taşkın et al., the frequency of *CYP6D1v1* in house fly population from Turkey was 39% [36]. In Kazakhstan, this allele was present in 3 out of 6 populations of *M. domestica* with a much lower frequency: 4.4–6.3% [22]. In our study, PCR-RFLP assay did not reveal an insertion characteristic of the resistant allele of *CYP6D1v*; however, a mutation described earlier for *M. domestica* laboratory culture was detected in flies from the Nov and Cha populations [42]. Freeman J. C. et al. rightly pointed out in their study that *CYP6D1v1* is only partially responsible for the increased expression level of *CYP6D1* [14]. Due to the high evolutionary plasticity of *CYPs*, their other representatives or other mutations not yet described may be involved in the formation of resistance to insecticides – in general, and pyrethroids – in particular, in local *M. domestica* populations.

Detection of a rather large percentage of flies with the *kdr* mutation among *M. domestica* of the three field populations under study is not surprising, since, according to the surveys, pyrethroids (mainly deltamethrin and cyfluthrin) had been used for premise disinsection and animal protection from annoying insects for several seasons in livestock farms where the flies were collected. The use of these insecticides in this case served as a selection factor that apparently allowed the *kdr* (L1014F) mutation to gain a foothold in the populations under study. It is believed that in the presence of the *kdr* (L1014F) mutation, a higher level of pyrethroid resistance is formed than in the presence of the *kdr-his* (L1014H) mutation [36, 37]. In order to slow down the emergence of populations highly resistant to pyrethroids, it is advisable to replace pyrethroids with insecticides with a different mechanism of action (e.g., pyrroles, oxadiazines, insect growth regulators, etc.) in the studied livestock farms.

The higher *Diptera* have only one AChE-encoding gene and, accordingly, mutations providing resistance to OPCs and carbamates in this group of insects were found only in the *ace-2* gene. Such mutations individually or in combination lead to amino acid substitutions close to the catalytic triad of the active centre of the enzyme, affecting the orientation of the amino acids of the triad and limiting the access and/or binding of bulk insecticides (enzyme inhibitors) in the substrate centre of the protein [25]. Six such mutations have been described in detail for *M. domestica*: V260L, A316S, G342A, G342V, F407Y and G445A [25, 28]. In addition to *M. domestica*, resistance to OPCs and carbamates is known to be formed by a similar mechanism in other insect species, such as the green meat fly *Lucilia*

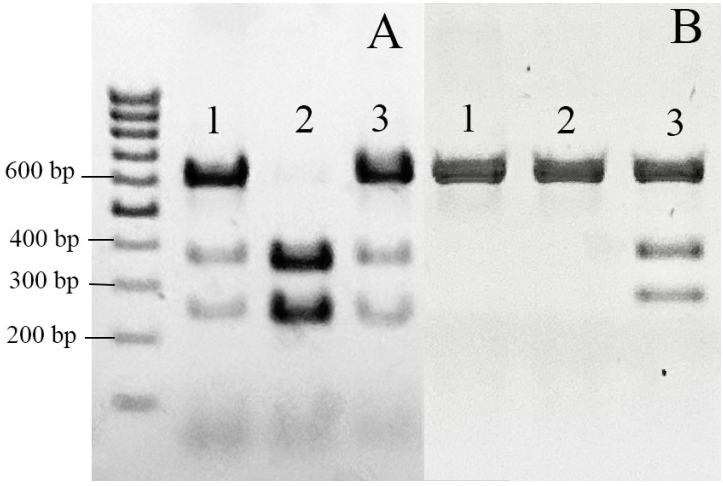

Fig. 3. Electrophoregram for PCR-RFLP amplification products of ace-2 gene region: A – using Mh1 I restrictase; B – using Aco I restrictase; 1 – 342 (G/V), 2 – 342 (G/G), 3 – 342 (G/A)

cuprina [43], *Drosophila melanogaster* [44, 45], and tephritid fruit flies *Bactrocera oleae* [46] and *Bactrocera dorsalis* [47]. In their study S. Başkurt et al. [48] indicated equivalent substitutions of amino acid residues in the AChE molecule for *M. domestica* and *D. melanogaster*. Literature data indicate that mutations underlying resistance of the house fly to OPCs and carbamates are widespread worldwide. Thus, resistant alleles G342A and G342V were found in flies of field populations of *M. domestica* of the USA, China, Iran, Kazakhstan [14, 22, 26, 49]. In house fly populations from Kazakhstan, G342A and G342V resistant alleles were found with a frequency of 27–48 and 0–20%, respectively [22]. G342A and G342V mutations were detected in 30 and 40% of *M. domestica* flies from Iran, respectively [26]. In our study, the G342V resistant allele was only present in the Nik population (the mutation was present in 40% of flies) with a frequency of 25%, the G342A allele in the Nik (in 10% of flies) and Cha (in 60% of flies) populations with a frequency of 5 and 30%, respectively, and these mutations were not detected in the Nov population. It is assumed that the allele with the G342V mutation plays a more significant role in AChE insensitivity and the formation of a high level of resistance to certain insecticides compared to that with G342A mutation [14, 25, 49].

CONCLUSION

In this study, PCR-RFLP assay showed presence of the *kdr* allele (L1014F), responsible for resistance to pyrethroids, with a frequency of 30–55% and the G342A/V alleles associated with resistance to OPCs and carbamates, with a frequency of 5–30% in flies from three and two field populations of *M. domestica* in the Tyumen Oblast, respectively. The presented data indicate the potential for formation of resistance to pyrethroids, OPCs and carbamates in the studied populations. On the basis of the obtained results it is possible to recommend replacement of these insecticides during disinsection of livestock facilities with preparations from other groups in order to mitigate the spread of resistant alleles in local populations of *M. domestica*. Further molecular studies of insects from different regions of the country are required to assess more fully the situation regarding resistance to pyrethroids, OPCs and carbamates and the potential for its formation in *M. domestica* in Russia.

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