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AIMS AND SCOPE

The mission of the publication is the delivery of information on basic development trends of veterinary science and practice and highlighting of vital issues and innovative developments in veterinary area for scientific community.

The journal is intended for scientists engaged in fundamental and applied research in the field of general and veterinary virology, epizootology, immunology, mycology, micotoxicology, bacteriology, as well as practicing veterinarians and doctors of veterinary laboratories and state veterinary services, university-level teachers for veterinary, biological, medical specializations, graduate and postgraduate students.

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Миссией издания является представление информации об основных направлениях развития российской и мировой ветеринарной науки и практики и привлечение внимания научной общественности к актуальным проблемам и инновационным разработкам в области ветеринарии.

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Implementation of the Joint Action Plan by the CIS Members to Prevent and Control Rabies

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ABSTRACT

Rabies is a fatal viral infection that attacks the central nervous system of mammals, including humans, leading to severe neurological dysfunction. Rabies continues to be a serious concern worldwide, including within the CIS countries. A comprehensive approach is essential to achieve and sustain freedom from rabies. On May 12, 2016, the Intergovernmental Council for Veterinary Cooperation of the Commonwealth of Independent States (CIS) tasked the Federal Centre for Animal Health with developing a draft Joint Action Plan for the CIS Member States on Rabies Prevention and Control, to cover the period through 2025. The set was developed and approved by the decision of the Council of Heads of the CIS Government on June 1, 2018. This paper reviews the implementation results of the Joint Action Plan to 2025 and presents the subsequent plan for 2025–2030, developed with the primary goal of mitigating the risk of rabies outbreak spread throughout the CIS members.

Keywords: FGBI "ARRIAH", CIS members, rabies, Joint Action Plan, One Health, prevention

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О реализации Комплекса совместных действий государств – участников СНГ по профилактике и борьбе с бешенством

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РЕЗЮМЕ

Бешенство – широко распространенное инфекционное заболевание млекопитающих животных, в том числе человека. Заболевание проявляется нарушением функции центральной нервной системы. Эпизоотическая ситуация по бешенству в мире остается напряженной, не отличается ситуация и в странах СНГ. Для достижения стойкого благополучия по бешенству необходим комплексный подход. Решением Межправительственного совета по сотрудничеству в области ветеринарии Содружества Независимых Государств от 12 мая 2016 г. ФГБУ «ВНИИЗЖ» было поручено подготовить проект Комплекса совместных действий государств – участников СНГ по профилактике и борьбе с бешенством на период до 2025 г. Комплекс был разработан и утвержден решением Совета глав правительств СНГ от 1 июня 2018 г. В статье подводятся итоги реализации Комплекса совместных действий до 2025 г. и освещается разработка Комплекса совместных действий на 2025–2030 гг., основной целью которого является минимизация риска распространения очагов бешенства на территории государств – участников СНГ.

Ключевые слова: ФГБУ «ВНИИЗЖ», страны СНГ, бешенство, Комплекс совместных действий, концепция «Единое здоровье», профилактика

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Конфликт интересов: Чвала И. А. и Метлин А. Е. являются членами редколлегии, но не имеют никакого отношения к решению опубликовать эту статью. Об иных конфликтах интересов авторы не заявляли.

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Rabies is a widespread, often fatal infectious disease reported on all continents except Australia and Antarctica (Fig. 1). It affects the central nervous systems of warm-blooded animals and humans, leading to encephalomyelitis, paralysis, and death.

Although the disease has been eradicated in some European countries, it remains a serious problem across much of Eurasia, Africa, and the Americas. According to the World Health Organization (WHO), approximately 70,000 people die from rabies annually in over 150 countries, with 95% of these cases concentrated in Africa and Asia. This disparity is due to a lack of awareness about rabies and limited or absent access to essential preventive measures. The disease is reported in both wild carnivores and domestic animals, being the main reservoir of the virus. Asian countries hold the lead in the number of human and animal rabies cases. International conferences are organized for World Rabies Day (September 28) to facilitate the exchange of data on infection control, diagnostic methods, and up-to-date information on related drugs. In 2024, the Federal Centre for Animal Health hosted an international scientific and practical conference "Breaking Rabies Boundaries" within the framework of a four-party partnership between WHO, the World Organization for Animal Health (WOAH), the Food and Agriculture Organization of the United Nations (FAO) and the United Nations Environment Programme (UNEP), dedicated to this day. The conference was attended by Thanawat Tiensin, FAO Assistant Director-General, and Chief Veterinarian; Oleg Kobiakov, the Director of Liaison Office with the Russian Federation; Batyr Berdyklychev, Head of the WHO Country Office in the Russian Federation; Vladimir Moshkalo, Head of the UNEP Regional Office in the Russian Federation; and Budimir Plavsic, Head of the WOAH Regional Representation for Europe in Moscow. The conference brought together more than 400 participants, including experts from international organizations, leading veterinary specialists from Russian institutions, government officials, and members of the public. Based on the conference outcomes, experts established a rabies control strategy centered on three key components: public awareness campaigns, managing stray animal populations via mandatory pet identification, and the widespread vaccination of cats and dogs.

The animal rabies situation is tense in many countries bordering Russia, such as Belarus, Azerbaijan, Kazakhstan, Mongolia, and China. The Baltic countries successfully eliminated rabies a few years ago through an intensive, large-scale oral vaccination campaign. This approach has since served as an effective model for nations in the Balkan Peninsula, such as Slovenia, Croatia, Serbia, North Macedonia, Montenegro, Albania and Bulgaria, which are now also exemplary cases of disease eradication. Through comprehensive programs focused on vaccination, monitoring, and surveillance, they have successfully eradicated rabies within their territories. Thanks to strict epizootic surveillance, these countries have reported no major outbreaks for many years, with only isolated, imported cases of rabies occurring.

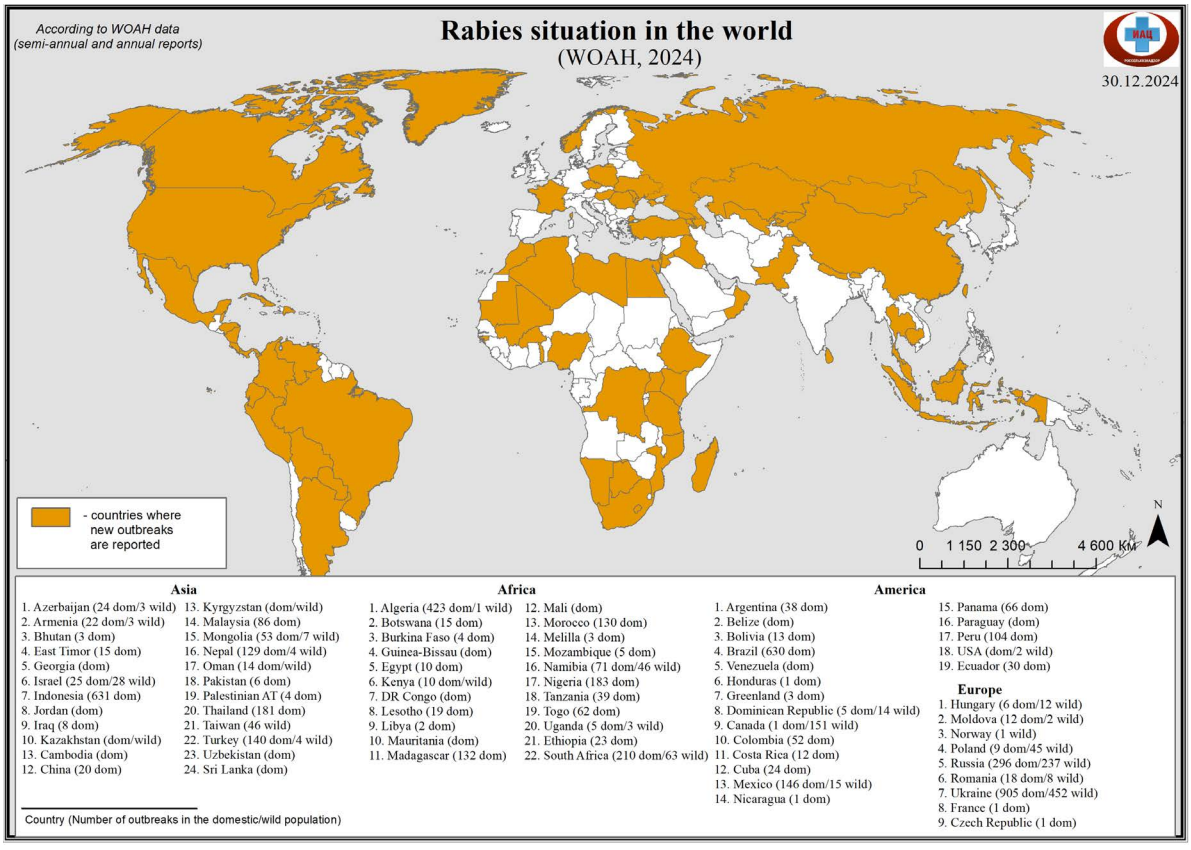

Fig. 1. Global rabies situation as of December 30, 2024


МИНИСТЕРСТВО СЕЛЬСКОГО ХОЗЯЙСТВА РОССИЙСКОЙ ФЕДЕРАЦИИ

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Москва ВДНХ

Fig. 2. Golden Autumn 2018 Diploma

The global increase in dog rabies cases is amplifying the role of dogs as a viral source, directly endangering human health. In response, the Russian Federation, as a member of the WHO, WOAH, and FAO, is taking steps to meet the goals of a 2030 global initiative to eliminate dog-mediated human rabies. This initiative, launched at the 2015 International Conference on Rabies, requires action in research, policy, and practical areas. The Russian Federation's first steps began in 2000 when the Veterinary Department of the Ministry of Agriculture Russia designated the All-Russia Research Institute for Animal Health (now the Federal Centre for Animal Health) as the national diagnostic center for rabies testing by Order No. 32 on October 19, 2000. Through this order, the Federal Centre for Animal Health was entrusted with the responsibility of monitoring the epizootic situation and was granted the authority to submit proposals for enhancing diagnostic methods, pharmaceuticals, and preventive measures for rabies in domestic and wild animals. The decision of the Intergovernmental Council for Veterinary Cooperation of the Commonwealth of Independent States (CIS) of May 12, 2016 tasked the Federal Centre for Animal Health with developing a draft Joint Action Plan for the CIS Member States on Rabies Prevention and Control, to cover the period through 2025 (hereinafter referred to as the Joint Action Plan). It was determined that a sustainable, rabies-free status and a reduction in economic losses require an integrated approach, encompassing the development of diagnostic protocols, evaluation of immunization efficacy, large-scale molecular research in Russia and in the CIS neighboring states, and analysis of vaccine strain properties. Addressing these tasks requires active international cooperation through the exchange of experience, knowledge, and the pursuit of joint research. The Joint Action Plan was developed and approved by the decision of the CIS Council of Government Heads on June 1, 2018. This decision was signed by the Governments of the Republics of Armenia, Belarus, Kazakhstan, Tajikistan, Uzbekistan, Kyrgyzstan and the Russian Federation.

For the development of the Joint Action Plan, the Federal Centre for Animal Health was awarded the gold medal of the Golden Autumn contest in 2018 (Fig. 2).

During the implementation of the 2018–2025 Joint Action Plan, all CIS members took significant steps to combat rabies. These included developing and executing national control plans, conducting diagnostic tests on wild and domestic animals, and accrediting laboratories to the GOST ISO/IEC 17025-2019 standard. Furthermore, countries assessed the epizootic situation, carried out preventive immunization, and monitored immune status. These comprehensive measures have been instrumental in advancing disease freedom, reducing infection risk for humans and animals, and minimizing the economic impact of the disease.

The work of the Federal Centre for Animal Health has also been crucial to rabies control successes. As the CIS's designated training organization, the Federal Centre for Animal Health enhances the skills of veterinary specialists from member states in modern rabies diagnosis, prevention, and control.

Several countries have established a reserve stock of essential veterinary drugs to control infectious diseases. This stock, which includes rabies vaccines and immunoglobulins for public immunization, supports measures for prevention, diagnosis, outbreak response, and treatment. Laboratories involved into rabies diagnostics participated in interlaboratory comparison tests on a regular basis.

Scientific research into the molecular epidemiology of rabies involved creating and replenishing a national collection of field isolates. Analysis confirmed the absence of vaccine strains among these isolates, demonstrating the safety of the rabies vaccines in use.

The knowledge gained from this accumulated experience was disseminated annually through open-source publications (scientific journals, collections of papers, conference proceedings) and presentations at international conferences, roundtables, webinars and seminars.

The veterinary services of the CIS members consistently informed the public about rabies prevention and bite response protocols through articles, informational leaflets, and website postings.

To summarize the results of the Joint Action Plan implementation, the CIS Executive Committee sent letters to the CIS governments. Responses were received from the Republics of Armenia, Belarus, Kazakhstan, Tajikistan, Uzbekistan, the Kyrgyz Republic and the Russian Federation.

A review of reports from CIS member states indicates that the rabies epizootic situation remains tense (Fig. 3). Despite this, the implemented measures have yielded significant results, including a reduction in animal rabies cases and the prompt execution of laboratory diagnostics and animal health measures as outlined in the Joint Action Plan.

This work has established a long-term foundation for effective rabies prevention and control cooperation among the CIS members. These results provide a foundation to continue working consistently toward rabies freedom and eventual eradication throughout the CIS. Although the territories of the CIS members remain infected due to their natural geography, the work done in recent years has had a positive impact.

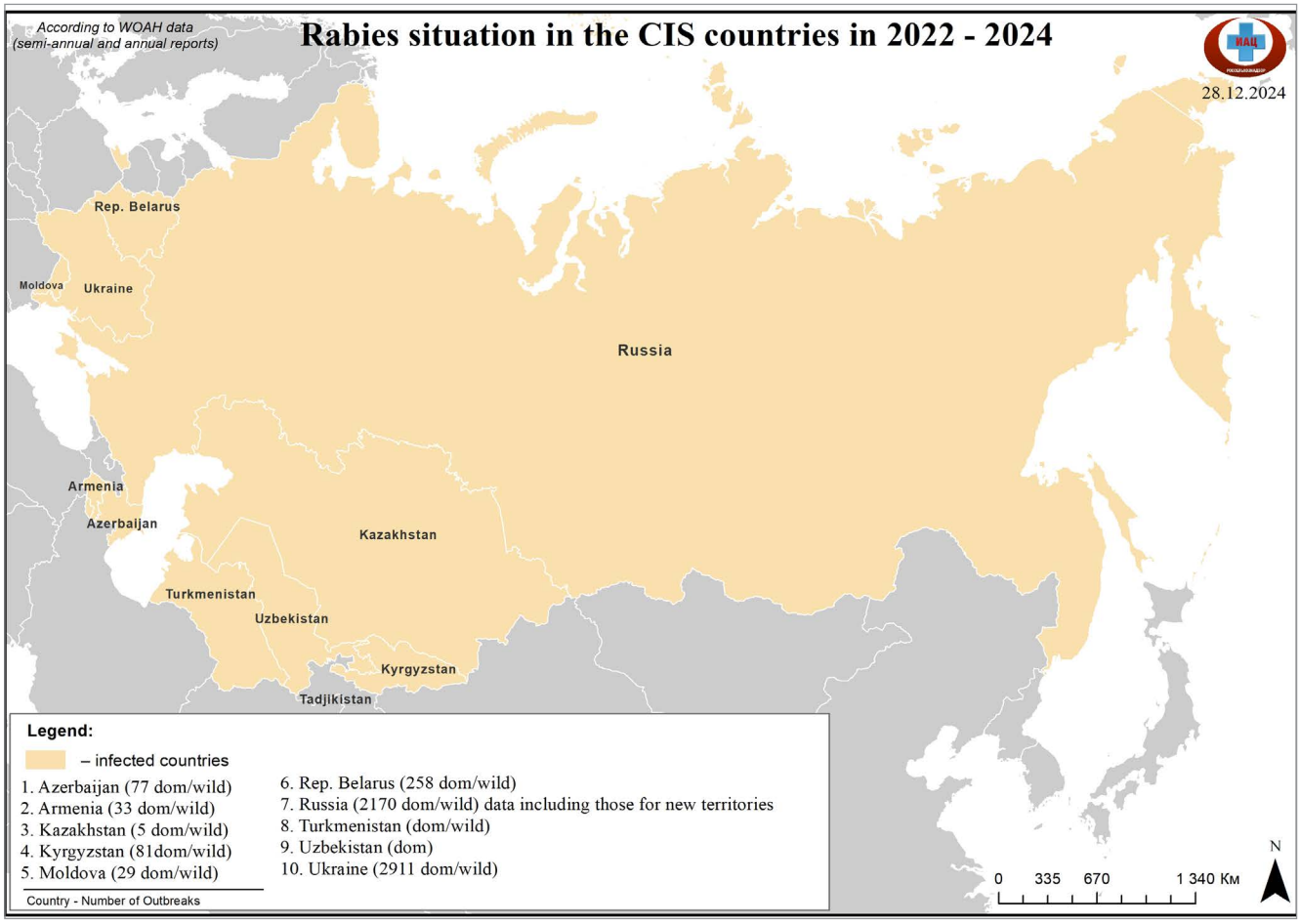

Fig. 3. Rabies situation in the CIS members as of December 28, 2024

Since rabies remains a serious problem in the CIS and the current Joint Action Plan ends in 2025, the Intergovernmental Council for Veterinary Cooperation has endorsed a proposal by the Federal Centre for Animal Health to extend the plan to 2030. The proposal emphasized the need to strengthen the close cooperation among the CIS members to achieve and maintain rabies freedom in each state and across the Commonwealth.

Draft Joint Action Plan for 2025–2030 developed by the Federal Centre for Animal Health was discussed in October 2024 at the 46th regular meeting of the Intergovernmental Council for Veterinary Cooperation in Turkmenistan (Ashgabat) and approved by the representatives of the CIS Veterinary Services. In the period from January 31 to February 21, 2025, the CIS Executive Committee office (Moscow) approved it at expert group meetings and on July 18, 2025, it was approved at a meeting of the CIS Economic Council. The approval of the Joint Action Plan is on the agenda of the meeting of the Council of Government Heads on September 30, 2025.

The Joint Action Plan for 2025–2030 builds upon foundational CIS veterinary safety documents, maintaining continuity with the goals, objectives, and cooperative frameworks established in the previous plan up to 2025. The plan incorporates proposals from the CIS Veterinary Services, recommendations from international organizations, and lessons learned from rabies control programs in the EU, US, and Canada. Furthermore, the new Joint Action Plan reflects the core principles of the "Zero by 30" strategy. This strategy is underpinned by a Tripartite Memorandum of Understanding between the WHO, FAO, and WOAH, which guides countries in developing national rabies eradication plans through a One Health and intersectoral cooperation framework.

The main goal of the Joint Action Plan for 2025–2030 is to work towards the eradication of dog-mediated rabies in CIS members by minimizing the risk of outbreaks and their spread.

The implementation of the Joint Action Plan on Rabies Prevention and Control through 2030 will enable the CIS members to develop national strategies to stop rabies circulation among domestic and stray carnivores, primarily dogs; to continue veterinary measures that reduce rabies incidence in wild carnivore populations; to strengthen veterinary services in fulfilling the requirements of Article 8.15 of the WOAH Terrestrial Animal Health Code and to minimize the risk of human rabies infections.

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Basics of rabies prevention in the Republic of Belarus (review)

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ABSTRACT

Introduction. Rabies remains one of the most dangerous zoonotic diseases. The Republic of Belarus is affected by animal rabies and the disease is reported in all regions of the country.

Objective. The work was aimed at assessment of rabies situation in the Republic and summarizing up-to-date approaches to the disease diagnosis and prevention.

Results. Current data on rabies situation in animals and humans in the Republic of Belarus are presented. In the Republic of Belarus, rabies is commonly reported in wildlife and both the country territory and its neighbouring countries (Poland, Lithuania, Latvia, Ukraine, Russia). Wild carnivores, foxes, raccoon dogs and wolves are the main reservoir of rabies virus (70% of all reported cases). Domestic carnivores (cats, dogs) are the second rabies reservoir, and sporadic rabies cases are also reported in livestock animals. The main activities of the Veterinary and Sanitary-Epidemiological Service of the Republic of Belarus, including specific rabies prevention in domestic carnivores, oral rabies vaccination of wild carnivores and public awareness campaigns are described in the paper, historical and modern data on the vaccination and its effectiveness, main strategies laid down in the Comprehensive Rabies Prevention Plan for 2021–2025 as well as data on the effectiveness of the specific activities included in the plan are presented.

Conclusion. In the Republic of Belarus, rabies remains a significant concern, particularly in wild animals. Key rabies control measures primarily involve the oral vaccination of wild animals and public awareness campaigns. To achieve a sustained reduction in rabies cases in wild and domestic animals and to minimize human exposure risks, the areas of wildlife oral vaccination shall be expanded, domestic carnivore vaccination programs shall be intensified and their control shall be enhanced.

Keywords: review, Republic of Belarus, rabies, domestic and wild animals, prevention, oral vaccination

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Основы профилактики бешенства в Республике Беларусь (обзор)

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ABSTRACT

Введение. На современном этапе бешенство продолжает оставаться одним из самых опасных инфекционных заболеваний зоонозной природы. Республика Беларусь является неблагополучной по бешенству животных, при этом заболевание регистрируется во всех регионах страны.

Цель исследования. Целью работы являлись оценка эпизоотической ситуации по бешенству в республике и обобщение современных подходов к диагностике и профилактике заболевания.

Результаты. Приведены актуальные данные об эпизоотической и эпидемиологической ситуации по бешенству в Республике Беларусь. Бешенство на территории республики протекает в виде эпизоотии природного типа, в которую она была вовлечена совместно с граничащими государствами (Польша, Литва, Латвия, Украина, Россия). Чаще всего резервуаром вируса бешенства являются дикие плотоядные животные (70% от всех зарегистрированных случаев): лисицы, енотовидные собаки и волки. На втором месте – домашние плотоядные (кошки, собаки), в единичных случаях –

сельскохозяйственные животные. В статье описаны основные направления работы ветеринарной и санитарно-эпидемиологической службы Республики Беларусь, включая специфическую профилактику бешенства среди домашних плотоядных животных, оральную антирабическую вакцинацию диких плотоядных животных и информационную работу с населением, приводятся исторические и современные данные о вакцинации и ее эффективности, представлены основные стратегические направления Комплексного плана профилактики бешенства на 2021–2025 гг., приведены данные об эффективности отдельных мероприятий плана.

Заключение. В Республике Беларусь проблема бешенства остается актуальной, в первую очередь она касается дикой фауны. Основополагающими методами борьбы с заболеванием являются оральная антирабическая вакцинация диких животных и информационная работа с населением. Для стойкого уменьшения количества случаев бешенства в дикой и домашней фауне, а также для снижения рисков заболевания людей считаем необходимым дальнейшее расширение площадей оральной антирабической вакцинации, а также усиление контроля и интенсивности программы антирабической вакцинации домашних плотоядных животных.

Ключевые слова: обзор, Республика Беларусь, бешенство, домашние и дикие животные, профилактика, оральная вакцинация

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INTRODUCTION

The Republic of Belarus is a country affected by animal rabies, the disease is reported in all its regions. In the Republic of Belarus, rabies is commonly reported in wildlife and affects both the country territory and territories of its neighbours (Poland, Lithuania, Latvia, Ukraine, Russia) [1, 2, 3, 4, 5, 6].

The main reservoir of the rabies virus is wild carnivores, 70% of all reported cases. Among them, foxes are primary carriers. Raccoon dogs and wolves are also involved in the epizooty; their high population densities are supported by favourable environmental conditions for these species. Domestic carnivores (cats, dogs) are the second rabies reservoir; sporadic rabies cases are also reported in livestock animals. However, despite the fact that rabies cases are reported in animals annually, no rabies cases have been reported in humans since 2012 [3, 7, 8, 9, 10].

For the purpose of rabies prevention, mandatory anti-rabies vaccination of domestic carnivores (cats and dogs) and livestock animals is annually carried out in rabies-affected localities in Belarus. Persons at high-risk occupations for rabies exposure – including veterinarians, animal control officers, foresters, gamekeepers, and hunters – also receive pre-exposure rabies vaccination [9, 11, 12, 13, 14].

The ongoing oral immunization of wild carnivores, implemented since 2004, has significantly improved the rabies situation in the Republic of Belarus. It should be noted that chicken heads, into which the vaccine was directly injected, were used for the first vaccination programs. In 2007–2010, vaccination of wild carnivores was carried out throughout the country, and in 2010, the country's veterinary service decided to centralize the vaccination efforts and the baits containing the vaccine in a blister were developed and put into practice. Since 2011, vaccination has been carried out along the border of the Republic of Belarus with Lithuania, and since 2012 – with Latvia and Poland.

In 2014, the number of animal rabies cases in the country decreased. In 2015 the number of animal rabies cases increased due to the rabies cases detected in uncultivated territories of the Gomel Oblast (230 cases) and Mogilev Oblast (138 cases). In 2016, the number of animal rabies cases decreased again, but in 2017–2018 there was an increase in rabies incidence, 539 cases were reported in 2019, and 752 cases were reported in 2020. The largest number of rabies cases was reported in the Mogilev (199), Minsk (177) and Gomel (168) Oblasts. A total of 123 rabies cases were reported in the Vitebsk Oblast, 52 rabies cases were reported in the Brest Oblast. The lowest number of rabies cases (33) was reported in the Grodno Oblast. Analysis of the data clearly indicates the dependence of the number of infected animals in different oblasts of Belarus on the performed/non-performed oral vaccination of wild carnivores [9, 11, 12]. In the Republic of Belarus, the oral vaccination is targeted and limited. Over the past two years, oral vaccination of wild carnivores has not been carried out in the Gomel and Mogilev Oblasts, which have traditionally been leaders in the number of reported animal rabies cases in recent years (for example, in 2020 there was a twofold increase compared to the previous year, followed by a gradual decline). Vaccination of wild animals in the Grodno Oblast, including vaccination performed within the transboundary collaborative actions, resulted in 2.8 fold-decrease in animal rabies cases in the Oblast in 2020 as compared to the previous year, and the number of infected animals was practically reduced to zero by the reporting year 2024 [9, 10].

A total of 2.75 million vaccine baits are distributed annually (1,377 thousands baits in spring and 1,377 thousands baits in autumn) in the Grodno Oblasts, as well as in

Table
Dynamics of rabies incidence in the regions where oral rabies vaccination is carried out

Region	2020	2024
Brest Oblast	52 (39 wild + 13 dom.)	9 (8 wild + 1 dom.)
Vitebsk Oblast	123 (73 wild + 50 dom.)	28 (20 wild+ 8 dom.)
Grodno Oblast	33 (25 wild + 8 dom.)	1 wild

wild – wild animals, dom. – domestic animals.

the areas of the Brest and Vitebsk Oblasts bordering the EU countries (about 56 thousand km^2). It should be noted that there is a steady trend towards a decrease in rabies cases in wildlife, owing to, among other things, the annually expanding area of oral vaccination of wild carnivores. Thus, reported data shows a 4-fold decrease in rabies cases in the Brest and Vitebsk Oblasts and a 30-fold decrease in the Grodno Oblast for the past five years (2020–2024). In the latter, the disease has been nearly eliminated (Table).

Therewith, in the oblasts where oral vaccination was not carried out (Minsk and Mogilev Oblasts) or was performed manually with minimal bait distribution in some areas (Gomel Oblast), the number of rabies cases was significantly higher in 2024. This was particularly evident in the city of Minsk and Minsk Oblast and Mogilev Oblast, where 75 and 49 cases were reported, respectively.

To achieve full coverage of the country's territory the area of aerial wild carnivore vaccination must be expanded to 129,000 km^2. For the Republic of Belarus, the estimated vaccination coverage area is 185,000 km^2 where 8.52 million baits are to be distributed annually – 4.26 million baits in spring and 4.26 million baits in autumn – at a distribution density of (24 ± 1) baits per km^2. It should be noted that the Republic authorities pay special attention to the ongoing monitoring and assessment of oral vaccination effectiveness in wild carnivores, which is critical for making evidence-based decisions on the vaccination program continuation, modification, or cessation.

The comprehensive scheme (program) for assessment of oral rabies vaccination effectiveness in wild carnivores includes the following key points.

1. Estimation of the vaccination coverage (the area where the oral rabies vaccination of wild carnivores is carried out).

2. Registration of oral rabies vaccination duration and continuity in wild carnivores.

3. Selection of target (sentinel) animal species for assessing the effectiveness of oral rabies vaccination of wild carnivores.

4. Assessment of the vaccines used for oral rabies vaccination for their effectiveness and safety.

5. Calculation of optimal of rabies vaccine bait placement (pattern and quantity) in wild carnivore habitats.

6. Monitoring for evaluation of the oral rabies vaccination results in wild carnivores.

7. Making adjustments to the oral vaccination program, if necessary, based on the monitoring results.

The State Veterinary Service is responsible for organizing the effectiveness assessment, which includes the following tasks:

– determination of the sampling size and sampling areas for monitoring purposes;

– determination of the funding source and designation of the testing laboratory;

– determination of the sampling procedure: persons involved in the shooting of animals, the place of sample storage, procedure for sample transportation to the testing laboratory.

The laboratory tests are aimed at:

– assessment of the level of animal protection based on the presence of anti-rabies virus antibodies;

– assessment of the vaccine bait uptake by identifying (counting) the animals containing the tetracycline marker in bone tissue (jaw bone and teeth);

– estimation of morbidity (number of diseased animals detected during monitoring and number of rabies cases detected during the year).

Based on the assessment and the above tasks fulfilment outcomes conclusions on the vaccination quality and on need for its correction or cessation are made.

During the development of oral vaccination programs, target animal species, being the main disease vectors in a particular territory, are identified and further used as sentinels during oral vaccination monitoring. In the Republic of Belarus, these are foxes, wolves and raccoon dogs.

The following sampling size for oral vaccination monitoring was determined: 4 animals per 100 km^2 per year, i.e. 2 animals per spring and autumn campaign (according to the resolution "Towards the elimination of Rabies in Eurasia", May 27–30, 2007), providing that principle of sample uniformity (homogeneity) is met [15].

Non-representative sampling repeated annually alongside the lack of serological monitoring for antibodies may obstruct a comprehensive evaluation of oral vaccination effectiveness. This data gap compromises the ability to make evidence-based decisions regarding the expansion or reduction of vaccination areas. It can also lead to an erroneous conclusion on rabies eradication in the area upon the oral vaccination program completion.

The Department of Veterinary and Food Supervision of the Ministry of Agriculture and Food of the Republic of Belarus designated the Medical and Diagnostic

Institution "Vitebsk Oblast Veterinary Laboratory" [9] accredited according to GOST ISO/IEC 17025-2019[1] to perform laboratory monitoring of the oral vaccination effectiveness based on the following parameters:

– detection of antibodies to rabies virus in sera, blood plasma or biological fluids by enzyme-linked immunosorbent assay (ELISA);

– detection of a biomarker (tetracycline) in wild carnivore teeth;

– detection of the antigen – antibody complex by fluorescent antibody test (FAT).

For quality assurance purpose, the MDI "Vitebsk Oblast Veterinary Laboratory" took part in international comparative tests using the following parameters in 2021:

– detection of antibodies against rabies virus in carnivorous sera in the framework of proficiency testing program (provided by VETQAS, Animal & Plant Health Agency, Great Britain);

– detection of a marker (tetracycline) in carnivore teeth/jaws (organized by the Federal Centre for Animal Health, Russia).

Laboratory tests for rabies diagnosis are carried out in accordance with the regulatory documentation: GOST 26075-2013[2]; Methodical guidelines for the detection of antibodies to rabies virus with ELISA in sera, blood plasma or biological fluids[3] and for the detection of a marker (tetracycline) in wild carnivore teeth[4].

When monitoring oral vaccination effectiveness, ensuring the quality of the samples collected for laboratory testing is crucial. The samples are collected jointly by the Veterinary Service and hunting farms (e.g., hunters, rangers) or environmental protection agencies (e.g., foresters) representatives. Jaw sections with canines and incisors as well as biological fluids of the animal (blood, transudate, thoracic fluid) are collected for laboratory tests.

The oral rabies vaccine uptake is determined by the fluorescent method: a marker (tetracycline) contained in baits is detected in teeth and mandibular bone tissue. Test materials (lower jaw with canines and incisors) are stored and transported in frozen state.

The age is taken into account when bait uptake is determined. Because the tetracycline marker is permanent, it is not possible to ascertain when the bait was consumed. Therefore, the proportion of tetracycline-positive results is usually higher in adults than in young animals. To evaluate the outcomes of the latest vaccination round, a cohort of young animals should be selected. However, age assessment is often neglected since young animals constitute a significant proportion of the population.

The level of animal protection (seroprevalence) is assessed by determination of virus neutralizing antibody levels (IU/mL). It should be taken into account that 0.5 IU/mL is considered to be the level of antibodies (determined by virus neutralization test) that reliably protects against infection with rabies virus. For rabies eradication, the proportion of protected animals (target species) in the population should be at least 70%. This level of protection prevents infection, even after contact between a rabid animal and vaccinated animals. When the virus transmission rate falls below one, the epizootic subsides. Maintaining a high and uniform level of animal protection in a vaccinated area is crucial because low-immunity pockets can serve as persistent reservoirs for natural infection. This can facilitate the spread of disease to other regions after oral vaccination program cessation.

The quality of biological samples collected for laboratory diagnosis is of great importance. The main biological sample to be collected for tests for rabies is the animal brain, its most important parts: medulla oblongata and cerebellum as well as Ammon's horn and cerebral cortex.

For optimal preservation, biological samples shall be stored frozen or chilled. Preservation in buffered saline supplemented with 10% formalin is acceptable only in extreme cases. It is critical to note that this latter method affects the test result quality, making virus isolation impossible. Preservation in buffered saline solution supplemented with glycerine (50%) is also allowed, however, this method also produces lower-quality results [16, 17].

The MDI "Vitebsk Oblast Veterinary Laboratory" carries out the following tests: fluorescent antibody test, mouse inoculation test (bioassay), ELISA and neutralization test.

Fluorescent antibody test (other names: immunofluorescence assay (IFA), direct fluorescent antibody test (dFAT) offers the following advantages being rapid (providing results within 2–3 hours), highly sensitive and specific, relatively cheap. Disadvantages of the FAT include the lack of instrumental recording, which introduces subjectivity in result interpretation.

The mouse inoculation test (bioassay) demonstrates high sensitivity and specificity when performed on fresh, non-decomposed samples. However, this method has several significant disadvantages: it is a prolonged procedure, requiring up to 30 days to complete; it is costly; and its sensitivity drastically decreases with putrefied samples leading to false-negative results. Furthermore, the technique poses an increased biohazard risk to the operator due to handling infected animals and syringe as well as a potential risk to the environment. It also faces growing ethical concerns and public opposition to the use of tests in animals. It should be noted that bioassay for rabies detects only infectious (active) virus. Therefore, if a pathogen has been inactivated, a bioassay might show a negative result (no live virus) even with positive antigen or genome detection, because the components of the virus are still present but the virus itself is not active. In addition, if the biomaterial has

[1] https://nil39.ru/docs/GOST-17025.pdf (in Russ.)

[2] https://docs.cntd.ru/document/1200104625 (in Russ.)

[3] Methodical guidelines for the detection of antibodies to rabies virus with ELISA in sera, blood plasma or biological fluids: approved by the MDI "Vitebsk Oblast Veterinary Laboratory" on 12 May 2021. (in Russ.)

[4] Methodological guidelines. Detection of a marker (tetracycline) in wild carnivore teeth: approved by the MDI "Vitebsk Oblast Veterinary Laboratory" on 11 June 2021. (in Russ.)

a toxic effect, it is impossible to obtain a result. Mouse deaths are confirmed as rabies-specific using the FAT.

The enzyme-linked immunosorbent assay (ELISA) designed for virus antigen detection provides significant advantages, including high specificity, a high-throughput capacity for processing numerous samples quickly, and a simple test procedure, instrumental recording of the results; undiminished sensitivity when testing non-fresh (decomposed) pathological samples. But at the same time, ELISA demonstrates lower sensitivity when used for testing fresh pathological samples compared to FAT. However, the World Organisation for Animal Health (WOAH) recommends that ELISA, due to its often lower sensitivity compared to gold-standard methods, be used for rabies diagnosis only when followed by confirmatory testing.

Virus neutralization test – VNT (such as rapid fluorescent focus inhibition test – RFFIT) is used for testing anti-rabies immunity level for the purpose of assessment of oral vaccination effectiveness. The principal advantage of the VNT is its ability to precisely quantify virus-neutralizing antibodies, a key feature that establishes it as the diagnostic "gold standard". However, the VNT has several disadvantages. These include a prolonged procedure lasting several days, stringent requirements for operators' qualifications and experience, cell culture maintenance, high operational costs. Furthermore, the test can be compromised by the toxic effects of certain samples on the cell monolayer and necessitates the use of fixed rabies virus.

When assessing rabies occurrence in wildlife, it is essential to recognize that monitoring and epizootological surveillance are interdependent and serve as key components of successful oral vaccination programs. A decrease in reported rabies cases is a key indicator of successful oral vaccination programs (provided that ongoing passive surveillance is carried out and laboratory testing of suspected animals is performed).

A decision on oral rabies vaccination cessation is made when no rabies cases have been reported for at least two years.

The rabies situation in the country requires the constant and vigilant attention of the Sanitary and Epidemiological Service of the Ministry of Health of the Republic of Belarus [10, 13, 18]. A change in the epizootic pattern increases human exposure to rabid animals, including mass exposure events (more than 5 injured persons), thereby raising the risk of rabies in the human population. Annually, approximately 18,000 to 20,000 people receive post-exposure prophylaxis for rabies following contact with animals. Of these, 400 to 700 human cases involve confirmed exposure to rabid animals. In 2024, the largest number of post-exposure treatments was reported in the Minsk and Mogilev Oblasts, while the minimum number of post-exposure treatments was registered in the Grodno Oblast [10, 18, 19, 20].

Control of natural rabies reservoirs, primarily through the mass oral vaccination of wild carnivores, is the most effective strategy for ensuring public safety. This approach is essential given the rising number of animal rabies cases and the central role wild carnivores play as the main reservoir of the virus.

Successful rabies prevention requires constant and coordinated actions taken by the concerned ministries and departments, executive authorities, and research institutions. Only an interdepartmental approach can successfully combine the efforts of these organizations. This strategy served as the basis for the Comprehensive Rabies Prevention Action Plan (hereinafter referred to as the Comprehensive Action Plan) in the Republic of Belarus (2021–2025)[5]. The Plan was approved in 2021 (No. 06/204-211/321). One of its main tasks was to organize the vaccination of wild carnivores against rabies.

The Comprehensive Action Plan goal is to prevent rabies in both wild and domestic animals, as well as to ensure epidemiological and public health safety, to prevent human rabies by drastically reducing natural rabies cases through vaccination of wild carnivores; to carry out specific anti-rabies preventive measures in pets and livestock animals; to reduce human exposures to rabies following the contact with animals, including rabid animals, that will ultimately reduce the number of persons receiving rabies post-exposure treatment.

According to the Comprehensive Action Plan, responsible authorities (Ministry of Agriculture and Food, Ministry of Health, Ministry of Nature, Ministry of Forestry, Ministry of Housing and Communal Services, executive committees in oblasts and raions of the Republic of Belarus) shall carry out relevant measures, the main of which are the following:

– censusing of wild carnivores including foxes, raccoon dogs, wolves: number of animals per km^2 of hunting grounds;

– monitoring of rabies situation in the world including rabies situation in border areas;

– mapping of wild carnivore density in hunting grounds;

– procurement of blister vaccine baits for oral immunization of wild carnivores taking into account the bait distribution area;

– briefing for persons involved in oral vaccination of wild carnivores;

– aerial distribution of blister vaccine baits across the target vaccination zone and recording the bait release coordinates;

– control shooting of wild carnivores being natural rabies reservoirs in the target zone, at least 2 animals per 100 km^2 of hunting grounds and delivery of samples to the diagnostic departments of regional veterinary stations (taking into account the population size);

– sampling and delivery of pathological samples (jaws and blood) collected from the animals shot within the vaccine quality monitoring and control to the laboratories;

– laboratory tests of the samples (blood – for antibodies, jaws – for markers) collected from the shot animals;

– collection and delivery of biological samples (brains of dead rabies suspects) for laboratory testing to monitor and record animal rabies cases;

– laboratory tests of brain samples from dead rabies suspects for animal rabies case monitoring and recording;

[5] https://s3-minsk.cloud.mts.by/datastorage/belitsa/library/321_Plan_for_rabies_prevention.pdf (in Russ.)

Fig. 1. Feeding oral immunization baits to wolves

– capture of stray dogs and cats in residential areas;

– measures taken to prevent the incursion of wild and stray animals on organizational facilities and premises;

– censusing of pets (dogs and cats) with annual data updating;

– oversight of adherence to pet and livestock keeping regulations by private owners and agricultural organizations;

– procurement of anti-rabies parenteral vaccine for immunization of pets and farmed animals;

– vaccination of pets (dogs and cats) against rabies;

– establishing and maintaining continuously renewed stock of anti-rabies vaccine and immunoglobulins in the regions and in city of Minsk sufficient to meet simultaneous demand for at least 20 persons;

– provision of post-exposure treatment to the exposed persons following the contact with animals;

– compiling the lists of persons at high risk of rabies virus infection (veterinarians, hunters, foresters, persons engaged in trapping and keeping stray animals, and others) and submission of these lists to regional public health authorities, as well as authorities responsible for official sanitary surveillance;

– preventive vaccination of persons at high risk of rabies virus infection;

– public awareness campaign across all media (including Internet resources) to raise awareness of rabies prevention measures among the persons of various age and professional groups, including: manifestations of rabies virus infection, actions to be taken in case of contact with animals, including rabid and rabies suspected animals, the consequences of refusing or interrupting rabies post-exposure treatment, as well as the rules for pet and livestock keeping, such as timely registration of animals and timely vaccination of animals against rabies.

Another area of the State Veterinary Service activities is the continuous monitoring and specific rabies prophylaxis improvement, when necessary, among wild carnivores kept in zoos, stray animals and animals kept in shelters. These animals pose a substantial risk to staff, and their rabies vaccination history is often uncertain. In such situations, parenteral rabies prophylaxis is highly risky and difficult. Consequently, current efforts include analysing and evaluating existing rabies vaccination methods for carnivores in shelters and zoos.

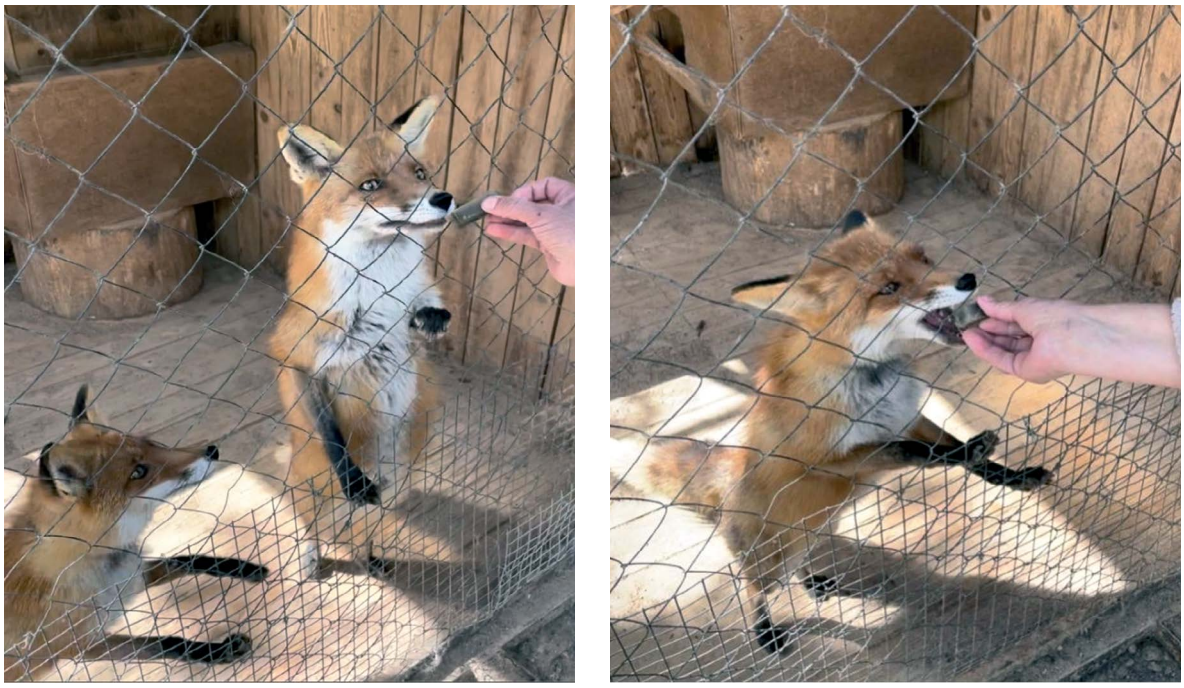

Fig. 2. Feeding oral immunization baits to foxes

This work is coupled with the development of improved anti-rabies vaccine baits and an assessment of their efficacy in these controlled environments (Figs. 1 and 2).

To enhance disease control and cost-effectiveness, the efforts are focused on development of improved designs of the baits for captive and stray carnivores and wildlife. Experimental bait models are being developed by the OJSC "BelVitunipharm" specialists. "Rabivit-VBF", rabies vaccine-containing bait, series No. 19 (22 g), was taken as a basis (prototype) and improved: its shape, size and composition were changed. New types of baits were experimentally tested in various groups of animals in order to identify the most effective form for the bait uptake. The tests were carried out in the Vitebsk Zoo and "Dobrik", Vitebsk shelter for stray animals, where oral baits were fed to wolves, foxes, raccoon dogs, domestic dogs and cats.

Our own tests performed for evaluation of oral rabies vaccination for its effectiveness yielded the following results.

1. According to video surveillance, animals actively consumed improved baits of all types (both old and new) within the first two days, but showed a distinct preference for the new ones.

2. It was observed that wild animals, particularly foxes, often do not consume baits at the drop point but instead relocate them for later consumption. This behaviour is a critical factor to consider during the visual assessment of bait uptake.

3. In the experiment conducted under near-natural conditions in the Vitebsk Zoo, the average uptake of all new-formulation baits was high, reaching 82%.

4. It was found that under adverse weather conditions, including heavy rain, the tested baits retained their original shape and form. Bait uptake was the highest in the evening and at night.

Thus, the experiment results indicate that the most modified baits tested meet the specified parameters and are suitable for the oral rabies vaccination of carnivores and wild animals in the Republic of Belarus. The new-formulation baits are cost-effective and will increase the effectiveness of oral rabies vaccination for wild carnivores across the country.

According to the Ministry of Health of the Republic of Belarus, in recent years, mass exposures of humans to rabid animals have become an alarming trend. While most incidents involve pets (dogs and cats) and livestock (sheep, goats and cattle), exposures to wild animals (foxes, raccoon dogs, hedgehogs), though less frequent, are also a significant concern. In agriculture, mass exposures are often occupational, whereas those with pets and wild animals typically result from human negligence, a lack of understanding about the severity of the risk, and the potential threat of rabies. Particularly noteworthy is the contemporary "selfie epidemic" spreading among the public. In several cases, it was the reckless pursuit of a photo with a wild animal – not a lack of knowledge about the disease – that led to mass exposures to a rabid animal. The public's frequent refusal to vaccinate pets was the second major factor contributing to their infection and the most common cause of mass exposures to rabid domestic animals. The reason for this is the pet's so-called "indoor lifestyle" and the pet owner's belief that it has little to no contact with the outside environment or other animals. Ignoring the potential risks of infection, despite possible or actual contact with wild or stray animals, leads to pet infections and, subsequently, to mass human exposures.

The growing populations of wild animals including ungulates (e.g., deer, roe deer), carnivores, and omnivores (e.g., foxes, raccoon dogs, wolves, lynx, bears) in several regions of the Republic of Belarus is a significant

contributing factor. This increase elevates the risk of rabies spread among animal populations, thereby amplifying the biological threat to humans. This is particularly true for high-risk groups such as hunters, gamekeepers, foresters, staff of nature reserves and national parks, zoologists, and others whose professional or scientific activities involve contact with wildlife.

It is also crucial to intensify public awareness campaigns on rabies prevention, targeting various demographic and professional groups, with a primary focus on children. The most vulnerable group – elementary school students – is of particular concern. This includes personal meetings between humane medicine practitioner, veterinarians and parents as well as dissemination of information through mass media platforms.

To facilitate easy and accessible understanding of the importance and danger of rabies for various demographic and professional groups, informational materials (posters, presentations, educational films) are being developed. Furthermore, different speakers are selected to best engage each target audience: educational sessions for children are conducted by students, Master's Degree candidates, or postgraduates from the Vitebsk State Academy of Veterinary Medicine, educational sessions for adults are conducted by the State Veterinary Service stuff-members and officers of the Epidemiological Service of the Ministry of Health of the Republic of Belarus. This tailored approach ensures the message is delivered in the most relatable and effective way for each audience, maximizing both comprehension and retention of critical information about rabies [10].

Special attention is also given to public awareness campaign targeting pet owners and to control of pet registration and vaccination by municipal services and the State Veterinary Service. These activities aim to clarify pet keeping regulations for the public, emphasizing the critical importance of timely animal registration and anti-rabies vaccination.

CONCLUSION

Thus, rabies remains a significant concern in the Republic of Belarus, particularly in wildlife. Given the increasing density of wild ungulate, carnivore, and omnivore populations, the risk of rabies infection is rising among both livestock animals and pets as well as humans. Intensive ongoing efforts for rabies prevention are taken. Key rabies control measures primarily involve the oral vaccination of wild animals and public awareness campaigns. To achieve a sustained reduction in rabies cases in both wild and domestic animals, and to lower the disease risk to humans, we consider it essential to further expand the geographic coverage of oral rabies vaccination campaigns, ultimately encompassing the entire territory of the Republic of Belarus. Concurrently with the expansion of the oral rabies vaccination program for wild carnivores, it is necessary to enhance the control and intensity of the anti-rabies vaccination program for dogs and cats.

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Rabies in the Russian Federation: A 35-year review of trends, patterns, and influencing factors

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ABSTRACT

Introduction. Sylvatic rabies cases characterized by a consistently high incidence among wild and domestic animals are reported in the Russian Federation. The epizootic cycle of rabies is maintained through the biological reservoir of *Lyssavirus rabies* in wild canid predators, primarily the red fox (*Vulpes vulpes*). Fox ecology and behavior determine the spatial spread of rabies, its seasonal incidence patterns, and the species composition of animals involved in the epizootic cycle.

Objective. The rabies spatiotemporal analysis of 35-year monitoring data to study determinants and patters of the current disease situation.

Materials and methods. Using Microsoft Access (relational database management system www.microsoft.com) the data on rabies outbreaks in the Russian Federation, rabies vaccination among wild animals and natural and agricultural zoning were aggregated. For spatial analysis, all epizootiological data were geocoded and visualized as vector map layers within the GIS thematic project. The GIS project was constructed using QGIS Desktop platform (www.qgis.org).

Results. The current rabies distribution area covers most of the Russian regions. The area of persistently high rabies incidence primarily encompasses the forest-steppe, mixed forest, and broadleaf forest biomes of the East European Plain. In the Russian Federation, the maximum number of rabies cases is reported among foxes. Rabies epizootics in natural ecosystems exhibit spillover effects, leading to active transmission among multiple domestic animal species. The primary risk group involves dogs, cats and cattle. Oral rabies vaccination of wild carnivores established a significant downward trend in animal rabies incidence while reducing amplitude fluctuations in long-term epizootic cycle.

Conclusion. The observed decline in rabies incidence across the Russian Federation has not been accompanied by a proportional reduction in the disease's geographic distribution. These findings underscore the need to modify current control measures and implement a comprehensive program for complete elimination of circulating *Lyssavirus rabies* strains from infected ecosystems.

Keywords: epizootic process, rabies, *Lyssavirus rabies*, disease distribution area, virus reservoir, database, geoinformation system, spatial analysis

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Обзор современной эпизоотической ситуации по бешенству в Российской Федерации. Динамика изменений за 35-летний период, закономерности, влияющие факторы

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РЕЗЮМЕ

Введение. На территории Российской Федерации регистрируются эпизоотии бешенства природного типа, которые характеризуются стабильно высокой инцидентностью среди диких и домашних животных. Непрерывность эпизоотического процесса бешенства обеспечивается биологической резервацией вируса *Lyssavirus rabies* в популяциях диких хищников семейства псовых, преимущественно в популяции рыжей лисицы *(Vulpes vulpes)*. Экология и этология лисиц предопределяют территорию распространения бешенства, сезонную динамику инцидентности и видовой состав животных, вовлекаемых в эпизоотический процесс.

Цель исследования. Проведение пространственно-временного анализа данных мониторинга бешенства за 35-летний период для исследования факторов, определяющих особенности современной эпизоотической ситуации.

Материалы и методы. На платформе реляционной системы управления базами данных Microsoft Access (www.microsoft.com) были агрегированы данные о вспышках бешенства на территории Российской Федерации, о проведении антирабической вакцинации среди диких животных, а также о природно-сельскохозяйственном районировании. Для выполнения пространственного анализа вся эпизоотологическая информация базы данных была геокодирована и представлена в виде наборов векторных карт в тематическом проекте геоинформационной системы. Построение ГИС-проекта

проводилось на платформе QGIS Desktop (www.qgis.org).

Результаты. Современный нозоареал бешенства охватывает территорию, которая затрагивает большую часть регионов Российской Федерации. Основная зона стабильно высокой инцидентности бешенства приходится на биомы лесостепей, смешанных и широколиственных лесов Восточно-Европейской равнины. На территории Российской Федерации максимальное количество случаев бешенства регистрируется среди лисиц. Эпизоотии бешенства в природных экосистемах сопровождаются эффектом «перелива» (spillover effect) с активным распространением болезни среди различных видов домашних животных. Основную группу эпизоотологического риска составляют собаки, кошки и крупный рогатый скот. Оральная антирабическая вакцинация диких хищников обеспечила формирование выраженного тренда на снижение заболеваемости животных бешенством с одновременным уменьшением амплитуды колебаний кривой инцидентности в периоды многолетних эпизоотических циклов.

Заключение. Регистрируемое снижение инцидентности бешенства на территории Российской Федерации не сопровождается значимым уменьшением площади нозоареала. Это свидетельствует о необходимости коррекции проводимых противоэпизоотических мероприятий с разработкой программы по обеспечению полной элиминации циркулирующих полевых штаммов *Lyssavirus rabies* из неблагополучных экосистем.

Ключевые слова: эпизоотический процесс, бешенство, *Lyssavirus rabies,* нозоареал, резервуар вируса, база данных, геоинформационная система, пространственный анализ

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Конфликт интересов: Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Rabies is a viral disease of the sylvatic cycle that causes fatal meningoencephalitis in all mammalian species [1]. Clinical rabies in animals and humans can be induced by infection with any of the 18 known types of viruses of *Lyssavirus* genus [2]. Lyssaviruses circulate within conspecific populations of mammals, known as biological reservoirs, and are maintained in natural ecosystems. The vast majority of lyssaviruses circulate in bat populations *(Chiroptera)* [3]. An exception is the *Lyssavirus rabies* species, which possessed biological mechanisms that ensure the replacement of the reservoir host [4, 5, 6]. The adaptation of *Lyssavirus rabies* to sustained circulation within terrestrial *Carnivora* populations has facilitated its global spread and establishment in diverse ecosystems worldwide [7].

Lyssavirus rabies is responsible for the overwhelming majority of all reported rabies cases [8, 9]. Since the mid-20th century, the red fox *(Vulpes vulpes)* has served as the primary reservoir host and main vector for *Lyssavirus rabies* transmission [10, 11]. In Arctic ecosystems, the Arctic fox *(Alopex lagopus)* is the primary reservoir for *Lyssavirus rabies* [12]. In forest biomes, the *Lyssavirus rabies* may be maintained in fox and raccoon dog *(Nyctereutes procyonoides)* populations [13]. However, the role of raccoon dogs as a key biological reservoir of rabies virus in the Russian Federation remains debated [14].

Although sylvatic (fox) rabies results in fewer human infections compared to urban canine rabies, it causes significant morbidity and mortality among diverse domestic and wild animal species [15]. Infection of non-reservoir animal species represents a spillover event that terminates the epizootic chain, as these dead-end hosts cannot sustain further transmission [16].

It was proved that three species of lyssaviruses are circulating in bat populations in the Russian Federation [17, 18, 19]. However, given the diversity of bat species and their habitats, this list is likely incomplete. Strains of *European bat lyssavirus 1* (EBLV-1, formerly *Lyssavirus hamburg*) have been confirmed to circulate in serotine bat *(Eptesicus serotinus)* populations across the European subcontinent. The circulation of *Lyssavirus Irkut* virus strains was detected in a population of the greater tube-nosed bat *(Murina leucogaster)*. In the Russian Federation, the distribution of this bat species and its maintained lyssavirus extends into climatically suitable areas of Siberia and the Far East. In the foothills of the Caucasus, the circulation of *Lyssavirus caucasicus* strains *(West Caucasian bat lyssavirus)* was detected in the population of the common bent-wing bat *(Miniopterus schreibersii)*.

Due to the largely isolated nature of insectivorous bat colonies, lyssavirus transmission from bats to other species or humans occurs only sporadically and does

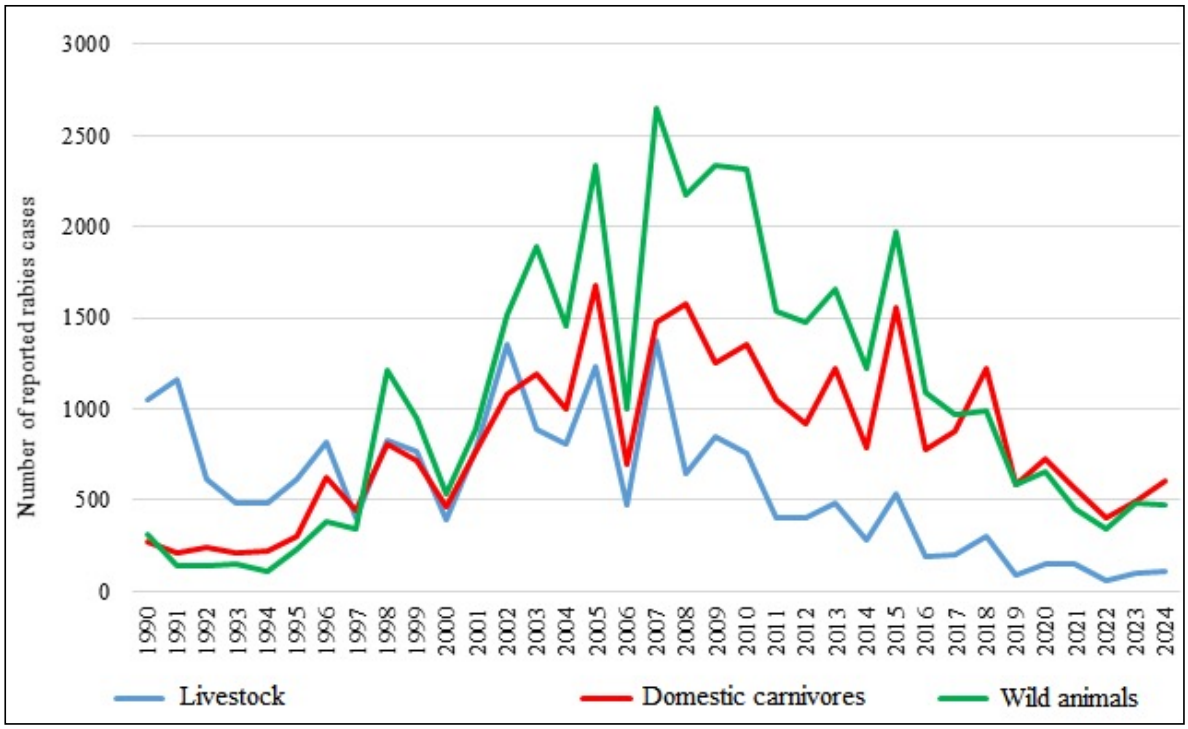

Fig. 1. Annual rabies incidence curves in the major species groups

not currently represent a widespread epizootic or epidemic threat on a global scale.

The preservation and amplification of lyssavirus strains within their natural biocenoses depend on their sustained circulation among reservoir host populations. Furthermore, the epizootic dynamics of rabies are shaped by the landscape and climatic conditions of ecologically favorable yet epidemiologically high-risk ecosystems [20]. Analyzing the spatiotemporal dynamics of animal rabies incidence across natural and agricultural zones enables the identification of high-risk areas and key ecological factors influencing epizootic stability in different ecosystems [21].

MATERIALS AND METHODS

Using Microsoft Access (relational database management system www.microsoft.com), data from reports of the Veterinary Department under the Ministry of Agriculture of the Russian Federation on rabies outbreaks and rabies vaccination among wild animals were aggregated. The database was structured to categorize all records by time period, administrative region, and affected animal species. All epizootiological data were georeferenced to administrative regions and cross-referenced with Russia's natural and agricultural zoning data [22]. Graphical visualization and statistical data were processed using Microsoft Excel Data Analysis ToolPak (www.microsoft.com).

For spatial analysis, records of all rabies outbreaks were georeferenced and linked to unique identifier (ID) codes from the attribute tables of administrative boundary vector layers of the Russian Federation. This enabled the creation of thematic vector layers incorporating epizootiological data within the GIS project. The GIS project was constructed using QGIS Desktop platform (www.qgis.org).

STUDY RESULTS

Analysis of 35 years of epizootiological monitoring data reveals continuous temporal variation in rabies epizootic indicators across the Russian Federation. These fluctuations affected all key epidemiological parameters, particularly annual incidence rates and the relative contribution of different animal species to epizootic transmission.

As shown in Figure 1, the most pronounced incidence fluctuations occurred in wildlife populations. Until 1997, the number of reported rabies cases among wild animals was significantly lower than the number of cases among livestock and pets, which can be explained by the lack of active and passive monitoring tests among wildlife. Over the following decade, diagnostic methods improved and the number of tests among wild animals increased. During this period, in the absence of oral vaccination for wild carnivores, the epizootic process exhibited distinct multi-year cycles characterized by 2–4 year intervals between peak incidence rates. This pattern reflects autocorrelation in the epizootic process, wherein pathogen-induced mortality affects reservoir host population density.

In the middle of the 35-year period under review, the use of rabies oral vaccines of wild carnivores was initiated. Following the intensification of wildlife rabies vaccination efforts, both a progressive decline in disease incidence and stabilization of epizootic cycle amplitudes were observed [9].

Comparison of the incidence curves in Figure 1 reveals that the rabies incidence in domestic carnivores and farm animals directly correlates with the number

of cases in wild animals. This correlation is evident within epizootic cycles, though long-term trends. Following 2007, incidence rates declined more substantially in livestock populations compared to domestic carnivores. The technological modernization of livestock production in recent decades has coincided with a reduction in total herd sizes, including grazing animal populations. The total number of cattle, according to Rosstat, was: in 1990 – 57,043.0 thousand animals; in 2007 – 21,501.6 thousand animals; in 2023 – 17,068.2 thousand animals (as of the end of the year). While farm animal populations have remained stable or decreased in some areas, the number of cats and dogs, particularly in rural areas and small towns – has increased, raising the potential for wildlife interactions [23].

The past decade to decade-and-a-half has represented a period of relative stability in ecological and epidemiological drivers influencing rabies epizootic dynamics. During this period in the Russian Federation, oral rabies vaccination campaigns targeting wild predators have been conducted on a regular basis. The minimum amount of oral vaccine used per year was in 2014–2015 (5.5 and 5.0 million doses, respectively). In other years, this figure ranged from 12.0 million doses (2013) to 26.4 million doses (2020). On average, about 16 million doses of oral rabies vaccine were used annually. That is why, 2013–2024 period was chosen for the study of the rabies epizootic process patterns.

It was found that rabies was most often detected among foxes, dogs, cats, cattle and raccoon dogs (Fig. 2).

Contemporary rabies epizootics in the Russian Federation are characterized not only by multi-annual incidence cycles but also by distinct seasonal patterns, largely determined by the biological and behavioral characteristics of the red fox as the principal viral reservoir and vector.

Rabies incidence reaches its annual nadir in early summer, corresponding to the fox cub-rearing period when adult vulpine hosts exhibit minimal migratory activity and restricted home ranges. At the end of summer, young foxes become more active, dispersing from their birth areas to establish their own territories, thus inducing an autumn increase in morbidity. The sharpest increase in rabies cases among foxes typically occurs in March, due to the increased activity and interactions between animals during the winter mating season (Fig. 3).

An analysis of data on rabies-infected localities over this period shows that the current rabies distribution area involves most of the Russian Federation regions. Excluding the four new regions, rabies cases have been reported in 78 regions of the country over the past 12 years, and rabies outbreaks have been reported annually in 39 of them. The high incidence and spatial density of rabies outbreaks in the new regions, based on the observation data for the last two years, makes it possible to classify these territories as a high-risk zone.

Over the 12-year period, 30 regions of the country can be classified as the most severely infected, with over 80% of rabies outbreaks reported (Table 1).

The highest incidence rates over the 12 years were reported in the Central and Volga Federal Districts. Herewith, a comparative analysis of six-year intervals

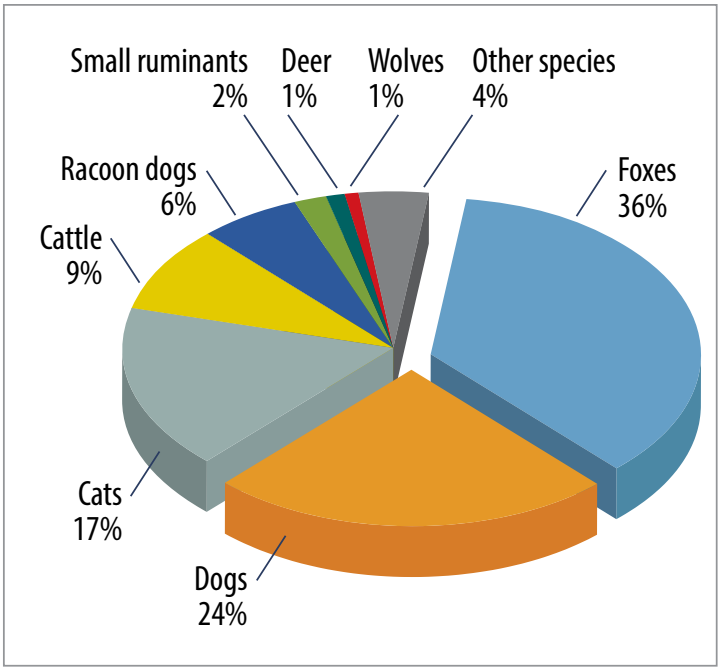

Fig. 2. Percentage of rabies cases by animal species in 2013–2024

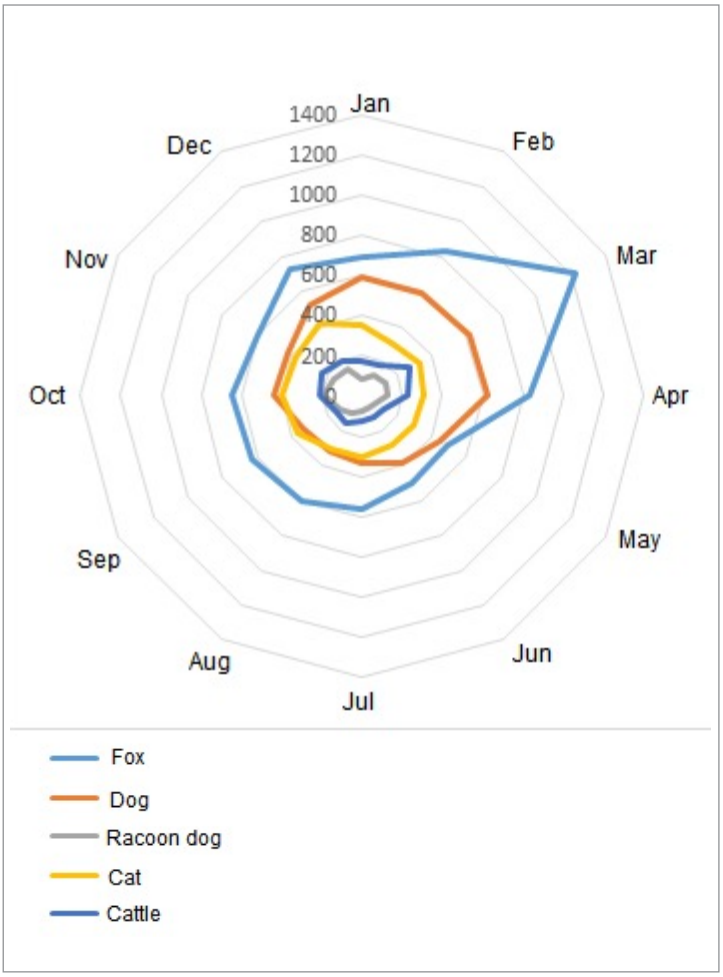

Fig. 3. Dynamics of seasonal rabies incidence in animals involved in the rabies process (according to monitoring data for 2013–2024)

in these regions demonstrated substantial decline in rabies incidence.

As evidenced by Table 1, the decline in disease incidence occurred asynchronously across regions, with some areas showing minimal change or even increased case numbers.

The current rabies distribution area is mainly associated with warmer regions of the Russian Federation. A negligible number of cases was reported in the biomes of the northern territories.

A characteristic geographical pattern shows that rabies outbreaks have lower spatial density in the subzones of the northern and middle taiga regions. The limited monitoring data from boreal forests reflects both low human population density and inherently low rabies prevalence, as evidenced by consistently minimal cases among domestic carnivores – suggesting ecological factors naturally limit disease transmission in these ecosystems. Rabies epizootics in boreal forests naturally attenuate due to three key ecological factors:

Table 1
The subjects of the Russian Federation with the highest rabies incidence in 2013–2024

Federal District	Russian Federation Subject	Rabies cases reported			Percentage relationship between the two periods, %
		In total	2013–2018	2019–2024	
Central	Moscow Oblast.	1,501	1,221	280	23
Volga	Saratov Oblast	1,229	842	387	46
Central	Belgorod Oblast	1,220	996	224	22
Central	Lipetsk Oblast	931	852	79	9
Volga	Republic of Tatarstan	811	719	92	13
Ural	Chelyabinsk Oblast	797	437	360	82
Volga	Penza Oblast	791	470	321	68
Central	Voronezh Oblast	734	522	212	41
Central	Tambov Oblast	733	493	240	49
Central	Vladimir Oblast	684	443	241	54
Southern	Volgograd Oblast	680	478	202	42
Central	Smolensk Oblast	665	371	294	79
Central	Tver Oblast	656	501	155	31
Volga	Republic of Udmurtia	646	524	122	23
Volga	Orenburg Oblast	565	453	112	25
Volga	Nizhny Novgorod Oblast	563	309	254	82
Volga	Samara Oblast	530	255	275	108
Central	Bryansk Oblast	509	434	75	17
Central	Yaroslavl Oblast	507	411	96	23
Ural	Sverdlovsk Oblast	466	321	145	45
Ural	Tyumen Oblast	425	214	211	99
Central	Tula Oblast	414	365	49	13
Central	Ryazan Oblast	382	245	137	56
Volga	Kirov Oblast	370	315	55	17
Siberian	Krasnoyarsk Krai	309	110	199	181
Siberian	Novosibirsk Oblast	298	184	114	62
Central	Kursk Oblast	295	257	38	15
Siberian	Republic of Khakassia	287	156	131	84
Southern	Astrakhan Oblast	248	180	68	38
Central	Kaluga Oblast	238	150	88	59

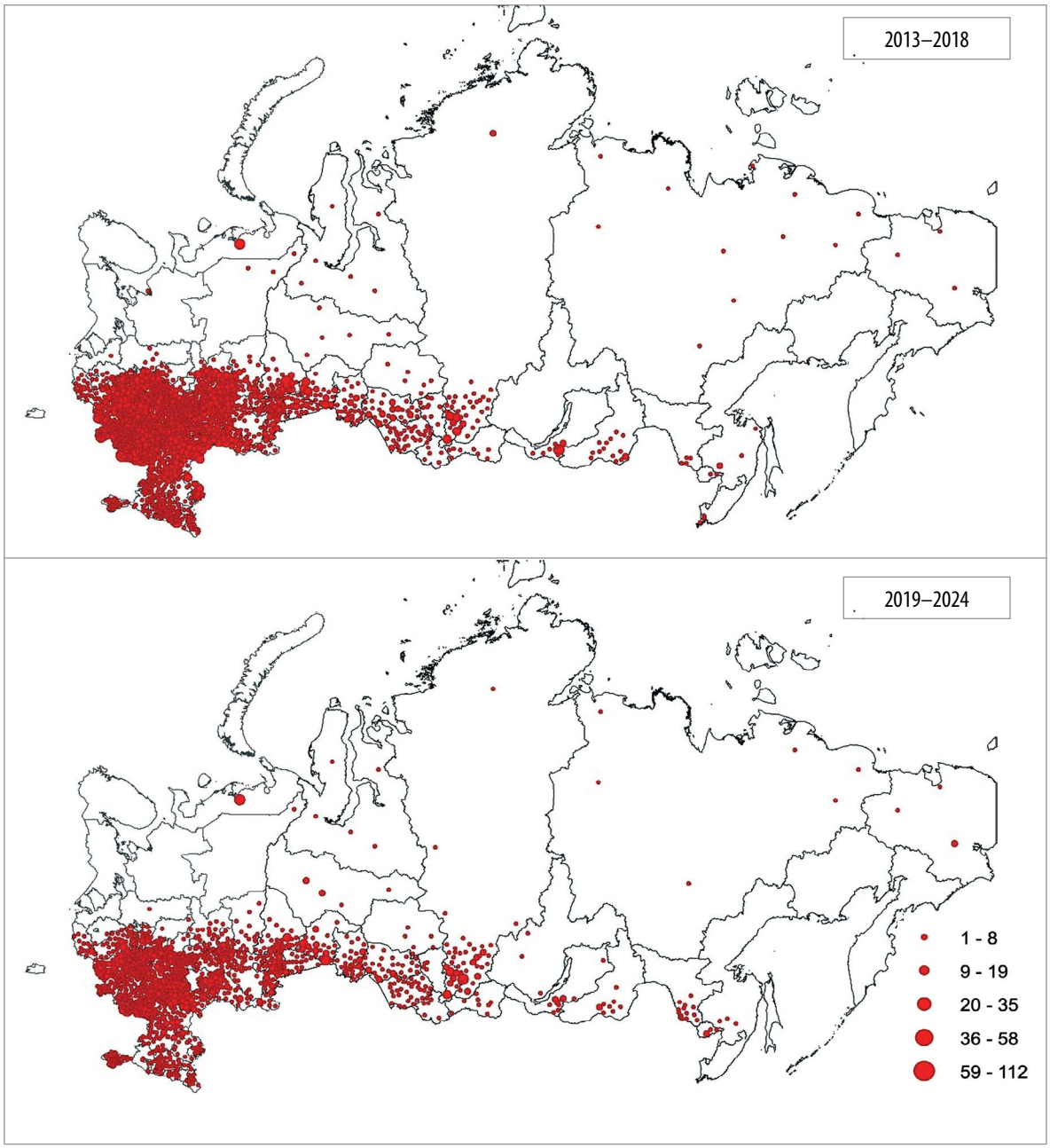

Fig. 4. Location and number of rabies outbreaks reported in the administrative regions of the Russian Federation in 2013–2018 and 2019–2024

low predator population densities resulting from limited prey availability, landscape barriers that restrict host movement, and winter snow cover that impedes migratory transmission.

Comparative spatial analysis of rabies outbreaks between 2013–2018 and 2019–2024 revealed decreased outbreak density (cases per unit area) while the geographic extent of affected regions remained stable (Fig. 4).

The observed spatial heterogeneity in rabies outbreak distribution reflects environmental factors influencing wild predator ecology – particularly those affecting host abundance and dispersal patterns. Consequently, current epizootic risk zones show a strong correlation with agroecological provinces and zones.

Between 2013 and 2024, rabies was detected in 42 agroecological provinces of Russia, with over 50% of cases concentrated in two high-risk zones: the Middle Russian forest-steppe and the Southern Taiga Forest ecosystems (Fig. 5).

The association between rabies occurrence and natural ecosystem characteristics influences both the spatial distribution of outbreaks across administrative units and regional variations in affected animal species (Table 2).

The spatial distribution of rabies cases varies significantly among different species. In colder regions, rabies cases predominantly occur in wild animals, whereas in warmer regions, domestic carnivores represent the main epizootiological risk group. This pattern can be

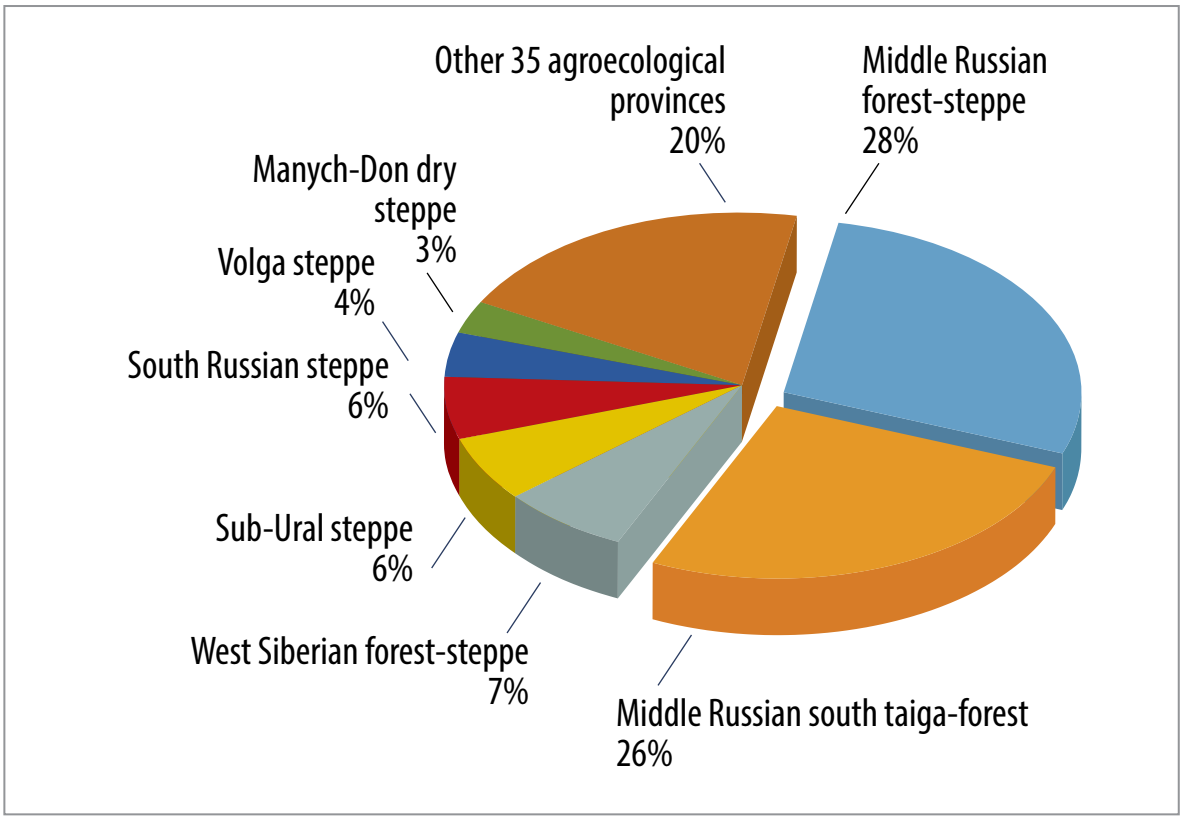

Fig. 5. Distribution of rabies outbreaks reported in 2013–2024 by agroecological provinces of the Russian Federation

attributed to the intensive interpenetration of natural and anthropogenic landscapes in southern regions, where human activity and ecosystems are deeply intertwined.

The lowest rabies incidence among foxes during 2013–2024 was reported in the Caucasian Mountain natural area. Of the 192 recorded rabies cases, only 7 (4%) occurred in foxes. Furthermore, this area shows no seasonal peak in animal rabies incidence during March, which is characteristic of fox rabies in lowland biomes.

DISCUSSION AND CONCLUSIONS

Rabies persistence in the Russian Federation is maintained by the virus's reservoir in wild fox populations. Between 2013 and 2024, foxes accounted for 36% of all reported rabies cases. The most extensive and infected zone comprises two agroecological provinces: the Middle Russian forest-steppe and Middle Russian south taiga-forest. The ecological conditions of the East European Plain's forest-steppe zone and adjacent southern taiga (mixed and deciduous forests) support robust canid

Table 2
Rabies infected animals of various groups by territories (according to monitoring data for 2013–2024)

Federal District	Wild animals		Pet animals		Farm animals	
	Total animals	Infected, %	Total animals	Infected, %	In total	Infected, %
North-Western	218	60	61	17	85	23
Central	4,909	49	4,410	44	711	7
Volga	2,771	43	2,823	44	876	14
Southern	362	24	935	61	243	16
North Caucasian	61	11	290	51	223	39
Ural	1,244	60	559	27	259	13
Siberian	928	53	359	21	448	26
Far Eastern	110	48	61	27	57	25

predator populations through abundant food resources while facilitating widespread rabies transmission across the region. The synergistic effect of these ecological and epidemiological factors sustains the epizootic cycle, maintaining consistently high rabies incidence rates across the region.

Domestic animals – particularly dogs, cats, and cattle – collectively represent the most frequently reported rabies cases among other species, collectively comprising approximately 50% of total disease incidence. All cases of infection among these species can be regarded as a side effect of the sylvatic cycle spillover. While dogs account for approximately 24% of animal rabies cases in Russia, the question of a sustained urban rabies cycle (urban rabies) in the Russian Federation remains complex. However, with a well-developed veterinary service and a government-funded rabies vaccination program, the probability of the virus establishing stable, independent circulation in the dog population is extremely low. Due to their behavior, domestic carnivores are the most common victims of rabid foxes invading settlements. As such, they serve as clear sentinels for sylvatic rabies outbreaks, especially in areas with insufficient disease monitoring. However, in some territories, primarily in the North Caucasus, the epidemiology of rabies exhibits distinct characteristics that sharply contrast with the rest of the country. In the Caucasian Mountain natural area, rabies incidence in foxes is reported as 4%, with no observed seasonal increase in March. This evidence supports the hypothesis of persistent, independent canine rabies circulation in localized zones [24]. This possibility is supported by studies demonstrating the genetic isolation of Caucasus-region rabies virus strains. Furthermore, the region's geographical proximity to the canine rabies enzootic area of Asia Minor provides a plausible explanation for this distinct epidemiological pattern [25, 26].

Oral vaccination campaigns targeting wild predators over the past 15 years have driven a significant reduction in the overall incidence of animal rabies in the Russian Federation [27]. The most pronounced decline occurred in fox population. During this period, several short-term increases in incidence interrupted the overall downward trend. The most significant peak in animal rabies incidence occurred in 2015, resulting from a dramatic reduction in the oral vaccination of wild predators during the preceding year (2014–2015). The decline in rabies incidence among domestic dogs and cats has been less pronounced in recent years, which is likely attributable to their actively growing populations.

A comparative spatial analysis of rabies outbreaks over the last two six-year periods reveals that, despite the overall incidence decline, the virus persists in historically endemic areas and has not been eliminated. While this strategy significantly reduces epizootic and epidemiological risks, any reduction in the oral vaccination of wild predators would likely lead to a rapid deterioration of the rabies situation. To date, Russia's successful rabies eradication using oral vaccine baits has been confined to regions that are either geographically isolated from the main disease distribution area, like Kaliningrad Oblast, or located on its periphery, such as Leningrad Oblast and the Republic of Karelia [28, 29].

The current epizootic situation necessitates a new disease control strategy focused on containing and reducing the disease distribution area. The scientific foundation for territorial rabies eradication programs should be based on the principles of systemic epizootology. This discipline analyzes pathogen spread within the context of local ecosystems and the impact of existing disease control measures [21].

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Phylogenetic analysis of rabies virus isolates recovered from animals in Volgograd Oblast

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ABSTRACT

Introduction. The Lower Volga region, including the Volgograd Oblast, remains one of Russia's most rabies-affected areas to date. Data on the genetic diversity of rabies viruses (RABVs) currently circulating in the Volgograd Oblast are insufficient, making phylogenetic analysis of RABV isolates from this region a relevant scientific objective.

Objective. The study aims to conduct a phylogenetic analysis of current RABV isolates recovered from animals in the Volgograd Oblast, based on the full-length nucleoprotein gene sequence.

Materials and methods. Brain tissue samples from animals diagnosed with rabies were used. The obtained nucleotide sequences of the RABV nucleoprotein gene were analyzed using the Bayesian strict molecular clock method. The spatial distribution of RABV isolates was described using Natural Earth physical map.

Results. Full-length nucleoprotein gene sequencing was performed for 13 RABV isolates collected in the Volgograd Oblast. Phylogenetic analysis revealed that the RABV population in this region comprises distinct genetic variants of genetic group C, formed at different times. Genetic relationship to the isolates from Kazakhstan, Ukraine, Moldova and Central/Southern Russia indicates intensive RABV circulation in Southern European Russia. Notably, distinct virus variants were detected on the left and right banks of the Volga River.

Conclusion. All studied RABV isolates collected in the Volgograd Oblast belonged to genetic group C and exhibited high genetic diversity among variants.

Keywords: rabies virus (RABV), phylogenetic analysis, genetic group C, Volgograd Oblast

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Филогенетический анализ изолятов вируса бешенства, выделенных от животных на территории Волгоградской области

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РЕЗЮМЕ

Введение. Нижнее Поволжье, в том числе Волгоградская область, вплоть до настоящего времени относится к числу наиболее неблагополучных по бешенству регионов России. Сведения о генетическом разнообразии возбудителя бешенства в современный период для Волгоградской области представлены недостаточно, поэтому филогенетический анализ изолятов вируса бешенства из этого региона представляется актуальной научной задачей.

Цель исследования. Проведение филогенетического анализа современных изолятов вируса бешенства, выделенных от животных на территории Волгоградской области, на основании полноразмерной последовательности гена нуклеопротеина.

Материалы и методы. Использовали головной мозг животных с диагнозом «бешенство». Анализ полученных нуклеотидных последовательностей

гена нуклеопротеина вируса бешенства проводили с помощью байесовского метода строгих молекулярных часов. Ландшафтно-географическая карта Natural Earth использована для описания пространственного распределения изолятов возбудителя бешенства.

Результаты. Определена полноразмерная последовательность гена нуклеопротеина 13 изолятов вируса бешенства из Волгоградской области. Филогенетический анализ позволил установить, что представители популяции возбудителя бешенства из этого региона относятся к различным генетическим вариантам группы С, сформировавшимся в разное время. Генетическое родство с изолятами из Казахстана, Молдовы, центральных и южных регионов России, с Украины свидетельствует об интенсивном перемещении вируса бешенства на юге европейской части России. В то же время разные варианты вируса бешенства обнаружены на левом и правом берегах Волги.

Заключение. Все изученные изоляты возбудителя бешенства из Волгоградской области принадлежали к генетической группе С и отличались высоким генетическим разнообразием вариантов.

Ключевые слова: вирус бешенства, филогенетический анализ, генетическая группа С, Волгоградская область

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Конфликт интересов: Груздев К. Н. является главным редактором журнала «Ветеринария сегодня», но не имеет никакого отношения к решению опубликовать эту статью. Рукопись прошла принятую в журнале процедуру рецензирования. Об иных конфликтах интересов авторы не заявляли.

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INTRODUCTION

The application of molecular genetic studies in epizootology has significantly enhanced surveillance capabilities for zoonotic infections. Data on intra-species diversity and genetic lineage relationships of pathogens, obtained through phylogenetic analysis of both individual gene sequences and complete genomes, provide critical insights into pathogen origins, spatial distribution patterns, and their significance in human and veterinary pathology. These advances have established and continue to develop two emerging fields: molecular epidemiology and genomic epidemiological surveillance [1, 2].

Multiple genetically distinct rabies virus (RABV) groups with distinct geographical distributions have been identified in the Russian Federation [3, 4, 5, 6, 7]. Representatives of genetic group C ("steppe") [3] demonstrate the widest distribution, currently spanning territories from Eastern Europe through southern Siberia to Kazakhstan and northeastern China [6, 8, 9, 10, 11]. Since the mid-20th century, the red fox (*Vulpes vulpes*) has served as the primary RABV reservoir in this region. Current epidemiological models suggest that fox-mediated rabies epizootics originated in 1939 in East Prussia (Polish Corridor), subsequently spreading westward to France and Belgium and eastward to the Baltics, Ukraine, Belarus and western Russia within 20–30 years [12, 13]. Cartographic analyses clearly document this progressive expansion of fox rabies across the European territories of the former USSR [14]. By the early 21st century, these epizootics had reached previously rabies-free Siberian regions, including Krasnoyarsk Krai and Transbaikalia, after decades of absence [7, 15].

A wild rabies outbreak was documented in the Volga River Delta in 1942 [13]. This region, along with East Prussia, is believed to have been one of the epicenters of fox rabies spread in the USSR [13, 16]. To this day, the Lower Volga region, including the Volgograd Oblast, remains one of Russia's most rabies-endemic areas [7, 16]. Nearly the entire territory of the Volgograd Oblast is enzootic for the disease [17, 18]. Although the fox is considered the RABV primary reservoir in this region, up to 38% of all recorded cases in recent decades have involved dogs and cats, while wild animals accounted for no more than 25% [19]. Notably, in 4 out of 6 human rabies cases reported after 2000, transmission occurred via dogs and cats [7, 20]. To date, nucleotide sequences of the nucleoprotein gene (N gene) have been published for only two RABV isolates from animals in the Volgograd Oblast, both belonging to genetic group C [3]. Given the limited data on the genetic diversity of current RABVs in the region, a phylogenetic analysis of the virus isolates from the Volgograd Oblast remains a relevant and necessary research objective.

The aim of the study was to conduct a phylogenetic analysis of currently circulating RABV isolates recovered from animals in the Volgograd Oblast, based on the complete nucleoprotein (N) gene sequence.

MATERIALS AND METHODS

A total of 13 RABV isolates collected from animals in the Volgograd Oblast in 2018–2021 were studied (Table 1, Fig. 1).

RNA was extracted from animal brain tissue, and two overlapping genome fragments containing the full-length nucleoprotein (N) gene of RABV were amplified by reverse transcription polymerase chain reaction. Following purification, the fragments were subjected to Sanger sequencing as previously described [5]. The resulting sequences were deposited in the international GenBank database (accession numbers provided in Table 1).

The phylogenetic analysis was performed using the Bayesian approach in the Bayesian Evolutionary Analysis Sampling Trees (BEAST X) software package [21]. A model suitable for constructing a phylogenetic tree was preliminarily evaluated using the MEGA X program, with the Bayesian information criterion (BIC) and the corrected Akaike information criterion (AICc) determined. Based on the test results, the Hasegawa-Kishino-Yano model with

Table 1
Information on RABV isolates recovered from animals in the Volgograd Oblast

№	Isolate full name	Coordinates of populated localitites		Year	Host animal	GenBank accession number
		latitude	longitude			
1	490_2/2018/Volgograd	50.19760	42.69182	2018	dog	OP311892
2	490_4/2018/Volgograd	49.98861	46.68835	2018	dog	OP311893
3	1563/2018/Volgograd	50.99677	44.35670	2018	dog	OP311840
4	1299/138/2021/Volgograd	51.07931	42.51306	2021	cattle	OP311869
5	1299/139/2021/Volgograd	50.08529	45.40216	2021	dog	OP311870
6	1299/141/2021/Volgograd	48.38944	42.35912	2021	dog	OP311871
7	1299/142/2021/Volgograd	48.09285	42.59016	2021	cattle	OP311872
8	1299/143/2021/Volgograd	50.24182	41.83726	2021	dog	OP311873
9	1299/144/2021/Volgograd	49.85802	42.33575	2021	dog	OP311874
10	1299/145/2021/Volgograd	49.35021	45.07359	2021	marten*	OP311875
11	1299/146/2021/Volgograd	49.73250	43.48296	2021	cattle	OP311876
12	1299/147/2021/Volgograd	48.24536	42.64595	2021	cat	OP311877
13	1299/148/2021/Volgograd	49.85802	42.33575	2021	wolf**	OP311878

* marten – *Martes* sp.; ** wolf – *Canis lupus.*

a 4-category gamma distribution (HKY + G; BIC = 21,360.059; AICc = 17,664.682) was selected [22]. A phylogenetic tree reflecting the posterior probability of the formation time of internal (ancestral) and terminal nodes with a 95% confidence interval (95% CI) was constructed using the strict molecular clock method, with heterochronic calibration to the year of sample isolation, a random initial tree model with a constant population size, and a Markov chain Monte Carlo (MCMC) chain length of 1×10^8 with sampling every 1×10^5 steps. The posterior prognostic verification was performed for all taxa belonging to the genetic group C of the RABV.

The reliability of the MCMC was assessed using the Tracer program by analyzing the BEAST X output data [23].

The tree was annotated using the TreeAnnotator utility and the settings of outlier removal (burn-in = 10%, 10,000,000 states), maximum clade confidence (MCC), and maintaining a given length.

Visualization of the phylogenetic tree was performed using the FigTree v.1.4.5 program [24].

The QGIS 3.2.1 program and the Natural Earth electronic landscape-geographic map were used for cartography. The points were plotted on the map according to the geographic coordinates of settlements where rabies cases were detected.

RESULTS AND DISCUSSION

The full-length nucleotide sequence of the N gene (1353 nt) was determined for 13 RABV field isolates collected from animals in ten raions of the Volgograd Oblast in 2018–2021. The map shows the locations where isolates were detected (Fig. 1).

According to preliminary analysis, all studied isolates belong to RABV genetic group C. Therefore, to construct the phylogenetic tree, full-length sequences of all available isolates from this group identified in the Russian Federation with known collection years were retrieved from the GenBank database. Additionally, BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of the studied sequences revealed closely related isolates from Kazakhstan, Moldova, Poland, and Ukraine, which were also included in the phylogenetic analysis. To clearly represent group C, several isolates from other genetic groups circulating in Europe and European Russia were included as genetic variants (excluding Arctic-related clade representatives due to their distant genetic relationship). In total, 178 full-length N gene sequences were analyzed, including 121 sequences from genetic group C. For improved figure readability, the isolate groups phylogenetically distant from the tested ones were collapsed. The resulting phylogenetic tree is presented in Figure 2.

According to the tree topology, all the studied isolates belong to the genetic group C, the modern range of which within the Russian Federation extends from the western borders and regions of the central zone of the European part of the country to the steppe and forest-steppe territories of Siberia, everywhere going beyond the borders of Russia in the south [3, 6, 5, 11]. RABV of genetic lineage C was isolated from both wild carnivores and domestic and farm animals in the Volgograd Oblast.

All studied isolates are unique compared to previously identified isolates, including those from the Volgograd Oblast, and form several groups with a high posterior probability estimate (PPE) with each other and with previously studied isolates from Russian and foreign regions.

Thus, isolate 490_4/2018/Volgograd shows significant similarity (99.85%) with isolate Rab-8-4, identified in the Turkestan Oblast of Kazakhstan. The estimated time of evidence of the most recent common ancestor (MRCA) for this group is 2015.9 (95% CI – from 2012.1 to 2016.93), the PPE of the group is 1.0.

Isolates 1299/143/2021/Volgograd and 1299/148/2021/Volgograd show the greatest relationship

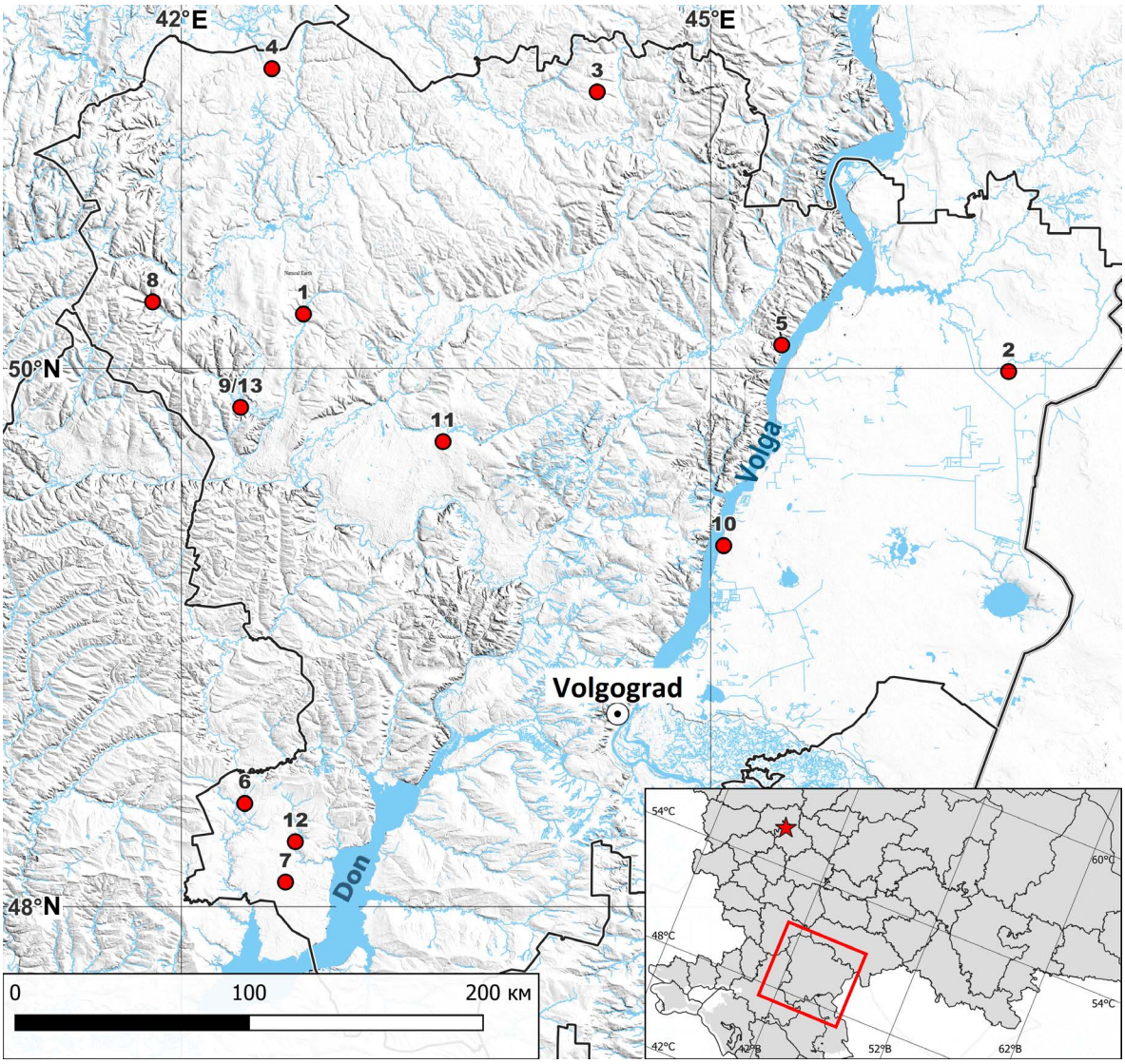

Fig. 1. Distribution of RABV isolates collected from animals in the Volgograd Oblast (the isolates listed in Table 1 are numbered)

with isolates previously identified in the Vladimir, Lipetsk, Nizhny Novgorod and Ryazan Oblasts (more than 98.67%). The MRCA for this group is 2007.95 (95% CI – from 2002.48 to 2009.67), the PPE of the group is 1.0.

The group of isolates (490_2/2018/Volgograd, 1299/138/2021/Volgograd, 1299/139/2021/Volgograd, 1563/2018/Volgograd) shows the greatest affinity (more than 98.9%) with the isolates previously identified in the Nizhny Novgorod and Lipetsk Oblasts (but with others than in previous cases). The MRCA for this group is 2001.43 (95% CI – from 1994.02 to 2002.65), the PPE of the group is 1.0.

Another five isolates from the Volgograd Oblast (1299/142/2021/Volgograd, 1299/147/2021/Volgograd, 1299/141/2021/Volgograd, 1299/144/2021/Volgograd, 1299/146/2021/Volgograd) form a group with a high PPE (1.0) with isolates from the Belgorod, Lipetsk, Ryazan, Smolensk, Kursk, Nizhny Novgorod, Moscow Oblasts, as well as with isolates identified in other countries – Moldova, Ukraine and Poland. It is noteworthy that this relatively genetically dense group occupies an extremely vast area, stretching at least 1,000 km from north to south and 2,000 km from west to east. The MRCA for this group was 1996.13 (95% CI from 1989.12 to 2003.42).

Isolate 1299/145/2021/Volgograd genetically differs significantly from other Volgograd isolates and shows the greatest (although not very close) relationship with isolates identified in the Astrakhan Oblast (98.74%). It forms a group supported by a high PPE (0.98) with RABV isolates identified in the Orenburg and Astrakhan Oblasts, as well as in Kazakhstan. The MRCA for this group is 1978.98 (1974.35–1983.97).

Two isolates (2070f and 2072f) identified in the Volgograd Oblast by I. V. Kuzmin et al. [3] in 2003 differ significantly from the studied isolates from this region. Isolate 2070f forms a group with isolate 1350/2008/Krasnodar from the Krasnodar Krai (PPE – 1.0; MRCA – 1984.33; 95% CI –1977.17–1989.78), and isolate 2072f, although having a genetic relationship with isolate 2070f (99.1%), but their group has a very low posterior probability support – 0.19.

The number of analyzed isolates collected from various animal groups generally reflects the morbidity structure

observed during 2020–2024. Over 50% of these isolates were derived from canine and feline specimens (Table 2).

Thus, the key characteristic of the RABV population in the Volgograd Oblast is its remarkable heterogeneity, possibly the highest among well-studied regions to date. At the same time, nearly all representatives of the Volgograd RABV population have close genetic relatives in other regions of Russia and abroad, suggesting active viral spread. According to published data, increased epizootic activity in the Volgograd Oblast was recorded in 1988, 1991, 1997, 1998, and 2001 [17]. The Volgograd RABV population comprises multiple genetic variants, which emerged at different times, as evidenced by the estimated age of their last common ancestors. It is plausible that peaks in epizootic activity coincide with the introduction of new viral variants. Following their spread, these variants establish themselves among susceptible hosts in the region, continue to evolve, and ultimately contribute to the highly diverse genetic profile observed in the current population.

The localization features of different RABV subgroups are noteworthy. The Volgograd Oblast occupies the central part of the southeast of the Russian (East European) Plain within three natural zones: forest-steppe, steppe and semi-desert [25]. Isolates collected in the forest-steppe and steppe regions of the northern part of the Volgograd Oblast on the right bank of the Volga (490_2/2018/Volgograd, 1563/2018/Volgograd, 1299/138/2021/Volgograd, 1299/139/2021/Volgograd, 1299/143/2021/Volgograd, 1299/144/2021/Volgograd, 1299/148/2021/Volgograd) are genetically related to isolates from the Middle Volga and central regions of Russia. This likely results from the absence of natural barriers to wild carnivore migration in northern directions, combined with high fox population densities in adjacent areas [26]. RABV isolates 1299/141/2021/Volgograd, 1299/142/2021/Volgograd, 1299/147/2021/Volgograd, which are similar to the isolates found in Ukraine, Moldova, Poland, and western/central regions of European Russia, were identified in the

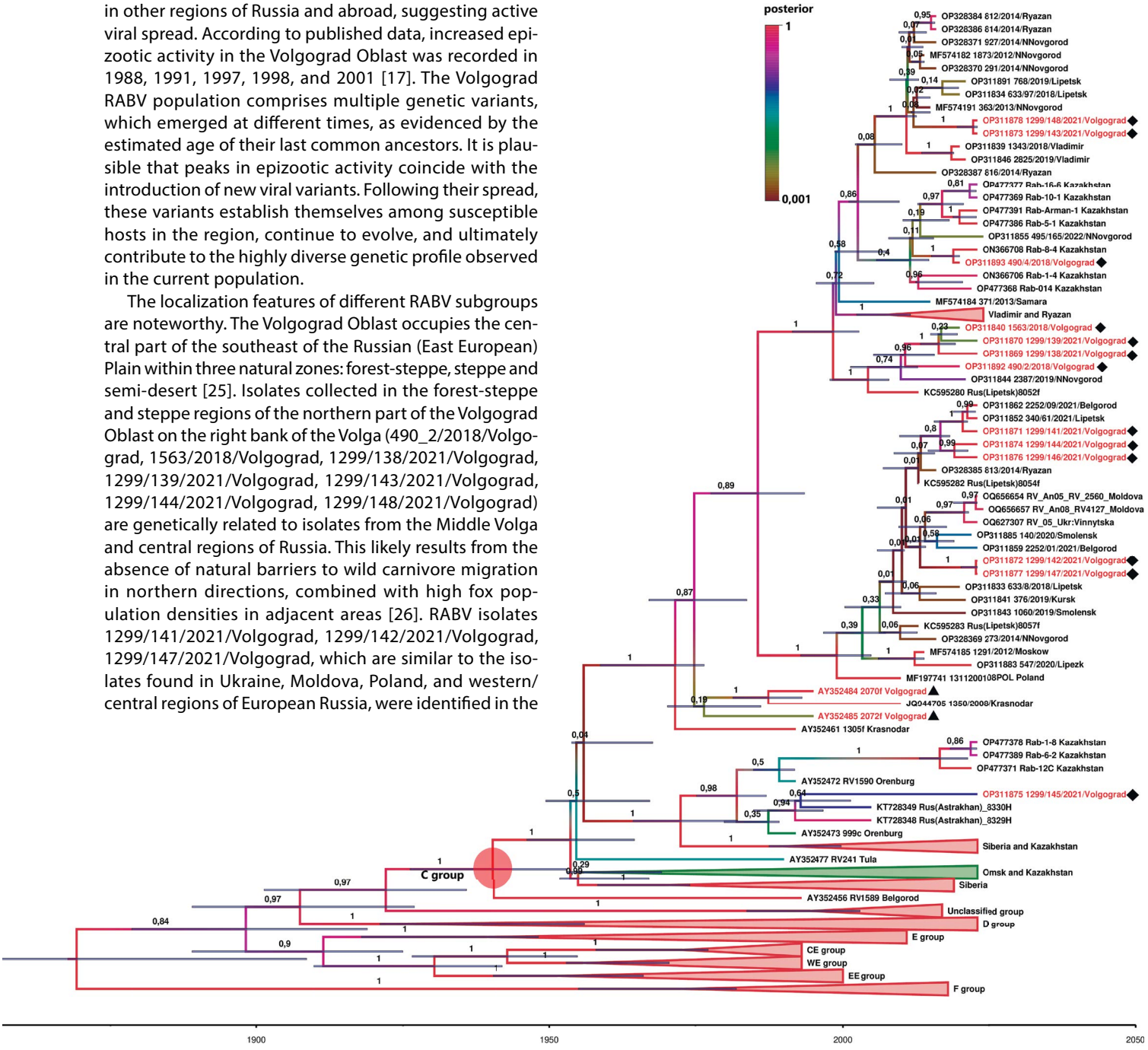

Fig. 2. Phylogenetic tree constructed using the full-length nucleotide sequence of RABV N gene and a strict molecular clock algorithm. Isolates collected from animals in the Volgograd Oblast are highlighted in red. Numbers (0–1) indicate posterior probability values for specific groups; the same parameter is indicated with the color of branches (see color scale for interpretation). The horizontal timeline at the bottom shows the estimated years of the most recent common ancestor for this or that group, with blue bars at nodes representing 95% confidence intervals for this value. The pink circle marks the most recent common ancestor of genetic group C. Black diamonds denote the studied isolates, while triangles represent two previously characterized isolates from the Volgograd Oblast

Table 2
Data on rabies confirmation in animals from the Volgograd Region (2020–2024*) and sources of virus isolates for phylogenetic analysis

Animal categories	Confirmed rabies cases		Number of tested isolates	
	n	%	*n*	%
Livestock (cattle, small ruminants, horses)	42	23.3	3	23.1
Domestic carnivores (dogs, cats)	109	60.6	8	61.5
Wild carnivores (foxes, wolves, etc.)	29	16.1	2	15.4
Total	180	100	13	100

* Data for 2024 (9 months).

southwestern Volgograd Oblast on the right bank of the Tsimlyansk Reservoir. Isolates from the left bank of the Volga (490_4/2018/Volgograd, 1299/145/2021/Volgograd) show genetic similarity to strains from southern and eastern neighboring regions, reflecting shared landscape characteristics with the semi-deserts and dry steppes of Kazakhstan and Astrakhan Oblast. Major water barriers (Tsimlyansk and Volgograd Reservoirs and the Volga-Don Canal), which cross the entire region northeast to southwest, freeze for only 2–3 months annually [25] and consequently serve as effective natural barriers to rabies spread for most of the year. We obtained only two isolates from the left bank of the Volga, both distinct from the right-bank strains. Thus, isolate 1299/145/2021/Volgograd (recovered in the Bykovsky Raion), clusters with strains from Orenburg and Astrakhan Oblasts (exact origins unknown) and Kazakhstan. Isolate 490/4/2018/Volgograd (identified in the Pallasovsky Raion) shows closest relations to some Kazakhstan strains and one strain (being the only exception) from the Nizhny Novgorod Oblast. Notably, raions with highest animal rabies incidence (Nikolaevsky, Bykovsky, Leninsky, Oktyabrsky) [19] are all located on the left Volga bank. The Volga-Akhtuba floodplain appears to function as a significant ecological corridor connecting Volgograd and Astrakhan Oblasts, which is supported by the genetic similarity of the isolates from these areas (1299/145/2021/Volgograd). The limited number of studied isolates prevents definitive conclusions. However, these findings warrant attention for ongoing research and strategic planning of fox oral vaccination programs.

CONCLUSION

All studied RABV isolates collected from animals in the Volgograd Oblast belong to genetic group C. Within this lineage, the RABV population in this region demonstrates exceptionally high genetic diversity. The genetic relatedness of these isolates to strains from Kazakhstan, Ukraine, Moldova and central/southern Russian regions suggests intensive host-mediated viral movement. This epidemiological pattern requires careful consideration when planning and implementing anti-epidemic measures.

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Construction of Newcastle disease virus LaSota strain-based internal sample for rabies diagnosis with RT-PCR

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ABSTRACT

Introduction. The following factors can impact the reliability of polymerase chain reaction results: operator errors, amplifier malfunction, presence of reaction inhibitors in the sample, poor reagent quality and others. All this can lead to the so-called false negative results.

Objective. Construction of the internal control based on heterologous Newcastle disease virus for detection of rabies virus by polymerase chain reaction.

Materials and methods. Freeze-dried live vaccine against Newcastle disease based on LaSota strain produced by the Federal Centre for Animal Health (Russia) was used as an internal control. RNA extraction from samples was performed with "Ribosorb" reagent kit (Central Research Institute for Epidemiology of the Rospotrebnadzor, Russia). Promega Corporation reagents (USA) and oligonucleotides manufactured by the Syntol Company (Russia) were used for the reverse transcription polymerase chain reaction.

Results. LaSota strain of Newcastle disease virus was selected as the target for the internal control. The corresponding primers were designed. Experiments showed that the PCR system for the internal control did not compete with the PCR system for the rabies virus when they were used together. The main parameters of reverse transcription and polymerase chain reaction were optimized. The developed method was validated using several key parameters: correctness, specificity, sensitivity, repeatability (intermediate precision under same conditions), and reproducibility (intermediate precision under different conditions). Validation results have shown that the method characteristics comply with the required ones.

Conclusion. Newcastle disease virus LaSota strain-based internal control system has been constructed for use together with reverse transcription polymerase chain reaction assay for rabies virus detection that allows control of the assay stages in each reaction tube. This internal control after its proper optimization can be also used in experimental studies carried out at relevant research institutions for PCR diagnosis of the diseases caused by other RNA-viruses.

Keywords: reverse transcription polymerase chain reaction, rabies virus, internal control, Newcastle disease virus, NDV, diagnosis

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Разработка системы внутреннего контроля на основе штамма «Ла-Сота» вируса ньюкаслской болезни при диагностике бешенства методом ОТ-ПЦР

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РЕЗЮМЕ

Введение. На достоверность результатов, получаемых при проведении ПЦР-диагностики, могут влиять такие факторы, как ошибки оператора, неполадки в работе амплификатора, наличие в образце ингибиторов реакции, низкое качество реактивов и другое. Все это может приводить к появлению так называемых ложноотрицательных результатов.

Цель исследования. Разработка системы внутреннего контроля на основе гетерологичного вируса ньюкаслской болезни при детекции вируса бешенства методом полимеразной цепной реакции.

Материалы и методы. В качестве внутреннего контрольного образца использовалась «Вакцина против ньюкаслской болезни из штамма «Ла-Сота» сухая живая» производства ФГБУ «ВНИИЗЖ» (Россия). РНК из образцов выделяли с помощью набора реагентов «РИБО-сорб» (ФБУН «Центральный научно-исследовательский институт эпидемиологии» Роспотребнадзора, Россия). Для полимеразной цепной реакции с обратной транскрипцией использовали реактивы фирмы Promega (США) и олигонуклеотиды производства ООО «Синтол» (Россия).

Результаты. В качестве объекта для внутреннего контрольного образца выбран штамм «Ла-Сота» вируса ньюкаслской болезни. Проведен дизайн праймеров. В серии экспериментов установлено, что ПЦР-система для внутреннего контрольного образца не конкурирует с ПЦР-системой для вируса бешенства при их совместном использовании. Оптимизированы основные параметры обратной транскрипции и полимеразной реакции. Проведена валидация разработанной методики, в ходе которой определялись такие характеристики, как правильность, специфичность, чувствительность, промежуточная прецизионность в условиях повторяемости (сходимость) и промежуточная прецизионность в условиях воспроизводимости (воспроизводимость). По результатам валидации полученные характеристики метода соответствуют требуемым.

Заключение. На основе штамма «Ла-Сота» вируса ньюкаслской болезни разработана система внутреннего контрольного образца для использования совместно с методикой выявления вируса бешенства методом полимеразной цепной реакции с обратной транскрипцией, позволяющая контролировать ход всех этапов анализа в каждой реакционной пробирке. Данная система при надлежащей оптимизации потенциально может применяться также в экспериментальных научных исследованиях в соответствующих профильных научных организациях при ПЦР-диагностике заболеваний, вызываемых другими РНК-содержащими вирусами.

Ключевые слова: полимеразная цепная реакция с обратной транскрипцией, вирус бешенства, внутренний контрольный образец, вирус ньюкаслской болезни, диагностика

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INTRODUCTION

The following factors can impact the reliability of polymerase chain reaction (PCR) results: operator errors, amplifier malfunction, presence of PCR inhibitors in the sample, poor reagent quality and others [1]. This can produce false negative results, where the test reads negative despite the presence of the target agent in the sample. Use of internal controls is one of the most effective approaches aimed at making PCR assays more reliable [2]. Internal control is a nucleic acid that is added to the tested sample and undergoes all or some assay stages alongside the tested sample. In this case, specific amplification of the internal control nucleic acid fragment is observed, confirming that the entire PCR process functioned correctly.

To date, various PCR-based methods for rabies virus genome detection, using both classical PCR [3, 4, 5, 6, 7, 8, 9], and real-time PCR [10, 11, 12, 13, 14, 15, 16, 17, 18], have been described in the literature. However, only some of them include internal control use.

In general, internal controls are widely used in PCR diagnostics of various pathogens. There are various strategies for designing internal controls. Thus, J. Coertse et al. used artificially synthesized RNA, the sequence of which corresponded to a fragment of the rabies virus CVS strain genome [19]. Smith J. et al. used ribosomal RNA as an internal control, which, according to them, has degradation kinetics similar to viral RNA [20]. Some studies have described the internal control constructed from MS2 bacteriophage [21, 22, 23, 24]. Genetically engineered virus-like particles were also used [25, 26, 27]. Plasmids containing an insert with sequences complementary to the target PCR primers are a common and effective tool for constructing internal controls [28, 29, 30, 31]. Also, internal controls based heterologous viruses are described [32, 33].

Polymerase chain reaction assay developed and described by A. E. Metlin et al. [34] having high sensitivity and specificity and providing reliable results have been used together with other methods at the Reference Laboratory for Rabies and BSE of the Federal Centre for Animal Health (Vladimir) for several years. However, modern quality standards mandate higher reliability of test results. Therefore, the study was aimed at construction of internal control system and subsequent its use in PCR diagnosis of rabies for the purpose of testing quality improvement.

MATERIALS AND METHODS

Animal brain samples submitted at the Reference Laboratory for Rabies and BSE of the Federal Centre for Animal Health (Vladimir) for testing for rabies virus were used.

ARRIAH strain of rabies virus (infectious activity in cell culture – 6.0 lg $TCID_{50}$/mL) from the Federal Centre for Animal Health Collection of Microorganism Strains was used for optimization of reverse transcription polymerase chain reaction (RT-PCR) and as a positive control.

Freeze-dried live vaccine against Newcastle disease based on Newcastle disease virus LaSota strain manufactured by the Federal Centre for Animal Health (Vladimir) was used as an internal control: the vaccine filling volume – 4,000 doses, Newcastle disease virus (NDV) content – $4 \times 10^{9.7}$ EID_{50}).

RNA was extracted from the samples with RIBO-sorb reagent kit (Central Research Institute of Epidemiology of the Rospotrebnadzor, Russia) according to manufacturer's instruction.

RT-PCR was performed as described by A. E. Metlin et al. [34].

Amplicon software tool (version b08) [35] and the sequence of NDV LaSota strain (GenBank accession number – JF950510) were used for the designing primers.

Promega reagents (USA) were used for RT-PCR.

The primers were synthesized at the LLC Syntol (Russia).

PCR products were analysed by electrophoresis in 2% agarose gel with ethidium bromide staining. GelDoc gel-documenting system (Bio-Rad Laboratories, Inc., USA) was used for electrophoregram capturing.

Resulting amplicons were sequenced with the primers used for RT-PCR and BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) on ABI Prism 3100 capillary DNA sequencer (Applied Biosystems, USA).

For validation, rabies virus RV-97 strain (obtained from the Federal Centre for Animal Health Collection of Microorganism Strains) was used as a positive standard control sample and distilled water was used as a negative standard control sample. Brain samples from various animals that tested positive for rabies virus with immunofluorescence assay (IFA) served as positive controls. Brain samples from various animals that tested negative for rabies virus with IFA served as negative control samples.

The method was assessed for its accuracy by testing the positive standard control sample in ten repeats and the negative standard control sample in ten repeats.

Ten brain samples confirmed negative for rabies virus with IFA were tested to determine the method specificity. Specificity was calculated as the percentage of true negative results to the total number of tests according to the following formula:

$$Sp = (TN / (TN + FP)) \times 100\%,$$

where TN – true negative result;
FP – false positive result.

Ten brain samples confirmed positive for rabies virus with IFA were tested to determine the method sensitivity. Sensitivity was calculated as the percentage of true positive results to the total number of tests according to the following formula:

$$Se = (TP/ (TP + FN)) \times 100\%,$$

where TP – true positive result;
FN – false negative result.

To assess the intermediate precision under same conditions (repeatability), single positive sample was analysed in triplicate under the same measurement conditions using the same equipment, operator, laboratory, and within a short time period.

To assess intermediate precision under different conditions (reproducibility), single positive sample was analysed under varying conditions. This included testing the sample in triplicate on three different days by the same operator, and in triplicate on the same day by two different operators.

RESULTS AND DISCUSSION

Heterologous viruses with similar genome structure (DNA/RNA, one/two strands) are one of the most optimal types of internal controls for detection of viruses with molecular diagnostic methods. This strategy provides end-to-end control over the assay workflow, from nucleic acid extraction through reverse transcription to PCR. In this case, the control is designed to be as similar as possible to the test sample and is processed through all analytical stages in the same reaction vessel (tube).

LaSota strain of NDV included in freeze-dried live vaccine against this disease, was selected as an internal control. The reason was that NDV, being a member of *Paramyxoviridae* family, has single-stranded negative-sense RNA genome similar to the rabies virus. Moreover, the vaccine can be served as a ready-to-use internal control requiring only dilution with water. This eliminates time-consuming steps such as virus cultivation and titration.

Six different pairs of primers were designed. The primers corresponded to the region of genes encoding nucleoprotein, phosphoprotein, and matrix protein, as well as to the 3'-non-coding region of NDV LaSota strain genome. The primers have been designed taking into account the annealing temperature at which the target reaction takes place (55 °C). The internal control amplicon (approximately 700 bp) was deliberately designed to be longer than the target fragment (384 bp). This ensured that the target fragment was amplified with a competitive advantage over the internal control during PCR assay [36]. The primer design was tested for its specificity using the Primer-BLAST Internet service (www.ncbi.nlm.nih.gov/tools/primer-blast).

The synthesized primer pairs were tested for their functionality with anti-ND vaccine based on NDV LaSota strain (Fig. 1).

Three best combinations of primers were selected based on the test results: LASF107 – LASR800, LASF2086 – LASR2793, LASF2318 – LASR3026. Each primer pair was tested in conjunction with the target PCR for rabies virus to assess potential interference between the two amplification systems. The combined use of the two systems, in all three variants, was shown to produce no undesirable effects. In addition, a ten-fold serial dilution of the vaccine was used to assess the analytical sensitivity of the internal control detection with the selected primer pairs. The pair of LASF107 – LASR800 primers showed the highest sensitivity (dilution of the initial vaccine – 1:10,000) so they were finally selected for the internal control. This pair of primers enabled amplification of a 693 nt-fragment of NDV genome. The specificity of the amplified internal control fragment was confirmed by nucleotide sequencing.

A series of experiments demonstrated that the sensitivity of the internal control detection system decreases as the concentration of rabies virus increases. In contrast, the sensitivity of the rabies virus detection system remains unchanged with increasing the internal control concentration. Thus, the internal control system does not compete with the rabies virus detection system. This is particularly important for diagnostic accuracy when the viral load in the sample is low. However, to increase the reliability of the target PCR, it was decided to use the internal control at working concentration slightly above the minimum – 1:1,000 dilution of the original vaccine. Figure 2 demonstrates the operation of both primer systems in the presence of the internal control.

A series of experiments was performed to optimize the key parameters of reverse transcription and polymerase chain reaction. The optimal PCR parameters were found as follows: concentration of magnesium ions – 3 mM, concentration of primers – 40 mM. The internal control

(10 μL of the vaccine diluted to 1:1,000 with water) was added to the test sample (50 μL of brain suspension or 100 μL of culture fluid) at the stage of RNA extraction.

Thus, the method enabling rabies virus detection in the samples with simultaneous monitoring of the assay workflow quality in each reaction tube has been developed. During the assay, known rabies virus-positive and known rabies virus-negative samples (external positive and negative controls) were also tested along with test samples. Internal control was also added to the external positive and negative controls, 10 μL per each. The presence of a 693 bp DNA fragment and the absence of a 384 bp fragment in the PCR products confirmed that the assay had been performed correctly.

Fifty-two samples confirmed positive for rabies virus with IFA and forty-eight samples confirmed negative with IFA were tested with the developed method. In all cases, the rabies virus detection results were identical to IFA results. A 693 bp fragment was always present in the PCR products when negative samples were tested, which was indicative of the internal control genome fragment amplification, i.e. the absence of a false positive result. When rabies virus-positive samples were tested, a 693 bp fragment was not always amplified. That was expected since the target reaction had a competitive advantage over the reaction with the internal control – all the resources in the reaction tube were consumed for the target fragment synthesizing. However, the 384 bp fragment was consistently detected in PCR products when the rabies virus-positive samples were tested. That was indicative of the presence of the rabies virus in the sample.

The developed method was validated using several key parameters: accuracy, specificity, sensitivity, repeatability (intermediate precision under same conditions), and reproducibility (intermediate precision under different conditions) to confirm its reliability. When the method was tested for its accuracy, the results consistent to the sample statuses (positive or negative) were obtained for all samples. Thus, the calculated accuracy of the validated method was 100%. When the method was tested for its specificity, all known rabies virus-negative samples were tested negative with the method under validation. Therefore, the calculated specificity of the method under validation was 100%. When the method was tested for its sensitivity, all positive rabies virus-positive samples were tested positive with the method under validation, so the calculated sensitivity of the method under validation was 100%. The method demonstrated good repeatability in triplicate tests. Thus, the method under validation was shown to have absolute repeatability. The method demonstrated good reproducibility in all cases. Thus, the method under validation was shown to have absolute reproducibility. Validation results showed that defined characteristics of the method comply with the required ones.

Despite the variety of internal control designs available, each has its own advantages and disadvantages. Thus, artificially synthesized RNA [19] is prone to degradation by RNases, enzymes that are typically ubiquitous in samples. The ribosomal RNA used by J. Smith et al. [20] is, in principle, more resistant to RNases due to its well-developed tertiary structures. However, the reliability and effective range of this resistance remain to be determined. MS2 bacteriophage, being a viral particle, lacks this disadvantage as it has a protein shell that protects it from RNases. However, phage cultivation is an additional stage of the work. Artificially created virus-like particles offer many advantages, including presence of protective protein shell, customizing the genome nucleotide sequence with tailored properties, and incorporation of the desired nucleic acid type. However, the creation of such particles is technologically complex and their maintaining is labour-intensive. The development of plasmids serving as internal controls in numerous domestically produced commercial diagnostic kits requires minimal economic investment, as the production processes are already well-established. However, this plasmid-based strategy limits the type of nucleic acid to DNA. When used for RNA virus diagnosis, it fails to control for critical steps such as reverse transcription. In our opinion, the use of heterologous viruses is one of the most successful strategies for designing internal controls, as it combines the advantages offered by multiple alternative approaches. Firstly, they possess a robust protein shell that

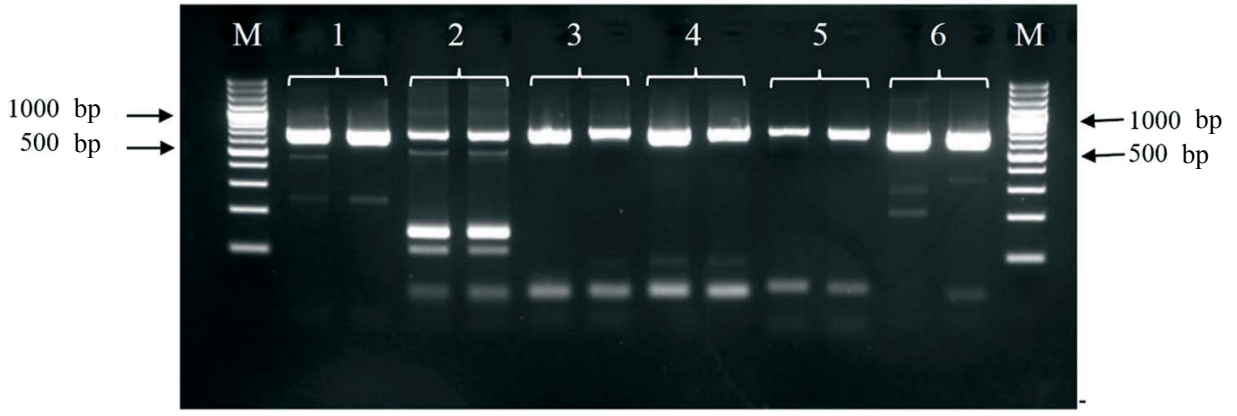

Fig. 1. Electropherogram of the PCR products using different combinations of primers for amplification Newcastle disease virus LaSota strain genome fragment. Each combination is given in duplicate. The numbers indicate the lanes with the following primer combinations: 1 – LASF815 – LASR1500; 2 – LASF1632 – LASR2337; 3 – LASF2086 – LASR2793; 4 – LASF2318 – LASR3026; 5 – LASF2598 – LASR3296; 6 – LASF107 – LASR800. M – molecular weight marker GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) fragment length is 100 bp

provides protection against RNases. Secondly, a virus can be selected whose nucleic acid type – whether DNA or RNA, single-stranded or double-stranded-matches that of the target virus. Thirdly, these are ready-made biological objects that are relatively easily propagated in cell cultures. When using a finished product such as a vaccine (as in our case), even the cell culture cultivation and titration stages are omitted.

It should be noted that the developed internal control based on LaSota strain of NDV can also be used either directly or after optimization (e.g., if PCR parameters of the internal control system mismatch the ones of the target system) for diagnosing diseases caused by other RNA viruses.

CONCLUSION

Newcastle disease virus LaSota strain-based internal control has been constructed for use together with reverse transcription-polymerase chain reaction assay for rabies virus detection that enables control of the assay workflow in each reaction tube. This internal control system was tested for its reliability using clinical samples containing and not containing rabies virus. The constructed internal sample was successfully validated. This internal control after proper optimization can be also used in experimental studies aimed at PCR diagnosis of the diseases caused by other RNA viruses carried out at relevant research institutions.

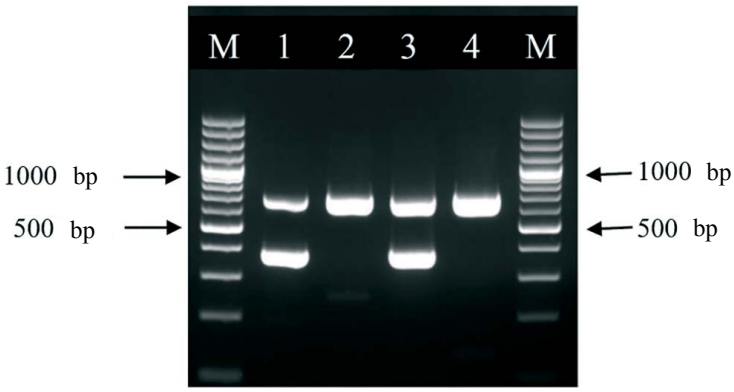

Fig. 2. Electropherogram of PCR products from rabies virus-positive samples and rabies virus-negative samples. The numbers 1 and 3 indicate the lanes with rabies virus-positive samples; the numbers 2 and 4 indicate the lanes with rabies virus-negative samples. M – molecular weight marker GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) fragments length is 100 bp

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Вклад автора: Чупин С. А. – формирование идеи, формулировка и развитие ключевых целей и задач, проведение исследований, анализ и интерпретация полученных данных, подготовка и редактирование текста.

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Drift of antibiotic resistance genes in pathogenic *Enterobacteriaceae*: a case study of *Escherichia coli*

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ABSTRACT

Introduction. The widespread antibiotic resistance among representatives of the *Escherichia coli* species is an acute problem for livestock and poultry farms, since this pathogen is the most frequently registered component of the etiological structure of gastrointestinal diseases in young farm animals and poultry, and is also often detected in diseases of other organs and systems. Even now, in many farms, the use of antibacterial drugs to treat diseases caused by this pathogen is difficult due to the circulation of strains with multiple resistance to most antibiotics used in veterinary practice. It is known that over time, the sensitivity of a microorganism to various groups of antibacterial drugs changes, often quite significantly. Sensitivity monitoring can help contain the spread of antibiotic resistance and optimally select drugs for use in therapy.

Objective. Analysis and systematization of the research results presented in the scientific literature on the resistance of *Escherichia coli* to antibacterial drugs.

Materials and methods. A search was conducted for scientific papers on this topic in scientific journals and materials of scientific and practical conferences.

Results. This article presents and summarizes literature data on trends in *Escherichia coli* resistance to antibacterial drugs.

Conclusion. *Escherichia coli* resistance is most often demonstrated to *β*-lactam antibacterial drugs, aminoglycosides, as well as tetracyclines, macrolides (erythromycin) and lincosamides (lincomycin). In almost all studies, *Escherichia coli* exhibits polyresistance (resistance to two or more drugs) and in some cases, multiresistance (resistance to at least one drug from three or more groups). The results of susceptibility determination in many studies differ significantly from each other, which is associated with different conditions for the formation of antibiotic resistance in bacteria on different livestock enterprises. For a more accurate assessment of the dynamics of the spread of antibiotic resistance within the *Escherichia coli* species, it is necessary to continue studying the sensitivity to antimicrobial drugs of various strains detected in livestock and poultry farms, as well as from environmental objects.

Keywords: review, antibiotics, resistance, *Escherichia coli*, colibacteriosis

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Дрейф генов антибиотикорезистентности патогенных энтеробактерий на примере *Escherichia coli*

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РЕЗЮМЕ

Введение. Широкое распространение антибиотикорезистентности среди представителей вида *Escherichia coli* является острой проблемой животноводческих и птицеводческих хозяйств, поскольку этот возбудитель является наиболее часто регистрируемым компонентом этиологической структуры желудочно-кишечных заболеваний молодняка сельскохозяйственных животных и птицы, а также нередко выявляется при заболеваниях других органов и систем. Уже сейчас во многих хозяйствах затруднено применение антибактериальных препаратов для лечения заболеваний, вызванных данным патогеном,

ввиду циркуляции штаммов, обладающих множественной устойчивостью к большинству применяемых в ветеринарной практике антибиотиков. Известно, что со временем чувствительность микроорганизма к различным группам антибактериальных препаратов изменяется, и нередко весьма значительно. Мониторинг чувствительности может помочь в сдерживании распространения антибиотикорезистентности и оптимальном подборе препаратов для применения их в терапии.

Цель исследования. Анализ и систематизация представленных в научной литературе результатов исследований по изучению устойчивости *Escherichia coli* к антибактериальным препаратам.

Материалы и методы. Проведен поиск работ по заявленной теме в научных журналах и материалах научно-практических конференций.

Результаты. В статье представлены и обобщены литературные данные о тенденциях в резистентности *Escherichia coli* к антибактериальным препаратам.

Заключение. Устойчивость *Escherichia coli* чаще проявляется к *β*-лактамным антибактериальным препаратам, аминогликозидам, а также тетрациклинам, макролидам (эритромицину) и линкозамидам (линкомицину). Практически во всех исследованиях установлено, что выделенные изоляты *Escherichia coli* характеризуются полирезистентностью (устойчивостью к двум и более препаратам), а в некоторых случаях и мультирезистентностью (устойчивостью по крайней мере к одному препарату из трех и более групп). Результаты определения чувствительности во многих исследованиях значительно отличаются друг от друга, что связано с неодинаковыми условиями формирования антибиотикорезистентности у бактерий на разных животноводческих предприятиях. Для более точной оценки динамики распространения антибиотикорезистентности внутри вида *Escherichia coli* необходимо продолжать изучение чувствительности к антимикробным препаратам различных штаммов, выявляемых в животноводческих и птицеводческих хозяйствах, а также из объектов окружающей среды.

Ключевые слова: обзор, антибиотики, резистентность, *Escherichia coli*, колибактериоз

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INTRODUCTION

Escherichia coli is a significant opportunistic pathogen in animals, often causing colibacillosis, but its role extends beyond this primary disease. This microorganism is frequently found in diseased animals and humans, particularly in gastrointestinal, obstetric, gynecological, respiratory, and urinary tract infections. *E. coli* are categorized into 7 pathotypes, including enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), and shigatoxin-producing (STEC). Additionally, there's a category for strains that are pathogenic to birds.

Colibacillosis remains a major challenge in animal husbandry, particularly in industrial poultry farming, where it ranks among the most prevalent bacterial infections in birds (is detected in 40–70% of disease cases). A critical complicating factor is the widespread antimicrobial resistance (AMR) in *E. coli* isolates, with resistance observed against all major classes of antibacterial drugs – in some cases reaching 100% [1, 2, 3].

The significance of this study is underscored by two critical factors: the substantial economic losses inflicted on livestock production by colibacillosis, and the escalating global prevalence of antimicrobial-resistant *E. coli* strains associated with this disease. The sensitivity of pathogens to antibiotics can change over time, making ongoing monitoring of antimicrobial resistance crucial, especially for pathogens like *E. coli*.

This study summarizes the data available in the literature on the resistance dynamics of *E. coli* isolated from different farm animal species to antibacterial drugs.

Antimicrobial resistance is the ability of microorganisms to resist the effects of antimicrobial agents, including antibiotics. The early years 1945–1963 following Fleming's 1945 discovery were marked by a belief that the pharmaceutical industry could constantly develop new antibiotics faster than bacteria could develop resistance. Later, the discovery of plasmids, which can transfer resistance genes between bacteria, highlighted a major route for resistance spread and increased concern (1963–1981). This concern grew into a global problem perception from 1981–1992, leading to increased research and funding (1992–2013). Since 2013 the problem continues to worsen due to the emergence of new resistance mechanisms and the increasing spread of resistant microorganisms among populations [4, 5].

The use of alternative products like bacteriophages, probiotics, phytobiotics, and antimicrobial peptides is increasingly important in the treatment of bacterial diseases. However, antibacterial drugs are still very widely used for treatment in livestock and poultry farming. Moreover, the use of antibiotics to prevent disease and boost productivity in agriculture is the reason for the creation and accumulation of AMR genetic determinants in *Escherichia* species. This, in turn, leads to AMR

developement and contamination of raw materials and food products [6, 7].

The purpose of this study was to summarize the scientific literature data on *E. coli* AMR resistance dynamics.

MATERIALS AND METHODS

This study was conducted through systematic analysis of literature data on antibiotic resistance patterns in diverse *E. coli* strains isolated from pathological specimens and biological samples obtained from companion animals, livestock, and poultry.

RESULTS AND DISCUSSION

In 2011 N. N. Shkil presented the results of testing of 21 pathological and biological samples from aborted and stillborn bovine fetuses; 71 samples from newborn calves; 67 samples from calves aged 10 days to 1 month; 47 samples from 1–3-month-old calves and 18 samples from > 3 month old calves. Pathogenic microorganisms were isolated in 32% of samples from animals exhibiting clinical signs of gastrointestinal disease, while respiratory syndrome was observed in 68% of affected calves. *Escherichia* were isolated from 38% of the samples. Isolation tests were conducted annually from 2001 to 2010. As established by the author, in 2001, 50% of the isolated *Escherichia* bacteria exhibited high sensitivity to aminoglycosides. In subsequent years, the increased sensitivity to quinolone/fluoroquinolone drugs was established, which peaked in 2006 (66%). By 2007, resistance rates to these agents had reached parity with aminoglycosides. Subsequently, in 2009–2010, bacterial isolates demonstrated significantly greater sensitivity to aminoglycosides (50%) compared to fluoroquinolones (15%). The author observed a consistent inverse relationship in antimicrobial susceptibility patterns, where increased sensitivity to one drug class frequently corresponds with decreased sensitivity to another class within the same temporal period. Furthermore, these susceptibility trends demonstrate cyclical fluctuations, characterized by multi-year periods of increasing sensitivity followed by subsequent declines in subsequent years [8]. *E. coli* have, apparently, developed a robust mechanism for acquiring resistance to fluoroquinolone antibiotics. Antisense RNA produced by the *micF* gene does indeed inhibit porin protein synthesis at the translation level. This has a positive effect on the sigma factor content related to multiple stress resistance σ^S in cells. The most significant fluctuations in *E. coli* resistance are associated with this phenomenon [9].

The analysis of dynamics in sensitivity of *E. coli* strains from diseased calves to antimicrobial drugs by N. E. Gorkovenko and Yu. A. Makarov revealed resistance to enrofloxacin in 6.5% isolates in 2006; by 2007 their number increased to 36.4%, and in 2010 reached 90.0%. The number of polymyxin-resistant *E. coli* isolates in 2006 was 23.3%, in 2010 – 75%. Neomycin resistance was revealed in 64.0 and 81.8% in 2006 and 2010, respectively. Thus, an increasing resistance to enrofloxacin, polymyxin and neomycin, which significantly reduces the therapeutic effectiveness of these antibiotics is observed. Tetracycline resistance in 2006–2008 was approximately 70%, in 2009 it decreased to 60%, and in 2010 it reached 100%. Resistance to chloramphenicol in 2006 and 2007 was 60 and 55%, respectively; in 2008–2009 it increased to 80%, and then decreased significantly. Resistance to streptomycin and kanamycin was increasing from 2006 to 2008, an then decreased in 2009. It reached 100% for both drugs in 2010.

Consistent with previous findings, these data reveal cyclical fluctuations in antibacterial resistance patterns. Despite these periodic variations, the overall trend demonstrates progressive escalation of resistance, ultimately culminating in pan-resistance and complete loss of clinical efficacy for some antimicrobial agents [10].

The research presented by D. A. Zhelyabovskaya et al. in 2017 suggests that 71.4% of the studied *E. coli* strains (O15, O18, O26) isolated from the intestines of newborn calves are polyresistant. These isolates demonstrated resistance to erythromycin (95.2%), tetracycline and penicillin (90.5%), kanamycin (85.7%), ampicillin (76.2%), streptomycin and gentamicin (71.4%) [11].

Analysis by N. M. Al-Hammash and A. V. Ignatenko of *E. coli* isolates from a dairy farm revealed high resistance prevalence: 94% to benzylpenicillin, erythromycin, and lincomycin; 83% to tetracycline; 61% to ampicillin; 56% to neomycin; 44% to chloramphenicol; 37% to pefloxacin; 33% to polymyxin; and 28% to cephalexin. The isolates demonstrated intermediate susceptibility to neomycin (55%), polymyxin (50%), furadonin (27%), chloramphenicol (16%), and kanamycin (14%). They were susceptible to the following antibacterials: gentamicin (83%), kanamycin (78%), cephalexin (74%), furadonin (72%), pefloxacin (62%), chloramphenicol (39%), neomycin (39%), ampicillin (33%). Isolates showed 100% susceptibility only to ceftriaxone, while absolute resistance was observed to oleandomycin, clindamycin, and oxacillin [12].

In the study by S. N. Zolotukhin et al. 34.8% of *E. coli* isolates showed susceptibility to gentamicin, 34.2% were resistant and 31.5% were moderately resistant. Ampicillin was active against 57.8% of *E. coli* isolates, 27.3% were resistant to it, and 14.4% showed moderate resistance. The highest sensitivity was found to ceftriaxone (84.7%), ciprofloxacin (74.2%) and chloramphenicol (60.6%). The test results demonstrated that none of the antibiotics completely inhibited microbial growth (100% inhibition). Most strains were polyresistant to erythromycin, chloramphenicol, streptomycin, tetracycline, neomycin, ampicillin, gentamicin, and penicillin [13].

Between 2016 and 2020, M. E. Ostyakova and I. S. Shul'ga examined the gut microbiota (enterobiocenosis) of newborn calves affected by gastrointestinal disorders. In the course of this work, the resistance of *E. coli* strains to certain antibacterials was analyzed. The results of the study are the following: isolates showed resistance to benzylpenicillin, ofloxacin, ciprofloxacin and erythromycin. This suggests multidrug resistance of the isolated strains. 91.7% of the isolates were sensitive to polymyxin, 70.6% to cefazolin, 65.5% to streptomycin, 62.5% to amoxicillin / clavulanic acid combination. Therefore, these antibiotics are the drugs of choice for the treatment of intestinal infection caused by *E. coli* [14].

In the characterization of diarrheagenic *E. coli* museum strains for pathotypes and AMR genes by Yu. I. Pobolelova

and S. P. Yatsentyuk, EPEC *E. coli* was the most prevalent pathotype, accounting for 29% of cases relative to other pathotypes. The determination relied on fragments of AMR determinants to *β*-lactam atibiotics (*blaTEM*, *blaSHV* genes), florfenicol (*floR*), chloramphenicol (*cat1*, *cml*A), streptomycin (*aadA1* gene), gentamicin (*aac3-IV* gene). Among the studied strains, 36% had resistance genes to at least one of the antimicrobials under study, and 5 strains demonstrated resistance to two antibiotics simultaneously: 2 strains to chloramphenicol and streptomycin, 2 more to streptomycin and florfenicol, and 1 strain to chloramphenicol and florfenicol. In total, the chloramphenicol resistance genes *cat1* and *cmlA* were identified in 3 and 4 strains, respectively; the streptomycin resistance gene *aadA1* was identified in 17 strains, and the florfenicol resistance gene *floR* was identified in 4 strains. Resistance genes to gentamicin and *β*-lactams were not detected [15].

Researchers A. A. Golikova and O. A. Manzhurina conducted experiments on susceptibility of *E. coli* strains isolated from colibacillosis-affected calves to 16 antimicrobials of various pharmacological classes. The authors established the susceptibility of *E. coli* O20 strain to the following antibiotics: ampicillin, amoxicillin, tetracycline, chloramphenicol, gentamicin, polymyxin, norfloxacin, enrofloxacin and streptomycin. Strain O33 showed sensitivity to the same antimicrobials as O22, but was resistant to gentamicin and streptomycin and sensitive to furazolidone and furadonin. *E. coli* O137 demonstrated susceptibility to ampicillin, amoxicillin, tetracycline, chloramphenicol, gentamicin, polymyxin, furazolidone, furadonin, norfloxacin, enrofloxacin, and streptomycin [16].

According to E. A. Sazonova, in 2020–2022, *E. coli* strains tended to develop multidrug resistance to first-generation cephalosporins, penicillins, tetracyclines, macrolides, lincosamides, sulfonamides, and streptomycin. Antimicrobial resistance was reveled in O2, O78, O115, O126, O15, O18, O119, O33, O41, O101, O137, O157:H7 serovariants isolated from swine colibacillosis cases.

At the same time, the *E. coli* resistance to various antibacterial drugs changed as follows: 44.0% to cephalexin in 2020, 71.4% in 2021, 100.0% in 2022; 29.1% to cefazolin in 2020, 50.0% in 2021, 31.5% in 2022; 6.9, 14.3, 15.3% to ceftriaxone; 73.7, 50.0, 48.7% to amoxicillin; 73.7, 78.7, 81.3% to ampicillin; 80.5, 57.1, 64.3% to tetracycline; 84.5, 100.0, 99.1% to doxycycline; 30.9, 71.4, 72.5% to streptomycin; 83.4, 100.0, 92.3% to erythromycin; 85.7, 92.8, 91.3% to rifampicin; 11.4, 7.1, 10.6% to norfloxanin; 18.3, 7.4, 2.3% to enrofloxacin; 10.3, 7.2, 11.3% to ciprofloxacin in 2020, 2021 and 2022 accordingly. These figures demonstrate that antibiotic-resistant *E. coli* strains are highly prevalent. Moreover, the antimicrobial resistance increases over time, sometimes (for example, to cephalexin, doxycycline, erythromycin) reaching 100% [17].

Tishchenko A. S. et al. reported that *E. coli* strains (K99:O141, F41:O26, F41, K88:O157) from calves and piglets with enteric diseases exhibited resistance to amoxiclav, tetracycline, gentamicin, oxacillin, azithromycin, and ceftazidime, highlighting challenges in veterinary antimicrobial therapy. While fluoroquinolones (ciprofloxacin and pefloxacin) demonstrated the highest antibacterial activity among tested agents, the observed intermediate resistance levels diminish their clinical efficacy. Amoxiclav, oxacillin, gentamicin, and azithromycin demonstrated the lowest antibacterial effect [18].

According to I. N. Zhdanova et al., *E. coli* strains O8, O15, O20, O101, O115, O157 were isolated from calves and adult cattle in the farms of the Perm Krai in 2020–2021. These strains revealed resistance to ampicillin and cefazolin (61.5% for each) and high resistance to ceftriaxone (23.1%), cefoxitin (30.7%), chloramphenicol (61.5%) and tetracycline (79.5%). The isolates were sensitive to imipenem and tobramycin (100%), meropenem (97.4%), amikacin and moxifloxacin (92.3%) [19].

Makavchik S. A. and Sukhinin A. A. studied microorganisms isolated from the milk of mastitic cows in 2021–2022. *E. coli* cultures were susceptible to neomycin and carbapenems (100%) and resistant to cephalexin (75%), tetracycline (30%), cefotaxime (30%), gentamicin (14%) and ciprofloxacin (7%). The collected data demonstrate a concerning trend of rapidly increasing resistance to cephalosporins, tetracyclines, and aminoglycosides among clinical isolates [20].

The results of studies conducted by A. S. Lokteva et al. demonstrated that *E. coli* O141 and O33 strains isolated from samples from dead pigs in 2017–2022 turned out to be pan-resistant. Over 90% of pathogenic isolates exhibited polyresistance, with this concerning prevalence persisting throughout the entire study period [21].

The article by I. M. Donnik describes that the majority of bacteria isolated from samples of cervical scrapings, from mammary secretions, nasal and oral swabs of animals, manure, feed and contact surfaces and equipment were resistant to antimicrobials. *Escherichia* were resistant to rifampicin, semi-synthetic penicillins and tetracyclines (64–67%); approximately 44% of isolates demonstrated low sensitivity to 3–5 antibiotics of different classes; 28% among them to third-generation cephalosporins: ceftriaxone and cefotaxime. The bacteria exhibited high sensitivity to fluoroquinolones: ciprofloxacin, enrofloxacin, and ofloxacin (82%) [22].

Kochkina E. E. and Morozova N. V. assessed antimicrobial resistance in *E. coli* strains isolated from cats with genitourinary tract disorders. The study revealed that isolates exhibited the sensitivity to cephalosporins (71 ± 10.7%), moderate resistance was observed to aminoglycosides (83.3 ± 8.8%) and synthetic penicillins (66 ± 11.1%) and resistance to macrolides and fluoroquinolones. The sensitivity of the strains to individual antibiotics is presented as follows: all studied cultures were sensitive to cefepime; 83.3 ± 8.8% were sensitive to ceftriaxone and cefazolin; 83.3 ± 8.8% showed moderate resistance to cefotaxime, enrofloxacin, and gentamicin; 66.7 ± 11.1% showed moderate resistance to amoxiclav; and 50 ± 11.8% showed moderate resistance to ciprofloxacin; 16.7 ± 8.8% exhibited moderate resistance to ceftriaxone and cefazolin. The authors report the absolute resistance to tylosin [23].

When assessing antibiotic resistance before using antibacterial drugs, N. N. Muzyka and A. V. Beletskaya isolated *E. coli* from various bird species. The isolates exhibited sensitivity to gentamicin (19.0%), florfenicol (16.6%), enrofloxacin (14.3%), spectomycin (14.3%),

norfloxacin (7.1%), trimethoprim (4.7%), tilmicosin (4.7%), doxycycline (2.4%) and lincomycin (2.4%). Moderate sensitivity was observed for tilmicosin (11.9%), doxycycline, florfenicol, norfloxacin and spectinomycin (4.7% each), trimethoprim, lincomycin and gentamicin (2.4% each). Thus, the overall sensitivity rate to antibacterial drugs was 20% or lower [24].

In 2023, A. S. Krivonogova et al. published the article "Antibiotic resistance of *Enterobacteriacea* in the microbiomes associated with poultry farming". In this study, the minimum inhibitory concentration (MIC) was determined for the reference strain *E. coli* ATCC 25922 against ciprofloxacin, meropenem, cefepime, and ampicillin. The control strains were cultured during 37 days. This period corresponds to the period of broiler raising in commercial poultry farms from hatching to slaughter. The study demonstrated that the reference strain *E. coli* ATCC 25922 exhibited resistance to ciprofloxacin (MIC = 0.06–0.12 mg/L), meropenem (MIC = 0.12 mg/L), ampicillin (MIC = 2–4 mg/L), and cefepime (MIC = 0.5 mg/L). Under these conditions, antibiotic resistance did not arise because the studied microorganisms lacked active resistance determinants in their genomes and had no opportunity for horizontal gene transfer due to isolation from other microbes.

The sensitivity to antibacterial drugs of microflora isolated from chicken cloacal swabs and litter at different stages of poultry rearing was also analyzed. All *E. coli* isolates exhibited sensitivity to ampicillin (MIC: 2.0–4.0 mg/L) and ciprofloxacin (MIC: 0.06–0.12 mg/L). Meropenem was active against 74% of isolates at MIC 0.06 mg/L and against all isolates at MIC 0.12 mg/L. 50% of isolates were resistant to cefepime at MIC 0.125 mg/L, while 100% of isolates were susceptible to it at MIC 0.5 mg/L [25].

The results demonstrate that subinhibitory antibiotic concentrations promote pathogen survival and facilitate the development of resistance through vertical gene transfer. These studies demonstrate that carbapenem resistance in pathogenic *E. coli* serotypes severely limits therapeutic options, underscoring their critical status in clinical management and necessitating improved antimicrobial stewardship and infection control measures.

A group of authors studied the antibiotic sensitivity of pathogenic coliform cultures circulating in a commercial poultry farm in the Omsk Oblast. *E. coli* O37, O115, and O2 serovariants were isolated from pathological samples from chickens and chicks of different ages in 2018. These isolates exhibited 100% sensitivity to fluoroquinolone formulated into "Triflon" and "Enroflon K" drugs. At the same time, the strains showed absolute resistance to tetracycline, and most strains were resistant to tylosin, gentamicin, doxycycline, and chloramphenicol [26].

Isakova M. N. et al. studied 127 *E. coli* isolates from bovine mammary secretions and cervical swabs. Phenotypic resistance to rifampicin, semi-synthetic penicillins, and tetracyclines was prevalent among the studied isolates. The cultures showed a weaker resistance to azithromycin, chloramphenicol and tobramycin. Among the tested isolates, 28.46% demonstrated intermediate resistance to third-generation cephalosporins, while 49.02% carried the *blaDHA* resistance gene [27].

In 2023 M. S. Alexyuk et al. conducted the monitoring of *E. coli* antimicrobial resistance in the Republic of Kazakhstan. During 3 months, fecal samples from calves with escherichiosis clinical signs were collected on private farms in the Almaty region. 30 *E. coli* isolates were recovered from the biological samples; 6 of them were presumably identified as O157:H7. Study results revealed that only 4 isolates were susceptible to all tested antibiotic classes, while 7 isolates exhibited resistance to a single antibiotic. The majority demonstrated multidrug resistance (defined as non-susceptibility to ≥ 3 antibiotic classes). Five isolates showed resistance to 7 antibiotic classes, and one of the isolates proved to be resistant to all 8 classes of antibiotics. Most isolates were resistant to ampicillin, tetracycline, gentamicin, florfenicol, and trimethoprim. Resistance to enrofloxacin and amoxicillin / clavulanic acid combination was less prevalent. Almost all isolates were found to be sensitive to colistin. Some strains exhibited intermediate resistance to gentamicin and amoxicillin / clavulanic acid combination [28].

In the study by M. Yu. Syromyatnikov et al. the antimicrobial resistance genes of *E. coli* isolated from the intestines of 2–5-day-old diarrheic piglets were analyzed. Bioinformatic analysis identified 26 antibiotic resistance genes, including: aminoglycoside resistance (*Aac6-Aph2*, *Aac6-If*; *StrA*, *StrB*); β-lactam resistance (*AmpC1_Ecoli*; *OXA-10*, *OXA-14*, *OXA-16*, *Penicillin_Binding_Protein_Ecoli*, *TEM-143*, *TEM-166*, *TEM-215*, *TEM-76*, *TEM-95*); quinolone resistance (*QnrB19*, *QnrB5*, *QnrD*, *QnrVC4*); sulfonamide resistance (*SulI*); tetracycline resistance (*TetD*); trimethoprim resistance (*DfrA1*, *DfrA14*, *DfrA27);* phenicol resistance (*CmlA5*, *CmlA1*; *FloR*). Among 4 detected quinolone resistance genes, *QnrD* turned out to be prevalent: almost 60% of the genes in this sample. *E. coli* strains harboring this resistance plasmid are predicted to exhibit broad-spectrum resistance to most antimicrobial agents commonly used in veterinary practice. Among 10 identified β-lactam resistance genes, *Penicillin_Binding_Protein_Ecoli* was the most prevalent (24%). The prevalence of *OXA-16* was 9%, *AmpC1_Ecoli* – 15%, *OXA-10* – 12%, *OXA-14* – 11%. The prevalence of *TEM-143*, *TEM-166*, *TEM-76*, and *TEM-95* genes was 6% in total and only 1% of the findings were for *TEM-215*. Among the phenicol resistance genes, *CmlA5* (52%) and *CmlA1* (44%) were most prevalent. The most common trimethoprim resistance determinant was *DfrA14* gene (64%). Among the aminoglycoside-associated genes, *StrA* (35%) and *StrB* (31%) were the most prevalent. Tetracycline and sulfonamide resistance genes collectively represented 3% of the total relative abundance of resistance genes. Among the remaining sequences, no individual resistance group exceeded 10% in relative abundance. Specifically, resistance genes for tetracyclines, aminoglycosides, and sulfonamides each accounted for less than 1% of the total [29].

High-throughput sequencing revealed that *QnrD*, encoding quinolone resistance, was the most prevalent resistance gene identified.

CONCLUSION

The literature review indicates that *E. coli* frequently demonstrates resistance to: β-lactam antibiotics (particularly benzylpenicillin, penicillin, and cephalexin); aminoglycosides (primarily streptomycin and gentamicin);

tetracyclines, macrolides (erythromycin) and lincosamides (lincomycin). The majority of studies demonstrate that *E. coli* exhibit polyresistance (resistance to ≥ 2 antimicrobial agents), with many strains showing multiresistance (non-susceptibility to ≥ 1 agent from ≥ 3 antimicrobial classes).

Nevertheless, antibiogram results can vary significantly between studies. This can be attributed to the uneven distribution of antibiotic resistance mechanisms within the microbial populations (microbiocenosis) of individual livestock farms. The specific combination of resistance mechanisms in *E. coli* populations varies between farms and depends on multiple factors: circulating serovars and their genetic backgrounds; spectrum of antibiotics used and their application methods; presence of resistance determinants in farm environments; disinfection efficacy, as subinhibitory disinfectant concentrations may promote microbial adaptation. These factors collectively shape: the specific array of antibiotic resistance genes circulating within the farm's microbial community; phenotypic resistance mechanisms (including biofilm formation and bacterial persistence) and adaptive resistance (transient survival advantages under antimicrobial pressure). This represents one of the most critical challenges in treating *E. coli* infections, as the expanding resistance profile increasingly compromises optimal antimicrobial selection.

The literature review revealed key trends in *E. coli* antimicrobial resistance and confirmed the escalating challenge of antibiotic resistance in animal production systems. Consequently, comprehensive antimicrobial resistance surveillance is required, encompassing both livestock production facilities and their surrounding environments where resistant microorganisms may persist and spread. Monitoring of antimicrobial susceptibility trends will enable evidence-based updates to therapeutic guidelines for *E. coli* infections in livestock. In addition, it's crucial to continue the development and implementation of alternative therapies for infectious diseases that don't rely on antibiotics.

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The role of purinergic signaling and cytokine network in the inflammatory process

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ABSTRACT

Introduction. Inflammation is a complex biological process essential for host defense against pathogens and tissue repair. This process is regulated by a variety of signaling molecules, among which purines and cytokines play an important role. Purinergic signaling mediated by adenosine triphosphate, adenosine monophosphate, and other nucleotides plays a key role in regulating immune responses and inflammatory processes. The cytokine network, including interleukins, tumor necrosis factor *α* and other molecules, is also an important component of inflammation, providing communication between cells of the immune system and regulating their activity. Understanding the purinergic signaling and the cytokine network interaction mechanisms is crucial for developing innovative treatments for inflammatory diseases.

Objective. To synthesize current research findings on the role of purinergic signaling and the cytokine network in inflammatory processes within animal models.

Materials and methods. 55 scientific publications by Russian and international authors (2000–2021) investigating the effects of nucleotides, nucleosides, and purinergic receptors on immune response development, macrophage activation, and cytokine release mechanisms were analyzed. Source databases included eLIBRARY.RU, CyberLeninka, PubMed, NCBI, ResearchGate, CABI, and Google Scholar.

Results. The analysis explored mechanisms of the inflammatory response, including the role of various cells and molecules – cytokines and receptors – in the regulation of the immune response. The latter plays an important role in activating immune system cells and regulating inflammatory reactions. The process of adenosine triphosphate dephosphorylation by CD39 and CD73 enzymes, which promotes the production of adenosine and the activation of anti-inflammatory mechanisms, is discussed. The functions of pro-inflammatory cytokines such as interleukin-1, tumor necrosis factor *α* and interleukin-6 are analyzed in the context of macrophage activation and neutrophil migration to the site of inflammation. The importance of regulating these processes is emphasized in order to prevent excessive inflammatory response and ensure homeostasis. The mechanisms of transition between the phases of inflammation are examined, including the role of anti-inflammatory cytokines such as interleukin-10 and transforming growth factor *β* in controlling neutrophil activity and resolving the inflammatory process.

Conclusion. Further study of this topic can deepen the modern knowledge of scientists about the mechanisms of inflammation and create the basis for the development of innovative therapeutic strategies aimed at treating diseases caused by disorders of the immune system.

Keywords: review, nucleotides, receptors, purinergic regulation, CD39, CD73, ATP, ADP, AMP, adenosine, macrophages, cytokines, interleukins

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Роль пуринергической сигнализации и цитокиновой сети в воспалительном процессе

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РЕЗЮМЕ

Введение. Воспаление представляет собой сложный биологический процесс, направленный на защиту организма от патогенов и восстановление поврежденных тканей. Этот процесс регулируется множеством сигнальных молекул, среди которых важное значение имеют пурины и цитокины.

Пуринергическая сигнализация, опосредованная аденозинтрифосфатом, аденозинмонофосфатом и другими нуклеотидами, играет ключевую роль в регуляции иммунных реакций и воспалительных процессов. Цитокиновая сеть, включающая интерлейкины, фактор некроза опухоли *α* и другие молекулы, также является важным компонентом воспаления, обеспечивая коммуникацию между клетками иммунной системы и регулируя их активность. Понимание механизмов взаимодействия между пуринергической сигнализацией и цитокиновой сетью имеет важное значение для разработки новых стратегий лечения воспалительных заболеваний.

Цель исследования. Обобщение результатов исследований по значению роли пуринергической сигнализации и цитокиновой сети в воспалительном процессе у животных.

Материалы и методы. Проведен анализ 55 научных публикаций российских и иностранных авторов за период с 2000 по 2021 г., исследующих влияние нуклеотидов, нуклеозидов и пуринергических рецепторов на формирование иммунного ответа, а также механизмы активации макрофагов и выделения цитокинов. Для поиска источников использовались базы данных eLIBRARY.RU, CyberLeninka, PubMed, NCBI, ResearchGate, CABI и Google Scholar.

Результаты. Рассмотрены механизмы воспалительного ответа, включая роль различных клеток и молекул, таких как цитокины и рецепторы, в регуляции иммунной реакции. Последние имеют важное значение в активации клеток иммунной системы и регуляции воспалительных реакций. Обсуждается процесс дефосфорилирования аденозинтрифосфата с участием ферментов CD39 и CD73, что способствует образованию аденозина и активации противовоспалительных механизмов. Проанализированы функции провоспалительных цитокинов, таких как интерлейкин-1, фактор некроза опухоли *α* и интерлейкин-6, в контексте активации макрофагов и миграции нейтрофилов к месту воспаления. Подчеркивается важность регуляции этих процессов для предотвращения чрезмерного воспалительного ответа и обеспечения гомеостаза. Рассмотрены механизмы перехода между фазами воспаления, включающие роль противовоспалительных цитокинов, таких как интерлейкин-10 и трансформирующий фактор роста *β*, в контроле активности нейтрофилов и разрешении воспалительного процесса.

Заключение. Дальнейшее изучение данной темы способно углубить современные знания ученых о механизмах возникновения воспаления и создать основу для разработки инновационных терапевтических стратегий, направленных на лечение болезней, обусловленных нарушениями иммунной системы.

Ключевые слова: обзор, нуклеотиды, рецепторы, пуринергическая регуляция, CD39, CD73, АТФ, АДФ, АМФ, аденозин, макрофаги, цитокины, интерлейкины

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INTRODUCTION

Currently, innovative medicinal products are widely used in human medicine and veterinary medicine. The development of liposome-based medicinal products for targeted delivery of medicinal substance attracts the attention of scientists all over the world. Special attention is paid to the creation of immunomodulating liposomal medicinal products that specifically affect the macrophage component of the immune system. Scientists from the Laboratory of Veterinary Medicine and Biotechnology of the Federal Agricultural Kursk Research Center are working in this field. They proposed and tested a method for obtaining liposomal immunotropic composition for the pre-nosological prevention of pathologies in cattle of different ages in the settings of the Uchkhoz Znamenskoye of the Kursk State Agrarian University.

The immune system is a complex mechanism that helps to protect the body from pathogenic microorganisms. Nucleic acids, purinergic receptors, and cytokines play an important role in regulating immune system functions. These components are involved in a wide range of processes: from cell signaling to providing energy for active immune responses. Nucleotides such as adenosine triphosphate (ATP) act as key mediators in intercellular communication and intracellular signaling. Purinergic receptors that respond to extracellular nucleotides promote the activation of inflammatory processes and regulate the behavior of immune cells. Nucleosides, required for DNA and RNA synthesis, support the division and growth of immunocompetent cells, thus playing a vital role in adaptive immunity [1, 2]. By understanding how these mechanisms function, we can simulate potential inflammatory processes in animal models and develop methods to prevent pathologies.

Inflammation plays a key role in the animal immune response, protecting against infection, injury, and other harmful factors. This process is a complex and well-coordinated reaction that includes the activation of various cells of the immune system, release of cytokines and other mediators, as well as changes

in blood circulation and vascular permeability. Inflammation is the body's initial defense against infection. When pathogens like bacteria, viruses, or fungi invade tissues, local immune cells such as macrophages and dendritic cells are triggered to initiate an immune response. When cells recognize a pathogen, they start to release pro-inflammatory cytokines, signaling molecules that attract other white blood cells to the site of infection. The inflammatory process recruits neutrophils and monocytes from the bloodstream to affected tissues. Neutrophils are the first responders to inflammation, phagocytosis of pathogens and releasing antimicrobial substances. Once in the tissues, monocytes differentiate into macrophages, which continue to destroy pathogens and promote tissue repair. Inflammation is not only aimed at fighting infection, but also at eliminating tissue damage [3, 4, 5].

Macrophages play an important role in the healing process by removing dead cells and cellular debris. They also release growth factors involved in tissue regeneration [4, 6, 7, 8, 9]. By presenting antigens to T lymphocytes, inflammation helps activate the adaptive immune response. Dendritic cells capturing pathogens during inflammation migrate to the lymph nodes, where they present antigens to T cells. This leads to the development of a specific immune response against specific pathogens. Although inflammation is necessary to protect the body, but its excessive or prolonged activity can lead to tissue damage and the development of chronic diseases. Therefore, it is important to have mechanisms for regulating the inflammatory response, such as anti-inflammatory cytokines (for example, interleukin IL-10) and other molecules that help resolve inflammation once the threat is eliminated. For veterinary medicine it is important to understanding the mechanisms of inflammation. Chronic inflammation is associated with a number of diseases in animals, including allergies, autoimmune disorders and metabolic diseases [2, 10].

The interaction of purinergic receptors with the cytokine network is an important aspect of the regulation of the immune response and inflammatory processes in the body. Purinergic receptors such as P2X and P2Y are activated by nucleotides such as adenosine and ATP and are important in the immune response in animals. Purinergic receptors regulate macrophage phagocytosis. This leads to the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), which play an important role in initiating and maintaining the inflammatory response. Adenosine signaling through P1 receptors (A2A and A2B) can suppress pro-inflammatory cytokines and promote anti-inflammatory cytokines, notably IL-10. This establishes a balance between pro-inflammatory and anti-inflammatory signals, that is crucial for preventing excessive inflammation and subsequent tissue damage. Purinergic receptors regulate leukocyte migration to inflammatory sites by detecting extracellular nucleotides like ATP released from damaged cells. This activation triggers immune cells to release chemoattractants such as IL-6 and IL-8, which in turn recruit and increase the number of immune cells at the site of inflammation [11]. Purinergic signaling interacts with other cellular pathways, including those associated with Toll-like receptors (TLRs). This interaction can enhance or modulate the response to infection or tissue damage. Purinergic receptors play an important role in maintaining immune homeostasis. They help regulate immune response activation and suppression, which is crucial for preventing autoimmune diseases and chronic inflammation [12, 13, 14, 15].

Thus, the interaction between purinergic receptors and the cytokine network is a complex, dynamic system vital for regulating immune responses and inflammatory processes. Understanding these interactions could facilitate the development of novel therapeutics for inflammatory and autoimmune diseases.

This study aims to systematize data reflecting the importance of purinergic signaling and the cytokine network in the development of inflammatory processes.

MATERIALS AND METHODS

This review is based on an analysis of 55 scientific publications by Russian and international authors, investigating the role of purinergic signaling and cytokine networks in regulating inflammatory responses. The literature search was conducted using major digital repositories, including eLIBRARY.RU, CyberLeninka, PubMed, NCBI, ResearchGate, CABI, and Google Scholar. Key search terms comprised the following: "purinergic regulation", "cytokine network", "CD39", "CD73", "ATP", "ADP", "AMP", "adenosine", "macrophages", "cytokines", "interleukins" and "tumor necrosis factor".

Publications were selected based on the following criteria: relevance to the research topic, scientific significance, methodological clarity, and the inclusion of a robust analytical discussion comparing the results with existing literature.

RESULTS AND DISCUSSION

Purinergic signaling in animals involves the interaction of purines, such as adenosine and ATP, with purine receptors that are located on the surface of cells. These receptors are divided into two main groups: P1 (adenosine) and P2 (ATP receptors) [2, 3, 4]. Adenosine functions as an anti-inflammatory and pro-healing molecule by interacting with P1 receptors, leading to reduced pro-inflammatory signals and enhanced immune cell regulation. Specifically, it suppresses the production of inflammatory cytokines and promotes tissue repair, playing a vital role

in healing processes. On the contrary, ATP, particularly through P2 receptors, acts as a "danger signal" that activates immune cells like macrophages, leading to the release of pro-inflammatory cytokines such as IL-1β and TNF-α, thus promoting inflammation even tissue damage. ATP release is triggered by various cellular stress factors, including inflammation, hypoxia, apoptosis, and necrosis [3, 4, 5].

Inflammation and hypoxia (low oxygen) conditions promote the release of ATP and ADP (adenosine diphosphate) from cells, leading to increased extracellular adenosine levels [1, 11]. Hypoxia and HIFs increase extracellular adenosine concentrations by transcriptionally regulated genes involved in its adenosine metabolism and receptor expression [12, 13, 14, 15, 16, 17]. The critical role of extracellular adenosine metabolism is demonstrated in mice. Mice with genetic defects in ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73), exhibit reduced extracellular adenosine, leading to impaired adenosine signaling, even when ATP levels are normal or high. Adenosine signaling plays a key role in the lung's response to damage. Adenosine has complex and multifaceted effects on inflammation, repair, and remodeling by binding to G protein-coupled receptors on cell surfaces [18, 19, 20], causing both protective and destructive reactions. Its anti-inflammatory and protective-regenerative roles are primarily mediated through A2A and A2B receptor activation (A2AAR, A2BAR). Conversely, elevated adenosine levels can activate A1 (A1AR), A2BAR, and A3 (A3AR) receptors, promoting a pro-inflammatory state and dysregulated tissue remodeling that exacerbates chronic lung diseases [10, 21].

The role of purine nucleotides, nucleosides and purinergic signaling in acute and chronic inflammation has been extensively studied, particularly with regard to ATP, ADP and adenosine. In health ATP is found in mammalian cells. In disease, such as inflammation or ischemia, ATP is released from intracellular stores due to cellular necrosis [19, 20, 22, 23]. During apoptosis, pannexin hemichannels control ATP release into the extracellular space, where ATP serves as a phagocyte chemotactic signal [24]. Inflammatory cells, like neutrophils, and endothelial cells can release ATP into the extracellular space through connexin hemichannels [25, 26, 27, 28]. ADP can be released from intracellular platelet granules. ATP signals pass through receptors, initially designated as P2 receptors [25], and then reclassified into P2X receptors (ligand-gated ion channels) and P2Y receptors (G protein-coupled receptors). Mice with deleted P2 receptors are viable and they exhibit protection against inflammatory diseases like asthma, vascular inflammation, and "graft-versus-host disease" [18, 29, 30, 31, 32]. Pharmacological antagonism of P2 receptors has been shown to suppress inflammation in various conditions, including inflammatory bowel diseases, lung inflammation and ischemic reperfusion injury [26, 31, 33].

Adenosine's interaction with P1 receptors, which are G-protein coupled receptors, occurs in the extracellular environment. These receptors are categorized into four subtypes: A1, A2A, A2B, and A3. A1 and A2A receptors are known for their high affinity, while A2B and A3 receptors exhibit significantly lower affinity. All subtypes of adenylate cyclase affect the enzyme's function and the subsequent production of cyclic adenosine monophosphate (cAMP). The A2A and A2B receptors activate the process, while the A1 and A3 receptors inhibit it [2, 10, 11, 12].

The ATP molecule interacts with P2-purinergic receptors on the cell surface. These receptors are divided into two main types: P2X and P2Y. The P2X receptors are ion channels. This means that when ATP binds to the P2X receptor, it opens a channel allowing certain ions to enter the cell. Sodium (Na^+), calcium (Ca^{2+}), and potassium (K^+) ions pass through these channels. Thus, ATP, binding to the P2X receptor, triggers the process of moving these ions inside the cell (the flow of ions depends on the concentration gradient and electrical potential). This process plays an important role in cell signaling and various cellular functions [4, 5]. P2Y receptors are specialized proteins located on the cell's outer membrane and capable of recognizing ATP and ADP, as well as a number of similar substances. Upon binding a substance, the receptor activates a G protein, which then influences enzymes like adenylate cyclase and phospholipase C, and regulates ion channels, ultimately altering ion movement and thereby affecting degree of ion penetration into the cell. These receptors are present on the surfaces of the immune system cells and the endothelium – a layer of cells lining the walls of blood vessels from the inside [14, 15, 16].

Experiments on genetically modified mice lacking P2X and P2Y receptors have demonstrated that their absence does not impair normal development or reaching adulthood in animals. However, studies focusing on mice deficient in the P2X2 and P2X3 receptor (P2X receptor subtype) revealed immune dysregulation, characterized by an increased number of immune cells and enlarged spleen (that is an important part of the body's immune system). This phenotype indicates that P2X2 and P2X3 receptors play a critical role in modulating immune function; their absence may lead to an overly reactive immune response and immune system hypertrophy. These findings are supported by the work of A. Surprenant et al. [6] and E. Kaniewska et al. [7].

Research demonstrates that P2Y receptors play a complex role in regulating cellular activity and immune responses. Although the absence of P2X/P2Y receptors is not lethal, specific loss of the P2X2 and P2X3 subtypes results in pronounced alterations in immune system function [6, 15].

It has been established that ATP can be actively released from intact cells in response to stimuli like mechanical deformation, hypoxia (low oxygen), and acetylcholine, without causing cell damage [7, 11, 12]. For example, ATP release from intact cells was first observed in neurons secreting ATP into the synaptic cleft [13]. However, it has been shown that the underlying mechanism is very complex and includes stretch-activated channels, potential-dependent anion channels, P2X7 receptors, as well as connexin and pannexin hemichannels [14].

Contrasting to intracellular ATP, primarily utilized as energy, extracellular ATP is considered to be a powerful signaling molecule through the nucleotide-selective P2 receptors. Extracellular ATP is rapidly metabolized to adenosine by ectonucleotidases [25]. The ectonucleotidases consist of four family types including ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family, ectonucleoside triphosphate diphosphohydrolase (ENTPDase) family, alkaline phosphatases (AP), and CD73 [15, 16]. Extracellular adenosine, an intermediate metabolite of nucleotides, can undergo three processes: conversion to inosine by adenosine deaminase, reconversion to AMP by adenosine kinase, and cellular reuptake through concentrative nucleoside transporters (CNTs) or equilibrative nucleoside transporters (ENTs) [15, 17, 18].

Purinergic receptors have been widely studied in signaling systems in response to extracellular ATP and related nucleotides. Purinergic receptors consist of three major families based on their structural and biological properties [19]. The G-protein-coupled P2Y receptors recognize ATP and several other nucleotides, including ADP, uridine triphosphate (UTP), uridine diphosphate (UDP), and UDP-glucose [20]. P2X receptors function as ATP-gated ion channels that facilitate the influx and efflux of extracellular cations, including calcium ions, which only respond to ATP [20, 22]. To date, P2Y receptors consist of eight subtypes, a family of P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. P2X receptors have seven subunits that may form six homomeric (P2X1–P2X5 and P2X7R), and at least seven heteromeric (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/5, P2X2/6, and P2X4/6) [21, 22, 23]. The conversion of ATP/ADP to adenosine by ectonucleotidases terminates P2R signaling within the extracellular compartment. Adenosine can signal through four distinct G-protein-coupled receptors (P1 receptors): A1, A2A, A2B, and A3 [24, 25, 26]. The purinergic receptor subtypes are widely distributed throughout the immune cells and the central nervous system (CNS) [25, 27, 28].

Extracellular ATP and ADP are rapidly converted to AMP, which is then further metabolized to adenosine [30].

Adenosine can signal through four distinct G-protein-coupled receptors: A1AR, A2AAR, A2BAR, and A3AR [1, 34]. The subtypes of adenosine receptors are expressed differently in each target cell. A2AAR is largely expressed in immune cells such as neutrophils [35, 36] and lymphocytes, while A2BAR is largely expressed in vascular endothelial cells [37, 38]. Adenosine receptor knockout mice are viable, and no human pathologies have been attributed to mutations and defects of adenosine receptors. However, adenosine receptors, beyond their basic physiological roles, have been extensively studied in the context of various diseases and pathological conditions. For example, adenosine's chronotropic effects via A1AR is essential in the treatment of supraventricular tachycardia. A2AAR serves anti-inflammatory functions in neutrophils, diminishing inflammatory cell activation at various sites [19, 39, 40, 41]. A2AAR antagonists exert benefits in Parkinson's disease. A2BAR contribute to tissue adaptation in response to inflammation, ischemia, and hypoxia [42, 43, 44]. A3AR functions in aqueous humor production in the eye [45], and agonism of A3AR has proven effective in the treatment of dry eye [46].

Extracellular adenosine can be transported into the cell via concentrative or equilibrative nucleoside transporters known as CNTs and ENTs. Diffusion-limited, these channels allow adenosine to diffuse freely across the cellular membrane, following its concentration gradient [21]. Adenosine movement into intracellular space diminishes adenosine signaling [45]. Adenosine signaling can also be terminated by deamination of extracellular adenosine to inosine by cell surface CD26-conjugated adenosine deaminase (ADA) or via phosphorylation back into AMP via adenosine kinase [22]. Genetic deficiency of ENTs is not lethal. ENT-deficient mice exhibit elevated adenosine levels that provide protection during disease states like organ ischemia [41]. Pharmacologic blockade of ENT with dipyridamole, resulting in accumulation of extracellular adenosine causing coronary artery vasodilation, is employed in stress echocardiography to identify coronary atherosclerotic lesions. ENT antagonism is also used to inhibit platelet aggregation and prevent recurrence of stroke and to preserve the patency of hemodialysis grafts. ADA-deficient mice exhibit elevated extracellular adenosine levels, which result in severe pulmonary inflammation and fibrosis. In human, a defect in the ADA gene causes severe combined immunodeficiency (SCID) resulting from metabolites of adenosine exerting cytotoxic effects on lymphocytes. ADA-deficient associated SCID has been successfully treated with ADA gene therapy [39]. The anti-inflammatory effects of cyclosporine may be partially due to inhibition of adenosine kinase, resulting in elevated adenosine levels [40].

Extracellular nucleotides, which activate purinergic receptors, are regulated by enzymes like CD39 and ENPP. CD39 breaks down ATP to AMP and phosphates, influencing hydroxyapatite formation

(calcification), while the inorganic pyrophosphate (PPi) formed by ENPP can inhibit calcification. However, the activity of tissue alkaline phosphatase can convert PPi to phosphate, which initiates calcification [44, 47].

Experiments show that the purinergic signaling system, including CD39 and CD73, is actively involved in aortic valve calcification. In particular, studies on porcine aortic valves have shown high expression of CD39 and CD73 in both endothelial and interstitial cells. Adding extracellular nucleotides to cell cultures reveals distinct activity levels for CD39 and CD73, suggesting their different roles in processes like calcification.

In vitro experiments using vascular smooth muscle cells have demonstrated that *β*-glycerophosphate and uridine adenosine tetraphosphate (Up4A) promote calcification by activating P2X and P2Y receptors. Conversely, inhibition of specific purinergic receptors, such as P2Y, has been shown to reduce valve calcification, highlighting their potential as therapeutic targets.

It has been shown that P2Y2 receptor, activated in various tissues by stress or damage, promotes tissue regeneration by activating multiple signaling pathways. Many studies show that ATP and P2Y2 receptor-mediated signaling influence diverse biological processes, including chemotactic signal production and immune cell activation, thereby promoting migration, proliferation, differentiation, and inflammatory mediator release [48].

Adenosine triphosphate has also been implicated to induce chemotaxis of neutrophils via actin polymerization and direct cell orientation by feedback signaling involving P2Y2R [49]. The subsequent P2Y2 receptor activation will amplify gradient sensing of chemotactic signals (e.g., N-formyl peptides and IL-8) by stimulating F-actin to the leading edge. Chemotaxis of neutrophils to sites of infection is critical for immune defense and for the physiological downregulation of neutrophil-driven inflammation [50].

Therefore, the ATP-P2Y2 receptor signaling system exerts a dual effect. It protects the host from infection, promotes the repair of damaged tissues, and enhances pulmonary clearance of harmful substances. However, if dysregulated, this same healing response can drive chronic inflammation and pathological fibrosis [19, 34].

The P2Y6 receptor also plays an ambivalent role in inflammatory diseases. The receptor is crucial for innate immune responses against bacterial infection any studies show that P2Y6 receptor activation is involved in the release of chemokines from immune cells, such as monocytes, dendritic cells, eosinophils, and recruiting monocytes/macrophages during inflammation or infection [48].

In neurodegenerative diseases, microglia are engaged in the clearance of dead cells or dangerous debris, which is crucial for the maintenance of brain functions. Extracellular ATP regulates microglial motility dynamics in the intact brain, and its release from the damaged tissues mediates a rapid microglial response toward injury [43]. Moreover, UTP and UDP released from injured neurons have been shown to enhance microglial phagocytic capacity for dying cells via activation of P2Y6 receptor, serving as an "eat-me" signal for microglia. This signal is considered to be an important initiator of the clearance of dying cells or debris in the CNS [48].

When the endothelium or epithelium is inflamed, the signaling pathway mediated by the P2Y6 receptor exert a negative effect. Idiopathic inflammatory bowel diseases include, in particular, Crohn's disease and ulcerative colitis. These chronic conditions arise from a dysregulated inflammatory response to intestinal microbiota in genetically susceptible individuals. Experimental studies of colitis have shown an increase in the expression of both P2Y2 and P2Y6 receptors in intestinal epithelial cells [41, 45, 46].

Similarly, P2Y6 receptor plays an important role in acute and chronic allergic airway inflammation, and selective blocking of P2Y6 receptor or P2Y6 receptor deficiency in structural cells reduces symptoms of experimental asthma. Recently, P2Y6 receptors have not only been found to be up-regulated in murine atherosclerotic plaques, but also to play a key role in inflammatory diseases.

Thus, P2Y6 receptor activation plays a role in innate immunity against infection whereas P2Y6 receptor over-activation can result in harmful immune responses and chronic inflammation [48].

P2X7 receptor is highly expressed in immune cells, in particular, mast cells, macrophages, microglia cells and dendritic cells. The best-investigated and most widely accepted P2X7 functions are it role in inflammation and immune signaling. The P2X7 receptor plays a central role in the immune system's response to bacterial and parasitic infections by acting as a crucial link in the signaling pathways involved in inflammation and pathogen control. It has been shown to be involved in the killing of intracellular pathogens such as *Mycobacterium tuberculosis, Chlamydia trachomatis,* and *Leishmania amazonensis*, either by killing the microorganism or by inducing apoptosis of infected macrophages [19]. In addition, this receptor is involved in the development of fever by stimulating the production of prostaglandin E2 (PGE2) and IL-1*β* [20].

The P2X7 receptor is widely recognized to mediate the pro-inflammatory effects of extracellular ATP. However, as a recent study has shown, this receptor can function as one of the scavenger receptors involved in the recognition and removal of apoptotic cells in the absence of extracellular ATP and the P2X7 receptor a promising target for the development of new drugs [30, 44].

In the CNS, P2X7 receptor activation promotes neuroinflammation by causing the release of proinflammatory cytokines, such as IL-1β and TNF-α as well as activation of mitogen-activated protein kinases and nuclear factor kappa-light-chain-enhancer of activated B cells, resulting in upregulation of proinflammatory gene products, including cyclooxygenase-2 and the P2Y2 receptor. In P2X7 receptor knock-out mice, amyloid β (Aβ) triggered increase of intracellular Ca^{2+}, ATP release, IL-1β secretion, and plasma membrane permeabilization in microglia [25]. In fact, *in vivo* inhibition of P2X7 receptor in mice transgenic for mutant human amyloid precursor protein indicated a significant decrease of the number of hippocampal amyloid plaques [48]. Thus, the identification of extracellular ATP and P2X7 receptor as key factors in Aβ-dependent microglia activation unveils a non-conventional mechanism in neuroinflammation and suggests new possible pharmacological targets.

Extracellular ATP and P2X7 receptor signaling also contributes to the development of smoking-induced lung inflammation and emphysema. P2X7 receptor knock-out mice exhibit decreased inflammatory responses, including a reduction in pulmonary fibrosis in a mouse model of lung inflammation. Inhibition of this receptor may be a new possible therapeutic target for the treatment of chronic obstructive pulmonary disease [29, 30].

The purinergic P2X7 receptor is associated with activation and release of IL-1 and IL-18, which is strongly implicated in the multiple inflammatory pathways involved in the pathogenesis of rheumatoid arthritis (RA). P2X7 receptor has also been shown to be expressed by synoviocytes from RA joints and contributes to modulation of IL-6 release. P2X7 receptor activation also plays a novel and direct role in tissue damage through release of cathepsins in joint diseases. Although, AZD9056, a P2X7 receptor antagonist, has been shown to reduce articular inflammation and erosive progression [38], clinical trials with the P2X7 receptor antagonist in patients with RA failed to inhibit disease progression [19, 39]. Similarly, the effect and safety of AZD9056 in Crohn's disease is still under clinical trial.

Taken together, P2X7 receptor signaling not only plays a critical role in mediating appropriate inflammatory and immunological responses against invading pathogens, but also contributes to a wide range of chronic inflammatory diseases when activated inappropriately.

Studies have also shown that pathological dysregulation of purinergic signaling can lead to serious diseases such as autoimmune disorders, cardiovascular problems, and cancers [51]. Elucidating the molecular mechanisms underlying this dysregulation will enable the development of novel diagnostic and therapeutic strategies. Consequently, further research into the purinergic regulation of inflammation is paramount, as it opens avenues for innovative treatments aimed at improving patient quality of life and reducing the population-level disease burden.

In addition, it was found that adenosine, formed as a result of ATP metabolism, has an anti-inflammatory effect, suppressing the activity of immune cells and reducing the severity of the inflammatory process. Adenosine signalling has long been a target for drug development.

Thus, the study of the mechanisms of purinergic regulation of inflammation is an important aspect of modern medicine and biology. It has been established that ATP and ADP play a significant role in activating immune cells and initiating inflammatory responses. These nucleotides activate P2X and P2Y receptors, which initiates a series of signaling pathways that lead to the release of cytokines and chemokines.

Cytokines are a diverse group of small signaling proteins that produced by a wide variety of cells, including immune cells, endothelial cells, and fibroblasts. They perform multiple functions, including attracting and activating immune cells, stimulating cell proliferation and differentiation, and inducing the synthesis of other inflammatory mediators.

It is important to note that the purinergic system closely interacts with the cytokine network. For example, ATP and ADP can stimulate the production of cytokines such as IL-1β, TNF-α as well as interferon-γ through activation of the corresponding receptors. In their turn, cytokines can modulate the expression of purinergic receptors and influence the metabolism of ATP and adenosine. Thus, the interaction between these two systems is a key factor in determining the nature and intensity of the inflammatory response. Cytokines are key players in orchestrating cellular interactions during inflammation, both promoting and suppressing the cooperation between different cell types [4]. Acting as a chemoattractant, ATP stimulates phagocytes to produce reactive oxygen species and macrophages to produce pro-inflammatory cytokines [1, 2, 3, 4].

Pro-inflammatory cytokines are small protein molecules produced by cells of the body's immune system, mainly by macrophages, T cells, and dendritic cells. They are pivotal in initiating and sustaining inflammation, orchestrating interactions among immune components, and stimulating protective responses to infections and tissue damage. These cytokines, acting together in a cascade, aim to eliminate pathogens and repair damaged tissues. Excessive production of pro-inflammatory cytokines can lead to harmful consequences if not properly regulated. This overproduction can damage tissues and contribute to the development of chronic inflammatory diseases, autoimmune disorders, and even sepsis.

Pro-inflammatory cytokines include IL-1, TNF-α, and IL-6. These substances cause local and systemic changes characteristic of acute inflammation

[9, 10]. IL-1 plays a pivotal role in the immune response by orchestrating key defense mechanisms and initiating tissue repair [2]. Following antigen exposure, secretion of mature interleukins begins after approximately 2 hours, peaks between 24 and 48 hours, and subsequently declines rapidly [9, 11].

The chain of inflammatory processes is triggered by the activation of endothelial cells [11, 12, 13, 14, 15]. Cytokines released at the inflammatory site act on the endothelium (the inner blood vessel lining), triggering the recruitment of specific immune cells to form an infiltrate tailored to the stimulus. The appearance and accumulation of leukocytes at an inflammatory site is a process involving changes in the activity of adhesion molecules both on the surface of leukocytes and endothelial cells, as well as variations in the inflammatory substances produced by these cells [16, 17]. Tissue damage triggers a cascade of events leading to acute inflammation, characterized by neutrophil migration from blood vessels [17, 18, 19]. Specific interleukins (IL-1, IL-6, IL-8, IL-12) activate endothelial cells to produce adhesion molecules (E-selectin, P-selectin, ICAM1, VCAM1), these adhesion molecules then facilitate the movement and passage of white blood cells (leukocytes) from the bloodstream into inflamed tissues, a process essential for immune response and tissue repair [11, 18, 19, 20]. This process is further enhanced by endothelial cell contraction and widening of intercellular space. In an inflammatory response, activated endothelial cells are essential by releasing chemokines like MCP1 and IL-8, and cytokines such as IL-1, IL-6, and GM-CSF, which attract and activate immune cells like neutrophils and monocytes to the site of injury [18]. Endothelial cell stimulation also leads to the expression of phospholipids on their surface [11, 12, 13].

Cytokines IL-1, IL-6, GM-CSF, and TNF-*α* increase circulating neutrophil counts by stimulating their production in the bone marrow, promoting their release, and enhancing their survival [21, 22, 23].

Neutrophils are the initial responders to injury and are later replaced by monocyte-derived macrophages, a process driven by chemokines secreted by the neutrophils themselves that attract these subsequent immune cells to the site of inflammation [21, 23]. Neutrophils play a crucial role in the inflammatory process by migrating from the bone marrow into the bloodstream, adhering to blood vessel walls, and ultimately penetrating the tissue to reach the site of inflammation. Once at the site, they form a leukocyte "shaft", engage in phagocytosis, release destructive lysosomal enzymes, and ultimately undergo self-destruction [23]. Chemokines, secreted by neutrophils, act as signaling molecules that trigger the release of neutrophils from the bloodstream and guide their migration towards the site of infection. IL-8 plays an important role in attracting white blood cells [21, 24, 25].

The first effect of IL-8 on neutrophils is to trigger their activation, specifically by activating their contractile cytoskeleton and causing the formation of broad cytoplasmic extensions. These changes are noticeable after 20–30 seconds and they reach their highest intensity after about 1.52 minutes [26]. Neutrophils use constitutively expressed L-selectin on their membrane to initiate rolling and slow down within capillaries. IL-8, released in the affected area, binds to receptors on neutrophils, prompting them to rearrange their surface molecules to better adhere to the blood vessel lining (endothelium) and to activate expression of integrins LFA-1 and Integrin-1 on the surface of macrophages (Mac1). The interaction of specific adhesive molecules on neutrophils and endothelial cells causes rolling neutrophils to stop their movement, allowing them to penetrate the tissue and migrate towards a chemoattractant source [27]. IL-8 stimulates the release of neutrophils from postcapillary venules [2], leading to their migration into acute inflamed areas [28]. IL-8 also increases calcium levels within neutrophils, prompting their migration and activating the pentose phosphate pathway, which, in turn, boosts the production of reactive oxygen species, which are involved in fighting off pathogens. Furthermore, IL-8 causes the release of enzymes from neutrophils through degranulation. IL-8 production is increased by the paracrine action of TNF-*α* and IL-1 on nearby macrophages [2, 9, 10].

Anti-inflammatory cytokines are small signaling molecules secreted by immunocompetent cells, including regulatory T cells, macrophages and others, to limit inflammation and prevent excessive immune responses. By doing so, they help restore immunological homeostasis and mitigate the risk of damage to healthy tissues [49, 50, 52].

Anti-inflammatory cytokines are vital components of the immunoregulatory system, preventing excessive immune responses (hyperinflammation) and mitigating damage from chronic inflammation. For instance, transforming growth factor *β* (TGF-*β*) and IL-10 are key cytokines that protect the gastrointestinal tract's mucous membranes from the aggressive effects of the intestinal microflora [40, 53].

An imbalance between pro-inflammatory and anti-inflammatory cytokines can disrupt the body's ability to regulate the immune response, leading to a range of diseases, such as chronic inflammation, autoimmune diseases, and allergies.

Some anti-inflammatory cytokines exert an inhibitory effect on neutrophils, significantly suppressing their production of pro-inflammatory cytokines [54]. TGF-*β* further inhibits inflammation by preventing leukocyte adhesion to the endothelium and reducing the secretion of superoxide radicals and monokines (IL-1, IL-6, TNF-*α*) [2, 4, 18, 21, 29]. IL-10 and TGF-*β* suppress both monocyte and neutrophil-mediated inflammation by blocking the transcription of genes responsible for producing inflammatory

cytokines in neutrophils [18]. IL-6 inhibits the synthesis of IL-1 and TNF-*α* [2, 4, 18, 21]. Furthermore, it stimulates the production of the IL-1 receptor antagonist and promotes neutrophil apoptosis. Thus, IL-6 acts as a negative regulator in the complex network of cytokines, it helps to reduce inflammation by controlling neutrophil activity and shaping their functional phenotype during the resolution of an inflammatory response [4, 30]. Furthermore, neutrophils can modify their response to monokines by altering their receptor expression, specifically, they can reset their receptors into a state of increased activity, which potentially alters their susceptibility to monokine signaling. Additionally, detached neutrophil receptors act as "traps", reducing the availability of cytokines and thus diminishing their effect on other cells.

Neutrophil recruitment is a tightly controlled process essential for shifting inflammation from its acute phase to the resolution and repair phase carried out by monocytes and macrophages. Excessive neutrophil activity, however, can be harmful, leading to impaired function and undesirable immune reactions [4]. In mammals, the leukocytic stage of inflammation – spanning from the initial insult to the peak of neutrophil death in the affected tissue – typically lasts 12 to 24 hours [23]. Cytokines thus play a central role in orchestrating an effective inflammatory response. They ensure balance through bidirectional regulation – both enhancing and suppressing signals – and by determining the precise sequence of the process' stages.

The interaction between purinergic signaling and the cytokine network is a critical regulatory mechanism for inflammation. For instance, ATP activation of P2 receptors stimulates pro-inflammatory cytokine release. These cytokines then elevate ATP levels, further activating purinergic signaling and creating a self-amplifying, pro-inflammatory cycle that can contribute to various diseases.

Conversely, adenosine counteracts this by activating P1 receptors, which suppresses pro-inflammatory cytokines and promotes anti-inflammatory ones [43, 55].

CONCLUSION

The investigation of purinergic signaling and cytokine networks is a rapidly growing field in modern medicine and biology. A review of extensive literature reveals a remarkable diversity of molecular mechanisms regulating inflammation, confirming the pivotal role of purinergic receptors and cytokines in both maintaining homeostasis and driving pathology.

A key aspect of this regulation is the crosstalk between purinergic signaling and cytokines, which fine-tunes macrophage and T cell activity. This interaction determines outcomes in infectious diseases, autoimmunity, and cancer. Critically, an imbalance between pro-inflammatory and anti-inflammatory signals can lead to chronic inflammation, a key precursor to serious conditions like cardiovascular disease, diabetes, arthritis, and cancer.

The data obtained indicate that drugs modulating purinergic receptors and cytokine levels could effectively treat inflammatory diseases of various origins. For instance, compounds stimulating the synthesis of anti-inflammatory metabolites like adenosine may suppress excessive immune activation and mitigate tissue damage in autoimmune pathologies. Conversely, blocking receptors for pro-inflammatory agents can help prevent both acute and chronic inflammation.

Consequently, elucidating the interactions between purinergic systems and cytokine networks is vital for creating innovative medicinal products to treat immune regulatory disorders and mitigate the complications of excessive inflammation. Progress in this field promises to enhance quality of life and reduce the societal burden of common diseases driven by pronounced inflammatory processes.

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Validation and application of qPCR test kit for detection of *Mycoplasma dispar* DNA

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ABSTRACT

Introduction. Currently, *Mycoplasma dispar* is widely spread and circulating in livestock farms around the world, including in the Russian Federation. The implementation of a real-time polymerase chain reaction test kit for detecting *Mycoplasma dispar* DNA in veterinary practice is highly relevant, as this pathogen can cause respiratory diseases in cattle and contribute to calf mortality, leading to significant economic losses in livestock production.

Objective. To introduce a newly developed real-time polymerase chain reaction test kit *Mycoplasma dispar* DNA detection kit into veterinary practice and determine its major validation parameters.

Materials and methods. *Mycoplasma dispar* reference strain (ATCC No. 27140) was cultured in 1699 Revised Mycoplasma Medium recommended by the American Type Culture Collection. DNA was extracted using a commercial kit, real-time polymerase chain reaction was performed using pre-selected parameters. The major validation parameters of the test kit were determined: analytical sensitivity, analytical specificity, amplification efficiency, repeatability and reproducibility. Applicability of real-time polymerase chain reaction test kit for detection of *Mycoplasma dispar* DNA was demonstrated.

Results. The *Mycoplasma dispar* DNA detection test kit demonstrated an analytical sensitivity (detection limit) of 10 copies/μL (100 copies/reaction), 100% specificity (exclusive to *Mycoplasma dispar* DNA), 99.01% amplification efficiency, and an average repeatability coefficient of variation of 0.91%. Reproducibility coefficient of variation ranged from 0.66% to 1.26% across 5 replicates and was 0.91% across 15 replicates. The test kit was validated using 228 biological samples from cattle from 13 regions of the Russian Federation, while *Mycoplasma dispar* DNA was detected in 39.47% of the samples tested.

Conclusion. The developed *Mycoplasma dispar* DNA test kit has demonstrated high validation performance and is suitable for diagnosing bovine mycoplasmosis.

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Валидация и применение тест-системы на основе метода ПЦР в режиме реального времени для выявления ДНК *Mycoplasma dispar*

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РЕЗЮМЕ

Введение. В настоящее время наблюдается широкое распространение и активная циркуляция *Mycoplasma dispar* в животноводческих хозяйствах по всему миру, в том числе и в Российской Федерации. Актуальность внедрения в ветеринарную практику тест-системы на основе полимеразной цепной реакции в режиме реального времени для выявления ДНК *Mycoplasma dispar* обусловлена важной ролью данного возбудителя в развитии комплекса респираторных болезней крупного рогатого скота, приводящих к значительным экономическим потерям в животноводстве, в том числе являющихся причиной падежа телят.

Цель исследования. Определение основных валидационных характеристик и внедрение в ветеринарную практику разработанной тест-системы для выявления ДНК *Mycoplasma dispar* методом полимеразной цепной реакции в режиме реального времени.

Материалы и методы. Референтный штамм *Mycoplasma dispar* (АТСС № 27140) культивировали на питательной среде 1699 Revised Mycoplasma Medium, рекомендованной American Type Culture Collection. ДНК выделяли с использованием коммерческого набора, постановка ПЦР в режиме реального времени осуществлялась по заранее подобранным параметрам. Были определены основные валидационные характеристики тест-системы: аналитическая чувствительность, аналитическая специфичность, эффективность амплификации, повторяемость и воспроизводимость. Показана возможность практического применения тест-системы на основе ПЦР в режиме реального времени для выявления ДНК *Mycoplasma dispar.*

Результаты. Аналитическая чувствительность (предел обнаружения) тест-системы по выявлению ДНК *Mycoplasma dispar* составила 10 копий ДНК/мкл (или 100 копий ДНК/реакцию), специфичность – 100% (детектирует только ДНК *Mycoplasma dispar*), эффективность амплификации – 99,01%, среднее

значение коэффициента вариации при оценке повторяемости – 0,91%, воспроизводимости – 0,66–1,26% в рамках 5 повторений и 0,91% в рамках 15 повторений. Апробацию тест-системы проводили на 228 пробах биоматериала, отобранных от крупного рогатого скота из 13 регионов Российской Федерации, при этом ДНК *Mycoplasma dispar* была обнаружена в 39,47% исследуемых проб.

Заключение. Разработанная тест-система для выявления ДНК *Mycoplasma dispar* продемонстрировала высокие валидационные показатели и может быть использована в диагностике микоплазмоза крупного рогатого скота.

Ключевые слова: *Mycoplasma dispar,* тест-система, ПЦР-РВ, внутренний контрольный образец, чувствительность, специфичность, эффективность амплификации

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INTRODUCTION

Mycoplasma dispar is a globally prevalent pathogen responsible for respiratory diseases in cattle, particularly in calves. *M. dispar* infection typically manifests as mucopurulent inflammation of the upper respiratory tract with frequent lung involvement, resulting in substantial economic losses in cattle production [1, 2, 3].

Mycoplasma dispar, a member of the genus *Mycoplasma* (class *Mollicutes*), lacks a cell wall, is pleomorphic, and has a compact 803 kb genome with a low G + C composition (28.5–29.3 mol%) [4, 5].

Mycoplasma dispar requires a specific medium to enable growth and its colonies do not show – especially during the early passages – the typical "fried-egg" appearance. In addition, *M. dispar* are not always inhibited by hyperimmune serum, making conventional identification difficult in the early stages [5]. *M. dispar* glucose fermentation aligns with its phylogenetic cluster (hominis group), but unlike some mycoplasmas, it lacks arginine hydrolysis. Tetrazolium reduction occurs under both oxygen conditions. The bacterium has no phosphatase activity [6, 7, 8]. *M. dispar* produces an outer capsule consisting of a polysaccharide identified as a polymer of galacturonic acid [9]. The capsule appears to be produced during infection in response to mammalian host cells, and may exert an inhibitory effect on the activity of bovine alveolar macrophages and prevent their activation [10].

It has been reported that mycoplasma can be inhibited by alveolar macrophages if anti-capsular polysaccharide antibodies are present [9].

Mycoplasma dispar was first isolated from the lungs of pneumonic calves in England in 1969. Then these mycoplasmas were reported from Denmark, Belgium, the Netherlands, France, Australia, the USA, Canada, Korea and Japan [8]. In Europe, *M. dispar* infection was reported from the UK, and recently from Brazil and Italy [11, 12, 13, 14, 15]. In the Russian Federation, testing of 1,186 biological samples collected from cattle with respiratory and/or reproductive diseases from 34 different regions during 2015–2018 revealed *M. dispar* DNA in 37.15% of samples by agarose gel electrophoresis of polymerase chain reaction (PCR) products [16].

Mycoplasma dispar is transmitted between animals through respiratory secretions and can be detected in the respiratory tracts of both healthy calves and those with pneumonia [3]. Mycoplasma infections, particularly when combined with other respiratory pathogens under suboptimal conditions, represent a significant threat to animal health and productivity in high-density livestock operations [17, 18]. In the Netherlands, *M. dispar* was isolated from 92% of pneumonic calves and from only 40% of healthy calves [7, 19]. In Denmark *M. dispar* was found in over half of calf lungs showing either fibrino-necrotising or suppurative bronchopneumonia [20]. Other bacterial pathogens, including *Histophilus somni, Pasteurella multocida, Arcanobacterium pyogenes,* and *Mannheimia haemolytica*, were co-detected with mycoplasmas. *M. dispar* is detected frequently from pneumonic calves in the UK, and is believed to be the cause of a severe pleuropneumonia similar to the WOAH (World Organization for Animal Health) listed contagious bovine pleuropneumonia [21].

Few detailed studies have been carried out to establish the exact role of *M. dispar* in bovine respiratory disease (BRD) which is a chronic condition affecting beef cattle in feedlots and dairy calves. It is estimated to cost the USA cattle industry alone over US $4 billion in production losses, treatment and prevention [21].

Calves affected by BRD are usually treated with antimicrobials even though some of these are not effective against mycoplasmas [22]. Consequently, there is a real danger of the emergence of resistance as a result of chronic therapies with multiple antimicrobials, which is already observed for the other BRD bacterial pathogens [23].

The pathogenicity mechanisms have been identified in *M. dispar* and include its ability to produce hydrogen peroxide and biofilm, both well-known virulence factors.

Studies have shown that *M. dispar* is able to colonize the epithelial cells of the respiratory tract exerting cytostatic and even cytopathic effects on bronchial and bronchiole cells, potentially impairing the clearance of bacteria [9, 21, 24]. Furthermore, *M. dispar* has been shown to be immunosuppressive in the host [25]. For these reasons *M. dispar* is included with *M. bovis* amongst the agents that cause or exacerbate BRD [8, 21]. *M. dispar* is causally associated with exudative bronchitis and interstitial pneumonia in calves, with transmission occurring primarily via airborne respiratory droplets and sustained close contact [26].

Mycoplasma dispar causes purple to red consolidation mainly in the cranioventral areas of the lung [27]. *M. dispar* were shown to be capable of causing a mild sub-clinical bronchiolitis with lymphoid cuffing in gnotobiotic calves [10]; occasional cases of mastitis were also reported [28]. Other authors report that *M. dispar* is frequently related to alveolitis, in which neutrophils, macrophages, and edema liquid aggregate in the alveolar wall and spaces [26, 29]. Field cases of subclinical pneumonia from which *M. dispar* was isolated had similar lesions [30].

The genomic information of *M. dispar* in publicly available databases is limited. The genome succession of *M. dispar* reference strain ATCC 27140 was delivered in the GenBank in 2015. Based on the phylogenetic analysis of 16S rRNA gene sequence *M. dispar* was clustered with *M. ovipneumoniae, M. flocculare* and *M. hyopneumoniae,* isolated from sheep and goats [31].

Controlling mycoplasmosis effectively requires a multi-faceted approach that includes minimizing environmental stress, ensuring proper animal husbandry, and maintaining good air circulation within facilities. Measures to prevent infection of calves from adult animals are required [8].

Timely diagnosis is one of the key components in bovine mycoplasmosis control. Laboratory confirmation of *M. dispar* infection is clinically important, as this globally distributed pathogen significantly impacts livestock health and production [18, 32, 33, 34].

Conventional identifying bovine mycoplasmas through isolation on nutrient media is a crucial first step, as it allows for detailed examination of their cultural, morphological, and biological characteristics. This method also enables the establishment of a clinical isolate bank, which can support future development of improved prevention, control, and eradication strategies for mycoplasmosis, as well as facilitate antimicrobial resistance monitoring [8, 18, 33, 34]. However, it should also be noted that this method is laborious and requires 7–10 days for diagnosis [3, 18, 34].

While not widely used, serological tests like radial hemolysis, ELISA [35], and passive hemagglutination have been reported for detecting *M. dispar* antibodies [36, 37]. The authors described that weak antibody presence against *M. dispar* in cattle, despite its surface-lung location, might be due to the relatively low sensitivity of the serological tests used, rather than the animal's immune system failing to mount an adequate response [35].

While PCR has significantly improved mycoplasma detection, it was not until 2004 that a PCR method specific to the *M. dispar* genome was reported [38]. Both specific and universal oligonucleotides were utilized to detect single nucleotide polymorphisms in the 16S rRNA gene sequence. The PCR/DGGE technique (denaturing gradient gel electrophoresis) was also described, which can detect and identify more than 70 different mycoplasmas, including *M. dispar* [39, 40].

Contemporary diagnostics can achieve high efficiency through real-time PCR (qPCR), enabling rapid and precise detection of specific mycoplasma genomic loci in biological samples [18, 32, 34, 41].

The use of fluorescently labeled TaqMan probes that bind to a specific sequence within the amplified DNA region (the interprimer segment) significantly enhances the specificity of PCR-based assays. Real-time PCR, unlike traditional PCR, doesn't require post-amplification handling of the sample, reducing the risk of contamination and leading to faster, more efficient analysis.

The incorporation of an internal control sample (ICS) in commercial qPCR assays ensures result reliability by detecting PCR inhibition, thereby preventing false-negative interpretations [42]. An important need in qPCR test kit development is incorporating ICSs to monitor both nucleic acid extraction and the subsequent amplification process [43, 44, 45, 46].

It is also worth noting that, in accordance with the WOAH requirements, it is recommended to include ICS into each PCR test for quality control [47].

Tools for the molecular identification of mycoplasma genomes, including of *M. dispar*, enable not only to monitor the animal disease situation in domestic farms, but also to control both the import of animals and their use for production purposes [34, 48].

Currently, there are no commercially available domestic qPCR test kits to detect *M. dispar* DNA in the Russian Federation, and therefore the development of a specific qPCR diagnostic test kit and its introduction into veterinary practice is an urgent task [34].

The purpose of this work was to determine the main validation characteristics of the qPCR test kit for detection of *M. dispar* DNA developed by the Federal Centre for Animal Health, and to introduce it into veterinary practice.

MATERIALS AND METHODS

Bacteria and viruses. The *M. dispar* reference strain (ATCC No. 27140), delivered from the collection of strains of microorganisms of the Federal Centre for Animal Health, and biological samples collected from cattle of various age groups (stabilized blood; nasal and tracheal swabs; pieces of lungs, trachea and lymph nodes; pleural fluid) were used.

To assess the analytical specificity of the developed test kit, the following bacterial strains were used: *M. bovis* ATCC No. 25523, *M. bovigenitalium* ATCC No. 19852, *M. bovis* Kaluga 2020, *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) Madugri-8 DNA (Federal Research Center of Virology and Microbiology, Russia), *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) "T1/44/ARRIAH", and other bacterial and viral agents responsible for analogous diseases in cattle: *Escherichia coli* "EC-21", *Mannheimia haemolytica* "No. 1412", *Pasteurella multocida* " No. 1414", *M. bovigenitalium* isolate, *M. dispar* isolate, *M. bovis* isolate, bovine parainfluenza 3 virus "VGNKI-4", bovine respiratory syncytial virus "Vologda/2020", bovine viral diarrhea virus "NADL-ARRIAH".

Mycoplasma dispar culture. The *M. dispar* reference strain (ATCC No. 27140) was cultured on the 1699 Revised Mycoplasma Medium nutrient medium recommended by the American Type Culture Collection, which contained 7.5 g of brain

Table 1
Threshold cycle (Ct) values of optimized real-time polymerase chain reaction temperature-time profile for *M. dispar* DNA detection ($n = 3$)

Stage	Temperature	Duration	Number of cycles	Mean value Ct ± SD
Profile 1				
Heating of the reaction mixture	95 °C	5 minutes	1	25.46 ± 0.37
Denaturation	95 °C	15 seconds	40	
Primer annealing and elongation	60 °C	60 seconds (fluorescence measurement of Green/FAM, Red/Cy5)		
Profile 2				
Heating of the reaction mixture	95 °C	5 minutes	1	26.30 ± 0.86
Denaturation	95 °C	10 seconds	40	
Primer annealing	60 °C	20 seconds (fluorescence measurement of Green/FAM, Red/Cy5)		
Elongation	72 °C	20 seconds		
Profile 3				
Heating of the reaction mixture	95 °C	5 minutes	1	28.87 ± 0.15
Denaturation	95 °C	10 seconds	45	
Primer annealing	58 °C	20 seconds (fluorescence measurement of Green/FAM, Red/Cy5)		
Elongation	72 °C	20 seconds		

heart infusion broth; 40.0 µL of 10× Hank's Balanced Salts Solution; 10.0 µL of 0.25% phenol red solution; 200.0 µL of heat inactivated porcine serum; 100.0 µL of 5% lactalbumin hydrolysate in 1× phosphate buffer solution; 20.0 µL of yeast extract and 660.0 µL of distilled water. The bacteria were cultured in an incubator at (37 ± 0.5) °C and 5% CO_2 for 5 days. After incubation, turbidity in test tubes and discoloration of the medium (yellowing) were observed, *M. dispar*-like colonies formed on a solid nutrient medium; *M. dispar* biological activity was determined by colony-forming unit counting (CFU/µL) [49, 50].

DNA was extracted using a commercial Ampli Prime Ribosorb kit (Central Research Institute of Epidemiology, Rospotrebnadzor, Russia) in accordance with the manufacturer's instruction.

Real-time PCR protocol. The reaction mixture for amplification per one reaction contained the following components: PCR-buffer-B for Taq DNA polymerase 10× (Syntol, Russia); 5 U/µL of SynTaq DNA polymerase with enzyme-inhibiting antibodies (Syntol, Russia); 25 mM of aqueous solution of magnesium chloride $MgCl_2$ (Syntol, Russia); 100 mM of aqueous solutions of four deoxynucleoside triphosphates (dNTP): dATP, dGTP, dTTP, dCTP (a common mixture of dNTP is prepared and diluted with nuclease-free water to a concentration of 10 mM of each dNTP; Fermentas, Lithuania); direct primer (100 pmol/µL), reverse primer (100 pmol/µL) and a TaqMan probe (100 pmol/µL) to detect a region of the gene encoding *M. dispar*16S rRNA (Syntol, Russia); direct primer (100 pmol/µL), reverse primer (100 pmol/µL) and TaqMan probe (100 pmol/µL) for detection of artificially synthesized ICS (Syntol, Russia) [51]. The resulting volume of the reaction mixture was adjusted to 15 µL with deionized, nuclease-free water (Eurogen, Russia). After that, 15 µL of the reaction mixture and 10 µL of the DNA matrix of the tested samples were added to the prepared tubes. Amplification was performed in a Rotor-Gene real-time PCR cycler (QIAGEN, Germany).

A plasmid construct containing the oligonucleotide sequence of the genome region (with an initial concentration of 2×10^7 copies/µL) and *M. dispar* genome specific region (target fragment) was used as a positive control sample.

Deionized, nuclease-free water (Eurogen, Russia) was used as a negative control sample (NCS) and as a negative PCR control.

Validation. The validation parameters of the test kit were determined according to the recommended method by S. A. Bustin et al. guidelines for publication of developed quantitative PCR protocols [52].

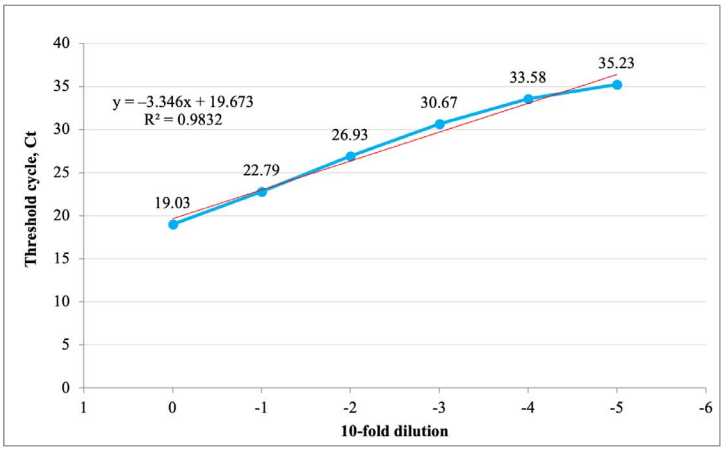

Fig. 1. Linear correlation of real-time polymerase chain reaction results for 10-fold dilutions of the M. dispar genome DNA

Table 2
Specificity assessment of the developed real-time polymerase chain reaction kit for *M. dispar* DNA detection (*n* = 3)

Genetic material	Strain	qPCR result, Green/FAM (*M. dispar* DNA)	qPCR result, Red/Cy5 (ICS DNA)
Escherichia coli	"EC-21"	neg.	+
Mannheimia haemolytica	"No. 1412"	neg.	+
Pasteurella multocida	"No. 1414"	neg.	+
Mycoplasma bovigenitalium	ATCC No. 19852	neg.	+
Mycoplasma bovigenitalium	isolate	neg.	+
Mycoplasma dispar	ATCC No. 27140	pos.	+
Mycoplasma dispar	isolate	pos.	+
Mycoplasma bovis	ATCC No. 25523	neg.	+
Mycoplasma bovis	"Kaluga 2020"	neg.	+
Mycoplasma bovis	isolate	neg.	+
Mycoplasma mycoides subsp. *mycoides* SC (MmmSC)	"T1/44/ARRIAH"	neg.	+
Mycoplasma mycoides subsp. *mycoides* SC (MmmSC)	Madugri-8	neg.	+
Bovine parainfluenza virus-3	"VGNKI-4"	neg.	+
Bovine respiratory syncytial virus	"Vologda/2020"	neg.	+
Bovine viral diarrhea virus	"NADL-ARRIAH"	neg.	+
Nuclease-free water	–	neg.	+

neg. – *M. dispar* DNA was not detected; pos. – *M. dispar* DNA was detected; "+" – ICS DNA was detected.

Table 3
Variability of real-time polymerase chain reaction Ct values for *M. dispar* (*n* = 15)

Run	Replicate	Ct value	Mean Ct value	Standard deviation (SD)	Coefficient of variation (Cv, %)
I	1	27.09	26.74	0.22	0.82
	2	26.76			
	3	26.51			
	4	26.68			
	5	26.64			
II	1	27.41	27.36	0.18	0.66
	2	27.50			
	3	27.54			
	4	27.13			
	5	27.20			
III	1	26.58	27.00	0.34	1.26
	2	26.93			
	3	27.32			
	4	27.38			
	5	26.77			
		Total	27.03	0.25	0.91

To assess the specificity of the developed qPCR, biological samples containing *Mycoplasma* DNA and viral and bacterial nucleic acids which can cause similar diseases in cattle were tested. The test kit sensitivity was assessed using known positive biological samples containing *M. dispar* DNA.

The limit of *M. dispar* DNA detection (analytical sensitivity) of the developed test kit was assessed using a positive control sample containing *M. dispar* DNA with an initial concentration of 2×10^7 copies/μL, each dilution was tested in 5 replicates.

PCR amplification efficiency was assessed using serial 10-fold dilutions of a positive sample of biological material containing *M. dispar* DNA in 3 replicates and calculated according to the formula:

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

where slope is standard curve (plot of Ct vs. $\log_{10}$ input template concentration).

The data were statistically analyzed using Microsoft Excel, including calculations of mean values with standard deviations (± SD), regression analysis, and coefficients of variation. The coefficient of variation (CV) for reproducibility and repeatability should not exceed 10%.

Intermediate precision (reproducibility) was assessed by testing the same sample in 5 replicates in 3 independent qPCR runs (*n* = 15 total replicates).

RESULTS AND DISCUSSION

The Federal Centre for Animal Health developed and validated Russia's first qPCR test kit for *Mycoplasma dispar* DNA detection, incorporating an ICS.

The use of ICS improve PCR reliability and accuracy, particularly by identifying reaction inhibition. The Laboratory Technology Committee of the American Association of Veterinary Laboratory Diagnosticians (AAVLD) has recommended to its membership that all new molecular assays being validated and implemented include an inhibition monitoring strategy based on internal validation for the host, target species, and sample matrix combination being tested [53].

The initial validation step involved identifying a temperature profile that optimizes both the sensitivity and amplification efficiency of the test kit.

The qPCR temperature-time profile was optimized using pre-established reaction component concentrations to achieve consistent threshold cycle (Ct) values. The results are given in Table 1.

The following qPCR temperature-time profile was considered optimal for *M. dispar* DNA detection: 5 minutes at 95 °C (heating of the reaction mixture), followed by 40 PCR cycles consisting of DNA denaturation for 15 seconds at 95 °C, primer annealing and cDNA elongation for 60 seconds at 60 °C.

Validation testing confirmed that the developed kit eliminates false-positive results when challenged with DNA from bovine respiratory disease-associated bacteria. It was shown that the test kit has 100% analytical specificity for *M. dispar* (Table 2), which is higher than demonstrated by J. B. W. J. Cornelissen et al., in which the specificity was 98.2% [54]. The oligonucleotides were verified using the NCBI BLAST database, confirming no significant homology with other *Mycoplasma* species and demonstrating specificity for the *M. dispar* genome.

The amplification efficiency was evaluated using serial 10-fold dilutions of a positive sample containing *M. dispar* DNA. Based on the obtained Ct values for each dilution, the amplification efficiency (E) was 99.01%, which was more significant than the 97.49% efficiency demonstrated by J. B. W. J. Cornelissen et al. during the development of a single PCR [54]. Linear correlation value (R2) was 0.9832 (Fig. 1).

In-laboratory precision under reproducibility conditions was evaluated by examining how consistently measurements are obtained when varying personnel, time, and equipment. The tests were performed using biological material containing and not containing *M. dispar* DNA. Recent studies have shown that the results of *M. dispar* DNA detection using the developed test kit are fully consistent with the expected results and do not depend on time, personnel, or equipment.

Intermediate precision under reproducibility (repeatability) conditions was assessed by testing the same sample in five replicates in three independent qPCR runs ($n = 15$ total replicates).

The threshold cycle (Ct) mean value in three PCR runs ranged from 26.74 to 27.36 with a standard deviation (SD) of 0.18 to 0.34. The coefficient of variation (CV) of 0.91% is well within the acceptable range of 10%. When summing up the results of three qPCR runs, the mean Ct value and the standard deviation was 27.03 and ± 0.25, respectively (Table 3).

At the next stage, the detection limit (analytical sensitivity) was determined for *M. dispar* DNA isolated from serial 10-fold dilutions of an artificially synthesized DNA sequence corresponding to a specific region of the *M. dispar* genome, starting at an initial concentration of 2×10^7 DNA copies/µL. Each dilution was tested in five replicates (Table 4).

It has been established that for the developed qPCR-based test kit, the detection limit of *M. dispar* DNA is 10 DNA copies/µL (100 DNA copies/reaction).

In studies conducted by L. McAuliffe et al., DGGE of the 16S ribosomal DNA PCR product was used to differentiate 32 mycoplasma species. This method is a valuable tool for quickly identifying various *Mycoplasma* species, especially when specific PCR tests are not available [39, 40]. However, artifacts introduced during PCR or the subsequent DGGE analysis lead to skewed results when this method is used for quantitative analysis of *α*-diversity or relative operational taxonomic unit abundance [55].

While some researchers have used conventional PCR to detect *M. dispar* [31, 38, 56], qPCR offers faster and more accurate results without the need for gel electrophoresis [57].

Table 4
Analytical sensitivity (detection limit) of the real-time polymerase chain reaction test kit for *M. dispar* DNA detection

Matrix	*M. dispar* DNA detection, replicate					Matching the expected result, %
	1	2	3	4	5	
2×10^7 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^6 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^5 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^4 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^3 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^2 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
20 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
10 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
5 DNA copies/µL	neg.	pos.	neg.	pos.	neg.	40

pos. – *M. dispar* DNA was detected;
neg. – *M. dispar* DNA was not detected.

Table 5
Requirements for the control results following amplification

Control	Threshold cycle (Ct) value	
	Green/FAM channel	Red/Cy5 channel
Negative PCR control	absent	absent
Negative control sample NCS	absent	≤ 35
M. dispar positive control	≤ 35	≤ 35

Analysis and interpretation of the results. Both fluorescence channels (Green/FAM and Red/Cy5) shared identical qPCR parameters: dynamic background adjustment, slope correction, 10% emission cutoff, linear scale quantification, and a detection limit of 0.05 (Fig. 2). Results are interpreted according to whether fluorescence curves intersect the threshold line, corresponding to the presence or absence of threshold cycles (Ct) in both Green/FAM and Red/Cy5 channels. These data are displayed in the corresponding amplification plots and result tables generated by the thermocycler.

The result of qPCR is considered reliable provided that the correct results are obtained for the negative PCR control,

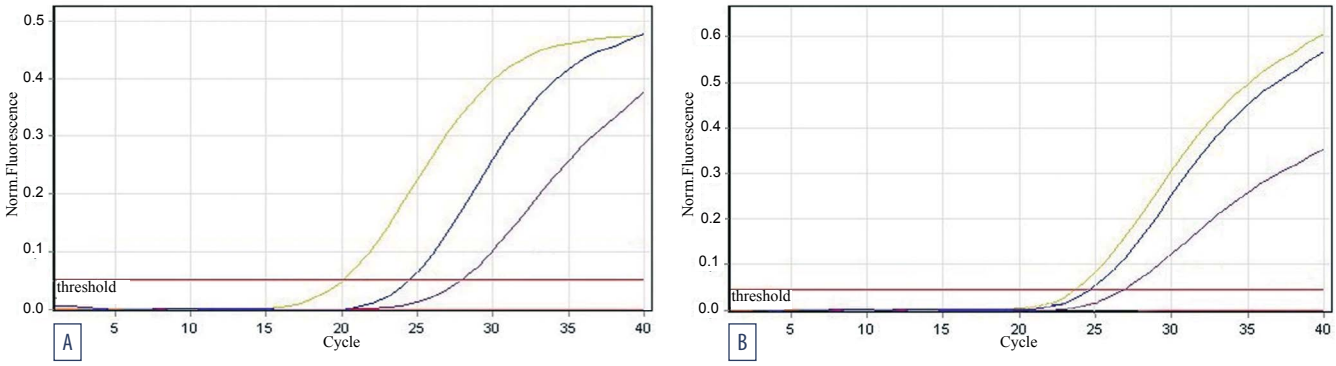

Fig. 2. Fluorescence curves: A – Green/FAM channel (M. dispar DNA); B – Red/Cy5 channel (internal control sample DNA)

the NCS (extraction control) and *M. dispar* positive control. The control requirements are described in Table 5.

The results for the tested samples should be interpreted in accordance with Table 6.

Using the developed test kit, 228 biological samples collected from cattle with respiratory clinical signs were tested, which were received by the Federal Centre for Animal Health, in 2024 from 13 regions of the Russian Federation. The results are given in Table 7.

Table 6
Interpretation of the results for the tested samples

Threshold cycle (Ct) value		Result
Green/FAM channel (*M. dispar* DNA)	Red/Cy5 channel (ICS)	
absent	≤ 35	*M. dispar* DNA not detected
≤ 35	detected or absent	*M. dispar* DNA detected
> 35	≤ 35	inconclusive[1]
absent or > 35	absent or > 35	not reliable[2]

[1] re-test relevant samples, starting from the DNA extraction stage, if the test yields the same results, it can be assumed that *M. dispar* DNA has been detected in the original sample;
[2] re-test the relevant samples, starting from the DNA extraction stage.

Table 7
Identification and detection of *M. dispar* genome in biological samples using the developed test kit in 2024

Region	Swabs (nasal, tracheal)		Pathological samples (stabilized blood, pieces of lungs, trachea and lymph nodes, pleural fluid)	
	Total number of samples	Number of positive samples	Total number of samples	Number of positive samples
Voronezh Oblast	–	–	52	0
Vologda Oblast	–	–	2	0
Vladimir Oblast	12	10	24	4
Moscow Oblast	22	8	2	2
Nizhny Novgorod Oblast	10	0	10	4
Republic of Chuvashia	10	10	–	–
Krasnodar Krai	–		2	0
Republic of Tatarstan	24	24	2	2
Ryazan Oblast	10	0	4	4
Yaroslavl Oblast	–	–	4	0
Republic of Mari El	–	–	2	2
Orenburg Oblast	6	2	8	6
Samara Oblast	16	6	6	6
Total	110	60	118	30

"–" – samples from this region were not received for testing.

It was established that *M. dispar* DNA detection rate in biological samples was 39.47%. The findings demonstrate widespread prevalence of *M. dispar* among cattle populations across Russian farms in 2024.

CONCLUSION

This study established the validation parameters and demonstrated the utility of the "MIC-DISPAR qPCR" kit for detecting *Mycoplasma dispar* DNA via real-time polymerase chain reaction. The assay exhibited high analytical specificity and sensitivity, with excellent repeatability and reproducibility across various test conditions (coefficient of variation range: 0.66–0.91%) The amplification efficiency (E) was 99.01%, and the detection limit was 100 DNA copies/reaction. These validation parameters represent essential criteria for assessing polymerase chain reaction test kit reliability.

Inclusion of an exogenous internal controls example prevents false-negative result interpretation. Using the developed test kit *M. dispar* DNA was detected in 39.47% of the tested samples received from different regions of the Russian Federation by the Federal Centre for Animal Health, in 2024, which indicates the relevance of *M. dispar* diagnostic tests. In this case, the internal controls example serves as an indicator of the extraction stage and the presence of possible inhibitors.

While the test's validation framework could be extended to include related mycoplasmas (e.g., *M. bovirhinis, M. flocculare,* and *M. ovipneumoniae*), current data lack evidence of these pathogens circulating in cattle and small ruminants within the Russian Federation. *M. ovipneumoniae* is the primary cause of mycoplasmal pneumonia in sheep and goats, and the risk of its transmission to cattle is minimal [58, 59, 60]. The demonstrated high specificity of the oligonucleotides (confirmed by BLAST analysis) supports the test kit's suitability for *M. dispar* diagnostics in cattle.

Thus, this test kit can serve as an effective tool in veterinary laboratory practice for the detection of *M. dispar* DNA in biological samples from cattle.

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Development and application of ELISA test system for assessing humoral immunity against SAT2 topotype XIV foot-and-mouth disease virus

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ABSTRACT

Introduction. Foot-and-mouth disease (FMD) is a highly contagious, economically significant disease of cloven-hoofed animals, characterized by vesicular symptoms. There are seven known foot-and-mouth disease virus (FMDV) serotypes (A, O, C, SAT 1, SAT 2, SAT 3 and Asia 1), which immunologically differ from each other. Special attention has been recently paid to FMDV of SAT2/XIV topotype (South African Territories 2) due to its rapid spread. One of the key methods of FMD control is vaccination and assessment of the susceptible animal immune status.

Objective. Development and testing of an enzyme-linked immunosorbent assay (ELISA) system based on the indirect liquid-phase blocking ELISA for the determination of antibodies to structural proteins of SAT2/XIV FMDV in order to evaluate the effectiveness of an FMD vaccine based on SAT-2/XIV/2023 FMDV antigen in the process of its production and subsequent use.

Materials and methods. The test material included experimental serum samples collected from cattle, pigs and white mice. The developed ELISA test system for assessing the level of post-vaccination antibodies against SAT2/XIV FMDV was validated through comparative testing with commercial test-kits: Test-kit for the determination of SAT 2 FMDV antibodies (Federal Centre for Animal Health, Russia) and "Solid-phase competitive ELISA for antibodies specific to FMDV serotype SAT 2" (IZSLER & The Pirbright Institute, Italy/Great Britain).

Results. The effectiveness of the proposed test system in detecting the induction of antibodies against SAT2/XIV FMDV was higher than that of other ELISA systems with the pronounced topotype specificity to the SAT2/VII FMD agent. Specific antibodies were detected in individual cattle on day 7 post vaccination. High diagnostic sensitivity (90%), specificity (98%) and accuracy (95%) ensured high degree of the ELISA results consistency with the known diagnostic status of the tested animals (κ-criterion – 0.896).

Conclusion. Thus, the ELISA system for assessing humoral immunity against SAT2/XIV FMDV, which is 100% homologous with the vaccine strain and demonstrates high diagnostic parameters, is a reliable tool for assessing the quality of the SAT2/XIV FMD vaccine.

Keywords: serotype SAT 2 FMDV, ELISA, humoral immunity

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Разработка и применение иммуноферментной тест-системы для оценки гуморального иммунитета против вируса ящура топотипа SAT2/XIV

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РЕЗЮМЕ

Введение. Ящур – высококонтагиозная, экономически значимая болезнь парнокопытных животных, характеризующаяся везикулярными симптомами. Известны 7 серотипов вируса ящура (A, O, C, SAT 1, SAT 2, SAT 3 и Asia 1), иммунологически отличающихся между собой. В последнее время особое внимание уделяется топотипу вируса ящура SAT2/XIV (South African Territories 2) ввиду его быстрого распространения. Одними из основных методов борьбы с ящуром являются вакцинопрофилактика и оценка иммунного статуса поголовья восприимчивых животных.

Цель исследования. Разработка и испытание иммуноферментной тест-системы на основе непрямого жидкофазного блокирующего варианта иммуноферментного анализа для определения антител к структурным белкам вируса ящура топотипа SAT2/XIV для оценки эффективности противоящурной вакцины на основе антигена вируса ящура штамма SAT-2/XIV/2023 при ее производстве и последующем применении.

Материалы и методы. Исследуемым материалом служили экспериментальные образцы сыворотки крови крупного рогатого скота, свиней и белых мышей. Испытание разработанной иммуноферментной тест-системы для оценки уровня поствакцинальных антител к вирусу ящура топотипа SAT2/XIV проводили в сравнении с коммерческими наборами: для определения антител к вирусу ящура SAT 2 (ФГБУ «ВНИИЗЖ», Россия) и Solid-phase competitive ELISA for antibodies specific to FMDV serotype SAT 2 (IZSLER & The Pirbright Institute, Италия/Великобритания).

Результаты. Эффективность предложенной тест-системы в регистрации выработки антител к вирусу ящура топотипа SAT2/XIV была выше, чем у других иммуноферментных тест-систем, обладающих выраженной топотипоспецифичностью к возбудителю ящура топотипа SAT2/VII. У отдельных особей крупного рогатого скота специфические антитела выявляли на 7-е сут после вакцинации. Высокие значения диагностической чувствительности (90%), диагностической специфичности (98%) и диагностической точности (95%) определили высокую степень согласованности результатов реакции иммуноферментного анализа с известным диагностическим статусом обследуемых животных (*к*-критерий – 0,896).

Заключение. Таким образом, иммуноферментная тест-система для оценки гуморального иммунитета против вируса ящура топотипа SAT2/XIV, имеющая 100%-ю степень гомологии с вакцинным штаммом, обладающая высокими диагностическими показателями, является надежным инструментом оценки качества вакцины против ящура SAT2/XIV.

Ключевые слова: вирус ящура серотипа SAT 2, иммуноферментный анализ, гуморальный иммунитет

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious, economically significant disease of cloven-hoofed animals, which is characterized by vesicular symptoms. There are seven known FMDV serotypes: A, O, C, SAT 1, SAT 2, SAT 3 and Asia 1, which immunologically differ from each other.

The disease causative agent in the susceptible animals is an *Aphthovirus* of the *Picornaviridae* family, which is a capsid enclosing a positive sense RNA chain. The capsid of an icosahedron structure with a sedimentation coefficient 146S, is formed of 60 copies of a protomeric subunit consisting of four structural proteins (SP): VP1–VP4. VP1–VP3 surface polypeptides carry epitopes responsible for the FMDV serotype specificity and inducing production of the virus neutralizing antibodies. The priority polypeptide is VP1, which features the major antigenic site located within the G-H loop region. The internal protein VP4 is highly conserved across the serotypes of the pathogen, and antibodies against VP4 epitopes do not confer protection against the infection [1, 2].

Domesticated animals, mainly cattle, pigs, sheep, goats, buffaloes (*Bubalus bubalis)*, as well as camels and New World camelids, are FMD susceptible. However, the FMDV is sometimes isolated from wild cloven-hoofed animals such as wild pigs, antelopes and deer. Cross-infection between wild and domestic cloven-hoofed animals is therefore possible through direct or indirect contact. In sympatric species, this is one of the most likely routes of FMDV transmission. The primary FMDV reservoir is cattle, as this species is characterized by the virus carrier phenomenon. The virus carriers are individuals in whose oropharynx the virus persists for more than four weeks after infection. In cattle, this condition usually persists for up to six months, but in some individuals it lasts for up to three years. In wild animals, the virus carrier state was proven only for African buffaloes (*Syncerus caffer*), some individuals of which maintained the virus for five years, and in the herd the pathogen can circulate for 24 years or more. This characteristic of cattle significantly complicates FMD control efforts, particularly in the disease endemic areas. Such areas include a number of countries in Africa, Middle East, and Asia, as well as in a limited area of South America [1], which in turn creates a serious risk of FMD spread outside the endemic territories.

The FMD control consists in timely preventive measures, such as effective epidemiological disease control; vaccination of domestic and farm animals against FMD; movement control of wild cloven-hoofed animals in border areas at risk of the pathogen introduction from the FMD-infected countries; monitoring tests to assess the immunity level of farm and domestic animals, including retrospective FMD diagnosis. Since the viruses of different serotypes induce virtually no cross-immunity, and the vaccines, which are based on strains different from the field ones, may provide weak protection even within the same serotype, the development of vaccine formulations requires meticulous strain selection to ensure maximum possible protection of the animal population against FMD in a specific region [1, 2].

Recently, special attention has been paid to serotype SAT 2 FMDV, which is exotic for many regions of the world, including for Russia. The name itself, which is an English abbreviation of South African Territories, indicates the origin of this serotype. FMD outbreaks caused by serotype SAT 2 virus, whose representatives are characterized by high genetic variability and are divided into 14 topotypes (I-XIV), are periodically reported in the African continent [3]. Until recently, the FMD epidemic situation in Africa was generally associated with the members of serotype SAT 2 topotype VII (SAT2/VII), but in 2022, rapid spread of SAT 2 topotype XIV (SAT2/XIV) virus started, which resulted in its spread outside the endemic territories. FMD outbreaks caused by SAT2/XIV topotype virus were reported in 2023 in some Eurasian countries, including Iraq, Jordan, and Turkey.

Serotype SAT 2 FMDV, which circulated in Iraq in 2023, is closely related to the virus isolates recovered in Ethiopia in 2022 (SAT2/ETH/3/22 and SAT2/ETH/2/22). Both viruses were isolated from the infected cattle in March 2022. According to the reports, the virus currently isolated in Jordan is also related to the specified Iraqi virus. The spread of the exotic foot-and-mouth disease virus to large susceptible cattle and buffalo populations in Iraq poses risk to numerous animal populations in Iran, Turkey, as well as a number of other countries in the Middle East, and raises serious concern [4].

Close cooperation with the CIS and Middle East countries, trade and economic ties of the Russian Federation with the African countries, where preventive vaccination of livestock against serotype SAT 2 FMDV is not carried out, increase the risk of this serotype FMDV introduction into our country. This is why greater relevance is now placed on the production of the vaccines against serotype SAT 2 FMDV, including SAT2/XIV FMDV, as well as on the development and introduction of diagnostic test systems to assess the effectiveness of the vaccination and the strength of the immunity against this topotype virus in farm and domestic cloven-hoofed animals and to detect post-infection FMDV antibodies in the sera of the unvaccinated animals.

The Federal Centre for Animal Health has developed and successfully tested a mono- and polyvalent vaccine based on the antigen of FMDV strain SAT-2/XIV/2023 [4]. The strain was obtained by adapting the FMDV isolate recovered at the Federal Centre for Animal Health from the bovine pathological material delivered from the Hashemite Kingdom of Jordan in August 2023 to the reproduction in the primarily trypsinized porcine kidney monolayer cell line, in the continuous cell cultures BHK-21/SUSP/ARRIAH, IB-RS-2, PSGK-30, as well as in cattle and pigs. SAT-2/XIV/2023 strain was deposited in the All-Russian State Collection of Exotic FMDV Types and Other Animal Pathogens of the Federal Centre for Animal Health in 2023 and was proposed for the manufacture of vaccine products and diagnostic tools [5, 6]. This necessitated the development of an enzyme-linked immunosorbent assay (ELISA) system to objectively and reliably evaluate the antigenic and immunogenic properties of the FMD vaccine based on SAT-2/XIV/2023 virus antigen, as well as the immune background and intensity of post-vaccination immunity in the susceptible animals.

The paper describes the stages of the development and testing of the ELISA system based on an indirect liquid-phase blocking enzyme-linked immunosorbent assay intended for assessment of the humoral immunity against SAT2/XIV FMDV.

MATERIALS AND METHODS

Serum samples. Experimental serum samples collected from cattle, pigs, and white mice served as the ELISA test material.

Preparation of immunospecific reagents. The antigen of FMDV strain SAT-2/XIV/2023 intended for the production of ELISA-specific components (antigen, capture (coating) and detector antibodies) was isolated from the inactivated virus-containing BHK-21 cell suspension according to the following procedure. At the first stage, the antigenic source materials were purified from cellular debris by low-speed centrifugation (Avanti J-26 XP; Beckman Coulter, USA) for 30 minutes. The supernatant was discarded and used to precipitate the viral antigen with 8% polyethylene glycol of molecular weight 6,000 g/mol (PEG 6,000) and 0.85% NaCl for 18–20 hours at (6 ± 2) °C. The precipitated antigen was pelleted using Avanti J-26 XP centrifuge (Beckman Coulter, USA) at 6,000 rpm and 4 °C for 60 minutes. The supernatant was removed, and the pellet was re-suspended in 1/500 of the original volume of the starting material using phosphate buffered saline solution (PBS) at pH 7.4. The resulting suspension was then thoroughly homogenized with 50% chloroform and fractionated at 3,000 rpm and 4 °C for 15 minutes using Allegra X-22R Centrifuge (Beckman Coulter, USA). The upper aqueous fraction was an intermediate substance in the form of a concentrated, partially purified antigen, hereinafter referred to as a precipitate of the antigen of FMDV strain SAT-2/XIV/2023 ($Ag_{precipitate}$ SAT2/XIV). $Ag_{precipitate}$ SAT2/XIV was then fractionated using a discontinuous sucrose density gradient comprising successive layers of 20, 30, 40, 50% sucrose in PBS on Optima L-80 XP Ultracentrifuge (Beckman Coulter, USA) at 24,000 rpm and 4 °C for 3 hours.

The antigenic fractions were analyzed spectrophotometrically at 260 nm wavelength and by electrophoretic separation of protein molecules in the polyacrylamide gel. 30–50% sucrose fractions containing VP1–VP3 SP (an intact FMDV SAT-2/XIV/2023 antigen representing 146S particles), free from impurities or comprising their minimal amount, were combined, labeled as 146S-Ag SAT2/XIV and then used to manufacture the immunospecific components of the liquid-phase blocking ELISA (LPB-ELISA): FMDV capture and detector antibodies as well as antigen.

Rabbits and guinea pigs were immunized twice with 146S-Ag SAT2/XIV at a dose of approximately 0.3 and 0.15 mg/animal, respectively. The antigen intended for administration was mixed with ISA-206 adjuvant in equal proportions. The bleeding was performed on day 35 post the first vaccination.

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

Standard panel of strain-specific bovine and porcine serum samples was used to select the working dilutions of the LPB-ELISA components, including capture and detector antibodies, antigen and rabbit anti-guinea pig IgG secondary antibody immunoperoxidase conjugate. The panel included both homologous and heterologous samples obtained for other FMDV serotypes, as well as normal serum, collected from the animals not vaccinated against FMD.

ELISA. The test was carried out according to the recommendations of the World Organization for Animal Health (WOAH) for LPB-ELISA [1] and instructions for use of the ELISA test kit for detection of antibodies against FMDV SP using the optimal concentration of immunospecific reagents selected during the test system development. Diagnostic LPB-ELISA test-kit for the detection of antibodies to SAT 2 FMDV (SAT2/VII topotype) SP manufactured by the Federal Centre for Animal Health, as well as a commercial test-kit for the detection of antibodies to serotype SAT 2 FMDV "Solid-phase competitive ELISA for antibodies specific to FMDV serotype SAT 2, hereafter SAT2-IZSLER" (IZSLER & The Pirbright Institute, Italy/UK) were also used according to the manufacturer's instructions.

The virus neutralization test (VNT) of the FMDV was performed in the continuous porcine kidney cell line IB-RS-2 in accordance with the WOAH recommendations. Microneutralization technique with 96-well flat-bottom cell culture plates was used for the virus neutralization. Approximately 10^2 $TCID_{50}$/0.05 mL (tissue culture infective dose of the virus, causing death in 50% of the cells) of the virus was added to the tested and control serum samples two-fold diluted with Eagle's nutrient medium (permissible range $10^{1.5}$–$10^{2.5}$ $TCID_{50}$/0.05 mL. The sera were incubated for 1 hour at 37 °C with 5% CO_2, then 0.05 mL of IB-RS-2 cell culture suspensions at a concentration of 10^6 cells/mL were added to each plate well and incubated under the same conditions for 48–78 hours. The test results were recorded as the virus cytopathic effect (CPE) developed in the control wells with the infected cell culture and without test animal sera. The virus-neutralizing antibody (VNA) titer was defined as the highest serum dilution that inhibits the viral cytopathic effect (CPE) by 50% [1].

Alignment of amino acid sequences. The amino acid sequences of VP1 protein of the FMDV of different SAT 2 topotypes were aligned according to information obtained from GenBank NCBI and the Federal Centre for Animal Health database: SAT2/ETH/2/2022 (GenBank: WKE35517.1); SAT2/JOR/11/2023 (GenBank: WUR05443.1); SAT-2/XIV/2023 (the Federal Centre for Animal Health database); SAT2/ETH/2/91 (GenBank: WKE35516.1); SAT2/ERI/4/98 (GenBank: AAR09103.1); SAT2/LIB/39/2012 (GenBank: AFU55195.1); SAT2/EGY/Ismailia/2018 (GenBank: QZE50286.1); SAT2/EGY/Beni-Suef-2/2018 (GenBank: QEI49588.1); SAT2/EGY/Dakahlia/2017 (GenBank: AXR97922.1) [7, 8, 9, 10, 11, 12, 13, 14].

FMDV isolate	Topotype	Location	Amino acid sequence
SAT2/ETH/2/2022	XIV	1	ttsagegadv vtidptthgg aarptrrvht dvafldrst hvhtnkttfn idlmdtkeka
SAT2/JOR/11/2023	XIV	1	---------- ---------- ---------- ---------- ---------- ----------
SAT2/XIV/2023	XIV	1	---------- ---------- ---------- ---------- ---------- ----------
SAT2/ETH/2/91	XIV	1	---------- ---------- svt-a--i-- ---------- ---------- ----------
SAT2/ERI/4/98	VII	1	---------- --t--s---- nvqeg--k-- e--------- -------s-v v------k--
SAT2/LIB/39/2012	VII	1	---------- --t--s---- nvqeg--k-- e--------- ----g--s-v v------k--
SAT2/EGY/Ismalia/2018	VII	1	---------- --t--s---- nvqeg--k-- e--------- ----g--s-v v---n-----
SAT2/EGY/Beni-Suef-2/2018	VII	1	
SAT2/EGY/Dakahlia/2017	VII	1	
SAT2/ETH/2/2022	XIV	61	lvgailrsat yyfcdleiac vgdhkrvywq pngapratql gdnpmvfshn rvtrfaipyt
SAT2/JOR/11/2023	XIV	61	---------- ---------- ---------- ---------- ---------- ----------
SAT2/XIV/2023	XIV	61	---------- ---------- ---------- ---------- ---------- ----------
SAT2/ETH/2/91	XIV	61	---------- ---------- --e----f-- ------t-t- ------y--- g---------
SAT2/ERI/4/98	VII	61	-------as- ---------- ----t--f-- ------t--- ------yakg g-------f-
SAT2/LIB/39/2012	VII	61	-------as- ---------- ----t--f-- ------t--p ------akg g-------f-
SAT2/EGY/Ismalia/2018	VII	61	-------as- ---------- --e-t--f-- ---------- ------akg g-------f-
SAT2/EGY/Beni-Suef-2/2018	VII	61	-- --e-tk-f-- ------t--- ------akg g-------f-
SAT2/EGY/Dakahlia/2017	VII	61	s- ---------- ----t-af-- ---------- ------akg g-------f-
SAT2/ETH/2/2022	XIV	121	aphrllstvy ngectynkss tairgdravl vakyadnkht lpstfnfghv tadapvdvyy
SAT2/JOR/11/2023	XIV	121	---------- ------s--- ---------- ---------- ---------- ----------
SAT2/XIV/2023	XIV	121	---------- ------s--- ---------- ---------- ---------- ----------
SAT2/ETH/2/91	XIV	121	---------- ----d-k-a- ---------- a----nt--- ---------- ----------
SAT2/ERI/4/98	VII	121	---------- ------t-ta --------a- a------vv- --q-----f- -v-k------
SAT2/LIB/39/2012	VII	121	---------- ----d---tv --------a- a------t-- --------f- -v-k------
SAT2/EGY/Ismalia/2018	VII	121	---------- ----v-k-pv as------a- a-----st-- --p------- -v-k------
SAT2/EGY/Beni-Suef-2/2018	VII	121	---------- ----v-t-pv ap------a- a-----st-- --p-----s- -v-k------
SAT2/EGY/Dakahlia/2017	VII	121	---------- ----v-ketp --------a- a----gtn-- --------f- -v-k------
SAT2/ETH/2/2022	XIV	181	rmkraelycp rallpaynha grdrfdapig vekql
SAT2/JOR/11/2023	XIV	181	---------- ---------- ---------- ----
SAT2/XIV/2023	XIV	181	---------- ---------- ---------- ----
SAT2/ETH/2/91	XIV	181	---------- -------d-v ---------- --r--
SAT2/ERI/4/98	VII	181	---------- -p-----d-- s--------- --rqtl
SAT2/LIB/39/2012	VII	181	---------- -p---t-d-- ---------- --r-
SAT2/EGY/Ismalia/2018	VII	181	---------- -p-----d-- ---------- --r-
SAT2/EGY/Beni-Suef-2/2018	VII	181	---------- -p-----d-- --y------- --r-t
SAT2/EGY/Dakahlia/2017	VII	181	---------- -p-----e-- s--------- --r-t

Fig. 1. Amino acid sequences of VP1 polypeptide of serotype SAT 2 topotype XIV and VII FMDV obtained from NCBI and Federal Centre for Animal Health databases (up to 216 a.r.), G-H loop area is highlighted in red

Fig. 2. Location of SAT-2/XIV/2023 strain on serotype SAT 2 FMDV phylogenetic tree. The dendrogram is based on the comparison of VP1 gene nucleotide sequences. Topotypes I–XIV are in square brackets

Polyacrylamide gel electrophoresis. Electrophoretic separation of proteins in polyacrylamide gel was performed as previously described [15, 16].

Statistical analysis was carried out using online resources, including MedCalc's Diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php), Kappa Calculator (https://www.easycalculation.com/statistics/cohens-kappa-index.php).

RESULTS AND DISCUSSION

Development of SAT2/XIV-ARRIAH test system. The effectiveness of the vaccine, immune background and anti-FMDV immunity strength in the susceptible animals are assessed by the level of the specific virus neutralizing (protective) antibodies in the sera of animals after vaccination or infection. In the laboratory diagnostics, two key methods are used to examine serum samples for the antibodies to the FMDV SP: VNT and ELISA [1, 2, 17, 18, 19].

The developed ELISA system, designed primarily to evaluate the antigenic and immunogenic activity of the FMD vaccine containing genotype SAT2/XIV FMDV antigen, is based on LPB-ELISA. This ELISA variant, recommended by the WOAH as one of the key methods of retrospective FMD diagnosis aimed at the monitoring the FMD vaccine immunogenicity, assessing the immunity strength, and FMD monitoring tests, has been adapted for the Federal Centre for Animal Health, optimized, and modified. An undeniable advantage of the LPB-ELISA is that the formation of the 'antigen – antibody' complex occurs in the liquid phase, i.e., under conditions that closely mimic the natural environment. In this case, there is no deformation of the virion and the antigenic sites do not change, which makes it similar to the VNT in cell culture or chicken embryonated eggs [1, 17, 18, 19].

When creating the test systems for FMD diagnosis, the developers strive for the ELISA serotype specificity. Indeed, within the serotype, the FMDVs of different genetic lineages, as a rule, reveal a serological relationship. However, when testing the primarily vaccinated young animals, the test systems for detecting the certain serotype FMDV antibodies produced by different manufacturers may demonstrate strain/genotype specificity. This is of great importance in assessing the antigenic and immunogenic activity of a FMD vaccine. It is essential to carefully select the tool to obtain the most objective and reliable information.

Two test systems were earlier developed at the Federal Centre for Animal Health to assess humoral immunity against SAT 2 topotype VII FMDV based on SAT2/LIB/39/2012 and SAT2/ERI/98 strains, which demonstrated a high degree of relatedness and, as a result, interchangeability [3]. These test systems were

Table 1
Antigenic relatedness of subtype SAT 2 FMDV strains determined in the virus neutralization test, r_1

Serum samples	r_{1mean} in FMDV neutralization test*		
	SAT2/LIB/39/2012	SAT2/ERI/98	SAT-2/XIV/2023
SAT2/LIB/39/2012	1.0	**0.36**	0.06
SAT2/ERI/98	**0.69**	1.0	0.16
SAT-2/XIV/2023	0.14	0.16	1.0

* $r_1 \geq 0.3$ – close relatedness; $r_1 < 0.3$ – low relatedness.

supposed to be able to provide a full-scale ELISA monitoring of the effectiveness of the vaccine containing the antigen of the new FMDV strain. Data on the genetic and serological matching of SAT 2 FMDV strains and isolates of topotypes VII and XIV were analyzed, and the feasibility of developing a new test system for SAT 2 FMDV of topotype XIV was considered.

To identify the genetic relationship (homology) between topotypes VII and XIV, amino acid sequences of SAT 2 FMDV VP1 protein were compared. As can be seen in Figure 1, the vaccine FMDV strain SAT-2/XIV/2023 had 100% homology with the FMDV isolate recovered in the Hashemite Kingdom of Jordan in 2023 (SAT2/JOR/11/2023) in the amino acid sequence of VP1 polypeptide. Both viruses differed from SAT2/ETH/2/2022 isolate recovered in Ethiopia in 2022 by one amino acid substitution in the G-H loop region and by 20 substitutions from another Ethiopian isolate SAT2/ETH/2/91.

These viruses belong to topotype XIV of SAT 2 serotype and significantly differ from the isolates of SAT 2 topotype VII virus, which directly affects the antigenic matching of SAT 2 virus strains belonging to different topotypes. The phylogenetic tree of the SAT 2 FMDV in Figure 2, based on the comparison of VP1 gene nucleotide sequences clearly demonstrates the topotypic (genotypic) differences between the virus strains.

When studying the antigenic matching of the vaccine strain SAT-2/XIV/2023 (topotype XIV) and FMDV strains SAT2/LIB/39/2012 (topotype VII) and SAT2/ERI/98 (topotype VII), a low degree of relatedness between the strains of different topotypes was established in VNT in IB-RS-2 cell culture: 0.06–0.16. Meanwhile, strains SAT2/LIB/39/2012 and SAT2/ERI/98 showed close relatedness between each other: 0.36 and 0.69, respectively (Table 1), which indicated good cross-protection between these FMDVs.

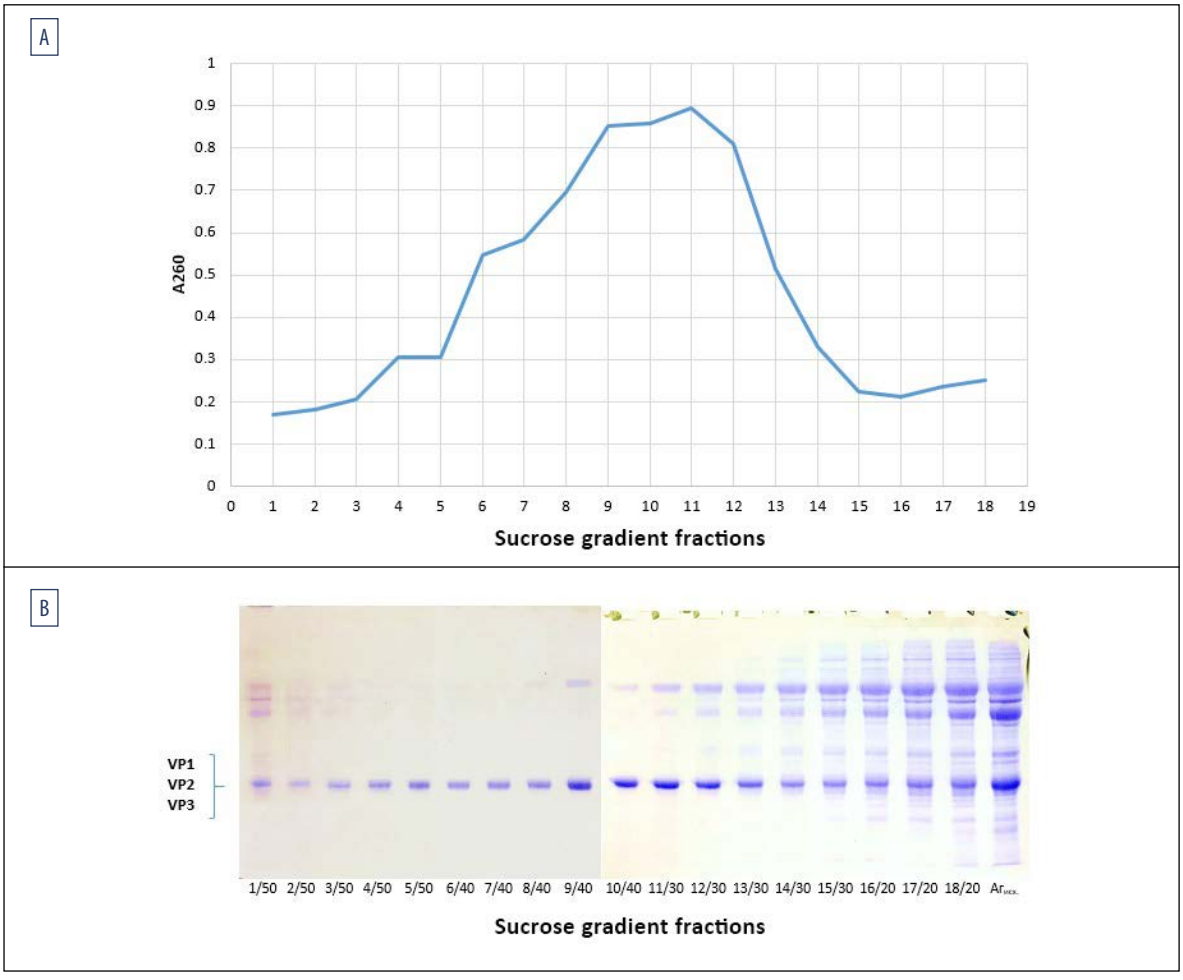

Fig. 3. Isolation of 146S-Ag SAT2/XIV during fractionation of SAT2/XIV $Ag_{precipitate}$ using sucrose gradient ultracentrifugation:
A – sedimentation profile of SAT2/XIV $Ag_{precipitate}$ with wavelength 260 nm at 1:10 dilution;
B – electrophoregram of sucrose gradient fractions, including SP VP1, VP2, VP3 of SAT-2/XIV/2023 FMDV strain

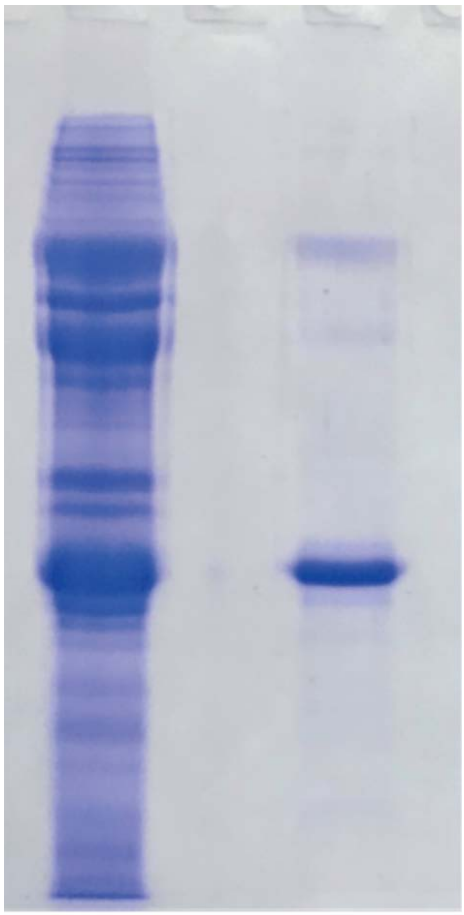

Fig. 4. 12% polyacrylamide gel electrophoresis of SAT-2/XIV/2023 FMDV antigen: 1 – SAT2/XIV $Ag_{precipitate}$ before fractionation by sucrose gradient ultracentrifugation; 2 – 146S-Ag SAT2/XIV

Thus, when developing an enzyme-linked immunosorbent assay system to assess the antigenicity and immunogenicity of the vaccine against SAT2/XIV FMD, the production strain SAT-2/XIV/2023 was selected.

Special attention was paid to the extraction of 146S component from the inactivated virus-containing suspension of BHK-21 cell culture (146S-Ag SAT2/XIV) when preparing specific LPB-ELISA reagents for the detection of antibodies to SAT2/XIV FMDV SP. It is an intact antigen with sedimentation coefficient of 146S, that is, an antigen with an unaltered structure, representing virions that have lost their infectivity during the inactivation process. This antigen consists of a capsid enclosing inactivated RNA. The integrity of the capsid ensures the immunogenicity of the FMD vaccine, since the surface polypeptides VP1–VP3 induce the production of the virus neutralizing strain/serotype-specific antibodies. Use of highly purified 146S antigen during the ELISA "liquid phase", when an immune complex with specific antibodies is formed in the test sample, as well as for the immunization of rabbits and guinea pigs for the production of capture (coating) and detector antibodies, respectively, allows for the determination of the level of humoral immunity against FMD with the highest possible reliability.

The use of antigens with a lower degree of purification in the ELISA, i.e. partially purified antigen (antigenic precipitate) or antigen-containing cell suspension, which are characterized by the presence of ballast proteins in the form of residual cell debris and serum albumin, can lead to biased test results.

During antigen fractionation by ultracentrifugation in a 20–50% sucrose gradient, the majority of 146S particles accumulated at the interface between the 30 and 40% sucrose layers as an opalescent band. The gradient fractions collected at 1 mL were analyzed spectrophotometrically at a wavelength of 260 nm to construct SAT2/XIV $Ag_{precipitate}$ sedimentation profile and by electrophoretic separation of protein molecules in a 12% polyacrylamide gel (Fig. 3B).

Fractions with the highest accumulation of VP1–VP3 structural polypeptides and the lowest content or absence of impurities were selected for 146S antigen. As a result, after combining the fractions, 146S-Ag SAT2/XIV was obtained with a protein concentration of approximately 0.55 mg/mL. Figure 4 shows an electrophoregram of SAT-2/XIV/2023 FMDV antigen in 12% polyacrylamide gel before and after fractionation by sucrose-gradient ultracentrifugation.

Table 2
ELISA and VNT results of serum samples collected from cattle vaccinated against SAT2/XIV FMD (adsorbed monovalent vaccine)

Serum samples	Vaccine dose	Test system			
		SAT2/XIV-ARRIAH $PI_{pos} \geq 50\%$, pos/n	SAT2/VII-ARRIAH $PI_{pos} \geq 50\%$, pos/n	SAT2-IZSLER $PI_{pos} \geq 70\%$, pos/n	SAT2/XIV-VNT T_{pos} VNA $\geq 5 \log_2$, pos/n
S_{bov} 0 dpv	before vaccination	0/16	0/16	0/16	0/16
S_{bov} 3 dpv	whole dose	0/5	0/5	0/5	0/5
	1/4	0/5	0/5	0/5	0/5
	1/16	0/5	0/5	0/5	0/5
	control	0/1	0/1	0/1	0/1
S_{bov} 7 dpv	whole dose	1/5	0/5	0/5	0/5
	1/4	1/5	0/5	0/5	0/5
	1/16	1/5	1/5	0/5	0/5
	control	0/1	0/1	0/1	0/1
S_{bov} 14 dpv	whole dose	5/5	4/5	3/5	3/5
	1/4	4/5	2/5	1/5	3/5
	1/16	2/5	1/5	0/5	1/5
	control	0/1	0/1	0/1	0/1
S_{bov} 21 dpv	whole dose	5/5	4/5	3/5	4/5
	1/4	4/5	1/5	1/5	2/5
	1/16	2/5	1/5	0/5	1/5
	control	0/1	0/1	0/1	0/1

S_{bov} – bovine sera; PI – inhibition percent; T_{pos} VNA – positive (protective) titre of virus neutralizing antibodies; pos/n – number of positive reactors against total number of the animals in the group; dpv – day post vaccination.

Table 3
ELISA results of serum samples collected from white mice immunized with an adsorbed monovalent vaccine against SAT2/XIV FMDV

Serum samples	Vaccine dose	Test system		
		SAT2/XIV-ARRIAH $PI_{pos} \geq 50\%$	SAT2/VII-ARRIAH $PI_{pos} \geq 50\%$	SAT2-IZSLER $PI_{pos} \geq 70\%$
S_{mur} 14 dpv	whole dose	**67.5%**	**64.4%**	30.6%
	1/4	**64.9%**	**50.1%**	14.9%
	1/16	21.1%	5.9%	22.3%
S_{mur} 21 dpv	whole dose	**71.6%**	**65.3%**	32.5%
	1/4	**63.5%**	48.0%	43.7%
	1/16	12.5%	1.7%	1.8%
	control	6.1%	0.35%	2.4%

S_{mur} – white mouse sera; PI – inhibition percent; dpv – day post vaccination; positive PI values are in bold.

The resulting 146S-Ag SAT2/XIV was used to manufacture immunospecific components of LPB-ELISA: capture and detector antibodies, and FMDV antigen.

The test system for the determination of SAT2/XIV FMDV SP antibodies demonstrated specificity and sensitivity in determining the immune status of the tested animals when using immunospecific reagents in the following working dilutions: capture antibodies – 1:1000, detector antibodies – 1:5000, FMDV antigen – 1:1000, or 0.55 μg/mL, commercial anti-species conjugate – 1:1500, or 0.75 μg/mL.

When interpreting the ELISA results, it is necessary to establish qualitative assessment criteria. For this purpose, the cut-off value is determined – a positive and negative threshold (PNT) that allows classifying the tested biological samples as positive or negative for a specific infectious agent.

In the developed SAT2/XIV-ARRIAH test system, the qualitative analysis of the ELISA results is based on a PNT of 50% PI (percentage of inhibition) [1]. The calculation was performed based on the data obtained during testing of 176 serum samples collected from clinically healthy cattle and pigs not vaccinated against FMD. Mean PI and standard deviation from the mean (SD) were obtained. The mean PI for this serum sample panel (n = 176) was 21.09%, SD – 14.67%. The PNT was calculated by the formula: PNT = PI_{mean} + 2SD and amounted to 50.43%.

Thus, the PNT value for the qualitative characteristics of the serum samples tested for the presence of SAT2/XIV FMDV antibodies using the proposed test system corresponded to the predicted 50% PI, which indicated the optimal choice of working dilutions of the immunospecific components that determined the accuracy and objectivity of the analysis.

Trials of SAT2/XIV-ARRIAH test system. SAT2/XIV-ARRIAH ELISA system was evaluated during the assessment of the antigenicity and immunogenicity of SAT2/XIV monovalent adsorbed FMD vaccine in comparison with other test systems for SAT 2 FMD diagnosis (Table 2). For this purpose, fifteen black-and-white bull calves were vaccinated once with the vaccine at different doses: whole dose, 1/4 dose and 1/16 dose, with five animals per each vaccine dose. One control animal was not vaccinated. Blood for testing in ELISA and VNT was collected from all animals on day 0, 3, 7, 14, 21 post administration of the vaccine against SAT2/XIV FMD. The serum samples were tested for SAT 2 FMDV antibodies using the following ELISA systems: SAT2/XIV-ARRIAH based on SAT-2/XIV/2023 strain; SAT2/VII-ARRIAH based on SAT2/LIB/39/2012 strain; SAT2-IZSLER, and SAT-2/XIV/2023 VNT.

As can be seen from Table 2, the effectiveness of SAT2/XIV-ARRIAH test system was higher in recording the specific antibody production as compared to other test systems under study. Already on day 7, one seropositive animal was detected in each of the three groups of vaccinated cattle, and one positive reactor was detected using SAT2/VII-ARRIAH test system. In the other two reactions, the specific antibodies were detected only on day 14. As a result, the number of positive reactors detected using ELISA and VNT on day 14–21 post administration of different vaccine doses

Table 4
ELISA results of serum samples collected from cattle and pigs before and after vaccination against serotype SAT 2 FMD and challenge

Samples serum	Test system								
	SAT2/XIV-ARRIAH $PI_{pos} \geq 50\%$			SAT2/VII-ARRIAH $PI_{pos} \geq 50\%$			SAT2-IZSLER $PI_{pos} \geq 70\%$		
	PI_{mean}	pos/n	SP, %	PI_{mean}	pos/n	SP, %	PI_{mean}	pos/n	SP, %
S_{por} 0 dpv	23.2	0/16	–	13.9	0/16	–	22.3	0/16	–
S_{bov} 0 dpv	25.1	0/37	–	30.3	0/37	–	20.2	0/37	–
S_{por} 16–28 dpv SAT2/LIB/39/2012	68.2	28/30	93.3	83.7	29/30	96.7	90.2	29/30	96.7
S_{bov} 14–35 dpv SAT2/LIB/39/2012	70.1	14/15	93.3	89.5	15/15	100.0	87.6	15/15	100.0
S_{bov} 21–45 dpv/dpi SAT2/ERI/98	59.5	23/35	65.7	80.3	27/35	77.1	89.1	29/35	82.9
S_{bov} 14–21 dpv SAT-2/XIV/2023	76.8	30/30	100.0	63.8	24/30	80.0	72.6	18/30	60.0

S_{bov} – bovine sera; S_{por} – porcine sera; PI – inhibition percent; pos/n – number of positive reactors against total number of the animals in the group; SP – seropositivity (number of positive reactors); dpv/dpi – day post vaccination/infection.

was the following: SAT2/XIV-ARRIAH – 22/30 (73.3%), SAT2/VII-ARRIAH – 13/30 (43.3%), SAT2-IZSLER – 8/30 (26.7%), SAT2/XIV-VNT – 14/30 (46.7%), which indicated an undeniable advantage of SAT2/XIV-ARRIAH test system as for its diagnostic sensitivity.

An experimental saponin-containing vaccine with inactivated FMDV strain SAT-2/XIV/2023 also induced humoral response when administered to the laboratory white mice. Animals weighing 24–26 g were vaccinated once with the vaccine at different doses: whole dose, 1/4 dose and 1/16 dose, at 0.4 mL per animal (13 animals for each vaccine dose). Eleven control mice were not vaccinated. Blood was collected in pools on days 14 and 21 after vaccination. The results are presented in Table 3. As in the experiment with cattle, SAT2/XIV-ARRIAH test system demonstrated higher sensitivity as compared to other test systems for SAT2/VII topotype.

The vaccine containing SAT-2/XIV/2023 FMDV antigen, as well as SAT2/XIV-ARRIAH diagnostic test kit supplied with the vaccine, were tested in the field, namely in Jordan. According to the data provided by the Jordanian side, both the monovalent adsorbed vaccine against SAT2/XIV and the homologous test system for evaluating the effectiveness of manufactured by the Federal Centre for Animal Health vaccine demonstrated convincing results of the vaccine's antigenicity after double vaccination of cattle. All vaccinated animals (10 animals) demonstrated protective antibody level with PI_{mean} = 84.58 ± 12.56, while the unvaccinated controls (2 animals) did not show any antibodies to the SAT2/XIV FMDV (PI_{mean} = 35.34 ± 2.21).

To confirm the reliability of the ELISA data, 163 serum samples collected from pigs and cattle were tested using the following test systems: SAT2/XIV-ARRIAH, SAT2/VII-ARRIAH, SAT2-IZSLER. The results of parallel serum tests before and after whole-dose vaccination against SAT2/XIV FMD agent (vaccine based on strain SAT-2/XIV/2023) and SAT2/VII FMD agent (vaccines based on strains SAT2/LIB/39/2012 and SAT2/ERI/98), as well as after challenge with FMDV strain SAT2/ERI/98 are shown in Table 4.

The analysis of the resulted data demonstrated pronounced topotype specificity of the test systems for this panel of samples. The bovine sera against FMDV SAT-2/XIV/2023 antigen tested positive in 100% of cases using ELISA SAT2/XIV-ARRIAH test system, and in 80 and 60% cases using SAT2/VII-ARRIAH and SAT2-IZSLER, respectively. While seropositivity of animals against SAT2 topotype VII FMDV in SAT2/VII-ARRIAH and SAT2-IZSLER test systems was 91.27 and 93.2%, respectively, in SAT2/XIV-ARRIAH test system it amounted to 84.1%.

Determining diagnostic parameters of SAT2/XIV-ARRIAH test system. During SAT2/XIV-ARRIAH validation, such basic diagnostic parameters as sensitivity, specificity, accuracy, and the κ-criterion were determined as described earlier [20]. Table 5 shows the data on statistical processing of ELISA results for 301 serum samples collected from unvaccinated cattle and from cattle vaccinated against SAT2/XIV FMDV.

As evident from the results, 90% diagnostic sensitivity, 98% diagnostic specificity, and 95% diagnostic accuracy of SAT2/XIV-ARRIAH ELISA system demonstrated a high degree of consistency between the ELISA results and the known diagnostic status of the tested animals (κ-criterion – 0.896).

Table 5
Diagnostic parameters of the test systems for detecting antibodies to SAT 2 FMDV SP, defined for the SAT2/XIV topotype

Test systems used	Diagnostic parameters (n = 301), (95% confidence interval)			
	sensitivity	specificity	accuracy	κ-criterion*
SAT2/XIV-ARRIAH	90.24% (83.58–94.86%)	98.31% (95.15–99.65%)	95.02% (91.91–97.18%)	0.896
SAT2/VII-ARRIAH	55,0% (41.6–67.9%)	98,1% (89.9–100.0%)	75.2% (66.2–82.9%)	0.516
SAT2-IZSLER	40,0% (27.6–53.5%)	100,0% (93.3–100.0%)	68.1% (58.7–76.6%)	0.385

*κ-criterion – consistency between ELISA test results of individual serum samples and the animals' diagnostic status; < 0 – no consistency; 0–0.20 – insignificant; 0.21–0.40 – low; 0.41–0.60 – moderate; 0.61–0.80 – significant; 0.81–1.00 – high.

Currently only one test system for retrospective FMD diagnosis, capable of detecting SAT2 FMDV SP antibodies using ELISA is reliably known to be available on the global market. This is Solid-phase competitive ELISA test kit for antibodies specific to FMDV serotype SAT 2 produced by IZSLER & The Pirbright Institute (Italy/United Kingdom) [21].

The use of monoclonal antibodies in diagnostic ELISA systems for serotyping specific FMDV antibodies is designed to create the versatility of analysis within a specific FMDV serotype (the possibility of detecting antibodies induced against different FMDV strains of the same serotype in the tested sera or blood plasma with the same sensitivity), that is, the test system should have wide serotype specificity.

However, in the case of SAT2-IZSLER test system, pronounced topotype specificity was observed. When testing samples of monospecific bovine or porcine sera against SAT2/VII and SAT2/XIV FMDV using SAT2-IZSLER, the highest number of positive results was obtained for sera against topotype SAT2/VII, while antibodies to topotype SAT2/XIV were detected with lower efficiency than in case of SAT2/XIV-ARRIAH test system. This indicates that the use of SAT2-IZSLER kit to study the antigenicity and immunogenicity of the FMD vaccine comprising SAT-2/XIV/2023 FMDV antigen, does not allow for an objective and reliable assessment of the vaccine effectiveness using ELISA. Thus, the ELISA system for assessing humoral immunity against SAT2/XIV FMDV, which has a 100% degree of homology with the vaccine strain and high diagnostic performance, is an indispensable and currently irreplaceable tool for evaluating vaccine quality in ELISA.

CONCLUSION

The developed test system based on a liquid-phase blocking ELISA variant for detection of SAT2/XIV FMDV SP antibodies is specific and sensitive. The uniqueness

of the test system consists in a 100% degree of homology with the vaccine products, which allows for the most reliable and effective assessment of the antigenic and immunogenic properties of the vaccine during its production and subsequent use in the field. During validation trials, this test system demonstrated high diagnostic sensitivity (90%), diagnostic specificity (98%), and diagnostic accuracy (95%), indicating a strong consistency between ELISA results and the known diagnostic status of the tested animals (*κ*-criterion – 0.896).

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Microbial species diversity and antibiotic-resistant *Enterobacteriaceae* spread on dairy farms

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ABSTRACT

Introduction. Bacterial communities significantly affect the overall productivity of agricultural establishments, as animal health, milk production, and food quality and safety depend on them. Zoonotic bacteria not only have a negative impact on animal health, but also pose a risk to public health, so monitoring of the microbial species diversity on dairy farms to determine the predominant pathogen species and antibiotic resistance profiles is essential.

Objective. Study of bacterial species diversity on a dairy farm and monitoring of antibiotic resistance spread in *Escherichia coli* and *Proteus mirabilis* isolates in order to enable timely development of measures containing the spread of antibiotic-resistant microorganisms.

Materials and methods. To achieve this goal, microorganisms were identified by MALDI-ToF mass spectrometry and antibiotic susceptibility of the isolated cultures was determined using the disc diffusion test.

Results. The species diversity of microorganisms isolated from samples of cattle limb wound exudates, feces, and feed was established. Opportunistic and pathogenic *Escherichia coli* and *Proteus mirabilis* turned out to be the predominant microorganisms, and their antibiotic resistance profiles were determined. One of the *Escherichia coli* isolates was found to be multi-resistant; only a combination of amoxicillin and clavulanic acid proved effective in inhibiting the growth of this culture. A large proportion of *Proteus mirabilis* isolates were resistant to drugs included in the group of fluoroquinolones and sensitive to all other tested antibacterial agents.

Conclusion. The factors influencing the microbial species diversity in wound exudate, feces and feed were reported. Determination of *Enterobacteriaceae* antibiotic resistance profiles will allow for the rotation of antibacterial drugs on the studied livestock farms.

Keywords: microbial communities, antibiotic resistance, bacterial species diversity, cattle, environmental pathogens

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Видовое разнообразие микроорганизмов и распространение антибиотикорезистентных энтеробактерий на молочных фермах

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РЕЗЮМЕ

Введение. Бактериальные сообщества существенно влияют на общую производительность сельскохозяйственных предприятий, от них зависит здоровье животных, производство молока, качество и безопасность пищевых продуктов. Зоонозные бактерии не только оказывают негативное воздействие на благополучие животных, но и представляют риск для общественного здравоохранения, поэтому мониторинг видового разнообразия микроорганизмов на молочных фермах для определения преобладающих видов возбудителей и профилей антибиотикорезистентности имеет важное значение.

Цель исследования. Изучение видового разнообразия бактериальных сообществ на молочной ферме и мониторинг распространения антибиотикорезистентности у изолятов *Escherichia coli* и *Proteus mirabilis* для своевременной разработки мер по сдерживанию распространения устойчивых к антибактериальным препаратам микроорганизмов.

Материалы и методы. Для достижения поставленной цели проводили идентификацию микроорганизмов методом MALDI-ToF масс-спектрометрии и определяли антибиотикочувствительность выделенных культур с помощью диско-диффузионного метода.

Результаты. Установлено видовое разнообразие микроорганизмов, выделенных из проб экссудата с поверхности ран конечностей крупного рогатого скота, фекалий и образцов корма. Преобладающими микроорганизмами оказались оппортунистические и патогенные *Escherichia coli* и *Proteus mirabilis*, для них определены профили антибиотикорезистентности. Один из изолятов *Escherichia coli* был мультирезистентным, только комбинация амоксициллина и клавулановой кислоты проявила эффективность в подавлении роста данной культуры. Большая доля изолятов *Proteus mirabilis* обладала устойчивостью к препаратам из группы фторхинолонов и чувствительностью ко всем остальным исследованным антибактериальным средствам.

Заключение. Отмечены факторы, влияющие на видовое разнообразие микроорганизмов в раневом экссудате, фекалиях и кормах. Определение профилей антибиотикорезистентности энтеробактерий позволит провести ротацию антибактериальных препаратов в исследованных животноводческих организациях.

Ключевые слова: микробные сообщества, антибиотикорезистентность, видовое разнообразие бактерий, крупный рогатый скот, патогены окружающей среды

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INTRODUCTION

Bacterial communities circulating on dairy farms significantly affect food safety, quality of dairy products, and animal health [1]. "One Health" is critically important for understanding the antibiotic resistance spread; it implies interaction between humans, animals and environment, which is especially important due to the common nature of antimicrobial-resistant bacteria in humans and animals [2]. Half a billion people in the world are engaged in animal husbandry and are directly exposed to zoonotic microorganisms [3]. Residual amounts of antimicrobials and antibiotic-resistant pathogens are often found in animal waste and pollute the soil environment and wastewater. *Escherichia coli* serves as a reservoir for multiple antibiotic resistance determinants, which can transmit to animals and humans through various transmission routes: direct animal contact, products of animal origin, and environmental exposure [4]. *Proteus mirabilis* is an opportunistic pathogenic microorganism of the *Enterobacteriaceae* family that causes inflammatory diseases of the skin, respiratory tract, urinary tract and gastrointestinal tract. After *E. coli*, it is the most common opportunistic and zoonotic bacterium found in various animals such as chickens, ducks, turtles, cattle and other domestic animals [5]. *P. mirabilis* is found in various environments: wastewater, soil, and gastrointestinal tract of animals and humans [6]. Failure to comply with the recommendations for prescribing antimicrobials in animal husbandry contributes to the spread of antibiotic resistance.

The relevance of this work is determined by the fact that studying the composition of bacterial communities circulating in livestock facilities will allow identifying priority microorganisms affecting animal health. The novelty of the research lies in acquiring new data on the composition of the microbiota of cattle feed, wound exudate and feces and identifying the prevalence of *Enterobacteriaceae* antibiotic resistance in agricultural organizations across the Sverdlovsk Oblast.

The aim of the study was to examine the species diversity of bacterial communities, as well as to monitor the spread of antibiotic-resistant enterobacteria (*E. coli* and *P. mirabilis*) on dairy farms.

MATERIALS AND METHODS

The research was conducted in 2023–2024 at the Department of Animal Genomics and Selection of Ural Federal Agrarian Scientific Research Center, Ural Branch of the Russian Academy of Sciences and at the laboratory of Quality Med LLC (Ekaterinburg). The activities were carried out in four agricultural organizations in the Sverdlovsk Oblast that are engaged in Holstein cattle breeding. A total of 61 samples were collected: exudate from the surfaces of limb wounds (25), feces (22), and feed samples (14).

Fecal samples from cows were collected with a swab probe into the tubes with modified Cary-Blair medium, which is specifically designed for intestinal pathogen transportation and their viability preservation (FecalSwab™, Copan, Italy). Samples of the remaining biological materials were placed in tubes with Amies transport medium (ESwab®, Copan, Italy).

In the laboratory of Quality Med LLC, the quadrant streaking method was used to inoculate 10 µL of the biomaterial suspension with a sterile calibrated loop onto the following nutrient media: Columbia agar (Bio-Rad Laboratories, Inc., France) with 5% defibrinated sheep blood (E&O Laboratories Ltd., Scotland); Ploskirev agar

(State Research Center for Applied Microbiology and Biotechnology, Russia); GRM-agar (State Research Center for Applied Microbiology and Biotechnology, Russia); UriSelect 4 chromogenic medium (Bio-Rad Laboratories, Inc., France); Sabouraud agar with 2% glucose and chloramphenicol (SIFIN diagnostics GmbH, Germany). The inoculated Petri dishes were transferred into the thermostat at (37 ± 1) °C and incubated for 24 hours.

The grown colonies were identified by MALDI-ToF mass spectrometry using a Vitek® MS instrument (bioMérieux, France). For this purpose, the bacterial biomass was applied to a slide spot, covered with 1 µL of matrix solution (*α*-cyano-3-hydroxycinnamic acid), air-dried at room temperature, and analyzed by mass spectrometry to acquire ribosomal protein spectra. The data were compared against reference databases using MYLA® software (bioMérieux, France). Genus and species identification of the isolates was performed with semi-quantitative and quantitative characterization (CFU/gram and CFU/sample).

Antibiotic susceptibility was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard method, using Mueller – Hinton agar (Bio-Rad Laboratories, Inc., France) and antibiotic discs (Bio-Rad Laboratories, Inc., France) impregnated with predefined drug concentrations (Table). The ADAGIO automatic analyzer (Bio-Rad Laboratories, Inc., France) was used to read the antibiograms. When interpreting the susceptibility categories, the EUCAST criteria were applied (E. A. Elshafiee, S. M. Nader, S. M. Dorgham, D. A. Hamza; version 12.0, effective from 01.01.2022). Despite the fact that, according to Order No. 771 of the Ministry of Agriculture of the Russian Federation of 18 November 2021, ceftazidime, cefepime, cefotaxime, ceftriaxone, and cefoperazone are prohibited for veterinary use, many of these antibacterial substances were previously included in the medicines used in the inflammatory diseases therapy in cattle, and therefore these drugs were also used to evaluate the antibiotic resistance profile of *E. coli* and *P. mirabilis* isolates.

Table
Antibacterial drugs used for assessing the antibiotic susceptibility of microorganisms

Drug	Group of antibiotics	Active substance concentration, µg
Cefixime	third-generation cephalosporins	5
Cefpodoxime Ceftazidime	third-generation cephalosporins	10
Cefepime Cefotaxime Cefuroxime Ceftriaxone	second-, third-, fourth-generation cephalosporins	30
Cefoperazone	third-generation cephalosporins	75
Marbofloxacin Enrofloxacin Levofloxacin Ciprofloxacin Norfloxacin	fluoroquinolones	5
Gentamicin	aminoglycosides	10
Amoxicillin	semi-synthetic penicillins	30
Amoxicillin / Clavulanic acid	semi-synthetic penicillins / beta-lactamase inhibitors	20/10

RESULTS AND DISCUSSION

A total of 189 isolates were identified using MALDI-ToF method.

Among opportunistic and pathogenic microorganisms, the largest proportion in the tested wound exudate samples (n = 86) was taken by (Fig. 1): *P. mirabilis* (9.30%), *E. coli* and *Trueperella pyogenes* (8.14% each), *Bacteroides pyogenes* (6.98%), *Aerococcus viridans* and *Prevotella melaninogenica* (5.81% each), *Aeromonas hydrophila* and *Candida catenulata* (4.65% each), *Bacillus altitudinis/pumilus*, *Mannheimia haemolytica*, *Streptococcus agalactiae* (3.49%).

According to S. C. Liegenfeld et al. [7], the following microorganisms are most often detected in the infected wound microbiota: gram-negative organisms – *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterobacteriaceae*, *E. coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter* spp., *Proteus* spp. and *Bacteroides* spp.; gram-positive organisms – *Staphylococcus aureus*, *Streptococcus* spp., *Enterococcus* spp., *Micrococcus* spp., *Corynebacterium* spp., *Streptococcus pyogenes*, *Corynebacterium diphtheriae* and coagulase-negative staphylococci. The information on the species diversity of wound microflora published by the authors, therefore, differs from our study results.

Prevalence of *P. mirabilis* in animal food products and by-products is understudied [8]. *P. mirabilis*, as well as *E. coli* detections in wound exudate are most likely associated with fecal contamination of the wound surface. *T. pyogenes* spp. bacteria are part of the microbiota of the skin and mucous membranes of the upper respiratory tract, gastrointestinal tract, and genitourinary tract of animals and they are opportunistic microorganisms. They cause various purulent infections such as metritis, mastitis, pneumonia and abscesses leading to significant economic damage to the livestock production [9]. *B. pyogenes* is a representative of the oral microbiota of cats and dogs, the bites of these animals are the main risk factors of human infection. This bacterium can cause a number of inflammatory diseases, including skin and soft tissue infections, osteomyelitis, metritis, and liver abscesses [10, 11]. Anaerobic bacterium *P. melaninogenica*, detected during the study, is involved in the footrot development and progression in cattle [12]. Representatives of the phylum *Aeromonas* cause diarrhea-associated diseases in piglets and pigs, colts and horses; abortions and reproductive diseases in mares, septic arthritis in colts, septicemia in dogs, mastitis in cows, polyarthritis in calves; and *A. hydrophila* has been identified as the only source of infection in bovine wounds [13]. *S. agalactiae* is a microorganism capable of inducing chronic mastitis in cows. Moreover, streptococcus of this species can colonize the gastrointestinal tract of dairy cows, and in case the wound surfaces on the limbs are contaminated with the feces, the microorganism can be detected in the wound exudate [14].

A significantly lower species diversity of opportunistic and pathogenic microorganisms was identified in bovine fecal samples (n = 59): *E. coli* – 28.81%, *Aspergillus niger* complex – 15.25%, *Bacillus licheniformis* – 8.47%, *P. mirabilis* and *Enterococcus hirae* – 6.78% each, other representatives of the fungal microbiota were reported (Fig. 2).

A decrease in the diversity of the fecal microbiota may be associated with an increase in concentrated feed in the diet [15]. A high-concentrate diet reduces the acetate-to-propionate ratio and rumen pH, adversely

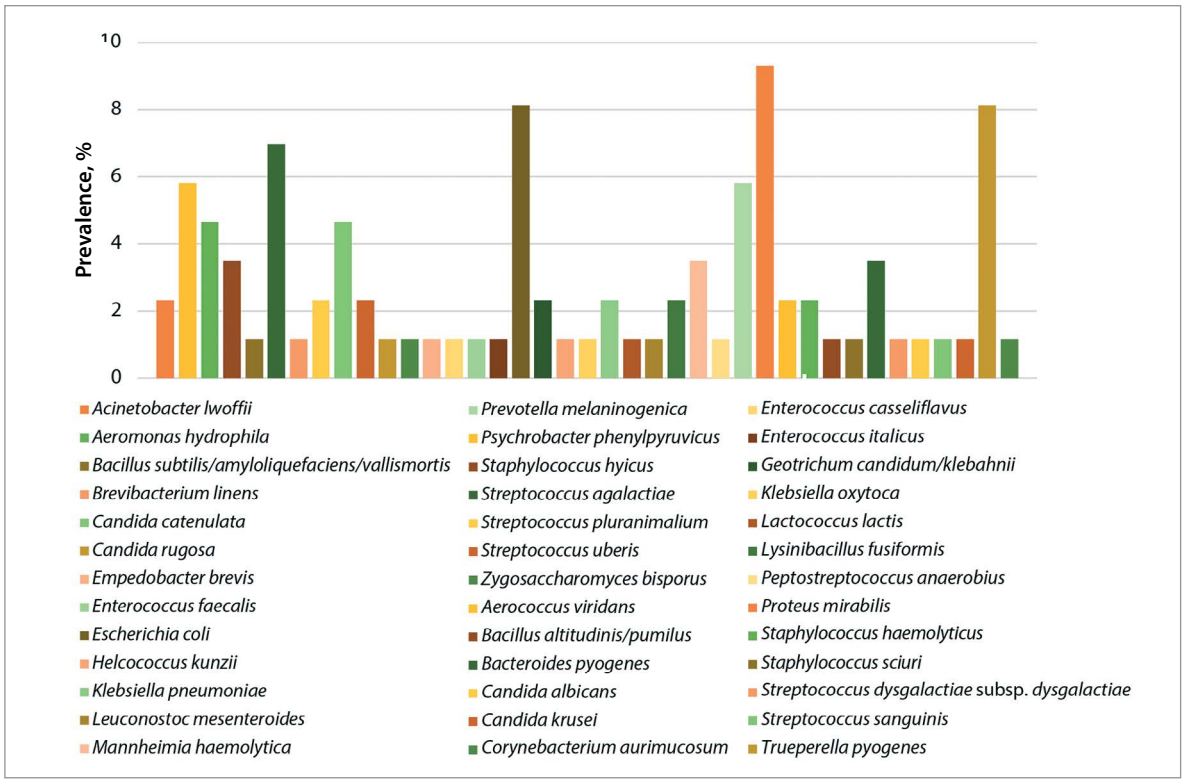

Fig. 1. Species diversity of bacterial communities isolated from wound exudates of cattle (n = 86)

affecting overall health and performance of cattle [16]. Against this background, dysbiosis develops, which can lead to the increased populations of specific bacteria, such as opportunistic *E. coli* [16], whose predominance in the fecal microbiota of cattle was identified in our studies. Environmental contamination with fecal *E. coli* increases the risk of coliform mastitis in cows [17] and inflammatory diseases of the reproductive system [18].

In the feed samples (silage, haylage, and compound feed, *n* = 44) the predominant opportunistic and pathogenic species were the following: *Bacillus altitudinis/pumilus* – 9.09%, *Aspergillus niger* complex, *Bacillus cereus*, *Candida krusei*, *Candida rugosa*, and *P. mirabilis* – 6.82% each (Fig. 3).

The microorganisms typically found in silage include: *Bacillus pumilus*, *B. licheniformis*, *B. coagulans*, *B. sphaericus*, and *B. cereus*. Emergence of *B. cereus* spores in the silage is inevitable when the proper feed harvesting and storing technologies are violated. *B. cereus* spores pass through the gastrointestinal tract of cattle unchanged and are excreted with the feces; during milking, they can be transferred to the raw milk via contaminated nipple surfaces [19]. Presence of *Candida* spp., in particular *C. krusei*, in feed samples is an adverse indicator, since these

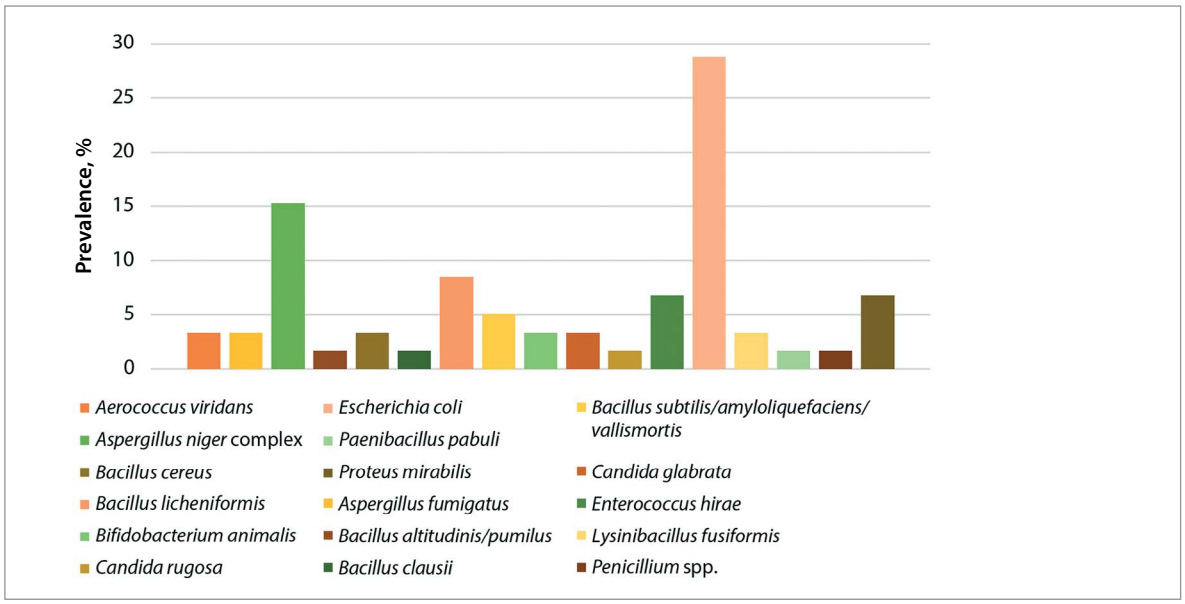

Fig. 2. Fecal microbiota species diversity in cattle (n = 59)

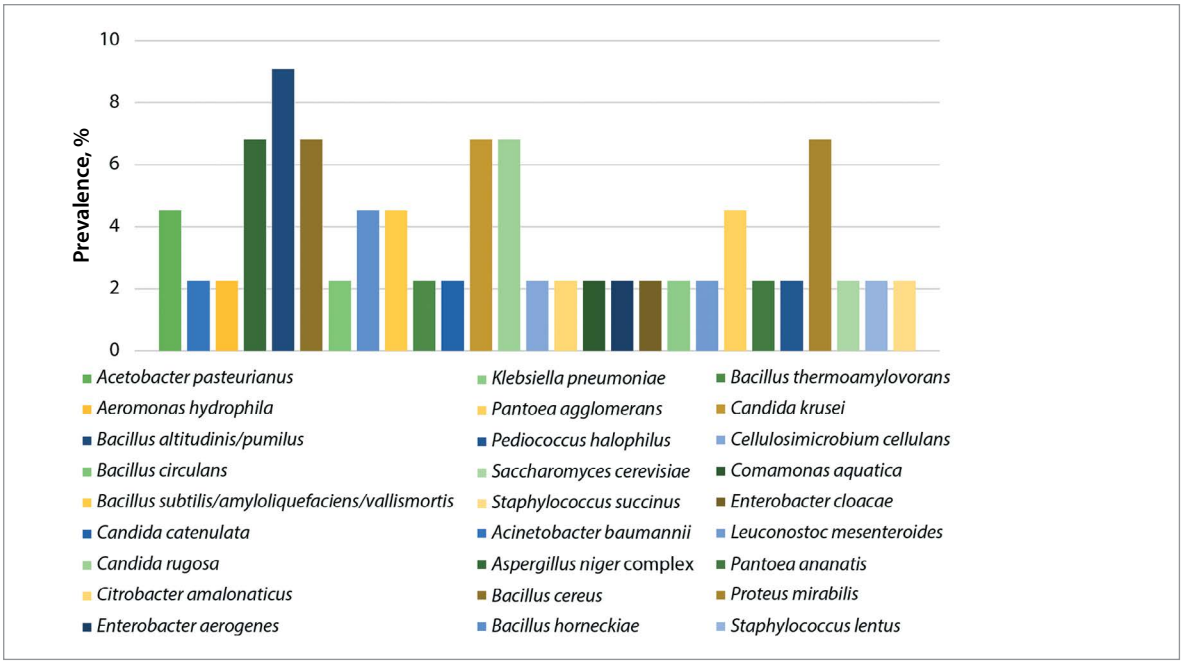

Fig. 3. Species diversity of microorganisms isolated from feed samples (n = 44)

microscopic fungi can induce mycotic mastitis in cows [20]. During our study, *A. niger* was detected in silage samples, which can have both positive and negative effects on cows' health. This aspergillus species produces *β*-glucosidase, an enzyme that breaks down cyanogenic glycosides (toxic to cattle) thus reducing the cyanide poisoning risk and improving the silage quality [21]. At the same time, aspergillosis in cattle, especially caused by *A. niger*, can lead to mycotic abortions and mastitis [22]. Detection of pathogenic microorganisms in the feed samples indicates the need to develop and implement measures aimed at improving the technological processes of feed harvesting, storage and quality control in the examined livestock raising institutions.

E. coli and *P. mirabilis* isolates were selected to determine the antibiotic susceptibility, since these microorganisms proved to be predominant in almost all types of tested biological materials. Moreover, they are important in inducing diseases in both animals and humans.

The disk diffusion test results demonstrated that *E. coli* cultures (*n* = 17) were resistant to fluoroquinolones (35.29%) and cephalosporins (11.76%). At the same time, 80.0% of *Escherichia coli* isolates recovered from feces were sensitive to cefuroxime only after the increased exposure. The highest isolates' resistance was detected to amoxicillin – 41.18%, but their resistance to amoxicillin / clavulanic acid decreased by 29.42%. Resistance to gentamicin was found in 17.65% of the isolates (Fig. 4).

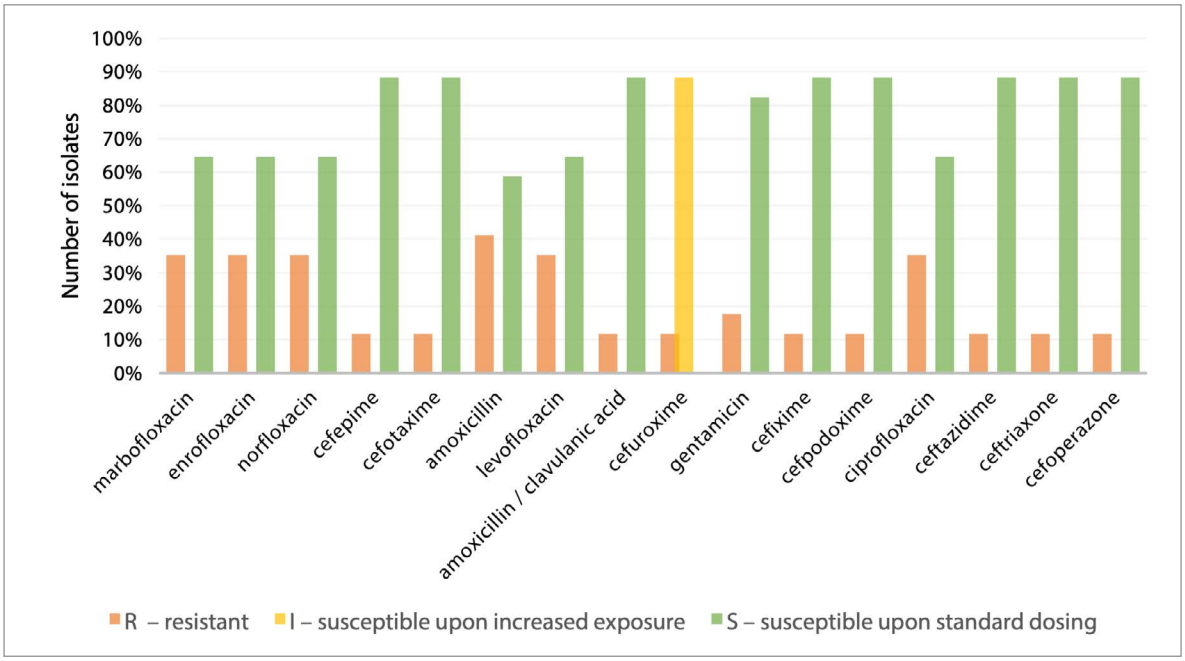

Fig. 4. Resistance of E. coli isolates to antibacterial drugs (n = 17)

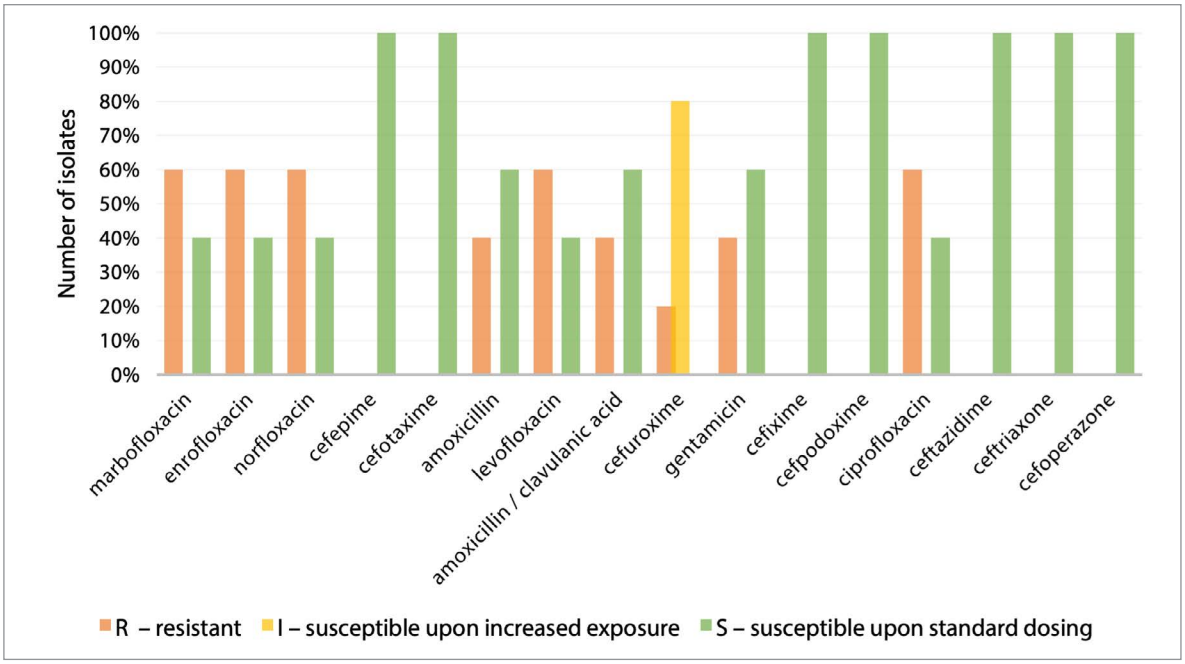

Fig. 5. Resistance of P. mirabilis isolates to antibacterial drugs (n = 10)

The multidrug-resistant phenotype, which is defined as resistance to at least one agent from three or more antibiotic classes, was identified in one *E. coli* isolate. A combination of amoxicillin and clavulanic acid proved effective in suppressing this isolate's growth. Among the multidrug-resistant pathogens widespread on dairy farms, *E. coli* is of particular concern, as some strains can cause foodborne infections in humans [4]. During R. Manishimwe et al. experiments [23], *E. coli* isolates demonstrated resistance to tetracycline (8.2%), ceftriaxone (56.8%), ciprofloxacin (77.3%) and a combination of nalidixic acid and ciprofloxacin (54.5%); that is, the frequency of resistant *E. coli* isolate occurrence was significantly higher than that detected in our studies.

All *E. coli* isolates recovered from wound exudate samples, with the exception of one resistant to amoxicillin, were found to be sensitive to all tested antibiotics. Alharbi N. S. et al. revealed that more than 50% of *E. coli* isolates recovered from wound discharge were resistant to cefazolin, ampicillin, cefuroxime, ciprofloxacin, mezlocillin, moxifloxacin, piperacillin, and tetracycline; 70% of the isolates produced extended-spectrum beta-lactamases [24].

Resistance to fluoroquinolones was observed in 60.0% of *P. mirabilis* isolates (n = 10) recovered from the wound exudate samples (Fig. 5). All isolates were susceptible to drugs of the cephalosporin group, with the exception of cefuroxime, to which 80% of isolates demonstrated susceptibility only upon the increased exposure. Resistance to amoxicillin and its combination with clavulanic acid was reported in 40% of the isolates recovered from the feces and wound exudate; 40% of isolates recovered from the wound discharge were resistant to gentamicin. In general, it is worth noting that the drugs of the cephalosporin group are effective against *P. mirailis*.

Previous studies have revealed that high prevalence of *P. mirabilis* strains resistant to penicillins, cephalosporins, and sulfonamides in chickens is a direct consequence of the use of antimicrobials in poultry farming [25, 26]. The spread of antibiotic-resistant *P. mirabilis* in food-producing animals and in the environment of the agricultural establishments is an urgent public health problem. Transmission of *P. mirabilis* of multidrug resistant phenotype from animals to humans through the consumption of contaminated food or through close contact with animals has already been described [8, 25].

During the studies, the species diversity of bacteria isolated from exudate samples collected from the wound surfaces on cattle limbs, feces and feed was established using the MALDI-ToF method. The data obtained can be used at the agricultural establishments to prescribe rational antibiotic therapy for both wound infections and other inflammatory diseases associated with these pathogens.

CONCLUSION

As a result of the studies, a significantly greater species diversity was revealed in the samples of wound exudate, which may mainly be associated with fecal contamination of limb wounds. The fecal microbiota was characterized by a lower species composition, which may be due to the occurrence of dysbiosis resulted from the increased proportion of the concentrated feed in the diet of cattle, while the prevalence of opportunistic *E. coli* (28.81%) was found in fecal samples. The predominance of pathogenic *A. niger*, *B. cereus*, and *C. krusei* species in feed samples indicates the need to change the technological processes of feed harvesting, storing, and quality control in the studied livestock organizations.

The antibiotic resistance profiles of *E. coli* and *P. mirabilis* were established. In *E. coli* cultures isolated from feces, the resistance was mainly found to drugs from the groups of fluoroquinolones (35.29%) and cephalosporins (11.76%). Notably, nearly all *Escherichia* isolates recovered from wound exudates demonstrated susceptibility to all tested antimicrobial agents. One *E. coli* isolate revealed a multidrug-resistant phenotype, and a combination of amoxicillin and clavulanic acid proved effective in suppressing its growth. It was found that almost all *P. mirabilis* isolates were resistant to antibacterial drugs

from the group of fluoroquinolones, while the drugs from the group of cephalosporins proved effective against *P. mirabilis*. To prevent further growth of antimicrobial resistance, rotational use of antibacterial drugs on dairy farms should be implemented according to the identified *Enterobacteriaceae* resistance profiles.

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Identification and some biological characteristics of *Bacillus* strains isolated from poultry large intestine

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ABSTRACT

Introduction. Censuses of phylogenetic diversity of bacteria colonizing the intestinal tract of clinically healthy poultry conducted over the past decade indicate that up to 60% of genera present in the gut microbiome contain spore-forming bacteria, accounting for 30% of total gut microbiota. Benefits associated with using probiotics containing *Bacillus* spore-forming bacteria have been documented. Analysis of the prevalence of hemolytic and potential biofilm-forming activity, as well as antibiotic resistance in poultry gut spore-forming microbiota is essential for understanding the true role of aerobic spore-formers of the *Bacillus* genus in avian gut microbiome ecology.

Objective. Identification and investigation of biological characteristics (hemolytic activity, potential biofilm-forming capacity and antibiotic resistance) of *Bacillus* bacterial isolates obtained from the large intestine of poultry.

Materials and methods. Spore-forming bacteria were isolated from cecal content through sample heat treatment. Phenotypic identification was performed using API 50CHB biochemical test panels (bioMérieux, France). Hemolytic properties were assessed using Columbia agar (HiMedia Laboratories Pvt Ltd., India) supplemented with 5% sterile defibrinated sheep blood; catalase activity was assessed using 10% hydrogen peroxide according to General Pharmacopoeia Monograph GPM.1.7.2.0012.15; antibiotic sensitivity was assessed with disk diffusion test involving standard antibiotic-impregnated disks (5–30 µg/disk). Biofilm-producing, spore-forming bacteria were tested qualitatively using brain-heart infusion agar (BHI; HiMedia Laboratories Pvt Ltd., India) supplemented with Congo red and 5% sucrose.

Results. It was established that the cecal aerobic spore-forming microbiota in poultry comprised *B. licheniformis, B. subtilis/amyloliquefaciens, B. mycoides, B. megaterium* and *B. cereus*. All tested isolates were catalase-positive and lacked *α*-hemolytic activity. Some isolates demonstrated *β*-hemolytic activity. The overwhelming majority exhibited biofilm-forming phenotypes and showed susceptibility to tested antibiotics.

Conclusion. Vegetative forms of *Bacillus* spore-forming bacteria may potentially persist in or temporarily associate with the complex gut ecosystem. Hemolytically active intestinal isolates cannot be considered safe until the effects of this virulence factor on animals are clarified. These findings provide a basis for selecting candidate *Bacillus* strains for probiotic development.

Keywords: sporobiota, hemolysis, biofilm, antibiotic resistance

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Идентификация и некоторые биологические свойства изолятов бактерий рода *Bacillus*, выделенных из толстого отдела кишечника птицы

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РЕЗЮМЕ

Введение. Проведенная за последнее десятилетие перепись филогенетического разнообразия бактерий, колонизирующих кишечный тракт клинически здоровой птицы, показывает, что до 60% родов, присутствующих в микробиоме кишечника, содержат спорообразующие бактерии, и эти роды составляют 30% от общей кишечной микробиоты. Были зарегистрированы преимущества, связанные с использованием пробиотиков, содержащих спорообразующие бактерии рода *Bacillus*. Анализ широты распространения гемолитической и потенциальной биопленкообразующей активности, а также антибиотикорезистентности у кишечной популяции споробиоты птицы необходим для понимания истинной роли аэробных спорообразователей рода *Bacillus* в экологии кишечного микробиома птицы.

Цель работы. Идентификация и исследование биологических характеристик (гемолитическая активность, потенциальная способность к биопленкообразованию и антибиотикорезистентность) изолятов бактерий рода *Bacillus*, выделенных из толстого отдела кишечника птицы.

Материалы и методы. Выделение спорообразующих бактерий из содержимого слепых отростков толстого кишечника птицы проводили путем прогревания образцов. Фенотипическую идентификацию изолятов осуществляли с использованием биохимических тест-панелей API 50CHB (bioMérieux, Франция). Гемолитические свойства определяли на колумбийском агаре (HiMedia Laboratories Pvt Ltd., Индия) с добавлением 5% стерильной дефибринированной крови барана; каталазную активность – в тесте с 10%-й перекисью водорода по ОФС.1.7.2.0012.15; чувствительность к антибиотикам – дискодиффузионным методом со стандартными дисками, импрегнированными антибиотиками в концентрациях от 5 до 30 µg/disk. Скрининг спорообразующих бактерий – продуцентов биопленки проводили качественным методом на сердечно-мозговом агаре (HiMedia Laboratories Pvt Ltd., Индия) с добавлением индикатора конго красного и 5% сахарозы.

Результаты. Установлено, что кишечная популяция аэробной споробиоты слепых отростков толстого кишечника птицы представлена видами *B. licheniformis, B. subtilis/amyloliquefaciens, B. mycoides, B. megaterium* и *B. cereus.* Все изученные изоляты были каталазоположительными, не обладали *α*-гемолитической активностью. У части изолятов отмечена *β*-гемолитическая активность. Подавляющее большинство изолятов относились к биопленкообразующим фенотипам и проявляли чувствительность к тестируемым антибиотикам.

Заключение. Вегетативные формы спорообразующих бактерий рода *Bacillus* потенциально могут сохраняться в сложной экосистеме кишечника или временно ассоциироваться с ней. Гемолитически активные кишечные изоляты спорообразующих бактерий не могут считаться безопасными до выяснения действия этого фактора вирулентности на организм животных. Результаты исследований могут быть использованы при отборе кандидатных штаммов бактерий рода *Bacillus*, выбранных в качестве пробиотиков.

Ключевые слова: споробиота, гемолиз, биопленка, антибиотикорезистентность

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INTRODUCTION

The gut microbiota is crucial for maintaining host health by supplying essential nutrients, regulating energy balance, modulating immune responses and protecting against pathogens. Consequently, restoring and maintaining its stability with beneficial bacteria – rather than antibiotics – has been proposed as a strategy to prevent adverse effects on gut health [1].

A census of the phylogenetic diversity of bacteria identified in the intestinal tract of healthy poultry conducted over the past decade shows that up to 60% of the genera present in the gut microbiome contain spore-forming bacteria, and these genera account for 30% of the total gut microbiota [2, 3].

In microbiome research, spore-forming bacteria are considered a distinct functional group within the broader microbiota. The term "sporobiota" has been proposed to describe the total collection of these bacteria in a given microbial community [4].

Endospores are formed by members of *Firmicutes*, a large, diverse and morphologically complex bacterial phylum [5]. Within it, species of the genus *Bacillus* (family *Bacillaceae*) have attracted the most scientific attention due to their significant role in modulating the intestinal microbiota [6].

While spore-forming bacteria of the genus *Bacillus* are primarily associated with the soil microbiome [7], their presence in the intestine is attributed to the consumption of feed and water. It has been conventionally assumed that *Bacillus* species enter the intestine exclusively in their spore form [8]. However, accumulating evidence suggests that some *Bacillus* species exhibit a bimodal life cycle, capable of both growth and sporulation not only in the environment but also within the gastrointestinal tract [9, 10].

Spore-forming bacteria of the genus *Bacillus* are widely used in animal feed to promote animal growth and inhibit pathogens [11, 12]. The probiotic mechanisms of these microorganisms involve synthesizing antimicrobial compounds and lactic acid [13], enhancing non-specific immunity [14], and producing enzymes such as amylase, lipase, protease, pectinase and cellulose [15]. Specifically, *Bacillus subtilis* stimulates growth of beneficial gut microbiota, increases intestinal microbiome diversity [16], modulates regulatory systems for intestinal epithelium renewal and immune cell activity [17], and mitigates the adverse effects of various factors, particularly antibiotic therapy [18, 19].

It has also been shown that *B. subtilis* plays an important role in stimulating the development of gut-associated lymphoid tissue and that sporulation of live bacilli is considered critical for this process [20, 21].

Survival and colonization within the digestive tract are essential for the microbiota to exert its physiolog-

Table 1
Hemolytic activity and biofilm-forming capacity of spore-forming bacterial isolates from poultry cecal appendages

Isolate species identification using the standardized API 50CHB system	Total number of isolates	Number of hemolytically active isolates			Number of isolates – potential biofilm producers
		Type of hemolysis			
		α	*β*	*γ*	
B. licheniformis	26	0	4	6	23
B. subtilis/amyloliquefaciens	16	0	3	1	16
B. megaterium	3	0	2	1	2
B. mycoides	5	0	0	0	5
B. cereus	7	0	5	1	5
Unidentified species	11	0	5	4	not tested
Total	68	0	19	13	51

ical functions [22]. For spore-forming bacteria, a key prerequisite for intestinal colonization is the adhesion of spores to the intestinal mucosa and subsequent biofilm formation, a process that enables the transition of planktonic cells to attached forms [21, 23].

The term "biofilm" is used to describe a structured community of bacterial cells embedded in a self-produced polymeric extracellular matrix and adhered to an inert or living surface [24]. This polymeric extracellular matrix is a complex system composed of exopolysaccharides, the TasA protein, lipids, nucleic acids and various heteropolymers, all of which are secreted by microorganisms into the extracellular environment [25]. Studies have shown that the TasA protein and exopolysaccharides are key components of the *Bacillus* biofilm matrix. TasA is an amyloid protein that has been implicated in the formation of the biofilm matrix by various microorganisms, including spore-forming bacteria [26, 27, 28, 29].

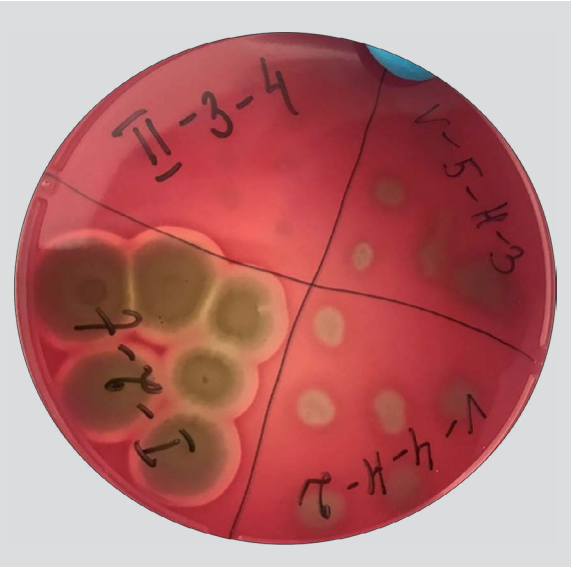

Fig. 1. Hemolytic activity of Bacillus isolates cultured on sheep blood-supplemented SCD agar at (37 ± 1) °C for 24 hours

Bacterial biofilms can protect bacteria from environmental stressors, host immune responses and antimicrobial agents, including antibiotics [30].

The use of Congo red dye to assess bacterial amyloid synthesis, and thus identify mucus or biofilm-forming organisms like *Staphylococcus* sp., was first proposed by D. J. Freeman et al. [31]. This method distinguishes biofilm-forming *Staphylococcus* colonies from non-forming ones based on their morphology and color on Congo red agar. Subsequent studies confirmed the utility of this assay for detecting potential biofilm formation in a broad spectrum of gram-positive and gram-negative microorganisms, including spore-forming bacteria of the genus *Bacillus* [32, 33, 34].

Recent data indicates that *Bacillus* sporulation is more active within biofilms than in planktonic cultures [35].

The growing threat of antibiotic-resistant pathobiont spread has brought to the forefront research focused on assessing the antibiotic resistance of the commensal gut microbiota as potential donors of resistance determinants.

In light of this problem, it has been suggested that spore-forming bacteria may play a significant role in the dissemination and accumulation of antibiotic resistance genes due to their ability to withstand antibiotic treatment [36, 37]. However, the extent of antibiotic resistance and the biofilm-forming capacity among members of the intestinal sporobiota remain largely unknown.

Previous studies on the spread of antibiotic resistance and the biofilm-forming ability of *Bacillus* bacteria were focused on a limited number of species and did not include isolates derived from poultry gut microbiota [38, 39, 40, 41].

The prevalence of hemolytic activity within the intestinal population of aerobic *Bacillus* bacteria remains insufficiently studied. Furthermore, the role of their hemolysins in maintaining intestinal mucosal integrity is unclear, and it has not been established that hemolysin production is harmless to the host. Non-hemolytic (*γ*-hemolytic) strains are generally considered safe for their hosts, while strains exhibiting hemolytic activity are deemed pathogenic [42, 43].

Given the modern understanding of the gut microbiota's role in maintaining gut colonization resistance, this knowledge gap leads to a lack of understanding regarding the true role of aerobic *Bacillus* spore-formers in the poultry gut microbiome and the specificity required for their selection and application as probiotics [44, 45].

The relevance of this work is that the safety assessment of aerobic spore-forming *Bacillus* bacteria, which are widely used in the production of probiotic feed additives, is associated not only with species identification but also with the individual characteristics of the strains.

The novelty of the obtained data lies in the study of specific biological properties of isolates from the ceca of commercial poultry, thereby evaluating the pros-

pects of using *Bacillus* bacteria for developing probiotic feed supplements.

The aim of this work is to investigate the biological properties of *Bacillus* isolates (hemolytic activity, potential for biofilm formation, and antibiotic resistance) obtained from the large intestine of poultry, with the goal of selecting promising strains for use in the biotechnology of probiotic production.

MATERIALS AND METHODS

Spore-forming bacteria used in this study were isolated from samples of the cecal content of clinically healthy poultry obtained from farms in the Moscow Oblast that are free from infectious diseases. Birds were euthanized in the animal facility using CO_2 and subjected to necropsy according to a standard protocol. All animal procedures complied with the ethical standards set by European Convention ETS No. 123.

The ceca were isolated by applying ligatures at the junction with the rectum, excised, placed in separate containers and transported to the laboratory under a cold chain (2–8 °C). Within maximum 30 minutes of obtaining the isolated ceca, the contents of each were extruded into sterile disposable dishes and labeled. The resulting aliquots of each chyme sample were diluted 1:100 with buffered peptone water and resuspended by vigorous mixing to obtain a homogeneous suspension.

To isolate spore-forming bacteria, the aliquots were heated in a water bath at 65 °C for 45 minutes [46].

To obtain isolated colonies, the heated aliquots were streaked onto Tryptic Soy Agar (SCD agar; HiMedia Laboratories Pvt Ltd., India). Cultivation was carried out under aerobic conditions at (37 ± 1) °C. The cultures were examined after 18, 24, and 36 hours of incubation.

Colonies exhibiting morphological characteristics of the *Bacillus* genus were selected and purified by repeated streaking on SCD agar. After 18 and 24 hours of cultivation, following visual assessment of growth and microscopy of gram-stained smears, isolates of spore-forming bacteria were subcultured into Tryptic Soy Broth (TSB; SCD broth; HiMedia Laboratories Pvt Ltd., India), and broth cultures of vegetative cells were used for further work.

Phenotypic characterization. A phenotypic characterization of the spore-forming bacterial isolates was performed based on morphological properties, Gram staining,hemolytic activity, catalase activity, biofilm-forming capacity and antibiotic susceptibility. All tests were performed in duplicate.

Isolate identification. Phenotypic identification of the spore-forming bacterial isolates was conducted using commercial disposable biochemical diagnostic test panels API 50CHB (bioMérieux, France). This test classifies bacterial strains based on their ability to ferment 49 different carbohydrates. Results were analyzed using the APIWEB™ software (bioMérieux, France).

Hemolytic activity test. The hemolytic properties of the spore-forming bacterial isolates were determined using Columbia agar (HiMedia Laboratories Pvt Ltd., India) supplemented with 5% sterile defibrinated sheep blood. Twenty-four-hour cultures of the isolates were stab-inoculated onto the agar surface. The reaction was recorded 24 hours after incubation at (37 ± 1) °C. An isolate was considered *α*-hemolytic if colonies caused a green or brown discoloration of the surrounding medium, *β*-hemolytic if true lysis of erythrocytes resulted in a clear, transparent zone surrounding the colonies, and *γ*-hemolytic (non-hemolytic) if no reaction was observed in the surrounding medium [47].

Catalase test. The catalase activity of bacterial isolates was determined using a 10% hydrogen peroxide solution [48]. The liberation of oxygen, evident by the formation of gas bubbles, indicated catalase production.

Antibiotic susceptibility test. The antibiotic susceptibility of *Bacillus* sp. isolates was tested using the disk diffusion method [49]. Standard antibiotic-impregnated discs (HiMedia Laboratories Pvt Ltd., India) with the following concentrations (5 to 30 µg/disk) were used: ciprofloxacin (Cip 5), rifampicin (Rif 5), enrofloxacin (Ex5), doxycycline (Do10), gentamicin (Gen10), neomycin (N30), cefazolin (Cz30), norfloxacin (Nx10), benzylpenicillin (P100), pefloxacin (Pf5), kanamycin (K30), lincomycin (L15), azithromycin (AZM15), nalidixic acid (NA30), chloramphenicol (C30), oxytetracycline (O30), imipenem (Ipm10), oleandomycin (Ol15), clindamycin (Cd2), clarithromycin (Clr15), oxacillin (Ox1), ampicillin (Amp25).

Eighteen-hour agar cultures of spore-forming bacteria were adjusted to a 0.5 McFarland standard; 250 µL of the culture was swabbed onto the surface of SCD agar plates, and antibiotic discs were dispensed (8 per plate)

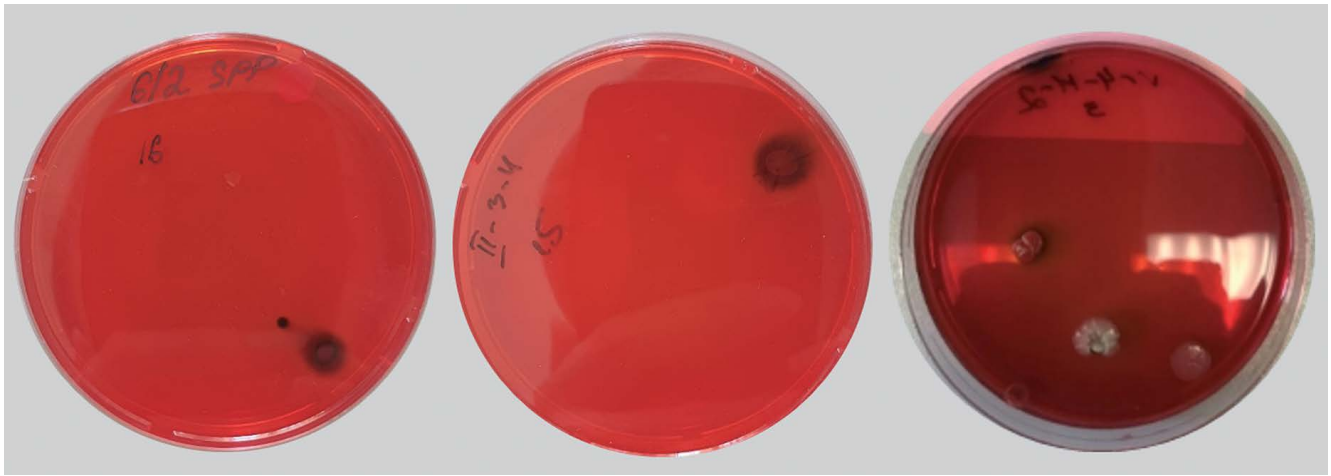

Fig. 2. Bacillus isolates cultured on Congo red-supplemented BHI agar at (37 ± 1) °C for 24 hours

Table 2
Antibiotic sensitivity profiles of 57 spore-forming isolates determined using disk diffusion method

№ п/п	Antibiotic disc type	Number of antibiotic-sensitive isolates				
		B. licheniformis (*n* = 26)	*B. subtilis/ amyloliquefaciens* (*n* = 16)	*B. megaterium* (*n* = 3)	*B. mycoides* (*n* = 5)	*B. cereus* (*n* = 7)
1	Ciprofloxacin (5 µg/disk)	+	+	+	+	+
2	Rifampicin (5 µg/disk)	2	1	1	+	+
3	Enrofloxacin (5 µg/disk)	+	+	+	+	+
4	Doxycycline (10 µg/disk)	+	+	+	+	+
5	Gentamicin (10 µg/disk)	+	+	+	+	+
6	Neomycin (30 µg/disk)	+	+	+	+	+
7	Cefazolin (30 µg/disk)	3	+	1	+	3
8	Norfloxacin (10 µg/disk)	+	+	+	+	+
9	Benzylpenicillin (100 µg/disk)	+	+	+	+	+
10	Pefloxacin (5 µg/disk)	+	+	+	+	+
11	Kanamycin (30 µg/disk)	+	+	+	1	+
12	Lincomycin (15 µg/disk)	8	3	1	+	+
13	Azithromycin (15 µg/disk)	+	2	+	+	+
14	Nalidixic acid (30 µg/disk)	+	+	+	+	+
15	Chloramphenicol (30 µg/disk)	+	+	+	+	+
16	Oxytetracycline (30 µg/disk)	2	2	+	1	1
17	Imipenem (10 µg/disk)	+	+	+	+	+
18	Oleandomycin (15 µg/disk)	+	+	+	+	+
19	Ampicillin (25 µg/disk)	+	+	+	+	+
20	Clindamycin (2 µg/disk)	+	+	+	+	+
21	Clarithromycin (15 µg/disk)	+	+	+	+	+
22	Oxacillin (1 µg/disk)	+	+	+	+	+

using a dispenser (HiMedia Laboratories Pvt Ltd., India). Plates were then incubated at (37 ± 1) °C for 20–24 hours.

Strains were classified as resistant (R) if the inhibition zone diameters for the antibiotics listed in EFSA (European Food Safety Authority) documents exceeded the threshold level for *Bacillus* strains [50]. For antibiotics lacking reliable interpretive criteria, *Bacillus* strains exhibiting inhibition zones of less than 12 mm around the antibiotic disc were considered resistant.

Determination of biofilm producers. Screening of spore-forming bacteria as potential biofilm producers was performed using a qualitative method by detecting extracellular amyloid proteins on Brain Heart Infusion Agar (BHI agar; HiMedia Laboratories Pvt Ltd., India) supplemented with Congo red indicator and 5% sucrose [31].

A 24-hour broth culture of the test isolate was stab-inoculated onto Congo red BHI agar and incubated for 48 hours at (37 ± 1) °C. The color and morphology of the resulting colonies were assessed visually.

The interaction of Congo red with amyloid proteins of the biofilm produces a compound that gives colonies a dark red or dark brown color with a black base. Weak biofilm producers typically remain pink, though some

darkening in the center may be observed. Isolates incapable of forming biofilms produce white or very light pink colonies [51].

RESULTS AND DISCUSSION

During the study, a total of 68 strains of aerobic spore-forming bacteria of the genus *Bacillus* were isolated and identified to the species level.

Phenotypic data confirmed that the isolates obtained from the contents of the ceca of the large intestine of clinically healthy birds belonged to spore-forming bacteria.

The identified isolates of spore-forming bacteria were represented by 5 species: *Bacillus licheniformis, Bacillus subtilis/amyloliquefaciens, Bacillus megaterium, Bacillus cereus* and *Bacillus mucoides*. A number of isolates could not be identified using the standardized API 50CHB system.

Microscopic examination of the isolated cells revealed a diverse collection of rod-shaped bacteria producing endospores of various sizes and shapes.

The colonies of spore-forming bacteria exhibited significant polymorphism. *B. licheniformis* isolates, after 18–24 hours of growth on SCD agar, formed raised, medium-sized colonies colored white or beige, with a flower-like shape.

When cultured on SCD agar, *B. subtilis/amyloliquefaciens* isolates grew into large white, medium-sized colonies with a lighter central point, or rough undulating colonies of a white-cream color with a dry, finely wrinkled structure, slightly raised above the agar surface.

Bacillus megaterium isolates on SCD agar formed sharply defined colonies of a dirty white color.

Morphologically, the colonies of *B. mucoides* and *B. cereus* isolates were identical and appeared as rough, spreading, rhizoid, greyish-white colonies with undulating edges, or as wrinkled, milky-colored colonies with wavy edges.

The isolated spore-forming bacteria were assessed for their safety based on hemolytic activity (Table 1).

All isolates of spore-forming bacteria obtained from the chyme samples of birds' ceca lacked *α*-hemolytic activity. Growth on blood agar revealed a wide zone of hemolysis, characteristic of *β*-hemolytic bacteria, in 14 out of the 57 studied isolates of the species *B. licheniformis, B. subtilis/amyloliquefaciens, B. megaterium,* and *B. cereus* (Fig. 1). It was found that 9 isolates of spore-forming bacteria exhibited *γ*-hemolytic activity.

The majority of the studied isolates of spore-forming microorganisms grown on Congo red BHI agar were classified as potential biofilm producers based on their dark red colonies with a black base, with the exception of 3 isolates of *B. licheniformis,* 1 isolate of *B. megaterium,* and 2 isolates of *B. cereus*, which formed light pink colonies (Fig. 2).

Antibiotic susceptibility tests showed that the majority of the studied isolates of spore-forming bacteria were sensitive to all 22 antibiotics tested (Table 2).

Only 4 isolates of spore-forming bacteria were resistant to rifampicin, 7 – to cefazolin, 12 – to lincomycin, and 6 – to oxytetracycline. Some isolates were resistant to 2 antibiotics, but the majority of isolates exhibited resistance to maximum one antibiotic.

CONCLUSION

Based on the test results, the identified species of spore-forming bacteria of the genus *Bacillus* isolated from the ceca of commercial poultry included: *B. licheniformis, B. subtilis/amyloliquefaciens, B. mycoides, B. megaterium* and *B. cereus*; a number of isolates could not be identified.

All studied isolates were catalase-positive and lacked *α*-hemolytic activity. Some isolates exhibited *β*-hemolytic activity, which precludes their classification as non-pathogenic.

The potential ability of the isolated strains to form biofilms, which indirectly characterizes their capacity to survive in the intestine, was additionally investigated. The vast majority of isolates were classified as potential biofilm-forming phenotypes and demonstrated susceptibility to all 22 tested antibiotics.

Hemolytically active intestinal isolates of spore-forming bacteria cannot be considered safe until the effect of this virulence factor on the animal organism is elucidated.

The research results can be used for the selection of candidate strains of bacteria of the genus *Bacillus* chosen as probiotics

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Antimicrobial resistance of *Salmonella* spp. detected in animal products in 2022–2024

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ABSTRACT

Introduction. Although antibiotics represent one of humanity's greatest discoveries, their improper use can cause significant harm and lead to severe consequences.

Objective. Testing of animal product samples followed by *Salmonella* spp. isolation, typing, identification and assessment of their antimicrobial resistance dynamics.

Materials and methods. The study was carried out at the Department for Microbiological Testing of the Vladimir Testing Laboratory of the Federal Centre for Animal Health. The disc diffusion test was used to determine bacteria resistance to antibiotics. The sizes of the microorganism growth inhibition zones were interpreted according to the Russian recommendations "Determination of the sensitivity of microorganisms to antimicrobial drugs" (IACMAC, version 2025-01), prepared on the basis of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations and using CLSI M100 standard. PETSAL® monovalent and polyvalent O- and H-sera (The Saint Petersburg Scientific Research Institute of Vaccines and Serums of the FMBA of Russia) were used for serological identification. Antimicrobial resistance genes (blaCTX-M, blaOXA10, blaDHA, blaDES, blaKPC, blaOXA48-like, blaNDM, blaVIM) were identified by real-time polymerase chain reaction using the RESISTOM test systems ("LITECH" Co. Ltd., Russia).

Results. Forty-two *Salmonella* spp. isolates were recovered from animal product samples in 2022–2024. *S. Enteritidis* was the most frequently isolated serovar, and *Salmonella* spp. were predominantly isolated from poultry meat products. The detected isolates demonstrated maximum resistance to benzylpenicillin, erythromycin, norfloxacin, and tetracycline. Most of the isolates showed multiple resistance to several antimicrobials. Increased resistance to cephalosporins, fluoroquinolones, tetracyclines, aminoglycosides, chloramphenicol/levomycetin and sulfamethoxazole/trimethoprim was demonstrated for *Salmonella* spp. isolates. No antimicrobial resistance genes were detected when the isolates were tested with real-time polymerase chain reaction.

Conclusion. The study demonstrated widespread antimicrobial resistance, including multiple resistance, among *Salmonella* spp. isolates detected in animal products in 2022–2024.

Keywords: *Salmonella* spp., antimicrobial resistance, antimicrobial resistance genes, antibiotics, antimicrobial susceptibility, real-time polymerase chain reaction

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Антибиотикорезистентность бактерий рода *Salmonella*, выявленных в продукции животного происхождения в 2022–2024 гг.

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РЕЗЮМЕ

Введение. Несмотря на тот факт, что антибиотики – величайшее открытие человечества, они могут нанести огромный ущерб и привести к серьезным последствиям при нецелесообразном использовании.

Цель исследования. Исследование образцов животноводческой продукции с последующим выделением изолятов бактерий рода *Salmonella*, их типированием, определением и оценкой динамики развития антибиотикорезистентности.

Материалы и методы. Работу выполняли на базе отдела микробиологических исследований Владимирской испытательной лаборатории ФГБУ «ВНИИЗЖ». Для определения устойчивости бактерий к антибиотикам применяли диско-диффузионный метод. Значения зон задержки роста микроорганизмов интерпретировались согласно российским рекомендациям «Определение чувствительности микроорганизмов к антимикробным препаратам» (МАКМАХ, версия 2025-01), подготовленным на основе рекомендаций Европейского комитета по определению чувствительности к антимикробным препаратам (EUCAST) и с использованием стандарта CLSI M100. Серологическую идентификацию проводили с помощью моно- и поливалентных О- и Н-сывороток «ПЕТСАЛ»® (ФГУП СПбНИИВС ФМБА России). Гены антибиотикорезистентности (blaCTX-M, blaOXA10, blaDHA, blaGES, blaKPC, blaOXA48-like, blaNDM, blaVIM) определяли методом полимеразной цепной реакции в режиме реального времени с применением тест-систем серии

«РЕЗИСТОМ» (ООО НПФ «Литех», Россия).

Результаты. При исследовании образцов продукции животного происхождения в 2022–2024 гг. выявлено 42 изолята бактерий рода *Salmonella*. Наиболее часто детектируемый изолят – *S. Enteritidis*, а превалирующий продукт, в котором обнаруживали бактерии рода *Salmonella*, – продукция из мяса птицы. Выявленные изоляты имели максимальную резистентность к бензилпенициллину, эритромицину, норфлоксацину и тетрациклину. Большинство из них проявили множественную устойчивость сразу к нескольким антимикробным препаратам. Отмечен рост резистентности к цефалоспоринам, фторхинолонам, тетрациклинам, аминогликозидам, хлорамфениколу/левомицетину и сульфаметоксазолу/триметоприму. При исследовании методом полимеразной цепной реакции в режиме реального времени гены антибиотикорезистентности не обнаружены.

Заключение. Показано значительное распространение устойчивости к антимикробным препаратам, в том числе множественной, среди изолятов бактерий рода *Salmonella*, выявленных в продукции животного происхождения в 2022–2024 гг.

Ключевые слова: бактерии рода *Salmonella*, антибиотикорезистентность, гены антибиотикорезистентности, антибиотики, чувствительность к антимикробным препаратам, полимеразная цепная реакция в режиме реального времени

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INTRODUCTION

In 2024, the World Health Organization (WHO) published a list of 24 antibiotic-resistant bacterial pathogens. Listed gram-negative bacteria, including *Salmonella* spp., are of great concern due to their resistance to the latest generation of antimicrobials [1].

Antimicrobial resistance is a global challenge requiring a coordinated approach and actions at all levels: human health, agriculture, environment management, food production (WHO classified antimicrobial resistance among the top ten global public health threats in 2019) [2, 3, 4].

Incorrect and uncontrolled use of antibiotics is the main factor leading to the antimicrobial resistance development. Agricultural workers are particularly at risk given the fact that up to 50–80% of all antibiotics are used in the agriculture sector [5, 6].

Considering that human, animal, plant and environmental health, including ecosystems, are closely interrelated and interdependent, the WHO, FAO (Food and Agriculture Organization of the United Nations), UNEP (United Nations Environment Programme) and WOAH (World Organization for Animal Health) have joined efforts to combat antimicrobial resistance within the framework of the One Health concept. The WHO established the Global Antimicrobial Resistance and Use Surveillance System (GLASS) providing standardized methods and in 2001 published the WHO Global Strategy for Containment of Antimicrobial Resistance. In 2022 the WHO launched the Global Genomic Surveillance Strategy for Pathogens with Pandemic and Epidemic Potential (2022–2032) [7].

The FAO has called for halting the use of antimicrobials for the infection prevention and as growth promoters in livestock and aquaculture sectors as a part of the combating antimicrobial resistance. Additionally, the FAO/WHO Codex Alimentarius Commission on Food Standards adopted strict standards on the maximum permissible limits for medicinal product residues.

Moreover, world leaders adopted a political declaration at the 79th session of the United Nations General Assembly, in which they committed to achieving specific targets to combat antimicrobial resistance.

In Russia, antimicrobial resistance is also regulated by legislation. In 2017, the National Strategy of the Russian Federation for Preventing the Spread of Antimicrobial Resistance in the Russian Federation to 2030 outlining the official policy aimed at limiting the spread of antimicrobial resistance was approved by Russian Federation Government Order No. 2045-r. In 2024, the Action Plan for the Implementation of the Strategy for the Prevention of the Spread of Antimicrobial Resistance in the Russian Federation by 2030 addressing the key areas such as regulatory measures, public awareness, systemic monitoring and other aspects related to antimicrobial resistance for 2025–2030 was approved by Russian Government Order No. 2214-r.

Therewith, list of veterinary medicinal products restricted for therapeutic use (approved by Order No. 771 of the Ministry of Agriculture of the Russian Federation of 18 November 2021) has been put in effect since 2022, and procedure for prescribing veterinary medicinal products

and the list of prescription veterinary medicinal products (approved by Order No. 776 of the Russian Federation Ministry of Agriculture of 2 November 2022) have entered into force since 2025.

In 2024, the first BRICS International Conference on Antimicrobial Resistance was held in Moscow.

For the purpose of medicinal product residue control in food products, the following amendments were made to the Technical Regulations of the Customs Union by Decision No. 70 of the Council of the Eurasian Economic Commission of 23 June 2023: permissible limits for 75 medicinal products were established, as well as new requirements for providing information on used veterinary medicinal products were laid down.

Continuous monitoring for changes in the antimicrobial susceptibility of pathogens is one of the measures for combating the antimicrobial resistance spread. There is an on-line antimicrobial resistance map platform and other antimicrobial resistance services: web products designed for antimicrobial resistance surveillance for analysis of the data on antimicrobial resistance in Russia.

Salmonella spp. are one of the four main causes of diarrheal diseases in the world. Annually, diseases caused exclusively by *Salmonella* spp. claim the lives of more than 200,000 people worldwide [8, 9].

Over the last 10 years, salmonellosis has remained a significant concern in the Russian Federation due to the existing risks of infection in intensively developing agricultural sector. Thus, according to the Federal Service for the Oversight of Consumer Protection and Welfare (Rospotrebnadzor) reports, salmonellosis incidence per 100,000 population was as follows in the Russian Federation: 24.68 cases in 2024; 21.45 cases in 2023; 17.10 cases in 2022 and 13.61 cases in 2021[1, 2, 3, 4].

The most frequently detected serovars causing diseases in all countries are: *S. Enteritidis, S. Typhimurium* and *S. Infantis*. The frequency of other serovars detection depends on the region [10, 11].

Currently, there is a rise in antibiotic-resistant infections, including those caused by *Salmonella*. At the same time, antimicrobial-resistant *Salmonella* pose a significant threat to the human and animal life due to their widespread prevalence and ability to contaminate water sources, among other transmission routes. Food products are the main factor in *Salmonella* transmission [12, 13, 14].

In the view of the above, the study was aimed at testing samples of animal products from three Central Russian regions (Vladimir, Kostroma and Ivanovo Oblasts), followed by *Salmonella* isolation, identification, typing and their assessment for antimicrobial resistance dynamics in 2022–2024.

MATERIALS AND METHODS

Tests were carried out at the Department for Microbiological Testing of the Vladimir Testing Laboratory, Federal Centre for Animal Health. Forty-two *Salmonella* isolates recovered from animal products in 2022–2024 were used for tests.

Reagents and nutrient media: buffered peptone water (HiMedia Laboratories Pvt Ltd., India), Rappaport – Vassiliadis magnesium medium (RVS-broth; Merck KGaA, Germany), selenite broth (Merck KGaA, Germany), tryptic soy agar (TSA; Scharlab S.L., Spain), xylose lysine deoxycholate agar (XLD-agar; State Research Center for Applied Microbiology and Biotechnology, Russia), bismuth sulphite agar (Merck KGaA, Germany), Mueller – Hinton agar (State Research Center for Applied Microbiology and Biotechnology, Russia).

Microbiological tests were performed according to GOST 31659-2012 "Food products. Method for the detection of *Salmonella* spp.". The weighted portion of the product (25 g) was placed in a sterile bag containing 225 cm^3 of buffered peptone water, homogenized for 1 min and then incubated at 37 °C for 18–20 hours.

The prepared cultures (1 mL) were re-inoculated into selective enrichment media: RVS-broth (10 mL) and selenite broth (10 mL) and incubated at temperature of (41.5 ± 1.0) °C and 37 °C, respectively, for 24 hours. Then, the material from each tube was re-inoculated by streaking using bacteriological loop according to GOST 26670-91 "Food products. Methods for cultivation of microorganisms" onto two selective agar media: XLD-agar and bismuth sulfite agar, and then incubated at 37 °C for (24 ± 3) hours.

To identify selected colonies demonstrating growth characteristic of *Salmonella* spp. and to prepare isolated colonies the materials were re-inoculated and then cultivated onto dried TSA supplemented with yeast extract at a temperature of 37 °C for (24 ± 3) hours.

The grown colonies were typed as *Salmonella* with API 20E biochemical tests (bioMérieux, France) and enzyme-linked immunosorbent assay using a Mini Vidas analyser (bioMérieux, France).

Serological identification. During the study, *Salmonella* spp. isolates recovered using slide agglutination test with PETSAL® dry diagnostic adsorbed mono- and polyvalent O- and H-sera (The Saint Petersburg Scientific Research Institute of Vaccines and Serums of the FMBA of Russia) were serologically identified. The serological variant of the strain was identified using serological formula in accordance with Kauffman – White scheme according to MG 4.2.4070-24 "Laboratory diagnosis of salmonellosis, detection of *Salmonella* in food products and environmental objects: methodical guidelines" (approved by the Chief Medical Officer of the Russian Federation on 27 September 2024).

Determination of antimicrobial resistance. The recovered *Salmonella* spp. isolates were tested for their susceptibility to antimicrobials with disc diffusion test according to MG 4.2.1890-04 "Determination of the susceptibility of microorganisms to antibacterial drugs: methodical guidelines".

Antibiotics (paper disks produced by the Saint Petersburg Pasteur Institute, Russia): azithromycin 15 μg; amikacin 30 μg; amoxicillin 20 μg; ampicillin/sulbactam 10 μg; benzylpenicillin 10 U/6 μg; gentamicin 10 μg; doxycycline 30 μg; imipenem 10 μg; kanamycin 30 μg; levofloxacin 5 μg; meropenem 10 μg; norfloxacin 10 μg; sulfamethoxazole/trimethoprim 23.75/1.25 μg; streptomycin 10 μg; tetracycline 30 μg; chloramphenicol/levomycetin 30 μg; cefazolin 30 μg; cefotaxime 30 μg; cefuroxime 30 μg; ciprofloxacin 5 μg; erythromycin 15 μg.

Bacterial suspension (optical density – 0.5 according to the McFarland standard) prepared from day-old cultures of *Salmonella* spp. isolates grown on TSA was used

[1] https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=21796 (in Russ.)
[2] https://www.rospotrebnadzor.ru/upload/iblock/b50/t4kqksh4b12a2iwjnha29922vu7naki5/GD-SEB.pdf (in Russ.)
[3] https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=27779 (in Russ.)
[4] https://www.rospotrebnadzor.ru/upload/iblock/b8a/u6lsxjabw032jkdf837nlaezxu3ue09m/GD_SEB.pdf (in Russ.)

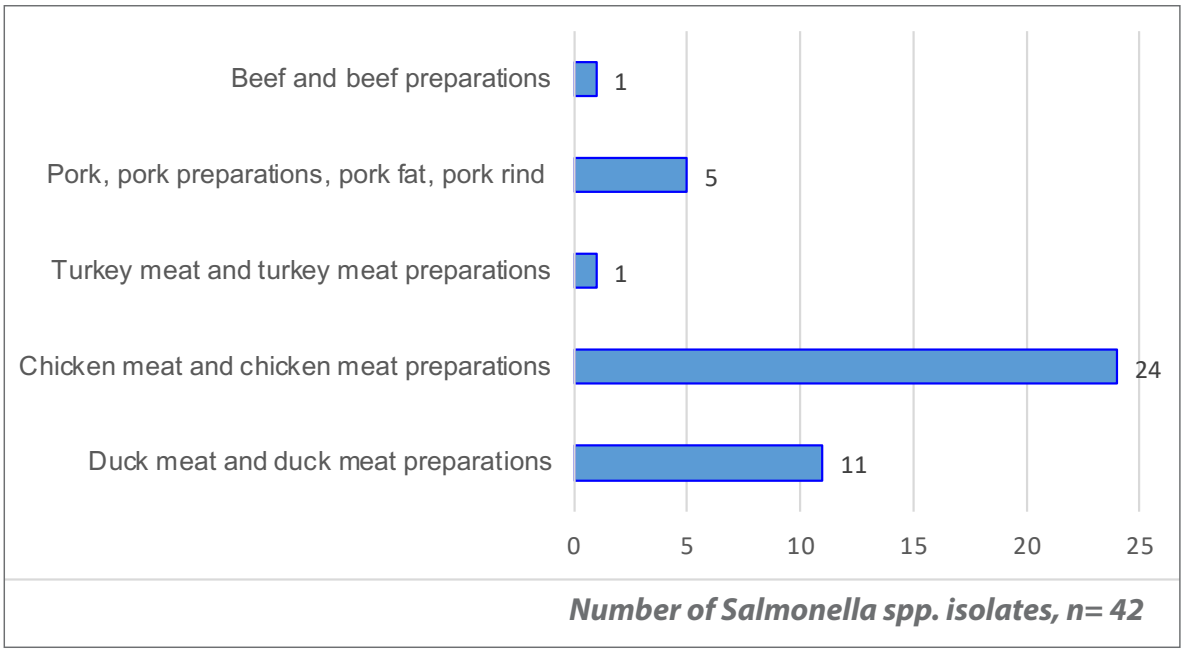

Fig. 1. Frequency of Salmonella spp. isolate detection in animal product samples in 2022–2024, by isolation source

for antibiotic resistance determination. VITEK BIOMERIEUX model DENSICHEK (France) densitometer was used for the suspension density determination.

Molten TSA not later than 15 minutes after its preparation was poured into sterile Petri dishes, (100 mm in diameter), 20 mL per Petri dish. The bacterial suspension, was inoculated by streaking onto dried Mueller – Hinton agar with sterile cotton swab, then the discs were placed onto the agar (4 discs per Petri dish). After placing antibiotic discs, the Petri dishes were incubated at 37 °C for (18 ± 2) hours. The results were assessed according to the microbial growth inhibition zones formed around the discs, measured at accuracy of 1 mm on a dark matte surface at a distance of about 30 cm from the eyes using a ruler at an angle of 45°.

The results were interpreted according to the Russian recommendations "Determination of microorganism susceptibility to antimicrobials" (IACMAC, version 2025-01) prepared on the basis of the recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST), and using CLSI M100 standard. The EUCAST susceptibility assessment and interpretation approaches are currently considered theoretically sound [15, 16, 17].

Real-time polymerase chain reaction. Sorb-GMO-B kit (Syntol, Russia) was used for the extraction of *Salmonella* spp. DNAs.

Salmonella spp. isolates were examined for their molecular and genetic properties as well as for antimicrobial resistance genes (blaCTX-M, blaOXA10, blaDHA, blaGES, blaKPC, blaOXA48-like, blaNDM, blaVIM) using RESISTOM test systems (Lytech, Russia) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Forty-two *Salmonella* spp. isolates were detected in animal product samples: 15 isolates in 2022, 11 isolates in 2023, 16 isolates in 2024.

The morphological and cultural properties of *Samonella* spp. isolates were characteristic of their family and genus.

Figure 1 shows that *Salmonella* spp. were most often detected in poultry meat products – 36 isolates (85.7%), particularly in chicken meat – 24 isolates (57.1%). Therewith, according to the European Centre for Disease Prevention and Control (ECDC), poultry meat and poultry meat preparations are the most commonly infected with *Salmonella* spp. at the stage of their distribution in the European Union. Turkey meat and turkey meat products as well as pork were also found to be highly contaminated [10, 18].

Serological identification showed that the most *Salmonella* spp. isolates belonged to O:9 (D1) group – 18 isolates (42.9%) and to O:7 (C1) group – 13 isolates (30.9%), 7 isolates (16.7%) belonged to O:4 (B) group, 4 isolates (9.5%) belonged to O:8 (C2–C3) group. The results are shown in Figure 2.

Serotyping showed that the most often detected *Salmonella* spp. were as follows (Fig. 3): *S. Enteritidis* – 14 (33.3%), *S. Blegdam* – 3 (7.1%), *S. Derby* – 2 (4.8%). Therewith, *S. Enteritidis* and *S. Derby* were more often detected in duck products, while *S. Blegdam* – in chicken meat. Also,

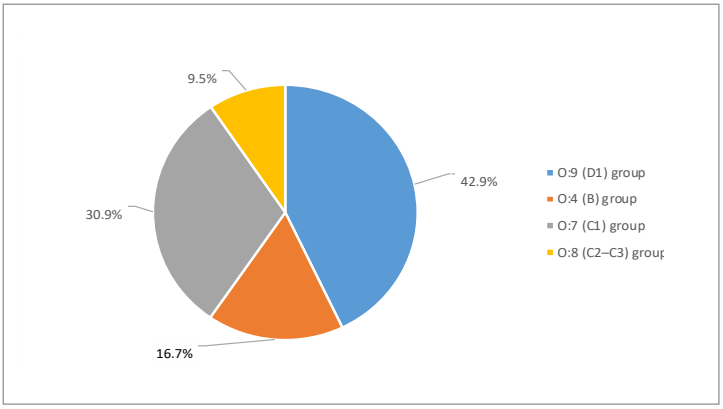

Fig. 2. Identification of O-groups of Salmonella spp. isolates recovered from samples of animal products in 2022–2024

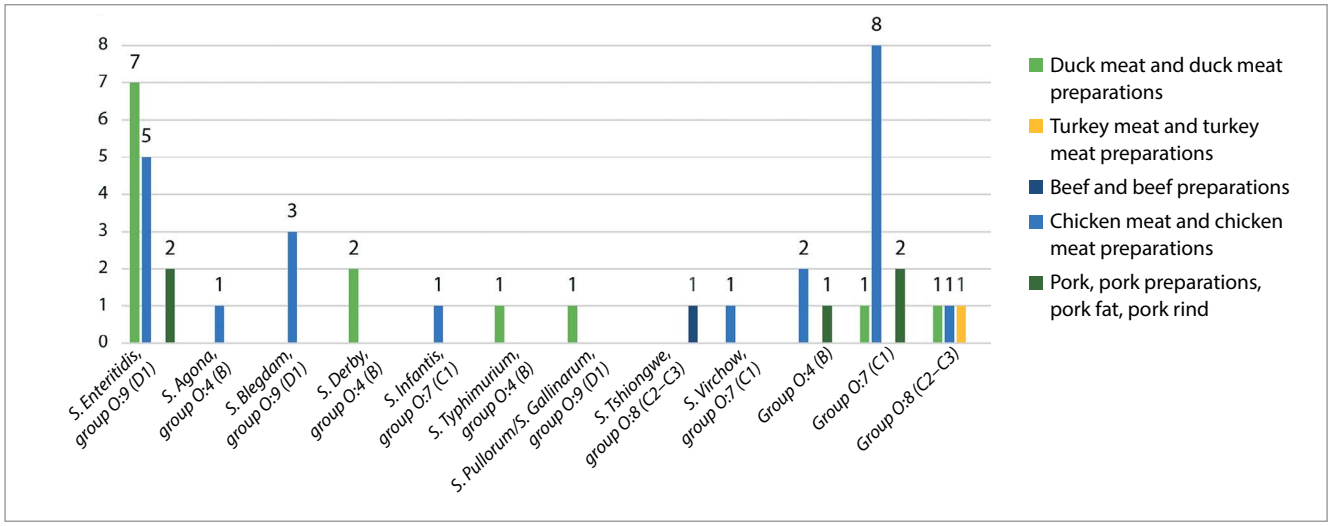

Fig. 3. Serotyping of Salmonella spp. isolates recovered from animal product samples in 2022–2024

among 17 non-typeable *Salmonella* spp. isolates (40.5%), 11 isolates (26.2%) were classified to the O:7 (C1) group.

During the study, *Salmonella* spp. isolates were tested for their resistance to 21 antimicrobials. The results are shown in Figure 4.

Salmonella spp. isolates recovered from animal products in 2022–2024 demonstrated relatively high level of common resistance to some antibiotics.

Examined *Salmonella* spp. isolates were the most frequently resistant to erythromycin (80.9%), benzylpenicillin (78.6%), norfloxacin (69.0%) and tetracycline (40.5%).

It should be noted that all *Salmonella* spp. isolates were susceptible to meropenem and imipenem.

In 2025, the "European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2022–2023" was published. It states that most *Salmonella* spp. isolates are also resistant to tetracycline and sulfonamides. Moreover, there is a trend for increase in resistance to ciprofloxacin and third generation cephalosporins in several countries [19].

These and other studies [20] indicate that *Salmonella* spp. susceptibility monitoring is crucial because of their increasing resistance to some critically important antimicrobials.

Also, tests showed that 90% of *Salmonella* spp. isolates were resistant to more than one of the tested antibiotics. In addition, 38% of the isolates were resistant to more than three classes of antimicrobials.

Figure 5 shows that 13 isolates (31.0%) demonstrated resistance to three antimicrobials, 8 isolates (19.0%) – to two antimicrobials and 5 isolates (11.9%) – to eight antimicrobials.

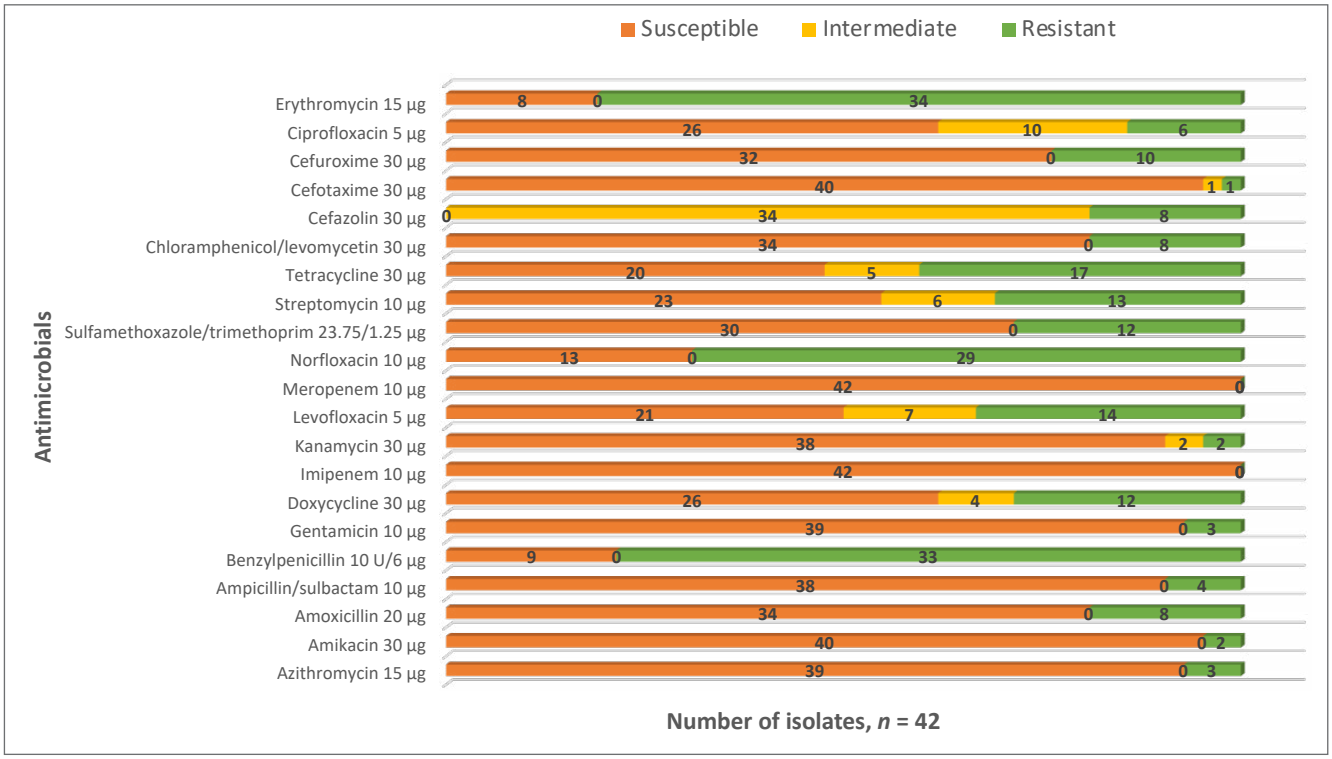

Fig. 4. Antimicrobial resistance of Salmonella spp. isolates recovered from animal products in 2022–2024

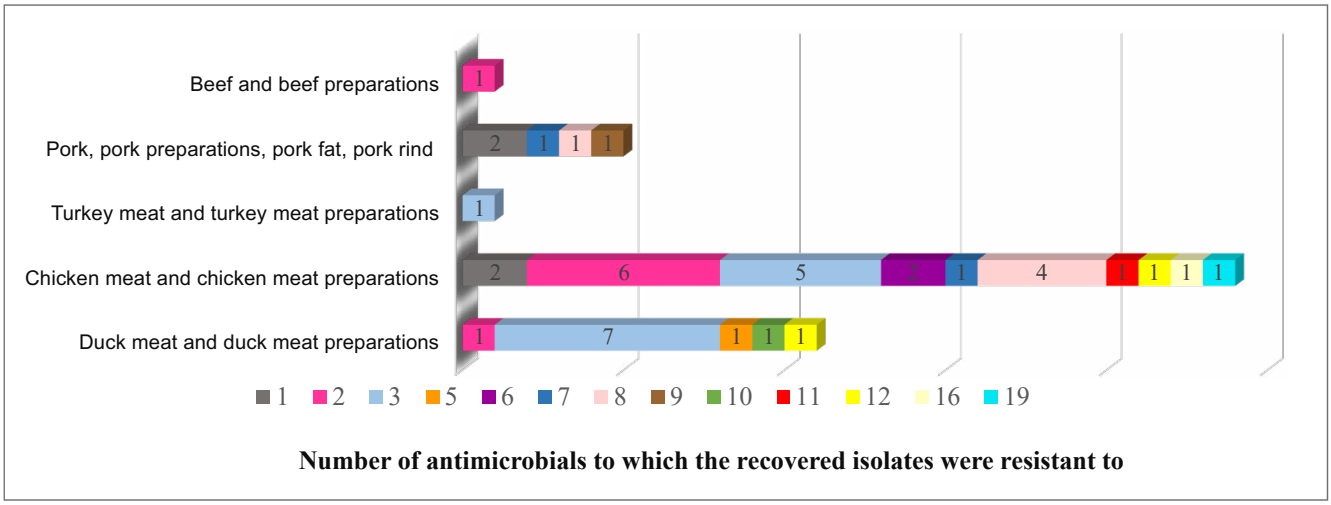

Fig. 5. Number of Salmonella spp. isolates demonstrating multiple antimicrobial resistance recovered from animal product samples in 2022–2024

The isolate resistant to 6 antibiotics and the isolate resistant to 19 out of tested 21 antibiotics were detected during the study. Both isolates belonged to O:4 (B) group and were detected in chicken meat.

According to the official report "On the state of sanitary and epidemiological welfare of the population in the Russian Federation in 2024", the majority of resistant *Salmonella* spp. were detected in poultry meat, eggs and products thereof (97.2%) and in meat and meat products (95.1%) [5].

According to some authors, most of the *Salmonella* spp. isolated from the products are resistant to at least one class of antimicrobials. For example, the ECDC informs that in the European Union *Salmonella*-infected people often demonstrate resistance to antimicrobials, therewith more than 20% of such patients demonstrate resistance to at least three classes of antimicrobials. Moreover, according to the WHO, resistance to fluoroquinolones and cephalosporins is increasing every year [1, 18, 21, 22].

Currently, reports on multiple resistance of *Salmonella* spp. are appearing more frequently. Tests of *Salmonella* spp. detected in pig products have shown their high resistance to tetracycline, streptomycin, and sulfamethoxazole/trimethoprim [23, 24].

Tests of *Salmonella* spp. isolates recovered from pig products performed during this study showed similar results: 80% of the isolates demonstrated high resistance to erythromycin.

The Federal Service for Supervision of Consumer Rights Protection and Human Welfare in its official report for 2024 indicated that *Salmonella* spp. demonstrated resistance to one or more antimicrobials with the highest resistance observed to tetracycline, ciprofloxacin and sulfamethoxazole/trimethoprim.

Thus, excessive antibiotic use fuels multiple antimicrobial resistance leading to longer and more expensive treatment, as well as fatal outcomes and economic losses, thereby posing a great threat [25, 26].

Figure 6 shows dynamics of increase in *Salmonella* spp. isolates resistant to antimicrobials of the same class in 2022 to 2024.

[5] https://www.rospotrebnadzor.ru/upload/iblock/b8a/u6lsxjabw032jkdf837nlaezxu3ue09m/GD_SEB.pdf (in Russ.)

The use of cephalosporins, in particular the third generation cephalosporins, for salmonellosis treatment has long been the most promising option owing to their high efficacy against *Salmonella* spp., resistance to bacterial beta-lactamases, excellent bioavailability, and favourable safety profile, especially in short-term treatment regimens. Fluoroquinolones are also effective antimicrobials against salmonellosis owing to their good cellular penetration. However, *Salmonella* spp. are increasingly developing resistance to this class of antimicrobials worldwide [27, 28].

The study results (Fig. 6A) have shown that resistance of *Salmonella* spp. to cephalosporins has increased since 2022: resistance to the first generation cephalosporin (cefazolin) has increased by 18.0% (5 isolates (31.3%) out of 16 recovered isolates were resistant in 2024), resistance to the second generation cephalosporin (cefuroxime) has increased by 50.0% (8 isolates (50.0%) out of 16 recovered isolates were resistant in 2024) and resistance to the third generation cephalosporin (cefotaxime) has increased by 6.3% (1 isolate (6.3%) out of 16 recovered isolates was resistant in 2024, previously no resistant isolates were detected).

Figure 6B shows the similar results for norfloxacin (second generation fluoroquinolone): the resistance has increased by 15.0% (12 isolates (75.0%) out of 16 recovered isolates were resistant in 2024), as well as resistance to levofloxacin (third generation fluoroquinolone) has increased by 49.2% (10 isolates (62.5%) out of 16 recovered isolates were resistant in 2024).

The resistance of isolates to chloramphenicol/levomycetin (increased by 37.5%) and sulfamethoxazole/trimethoprim (increased by 17.5%) has changed in 2022–2024 (Fig. 6C).

High increase in resistance to tetracyclines was detected in 2022–2024 (Fig. 6D): resistance to tetracycline (first generation) increased by 29.6% (9 isolates (56.3%) out of 16 recovered isolates were resistant in 2024), resistance to doxycycline (second generation) increased by 30.0% (8 isolates (50.0%) out of 16 recovered isolates were resistant in 2024).

At the same time, an increase in aminoglycosides resistance was observed (Fig. 6E). Thus, amikacin (third generation) and kanamycin (first generation), resistance has

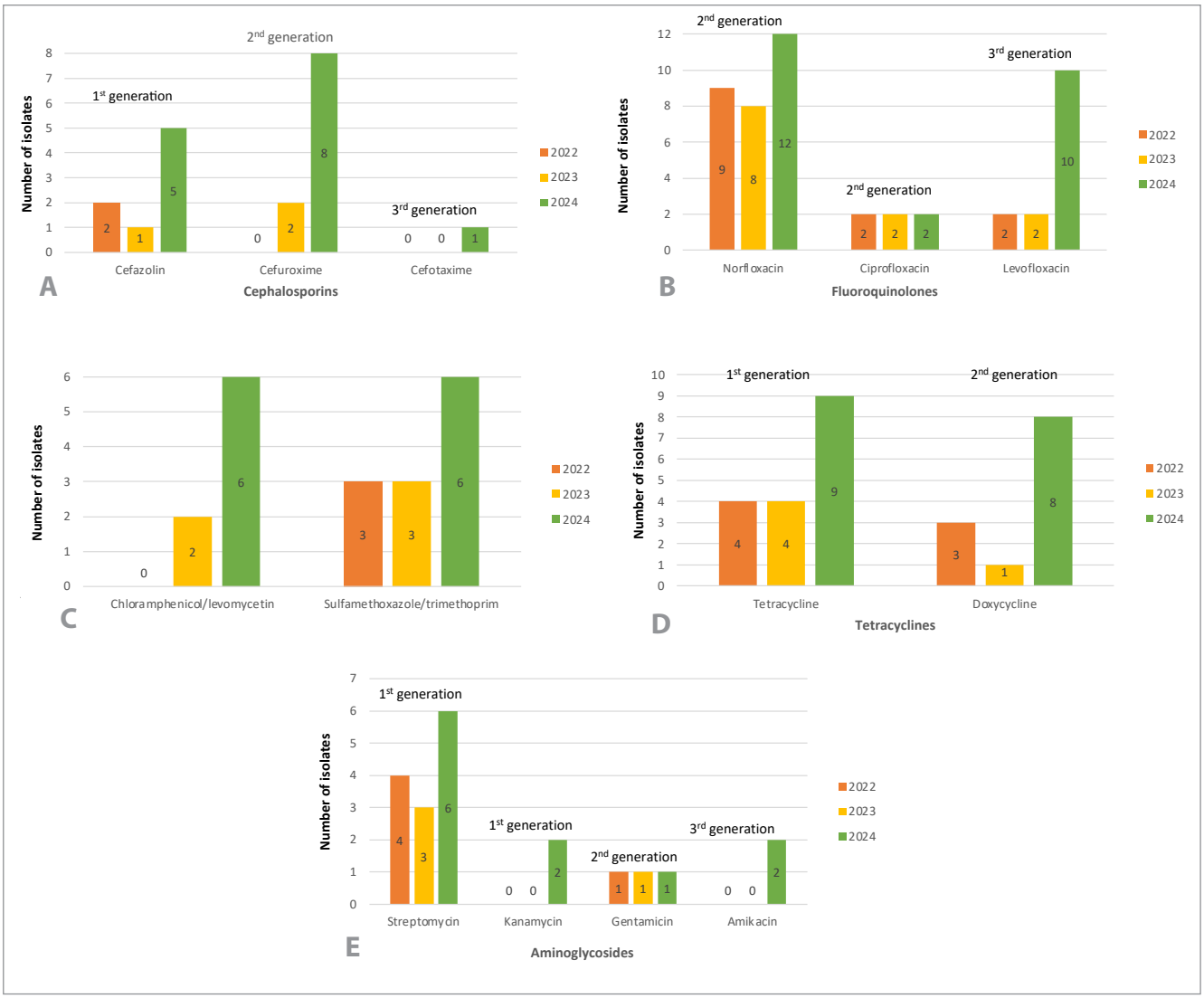

Fig. 6. Resistance of Salmonella spp. isolates to the following antibiotics: A – cephalosporins, B – fluoroquinolones, C – chloramphenicol/levomycetin and sulfamethoxazole/trimethoprim, D – tetracyclines, E – aminoglycosides

increased by 12.5% (2 isolates (12.5%) out of 16 recovered isolates were resistant in 2024). Streptomycin (first generation) resistance increased by 10.8% (6 isolates (37.5%) out of 16 recovered isolates were resistant in 2024).

No antimicrobial resistance genes, blaCTX-M, blaOXA10, blaDHA, blaGES, blaKPC, blaOXA48-like, blaNDM, or blaVIM, were found in the recovered *Salmonella* spp. isolates during the study.

CONCLUSION

Forty-two *Salmonella* spp. isolates were detected during the study; the predominant serovars were as follows: *S. Enteritidis* – 14 (33.3%), *S. Blegdam* – 3 (7.1%), *S. Derby* – 2 (4.8%).

Significant spread of resistance, including multiple resistance, has been shown. *Salmonella* spp. isolates demonstrated maximum resistance to erythromycin (80.9%), benzylpenicillin (78.6%), norfloxacin (69.0%) and tetracycline (40.5%). All *Salmonella* spp. isolates were susceptible to meropenem and imipenem.

Most isolates of *Salmonella* spp. demonstrated resistance to three antimicrobials at once (31.0%), and one isolate was also found to be resistant to 19 out of 21 antimicrobials used for the study.

In addition, an increase in resistance of *Salmonella* spp. isolates to the following antimicrobials was shown: cephalosporins – resistance to the first generation (cefazolin) increased by 18.0%, resistance to the second generation (cefuroxime) increased by 50.0%, resistance to the third generation (cefotaxime) increased by 6.3%; fluoroquinolones – resistance to the second generation (norfloxacin) increased by 15.0%, resistance to the third generation (levofloxacin) increased by 49.2%; tetracyclines – resistance to the first generation (tetracycline) increased by 29.6%, resistance to the second generation (doxycycline) increased by 30.0%.

Besides, resistance of isolates to chloramphenicol/levomycetin increased by 37.5% and to sulfamethoxazole/trimethoprim increased by 17.5% during the study period, 2022 –2024.

Resistance to aminoglycosides has also increased: resistance to amikacin (third generation) and kanamycin (first generation) has increased by 12.5%; resistance to streptomycin (first generation) has increased by 10.8%.

No antimicrobial resistance genes (blaCTX-M, blaOXA10, blaDHA, blaGES, blaKPC, blaOXA48-like, blaNDM, blaVIM) have been detected in the recovered *Salmonella* spp.

Continuous monitoring of animal product quality enables prompt detection of shifts in bacterial populations, facilitating the development of effective strategies for reducing the transmission of resistant strains and genes to humans. However, monitoring for antimicrobial resistance trends in isolated strains supports rational antibiotic use in veterinary and clinical medicine. This is essential for effective salmonellosis surveillance under the One Health framework

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On the 75th anniversary of Vladimir S. Rusaleyev

Vladimir S. Rusaleyev was born on July 6, 1950 in Murmansk. After graduating from an agricultural technical school and completing military service, he studied at the Troitsk Veterinary Institute in the Chelyabinsk Oblast from 1971 to 1976. From 1976 to 1980, he worked at the Kostanay Regional Veterinary Laboratory, first as a veterinarian and later as a head of a department.

From January 1980 to December 1982, he took a postgraduate course at the All-Union Research Institute of Veterinary Virology and Microbiology (Volginsky, the Vladimir Oblast), working as a junior researcher. In 1983, he defended his candidate's thesis.

From 1984 to 1985, V. S. Rusaleyev completed advanced training courses in molecular biology and genetic engineering at the Biological Center of the USSR Academy of Sciences (Pushchino, the Moscow Oblast). In 1986, he took a training course in highly dangerous infections at the Stavropol Anti-Plague Research Institute of the USSR Ministry of Health.

Starting in 1987, Vladimir Rusaleyev worked as a senior lecturer and later as an Associate Professor at the Kostanay Agricultural Institute. In January 1991, he was admitted as a doctoral candidate at the Laboratory of Bacterial Infections of the All-Russian Research Veterinary Institute (Kazan). In October 1992, he successfully defended his doctoral thesis.

In September 1993, Dr. Rusaleyev joined the All-Russian Research Institute for Animal Health as a senior researcher to facilitate research in the bacteriological field. In 1994, he was elected as a leading researcher, and in 1995, he was appointed as head of the Microbiology Laboratory, which he ran until 2012. During this period, Dr. Rusaleyev supervised training of 10 Candidates and 1 Doctor of Science. In 2005, he was awarded the academic title of professor.

Vladimir S. Rusaleyev's research work mainly focused on studying pathogens of economically significant animal diseases, such as salmonellosis, pasteurellosis, porcine polyserositis, porcine pleuropneumonia, atrophic rhinitis, and others. Under his supervision, some bacterial isolates were recovered and studied, and were later on deposited as industrial strains. Inactivated antibacterial vaccines were developed, registered, and produced, which were widely and successfully used to prevent the aforementioned diseases.

Dr. Rusaleyev has authored over 200 scientific papers and patents for inventions and has been awarded medals from the Exhibition of Achievements of National Economy (VDNKh).

From 2012 to 2022, V. S. Rusaleyev held the position of Scientific Secretary and actively participated in training personnel through postgraduate programs and the dissertation council of the Federal Centre for Animal Health.

Dr. Rusaleyev is now retired after a distinguished career, while his successors continue to work productively in various fields of veterinary science.

We extend our heartfelt congratulations to Dr. Rusaleyev on this significant milestone and wish him prosperity, enduring vitality, and a long, joyful life!

On the 75th anniversary of Konstantin N. Gruzdev

On July 24, 2025, Konstantin N. Gruzdev – Doctor of Biological Sciences, Professor, three-time laureate of the Russian Federation Government Prize in Science and Technology, and Honored Veterinarian of the Russian Federation – celebrated his 75th birthday.

Dr. Gruzdev was born on July 24, 1950, in Nurlat, Tatar ASSR. In 1972, he graduated with honors from the Kazan Veterinary Institute named after N. E. Bauman. Following his studies, he served in the Soviet Army and later worked as the chief veterinarian of the Yermishinsky Raion in the Ryazan Oblast. From 1974 to 2003, he was associated with the All-Russian State Center for Quality and Standardization of Veterinary Drugs and Feed (Moscow), where he pursued postgraduate studies and advanced through various roles – from junior and senior researcher to head of the laboratory, head of the virology department, and deputy director of the institute.

In 1979, he defended his dissertation and earned the degree of Candidate of Veterinary Sciences. By 1993, he had attained the title of Doctor of Biological Sciences, and in 1999, he became a Professor of Virology.

From 2003 to 2007, he served as Director of the Federal State-Financed Institution Federal Center for Animal Health Protection (Vladimir). During this challenging period in the institute's history, Dr. Gruzdev dedicated immense effort and energy to implementing sweeping reforms. He spearheaded fundamental changes in the institution's operational framework, including revisions to its governing statutes, the establishment of new research and production divisions, and the creation of innovative structural subdivisions.

From 2007 to 2010, he served as Head of the Veterinary Service and Director of the Veterinary Department of the Vladimir Oblast Administration.

In 2011, he founded and led the laboratory for African swine fever at the Federal Centre for Animal Health. Since 2013, Dr. Gruzdev has been the Chief Scientist of the Information and Analytical Center at the Federal Centre for Animal Health, where he continues to pursue multifaceted scientific research. Additionally, he serves as Editor-in-Chief of the quarterly journal "Veterinary Science Today", which is listed among the peer-reviewed scientific publications recommended by the Higher Attestation Commission (VAK).

Under the direct supervision and personal involvement of Dr. Gruzdev, studies were conducted on pathogenicity and immunogenicity factors, genetic markers of classical swine fever, transmissible gastroenteritis, porcine reproductive and respiratory syndrome, African swine fever, and rabies. Additionally, he also developed groundbreaking methods for the genodiagnostics of viral animal diseases.

Dr. Gruzdev is the author or co-author of over 350 works, including 9 monographs, a scientific manual, a reference book, and 2 popular science editions. The scientific priority of his research is supported by 9 authorship certificates and 15 patents.

Under his supervision, 4 doctor's and 12 candidate's theses were prepared and successfully defended.

Dr. Gruzdev served as Russia's focal point in the European Association of Veterinary Virologists and held multiple key roles, including:

- Member of the Expert Council on Biosafety under the Russian Ministry of Industry and Science;
- Member of the Operational Headquarters of the Russian Government for coordinating measures to prevent the spread of avian influenza and African swine fever;
- Member of the Expert Council of the Higher Attestation Commission (VAK).

Currently, he collaborates with the World Organization for Animal Health (WOAH) on African swine fever and serves as an expert for GF-TADs on the same issue.

For outstanding professional achievements in the field of veterinary medicine, Dr. Gruzdev was awarded a WOAH (Paris) certificate on October 11, 2018.

Dear Dr. Gruzdev, we wish you good health and many more years of fruitful scientific work!

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