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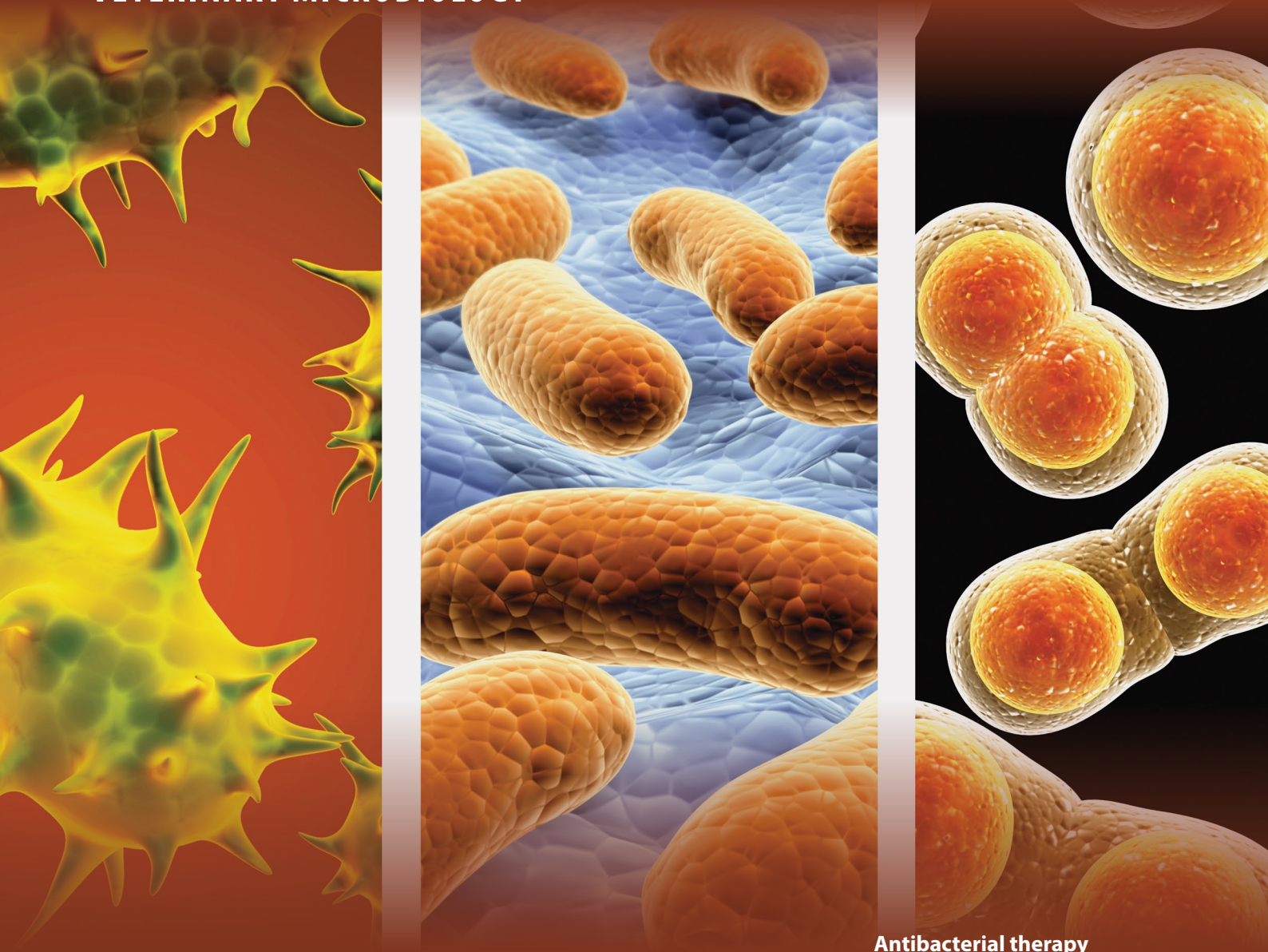

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AIMS AND SCOPE

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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Methodological approaches for the prevention and eradication of caprine arthritis-encephalitis in international goat herds: A review

Vyacheslav Yu. Koptev[1], Natalia O. Likhacheva[2], Nikolay A. Shkil[1], Irina M. Yurkova[2]

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ABSTRACT

Introduction. Caprine arthritis-encephalitis (CAE) is a chronic viral disease of goats caused by a lentivirus from the *Retroviridae* family (small ruminant lentivirus group, SRLV). Infection typically involves prolonged asymptomatic carriage, progressing to debilitating lesions in the joints, central nervous system, lungs, and mammary gland. Transmission occurs primarily via colostrum, milk, and direct contact, including fomites and co-mingling with infected animals. Diagnosis relies on serological and molecular methods.

Objective. To summarize international methodological approaches for the prevention of CAE.

Materials and methods. This review was conducted based on an analysis of publications in peer-reviewed scientific journals, official international guidelines, and reports from key organizations such as the Food and Agriculture Organization and the World Organisation for Animal Health. Literature was identified and selected through searches in major scientific databases, including Web of Science, Scopus, PubMed, Google Scholar, Science Hub, CyberLeninka and others.

Results. Analysis of the literature indicates that the most effective outcomes in controlling CAE have been achieved in countries with mandatory national eradication programs, which integrate comprehensive preventive, diagnostic, and administrative measures (e.g., Norway, Switzerland). In nations with voluntary participation programs (e.g., Australia, New Zealand, Canada, Italy, France), positive results are also evident, particularly when supported by incentive mechanisms and sustained educational outreach. Conversely, in countries lacking coordinated national programs (e.g., Turkey, Brazil, Iran, and most African nations), disease control remains inadequate, and seroprevalence rates are consistently high.

Conclusion. The most effective outcomes in controlling CAE have been achieved in countries with mandatory national eradication programs, which integrate comprehensive preventive, diagnostic, and administrative measures (e.g., Norway, Switzerland).

Keywords: review, caprine arthritis-encephalitis, prevention, serological monitoring, eradication programs

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Методические подходы к профилактике и оздоровлению популяции коз от артрита-энцефалита в зарубежных странах (обзор)

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РЕЗЮМЕ

Введение. Артрит-энцефалит коз – хроническое вирусное заболевание, вызываемое лентивирусами семейства *Retroviridae*, относящимися к группе SRLV (лентивирусы мелкого рогатого скота). Заболевание характеризуется длительным бессимптомным вирусоносительством с последующим прогрессирующим поражением суставов, центральной нервной системы, легких и ткани молочных желез. Вирус может передаваться с молозивом и молоком, при прямом контакте, через оборудование и при совместном содержании здоровых и инфицированных животных. Диагностика заболевания основана на использовании иммунологических и молекулярно-биологических методов.

Цель обзора. Обобщение зарубежных методических подходов к профилактике артрита-энцефалита коз.

Материалы и методы. При написании обзора были использованы публикации в рецензируемых журналах, официальные зарубежные методические рекомендации, а также отчеты Продовольственной и сельскохозяйственной организации Объединенных Наций, Всемирной организации здравоохранения животных. Отбор источников производился в базах данных Web of Science, Scopus, Google Scholar, PubMed, Science Hub, КиберЛенинка и др.

Результаты. Анализ литературных источников показывает, что наиболее эффективные результаты по борьбе с данным заболеванием достигнуты в странах, где разработаны обязательные государственные программы, включающие комплекс профилактических, диагностических и административных мер (Норвегия, Швейцария). В странах с добровольным участием в программах оздоровления (Австралия, Новая Зеландия, Канада, Италия, Франция) при наличии практики стимулирующих механизмов и информационной поддержки также наблюдаются положительные результаты. В странах с отсутствием координированных программ (Турция, Бразилия, Иран, большинство африканских стран) уровень контроля за распространением заболевания остается низким, а серопревалентность – высокой.

Заключение. Наибольших успехов в борьбе с данным заболеванием удалось достичь в странах, где разработаны обязательные государственные программы по контролю и искоренению, включающие комплекс профилактических, диагностических и административных мер (Норвегия, Швейцария).

Ключевые слова: обзор, артрит-энцефалит коз, профилактика, серомониторинг, программы по искоренению заболевания

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INTRODUCTION

Caprine arthritis-encephalitis (CAE) is a persistent viral infection caused by small ruminant lentiviruses (SRLV) within the *Retroviridae* family. SRLVs are classified into five genotypes (A, B, C, D, E) with further subtype divisions [1, 2, 3].

CAE pathogenesis is described in detail in several scientific publications [4, 5, 6] and in dedicated chapters of various monographs [7, 8, 9]. Infection typically involves prolonged asymptomatic carriage, progressing to debilitating lesions in the joints, central nervous system, lungs, and mammary gland.

Several authors [10, 11, 12, 13, 14] confirm that CAEV transmission occurs primarily via colostrum, milk, and direct contact, including fomites and co-mingling with infected animals. Cases of interspecies transmission of the virus between goats and sheep have also been documented [15, 16].

Diagnosis relies on serological methods such as enzyme-linked immunosorbent assay (ELISA) and the agar gel immunodiffusion (AGID), and polymerase chain reaction (PCR) and sequencing [17, 18, 19].

The key risk factors amplifying outbreaks include retaining seropositive animals without segregation or culling; mixing infected and healthy goats in crowded housing; communal use of kidding pens and lack of disease preventive measures.

Serological studies using AGID have documented high CAE virus (CAEV) seroprevalence exceeding 65% in dairy goat populations of several developed countries with advanced goat industries, including Canada, France, Norway, Switzerland, and the United States [20].

The purpose of this review is to summarize international methodological approaches for the prevention of caprine arthritis-encephalitis in search of most effective ways.

EUROPE

Norway. Norway launched the Healthier Goats program as a voluntary initiative in the early 2000s, and it transitioned to mandatory participation in high-prevalence regions from 2012. The integrated strategy involved culling seropositive animals, immediate separation ("snatching") of kids post-birth for rearing on cow colostrum or milk replacers in isolated barns, serological testing and herd certification.

Comprehensive studies confirmed the high effectiveness of the program: circulating CAEV variants were effectively eliminated [21].

Over 15 years of the program, the proportion of CAE-positive dairy goat herds plummeted from 30% to under 1% [22], demonstrating marked improvements in herd health and productivity, with financial viability hinged on state subsidies [23].

Switzerland. This country pioneered a national CAE eradication program. The program started in the early 1980s and official data indicate over 90% of farms attained CAE-free certification by 2008 [24].

The program's target was complete eradication of the infection by rigorous serological testing, culling of seropositive goats, and strict animal movement controls to achieve virus-free status [25].

The control measures were monitored by the Swiss Goat Breeders' Association (SZZV) and federal veterinary authorities [26, 27].

France. France manages CAE through voluntary regional and national programs coordinated by veterinary services, with farmer cooperatives and networks. Since around 2020, voluntary herd certification schemes and farm hygiene certification have gained traction [28].

One of the key initiatives is Plan de qualification sanitaire des élevages caprins, which is a tool for goat farm biosecurity assessment and improvement [29].

Italy (South Tyrol, Northern Italy). Italy implements CAE control through regional programs, particularly in South Tyrol, where veterinary authorities and laboratory networks collaborate on compulsory eradication efforts since late 2000s.

The main approach is identification of positives by serological monitoring for selective removal and culling seropositive animals individually without mass slaughter [30]. This strategy has proved effective: by 2020, most farms in the region achieved CAE-free status [31].

Other Italian regions maintain less uniform, voluntary efforts with variable coverage.

Spain. Spain lacks official recognition of CAE as a notifiable infectious disease under national legislation, resulting in no mandatory control or eradication programs. Prevention relies entirely on voluntary farmer initiatives.

Seroprevalence in dairy goat herds remains elevated at 12–23% [32].

A similar situation can be observed in ***Germany.*** The country maintains around 120,000 goats, representing about 1% of the EU's total goat population [33], with no federal notifiable status or mandatory control program for CAE [34].

NORTH AMERICA

The USA. A 1981 study across 24 states reported a strikingly high CAE seroprevalence of 81% in goat populations [35]. A 1992 study published in the Journal of the American Veterinary Medical Association confirmed widespread CAE infection in U.S. dairy goat herds, particularly in California and northwestern states. Researchers tested 3,790 goats across 28 states using identifying 1,175 seropositive animals – a 31% prevalence rate [36].

The United States lacks a federal mandatory program for CAE despite research since the 1980s.

The programs rely on voluntary initiatives at state, regional, and farm levels informed by scientific studies and international guidelines and are developed in partnership with state veterinary services and universities.

Minnesota exemplifies U.S. state-level voluntary CAE control, where since 2006 farmers have participated in testing and registration to maintain negative herd status [37]. Iowa supports voluntary CAE control through regular training seminars educating goat owners in serological monitoring, culling or segregating seropositive goats, artificial rearing of newborns with heat-treated colostrum or replacers [38]. In the state of Michigan, owners of seropositive animals are recommended to carry out "sterile lambing" (immediate kid-dam separation at birth to prevent colostrum/milk exposure), regular serological testing and culling of positives, and establishment/maintenance of CAE-negative herds [39].

A number of universities, in particular Washington State University, Iowa State University, Michigan State University, Alabama Agricultural and Mechanical University, provide consultations, manuals, and training on CAE eradication for goat farms [40, 41].

Standard protocols for goat herd owners include the following:

– serological screening starting at 6 months of age;

– segregation of seropositive and seronegative animals followed by culling infected ones;

– "sterile lambing" via immediate post-birth kid separation with pasteurized colostrum/milk or replacers;

– rigorous disinfection of equipment and facilities;

– and quarantine/testing bans on new imports.

The United States Department of Agriculture (USDA) and its Animal and Plant Health Inspection Service (APHIS) collaborate with state veterinary services on animal health surveillance, providing diagnostic support and research for CAE, but maintain no centralized mandatory control or eradication program. In the United States, goat owners have widespread access to CAE diagnostic testing through state and university veterinary labs, backed by scientific guidance, financial grants and coordination with state services for lab cooperation [42, 43].

The American Dairy Goat Association (ADGA) does not run a standalone CAE control program but promotes established veterinary protocols and measures to control and to curb spread in dairy herds.

Canada. Canada lacks a unified federal CAE control program, relying instead on effective provincial initiatives and industry-led efforts through associations like the Ontario Goat Association [44]. The Ontario Goat Association's GoGen Dairy Genetic Improvement Program offers detailed guidance:

– whole-herd diagnostics with kids tested at 4–6 months, 8–10 months, and post-12 months pre-kidding, plus semi-annual post-partum checks [44];

– "sterile lambing" via immediate weaning, separate rearing on pasteurized colostrum/milk or replacers;

– isolation/culling of seropositives;

– sourcing CAE-free breeders;

– quarantining new arrivals until testing clears them;

– "low-risk" farm certification.

SOUTH AMERICA

South American countries report elevated CAE seroprevalence. Regular PCR and serological investigations map strains and risk factors, but no national eradication initiatives exist [45, 46, 47].

Brazil. Brazil's 2019 epizootological surveys reported CAE seroprevalence ranging from 2–17% nationally, with different rates among regions and farms. The peaks above 10% were reported in Alagoas, Ceará and São Paulo regions, versus lower rates in Maranhão and Paraíba [45].

Brazil lacks a centralized national CAE eradication program, but there are a number of mandatory measures and initiatives implemented at the regional and federal levels, including mandatory confirmation and reporting. The Ministry of Agriculture and Livestock (MAPA) mandates case confirmation, reporting, and surveillance registration to track spread, integrating CAE into veterinary monitoring. The Brazilian Agricultural Research Corporation (Embrapa) has produced technical manuals detailing symptoms, diagnostics (ELISA, PCR), prevention (testing, quarantine), breeding practices, annual screening, and farm-specific control plans tailored to technological capacity [45].

Some states apply partial on-farm eradication via selective measures.

Argentina. Argentina reported a 3.86% seroprevalence of CAEV in goat herds in a 2011 nationwide study [46].

The National Food Safety and Quality Service (SENASA) oversees voluntary CAE control, which includes the following:

– epizootological control: mandatory farm registration, case notifications, and epizootological investigations upon positives;

– diagnostics and monitoring: ELISA/AGID serological diagnostics with routine surveys for CAEV antibodies in accredited SENASA laboratories;

– spread prevention: seropositive isolation/culling, movement controls requiring CAE-negative certification, bans on raw infected milk for kids, "clean herd" formation via testing/selection;

– eradication measures: gradual eradication through status segregation and seronegative breeding;

– educational work: farmer education on biosecurity, veterinary surveillance over program implementation, public awareness campaigns concerning CAE risks and control measures [47].

ASIA AND MIDDLE EAST

Studies by several authors confirm CAE presence across Asia and the Middle East, with seropositive goats in Turkey, Iran, Iraq, Saudi Arabia, Jordan, and Lebanon. The research

highlights genetic diversity in circulating CAEV strains. Preventive measures remain minimal [48, 49, 50].

Turkey. Turkey lacks a nationwide mandatory program for CAE. Monitoring and control are carried out at the local level, mainly in accredited public and private veterinary organizations and laboratories.

A study of 808 goats across three public and seven private farms detected CAEV antibodies in 1.9% (16/808) of samples, predominantly on state farms [51]. Siirt province showed 0% seropositivity suggesting low-prevalence [52]. While Hatay region's two of six provinces had 1.03% via AGID and competitive ELISA [53]. These results findings align with prior reports from Turkey.

Although there is no nationwide program in the country, measures that have proved effective in other countries are applied in practice:

– routine diagnostic testing of animals;
– restricting contact with seropositives;
– mandatory veterinary inspections with serology for all domestic or imported goats before herd integration.

Iran. Iran first detected CAEV via PCR in 2014, with 15.7% prevalence among 95 tested goats [49]. No centralized or government-led CAE eradication programs exist in Iran. Preventive measures are aimed at monitoring and research.

Iraq. Researchers from Al-Qasim Green University first reported CAEV in Iraq in 2022 through PCR analysis of 85 goat blood samples yielding 5.9% (5/85) positives [50]. At the moment, there is no data in open sources on special government programs for CAE control or eradication.

Saudi Arabia. Saudi Arabia maintains no official government programs for CAE eradication. A 1990 serological study on indigenous sheep detected CAEV antibodies only in 0.8% of samples [54].

Jordan. Jordan conducted a 2006 serological survey examining 1,100 goats from 69 herds, revealing 23.2% herd-level seropositivity and 8.9% individual animal prevalence [55]. It should be noted that no national government eradication program exists in Jordan.

Lebanon. Lebanon has no confirmed national government program for CAE eradication, with control limited to research and voluntary farm practices. A 2015 serological study found 13.1% individual seropositivity and 51.7% herd-level infection. Local Baladi breed exhibited greater resistance to the infection [56].

Ongoing Asian studies identify SRLV genetic clusters via sequencing.

China. A 2024 study in eastern China documented approximately 0.8% seropositivity among tested goats [57].

China lacks a dedicated national program exclusively targeting CAE eradication. However, the disease falls under mandatory registration and surveillance as a notifiable condition. The main CAE control measures are the following:

– comprehensive ELISA screening (with confirmatory PCR if needed);
– isolation of animals pending negative status;
– culling seropositives;
– segregation by status;
– rearing kids on pasteurized colostrum/milk from seronegative dams or substitutes.

Japan. From 2002–2004, testing of 3,102 goats showed nearly 20% seropositivity. A 2006–2007 survey of 857 animals from 113 herds across 28 prefectures found 15% herd-level infection and 10% individual seroprevalence [58].

Japan lacks a national CAE eradication program, with control decentralized across regions supported by research institutes like the National Institute of Animal Health (NIAH). NIAH conducts scientifically validated diagnostics (AGID, ELISA, PCR) at a national level, offering recommendations in collaboration with prefectural veterinary services to aid farmers.

India. India reported 3.33% CAE seroprevalence in 2015 studies, with 12 of 360 tested goats seropositive, and clinical cases noted in affected regions [59].

No national CAE control program exists in India. Monitoring occurs via regional veterinary initiatives, which include the following activities: screening via AGID, ELISA, and PCR diagnostics; isolation of seropositives; pasteurization of milk for kids; and equipment hygiene to curb horizontal/vertical spread.

AFRICA

CAEV circulates across multiple African countries, confirmed through serological and molecular evidence, yet systematic monitoring and diagnostic infrastructure remain inadequate.

Algeria. A 2013–2015 serological study in Algeria using ELISA detected CAEV antibodies in 29.7% of tested goats [60].

Sudan. A 2009–2010 serological survey using ELISA across five Sudanese states detected CAEV antibodies in 5.8% of examined goats [61].

Ethiopia. A 2013–2019 serological study in multiple districts of Ethiopia's Amhara State using ELISA identified 4.7% seropositivity for CAEV among tested goats [62].

Our analysis of available data indicates that no country on the continent has implemented a national program for the eradication of CAE. Furthermore, widespread practices such as mass screening of goats, systematic milk pasteurization, animal isolation, or compensation for culling are not established.

OCEANIA

Australia. CAE was first detected in Australia during the 1980s. A 1995 survey across 14 New South Wales dairy farms revealed 56.8% seropositivity [63]. Current data indicate significant declines over 30 years through voluntary controls, though precise figures remain sparse. In 2021, AgriFutures Australia funded a report "Development of innovative tools for the detection and control of caprine arthritis encephalitis virus" for developing affordable serological and molecular diagnostics to enhance on-farm detection and management [64].

In 2022 Animal Health Australia (AHA), in collaboration with the Australian Veterinary Association (AVA) launched the GoatMAP (Market Assurance Program for Goats), a voluntary national scheme to monitor, manage, and minimize CAE and Johne's disease spread in goat herds [65].

A National Kid Rearing Plan was developed. Based on the plan the following measures are used to prevent and eradicate CAE in Australia.

1. Animal identification: individual animal identification with movement tracking.
2. Mandatory health certificates retained for 7 years.
3. Regular (biannual) veterinary inspections for enrolled farms.
4. Kid hygiene: immediate post-kidding isolation of kids fed pasteurized colostrum/milk from seronegative dams to curb horizontal transmission.

5. Segregation: strict seronegative-seropositive segregation.

6. Testing: regular ELISA screening (CAE-free status requires two negatives 6 months apart in goats > 6 months).

7. Culling: culling of clinically affected seropositives [66].

New Zealand. New Zealand first detected CAEV in the early 1980s. The Ministry for Primary Industries (MPI) describes current prevalence as low despite steady presence in goat populations. Currently, the CAE is under control due to strict export and breeding farm requirements, while enforcing an integrated approach [67].

New Zealand Dairy Goat Breeders Association (NZDGBA) accreditation is aimed for certified CAE-free herds. It requires annual ELISA testing of all goats > 1 year for three years. Then testing is performed every three years upon three consecutive negatives. The goal is to gradually establish certified CAE-free herds in New Zealand [68].

Key measures include immediate post-birth kid weaning with pasteurized colostrum/milk from CAE-negative goats or replacers; seronegative-seropositive segregation; dedicated equipment for negatives milked first; pre-entry testing/quarantine for new animals; closed herd policies; and culling seropositives on commercial farms [69].

CONCLUSION

An analysis of literature sources indicates that caprine arthritis-encephalitis is widespread throughout the world. In the fight against this disease, the most effective results have been achieved in countries where mandatory government programs have been developed. These programs typically include a comprehensive set of preventive, diagnostic, and administrative measures. The national programs of Norway and Switzerland serve as exemplary models of this approach. Their success can be attributed to the following key factors:

– mandatory participation of all goat owners;
– centralized control and coordination at the national level;
– mandatory culling of seropositive animals;
– isolation of young stock and feeding of pasteurized milk;
– regular serological monitoring and accurate farm status recording.

In countries with voluntary participation in eradication programs, such as Australia, New Zealand, Canada, and specific regions of Italy and France, positive results have also been observed. These outcomes are particularly evident where well-designed incentive structures and educational support for animal owners are in place.

In countries lacking coordinated government programs, such as Turkey, Brazil, Iran, and most African nations, control over disease spread remains limited, while seroprevalence levels stay persistently high.

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Cross-species transmission of avian influenza A(H5N1) virus to mammals: lessons learnt from 2024–2025 outbreaks in cattle

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ABSTRACT

Introduction. In 2024–2025, a number of high pathogenicity avian influenza A(H5N1) outbreaks were reported in dairy cattle. Such an expansion of the virus' hosts range increases global risks for livestock farming and public health, which requires strengthening animal disease monitoring and control systems.

Objective. Analysis of the epizootological characteristics of avian influenza A(H5N1) virus infection in dairy cattle and other mammals in 2024–2025, as well as a summary of the response measures taken and recommendations of international organizations.

Materials and methods. Analytical research methods were used utilizing PubMed, Scopus, Web of Science, Springer, Wiley Online Library databases and materials from international organizations (FAO, EFSA, WOAH, OFFLU, CDC).

Results. Outbreaks of avian influenza A(H5N1) caused by clade 2.3.4.4b virus of genotypes B3.13 and D1.1 in dairy cattle in 2024–2025 occurred as a result of three independent confirmed cases of the pathogen transmission from wild birds. The infection was detected on 1,078 cattle farms in 17 US states. Subsequent virus transmission to poultry, wild and domestic animals, including cats, as well as to humans (70 people), mainly those working on livestock and poultry farms, was reported. Reverse transmission of the pathogen from cows back to birds was documented as well. Rapid and wide spread of the virus is associated with extensive animal movements and insufficient biosafety measures. Influenza A(H5) is recommended for the inclusion in the differential diagnosis for cattle, pigs, domestic and wild animals.

Conclusion. Avian influenza A(H5N1) epizootic in cattle and the infection transmission to other mammals pose a serious threat to livestock industry and public health. In response to the existing risks, it is necessary to strengthen biosafety measures and surveillance in epidemiologically significant animal populations, incorporate the experience of other countries and establish international cooperation to study the trends of the virus evolution.

Keywords: review, avian influenza A(H5N1), cross-species transmission, cattle, cats, pigs, biosafety, animal movement, milk, epizootological surveillance

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Межвидовая передача вируса гриппа птиц A(H5N1) млекопитающим: уроки вспышек среди крупного рогатого скота в 2024–2025 гг.

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РЕЗЮМЕ

Введение. В 2024–2025 гг. произошла серия вспышек высокопатогенного гриппа птиц A(H5N1) среди молочного скота. Подобное изменение круга хозяев вируса повышает глобальные риски для животноводства и общественного здравоохранения, что требует усиления систем эпизоотологического мониторинга и контроля.

Цель исследования. Анализ эпизоотологических характеристик инфекции молочного скота и других млекопитающих, вызванной вирусом гриппа птиц A(H5N1) в 2024–2025 гг., а также обобщение принятых мер реагирования и рекомендаций международных организаций.

Материалы и методы. Применялись аналитические методы исследований с использованием баз данных PubMed, Scopus, Web of Science, Springer, Wiley Online Library и материалов международных организаций (FAO, EFSA, WOAH, OFFLU, CDC).

Результаты. Вспышки гриппа птиц A(H5N1), обусловленные вирусом клады 2.3.4.4b генотипов B3.13 и D1.1, среди молочного скота в 2024–2025 гг. произошли в результате трех установленных независимых случаев передачи возбудителя из популяции дикой птицы. Инфекция была выявлена на 1078 фермах у крупного рогатого скота в 17 штатах США. Зафиксирована последующая передача вируса домашней птице, диким и домашним животным, включая кошек, и людям (70 человек), в основном работникам ферм и птицефабрик, а также обратная передача возбудителя от коров к птицам. Повсеместное быстрое распространение вируса связано с массовыми перемещениями скота и недостаточностью мер обеспечения биобезопасности. Рекомендовано проводить исследования на грипп A(H5) при дифференциальной диагностике заболеваний крупного рогатого скота, свиней, домашних и диких животных.
Заключение. Эпизоотия гриппа птиц A(H5N1) среди крупного рогатого скота и передача инфекции другим млекопитающим демонстрирует серьезную угрозу для животноводства и общественного здоровья. В ответ на существующие риски необходимо усилить меры биобезопасности и надзор в эпидемиологически важных популяциях животных, учесть опыт других стран и наладить международное сотрудничество для изучения направлений эволюции вируса.

Ключевые слова: обзор, грипп птиц A(H5N1), межвидовая передача, крупный рогатый скот, кошки, свиньи, биобезопасность, перемещение скота, молоко, эпизоотологический надзор

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INTRODUCTION

A highly alarming development in recent years has been the worldwide reports of high pathogenicity avian influenza virus (HPAIV) detection in mammals (Fig.). Previously considered primarily as a threat to poultry, HPAIV has established a "new normal", characterized by transmission to mammals [1, 2, 3]. Since 2022, a significant increase in the number of H5 avian influenza cases has been reported in terrestrial animals (foxes, raccoons, minks, bears, tigers, lynxes, ermines, squirrels, etc.) and aquatic mammals (dolphins, seals, sea lions, walruses, etc.) [4]. In 2023, HPAI transmission with wild virus-infected mammals from North and South America to Antarctica was first reported [5]. Mass animal mortality was reported: from hundreds to thousands of sea lions in Peru, Argentina, Uruguay, and Brazil, fur seals in Chile and Brazil, and elephant seals in Argentina [3, 6]. In early 2024, mass mortality of southern elephant seals and fur seals caused by HPAI was reported in the sub-Antarctic zone, which was probably facilitated by their colonial lifestyle and high susceptibility to the pathogen [7]. In 2024, the situation worsened regarding HPAIV infection of domestic animals (cats) and humans from livestock, including cattle. This indicates a continuing threat of mammal-to-mammal transmission of the avian influenza agent and presents a significant risk for further viral mutation and the potential acquisition of human-to-human transmissibility [8].

According to the Food and Agriculture Organization of the United Nations (FAO), in the first quarter of 2025, the H5Nx subtype influenza virus was confirmed in almost 100 mammalian species, mainly carnivorous. Thus, in a relatively short period (2021–2025) of subtype H5Nx virus spread, the number of susceptible species of mammals and wild birds has doubled[1]. The expansion of the range of susceptible mammalian species and the geographical spread of the virus increase the risk of its spillover to humans, according to the World Organisation for Animal Health (WOAH). Therefore, it is extremely important to maintain reliable surveillance and comply with biosafety measures, especially for new and atypical avian influenza virus hosts [6].

The increased risk of the interspecies HPAIV transition to new mammalian species and from mammals to humans is facilitated by the following factors: high activity of type A(H5N1) virus circulation, increase in the genetic diversity within clade 2.3.4.4, accumulation of mutations resulting in adaptation to mammals (including increased viral replication and changes in virulence, host-specific polymerase activity, *inter alia* binding to human-type α2,6-linked sialic acid receptors, etc.) in gene segments encoding the key surface protein hemagglutinin (HA), another surface protein neuraminidase (NA), matrix proteins (M1 and M2), nucleoprotein (NP) and polymerase basic protein 2 (PB2) [9, 10, 11, 12].

Cases of infection in mammals that are in close contact with humans draw particular attention to the issue of the virus overcoming the interspecies barrier and increasing its pandemic potential. Such animals include farm animals (cattle, pigs) and pets (cats) [8, 9, 13, 14, 15].

In order to study the epizootological characteristics of the infection caused by avian influenza A(H5N1) virus in dairy cattle and other mammals in 2024–2025, as well as the response measures taken and recommendations from international organizations, an analysis of available scientific publications and materials from international organizations such as WOAH, FAO, European Food Safety Agency (EFSA), Global WOAH/FAO Network of Expertise on Animal Influenza (OFFLU), US Centers for Disease Control and Prevention (CDC) was made. Examination of the current HPAI pattern and experience in its control are essential for the improvement of the comprehensive disease control.

AVIAN INFLUENZA OUTBREAKS IN DAIRY CATTLE IN THE USA

Infection of cattle with influenza A(H5) virus is a dramatic change in the range of the virus hosts and the disease pattern, that has led to the sustained pathogen transmission

[1] Global avian influenza viruses with zoonotic potential situation update: Bird & mammal species affected by H5Nx HPAI. https://www.fao.org/animal-health/situation-updates/global-aiv-with-zoonotic-potential/bird-species-affected-by-h5nx-hpai/en

from one mammal to another and an increased risk for wild and domestic animals, as well as to agricultural workers, thus increasing global public health risks [14]. According to FAO, the detection of the virus in dairy cattle and cases of the infection in farm workers who have come into contact with the infected animals emphasize the need to strengthen the disease monitoring and control systems [16].

In March 2024, after reports of unexplained symptoms in dairy cattle in the United States, influenza A(H5N1) virus of clade 2.3.4.4b, genotype B3.13 was isolated from unpasteurized milk samples and oropharyngeal swabs collected from cattle [13]. Genotype B3.13 influenza causative agent is a reassortant of the European highly pathogenic strain of subtype H5N1 virus and the North American low pathogenic strain [1]. Sequencing and phylogenetic analysis of the isolated virus strains demonstrated that initially there was a single event of this pathogen (genotype B3.13) spillover from wild birds to cattle in late 2023 – early 2024, followed by its wide and rapid spread across the United States, mainly due to the peculiarities of cattle farming in the country. Subsequent transmission of genotype B3.13 influenza A(H5N1) virus from infected cattle to wild birds, poultry (chickens), wild mammals (raccoons), synanthropic rodents (mice), pets (cats) and humans (animal and poultry farm operators) was reported. Moreover, reverse transmission of the virus from cows to birds was registered [17].

In early 2025, the second and third independent cases of highly pathogenic clade 2.3.4.4b influenza virus spillover from wild birds to cattle were confirmed, with the pathogen belonging to genotype D1.1[2]. Currently, D1.1 genotype virus is the most commonly detected in North America and it infects wild birds and poultry, as well as mammals.

The website of the United States Department of Agriculture (USDA) features an updated map with the quantitative and territorial (by state) distribution of HPAI cases in animals (cattle, swine, alpacas)[3]. As of 01 August 2025, 1,078 cattle herds in 17 states were HPAIV infected. On the CDC website, the current situation in humans is displayed[4]. As of 07 July 2025, there were 70 confirmed cases of human infection with the avian influenza virus (mostly in animal and poultry farm employees). So far, the current public health risk is assessed as low.

The widespread transmission of highly pathogenic avian influenza A(H5N1) virus in cattle challenges long-established theories of influenza ecological dynamics and highlights substantial gaps in the global preparedness system for responding to such biological threats, necessitating immediate measures to address the identified deficiencies [14].

Infection transmission routes and risk factors. On-farm virus transmission mainly occurred through contaminated milk and during milking procedures (shared milking equipment), rather than by respiratory route [18], while the farm-to-farm spread is mainly due to the animal movements and use of shared equipment [13]. It was demonstrated that influenza A (H5N1) virus retains its infectivity for several hours in raw milk from infected animals remaining on milking machines and is detected in environmental samples from milking parlors, which highlights the risk of indirect transmission during routine milking. However, under experimental conditions involving prolonged co-housing (14 days) of infected and control cows, as well as shared use of milking equipment, the reproduction of the virus transmission failed [19]. During the model experiment, the possibility of calves becoming infected through the alimentary route by being fed raw milk from cows infected with the highly pathogenic subtype H5N1 genotype B3.13 avian influenza virus was confirmed [20]. At the same time, the calves' clinical signs were mild (nasal discharge, mild fever and lethargy, loose stools and rapid breathing) and similar to signs of other common diseases, which would complicate the diagnosis in the field.

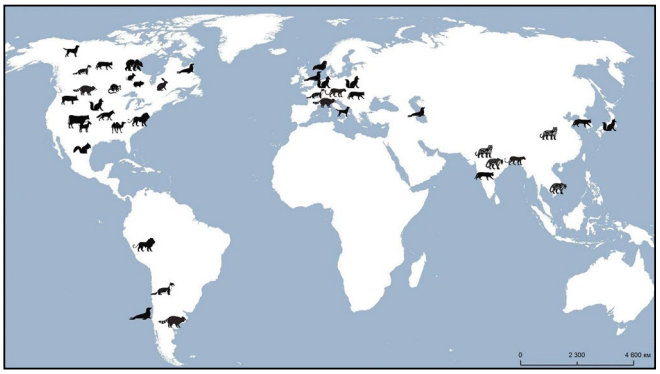

Fig. Geographic distribution of HPAI in mammals, 2022–2025 (symbols on the map indicate main mammalian families affected by HPAI outbreaks during this period; World Animal Health Information System, 01 August 2025)

Specifics farming practices in the United States imply extensive animal movements, both on-farm and from farm to farm, at different production stages [9]. For example, about 40% of the young replacement dairy cows are reared outside the farm. According to the National Epidemiological Survey of the United States Department of Agriculture (USDA), the onset of clinical signs in livestock on more than 50% of affected farms was preceded by the introduction of new cattle into the herd within the previous 30 days. Furthermore, over 45% of farms continued to move cattle even after animals had begun to demonstrate clinical signs[5]. Farm animal movements are allowed in the United States and the animals were moved without prior laboratory tests, which contributed to the virus spread in the absence of clinical signs.

Another highly probable route of the infection transmission between farms and states is non-compliance with biosafety requirements: use of shared transport and equipment (including those used for cleaning and feed and waste handling), lack of their disinfection; the clothes and shoes of regular staff who are simultaneously employed on other farms or have their own livestock and poultry, as well as farm visitors, who have access to animals (including veterinarians, feed consultants, animal breeders and hoof trimming specialists, carriers, etc.) [13, 16].

[2] Updated joint FAO/WHO/WOAH public health assessment of recent influenza A(H5) virus events in animals and people (Assessment based on data as of 1 March 2025). https://www.woah.org/app/uploads/2025/04/2025-04-17-fao-woah-who-h5n1-assessment.pdf

[3] HPAI confirmed cases in livestock. https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/HPAI-detections/HPAI-confirmed-cases-livestock

[4] H5 bird flu: Current Situation. https://www.cdc.gov/bird-flu/situation-summary/index.html

[5] Animal and Plant Health Inspection Service, U.S.D.A. Highly Pathogenic Avian Influenza H5N1 Genotype B3.13 in Dairy Cattle: National Epidemiologic Brief. https://www.aphis.usda.gov/sites/default/files/highly-pathogenic-avian-influenza-national-epidemiological-brief-09-24-2024.pdf

An additional risk factor involves mixed animal breeding and presence of pets in the animal housing areas, as well as feeding them and young cattle with unpasteurized milk. For example, cats were present on 75% of HPAI infected farms, and poultry was kept on 19% of the farms[6].

Thus, the virus widespread in the U.S. dairy herds was mainly due to asymptomatic transmission, lack of surveillance in epizootologically important populations, and insufficient compliance with biosafety measures.

Currently, the routes and means of the pathogen transmission in cattle as well as the virus shedding duration, etc. are being studied. A preliminary model study demonstrates that the duration of the infection period can vary from 2.8 to 13.1 days, with a median of 6.2 days [21].

Clinical signs were reported on average in less than 20% of cases, and mortality did not exceed 2%. The following prevailing clinical signs are described in cattle [13, 14]:

– decrease in milk production and change in milk quality (color, consistency, coagulation);
– loss of appetite and reduced rumen activity;
– fever;
– mastitis;
– dehydration;
– changed feces consistency;
– nasal discharge and respiratory distress.

During the outbreaks in the United States, clinical signs in cattle persisted for up to 21 days (an average of 6 days), with the exception of changes in milk quality. Milk production was reported to be reduced for up to 45 days (an average of 12 days). Despite the fact that viral RNA was detected in milk, nasal swabs, urine, and sera of infected cattle, the highest concentrations of the infectious virus were consistently detected in milk and mammary gland tissues [22].

Milk tests. The H5 avian influenza virus actively replicates in the mammary glands, and the infected cows shed high quantities of the virus in milk for up to 3 weeks, even in the absence of clinical signs [13]. The widespread expression of receptors for both avian and human influenza viruses in cow mammary glands combined with a high viral load in milk (from $10^{4.0}$ to $10^{8.8}$ $TCID_{50}$/mL) suggests local replication of the virus. It was experimentally demonstrated that intramammary exposure to even low doses of genotype B3.13 influenza A(H5N1) virus (from 10^{1} to 10^{3} $TCID_{50}$) is sufficient for stable infection establishment, shedding high titers of the virus in milk, and clinical mastitis development [19]. Intramammary exposure to influenza A(H5N1) virus in high doses leads to severe clinical outcomes and death observed in dairy cows on farms, while respiratory and oral exposure are less likely to result in productive infection and related morbidity.

During laboratory studies, various methods were used to isolate influenza virus RNA from raw milk samples, including column-based and magnetic sorbent extraction, as well as phenol-chloroform extraction method (Trizol LS). The efficiency of isolation can be influenced by pretreatment of milk samples, as well as their storage conditions [8].

Studies have shown that milk increases the thermal stability of the influenza viruses, but results of the experiments on the pathogen inactivation under different heating conditions and processing time are contradictory [23]. A number of studies indicate that industrial pasteurization of milk is a reliable method of the avian influenza virus inactivation [24]. Other studies mention that neither pasteurization at 72 °C for 15 seconds nor at 63 °C for 30 minutes can completely kill the virus in milk, whereas heat treatment at 80 °C for 15 seconds completely inactivates H5N1 influenza virus in milk [23, 25]. Despite the fact that viral RNA is detected in the samples of pasteurized dairy products, no infectious virus has been detected so far [26]. This further highlights the potential dangers of unpasteurized milk and dairy products made from it. Laboratory experiments have demonstrated that the HPAIV remains viable in raw cow and sheep milk for over 24 hours at room temperature and for more than 7 days under refrigeration [27]. Furthermore, studies of raw milk cheese showed that infectious avian influenza A(H5N1) virus can persist in such a product for several months (more than 60 days)[7]. The results obtained indicate the need to implement additional measures to reduce the risks of infection for both animals and humans during the production and consumption of raw milk products [28].

AVIAN INFLUENZA IN PIGS AND CATS

Pigs have unique anatomical and physiological characteristics of the respiratory tract, which may facilitate infections caused by influenza A virus strains that are not adapted to them [29]. Thus, in October 2024, the U.S. Department of Agriculture confirmed the presence of the avian influenza A(H5N1) pathogen in a pig on a backyard farm in Oregon[8]. On this farm, both poultry and livestock – including pigs – were kept together and shared common water sources, facilities, and equipment. The pig did not demonstrate any clinical signs of infection caused by influenza A(H5) pathogen. No specific adaptation of the virus to humans or mammals has been identified. The detection of H5N1 viral genetic material in pig tissues and excrement indicates their potential role in maintaining and amplifying the pathogen transmission [14].

A preliminary experimental study in pigs demonstrated limited replication of genotype B3.13 influenza A(H5N1) virus isolated from cattle and no pathogen transmission through direct contact from the infected pigs to the uninfected ones. Experimental intranasal and oral infection of pigs with another genotype of influenza A(H5N1) virus of clade 2.3.4.4b revealed that the strains isolated from mammals demonstrated a higher potential for replication, pathogenicity, and transmissibility compared with the strains isolated from birds [30, 31]. Thus, pigs represent a critically important element in the mechanism of interspecies transmission, especially under conditions of mixed livestock farming. This factor requires close attention and the need to test pigs for avian influenza virus due to its genetic diversity and scale of circulation, since pigs act as "mixing vessels" for genetic reassortment of avian and human influenza viruses, potentially contributing to the emergence of new strains with pandemic potential. Pigs must

[6] Animal and Plant Health Inspection Service, U.S.D.A. Highly pathogenic avian influenza H5N1 genotype B3.13 in dairy cattle: National epidemiologic brief. https://www.aphis.usda.gov/sites/default/files/highly-pathogenic-avian-influenza-national-epidemiologicalbrief-09-24-2024.pdf

[7] United States Food and Drug Administration. Investigation of Avian Influenza A (H5N1) Virus in Dairy Cattle. https://www.fda.gov/food/alerts-advisories-safety-information/investigation-avian-influenza-h5n1-virus-dairy-cattle

[8] Federal and State Veterinary Agencies Share Update on HPAI Detections in Oregon Backyard Farm, Including First H5N1 Detections in Swine. https://www.aphis.usda.gov/news/agency-announcements/federal-state-veterinary-agencies-share-update-HPAI-detections-oregon

be incorporated into comprehensive epizootiological surveillance systems to more accurately assess the ecological characteristics of the H5N1 influenza virus as it circulates in domestic animal populations and to analyze their potential role in zoonotic outbreaks.

Importantly, in recent years there has been an increase in interspecies transmission of avian influenza A(H5N1) virus to cats. Since 2022, at least 88 cases of domestic cat infection with this subtype virus have been reported in the United States alone. In 2024–2025, infection and mortality cases were reported in domestic and wild members of the *Felidae* family in India, Vietnam, the Netherlands and other countries [32]. Cats can act as the virus carriers or intermediate hosts, as they are in close contact with humans and animals of other species. Infection of cats with both avian influenza and human influenza viruses is possible, which can lead to the virus adaptation and emergence of recombinant strains with zoonotic potential [8, 15, 32, 33].

During influenza outbreaks caused by subtype A(H5N1) virus of clade 2.3.4.4b in 2024–2025, neurological and respiratory signs, as well as high mortality, were reported in infected cats in the United States, indicating the susceptibility of the *Felidae* family members to the virus and their potential role in its transmission. Cats kept at home, as well as animals from shelters, are at increased risk of infection, especially when they come into contact with infected wild birds, consume raw infected poultry meet or unpasteurized milk from cattle infected with influenza A virus [15, 34]. For example, in raw milk consumed by cats with clinical signs of avian influenza virus infection, reverse transcription polymerase chain reaction (RT-PCR) revealed the RNA of subtype H5N1 pathogen. After consuming raw milk derived from infected cattle, about 50% of cats became diseased and died [13]. HPAI A(H5N1) virus was detected in domestic cats in the homes of dairy farm workers[9], while there was no direct contact of animals with the avian influenza affected farm, which suggests the pathogen transmission with the humans (for example, with contaminated footwear and clothing) [35]. Clinical signs in infected cats included depression, body stiffness, ataxia, blindness, circling, ocular and nasal discharge. The influenza A virus antigen was detected in the brain, lungs, heart, and retina of the diseased cats [13]. Therefore, when examining cats that have had contact with wild birds or have consumed raw poultry or dairy products, and that present with acute neurological and respiratory symptoms, veterinarians should include avian influenza in the list of differential diagnoses [34].

MEASURES TAKEN AND RECOMMENDATIONS

In response to the unprecedented HPAIV spread in cattle in the United States, the following response measures have been implemented at the governmental level: restrictions on the import of cattle with clinical signs of the disease, as well as on the movement of animals from infected states; requirements for veterinary inspection certificates prior to transport; testing of dairy cattle before interstate movement; enhanced biosafety measures; restrictions on exhibitions; and the imposition of quarantine [13, 16]. State financial support was offered to dairy producers to ensure the necessary biosafety level and to compensate for the costs associated with HPAI outbreaks. In addition to federal decrees, additional response measures were implemented at the state level.

On 06 December 2024, the USDA announced the launch of the National Milk Testing Strategy (NMTS)[10, 11]. The total number of PCR tests conducted in the first year, from April 2024 to April 2025, amounted to 210,146. The NMTS specifies that before transporting dairy cattle from one state to another, a negative test result for influenza A virus must be obtained from an accredited laboratory; the movement of cattle with influenza clinical signs and their transport for slaughter are not permitted. Milk sampling should be carried out under the supervision of a licensed or accredited veterinarian. The sample size should be from 3 to 10 mL and contain milk from each udder lobe. Milk samples can only be pooled in a laboratory. All animals moved in a group (consignment) of 30 or fewer animals must be tested. If larger numbers of animals are being moved, then a total of only 30 animals shall to be tested. Samples shall be collected and tested no later than 7 days before the movement. If positive results for influenza A are obtained, dairy cattle are not permitted to be transported for 30 days from the date of the last positive sample collected from any animal in the herd. After the 30-day period, the animals must be retested. Dairy cattle without clinical signs transported directly to slaughter are not required to be subjected to the pre-movement test, but they must have a veterinary inspection certificate.

Based on the analysis of epizootological study results of the animals infected with influenza A(H5N1) virus, the USDA recommends strengthening biosafety measures on dairy farms:

– avoid sharing equipment and vehicles, and disinfect them;

– comply with biosafety requirements during any contact between people (personnel, visitors) and animals on the farm;

– exclude open storage of feed and litter material and prevent contact of these materials with domestic and wild animals and birds;

– avoid housing animals of different species together on the farm;

– utilize advanced waste management practices to prevent infected manure from entering compost;

– carry out pasteurization, chemical, or heat treatment of dairy waste;

– disinfect raw milk (pasteurization) if it is subsequently fed to calves or other animal species;

– quarantine new animals added to the herd for minimum 30 days, and isolate any cattle displaying the disease clinical signs.

The literature also emphasizes the importance of proactive control of HPAI in wildlife [7, 12] and in domestic animals [9]. The need is emphasized for monitoring wild animal populations for subtype H5 HPAIV, based on the data on unusual morbidity levels and recorded mortality cases, as well as on the results of virological and serological

[9] Naraharisetti R., Weinberg M., Stoddard B., Stobierski M. G., Dodd K. A., Wineland N., et al. Highly pathogenic avian influenza A(H5N1) virus infection of indoor domestic cats within dairy industry worker households – Michigan, May 2024. https://www.cdc.gov/mmwr/volumes/74/wr/mm7405a2.htm?s_cid=mm7405a2_w

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analyses, including timely exchange of diagnostic information on the disease and viral genome sequences for the rapid detection of new incursions of the virus and tracking its evolution through phylogenetic analyses. Enhanced biosafety measures are coming to the fore for domestic and farm animals.

In light of reports of cattle and other mammalian species being infected with avian influenza virus, the FAO has published updated recommendations. These recommendations include guidance on implementing effective monitoring programs for the timely detection of avian influenza cases in cattle [16]. Among them are the following:

– strengthen epizootological surveillance and timely report with a view to early detection of influenza A(H5) virus in poultry, wild birds and mammals;

– include influenza A(H5) in differential diagnosis for cattle, pigs and farm animals, as well as for domestic and wild animals;

– promptly report HPAI cases in all animal species, including cattle and other domestic and wild mammals, to international organizations (WOAH, FAO);

– carry out active monitoring/detection of the disease cases, using *inter alia* molecular and serological tools;

– provide the persons in contact with animals with appropriate personal protective equipment, as well as provide them with the capacities for testing;

– perform genome sequencing and deposit the influenza virus genetic sequences and associated metadata in the publicly accessible databases;

– implement and/or strengthen biosafety systems on the animal farms/premises and throughout the production chain;

– take preventive measures and early response measures to interrupt the infection chain in pets.

CONCLUSION

The avian influenza A(H5N1) outbreak in dairy cattle in the United States represents a significant change in the epidemic potential of the virus and its ability to interspecies transmission. This event highlights the need to strengthen the epizootological monitoring systems and mandatory compliance with biosafety measures in animal husbandry. The atypical and vague clinical manifestations of avian influenza in cattle complicate the disease diagnosis and timely measures. It is important that the virus can be excreted in milk for several weeks even in the absence of clinical signs. This fact underscores the need for strict compliance with dairy processing protocols, including pasteurization as an effective method of virus inactivation. Pigs and cats have also been found to be susceptible to the infection caused by influenza A(H5N1) pathogen, thus raising concerns about their potential role in the virus evolution and spread.

Further efforts are needed to address gaps in the biological threat monitoring and response system. To effectively control the subtype H5N1 influenza virus spread in animals and minimize the risk of zoonotic infections, the experience of other countries should be taken into account and comprehensive measures aimed at the pathogen spread prevention should be taken. The inclusion of influenza A(H5) virus infection in the differential diagnosis for cattle, pigs and other mammals, as well as increased epizootological surveillance, are key steps in ensuring safety of animal husbandry and public health. When studying the avian influenza virus evolution and developing effective prevention strategies, it is important to carry out genomic monitoring and establish international scientific cooperation.

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Applying One Health approach to the study of West Nile fever in the Russian Federation

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ABSTRACT

Introduction. West Nile fever is a zoonotic transmissible disease caused by flavivirus that primarily circulates in nature within an enzootic cycle between mosquitoes and birds, and causes disease cases in humans, horses, and other mammals. The rapid expansion of the West Nile fever pathogen range, development of outbreaks with severe clinical manifestations, and the lack of specific preventive tools have been the main arguments for classifying it as a potentially dangerous threat to global health. The close interconnection between human health, animal health, and ecosystems necessitates communication and coordination across the relevant sectors. One Health is an integrated, unifying approach aimed at optimizing the health of humans, animals, and ecosystems involving public health, veterinary and environment protection authorities.

Objective. Analysis of basic epizootological data on the spread of West Nile fever in the Russian Federation.

Materials and methods. The following international and Russian databases were used for literature searching: PubMed, Springer, Google Scholar, CrossRef and Russian Science Citation Index (RSCI), eLibrary, CyberLeninka, respectively. The searching was performed based on the following key words: West Nile fever, One Health, migration of birds, invertebrate hosts, control measures.

Results. In Russia, West Nile virus was first isolated in Astrakhan Oblast in 1963. Currently, presence of the pathogen has been proven in the southern and central regions of the European part of the country, in the south of Western Siberia and the Far East. The lack of disease monitoring in some Russian regions and small numbers of samples tested in most subjects of the Russian Federation hinder an objective assessment of the disease situation, so there is a need to increase the number of tests. It has been shown that the main carriers of the pathogen in our country are mosquitoes of the genera *Culex, Anopheles* and *Aedes*, ixodid, argasid and gamasid ticks are also involved in maintaining the virus circulation. The review describes the role of birds in the pathogen transmission, provides data on susceptibility of animals to the infection, discusses modern aspects of West Nile fever diagnosis, prevention and control.

Conclusion. Surveillance of West Nile fever presents a considerable challenge, as the virus circulates among humans, arthropods, and birds. While vaccination is an effective preventive tool, no vaccines against the disease have yet been developed in Russia. In this context, strengthening inter-authority coordination and implementing environmental control measures to limit the virus spread are essential priorities.

Keywords: review, West Nile fever, One Health, migration of birds, invertebrate hosts, control measures

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Концепция «Единое здоровье» в изучении лихорадки Западного Нила на территории Российской Федерации

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РЕЗЮМЕ

Введение. Лихорадка Западного Нила – зоонозное трансмиссивное заболевание, вызываемое флавивирусом, который чаще всего циркулирует в природе в энзоотическом цикле между комарами и птицами и вызывает случаи болезни у людей, лошадей и других млекопитающих. Стремительное расширение ареала возбудителя лихорадки Западного Нила, развитие вспышек с тяжелым течением болезни, отсутствие средств специфической профилактики послужили главными аргументами при отнесении его к группе потенциально опасных угроз глобальному здравоохранению. Тесная взаимосвязь между здоровьем людей, животных и экосистемой требует коммуникации и координации между соответствующими секторами. Принцип «Единое

здоровье» – это интегрированный, объединяющий подход, направленный на оптимизацию здоровья людей, животных и экосистем, включающий в себя привлечение органов общественного здравоохранения, ветеринарии и охраны окружающей среды.

Цель исследования. Анализ основных эпизоотологических данных о распространении лихорадки Западного Нила на территории Российской Федерации.

Материалы и методы. Поиск источников литературы осуществлялся с использованием международных (PubMed, Springer, Google Scholar, CrossRef) и российских (РИНЦ, eLibrary, КиберЛенинка) баз научного цитирования. Отбор материала проводился по ключевым словам: лихорадка Западного Нила, единое здоровье, миграция птиц, беспозвоночные хозяева, меры контроля.

Результаты. В России вирус Западного Нила был впервые изолирован в 1963 г. в Астраханской области. В настоящее время присутствие возбудителя доказано в южных и центральных регионах европейской части страны, на юге Западной Сибири и Дальнего Востока. Отсутствие эпизоотологического мониторинга заболевания в отдельных регионах России и низкие объемы исследуемого материала в большинстве субъектов не позволяют дать объективную оценку эпизоотической ситуации, поэтому имеется необходимость в увеличении числа проводимых исследований. Показано, что основными переносчиками возбудителя на территории нашей страны являются комары родов *Culex, Anopheles* и *Aedes,* также в поддержании циркуляции вируса принимают участие иксодовые, аргасовые и гамазовые клещи. В обзоре описана роль птиц в передаче возбудителя, приводятся сведения о восприимчивости животных к инфекции, рассмотрены современные аспекты диагностики, профилактики лихорадки Западного Нила и меры борьбы.

Заключение. Надзор за лихорадкой Западного Нила является достаточно сложной задачей, поскольку циркуляция вируса происходит среди людей, членистоногих и птиц. Вакцинация является эффективным средством профилактики, однако иммунологические профилактические препараты против заболевания в России пока не разработаны. В связи с этим особое внимание должно уделяться взаимодействию между различными ведомствами, а также профилактическим мерам, направленным на снижение распространения вируса в окружающей среде.

Ключевые слова: обзор, лихорадка Западного Нила, «Единое здоровье», миграция птиц, беспозвоночные хозяева, меры контроля

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

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INTRODUCTION

Zoonoses cause billions of human infections annually, representing a major global public health challenge [1]. The vast diversity of animal species living on the territory of the Russian Federation (RF) creates favourable conditions for the development of infectious diseases, including those transmitted by arthropods. West Nile fever (WNF), caused by a flavivirus, is a zoonotic vector-borne disease. It primarily circulates in an enzootic cycle between mosquitoes and birds, with spillover causing the disease in humans, horses, and other mammals [2, 3, 4]. West Nile virus (WNV) has been detected in more than 300 bird species, including wild, domestic, and synanthropic species. The migration routes of birds that nest across vast areas of Russia and winter in southern countries create a pathway for the introduction of the virus from wintering grounds, potentially leading to the formation of seasonal or persistent natural foci [5]. The rapid expansion of the WNV range, the emergence of severe disease outbreaks, and the absence of specific preventive measures were the primary factors justifying its classification as a potentially dangerous threat to global health – a consideration reflected in the World Health Organization's (WHO) International Health Regulations[1] [6]. The interdependence of human, animal, and ecosystem health, necessitating cooperation, communication, and coordination between the relevant sectors. One Health principle is an integrated, unifying approach aimed at sustainable balance and optimization of human, animal and ecosystem health, including the involvement of public health, veterinary and environmental protection authorities [7, 8]. The application of this approach is the key to obtaining a comprehensive understanding of WNF situation and measures for its control.

[1] International Health Regulations (2005). 3rd ed. WHO; 2016. 74 p. https://www.who.int/publications/i/item/9789241580496

The purpose of the review is to analyse the main epizootological data on WNF spread in the RF.

MATERIALS AND METHODS

The literature sources were searched using international (PubMed, Springer, Wiley Online Library, Google Scholar, CrossRef) and Russian (RSCI, eLibrary, CyberLeninka) scientific citation databases. The selection of the material was carried out according to the keywords: West Nile fever, One Health, bird migration, invertebrate hosts, control measures.

RESULTS AND DISCUSSION

West Nile fever is caused by a flavivirus maintained in a natural transmission cycle between birds and mosquitoes. Humans, horses, and other mammals are considered incidental or dead-end hosts [9, 10]. According to the classification of the International Committee on Taxonomy of Viruses (Release 2022), WNV belongs to the genus *Orthoflavivirus* of the *Flaviviridae* family and is a small (about 50 nm in diameter) spherical enveloped flavivirus, the genome of which consists of a single-stranded positive-sense RNA molecule encoding three structural and seven nonstructural proteins [11, 12]. Based on phylogenetic analysis, WNV was grouped into 9 lineages [13]. Strains of lineages 1 and 2 are the most virulent and are capable of causing outbreaks of infection with severe neurological signs [14, 15, 16].

In 1937, WNF agent was first isolated from a woman in West Nile District in Uganda (Africa). Since then, the virus has spread widely around the world, causing outbreaks in humans and animals on all continents, including most of Africa, Eastern and Southern Europe, North America, Western Asia and the Middle East. Outbreaks in humans have been reported in South Africa, the USA, Algeria, Tunisia, Morocco, Romania, Israel, Italy, and Greece. Horse-related epizootics

Fig. 1. Administrative territories of the Russian Federation where West Nile fever human cases were reported in 2024 (according to the data of the Volgograd Plague Control Research Institute of the Rospotrebnadzor[2])

have occurred in Morocco and Italy. In Russia, direct evidence of WNV presence was first obtained in 1963 when studying Crimean-Congo hemorrhagic fever foci in Astrakhan Oblast. Southern Russian territories are historically endemic for WNF; however, global climate change has facilitated northward spread, with recent outbreaks documented in central regions and the Volga basin. Currently, the presence of the WNF agent has been proven in the southern and central regions of the European part of Russia, in the south of Western Siberia and the Far East [6, 17, 18, 19, 20].

Humans are very susceptible to WNV, but they are considered dead-end hosts. Approximately 80% of human infections with WNV are asymptomatic. In the majority of symptomatic cases (20%), patients have mild fever associated with myalgia, arthralgia, headache, fatigue, intestinal disorders, rash, and enlarged lymph nodes. Less than 1% of them develop serious neurological complications, manifested by various pathologies such as meningitis or meningoencephalitis, acute flaccid paralysis, and eye diseases. Encephalitis is the most severe neurological form, which can sometimes be fatal, especially in the elderly people and people with compromised immunity. Assessing persistent WNV infection in humans remains challenging; however, detection of the virus in urine up to 9 years post-infection demonstrates long-term renal persistence. Consequently, WNV can cause chronic human disease [11, 19].

Epidemiological surveillance of sylvatic cycle infections having transmission mechanism in our country is carried out by the Volgograd Plague Control Research Institute of the Rospotrebnadzor. There are Reference Centres for WNF, Zika fever, tick-borne infections, etc. at the Institute. According to the Reference Centre data on monitoring of WNF agent, human cases were reported in 33 Subjects of the RF in 2024 (Fig. 1). Laboratory tests of birds for WNV markers in 2024 were carried out in 34 RF Subjects, which is only 38.2% of the total number of administrative territories of the RF. Mosquitos were tested for the infection in 76 RF Subjects (85.4% of the total number of administrative territories of the RF), ticks were tested for the infection in 58 RF Subjects (65.2%). Tests of large mammals for antibodies to WNV were carried out in 5 RF Subjects, the virus markers detection rate was 3.4%.

The absence of epizootological monitoring for WNV in some regions of the country, combined with the low volume of samples tested in most RF Subjects, precludes an objective assessment of the disease situation. Consequently, there is a clear need to increase the volume of testing. Therewith, the exchange of data on the WNF surveillance between different authorities would provide a more complete understanding of the epidemiology and epizootology of the pathogen and improve disease prevention and control measures. To do this, it is necessary to create a single cross-departmental database based on integrated animal and environmental monitoring systems, and the data exchange mechanism should be transparent and standardized [21].

Ecology of the virus. WNV is maintained in a "bird – mosquito – bird" enzootic cycle, with birds serving as amplifying hosts. Mosquitoes become infected by feeding on the blood of infected birds and remain contagious throughout their lives. The disease agent is transmitted to animals and humans through mosquito bites (mainly of the genus *Culex*), but it can also be transmitted through organ transplantation, blood transfusion, and from mother to foetus during pregnancy [2, 22, 23]. The biological cycle of WNV spread is shown in Figure 2.

After entering the mosquito's body, the virus first infects the midgut. It then crosses the midgut barrier, disseminates via the hemolymph to other organs, and ultimately infects the salivary glands – a prerequisite for transmission to new susceptible vertebrate hosts. The virus is transmitted to humans and animals through the bites of infected mosquitoes. Once inside the host, it can replicate and lead to clinical disease [19]. At the same time, WNV does not cause apparent disease in mosquitoes [24]. The virus may also be maintained in mosquito populations via vertical

[2] Results of epizootological monitoring of West Nile fever in the Russian Federation in 2024 (according to the Volgograd Plague Control Research Institute of the Rospotrebnadzor). http://vnipchi.rospotrebnadzor.ru/s/203/files/directions/centre/lixoradka/analiz/145607_505.pdf (in Russ.)

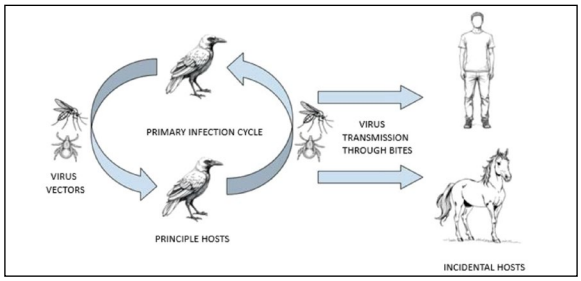

Fig. 2. Biological cycle of West Nile virus transmission (the drawing was prepared by D. A. Lunina)

transmission, in which an infected female passes the pathogen directly to her progeny. Mosquito pupae can become infected through experimental exposure to infectious mosquito secretions, potentially establishing a reservoir of infection within the mosquito population without the involvement of vertebrate hosts [25].

In humans, other vertebrates, and birds, WNV multiplies in various cell types – including keratinocytes, neutrophils, and monocytes – following the bite of an infected mosquito. The virus then disseminates via the bloodstream to peripheral organs such as liver, kidneys, and spleen. To reach the brain, the virus must cross the blood-brain barrier, which can occur in two main ways: the first involves axonal retrograde transport through the spinal cord, and the second involves transendothelial migration from the bloodstream [19].

Of approximately 100 blood-sucking mosquito species living in Russia, WNV markers have been detected in representatives of the following species: *Culex modestus* Fic., *Cx. pipiens* L. (non-autogenous form of *Cx. pipiens f. pipiens* and autogenous form of *Cx. pipiens f. molestus*), *Anopheles maculipennis* Mg., *An. claviger* Mg., *An. hyrcanus* Pall., *An. messeae* Pall., *Aedes cinereus* Mg., *Ae. geniculatus* Oliv., *Ae. vexans* Mg., *Ae. caspius* Pall., *Ae. pulchritarsis* Rond., *Ae. albopictus* Sk., *Ae. cataphylla* Dyar, *Ae. flavescens* Mull., *Ae. excrucians* Walk., *Ae. cantans* Mg., *Culiseta annulata* Schr., *Coquillettidia richiardii* Fic., *Uranotaenia unguiculata* Edw. Using Astrakhan Oblast as a model, it was shown that in anthropogenic biocenoses, the epidemiologically significant vectors include *Cx. pipiens*, *An. hyrcanus*, *Coq. richiardii*, and *An. messeae*. In natural biocenoses, *An. hyrcanus* and *Coq. richiardii* play this role. In another active WNF focus located in Volgograd Oblast, high WNV infection rates were found for mosquitoes *Cx. modestus*, *Cx. pipiens*, *An. maculipennis*, and *An. hyrcanus* [6, 26]. Thus, mosquitoes of the genera *Culex*, *Anopheles* and *Aedes* are the main vectors of WNV in the RF. The genus *Culex* is ornithophilic and very aggressive towards humans. The largest number of *Culex* mosquitoes is observed in July and August. During the same period, an increase in human morbidity is observed, which is typically preceded by epizootics first among wild birds, and subsequently among domestic and synanthropic bird populations [27].

The high number of ticks in the region is also of epizootological importance. Ixodes ticks, argas ticks and gamasid mites take part in maintaining WNV circulation in Russia. Ticks of the families *Ixodidae* and *Argasidae* are believed to play a secondary role in virus transmission; their primary importance may lie in serving as overwintering reservoirs for the virus. Virus markers (antigen, RNA) were identified in entomological material and WNV isolates were recovered from ticks of 12 species, of which most often from *Hyalomma marginatum*, *H. scupense*, *Rhipicephalus rossicus*, and *Dermacentor reticulatus* in the south of the European part of the country [6, 28]. In some years, the infection rate in *H. marginatum* ticks in anthropogenic biocenoses of the Volga Delta significantly exceeded that of mosquitoes. In addition, corvids exhibited heavy infestations with *H. marginatum* larvae and nymphs (up to 300 individuals per bird), suggesting a key role for these ticks in viral maintenance [29].

Higher ambient temperatures enhance viral replication within vectors and shorten extrinsic incubation periods, thereby promoting virus circulation and outbreak occurrence [30]. Drought reduces water flow, forming stagnant pools enriched with organic matter – optimal breeding sites for mosquitoes. Birds gather around small water bodies during droughts, and this enhances the interaction of birds and mosquitoes. Although infected mosquitoes can spread WNV over long distances – both unaided (e.g., via wind currents) and through human-mediated transport (e.g., on boats or airplanes) – infected migratory birds that carry the virus to new areas remain the primary route of WNV dissemination [31].

Role of birds in WNV transmission. Birds have the physical ability to travel thousands of kilometres across continents in a few days, crossing geographical barriers such as mountains, deserts, and seas. The intercontinental movement of wild birds across vast geographical areas facilitates the exchange of pathogens between regions and populations. Migratory birds transport various infectious agents en route and likely drive the wide-scale spread of some arboviruses. WNF circulates in nature via a sylvatic cycle involving wild birds and ornithophilic mosquitoes, alongside an urban cycle involving synanthropic and domestic birds. Migratory birds play a crucial role in WNV introduction, while resident species living in a certain territory participate in the virus amplification and local circulation. WNV persists through winter in infected female mosquitoes and avian hosts, reducing reliance on migratory birds for seasonal reintroduction [32, 33].

The most common route of infection in birds is through the bite of a mosquito that has previously fed on an infected bird. Many bird species do not develop any symptoms of the disease after infection. However, some species, such as crows, jays, and predatory birds, may die from infection. Another WNV transmission mechanism is contact. It can lead to infection, as some bird species shed large amounts of the virus with their cloacal excretions. Contact transmission can be epizootically significant when a large numbers of birds are concentrated in one area (in nesting colonies or at stopover sites during migration). Predatory birds such as hawks and owls, can transmit WNV via predation on infected birds and other susceptible vertebrates, which serve as either live prey or carrion. The long-term persistence of WNV in the tissues of infected animals can increase the likelihood of infection in predatory birds, even several months after the end of the mosquito season – providing a potential mechanism for the virus overwintering [34].

Five main wild bird migratory routes pass through the territory of Russia: East Atlantic, Mediterranean-Black Sea, West Asian-African, Central Asian and East Asian-Australasian[3]. Different populations of the same species may use different migratory routes, or they may use one. Birds

[3] Krasnova E. D. About bird migrations. ROSIP. https://birdsrussia.ru/about/articles/e-d-krasnova-o-ptichikh-pereletakh (in Russ.)

introduce the virus into the territory of the RF from Africa, Southwest Asia, and Southeast Asia. WNV is introduced into the European part of Russia from Africa by migratory birds that follow southern and south-western flight paths in autumn. These include lake gulls, quails, swallows, ducks, sandpipers, rooks, starlings, and many other species. Birds of Western Siberia: geese, ducks, sandpipers, gulls, passerines, also follow the south-western direction during the autumn migration. Differences in avian migratory routes drive the formation of distinct WNV genotype foci in adjacent territories across Europe and Russia. Even between Volgograd and Astrakhan Oblasts, both the species composition and migratory routes of birds differ [27].

Bird migration routes in the central regions of the European part of Russia run in a meridional direction, mainly along river valleys. The bird aggregation sites are located in water-storage basins, lakes, and wetlands. Spring migration takes place between mid-March and May, while autumn migration occurs from September through October. During these periods, near-water birds – including ducks, geese, cranes, gulls, and others – undertake continuous, round-the-clock flights. More than 50 bird species have been implicated in the virus transmission in wetlands and in the vicinity of the Volga Delta. In wild birds, storks and other birds of the order *Ciconiiformes*, as well as great cormorants (*Phalacrocorax carbo*), Eurasian coots (*Fulica atra*), great cormorant (*Gallinula chloropus*), great grebes (*Podiceps cristatus*), gulls and terns (family *Laridae*) were most often identified as the virus hosts [26].

Avian species exhibit marked variation in both susceptibility to WNV infection and the clinical severity of the disease. Some avian species show virtually no symptoms of infection, but remain viremic for several days after infection, and then develop lifelong immunity. In other bird species, infection can lead to severe neurological disease and sudden death [18, 22, 35]. Most poultry species are not susceptible to the disease. In galliform birds – such as chickens, pheasants, guinea fowl, and turkeys – infection does not result in morbidity or mortality, and viremia is minimal. In contrast, predatory birds (such as hawks, owls, and eagles) appear to be susceptible to natural infection and exhibit a wide range of clinical signs [34]. In some avian species, the virus affects the central nervous system and organs such as the heart, liver, spleen, and kidneys. WNF clinical signs in birds range from nonspecific symptoms – weight loss, lethargy, and ocular disturbances – to neurological manifestations, including weakness, ataxia, head tilt, and tremors [19].

Corvids (crows, jays, magpies) serve as key sentinels for WNV circulation, as they develop fatal encephalitis – an indicator of active viral spread in emerging endemic areas. For example, in 1999, the deaths of American crows (*Corvus brachyrhynchos*) near Bronx Zoo marked the first indication of WNV introduction into the region. Since that time, these birds have been recognized as effective sentinels for the disease [36].

Susceptible animals. Some wild vertebrates, such as squirrels, chipmunks, house mice, hamsters, bats, bears, wolves, tigers, lions, striped skunks, raccoons, and crocodiles, are also affected by WNV. Wild boars serve as ideal sentinels for WNV circulation, frequently encountering infected mosquitoes in forest habitats while possessing favourable physical traits – sparse coat density and thin epidermis – that enhance mosquito feeding success. The use of alternative sentinels can help in the detection of WNV transmission routes outside of the "bird – mosquito – bird" enzootic cycle. In particular, monitoring of wild mammals can cover a variety of habitats and time intervals during which the virus transmission may occur [37]. Among domestic animals, horses, cattle, sheep, pigs, dogs, cats, and other species are susceptible to WNV infection; however, their role in the maintenance of the virus in nature has not yet been determined [11]. As a rule, these species develop insufficient viremia for the virus transmission, and they are considered accidental or dead-end hosts [17].

Horse infections with WNV are predominantly subclinical, though some cases manifest clinical signs of the disease. Experimental infection has shown that the incubation period before onset of the first neurological signs in horses lasts for 7–9 days. The most common clinical signs are weakness, anorexia, and lethargy. Eye disorders may also develop, although blindness is considered one of the rarest clinical manifestations. Common clinical signs include enterocolitis, colic, rectal prolapse, lameness, cervical/thoracic pain, anaemia, glossitis, jaundice (suggesting hepatopathy), and dysuria (indicating nephropathology). The onset of neuroinvasive disease occurs around day 7 post-experimental infection, the clinical manifestations include ataxia, muscle weakness, fever, anorexia, lethargy, gnashing of teeth, hydrophobia, anxiety, circular movements, systemic muscle tremor and facial paralysis. WNV predominantly targets motor neurons in the brainstem (midbrain and rhombencephalon) cranial nerve nuclei, resulting in clinical signs such as dysphagia, hypersalivation, and unilateral facial nerve paralysis. In horses, the spinal cord and grey matter of the medulla oblongata and rhombencephalon are most affected, while the cerebral cortex appears to be less involved. Mortality in neuroinvasive equine cases varies from 22 to 36%; higher in older, immunocompromised horses, and foals < 12 months [38, 39].

Virus identification. WNF diagnosis plays a key role in the virus studying and disease situation monitoring. Given the risk of subclinical infection, diagnostic testing includes laboratory confirmation of the virus, as well as tests for differential diagnosis from other orthoflaviviral diseases. Accurate diagnosis requires a combination of molecular, serological, and virological methods, which provides an integrated approach to the disease detection.

Recently, WNF diagnosis has undergone significant changes with the introduction of modern molecular diagnostic methods. These techniques have significantly improved the sensitivity, specificity, and overall effectiveness of detecting this potentially dangerous infection. In particular, reverse transcription polymerase chain reaction (RT-PCR) is a molecular diagnostic tool that enables the detection of WNV RNA in blood, cerebrospinal fluid, or tissue samples during the early stages of infection, particularly in cases with severe neurological symptoms such as encephalitis or meningitis. This method is highly valuable for detecting WNV during acute-phase of the disease. However, its diagnostic window is narrow, generally limited to the first few days after infection. Real-time RT-PCR offers enhanced diagnostic capability by enabling viral load quantification, a critical parameter for monitoring of the virus circulation among humans, animals, and mosquito vectors [38]. The introduction of real-time RT-PCR for the detection of WNV RNA in urine has become an important diagnostic tool, particularly for confirming neuroinvasive WNF forms. Compared to blood testing, this approach enables detection of the virus in urine at higher concentrations and over a longer period, thereby increasing

the number of detected cases. Moreover, the use of whole blood for PCR testing proved to be more effective than using cerebrospinal fluid or serum as demonstrated during the outbreak in Arizona in 2021. This approach provides faster and more accurate diagnostic data, which is critically important for timely and effective treatment of neuroinvasive forms of the disease [40].

Enzyme-linked immunosorbent assay (ELISA) and virus neutralization tests are the most commonly used serological methods for detection of antibodies to WNV. Antibodies can be detected in horse sera with solid-phase ELISA with IgM and IgG capture, hemagglutination inhibition test (HI test), plaque reduction neutralization test (PRNT) and virus microneutralization test (MNT). IgM-ELISA detects antibodies in horse serum at clinical onset. These immunoglobulins usually develop on 7 or 10 day after infection and persist for up to 1–2 months. IgG antibodies to WNV develop shortly after the IgM response and can persist for many years. Thus, the presence of IgG without IgM is indicative of a previous infection only [11]. WNV-neutralizing antibodies are detected in horse serum two weeks after infection and can persist for more than a year. Serological assays for WNV may exhibit cross-reactivity with closely-related flaviviruses, including Usutu virus, St. Louis encephalitis virus, Japanese encephalitis virus, and tick-borne encephalitis virus. PRNT is used for differentiation of WNV from other flaviviruses.

The virus is able to replicate in susceptible cell cultures such as rabbit kidney cells (RK-13) and African green monkey kidney cells (Vero), as well as in embryonated chicken eggs. Embryonated chicken eggs or *Aedes albopictus* C6/36 cell lines can be used for the initial virus isolation with subsequent virus passaging in mammalian cells. Several passages may be required for the cytopathic effect to become apparent. Indirect immunofluorescence assay of infected cell cultures or nucleic acid detection technique are used for WNV confirmation [41].

Immunohistochemistry enables detection of macroscopic and microscopic lesions associated with West Nile encephalitis in the central nervous system tissues [42].

Treatment and prevention of the disease. The principles of WNF treatment are purely symptomatic and include maintaining adequate hydration and relieving pain associated with inflammation. Patients with severe neurological manifestations frequently require anticonvulsive therapy and respiratory assistance [34].

Global strategies for WNF prevention include two approaches: vaccination and mosquito control. Vaccines are the most effective measures against flaviviruses, especially in high-risk groups. However, despite the fact that several candidate vaccines against WNF have been developed for humans, none of them have been licensed. Currently, several vaccines for humans are undergoing phase I and II clinical trials. Development strategies include live attenuated vaccines, subunit vaccines, and recombinant DNA vaccines [43].

Vaccines for veterinary use are aimed at preventing infection of WNV-susceptible animals. Vaccines against WNF for horses have already been licensed and are available on markets of different countries. According to American scientists, four anti-WNF vaccines for horses are licensed in the United States: two inactivated whole-virion vaccines, modified live recombinant vaccine containing the canarypox virus as a vector, and a chimeric one that combines the WNV antigen with an inactivated flavivirus [44, 45]. Vaccines against WNF have proven protective efficacy, and their use has significantly contributed to reducing the disease incidence in horses in the United States. However, despite their proven effectiveness, these vaccines still have limitations, including the need for multiple doses during primary immunization and the relatively short duration of immunity, which necessitates annual revaccination. Therefore, these aspects should be taken into account in order to improve existing vaccines and candidate vaccines under development [11]. Products intended for protection of dogs, cats, cattle, pigs, crocodiles and birds from WNV infection are under development.

To date, no safe and effective vaccine against WNF has been developed in Russia. This situation contributes to the regular occurrence of outbreaks in both endemic and non-endemic areas. Therefore, special attention should be paid to preventive measures aimed at reducing the virus spread in the environment, including among birds, animals and mosquitoes. To prevent WNV infection, individuals should take the following precautions: protect themselves against mosquito bites by using repellents, mosquito nets, and fumigators; eliminate standing water in containers around gardens and suburban areas to reduce mosquito breeding sites; avoid swampy areas, and wear protective clothing when visiting forests or the shores of water bodies. Avoid direct contact with sick or dead birds, including their faeces and feathers. When it is necessary to pick up a bird (e.g., to transport it to a veterinary centre), the persons should wear gloves and wash hands thoroughly afterward, along with any surfaces that may have been contaminated. When WNF is suspected in humans, pets, or birds, official health authorities and the state veterinary supervision service should be promptly notified [22].

CONCLUSION

West Nile virus is not classified as a candidate virus for a pandemic, as its spread and clinical significance vary depending on the region and environmental conditions. Nevertheless, it is of considerable interest to researchers in various fields, including physicians, public health workers, environmentalists, veterinarians, epidemiologists, and specialists in infectious diseases. This is due to the ability of WNV to cause severe neurological conditions in humans, especially in the elderly and immunocompromised patients, which makes it a potential threat to vulnerable population groups. In addition, clinical manifestations of WNF can be observed in animals and birds, which underlines the importance of WNV as a zoonotic pathogen. The trend for global climate change observed in recent decades contributes to the expansion of the habitat of arbovirus vectors and, consequently, increases the risk of pathogens spreading to new, previously non-endemic areas. This can lead to changes in the epidemiological context and the appearance of new foci of infection. Given these factors, WNV represents a significant concern that demands ongoing attention from the scientific community and health authorities. It can be classified to the group of potentially dangerous threats to global health, especially in the context of sustainable climate change [46].

Currently, research activities are focused on the development and improvement of new methods for WNF diagnosis, treatment and prevention. Significant emphasis is placed on molecular diagnostic techniques, as they allow for quick and reliable detection of the virus in biological samples. The immunological aspects of the disease are also being

actively studied, including the mechanism of immunity development and possible ways for effective vaccine creation. Vaccination is one of the most effective tools for disease prevention, and the development of a vaccine against WNF could be an important breakthrough for combating this infection, especially for at-risk groups. However, to date, no immunological preventive products against WNF have been developed or certified in Russia. Hence, a comprehensive, unifying One Health approach involving public health, veterinary and environmental authorities, could become the most effective and efficient mechanism to combat WNF.

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Pathomorphological, bacteriological and virological features of pneumonia in captive monkeys

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ABSTRACT

Introduction. Respiratory infections pose a significant challenge in veterinary practice due to their high prevalence across various animal species. Pneumonia and gastrointestinal diseases are leading causes of mortality in captive primates.

Objective. Study of pneumonia incidence in monkeys, the analysis of pulmonary microbiota composition, study of pathomorphological lesions in lung tissue.

Materials and methods. Common methods were used for pathomorphological, microscopic and bacteriological examinations of 1,862 dead monkeys. Lung samples and serum samples from 126 monkeys died of pneumonia in 2021–2024 were tested for acute respiratory viral pathogens as well as for antibodies to them with polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).

Results. Pneumonia was postmortem diagnosed in 865 monkeys (46.5%). The mortality rate for pneumonia in baby monkeys during their first month of life reached 100%. In baby monkeys under one year of age, the mortality rate was 65.4%. The obtained data showed that the disease incidence in these age groups was high. Deaths of monkeys due to pneumonia were reported throughout the year. Based on postmortem examinations, bilateral polysegmental broncho-pneumonia was the most frequent finding, lobar fibrinous pneumonia affecting the right lung was less common. Microscopic analysis detected purulent exudate and cocci bacteria in the bronchial lumen. The predominant bacteria isolated from lung tissue were enterobacteria (58.5%) and Gram-positive cocci (36.6%). Various microorganisms were isolated but the most frequently enteric bacteria were as follows: *Escherichia coli* (66.1%), *Enterococcus* spp. (27.5%) and *Proteus* spp. (31.5%). The following bacterial pathogens associated with pneumonia were detected: *Staphylococcus aureus* (31.5%), *Klebsiella pneumoniae* (2.2%), *Pseudomonas aeruginosa* (0.8%) and *Streptococcus pneumoniae* (0.6%). Adenoviruses, human parainfluenza viruses of type 1 and type 3 and respiratory syncytial virus (RSV) were also circulated in the monkey colony.

Conclusion. During analysis of microbial etiology of pneumonia in monkeys it shall be considered that pneumonia is frequently arisen as a secondary infection, heavily influenced by underlying gastrointestinal pathologies and immunosuppression.

Keywords: monkeys, pneumonia, pathomorphology, bacteriology, virology

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Патоморфологические, бактериологические и вирусологические особенности пневмоний у обезьян, содержащихся в неволе

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РЕЗЮМЕ

Введение. В ветеринарии проблема возникновения и лечения респираторных инфекций актуальна в связи с высоким уровнем заболеваемости среди разных видов животных. У обезьян, содержащихся в условиях неволи, пневмонии, наряду с желудочно-кишечными заболеваниями, являются одной из главных причин гибели.

Цель исследования. Изучение заболеваемости обезьян пневмонией, анализ спектра легочной микрофлоры, изучение патоморфологических изменений в легочной ткани.

Материалы и методы. При изучении 1862 случаев гибели обезьян патоморфологические, микроскопические и бактериологические исследования проводили по общепринятым методикам. Выявление возбудителей острых респираторных вирусных инфекций и антител к ним осуществляли методами полимеразной цепной реакции и иммуноферментного анализа при исследовании образцов легких и сывороток крови от 126 обезьян, погибших с диагнозом «пневмония» в 2021–2024 гг.

Результаты. У 865 обезьян postmortem поставлен диагноз «пневмония» (46,5%). Гибель от пневмоний малышей первого месяца жизни приближается к 100%. У детенышей возрастом до одного года показатель смертности составляет 65,4%. Полученные данные свидетельствуют о высокой частоте заболевания в этих возрастных группах. Гибель обезьян от пневмоний регистрируется на протяжении всего года. На основании патолого-анатомических исследований установлено, что чаще возникает двусторонняя полисегментарная бронхопневмония, реже – долевая фибринозная пневмония с развитием воспалительного процесса в правом легком. При микроскопическом исследовании в просвете бронхов выявляются гнойные экссудаты и кокковая микрофлора. Среди бактерий, выделенных из ткани легких, наибольший процент составляют энтеробактерии (58,5%) и грамположительные кокки (36,6%). Спектр выделенных видов микроорганизмов разнообразный, но в большинстве случаев представлен кишечными бактериями: *Escherichia coli* (66,1%), *Enterococcus* spp. (27,5%), *Proteus* spp. (31,5%). Из возможных бактериальных возбудителей пневмоний обнаружены *Staphylococcus aureus* (31,5%), *Klebsiella pneumoniae* (2,2%), *Pseudomonas aeruginosa* (0,8%), *Streptococcus pneumoniae* (0,6%). В стаде обезьян также циркулируют аденовирусы, вирусы парагриппа человека типа 1 и 3 и респираторно-синцитиальный вирус.

Заключение. При анализе возможной роли микроорганизмов в развитии пневмоний у обезьян необходимо учитывать, что у большинства животных заболевание развивалось на фоне патологий желудочно-кишечного тракта и при сниженном иммунитете.

Ключевые слова: обезьяны, пневмония, патоморфология, бактериология, вирусология

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INTRODUCTION

In veterinary practice, pneumonia occurrence and treatment is of current importance due to the its high incidence rate among wild, domestic, farm animals (cats, dogs, cattle, pigs, horses, etc.) and birds. Pneumonia mainly affects baby and young animals, while the disease frequency and severity depend on the keeping conditions, stress, density of animals, their immune status, diet, climate and production practice, as well as coinfection with various pathogens [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11]. In domestic animals, adults and elderly animals are often susceptible to the disease [4, 5, 6].

Low primates are also susceptible to most human pathogens, so it is easy to reproduce some human infectious diseases in them in a similar form [12]. In recent decades, due to the sharp decline in monkey populations in natural habitats and the ban on trapping, primate breeding centres have become the main source of laboratory primates. Animals kept in captivity are in most cases susceptible to intestinal and respiratory diseases. Pneumonia is one of the leading causes of morbidity and often leads to the death of animals [13, 14, 15]. Pneumonia may act either as an independent pathological process or as a concomitant (secondary) disease arising from gastrointestinal disorders, including acute or chronic gastritis and gastroenterocolitis [12, 16]. In addition, pneumonia often occurs in animals recently imported from their natural habitats and undergoing acclimatization [17, 18].

The lungs are a complex ecosystem with a large number of diverse microorganisms interacting both with each other and with the host organism [18, 19]. A functional relationship exists between the microbiomes of the lungs, oropharynx, and intestines – an interconnection that significantly influences the development of both pulmonary and intestinal diseases through the modulation of metabolic, immune, and other physiological processes. Because of the ongoing microbial exchange between the oropharynx and the upper and lower respiratory tracts, the microbiome of the lungs is never static. The dominant microbial population in the microbiome and its size differ significantly between healthy and pathologically altered organs. According to this concept, infectious lung lesions are viewed as a disruption of the existing microbial balance, with the course and outcome of pneumonia being largely determined by interactions among the microorganisms themselves [19].

A critical step in pneumonia diagnosis is the isolation and identification of the involved infectious agents – including bacteria, viruses, and fungi – from the respiratory tract [2, 20]. According to literature data, less than 10% of pneumonia cases are described as polymicrobial, and in more than 50% of cases, the etiological agents of pneumonia in monkeys remain unidentified [19]. According to the literature data, the most common causes of pneumonia in captive monkeys of various species are *Streptococcus* spp., less commonly *Klebsiella pneumoniae*, *Staphylococcus aureus* and other bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus* spp., *Morganella morganii*) [21, 22, 23, 24, 25]. Earlier, staff-members of the Kurchatov Complex of Medical Primatology of National Research Centre "Kurchatov Institute" demonstrated the involvement

of human parainfluenza virus type 3 in the respiratory tract pathology in *Papio anubis* [26].

The importance of this study is highlighted by the fact that respiratory diseases are predominant in the etiological structure of diseases in monkeys and constitute one of the primary causes of mortality for animals in captivity.

This study presents novel data by consolidating the results of pneumonia mortality monitoring in captive monkeys in the primate breeding centre, representing the first such investigation carried out in Russia. Accordingly, the present study was aimed at not only bacterial microflora detection in the lungs of dead animals and assessment of serological and molecular-genetic markers of acute viral respiratory infections (AVRIs), but also at description of pathologically altered lung features.

The purpose of the study was to study the pneumonia incidence, to analyse lung microbiota landscape, and to examine lung lesions.

MATERIALS AND METHODS

Animals. A total of 1,862 deaths of monkeys of various species from spontaneous diseases were studied in the period from January 2019 to December 2024. All animals were kept in the primate breeding centre of the Kurchatov Complex of Medical Primatology of National Research Centre "Kurchatov Institute". Dead male (n = 726) and female (n = 1,136) monkeys ranged in age from new-born to 38 years (Table 1).

Postmortem and microscopic examinations. Dead animals were necropsied in the necropsy room of the Laboratory for Pathological Anatomy of the Kurchatov Complex of Medical Primatology. Internal organs and tissues from dead animals were examined to detect pathological lesions. When gross lung inflammation signs were detected, tissue samples were collected for histological analysis. The samples were fixed in a 10% neutral (pH 7.4) formalin solution, then subjected to standard histological processing, followed by mounting into Histomix paraffin medium (BioVitrum, Russia). Histological sections, 4 µm thick, were prepared from the paraffin-embedded tissues and stained with Hansen's hematoxylin and eosin, as well as with Van Gieson's picrofuchsin [27].

Morphological analysis (microscopic examination) was carried out with Axiolab.A1 laboratory microscope (Carl Zeiss Microscope GmbH, Germany). Axiocam 105 colour digital camera (Carl Zeiss Microscopy GmbH, Germany) was used for microphotography.

Bacteriological examination was carried out according to a common technique: a lung smear was made on a slide, Gram-stained, and inoculated in diagnostic nutrient media, followed by biochemical identification of the grown colonies of microorganisms, as described earlier [28].

Virological examination. Serum and lung samples collected in 2021–2024 from 126 dead monkeys with diagnosed pneumonia including 48 *Macaca mulatta*, 22 *Macaca fascicularis*, 7 *Chlorocebus aethiops* ssp., 38 *Papio hamadryas* and 11 *Papio anubis* were used for the virological examination. The lung suspension was prepared using Minilys homogenizer (Bertin Technologies, France) at a ratio of 5–6 g of the material per 1 mL of 0.1M sodium phosphate buffer, pH 7.4; centrifuged with Allegra cold centrifuge (Beckman Coulter, USA) at 3,000 rpm for 30 min for clarification. The resulting 10% supernatant was used for further tests.

Test kits manufactured by ECOlab (Russia) were used for detection of IgG, IgM and IgA antibodies to parainfluenza virus types 1 and 3, respiratory syncytial virus (RSV) and adenovirus. The results of enzyme-linked immunosorbent assay (ELISA) were read using Immunochem-2100 laboratory spectrophotometer (High Technology Inc., USA) at wavelength of 450 nm. The sera reactivity to respiratory viruses was assessed based on OD_{450} values (optical density of ELISA-tested serum samples at a wavelength of 450 nm). The test results were interpreted according to the test-kit manufacturer's instructions.

Table 1
Characterization of dead monkeys by age groups

Monkey species	Age groups						Total
	under 1 month of age	under 11 months of age	1–3 years of age	4–10 years of age	11–15 years of age	16 years of age and older	
Macaca mulatta	52	45	116	176	86	129	604
Macaca fascicularis	73	32	78	148	76	94	501
Macaca nemestrina	15	5	2	8	6	17	53
Chlorocebus aethiops ssp.	11	7	7	18	14	18	75
Papio anubis	30	13	27	40	21	20	151
Papio hamadryas	109	49	47	110	58	71	444
Other species	5	2	7	9	3	8	34
Total	295	153	284	509	264	357	1,862

Table 2
Characterization of fatal pneumonia cases in monkeys

Monkey species	Quantity/%		
	total	females	males
Macaca mulatta	249/41.2	156/40.7	93/42.1
Macaca fascicularis	221/44.1	129/39.5	92/52.9
Macaca nemestrina	33/62.3	21/70.0	12/52.2
Chlorocebus aethiops ssp.	32/42.7	22/44.0	10/40.0
Papio anubis	74/49.0	37/40.2	37/62.7
Papio hamadryas	236/53.2	109/45.8	127/61.7
Other species	20/58.8	8/50.0	12/66.7
Total	865/46.5	482/42.4	383/52.8

Table 3
Characterization of monkeys died of pneumonia by age groups

Monkey species	Age groups												p, χ^2 for trend*
	under 1 month of age		under 11 month of age		1–3 years of age		4–10 years of age		11–15 years of age		16 years of age and older		
	number of animals	%	number of animals	%	number of animals	%	number of animals	%	number of animals	%	number of animals	%	
Macaca mulatta	47	90.4	25	55.6	46	39.7	62	35.2	24	27.9	45	34.9	**0.0005**↑↓
Macaca fascicularis	72	**98.6**	22	68.8	22	28.2	41	27.7	23	30.3	41	43.6	0.7675
Macaca nemestrina	14	93.3	1	20.0	1	50.0	6	75.0	4	66.7	7	41.2	0.9643
Chlorocebus aethiops ssp.	9	81.8	1	14.3	1	14.3	5	27.8	6	42.9	10	55.6	**0.0258**↓↑
Papio anubis	27	90.0	9	69.2	9	33.3	13	32.5	8	38.1	8	40.0	0.1357
Papio hamadryas	100	91.7	40	**81.6**	29	**61.7**	25	22.7	17	29.3	25	35.2	< **0.0001**↓
Other species	5	100	2	100	5	71.4	4	44.4	1	33.3	3	37.5	0.9648
Total	274	92.9	100	65.4	113	39.8	156	30.6	83	31.4	139	38.9	< **0.0001**↑↓

* $p < 0.05$ (χ^2 test – statistical difference in pneumonia diagnosis rates between monkey species). Arrows indicate the trend in detection frequency by age, providing that the test result was statistically significant.

Table 4
Number of monkeys died of pneumonia by year

Monkey species	Year of testing						p, χ^2 for trend*
	2019	2020	2021	2022	2023	2024	
Macaca mulatta	42	32	43	36	44	52	**0.0075**↑↓
Macaca fascicularis	42	44	39	27	32	37	0.4535
Macaca nemestrina	6	9	3	3	8	4	0.7173
Chlorocebus aethiops ssp.	5	5	2	6	10	4	0.3061
Papio anubis	20	8	15	7	14	10	0.3494
Papio hamadryas	43	42	40	45	38	28	0.3828
Other species	8	4	3	2	1	2	**0.0177**↓
Total	166/46.6%	144/45.4%	145/38.2%	126/49.2%	147/51.9%	137/50.8%	0.0910

* $p < 0.05$ (χ^2 test – statistical difference in pneumonia diagnosis rates between monkey species). Arrows indicate the trend in detection frequency by year, providing that the test result was statistically significant.

Nucleic acids were extracted from the prepared 10% lung supernatant using RIBO-prep kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) according to the manufacturer's instructions. Complementary DNA was synthesized on a total RNA matrix using Reverta-L reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) in accordance with the manufacturer's instructions. Resulting cDNAs were amplified using "AmpliSens® AVRI-screen-FL" real-time PCR kit to identify acute respiratory viral infection pathogens (RSV; metapneumovirus; parainfluenza virus types 1, 2, 3 and 4; coronavirus; rhinovirus; adenovirus groups B, C and E; bocavirus) according to the manufacturer's instructions. Amplification and analysis of the results were performed using Rotor-Gene Q device (QIAGEN GmbH, Germany).

Statistical data processing. Statistical processing of the data and calculations were carried out using GraphPad-Prism 8 software. To detect changes in frequency metrics across study years or age groups, a χ^2 trend test (Pearson's chi-square) was applied. All differences were interpreted as significant at $p < 0.05$.

Table 5
Number of microorganisms isolated from lungs of the monkeys died of pneumonia (2019–2024)

Microorganism	Year of testing						p, χ^2 for trend	Total
	2019	2020	2021	2022	2023	2024		
E. coli	112	100	106	85	87	82	0.2274	572
Representatives of tribe *Proteeae*	51	50	52	42	62	44	**0.0002**↑↓	301
Klebsiella spp.	18	8	5	4	3	3	**0.0048**↓	41
Enterobacter spp.	7	4	4	1	4	6	0.5613	26
Citrobacter spp.	7	4	0	2	2	0	**0.0344**↑↓	15
Other enterobacteria	4	5	1	4	4	1	0.9126	19
Ps. aeruginosa	1	2	2	0	1	1	0.9926	7
Other non-fermenting bacteria	0	0	1	0	2	6	< **0.0001**↑	9
Bacillus spp.	1	1	6	4	3	7	**0.0024**↓↑	22
Other Gram-positive rods	27	10	0	0	3	2	< **0.0001**↓	42
Staphylococcus spp.	123	64	41	40	62	25	< **0.0001**↓	355
Enterococcus spp.	53	49	40	31	36	29	0.9856	238
Other Gram-positive cocci, including *Streptococcus pneumoniae*	6	3	0	1	2	0	0.0503	12
Candida spp.	0	0	1	0	0	0	0.9320	1
Total	410	300	259	214	271	206	< **0.0001**↓	1,660

* $p < 0.05$ (χ^2 test – statistical difference in detection frequency between microorganism species). Arrows indicate the trend in detection frequency by year, where the test result was statistically significant.

RESULTS AND DISCUSSION

A total of 865 pneumonia cases (46.5% of the total number of dead animals) were detected over a six-year period based on the results of postmortem examinations (Table 2). Pneumonia was more frequently diagnosed in males than in females (52.8 and 42.4%, respectively). Pneumonia mortality rates were generally similar across monkey species, exception for *Papio hamadryas* and *Macaca nemestrina*. In these monkey species, pneumonia caused more than 50% of deaths.

According to the findings, the highest mortality for pneumonia was observed in monkeys during their first month of life (Table 3).

The total number of monkeys that died due to pneumonia averaged between 126 and 166 cases per year, remaining relatively stable in percentage terms over a six-year period (Table 4).

Over the six-year study period, neonatal mortality in monkeys exhibited a steady increase, with pneumonia accounting for 100% of deaths in this age group by 2024.

Analysis of seasonal patterns in monkey mortality due to pneumonia revealed that more than 50% of cases occurred during the autumn-winter period of 2020 and 2024, and during the summer-spring period of 2023. In 2022, mortality due to pneumonia in these animals remained high throughout all seasons.

Pathomorphological features of pneumonia. Polysegmental bronchopneumonia was more frequently observed in monkeys living at the primate breeding centre, whereas lobar fibrinous pneumonia – similar to human croupous pneumonia – was considerably less common. Macroscopically, bilateral pulmonary involvement was the most common finding, with the right lung being affected more extensively or more frequently. In cases of unilateral involvement, pneumonia was observed more frequently in the right lung alone (Fig. 1).

Croupous pneumonia in monkeys was characterized by lobar fibrinous inflammation with pleura lesions. In addition to lobar involvement, large focal pneumonic lesions were detected in the centre of the lobes or extending to the pleura. The affected lung tissue was airless, of dense consistency and greyish-reddish colour. A cloudy, foamy fluid exuded from the cut surface when the affected lung was compressed. The lung lymph nodes were enlarged. Cases of total, bilateral lung damage with alveolar filling by fibrinous-leukocytic exudate and serous fluid buildup were recorded (Fig. 2).

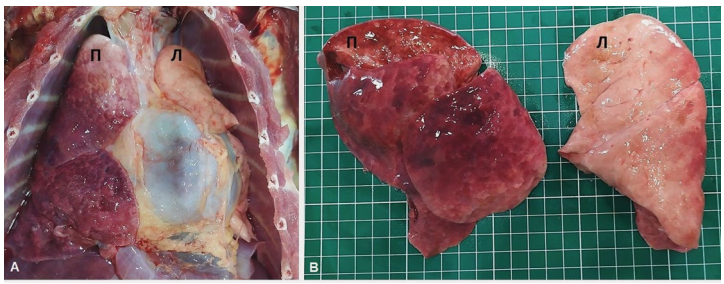

Fig. 1. Macrophotography of heart – lung complex in the monkey with polysegmental bronchopneumonia (Macaca fascicularis, ♀, 3 years old): A – general view; B – all lobes of the right lung are affected; П – right lung; Л – left lung

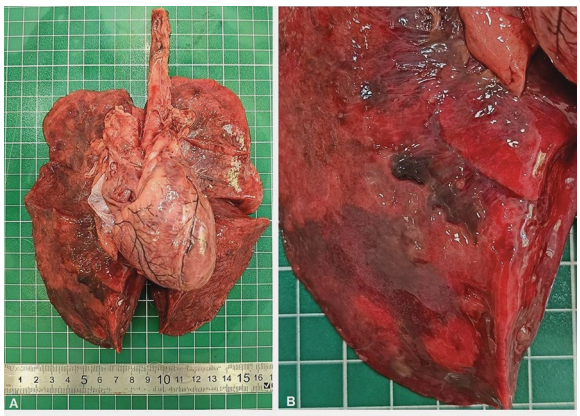

Fig. 2. Macrophotography of heart – lung complex in the monkey with fibrinous pneumonia (Erythrocebus patas, ♂, 10 years old), all lobes of the right and left lungs are completely affected: A – general view; B – fibrin threads on the visceral pleura of the right lung

Fibrin, degenerated leukocytes, and other cell debris were detected within the alveoli in croupous pneumonia cases. There were areas of lung tissue containing only red blood cells in the alveoli. Apparent vascular hyperemia with parietal leukocyte accumulation and capillary stasis within the interalveolar septa were observed (Fig. 3).

Purulent tissue melting foci located generally around the affected bronchi were observed in all croupous pneumonia cases. The bronchial lumen contained purulent exudate and coccoid microflora.

Bronchopneumonia occurred both independently and as a concomitant disease complicating gastrointestinal pathologies. In bronchopneumonia cases, multiple small inflammation foci of grey-red or bluish-purple colour, often merging, that located along the branching bronchi were found in the lungs. The lung tissue around inflammation foci was edematous with hyperemia or severe emphysema, which gave the incision surface a mottled appearance (Fig. 4).

Similar to croupous pneumonia, the microscopic inflammation lesions in these cases were characterized by accumulation of the exudate of various types. Foci of serous fluid mixed with red blood cells as well as polymorphic cellular exudate were found in the alveoli. Leukocytes and mucous with a large amount of bacterial microflora often predominated in the exudate. In all cases, inflammatory lung lesions were combined with the development of focal atelectasis and focal emphysema (Fig. 5).

Thus, croupous pneumonia and bronchopneumonia in monkeys kept in the Kurchatov Complex of Medical Primatology primate breeding centre were characterized by a variety of morphological changes in the lungs. This appears related to the different properties of the disease pathogens.

Asymptomatic pneumonia diagnosed at necropsy only were found in most dead animals. In some cases, differentiation between croupous pneumonia and bronchopneumonia was difficult, as the presenting symptoms were subtle or nonspecific. Intrapulmonary complications including purulent bronchitis, lymphangitis, purulent inflammation foci, extremely rarely – pleural empyema were observed in some animals. The following extrapulmonary lesions were recorded – purulent meningitis, serous-purulent pericarditis.

Microbial landscape in lungs. Gram-positive cocci were found in all smears during examination of lung

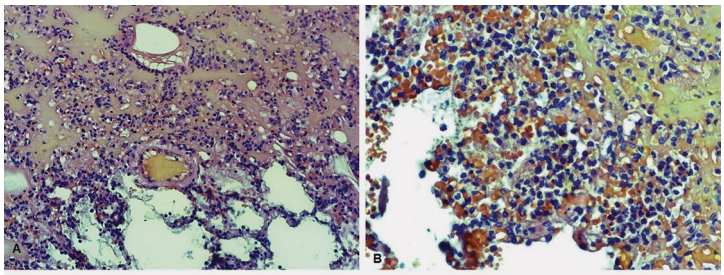

Fig. 3. Microscopic lesions in lungs of the monkey with lobar pneumonia (Macaca mulatta, ♂, 5 years old): A – alveolar edema, fibrin threads in the alveolar lumen, degenerated leukocytes (hematoxylin and eosin staining, magnification 200×); B – alveolar edema, erythrocyte and leukocyte accumulations in alveolar lumen (hematoxylin and eosin staining, magnification 400×)

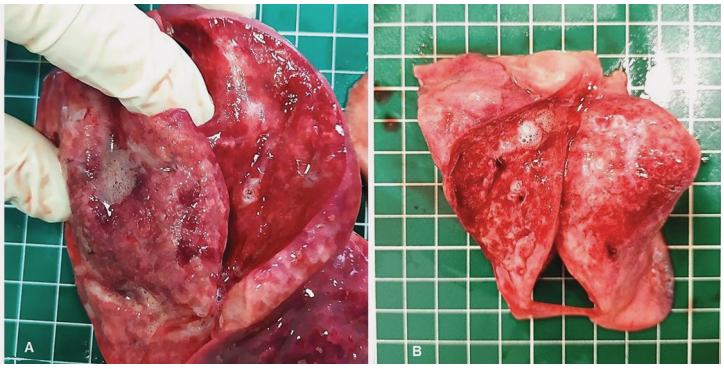

Fig. 4. Macroscopic lesions in lungs of the monkey with bronchopneumonia (Papio anubis, ♂, 5 years old): A, B – alveolar edema (foamy fluid on section), mottled pattern in lung tissue

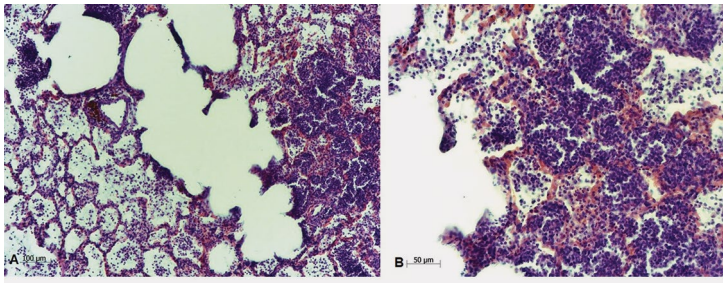

Fig. 5. Microscopic lesions in lungs of the monkey with bronchopneumonia (Papio anubis, ♂, 5 years old): A – focal emphysema (dilated alveoli, thinned and partly disrupted alveolar walls), polymorphic cellular exudate in the alveoli lumen (hematoxylin and eosin staining, magnification 100×); B – polymorphic cellular exudate in the alveoli lumen (hematoxylin and eosin staining, magnification 200×)

Table 6
Indicators of viral infections

Indicators / Monkey species	Parainfluenza virus type 1		Parainfluenza virus type 3		RSV		Adenovirus
	IgG	IgA	IgG	IgA	IgG	IgM	IgG
Monkeys that died in 2021–2022							
Macaca mulatta	10/15* 66.7%	0/15 0%	0/15 0%	0/15 0%	n/d	n/d	2/15 13.3%
Macaca fascicularis	3/4 75.0%	0/4 0%	0/4 0%	0/4 0%	n/d	n/d	0/4 0%
Chlorocebus aethiops ssp.	1/3 33.3%	0/3 0%	0/3 0%	0/3 0%	n/d	n/d	0/3 0%
Papio anubis	3/15 20.0%	0/15 0%	0/15 0%	0/15 0%	n/d	n/d	0/15 0%
Papio hamadryas	0/4 0%	0/4 0%	0/4 0%	0/4 0%	n/d	n/d	0/4 0%
Total	17/41 41.5%	0/41 0%	0/41 0%	0/41 0%	n/d	n/d	2/41 4.9%
Monkeys that died in 2023–2024							
Macaca mulatta	0/33 0%	0/33 0%	0/33 0%	0/33 0%	0/33 0%	8/33 24.2%	4/33 12.1%
Macaca fascicularis	1/18 5.6%	0/18 0%	0/18 0%	0/18 0%	0/18 0%	9/18 50.0%	1/18 5.6%
Chlorocebus aethiops ssp.	0/4 0%	0/4 0%	2/4 50.0%	0/4 0%	1/4 25.0%	2/4 50.0%	1/4 25.0%
Papio anubis	0/23 0%	0/23 0%	3/23 13.0%	0/23 0%	0/23 0%	8/23 34.8%	2/23 8.7%
Papio hamadryas	0/7 0%	0/7 0%	0/7 0%	0/7 0%	0/7 0%	3/7 42.9%	1/7 14.3%
Subtotal	1/85 1.2%	0/85 0%	5/85 5.9%	0/85 0%	1/85 1.2%	30/85 35.3%	9/85 10.6%
Total	18/126 14.3%	0/126 0%	5/126 4.0%	0/126 0%	1/85 1.2%	30/85 35.3%	11/126 8.7%

* positive serum samples / number of tested serum samples; n/d – no data.

microbiota of monkeys. Over six years, 1,660 microorganisms were isolated during bacteriological tests, the majority of which were representatives of the family *Enterobacteriaceae* (58.5%). Gram-positive cocci were detected in 36.6% of cases, the proportion of non-fermenting bacteria, including *Pseudomonas aeruginosa*, and non-differentiated Gram-positive rods was 1.0 and 3.8%, respectively (Table 5).

Statistical analysis showed significant changes in the detection rates of certain microorganisms over the years of the study ($p < 0.0001$), which indicated shifts in lung microbiota composition.

The number of representatives of tribe *Proteeae* varied without any clear tendency to increase or decrease, which was indicative of a dynamic change in the role of these microorganisms ($p = 0.0002$). The number of *Klebsiella* spp. detections decreased over the years ($p = 0.0048$), potentially indicating a decline in the pathogen's role in pneumonia development. The number of *Citrobacter* spp. ($p = 0.0344$) and *Bacillus* spp. ($p = 0.0024$) detections varied over the years. Notwithstanding the high isolation rate of *Staphylococcus* spp., a significant decline has been recorded in recent years, from 123 to 25 cases ($p < 0.0001$). Gram-positive rods detection rate also decreased ($p < 0.0001$). Non-fermenting Gram-negative rods were rarely isolated from the lungs, however, they were detected in 6 cases ($p < 0.0001$) in 2024. The total level of the isolated microorganisms decreased over the years (from 410 in 2019 to 206 in 2024), which was confirmed by statistical data ($p < 0.0001$).

As for microbial landscape, the leading position was occupied by *E. coli*, which were found in the lungs of 66.1% of monkeys, the second position was occupied by *Staphylococcus* spp., including *S. aureus* (41.1%), followed by representatives of tribe *Proteeae* (*Proteus* spp., *Providencia* spp., *M. morganii*) and *Enterococcus* spp. (34.8 and 27.5%, respectively). Other microorganisms were isolated rarely. During the tested period, *S. aureus* was more often isolated

as compared to other main bacterial pathogens of pneumonia – in 267 monkeys (31.5%), other pathogens were found in some cases, namely: *K. pneumoniae* – in 19 monkeys (2.2%), *Ps. aeruginosa* – in 7 monkeys (0.8%), *St. pneumoniae* – in 5 monkeys (0.6%). No bacterial growth was observed on the nutrient media inoculated by the samples from 7 cases (0.8%). Also, no materials were collected for examination from 17 monkeys with pneumonia due to postmortem decomposition. Bacterial associations were observed, however, the number of detected associations decreased during the tested period. In 2019, 89% of the detected microbial isolates were associations, compared to 52% in 2024, with the frequency of 4-component associations apparently decreasing.

Any analysis of the etiological role of the isolated microorganisms in monkey pneumonia shall account for the fact that, in the majority of cases, the disease has arisen concurrently with gastrointestinal pathology and immunosuppression. This was evidenced by the detection of coliforms in lungs, as well as the prolonged pneumonia course with subtle or non-specific clinical signs. *S. aureus* represented an exception, based on previous molecular genetic evidence indicating its high pathogenic potential and lung tissue tropism of some strains [29, 30].

When 126 monkeys with diagnosed pneumonia who died in 2021–2024 were tested for AVRI indicators, IgG antibodies indicative of post-infection immunity against the following viruses were detected: parainfluenza virus type 1 and 3 (14.3 and 4.0% of the monkeys, respectively), RSV (1.2%), and adenovirus (8.7%). IgA and IgM antibodies indicative of acute infection period were detected only against RSV (35.3% of the monkeys). No AVRI pathogen RNAs/DNAs were detected with polymerase chain reaction (PCR) in the lung parenchyma (Table 6).

In monkeys that died in 2021–2022 ($n = 41$), IgG antibodies against parainfluenza virus type 1 (41.5%) only and adenovirus (4.9%) were detected, while no IgG antibodies against parainfluenza virus type 3 were detected. Also, no serological indicators of acute infection were detected.

In animals with diagnosed pneumonia that died in 2023–2024 ($n = 85$), IgM antibodies against RSV (35.3%) were detected in their sera, while the no IgA antibodies against parainfluenza virus type 1 and 3 were detected. In addition, IgG antibodies indicative of postinfection immunity were detected: IgG against parainfluenza virus type 1 and 3 (1.2 and 5.9%, respectively), RSV (1.2%), and adenovirus (10.6%).

CONCLUSIONS

1. Pneumonia is the most common cause of death of monkeys kept in the Kurchatov Complex of Medical Primatology breeding centre (46.5%) and often occurs concurrently with gastrointestinal diseases in weakened animals, which is consistent with previously published data.

2. The highest mortality due to pneumonia was observed in baby monkeys under the age of one month (92.9%) and during the first year of life (65.4%). Consequently, captive monkeys from birth to 1 year of age were the most susceptible to pneumonia.

3. The most frequent finding was bilateral polysegmental bronchopneumonia, and less frequently, lobar fibrinous pneumonia resembling human croupous pneumonia, with predominant involvement of the right lung.

4. The bacterial microflora isolated from pneumonia-affected lungs consisted of enterobacteria (58.5%) and Gram-positive cocci (36.6%), with *S. aureus* being isolated in 41.1% of cases. Microscopic examination also revealed cocci in the lung tissues.

5. Circulation of adenovirus, human parainfluenza virus types 1 and 3, as well as RSV in monkeys who died from pneumonia was shown. A high percentage of IgM against RSV indicated the possible involvement of this virus in the respiratory pathologies in monkeys.

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Serotype identification and antibiotic resistance analysis of *Listeria monocytogenes* isolates recovered from animal products in 2021–2024

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ABSTRACT

Introduction. *Listeria monocytogenes* is a pathogenic microorganism that causes a large number of deaths due to the consumption of contaminated animal products. This underpins the relevance of monitoring the spread of the listeriosis agent in raw materials, animal products, and environmental objects, as well as the antibiotic resistance of isolates.

Objective. Serotype identification and antibiotic resistance analysis of *Listeria monocytogenes* isolates recovered from animal products in 2021–2024.

Materials and methods. The work was performed at the Microbiology Unit of the Vladimir Testing Laboratory, the Federal Centre for Animal Health. *Listeria* genus bacteria isolates were identified using time-of-flight mass spectrometry. Antibiotic resistance of isolates belonging to the species *Listeria monocytogenes* was determined by the disk diffusion method. The values of the growth retardation zones were interpreted according to the Russian recommendations "Determination of the sensitivity of microorganisms to antimicrobial drugs" (IACMAC, version 2025-01). *Listeria* serological groups were identified using real-time polymerase chain reaction (qPCR) with primers manufactured by Syntol (Russia).

Results. The article presents the results of antibiotic resistance testing of 77 *Listeria monocytogenes* isolates detected in animal products in 2021–2024, as well as their differentiation by serogroups. *Listeria monocytogenes* was most frequently detected in poultry products. The detected isolates showed maximum resistance to cefuroxime, sulfamethoxazole/trimethoprim, norfloxacin, rifampicin, levofloxacin, and kanamycin. Moreover, most isolates exhibited resistance to more than one antimicrobial medicinal product. The study established the belonging of the *Listeria monocytogenes* isolates to the following serogroups: IIa (serotypes 1/2a, 3a) – 92.2%; IIc (serotypes 1/2c, 3c) – 5.2%; IVb (serotypes 4b, 4d, 4e) – 2.6%.

Conclusion. The spread of resistance, including multidrug resistance, among *Listeria monocytogenes* isolates detected in animal products in 2021–2024 was demonstrated. The study identified the presence of listeria belonging to group IVb (serotypes 4b, 4d, 4e). However, the dominant part of *Listeria monocytogenes* isolates was classified as group IIa (serotypes 1/2a, 3a).

Keywords: *Listeria monocytogenes*, antibiotic resistance, serotype, antimicrobial susceptibility, real-time polymerase chain reaction

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Идентификация серотипов и анализ антибиотикорезистентности изолятов *Listeria monocytogenes*, выделенных из продукции животного происхождения за период с 2021 по 2024 г.

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РЕЗЮМЕ

Введение. *Listeria monocytogenes* – патогенный микроорганизм, вызывающий большое количество летальных исходов вследствие потребления контаминированной продукции животного происхождения. Этим обусловлена актуальность мониторинга распространения возбудителя листериоза в сырье, продукции животного происхождения и объектах окружающей среды, а также антибиотикорезистентности изолятов.

Цель исследования. Идентификация серотипов и анализ антибиотикорезистентности изолятов *Listeria monocytogenes*, выделенных из продукции животного происхождения за период с 2021 по 2024 г.

Материалы и методы. Работа была выполнена на базе отдела микробиологических исследований Владимирской испытательной лаборатории ФГБУ «ВНИИЗЖ». Изоляты бактерий рода *Listeria* идентифицировали с использованием метода времяпролетной масс-спектрометрии. Определение антибиотикорезистентности изолятов, относящихся к виду *Listeria monocytogenes*, проводили диско-диффузионным методом. Значения зон задержки роста интерпретировали согласно российским рекомендациям «Определение чувствительности микроорганизмов к антимикробным препаратам» (МАКМАХ, версия 2025-01). Серологические группы листерий были идентифицированы с использованием метода полимеразной цепной реакции в режиме реального времени с применением праймеров производства НПК «Синтол» (Россия).

Результаты. В статье представлены результаты исследований антибиотикорезистентности 77 изолятов *Listeria monocytogenes*, выявленных в продукции животного происхождения в 2021–2024 гг., а также их дифференциации по серогруппам. Чаще всего *Listeria monocytogenes* определяли в продукции из мяса птицы. Выявленные изоляты имели максимальную резистентность к цефуроксиму, сульфаметоксазолу/триметоприму, норфлоксацину, рифампицину, левофлоксацину и канамицину. При этом большинство изолятов проявило устойчивость более чем к одному антимикробному препарату. В рамках исследования установлена принадлежность изолятов *Listeria monocytogenes* к следующим серогруппам: IIa (серотипы 1/2a, 3a) – 92,2%; IIc (серотипы 1/2c, 3c) – 5,2%; IVb (серотипы 4b, 4d, 4e) – 2,6%.

Заключение. Показано распространение устойчивости, в том числе множественной, среди изолятов *Listeria monocytogenes*, выявленных в продукции животного происхождения в 2021–2024 гг. В результате проведенного исследования было определено присутствие листерий, относящихся к группе IVb (серотипы 4b, 4d, 4e). Однако доминирующая часть изолятов рода *Listeria monocytogenes* была классифицирована как группа IIa (серотипы 1/2a, 3a).

Ключевые слова: *Listeria monocytogenes*, антибиотикорезистентность, серотип, чувствительность к антимикробным препаратам, полимеразная цепная реакция в режиме реального времени

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INTRODUCTION

Listeria monocytogenes is a gram-positive motile facultative anaerobic rod that does not form spores or capsules and causes listeriosis, a life-threatening disease manifesting, other things, as central nervous system damage and meningoencephalitis. Children, including newborns, pregnant women (leading to fetal death), and the elderly are at high risk. In healthy individuals, *L. monocytogenes* causes mild symptoms, but listeriosis poses a serious threat to immunocompromised individuals and those with chronic diseases, as it can lead to sepsis and other complications [1, 2, 3, 4].

This microorganism is widespread in terrestrial and aquatic ecosystems, being transmitted to animals and humans both directly and indirectly through the consumption of contaminated food or water. *L. monocytogenes* infection mainly characterized by foodborne transmission, and according to the World Health Organization, listeriosis is one of the most serious and severe foodborne diseases, with the highest case fatality rates in foodborne infections (over 20%) [5, 6, 7].

L. monocytogenes bacteria can adapt to various conditions: they can survive at temperatures below 7 °C. They are resistant to low pH values and high salt concentrations. Furthermore, *Listeria* can form biofilms, that allow them not only to persist in food production environments, but also to accumulate, even if their initial concentration was low. Animal products with a high risk of *L. monocytogenes* contamination include meat preparations and ready-to-eat meat products, including vacuum-packed products, dairy products, including soft cheeses, and cold-smoked fish products. Chilled ready-to-eat foods pose a particular risk [8, 9, 10].

The disease is reported in more than 65 countries on all continents. The number of recorded cases per year is 0.1–10.0 per 1 million people, depending on the country [9, 11].

In the European Union and European Economic Area (EU/EEA) countries, 2,993 confirmed cases were reported in 2023 (0.67 cases per 100,000 population), the highest annual rate since 2007. Incidence peaks during the summer months, with the number of cases increasing annually, showing a statistically significant rise and upward trend [12, 13].

Analysis of listeriosis epidemiology demonstrates that most sporadic cases and all major epidemic outbreaks are associated with pathogen isolates belonging to the first two phylogenetic lineages, designated I and II. In total, four phylogenetic lineages are distinguished within the species *L. monocytogenes*, differing in genetic and phenotypic characteristics.

Traditionally, five serogroups are distinguished: I (serotypes 1/2a and 3a; with 3a rarely detected in clinical cases); II (serotypes 1/2b, 3b and 7); III (serotypes 1/2c, 3c; with 3c being an extremely rare form); IV (serotypes 4b, 4d and 4e; with 4e playing a minor role in disease development); V (serotypes 4ab, 4a, 4c, which are practically not associated with clinical cases).

Listeria serotypes, classified based on variations in somatic (O) and flagellar (H) antigens, show significant differences in their epidemic potential and pathogenicity. These differences are due to the heterogeneity of antigenic structure, allowing serotypes to adapt to different environmental conditions and interact with the host's immune system [3, 5, 6, 14, 15].

Based on molecular typing, *L. monocytogenes* is divided into the following serogroups: IIa (serotypes 1/2a and 3a), IIb (serotypes 1/2b, 3b), IIc (serotypes 1/2c, 3c and 7), and IVb (serotypes 4b, 4d and 4e) [12, 16].

Human listeriosis is most commonly caused by serotypes 1/2a, 1/2b, and 4b, which account for over 90% of detected isolates. Serotype 4b is associated with most major listeriosis outbreaks, making it one of the most dangerous. Meanwhile, the vast majority of isolates belonging to serotypes 1/2 are widely distributed in food products and ecological niches where *Listeria* spp. are found. In particular, serotype 1/2a is most often detected in food products [6, 17, 18, 19].

In the Russian Federation, the number of listeriosis cases and the percentage of fatalities are also increasing annually (an increase in severe and moderate forms of the disease is noted), despite the fact that listeriosis is generally reported as sporadic cases [2, 10].

Thus, according to the official reports of the Federal Service for the Oversight of Consumer Protection and Welfare, in 2021, the incidence of listeriosis in the Russian Federation was 45 cases[1]; in 2022 – 81 cases, including 14 fatalities[2]; in 2023 – 100 cases, including 18 fatalities[3]; in 2024 – 208 cases, 49 of which were fatal[4]. The majority of cases are reported annually in large cities, Moscow and Saint Petersburg, with no pronounced seasonal fluctuations of the disease established [1].

The high mortality rate of listeriosis requires prompt treatment, specifically the use of antibiotics. Regarding antimicrobial susceptibility, *L. monocytogenes*, despite its widespread distribution in the environment, generally exhibits relatively low resistance rates. However, recent studies have shown the acquisition of antibiotic resistance in *Listeria* strains, including those isolated from food products [3, 16, 20, 21, 22].

At the same time, antibiotic resistance is currently considered one of the main threats to global health, to combat which the World Health Organization, the Food and Agriculture Organization of the United Nations, the United Nations Environment Programme, and the World Organisation for Animal Health have united under the "One Health" concept [23, 24, 25].

To combat antimicrobial resistance in the Russian Federation, the "Strategy for Preventing the Spread of Antimicrobial Resistance in the Russian Federation for the Period up to 2030"[5] was approved in 2017. Furthermore, in 2024, an "Action Plan for 2025–2030"[6] for the implementation of this strategy was approved, determining legal regulation, public awareness, systematic monitoring, and more.

Thus, the relevance of this study stems from the importance of monitoring the spread of *L. monocytogenes* by testing food products to trace the epidemiological pathways of the pathogen, including resistant ones, prevent its transmission to humans, and occurrence of listeriosis outbreaks.

The novelty of the work lies in the results of tested samples of livestock products obtained from three regions of Central Russia (Vladimir, Kostroma, and Ivanovo Oblasts), with subsequent isolation of *L. monocytogenes* isolates, typing using qPCR, determination of antibiotic resistance, and evaluation of trends in antibiotic resistance.

The objective of the work is to identify serotypes and analyze the antibiotic resistance of *L. monocytogenes* isolates recovered from animal products between 2021 and 2024.

[1] https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=21796 (in Russ.)

[2] https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=25076 (in Russ.)

[3] https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=27779 (in Russ.)

[4] https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=30171 (in Russ.)

[5] http://static.government.ru/media/files/onJ3GY3ObDGqLDvrED7AhpLF3ywRRFpp.pdf (in Russ.)

[6] https://www.garant.ru/products/ipo/prime/doc/409448585/?ysclid=mhymceuaxf332408554 (in Russ.)

Table
Primers for the molecular identification and differentiation of *L. monocytogenes* isolates by serogroups

Serogroups	Genes	Sequence (5′–3′)
4b, 4d, 4e	ORF0799F	5′-GCTGGGTTTCTTACGA-3′
	ORF0799R	5′-CAACCGTTCATTTAGCTCAT-3′
	ORF0799P	FAM-TCTGCTGTTCAGTTATTGGAGTGGGA-BHQ1
1/2a, 1/2c, 3a, 3c	Lmo0737F	5′-GCGGATGTGATTGATTTAC-3′
	Lmo0737R	5′-AAACTGCACTAACTCTTGAAT-3′
	Lmo0737P	ROX -TGCTCCAGGATCAAGACACGGTA-BHQ2
1/2c, 3c	Lmo1118F	5′-CTTAGTATTCCAGGATTTAAGACC-3′
	Lmo1118R	5′-CCAAAGAACCAAATTGATCGAATC-3′
	Lmo1118P	FAM-CCTTTATCTTCTCCTGAGTGTATACGCCTC-RTQ1

MATERIALS AND METHODS

The testing was conducted in the Microbiological Testing of the Vladimir Testing Laboratory of the Federal Centre for Animal Health. A total of 77 *L. monocytogenes* isolates, recovered from animal products during 2021–2024, were used in this testing.

Reagents and nutrient media: Fraser broth for primary enrichment (Merck KGaA, Germany), Fraser broth for secondary enrichment (Merck KGaA, Germany), Ottaviani – Agosti agar (ALOA; Merck KGaA, Germany), Oxford agar (Merck KGaA, Germany), tryptone soya agar (TSA; State Research Centre for Applied Microbiology and Biotechnology, Russia), and Mueller – Hinton agar (MHA; State Research Centre for Applied Microbiology and Biotechnology, Russia).

Microbiological testing was performed according to GOST 32031-2022 "Food products. Methods for detection of *Listeria monocytogenes* and other species of *Listeria* (*Listeria* spp.)"[7].

A 25 g test sample of the product was placed into a sterile bag containing 225 mL of Fraser broth for primary enrichment, homogenized for 1 min, and incubated at (30 ± 1) °C for (25 ± 1) hours. After primary enrichment, 0.1 mL of the culture was transferred into 10 mL of Fraser broth and incubated at (37 ± 1) °C for (24 ± 2) hours.

Following incubation, samples subcultured using a bacteriological loop onto the surface of two solid selective media (ALOA and Oxford agar) and cultured at (37 ± 1) °C for 48–24 hours, monitoring for the presence of colonies characteristic of *Listeria* spp.

Colonies exhibiting growth characteristic of *Listeria* spp. were subcultured onto the surface of tryptone soya agar with yeast extract to obtain isolated colonies and incubated at (37 ± 1) °C for (24 ± 3) hours.

Simultaneously, rapid identification of the isolated microorganisms was performed using time-of-flight mass spectrometry (Autof MS1000, Autobio Diagnostics Co., Ltd, China), as well as by determining culture motility, Gram stain, and catalase activity.

Determination of antibiotic resistance. Antimicrobial susceptibility testing of *L. monocytogenes* isolates was

[7] https://docs.cntd.ru/document/1200193714?ysclid=mhyosx40nk732110910 (in Russ.)

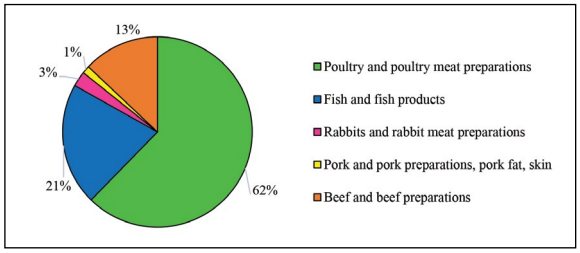

Fig. 1. Distribution frequency of L. monocytogenes isolates detected in animal product samples in 2021–2024

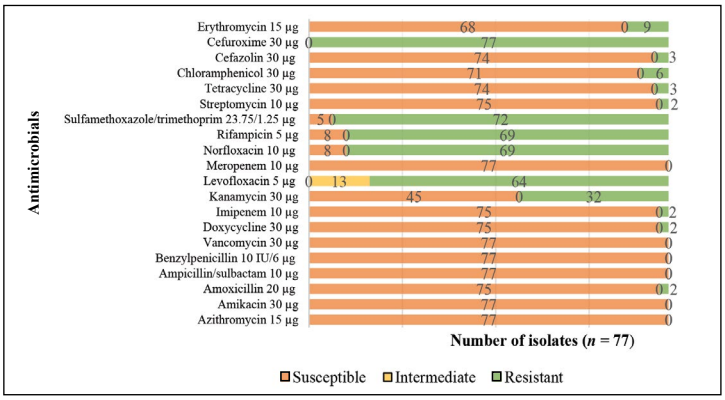

Fig. 2. Antibiotic resistance of L. monocytogenes isolates detected in animal products in 2021–2024

performed using the disk diffusion method according to the methodological guidelines MUK 4.2.1890-04 "Determination of the Sensitivity of Microorganisms to Antimicrobials"[8].

The following antibiotics (paper disks manufactured by the Saint-Petersburg Pasteur Institute, Russia) were used: azithromycin (15 μg), amikacin (30 μg), amoxicillin (20 μg), ampicillin/sulbactam (10 μg), benzylpenicillin (10 IU/6 μg), vancomycin (30 μg), doxycycline (30 μg), imipenem (10 μg), kanamycin (30 μg), levofloxacin (5 μg), meropenem (10 μg), norfloxacin (10 μg), rifampicin (5 μg), sulfamethoxazole/trimethoprim (23.75/1.25 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), cefazolin (30 μg), cefuroxime (30 μg), and erythromycin (15 μg).

The choice of antibacterial agents was driven by the fact that penicillins (ampicillin, benzylpenicillin, penicillin, amoxicillin) are often used in combination with aminoglycosides (gentamicin, streptomycin) in therapy of animals, as well as in the treatment of listeriosis in humans. Alternative antibiotics (second-line treatment agents) may include: sulfamethoxazole/trimethoprim, macrolides (erythromycin), fluoroquinolones (levofloxacin), tetracyclines (tetracycline, doxycycline), carbapenems (meropenem, imipenem), rifampicin, and vancomycin. Thus, detection of resistance to these agents may limit treatment options, particularly for patients with allergic reactions to certain antimicrobials [5, 16, 21, 22, 26].

For antibiotic susceptibility testing, a bacterial suspension with an optical density of 0.5 McFarland standard was prepared from a 24-hour culture of *L. monocytogenes* isolates grown on MHA.

The density of the suspension was measured using a densitometer (VITEK® bioMérieux model Densichek, France). Subsequently, it was inoculated onto sterile Petri plates (on dried surface of tryptone soya agar) with a sterile cotton swab by streaking with no gaps. After applying antibiotic disks (4 disks per Petri plate), the plates were incubated at 37 °C for (18 ± 2) hours. The growth retardation zones of microorganisms around the discs were measured with an accuracy of 1 mm.

Results were interpreted using the Russian guidelines "Determination of the Sensitivity of Microorganisms to Antimicrobials" (IACMAC, version 2025-01), prepared based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [27, 28].

Since EUCAST recommendations do not provide criteria for interpretation of *L. monocytogenes* antibiotic resistance for the entire list of antimicrobials used in this work, the zone diameter breakpoints for most antibiotics were based on data for *Staphylococcus* spp. For the analysis of *Listeria* susceptibility to vancomycin and streptomycin, values for *Enterococcus* spp. were used [5, 16, 29, 30].

Real-time PCR (qPCR). For DNA extraction, the "RIBO-prep" reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) was used according to the manufacturer's instruction.

Serological groups of *L. monocytogenes* were identified according to the methodological recommendations for the differentiation of bacterial genome regions of serogroups (1/2a, 3a), (1/2c, 3c), and (4b, 4d, 4e) in animal products using qPCR, developed at the Federal Centre for Animal Health.

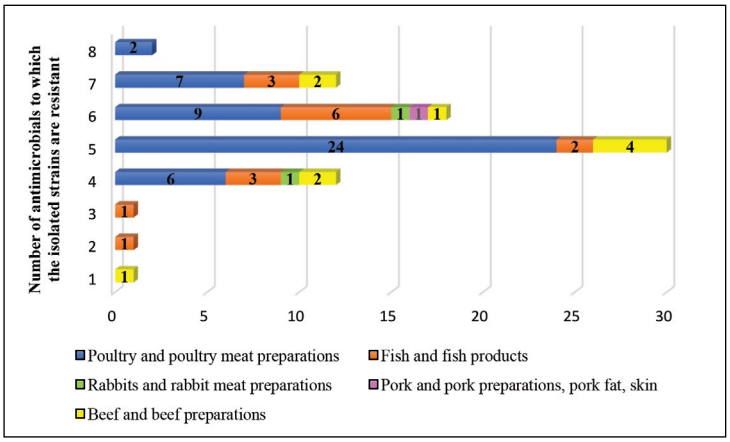

Fig. 3. L. monocytogenes isolates with multidrug resistance detected in samples of animal products in 2021–2024

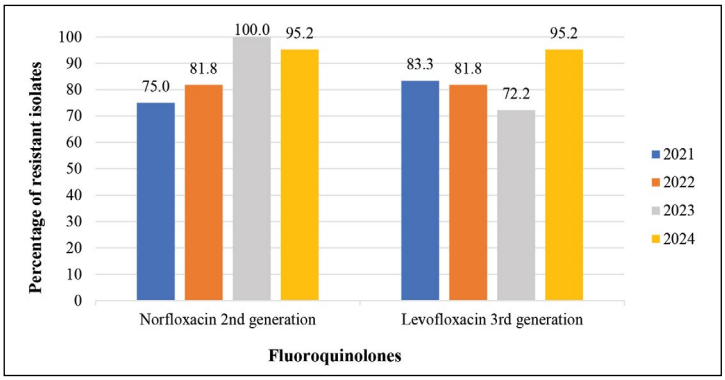

Fig. 4. Resistance of L. monocytogenes isolates to fluoroquinolones

[8] https://docs.cntd.ru/document/1200038583?ysclid=mhypcum520443253115 (in Russ.)

Serogroup IIa (serotypes 1/2a and 3a) was identified by amplification of the lmo0737 gene fragment; the lmo0737 and lmo1118 genes allowed identification of serogroup IIc (serotypes 1/2c, 3c); serogroup IVb (serotypes 4b, 4d, and 4e) was determined by amplification of the ORF0799 gene. Primers were manufactured by order of Syntol (Russia).

The following strains were used as positive controls:

– serotype 1/2a – DNA of *L. monocytogenes* No. 15 (Federal Research Center for Virology and Microbiology, Russia);

– serotype 1/2c – DNA of *L. monocytogenes* 5348 No. 20 (Federal Research Center for Virology and Microbiology);

– serotype 3a – DNA of *L. monocytogenes* No. 39 (Federal Research Center for Virology and Microbiology);

– serotype 3c – DNA of *L. monocytogenes* No. 46 (Federal Research Center for Virology and Microbiology);

– serotype 4b – DNA of *L. monocytogenes* ATCC 19115 (State Research Center for Applied Microbiology and Biotechnology, Russia);

– serotype 4d – DNA of *L. monocytogenes* 10888 No. 72 (Federal Research Center for Virology and Microbiology);

– serotype 4e – DNA of *L. monocytogenes* 19118 No. 75 (Federal Research Center for Virology and Microbiology).

The nucleotide sequences of primers and probes for differentiating genomic regions of *L. monocytogenes* serogroups (1/2a, 3a), (1/2c, 3c), and (4b, 4d, 4e) are presented in the Table [14, 19].

When preparing the reaction mixture, the following volumes of components per sample were used: 10× PCR buffer B – 2.5 µL; dNTP 2.5 mM – 2.5 µL; $MgCl_2$ 25 mM – 2.5 µL; a mixture of primers and a probe (10 pmol/µL each) – 0.5 µL each; SynTaq DNA polymerase 5 U/µL – 0.2 µL; ddH_2O – 11.8 µL (a set of reagents for qPCR manufactured by Syntol, Russia).

The qPCR was performed in a thermal cycler (CFX module, C1000 Touch, Bio-Rad Laboratories, Inc., USA) in a volume of 25 µL containing 20 µL of a mixture and 5 µL of *L. monocytogenes* DNA isolates.

The mixture preparation protocol included heating of the reaction mixture at 94 °C for 3 minutes, 40 cycles with denaturation at 94 °C for 20 seconds, annealing at 58 °C for 30 seconds and elongation at 72 °C for 25 seconds, and then completion of the reaction at 72 °C for 10 minutes.

Statistical processing of the research results was performed using the Microsoft Excel program.

RESULTS AND DISCUSSION

During testing of samples of animal products from 2021 to 2024, a total of 77 isolates of *L. monocytogenes* were detected (12 isolates in 2021, 22 isolates in 2022, 22 isolates in 2023, 21 isolates in 2024).

Figure 1 presents a graphical interpretation of the distribution frequency of *L. monocytogenes* isolates detected in animal products. The pathogen was most frequently found in poultry meat, with 48 *Listeria* isolates, accounting for a significant proportion (62%) of the total detected isolates. Fish and fish products, as well as beef and meat preparations derived from it, also represented a significant source of *L. monocytogenes* contamination (16 isolates – 21% and 10 isolates – 13%, respectively).

Data from the European Food Safety Authority (EFSA) indicate that outbreaks of foodborne infections caused by *L. monocytogenes* in Europe were mainly attributed to contamination of food products from these same categories: broiler meat, beef, pork, and products thereof; fish and fishery products; and cheeses [7].

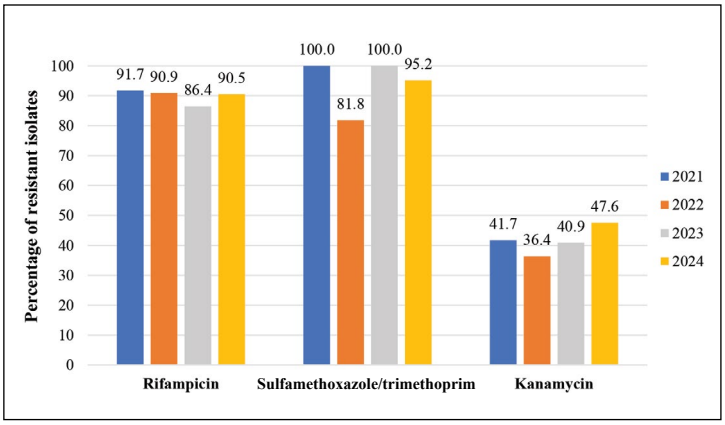

Fig. 5. Resistance of L. monocytogenes isolates to sulfamethoxazole/trimethoprim, rifampicin, and kanamycin

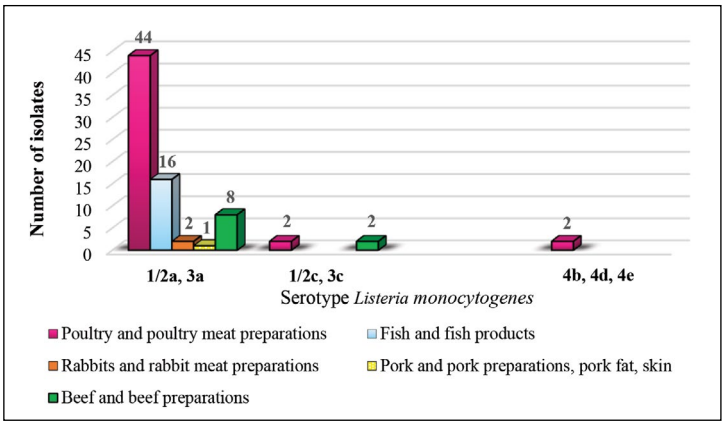

Fig. 6. Determination of L. monocytogenes serotypes in animal product samples by qPCR

As part of this work, evaluation of the resistance of *L. monocytogenes* isolates to 20 antimicrobial agents was conducted. The results are presented in Figure 2.

The research demonstrated a high level of resistance in *L. monocytogenes* isolates to several antibacterials. Thus, the maximum frequency of resistance was observed to cefuroxime (100.0%), sulfamethoxazole/trimethoprim (93.5%), norfloxacin (89.6%), rifampicin (89.6%), levofloxacin (83.1%), kanamycin (41.6%). Concurrently, all *L. monocytogenes* isolates were susceptible to ampicillin/sulbactam, benzylpenicillin, azithromycin, amikacin, vancomycin, and meropenem.

The data obtained correlate with findings of other authors that show the susceptibility of *L. monocytogenes* isolates to ampicillin, benzylpenicillin, vancomycin and resistance to rifampicin, sulfamethoxazole/trimethoprim, kanamycin, norfloxacin and erythromycin [4, 5, 22, 31]. Furthermore, the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing, in its official report for 2024, provided information on the resistance of *L. monocytogenes* strains isolated from food products to sulfamethoxazole/trimethoprim[9].

These and other results presented by individual authors emphasize the importance of monitoring the susceptibility

[9] https://www.garant.ru/products/ipo/prime/doc/409448585/?ysclid=mhymceuaxf332408554 (in Russ.)

of *L. monocytogenes* due to the increasing resistance to a number of antimicrobials, including tetracycline and erythromycin [3]. In our work, 3.9% of isolates were found to be resistant to tetracycline, and 11.7% to erythromycin.

Additionally, several researchers note the diversity of genetic profiles of *L. monocytogenes*, which leads to variability in antimicrobial susceptibility [16].

In the current tests, it was found that 98.7% of *L. monocytogenes* isolates were resistant to more than one antibiotic. No isolates resistant to all tested antimicrobials were detected.

Results from other authors not only confirm the fact of resistance of *L. monocytogenes* isolated from food products to at least one antibiotic, but also demonstrate an increase in the number of strains with multidrug resistance, posing a serious challenge for modern medicine [5, 16, 32].

Furthermore, as shown in Figure 3, resistance to five antimicrobials was identified in 30 isolates (38.9%), to six in 18 isolates (23.4%), and to four in 12 isolates (15.6%).

Also, as part of the work, 2 *L. monocytogenes* isolates resistant to eight antibiotics (2.6%) and 12 isolates resistant to seven antibiotics (15.6%) were identified.

Nearly half of the isolated *L. monocytogenes* strains (46.8%) were resistant to three classes of antibiotics (cephalosporins, sulfonamides, fluoroquinolones) and rifampicin. Isolates with multidrug resistance were most frequently detected in poultry meat products.

The emergence of strains resistant to fluoroquinolones was first reported in the early 1990s; however, multidrug resistance in *L. monocytogenes* was rare until the mid-2000s [5].

Figure 4 demonstrates the increase in the number of *L. monocytogenes* isolates resistant to certain antimicrobials within a single class (fluoroquinolones) during the period from 2021 to 2024. Specifically, in 2024, isolate resistance to levofloxacin (a third-generation fluoroquinolone) increased by 11.9% compared to 2021. Similar results were observed for norfloxacin (a second-generation fluoroquinolone), with a 20.2% increase in resistance over the four-year period. Similar results were observed for norfloxacin (a second-generation fluoroquinolone), with a 20.2% increase in resistance over the four-year period.

Resistance to kanamycin (a first-generation aminoglycoside) increased by 5.9% between 2021 and 2024. A high level (ranging from 81.8% to 100.0%) of resistance to sulfamethoxazole/trimethoprim and rifampicin was noted (Fig. 5).

In the next stage of our work, the serological groups of *L. monocytogenes* were identified. According to findings from other authors, a significant proportion of *Listeria* isolates detected in products belong to serogroup IIa, particularly serotype 1/2a, which demonstrates higher adaptability and resistance to disinfectants or other environmental factors [3, 6, 16, 32, 33]. However, according to data from the European Centre for Disease Prevention and Control (ECDC European Surveillance System, TESSy) report, in 2023, the most prevalent serogroup was IVb (47.8%), followed by IIa (41.7%), IIb (9.0%), and IIc (1.6%) [12].

Determining *Listeria* serotypes using traditional serological methods is time-consuming, lacks specificity, and is not widespread in the Russian Federation due to the absence of specific sera. Several authors recommend using qPCR for determining *L. monocytogenes* serogroups [15, 17].

In our work, using qPCR with three pairs of primers for the serological identification of 77 *L. monocytogenes* isolates, it was determined that 71 isolates (92.2%) belonged to serotypes 1/2a, 3a and were assigned to serogroup IIa; 4 isolates (5.2%) belonged to serotypes 1/2c, 3c and serogroup IIc; and 2 isolates (2.6%) belonged to serotypes 4b, 4d, 4e and serogroup IVb (Fig. 6). An isolate of the most dangerous serotype, *L. monocytogenes* 4b, was detected in poultry meat, which may pose a potential epidemiological hazard.

CONCLUSION

In this work, 77 *L. monocytogenes* isolates were identified, and it was found that poultry meat was the main source of *Listeria* contamination, accounting for 62% of the total isolates detected.

Data were obtained indicating increasing resistance in *Listeria* isolates, including multidrug resistance.

The *L. monocytogenes* isolates exhibited the highest resistance rates to cefuroxime (100.0%), sulfamethoxazole/trimethoprim (93.5%), norfloxacin (89.6%), rifampicin (89.6%), levofloxacin (83.1%), and kanamycin (41.6%). At the same time, all isolates were susceptible to azithromycin, amikacin, ampicillin/sulbactam, benzylpenicillin, vancomycin, and meropenem.

The vast majority of *L. monocytogenes* isolates (98.7%) demonstrated resistance to more than one antibiotic. Thus, resistance to five antimicrobials was observed in 30 isolates (38.9%), to six in 18 isolates (23.4%), and to four in 12 isolates (15.6%). Additionally, 2 isolates (2.6%) were found to be resistant to eight antibiotics, and 12 isolates (15.6%) to seven antibacterials.

Analysis of the data obtained from 2021 to 2024 revealed an increase in resistance in *L. monocytogenes* isolates to medicines from the fluoroquinolone group: resistance to norfloxacin (second-generation fluoroquinolone) increased by 20.2%, and to levofloxacin (third-generation fluoroquinolone) by 11.9%. During the same period, an increase in resistance to kanamycin by 5.9% was also observed. Resistance to sulfamethoxazole/trimethoprim and rifampicin remained at levels between 81.8 and 100.0%.

Using qPCR, it was determined that 92.2% of the studied *L. monocytogenes* isolates belong to serogroup (1/2a, 3a). Isolates belonging to serogroup IVb, which includes the most epidemiologically dangerous *Listeria* serotype 4b, were detected in poultry meat.

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Antibacterial therapy in dairy herds and the approach of veterinarians towards the issue of antimicrobial resistance in Nizhny Novgorod Oblast

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ABSTRACT

Introduction. A rational approach adopted by veterinary specialists to the selection of antibiotics is essential for successful treatment of infectious animal diseases, *inter alia* reducing the risk of developing antimicrobial resistance. Understanding the position of veterinarians regarding antimicrobial resistance and information about the extent of their knowledge on this issue are necessary for revising plans for the optimal use of antimicrobials in the field of animal husbandry.

Objective. The objective of the work was to determine the main reasons and the soundness of the use of antibacterials in dairy farming, as well as to assess the awareness of the issue of antimicrobial resistance and ways to overcome it.

Materials and methods. Rational use of antibiotics was assessed by surveying veterinarians from 44 dairy farms located in 12 raions and municipalities of Nizhny Novgorod Oblast, followed by statistical processing of the obtained data.

Results. It was found that 90.0% of the surveyed farm veterinarians kept an animal treatment log with records of antibiotic treatments, while 10.0% did not always record antibiotic prescriptions in the log. Of them, 63.0% use antibacterials to protect animals from diseases, 21.0% – to increase livestock profitability, and 16.0% – to prevent diseases. Most often, antibacterials were prescribed for treating diseases of the respiratory system (21.4%), the mammary gland (19.0%), and the reproductive organs (22.1%). More than half of the respondents stated that they do not use antibiotics for animal disease prevention, 17.3% use antimicrobials for preventing mammary gland diseases (mainly during the dry period), and 9.6% for preventing diseases of female reproductive organs after calving. Meanwhile, 50.0% of respondents do not aim to reduce their use of antibiotics. This creates high risks of the emergence of antibiotic-resistant strains of microorganisms and their spread through the food chain. The majority of veterinarians (68.0%) noted that the monitoring of residual concentrations of antibiotics in milk is a limiting factor for the widespread use of antibiotics in dairy cattle.

Conclusion. Despite the absence of a comprehensive program to reduce antibiotic use, the surveyed specialists acknowledge the existence of a mechanism that limits the spread of antimicrobial resistance. The only condition for curbing antibiotic use is the control of their content in raw milk, which dictates the need for further regulation in this area, as well as the optimization and prudent use of antibiotics.

Keywords: veterinarians, survey, use of antibiotics, antimicrobial resistance (AMR), dairy farming

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Антибактериальная терапия в молочных стадах и отношение ветеринарных врачей к проблеме антибиотикорезистентности в Нижегородской области

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РЕЗЮМЕ

Введение. Рациональный подход ветеринарных специалистов к выбору антибиотика является одним из условий успешного лечения инфекционных болезней животных, в том числе снижает риск формирования антибиотикорезистентности. Понимание позиции ветеринарных врачей относительно антимикробной резистентности и информация об объеме их знаний по данной теме необходимы для пересмотра планов по оптимальному применению противомикробных препаратов в области животноводства.

Цель исследования. Определение основных причин и степени обоснованности применения антибактериальных препаратов в молочном животноводстве, а также выяснение информированности ветеринарных врачей о проблеме антибиотикорезистентности и путях ее преодоления.

Материалы и методы. Оценку рациональности использования антибиотиков проводили методом анкетирования ветеринарных врачей 44 молочных хозяйств, расположенных в 12 районах и городских округах Нижегородской области, с последующей статистической обработкой полученных данных.
Результаты. Установлено, что 90,0% опрошенных ветеринарных врачей хозяйств вели амбулаторный журнал с записью о лечении антибиотиками, а 10,0% не всегда регистрировали назначения антибиотиков в журнале. Больше половины (63,0%) ветеринаров используют антибактериальные препараты для обеспечения благополучия животных, 21,0% – для повышения рентабельности животноводства и 16,0% – для профилактики заболеваний. Чаще всего антибактериальные препараты назначались для лечения органов дыхания (21,4%), молочной железы (19,0%) и репродуктивных органов (22,1%). Около 52,0% респондентов заявили, что не используют антибиотики для профилактики заболеваний животных, 17,3% – применяют антимикробные препараты для профилактики заболеваний молочной железы (в основном в сухостойный период) и 9,6% – для профилактики заболеваний репродуктивных органов самок после отела, при этом 50,0% опрошенных не ставят перед собой задачу сокращения применения антибактериальных средств. Это создает высокие риски появления антибиотикорезистентных штаммов микроорганизмов и распространения их по пищевой цепи. Большинство ветеринарных врачей (68,0%) отметили, что ограничивающим условием для широкого применения антибиотиков для молочного скота является контроль их остаточного содержания в молоке.
Заключение. Несмотря на отсутствие комплексной программы по сокращению использования антибиотиков, опрошенные специалисты признают существование механизма, ограничивающего распространение антибиотикорезистентности. Единственным условием сдерживания использования антибиотиков является контроль их содержания в сыром молоке, что диктует необходимость дальнейшего регулирования в данной сфере, оптимизации и рационализации антибиотикотерапии.

Ключевые слова: ветеринары, опрос, использование антибиотиков, устойчивость к противомикробным препаратам, молочное животноводство

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INTRODUCTION

Since its discovery, antibacterial substances have been widely used in medicine and agriculture. Antibiotics are used in livestock farming for the treatment and prevention of diseases and like growth promoters. For therapeutic purposes, antibacterials are typically administered in doses and courses prescribed by their manufacturers. To stimulate growth, they are introduced into the animal's body over a long period, and for prevention, they are used in small doses [1, 2, 3, 4]. More frequent use of antibiotics and their improper prescription exert selective pressure on clinically significant bacteria, which causes the development of antimicrobial resistance (AMR). This complicates the treatment of diseases they cause and makes first-line antimicrobial agents ineffective, leading to the overuse of second- and third-line antibacterials [4, 5]. Furthermore, the use of antibiotics leads to their accumulation in animal products, through which they can enter the human body [6, 7]. Also, the presence of antibiotics in milk reduces its technological properties, hindering fermentation by bacterial cultures when producing fermented dairy products and cheese [8]. Antibiotics can enter aquatic and terrestrial ecosystems through farm wastewater [9]. The close attention of scientists and practitioners to the problem of AMR has contributed to the adoption of documents at the global and national levels aimed at prudent use of antibiotics. For example, in 2017, the World Health Organization (WHO) developed a classification of antibiotics designed to systematize their use for treatment purposes. All antibiotics were divided into three groups: Access, Watch, and Reserve [10].

In our country, in 2017, the Strategy to Prevent the Spread of Antimicrobal Resistance in the Russian Federation to 2030 was approved. It is aimed at preventing and limiting the spread of microorganism resistance to antimicrobials, as well as informing and increasing the knowledge level of physicians and veterinary specialists on these issues [11]. In addition, Russian national standards do not permit the presence of antibiotics in milk and cattle meat [12]. Despite various existing regulatory documents, residual amounts of antibacterials are sometimes detected in milk and beef, indicating their uncontrolled use on livestock farms.

To understand risks to human and animal health associated with the use of antibiotics for therapeutic and preventive purposes in dairy farming, it is important to determine conditions and motivations for the use of antimicrobials on farms. Consequently, global efforts are currently underway to assess the awareness of the general public, physicians, and veterinary specialists about antibiotic use and AMR. In 2015, the WHO developed a questionnaire to evaluate public awareness and behavior related to antibiotic use [13]. Studies using surveys and interviews among the public, physicians, and veterinarians have been conducted in several European and Asian countries [14]. Such research is part of the system aimed at monitoring and evaluating knowledge about AMR.

In Russia, no similar investigations involving surveys of veterinarians working with food producing animals have been conducted. It is necessary to understand the perspective of veterinarians on AMR and the level of their awareness about the issue in order to revise plans for the prudent

use of antimicrobials in livestock farming. Therefore, this study was conducted among veterinarians on livestock farms to assess their practical and theoretical knowledge of antibiotic use and the emergence of resistance resulting from their use.

MATERIALS AND METHODS

Study area. The study was conducted in Nizhny Novgorod Oblast, an area with developed dairy and beef cattle, poultry, and pig farming. In total, there are 383 industrial livestock farms in the region, including 345 dairy farms with a total population of 243,400 cattle, of which 104,300 are cows (including heifers and beef cows). The average milk yield per dairy cow is 7,306.0 kg per year, with a total yield of 536.9 million kg per year. The consumption of milk and dairy products per person is 289.2 kg per year, and for the entire population of the region, it is 890.7 million kg per year, which is 65.9% more than what is produced [15].

Questionnaire survey. Researchers from the Nizhny Novgorod Research Veterinary Institute – Branch of Federal Research Center for Virology and Microbiology developed a research tool (questionnaire) using the "Yandex Forms" service (https://forms.yandex.ru/u/6800be6084227c3e18e6892c) to conduct an online survey and collect information on antibiotic use on livestock farms. The authors of this article surveyed 44 veterinarians serving dairy farms in 12 raions and municipalities of Nizhny Novgorod Oblast. Additionally, staff from the Veterinary Committee of Nizhny Novgorod Oblast orally informed veterinarians about the online survey and the study's objectives, providing guarantees of complete anonymity for the responses. No exclusion criteria were applied to the survey participants.

Questionnaire content. The questionnaire items provided information on the livestock industry and the number of animals serviced by a specific veterinarian; the objectives and motivations for using antibacterials in practice; their types and pharmacological groups; and the age and sex structure of the animals for which they are prescribed, as well as the symptoms and diseases involved. The questions also covered methods of medicinal product administration, record-keeping of their use, and assessment of microbial sensitivity to antibiotics. The final questions aimed to ascertain the veterinarians' opinions on the problem of bacterial resistance to antibacterials and the possibility of reducing or discontinuing antibiotic use.

Data analysis. Only questionnaires containing answers to questions with single or multiple response options were used for subsequent analysis. The analysis of the collected survey data was performed using free R software (version 4.3.1, available at https://www.r-project.org). Data processing involved methods for analyzing categorical data, as well as calculating percentage distributions using the "table" function in combination with the prop.table function. Statistical significance was estimated based on the *p*-value: if it was less than 0.05, this indicated statistical significance.

RESULTS AND DISCUSSION

The survey found that 90.0% of veterinary specialists maintain a clinic log, where they record antibiotic treatments, providing verifiable documentation.

Further analysis of the reasons for using antibacterials (Fig. 1) showed that 63.0% of respondents use antibiotics

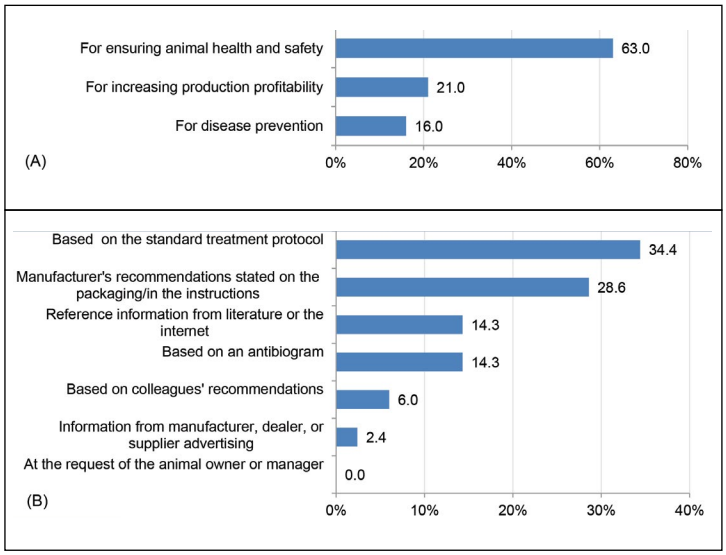

Fig. 1. Factors contributing to use of antibiotics: (A) purpose of use; (B) grounds for prescription

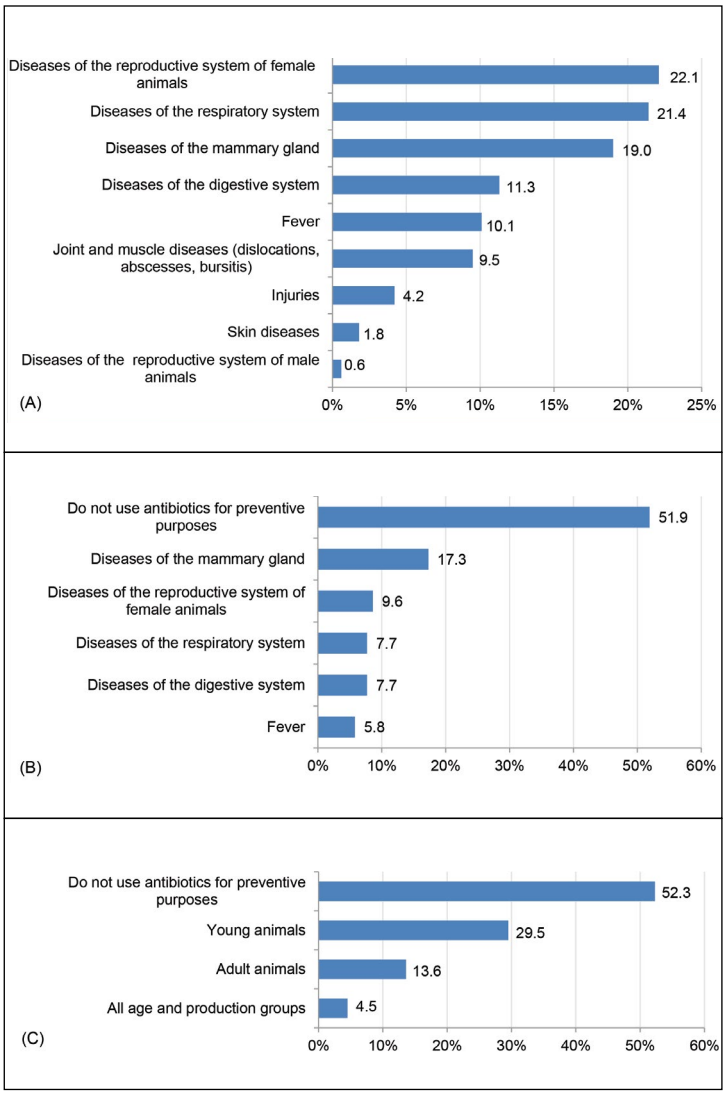

Fig. 2. Use of antibiotics: (A) for treating diseases in organs and systems; (B) for disease prevention in organs and systems; (C) for prevention purposes in animal groups. (Multiple responses allowed.)

to treat sick animals, 21.0% to ensure farm profitability, and 16.0% to prevent diseases. The majority of veterinary specialists (34.4%) used antibacterials according to a standard treatment protocol, while 28.6% followed the manufacturer's recommendations specified in the instructions for use. Additionally, 14.3% of respondents prescribed medicinal products after consulting reference literature or the internet and based on an antibiogram. Only 6.0% acted on the recommendations of colleagues, and a mere 2.4% relied on information from manufacturer (supplier) advertising.

The survey results established (Fig. 2A) that antibacterials were most frequently used to treat the reproductive system of female animals (22.1%), the respiratory system (21.4%), and the mammary gland (19.0%). Additionally, 11.3% of respondents used these medicinal products for digestive tract diseases, 10.1% for fever, 9.5% for joint and muscle conditions (dislocations, abscesses, bursitis), and 4.2% for injuries. A small number of respondents used antibacterials for treating skin diseases (1.8%) and diseases of the reproductive system of male animals (0.6%).

A little more than half of the surveyed veterinary specialists (51.9%) did not use antibiotics for preventive purposes (Fig. 2B). When used, they were primarily administered to prevent diseases of the mammary gland (17.3%) and the reproductive system of female animals (9.6%), respiratory and digestive organs (7.7% each), and fever (5.8%).

More than half of the veterinarians in Nizhny Novgorod Oblast (52.3%) did not prescribe antibacterials to any specific group of animals for prevention. However, 29.5% of respondents prescribed them to young stock, 13.6% to adult animals, and only 4.5% used these medicinal products for preventive purposes across all age and production groups (Fig. 2C).

Antibiotics (according to the AWaRe classification) from the Access group were used by 59.5% of veterinarians (Fig. 3). Specifically, they used penicillins, penicillins with *β*-lactamase inhibitors, ampicillins and amoxicillins, tetracyclines (oxytetracycline, Nitox®, etc.), 1st generation cephalosporins (cephaloridine, cephalothin, cephapirin, cephradine, cefazolin, cefalexin, cefadroxil), aminoglycosides (streptomycin, gentamicin, kanamycin, neomycin/gentamicin, tobramycin, netilmicin, sisomicin/amikacin/isepamicin), metronidazole, chloramphenicol (Levomekol®), lincomycin, sulfonamides (streptocide, Ditrim®, Norsulfazole, Ethazole, Sulfadimezin, sulfadimethoxine, trimethoprim, etc.), nitrofuran medicinal products (furacilin, furazolidone, etc.), and feed antibiotics.

Antibacterials from the Watch group were used by veterinarians in 36.7% of cases. This group was represented by medicinal products such as 2nd generation cephalosporins (cefuroxime, cefaclor, cefamandole, cefotiam, cefsulodin, cefoxitin), 3rd generation cephalosporins (cefotaxime, cefoperazone, ceftriaxone, ceftibuten, ceftazidime, cefixime, cefpodoxime, cefodizime, cefetamet), fluoroquinolones (ciprofloxacin, norfloxacin, ofloxacin, pefloxacin, lomefloxacin, sparfloxacin, levofloxacin, moxifloxacin, gemifloxacin, gatifloxacin, sitafloxacin, trovafloxacin, delafloxacin, enrofloxacin: Enroflon®, Enroflox, Enroxil®, Renrovet, Iroflox, Baytril, Enrofarm, Enrosept, etc.), macrolides (tylosin, Pharmazin®, Tylan, Tylanic, Disparcol, Draxxin, Floritil, Endometramag-T®, Spirovim, Pulmotil®, Aquatyl, Aivlosin®, etc.), and rifamycins (rifampicin, Rifacyclin, Rifapol, etc.).

The use of Reserve group antibiotics was minimal – 3.8%, including 4th generation cephalosporins (cefpirome,

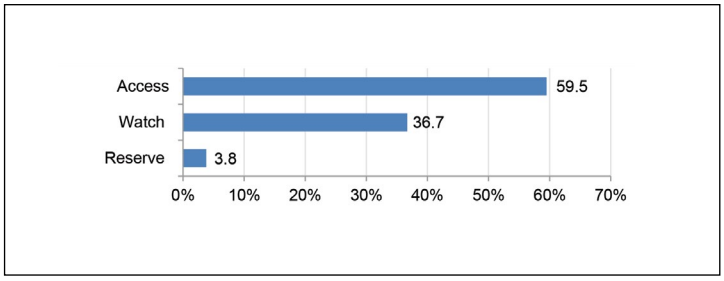

Fig. 3. Antibacterials of the Access, Watch, and Reserve groups used for livestock treatment on farms in Nizhny Novgorod Oblast

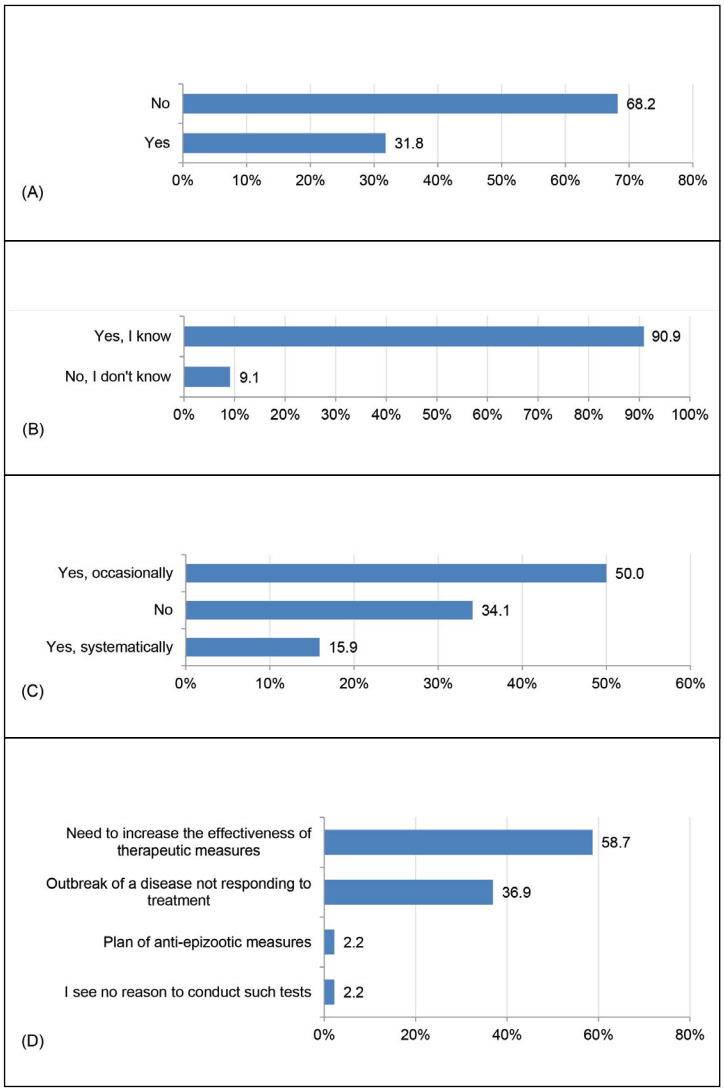

Fig. 4. Antibiotic resistance awareness analysis: (A) Have you ever participated in a survey regarding the prevention of antimicrobial resistance? (B) Are you aware of the issue of bacterial resistance to antibiotics? (C) Do you perform laboratory tests to determine the sensitivity of microorganisms to antibiotics? (D) In which cases do you conduct antibiotic sensitivity testing (multiple responses allowed)?

cefepime), 5th generation cephalosporins (ceftobiprole, ceftaroline, ceftolozane), and polymyxins (polymyxin M, polymyxin B).

Most of the surveyed veterinary specialists used antibacterials most frequently during transitional seasons: 46.1% in the spring and 35.2% in the autumn. This is explained by the fact that this period is one of the most challenging stages in the production cycle of dairy cattle and their offspring, as the main problem for cows after calving is the imbalance between the body's reserves and the nutrient requirements for milk production. The sharp increase in metabolic rate associated with calving and the onset of lactation leads to greater susceptibility to stress, which contributes to the occurrence of various disorders in cows and even calves.

The problem of AMR is highly relevant; therefore, several questionnaire items addressed this topic. 90.9% of the surveyed veterinary specialists are aware of the issue of AMR, while 9.1% reported being unaware. Regarding the prevention of AMR, 68.2% of respondents had been previously surveyed on the matter, while 31.8% had not. Only 15.9% of veterinary specialists consistently determine microbial sensitivity to antibiotics, 50.0% perform resistance testing occasionally, and 34.1% of specialists do not conduct such tests at all.

For specialists who perform antibiotic sensitivity testing, the primary reason in most cases (58.7%) is the need to increase the efficacy of therapeutic measures. In 36.9% of cases, the reason is an outbreak of a disease unresponsive to treatment, in 2.2% of cases – it is the fulfillment of an anti-epizootic measures plan, and in 2.2% of cases – respondents see no grounds for conducting the aforementioned testing (Fig. 4).

When studying the conditions for the possible reduction of antibiotic use in the future, it was found that the majority of respondents (47.1%) believe that the use of vaccines and sera would contribute to this, 41.1% – the implementation of veterinary and sanitary measures (disinfection, disinsection, and deratization), 4.0% – suggested other options (adherence to animal feeding and animal keeping rules), and 7.8% of specialists stated that there are no grounds for reducing the volume or changing the strategy of antibiotic use (Fig. 5A).

The majority of veterinary specialists (40.9%) believe that the antibiotics used for animals are becoming ineffective, meaning microorganisms develop resistance to them (based on laboratory sensitivity testing), 36.4% of respondents doubt this (judging by the reduced effectiveness of therapeutic measures), and 22.7% of respondents believe that resistance to antimicrobials does not develop (Fig. 5B).

None of the respondents would be able to completely stop using antibacterials; 47.7% of respondents definitely would not stop using them, and 52.3% believe they could reduce the use of antibiotics (Fig. 5C).

An analysis of awareness regarding antibiotic use recommendations revealed that in the majority of cases (50.0%), respondents had not been tasked with reducing antibiotic use in animals; 31.8% of veterinarians were instructed by farm management, and 18.2% by the raion/municipality veterinary administration.

An analysis of the reasons for refusing or reducing antibiotic use in dairy farming showed that most surveyed veterinarians (68.0%) limit their use due to the monitoring of residual amounts in milk and meat. 26.0% of specialists

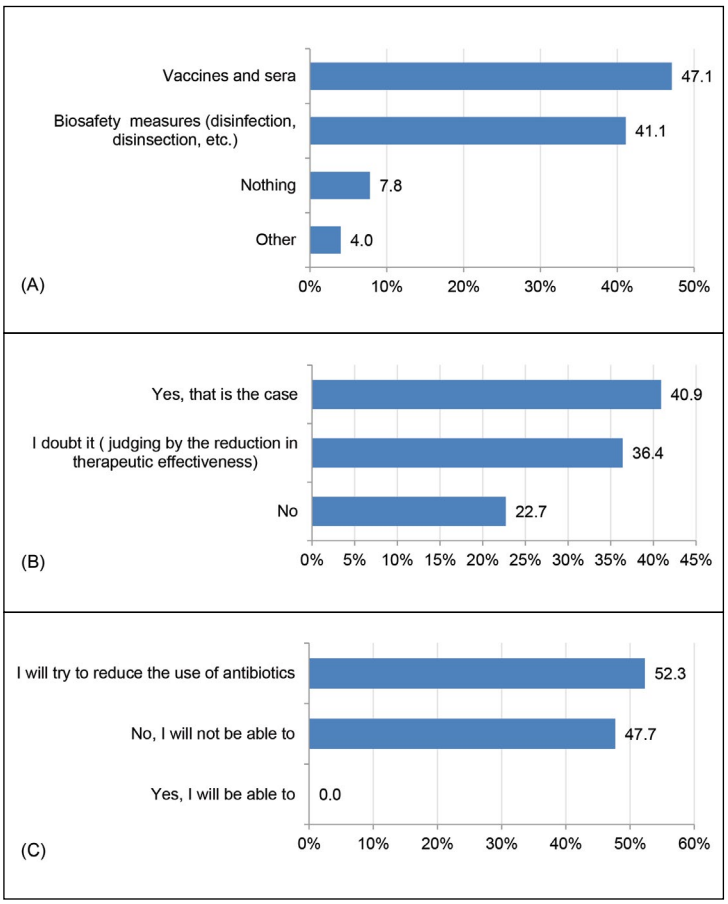

Fig. 5. Analysis of factors reducing future use of antibiotics: (A) In your opinion, what factors would lead to a reduction in antibiotic use (multiple responses allowed)? (B) Do you believe that some antibiotics you use for livestock treatment have become ineffective (due to the development of resistance)? (C) Will you be able to completely stop using antibiotics in your practice in the foreseeable future?

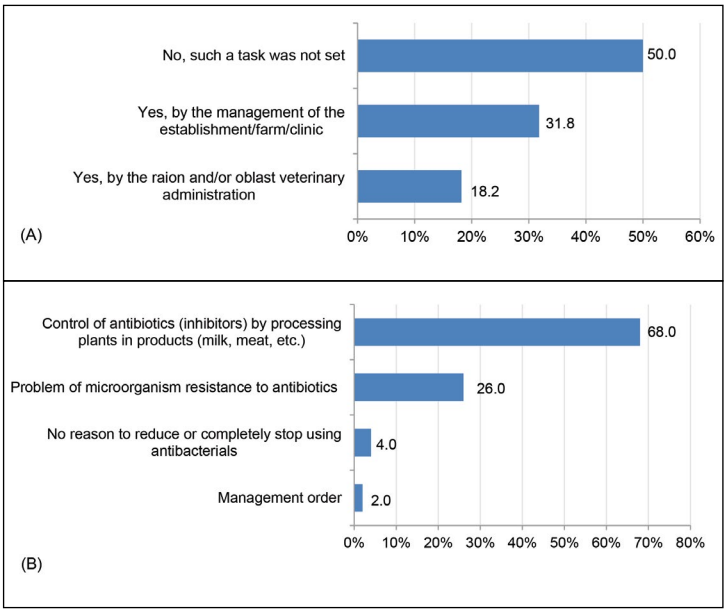

Fig. 6. Analysis of information on the use of antibacterials by veterinary specialists in Nizhny Novgorod Oblast: (A) Has management set a goal for you to reduce the use of antibiotics in animals? (B) What is the reason for the reduction or complete cessation of antibiotic use (multiple responses allowed)?

attribute their refusal of widespread antibiotic use to the development of microbial resistance, 2.0% – to instructions from farm management, and 4.0% believe there are no grounds for reducing or completely abandoning the use of antibiotics in dairy farming (Fig. 6).

This is the first documented study in Russia involving only veterinary practitioners working on dairy farms. The survey showed that 100.0% of specialists in Nizhny Novgorod Oblast use antibiotics in their practice. At the same time, 59.5% of veterinarians prescribed antibiotics from the Access group, 36.7% – from the Watch group, and the use of Reserve antibiotics was minimal (3.8%). Veterinarians in Nizhny Novgorod Oblast did not use medicinal products from the 'not recommended' group. The most frequently prescribed antibiotics were tetracyclines (12.4%), macrolides (12.4%), and 3rd generation cephalosporins (10.0%). These findings are similar to data on antibiotic use in European countries. For instance, in Sweden, Access group antibiotics, particularly penicillins, were used significantly more often than others (84.0%). The use of Watch group antibiotics was more frequently reported in Germany (42.0%), while in Sweden, medicinal products from this group were not used [16].

More than a third of the surveyed veterinarians (34.4%) prescribe antibiotics based on a standard treatment protocol, and 28.6% based on manufacturer recommendations. Only 14.3% base their choice of medicinal products on the results of laboratory sensitivity testing of the herd microflora. We assume that an important factor in choosing an antibacterial could be its cost.

Data from other researchers indicate that antibiotics are most commonly used to treat infectious diseases in cows (mastitis, endometritis) and young stock (respiratory and digestive diseases) [17]. Our results show that veterinary specialists most frequently use antimicrobials to treat diseases of the reproductive organs of female animals (22.1%), respiratory system (21.4%), and mammary glands (19.0%).

The preventive use of antibiotics is also very widespread all over the world [18]. Researchers believe that using antibiotics for prevention has both advantages and disadvantages. The main advantage is maintaining the maximum number of healthy animals, while the risk lies in the fact that antimicrobial use leads to the emergence of resistant bacterial strains and serotypes, which can ultimately enter the human body [18, 19]. The most favorable time for antibiotic therapy of bovine mastitis is the dry period [17]; therefore, 17.3% of the surveyed veterinarians prescribe antibiotics for mastitis prevention specifically during this timeframe. After calving, the period of milking and calf feeding begins. During the time when milk is not sent to processing plants and there is no risk of a milk batch being returned, treatment and prevention of uterine and birth canal diseases are performed. Our results show that antimicrobials are used for the treatment and prevention of reproductive organ infections in cows (22.1 and 9.6% of cases, respectively). However, this leads to the excretion of residual amounts of the medicinal product in milk, which is then fed to calves.

Calves are the group of animals most susceptible to diseases on dairy farms. In the first weeks of life, there is a high risk of digestive system diseases, and in the first 2–3 months, respiratory diseases [20]. Therefore, various therapeutic and preventive measures are performed for this animal group, which may include the use of antibiotics. We have previously demonstrated [21] that 21.4 and 11.3% of veterinarians use antibacterials to treat respiratory and digestive diseases, while antibiotics are most frequently used for preventive purposes in young stock (29.5% of cases). In particular, 7.7% of respondents consciously use them for the prevention of digestive and respiratory diseases.

As can be seen, antibacterials are deliberately and widely used in dairy farming to increase the effectiveness of therapeutic measures or disease prevention. Almost half (50.0%) of the surveyed veterinarians did not set a goal to reduce antibiotic use. At the same time, monitoring the development of AMR in the herd microflora is weak: only 15.9% of veterinarians regularly determine the sensitivity of isolated microorganisms to antibacterials, while a third never do so; 47.7% of veterinarians affirmatively state that they will never stop using antibiotics, even though they are aware of the AMR problem. Thus, despite awareness of the problem, there are no restrictions on the use of antibacterials at the farm and establishment level.

There are several factors limiting the use of antibiotics, such as administrative or industry-specific restrictions. For instance, the Strategy to Prevent the Spread of Antimicrobal Resistance in the Russian Federation to 2030 outlines several key areas, such as informing the public about the use of antimicrobials and resistance issues, and improving the training and awareness of physicians and veterinarians on these issues [11]. The survey revealed that 68.0% of veterinary specialists limit antibiotic use only because of the monitoring of their presence in raw milk by dairies. Thus, an important production factor restraining the use of antibacterials is GOST 31449-2013 Raw cow's milk. Specifications'[1], which do not allow residual amounts of antibiotics in milk; if they are detected, farms suffer losses. Despite this, only 26.0% of surveyed veterinarians, recognizing the significance of AMR, are reducing antibiotic use in cows and calves. Such an attitude among veterinarians contributes to the development of AMR and the spread of resistant microorganisms through the food chain, creating a risk of untreatable bacterial infections in both animals and humans.

CONCLUSION

Thus, the use of antibiotics in dairy farming in Nizhny Novgorod Oblast is widespread. Antibacterials are integrated into treatment protocols for most common diseases in dairy cows and calves and are also used for preventive purposes. This contributes to the development of AMR in on-farm bacteria and the dissemination of resistant strains beyond the farm environment via raw milk. The only functional tool for restraining antibiotic use is the monitoring of residual levels in raw milk by dairies. However, the concept of a conscious refusal to use antibiotics is not widely supported by veterinarians, highlighting the urgent need for further regulatory measures in this sector, as well as enhanced training and outreach for both veterinary specialists and farm management.

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Yak thelaziasis in the Orenburg Oblast: *Musca autumnalis* (De Geer, 1776) as a vector and *Thelazia rhodesi* (Desmarest, 1827) as the causative agent of infestation

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ABSTRACT

Introduction. Thelaziasis remains a widespread vector-borne parasitic zoonosis both within the Russian Federation and globally. Thelaziasis in yaks remains insufficiently studied, with the available data being fragmentary. It is the first time a thelaziasis clinical case in yaks from the Orenburg Oblast is described and it is of significant interest to a broad range of specialists.

Objective. Analysis and clinical case description of thelaziasis in yaks from the Orenburg Oblast, including the study of its causative agent and vector.

Materials and methods. Studies conducted from 2021 to 2023 at the steppe field station of the Institute of Steppe of the Ural Branch of the Russian Academy of Sciences in the Belyayevsky Raion of the Orenburg Oblast included clinical examinations and assessment of pathological lesions and severity of inflammation in the eyes and conjunctiva of yaks. Parasitic secretophagous dipterans (flies) from the ocular region were collected and counted, and their abundance, species and sex ratios were determined. Helminthoscopy was performed, and the nematode species was determined morphologically.

Results. Clinically, thelaziasis in yaks manifested as profuse lacrimation and recurrent keratoconjunctivitis. The extent of invasion (EI) was 100%, and the intensity of invasion (II) was 5. The detected helminths belonged to *Thelazia rhodesi* species. The intermediate hosts and vectors of *Thelazia* were facultative hematophages, specifically *Musca autumnalis*, a synbovine fly species ubiquitous in the steppe landscapes of the Orenburg Oblast. The ratio of females to males collected from the head region of yaks was 83 and 17%, respectively, confirming the leading role of female *Musca autumnalis* as vectors of nematodes of the genus *Thelazia*.

Conclusion. Domestic yaks in the natural and climatic conditions of the Orenburg Oblast are susceptible to thelaziasis. The disease progress, its clinical manifestations, as well as extent and intensity of invasion are likely influenced by acclimatization of yaks, who are not indigenous to this region.

Keywords: ocular infection, thelaziasis, zoonosis, yaks, *Musca autumnalis*, *Thelazia rhodesi*

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Телязиоз яков в Оренбургской области: *Musca autumnalis* (De Geer, 1776) как переносчик и *Thelazia rhodesi* (Desmarest, 1827) как возбудитель инвазии

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РЕЗЮМЕ

Введение. Телязиоз остается широко распространенным трансмиссивным паразитарным зоонозом как на территории Российской Федерации, так и в мире. Заболевания телязиозом яков изучены недостаточно, имеющиеся сведения фрагментарны. Клинический случай телязиоза яков в Оренбургской области описывается впервые и представляет собой большой интерес для широкого круга специалистов.

Цель исследования. Анализ и описание клинического случая телязиоза яков в Оренбургской области, изучение возбудителя и переносчика данного инвазионного заболевания.

Материалы и методы. Исследования, проведенные в 2021–2023 гг. в степном стационаре Института степи Уральского отделения Российской академии наук в Беляевском районе Оренбургской области, включали клинический осмотр, оценку патологических процессов и степени воспалительных процессов глаз и конъюнктивы яков. Произведен отлов с области глаз и учет паразитических двукрылых мух-секретофагов, определены их количественные, видовые и половые характеристики. Проведена гельминтоскопия, видовая принадлежность обнаруженных нематод установлена морфологически.

Результаты. Клинически телязиоз яков проявлялся в обильном слезотечении и рецидивирующем кератоконъюнктивите. Экстенсивность инвазии составила 100%, интенсивность инвазии равнялась 5. Обнаруженные гельминты принадлежали виду *Thelazia rhodesi.* Промежуточными хозяевами и переносчиками телязий являлись факультативные гематофаги, представители синбовинной фауны мухи *Musca autumnalis*, повсеместно распространенные в степных ландшафтах Оренбургской области. Соотношение самок и самцов, снятых в области головы яков, составило 83 и 17% соответственно, что подтверждает ведущую роль самок *Musca autumnalis* как вектора передачи нематод рода *Thelazia.*

Заключение. Домашние яки в природно-климатических условиях Оренбургской области подвержены телязиозу. Течение болезни, клинические проявления, экстенсивность и интенсивность инвазии, вероятно, обусловлены процессами акклиматизации яков, которые не являются аборигенными для данной местности.

Ключевые слова: глазная инвазия, телязиоз, зооноз, яки, *Musca autumnalis*, *Thelazia rhodesi*

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INTRODUCTION

Thelaziasis, caused by "eyeworms", is a seasonal parasitic keratoconjunctivitis spread across both Europe (England, Italy, Spain, France, Croatia, Serbia, Germany, Romania, Poland) and Asia (India, Korea, Taiwan, Thailand, Bangladesh, Mongolia, Indonesia, China, Myanmar, Japan) [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. Sporadically, the invasion was noted in Africa, Australia, North and South America [2, 10].

According to the literature, bovine thelaziasis in the Russian Federation occurs in the Northwestern, Volga, West Siberian, and Far Eastern regions, as well as in the Middle and Southern Urals [11, 12, 13]. In the Orenburg Oblast, bovine thelaziasis is registered everywhere, the causative agent is *Thelazia rhodesi* (Desmarest, 1827) [1, 12, 14].

Currently, 16 *Thelazia* species have been described [10], the most common are:

– *Th. callipaeda* (Railliet et Henry, 1910) – oriental eye worm;
– *Th. californiensis* (Price, 1930) – California eye worm;
– *Th. gulosa* (Railiet et Henry, 1910) – cattle eye worm;
– *Th. lacrymalis* (Gurlt, 1831) – eyeworm in horses;
– *Th. rhodesi* (Desmarest, 1827) parasitic nematode of cattle;
– *Th. leesei* (Railliet et Henry, 1910);
– *Th. alfortensis* (Railliet et Henry, 1910);
– *Th. skrjabini* (Erschov, 1928);
– *Th. ershowi* (Oserskaja, 1931);
– *Th. bubalis* (Ramanujachari et Alwar, 1952);
– *Th. anolabiata* (Molin, 1860).

Nematodes of the genus *Thelazia* parasitize cattle, domestic horses, Przewalski's horses, donkeys, mules, and European bison [2, 3, 4, 6, 7, 8, 15]. The infection also affects small ruminants, pigs, cats, dogs, foxes, and rabbits [1, 5, 9]. Reports in the literature describe infection in deer, badgers, monkeys, and wolves [10]. Several cases of avian infestation have been described [1, 2, 16].

Thelaziasis in yaks has been confirmed in the Kabardino-Balkarian Republic. The extent of invasion (EI) was 2.7%, and the causative agent was *Thelazia gulosa* [17, 18]. There is evidence that *Th. skrjabini* can infest yaks [1].

In disadvantaged socioeconomic environments, humans can also become an accidental host of *Th. californiensis*, *Th. gulosa* or *Th. callipaeda*. Thus, thelaziasis is a parasitic zoonosis, which is consistent with the literature data [1, 10, 19, 20, 21].

The role of *Musca autumnalis* (De Geer, 1776) as an intermediate host for nematodes of the genus *Thelazia* has been extensively described in both Russian [11, 12, 16] and international literature [2, 3, 4, 10, 22].

The aim of this work was to study the vector *(Musca autumnalis)* and the causative agent (*Thelazia rhodesi*) of thelaziasis in yaks from the Orenburg Oblast. It is the first time a thelaziasis clinical case in yaks from the Orenburg Oblast is described and it is of significant interest to a broad range of specialists.

MATERIALS AND METHODS

The studies were carried out at the Orenburg Tarpania Steppe Field Station (Institute of Steppe of the Ural Branch of the Russian Academy of Sciences, Orenburg Federal Research Center of the Ural Branch of the Russian Academy of Sciences) in the Belyayevsky Raion of the Orenburg Oblast. This location is home to an assembled collection of ungulates: in addition to domestic yaks, the site supports populations of Przewalski's horses, Tibetan kiangs, Bactrian camels and wool goats.

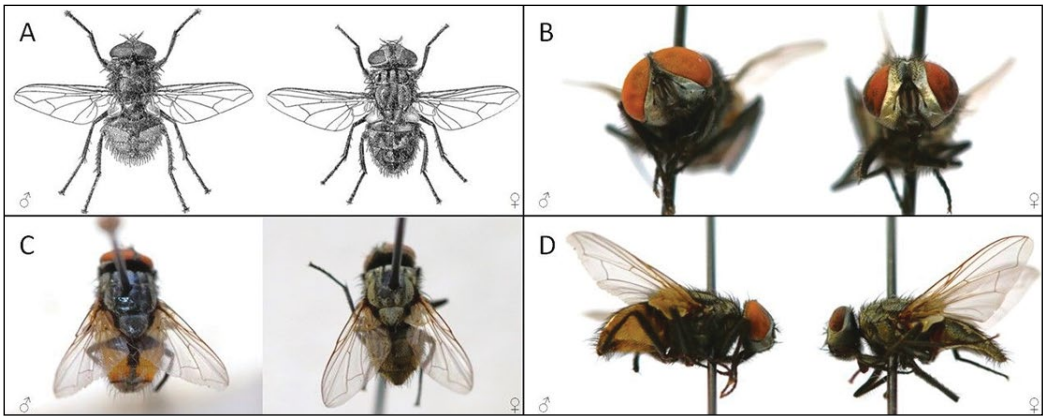

Fig. 1. M. autumnalis: A – dorsal view (adapted from A. A. Stackelberg, 1956, fig. 56, p. 75); B – head, illustrating holoptic eyes in the male and dichoptic eyes in the female; C – dorsal view; D – lateral view

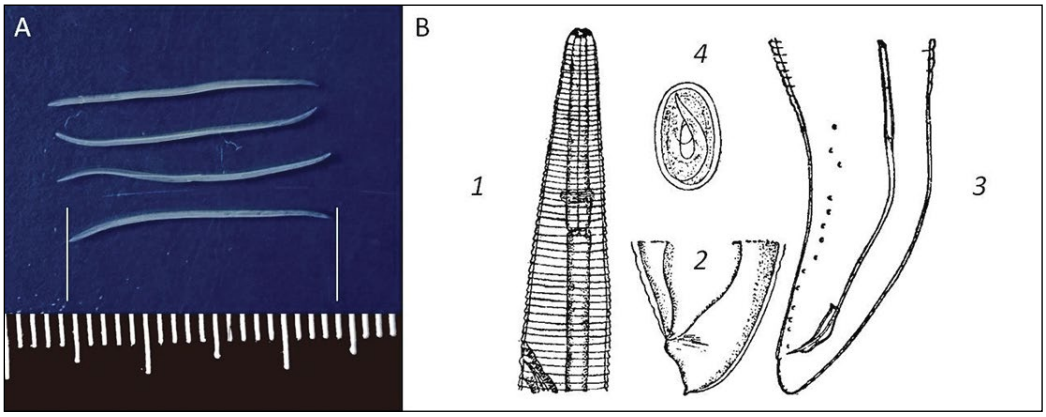

Fig. 2. Th. rhodesi: A – adult females from the yak conjunctival sac (external view and size); B – morphological details: 1 – anterior end, 2 – female posterior end, 3 – male posterior end, 4 – egg (adapted from K. I. Skryabin et al., 1934, fig. 277, p. 311)

In 2021–2023, yak were clinically examined to assess pathological lesions and inflammation degree in the eyes and conjunctiva.

Parasitic secretophagous dipterans (flies) from the ocular region were collected and counted, and their abundance, species and sex ratios were determined. Collection and counting of insects from the suborder *Brachycera Orthorrhapha* were carried out throughout the entire flight period of imago using an entomological sweep net directly on the animals, following standard methods for collecting *Diptera* [23]. Insects were identified using taxonomic keys [24, 25, 26].

Adult *Thelazia* nematodes were recovered by irrigating the conjunctival sacs of the yaks. The animal's head was secured, the eyelids were retracted, and the third eyelid and conjunctival cavity were flushed with a 3% boric acid solution. Strong jets of fluid were produced using a rubber bulb syringe to ensure thorough flushing. Next, contents of the conjunctival cavities were collected [16]. The nematode species were identified morphologically.

The study assessed both the extent of invasion, defined as the percentage of infected animals out of the total examined, and the intensity of invasion, defined as the number of parasite specimens recovered from an individual host.

Photographs were obtained using a Canon 760D camera (Japan) and a Nikon Eclipse E200 microscope (Japan).

RESULTS AND DISCUSSION

Musca autumnalis (the face fly) belongs to the family *Muscidae* (Latreille, 1802), which includes house flies and stable flies. It belongs to the superfamily *Muscoidea*, section *Calyptratae*, suborder *Brachycera (Cyclorrhapha)*, order *Diptera* [26]. *M. autumnalis* is widespread in the Palearctic region, throughout Western Europe (Sweden, Norway, Spain, Italy), the Caucasus and Central Asia. These flies inhabit steppe, semi-desert, forest-steppe, and forest landscapes, and are a component of pasture fauna [1].

Fifty-eight species of *Diptera – Brachycera* from the synbovine complex have been identified in the Orenburg Cis-Urals, 40 of which are capable of mechanically transmitting helminths of medical and veterinary importance (including pinworms, ascarids, whipworms, tapeworms, hookworms, *Drascheia megastoma*, *Habronema*, *Parabronema*, *Parafilaria*, *Setaria*, *Stephanofilaria*, and *Thelazia*) [27]. *M. autumnalis* was found to be active throughout the flight

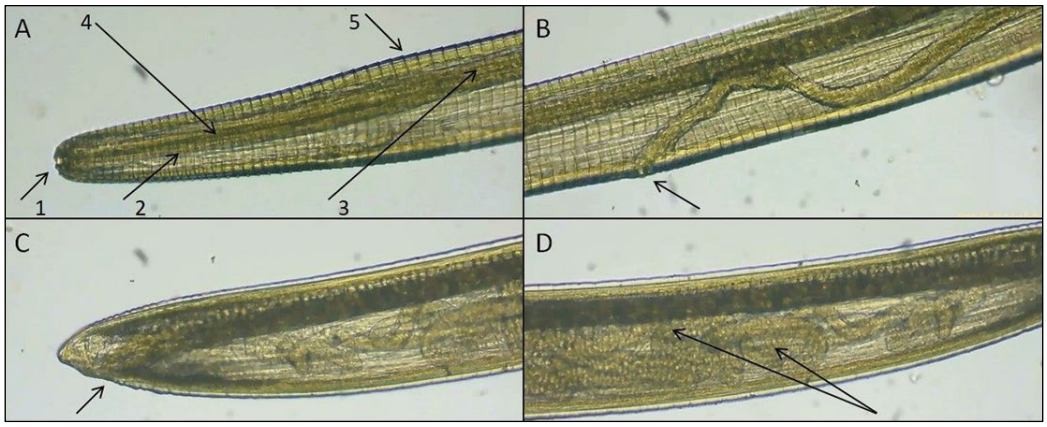

Fig. 3. Morphology of Th. rhodesi female. A – anterior end: 1 – oral opening (mouth), 2 – esophagus, 3 – intestinal tube, 4 – nerve ring, 5 – cuticular striations; B – vulva region, genital opening situated near the anterior end; C – posterior end, anal opening (anus); D – uterus containing eggs within the nematode body

period, from early spring to late autumn, confirming its status as a thermophilic species. Figure 1 shows the pronounced sexual dimorphism in eye color and structure between male and female flies. Holoptic eyes in males are adapted for swarming and mating in flight. The male exhibits symmetrical, translucent dark yellow spots located on the third and fourth abdominal tergites. The female has an abdomen entirely covered in a grayish bloom with iridescent spots; setae are developed on the first sternite [26].

Females constituted the overwhelming majority of the insects captured. The ratio of females to males collected from the head region was 83 and 17%, respectively, confirming the data of G. A. Kotelnikov [16] and F. Gregor et al. [22] regarding the leading role of females from an ecological and veterinary perspective. *M. autumnalis* serves as a transmission agent for various pathogens, particularly nematodes of the genus *Thelazia*.

Musca autumnalis is a typical representative of the synbovine, zoophilic fauna. In the imaginal stage, for example, members of the family *Muscidae* frequently feed on secretions from wounds and the ocular, nasal, and oral mucosa of grazing animals [26].

Nartshuk E. P. [26] and Ageeva T. Yu. [27] regard female *M. autumnalis* as facultative hematophages, a characterization that does not contradict the data obtained in the present study. *M. autumnalis* lacks a piercing proboscis capable of actively penetrating the skin of mammals. However, females possess prestomal teeth, with which they can damage healing wounds and mucous membranes, thereby sustaining inflammatory processes and obtaining nourishment.

Larvae of *M. autumnalis* are specialized saprophages that develop in and feed on vertebrate dung, exhibiting a coprophagous habit specific to the *Muscidae* [11, 26].

During the summer, the animals under study were kept together as a single herd, managed under semi-wild conditions on natural pasture. The general condition of the experimental animals was satisfactory; appetite and movement were normal. Water was provided from an open source, the Sazan stream. In winter, the animals were maintained under covered shelters. No antiparasitic drugs were administered to the animals.

Profuse lacrimation was recorded as one of the clinical signs of thelaziasis in the yaks. The animals presented with chronic recurrent keratoconjunctivitis and exhibited visual impairment, which is consistent with the findings of D. F. L. Djungu et al. [7].

Other clinical signs associated with thelaziasis are photophobia, blepharospasm, and ulcerative as well as non-ulcerative keratitis. Reported complications include granulomas and corneal perforations, inflammation of the lens, ectropion of the eyeball, corneal opacity (leukoma) resulting from nematode migration through the cornea, fibrohemorrhagic iridocyclitis, and blindness [1, 9, 10, 11]. The clinical course of thelaziasis is frequently complicated by secondary infections, further compromising the animals' health [10, 11, 12, 13, 14].

In the overwhelming majority of cases, thelaziasis-associated keratoconjunctivitis in yaks was observed to be bilateral. No age- or sex-related differences were observed, which contrasts with the findings of D. M. Tweedle et al. [8] that cattle aged 21 to 38 months were more commonly affected.

The intensity of invasion was determined to be 5, and the extent of invasion was 100%; this contrasts with the findings of A. K. Oshkhunov et al. [17]. In conclusion, yaks introduced into the natural and climatic conditions of the Orenburg steppes acquired the infection during the process of acclimatization.

Thelaziasis clinical signs were observed throughout the entire *M. autumnalis* flight period, thereby determining the disease seasonality. This finding is consistent with the data reported by D. M. Tweedle et al. [8], E. Kim et al. [9], and R. R. Kasarla et al. [10].

Nematodes were isolated from the conjunctival sacs of yaks during helminthoscopic examination by flushing, followed by collection and microscopic analysis. The parasites were identified as belonging to the species *Th. rhodesi*, and all nematodes recovered were female. Figure 2 illustrates that the females were approximately 20 mm long. They were highly motile, whitish in color, and barely visible to the naked eye within the conjunctival sac. The nematodes were most visible in the medial canthus (inner corner) of the yaks' eyes.

The species *Th. rhodesi* belongs to the genus *Thelazia*, the order *Spirurida*, the family *Thelaziidae*, the type *Nematoda*, nematodes, or roundworms. Microscopically, the

head end of *Th. rhodesi* has a cross-striations, as shown in Figure 3. The serrated cuticle of the nematodes inflicts mechanical damage to the corneal and conjunctival surfaces, resulting in inflammation.

Excessive lacrimation is a response to damage to the eye tissues. The blood-feeding fly *M. autumnalis*, acting as a facultative hematophage, induces profuse lacrimation in animals both through mechanical trauma to the cornea and by transmitting *Thelazia* infestation. In addition to the mechanical effects caused by the nematodes, there is also evidence (Glazunova L. A. et al. [1]) of their allergic and toxic pathological influence on the host. Khristianovsky P. I. et al. describe the phenomenon of parasite carriage by definitive hosts as a cause of annual infection in animals [12].

Larvae of *Thelazia* are excreted from the bodies of afflicted yaks through the lacrimal passages, namely from the lacrimal gland ducts, the conjunctival cavity, and the area under the third eyelid. *M. autumnalis* consumes the secretion of the lacrimal glands together with *Thelazia* larvae, thereby acting as a specific thelaziasis intermediate host. The nematodes reach the final stage of their development within the host organism over a period of approximately one month. Transmission of infective larvae to the definitive host occurs when flies ingest (take up) the larvae while feeding on yaks' tears and mucous secretions with their proboscises.

CONCLUSION

As a vector-borne parasitic zoonosis, thelaziasis continues to be of considerable importance in both human and veterinary medicine, necessitating continued research.

Thelaziasis in yaks is documented for the first time in the Belyayevsky Raion of the Orenburg Oblast, contributing both theoretical and practical value to the understanding of this disease.

The isolated nematodes were identified as *Th. rhodesi*. *M. autumnalis*, a species ubiquitous in the steppe ecosystems of Orenburg, served as the vector for *Thelazia* transmission. The chronic nature of the disease is linked to pathogen carriage over the winter stall period. Clinical manifestations and disease progression in yaks were driven by the mechanical, allergic, and toxic effects of *Th. rhodesi*, combined with a relatively low infestation intensity.

No distinct differences in clinical signs, diagnostic methods, prevention, or treatment were observed between thelaziasis in yaks and that in cattle. Preventive and therapeutic measures against thelaziasis in yaks should include seasonal applications of repellents and insecticides, as well as scheduled anthelmintic treatments. To control the incidence of thelaziasis, it is necessary to manage the population size of vector flies and maintain proper zoohygienic conditions on farms.

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Serological tests for lumpy skin disease in Republic of Tajikistan in 2023

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ABSTRACT

Introduction. Cattle farming plays a key role in the agriculture of the Republic of Tajikistan, satisfying not only the needs for meat and dairy products. Recently, the intensification of livestock production in Tajikistan has been facing serious problems related to infectious diseases, in particular those caused by capripoxviruses, including lumpy skin disease (LSD), which causes significant economic damage and compromises animal performance and health in the region and around the world. Studying and understanding the LSD epizootology in the Republic of Tajikistan climatic environment will facilitate better disease control.

Objective. Epizootological data collection and brief description of LSD outbreaks in the Republic of Tajikistan in 2023.

Materials and methods. Traditional epizootological analysis tools were used to collect data on LSD outbreaks, as well as serological tools for the disease retrospective diagnosis.

Results. In 2023, an LSD case was first reported in the Republic of Tajikistan in the region bordering Afghanistan. Analysis of the seasonal pattern of the disease occurrence in Tajikistan demonstrated that the LSD outbreaks were most often reported in summer and autumn: from July to November. The disease spread mainly in the areas with a high concentration of livestock, in particular in the Khatlon Region. The average animal density in the Khatlon Region is 46 cattle per 1 km^2 and 117 sheep and goats per 1 km^2. The peak of LSD epidemic was recorded in September – November 2023. Morbidity and mortality varied by districts, ranging from 10 to 55% and from 2 to 15%, respectively. Tests of 216 bovine serum samples demonstrated LSD virus antibodies in 109 animals, accounting for 50.5% of the total.

Conclusion. In 2023, a range of studies was conducted and measures were taken in the Republic of Tajikistan to prevent LSD spread. To effectively control the disease, it is necessary to strengthen epizootological monitoring, carry out timely vaccination of animals and implement measures for the identification and control of potential vectors. These steps will minimize economic losses and maintain the animal health in the republic.

Keywords: lumpy skin disease (LSD), epizootology, clinical signs, diagnosis, enzyme-linked immunosorbent assay (ELISA), vaccination, CIS

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Проведение серологических исследований на заразный узелковый дерматит в Республике Таджикистан в 2023 г.

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РЕЗЮМЕ

Введение. Скотоводство играет ключевую роль в сельском хозяйстве Республики Таджикистан, обеспечивая не только потребности в мясных и молочных продуктах. В последнее время интенсификация животноводческой деятельности в Таджикистане сталкивается с серьезными проблемами, связанными с инфекционными заболеваниями, в частности вызываемыми каприпоксвирусами, в том числе заразным узелковым дерматитом крупного рогатого скота, который наносит значительный экономический ущерб и ставит под угрозу продуктивность и здоровье животных в регионе и во всем мире. Изучение и понимание эпизоотологии заразного узелкового дерматита крупного рогатого скота в климатических условиях Республики Таджикистан позволит улучшить контроль за заболеванием.

Цель исследования. Эпизоотологическое описание и краткая характеристика вспышек заразного узелкового дерматита крупного рогатого скота в Республике Таджикистан в 2023 г.

Материалы и методы. В работе использованы классические методы эпизоотологического анализа по сбору данных о вспышках заразного узелкового дерматита крупного рогатого скота, а также серологические методы для ретроспективной диагностики заболевания.
Результаты. В 2023 г. в Республике Таджикистан впервые был зарегистрирован случай заболевания крупного рогатого скота заразным узелковым дерматитом в районе, граничащем с Афганистаном. Анализ сезонности проявления данного заболевания в Таджикистане показал, что вспышки заразного узелкового дерматита крупного рогатого скота происходили чаще всего в летне-осенний период: с июля по ноябрь. Болезнь преимущественно распространилась в регионах с высокой концентрацией скота, в частности в районах Хатлонской области. Средняя плотность животных в Хатлонской области составляет 46 голов крупного рогатого скота на 1 км^2 и 117 голов мелкого рогатого скота на 1 км^2. Пик эпизоотии заразного узелкового дерматита крупного рогатого скота был зафиксирован в сентябре – ноябре 2023 г. Заболеваемость и смертность варьировали по районам от 10 до 55% и от 2 до 15% соответственно. При исследовании 216 проб сывороток крови крупного рогатого скота антитела к вирусу заразного узелкового дерматита были обнаружены у 109 животных, что составило 50,5% от общего числа.
Заключение. В 2023 г. в Республике Таджикистан был проведен комплекс исследований и предприняты меры по предупреждению распространения заразного узелкового дерматита крупного рогатого скота. Для эффективной борьбы с заболеванием необходимо усилить эпизоотологический мониторинг, проводить своевременную вакцинацию животных и внедрять меры по идентификации потенциальных переносчиков и их контролю. Эти шаги позволят минимизировать экономические потери и сохранить здоровье скота в республике.

Ключевые слова: заразный узелковый дерматит, эпизоотология, клинические признаки, диагностика, иммуноферментный анализ, вакцинация, СНГ

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INTRODUCTION

Lumpy skin disease (LSD, Neethling virus infection) is a viral transmissible disease of cattle characterized by fever, damage to the lymphatic system, edema of subcutaneous tissue and internal organs, formation of skin nodules, damage to the eyes and mucous membranes of the respiratory and digestive systems, loss of productive performance and body weight [1]. The disease causative agent is a DNA-containing virus of the genus *Capripoxvirus*, family *Poxviridae*, which is closely related to sheep pox virus and goat pox virus [2].

Lumpy skin disease virus (LSDV) spread outside the disease outbreak is possible in two ways. Firstly, the infected animals demonstrating clinical signs and being in the incubation period are active shedders of the pathogen in an unapparent form [3]. In this case, subclinically infected animals serve not only as an active source of infection, but also as an important factor in the virus spread over long distances, which is most often associated with cattle driving or illegal transportation by road vehicles [4]. It should be noted that LSD causes major economic losses, such as a sharp decline in milk production, reduced milk and raw hide quality, loss of body weight, abortions, infertility in bulls and reduced fertility in cows. As a result, trade relations are prohibited, which negatively affects the economies of countries focused on exporting animal products [5].

It is assumed that the main factor of the disease agent transmission is blood-sucking insects and ticks [6], although it has now been experimentally proven that mosquitoes of the genus *Aedes*, flies (for example, *Stomoxys calcitrans* and *Biomyia fasciata*) and ticks (*Rhipicephalus appendiculatus* and *Amblyomma hebraeum*) are capable of participating in the virus spread [7]. The importance of various arthropod vectors may vary in climatically different regions depending on their abundance and trophic behavior, however, no relative entomological studies from different geographical and climatic zones have been described. The virus transmission via contaminated semen is possible [8].

Direct contact was not previously considered an effective route of the infection transmission [9], however, given the disease epizootological profile, when the outbreaks can be reported in early spring or autumn in the absence of flight activity of blood-sucking insects, alimentary and air-borne routes of the infection are feasible, since a diseased animal sheds the virus with the nasal discharge and scabs from the necrotized nodes [10]. Evidence of the contact virus transmission has been obtained in a number of studies involving experimental infection of susceptible animals [11].

In the past, the LSD distribution range was limited only to South Africa. However, by 1956, the disease quickly spread to Central and East Africa. The first case of LSD infection outside the African continent was reported in Israel, which marked the beginning of the intercontinental spread of the disease [12, 13, 14, 15, 16]. The disease with characteristic signs was detected in Albania, Greece, Georgia, Iran, Macedonia, Bulgaria, Turkey and other countries of the world with numerous outbreaks being reported in recent years [17, 18, 19]. In the CIS countries (Fig. 1), LSD was officially reported in Azerbaijan (2014), Armenia (2015), Russia (2015), Kazakhstan (2016) [20, 21, 22].

Since 2019, LSD has begun to spread in Southeast Asian countries such as Thailand, Vietnam, India, Pakistan, Indonesia, and Singapore; China, Japan, and South Korea have also reported outbreaks [15, 23, 24, 25, 26]. This created a threat of the disease cross-border transmission to the

countries of Central Asia. LSD was not reported in the Republic of Tajikistan until 2023, but the massive spread of the infection to the Asian region since 2019 has resulted in the pathogen introduction into the country [25]. In 2023, LSD was for the first time diagnosed in cattle in the border region with Afghanistan, in the Panj District of Khatlon Region, Republic of Tajikistan. The infected animals comprised 30 cattle of varying ages of the Black Pied, Carpathian, Swedish, and local breeds. The disease was observed in animals in Mehnatobod village (Sughd Region), Kahramon village (Rudaki District of Republican Subordination), in Moskovsky settlement (Hamadoni District) as well as in the villages of Farkhor District of Khatlon Region, and then it spread to other regions of the Republic, causing significant economic damage to animal farmers.

The work was aimed at the epizootological description and brief characterization of the LSD outbreaks in the Republic of Tajikistan in 2023.

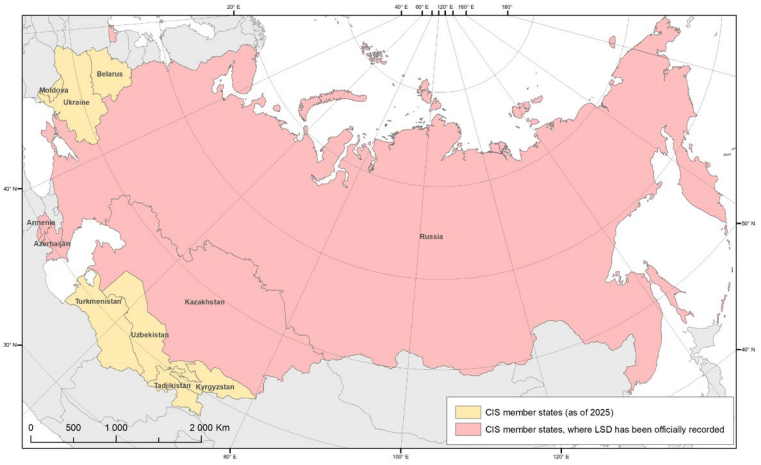

Fig. 1. CIS members, where LSD is reported

MATERIALS AND METHODS

LSD epizootic situation was studied on animal farms in the southwestern and northern regions of Tajikistan (Sughd Region, Khatlon Region, and the Districts of Republican Subordination), where a combined housing-and-pasture system of cattle management is practiced.

Standard clinical, epizootological, and serological research methods were used in the study. To assess morbidity and mortality, the proportion of diseased and dead animals out of the total susceptible population in the outbreak was calculated.

Serological analysis. To make a preliminary assessment of the situation, 216 serum samples of cattle of different age groups were serologically tested. The sera were collected from animals demonstrating LSD clinical signs in the regions of the Republic located in different climatic and geographical zones (Table 1).

Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used in the study. The study was performed using a test system for detecting antibodies to capripoxviruses in sera or plasma of cattle, sheep, goats or other susceptible species (IDvet, France) in accordance with the manufacturer's instructions. The serum samples were collected from infected (IN) and contact animals (C1 and C2) on days 0; 42 and 60 after the start of the experiment. The results were interpreted based on optical density (OD) measured at a wavelength of 450 nm using a Sunrise microplate reader (Tecan, Switzerland). They were expressed as the ratio of the optical density of the test sample to the optical density of the positive control (S/P%), calculated as follows: S/P% = (OD sample – OD negative control) / (OD positive control – OD negative control) × 100%. S/P ratio ≥ 30% was considered positive.

RESULTS AND DISCUSSION

The Republic of Tajikistan is a landlocked state in Central Asia, characterized by a predominantly mountainous terrain. The area of the country is 142.6 thousands km^2, the population is 10 million people. As of 2023, the cattle population in the country amounted to 2,605 thousands (FAOstat, 2025). The first-level administrative divisions consist of five units: Sughd Region, Khatlon Region, Gorno-Badakhshan Autonomous Region, the city of Dushanbe, and the Districts of Republican Subordination.

Tajikistan is located in zones of continental, sharply continental, and mountain climate. The country's climatic

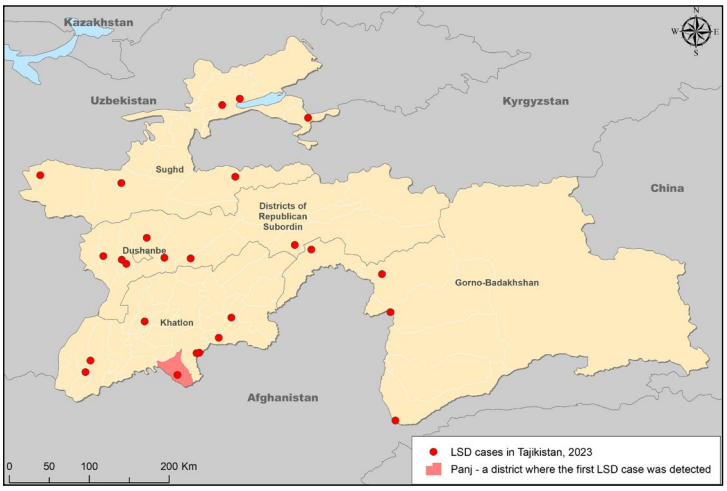

Fig. 2. LSD spread in the Republic of Tajikistan in 2023

conditions are determined by its topography, which encompasses both lowland and high mountain areas. Precipitation is distributed unevenly. The majority of the precipitation falls in winter and spring. Summer in lowland areas is dry.

The disease was first reported in the hot and arid Khatlon Region (in Panj, Farkhor and Hamadoni Districts), where high temperatures and dry climate contribute to the active reproduction of blood-sucking insects, the key virus carriers. The infection subsequently spread to other regions, including the Sughd Region, the city of Dushanbe and Districts of Republican Subordination, where the moderately warm climate, presence of pastures and water reservoirs also create conditions for the disease transmission. The location of the affected areas is shown in Figure 2.

In 2023, the majority of LSD outbreaks (14 outbreaks) were reported in the Khatlon Region, where 70% of the Republic's cattle population is concentrated. Morbidity and mortality of cattle in the Panj District reached 55 and 15%, respectively.

Analyzes of LSD situation in cattle demonstrated that during the further spread of the disease across the Republic in June – August 2023, the mortality in the Faizabad District was 30–40%, and mortality – 2–3%, while in the Gissar valley they were 20 and 3%, respectively.

In the Rasht Valley, where temperate climate prevails, the disease was reported in cattle in November – December.

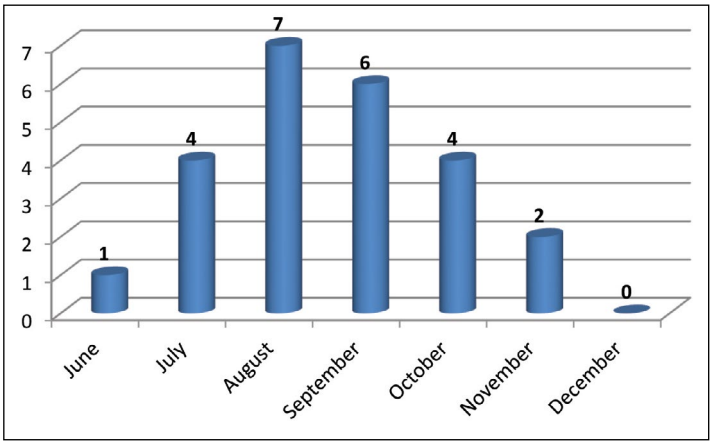

Fig. 3. Number of LSD outbreaks by month

Table 1
Regions of the republic where serological monitoring was carried out

Regions of the Republic	Type of pathological material	Number of tested samples
Khatlon Region	Serum	95
Sughd Region	Serum	58
Districts of Republican Subordination	Serum	63
	Total	216

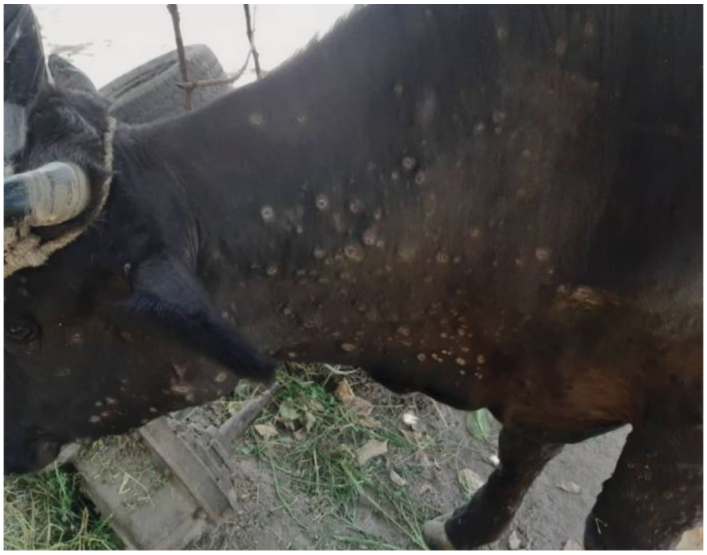

Fig. 4. Formation of nodular skin lesions on the neck and shoulder blade areas of LSD infected cattle

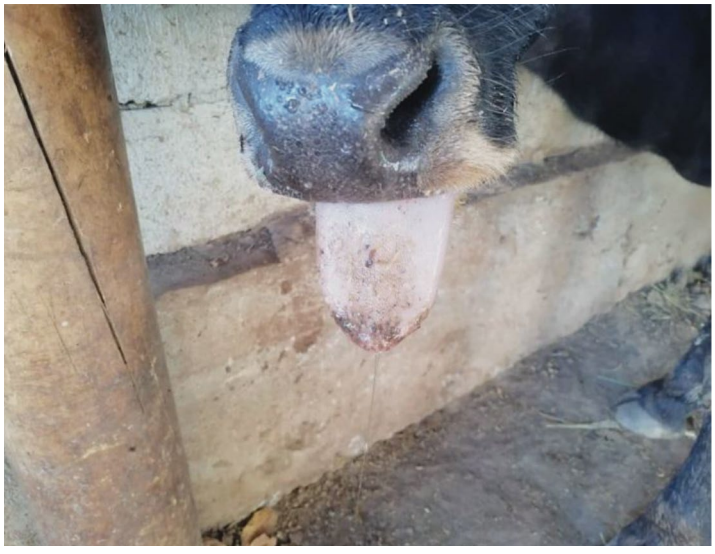

Fig. 5. Erosive lesions of the tongue mucosa of LSD infected cattle

Fig. 6. Multiple nodular skin lesions of LSD infected cattle

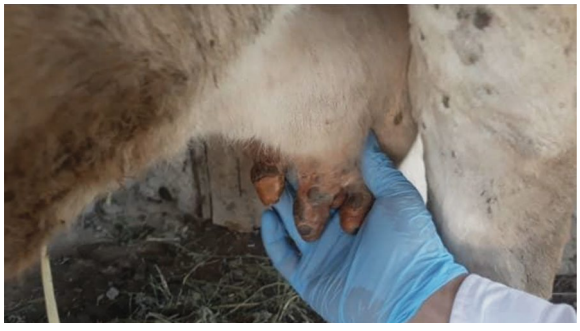

Fig. 7. Nodular and ulcerative lesions on the udder of LSD infected cattle

Table 2
LSD ELISA results

Districts	Number of kishlaks	Samples tested	Positive samples detected	Seroprevalence, %
Khatlon Region				
Panj	3	27	19	70.4
Hamadoni	4	19	11	57.9
Farkhor	4	15	9	60.0
Vose'	2	11	5	45.5
Kulob	3	8	3	37.5
Bokhtar city	3	15	8	53.3
Sughd Region				
Ayni	2	12	4	33.3
Ghafurov	3	10	2	20.0
Konibodom	1	7	2	28.6
Panjakent	1	16	7	43.8
Isfara	1	13	5	38.5
Districts of Republican Subordination				
Hisar	2	14	4	28.6
Rudaki	3	21	13	61.9
Faizabad	2	10	5	50.0
Rasht	2	18	12	66.7
Total	36	216	109	50.5

The mortality in this region did not exceed 10%, and the mortality of the infected animals amounted to 3–5%.

In northern Tajikistan, LSD was reported in Ayni, Ghafurov, Konibodom, Panjakent, and Isfara districts of Sughd Region. The cattle morbidity in these regions was 15–20%, and mortality ranged from 3 to 5%.

The disease outbreaks were less intense in the regions of the Badakhshan Mountainous Autonomous Region, which is associated with a lower activity of the insect vectors due to the specifics of the climate and terrain. Despite the fact that this area, located along the border with Afghanistan, occupies more than 43% of the territory of the Republic, it has the lowest animal population and density per 1 km^2.

The latest disease outbreaks in the Republic of Tajikistan were reported in December 2023 in the Districts of Republican Subordination. Overall, more than 20 outbreaks of LSD were reported during the year, most of them were detected in the summer-autumn period from July to November (Fig. 3). This may be attributed both to the activity of potential vectors and to the pasturing of livestock during this period. The peak of the LSD epizootic in cattle was recorded in September – November 2023. The information obtained is consistent with the published data [27, 28, 29].

It is important to note that, based on the field data, the incubation period for LSD was approximately 5 days and may have depended on the susceptibility of the animals [30].

Thus, the disease outbreaks were less intense in the mountainous regions of the country, where the harsh climate with low temperatures limits the insect activity. In the foothill areas and pastures, the infection could spread through livestock migration. Consequently, the combination of the hot climate, high animal density, and favorable conditions for insect reproduction have become key factors in the LSD epizootic process in Tajikistan [31].

The diseased animals demonstrated high body temperature (40.5–42.0 °C), nasal and ocular discharge, enlarged lymph nodes (especially patellar and scapular ones), refusal of food, as well as skin nodular eruptions of various shapes and sizes (Fig. 4), and erosive lesions in the oral cavity, including the tongue (Fig. 5).

The number of nodules ranged from ten to several hundred (Fig. 6). During LSD development in lactating cows, nodules of various shapes and sizes often appeared on the udder (Fig. 7). The diseased animals quickly became emaciated and lost their dairy and meat performance for a long time [32].

When ELISA was used to test 216 samples of bovine sera from animals from Khatlon and Sughd Regions and Districts of Republican Subordination, LSDV antibodies were detected in 109 of them, which accounted for 50.5% of the total number of animals (Table 2).

The highest seroprevalence was reported in the Khatlon Region: Panj District (70.4%), Farkhor District (60.0%) and Hamadoni District (57.9%), which indicates a significant spread of infection in this area. In Sughd Region, the highest rates were detected in Panjakent (43.8%) and Isfara Districts (38.5%), and as for the Districts of Republican Subordination, high level of seroprevalence was reported in Rasht (66.7%) and Rudaki Districts (61.9%).

Thus, the study results indicate the transboundary nature of LSD and emphasize the need for further monitoring and the implementation of preventive measures to control the disease situation with the aim of reducing the economic losses.

CONCLUSION

An analysis of the LSD situation in Tajikistan in 2023 showed that the infection outbreaks had seasonal pattern, reaching the peak in the summer and autumn period (July – November). The most affected areas were Khatlon Region, Districts of Republican Subordination and Sughd Region, where the seroprevalence varied from 20.0 to 70.4%. The infection outbreaks were reported both in the backyards and on commercial farms, which indicates the need for strict veterinary control and implementation of preventive measures.

To effectively control LSD, it is necessary to strengthen epizootological monitoring, carry out timely vaccination of animals, and implement measures for the identification and control of potential vectors. These steps will allow minimizing economic losses and maintaining animal health in the Republic.

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Optimizing transient transfection conditions in mammalian cell production lines for expression of classical swine fever virus E2 antigen

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ABSTRACT

Introduction. Despite no cases of classical swine fever (CSF) have been recorded in the Russian Federation since 2021, gaining official recognition, as a disease-free zone, will require adoption of effective, safe vaccines compatible with the DIVA strategy. A range of expression systems is being evaluated as potential platforms for a recombinant subunit vaccine; synthesizing the E2 antigen in mammalian cells appears to be a particularly promising approach.

Objective. Optimizing transient transfection conditions in mammalian cell production lines for expression of classical swine fever virus (CSFV) E2 antigen.

Materials and methods. The nucleotide sequence encoding a 188-amino acid fragment of the E2 antigen was cloned into the pVAX1 vector. Transient transfection was performed using two common methods – calcium-phosphate and cationic (employing branched polyethylenimine, PEI) – on three established mammalian production cell lines: CHO-K1, PK-15, and BHK-21/13. Expression efficiency was controlled using immunofluorescence, quantitative reverse transcription polymerase chain reaction, and enzyme-linked immunosorbent assay.

Results. It was determined that all the cell lines evaluated underwent transfection with an efficiency ranging from 60 to 90%. Cellular viability 24 hours post-transfection was at least 87%, with the lowest rates observed following calcium-phosphate transfection using an initial 12-hour incubation period. In all cases, transfection was accompanied by expression of specific messenger RNAs. The highest yield of the 17.3 kDa recombinant E2 protein was achieved in the CHO-K1 cell line (up to 47.4 mg/L), while the lowest yield was observed in the BHK-21/13 line (up to 24.1 mg/L). The specificity ratio in the antigen variant of the indirect enzyme-linked immunosorbent assay using specific antisera ranged from 5.1 to 6.2 units for all the expressed protein variants.

Conclusion. All the cell lines presented in the study demonstrated satisfactory transfection efficiency. Combined with their properties – such as high proliferation rates and adaptation to serum-free media – this makes them suitable for stable expression. Both the calcium-phosphate and cationic methods provide high transfection efficiency, relatively low cytotoxicity, and good reproducibility. The combined use of these control methods is advisable during the design phase of expression systems. In a production setting, however, the primary metric of their functionality is the overall yield of the specific recombinant protein, as determined by antigen-specific enzyme-linked immunosorbent assay.

Keywords: classical swine fever, mammalian cells, transient gene expression, recombinant antigen, immunofluorescence, messenger RNAs, enzyme-linked immunosorbent assay

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Оптимизация условий транзиентной трансфекции производственных линий клеток млекопитающих для экспрессии антигена Е2 вируса классической чумы свиней

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РЕЗЮМЕ

Введение. Несмотря на отсутствие в Российской Федерации с 2021 г. зарегистрированных случаев классической чумы свиней, для получения статуса зоны, свободной от данного заболевания, необходимо внедрение эффективных и безопасных вакцин, соответствующих стратегии DIVA. В качестве потенциальных инструментов для создания рекомбинантной субъединичной вакцины рассматриваются различные системы экспрессии; перспективным представляется синтез антигена E2 в клетках млекопитающих.

Цель исследования. Оптимизация условий транзиентной трансфекции производственных линий клеток млекопитающих для экспрессии антигена E2 вируса классической чумы свиней.

Материалы и методы. Нуклеотидная последовательность, кодирующая фрагмент антигена E2 протяженностью 188 а. о., была клонирована в вектор pVAX1. Транзиентная трансфекция проводилась двумя общедоступными методами: кальций-фосфатным и катионным (при помощи разветвленного полиэтиленимина) – в отношении трех производственных клеточных линий млекопитающих: CHO-K1, PK-15, BHK-21/13. Контроль эффективности экспрессии осуществлялся методами иммунофлуоресценции, количественной полимеразной цепной реакции с обратной транскрипцией, иммуноферментного анализа.

Результаты. Было установлено, что все рассматриваемые клеточные линии подвергались трансфекции с эффективностью от 60 до 90%. Выживаемость клеток через 24 ч после проведения трансфекции составляла не менее 87%, наименьшие показатели регистрировались при проведении кальций-фосфатной трансфекции с первичной инкубацией 12 ч. Проведение трансфекции во всех случаях сопровождалось экспрессией специфических матричных РНК. Наибольший выход рекомбинантного белка E2 молекулярной массой 17,3 кДа был характерен для линии CHO-K1 (до 47,4 мг/л), наименьший – для линии BHK-21/13 (до 24,1 мг/л). Коэффициент специфичности в антигенном варианте непрямого иммуноферментного анализа со специфическими антисыворотками для всех вариантов экспрессируемого белка варьировал в диапазоне 5,1–6,2 ед.

Заключение. Все представленные в исследовании клеточные линии обладали удовлетворительной трансфицируемостью, что в совокупности с их свойствами (высокой скоростью пролиферации, адаптацией к бессывороточным средам) позволяет использовать их для стабильной экспрессии. И кальций-фосфатный метод, и катионный обеспечивают высокую эффективность трансфекции, относительно низкую цитотоксичность и воспроизводимость. Применение рассматриваемых методов контроля целесообразно в совокупности на этапе конструирования экспрессионных систем, однако в производственных условиях основным критерием их функциональности является тотальный выход специфического рекомбинантного белка, регистрируемый при помощи антигенного иммуноферментного анализа.

Ключевые слова: классическая чума свиней, клетки млекопитающих, транзиентная экспрессия генов, рекомбинантный антиген, иммунофлуоресценция, матричные РНК, иммуноферментный анализ

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INTRODUCTION

Classical swine fever (CSF), caused by the RNA-containing *Pestivirus C*, is one of the most dangerous viral diseases of the *Suidae* family and is accompanied by hemorrhagic syndrome [1, 2]. Classical swine fever virus (CSFV) primarily targets endothelial cells and macrophages. Concurrently, it triggers T-cell apoptosis, leading to significant immunosuppression. In most cases, the infection follows an acute course, culminating in one of three outcomes: fatality, recovery with the development of virus-neutralizing antibodies, or progression to a chronic infection [3, 4]. In countries where CSF is endemic, effective live attenuated vaccines are commonly used. However, the ability of vaccine strains to replicate within the host complicates differentiating vaccinated animals from infected ones (the DIVA strategy). This challenge underlies the restrictive vaccination policies adopted in non-endemic regions. Thus, within the Russian Federation, there is a clear need to develop effective and safe marker vaccines. Such vaccines are essential for controlling potential CSF outbreaks and are a prerequisite for eventually achieving CSF-free zone status, officially recognized by the World Organisation for Animal Health [5, 6].

In the context of developing experimental subunit vaccines, various expression systems have been successfully employed for the biosynthesis of the major glycoprotein E2. We previously generated a prokaryotic equivalent of glycoprotein E2 [7]. Its synthesis predominantly resulted in inclusion body formation, necessitating extensive chromatographic purification and refolding steps. Consequently, the yield of purified, bioactive product was relatively low.

As an alternative to *Escherichia coli* expression, gene expression in mammalian cells can be considered. Its primary advantages are post-translational modifications closely resembling the native ones, along with recombinant protein solubility and folding compatible with high-level overexpression [8]. To facilitate future scale-up, mammalian cell expression must be cost-effective and high yielding. This necessitated development of an optimized genetic construct, selection of a suitable production cell line as a potential producer of the recombinant vaccine antigen, and adaptation of established transfection protocols to meet specific requirements of this bioprocess.

The objective of this study was to optimize transient transfection conditions in mammalian production cell lines for CSFV E2 antigen expression. The following tasks were completed to achieve this objective:

a) assess suitability of CHO-K1, PK-15, and BHK-21/13 cell lines for transient expression of a gene encoding a fragment of glycoprotein E2;

b) compare efficiency of widely available non-viral DNA delivery methods (calcium-phosphate and cationic transfection);

c) validate developed methods for monitoring expression efficiency: immunofluorescence assay (IFA) on cell culture, quantitative assessment of messenger RNA (mRNA), and antigen-capture enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Generation of recombinant DNA. A nucleotide sequence encoding a truncated E2 protein Shimen strain of CSFV (GenBank ID AF092448.2) was cloned into pVAX1 vector (Evrogen, Russia) using *BamHI* and *EcoRI* restriction sites. Sanger sequencing confirmed the identity of the generated construct. The recombinant plasmid was transformed into *E. coli* strain DH5α (Novagen, Germany), amplified, and isolated using a Midiprep system (Evrogen, Russia). Plasmid concentration was measured using a Nano-500 spectrophotometer (Allsheng, China). Sample purity was assessed by the absorbance ratio at 260 and 280 nm; a ratio between 1.8 and 2.0 indicates an absence of polypeptide or free-nucleotide contamination.

Cell culture. Monolayers of Chinese hamster ovary (CHO-K1, ATCC® CCL-61), Syrian hamster kidney (BHK-21/13, ATCC® CCL-10), and porcine kidney (PK-15, ATCC® CCL-33) cells were cultivated in 75 mL flasks and 100 mm diameter plates (Biologix Group Ltd., China). The growth medium consisted of DMEM-F12 (PanEco company, Russia) supplemented with 10% fetal bovine serum (HyClone, Australia), 100 IU/mL penicillin and streptomycin (PanEco company, Russia), and 20 mM L-glutamine (Sigma-Aldrich, USA). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2, starting at an initial concentration of 10×10^6 cells/mL. Routine contamination screening was performed. No contamination by bacteria, mycoplasma, or bovine viral diarrhea virus was detected.

Transfection process. The conditions for calcium-phosphate (CaP) and cationic (PEI) transfection of the production cell lines are detailed in the Table.

Cell viability was assessed 24 hours post-transfection in 96-well culture plates (Corning Incorporated, USA) using a colorimetric methyl thiazole tetrazolium (MTT) assay. To each well containing 100 µL of fresh culture medium, 10 µL of MTT reagent (Wuhan Servicebio Technology Co., Ltd., China) were added. The plates were then incubated for 3.5 hours under standard conditions. The formazan crystals that formed were dissolved by adding an equal volume of dimethyl sulfoxide. The absorbance in each well was then measured at a wavelength of 570 nm. The percentage of viable cells was calculated from the ratio of wells containing transfected cells to the wells with the intact cells.

Assessment of messenger RNA expression levels. The transcriptional activity of the E2 gene fragment was assessed by quantitatively determining its product – specific mRNAs. Nucleic acids were extracted from transfected cells using the RIBO-sorb reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). The isolated samples were treated with DNase (Syntol, Russia) at a ratio of 1 unit per 10 ng of DNA and incubated for 1 hour at 37 °C, followed by enzyme inactivation at 80 °C for 5 minutes. For amplification, the following reaction mixture was prepared (per one 20 µL sample): 4 µL of prepared PCR 5× qPCRmix-HS SYBR premix, 5 pM each of forward (5′-CGTCAACCAAT GAGATAGGGCTGT-3′) and reverse (5′-GCACAGCCCGAATC CGAAGT-3′) primers, 100 units of MMLV reverse transcriptase (Evrogen, Russia), 15 ng of RNA template, and ddH_2O to a final volume of 20 µL. A ten-fold serial dilution of the recombinant plasmid pVAX1-trE2 was used to generate a calibration curve. Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) was performed using a C1000 thermocycler with a CFX96 optical module (Bio-Rad Laboratories, Inc., USA) according to the following program: 1 – reverse transcription at 37 °C for 5 minutes; 2 – DNA denaturation at 95 °C for 5 minutes; 3 – 35 cycles of: 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 25 seconds; 4 – final elongation at 72 °C for 10 minutes. Melting curve analysis of the amplicons was performed using the CFX Manager software (Bio-Rad Laboratories, Inc.,

Table
Transfection conditions on culture plates (∅ 100 mm)

Indicator	Calcium phosphate transfection (CaP method)	Cationic transfection (PEI method)
Seeding density	$(6 – 10) \times 10^5$ cells/mL	
Cell confluency at the time of transfection (%)	70	70–85
Mixture composition:		
Solution A	2.5 M $CaCl_2$ – 30 µL Plasmid DNA – 10–20 µg ddH_2O – up to 300 µL	Serum-free medium – 100 µL Plasmid DNA – 2–4 µg
Solution B	HBS buffer, pH 7.05 – 300 µL Full medium – up to 5–6 mL	Serum-free medium – 100 µL PEI – 6–16 µL
Complex formation	Solution A was added drop by drop to solution B (3–5 min at room temperature)	Solution B was added drop by drop to solution A (15 min at room temperature)
Duration of incubation	6–12 hours	4–12 hours
Posttransfection cultivation	48–72 hours	48–72 hours

USA). The number of mRNA copies in each sample was normalized and is presented per 1 µg of the total RNA.

Immunofluorescence assay on cell culture. Transfected cells were stained with an FITC-conjugated polyclonal swine antiserum to CSFV (Federal Center for Toxicological, Radiation and Biological Safety) at a working dilution of 1:500, as previously described by the authors [9].

Isolation of the target protein. 48–72 hours after transfection, cells were mechanically removed from the surface of the culture vials using scrapers. Cells from the suspension were pelleted by centrifugation at 3,000 g for 15 minutes. The pellet was resuspended in 1 mL of chilled RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM ethylenediaminetetraacetic acid; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 0.1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 minutes. Subsequently, the cells were pelleted again by centrifugation. Protein presence in the supernatant was confirmed by analytical electrophoresis on a 15% polyacrylamide gel under denaturing conditions. Reactivity with specific antisera was assessed in ELISA using a previously developed test system designed to monitor the specificity of the recombinant CSFV antigen [10].

Statistical analysis was performed using GraphPad Prism 10.4 software (GraphPad Software, USA).

RESULTS AND DISCUSSION

Bioinformatic analysis identified a 188-amino acid fragment (residues 690–878) characterized by the highest density of CSFV-specific B-cell epitopes. According to pBLAST analysis results, the sequence identity of this fragment among representatives of CSFV genotypes 1 and 2 ranges from 91.19 to 98.74%. Furthermore, it exhibits no significant homology with the corresponding region of the bovine viral diarrhea virus proteome, which is a critical factor in selecting a diagnostic antigen sequence. The final amino acid sequence, flanked by a hexahistidine tag, was reverse-translated. The synthesized nucleotide sequence, containing a Kozak consensus sequence upstream of the start codon, was then cloned into the target pVAX1 vector. The construct scheme is given in Figure 1.

Cell viability was assessed 24 hours after transfection in the CHO-K1, PK-15, and BHK-21/13 cell lines (Fig. 2).

The chart shows that the percentage of viable cells following both transfection methods, under the specified incubation periods with the transfection agents, was at least 87%. Furthermore, cell viability exceeded 93.6% when using cationic transfection with incubation period of 6–12 hours, as well as with CaP transfection when the primary incubation period was limited to no more than 6 hours. Lower viability rates (87.2–92.8%) were recorded for CaP transfection when the primary incubation was extended up to 12 hours. This is likely attributable to the cytotoxic effect exerted by calcium precipitates during extended incubation periods.

The next phase of the study involved a comparative assessment of various methods for monitoring transfection efficiency, specifically direct IFA on transfected cells, quantitative mRNA analysis in real-time RT-PCR, and antigen-capture ELISA.

According to IFA performed on cell cultures, transfection efficiency at 48 hours post-transfection varied from 60 to 90%. The highest efficiency was recorded in monolayers of the CHO-K1 line: 87% for CaP transfection and 90% for PEI transfection. A slightly lower proportion of fluorescent

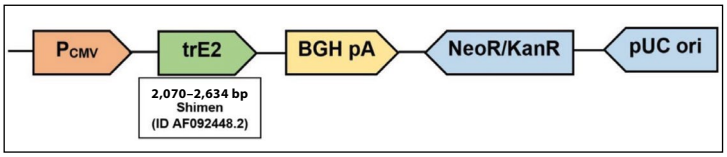

Fig. 1. Structure of the genetic construct based on the pVAX1 vector: P_{CMV} – cytomegalovirus promoter; trE2 – sequence encoding the truncated E2 protein of CSFV (nucleotides 2,070–2,634 of the Shimen strain); BGH pA – bovine growth hormone polyadenylation signal; NeoR/KanR – genes conferring resistance to selective antibiotics (neomycin and kanamycin); pUC ori – origin of replication

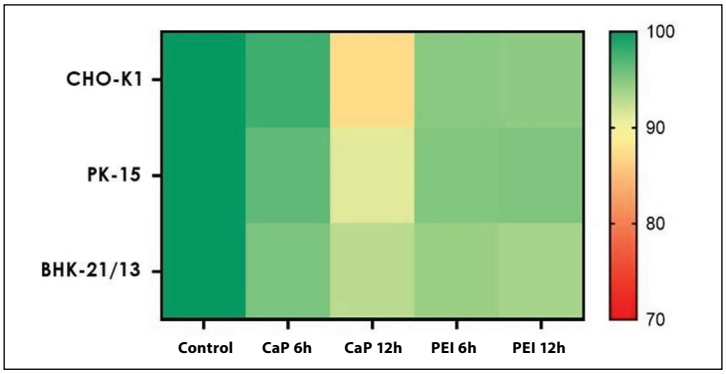

Fig. 2. Cell viability results for production cell lines 24 hours after calcium phosphate transfection and PEI using MTT assay

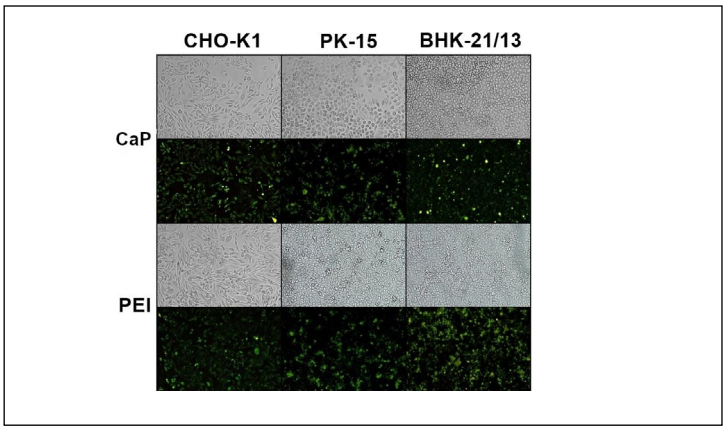

Fig. 3. Fluorescence-based monitoring of transfection (48 hours) in production cell lines

cells was observed for the PK-15 line: 82% for CaP and 85% for PEI transfection. The lowest efficiency was observed for the BHK-21/13 line: 80 and 65% for CaP and PEI transfection, respectively (Fig. 3).

In addition to IFA, which serves as a fundamental method for assessing transfection efficiency, an analysis of mRNA expression was conducted. It is established that measurements based on protein and mRNA levels are complementary and essential for a comprehensive assessment of gene expression. The real-time RT-PCR protocol was initially validated using a ten-fold serial dilution of the control plasmid pVAX1-trE2 (starting concentration 50 ng/µL). It was determined that the linearity of the reaction (R^2) was

0.994, the slope (Ct threshold between two DNA dilutions) was –3.748, the theoretical limit of detection (y-intercept) was 29.288, and the reaction efficiency (E) was 94.8%. The results of specific mRNA level quantification, determined by real-time RT-PCR, are given in Figure 4.

It is known that within a cell, the ratio of protein to mRNA is governed by translation and protein degradation processes, which are regulated at the gene-specific level [11]. The overall correlation between mRNA and protein concentrations in multicellular eukaryotes is considered significant but moderate compared to that in bacteria and yeasts: the number of protein molecules per transcript can vary substantially [12, 13]. Therefore, a more objective assessment of the expression system's functionality, particularly in a production setting, is the quantitative protein yield coupled with the determination of its specificity using an accessible serological method (Fig. 5).

According to the calculated data, the rE2 protein, with a predicted molecular mass of 17.3 kDa, is stable and soluble. These parameters did not differ among the protein variants expressed by all three producer lines, which may indicate similar patterns of post-translational modifications. This is supported by the comparable activity of all protein variants in ELISA, with a positiveness degree ranging from 5.1 to 6.2. The highest recombinant protein yield was achieved using the CHO-K1 line (35.6–47.4 mg/L), while the lowest yield was observed with BHK-21/13 (19.3–24.1 mg/L). This finding aligns with the established influence of cell origin on transfection efficiency under comparable conditions [14]. Although cationic transfection demonstrated greater efficiency in terms of transcript levels, the CaP transfection method yielded an average of (8.7 ± 1.4)% more functional product.

Literature describes production of the recombinant glycoprotein E2 of CSFV in mammalian cells. For instance, in the work of R.-H. Hua et al. [15], the generation of a stable BHK-21 cell line using the pCAG vector is reported, achieving a productivity of up to 45 mg/L. The recombinant protein provided 100% protection to pigs upon lethal challenge. Tian H. et al. [16] successfully created a truncated E2 protein containing CSFV-specific domains, which induced seroconversion in rabbits, i.e. in PK-15 cells transfected with

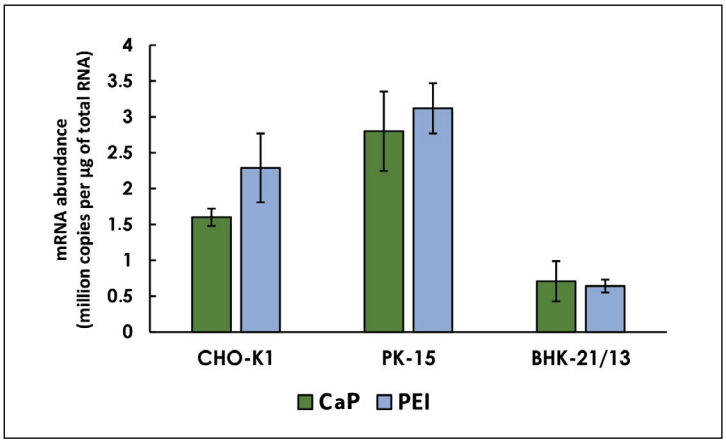

Fig. 4. Quantitative assessment of specific mRNA expression

the retroviral vector pBABE puro. The use of the CHO cell line also enabled significant yields of recombinant E2: L. Feng et al. [17] generated stable rCHO transgenic cells with a dynamic Txnip promoter, forming the basis for an inducible expression strategy. Thus, mammalian cell expression is an effective tool for the large-scale biosynthesis of complex glycoproteins and is applicable in the development of candidate subunit vaccines [18, 19, 20].

CONCLUSION

This study investigated the application of standard transfection methods on several mammalian production cell lines for the generation of the recombinant CSFV E2 antigen, along with methods for monitoring expression efficiency. It was established that the CHO-K1, PK-15, and BHK-21/13 cell lines are successfully transfected under standard conditions with an efficiency of up to 90%. Among these, the CHO-K1 line demonstrated the highest transfection efficiency as well as the highest overall yield of the target protein. Given that all cell lines considered are characterized by high proliferation rates, ease of cultivation (including in suspension), and adaptation to serum-free

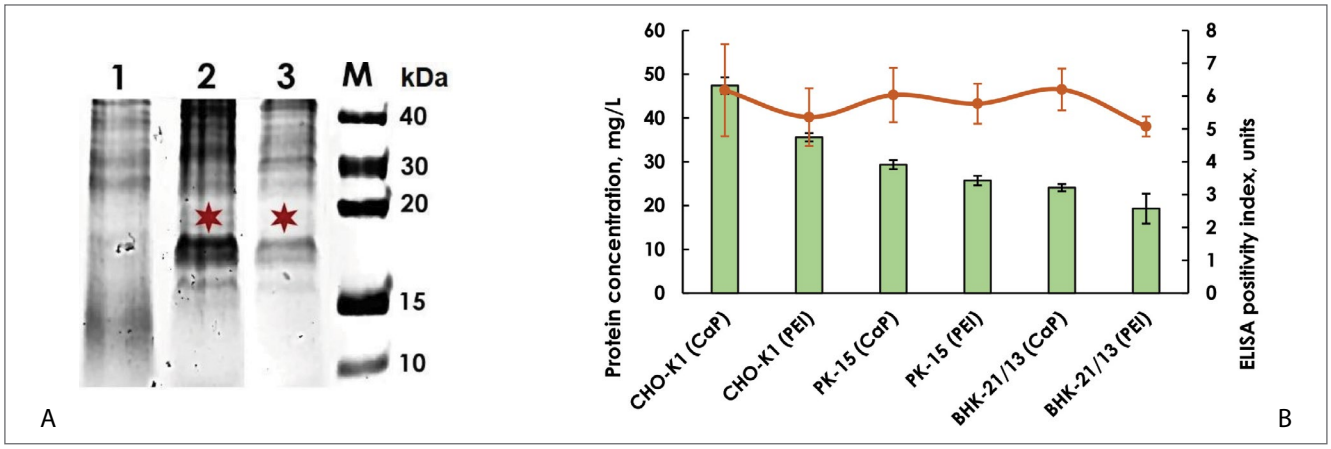

Fig. 5. Functional analysis of the expression systems based on the pVAX1 vector and cell lines CHO-K1, PK-15, and BHK-21/13.
A – electrophoretogram of cell lysates from the primary producer (CHO-K1): lane 1 – negative control (lysate of cells transfected with the original pVAX1 plasmid without insert); lanes 2 and 3 – lysates of cells transfected with the pVAX1-E2 plasmid (target polypeptide fraction marked with ✶);
M – molecular weight marker Prestained Protein Marker IV (Wuhan Servicebio Technology Co., Ltd., China);
B – assessment of the quantitative yield and specificity of the recombinant truncated E2 protein (ELISA data presented for antigen solutions at 1 µg/mL concentration)

media, they possess significant potential for generating clonal producer lines tailored to specific bioprocess requirements. It was also established that both transfection methods examined – CaP and PEI – provided sufficiently high transfection efficiency and comparable yields of the target protein. Their advantages include cost-effectiveness and reproducibility.

A comparative assessment of transfection efficiency monitoring methods – IFA, quantitative PCR, and indirect ELISA – demonstrated that their combined application is advisable during the expression system design phase. While conducting IFA with FITC-labeled polyclonal antibodies provides valuable information during the initial stages of protein synthesis, it necessitates continuous screening of cell lines and fetal bovine sera for contamination with bovine viral diarrhea virus, which can lead to false-positive results. Quantitative mRNA detection does not always correlate with target product yield; therefore, the use of this method is justified primarily for an initial assessment of gene transcriptional activity. In a production setting, the most reliable indicator is the total yield of the target protein. This is conveniently monitored using quantitative or semi-quantitative ELISA, particularly when working with stable expression cell lines.

Although this study utilized a nucleotide sequence encoding a truncated antigen intended for diagnostic purposes – specifically, a fragment of the E2 glycoprotein with the highest density of CSFV-specific domains – the described approaches are applicable to the development of a recombinant subunit vaccine against CSFV. This application is viable provided there is a well-designed construct encoding the full-length glycoprotein.

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Strengthening classical swine fever surveillance and control measures in the Russian Federation

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ABSTRACT

Introduction. Classical swine fever (CSF) remains a critical challenge in global pig production. In the Russian Federation the last reported outbreak occurred in 2020 among wild boar populations, but the risk of re-emergence is sustained. To reduce the existing threats the targeted disease surveillance and control measures are needed to be improved.

Objective. To analyze the current classical swine fever situation and the outcomes of epizootic monitoring in the Russian Federation, and to develop evidence-based proposals for its improvement.

Materials and methods. This analysis draws upon laboratory test results from 2020 to 2024, as recorded in the "Vesta" electronic state information system (part of the FGIS "VetIS"); epidemiological data from the World Organization for Animal Health (WOAH); and the official "Guidelines for planning laboratory testing and sampling to improve classical swine fever surveillance in the Russian Federation", developed and approved by the Federal Centre for Animal Health. Geospatial data were visualized using the MapChart platform, while statistical analyses were performed with Microsoft Excel.

Results. Drawing on international experience in disease eradication and control, this study outlines a phased approach for the eradication of classical swine fever in the Russian Federation, with a view toward achieving official recognition of disease-free status from the World Organization for Animal Health. The proposed disease surveillance strategy is comprehensive and multifaceted, comprising: early detection measures, including immediate notification of suspected cases, syndromic analysis, and clinical examinations with necropsies; routine monitoring at key control points, such as ante-mortem and post-mortem inspections; and confirmatory procedures, consisting of strategic sampling, laboratory diagnostics, and surveillance in sentinel units. The study further explores the prospects for a strategic transition, including the zoning of Russian territory, the phased discontinuation of immunization with live (attenuated) vaccines, and the potential introduction of marker vaccines.

Conclusion. The proposed approaches are fully aligned with international standards and are specifically designed to achieve classical swine fever freedom in the Russian Federation. The full implementation of the proposed measures will significantly strengthen classical swine fever control in Russia and, consequently, enhance the export potential of the domestic pork industry.

Keywords: classical swine fever, epizootological surveillance, monitoring, biosecurity, WOAH, freedom status, laboratory diagnostics, marker vaccines

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Совершенствование эпизоотологического наблюдения и контроля за классической чумой свиней в Российской Федерации

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РЕЗЮМЕ

Введение. Классическая чума свиней продолжает оставаться важной проблемой свиноводства. В Российской Федерации последняя вспышка была зарегистрирована в 2020 г. в популяции диких кабанов, однако риск возникновения новых случаев болезни сохраняется. Для снижения имеющейся угрозы необходимо совершенствование мер эпизоотологического наблюдения и контроля.

Цель исследования. Анализ текущей эпизоотической ситуации и результатов реализуемого на территории Российской Федерации эпизоотологического наблюдения за классической чумой свиней с разработкой предложений по его совершенствованию.

Материалы и методы. Для анализа использовались данные проведенных в 2020–2024 гг. лабораторных исследований, представленные в электронную государственную информационную систему «Веста» ФГИС «ВетИС», а также материалы Всемирной организации здравоохранения животных, «Методические

рекомендации по планированию лабораторных исследований и отбору проб для совершенствования эпизоотологического надзора за классической чумой свиней на территории Российской Федерации», разработанные и утвержденные в ФГБУ «ВНИИЗЖ». Картографирование осуществлялось с помощью онлайн-платформы MapChart, статистическая обработка проводилась в программе Microsoft Excel.

Результаты. На основании анализа международного опыта по ликвидации и контролю болезни описаны аспекты поэтапного оздоровления территории Российской Федерации от классической чумы свиней с перспективами получения соответствующего статуса Всемирной организации здравоохранения животных. Предлагаемая в исследовании стратегия эпизоотологического наблюдения включает комплексный подход с немедленными уведомлениями о подозрении на классическую чуму свиней, проведением синдромного анализа, клиническими исследованиями животных и патолого-анатомическим вскрытием трупов, ветеринарным предубойным осмотром животных и экспертизой продуктов убоя, наблюдением в дозорных единицах, проведением эффективного пробоотбора и лабораторной диагностики. Обсуждены перспективы зонирования территории России, поэтапного отказа от иммунизации живыми (аттенуированными) вакцинами, применения маркированных вакцин.

Заключение. Изложенные подходы гармонизированы с международными рекомендациями и соответствуют цели оздоровления страны от классической чумы свиней. Реализация всех предложенных этапов будет способствовать усилению контроля за болезнью в России и увеличению экспортного потенциала отечественной свиноводческой отрасли.

Ключевые слова: классическая чума свиней, эпизоотологическое наблюдение, мониторинг, биозащита, ВОЗЖ, статус благополучия, лабораторная диагностика, маркированные вакцины

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Конфликт интересов: Иголкин А. С. является членом редколлегии журнала «Ветеринария сегодня», но никакого отношения к решению опубликовать эту статью не имеет. Рукопись прошла принятую в журнале процедуру рецензирования. Об иных конфликтах интересов авторы не заявляли.

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INTRODUCTION

Classical swine fever (CSF) is one of the six priority diseases under the WOAH Terrestrial Animal Health Code (hereinafter referred to as the WOAH Terrestrial Code) for which countries or zones can apply for official recognition of disease-free status, along with foot-and-mouth disease, peste des petits ruminants, bovine spongiform encephalopathy, African horse sickness, and bovine contagious pleuropneumonia [1, 2].

According to Article 15.2.3 of the WOAH Terrestrial Code, a country or zone can achieve official recognition as free from CSF through complete cessation of vaccination against CSF in pigs, or use of validated DIVA (differentiating infected from vaccinated animals) vaccines as required by Chapter 3.9.2 of the WOAH Manual for Diagnostic Tests and Vaccines for Terrestrial Animals (hereinafter referred to as the WOAH Terrestrial Manual), followed by at least 12 months of continuous surveillance confirming no CSF cases in domestic pigs or wild boars during this period [2, 3].

In Russia's Primorsky Krai, the last CSF cases in domestic pigs were recorded in 2019, while wild boar infections extended into 2020. However, it is currently challenging to provide an objective assessment of the CSF situation in the country due to the absence of both an officially approved monitoring program and a state-led eradication strategy.

The persistent circulation of the virus in wild boar and domestic pig populations poses a significant threat for the re-emergence of CSF outbreaks. Individual CSF virus (CSFV) variants can persist in pigs, leading to asymptomatic carrier states that complicate disease detection, particularly in vaccinated animals [4].

Another threat is the contamination of pig products. The CSFV remains infectious for years in frozen or canned pork products under suitable conditions [5].

In addition to virus survival in abovementioned products, transboundary spread from infected neighboring countries poses a significant threat for CSF introduction, particularly in border regions [6].

Russia currently employs mass immunization of domestic pigs with live attenuated (non-marker) vaccines against CSF, which precludes DIVA strategy implementation and WOAH recognition of free status [7].

This work aims to analyze the CSF situation in the Russian Federation and propose improvements to surveillance, with the goal of strengthening the export potential of the pig industry, particularly following the official recognition of CSF-free status.

MATERIALS AND METHODS

This analysis utilized laboratory test results retrieved from the "Vesta" electronic information system (part of the

FGIS "VetIS") for the period 2020–2024, as well as data obtained from WOAH. The data were assessed and analyzed in accordance with "The recommendations for planning laboratory studies and sampling to improve epizootological surveillance over CSF in the Russian Federation", developed and approved by the Federal Center for Animal Health [8].

Geospatial analysis was conducted, with mapping performed using the online MapChart platform. Statistical analysis was performed using Microsoft Excel (Microsoft Office Professional Edition, 2003). This included correlation analysis with calculation of the Pearson correlation coefficient (R), the coefficient of determination (R^2), and the level of statistical significance (p); $R \geq 0.5$ was considered to indicate a strong positive correlation between the two variables; $R^2 \geq 0.5$ indicated a satisfactory level of explained variance.

RESULTS AND DISCUSSION

Analysis of CSF eradication strategies implemented in different countries. During the 91st WOAH General Session in May 2024, 38 countries were officially recognized as free from CSF, among them North American countries, Oceania, most European nations and specific zones in Brazil, Colombia and Ecuador. From 2020 to 2024, 17 countries reported CSF outbreaks worldwide, including Russia. In 2024, CSF persisted in parts of Asia, South America, Oceania and Madagascar (Fig. 1) [2].

There are several different approaches to CSF eradication.

Countries like Australia, Canada, the United States, and the European Union achieved and maintain WOAH-recognized CSF-free status through nationwide radical measures, throughout the country, including a strict no-vaccination policy, extensive serological and virological surveillance to detect carriers, and stamping-out (total culling of affected herds plus contacts), alongside enhanced farm biosecurity. All this imposed substantial economic costs [9].

To reduce costs, a less stringent approach is used – to adopt zoning and enhanced disease surveillance. The vaccination is maintained in high-risk zones and phased out in low-risk compartments, gradually expanding free zones. This approach is typical for Latin American countries (Brazil, Colombia and Ecuador).

For example, Brazil has reported historical CSF outbreaks since 1888 [10]. The country's efforts in the disease surveillance and control [11] achieved WOAH recognition for specific zones as free from CSF since 2001. The proportion of pigs raised in recognized areas was 95% by 2015 [10]. However, in 2018, the disease resurfaced in Brazil: 34 outbreaks were reported, followed by 15 outbreaks in 2023 and 2024 in backyards [2]. In response, the country developed and adopted a new Brazil CSF-Free Strategic Plan based on enhanced clinical observations, laboratory tests (using polymerase chain reaction, PCR), elimination of the infected animals and prophylactic vaccination (based on lapinized C-strain) in the zones at the highest risk. In Brazilian regions with the highest pig population density, CSF control measures included the cessation of vaccination, which enabled the use of serological diagnosis (i.e., the detection of specific

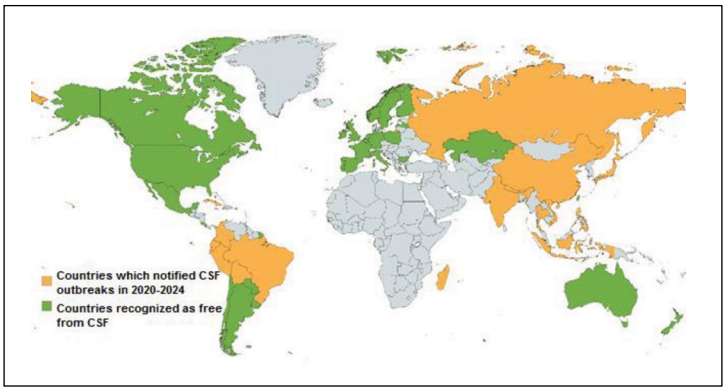

Fig. 1. CSF infected countries in 2020–2024

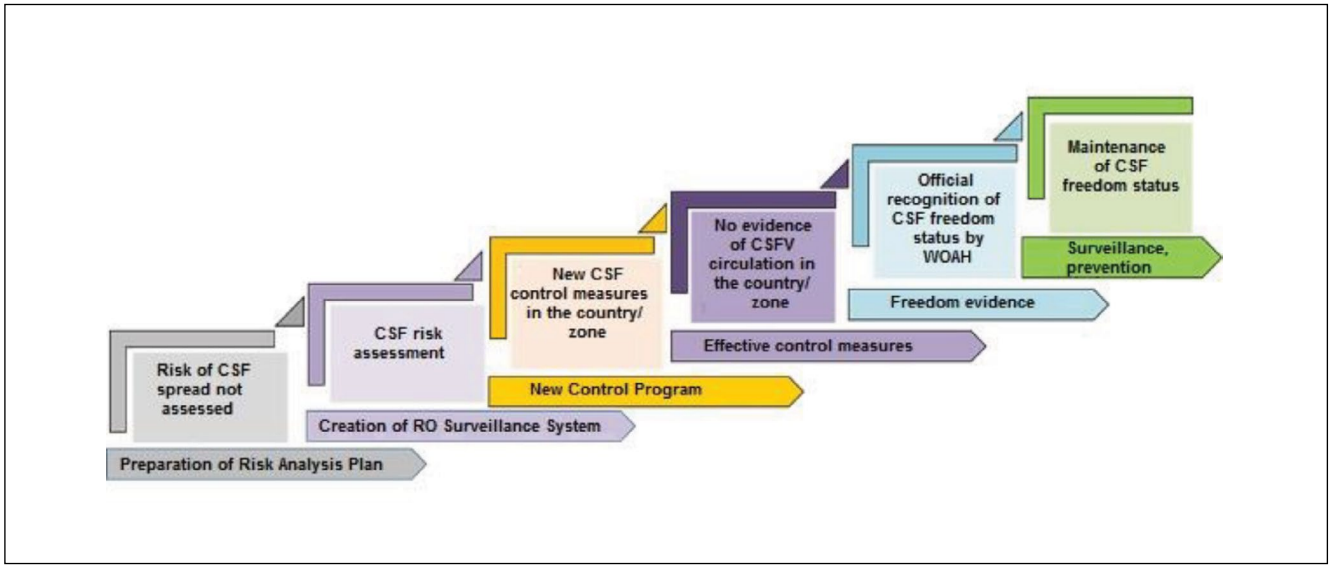

Fig. 2. Phased eradication plan for CSF in Russia [18]

antibodies). Serological surveillance was carried out by fauna managers (hunters) who collected blood samples [10]. Moreover, strict biosafety measures are applied to prevent the disease's spread [12].

It should be noted, that CSF remains endemic in several Asian countries including Japan, China, Indonesia, Thailand, and Nepal. A dramatic CSF situation has persisted in Japan in recent years. Outbreaks of CSF have been reported in the country since 1992 [13]. Vaccination with live GPE- strain was used historically for domestic pigs and wild boar but was banned for routine domestic use in 2006 [14]. Japan was officially recognized as a CSF-free country by the WOAH in 2015 [13]. However, in 2018, the country again notified CSF outbreaks: the virus genome was detected in blood samples from asymptomatic wild boars [13, 14]. In the period from 2018 to 2024, 4,118 cases of CSF were reported in Japan among wild boars and 486 among domestic pigs. The country intensified surveillance in wild fauna, conducted depopulation of wild boar and environmental disinfection at carcass sites to eliminate fomites [15]. Oral vaccination of wild boars with Pestiporc Oral vaccine bait produced by Ceva Tiergesundheit (Reims, GmbH, Germany) was conducted followed by seroconversion monitoring [16]. Domestic pigs were immunized with live vaccines in high-risk areas. Based on 2025 data sporadic CSF cases persist in domestic pigs in Japan, but overall epizootic activity has significantly faded [6, 17].

Thus, cases of CSF continue to be registered in numerous territories worldwide (Japan, China, Southeast Asia and Latin America countries). To contain CSF spread and minimize economic losses, live attenuated vaccines are widely used in many affected countries. Final eradication of CSF requires implementing robust epizootological monitoring and control measures alongside complete cessation of vaccination. Experience from multiple countries demonstrates that drastic CSF eradication measures need not be applied nationwide immediately; gradual implementation by risk zones or compartments is both feasible and effective.

Development of CSF eradication approaches in the Russian Federation. In the 20th century, CSF outbreaks were reported in many Russian regions. However, a decrease in the number of reported outbreaks was already noted at the beginning of the 21st century. Between 2010 and 2020, the dynamics of the epizootic followed a downward trend. The most recent cases of CSF in Russia were recorded in the subjects bordering the People's Republic of China: Amur Oblast and Primorsky Krai [6, 18, 19, 20].

No CSF outbreaks have been reported in Russia since March 2020. The improvement of the situation in the country is attributed to the mass vaccination of pigs, along with the strengthening of biosecurity measures on pig farms and holdings [6, 18]. However, immunization against CSF has notable disadvantages, including failure to fully block virulent virus transmission and severe hindrance to detecting persistent infections in pigs [21]. Other disadvantages of CSF vaccination include post-vaccination reactions in some pigs, substantial financial and labor costs, export restrictions to free-status markets, and the inability to differentiate vaccinated from infected animals using conventional live vaccines [6, 7, 21].

The mentioned shortcomings fully justify the strict WOAH Terrestrial Code recommendations to abandon vaccination for obtaining official CSF-free country/zone status. Given Russia's widespread use of vaccines against CSF, the Russian Federation cannot currently apply for official CSF-free status. To eradicate CSF in Russia, developing and implementing a comprehensive federal program (roadmap) for epizootological monitoring and control would be preferable. Any regulatory document formalizing CSF control measures in Russia should account for diverse epizootic scenarios, tailored to disease manifestations across farm types: intensive industrial enterprises versus extensive backyard/collective systems [8].

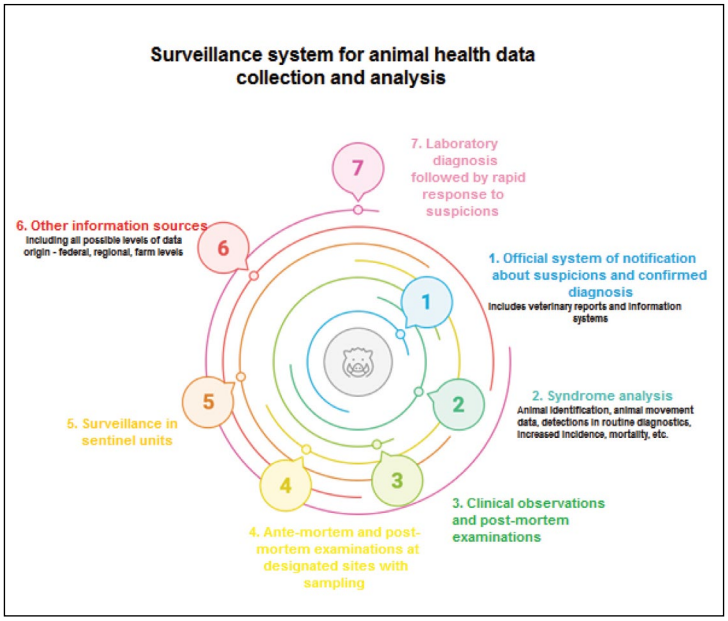

Fig. 3. Epizootological surveillance components

If Russia's CSF eradication strategy includes abandoning conventional vaccines, phased implementation – coupled with rigorous biosecurity enforcement across all farm types – is essential to avoid epizootic deterioration and new outbreaks [18].

Previously proposed phased eradication plan for CSF in Russia [18] is shown in Figure 2.

This plan includes conducting a risk analysis, establishing a risk-oriented (RO) epizootological surveillance system, and developing a new CSF control program with enhanced measures designed for a potential vaccination withdrawal. The final stages involve collecting and analyzing information to confirm the territory (or country) is free from the disease. This is followed by submitting an application to the WOAH to obtain official CSF-free status for the country or zone, and subsequently maintaining that status.

The implementation of the plan is feasible using "project management" approaches as a comprehensive process formalized by GOST R ISO 21500-2023, including risk analysis (regulated by the international standard ISO/IEC 31010:2009; GOST R ISO 58771-2019; GOST R ISO 31000-2019). In practice, simplified risk analysis approaches, including the use of only certain components, are often employed for applied purposes. For example, CSF eradication strategies can incorporate hazard identification and qualitative risk assessment to evaluate factors influencing outbreak likelihood or prevention, guiding targeted risk reduction measures.

Table 1
CSF diagnostic procedures recommended by the WOAH Manual [24]

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Post-vaccination control
Detection of the agent						
Virus isolation	–	+	–	+++	–	–
RT-PCR	+	+++	++	+++	++	–
ELISA (antigen)	++	+	+	+	–	–
FAT	–	–	+	+	–	–
Detection of virus-specific antibodies*						
ELISA (antibody)	+++	+++	+++	–	+++	+++
VN (FAVN or NPLA)	+	+++	++	++	+++	+++

* the availability of diagnostic tools that can differentiate between antibodies specific to CSF and those induced by other pestiviruses is essential.

When identifying CSF outbreak scenarios in risk assessments, criteria for delineating high-risk zones and populations must be incorporated to define boundaries effectively (per WOAH Terrestrial Code Articles 15.2.28–15.2.33) [3].

Another step in the eradication strategy is the preparation of the disease monitoring program.

Epizootological surveillance. Article 15.2.28 of the WOAH Terrestrial Code defines epizootological surveillance as a systematic, ongoing process of collecting, analyzing, and promptly reporting animal health data to enable timely intervention measures [3].

In the current context in Russia, the priority goal of epizootological surveillance is rationally considered to be the early detection of infection – including latent virus transmission in convalescent animals. An additional goal may be to demonstrate the absence of virus circulation within a herd, region, district, enterprise, or farm (information that is essential for regionalization purposes) [8].

Chapter 1.4 of the WOAH Terrestrial Code "On animal health surveillance" outlines a framework where the surveillance system comprises complementary components, which are shown in Figure 3 and discussed in detail in this paper below.

The components are the following:

1. The official notification system for suspicion and diagnosis, including the collection of information in accordance with Orders of the Ministry of Agriculture of the Russian Federation: No. 89 "On the Procedure for Information Submission to the State Information Agricultural System" of 21.02.2022[1]; No. 318 "On Approval of the Procedure for Information Submission to the Federal State Veterinary Information System and Information Retrieving From It" of 30.06.2017[2].

2. Syndrome analysis involves systematically collecting and evaluating data on changes in animal incidence, mortality, productivity, sales, and slaughter patterns to enable timely identification of disease causes through

[1] https://www.garant.ru/products/ipo/prime/doc/404424070 (in Russ.)

[2] https://www.garant.ru/products/ipo/prime/doc/71700754 (in Russ.)

complementary methods like clinical exams, necropsies, and laboratory tests [3].

In large pig farms, permanent production losses (mortality) are routine and stem from injuries, poisoning, non-contagious diseases, and contagious pathogens. Syndrome analysis enables timely detection of subpopulations (specific herds or pig groups) showing deteriorated indicators, allowing rapid response measures to investigate incident causes [8].

3. Clinical observation and necropsy. Regular and thorough clinical examination of all susceptible livestock, including necropsy, must be organized.

Paragraph 3 of the "Veterinary Rules for the Implementation of Preventive, Diagnostic, Restrictive and Other Measures, for Imposing and Lifting Quarantine and Other Restrictions to Prevent Spread of Classical Swine Fever and Eradicate Its Outbreaks"[3], approved by Order No. 580 of the Ministry of Agriculture on 29.09.2020 (hereinafter referred to as the Rules), Paragraph 3 provides an extensive list of clinical signs and post-mortem lesions which, as stipulated in Paragraph 9 of the Rules, constitute grounds for suspecting CSF.

However, practicing veterinarians often mistakenly believe that all or most of the symptoms listed in Paragraph 3 of the Rules must be present in order to suspect CSF. However, infection with the CSFV is not always accompanied by a pronounced or typical clinical and pathological picture. For example, at the beginning of an outbreak – when the acute course of the disease prevails – or in vaccinated pigs, where the disease may present atypically, animals may exhibit only isolated clinical signs. The situation may be further complicated by the presence of other diseases on farms that share some clinical signs with CSF.

Collectively, these factors underscore the necessity of laboratory testing with differential diagnosis to distinguish CSF from other infectious diseases, including African swine fever, circovirus infection, pasteurellosis, salmonellosis, Glässer's disease, infection with *Actinobacillus pleuropneumoniae*, and others. It is important to note that CSF can be complicated by concurrent infections with the aforementioned or other pathogens [22]. To avoid errors, such tests should be regular.

4. Veterinary ante-mortem examination of animals and post-mortem inspection of slaughter products must be conducted in accordance with established veterinary and sanitary requirements[4]. Furthermore, in high-risk areas for CSFV introduction where pigs are kept, it is advisable to pay particular attention to the lymph nodes, spleen, and kidneys during inspection. Carcasses, half-carcasses, quarters (including heads), and organs from wild boars must be submitted for mandatory veterinary-sanitary examination, where signs of lymphadenitis and hemorrhagic syndrome should raise immediate suspicion for CSF. If suspicious signs are detected during veterinary-sanitary examination, available samples are sent for differential laboratory testing, followed by decisions on disposal or destruction of slaughter products per veterinary-sanitary requirements[5].

5. Monitoring in sentinel units (for example farms in the settlement under high-risk of introduction or re-emergence; unvaccinated animals serve as most sensitive detectors)

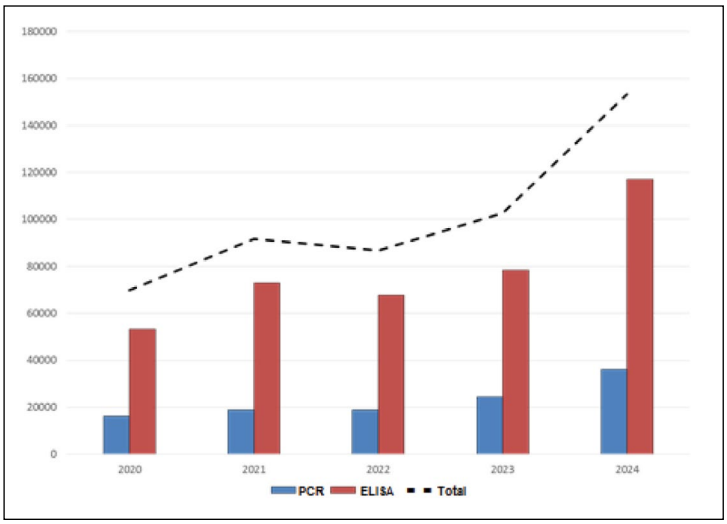

Fig. 4. Laboratory tests for CSF by PCR and ELISA conducted in Russia in 2020–2024 (retrieved from "Vesta" component, FGIS "VetIS")

relies on regular clinical observation of pigs, with immediate sampling and testing if CSF signs appear to detect early cases [8]. Hunting farms within wild boar populations should be designated as sentinel units due to their heightened risk of CSFV introduction from adjacent infected territories.

6. Other sources of information. Data from diverse sources – such as owner/hunter reports of suspicious cases, animal/product exports, pig and pig product sales, commercial mortality, and wild boar carcass discoveries – play a crucial role in comprehensive epizootological surveillance for CSF.

7. Laboratory diagnostics.

7.1. Sampling. Paragraph 17 of the Rules lists the requirements for sampling only in case of CSF suspicion. For such cases, random sampling of the suspected group of susceptible animals is necessary, aiming for a detection level of around 10% prevalence. Routine sampling and testing in other circumstances is not required.

When CSF is introduced into large vaccinated herds, initial infection prevalence can remain very low (around 0.1% or less, based on the observations of Federal Centre for Animal Health specialists during the disease outbreaks), especially in the herd where vaccination is practiced [8, 23]. Therefore, random sampling proves ineffective for early CSF detection in large herds. This is attributable to the fact that the representative sample size required for the aforementioned objective is virtually equivalent to the entire herd, effectively mandating comprehensive testing. Consequently, the financial burden of conducting such monitoring on a regular basis is substantial.

Methodological recommendations for planning laboratory testing and sampling to improve CSF surveillance in the Russian Federation advocate for a RO-approach. This strategy enhances testing effectiveness by combining passive and active surveillance components [8]. Passive surveillance must incorporate sampling and testing of live pigs and cadavers from suspected CSF cases, while active surveillance entails routine testing of samples from at-risk animals – including clinically healthy pigs. Active surveillance sampling should be rationalized by stratifying

[3] https://base.garant.ru/74901254 (in Russ.)

[4] https://legalacts.ru/doc/pravila-veterinarnogo-osmotra-uboinykh-zhivotnykh-i-veterinarno-sanitarnoi/ (in Russ.)

[5] https://docs.cntd.ru/document/350341002 (in Russ.)

Table 2
Number of tested samples from wild boar and domestic pigs

Year	PCR		FAT		ELISA	
	pos / No. of samples from boars	pos / No. of samples from pigs	pos / No. of samples from boars	pos / No. of samples from pigs	pos / No. of samples from boars	pos. in n / v / No. of samples from pigs
2020	7/2,893	13/13,286	0/17	0/279	0/142	0/53,187
2021	0/3,107	2/15,705	0/6	0/24	0/48	0/72,867
2022	0/2,251	3/16,518	0/0	0/0	0/12	0/67,860
2023	0/2,121	3/22,156	0/0	0/0	0/4	0/78,307
2024	0/3,040	3/33,090	0/0	0/160	0/25	0/116,977
Total	13,412	100,755	23	463	231	389,198

pos. – positive; n/v – non-vaccinated domestic pigs.

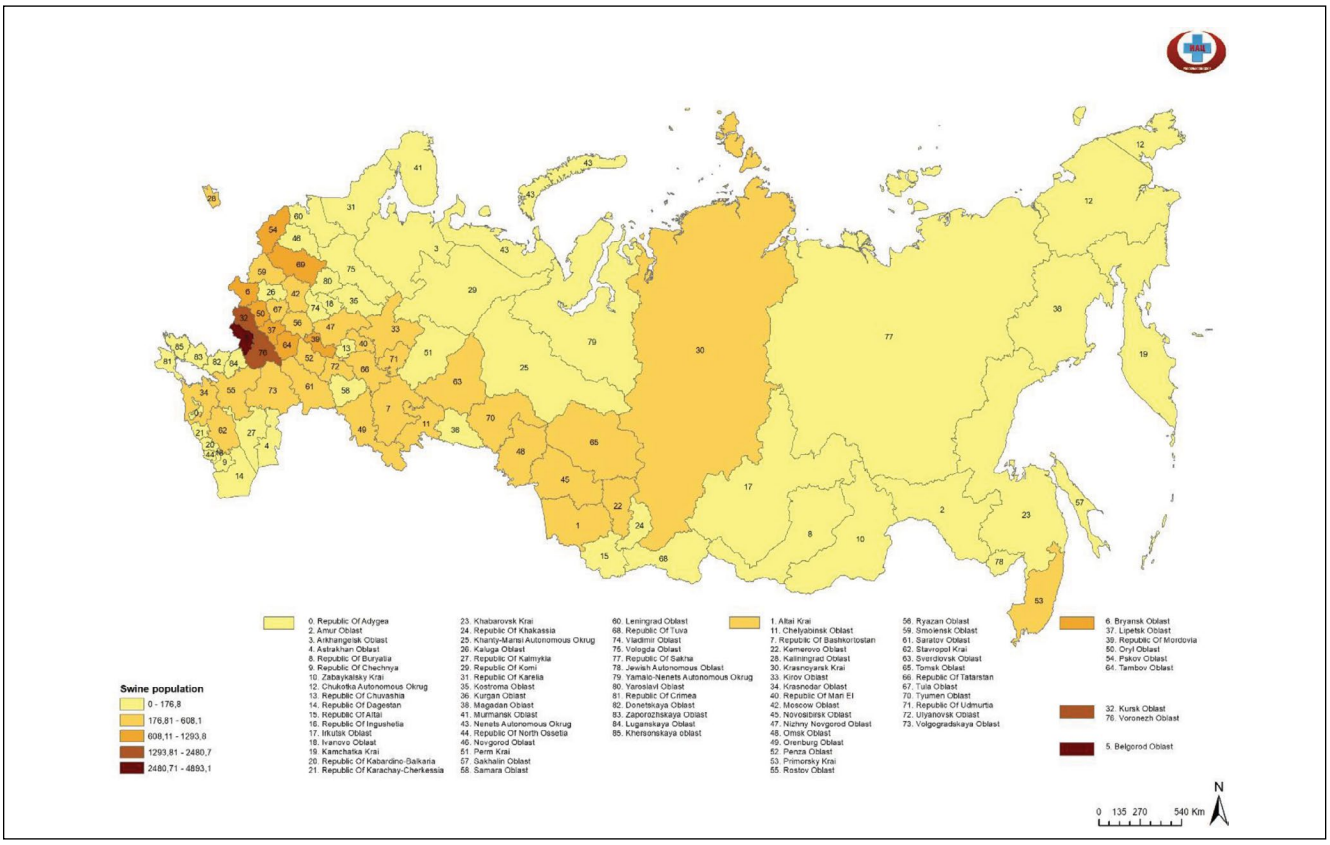

Fig. 5. Distribution of domestic pig population density and diagnostic testing across the Russian Federation in 2020–2024

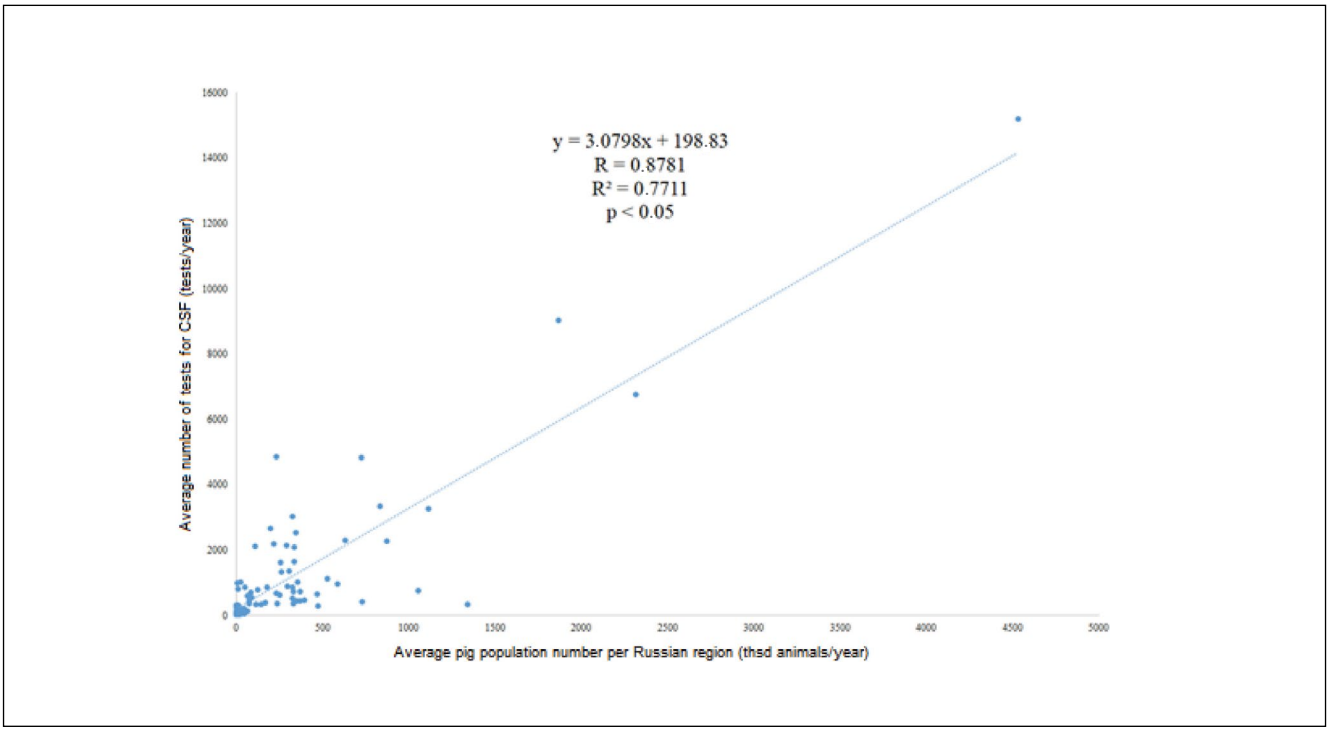

Fig. 6. Linear regression depicting the association between the average domestic pig population per region and the average number of CSF laboratory tests performed in that region (Russia, 2020–2024)

populations via syndrome analysis to target high-risk subgroups with rising morbidity/mortality. Syndrome data pinpoints deteriorating herds/sectors, prioritizing them over random sampling in large vaccinated systems. Within these groups, pigs exhibiting clinical or necropsy signs of infection are selected for testing[6]. In vaccinated pig herds, CSF introduction often leads to virus carriage in 1–3 month-old piglets, coinciding with waning colostral antibodies and immature post-vaccination immunity. It is recommended to consider such piglets as a high-risk group. Moreover, sampling vaccinated pigs should be delayed until 14–21 days post-vaccination to minimize detection of vaccine strain genomes, which could confound field virus identification.

7.2. Methods of laboratory diagnostics. According to Chapter 3.9.2 "On Classical Swine Fever" of the WOAH Terrestrial Manual, the methods of CSF laboratory diagnosis are divided into two groups:

a) direct – used to detect the virus (virus isolation in a sensitive cell culture), its antigen (fluorescence antibody test, FAT; enzyme-linked immunoassay, ELISA) and genome (reverse-transcription PCR, RT-PCR);

b) indirect – used to detect CSFV-specific antibodies: ELISA and virus neutralization (VN) tests (fluorescent antibody virus neutralization, FAVN, and neutralizing peroxidase-linked antibody, NPLA).

Recommendations for the use of direct and indirect techniques for CSFV diagnosis are given in the WOAH Terrestrial Manual (Table 1) [24].

The diagnostic techniques recommended by the WOAH are primarily designed for countries that have ceased using live vaccines. Using non-marker vaccines necessitates adjusted serological strategies, prioritizing laboratory tests, especially serological ones.

7.3. Analysis of laboratory tests for CSF conducted in 2020–2024 in Russia, and improvement of the laboratory diagnostic scheme. According to the data from "Vesta" component of the FGIS "VetIS", in 2020–2024, 504,140 tests for CSF were conducted, most of them tested by ELISA for specific antibodies (389,429 – 77.2%), and RT-PCR (114,167 – 22.6%), by FAT (486 – 0.1%) and virus isolation (58 – 0.01%) in 85 regions of the country (Fig. 4).

It was established that the number of ELISA tests for CSF (detection of CSFV antibodies) is significantly higher than by PCR (detection of the virus itself). At the same time, the feasibility of using indirect test methods for CSF is questionable, since in conditions of mass vaccination of pigs this fails the core goal of early virus detection in epizootological surveillance.

Details on the number of tests conducted on samples from wild and domestic pigs for CSF in 2020–2024 is presented in Table 2.

According to data from the "Vesta" component (FGIS "VetIS"), tests on wild boar samples account for only 2.7% of all tests conducted. In this context, direct testing methods (such as RT-PCR) are preferred, as serum sampling for ELISA is challenging.

The low number of tests conducted on wild boars is due to the absence of strict regulatory requirements. Thus, according to Paragraph 18 of the Rules, sampling from wild boars is regulated only in infected regions (status established in accordance with regionalization)[7]. We believe existing requirements should be adjusted to im-

[6] https://base.garant.ru/74901254 (in Russ.)

[7] https://base.garant.ru/74901254 (in Russ.)

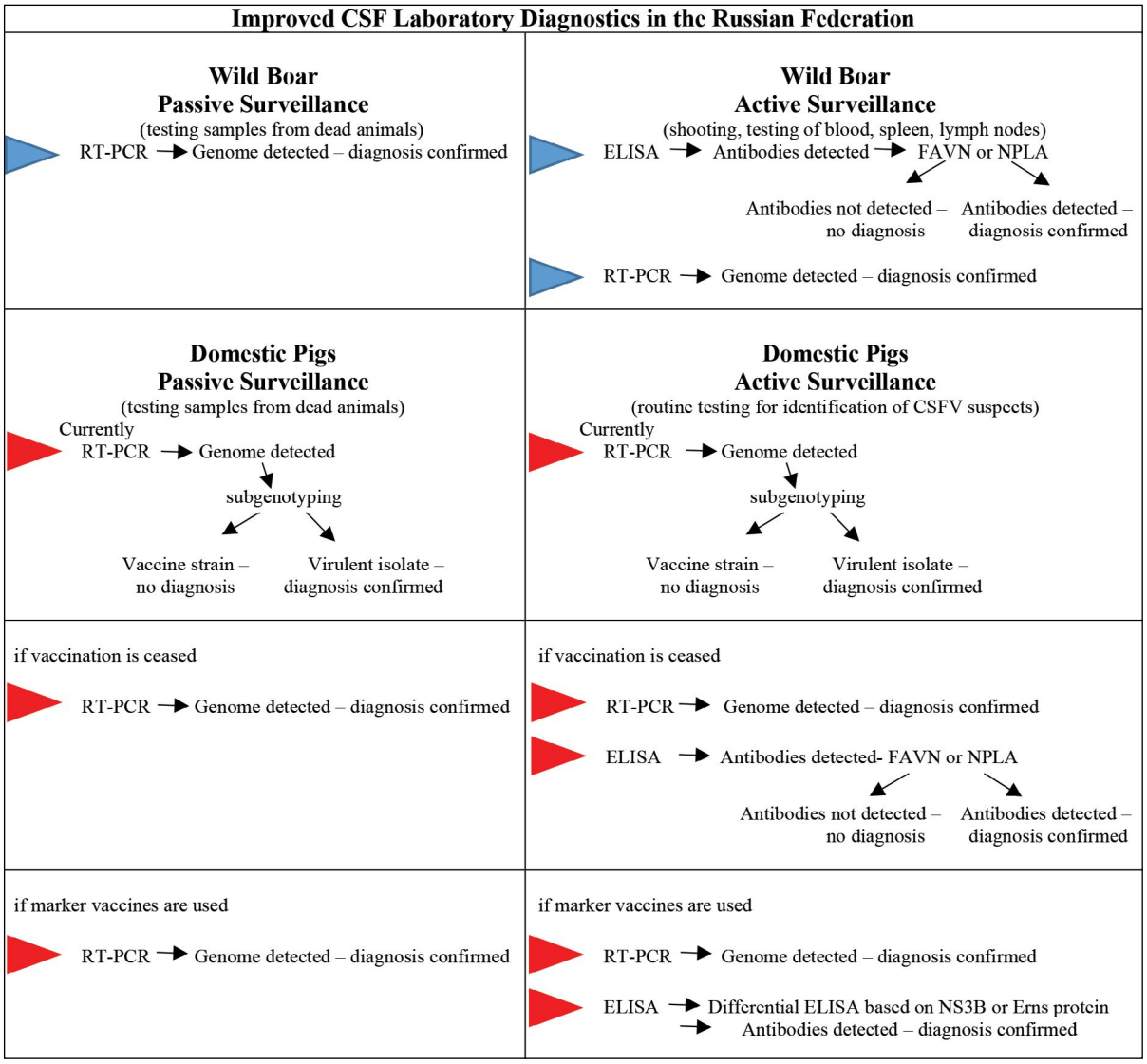

Fig. 7. A proposed strategy for CSF laboratory testing of samples from wild and domestic pigs in the Russian Federation
(If the CSF virus genome is detected and confirmed to be unrelated to vaccine strains, virus isolation is recommended to obtain the strain for further scientific research. In the current context, serological diagnostics in domestic pig populations are considered impractical due to the widespread use of live vaccine immunization.)

prove the reliability of data on CSF in the wild boar population.

The Rules should also be amended regarding the diagnosis of CSF in domestic pigs. There has been a five-year upward trend in the number of laboratory tests for CSF in domestic pigs. Thus, in 2024 compared to 2020, the number of tests using molecular genetic methods increased 2.5-fold, while serological tests increased 2.2-fold. Analysis of the spatial distribution of CSF laboratory tests revealed a direct correlation between the percentage of tests conducted and the density of domestic pigs in specific regions of Russia. For example, most of the tests (31.13%) for CSF were carried out in Belgorod (15.265%), Voronezh (9.079%) and Kursk (6.786%) Oblasts, while in the territories at risk, namely Amur Oblast (1.016%), the Republic of Buryatia (0.334%), Khabarovsk (1.006%) and Primorsky (0.881%) Krais accounted only for 3.237% of the total number of tests conducted (Fig. 5). Correlation analysis of the average pig population per region (thousand animals/year) and the average number of CSF laboratory tests conducted (tests/year) over 2020–2024 revealed a strong positive correlation ($R = 0.88$), with satisfactory variance explained ($R^2 = 0.77$) and statistical significance ($p < 0.05$), Fig. 6. The data indicate that, under current conditions, the primary criterion for sampling is the size of the livestock population in a given region of Russia. However, testing efforts should be redistributed in favor of regions at higher risk of CSFV introduction – particularly those bordering affected countries.

As shown in Figure 4 and Table 2, the majority of tests were performed using ELISA, which detects specific CSFV antibodies. In 2020–2024, no antibodies were detected in non-vaccinated pigs by ELISA. According to "Center of Veterinary Medicine", more than 90 million doses of CSF vaccine are administered to pigs annually in the Russian Federation. The serological tests mentioned in Table 2 (389.2 thousand) were also conducted among immunized pigs, which were seropositive. Serological methods fail for CSF detection in non-marker vaccine contexts, as they

cannot distinguish vaccine-induced from infection-derived antibodies, rendering positives non-specific [14].

At the same time, despite the limitations, the ELISA method is fast and widespread, which can play a significant role in laboratory diagnostics when vaccinations are ceased or marker vaccines are used. However, at present, its application for early infection detection is only justifiable when testing domestic pigs and wild boars that are confirmed to be unvaccinated. In addition, ELISA tests used in veterinary laboratories must incorporate recombinant E2 protein as the antigen and undergo full validation for accuracy, repeatability, reproducibility, sensitivity (Se), and specificity (Sp). Special emphasis is placed on validating the absence of cross-reactions with antibodies against other pestiviruses, such as bovine viral diarrhea virus and border disease virus in swine serum to ensure diagnostic specificity.

The WOAH Manual (Table 1) recommends using FAVN or NPLA to detect clinical cases rather than ELISA [24]. These methods offer the highest sensitivity for detecting CSFV antibodies but incur high costs for cell culture maintenance, virus propagation, specialized anti-specific conjugates, and monoclonal antibodies, rendering them unsuitable for routine diagnostics [25]. Although neutralization tests remain essential in reference laboratories to confirm and differentiate false-positive ELISA results. The integration of FAVN or NPLA into the workflows of accredited veterinary laboratories across Russia is a critical objective.

The next most frequently used method for CSF testing in Russia is RT-PCR, a technique specifically designed to rapidly and accurately detect fragments of the CSFV genome in test samples. Between 2020 and 2024, 31 positive results were obtained: 7 samples collected from wild boars in the Primorsky Krai – the site of Russia's last outbreak in 2020 – and 24 samples identified as vaccine strains.

Herewith, according to Paragraph 1.1.5 ("Molecular epidemiology and genetic typing") of Chapter 3.9.2 in the WOAH Terrestrial Manual, subgenotyping of CSFV upon a positive detection (virus isolation or genome confirmation) requires phylogenetic analysis targeting the 5'-nontranslated region, E2 glycoprotein gene, and NS5B polymerase gene [24, 26, 27]. However, these genotyping methods face limitations including prolonged processing times, requirements for costly equipment and reagents, and the need for highly qualified personnel.

To overcome these limitations, upgraded PCR assays have been developed for rapid and accurate differentiation of vaccine from field CSFV strains [28]. One advanced method is PCR with subsequent DNA melting analysis or PCR (PCR-DMA) high-resolution melt curve analysis (PCR-HRM) [29]. However, during new CSF outbreaks, Sanger sequencing followed by subgenotyping of isolates remains essential to trace virus origin, spread, and localization [2].

Furthermore, Paragraph 20 of the Rules stipulates the use of the FAT for detecting CSFV antigen in biological smears[8]. However, FAT usage for CSF diagnosis confirmation has declined in recent years due to high conjugate costs and domestic market shortages, risks of non-specific reactions, and subjective result interpretation errors.

Virus isolation serves as a direct diagnostic method for CSF confirmation, yet its application remains extremely limited nationally compared to total testing volume. Virus isolation for CSF diagnosis involves detecting viral replication in permissive porcine cell cultures such as the PK-15 kidney cell line or primary trypsinized testicular cells (TC), requiring confirmatory identification due to the absence of cytopathic effects. These are immunoperoxidase testing, FAT and RT-PCR [30].

According to WOAH recommendations, virus replication confirmation in cell cultures can be performed via FAT after 24–72 hours or immunoperoxidase testing 3–4 days post-inoculation. Virus isolation protocols recommend planning for 3–5 serial passages [24]. However, these methods face significant limitations, including costly cell culture bank maintenance, high prices and domestic shortages of specific conjugates, risks of non-specific staining, and subjective visualization errors.

RT-PCR simplifies and lowers the cost of confirming virus isolation results for CSF while minimizing false results compared to immunofluorescence or immunoperoxidase methods [31]. Virus isolation remains valuable for obtaining current CSFV isolates and strains to evaluate their biological properties – such as contagiousness, virulence, seroconversion, immunogenicity, and protectivity – in naturally susceptible animals. While these activities are integral to CSF surveillance, they are not directly involved in disease detection.

Virus isolation is preferably reserved for research within CSF surveillance frameworks, whereas RT-PCR has become the standard for routine diagnostics [31, 32].

Based on the discussion, a laboratory diagnostic scheme for CSF in domestic pig and wild boar populations is proposed for the current situation without routine vaccination but using marker vaccines (Fig. 7).

Together, the surveillance components provide reliable information, confirmed by several data sources. The integrated application of these methods will allow for the timely identification of and response to suspicious cases, as well as the formulation of an effective emergency control strategy.

CSF control. The next stage in Russia's CSF eradication strategy is to formulate an emergency response plan and incorporate the requisite amendments into the regulatory documentation. This necessitates the consistent refinement of the nation's current regulatory framework governing pig husbandry, as well as the prevention, diagnosis, and control of CSF.

For instance, it is already evident that to ensure the early detection of all infection cases and viral transmission among susceptible animals, the Rules must be amended to strengthen the epizootological surveillance measures they contain. To achieve this, one must account for the possibility of a prolonged latent period of the disease spread and the variability of clinical symptoms. We deem it advisable to approve the requirements based on the aforementioned methodological recommendations [8], mandating regular, rather than episodic, laboratory testing using direct methods. It should be noted that the transition to serological diagnostics as the primary method is contingent upon two prerequisites: the cessation of vaccine use (either regionally or nationally) and the removal of seropositive animals from the susceptible herd. This is necessitated by several factors:

– the duration of antibody persistence in previously immunized pig populations (post-vaccination antibodies to the vaccines used in Russia can be detected for at least two years, irrespective of the animal's age) [33];

– colostral antibody persistence (on average 5–7 weeks or more) [34];

[8] https://base.garant.ru/74901254 (in Russ.)

– inability to differentiate post-vaccination antibodies from post-infectious ones.

As part of the ongoing improvement of regulatory documentation, it is paramount to immediately introduce requirements mandating a comprehensive set of biosecurity measures for pig farms and enterprises, designed to reliably prevent the introduction and spread of infectious diseases.

Implementing the vaccination withdrawal phase will increase the risk of disease outbreaks on low-biosecurity farms throughout the country, and particularly in compartment III and IV pig farms situated on the border with countries that are currently or have historically been infected. Therefore, when adopting a non-vaccination policy, a phased approach is recommended, initiating the measure in a single region or even within a select group of farms (compartments) in the Central Federal District of Russia. Upon achieving positive results with the strategy outlined above, the list of such farms or regions can be expanded incrementally [18].

Upon successful cessation of vaccination, followed by 12 months of compliant surveillance and fulfillment of other requirements in Chapter 1.9 "Application for official recognition by WOAH of free status for classical swine fever" the official CSF-free status recognition becomes feasible.

In cases of high CSFV incursion risk precluding vaccination cessation, marker vaccine use is acceptable. For example, in 2024, a marker vaccine based on the recombinant E2 protein of the CSFV ("VERRES-CSF-E2", Vetbiokhim) was registered in the Russian Federation [35, 36]. In this case, post-vaccination antibodies can be differentiated from those resulting from infection using an ELISA based on recombinant E^{rns} and/or NS3B proteins (antibodies against these viral proteins are detectable only in infected pigs, not in vaccinated ones). For the implementation of a DIVA strategy in the country, it is feasible to utilize either the aforementioned marker vaccine or another registered alternative that has been validated in accordance with WOAH recommendations. For these purposes, the use of differentiating ELISA test kits will also be required.

In the future, our country will be required to substantiate its CSF-free status by submitting evidentiary documentation to the Scientific Committee.

CONCLUSION

Although no cases of CSF have been recorded in the Russian Federation in recent years, the absence of a legislatively mandated eradication policy and the continued practice of mass immunization with live vaccines render it impossible to apply for CSF-free status under current conditions.

Based on international experience and the current CSF situation in Russia, the article suggests approaches to improve the disease surveillance and control. These approaches are aligned with international recommendations and support the national disease eradication goal, which mandates amendments to the current veterinary regulations on CSF.

The methods for timely CSF laboratory diagnosis have been developed in Russia. However, the application of certain methods (e.g., ELISA) has only partial justification and does not consistently align with the national CSF eradication strategy. However, future development and implementation of the FAVN and/or NPLA tests will be necessary for differentiating false-positive sera in ELISA. These methods will be especially relevant following vaccination cessation. The proposed order and priority for using CSF diagnostic methods will enable more efficient allocation of available resources.

The framework proposed in this article for improving CSF surveillance and control in the Russian Federation will bring the country closer to attaining disease-free status recognized by WOAH. Success in this endeavor would, in turn, enhance the profitability of the pig industry and strengthen Russia's export potential.

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Differentiation of avian influenza and Newcastle disease viruses in organ samples from sick and dead chickens using a rapid test kit

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ABSTRACT

Introduction. Avian influenza virus (AIV) and Newcastle disease virus (NDV) pose serious threats to poultry health. Both pathogens are highly contagious and, due to their rapid spread, can lead to significant economic losses. Overlapping clinical signs complicate field differentiation of these diseases and delay response measures to isolate affected poultry. Rapid disease detection is critical for ensuring a timely response.

Objective. To develop a user-friendly test kit for detection of AIV and paramyxoviruses in organ samples from sick and dead chickens during disease outbreaks in commercial poultry operations.

Materials and methods. To differentiate AIV and paramyxoviruses in pathological samples collected from sick and dead birds, 96-well plates coated with fetuin and anti-NDV IgY in designated wells were used. The results obtained were compared with those from polymerase chain reaction (PCR) and virus titration in chicken embryos.

Results. The developed method for pathogen detection is based on distinct virus-binding principles: influenza virus binds to a receptor analog, while paramyxoviruses bind to NDV specific antibodies. Previous studies using hundreds of strains have demonstrated that influenza A virus of various subtypes binds to the sialoglycosyl residues of bovine fetal serum protein – fetuin. In contrast, none of the paramyxovirus isolates tested bound to this sialoglycoprotein. For paramyxovirus capture, immunoglobulins isolated from the egg yolks of chickens immunized against NDV were utilized. Binding was performed in 96-well plates using a test-kit analogous to enzyme-linked immunosorbent assay (ELISA).

Conclusion. The developed method enables the identification and differentiation of AIV and NDV in organ tissue homogenates from infected chickens within a few hours, representing a significant step toward preventing the spread and facilitating the eradication of dangerous disease outbreaks.

Keywords: Newcastle disease virus (NDV), avian influenza virus (AIV), differential diagnosis

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Тест-система для дифференциации вирусов гриппа птиц и ньюкаслской болезни в органах больных и павших кур

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РЕЗЮМЕ

Введение. Грипп птиц и ньюкаслская болезнь представляют собой серьезную угрозу для здоровья птиц. Оба заболевания характеризуются высокой контагиозностью и в условиях быстрого распространения могут привести к серьезным убыткам. Признаки болезней часто схожи, что затрудняет быструю диагностику и принятие экстренных мер по изоляции больных особей. Оперативное распознавание заболеваний является критически важным для своевременного реагирования.

Цель исследования. Целью работы является разработка простой тест-системы для детекции вируса гриппа птиц и парамиксовирусов в органах больных и павших птиц при возникновении вспышек заболеваний в птицеводческих хозяйствах.

Материалы и методы. Для дифференциации вирусов гриппа птиц и парамиксовирусов в патологическом материале, полученном от больной и павшей птицы, применяли 96-луночные планшеты, покрытые раствором фетуина и анти-ВНБ IgY в соответствующих лунках. Полученные данные сопоставляли с результатами, полученными методами полимеразной цепной реакции и титрования на куриных эмбрионах.

Результаты. Разработанный метод выявления возбудителей основан на разных принципах связывания вирусов. Вирус гриппа связывается с рецепторным аналогом, а парамиксовирусы – с антителами к вирусу ньюкаслской болезни. Ранее на примере сотен штаммов было показано, что вирус гриппа А разных субтипов связывается с сиалогликозильными остатками сывороточного белка эмбриона коровы – фетуина. В то же время ни один из исследованных при проведении работы изолятов парамиксовирусов не связывался с данным сиалогликопротеином. Для связывания парамиксовирусов использовали иммуноглобулины, выделенные из яичного желтка кур, иммунизированных против ньюкаслской болезни. Связывание проводили на 96-луночных плашках в системе, аналогичной иммуноферментному анализу.

Заключение. Разработанный способ выявления вирусов в гомогенатах тканей органов инфицированных кур позволяет за несколько часов идентифицировать и дифференцировать вирусы гриппа птиц и ньюкаслской болезни, что является важным шагом в предотвращении распространения и ликвидации очагов опасных болезней.

Ключевые слова: вирус ньюкаслской болезни, вирус гриппа птиц, дифференциальная диагностика

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INTRODUCTION

Newcastle disease (ND) ranks among the most economically significant infectious avian diseases worldwide and occurs on all continents except Antarctica. Newcastle disease virus (NDV) is an enveloped, non-segmented single-stranded negative-sense RNA virus belonging to the *Paramyxoviridae* family [1]. NDV strains are classified by chick pathogenicity into apathogenic, lentogenic, mesogenic, and velogenic pathotypes. Velogenic NDV strains cause either neurotropic or viscerotropic infections, depending on strain tropism. Infected birds exhibit fever, anorexia, depression, lethargy, somnolence, and respiratory distress with dyspnea. In viscerotropic NDV infection, the gastrointestinal tract bears the primary brunt of pathology. Lesions include splenic and hepatic necrosis along with hemorrhagic intestinal ulcers. Droppings become liquid and green-tinged. Viscous gray mucus discharge from the nares, poultry sneezes with swallowing efforts. Severe conjunctivitis may occur. Muscle tremor, cervical torticollis, and opisthotonus frequently precede death in neurotropic NDV infections. The most characteristic clinical picture of infection with NDV, which distinguishes it from other highly pathogenic avian viruses is severe lymphoid necrosis Mortality can reach 100% [2, 3]. Velogenic NDV belongs to *Orthoavulavirus javaense* species (OAVJ, formerly AOAV-1) within the genus *Orthoavulavirus*, comprising over two dozen genotypes. Since NDV discovery in the 1930s, four panzootics have occurred, with the ongoing fourth driven by genotypes V, VI, VII, VIII. In recent years, numerous ND outbreaks caused by genotype VII (subgenotype VII.1.1) have been reported in Africa and Eurasia [4]. Russia has notified dozens of Newcastle disease outbreaks, and numerous additional cases likely remain unregistered [5]. Wild birds introduce NDV into backyard poultry flocks by freely feeding alongside chickens in rural areas [6]. ND control measures cover vaccination and quarantine.

High pathogenicity avian influenza (HPAI) inflicts massive economic losses on poultry industries while posing serious zoonotic risks to animal and human health [7]. AIV H5 subtypes have triggered multiple outbreaks among North American mammals, including foxes, seals, raccoons, and others. Moreover, AIV has infected dairy cows with high viral loads shed in milk. The risk of interspecies AIV transmission and human adaptation has increased substantially [8].

H5 and H7 AIV subtypes have driven numerous outbreaks in wild birds and poultry, resulting in over 422 million poultry deaths since 2005 from culling and direct mortality. They have caused 2,634 human cases worldwide, including more than 1,000 deaths [9]. Migratory wild birds spread AIV globally, driving three major waves of influenza outbreaks across multiple continents. The third wave, which started in 2020, is still going on.

Early detection and the timely registration of ND and AI outbreaks are essential prerequisites for effective disease containment and preventing further viral spread. In recent

years, a range of rapid confirmatory tests for these diseases has become available. Immunochromatographic tests (ICT) using gold nanoparticle-labeled antibodies have been developed for detection of both AIV and NDV antigens. This method provides high sensitivity with results readable by naked eye within 15 minutes [10, 11]. Colloidal gold strip (CGS) immunochromatographic tests have been developed to detect AIV/H5 in field conditions [12]. ICTs have been developed for detecting AIV subtypes H9 [13], H7 (targeting A/H7N9 monoclonal antibodies) [14, 15], and H6 in field samples [16]. Strip-based immunochromatographic methods excel for outbreak control due to their low cost, rapid results, and high field sensitivity. However, these tests rely on monoclonal antibodies targeting specific subtypes, rendering them unsuitable for sudden outbreaks of unknown etiology.

We have previously reported a 96-well plate-based assay capable of detecting and differentiating AIV and avian paramyxoviruses [17]. Following cultivation in chicken embryos, the viruses were detected in the allantoic fluid. However, for rapid on-site diagnostics in small poultry farms, there is a growing demand for methods that eliminate the need for preliminary virus propagation.

The objective of this study was to develop a single-plate methodology capable of detecting various subtypes of avian influenza virus and paramyxovirus in samples collected from the organs of both sick and dead birds.

MATERIALS AND METHODS

Reagents and solutions. The following materials were used in the work: MycoKill AB (PAA Laboratories GmbH, Austria); horseradish peroxidase (#P8375, Sigma-Aldrich, USA); peroxidase-conjugated anti-mouse or chicken IgG; fetuin (#F3004, Sigma-Aldrich, USA).

Solutions:

– phosphate-buffered saline – 0.02 M, pH 7.2 (PBS);
– PBS supplemented with 0.1 mg/mL kanamycin, 0.4 mg/mL gentamicin, 0.01 mg/mL nystatin and 2% MycoKill AB solution;
– washing solution – 0.01% Tween 80 in PBS;
– blocking solution – 0.1% bovine serum albumin solution (BSA; 1 mg/mL) in PBS;
– reaction buffer – 0.02% Tween 80 and 0.1% BSA in PBS;
– substrate solution – 1 mg of 3,3′,5,5′-tetramethylbenzidine (TMB) + 10 µL 30% H_2O_2 in 10 mL 0.05 M sodium acetate buffer (pH ~5.0–5.5);
– stop solution – 3% sulfuric acid in water;
– horseradish peroxidase (HRP)-labeled synthesis solutions;
– freshly prepared 0.2 M $NaIO_4$ solution in water;
– 1 M and 0.1 M sodium carbonate buffers, pH 9.3;
– freshly prepared $NaBH_4$ solution in water (5 mg/mL);
– 1 M Tris buffer, pH 6.0;
– 0.1 M Tris buffer, pH 7.2.

Animals. Embryonated chicken eggs (CEs) were delivered from "Ptichnoye" poultry farm (Moscow), chickens – from "Tomilinskaya" poultry farm (Moscow Oblast). Live viruses were handled in biosafety level 3 facility. All tests were carried out in accordance with the standard governing the maintenance and care of laboratory animals GOST 33215-2014[1].

Viruses. Apathogenic AIV strains and paramyxoviruses were isolated from fecal samples during extended monitoring of avian influenza in a mallard population. Fresh feces collected on the shores of Moscow ponds [18] were suspended in two volumes of PBS supplemented with antibiotics (0.4 mg/mL gentamicin, 0.1 mg/mL kanamycin, 0.01 mg/mL nystatin) and 2% MycoKill AB. After centrifugation for 10 minutes at 4,000 rpm, 10-day CEs were infected with the supernatant. Infected allantoic fluid (IAF) was harvested at 48 hours, hemagglutinin (HA)-positive samples were then subjected to three additional passages.

The highly pathogenic NDV/Chicken/Moscow/6081/2022 strain was isolated from the kidneys of dead chickens [18]. For this purpose, the tissue was homogenized with fine glass powder. PBS supplemented with 0.1 mg/mL kanamycin, 0.4 mg/mL gentamicin, 0.01 mg/mL nystatin, and 2% MycoKill AB solution was then added. The mixture was centrifuged, and the supernatant was inoculated into CEs. Embryos were examined for mortality twice daily; IAF was harvested from dead CEs. The obtained virus was cloned and sequenced [19].

Highly virulent AIV A/chicken/Kurgan/3/2005 (H5N1) and A/FPV/Rostock/34 (H7N1) strains were kindly provided by S. S. Yamnikova, Dr. Sci (Biology), D. I. Ivanovskiy Institute of Virology, Moscow.

Production of NDV-specific immunoglobulins in eggs. Laying hens were infected with apathogenic NDV/Duck/Moscow/3639/2008 strain by adding 10^9 EID_{50} virus per chicken to the drinking bowl. After 2 weeks chickens were infected with highly pathogenic NDV/chicken/Moscow/6081/2022 strain. After 2 weeks eggs of immunized chickens were collected [20].

Extraction and purification of egg yolk immunoglobulins (IgY). Yolks from four eggs were separated from the whites. The chalazae were carefully removed, and the yolks were washed twice with cold distilled water before being transferred to a plastic container containing 40 mL of phosphate-buffered saline (PBS, pH 7.4). The mixture was homogenized thoroughly, brought to a final volume of 250 mL with water, and the pH was adjusted to 4.2 using 1 M hydrochloric acid. The preparation was then frozen at –30 °C. After 20 hours, the suspension was thawed and centrifuged at 10,000 g for 30 minutes. Activated carbon powder (1 g) was added to the supernatant. The mixture was stirred for 30 minutes and then filtered through filter paper [20]. Ammonium sulfate was added to the filtrate to achieve 25% saturation. The mixture was then held at 4 °C for 2 hours. The mixture was centrifuged at 10,000 g for 30 minutes. The resulting precipitate was dissolved in 10 mL of PBS, aliquoted, and stored at –20 °C.

Production of mouse antibodies. Mice were infected intranasally with 50 µL of IAF containing 10^7 EID_{50} of AIV or NDV. Mice were re-infected in 2 weeks. After another 2 weeks, total blood was collected from mice and serum was separated.

Detection of the influenza virus in IAF. 96-well plates were coated with 5 µ/mL fetuin solution, washed with water and blocked. Two-fold serial dilutions of IAF, starting from a 1:1 dilution, were prepared in the wells and incubated at 4 °C for 2 hours. Following washing, a solution of HRP-labeled fetuin (Fet-HRP) conjugate in reaction buffer was added to the wells, and the plates were incubated at 4 °C for 1 hour. After a final wash, the color reaction was developed with TMB. Absorbance was measured at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA).

Detection of viruses on plates coated for differential diagnosis. To detect AIV and NDV, a 96-well plate was coated

[1] https://docs.cntd.ru/document/1200127789 (in Russ.)

Coating	Fetuin											
Detection	Fet-HRP											
Dilution	H3N6	H3N6	H3N6	H3N8	H4N6	H4N6	NDV	NDV	APMV-4	APMV-4	APMV-4	APMV-4
IAF	5163	519	5172	5908	4781	4771	3639	3604	4096	3575	5268	4696
1:1	0.99	0.95	0.98	1.14	0.95	0.93	0.09	0.09	0.10	0.11	0.20	0.15
1:2	0.97	0.97	0.91	1.12	0.92	0.91	0.09	0.09	0.09	0.09	0.15	0.12
1:4	0.9	0.93	0.93	1.02	0.88	0.75	0.08	0.09	0.09	0.10	0.12	0.10
1:8	0.86	0.83	0.87	1.02	0.80	0.68	0.07	0.09	0.08	0.08	0.10	0.09
1:16	0.68	0.71	0.77	0.93	0.67	0.51	0.08	0.08	0.09	0.08	0.08	0.09
1:32	0.51	0.59	0.62	0.82	0.50	0.35	0.09	0.09	0.08	0.08	0.08	0.08
1:64	0.38	0.45	0.49	0.71	0.34	0.27	0.07	0.08	0.09	0.08	0.08	0.08
1:128	0.24	0.30	0.38	0.50	0.22	0.15	0.09	0.09	0.09	0.08	0.08	0.08

Fig. 1. Representative results showing the binding of HA-positive allantoic fluid samples to conjugated fetuin. Optical density was measured at 450 nm

Coating	Fetuin											
Detection	Murine sera											
Serum dilution	anti-H1N1	anti-H3N2	anti-H3N8	anti-H4N6	anti-H5N2	anti-H5N3	anti-H6N2	anti-H7N1	anti-H11N6	anti-H11N9	anti-H14N6	Normal serum
Control 1:2	0.34	0.35	0.35	0.43	0.38	0.41	0.23	0.17	0.24	0.44	0.22	0.24
1:2	0.70	1.48	1.64	0.72	0.76	0.98	1.06	0.60	0.55	0.73	0.62	0.44
1:4	0.55	1.51	1.57	0.63	0.77	0.94	1.18	0.55	0.49	0.62	0.53	0.36
1:8	0.46	1.42	1.51	0.55	0.59	0.82	0.97	0.51	0.37	0.59	0.48	0.27
1:16	0.38	1.31	1.44	0.59	0.42	0.69	0.84	0.46	0.41	0.55	0.31	0.22
1:32	0.36	1.26	1.47	0.35	0.37	0.61	0.59	0.34	0.38	0.48	0.34	0.20
1:64	0.29	1.18	1.42	0.25	0.42	0.41	0.35	0.36	0.29	0.43	0.22	0.15
1:128	0.28	1.08	1.37	0.27	0.36	0.34	0.25	0.32	0.22	0.37	0.19	0.11

Fig. 2. Representative results showing the binding of A/duck/Moscow/5908/2021-containing allantoic fluid to sera from mice immunized with different influenza virus subtypes. Optical density was measured at 450 nm

as follows. Rows 1 and 12 were not coated and served as negative controls. Rows 2–4 were coated with fetuin, while rows 5 and 6 were coated with anti-NDV IgY to serve as a negative control for AIV. Rows 7 and 8 were coated with fetuin to serve as a negative control for NDV, and rows 9–11 were coated with anti-NDV IgY. The plate, coated as described above, was then blocked with BSA solution. Then, 100 µL of a virus-containing solution at a uniform concentration was added to each well of the plate (the same solution was used for all 12 wells of a given row). Thus, the plate design enabled the analysis of up to 8 different samples. After incubation for 2 hours at +4 °C, the plate was washed and Fet-HRP conjugate was added to rows 1–6; anti-NDV IgY-HRP in a reaction buffer was added to rows 7–12 and incubated for 1 hour at +4 °C. After a final wash, the color reaction was developed with TMB. Absorbance was measured at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA). AIV was detected in rows 1–6, and NDV was detected in rows 7–12.

Detection of viruses in chicken tissues. 45 Leghorn chickens of the same age and weight were used for the experiment. The poultry were divided into equal groups: 10 chicks per experimental group and 5 chicks per control group. Two groups were infected with AIV (A/chicken/Kurgan/3/2005 H5N1 and A/FPV/Rostock/34 H7N1 strains, respectively), and one group was infected with NDV (NDV/Chicken/Moscow/6081/2022 strain). Each experimental group had a matched control group. The poultry were kept in separate cages in different rooms depending on the group. A total of 10 chickens per site were used. To assess viral pathogenicity, the poultry were deprived of water overnight prior to the experiment. The next day, 10 mL of water containing 10^8 EID_{50} of the viruses was placed in the drinkers and introduced into the cage with the birds. The control group received regular water.

Organs (kidneys, lungs, intestines) were extracted from dead or euthanized chickens, homogenized in a double volume of PBS, and centrifuged for 10 minutes at 5,000 rpm. The supernatant was used for further analysis. The organs of control poultry were prepared in the same way. Aliquots of the supernatants were retained for virus typing by polymerase chain reaction (PCR) and titration in CE. The remaining material was then added to 12 wells of a plate prepared as described above.

Coating	anti-NDV IgY											
Detection	anti-NDV IgY-HRP											
IAF dilution	H3N1 3554	H6N2 4031	H11N9 6454	NDV 6081	NDV 3639	NDV 3604	NDV LaSota	APMV-4 5268	APMV-4 4572	APMV-4 4096	APMV-4 3579	Virus-free control
1:1	0.22	0.21	0.19	1.22	1.35	1.11	1.03	0.19	0.16	0.16	0.15	0.14
1:2	0.19	0.16	0.16	1.11	1.23	1.06	0.89	0.15	0.15	0.15	0.15	0.14
1:4	0.17	0.15	0.15	1.02	1.10	0.97	0.83	0.15	0.15	0.16	0.14	0.15
1:8	0.17	0.15	0.15	0.56	1.00	0.82	0.70	0.15	0.15	0.15	0.15	0.15
1:16	0.18	0.15	0.16	0.36	0.74	0.61	0.53	0.15	0.15	0.15	0.14	0.15
1:32	0.17	0.15	0.15	0.28	0.53	0.44	0.44	0.14	0.15	0.15	0.16	0.15
1:64	0.16	0.15	0.14	0.22	0.38	0.31	0.37	0.13	0.14	0.15	0.15	0.14
1:128	0.17	0.16	0.16	0.18	0.27	0.19	0.30	0.12	0.13	0.13	0.14	0.15

Fig. 3. Detection of NDV on a plate coated with anti-NDV IgY, and treated with anti-NDV IgY-HRP. Optical density was measured at 450 nm

NDV detection with anti-NDV IgY and HRP conjugated anti-NDV IgY. A 96-well plate was coated with a 5 µg/mL solution of purified anti-NDV IgY. The plate was then washed with water and blocked with a BSA solution for 1 hour. Test supernatants from the homogenized samples were then added to the wells (100 µL per well) and incubated at 4 °C for 2 hours. The plate was washed, anti-NDC IgY-HRP conjugate in a reaction buffer was added and incubated for 1 hour at +4 °C. After a final wash, the color reaction was developed with TMB. Absorbance was measured at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA).

Synthesis of HRP-labeled conjugates. The synthesis Fet-HRP was described in detail by M. N. Matrosovich and A. S. Gambaryan [21]. Conjugation of HRP with anti-NDV IgY was performed similarly. A freshly prepared solution of 0.2 M $NaIO_4$ in water was added to HRP dissolved in bidistilled water. The mixture was then incubated in the dark at room temperature for 20 minutes. The reaction mixture was desalted on a Sephadex G-25 column. Solutions of fetuin or immune IgY in sodium carbonate buffer (pH 9.3) were then added, and the mixture was incubated in the dark for 4 hours. 5 mg/mL of freshly prepared $NaBH_4$ solution in water was added and incubated for 30 minutes on ice. The pH was adjusted to neutral using 1 M Tris buffer (pH 6.0) on ice. Chromatography was performed on a Sephacryl S-200 column. Fractions containing HRP were collected, pooled, aliquoted, and stored at –20 °C.

Detection of viruses by PCR. The presence of AIV and NDV RNA in the samples was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) with fluorescence detection, including an internal control. RNA was extracted from biological samples using a commercial magnetic particle-based kit for nucleic acid isolation via sorption "DNA/RNA-M-FLEX-FAKTOR" (VET FAKTOR, Russia). Commercial kits for detection of AIV and NDV RNA were used respectively – "PCR-INFLUENZA-A-FAKTOR" and "PCR-NEWCASTLE-FAKTOR" (VET FAKTOR, Russia). The matrix for RT-PCR was RNA samples extracted from the test material (feces, fragments of organs and tissues).

Ethics. Animal experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The study design was approved by the Ethics Committee of Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences (Resolution No. 4 dated 02.12.2014). All measures were taken to minimize animal suffering.

RESULTS

During the monitoring of migratory birds, we isolated dozens of AIV strains and paramyxoviruses [22]. In addition, during the investigation of the outbreak in Moscow Oblast NDV was isolated from chickens [18]. Final virus identification was based on complete or partial genome sequencing. However, at the initial stage, AIV was differentiated from NDV and avian paramyxovirus serotype 4 (APMV-4) using solid-phase ELISA. The materials presented herein describe the protocols employed for virus detection and identification.

Differentiation of AIV from paramyxoviruses. AIV detection relied on viral hemagglutinin binding to sialoglycosyl residues on fetuin [21]. IAF was incubated in wells of plated coated with fetuin. Following sorption and washing, plates were incubated with Fet-HRP conjugate solution and developed via chromogenic reaction. Final identification was confirmed by partial/complete genome sequencing of the virus isolates. All AIV samples were positive in this test, while all paramyxoviruses were negative (Fig. 1).

All viruses were isolated from duck feces in Moscow. AIV isolates were identified by subtype, while paramyxoviruses were classified by serotype. The second line contains the strains identified by the laboratory number. Eight wells of each row contain double IAF dilutions.

Subtyping of influenza A virus (AIV) using a combined assay with fetuin murine monoclonal sera against distinct AIV of different subtypes. The combination of universal fetuin capture and subtype-specific antibody detection simplifies AIV identification. An example of A/duck/Moscow/5908/2021 subtyping is shown in Figure 2. The influenza virus (H3N8) subtype was subsequently confirmed by sequencing.

IAF containing A/duck/Moscow/5908/2021 virus was added to rows B–H of plates coated with fetuin. Row A served as a negative control and contained no virus. Then, double dilutions of immune murine sera were added to each column. In the last column, normal murine serum was titrated. At the next stage, HRP-conjugated anti-mouse antibodies were incubated with plates. Following washing, plates were developed with TMB to visualize antibody binding.

Coating	---	Fetuin			anti-NDV IgY		Fetuin		anti-NDV IgY			---
Detection	Fet-HRP						anti-NDV IgY-HRP					
IAF AIV H5N1	0.19	1.11	1.26	1.21	0.37	0.29	0.15	0.15	0.14	0.15	0.15	0.12
IAF NDV 3639	0.12	0.12	0.17	0.20	0.22	0.20	0.45	0.44	1.30	1.38	1.35	0.13
Feces NDV 6081	0.15	0.22	0.24	0.23	0.25	0.27	0.19	0.21	0.58	0.63	0.55	0.14
Kidneys (control)	0.14	0.16	0.20	0.19	0.17	0.13	0.16	0.16	0.18	0.17	0.15	0.15
Kidneys AIV H5N1	0.12	0.92	0.97	1.00	0.22	0.20	0.25	0.28	0.19	0.21	0.23	0.12
Kidneys AIV H7N1	0.14	0.62	0.71	0.68	0.17	0.13	0.16	0.16	0.18	0.17	0.18	0.15
Kidneys NDV 6081	0.17	0.22	0.19	0.21	0.40	0.37	0.24	0.26	1.10	1.00	1.02	0.14
Kidneys NDV 6081	0.18	0.23	0.18	0.22	0.33	0.39	0.29	0.26	0.98	1.04	1.00	0.17

Fig. 4. Testing of preparations for AIV and NDV: A/chicken/Kurgan/3/2005 (H5N1) infectious allantoic fluid; NDV/Duck/Moscow/3639/2008 infectious allantoic fluid; faecal extract of chicks infected with NDV/Chicken/Moscow/6081/2022; kidney extract of uninfected chick; kidney extract of a chick infected with A/chicken/Kurgan/3/2005; kidney extract of a chick infected with A/FPV/Rostock/34 (H7N1); and kidney extracts of chicks infected with NDV/Chicken/Moscow/6081/2022

Table
Detection of NDV and AIV from feces and organs of sick chickens using solid-phase analysis, PCR and titration in chicken embryos

Method of detection	NDV							AIV
	IAF	Feces			Kidneys	Lungs	Intestine	Kidneys
		Day 3	Day 5	Day 7				
Solid-phase ELISA	6/6	5/10	0/4	0/8	10/10	2/2	4/6	5/5
PCR	6/6	10/10	4/4	6/8	4/4	5/5	6/6	5/5
Titration	6/6	7/7	2/2	1/1	4/4*	1/1	2/2	3/3*

* The virus infectivity titer was 10^9 EID_{50}/mL.

Detection of paramyxoviruses using solid-phase ELISA. Chickens were double-immunized with apathogenic NDV/duck/Moscow/3639/2008, then challenged with velogenic NDV/chicken/Moscow/6081/2022 (ch6081, subgenotype VII.1.1); eggs were harvested to purify anti-NDV IgY. IgY was concentrated and purified by 25% ammonium sulfate precipitation, then used to coat 96-well plates. Anti-NDV IgY was also conjugated to HRP. In tests with these antibodies, all NDV-infected allantoic fluids tested positive, while all APMV-4-infected allantoic fluids were negative (Fig. 3).

AIV isolates were identified by subtype, while paramyxoviruses were classified by serotype. The second line contained the strains identified by the laboratory number. Eight wells of each row contained double IAF dilutions.

Detection of viruses in organ samples from sick chickens. In the three aforementioned tests, AIV or NDV presence in allantoic fluid was confirmed following propagation in embryonated chicken eggs. However, the cultivation and isolation of viruses is not possible in small poultry farms, especially in backyards. Therefore, the possibility of detecting viruses in extracts obtained from organ tissues of infected chickens was investigated. The extracts were added to 12 wells of a 96-well plate prepared as described in section "*Detection of viruses in coated plates for differential diagnosis*" of "Materials and methods".

Figure 4 shows the results of this experiment.

Samples containing AIV (A/chicken/Kurgan/3/2005 IAF, kidney extracts from A/chicken/Kurgan/3/2005 and A/FPV/Rostock/34 – infected chickens) yielded positive signals in fetuin-coated wells detected by Fet-HRP conjugate. Only fetuin coating with Fet-HRP detection reliably detected AIV antigens across all tested strains. Samples containing NDV (NDV/Duck/Moscow/3639/2008 IAF, feces from NDV/Chicken/Moscow/6081/2022 infected chickens, and kidney extracts from NDV/Chicken/Moscow/6081/2022 infected chickens) give a positive signal in anti-NDV IgY-coated wells detected by anti-NDV IgY-HRP. Only IgY anti-NDV coating with IgY-HRP detection reliably detected NDV antigens across all tested strains. Thus, a single reaction differentiates HPAIV (H5N1, H7N9) from NDV in infected poultry.

Comparison of solid-phase ELISA with PCR and embryonated chicken egg infectivity titration. A comparison of the described solid-phase ELISA with PCR and titration in chicken embryos shows that the latter two are much more sensitive (Table). Using these methods, virus was detected in nearly all samples from days 3–7 post-ch6081 infection; solid-phase ELISA detected fecal shedding only at peak disease.

On days 5 and 7, no NDV/AIV was detected in fecal samples by this method; however, the proposed solid-phase ELISA achieved 100% detectability in avian organ tissues. The virus was detected in all kidney samples from chickens that died or were euthanized at the terminal disease stage. Highly pathogenic AIV and NDV accumulate in chicken kidneys at concentrations up to 10^9 EID_{50}/mL; this enables detection by solid-phase ELISA (analytical sensitivity

10^8 EID_{50}/mL). The developed method exhibits low sensitivity for fecal samples and is unsuitable for preliminary diagnosis at this stage. However, rapid poultry mortality on infected farms enables immediate and reliable cause determination using this test, facilitating timely control measures.

DISCUSSION

Recently, avian influenza and Newcastle disease have caused major global poultry industry losses. In recent decades, H5 subtype AIV has spread from Southeast Asia to all continents, including the Americas, underscoring the need for effective monitoring and diagnostics for this dangerous pathogen. NDV genotype VII has spread from Africa, with outbreaks reported from Japan to Northwestern Europe [23]. Since stamping out remains the primary containment strategy for HPAI and NDV timely outbreak detection is critically important [24].

The developed method enables simultaneous identification and differentiation of NDV and AIV, facilitating timely disease control measures. Since AIV detection uses a universal receptor analog and NDV detection employs polyclonal IgY from chickens immunized with different strains, the method should detect any poultry-pathogenic AIV/NDV strains responsible for mortality. The method enables on-site preliminary diagnostics. Commercial AIV test kits target specific subtypes, whereas our method simultaneously detects multiple AIV subtypes in feces/pathological material, significantly reducing diagnostic time and enabling timely pathogen control in poultry.

Development of a test kit for differential diagnosis of avian influenza and Newcastle disease represents a critical advance in poultry health management.

Future work will focus on validating this method and testing it in backyard flocks, as well as comparing it with existing methods for identifying these pathogens.

CONCLUSION

Global circulation of highly pathogenic H5N1 AIV and NDV outbreaks across Africa, Asia, and Europe necessitated development of a simple, rapid pathogen detection method for organs from sick/dead poultry. The developed method identifies and differentiates NDV from multiple AIV subtypes in feces and organ tissues of diseased/dead chickens.

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In vitro evaluation of chitosan cytotoxic properties

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ABSTRACT

Introduction. Chitosan immunomodulatory and mucoadhesive properties render it a promising vaccine adjuvant. Safety – particularly the absence of cytotoxicity – is a key requirement for adjuvant candidates. *In vitro* biocompatibility assessments enable evaluation of chitosan preparations prior to animal testing.

Objective. To evaluate low molecular weight chitosan solution at a concentration of 10 mg/mL for its cytotoxic effect on chicken embryo fibroblast (CEF) cultures and calf coronary artery epithelial-like cells (CCEC) to justify its further use as a vaccine adjuvant.

Materials and methods. Low molecular weight (LMW) chitosan (degree of deacetylation: 90%) prepared with a 1% glutamic acid solution (pH 6.9) was used. Cytotoxicity was comprehensively assessed using three methods: trypan blue vital staining (for cell viability), live-cell microscopy (for morphological evaluation), and calculation of the proliferation index after 72 hours of incubation at 37 °C in a 5% CO_2 atmosphere.

Results and discussion. Following 2-hour incubation with chitosan, viable CEF and CCEC were 97.4 and 98.7%, respectively, with no significant differences from controls (97.6 and 96.4%). Microscopy at 72 hours showed dense, homogeneous monolayers in test groups, free of cytopathic effects, vacuolization, or morphological changes – indistinguishable from controls. Proliferation indices aligned closely (CEF: 3.9 and 3.6; CCEC: 3.7 and 3.8), evidencing no cytostatic effect of the chitosan preparation.

Conclusion. Low-molecular-weight chitosan (10 mg/mL) exhibited no *in vitro* cytotoxic or cytostatic effects on the tested cell lines. The findings confirm its biocompatibility and justify advancement to *in vivo* studies for developing safe, effective vaccines for veterinary use.

Keywords: chitosan, cytotoxicity, adjuvant, cell viability, proliferation, cell cultures, primary chicken embryo fibroblasts (CEF), continuous calf coronary artery epithelial-like cells (CCEC), *in vitro*

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Цитотоксические свойства хитозана *in vitro*

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РЕЗЮМЕ

Введение. Хитозан, благодаря иммуномодулирующим и мукоадгезивным свойствам, является перспективным адъювантом для вакцин. Безопасность, в частности отсутствие цитотоксичности, – ключевое требование к адъювантам. Исследования *in vitro* позволяют определять биосовместимость препарата хитозана до тестирования на животных.

Цель исследований. Исследовать цитотоксическое действие раствора низкомолекулярного хитозана в концентрации 10 мг/мл на культурах фибробластов эмбриона кур и эпителиоподобных клеток коронарных сосудов теленка для обоснования его дальнейшего применения в качестве вакцинного адъюванта.

Материалы и методы. Применяли низкомолекулярный хитозан (степень деацетилирования – 90%) в 1%-м растворе глутаминовой кислоты, pH 6,9. Цитотоксичность определяли комплексно, используя метод витального окрашивания трипановым синим (оценка жизнеспособности), прижизненное микроскопическое наблюдение (оценка морфологии) и расчет индекса пролиферации после 72-часовой инкубации при температуре +37 °С в атмосфере 5%-го диоксида углерода.

Результаты и обсуждение. Количество жизнеспособных клеток фибробластов эмбриона кур и коронарных сосудов теленка после двухчасовой инкубации с хитозаном соответствовало значениям 97,4 и 98,7%, не имеющим статистически значимых отличий от контролей (97,6 и 96,4%). При микроскопическом наблюдении клетки в опытной группе через 72 ч инкубации формировали плотный однородный монослой без признаков цитопатического эффекта, вакуолизации, без изменений морфологии, аналогичный таковому в контрольных лунках. Индексы пролиферации в опытных и контрольных группах были сопоставимы: для фибробластов эмбриона кур – 3,9 и 3,6, для коронарных сосудов теленка – 3,7 и 3,8, что свидетельствует об отсутствии цитостатического действия изучаемого препарата.

Заключение. Хитозан низкомолекулярный в концентрации 10 мг/мл не проявляет цитотоксических или цитостатических свойств *in vitro* в отношении тестируемых клеток. Полученные данные подтверждают его биосовместимость и являются основанием для дальнейших исследований *in vivo* с целью разработки безопасных и действенных вакцин для ветеринарного применения.

Ключевые слова: хитозан, цитотоксичность, адъювант, жизнеспособность клеток, пролиферация, клеточные культуры, первичная культура фибробластов эмбриона кур, перевиваемая линия эпителиоподобных клеток коронарных сосудов теленка, *in vitro*

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INTRODUCTION

Avian infectious diseases such as avian influenza, Newcastle disease, infectious bronchitis, and avian metapneumovirus infection cause significant economic damage to poultry industry worldwide [1, 2]. Agents of these diseases are highly contagious and widespread, often causing mass mortality in poultry. This leads to significant economic losses and restrictions on international trade. Available commercial vaccines, although widely used, sometimes fail to confer sterilizing immunity and full protection for vaccinated flocks especially under the high pressure from field virus strains in intensive production system. This necessitates the exploration of novel approaches to enhance vaccine immunogenicity and prolong the duration of vaccine effectiveness [3, 4].

One of the promising areas of vaccinology is the development and application of modern adjuvants – substances capable of enhancing, prolonging, and modulating the immune response to a vaccine antigen when added to a vaccine formulation [5]. Modern adjuvants employ diverse mechanisms of action designed to activate innate immunity, ultimately leading to the development of a stronger and more potent adaptive immune response [6]. At the same time, safety, in particular the absence of cytotoxicity, remains a key requirement for any new adjuvant compositions [7, 8].

Chitosan is a natural biopolymer derived from the deacetylation of chitin, a structural component found in crustacean shells and fungal cell walls. Its key advantage is its low toxicity to warm-blooded organisms and its ability to degrade without harm to the environment [9, 10, 11]. Chitosan's favourable properties – coupled with its abundance of free reactive amino groups – underpin its extensive use in medicine and pharmacy. Key applications include wound dressings, drug delivery carriers, and, most notably, vaccine adjuvants [12].

In vaccinology, chitosan is considered as a multifunctional adjuvant, especially for mucosal use (intranasal, ocular, oral) [13, 14]. Its adjuvant properties are associated with a complex of mechanisms. Firstly, as a cationic polymer, chitosan can temporarily disrupt the integrity of tight junctions between epithelial cells in mucous membranes. This increases mucosal permeability and facilitates antigen penetration [15, 16]. Secondly, chitosan derivatives can form an antigen depot at the injection site, providing a prolonged release of the active substance. Thirdly, chitosan has a direct stimulating effect on innate immune cells, likely through interaction with pattern-recognition receptors such as TLR-2. This leads to the activation of antigen-presenting cells and the production of proinflammatory cytokines [17]. Foreign researchers have shown that chitosan is able to enhance both the humoral and cell-mediated immune response [16, 17, 18], which makes it particularly in demand for the development of new vaccines. It is important to note that the adjuvant effect may vary depending on the molecular weight and degree of polymer deacetylation [9, 14].

Use of LMW chitosan in avian vaccines, in particular against Newcastle disease is of particular interest. LMW fractions generally exhibit better solubility at physiological pH and, according to some studies, demonstrate lower potential toxicity compared to their high molecular weight counterparts [9]. However, prior to immunogenicity assessment, it is fundamentally important to establish the basic safety and absence of direct toxic effects of the tested product at the cellular level.

In vitro cytotoxicity determination in cell lines is the first mandatory step in preclinical studies of any new compound with potential biomedical or veterinary applications. This approach, governed by international standards like ISO 10993-5, allows for the quick, economical, and ethical collection of initial biocompatibility data in line with the 3R concept (Replacement, Reduction, Refinement). It thereby reduces or postpones the use of laboratory animals to later stages of the study [7]. This approach is widely used to assess the safety of a wide variety of compounds, from pesticides to pharmaceutical substances, which confirms its versatility and reliability [8, 19].

Cell lines were selected based on their representativeness. Primary CEF culture exhibits high sensitivity to toxic effects. Continuous CCEC line serves as a model for the cellular barrier encountered by an adjuvant upon mucosal administration. A concentration of 10 mg/mL was selected as it deliberately exceeds the estimated working concentrations in vaccines (1–2 mg/mL), allowing for stress testing and safety margin assessment.

Thus, the objective of this study was to evaluate the cytotoxic effect of a low molecular weight chitosan solution (10 mg/mL) on CEF and CCEC lines in order to justify its further use as a vaccine adjuvant.

MATERIALS AND METHODS

The study was carried out at the Department of Virology and Microbiology named after Academician V. N. Syurin of the Moscow State Academy of Veterinary Medicine and Biotechnology – MVA by K. I. Skryabin. All operations were performed in a laminar flow cabinet under sterile conditions, with strict adherence to standard aseptic techniques.

Preparation of chitosan solution. LMW water-soluble chitosan with a deacetylation degree of 90% (Bioprogress, Russia; dosage form – powder) was used. Working 2% (20 mg/mL) chitosan solution was prepared using 1% glutamic acid (Sigma-Aldrich, USA) in a ratio of 1:5 (w/v). The pH was adjusted to 6.9 ± 0.1 with a phosphate-buffered saline (PBS; BioloT, Russia) [9, 14].

Cell cultures and cultivation conditions. Two lines of adhesive cells of different origin were used for a comprehensive assessment of potential cytotoxicity:

– CCEC cells (BioloT Collection, Russia) are a continuous diploid line of epithelial-like cells derived from coronary vessels of calf. This cell line is characterized by stable growth and high sensitivity to external factors, making it a relevant model for toxicological studies;

– primary CEF culture was prepared in the laboratory from 11-day-old SPF embryo tissues using the standard trypsinization technique [19]. Cells at the 3rd passage, which retain high metabolic activity and sensitivity to toxic effects, characteristic of primary cultures were used [17].

The cells were cultivated under standard sterile conditions in an incubator (Binder, Germany) at a constant temperature of 37.0 ± 0.5 °C and 5% CO_2 to maintain a stable pH of the nutrient medium (7.2–7.4). The growth medium consisted of a 1:1 mixture of Eagle MEM and 199 media (BioloT, Russia), supplemented with 10% fetal bovine serum (St-Biol; BioloT, Russia) to provide essential adhesion and growth factors. Antibiotics were added to the medium: 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin (BioloT, Russia), to prevent bacterial contamination. The medium was changed every 48–72 hours depending on the cell growth rate. When cell monolayers reached 80–90% confluence, cultures were passaged using standard trypsinization with 0.25% Trypsin-Versene solution (BioloT, Russia). Cells in the logarithmic growth phase were selected for tests.

Test design. The cells were inoculated into 6-well plates (Jet Biofil, China) at a density of 1×10^6 cells/mL for CEF and 8×10^5 cells/mL for CCEC, which provided a 70–80% monolayer density by the beginning of the test. Separate plates were used for each cell line:

– "positive control" plate: cells were cultured in a standard growth medium;

– "test" plate: cells were cultured in a growth medium supplemented with the chitosan solution (final concentration – 10 mg/mL).

Table 1
Viability of cells after 2-hour incubation with chitosan (M ± SD, *n* = 3)

Type of the effect on cells	Cell viability, %	
	CEF	CCEC
Chitosan, 10 mg/mL in standard growth medium (test)	97.4 ± 1.2	98.7 ± 0.9
Standard growth medium (positive control)	97.6 ± 0.8	96.4 ± 1.1
70% ethanol solution (negative control)	0*	0*

* $p < 0.05$ as compared to all other groups.

Three parallel wells of the plate ($n = 3$) were used for each variant to ensure statistical reliability. The incubation was started once the cell monolayer covered 70–80% of the well bottom.

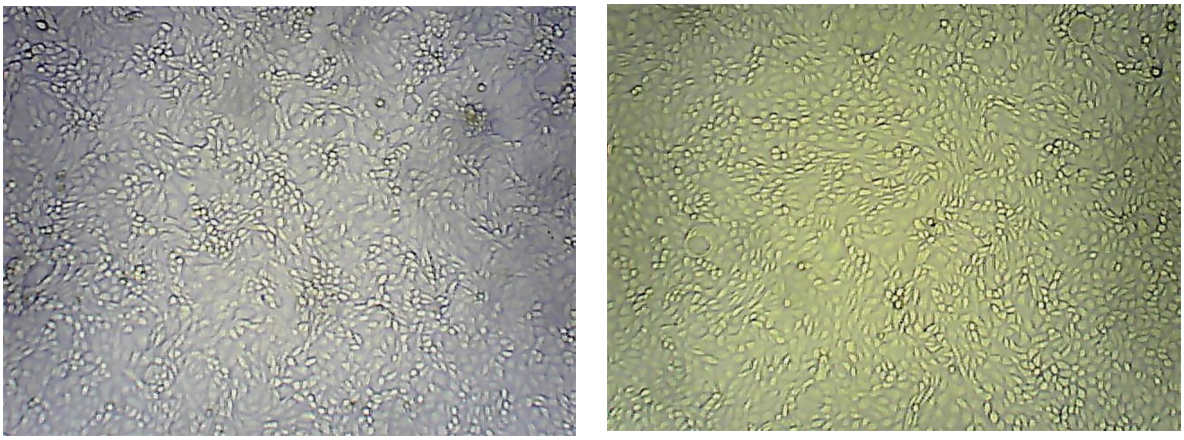
Determination of cell viability. Cell viability were assessed after 2-hour incubation with the test solutions using vital staining with 0.4% trypan blue solution (BioloT, Russia).

The counting was carried out in Goryaev counting chamber. Only cells with intact membranes (unstained) were taken into account [19]. Cells placed in a standard growth medium were used as a positive control, and cells treated with a 70% ethanol solution for 10 minutes were used as a negative control.

Morphological analysis (vital examination method). The cells were cultured with chitosan for 72 hours. Visual examination of the monolayer, cell morphology, and cytopathic effect manifestations was performed every 24 hours using Axio Observer A1 inverted microscope (Carl Zeiss, Germany) equipped with phase contrast optics and Axiocam 305 digital camera. Microscopy was performed at 120× magnification. The assessment parameters included adhesion degree, monolayer density, cytoplasmic vacuolization, shape alterations (rounding), substrate detachment, and lysis.

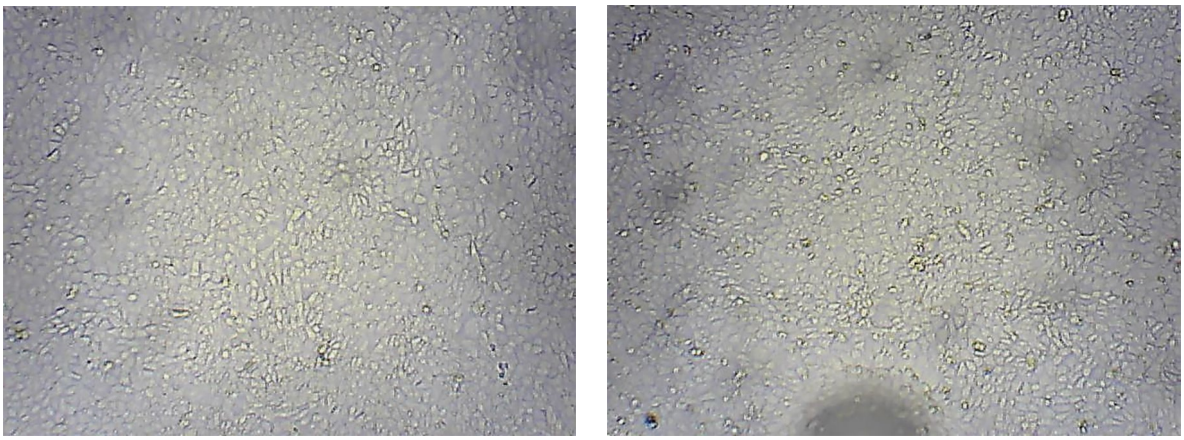
Determination of proliferative activity. The cells were inoculated into 6-well plates at known concentration (N_0). After 72 hours of co-cultivation with chitosan, the cells were dispersed with a 0.25% trypsin solution (from pig pancreas, activity: 1:250; BioloT, Russia) prepared using Versene solution (BioloT, Russia), and counted (N_{72}) in Goryaev counting chamber. The proliferation index (PI) was calculated using the formula: $PI = N_{72} / N_0$. To eliminate artifacts associated with potential errors during inoculation, the initial cell count (N_0) was performed in three additional wells immediately after cell adhesion, i.e., 4 hours post-inoculation.

Statistical analysis. Statistical processing was performed using Student's *t*-test for independent samples in Microsoft Excel 2019 and Statistica 10.0 (StatSoft, USA) software packages. The cell viability data obtained by direct counting in Goryaev counting chamber were analysed with a preliminary check of the data compliance with the normal



A B

Fig. 1. Morphology of CEF cells after 72 hours of cultivation (magnification 120×): A – control, B – test group (chitosan 10 mg/mL)



A B

Fig. 2. Morphology of CCEC after 72 hours of cultivation (magnification 120×): A – control, B – test group (chitosan 10 mg/mL)

distribution using the Shapiro – Wilk test (at $n = 3$). The uniformity of variances in the compared groups was checked using the F-test (Fisher's criterion). The following parameters were incorporated into the analysis: viability assessment was performed using absolute values of live and dead cell counts, from which the viability percentage was calculated; proliferative activity was analysed on the basis of absolute cell counts before and after incubation. For each test variant, data for three independent biological replicates ($n = 3$) were used, in each of which cell counts were performed in two analytical replications. Morphological examination findings were statistically analysed by qualitative assessment of a series of micrographs taken under identical conditions for all test groups. Quantitative results are presented in the following format: mean value ± standard deviation (M ± SD). The differences at the $p < 0.05$ level were considered statistically significant. All calculated *t*-test values and the corresponding *p*-significance levels were recorded in summary tables for further analysis.

RESULTS AND DISCUSSION

In the study, the cytotoxic effect of a low molecular weight chitosan solution was evaluated multilaterally using three complementary methods. At the first stage, cell viability was assessed after short-term incubation with the preparation. Then, dynamic microscopic examination of the cell morphology and monolayer state was carried out. At the final stage, the effect of chitosan on cell proliferative activity was assessed.

Cell viability. Results of tests of cells for their viability after 2-hour incubation are given in Table 1.

Obtained data indicate the absence of cytotoxic effects of low molecular weight chitosan (deacetylation rate – 90%) at a concentration of 10 mg/mL on two different cell models. For the CEF line, the viability index was 97.4 ± 1.2% versus 97.6 ± 0.8% in the control ($p > 0.05$). For CCEC line the values were also comparable: 98.7 ± 0.9% for cells in test plate and 96.4 ± 1.1% for the control ($p > 0.05$). Consequently, there were no statistically significant differences between the test and control groups for both tested cell lines.

High viability indices, intact morphology, and unaltered proliferative activity conclusively establish the biocompatibility of this preparation with animal cells.

Analysis of the data obtained (Table 1) allows us to conclude that the chitosan preparation under study does not have any cytotoxic effect under short-term incubation conditions. The minimal spread of values (SD = 0.8–1.2%) is noteworthy, as it indicates both high reproducibility of the results and uniformity of the cell populations. Complete

cell death was recorded in the negative control (70% ethanol treatment), thereby confirming both the adequacy of the applied technique and the sensitivity of the test system. Comparative analysis of the data for the two cell lines demonstrates that the response to chitosan exposure is neither species-specific nor tissue-specific. The comparably high viability indices observed in both the primary CEF line and the continuous CCEC line suggest that the biocompatibility of the preparation under study is universal.

Morphology of cells. Results of live-cell microscopic examination of cell cultures over time (after 24, 48, and 72 hours of incubation) clearly demonstrate the absence of any negative effect of chitosan on the morphofunctional state of the cells. Visual examination after 72 hours of incubation showed that cells in the test groups retained their typical morphology and demonstrated an active ability to form a monolayer, completely similar to control cells (Fig. 1, 2).

Typical fusiform (fibroblast-like) and stellate (epithelial-like) cells with long cytoplasmic protrusions firmly attached to the substrate were observed in the test group of CEF culture. The cells had smooth, well-defined margins and a homogeneous, non-vacuolated cytoplasm. The nuclei were clearly visible and had a regular oval or rounded shape without pyknosis or karyorrhexis signs. The resulting monolayer was dense and homogeneous, displaying the ordered cell arrangement characteristic of fibroblasts.

Both in the test group and the control group, CCEC cells had a flattened polygonal shape and formed a typical monolayer resembling "cobblestone pavement". Intercellular contacts were well developed, and there were no signs of cytoplasmic contraction or detachment from the plastic surface. Importantly, no cytopathic effects (CPE) were observed across any test groups: monolayers showed no cytoplasmic vacuolization, rounded or shrivelled cells, lysis, or zones of degeneration. Dynamic observation showed that the monolayer formed at the same rate in the test groups as in the control, reaching 90–95% confluence by 72 hours of cultivation. The morphological findings were entirely consistent with the viability and proliferative activity data, collectively confirming that chitosan exerts no cytotoxic effect at the concentration tested.

Proliferative activity. To evaluate the potential effect of chitosan on cell division, quantitative analysis of proliferative activity was carried out during a 72-hour period. Data given in Table 2 show that low molecular weight chitosan at a concentration of 10 mg/mL did not have any inhibitory effect on the proliferation of the tested cell cultures. Quantitative analysis showed that the proliferation indices in the test groups remained at a high level: for CEF – 3.9 ± 0.4, for CCEC – 3.7 ± 0.3. These values did not differ statistically ($p > 0.05$) from the control values, which were 3.6 ± 0.4 and 3.8 ± 0.3, respectively. Absolute cell counts confirmed that the cultures actively proliferated in the presence of chitosan: the CEF counts increased from 1.4×10^6 to 5.4×10^6 cells/mL, CCEC counts – from 1.2×10^6 to 4.4×10^6 cells/mL.

Comparative analysis of the two cell lines for their proliferative activity revealed an interesting trend for stimulation of CEF culture proliferation (3.9 in the test group vs. 3.6 in the control), though the difference was not statistically significant. CEF proliferation indices in the test and in the control were almost identical.

Thus, comprehensive proliferative activity analysis showed that chitosan at 10 mg/mL exhibits no cytostatic effects and fully preserves mitotic capacity and growth potential of cells.

Table 2
Effect of chitosan on cell proliferative activity (M ± SD, *n* = 3)

Cell culture	Parameters	Test (chitosan, 10 mg/mL)	Positive control
CEF	Number of cells added, cells/mL	1.4×10^6	1.4×10^6
	Number of cells after 72 hours, cells/mL	$(5.4 \pm 0.6) \times 10^6$	$(5.1 \pm 0.5) \times 10^6$
	Proliferation index	3.9 ± 0.4	3.6 ± 0.4
CCEC	Number of cells added, cells/mL	1.2×10^6	1.2×10^6
	Number of cells after 72 hours, cells/mL	$(4.4 \pm 0.4) \times 10^6$	$(4.6 \pm 0.3) \times 10^6$
	Proliferation index	3.7 ± 0.3	3.8 ± 0.3

Notably, all three methods employed to assess cytotoxicity – vital staining, morphological analysis, and proliferation assessment – produced consistent results, thereby strengthening the reliability of the conclusions. The revealed absence of toxic effects is consistent with the data of other authors, who also note the low cytotoxicity of chitosan and its oligomers [9, 10, 11]. It should be particularly noted that chitosan at a relatively high concentration (10 mg/mL) was used for our study. This concentration is much higher than the typical working concentrations of adjuvants in vaccine formulations, which are usually in the range of 0.1–2.0 mg/mL [14, 15, 18]. Despite this, the cells retained their normal viability and functional activity. Low molecular weight fractions of chitosan that are similar in physicochemical characteristics to the test sample typically exert the least toxicity, which is attributed to their milder interaction with cell membranes [11, 17]. This finding is especially significant for vaccine development, in which avoiding even minimal cellular damage is critically important. Chitosan's ability – at the concentration used – to preserve cell membrane integrity and not suppress proliferation is a key factor in its suitability as an adjuvant [12]. Furthermore, the lack of effect of chitosan on proliferative characteristics suggests that it does not interfere with the basic mechanisms of cell division and poses no risk of inducing pathological changes in proliferating tissues.

It is important to note that CEF and CCEC cultures are representative for *in vivo* modelling. CEF as a primary culture is more sensitive to toxic effects [8, 19]. The use of a primary fibroblast culture precludes artifacts associated with extended adaptation of cells to *in vitro* conditions – a phenomenon characteristic of continuous cell lines. Epithelial-like calf coronary cells (CCEC) are the first target for mucosal adjuvants [13]. Mucous epithelial cells represent the first barrier encountered by mucosally applied vaccines; therefore, preserving their integrity and functional activity is essential for effective vaccination. The lack of adverse effects on both cell lines suggests that chitosan will be well tolerated at the whole-organism level.

According to the literature, chitosan may exert its adjuvant effect not through direct cytotoxicity, but through

mild activation of cells and induction of chemokines and cytokines [14, 15]. Our findings on the preservation of cell morphology and proliferative activity are fully consistent with this concept, since they rule out non-specific cell damage as a mechanism underlying immunostimulation.

The immunostimulatory effect of chitosan is thought to be mediated by activation of innate immune signalling pathways [17, 18], a process that does not involve cell damage at the injection site. This mechanism – whereby a "beneficial" immunological effect is achieved without cell damage – is preferable for the development of modern, safe adjuvants [6]. Our findings indirectly confirm this hypothesis. Further studies should focus on the effects of chitosan on the functional activity of immunocompetent cells, specifically their antigen-presenting ability and cytokine production. Another area of interest is the evaluation of chitosan's synergistic effects in combination with other well-known adjuvants within combined adjuvant systems.

This study is limited by the testing of a single chitosan concentration. Further studies should involve the generation of a full concentration-response curve to accurately determine the toxicity threshold, that is consistent with modern paradigms for the preclinical safety evaluation of biomaterials [7, 8]. Notwithstanding this limitation, it can be confidently concluded that within the concentration range typically employed in vaccinology (generally not exceeding 1–2 mg/mL), chitosan demonstrates an excellent safety profile.

CONCLUSION

This comprehensive study demonstrates that low molecular weight chitosan (90% deacetylation) exhibits no cytotoxic or cytostatic effects *in vitro* on CEF and CCEC lines at a concentration of 10 mg/mL. These findings were corroborated by a range of complementary methods. Cell viability in the test groups remained between 97.4 and 98.7%, with no statistically significant difference from control values. Morphological analysis showed preserved normal cellular architecture and the capacity to form a dense, homogeneous monolayer. Proliferative activity assessment determined high proliferation indices (3.7–3.9), which were comparable to those of the control. The obtained data on the high chitosan biocompatibility are consistent with the results of other studies [9, 11, 14, 15] and provide justification for conducting further *in vivo* experiments to study its adjuvant activity. Importantly, the concentration of the tested chitosan preparation is significantly higher than the estimated working concentrations of adjuvants used in vaccines, suggesting a broad therapeutic range and a favourable safety profile for the preparation under study.

The inclusion of low molecular weight chitosan in vaccines against Newcastle disease and other avian infections, followed by assessment of the specific immune response, is a promising direction for further studies [4]. Future work should examine the adjuvant properties of chitosan administered by different routes (intranasal, oral, intramuscular) and its synergistic action with other immunostimulants. Further studies may be aimed at establishing the optimal working concentrations of chitosan in vaccine formulations, elucidating its impact on cellular and humoral immunity, and characterizing the duration of the resulting post-vaccination immune response.

The data obtained serve as the basis for the development of new safe and effective adjuvants based on low molecular weight chitosan. The proposed preparation meets modern requirements for the biocompatibility of immunostimulating products for use in veterinary medicine.

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On the anniversary of Olga V. Pruntova

On March 24, Olga V. Pruntova, Doctor of Biological Sciences, Professor, and Leading Researcher at the Information and Analytical Center of the Federal Centre for Animal Health, celebrated her anniversary.

Her name rightfully occupies a leading place in the scientific community. Olga V. Pruntova joined the Federal Centre for Animal Health team as a senior laboratory assistant immediately after graduating from university. Possessing a high level of training (secondary polytechnic school, Faculty of Biology of Perm State University named after A. M. Gorky, postgraduate studies, internships in leading research institutions of the country) and a genuine interest in science, Olga V. Pruntova successfully defended her dissertation for the Candidate degree in 1988 and for the Doctor degree in 2003. She is one of the creators of the first inactivated emulsion vaccines against salmonellosis and pasteurellosis in pigs registered in Russia, as well as enzyme immunoassay test-kits for detecting antibodies against *Salmonella* and *Pasteurella* in animal blood sera.

In 2006, Olga V. Pruntova headed the newly established Laboratory for the Diagnosis of Bacterial Infections in Animals. In addition to scientific research, her laboratory carried out epizootiological and serological monitoring of infections caused by bacteria of the families *Enterobacteriaceae* and *Pasteurellaceae* under state assignments and was involved in developing test-kits for detecting antibodies and antigens of these pathogens.

Her vast experience, professional skills, and strong life position contributed to the establishment of the Testing Centre of the Federal Centre for Animal Health, which she led for ten years and which subsequently received international accreditation for testing food products, feed, and products of animal origin for microbiological and physical-chemical parameters.

Olga V. Pruntova is not only a renowned scientist and a talented leader, but also a generous and wise mentor. Her gifted followers work successfully for the benefit of Russian science, particularly in the veterinary field. Under the supervision of Olga V. Pruntova, 7 candidate dissertations have been defended. For many years, she combined scientific research with teaching. She worked as a professor and head of the Biology Department at Vladimir State University, teaching such subjects as "Introductory Biology", "Ecology", "Introductory Microbiology with Fundamentals of Virology", and "Medical Microbiology", not only to future biologists, but also to students of other specializations. She continues to share her experience with young scientists: she lectures to postgraduates, participates in examination and attestation boards, acts as an academic supervisor for candidate dissertation applicants, and actively works in the dissertation council as deputy chairperson.

Her contribution to the development of Russian science is confirmed by over 300 scientific papers. These include articles in leading scientific journals, 3 monographs, textbooks and methodological guidelines, more than 20 patents and invention certificates, and abstracts of reports at scientific forums, symposia, and conferences.

For her active creative life, Olga V. Pruntova has been repeatedly awarded certificates and diplomas of various statuses and levels. In 2013, she was awarded the medal of the Federal Centre for Animal Health for her contribution to the development of the center; in 2018, she received the Rosselkhoznadzor medal for her contribution to veterinary science. For her scientific developments, she has repeatedly been among the winners of the "Golden Autumn" exhibition competition.

The entire large team of the Centre congratulates Olga V. Pruntova on her anniversary! We wish you good health, many years of active and fruitful life, inexhaustible creative energy, and well-being. May your research career lead to new outstanding achievements, and may your enthusiasm and wisdom inspire the new generation of scientists!

On the anniversary of Natalya S. Mudrak

February 8, 2026 is not only the Day of Russian Science, but also an anniversary date for Natalya S. Mudrak, Doctor of Science (Biology), Chief Researcher at the Reference Laboratory for Avian Viral Diseases at the Federal Centre for Animal Health.

Natalya S. Mudrak was born in Belebey town, Bashkir Autonomous Soviet Socialist Republic, in the family of an engineer and a teacher, who raised her to be a hardworking and determined person. In 1980, after graduating from the Faculty of Biology at the Bashkir State University, she began her career in science as a senior laboratory assistant at the All-Union Foot-and-Mouth Disease Research Institute. There she entered the postgraduate school and focused her research on the antigenic and immunogenic properties of synthetic FMDV peptides. In 1993, she successfully defended her Candidate of Science thesis and was soon appointed the Head of Laboratory for Avian Viral Disease Diagnosis. In 2010, Natalia S. Mudrak defended her Doctor of Science thesis.

The entire scientific career of Natalya S. Mudrak is dedicated to veterinary virology, development and improvement of tools and methods for diagnosing viral diseases of farm animals, based on advances in biotechnology and genetic engineering. The results of her research have made a significant contribution to the study of molecular epizootology of avian viral diseases, to the creation of a comprehensive serological monitoring system in poultry farming using domestic diagnostic kits, equipment, reagents and a computer software that ensures acquisition of reliable data on the immune status of the poultry population. Under the leadership of Dr. Mudrak and with her direct participation, enzyme-linked immunosorbent assay systems based on the indirect ELISA variant were developed and received state registration for the first time. These test systems are intended for the detection and quantification of specific antibodies to pathogens of the major economically significant infectious diseases of chickens. In order to introduce modern diagnostic methods into veterinary practice, Natalia S. Mudrak has taken much effort in equipping the workstations of regional, zonal and oblast (krai, republican) veterinary laboratories, diagnostic laboratories of business operators and veterinary institutions, providing them with ELISA and PCR-based diagnostic tools.

Dr. Mudrak has published over 300 scientific papers and has been granted 25 copyright certificates and patents for inventions of the Russian Federation.

A defining trait of Natalya S. Mudrak's character is her readiness to help everyone. Her sociability and communication skills command sincere respect from everyone she has worked with. She possesses vast experience and has the ability to identify new and promising trends

in science. Dr. Mudrak has supervised four Candidates and one Doctor of Science and continues to provide advisory, methodological, and often practical assistance to degree-seeking researchers.

Natalia S. Mudrak has been a member of the Federal Centre for Animal Health Council on the Thesis Defence for Doctor's and Candidate's Degrees since 2013 and Deputy Head of the Thesis Council since 2016.

Dr. Mudrak's scientific work in ensuring freedom from avian viral diseases has been highly appreciated. She was awarded a Certificate of Honor from the Ministry of Agriculture of Russia (2003); Federal Centre for Animal Health medal for contribution to the Centre's development (2013); Jubilee Medal "70 Years of Vladimir Oblast" (2014); Rosselkhoznadzor Commemorative Medal for the contribution to veterinary science (2018) and other awards of various levels and statuses.

The Federal Centre for Animal Health management and the entire team cordially congratulate Dr. Mudrak on her anniversary. We wish her robust health, new creative successes and achievements, happiness and well-being!

On the 75th anniversary of Vasily I. Belousov

In January 2026, Vasily I. Belousov, Doctor of Science (Veterinary Medicine), celebrated his 75th birthday.

Vasily I. Belousov was born on Druzhilinsky farm located in Serafimovichisky Raion, Volgograd Oblast, on January 4, 1951. In 1973, he graduated from the Moscow Veterinary Academy and was appointed Director of Kletsky Veterinary Laboratory of Volgograd Oblast. Over the years, he worked as a junior researcher at the Russian State Centre for Animal Feed and Drug Standardization and Quality, senior veterinarian at the Central Veterinary Laboratory of the Ministry of Agriculture of the Union of Soviet Socialist Republics, head of the Department for Serology and Leptospirosis at the Republican Scientific and Production Laboratory for Animal Diseases Control of the Ministry of Agriculture of the Russian Soviet Federative Socialistic Republic, Senior Researcher, Scientific Secretary, Head of the Laboratory for Immunology, Deputy Director for Research at the All-Russian Research and Technological Institute of Biological Industry. For more than 10 years, he was the Head of the Division for Veterinary and Sanitary Expertise and Laboratory Diagnostics of the Veterinary Department of the Ministry of Agriculture of the Russian Federation, and after the organization of the Federal Service for Veterinary and Phytosanitary Surveillance, he became the Head of the Department for Animal Product Safety and Laboratory Tests. In 2008, Vasily I. Belousov was transferred to the Central Scientific-Methodical Veterinary Laboratory as Deputy Director for Russian official veterinary laboratories relations. From 2021 to the present, Vasily I. Belousov has been working at the Federal Centre for Animal Health as Chief Researcher of the Research Coordination Department.

In 1980, Vasily I. Belousov successfully defended his thesis for the degree of Candidate of Science (Veterinary Medicine), and in 1997 – for the degree of Doctor of Science (Veterinary Medicine), in 2000 he was awarded the title of Professor.

Vasily I. Belousov is a leading expert in the field of microbiology, biotechnology as well as veterinary and sanitary expertise. His main research activities focus on the improvement and development of new technologies for veterinary medicinal products for animal disease prevention, methods of their diagnosis, safety control of food raw materials and food products of animal and plant origin as well as feed. With the participation of Vasily I. Belousov, the technology for large-scale production of combined vaccine against chlamydiosis, campylobacteriosis, salmonellosis and leptospirosis for sheep and goats, method for leptospira concentration and vaccine against leptospirosis as well as methods for ovine chlamydiosis (enzyme-linked immunosorbent assay) and leptospirosis (fluorescent immunoglobulin) diagnosis have been developed.

The Professor together with representatives of the Ministry of Economic Development, Federal Agency for Technical Regulation and Metrology of the Russian Federation and the Ministry of Health of the Russian Federation

actively participated in the interdepartmental working groups for discussion of draft federal laws in veterinary medicine and public medicine field, harmonization of Russian regulatory documents with international ones, certification of animal products and feed during the Russian Federation Veterinary Service preparation for the World Trade Organization accession.

Currently, Vasily I. Belousov is developing a procedure for laboratory control of products, facilities, activities subject to official veterinary surveillance, evaluates the foreign veterinary service performance related to control of animal products and feed supplied to Russia.

Vasily I. Belousov is the author and co-author of more than 250 scientific papers, including 7 monographs. Under the supervision of Vasily I. Belousov, 4 Doctor of Science theses and 14 Candidate of Science theses were defended, more than 30 technical regulations and methodological guidelines for the production and use of vaccines, diagnostic products and methods were prepared.

Vasily I. Belousov is a member of the Federal Centre for Animal Health Scientific Board, Council on the Thesis Defence for Doctor's and Candidate's Degrees of the Moscow State Academy of Veterinary Medicine and Biotechnology – MVA by K. I. Skryabin, a member of the Editorial Board of the "Compound feed" journal, a member of the Supervisory Board of the Association for the Development of Horse Breeding. He was a member of the Editorial Board of "Veterinary medicine" journal for more than 10 years.

Vasily I. Belousov has been awarded many medals and diplomas, and is Honoured Employee of the Federal Service for Veterinary and Phytosanitary Surveillance.

We wish you, dear Vasily I. Belousov, good health, inexhaustible energy and new scientific achievements!

VETERINARY SCIENCE TODAY JOURNAL INVITES AUTHORS TO SUBMIT THEIR PAPERS FOR PUBLICATION

Фото: Александр Плонский

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FEDERAL CENTRE FOR ANIMAL HEALTH (ARRIAH)
ФГБУ «ФЕДЕРАЛЬНЫЙ ЦЕНТР
ОХРАНЫ ЗДОРОВЬЯ ЖИВОТНЫХ»

WOAH REFERENCE LABORATORY FOR FOOT AND MOUTH DISEASE
РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ ВОЗЖ ПО ЯЩУРУ

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FAO REFERENCE CENTRE FOR FOOT-AND-MOUTH DISEASE
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CIS BASE CHAIR FOR ADVANCED TRAINING AND PROFESSIONAL RETRAINING IN THE FIELD OF ANIMAL DISEASE DIAGNOSIS AND CONTROL
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