FGBI "FEDERAL CENTRE FOR ANIMAL HEALTH" (FGBI "ARRIAH")

FEDERAL SERVICE FOR VETERINARY AND PHYTOSANITARY SURVEILLANCE (ROSSELKHOZNADZOR)

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SMALL RUMINANTS DISEASES

Clinical signs of caprine arthritis-encephalitis and disease-related pathomorphological changes p. 126

Bovine leukemia virus occurrence in Dagestan p. 111

Efficiency of the data generated by the robotic milking system for comprehensive diagnosis of mastitis in cows p. 119

Inactivation of foot and mouth disease virus for vaccine production p. 164

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Contents

Содержание

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History of the FGBI "ARRIAH" international cooperation on foot-and-mouth disease (on occasion of the 65th anniversary of the Institute)

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SUMMARY

The paper covers the long history of the FGBI "ARRIAH" international cooperation on foot-and-mouth disease, starting from 1964 when its name was the All-Union Foot-and-Mouth Disease Research Institute. Foot-and-mouth disease was the main focus of the Institute's research activities. Under of the auspices of the Institute, a Coordination Board was established. It consisted of the specialists of the veterinary services, research and educational institutions from all the republics of the USSR. A common research programme aimed at the development and implementation of effective tools and methods for FMD control was worked out. The next stage of international cooperation was related to the functioning of the Council for Mutual Economic Assistance (CMEA or COMECON). Starting from 1977, the Institute coordinated the CMEA member countries' research activities on 11 topics of the FMD issue, held meetings on the subject, as well as meetings of the Board of Commissioners. After formation of the Commonwealth of Independent States (CIS), the Institute developed the long-term "Programme of joint activities of the CIS member states for the prevention and control of foot-and-mouth disease in the CIS member states" (2004). Later on, the Programme was repeatedly altered and extended to address new priorities, with the Institute undertaking the coordinating role. The "Set of joint measures of the CIS member states for the prevention and control of foot-and-mouth disease for the period up to 2025" has now been adopted, and the FGBI "ARRIAH" also performs the functions of the base organization of the CIS member states for the advanced training and retraining in animal disease diagnosis and control. At present, much of the FGBI "ARRIAH" international cooperation on FMD takes place through its acting as the WOAH Reference Laboratory for Foot-and-Mouth Disease and the FAO Reference Centre for Foot-and-Mouth Disease.

Keywords: review, foot-and-mouth disease, international cooperation, FGBI "ARRIAH"

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История становления в ФГБУ «ВНИИЗЖ» международного сотрудничества по ящуру (к 65-летию института)

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РЕЗЮМЕ

Материалы статьи освещают многолетнюю историю осуществления международного сотрудничества по ящуру в ФГБУ «ВНИИЗЖ» начиная с 1964 г., когда учреждение еще носило название «Всесоюзный научно-исследовательский ящурный институт». Научная тематика по ящуру была главенствующей для учреждения, при институте был создан Координационный совет, в состав которого входили специалисты ветеринарных служб, исследовательских учреждений и учебных институтов из всех союзных республик СССР, прорабатывалась единая научная программа, направленная на разработку и внедрение эффективных средств и методов борьбы с ящуром. Следующий этап международного сотрудничества связан с функционированием Совета экономической взаимопомощи (СЭВ), когда институт с 1977 г. координировал исследования по 11 темам проблемы ящура стран – членов СЭВ, проводил совещания по тематике и заседания Совета уполномоченных. С созданием Содружества Независимых Государств (СНГ) учреждением в 2004 г. была разработана долгосрочная «Программа совместных действий государств – участников СНГ по профилактике и борьбе с ящуром в государствах Содружества», которая затем неоднократно изменялась и продлялась с появлением новых приоритетных целей, при этом координирующая роль отводилась институту. В настоящее время принят «Комплекс совместных мер государств – участников СНГ по профилактике и борьбе с ящуром на период до 2025 года», а ФГБУ «ВНИИЗЖ» выполняет также функции базовой организации государств – участников СНГ по повышению квалификации и переподготовке кадров в области диагностики и контроля болезней животных. Существенную роль в международном сотрудничестве института по ящуру на современном этапе играет исполнение функций Референтной лаборатории ВОЗЖ по ящуру, а также Референтного центра ФАО по ящуру.

Ключевые слова: обзор, ящур, международное сотрудничество, ФГБУ «ВНИИЗЖ»

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The analysis of the centuries-old experience in combating foot-and-mouth disease in the world has given full grounds to the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (WOAH, founded as OIE) to define this problem in the developed Global FMD Control Strategy (2012) as one of the classic global problems affecting animal farming industry, impeding free and safe movement of animal products, requiring huge and constant governmental and private investments in animal health protection in enzootic and disease free regions. That is, practically all countries, whether foot-and-mouth disease affected or free, have to bear the costs associated either with direct losses from the disease or expenditures for measures to prevent it [1].

The same position is clearly formulated in the final lines of the monograph "Foot-and-mouth disease" by the outstanding German scientist H. Röhrer: "... In order to achieve the set goal – the complete elimination of epizootics (of foot-and-mouth disease) ... with international relations growing and reviving everywhere, an effective fight against epizootics on the globe should become a common cause" [2].

Precisely in order to develop a scientifically sound system of foot-and-mouth disease control measures with uniform methodological guidance and unified coordination of research activities, Resolution of the Central Committee of the Communist Party of the Soviet Union and the Council of Ministers of the USSR No. 909-426 of 7 August 1958 was adopted. According to the Resolution, the Ministry of Agriculture of the USSR was to establish the All-Union Foot-and-Mouth Disease Institute with an experimental laboratory for the testing of biological products against highly dangerous infections in the RSFSR on the basis of the existing research and educational institutions and state-owned farms in 1958–1960 [3–8].

By the time of the Institute's establishment and launching (1962), the number of foot-and-mouth disease affected localities in the country annually amounted to hundreds and thousands (type A_7 FMD in 1951–1954 and type A_{22} FMD in 1964–1968). Before the Institute was established, more than 30 research institutions of the country had been studying various aspects of foot-and-mouth disease due to the urgency of this issue [9].

In June 1964, the All-Union Foot-and-Mouth Disease Research Institute (the new name of the Institute according to Order of the Ministry of Agriculture of the USSR No. 225 of 25 November 1960) hosted the first coordination meeting on this issue with participation of the representatives of 22 research and educational institutions, veterinary services. The meeting addressed the outcomes of research activities for 1963 and research activity plans for 1964–1965. The Institute was tasked with the coordination of foot-and-mouth disease research activities in the country.

In 1966, the Ministry of Agriculture of the USSR included the topic "Eradication of foot-and-mouth disease in the country" in the plan of research activities, and the All-Union Foot-and-Mouth Disease Research Institute was designated as the lead institution in that respect starting from January 1966. The following institutions were involved in addressing the issue:

– 16 research institutes of the republics of the Soviet Union;

– 8 veterinary research stations (those of Novosibirsk, Altai, Kuybyshev, Kursk, Voronezh, Gorky, Irkutsk, Saratov);

– in 1970, they were joined by the Transcaucasian (Yerevan, Armenia) and Central Asian (Dushanbe, Tajikistan) branches of the All-Union Foot-and-Mouth Disease Research Institute.

Under the auspices of the All-Union Foot-and-Mouth Disease Research Institute, a Coordination Board was established, which included the heads and specialists of the veterinary services of the USSR republics, researchers from research and educational institutes, staff members of establishments dealing with the foot-and-mouth disease issue. The meetings of the Board were held annually starting from 1966. The Board was headed by professor V. P. Onufriyev, the Director of the All-Union Foot-and-Mouth Disease Research Institute.

The common scientific programme for the Development and implementation of highly effective tools and a scientifically based system of measures for the control of foot-and-mouth disease in livestock taking into account zonal animal husbandry systems was worked out.

The meetings of the Coordination Board had obvious scientific significance and were also very important for furthering the relevant knowledge, especially for the early career staff members of the Institute, as they provided an opportunity to come into direct contact with practical problems, get acquainted with the country's leading foot-and-mouth disease experts. In particular, the following topics were addressed during the meetings: issues of regional epizootology, progress in foot-and-mouth disease vaccine development made by the All-Union Institute of Experimental Veterinary Medicine (VIEV) and the Soviet State Scientific and Control Institute for Veterinary Drugs Control (VGNKI), the activities of the VGNKI anti-epizootic mission led by I. A. Rostovtseva, etc. There were, however, some unsuccessful experiments and scientifically unsubstantiated statements, such as studies of

the non-aphthous (intestinal) form of foot-and-mouth disease and the transformation of one type of the virus into another in the refrigerator, the possibility of the virus typing "by eye", the failed experience of live foot-and-mouth disease vaccine development using cold mutants of the virus and attenuated strains.

The last, as it turned out, meeting of the Coordination Board was held in April 1991. During the meeting, the overall outcomes were summarized and a significant improvement of the foot-and-mouth disease situation in the USSR was acknowledged, which was a natural consequence of the immense work undertaken.

The next stage in the development of the Institute's international cooperation on foot-and-mouth disease was associated with the member countries of the Council for Mutual Economic Assistance (CMEA or COMECON). On 14 September 1974, the Ministries of Agriculture of the People's Republic of Bulgaria, the Hungarian People's Republic, the German Democratic Republic, the Republic of Cuba, the Mongolian People's Republic, the Polish People's Republic, the Socialist Republic of Romania, the USSR and the Czechoslovak Socialist Republic concluded the Agreement on the establishment of reference centres for the most important strains of viruses, bacteria and phages, their respective antigens and control sera, as well as certain laboratory chemicals, and in December of the same year – the Intergovernmental agreement on coordination of research activities and the establishment of reference centres in the topical areas of agricultural science, which included the Agreement on scientific and technical cooperation of the CMEA member countries for the prevention and effective control of foot-and-mouth disease, as well as the development of highly effective foot-and-mouth disease vaccines. The functions of the coordination centre were assigned to the VGNKI, and starting from September 1977 – to the All-Union Foot-and-Mouth Disease Research Institute, where a special unit – the Museum of strains with the coordination centre working group (A. I. Gritsenko, I. V. Zubov, V. M. Zakharov, L. A. Globenko) was established in 1978.

The first programme of cooperation for 1976–1980 was adopted in December 1975 at the first meeting of the Board of Commissioners (during all those years, the USSR was represented by Deputy Head of the Main Veterinary Department of the Ministry of Agriculture of the USSR P. P. Rakhmanin). The programme included 11 topics and involved 22 institutions of 9 CMEA member countries. The agreement provided for the coordination of activities carried out by national organizations on the agreed topics, according to the joint work plans, taking into account the mutual interest of the parties. During the coordination process, information was exchanged, mutual consultations and practical trainings were held.

The cooperation results were discussed at the annual meetings of the Board of Commissioners (Fig. 1). Every year, the coordination centre held meetings on specific topics of the foot-and-mouth disease issue, organized symposiums and seminars, publication of scientific materials [10].

During the symposiums, the cooperation outcomes were summed up, the key research activities for the upcoming five years were formulated. For example, the Detailed programme for 1986–1990 was developed, which was subsequently (at the meeting of the Board of Commissioners, Mongolia, 1988) transformed into the target project "Development and cooperative production of veterinary medicinal products for the prevention and control of foot-and-mouth disease", with the All-Union Foot-and-Mouth Disease Research Institute as the lead institution. The following activities were planned: studies on the molecular biology and genetics of foot-and-mouth disease virus, the development of technology and manufacture of diagnostics, in particular those based on monoclonal antibodies, the application of new adjuvants and inactivants in vaccine production, the establishment

Fig. 1. Participants of the meeting of the Board of Commissioners of the CMEA member countries on foot-and-mouth disease, Moscow, 1975 (V. P. Onufriyev is in the centre of the first row)

of a bank of vaccines, including dry shelf-stable ones, for the CMEA member countries at the facilities of the All-Union Foot-and-Mouth Disease Research Institute.

Based on the joint research activity results, a number of documents were developed, such as the CMEA standards for foot-and-mouth disease diagnosis methods and tools, Requirements for inactivated vaccines against foot-and-mouth disease, Instructions for foot-and-mouth disease control at pig breeding complexes, Recommendations for foot-and-mouth disease prevention and control at large livestock complexes, Draft measures to prevent the introduction of exotic foot-and-mouth disease types.

The main positive outcomes of the CMEA member countries' cooperation on foot-and-mouth disease include:

– maintenance of sustainable foot-and-mouth disease freedom of most CMEA member countries;

– acquisition of valuable experience in international cooperation on foot-and-mouth disease with foreign partners, including on a bilateral interstate basis;

– detailed familiarization of the researchers of the All-Union Foot-and-Mouth Disease Research Institute with foot-and-mouth disease diagnosticum and vaccine development and production at the facilities of the leading European foot-and-mouth disease centres (primarily in Czechoslovakia, Hungary, Romania);

– assembly of a large collection of production and epizootic strains of foot-and-mouth disease virus, which still makes up the "golden fund" of the Institute's museum of microorganism strains.

The working meetings on various topics of cooperation, symposiums, meetings of the Board of Commissioners held annually alternately in each cooperating country enabled the researchers of the Institute to establish personal and scientific contacts with foreign specialists and significantly expand their horizons.

But the main outcome was that the All-Union Foot-and-Mouth Disease Research Institute convincingly confirmed the high scientific level of its research activities, demonstrated the ability to ensure a clear coordination of comprehensive efforts with respect to the foot-and-mouth disease issue at both national and international levels, acquired the relevant organizational experience, gained international recognition and authority, which subsequently became the most important factors in the development of the Institute.

However, with the dissolution of the USSR in 1991, international cooperation in this direction ceased.

With the formation of the Commonwealth of Independent States (CIS) in 1991 and the termination of the functioning of the All-Union veterinary structures of the USSR, which had ensured the centralized implementation of the unified policy in the organization of anti-epizootic measures, the epizootic situation in the newly formed sovereign states worsened, which raised the question of the need for anti-epizootic measure coordination in the post-Soviet space.

The heads of the governments of 10 CIS member states (Armenia, Belarus, Kazakhstan, Kyrgyzstan, Moldova, Russia, Tajikistan, Turkmenistan, Uzbekistan and Ukraine) signed the Agreement on cooperation in the veterinary field[1] in Moscow on 12 March 1993. The agreement

Fig. 2. Leaders of the seminar: N. Belev (in the centre), Director of the FGBI "ARRIAH" V. A. Gruby (to his right)

served as a basis for launching joint activities for the control and prevention of highly dangerous animal diseases. Based on this agreement, the Intergovernmental Council for Cooperation in the Veterinary Field was established in the same 1993. During the meetings of the Council (Tbilisi, October 1996; Yerevan, October 1997; Alma-Ata, April 1998), the Institute, renamed the All-Russian Research Institute for Animal Health (ARRIAH) in 1992, repeatedly raised the question of the need for coordination of foot-and-mouth disease control measures in the CIS member countries [11–14].

Entrusted by the Intergovernmental Council for Cooperation in the Veterinary Field, the FGI "ARRIAH", with participation of the veterinary services of the CIS countries, developed the Programme of joint activities of the CIS member states for the prevention and control of foot-and-mouth disease in the CIS member states[2] for the period up to 2010. On 16 April 2004, the programme was approved by a decision of the Council of the CIS Heads of Governments (Cholpon-Ata, the Kyrgyz Republic).

The main goal of the programme was to ensure foot-and-mouth disease freedom of each member state and the Commonwealth as a whole. Its implementation became a practical measure for foot-and-mouth disease prevention and control in the CIS member states, allowing to minimize economic damage in case of an outbreak. On the basis of this programme, most of the CIS member states developed and adopted national programmes, rules (instructions) for foot-and-mouth disease prevention and control. The progress of the programme implementation was periodically addressed at the meetings of the Intergovernmental Council for Cooperation in the Veterinary Field.

The main foot-and-mouth disease control activities undertaken in the Russian Federation in 2007–2010 included:

– expert missions of the FGI "ARRIAH", the FGI "Veterinary Centre", the Rosselkhoznadzor, the Veterinary Department of the Ministry of Agriculture of the Russian Federation to different regions of the Russian Federation and other countries for the implementation of epizootological monitoring and participation in foot-and-mouth disease control activities;

[1] Agreement on cooperation in the veterinary field. Available at: https://fsvps.gov.ru/ru/fsvps/laws/203.html. (in Russ.)

[2] Programme of joint activities of the CIS member states for the prevention and control of foot-and-mouth disease in the CIS member states. Available at: https://e-ecolog.ru/docs/4ba3bDCZMR2FMLUJRmuS2/77. (in Russ.)

– manufacture of various diagnostic reagents and the complement fixation test, ELISA, etc. test kits by the FGI "ARRIAH" and their supplies to the Russian Federation regions, Azerbaijan, Armenia, Kazakhstan, Kyrgyzstan, Uzbekistan, Belarus, Mongolia;

– tests of pathological material and serum samples from animals from the Russian Federation, Kazakhstan, Kyrgyzstan, Tajikistan, Mongolia carried out at the FGI "ARRIAH";

– practical training of the veterinary specialists from Kazakhstan, Kyrgyzstan, Tajikistan, Poland at the FGI "ARRIAH";

– seminars, advanced training courses on animal foot-and-mouth disease diagnosis, prevention and control measures under modern conditions (the Russian Federation, Belarus, Kazakhstan, Kyrgyzstan, Moldova) [15–24].

As part of implementation of one of the programme items, in accordance with the Decision of the Intergovernmental Council for Cooperation in the Veterinary Field of 27 October 2010, joint foot-and-mouth disease response simulation exercises of the veterinary services of the Republic of Belarus, the Russian Federation and Ukraine were held on 16–18 June 2011. The exercises were also attended by the representatives of the veterinary services of Moldova and Tajikistan. The simulation exercises took place in the Gomel Oblast (Belarus), the Bryansk Oblast (the Russian Federation), the Chernigov Oblast (Ukraine), where preventive vaccination of animals against foot-and-mouth disease was not practised.

On 15–17 June 2011, the FGBI "ARRIAH" hosted a seminar led by Professor N. Belev, the President of the OIE Regional Commission for Europe. The seminar was aimed at giving insight into the World Animal Health Information System (WAHIS) operation principles (Fig. 2). The seminar was attended by the representatives (national coordinators) of 23 countries of Europe, Central Asia and Transcaucasia. The seminar was prompted by the introduction of new requirements for the submission of information on the epizootic situation in the countries to the OIE.

After the implementation of the programme, many of its activities were recognized as relevant for subsequent years. In the light of that fact, the Intergovernmental Council for Cooperation in the Veterinary Field recommended that the FGBI "ARRIAH" should develop the Set of joint measures of the CIS member states for the prevention and control of foot-and-mouth disease for the period up to 2020. The document was endorsed at the meeting of the Intergovernmental Council for Cooperation in the Veterinary Field held at the FGBI "ARRIAH" (Vladimir, 5 April 2013). Following a thorough discussion at the meeting of the CIS Economic Council on 13 December 2013, the Set of joint measures was approved by a decision of the Council of the CIS Heads of Governments (Minsk, 30 May 2014). The FGBI "ARRIAH" was designated as the coordinator of the activities.

The Set of joint measures included the in-depth analysis of the foot-and-mouth disease epizootic situation in the CIS member states and other countries of the world, the forecast of the risk of foot-and-mouth disease introduction, occurrence and spread in the CIS member states, the identification of regions with high risk of foot-and-mouth disease introduction and spread, measures for foot-and-mouth disease prevention and control in the CIS member states, proposals regarding measures aimed to improve the professional competencies of veterinary specialists, to coordinate the joint activities of the veterinary services of the CIS member states and international organizations (the OIE, the FAO, the EU) for the control of foot-and-mouth disease in animals, the assessment of the expected effectiveness of the activities planned.

An important point in the cooperation of the CIS countries was the decision taken following the meeting of the CIS Economic Council (Minsk, 21 June 2019) to grant the Federal State-Financed Institution "Federal Centre for Animal Health" the status of the base organization of the CIS member states for advanced training and retraining of personnel in animal disease diagnosis and control.

The main tasks of the base organization were defined as follows:

– advanced training and professional retraining of specialists in diagnosis and control of animal diseases;

– studying, summing up, dissemination of experience in personnel training in animal disease diagnosis and control;

– facilitation of dissemination of modern methods for training in animal disease diagnosis and control;

– coordination of the development and implementation of joint innovative educational and research programmes with relevant research institutions of the CIS member states;

– organization of comparative and applied studies of the problems of personnel training in animal disease diagnosis and control.

The granting of the base organization status to the FGBI "ARRIAH" contributed to the development of professional competencies of veterinary specialists and assurance of animal disease freedom in the CIS member states.

The meeting of the Intergovernmental Council for Cooperation in the Veterinary Field (Minsk, 11 October 2019) commended the outcomes of the programme activities. It was decided to extend the activities listed in the Set of joint measures until 2025, and the FGBI "ARRIAH" was tasked with the preparation of the relevant draft document taking into account the proposals of the member states.

The draft document was endorsed at the meeting of the Intergovernmental Council for Cooperation in the Veterinary Field and approved by a decision of the Council of the CIS Heads of Governments (Minsk, 28 May 2021). At the same time, it was stated that the progress made in the previous years allowed for the continuation of efforts to achieve foot-and-mouth disease freedom and eradicate the disease in the CIS countries. The implementation of the Set of joint measures of the CIS member states for the prevention and control of foot-and-mouth disease for the period up to 2025 will ensure the stability of the epizootic situation in the CIS member states, the effectiveness of implemented veterinary and sanitary measures and foot-and-mouth disease freedom of the CIS member states, the development of livestock industry and unhindered international trade.

On the whole, the Set of joint measures envisages the progressive expansion and enhancement of cooperation between the veterinary services of the CIS countries in the implementation of joint activities for the prevention and eradication of foot-and-mouth disease. During the entire period of consistent execution of this Set of

measures, the following main stages should be implemented:

– improvement of regulatory and communication framework for the activities;

– organization of the implementation of adopted regulatory documents and exchange of information concerning changes in the foot-and-mouth disease epizootic situation in each CIS member state;

– implementation of joint activities for foot-and-mouth disease monitoring, prevention and eradication in the CIS member states.

A separate aspect of the CIS countries' cooperation on foot-and-mouth disease is the interaction between the Eurasian Economic Union (EAEU) member states in the prevention, diagnosis, containment and eradication of outbreaks of highly dangerous, quarantinable and zoonotic animal diseases. The EAEU member states are Armenia, Belarus, Kazakhstan, Kyrgyzstan, Russia; Moldova, Uzbekistan, Cuba are observers. The Treaty on the Eurasian Economic Union, which entered into force on 1 January 2015, provides for the free movement of commodities, services, capital and labour, the implementation of coordinated, agreed or unified policy in economic sectors. The draft Procedure for the application of common principles and rules for the prevention, containment and eradication of foot-and-mouth disease outbreaks in the Eurasian Economic Union member states was developed (2017–2022).

As early as in the initial period after the dissolution of the USSR, in addition to cooperation with the CIS countries, the question came up regarding the establishment of a more intense interaction on foot-and-mouth disease with the European countries, the development of close-knit international cooperation with the main European institutions dealing with the foot-and-mouth disease issue within the framework of the FAO European Commission for the Control of Foot-and-Mouth Disease (EuFMD), as well as under the auspices of the OIE.

A fundamental event in this regard was the international scientific conference "Towards a new foot-and-mouth disease control strategy" with participation of the leading European foot-and-mouth disease experts, which was held in Vladimir and Suzdal in October 1991. The conference demonstrated the progress made by the researchers of the All-Union Foot-and-Mouth Disease Research Institute in foot-and-mouth disease vaccine improvement, giving grounds to really talk about a new strategy for combating the disease [25]. The conference allowed for the establishment of scientific contacts with specialists from Great Britain, France, Italy, Denmark, which became the prologue for granting the Institute an international status.

At the third meeting of the Intergovernmental Council for Cooperation in the Veterinary Field (Krasnodar, April 1993), it was decided that it would be appropriate to establish the OIE Reference Centre for Foot-and-Mouth Disease for Eastern Europe. In view of that, an application for the establishment of such centre at the FGBI "ARRIAH" was submitted to the International Committee of the OIE.

Taking into account the huge contribution of the Institute to the development and implementation of the set of measures to eradicate foot-and-mouth disease in the Russian Federation and the CMEA member countries, the OIE, at the 63rd General Session (1995), acknowledged the Institute's services as a scientific and methodological centre and granted it the status of the OIE Regional Reference Laboratory for Foot-and-Mouth Disease for Eastern Europe, Central Asia and Transcaucasia.

In August 2013, the FAO Animal Health Service designated the Institute as the FAO Reference Centre for Foot-and-Mouth Disease for Central Asia and Western Eurasia. In November 2020, this status was changed to a broader one – the FAO Reference Centre for Foot-and-Mouth Disease. Previously, only four research centres had been granted such status for foot-and-mouth disease by both the OIE and the FAO: the Plum Island Animal Disease Center (the USA), the Pirbright Institute (the UK), the University of Brescia (Italy) and the Onderstepoort Veterinary Institute (South Africa).

The following were the OIE FMD Experts in different years: Zh. A. Shazhko, Professor, Doctor of Science (Veterinary Medicine), 1995; S. A. Dudnikov, Candidate of Science (Veterinary Medicine), 1996–1997; V. M. Zakharov, Professor, Doctor of Science (Veterinary Medicine), from 1998 to the present.

The Institute's functions as the OIE/FAO Reference Centres for Foot-and-Mouth Disease include:

– assessment and standardization of test methods for foot-and-mouth disease;

– storage and distribution of reference biologicals and other reagents used for foot-and-mouth disease diagnosis and control;

– development of new foot-and-mouth disease diagnosis and control methods;

– collection, processing, analysis and dissemination of information on the epizootology of the disease;

– provision of expert consultants to the OIE/FAO;

– scientific and technical training of personnel from the OIE/FAO member countries;

– organization of scientific meetings on behalf of the OIE/FAO;

– coordination of scientific and technical research activities in cooperation with other laboratories (organizations);

– publication and dissemination of any information on foot-and-mouth disease that may be useful for the OIE/FAO member countries.

Reports on the activities of the OIE/FAO Regional Reference Laboratories and Collaboration Centres, which include the main outcomes of their activities, the number of workshops and conferences held, as well as the staff members' publications over the past year, are submitted annually.

An example of execution of these functions is cooperation carried out in 2006–2007 under the Altandi 1 and 2 international agreement with the Veterinary and Agrochemical Research Centre (Belgium) to study the correlation between blood antibody titres in foot-and-mouth disease vaccinated animals and their resistance to challenge with type O, A and Asia-1 foot-and-mouth disease virus. Based on the results of the studies, several papers were published in foreign journals, and the identified estimates are currently used in the European Pharmacopoeia [26–28].

Under the agreement between the FAO and the FGBI "ARRIAH" (Agreement PO No. 303836 – EuFMD of 19 June 2013, valid until 31 December 2013), the Institute received funding for experiments to study the clinical signs of foot-and-mouth disease in wild boars, determine the virus carrier state duration in foot-and-mouth

Fig. 3. Commemorative Medal of the FAO European Commission for the Control of Foot-and-Mouth Disease

disease-recovered animals, validate non-invasive sampling methods for foot-and-mouth disease virus genome detection.

Between 2002 and 2013, a large number of research activities were carried out together with the US scientific institutions through the International Science and Technology Center (ISTC), an intergovernmental organization. They were aimed at studying recovered foot-and-mouth disease virus isolates and some other viral animal disease agents. The experience of joint research activities, our staff members' visits to the leading foreign laboratories, joint publications of the results obtained contributed to the professional development of the ARRIAH specialists.

In connection with the termination of the ISTC activities in April 2015, a memorandum was signed on the transfer of the equipment obtained for use in the research activities within all the projects to the FGBI "ARRIAH" as gratuitous technical assistance, which allowed strengthening the Institute's capacities for conducting research at the state-of-the-art methodological level.

In view of the periodic occurrence of foot-and-mouth disease in the Siberian and Far Eastern regions, cooperation with China and Mongolia within the FAO Programme of technical cooperation "On cross-border trade and reduction of the risk of transboundary animal diseases (with a particular focus on foot-and-mouth disease)" was very important for the Russian Federation.

Intense cooperation with Mongolia in the field of foot-and-mouth disease control began back in 2011, when, pursuant to Decree of the Government of the Russian Federation No. 1427-rp of 12 August 2011, humanitarian assistance was provided to Mongolia by supplying 37 million doses of foot-and-mouth disease vaccine. The epizootological study of foot-and-mouth disease in Mongolia over an extended period of time allowed for the country's territory zoning based on the infection introduction risk level. The zoning was approved by Decree of the Government of Mongolia No. 247 of 7 August 2011. The country adopted the Programme for improving the health of livestock and expediting the work of the veterinary service of Mongolia. The Programme stipulated that foot-and-mouth disease-related activities would be implemented in three stages: the first stage – 2012, the second stage – from 2017 to 2018, the third stage – from 2019 to 2021. The activities included foot-and-mouth disease vaccine supplies on a contractual basis and immunity monitoring tests [29].

In 2019, the Ministry of Agriculture of the Russian Federation received a proposal from the Ministry of Food, Agriculture and Light Industry of Mongolia regarding the activities within the third stage of the programme for 2019–2021, which included:

– foot-and-mouth disease vaccine supplies;

– animal serum tests for the detection of possible foot-and-mouth disease virus circulation;

– provision of advice on the organization of operation of the Sangino biofactory (Mongolia);

– training of the biofactory's foot-and-mouth disease specialists at the FGBI "ARRIAH" postgraduate school;

– short-term training seminars for Mongolian specialists;

– periodic meetings of Mongolian and Russian specialists.

At the meeting of the heads of the veterinary services of the countries with participation of the FAO representatives (Vladimir, 23–25 January 2013), an Agreement of intent for cooperation in the field of animal health and quarantine was signed, which provided for the following:

– establishment of a mechanism for exchange of information on animal health situation;

– cooperation in the field of prevention and control;

– joint research activities for the improvement of methods for animal disease diagnosis.

In the period from 11 to 13 October 2016, the FGBI "ARRIAH" hosted the 6th Meeting on cross-border trade and reduction of the risk of transboundary animal diseases, which was held between China, Mongolia and Russia under the auspices of the FAO. The meeting was attended by the high-ranking representatives of the veterinary authorities of China, Mongolia and Russia, as well as highly professional teams from each country comprising laboratory experts, epizootologists, technical experts and representatives of aimags/provinces. It focused on strengthening cooperation and information exchange between the three participating countries: China, Mongolia and Russia. During the meeting, particular attention was given to five transboundary diseases: foot-and-mouth disease, African swine fever, highly pathogenic avian influenza, peste des petits ruminants and lumpy skin disease.

The information above only covers the history of the main areas of the Institute's international cooperation on foot-and-mouth disease. At present, intense cooperation is carried out on a multilateral, as well as bilateral basis in relation to the introduction into practice of the Institute's scientific developments with respect to foot-and-mouth disease and the marketing of foot-and-mouth disease diagnostica and vaccines.

The Institute's active international cooperation on foot-and-mouth disease has repeatedly been acknowledged by the agricultural authorities of the country, the World Organisation for Animal Health (WOAH, founded as OIE), the FAO Veterinary Service, the CIS Intergovernmental Council for Cooperation in the Veterinary Field. In particular, following the 19th Russian Agro-Industrial Exhibition "Golden Autumn" held in Moscow in October 2017, the Institute was awarded a gold medal for the development and implementation of the international project "The Set of joint measures of the CIS member states for the prevention and control of foot-and-mouth disease for the period up to 2020". But of particular significance is the commemorative medal dedicated to the 50th anniversary of the FAO European Commission for

the Control of Foot-and-Mouth Disease, awarded to the Institute as one of the world's five leading foot-and-mouth disease institutions in November 2004 with the wording "in appreciation of the outstanding contribution to the control of foot-and-mouth disease in Europe through leadership in technical areas and for the provision of services that helped combat the disease in the regions of the world where the foot-and-mouth disease situation posed a great threat to European countries" (Fig. 3).

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Bovine leukemia virus occurrence in Dagestan

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SUMMARY
Results of diagnostic tests for bovine leukosis carried out in the Dagestan Republic in 2022 (as of October 1, 2022) showing bovine leukemia virus (BLV) occurrence are presented in the paper for the whole Republic and for each municipality separately. In total, 632,454 susceptible animals were serologically tested with immunodiffusion assay; 3,573 reactor animals (0.6% of tested animals) were detected. Proportion of infected animals was as follows: 0.5% – in administrative raions, 1.6% – in urban districts and 1.1% – in distant pasture zones. Percentage of infected cattle varied from 0.01 to 4.9%. No hematological examinations for bovine leukosis were carried out because seropositive animals were timely culled. In the breeding sector, the proportion of bovine leukemia virus carriers was averagely 2.8%. Ninety-five bovine leukosis-affected localities were reported in the region as of 1 January 2022. Eighty-one new BLV-infected localities had been identified and bovine leukosis had been eliminated in 18 localities for 9 months of 2022. Totally, 158 localities were officially declared affected in 2022 (as of October 1, 2022): 36 agricultural holdings (including 5 breeding holdings), 18 small-scale farms and 104 backyard farms. The largest number of bovine leukosis-affected localities was registered in the Kizlyarsky (18), Tarumovsky (17), Babayurtovsky (16), Gunibsky (15), Tlyaratinsky (10) Raions and in the city of Makhachkala (9). One disease-affected locality was reported in each of the Bezhtinsky, Buynaksky, Derbentsky, Kazbekovsky, Kayakentsky, Kizilyurtovsky, Khasavyurtovsky Raions and towns of Khasavyurt and Yuzhno-Sukhokumsk. Two disease-affected localities were reported in each of the Rutulsky, Untsukulsky Raions, three disease-affected localities were reported in each of the Gergebilsky, Laksky, Novolaksky, Tsumadinsky Raions. Four disease-affected localities were reported in the Sergokalinsky Raion, five disease-affected localities were reported in the Charodinsky Raion, six disease-affected localities were reported in each of the Akhvakhsky, Dakhadaevsky, Karabudakhkentsky Raions, seven disease-affected localities were reported in each of the Botlikhsky, Kumtorkalinsky and Shamilsky Raions. Comparative analysis of serological and molecular genetic methods used for bovine leukosis diagnosis demonstrated the advantage of enzyme-linked immunosorbent assay and polymerase chain reaction as compared to immunodiffusion assay used in veterinary practice.

Keywords: bovine leucosis, bovine leukemia virus, occurrence, immunodiffusion assay, enzyme-linked immunosorbent assay, polymerase chain reaction, Republic of Dagestan

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Степень распространения вируса лейкоза крупного рогатого скота в Дагестане

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РЕЗЮМЕ
Изложены результаты диагностических исследований на лейкоз в Республике Дагестан в целом и отдельных муниципалитетах за 2022 год (по состоянию на 1 октября), характеризующие распространенность вируса бычьего лейкоза. Всего за анализируемый период подвергнуто серологическому исследованию в реакции иммунодиффузии 632 454 восприимчивых животных, выявлено 3573 положительно реагирующие особи, что составляет 0,6% от числа исследованных. Инфицированность животных в административных районах была равна 0,5%, городах – 1,6%, зонах отгонного животноводства – 1,1%. Степень зараженности поголовья скота варьировала от 0,01 до 4,9%. Ввиду своевременной выбраковки серопозитивных животных гематологические исследования на лейкоз не проводились. В племенном секторе носительство вируса лейкоза определили в среднем в 2,8% случаев. На 01.01.2022 в регионе было зарегистрировано 95 неблагополучных по лейкозу пунктов. За 9 месяцев 2022 г. выявили 81 новый пункт, оздоровили 18 и по состоянию на 1 октября неблагополучными официально объявлены 158 пунктов, в том числе на сельхозпредприятиях – 36 (из них 5 племхозы), в крестьянских (фермерских) – 18 и личных подсобных хозяйствах – 104. Наибольшее количество неблагополучных по лейкозу пунктов зафиксировано в Кизлярском (18), Тарумовском (17), Бабаюртовском (16), Гунибском (15), Тляратинском (10) районах и г. Махачкале (9). В Бежтинском участке, Буйнакском, Дербентском, Казбековском, Каякентском, Кизилюртовском, Хасавюртовском районах и городах Хасавюрте и Южно-Сухокумске регистрировалось по 1 очагу, в Рутульском, Унцукульском районах – по 2; в Гергебильском, Лакском, Новолакском, Цумадинском – по 3; в Сергокалинском – 4; в Чародинском – 5, в Ахвахском, Дахадаевском, Карабудахкентском – по 6; в Ботлихском, Кумторкалинском и Шамильском районах – по 7 очагов. При сравнительном

анализе серологических и молекулярно-генетических методов диагностики бычьего лейкоза установлено преимущество иммуноферментного анализа и полимеразной цепной реакции относительно применяемой в ветеринарной практике реакции иммунодиффузии.

Ключевые слова: лейкоз, вирус лейкоза крупного рогатого скота, распространение, реакция иммунодиффузии, иммуноферментный анализ, полимеразная цепная реакция, Республика Дагестан

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INTRODUCTION

Bovine leukosis (BL) is a chronic infectious disease caused by RNA-bovine leukaemia virus (BLV) belonging to *Retroviridae* family, *Deltaretrovirus* genus. The infection is widespread in many countries and remains an urgent problem in most Subjects of the Russian Federation. The numbers of diseased and infected animals have been reduced in many regions of the country owing to timely targeted preventive and elimination measures [1–3].

Losses caused by viral BL in animal industry are huge due to animal mortality rate, reduced performance, required elimination of diseased animals, destruction of animal carcasses and various organs with leukemic lesions, underproduction of young animals, costs for milk pasteurization, reduced sales of young animals, failed animal breeding activities [3–6].

According to the Information Analysis Centre of the Veterinary Surveillance Department (FGBI "ARRIAH") data, in 2021 viral BL was reported in 65 Subjects of the Russian Federation. A total of 2,070 affected localities were detected and 25 localities had remained affected since 2020. Totally 1,398.704 thousand animals were subjected to hematological examinations, 15,096 reactor animals (1.1%) were detected, 15,611 animals were sent to slaughter [7].

Since the official BL reporting (starting with the mid-60s of the last century) as a nosological disease and the use of agar gel immunodiffusion assay (AGID) for serological identification of the pathogen starting with the late 80s of the last century there has been a steady increase in the number of infected and diseased animals in the Republic of Dagestan. Therewith, numerous attempts to cope with this disease were mostly unsuccessful as the infection eradication on farms was not given due attention focusing on the eradication of such chronic diseases as brucellosis and tuberculosis [8, 9].

Clinical and hematological examinations and serological tests carried out in 1988–2017 showed high prevalence of the disease. Proportion of BLV-infected animals annually varied within 1.1–32.2% (on average – 13.3%), morbidity varied from 1.09% to 44.9% (on average – 15.3%). Proportion of BLV-seropositive animals in breeding holdings was high, 28.0% (from 6.4 to 41.5%), percentage of diseased animals was 30.6% (from 14.8 to 45.6%) [10, 11]. A small part of animals out of the cattle population was covered by routine diagnostic tests: 0.9% of animals was subjected to serological tests and 0.02% of animals was subjected to hematological examinations during the whole analyzed period [12, 13].

In line with the instruction of the Ministry of Agriculture of the Russian Federation[1], action plan[2] and draft Republican target subprogram "Prevention and elimination of bovine leukosis in holdings of the Republic of Dagestan"[3] for 2018–2020 were developed together with the Veterinary Committee experts and approved by the Ordinance of the Government of the Republic of Dagestan to ensure sustainable freedom of the region from bovine leukosis.

Effective study of the BLV infection occurrence and implementation of BL control measures in Dagestan has begun since 2018. Analysis of the disease spread dynamics for the last four years showed that the percentage of infected animals decreased and percentage of diseased animals increased. Thus, the proportion of BLV-carrier animals out of tested animals was 4.0% (proportion of diseased animals was 18.2%) in 2018; 2.9% (24.4%) in 2019; 1.4% (17.4%) in 2020 and 1.0% (19.0%) in 2021. In breeding holdings, the proportion of the virus carrier and diseased animals out of tested ones was 6.3 and 15.6%, respectively. Proportion of the cattle covered by serological tests and hematological examinations for BL in the municipal raions in 2018–2021 in dynamics was 22.9 and 0.1%; 57.4 and 0.6%; 64.8 and 0.3%; 76.2 and 0.1%, respectively [14–16].

[1] Development of the action plan for bovine leukosis control: letter of D. Kh. Khatyov, First Deputy Minister of Agriculture of the Russian Federation No. DKh-25-27/4786 of 27 April 2016. (in Russ.)

[2] Action plan for bovine leukosis prevention and control in the Republic of Dagestan for 2017–2020 approved by the Directive of the Government of the Republic of Dagestan No. 323-r of 11 September 2017. Available at: https://docs.cntd.ru/document/450340001. (in Russ.)

[3] Amendments to the official programme of the Republic of Dagestan: "Development of agriculture and regulation of agricultural product, raw material and food product markets for 2014–2020" approved by the Ordinance of the Government of the Republic of Dagestan No. 76 of 28 June 2018. Available at: https://docs.cntd.ru/document/550147549. (in Russ.)

It should be noted that number of seropositive animals was reduced owing to expanding the test coverage of cattle in previously BL-free municipal raions located in highland, mountainous and submountaine regions of the Republic, as well as the immediate culling of AGID-positive animals without confirmation by hematological examination. Increase in morbidity is associated with long-term BL persistence in the region, small coverage of AGID-positive cattle by hematological examinations and absence of systematic targeted measures for the disease eradication.

In 2018–2021, 152 new disease-affected localities were detected, the disease was eliminated in 65 affected localities within targeted subprogram implementation in the region; 95 BL-affected localities had been officially registered by the end of 2021.

Lifetime diagnosis is the basis of anti-epizootic measures for BL. The effectiveness of lifetime diagnostic method depends on its specificity, sensitivity, easy-to-use and low cost.

Imperfect methods for diseased and virus-carrier animal detection are one of the factors impeding the reduction of a period of BL elimination in affected localities.

Two serological methods: agar gel immunodiffusion assay (AGID) and enzyme-linked immunosorbent assay (ELISA) are currently used for detection of specific antibodies to BLV at veterinary laboratories of the country. Some researchers propose using polymerase chain reaction (PCR) enabling detection of proviral DNA or viral RNA directly in blood sample taken from the virus-infected animal together with AGID and ELISA for accelerated BL elimination in holdings [17–19].

These methods are aimed at identification of the infection or presence of the disease agent in animal. Some authors propose using AGID, ELISA and PCR in combination for effective implementation of measures for BL elimination in affected holdings [20–22].

Bovine leukosis epizootic situation in municipal raions and distant pasture zones in the Republic of Dagestan was objectively assessed and comparative analysis of serological and genetic-molecular methods for BL diagnosis for their effectiveness was carried out for the first time based on the results of large-scale serological tests of cattle.

Large-scale and multifaceted study of BL epizootic features and clarification of some theoretical and practical aspects are currently required for development of complex of measures for the disease prevention and control.

The study was aimed at examination of the extent of BLV occurrence in cattle in the whole territory of the Republic of Dagestan and in its separate municipalities for selection of optimal methods for further control of BLV infection.

MATERIALS AND METHODS

The study was performed in the Caspian Regional Research Veterinary Institute – Branch of the Dagestan Agriculture Scientific Center. Official reporting data of the Veterinary Committee of the Republic of Dagestan, Republic and Raion Veterinary Laboratories for 2022 (as of 1 October) were analyzed and statistically processed for assessment of epizootic situation on BL. Cattle of different ages were tested.

AGID was mainly used for tests aimed at detection of the virus carrier animals performed at the Republic Veterinary Laboratories. Serological tests were performed in accordance with the "Methodical guidelines for bovine leukosis diagnosis"[4] and epizootological investigations were carried out in accordance with the "Methodical guidelines for epizootological investigations of bovine leukosis cases"[5].

Whole blood samples and serum samples were collected from 258 cows aged over three years and kept in BL-affected holdings located in the Gergebilsky and Gunibsky Raions for comparative assessment of the laboratory tests (AGID, ELISA and PCR) used for BL diagnosis for their sensitivity. Tests were performed using certified equipment at the GBI "Kropotkinskaya Krai Veterinary Laboratory". Serum samples were AGID and ELISA tested for bovine leukemia virus using the "Test-kit for serological diagnosis of bovine leucosis" and "Test-kit for detection of antibodies against bovine leukaemia virus with enzyme-linked immunosorbent assay (ELISA) in serum and milk (variant No. 1 – screening)" produced by the Federal State-Owned Enterprise "Kursk Biofactory" – BIOK Co. (Russia), respectively. PCR tests were performed using "FACTOR-BOVINE LEUKOSIS- PCR kit" ("VET FACTOR", Ltd., Russia).

Obtained data were statistically processed and analyzed with conventional methods [23].

RESULTS AND DISCUSSION

Positive trend for a decrease in proportion of BLV-infected animals at an increasing number of affected localities indicates that BL remains a serious problem in Dagestan. The results of diagnostic tests for BLV performed in Dagestan municipalities within the analyzed period are presented in Table 1.

A total of 632,454 susceptible animals were subjected to serological AGID tests for BL in 42 municipal raions, 3 urban districts and 7 distant pasture zones in Dagestan and 3,573 (0.6%) reactor animals were detected. Proportion of infected animals was as follows: 0.5% in administrative raions, 1.6% in urban districts and 1.1% in distant pasture zones. Proportion of affected animals varied from 0.01 to 4.9%. Totally, 70.9% of cattle population in the region were covered by serological testing. No hematological examinations for BL were carried out in the view of timely culling of seropositive animals.

Proportion of BLV-infected animals significantly varied in tested raions, towns and distant pasture zone when the epizootic situation on BLV infection was analyzed in the context of the administrative territories of Dagestan.

Currently, livestock holdings located in 12 rural raions (Agulsky, Akhtynsky, Bezhtinsky site, Gumbetovsky, Dokuzparinsky, Kazbekovsky, Kaytagsky, Kurakhsky, Magaramkentsky, Suleyman-Stalsky, Khivsky, Tsuntinsky Raions), town of Kaspiysk and administrative territories of Bakresskaya, Derbentskaya, Kochubeyskaya and Ulankholskaya distant pasture zones are free from BLV infection. Thirty rural regions, city of Makhachkala, town of Khasavyurt

[4] Methodical guidelines for bovine leukosis diagnosis: approved by the Veterinary Department of the Ministry of Agriculture of the Russian Federation No. 13-7-2/2130 of 23 August 2000. Available at: https://docs.cntd.ru/document/1200118749. (in Russ.)

[5] Methodical guidelines for epizootological investigations of bovine leukosis cases: approved by A. M. Smirnov, Academician, Veterinary Medicine Division of the Russian Academy of Agricultural Sciences on 19 June 2001. M.; 2001. 26 p. eLIBRARY ID: 23892805. (in Russ.)

Table 1
Serological tests of cattle for bovine leukosis performed in the municipalities of Dagestan in 2022 (as of October 1)

No.	Raion/town/DPZ*	Number of cattle		Number of tested animals**	Number of ADIG-positive animals (+)	
		total	cows		Number of animals	%
	Raion					
1	Agulsky	10,795	3,434	4,136	–	0
2	Akushinsky	36,454	21,725	44,002	61	0.1
3	Akhvakhsky	19,531	8,686	13,060	92	0.7
4	Akhtynsky	9,778	5,585	8,993	–	0
5	Babayurtovsky	22,130	9,344	15,278	91	0.6
6	Bezhtinsky site	11,967	5,056	8,460	–	0
7	Botlikhsky	28,169	13,938	25,719	201	0.8
8	Buynaksky	43,730	12,544	20,653	24	0.1
9	Gergebilsky	21,896	9,768	13,028	52	0.4
10	Gumbetovsky	24,350	11,831	16,886	–	0
11	Gunibsky	31,506	17,488	21,668	382	1.8
12	Dakhadayevsky	27,927	12,593	16,271	77	0.5
13	Derbentsky	12,991	5,271	8,661	5	0.06
14	Dokuzparinsky	8,856	4,946	10,516	–	0
15	Kazbekovsky	13,587	6,964	10,154	–	0
16	Kaytagsky	10,439	3,754	5,808	–	0
17	Karabudakhkentsky	24,530	7,771	11,912	69	0.6
18	Kayakentsky	9,310	4,237	6,177	18	0.3
19	Kizilyurtovsky	12,730	7,569	11,969	2	0.02
20	Kizlyarsky	62,299	35,321	22,592	717	3.2
21	Kulinsky	17,089	9,299	14,141	4	0.03
22	Kumtorkalinsky	9,361	3,852	5,658	277	4.9
23	Kurakhsky	10,168	5,051	6,027	–	0
24	Laksky	30,141	12,403	24,910	38	0.2
25	Levashinsky	33,987	9,598	16,538	2	0.01
26	Magaramkentsky	18,716	8,461	18,089	–	0
27	Novolaksky	10,274	6,288	6,986	22	0.3
28	Nogaysky	22,495	16,229	15,018	3	0.02
29	Rutulsky	17,760	7,232	12,819	13	0.1
30	Sergokalinsky	9,732	4,605	7,136	56	0.8
31	Suleyman-Stalsky	11,438	9,553	13,184	–	0
32	Tabasaransky	22,376	9,661	11,497	1	0.01
33	Tarumovsky	31,171	19,236	26,335	423	1.6
34	Tlyaratinsky	8,692	3,953	15,018	3	0.02
35	Untsukulsky	19,082	9,646	11,665	89	0.8
36	Khasavyurtovsky	35,290	19,566	26,911	58	0.2
37	Khivsky	10,339	3,778	5,073	–	0
38	Khunzakhsky	24,262	11,483	13,425	11	0.1
39	Tsumadinsky	18,963	7,583	16,169	54	0.3
40	Tsuntinsky	8,439	4,403	3,203	–	0
41	Charodinsky	16,608	7,981	14,138	161	1.1
42	Shamilsky	28,192	10,967	22,125	161	0.7
	City/Town					
1	Kaspiysk	887	480	575	–	0
2	Makhachkala	10,908	5,928	11,733	238	2.0
3	Khasavyurt	3,547	2,100	2,422	3	0.1
	Distant pasture zone					
1	Babayurtovskaya	4,574	1,723	1,765	61	3.5
2	Bakresskaya	3,263	2,433	3,062	–	0
3	Derbentskaya	800	420	1,161	–	0
4	Kizilyurtovskaya	2,401	1,419	2,869	47	1.6
5	Kizlyarskaya	3,140	2,002	2,130	57	2.7
6	Kochubeyskaya	3,046	2,303	3,052	–	0
7	Ulankholskaya	1,756	1,095	1,677	–	0
	Total	891,872	428,556	632,454	3,573	0.6

*DPZ – distant pasture zone;
**blood samples from some cattle were retested for diagnosis confirmation.

and Babayurtovskaya, Kizilyurtovskaya and Kizlyarskaya distant pasture zones remain BLV-affected.

Single cases of BLV infection were detected in animals in the Derbentsky, Kizilyurtovsky, Kulinsky, Levashinsky, Nogaysky, Tobasaransky, Tlyaratinsky Raions and in town of Khasavyurt.

Up to 1.0% of BLV-infected animals was detected in territories of 18 administrative raions (Akushinsky, Akhvakhsky, Babayurtovsky, Botlikhsky, Buynaksky, Gergebilsky, Dakhadayevsky, Karabudakhkentsky, Kayakentsky, Laksky, Novolaksky, Rutulsky, Sergokalinsky, Untsukulsky, Khasavyurtovsky, Khunzaksky, Tsumadinsky, Shamilsky Raions).

From 1.0 to 4.9% of BLV-infected animals were reported in the Gunibsky, Kizlyarsky, Kumtorkalinsky, Tarumovsky, Charodinsky Raions, city of Makhachkala, Babayurtovskaya, Kizilyurtovskaya and Kizlyarskaya distant pasture zones.

In breeding holdings, 3.5% of dairy cattle and 0.6% of meat cattle (averagely 2.8% of the total number of tested animals) were found to be BLV-carriers. The proportion of infected animals by holding varied from 1.1 to 12.2% (Table 2). The coverage of breeding cattle with serological tests was 65.8%.

Eleven holdings were free from BLV infection, proportion of infected animals in five holdings was found to be low, from 1.1 to 3.6%. The proportion of infected animals

out of tested ones was 12.2% in two holdings only: SKhK "Agrofirma "Sogratl" (Gunibsky Raion) and AO "Kizlyaragrocomplex" (Kizlyarsky Raion).

Thirteen (72.2%) out of 18 tested breeding holdings were free from BLV infection. It should be noted that KKh "Agrofirma Chokh" and SPK Kolkhoz "Krasny partisan" where single BLV-infected animals had been detected were not declared disease-affected.

As of January 1, 2022 there were 95 BL-affected localities in Dagestan that had been initially reported in 2021. Eighty-one new leukosis-affected localities had been found for 9 months of 2022 and there were 158 reported BLV-infected localities as of the October 1, 2022: 36 agricultural holdings (including 5 breeding holdings), 18 small-scale farms, 104 backyard farms.

Maximum number of affected localities was reported in the Kizlyarsky Raion (18), Tarumovsky Raion (17), Babayurtovsky Raion (16), Gunibsky Raion (15), Tlyaratinsky Raion (10) and city of Makhachkala (9). One affected locality was reported in each of the following Raions: Bezhtinsky site, Buynaksky, Derbentsky, Kazbekovsky, Kayakentsky, Kizilyurtovsky, Khasavyurtovsky and in the towns of Khasavyurt and Yuzhno-Sukhokumsk. Two affected localities were reported in each of the Rutulsky and Untsukulsky Raions; three affected localities were reported in each of the Gergebilsky, Laksky, Novolaksky and Tsumadinsky

Table 2
Serological tests of cattle for bovine leukosis in breeding holdings located in Dagestan in 2022 (as of October 1)

No.	Holding	Holding type	Number of cattle		Number of tested cattle	Number of AGID positive animals (+)		Disease status
			total	cows		number of animals	%	
Gergebilsky Raion								
1	AO "Darada-Murada"	dairy	981	645	1,809	23	1.3	affected
2	PK "Murad "	meat	1,040	627	500	16	3.2	affected
3	KFKh "Kosulya"	meat	351	243	301	–	0	free
Gunibsky Raion								
4	SKhK "Agrofirma "Sogratl"	dairy	1,049	408	376	46	12.2	affected
5	KKh "Agrofirma Chokh"	dairy	1,302	617	614	7	1.1	free
Dakhadaeyvsky Raion								
6	SPK "Ulluchay"	dairy	129	107	114	–	0	free
Kizilyurtovsky Raion								
7	SPL "Agrofirma im. U. Buynakskogo"	dairy	770	450	573	–	0	free
8	OOO NPF "Plemservis"	dairy	360	338	558	20	3.6	affected
9	KFKh "Iman"	dairy	460	304	294	–	0	free
Kizlyarsky Raion								
10	AO "Kizlyaragrocomplex"	dairy	6,159	2,762	1,578	192	12.2	affected
Kulinsky Raion								
11	SPK "Kulinsky"	dairy	795	509	965	–	0	free
12	SPK "Plemkhoz im. B. Aminova"	dairy	257	96	273	–	0	free
Khunzakhsky Raion								
13	SPK Kolkhoz "Krasny partisan"	dairy	380	220	520	11	2.1	free
14	SPK "Alkhas Kuli"	meat	257	110	130	–	0	free
Shamilsky Raion								
15	SPA "Otgonnik"	dairy	285	110	280	–	0	free
16	SPK "Mesed"	dairy	356	244	628	–	0	free
Buynaksky Raion								
17	OOO "Kurbanservis"	meat	1,504	683	1,137	–	0	free
Kazbekovsky Raion								
18	OOO "Vympel-1"	meat	484	424	484	–	0	free
	Total		16,919	8,897	11,134	315	2.8	

Table 3
Results of diagnostic tests of cows for bovine leukosis with AGID, ELISA and PCR

Number of tested samples	Number of BLV-carrier animals detected with (number of animals/%)		
	AGID	ELISA	PCR
258	88/34.1	95/36.8	102/39.5

Raions; four affected localities were reported in the Sergokalinsky Raion; five affected localities were reported in the Charodinsky Raion; six affected localities were reported in each of the Akhvakhsky, Dakhadayevsky, Karabudakhkentsky Raions, seven affected localities were reported in each of the Botlikhsky, Kumtorkalinsky and Shamilsky Raions. Most of BL-affected localities (97.5%) were found in plain zone and other BL-affected localities (2.5%) were found in submountain zone. No BLV was found in tested animals located in highland and mountainous zones.

Bovine leukosis control is performed by governmental and regional authorities in accordance with current legal act, Order of the MOA of the Russian Federation No. 156 of 24 March 2021[6]. With putting the said Order in force the approach to BL diagnosis, declaring the localities as affected ones and elimination of the disease in BLV-affected population has significantly changed. As for disease diagnosis, the new veterinary rules provide for putting modern highly sensitive methods for early BL diagnosis (ELISA and PCR) into veterinary practice that facilitates prompt disease elimination in the affected holdings.

Results of tests of blood samples taken from cows using different methods are given in Table 3.

Comparative analysis of serological (AGID, ELISA) and molecular genetic (PCR) methods used for BL diagnosis showed the advantage of enzyme-linked immunosorbent assay and polymerase chain reaction as compared to immunodiffusion assay used in veterinary practice. Thus, 2.7 and 5.4% out of the cases previously AGID-tested negative were positive when tested with ELISA and PCR, respectively. It should be noted that all cases (100%) tested positive with ADIG were tested positive with ELISA and PCR.

"Methodical guidelines for bovine leukosis diagnosis and shortening the disease elimination period in the disease-affected holdings in the Republic of Dagestan" [24] has been developed and proposed for implementation; they provide for putting modern test methods into veterinary practice.

CONCLUSION

Analysis of data given in the paper shows that BL remains a serious problem for animal farming industry of the Republic of Dagestan as the number of affected localities especially backyard farms increases despite the positive trend for decrease in proportion of BLV-infected animals. The coverage of cattle population with diagnostic tests should be expanded for detection of all seropositive animals. Young animals at the age of 6, 12, 18 months and animals before their introduction to main herd should be tested.

In holdings where BLV-infected susceptible animals are still detected, measures for complete disease elimination are to be taken: frequency of serological tests in the group of seronegative animals should be increased until two successive negative test results are obtained.

Complex application of AGID, ELISA and PCR as a promising test system for BL control measures is proposed for detection of maximum number of BLV-carrier animals. Putting ELISA- and PCR-based diagnosis in veterinary practice in the Republic of Dagestan will allow early detection of BLV carrier calves (sera taken from calves before first feeding with colostrum are to be tested with ELISA and sera taken from calves at the age of 15–20 days are to be tested with PCR) and enhancement of the diseases elimination effectiveness and shortening the disease elimination period at final stages of BLV infection eradication in agricultural holdings.

[6] Veterinary rules for implementation of preventive, diagnostic and restrictive measures, quarantine imposition and lifting and other restrictions to prevent the bovine leukosis spread and eradication of bovine leucosis outbreaks: approved by Order of the Ministry of Agriculture of the Russian Federation No. 156 of 23 March, 2021. Available at: https://docs.cntd.ru/document/603433105. (in Russ.)

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Efficiency of the data generated by the robotic milking system for comprehensive diagnosis of mastitis in cows

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SUMMARY

Early mastitis diagnosis and treatment play a significant role in reducing the disease incidence in a dairy herd. Examination of the animals ($n = 61$) milked with VMS™ V300 automated voluntary milking system (DeLaval, Sweden) showed that mean milk yield was 15.03 kg (*min* – 4.50 kg, *max* – 24.52 kg); mean milking time in the group was 8 min 14 sec (*min* – 5 min 24 sec, *max* – 12 min 29 sec) during the observation period equal to 10,300 milkings. Milking time for the majority of the cows (67.2%) complied with the standards and equaled to 4–7 min, mean milking time for 32.7% of the animals was 8 minutes. Mean interval between milkings in the test animal group was 11 hours 30 minutes (*min* – 6 h 04 min, *max* – 18 h 54 min). Mean electrical conductivity of the milk was 4.14 1/Om×cm^3 for the whole group of animals. Determined mean mastitis detection index (MDi) was 1.6 and varied in the range of 1.03 to 1.41. Minimal and maximal MDi was 1.0 and 11.1, respectively. Diagnostically representative increase in MDi within 1.8–2.2 was observed in 24.6% of animals. Significant MDi increase to more than 2.2 was found in 21.3% of high-yielding cows. All animals with MDi higher than 1.8 (28 animals) were examined for mastitis. Inflammatory reactions in udder were detected in 28.6% of the animals, clinical and latent inflammations were detected in 7.1 and 21.4% of the cows, respectively. Tests of mammary gland secretion showed that average somatic cell count was up to 200 and 201–300 ths cells/mL in 45.9 and 37.7% of the animals, respectively. Udder secretions of 4.9% of cows contained 301–400 ths somatic cells/mL. In 9.8% of tested animals average somatic count was 401–700 ths somatic cells/mL, and in 1.6% of the animals – more than 701 ths somatic cells/mL. Microbiological and PCR tests of mammary gland secretion samples taken from the animals with mastitis detected the following contagious and coliform mastitis agents: *Staphylococcus* spp. (*St. epidermidis*, *St. saprophyticus*, *St. haemolyticus*), *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*. Various diagnostic techniques are found to be used for detection of mastitis in the herd and the data generated by robotic voluntary milking station such as mastitis detection index (MDi) can be used for earlier detection of changes in cow's mammary gland.

Keywords: high-yielding cows, mastitis, diagnosis, robotic voluntary milking systems, milk yields, milking time, interval between milkings, electrical conductivity, mastitis detection index (MDi), somatic cells, mastitis agents, contagious mastitis, coliform mastitis

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Эффективность использования данных, полученных с электронной системы роботизированного доения, при комплексной диагностике мастита у коров

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РЕЗЮМЕ

Важную роль в снижении заболеваемости молочного стада маститом играют ранняя диагностика и своевременные лечебные мероприятия. В результате исследования животных ($n = 61$), доение которых осуществлялось при помощи автоматизированной системы добровольного доения VMS™ V300 (DeLaval, Швеция), установлено, что за период наблюдения, равный 10 300 актам доения, средний надой составил 15,03 кг (*min* – 4,50 кг, *max* – 24,52 кг); средняя продолжительность доения по группе – 8 мин 14 сек (*min* – 5 мин 24 сек, *max* – 12 мин 29 сек). Период времени, за которое

происходил цикл доения большинства коров (67,2%), соответствовал нормативным показателям и составил 4–7 мин, у 32,7% животных средняя продолжительность доения была более 8 мин. Средний интервал между доениями в исследуемой группе животных равнялся 11 ч 30 мин (*min* – 6 ч 04 мин, *max* – 18 ч 54 мин). Средняя электропроводность молока по всей группе животных составила 4,14 1/Ом×см^3. Определили, что средний показатель MDi (индекс выявления мастита) был равен 1,16 с диапазоном от 1,03 до 1,41. Минимальное и максимальное значение MDi находилось на уровне 1,0 и 11,1 соответственно. Диагностическое увеличение индекса MDi в пределах 1,8–2,2 наблюдали у 24,6% животных. Достоверное повышение индекса более 2,2 установлено у 21,3% высокопродуктивных коров. Все животные с уровнем MDi более 1,8 (28 гол.) были обследованы на мастит, воспалительные реакции в вымени обнаружили у 28,6% особей, клиническое и скрытое воспаление имели 7,1 и 21,4% коров соответственно. При исследовании секрета молочной железы установили, что у 45,9 и 37,7% животных среднее содержание соматических клеток находилось в диапазоне до 200 и 201–300 тыс/мл соответственно. В секрете вымени 4,9% коров содержалось 301–400 тыс/мл соматических клеток, у 9,8% исследуемых животных показатель был на уровне 401–700 тыс/мл, у 1,6% – свыше 701 тыс/мл. Микробиологические и ПЦР-исследования проб секрета молочной железы от животных с маститом показали, что спектр возбудителей контагиозного и колиформного маститов представлен: *Staphylococcus* spp. (*St. epidermidis*, *St. saprophyticus*, *St. haemolyticus*), *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*. Установлено, что для выявления мастита в стаде должны быть использованы различные инструменты диагностики, а полученные данные с автоматизированных систем добровольного доения, такие как индекс выявления мастита (MDi), могут применяться для более раннего выявления изменений, происходящих в молочной железе коров.

Ключевые слова: высокопродуктивные коровы, мастит, диагностика, автоматизированные системы добровольного доения, надои, продолжительность доения, интервал между доениями, электропроводность, индекс выявления мастита (MDi), соматические клетки, возбудители мастита, контагиозный мастит, колиформный мастит

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INTRODUCTION

Milk production scaling-up focusing on raw milk quality improvement is of great importance in the modern dairy industry [1–3]. Inflammatory reactions of cow's mammary gland are one of the factors affecting the milk quality at the stage of primary milk production [3–5]. Mastitis in high-yielding cows is a significant financial problem especially in dairy industry. Inflammatory cow's mammary gland diseases are one of the obstacles to extra premium and premium milk production and marketing to processing establishments due to high somatic cell levels in the milk especially in animals with latent mastitis, increased milk contamination with pathogenic and opportunistic microflora and changes in milk fat-to-protein ratio [6]. Therewith, early diagnosis and treatment play an important role in reducing the disease incidence in dairy herd. Detection of somatic cells and analysis of their levels in cow's milk enable subclinical mastitis diagnosis at an earlier stage, when no clinical manifestations are observed. Microbiological and PCR tests of cow's mammary gland secretions allow for detection of a range of agents responsible for inflammatory processes in cow's udder and for identification of the disease etiology. In our country, there has been recently a steady trend for construction of large dairy holdings for keeping large lactating cow populations that hampers early diagnosis of the pathological process in the mammary gland. Automated milking systems capable of registering different parameters during milking can be used for solving this problem. Analysis of the said parameters allows for detection of changes in cow's mammary gland [7].

Automatic milking systems (AMS) were first introduced on dairy farms in 1990s [8]. For several decades, the dairy farming industry of our country has been transitioning to labor automation [9–14]. AMSs are increasingly being introduced into practice owing to their undoubted advantages, such as milk quality improvement and labor cost reduction [12–16]. This voluntary milking technology for dairy cattle provides for full automation of the process, that is based on computer control and significant average increase in milking frequency. AMSs are economically, technically and socially relevant for agriculture industry as well as animal physiology, health and welfare [17–21]. The cow's udder is not examined at every milking when the above said automatic milking systems are used. Therefore, analysis of online measurements is of great importance [22–24]. Parameters registered by robotic milking systems can vary depending on the robotic milking sustem model and equipment configuration. Standard parameters to be controlled are as follows: milk yields, milking time, interval between milkings, milk electrical conductivity, blood in milk [12, 24, 25]. Mastitis detection index (MDi) is less known value registered by robotic milking system. It is calculated based on three parameters: milk electrical

conductivity, interval between milkings and presence of blood in each udder quarter [12]. Currently, basic information on this index can only be found in the user manual for DeLaval VMS™ milking system (Sweden). MDi can be within the range of 0.8 to 4.0. When it is lower than 1.8 it means that this animal has no problems with its mammary glands. When MDi is higher than 1.8 this indicates that the particular cow should be examined for mastitis, MDi higher than 2.2 is indicative of inflammation in the cow's udder. However, there is no sufficient obvious scientific evidence of relationship between MDi and mastitis in high yielding cows.

The study was aimed at complex mastitis diagnosis in cows, analysis of parameters registered by automated voluntary milking system as well as assessment of MDi effectiveness for mastitis diagnosis in cows.

MATERIALS AND METHODS

The study was performed within the governmental programme of the Ministry of Science and Higher Education of the Russian Federation: research area No. 160 – Federal Research Programmes of the Governmental Academies of Sciences, research topic No. 0532-2021-0009 "Development of biological technologies for animal health management and lifetime animal and poultry product quality management" at the Reproductive Technologies Department of the Federal State Budgetary Scientific Institution "Ural Federal Agrarian Scientific Research Centre, Ural Branch of the Russian Academy of Sciences" (FSBSI UrFASRC, UrB of RAS) in 2020–2021.

The experiments were performed in high-yielding cows (milk production – more than 8,000 kg) kept in the breeding holding located in the Kamyshlovsky Raion, Sverdlovsk Oblast. VMS™ V300 automated voluntary milking system (DeLaval Company) was put into operation for group of 61 cows in September 2020. A total of 10,300 milkings were examined during the tested period (mean period – 4.9 months; *min* – 1 month, *max* – 7 months), the following parameters were examined: milking time, interval between milkings, milk electrical conductivity, MDi. The animals with MDi higher than 1.8 were additionally examined for clinical and subclinical mastitis. The animals were also examined for mastitis clinical signs by test milkings including examination for symmetry and size of udder quarters, changes in mammary gland skin and temperature. Special attention was paid to supramammary lymph nodes: they were examined for indurations. Changes in teat sphincters and drawn udder secretions were registered.

Tests for subclinical mastitis were performed using Keno™test diagnostic rapid test-kit (CID LINES, Belgium). Somatic cell counts in the mammary gland secretions were measured with viscometric milk analyzer "Somatos Mini" (Sibagropribor Ltd., Russia) and DeLaval DCC counter (Sweden). The method for somatic cell count quantification complies to the Russian Federation standards, GOST 23453-2014[1].

Samples of mammary gland secretions ($n = 8$) were collected from animals with mastitis during the observation period for further microbiological and PCR tests aimed at identification of the disease etiology. Tests were carried out with real-time polymerase chain reaction (PCR) using set of reagents: Vetscreen.STREPTOROL-V, Vetscreen.STAFIPOL, Vetscreen.KOLIPOL, Vetscreen.STREPTOROL (OOO IDS, Russia) and Rotor-Gene 3000 system (Corbett Research, Australia). Cow udder secretion samples were inoculated in liquid and solid nutrient media: meat peptone broth (MPB), meat peptone agar (MPA), Endo's medium, Sabouraud medium, mannitol-salt agar, enterococcus agar, Gissa's colour media, for bacteriological and mycological testing. The recovered isolates were identified in according to Bergey's Manual of Determinative Bacteriology and Manual for determination of pathogenic and opportunistic fungi.

[1] GOST 23453-2014 Milk. Methods for determination of somatic cells. Available at: https://docs.cntd.ru/document/1200115756. (in Russ.)

RESULTS AND DISCUSSION

Average milk yield in the group was 15.03 kg (*min* – 4.50 kg, *max* – 24.52 kg) during the observation period. The mean milking time in the group was 8 min 14 sec, minimal milking time was 5 min 24 sec, maximum milking time was 12 min 29 sec. Mean milking time was 5–7 min, and was consistent to physiological parameters of milk ejection reflex and complied with the limits required for machine cow milking. It was found that 67.2% of cows were milked out in 4–7 minutes and average milking time in 32.7% of cows was more than 8 minutes (Fig. 1).

Mean interval between milkings was 11 h 30 min in the tested group, ranging from minimal interval of 6 h 04 min up to maximum interval of 18 h 54 min. Therewith, the maximum interval of 20 h 11 min – 24 h 00 min was registered once or more times in 31.2% of cows during the observation period (Fig. 2).

Our previous tests showed that milk electrical conductivity in healthy cows was 3.5–4.5 1/Om×cm^3, milk electrical conductivity in cows with subclinical and clinical mastitis was 4.5–6.0 and 6.1–7.0 1/Om×cm^3, respectively [26].

Milking of cows with VMS™ V300 automatic voluntary milking system enables generation of the data on the electrical conductivity of the milk from each mammary gland quarter. Analysis of the data for the whole observation period showed that mean electrical conductivity in the test group was 4.14 1/Om×cm^3; therewith, in 16.4% of cows the milk electrical conductivity was 4.5–6.0 1/Om×cm^3. At the level of individual animal, electrical conductivity of the milk from left front quarter of the udder was 4.50–5.23 1/Om×cm^3 in 23.0% of the cows and more than 7.11 1/Om×cm^3 in 4.92% of cows; electrical conductivity of the milk from right front quarter of the udder was 4.52–5.05 1/Om×cm^3 in 13.1% of the cows and 6.24–9.39 1/Om×cm^3 in 8.2% of cows during the whole observation period. Similar analysis showed that the electrical conductivity of the milk from left hind quarter of the mammary gland was in the range of 4.54–5.20 and 6.06–9.14 1/Om×cm^3 in the same number of animals (13.1%). Increase in electrical conductivity of the milk from the right hind quarter of the udder from 4.51 to 5.73 1/Om×cm^3 and from 6.22 to 7.93 1/Om×cm^3 was registered in 21.31% and 4.92% of the animals, respectively.

Mean MDi was 1.16 (range: 1.03–1.41) during the 7-month test period and 10,300 milkings. Minimum and maximum MDi was 1.0 and 11.1, respectively (Fig. 3). Therewith, MDi was 1.8 up to 2.2 in 50.8% of high yielding cows, single index increase was registered in 26.2%

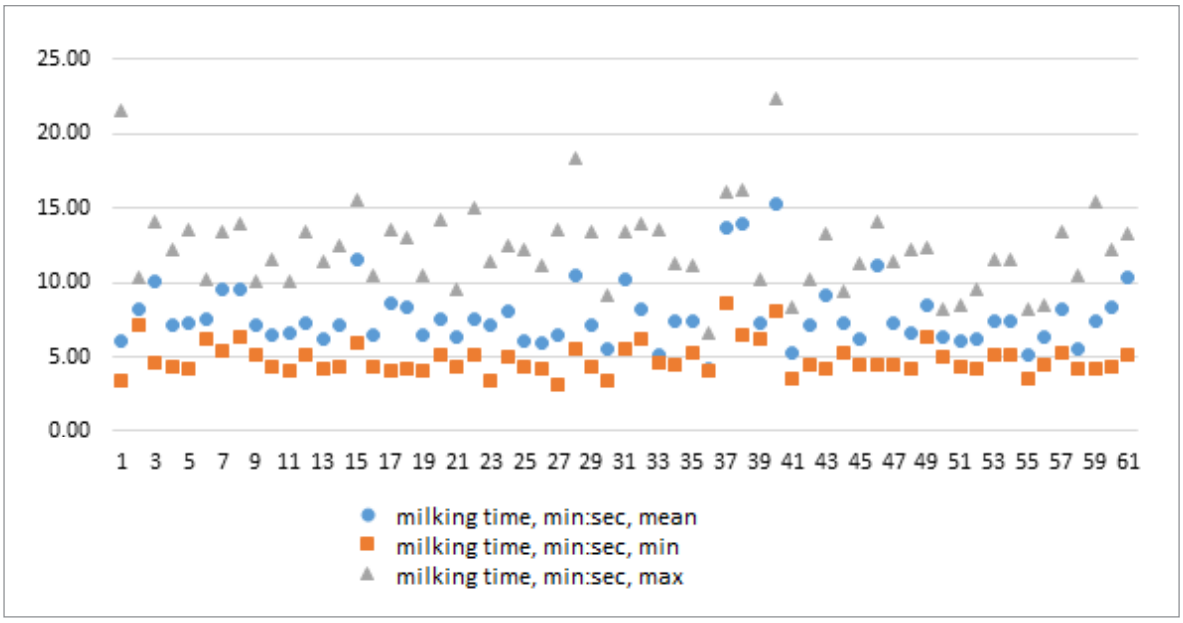

Fig. 1. Distribution of the time of cow milking with robotic milking system

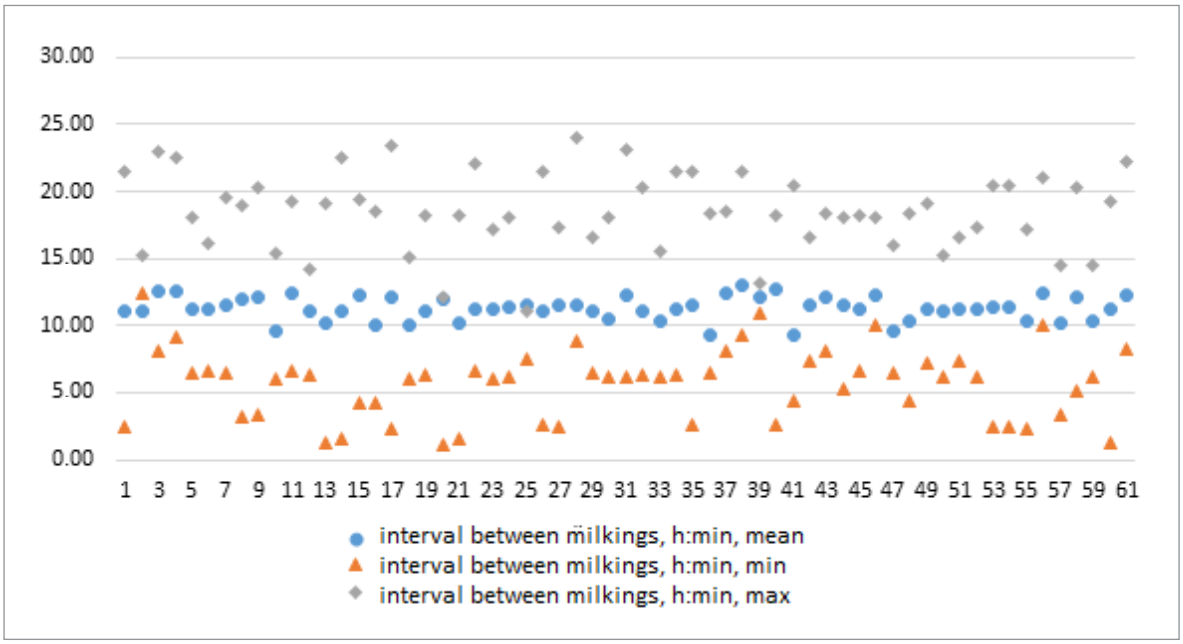

Fig. 2. Distribution of the interval between cow milkings with robotic milking system

of animals. Thereat, diagnostically significant MDi increase within the range of 1.8–2.2 was registered in 24.6% of cows. MDi higher than 2.2 was registered in 36.1% of animals; therewith increased MDi was observed in 14.8% of cows during one up to four milkings but subsequently found not be associated with mastitis in these animals. As a result, MDi higher than 2.2 in 21.3% of high-yielding cows was considered indicative of mastitis. Increased MDi, 1.8–2.2 and higher than 2.2, was registered during maximum 38 and 19 milkings, respectively.

All animals with MDi of 1.8–2.2 and higher than 2.2 (15 and 13 cows, respectively) registered during more than four milkings were examined for subclinical and clinical mastitis. Mastitis was diagnosed in 28.6% of 28 examined cows. Clinical and latent inflammation was detected in 7.1 and 21.4% of cows, respectively. Clinical mastitis was detected in the cows with MDi higher than 2.2. registered during 13–18 milkings. Subclinical mastitis was detected in cows with MDi of 1.8–2.2 registered during 17–28 milkings. No mastitis was detected in the cows with MDi less than 1.8 during the whole test period. Examinations for association between MDi and diagnosed mastitis showed positive correlation (correlation coefficient: $r = 0.78$).

Tests of mammary gland secretions showed that mean somatic cell count was 200 and 201–300 ths cell/mL in 45.9% and 37.7% of animals, respectively, that was indicative of absence of pathological processes in mammary glands. Therewith, MDi for these animals was less than 1.8. There were 301–400 ths/mL of somatic cells in the udder secretions from 4.9% of cows with MDi of 1.8–2.0. Clini-

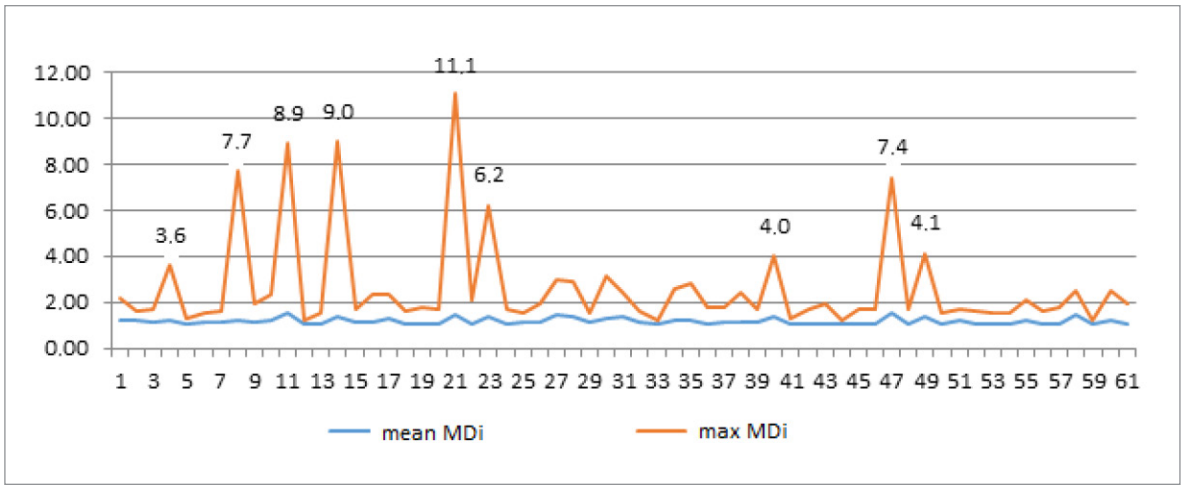

Fig. 3. Mastitis detection index (MDi)

cal examination of these cows revealed subclinical mastitis in 3.6% of them. Somatic cell count was 401–700 ths cell/mL in 9.8% of tested cows and their MDi was in the range of 2.0–2.2. All these animals had inflammation in the mammary gland and subclinical mastitis was detected in 7.1 and 14.3% of cows, respectively. More than 701 ths somatic cells/mL were detected in the milk from one cow (1.6% of tested animals) with MDi higher than 2.2. Clinical mastitis (3.6%) was detected during the clinical examination of the cow. Examination results are given in the Table below.

Fourteen bacterial isolates were recovered from mammary gland secretion samples collected from the cows with mastitis and tested with PCR. The following etiological agents of contagious mastitis were detected in the samples: *Staphylococcus* spp. (*St. epidermidis*, *St. saprophyticus*, *St. haemolyticus*) were detected 100% of samples; *Streptococcus agalactiae* and *Staphylococcus aureus* were detected in 25.0% and 12.5% of samples, respectively. *Escherichia coli* inducing coliform mastitis in cows was recovered from 37.5% of samples.

Microbiological tests of mammary gland secretion samples from the cows with diagnosed mastitis detected *Enterococcus faecium,* environmental microorganism, in 100% of samples as well as *Escherichia coli* and *Staphylococcus epidermidis* in 62.5% and 37.5% of samples, respectively, and *Staphylococcus aureus* in 12.5% of samples.

Thus, microbiological and PCR tests are complementary methods for mastitis etiology identification enabling detection of wide range of pathogens and selection of effective treatment.

CONCLUSION

All available diagnostic techniques (clinical examination, rapid tests, somatic cell counting, analysis of milk electrical conductivity and microbiological and PCR tests) should be used identification of mastitis of a particular type and for prescribing treatment and prediction of the disease course. Tests results showed that mean mastitis detection index (MDi) varied from 1.03 to 1.41, therewith, minimal and maximum MDi was 1,0 and 11.1, respectively. Diagnostically representative MDi increase within 1.8–2.2 was registered in 24.6% of animals. Significant MDi increase to more than 2.2 was found

Table
Somatic cell counts in mammary gland secretion collected from tested cows (*n*=61)

Somatic cell count, ths cells/mL	Number of animals	
	n	%
less than 200	28	45.9
201–300	23	37.7
301–400	3	4.9
401–700	6	9.8
more 701	1	1.6

in 21.3% of high yielding cows. Mastitis was detected in 28.6% of animals with MDi more than 1.8, therewith clinical and subclinical inflammations were detected in 7.1% and 21.4% of cows, respectively. Tests of cow udder secretions showed that mean somatic cell count was not more than 200 ths cells/mL in 45.9% of animals; somatic cell count was 201–300 ths cells/mL in 37.7% of cows, and MDi for these animals was less than 1.8. There were 301–400 ths somatic cells/mL in mammary gland secretions collected from 4.9% of cows with MDi within the range of 1.8–2.0. Somatic cell counts in 9.8% and 1.6% of tested cows with MDi within the range of 2.0–2.2 were 401–700 and more than 701 ths cells/mL, respectively. Microbiological and PCR tests of mammary gland secretion samples from the animals with mastitis detected the following pathogens responsible for contagious and coliform mastitis: *Staphylococcus* spp. (*St. epidermidis*, *St. saprophyticus*, *St. haemolyticus*), *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*. Estimated coefficient of correlation between MDi and diagnosed mastitis was +0.78. Taking into account recommendations given in VMS™ V300 Milking System Manual, where mastitis detection indices are roughly divided into the following ranges: less than 1.8 – "normal udder", 1.8–2.2 – "udder requires attention", more than 2.2 – "mastitis", performed experiments have confirmed that MDi can be used as an additional tool for mastitis diagnosis that allows early prompt measures to be taken.

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Clinical signs of caprine arthritis-encephalitis and disease-related pathomorphological changes

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SUMMARY
Over the past ten years, the small ruminant population in the Russian Federation grew sharply, especially goat population in backyards and on small-scale farms. Alongside with the population growth, clinical signs of some sporadic diseases or diseases that had not been previously registered were detected in animals. Caprine arthritis-encephalitis (CAE) is one of such diseases. It is a chronic infectious disease caused by a small ruminant lentivirus (SRLV) of the *Retroviridae* family, which includes four genotypes, of which genotypes A (maedi-visna – MVV virus) and B (caprine arthritis-encephalitis virus – CAEV) are of epizootic significance. The disease is characterized by long asymptomatic viral transmission and is associated with progressive lesions in the respiratory organs, joints and udder. The disease also affects nervous system in kid goats aged between 2 and 3 months. Clinical signs of caprine arthritis-encephalitis are not pathognomonic; therefore, it is often misdiagnosed, thus, resulting in a barrier to effective treatment. Given the fact, the issue of antemortem and postmortem diagnosis of caprine arthritis-encephalitis is still urgent, because most veterinary specialists have never encountered this disease and the data available in the literature often do not fully cover all clinical details and pathomorphological features. Therefore, the purpose of the work is to study CAE clinical signs and pathomorphological changes. The article describes in detail clinical manifestation of this disease, postmortem lesions in organs and tissues of the sick animals. The results obtained suggest that the destructive changes in the exposed organs are irreversible and, consequently, there is no effective treatment.

Keywords: caprine arthritis-encephalitis, lentivirus, pneumonia, pathomorphological features, small ruminants

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Клинические признаки и патоморфологические изменения при артрите-энцефалите коз

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РЕЗЮМЕ
Последнее десятилетие на территории Российской Федерации наблюдается резкое увеличение поголовья мелкого рогатого скота, в частности коз, содержащихся в личных подсобных и крестьянских фермерских хозяйствах. При этом все чаще ветеринарные специалисты начали сталкиваться с клиническими признаками заболеваний, ранее не регистрировавшихся в нашей стране либо встречавшихся в виде спорадических случаев. Одним из них является вирусный артрит-энцефалит коз – хроническое инфекционное заболевание, вызываемое лентивирусом мелких жвачных животных семейства *Retroviridae*, включающим в себя четыре генотипа, два из которых имеют эпизоотическое значение: генотип А (вирус маеди-висна – MVV) и генотип В (вирус артрита-энцефалита коз – CAEV). Артрит-энцефалит коз характеризуется длительным бессимптомным вирусоносительством с последующим развитием клинических признаков поражения органов дыхания, суставов конечностей и вымени, а также нервными явлениями у козлят 2–3-месячного возраста. Клинические признаки артрита-энцефалита коз не являются патогномоничными, вследствие чего ветеринарные специалисты, сталкиваясь с данной симптоматикой, часто ставят ложные диагнозы, что приводит к низкой эффективности терапевтических мероприятий. Учитывая вышеуказанный факт, вопрос прижизненной и посмертной диагностики артрита-энцефалита коз до сих пор остается актуальным, так как большинство ветеринарных специалистов никогда не сталкивались с данным заболеванием, а имеющиеся в литературе данные часто недостаточно подробно освещают все нюансы клинической и патоморфологической картины данной патологии. Исходя из этого, целью работы было изучить клинические признаки и патоморфологические изменения при вирусном артрите-энцефалите коз. В статье подробно рассматриваются клинические проявления данного заболевания, описаны патолого-анатомические изменения в органах и тканях больных животных. Полученные результаты указывают на необратимость деструктивных изменений в пораженных органах и, как следствие, на отсутствие эффективных способов терапии.

Ключевые слова: артрит-энцефалит коз, лентивирус, пневмония, патоморфологическая картина, мелкий рогатый скот

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INTRODUCTION

Easy and relatively cheap handling of backyard goats resulted in a sharp increase in their population in the Russian Federation, thus, forcing veterinarians to deal with clinical manifestations of the diseases that either had not been previously registered in the country, or occurred sporadically. The primary reason behind it is that, until recently, there has been no veterinary control over import of animals from Russia's near and far abroad and their movements between the regions of the country. Poor veterinary control arose from imperfect laws and outright negligence of some goat breeders.

Under these circumstances, veterinarians working with small ruminants (in particular with goats) have to deal with a growing number of lesions in respiratory tract, musculoskeletal system, as well as with mastitis that do not respond to standard therapy. The progeny produced by animals with these clinical signs can develop such neurological conditions as loss of coordination and head tilt. In most cases, a combination of the clinical features is indicative of caprine arthritis-encephalitis.

Caprine arthritis-encephalitis (CAE) is a chronic infectious disease of goats caused by a small ruminant lentivirus of the *Retroviridae* family. The family includes four genotypes, of which genotypes A (maedi-visna virus, MVV) and B (caprine arthritis-encephalitis virus, CAEV) are of epizootic significance [1, 2]. The virus can easily cross the species barrier between sheep and goats, causing a pathological process with similar clinical signs [3–8]. The disease is characterized by a long asymptomatic virus transmission, followed by development of lesions in the respiratory tract, joints and udder, as well as by neurological disorders [9]. At the same time, genotype A lentivirus is believed to affect respiratory organs, while genotype B causes a set of lesions in musculoskeletal system.

The disease is reported in all countries with well-developed goat farming, including Russia [10–12].

CAE clinical signs are not pathognomonic, therefore, it is often misdiagnosed by veterinary specialists, thus, resulting in a barrier to effective treatment.

Currently, CAE diagnosis can be confirmed only by serological and molecular biological methods, including enzyme-linked immunosorbent assay (ELISA) of blood sera for antibodies to CAE virus, or real time polymerase chain reaction (PCR) used to detect proviral DNA in pathological samples from animals [13–15].

No medicinal products for CAE specific prevention and therapy have been developed so far. Preventive measures are mainly technical, i.e. segregation of seropositives and seronegatives, kidding under sterile conditions and early weaning of kid goats, followed by feeding with pasteurized milk or whole milk substitute [16, 17]. Regular serological monitoring of herds for seropositive animals with the following culling is one of the main preventive measures [18, 19].

The situation is complicated by the fact that the Ministry of Agriculture of the Russian Federation has not so far developed any legal framework to regulate preventive measures in the field. It means that, if seropositive animals are detected, there is either no response on behalf of the state veterinary services, or they take hasty, often illegal, measures to eliminate the sick animals as soon as possible.

Despite a wide range of publications, the issue of antemortem and postmortem diagnosis of CAE is still urgent, because most veterinary specialists have never encountered this disease and the data available in the literature often do not fully cover all clinical signs and pathomorphological features [20–22].

Thus, the purpose of the work was to study clinical signs and pathomorphological changes caused by caprine arthritis-encephalitis.

MATERIALS AND METHODS

The following data are required to diagnose CAE antemortem: the anamnesis, clinical examination of animals and the results of serological tests.

Bodywin vacuum tubes (China) with coagulation activator and EDTA were used for blood sampling.

The antibodies in blood serum was detected using ID Screen® MVV/CAEV Indirect Screening Test (IDVet, France) and CAEV/MVV Antibody Test Kit (IDEXX B.V., the Netherlands) for indirect ELISA. The results were read with a semi-automated microplate absorbance reader Infinite® F50 (TECAN, Austria).

CAE virus was detected in blood with a set of reagents produced by "RealBest-Vet DNA CAEV (caprine arthritis-encephalitis virus)" (Vector-Best, Russia), on Bio-Rad (USA) amplification system.

The animals were autopsied using a generally accepted method of G. V. Shor [23]. Pathological material for histological studies was fixed in a 10% formalin solution. Histological preparations were stained with hematoxylin and eosin according to the standard procedure.

RESULTS AND DISCUSSION

Clinical manifestations and anatomical and morphological changes caused by caprine arthritis-encephalitis were described in two backyard animals of different age.

Example 1. A crossbreed kid goat of 2 months old. As the history taking shows, the animals at the age of 1.5 months have such neurological signs as head tilt, "circling". The animal clinical status deteriorated over time, as evidenced by the animal owners.

Fig. 1. Clinical manifestations of central nervous system lesions in a 2-month-old CAE infected kid goat

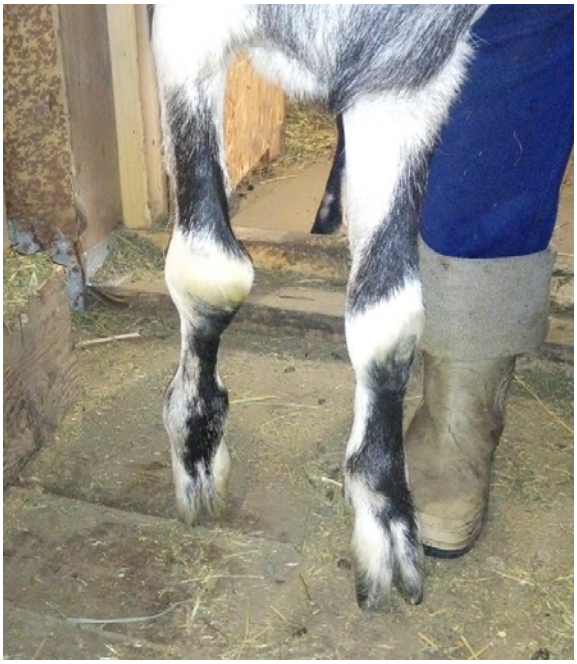

Fig. 2. Enlargement of carpal joints in a CAE-infected goat (5 years old)

The external examination demonstrated that the kid constantly tilted rightward. The animal starts circling and often gets lost in space. Coordination is bad, feed intake becomes difficult (Fig. 1).

To confirm the preliminary diagnosis of "caprine arthritis-encephalitis", a blood sample was taken from the mother goat to obtain serum and to test it in ELISA for antibodies to the disease agent. The test results revealed antibodies to CAE virus with S/P% – 603% (positive result according to the instruction for the kit: S/P% ≥ 60%).

Since the kid goat was fed with mother's milk since birth, he could have colostral antibodies to CAEV in sera. Therefore, in order to exclude a false-positive ELISA result, his blood sample was tested in PCR, which revealed a proviral DNA of CAE virus.

The analysis of clinical signs together with the laboratory diagnosis made it possible to ultimately diagnose "caprine arthritis-encephalitis". The animal owner decided to cull and destroy the baby goat and the mother goat.

Example 2. A Nubian goat, 5 years old. It was bought at the age of 2 months. As the history taking shows, a 3-year-old animal demonstrates low mobility, lameness, refused from mating.

As revealed by clinical examination, hock and carpal joints are enlarged. The animal finds it difficult to walk and weight-bearing lameness is observed (Fig. 2). During palpation, the affected joints were stiff and painless.

To confirm the CAE pre-diagnosis, a blood sample was taken from a goat after examination. An ELISA test for CAEV antibodies in sera showed S/P% of 592% (in accordance with the kit instructions, S/P% ≥ 60% stands for a positive result), which resulted in ultimate diagnosis of "caprine arthritis-encephalitis".

The goat's condition significantly deteriorated the following year. The animal practically did not walk and such respiratory signs as wheezing and dyspnea were seen.

After the emergency slaughter, the animal was autopsied and pathological material was sampled for histological tests, i.e. samples from different brain regions, medulla oblongata, meninges, lungs, articular capsule, and surface of the articular cartilage of the affected joint.

The autopsy and subsequent histological examination showed the following results.

The central nervous system. The dura mater has no visible changes. The brain vessels are injected; the petechial hemorrhages can be seen. Brain convolutions are smoothed (Fig. 3).

Histological analysis of the cerebral cortex, medulla oblongata and cerebellum revealed similar pathomorphological changes: hyperemia, pericellular and perivascular edema in brain tissue, mononuclears clusters around

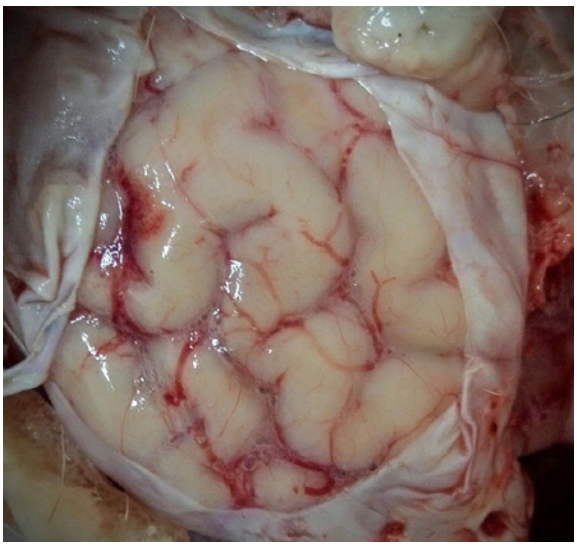

Fig. 3. Brain of a CAE-infected goat

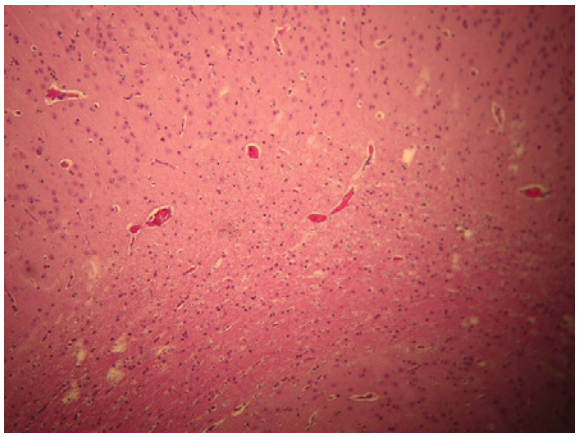

Fig. 4. Brain cortex of a CAE-infected goat. Histological section. Hemostasis. Hematoxylin and eosin stain (magnification 100×)

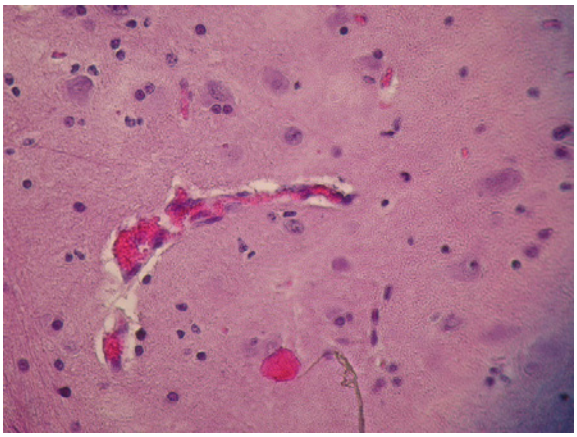

Fig. 5. Cerebellum of a CAE-infected goat. Histological section. Hemostasis. Hematoxylin and eosin stain (magnification 400×)

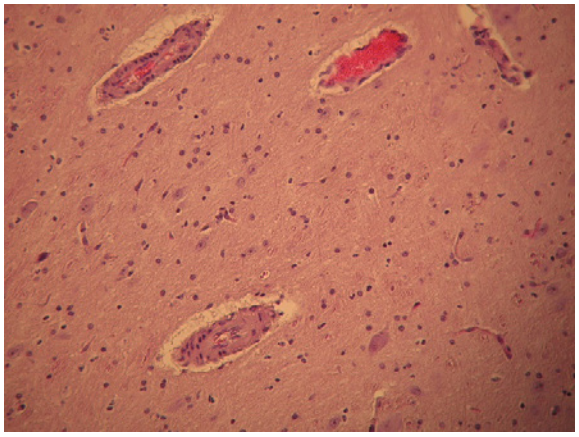

Fig. 6. Medulla oblongata of a CAE-infected goat. Histological section. Perivascular edema, hemostasis, mononuclear clusters. Hematoxylin and eosin stain (magnification 100×)

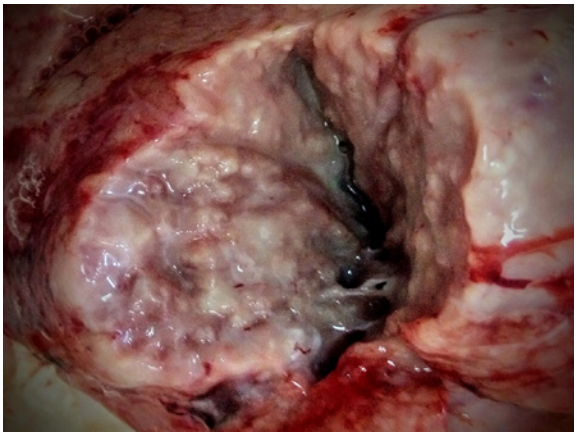

Fig. 7. Lung of a CAE-infected goat

blood vessels (lymphocytes and monocytes) – perivascular cellular sheaths, as well as scattered foci of cellular accumulations (Fig. 4–6).

Respiratory organs. Lungs are enlarged, become airless. The edges are rounded, the surface is rough. Fibrinous exudate is observed on the visceral pleura, local hemorrhages are found. Advanced fibrosis can be seen (Fig. 7).

The sections demonstrate broken lung architectonics. Lung parenchyma is dense, of "rubber" consistency, lobulated with local hemorrhages and extensive fibrosis.

Histological analysis of the lung tissue revealed signs of inflammation, the lumen of the alveoli is filled with exudate – reticulated fibrin masses and leukocytes. In some parts of the section, the exudate does not fit tightly

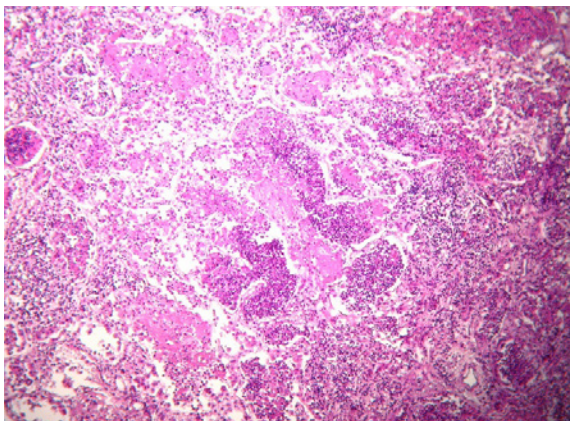

Fig. 8. Lung of a CAE-infected goat. Histological section. Croupous pneumonia. Hematoxylin and eosin stain (magnification 100×)

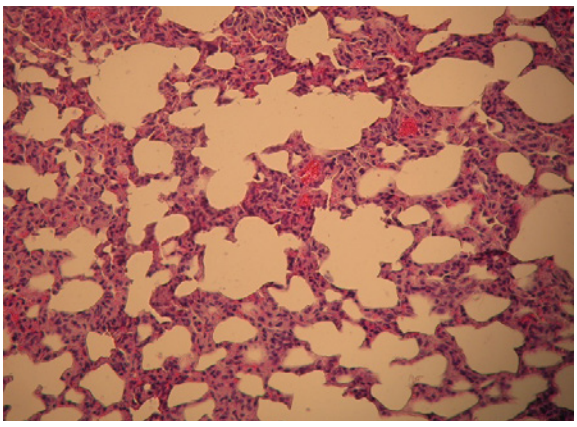

Fig. 9. Lung of a CAE-infected goat. Histological section. Croupous pneumonia. Hematoxylin and eosin stain (magnification 400×)

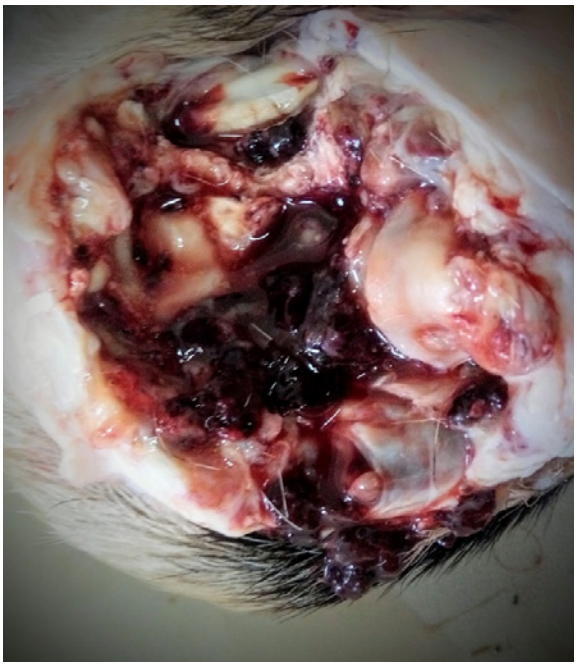

Fig. 10. Carpal joint of a CAE-infected goat.

to the alveoli walls – slit-like lumen can be seen. In the interalveolar septa, inflammation is not pronounced, vascular hyperemia, stasis and stroma edema are observed. Blood clots are visualized in the lumen of some small vessels. The whole combination of morphological changes observed in the micropreparation suggests croupous pneumonia in the animal (Fig. 8, 9).

Carpal joint. The carpal joint demonstrates a 1.5–2 times increase in size. The joint capsule edema with petechial hemorrhages is observed. A great number of blood clots is detected in the autopsied articular cavity (Fig. 10).

The synovial membrane is loose and blood-soaked. The surface of the articular cartilage of the radial and metacarpal bones is yellowish, matte, with degenerative sites.

The histological analysis revealed eroded surface of the articular capsule in the sick animal, which means degenerative changes and destruction of the joint surface due to arthritis (Fig. 11, 12).

The data obtained indicate that morphological changes in the CAE-affected tissues are irreversible, which can be a basis for animal culling and slaughter without any therapeutic support.

CONCLUSION

Caprine arthritis encephalitis is a chronic viral disease when infected goats remain asymptomatic carriers for a long time. The disease occurs in all the countries with well-developed goat farming. The main CAE clinical signs include arthritis of hock and carpal joints, respiratory lesions in the form of interstitial pneumonia, mastitis of one or both mammary glands. Clinical signs observed in animals younger than 3 months include lesions of the central nervous system, i.e. incoordination and head tilt. The symptoms manifest themselves only at the end stage at the age of 3–5 years, in young animals – within the first 2–3 months after birth. Due to poor awareness and taking into account the fact that CAE clinical manifestations are non-specific, the disease is often misdiagnosed, thus, resulting in a barrier to effective treatment.

CAE clinical manifestations and post-mortem lesions were studied during the research.

Necropsy of the CAE-infected animal revealed the following lesions in central nervous system: injection of vessels and petechial hemorrhages on the brain surface. The histological features included hyperemia, pericellular and perivascular edema of brain tissue, as well as accumulation of mononuclear cells (lymphocytes and monocytes) around blood vessels.

The lung edges are rounded, the surface is rough with advanced fibrosis. Fibrinous exudate is observed on the visceral pleura and local hemorrhages are found. Histological examination revealed that the lumen of the alveoli is filled with exudate – reticulated fibrin masses and leukocytes.

The joint capsule edema with petechial hemorrhages is observed. A great number of blood clots is detected in the articular cavity. The synovial membrane is loose. There were degenerative sites on the surface of the articular cartilage.

The histological microscopy revealed eroded surface of the articular capsule, which means there are arthritis-related degenerative changes of the joint surface.

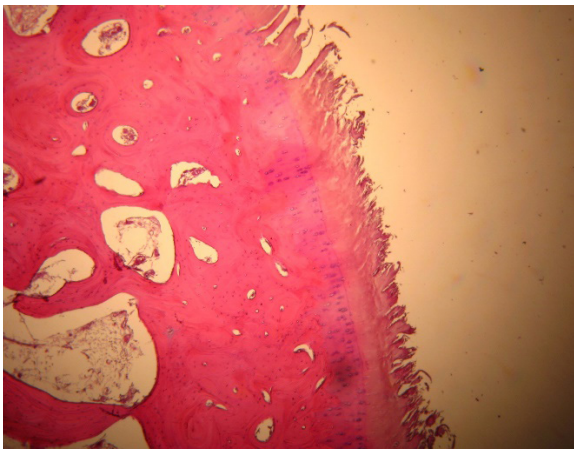

Fig. 11. Articular surface. Histological section. CAE-caused erosion of the articular surface. Hematoxylin and eosin stain (magnification 100×)

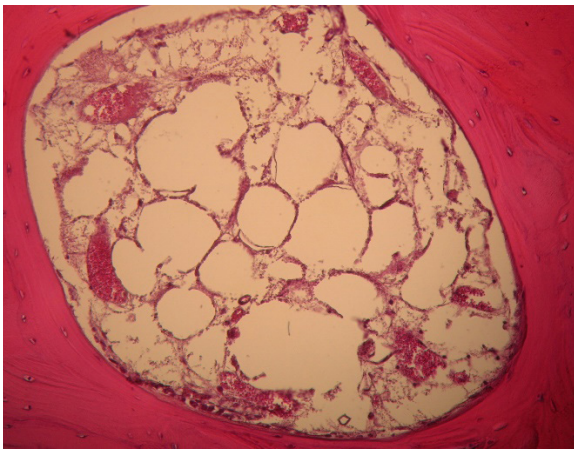

Fig. 12. Articular surface. Histological section. CAE-related hemostasis. Hematoxylin and eosin stain (magnification 100×)

In general, the results obtained significantly add to the earlier published data on CAE pathomorphological changes [17].

The disease symptoms are not pathognomonic, which may be a significant barrier to a correct diagnosis, if it is based only on history taking and clinical analysis. Consequently, the main diagnostic methods include ELISA for antibodies to CAE virus in the animal sera or real-time PCR for detection of proviral DNA.

Caprine arthritis-encephalitis clinically manifest itself only at the end stage. At the same time, pathological processes in tissues is irreversible. This fact makes it pointless to treat animals with obvious clinical signs; therefore, veterinary specialists should make the animal owners focus on the on-farm disease prophylaxis and prevention of the pathogen escape from the CAE-affected areas.

It is noteworthy that the Russian Federation has currently no regulations on CAE prevention approved by the Ministry of Agriculture.

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Torovirus infection in animals: a review

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SUMMARY

Massive digestive disorders of neonatal calves, clinically manifested as diarrhea causing severe dehydration, toxemia, immunodeficiency and metabolic disorders, induce huge economic losses in animal husbandry. Etiopathogenetic lesions of the digestive organs are characterized by significant polymorphism, including a wide range of various (physiological, sanitary and infectious) factors. Massive gastroenteritises in neonatal calves are primarily caused by such infectious agents as viruses, bacteria and protozoa. Massive diarrheas are registered in 70–80% of newborn calves by the end of the first day of life. Diseased newborn calves die on day 5–10 and mortality ranges from 15 to 55%. Rotavirus, coronavirus, pestivirus, parvovirus, enterovirus and kobuvirus, along with bacteria, are most frequently detected in faecal samples collected from neonatal calves with diarrhea. Diagnostic and vaccine products for prevention of these infections have been developed in the Russian Federation. At the end of the 20th – the beginning of the 21st century a large number of cattle were imported to Russia from the countries affected with different contagious diseases (USA, Denmark, France, Slovakia, Austria, Hungary, Germany, the Netherlands, Australia, Finland, etc.). Despite the high activity and field effectiveness of vaccines against rotavirus and coronavirus infections and viral diarrhea, massive neonatal calf diarrheas causing significant economic losses were registered in a number of large-scale livestock farms. Torovirus as well as the above-mentioned pathogens were detected in fecal samples from diseased calves. This report provides data on torovirus infection indicating a wide geographical distribution of animal torovirus in many countries of the world. All this suggests the need to take into account torovirus infection when conducting epizootological investigations in farms affected with massive gastrointestinal diseases of neonatal calves.

Keywords: review, torovirus, calves, piglets, horses, dogs, cats, electron microscopy, gastrointestinal pathology, fecal-oral transmission route

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Проблема торовирусной инфекции животных (обзор литературы)

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РЕЗЮМЕ

Большой экономический ущерб животноводству наносят массовые нарушения функции пищеварения новорожденных телят, клинически проявляющиеся диареей, обуславливающей развитие выраженной дегидратации, токсемии, иммунодефицитов и нарушения обмена веществ. Этиопатогенетические поражения органов пищеварения отличаются значительным полиморфизмом, включающим широкий спектр различных факторов, в том числе физиологических, санитарно-гигиенических и инфекционных. Главной причиной массовых гастроэнтеритов новорожденных телят являются такие инфекционные агенты, как вирусы, бактерии и простейшие. Массовые диареи регистрируются у 70–80% новорожденных телят уже к концу первых суток. Гибель больных новорожденных телят наступает на 5–10-е сут и составляет от 15 до 55%. Чаще всего в пробах фекалий, отобранных от больных диареей новорожденных телят, наряду с бактериями выявляют ротавирус, коронавирус, пестивирус, парвовирус, энтеровирус и кобувирус. Для профилактики указанных инфекций в Российской Федерации были разработаны диагностические и вакцинные препараты. В конце XX – начале XXI века на территорию России было завезено большое количество крупного рогатого скота из различных стран мира (США, Дания, Франция, Словакия,

Австрия, Венгрия, Германия, Нидерланды, Австралия, Финляндия и др.), неблагополучных по ряду инфекционных болезней. Несмотря на высокую активность и полевую эффективность вакцин против рота-, коронавирусной инфекций и вирусной диареи, в ряде крупных животноводческих хозяйств были зарегистрированы массовые диареи новорожденных телят, становившиеся причиной значительного экономического ущерба. В пробах фекалий от больных телят кроме перечисленных возбудителей был обнаружен торовирус. В данном сообщении приведены данные о торовирусной инфекции, свидетельствующие о широком географическом распространении торовируса животных во многих странах мира. Все это говорит о необходимости учета торовирусной инфекции при проведении эпизоотологических расследований в неблагополучных по массовым желудочно-кишечным заболеваниям новорожденных телят хозяйствах.

Ключевые слова: обзор, торовирус, телята, поросята, лошади, собаки, кошки, электронная микроскопия, патология желудочно-кишечного тракта, фекально-оральный путь заражения

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Gastrointestinal diseases of neonatal calves are ranked as the leading cause of economic damage and remain a topical issue in animal husbandry. This pathology is generally manifested as diarrhea and has an infectious nature, it is caused by various etiological agents and develops as mixed infections.

Along with bacteria, rotavirus, coronavirus, pestivirus (viral diarrhea virus – diseases of bovine mucosa), parvovirus, enterovirus and kobuvirus are most frequently detected in fecal samples collected from neonatal calves with diarrhea [1–9]. Inactivated vaccines were developed in the Russian Federation to prevent rotavirus and coronavirus infections, as well as viral diarrhea – a mucosal disease caused by genotype 1 virus [1–3, 10]. Currently, cases of the circulation of pestiviruses belonging to more than 15 subgenotypes of all 3 genotypes have been registered in the country [10]. All this indicates significant difficulties in determining the etiology of gastrointestinal pathology in neonatal calves. Such a variety of diarrhea agents significantly complicates the diagnosis, resulting in low effectiveness of prevention and leading to significant economic damage.

Torovirus was first isolated in Berne (Switzerland) in 1972 from a rectal swab of a newborn horse with diarrhea. At first this pathogen was named "Berne virus" by the place where sampling was performed, later it was classified as *Equine torovirus* (EToV) [11, 12]. In 1979, a virus similar in structure was detected in feces from diarrheal calves in Breda (USA), now known as *Bovine torovirus* (BToV). On this farm a severe form of diarrhea had been registered in young cattle for several months [13, 14].

In 1984 a virus with a similar structure was detected in rectal specimens from children with diarrhea [15]. A few years later, in 1997, torovirus was identified by electron microscopy in stool samples from 3-week-old piglets with diarrhea on a pig farm in the UK [16]. Subsequently, this pathogen (*Porcine torovirus*, PToV) was detected in 6–40% of fecal specimens collected from diarrheal piglets in the Netherlands, Canada, USA, South Africa, China, Belgium, Italy, Hungary, Spain and South Korea [17]. Antibodies to the torovirus were found in 50–100% of sera from piglets of different age. Toroviruses were also detected in fecal samples of other animal species with diarrhea [18–21]. It was found that there is a close genetic relationship between the toroviruses of pigs, cattle, horses, dogs and cats. It is believed that interspecies recombination between these pathogens is possible [16, 22, 23]. A number of researchers believe that toroviruses have zoonotic potential [13, 17, 24]. According to the current virus classification, toroviruses belong to the genus *Torovirus*, which is part of the family *Tobaniviridae*[1], although it was previously referred to the family *Coronaviridae* [23, 25, 26].

Toroviruses are polymorphic, enveloped, peplomer-bearing particles with a diameter of 120–140 nm. The shape of a virion is a biconcave disk (Fig.). The genome of toroviruses is represented by an infectious single-stranded positive RNA. The tubular nucleocapsid is bent into an open torus (swelling, node), hence the name of the pathogen is "torovirus" [2, 13, 25, 27, 28].

The buoyant density of virion in sucrose is 1.14–1.18 g/mL. Toroviruses are resistant to phospholipase C, trypsin, chymotrypsin. Triton X-100 and organic solvents destroy toroviruses. They persist for a long time at from minus 20 to minus 70 °C and pH of 2.5 to 10.5. Repeated cycles of freezing and thawing result in loss of peplomers and desintegration of virions [25].

Epidemiological properties of torovirus infection include long-term shedding of the pathogen from diseased animals and virus-carriers. Cattle, pigs and horses are the natural hosts of toroviruses. The main sources of the pathogen are diarrheal calves under 30 days of age [29, 30]. The virus sheds in feces and nasal discharge of diseased

[1] International Committee on Taxonomy of Viruses (ICTV). Available at: https://ictv.global/taxonomy.

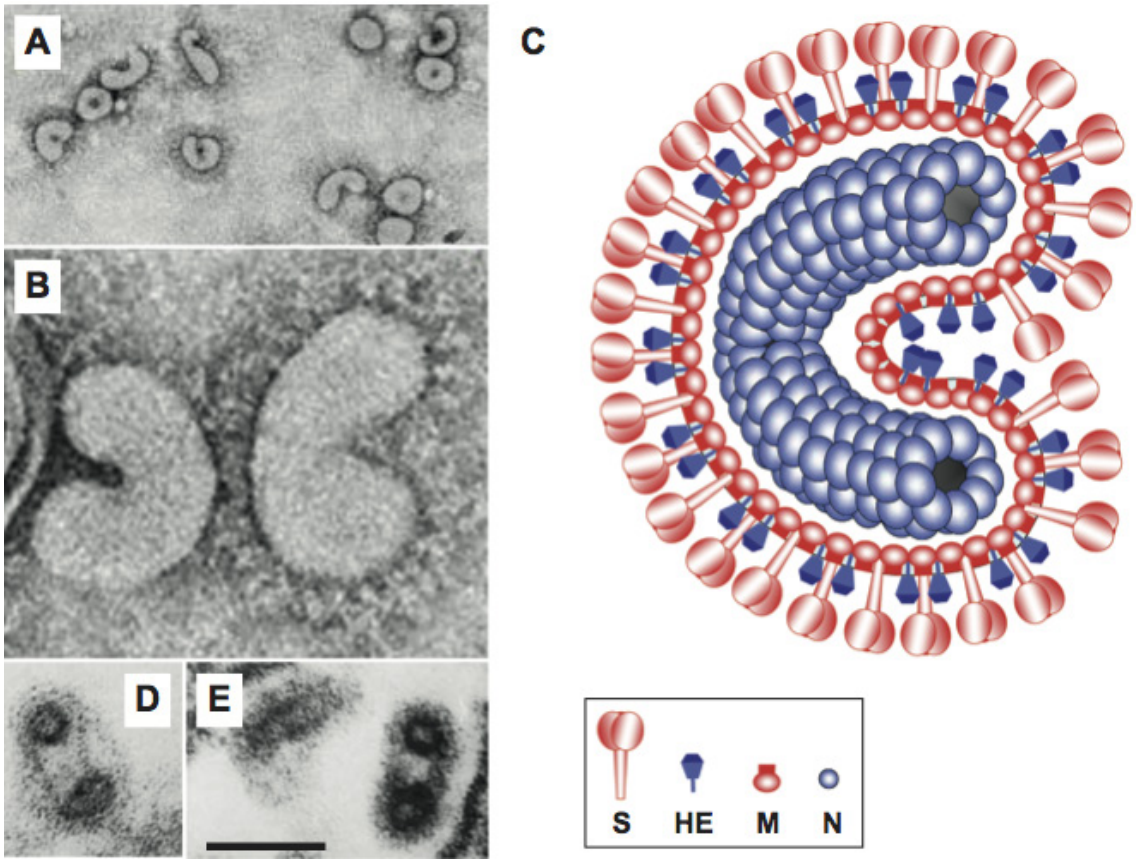

Fig. Electron micrograph of the virion and structure of the torovirus:
S-protein – glycoprotein spike;
HE-protein – hemagglutinin-esterase protein complex involved in virus-cell fusion and suppression of immune response;
M-protein – membrane protein;
N-protein – nucleoprotein (https://ictv.global/sites/default/files/inline-images/f68-08-9780123846846.png)

animals [14]. Feed and water contaminated with torovirus can be considered transmission factors. Calves are mainly infected via fecal and oral route [9, 31, 32].

Entering the gastrointestinal tract, toroviruses attach to enterocytes of the apical surface of the villi of the distal jejunum and ileum, as well as the large intestine [12, 33]. The virus enters enterocytes through receptor-mediated endocytosis. Replication occurs in the cytoplasm of enterocytes. The replication cycle of toroviruses takes around 10–12 hours to complete. Toroviruses are released from enterocytes into the intestinal lumen through pinocytosis. The characteristic torus morphology of BToV is only observed in extracellular viral particles or in vacuoles near the cell surface. Necrosis of the crypt epithelium, desquamation of villous enterocytes and their atrophy are recorded during torovirus infection. Intestinal lesions caused by the infection result in hypersecretory and malabsorptive diarrhea [27, 33, 34].

Torovirus is found in the feces of diseased calves, and in some cases also in fecal samples taken from clinically healthy young animals from farms affected with gastrointestinal diseases. It can be assumed that the test samples were collected from calves at different stages of the pathological process [35]. Torovirus was detected in fecal preparation after three weeks [13, 36]. Natural infection usually occurs in 2–5-day-old calves, but calves up to 4 months of age are apparently susceptible to infection as well [37–39].

This virus was detected in fecal specimens not only from newborn calves with diarrhea, but also from clinically healthy adult cattle [40–42]. The clinical signs observed during natural infection are identical to those demonstrated during rotavirus and coronavirus infections [3–6, 43]. Noroviruses, neboviruses and kobuviruses, along with toroviruses, were detected in feces of newborn calves with diarrhea [29, 31]. Toroviruses were detected in fecal specimens and nasal swabs of fattening cattle [8, 14, 31, 37].

The Japanese researchers first isolated the torovirus in a human rectal adenocarcinoma cell line (HRT-18) from the ileum contents of a calf with diarrhea. The cytopathic effect appeared on day 2–3 after virus inoculation. The torovirus accumulated in the titers of 5.8–6.8 lg $TCID_{50}$/mL after passage 3 in cell culture. Electron microscopy showed that toroviruses appear as oval particles ranging from 100 to 170 nm in diameter. Oval and elongated particles of approximately 100 to 170 nm in diameter with kidney-shaped projections were detected in the supernatant of the infected culture,

and torovirus-like (tubular and thorusnucleocapsid) structures were detected in infected cells using electron microscopy. An antiserum against bovine torovirus (BToV) reacted with the infected cells and neutralized the isolate of this pathogen [44].

Epidemiological investigations conducted in farms affected with mass diarrhea of newborn calves include testing of blood sera from diseased animals for the presence of virus antibodies using enzyme-linked immunosorbent assay (ELISA). To study the prevalence of torovirus infection in the Netherlands and the FRG, serological tests of blood serum samples (n = 1,313 and n = 716, respectively) collected from breeding and fattening herds were conducted. At the same time, antibodies were detected in 94% of adult cattle, 90% of newborn calves had high levels of maternal antibodies, which waned until the age of 3 months [45].

In order to determine the role of bovine torovirus in the development of diarrhea, the Japanese researchers studied the prevalence of this pathogen. Fecal samples of healthy and diarrheal calves were collected for testing using reverse transcription polymerase chain reaction. Torovirus was detected in 17.5% of samples from diseased animals and in 7.0% of samples from healthy calves. These data showed that BToV circulates in Japan mainly among calves under 2 weeks of age [35, 46]. This pathogen was also detected in fecal samples from newborn calves with diarrhea in Canada [29]. In 2009–2014 fecal specimens from 235 newborn calves were tested in Turkey. The torovirus RNA was detected in 4.7% of samples [32]. Subsequently, BToV was found in 16.7% of fecal samples collected from 72 calves from various farms in Turkey. In the phylogenetic tree, the virus isolates recovered from faecal samples of calves in Europe, America, Southeast Asia and Turkey were found to be divided into separate branches [47].

Neonatal calf diarrhea causes significant economic damage to livestock breeding in South Korea [38, 41]. Diarrhea was registered in one of the farms where 207 young cattle were kept. Genomes of pathogens of various infectious diseases were identified in fecal samples of 164 (79.2%) animals. Rotavirus, coronavirus, torovirus, parvovirus, norovirus, kobuvirus, pestivirus were detected in 69.9% of samples, *Escherichia coli* and *Clostridium* bacteria were detected in 31.8% of samples, protozoa (eimeria) in 31.7% of samples, fungi in 14.0% of cases [38]. These test results indicate a mixed etiology of this pathology.

Toroviruses were also detected in the tests for determining the etiology of diarrhea in newborn calves on Chinese farms [39]. BToV was isolated from faecal samples of calves with diarrhea in Croatia [28], Austria [22], from newborn calves and piglets in Hungary [36]. As numerous studies show, animal torovirus infection was diagnosed worldwide: in Switzerland, USA, India, Iran, Canada, Germany, France, Belgium, Great Britain, Costa Rica, the Netherlands, New Zealand, South Korea, Turkey, Japan, Brazil, Finland, Egypt, South Korea and the countries of South Africa. In infected farms, torovirus-induced diarrhea is registered in 50–60% of neonatal calves, resulting in death of 5–10% diseased calves. In most cases, the disease lasts for 5–10 days [14, 29, 32, 33, 35, 37, 38, 41, 42, 44–46, 48–51]. The obtained study results indicate a wide distribution of torovirus in livestock farms.

Tests of fecal samples collected from newborn calves with signs of diarrhea in several large livestock farms in the Russian Federation, conducted using electron microscopy, revealed, besides rotavirus and coronavirus, viral particles morphologically similar to astroviruses [43] and toroviruses [6].

Laboratory diagnosis of torovirus infection is based on test results of fecal samples by polymerase chain reaction and detection of the pathogen in epithelial cells of the small intestine by electron and immunoelectron microscopy. BToV was found to replicate in MDBK cells (calf kidney cell culture), HRT-18 (human rectum adenocarcinoma cell culture cell culture) and calf thyroid gland. Currently, there are no specific preventive tools for torovirus infection. Timely feeding with colostrum containing colostral antibodies induces protection of newborn calves from this infection. Along with this, compliance with sanitary and hygienic requirements and biosafety measures, as well as isolation of diseased animals are recommended. Toroviruses are believed by some researchers to play a certain role in the pathogenesis of diarrhea of mixed etiology in adult cattle [40–42, 45].

CONCLUSION

The presented data indicate a wide geographical distribution of torovirus in cattle, pigs, horses and other livestock species in different countries worldwide. Torovirus infection is manifested by diarrhea of neonatal animals, which leads to mass mortality of livestock and causes great economic damage to livestock farms. Torovirus sheds in faeces and nasopharyngeal secretions of diseased animals. The main route of infection of newborns is fecal-oral. Lesions of the intestinal mucosa in neonatal animals caused by torovirus infection result in the development of hypersecretory and malabsorption diarrhea. Clinical signs and pathologic-anatomical changes due to torovirus infection do not differ from those observed in rotavirus, coronavirus infections of newborn calves and viral diarrhea – diseases of bovine mucosa that are widely distributed in the Russian Federation. Toroviruses play a specific role in the pathogenesis of diarrhea in adult cattle. Data on the close genetic relationship of toroviruses of cattle, pigs, horses, cats and dogs suggest a high probability of cross-infection in these animals, which shall be taken into account when clarifying the etiology of mass diarrhea of these animal species. The torovirus infection is epizootologically characterised by long-term shedding of the pathogen in feces and nasal discharge of diseased animals and virus carriers in high concentrations. Factors of torovirus transmission may include feed, water and animal handling items contaminated with the pathogen. All this indicates the need to take into account torovirus infection when conducting epidemiological investigations in farms affected with mass gastrointestinal diseases of newborn calves, piglets, foals, as well as diseases of cats and dogs with diarrheal syndrome, based on the data of some researchers that toroviruses have zoonotic potential.

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Nontuberculous mycobacterium occurrence in biological material and environmental samples covered by epidemiological surveillance in the Republic of Dagestan

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SUMMARY

An investigation for causes of tuberculosis occurrence and persistence on farms, as well as of continuous presence of tuberculin reactor animals on tuberculosis-free farms impeding allergy diagnosis revealed that the major cause is the persistence of pathogenic and nontuberculous acid-fast mycobacteria in the environment. To determine the occurrence of typical and atypical mycobacteria in samples covered by epidemiological surveillance, 222 biological material samples from cattle, 248 environmental samples (manure, soil, water from different sources, feedstuffs), 44 milk samples from tuberculosis-affected farms, 20 vaginal discharge samples from endometritis-affected cows and 405 sputum samples from tuberculosis-affected humans were tested. Isolation and identification were performed in accordance with the guidelines. Thirty-nine cultures were isolated from the pathological material; of these, 7 (17.9%) were identified as *Mycobacterium bovis* and 32 (82.1%) were identified as atypical mycobacteria. Among nontuberculous mycobacterium cultures, 16 (50.0%) were classified as belonging to group II, 2 (6.2%) – as belonging to group III and 14 (43.8%) – as belonging to group IV according to the Runyon classification. The following species were found to be predominant: group II – *Mycobacterium scrofulaceum* and *Mycobacterium gordonae* (scotochromogenous), group IV – *Mycobacterium smegmatis* and *Mycobacterium fortuitum* (rapidly growing). No mycobacteria were detected in milk samples and vaginal discharge samples from tuberculin reactor cows. From 405 sputum samples from tuberculosis-affected humans, 64 (15.8%) cultures were isolated, of which 55 (85.9%) were classified as *Mycobacterium tuberculosis*, 9 (14.1%) – as *Mycobacterium bovis*. Out of 248 environmental samples tested, mycobacteria were detected in 65 (26.2%) samples, of which 58 (89.2%) were atypical mycobacteria of groups II, III and IV; *Mycobacterium bovis* was isolated from 7 (10.8%) samples (soil and manure). The attempts to isolate *Mycobacterium tuberculosis* failed. The tests demonstrated the wide spread of nontuberculous acid-fast mycobacteria in the environment irrespective of the altitudinal zone. These findings constitute a basis for further monitoring of mycobacterium circulation in the environment in the Republic of Dagestan with a view of optimizing preventive measures.

Keywords: tuberculosis, atypical (nontuberculous) mycobacteria, cattle, allergy diagnosis, environmental objects, biological material, tuberculin PPD for mammals, macroorganism

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Распространение нетуберкулезных микобактерий в объектах эпизоотологического надзора в Республике Дагестан

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РЕЗЮМЕ

При определении причин возникновения и длительного неблагополучия хозяйств по туберкулезу, а также наличия на постоянной основе реагирующих на туберкулин животных в благополучных хозяйствах, способствующих затруднению аллергической диагностики, установлено, что основной является сохранение в объектах внешней среды патогенных и нетуберкулезных кислотоустойчивых форм микобактерий. В целях определения распространенности микобактерий типичных и атипичных форм в объектах эпизоотологического надзора исследовано 222 пробы биологического материала от крупного рогатого скота, 248 проб, отобранных из объектов внешней среды (навоза, почвы, воды из разных источников, кормов), 44 пробы молока из

неблагополучных по туберкулезу хозяйств, 20 проб влагалищных выделений больных эндометритами коров и 405 проб мокроты больных туберкулезом людей. Выделение и идентификацию проводили в соответствии с рекомендациями. Из патматериала удалось выделить 39 культур, из которых 7 (17,9%) идентифицированы как *Mycobacterium bovis* и 32 (82,1%) – как атипичные. Из числа нетуберкулезных микобактерий 16 (50,0%) отнесены к группе II, 2 (6,2%) – к группе III и 14 (43,8%) – к группе IV по классификации Раньона. Установлено доминирующее значение видов из группы II – *Mycobacterium scrofulaceum* и *Mycobacterium gordonae* (скотохромогенные), группы IV – *Mycobacterium smegmatis* и *Mycobacterium fortuitum* (быстрорастущие). В пробах молока и влагалищных выделений от реагировавших на туберкулин коров микобактерии не обнаружили. Из 405 проб мокроты больных туберкулезом людей удалось изолировать 64 (15,8%) культуры, из которых 55 (85,9%) отнесены к *Mycobacterium tuberculosis*, 9 (14,1%) – к *Mycobacterium bovis*. В 65 (26,2%) образцах из объектов внешней среды из 248 исследованных обнаружены микобактерии, 58 (89,2%) из которых составляли атипичные виды II, III и IV групп, в 7 (10,8%) случаях из почвенных проб и навоза выделены *Mycobacterium bovis*. Изолировать *Mycobacterium tuberculosis* не удалось. Исследования показали широкое распространение нетуберкулезных кислотоустойчивых форм в объектах внешней среды, независимо от вертикальной зональности. Полученные данные представляют базовую основу для дальнейшего динамического слежения за циркуляцией микобактерий в природе в условиях Республики Дагестан в целях оптимизации профилактических мероприятий.

Ключевые слова: туберкулез, атипичные (нетуберкулезные) микобактерии, крупный рогатый скот, аллергическая диагностика, объекты внешней среды, биоматериал, ППД-туберкулин для млекопитающих, макроорганизм

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INTRODUCTION

The issue of tuberculosis prevention has become particularly relevant, given that large numbers of animals are kept in relatively small areas with deficient insolation. Such conditions, together with intensified performance, lead to decreased body resistance and facilitate the airborne transmission of tuberculosis agent [1–3].

The improvement of diagnosis system and the development of new reliable differential diagnosis tools, involving, *inter alia*, the investigation of the role of atypical acid-fast mycobacteria in macroorganism sensitization to tuberculin PPD for mammals, are important for enhancing the effectiveness of measures to combat bovine tuberculosis [4–7].

The wide spread of atypical acid-fast mycobacteria in the environment has rendered intradermal tuberculin test with PPD for mammals less indicative and significantly complicated the diagnosis of tuberculosis [8]. The number of reports on the isolation of nontuberculous mycobacteria from animals and humans has grown with the improvement of mycobacterium culture isolation methods and the deepening of relevant knowledge [9–11]. According to the abundant literature data, atypical mycobacteria are isolated from tuberculin reactor animals in 44.6% of cases, from non-reactors – in 48.8%. However, the question of what mycobacterium species can sensitize animals to tuberculin and under what conditions has not been fully elucidated; many researchers are of the view that the ecological relationships of such species have not been adequately investigated [12, 13].

The interest in this group of mycobacteria is attributed to their ability to sensitize a microorganism to tuberculin without causing any lesions associated with tuberculosis [14, 15]. In view of this, in the absence of pathological manifestations characteristic of tuberculosis, bacteriological tests are carried out; based on the test results, the diagnosis is either confirmed or excluded. It should be added that laboratory diagnosis methods are time-consuming and require the use of highly efficient nutrient media to obtain the most accurate result [7, 16]. Bioassay, a basic laboratory method used to differentiate between specific sensitization and non-specific one (caused by atypical mycobacteria), is time-consuming (up to 3 months or more). The analysis of literature shows that bioassay has low specificity for differentiation of most atypical mycobacterium species [17, 18].

It is known that not all atypical mycobacteria can sensitize animals to tuberculin. Therefore, the issues of mycobacterium culture isolation from materials collected from animals and identification thereof should be studied inseparably from detection of allergic reactions and tuberculosis-specific postmortem lesions [19]. The issues of human-animal interface and transmissibility, as well as the possibility of human and animal mycobacterium cross-circulation remain under-researched [20]. There are reports that, in some cases, atypical mycobacteria were found to be the etiological agents of different diseases in humans [21, 22]. Most researchers reject their pathogenicity for cattle and believe that such mycobacteria only cause sensitization to tuberculin [23, 24]. It is also important that some atypical mycobacteria can cause mastitis in cows and lymphadenitis in pigs [11, 25].

It is undisputed that, due to high resistance to various physical and chemical factors owing to the high lipid substance content in the bacterial cell, pathogenic forms

of mycobacteria are widely spread in nature and have extensive contact with the microorganism [26–29].

According to the numerous reports, nontuberculous acid-fast mycobacterium circulation monitoring results indicate that such mycobacteria are well-established in the environment and currently represent the major cause of cattle sensitization to tuberculin PPD for mammals [30–35].

Despite multiple papers concerning the occurrence of these taxons in nature and their relationships with macroorganisms, many aspects of this issue require further investigation.

The study was aimed at the determination of mycobacterium occurrence in biological material samples from animals and humans, as well as environmental samples in the Republic of Dagestan in relation to the altitudinal zonality and species composition.

MATERIALS AND METHODS

For testing, 222 biological material samples from cattle were used. Besides, 248 environmental samples such as manure, soil from livestock facilities and pastures, water from different sources, feedstuffs (straw, silage, haylage, mixed grasses) were collected. In addition, 44 milk samples, 20 vaginal swab samples from endometritis-affected cows and 405 sputum samples from tuberculosis-affected humans were tested.

Before inoculation, homogenized biological materials were treated with a mixture of a 3% sodium lauryl sulfate solution and a 3% sodium hydroxide solution, human sputum samples were treated with a 0.5% chlorhexidine bigluconate solution.

The isolation of cultures was carried out using egg and saline media most commonly applied under laboratory conditions and demonstrating different growth intensity and rate (the Löwenstein – Jensen, Petragnani, Sauton, Finn-II media). Differentiation between the human tuberculosis agent and other mycobacteria was performed based on cultural and morphological, as well as biochemical properties. In some cases, mycobacteria were typed with bioassay in guinea pigs and rabbits through animal inoculation with the test material suspension prepared with a sterile saline solution.

The identification of the isolated cultures was carried out using conventional methods in accordance with GOST 26072-89 "Agricultural animals and poultry. Methods of laboratory diagnostics of tuberculosis" (COMECON Standard 3457-81) and GOST 27318-87 "Agricultural animals. Methods of identification of non-typical mycobacteria" (COMECON Standard 5627-86).

TEST RESULTS

During the bacteriological tests of 222 samples from tuberculin reactor animals, 39 cultures were isolated, of which 7 (17.9%) were identified as *Mycobacterium bovis* and 32 (82.1%) – as atypical mycobacteria. Among 32 nontuberculous mycobacterium cultures, 16 (50.0%) were classified as belonging to group II, 2 (6.2%) – as

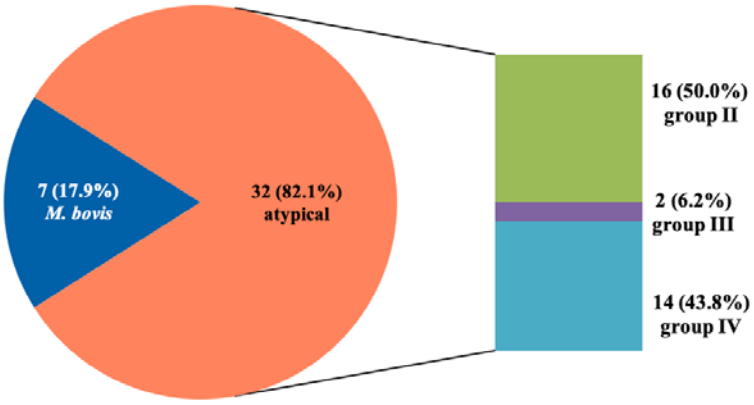

Fig. 1. Mycobacteria isolated from biological material samples from cattle

Table 1
Diversity of mycobacteria isolated from biological material samples from animals and humans

Type of sample	Number of samples	Number of isolated cultures	Type of mycobacteria					
			M. tuberculosis	*M. bovis*	Runyon's group			
					I	II	III	IV
Biological material from animals	222	39	–	7 (17.9%)	–	16 (50.0%)	2 (6.2%)	14 (43.8%)
Milk	44	–	–	–	–	–	–	–
Vaginal discharge	20	–	–	–	–	–	–	–
Sputum from humans	405	64 (15.8%)	55 (85.9%)	9 (14.1%)	–	–	–	–
Total	691	103	55	16	–	16	2	14

Table 2
Diversity of mycobacteria isolated from environmental samples

Type of sample	Number of samples	Number of isolated cultures	Type of mycobacteria					
			M. tuberculosis	*M. bovis*	Runyon's group			
					I	II	III	IV
Pasture soil	29	12	–	4	–	5	3	–
Farm soil	17	3	–	–	–	3	–	–
Stagnant water	22	2	–	–	–	–	–	2
Artesian water	26	–	–	–	–	–	–	–
River water	24	–	–	–	–	–	–	–
Mixed grass hay	30	6	–	–	–	5	1	–
Straw	20	4	–	–	–	1	3	–
Haylage	21	8	–	–	–	6	1	1
Silage	25	3	–	–	–	2	1	–
Manure	16	15	–	3	–	8	3	1
Samples from facilities	18	12	–	–	–	3	9	–
Total	248	65	–	7	–	33	21	4

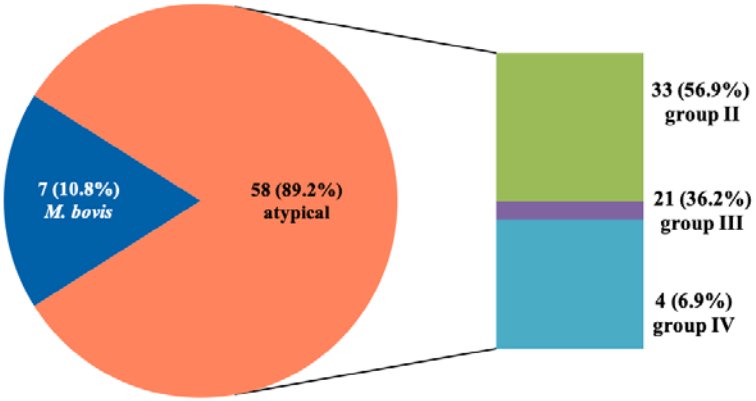

Fig. 2. Mycobacteria isolated from environmental samples

belonging to group III and 14 (43.8%) – as belonging to group IV (Fig. 1) according to the Runyon classification.

The analysis of isolated culture differentiation data allowed for identification of the predominant associations of atypical mycobacteria inhabiting the animal body. These were the combinations of *Mycobacterium scrofulaceum* and *Mycobacterium gordonae* (group II), *Mycobacterium smegmatis* and *Mycobacterium fortuitum* (group IV) according to the Runyon classification (Table 1).

No mycobacteria were detected in 44 milk samples from tuberculin reactor cows from tuberculosis-affected farms and in 20 vaginal discharge samples from endometritis-affected cows with positive tuberculin test results.

During the tests of 405 sputum samples from tuberculosis-affected humans using the Löwenstein – Jensen medium, 64 (15.8%) mycobacterium cultures were isolated, of which 55 (85.9%) were identified as *Mycobacterium tuberculosis* and 9 (14.1%) – as *Mycobacterium bovis*.

During the tests of 248 environmental samples, mycobacteria were detected in 65 (26.2%) of them; among these, 58 (89.2%) were classified as belonging to groups II, III and IV according to the Runyon classification. Bovine mycobacterium culture was isolated in 7 (10.8%) cases, the attempts to isolate *Mycobacterium tuberculosis* failed. The results are presented in Table 2 and Figure 2.

Quantitative distribution of isolated tuberculous cultures showed that such mycobacteria were isolated from pasture soil and manure samples only. Nontuberculous mycobacteria were detected in maize silage samples collected from the pit silo at the bovine tuberculosis-free dairy complex SPK "Dylym" in the Kazbekovsky Raion (submountain zone), even with direct microscopy, and this is indicative of their survivability and possible replication under the technological conditions of maize silage fermentation. Subsequently, a more detailed analysis found an association between permanent reactions to tuberculin PPD for mammals on this farm and continuous (based on the laboratory test results for a number of years) circulation of atypical mycobacteria in the environment.

The tests showed that nontuberculous mycobacteria are detected in samples collected on farms, whether tuberculosis-affected or not, irrespective of the altitudinal zone. In particular, *Mycobacterium smegmatis* and *Mycobacterium phlei*, the representatives of group IV of atypical mycobacteria, were isolated from manure samples and leftover feed samples collected from the feed bunks on the farms SPK im. Chapayeva (mountain zone) and KFKh "Rassvet" (submountain zone). *Mycobacterium scrofulaceum* (group II) was isolated from samples collected at the dairy complex SPK "Khamamatyurtovsky" (flatland zone) and those collected in the area adjacent to the farm SPK "Turchidag" (mountain zone).

The number of mycobacterium detections in the soil samples from the flatland zone pastures is higher than that in samples from the mountain zone. For example, no bacteria were isolated from samples collected in some pasture units of the farms SPK "Turchidag" and SPK im. Chapayeva (mountain zone); however, they were detected practically in all samples from the flatland zone pastures.

Also, no mycobacteria were detected in the mountain river and artesian well water samples. A group IV representative (*Mycobacterium fortuitum*) was isolated from water samples from the stagnant water bodies located near the area adjacent to the farm SPK "Rassvet" and the tuberculosis-affected dairy complex SPK "Tersky" (the Kizlyar zone of distant pastures) located in the flatland zone.

DISCUSSION AND CONCLUSIONS

The analysis of data from the study clearly reveals that atypical mycobacteria of groups II, III and IV (according to the Runyon classification) can be the major cause of macroorganism sensitization to tuberculin PPD for mammals. The predominance of group IV bacteria in soil, manure and stagnant water samples allows the conclusion that they are the typical obligate representatives of nontuberculous mycobacteria that have steadily established themselves in the environment of the Republic of Dagestan and shape the gastrointestinal mycobacterial landscape in cattle. Our findings are consistent with those of P. S. Guseynova et al., S. I. Dzhupina, M. Ridell, as well as E. Stackebrandt and B. M. Goebel from the determination of major causes of cattle sensitization to tuberculin for mammals [30–32, 35].

At the same time, in some cases during testing, no atypical mycobacteria were detected in the test samples. We believe that this is due to the imperfection of laboratory diagnosis, nontuberculous bacterium transition to a nonculturable state, various transformations and changes in the genetic structure. This fact is very important, since numerous studies, including those of recent years, show that atypical mycobacteria are isolated from bedding material and environmental samples, but they are not detected in the biological material samples from tuberculin reactor animals, and vice versa [17, 18, 22].

There is therefore a need to use specific tests for each particular typical and atypical mycobacterium species to enable the characterization of their isolation, cultivation, typing, as well as mycobacterium-like microorganisms' ability to sensitize a macroorganism to tuberculin.

Enhanced laboratory diagnosis and monitoring of nontuberculous mycobacterium circulation in the environment will allow for rapid response and interpretation of allergy test results for timely implementation of veterinary and sanitary measures.

Thus, the presented data show that atypical mycobacteria are widely spread in the environment. Bovine tuberculosis agents are isolated from the biological material samples from tuberculin reactor animals, environmental samples and soil samples from areas adjacent to tuberculosis-affected farms, as well as sputum samples from tuberculosis-affected humans. Further work will be needed to monitor the circulation of all types of mycobacteria in all physical and climatic zones of the Republic of Dagestan and to ensure control over the implementation of the veterinary and sanitary measures aimed to prevent mycobacterium spread in the natural reservoirs.

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Molecular identification of Newcastle disease virus isolated on the poultry farm of the Moscow Oblast in summer of 2022

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SUMMARY

In August 2022, a sudden death in backyard chickens was reported in the Moscow Oblast (urban district Chernogolovka, settlement Starki). As a result, within just a few days 45 chickens on this farm died or fell ill with the following signs – gray mucus discharge from nostrils and beak, coughing, gasping and rales. On day 1–3 after the onset of symptoms, the chicken died. The Newcastle disease virus, which is a representative of the *Paramyxoviruses* family, was isolated from the dead poultry. We determined the nucleotide sequences of fragments in F gene (encodes the fusion surface protein) and in NP gene (encodes the nucleocapsid protein). The motif of $_{109}$SGGRRQKRFIG$_{119}$ proteolysis site, typical for the velogenic pathotype, was determined for the F gene, and a phylogenetic analysis was carried out to demonstrate that the isolate belonged to Subgenotype VII, Class II of the subfamily *Avulavirinae*. The Basic Local Alignment Search Tool revealed that they are most genetically related with isolates from Iran. It was found that the average death time of developing chicken embryos, infected with a minimum infectious dose, was 52 hours, which is typical for the velogenic pathotype. The virus caused 100% death in six-week-old chickens after oral infection and 100% death in all contact chickens, including those kept in cages at a distance, which proves the high level of pathogenicity and contagiousness of the recovered isolate and its ability to transmit both via fecal-oral and aerosols–borne routes. No death cases were reported in mice after intranasal infection with high doses.

Keywords: Newcastle disease virus, pathogenicity, molecular genetic analysis, contagiousness

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Молекулярная идентификация вируса ньюкаслской болезни, выделенного в домашнем птицехозяйстве Подмосковья летом 2022 года

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РЕЗЮМЕ

В августе 2022 г. в Московской области (городской округ Черноголовка, деревня Старки) на личном подворье внезапно начался падеж кур, в результате которого в течение нескольких дней погибли или заболели все 45 голов этого хозяйства со следующими признаками: выделение серой слизи из ноздрей и клюва, резкие кашляющие звуки. Через 1–3 дня после появления симптомов птица погибала. Из тканей, полученных от павших кур, был выделен вирус ньюкаслской болезни, являющийся представителем обширного семейства парамиксовирусов. Определены нуклеотидные последовательности фрагментов гена F, кодирующего поверхностный белок слияния, и гена NP, кодирующего белок нуклеокапсида. Для гена F определен мотив сайта протеолиза $_{109}$SGGRRQKRFIG$_{119}$, типичный для велогенного патотипа, также проведен филогенетический анализ, по результатам которого установлена принадлежность изолята к субгенотипу VII класса II подсемейства *Avulavirinae*. С помощью Basic Local Alignment Search Tool выявлено наиболее генетически

близкое родство с изолятами из Ирана. Установлено, что среднее время смерти развивающихся куриных эмбрионов при заражении минимальной инфекционной дозой составило 52 ч, что характерно для велогенного патотипа. Вирус вызывал 100%-ю гибель цыплят шестинедельного возраста при оральном заражении, а также 100%-й падеж всего контактного молодняка, включая содержащихся в клетках на отдалении, что доказывает высокий уровень патогенности и контагиозности выделенного изолята и его способность распространяться не только фекально-оральным, но и аэрозольным путем. Гибель мышей при интраназальном заражении высокими дозами не наблюдалась.

Ключевые слова: вирус ньюкаслской болезни, патогенность, молекулярно-генетический анализ, контагиозность

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INTRODUCTION

Newcastle disease virus (NDV), or *Avian orthoavulavirus* 1 (AOAV-1), belongs to extensive *Paramixoviridae* family, *Avulavirinae* subfamily, its representatives cause avian diseases of various severity [1]. NDV affects more than 240 wild and domestic avian species and is an enveloped negative-sense single-stranded RNA virus. The genome is not segmented and is about 15,000 bases long, divided by conservative non-coding regions into six genes in 3'-NP-P-M-F-HN-L-5' sequence encoding eight proteins [2, 3].

One of the key factors that influence NDV pathogenicity is the number of basic acids in the F protein cleavage site [4]. Low pathogenic virus strains have sequence $_{109}$SGGGR(K)QGRLIG$_{119}$ in the proteolytic site and can be cleaved only by extracellular trypsin-like proteases. The motif of highly pathogenic NDV strains has several basic amino acids (lysine or arginine) and phenylalanine at amino acid position 117: $_{109}$SGGRRQ(K/R)RF(V/I)G$_{119}$. The protein of such a virus is able to transform into an active form with the help of furin-like proteases present in all body cells. This virus causes a generalized infection [5].

Phylogenetically, NDV is divided into 2 classes, Class I includes only one genotype and is represented mainly by apatogenic strains isolated from wild and domesticated birds [6]. Class II is divided into 21 genotypes and includes strains of different virulence affecting a wide range of hosts [7]. The class representatives are spread worldwide and have regularly induced epizooties.

NDV poses a serious threat to poultry. The mortality rate in the infected chickens can reach 100%. According to the pathogenicity for chickens, NDV can be lentogenic (low pathogenic), mesogenic (causing moderate disease in adult chickens and death in young birds) and velogenic (highly pathogenic for chickens of all ages) [3].

The virus may spread due to trade in poultry and poultry products, as well as during movement of workers from the infected poultry farms and during transportation [8]. Migration of wild birds is another way of the virus spread [9, 10]. Although most apatogenic NDV isolates recovered from wild birds do not pose a serious threat to chickens, a mutation accumulation increases the virus pathogenicity [11].

The aim of this research is to conduct a molecular and phylogenetic analysis of the Newcastle disease virus isolated from the chicken pathological material taken in one of the backyards located in the Moscow Region, to determine its pathotype; to study its pathogenicity and contagiousness.

MATERIALS AND METHODS

Reagents. MycoKill AB (PAA Laboratories GmbH, Austria); mini-kit for RNA virus isolation QIAamp Viral RNA Mini Kit (QIAGEN, Germany); set of reagents for reverse transcription MMLV RT kit, random decanucleotide primer, set of reagents Tersus Plus PCR kit, nuclease-free water, DNA markers and TAE buffer (Eurogen, Russia); ribonuclease inhibitor (Syntol, Russia); set reagents for sequencing ABI PRISM® BigDye™ Terminator v3.1 (ThermoFisher Scientific Inc., USA).

Virus isolation. Lung and kidney samples from dead chickens were used to isolate the virus. Tissue fragments were rubbed with fine glass powder; a fourfold phosphate buffered saline (PBS) solution was added containing 0.4 mg/mL gentamicin, 0.1 mg/mL kanamycin, 0.01 mg/mL nystatin and 2% MycoKill AB solution. The suspension was centrifuged for 10 min at 4,000 rpm, and 0.2 mL of the supernatant was inoculated through allantoic cavity into 10-day-old developing chicken embryos (DCE). Incubation lasted 72 hours at 37 °C; embryo death was controlled twice a day. Then, virus-containing allantoic fluid (VAF) was collected and tested in the hemagglutination test (HA test) using a 1% suspension of chicken erythrocytes according to the generally accepted method [12]. The amount of virus was expressed in hemagglutinating

units. The 50% infectious dose (EID_{50}) was determined by titration in developing chicken embryos.

RNA extraction, cDNA synthesis and sequencing. The viral RNA was extracted from virus-containing allantoic fluids using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The reverse transcription was carried out using a set of reagents MMLV RT kit (Eurogen, Russia) in presence of a random decanucleotide primer. The polymerase chain reaction (PCR) was carried out in a volume of 25 µL using a set of reagents Tersus Plus PCR Kit (Eurogen, Russia): sterile water for PCR – 17.5 µL; 10× Tersus Plus buffer – 2.5 µL; 50× dNTP mixture – 0.5 µL; direct primer fFapmv2 (10 µmol) – 1 µL; reverse primer rFapmv2 (10 µmol) – 1 µL, cDNA – 2 µL; 50× Tersus polymerase – 0.5 µL. Oligonucleotides used in the work: fFapmv2 (ATGGGCTCCAGACCTTCTAC); rFapmv2 (CTGCCACTGCTAGTTGCGATAATCC); fNPapmv (GGTATTCTGTCTTCGGATTG); rNPapmv (TCATCCGATATAAACGCAT). The PCR products were analysed by 2% agarose gel electrophoresis in a tris-acetate buffer. PCR fragments of about 500 bp were cut out for clean-up from the gel using the Qiagen MinElute Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's instructions. The nucleotide sequences of PCR fragments were determined using ABI PRISM® BigDye™ Terminator v3.1 reagent kit (ThermoFisher Scientific, USA), followed by PCR product analysis on the ABI PRISM 3130 sequencer (ThermoFisher Scientific, USA). The resulting chromatograms were analyzed using SnapGene Viewer program[1].

Phylogenetic analysis. All the sequences were downloaded from the GenBank database[2]. The sequences were processed using BioEdit 7.2[3] and MEGA X[4] [13, 14]. BEAST v1.10.4 software was used to perform a Bayesian Markov Chain Monte Carlo-based phylogenetic analysis (MCMC) of the nucleotide sequences (n = 150) using the General Time Reversible (GTR) model [15]. The iTOL v6[5] online service was used to visualize and annotate the tree. Genotype was identified using phylogenetic topology.

Determination of the virus pathotype. The virus pathotype was determined by the mean death time (MDT) method. 0.2 mL of 10-fold dilutions of fresh VAF on PBS (from 10^{-3} to 10^{-7}) were inoculated into the allantoic cavities of 9-day developing chicken embryos. After that, they were incubated for five days at 37 °C, checked 2 times a day to control embryo death. MDT was calculated as the mean death time of an embryo infected with a minimum lethal dose. If MDT is up to 60 hours, the virus pathotype is defined as velogenic, from 60–90 – as mesogenic, more than 90 – as lentogenic.

Analysis of pathogenicity and contagiousness of the virus for chickens. 6-week-old Leghorn chickens were infected orally, with the virus added to the drinking bowl. Virus-free fresh allantoic fluid was added into the drinking bowl for the control and contact groups. Each group included 5 chickens; the groups were initially put into separate cages. The chickens were daily examined for 10 days after infection.

Analysis of the virus pathogenicity for mice. BALB/c mice weighing 10–12 g were infected intranasally (under light ether anesthesia) with 50 µL containing doses from 10^3 to 10^6 EID_{50}/mouse. A group of 5–6 mice was infected with each viral dose. The control group received virus-free allantoic fluid diluted with saline solution. Survival rate and mice weight were recorded within 15 days after infection.

Ethical status. Animal experiments were conducted in accordance with the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, March 18, 1986)"[6]. The Ethics Committee of the FSASI "Chumakov FSC R&D IBP RAS" (Institute of Poliomyelitis), Resolution No. 4 of December 2, 2014, approved the experiment design. All measures were taken to reduce the suffering of animals.

RESULTS AND DISCUSSION

In late August 2022, mass death was reported in chickens in the Moscow region (urban district Chernogolovka, settlement Starki). Pathological material from the dead birds was sent for laboratory tests to the (FSASI "Chumakov FSC R&D IBP RAS" (Institute of Poliomyelitis). Lung and kidney tissue suspensions were inoculated into 10-day DCE through allantoic cavities. Newcastle disease virus was detected in all HA-positive VAF samples tested in RT-PCR. The isolate was named as NDV/chicken/Moscow/6081/2022.

Molecular and phylogenetic analysis. While studying the isolate, 438 bp F gene fragment (which includes a site encoding the F protein proteolytic sites) was amplified and sequenced. The obtained nucleotide sequence was analyzed using the Basic Local Alignment Search Tool (BLAST)[7] and the analysis showed that NDV/chicken/Moscow/6081/2022 isolate is most closely related to the viruses recovered in Iran from chickens in 2011–2013 (similarity is 97.03–97.48% with the first five sequences). Several basic amino acid residues (arginine/lysine) with $_{109}$SGGRRQKRFIG$_{119}$ motif were detected in F protein proteolytic site of the studied virus, as demonstrated by the obtained sequence. This sequence is specific for virulent strains based on the criteria used by the World Organisation for Animal Health to assess the virulence of NDV isolates [12].

Additionally, nucleotide sequence of the NP gene encoding the nucleocapsid protein (697 bp) was obtained. This gene may also indirectly affect NDV virulence. Thus, nucleotides 546 and 555 are different for lentogenic and velogenic strains [16]. According to the sequencing results, positions 546 and 555 in NP gene of the recovered isolate correspond to the velogenic variant (two thymines (T) in positions 546 and 555). Both sequences were uploaded to the GenBank database under numbers OQ190211 and OQ190212.

For phylogenetic analysis, a sample was taken from representative sequences of each genotype of Class II (n = 125) [7]. The sample was combined with

[1] SnapGene Viewer. Available at: https://www.snapgene.com/snapgene-viewer.

[2] GenBank. Available at: https://www.ncbi.nlm.nih.gov/genbank.

[3] BioEdit. Available at: https://bioedit.software.informer.com.

[4] MEGA X. Available at: https://www.megasoftware.net.

[5] iTOL. Available at: https://itol.embl.de.

[6] The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, March 18, 1986). Available at: rm.coe.int›168007a67b.

[7] BLAST. Available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

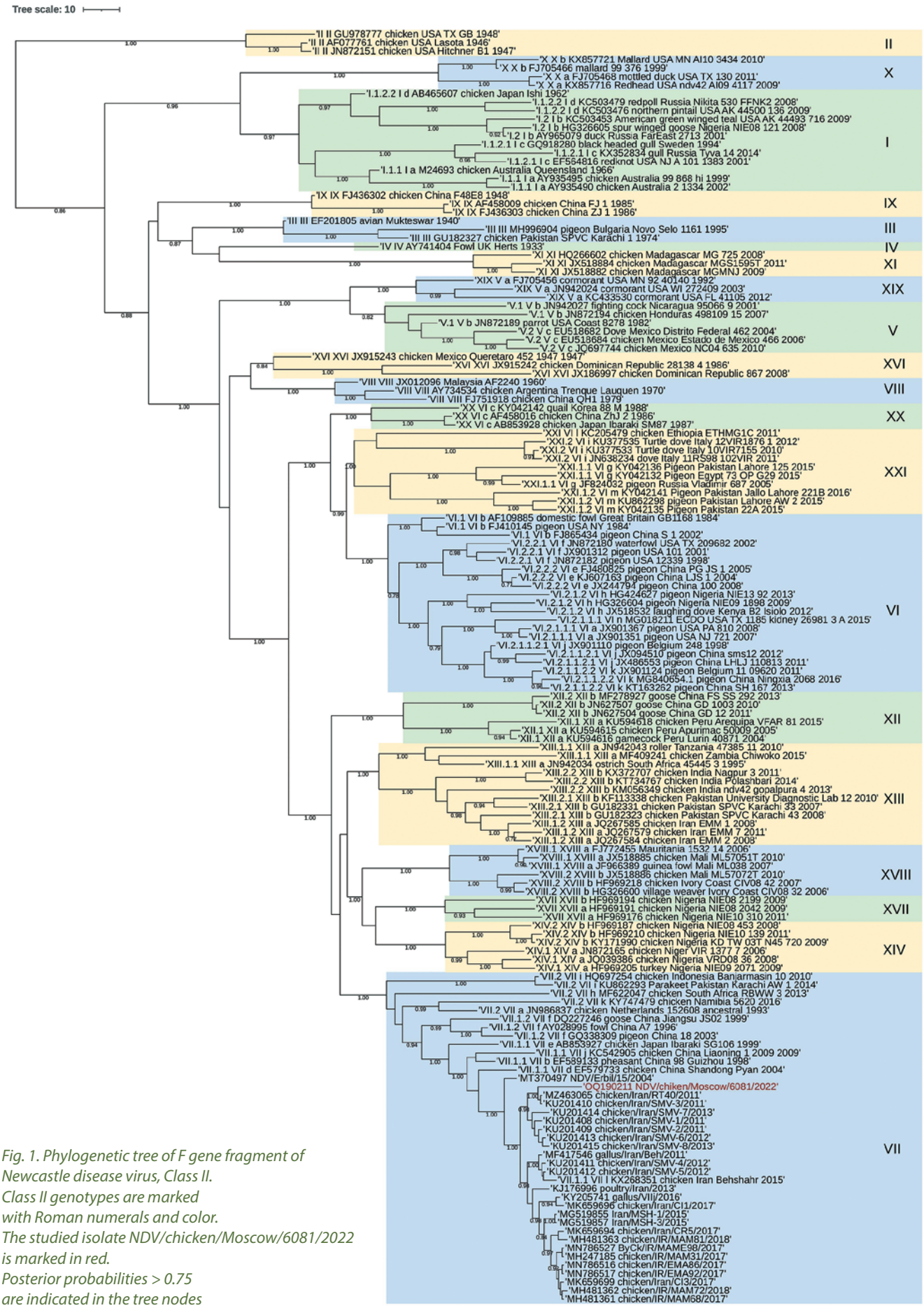

Fig. 1. Phylogenetic tree of F gene fragment of Newcastle disease virus, Class II. Class II genotypes are marked with Roman numerals and color. The studied isolate NDV/chicken/Moscow/6081/2022 is marked in red. Posterior probabilities > 0.75 are indicated in the tree nodes

the sequence of F gene fragment of the Moscow isolate and a sample from 24 most closely related viruses identified in BLAST. Phylogenetic analysis of the F gene fragment showed that NDV/chicken/Moscow/6081/2022 isolate belongs to Genotype VII, Class II (Fig. 1).

Genotype VII of Class II originated in Asia presumably in the 1980s and is now widespread in Eurasia and Africa; it is as well recorded in South America [17–19]. The virus was the cause of Newcastle disease Fourth Panzootic, which has been ongoing since the 1980s [9]. The genotype includes only velogenic NDV strains that cause high mortality in birds [20].

Determination of pathogenicity and contagiousness of NDV/chicken/Moscow/6081/2022 isolate for chickens. It was found that MDT for 9-day-old DCE is 52 hours, which corresponds to the velogenic type (up to 60 hours).

To study the isolate contagiousness, three groups of five 6-week-old chickens were formed. To infect the birds of Group 1, 10^8 EID_{50} of NDV/chicken/Moscow/6081/2022 virus were added to the drinking bowl. The next day, the infected chickens were put into a cage of Group 2. Cage No. 1 was removed and disinfected. The chickens from Group 3 were in Cage 3, located two meters away from Cages 1 and 2, so that to exclude contamination with feed particles and faeces, however, air and fine dust circulation between the cages was possible. The death dynamics in infected and contact birds is shown in Figure 2. All the infected chickens died by Day 5, contact birds from Group 2 died on Day 6, and in Group 3 chickens fell ill on Days 6 and 7, after that all the birds died by Day 10. NDV/chicken/Moscow/6081/2022 virus was detected in the organs of the dead birds using PCR.

Thus, contact chickens (Group 2) caught the disease from the infected ones almost immediately. The death of chickens from Group 3, who did not come into a direct contact with the sick ones, may be explained by the fact that at some point one of the chickens became infected through airborne droplets, thus causing the infection spread and rapid death of the entire group. The experiment results demonstrate a very high pathogenicity and contagiousness of NDV/chicken/Moscow/6081/2022 isolate and show that the birds are infected not only by fecal-oral route, but also through the air.

Pathogenicity of the virus for mice. Avulavirinae representatives, as a rule, are non-pathogenic to mammals. However, given the exceptionally high pathogenicity and contagiousness of NDV/chicken/Moscow/6081/2022 isolate for chickens, it was decided to make sure that it is safe for mammals.

The weight change dynamics in mice infected intranasally with NDV/chicken/Moscow/6081/2022 is shown in Figure 3. Groups 1, 2, 3 and 4 were infected at doses of 10^3, 10^4, 10^5 and 10^6 EID_{50}/mouse, respectively. The control group was given placebo (virus-free allantoic fluid, diluted with saline solution). Survival rate and mice weight were recorded within 12 days after infection. No mice died during the experiment. Figure 3 shows that groups infected with high viral doses slightly became slightly underweight on Days 2–5 after infection, but by Day 12 almost all the mice were healthy.

Thus, NDV/chicken/Moscow/6081/2022 isolate of Newcastle disease virus is practically not pathogenic for mice, despite its very high pathogenicity for chickens.

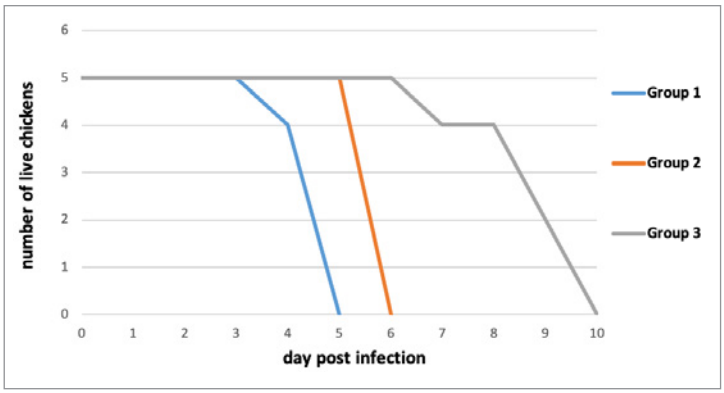

Fig. 2. Dynamics of death in infected and contact chickens

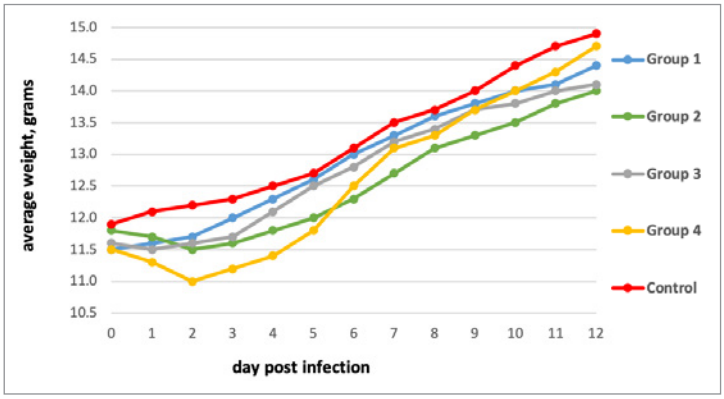

Fig. 3. Dynamics of the mouse weight changes upon infection with NDV/chicken/Moscow/6081/2022

CONCLUSION

In August 2022, all chickens died in one of the backyards of the Moscow region shortly after the first disease symptoms appeared. Newcastle disease virus was detected in the dead bird tissues. Laboratory tests showed high pathogenicity of the isolate for chickens and no pathogenicity for mice.

Molecular analysis together with the MDT test helped to check several factors indicating how NDV/chicken/Moscow/6081/2022 isolate is related to velogenic NDV: presence of a polybasic proteolytic site in F fusion protein, presence of two amino acids in NP nucleocapsid protein, which is typical for highly pathogenic NDV, MDT up to 60 hours.

The phylogenetically isolated virus belongs to Genotype VII, Class II. This genotype belongs to the "late" ones (emerged after the 1960s) and includes viruses of the velogenic pathotype only. Most current outbreaks in chickens in Asia and the Middle East are associated with this particular virus [21]. Its widespread is partly explained by the fact that the strains related to this genotype are able to spread in poultry vaccinated with popular commercial vaccines [20].

In the Russian Federation, NDV poses a potential economic threat to the poultry industry. As the Rosselkhoznadzor data show, more than 25 outbreaks of Newcastle disease have been registered in various regions of the country since 2019[8]. Serological data show an increase

[8] Rosselkhoznadzor. Available at: https://fsvps.gov.ru/ru. (in Russ.)

in the number of immune wild birds and non-vaccinated domestic ducks after 2017 [22–25]. To control the virus spread, it is required to continue monitoring, as well as to timely vaccinate poultry in backyards [26, 27].

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Dynamics of seasonal rabies incidence in animals in Azerbaijan

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SUMMARY

The increasing number of rabies outbreaks is currently one of the most important challenges in both human and animal health. The epidemiological and epizootic significance of rabies is determined by its absolute lethality in case of clinical manifestations, as well as global spread, latent incubation period and lack of specific treatment. Rabies is endemic in Azerbaijan; wild carnivores, stray dogs and cats determining the natural type of rabies are considered the main source of infection in the Republic. The dynamics of rabies natural cases has seasonal variability. As a rule, the number of disease cases increases in autumn, winter and spring, which is associated with the biological characteristics of the main disease vectors and the climatic conditions in the region. The main purpose of the study was to investigate the spread of rabies in Azerbaijan in different seasons of the year. For this purpose, the statistical data were collected based on animal incidence by month and season for the last four years (2018–2021). It was found that rabies cases were most often identified in the period from March to May: in 2018 – 21 (31%) cases, in 2019 – 24 (38%) cases, in 2021 – 8 (40%) cases. The exception was 2020, when the majority of rabies cases occurred in December – February. To assess the epizootological and epidemiological risks of rabies in the country, the disease frequency rate among various animal species was studied by year. The largest number of rabies cases (54%) was shown to be detected among dogs. Cattle accounted for 38.1% of cases, 5.7% of positive samples were derived from stray dogs, 1.6% – from sheep, 0.6% – from horses. The study results have shown that animal rabies exhibits a clearly pronounced seasonal pattern in the Republic of Azerbaijan.

Keywords: rabies, epizootic situation, seasonality, incidence, dynamics

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Динамика сезонной заболеваемости животных бешенством в Азербайджане

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РЕЗЮМЕ

На сегодняшний день одной из важнейших проблем как здравоохранения, так и ветеринарии является растущее количество очагов рабической инфекции. Эпидемиолого-эпизоотическая значимость бешенства определяется абсолютной летальностью при условии проявления клинических признаков, повсеместным распространением, латентным инкубационным периодом и отсутствием средств специфического лечения. В Азербайджане бешенство является эндемичным заболеванием, основным источником вируса считаются дикие плотоядные животные, бродячие собаки и кошки, обуславливающие природный тип инфекции в республике. Обычно динамика естественных случаев бешенства имеет сезонную изменчивость. Как правило, число случаев заболевания увеличивается осенью, зимой и весной, что связано с биологией основных переносчиков болезни и природно-климатическими условиями региона. Основной целью исследования было изучение распространения бешенства на территории Азербайджана в разные сезоны года. Для этого были собраны статистические данные за последние 4 года (2018–2021 гг.) с учетом заболеваемости животных по месяцам и сезонам. Установлено, что случаи заболевания бешенством чаще всего регистрировались в период с марта по май: в 2018 г. – 21 (31%), в 2019 г. – 24 (38%), в 2021 г. – 8 (40%). Исключение составил 2020 г., когда пик заболеваемости пришелся на декабрь – февраль. Для оценки эпизоотологических и эпидемиологических рисков возникновения бешенства в стране была изучена частота встречаемости заболевания среди животных разных видов по годам. Показано, что наибольшее количество случаев бешенства (54%) было выявлено среди собак. На долю крупного рогатого скота приходилось 38,1% случаев, 5,7% позитивных проб составляли образцы от бездомных собак, 1,6% – от овец, 0,6% – от лошадей. Результаты исследований показали, что заболевание бешенством животных на территории Азербайджанской Республики имеет четко выраженную сезонность.

Ключевые слова: бешенство, эпизоотическая ситуация, сезонность, заболеваемость, динамика

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INTRODUCTION

The World Health Organization (WHO) and other international organizations have set a goal to eliminate human deaths due to dog rabies by 2030. Despite detailed information on such a dangerous disease as rabies in the media, there is still a need to increase public awareness [1–4].

Humans, as a rule, become infected via the bite of a rabies-infected animal, most commonly dogs, cats, rabbits, ferrets, foxes, wolves, raccoons, bats, etc. [5–7].

Rabies is an endemic disease and is subject to mandatory reporting throughout the territory of Azerbaijan. Wild carnivores, stray dogs and cats are considered the main source of the virus and cause the natural type of rabies in the Republic [8]. The characteristics of stray dog populations (density, growth dynamics, etc.), most frequently causing infection in humans, are unknown [9–11]. Therefore, conducting research and epidemiological studies is important for disease control [12–14].

The number of stray dogs in Azerbaijan is currently quite large. Rabies cases in animals that attack people are registered annually. At the same time, bites of stray dogs are considered the main etiological factor [14–16]. The current program for managing the population of these animals is to conduct sterilization and vaccination against rabies. Killing of animals has been prohibited in Azerbaijan since the "European Convention for the Protection of Pet Animals"[1] was adopted in the country. Hence, it is very important to control the spread of zoonotic diseases (mainly, rabies) throughout the country, as well as in the population of stray and feral dogs bearing a number of problems with socio-economic, religious, environmental and political consequences [3, 9, 15].

Not enough studies have been conducted in Azerbaijan to assess the risks of the pathogen's spread. In addition to the fact that stray dogs are the main cause of human infection in the settlements of the Republic, forest areas on the border with other countries are considered risk zones. Epizootological monitoring, laboratory diagnosis, vaccination and public awareness campaigns are carried out in Azerbaijan within implementation of measures aimed at the disease elimination [17, 18].

According to the Ministry of Health of Azerbaijan, 18,702 cases of animal-bites in humans were registered in 2016, 18,470 cases in 2017, 31,060 cases in 2018, 40,234 cases in 2019 and 21,671 cases in 2020 were recorded. According to the data of the Center for Surveillance of Highly Dangerous Infections, in 2016 there were 7 human deaths caused by rabies, in 2017 – 3 cases, in 2018 – 5 cases, in 2019 – 5 cases, in 2020 – 2 cases. Despite the measures taken, the problem of rabies incidence is still relevant, so it is necessary to conduct risk analysis of rabies virus spread.

Table
Dynamics of seasonal rabies incidence in animals in 2018–2021

Year	Number of rabies cases				
	December – February	March – May	June – August	September – November	Total
2018	16	21	17	14	68
2019	18	24	8	13	63
2020	17	5	3	4	29
2021	4	8	4	4	20
Total	55	58	32	35	180

This study was aimed at investigating the dynamics of animal rabies transmission depending on the season. For this purpose, statistical data on animal disease cases in the Republic were analyzed for the period from 2018 to 2021.

MATERIALS AND METHODS

Seasonal changes in the population size, behavior and physiological parameters of animals are considered to be the factors that significantly affect the spread of wild animal diseases [19, 20]. Understanding the basic mechanisms and forecasting the seasonal spread of animal diseases such as rabies is crucial for the implementation and optimization of strategies aimed at their control [21, 22]. To determine the seasonal dynamics of rabies, the statistical data were collected for the last 4 years (2018–2021), taking into account the morbidity of animals by month and season. The rabies incidence in different animal species for the specified period was calculated based on the data provided by the National Veterinary Laboratory.

Rabies laboratory diagnosis is performed in accordance with the Guidelines on Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health (WOAH)[2]. The pathogen is identified using the fluorescent antibody test (FAT), which is considered the screening method. Positive samples are confirmed using polymerase chain reaction (PCR) [23].

TEST RESULTS

The table shows data on seasonal rabies incidence in animals for the last 4 years (2018–2021).

[1] European Convention for the Protection of Pet Animals (ETS No. 125). Available at: https://www.coe.int/ru/web/conventions/by-member-states-of-the-council-of-europe?module=treaty-detail&treatynum=125.

[2] Rabies (Infection with Rabies Virus and other Lyssaviruses). *In: WOAH. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.* Chapter 3.1.18. Available at: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.18_RABIES.pdf.

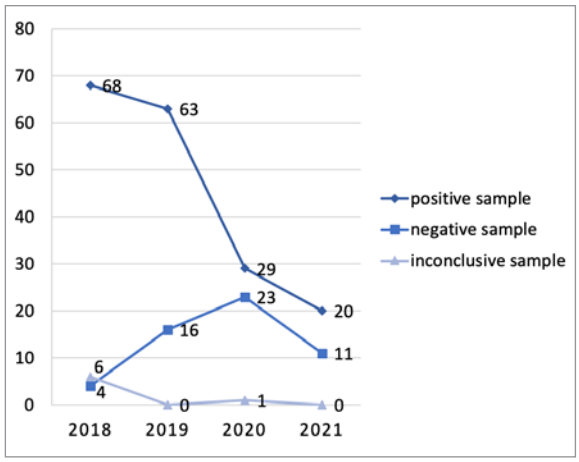


Fig. 1. Brain samples of suspected rabies cases submitted to the laboratory in 2018–2021

It was found that rabies cases were most often registered in the period from March to May: 21 (31%) cases – in 2018, 24 (38%) cases – in 2019, 8 (40%) cases – in 2021. The exception was 2020, when the majority of rabies cases occurred in December – February: 17 positive results were obtained, which constituted 59%.

Figure 1 shows test results for 241 brain samples submitted to the laboratory from rabies-suspected animals in 2018–2021. Among them, 180 samples were positive, 54 samples were negative, 7 samples were deteriorated and could not be tested due to late delivery to the laboratory.

To assess the epizootological and epidemiological risks of rabies in the country, the frequency of the disease occurrence among animals of different species by year was studied (Fig. 2). The statistical data provided by the National Veterinary Laboratory was analyzed and presented in the form of a graph reflecting information on rabies detection cases among different animal species for the period from 2018 to 2021.

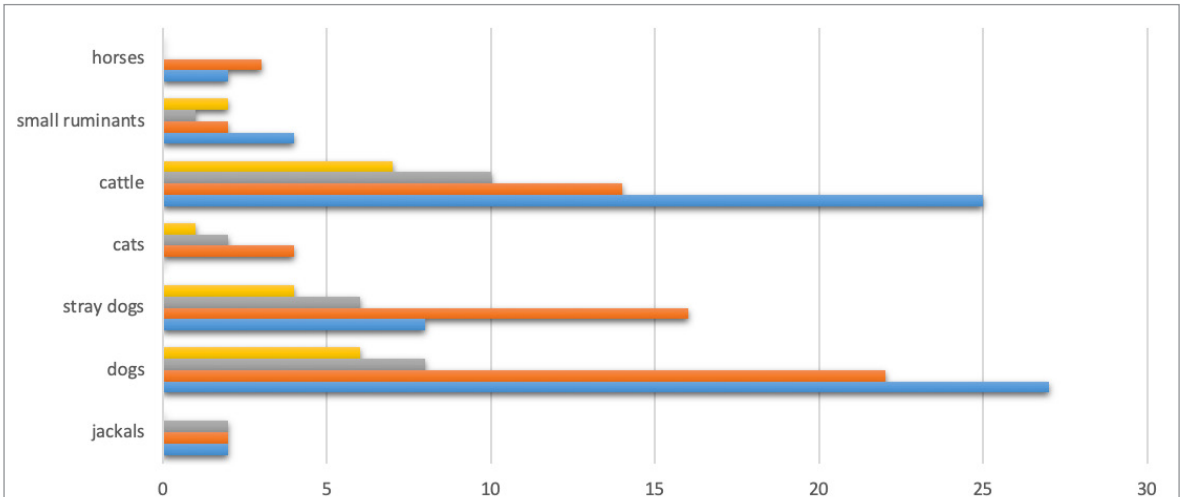
In total, during the period under review, out of 180 positive results in the December – May season, the largest number of rabies cases (54%) were detected in dogs. The share of cases in cattle accounted for 38.1%, 5.7% of positive samples were received from stray dogs, 1.6% – from sheep, 0.6% – from horses.

DISCUSSION

According to the generally accepted knowledge, natural rabies epizootics are characterized by seasonal variability. As a rule, the number of disease cases increases in autumn, winter and spring [24–26]. The highest frequency of rabies cases in our study was detected in the period from December to May 2018–2021. There is a possibility that this is due to the beginning of the bat breeding season [27]. It can be noted that the decrease in the number of rabies cases in 2020–2021 coincides with large-scale vaccination campaigns for pet dogs [18]. The revealed trend of seasonal morbidity requires further study in order to develop an effective strategy of rabies control.

Other expected causes of seasonal fluctuations in the incidence of animal infectious diseases are climatic conditions, frequency of breeding cycles, migration and lack of food [22]. Although domestic dogs do not exhibit seasonal breeding tendencies, under certain conditions their mating activity during the year may have a wave-like character [28].

The analysis of epizootological data and the laboratory test results allowed us to establish the nature of rabies seasonality in Azerbaijan. The monthly dynamics of incidence was revealed with the highest indicators in the period from December to May and the lowest – from June to September. These results are consistent with the recommendations on the scheme of preventive anti-rabies vaccination of pet dogs [18].



	jackals	dogs	stray dogs	cats	cattle	small ruminants	horses
2021	0	6	4	1	7	2	0
2020	2	8	6	2	10	1	0
2019	2	22	16	4	14	2	3
2018	2	27	8	0	25	4	2

Fig. 2. Incidence of rabies in different animal species in 2018–2021

CONCLUSION

The study results have shown that animal rabies has a clearly pronounced seasonality in the territory of the Republic of Azerbaijan. It was found that during the analyzed period, the largest number of animal rabies cases were recorded in the spring period, which is highly likely to be related to the animal breeding season.

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Development of submerged cultivation method for vaccine *Mycoplasma mycoides* subsp. *mycoides* strain

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SUMMARY

Members of *Mycoplasma* genus are widely spread in nature (soil, water, manure, cereals, food products), and there are ones pathogenic for humans, animals and birds. The group of highly dangerous diseases include contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides*. Risk of the disease agent introduction to Russia with imported livestock and raw materials still remains. Therefore, of topical importance is the improvement of the CBPP vaccine manufacturing technology. The studies were aimed at the development of the method of the submerged cultivation of the vaccine *Mycoplasma mycoides* subsp. *mycoides* strain. This method allows production of a large amount of the biological material and simplification of the biological product manufacturing technology. Dynamics of the mycoplasma mass accumulation during the submerged cultivation was examined within the studies. Four phases of the bacterial growth were clearly demonstrated. Insignificant decrease of the microbial cell concentration was reported in the first two days of cultivation; days 3 and 4 were specified by the increase of the microbial mass concentration by several orders of magnitude: from 2.5×10^8 to 4.5×10^9 cells/volume unit, on day 5 the concentration was in equilibrium and starting from day 6 the onset of the microorganism's death phase was reported. Similar dynamics was demonstrated during cultivation in the bioreactors. Singular fried egg-shaped colonies or their accumulations were observed at the visual examination of the submerged cultivated mycoplasma. Therefore, when using submerged cultivation method and such parameters as mycoplasma seeding at a dose of 10^5 microbial cells / volume unit; 2/3 filling volume; incubation at (37 ± 0.5) °C; agitation at 90 rpm and use of synthetic nutrient medium, the bacterium accumulates at the titre of 10^9 cell/volume unit.

Keywords: *Mycoplasma mycoides* subsp. *mycoides*, contagious bovine pleuropneumonia, cultivation, growth phases

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Разработка способа глубинного культивирования вакцинного штамма *Mycoplasma mycoides* subsp. *mycoides*

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РЕЗЮМЕ

Представители рода *Mycoplasma* широко распространены в природе (почве, воде, навозе, злаках, пищевых продуктах), среди них имеются виды, патогенные для человека, животных и птиц. К группе особо опасных инфекционных болезней отнесена контагиозная плевропневмония крупного рогатого скота, возбудителем которой является *Mycoplasma mycoides* subsp. *mycoides*. В настоящее время риск заноса возбудителя инфекции на территорию России с импортным скотом и сырьем из неблагополучных регионов сохраняется. В связи с этим совершенствование технологии изготовления вакцины против контагиозной плевропневмонии является актуальной задачей. Целью данного исследования явилась разработка глубинного способа культивирования вакцинного штамма *Mycoplasma mycoides* subsp. *mycoides*. Данный метод позволяет получать за короткий промежуток биомассу в больших объемах и упрощает технологию изготовления биопрепаратов. В процессе работы изучена динамика накопления биомассы микоплазмы при безопорном методе культивирования. Наглядно продемонстрированы 4 фазы роста бактерии. В первые двое суток выращивания отмечали незначительное снижение концентрации микробных клеток, третьи – четвертые сутки характеризовались увеличением биомассы на несколько порядков от $2,5 \times 10^8$ до $4,5 \times 10^9$ клеток в единице объема, на 5-е сутки концентрация находилась в равновесном положении, и начиная с 6-х суток регистрировали наступление фазы гибели микроорганизма. Аналогичная динамика прослеживалась и при культивировании в биореакторе. При визуализации микоплазмы, полученной при

выращивании глубинным способом, на твердой питательной среде наблюдали единичные колонии или их скопления, имеющие вид яичницы-глазуньи. Таким образом, используя безопорный метод культивирования и такие параметры, как засев микоплазмы в дозе 10^5 микробных клеток в единице объема, объем заполнения на 2/3, температура инкубирования (37 ± 0,5) °С, перемешивание при 90 об/мин, а также применение синтетической питательной среды бактерия накапливается в титре 10^9 клеток в единице объема.

Ключевые слова: *Mycoplasma mycoides* subsp. *mycoides*, контагиозная плевропневмония крупного рогатого скота, культивирование, фазы роста

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INTRODUCTION

Large amount of the submerged cultivated biomass is used in the manufacture of the anti-bacterial products. The method of the submerged cultivation was put into practice by N. E. Lebedev in 1950. The scientist demonstrated the possibility of *Enterobacteriaceae* family members' cultivation in the bioreactors with forced aeration and agitation [1].

This method allowed for significant achievements in the development of the industrial-scale biological product manufacturing technologies [2]. Growth technologies of tularemia bacteria [3], *Bacillus subtilis* and *Bacillus licheniformis* [4] used for probiotics production, etc. were, *inter alia*, improved.

When submerged cultivated the microbial cells are grown in liquid nutrient medium in special equipment – bioreactors of different modifications. The reservoir is equipped with the agitator of various configurations with adjusted rotation speed, sensors of temperature, pO_2, pH and foam levels, whose readings are transmitted to the computer display. Outside the bioreactor there are sampling ports and other devices [5].

Liquid nutrient media are used for submerged cultivation. The media in such physical condition facilitate access of the bacteria to the nutrient substances; they are easily mixed when incubated thus allowing for the replenishment of the nutrients essential for the culture [6].

Such bacteria as mycoplasmas are highly sensitive to the growth media. In natural environment they are in close contact with the host cells thus readily receiving all substances necessary for their development. The surface membranes of the mycoplasma and body cells are nearly similar in structure, therefore, close membrane contact occurs by way of dissolving in each other. This interaction facilitates transportation of the nutrients essential for mycoplasma from the host cell cytoplasm. Such cooperation resulted in the evolutionary loss by the microorganism of the genes coding these substances. Therefore, artificial nutrient media for mycoplasma cultivation shall contain complex components necessary for their growth. The protein base of the medium is the beef heart extract or peptone. Back in the 1970s, the mostly recognized media included media based on Martin's peptone and bovine heart broth. Yeast extract is used as the growth factor and source of vitamin B. The source of sterol is the equine serum (less frequently, porcine one), supplemented in large amounts. Bovine serum is not used as it can inhibit growth of some mycoplasma species [7]. Thus, porcine serum is used for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultivation as it allows production of a large amount of the mycoplasma biological material in the modified Frey's medium [8]. The source of energy involves glucose, L-arginine or urea [9].

The majority of mycoplasma are facultative parasites or commensals of animals and plants. The objects of the mycoplasma paratization are quite numerous starting from plants and up to mammals (including humans). There are mycoplasma species pathogenic for humans, animals and birds. They include *M. mycoides* – agent of bovine, sheep and goat pneumonia; *M. agalactiae* – agent of rheumatoid syndrome in sheep and goats, *M. pneumoniae* – agent of acute respiratory diseases and primary atypical pneumonia in humans, which demonstrates high hemolytic activity [10].

Among all mycoplasmas, *Mycoplasma mycoides* subsp. *Mycoides* should be specifically mentioned as it causes such highly contagious bovine disease as contagious pleuropneumonia. The disease is characterized by fever, fibrinous interstitial pneumonia, serofibrinous pleurisy followed by formation of anemic necrosis and sequestra in the lungs and accumulation of large amounts of exudate in the thoracic cavity [11–13].

The global community rates the disease as a highly dangerous one, and it is included in the list of notifiable diseases by the World Organization for Animal Health (WOAH). The disease is transboundary as well, and its spread can result in grave consequences at the national level. Annual economic losses due to contagious pleuropneumonia suffered by the African countries amount to 30 mln euros [14–16]. In the late XIX – early XX centuries,

the disease prevailed in European, Asian, African countries and in Australia. Severe animal health measures implemented in different countries allowed for the disease eradication in many localities of the globe. In the natural environment the abovementioned pathogen infects only ruminants, mostly cattle and zebu. In cattle contagious pleuropneumonia is manifested by changed general health condition (loss of appetite, pyrexia) and respiratory signs (dyspnoea, polypnea, coughing and nasal discharge). Sub-acute and acute disease is most often reported. The animals pose the highest risk of the infection transmission in case of chronic disease lacking obvious clinical signs [17].

The Russian Federation is the first EAEU country, which in 2020 was included in the WOAH list of 20 countries officially free from bovine contagious pleuropneumonia (there are total of 182 WOAH member countries). The key importance is currently given to the prevention of the disease agent introduction into the country with the animals and raw materials imported from the infected regions [18].

In this context, the vaccine production technology should be developed, and there are critical control points during the production process. Such enriched nutrient media as F_{66} and Gourlay are used for mycoplasma cultivation that in turn leads to possible bacterial contamination (unintentional contamination). Use of antibiotics during the medium preparation is very complicated due to high sensitivity of mycoplasma to them (apart from penicillins and sulfanilamides). This microorganism is demanding to the medium pH, thus, hydrogen ion exponent should be set within 7.8–8.0. The pH is rapidly decreasing during cultivation that affects the mycoplasma harvest and its viability outside the cultivation environment [18].

Therefore, there are a number of difficulties in the mycoplasma cultivation procedure, which should be given particular attention; and use of submerged cultivation in biotechnology is of topical importance.

The study was aimed at the development of the submerged cultivation method for *Mycoplasma mycoides* subsp. *mycoides*.

MATERIALS AND METHODS

Mycoplasma mycoides subsp. *mycoides* "MA-VNIIVVIM" strain was used in the study. The strain was received from the State collection of microorganisms causing dangerous or highly dangerous diseases, including zooanthroponoses, and exotic animal diseases (CCP register number – 441429)[1].

Nutrient media: nutrient broth and nutrient agar (Oxoid Ltd., Great Britain), yeast extract (Sanofi Diagnostics Pasteur, France) and equine serum (BioloT Ltd., Russia). Nutrient media were prepared according to the manufacturer's instructions. The media were sterilized by autoclaving at 121 °C for 15 min.

Equipment: bioreactor, 5 L (NBS, USA), shaker. In incubator shaker, cultivation was performed in 250 cm^3 Erlenmeyer flasks with 50 cm^3 of the nutrient medium. Key parameters were automatically controlled during cultivation in bioreactor: temperature, agitation speed, pH of the media.

Cultivation in flasks in incubator shaker. The nutrient medium was based on liquid broth and 10% yeast extract. The medium was supplemented with 20% of inactivated equine serum and filled at 50 cm^3 in 250 cm^3 flasks. The bacterial inoculum was reconstituted to the initial volume and 10-fold dilutions (10^{-1} and 10^{-2}) were prepared. Then 5.0 cm^3 of the 10^{-2} inoculum were transferred to the ready nutrient medium. The flasks with the infected material and control flask were incubated at (37 ± 0.5) °C and static shaking of 100 rpm.

Cultivation in bioreactor. The ready nutrient medium (3 L) was infected with working inoculum (300 cm^3) and transferred to the closed system bioreactor. Automated cultivation mode was set: temperature – (37 ± 0.5) °C, agitation – 90 rpm, bubble aeration followed by agitation – from day 2 of cultivation.

Upon the cultivation completion, the cell viability was assessed, the microorganism was visualized and microbiological purity of the culture was determined.

The microbiological purity was controlled by inoculation into the nutrient media according to GOST 28085-2013[2].

The viable cell concentration was determined by endpoint dilution method. Pooled sample was used to prepare the ten-fold dilutions (10^{-1}–10^{-9}) with phosphate buffered solution (pH 7.2–7.4). Then 1.0 cm^3 of each dilution starting from 10^{-4} and up to 10^{-9} were inoculated in 3 tubes with ready nutrient medium. The tubes were incubated at (37 ± 0.5) °C for 14 days. After that presence or absence of the microorganism growth was visually recorded by the opalescence appearance. The most probable number of the cells in the volume unit was calculated according to McCredy's table.

The strain was visualized by inoculating 0.1 cm^3 of each endpoint dilution demonstrating well-expressed opalescence in the broth into the dishes with solid synthetic nutrient medium. After the complete absorption, the dishes with the inoculates were transferred to the CO_2-incubator. The inoculates were incubated for 5–7 days at (37 ± 0.5) °C in the 5% CO_2 environment at 95% relative humidity. The resulted colonies grown on the solid media were microscopically examined using Opton ID 03 optical microscope (ZEISS, Germany).

RESULTS AND DISCUSSION

During the first round of the experiments, the dynamics of mycoplasma material accumulation was determined when cultivated in the suspension containing flasks in the shaker. The cultivation was proceeded until the culture reached the stationary growth phase. The initial mycoplasma concentration in the nutrient medium at inoculation was determined as 11.5×10^6 of the most probable number of microbial cells per volume unit. Hereafter, during the subsequent 7 days of cultivation, samples were collected for the assessment of the biological material accumulation. The results are demonstrated in Figure 1.

The graph demonstrates that the lag phase continued for 2 days of growth, when insignificant decrease of the microbial cell concentration was observed and the titres amounted to 2.5×10^5 – 9.5×10^5. Day 3 and 4 were viewed as logarithmic growth phase when bacterial population increased by several orders of magnitude.

[1] State collection of microorganisms causing dangerous or highly dangerous diseases, including zooanthroponoses, and exotic animal diseases. Available at: https://ckp-rf.ru/catalog/ckp/441429.

[2] GOST 28085-2013 Medicine remedies biological for veterinary use. Method of bacteriological control of sterility. Available at: https://meganorm.ru/Data2/1/4293775/4293775115.pdf.

During this period, the mycoplasma accumulated up to 2.5×10^8 – 4.5×10^9 cells per volume unit. On day 5, the concentration was in equilibrium with the log phase and amounted to 4.5×10^9 being indicative of the transmission of the culture to the stationary growth phase. Starting from day 6 of cultivation, the significant decrease of the bacterial material was observed that specified the onset of the death phase.

The results of the experiment, therefore, demonstrated that during the submerged cultivation the mycoplasma passes through all four growth phases. The maximal cell accumulation in the volume unit was observed on day 4 of cultivation during the exponential growth phase, the duration of which amounted to 48 hours for the tested strain.

Optimal pH levels of the medium used for *Mycoplasma mycoides* subsp. *mycoides* "MA-VNIIVVIM" strain propagation ranged from 7.8 to 8.0. Changes of pH according to the oncoming growth phase were reported during cultivation in flasks. Within the first 2 days, the medium acidity matched the original pH level of the prepared cultivation medium and equaled 8.0. On day 3–4, the pH level amounted to 7.8. Upon the new phase onset, the pH level gradually declined thus being indicative of active biomass accumulation. On day 5–6, the pH varied from 7.7 to 7.5 that was associated with the accumulation of bacteria waste products and decrease of nutrient substances essential for the growth of the microorganism, which in turn affected the culture growth.

Therefore, during the mycoplasma cultivation the hydrogen ion concentration in the cultivation medium changes depending on the bacterial growth phase.

The parameters selected for cultivation in flasks (filling level, infection dose, cultivation duration, agitation speed) were applied for propagation in 5-liter bioreactor. During cultivation the major attention was given to the babbling as the nutrient medium contained high percentage of serum that could result in foam formation. The results of the studies of the microbial cell accumulation dynamics are shown in the table.

As you can see, in the bioreactor all bacterial growth phases can be traced, which are typically observed during cultivation in flasks in the shaker. On day 4 of cultivation, high accumulation of the mycoplasma was reported – 2.8×10^9 cell/ volume unit. The duration of the exponential growth phase in the bioreactor also amounted to 48 hours with equal accumulation of the biomass.

Therefore, the submerged cultivation method can be used for the bacteria propagation during the contagious bovine pleuropneumonia vaccine production.

To visualize the mycoplasma produced by the submerged cultivation method, the tested sample was inoculated onto the solid nutrient medium. On day 5 of cultivation, the grown mycoplasma colonies were well seen by microscopy. Both singular colonies and their accumulation were in sight (Fig. 2).

The colonies were of regular round saucer-like shape with yellowish core and transparent gray edges (Fig. 3). There were colonies in the stage of division by budding (Fig. 4).

Hence, the biomass with high accumulation rate was obtained during the submerged cultivation, and when inoculated onto the solid nutrient media the mycoplasma had typical fried egg-shaped appearance.

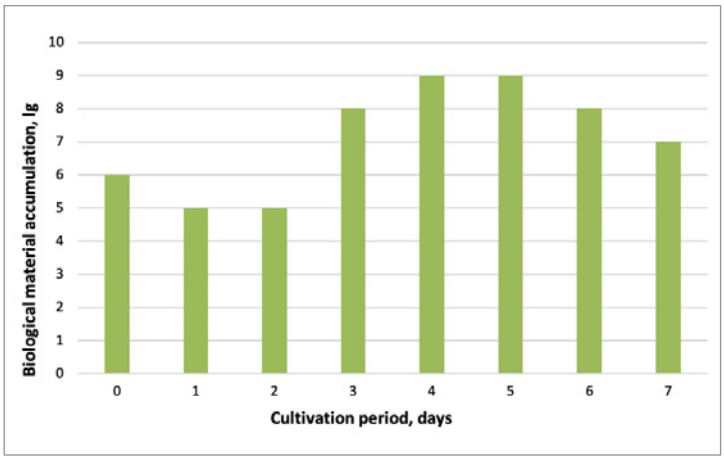

Fig. 1. Dynamics of mycoplasma population growth at suspension cultivation

Table
Accumulation of mycoplasma vaccine strain "MA-VNIIVVIM" in the fermenter

	Cultivation duration (day)			
	0	2	3	4
Activity (MPN)	9.5×10^5	2.5×10^4	9.5×10^8	2.8×10^9

MPN – most probable number.

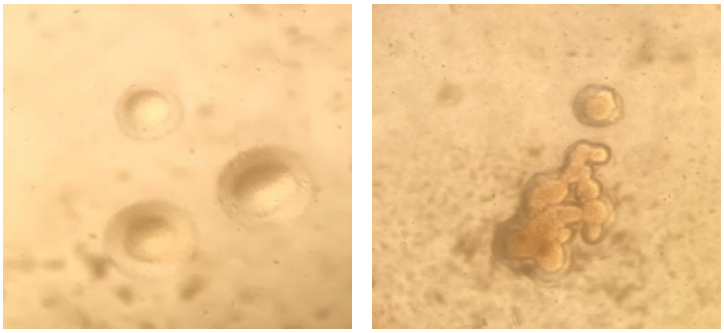

Fig. 2. Mycoplasma colonies

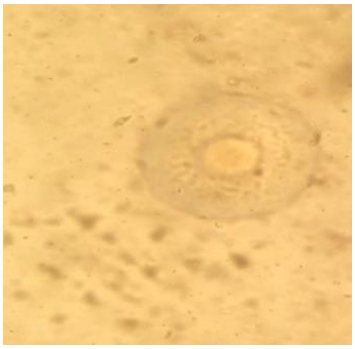

Fig. 3. Colony center

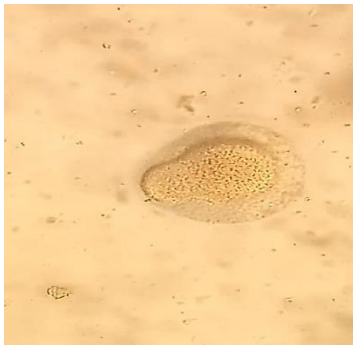

Fig. 4. Colony in the process of replication

As well as continuous cell cultures, the bacteria, and mycoplasmas in particular, are suspension cultivated and pass through all growth cycle phases [19]. The mycoplasmas are cultivated for a relatively long time in contrast to other bacteria, whose cultivation can take 18 hours, e.g. salmonella [20].

When mycoplasma is cultivated in suspension, the growth process is clearly demonstrated both in shaker and in bioreactor. Each of four stages took a certain time period. It was determined that loss of the bacterial mass by one order of magnitude was observed in the lag-phase, which continued for two days. Exponential, or log-phase, also lasted for two days as distinct from other bacteria, which need from several minutes to 24 hours for the phase. For instance, in case of salmonellas this phase takes 20–30 min; coliforms – 15–17 min, staphylococcus – 25–35 min, bacillus Kochii – 19–20 hours [19]. The stationary phase, during which equilibrium between the bacterial growth and death was observed, lasted for one day and the death phase further occurred.

Use of bioreactor for the mycoplasma cultivation allowed to automatize the key cultivation parameters: temperature, agitation of the liquid and air phases. Due to continuous agitation all components of the medium as well as microbial cells were evenly distributed all over the working space of the bioreactor thus allowing for homogenous bacterial mass production [21].

CONCLUSION

The study results demonstrated that submerged cultivation method can be used for mycoplasma biomass production. Such cultivation parameters as mycoplasma seeding at 10^5 microbial cells/volume unit, filling at 2/3 of the volume, incubation temperature (37 ± 0.5) °C, agitation at 90 rpm and use of synthetic nutrient medium resulted in the bacterium accumulation at the titre of 10^9 cells/volume unit at logarithmic growth stage.

It was determined that when submerged cultivated the vaccine strain *Mycoplasma mycoides* subsp. *mycoides* "MA-VNIIVVIM" follows routine patterns during the four phases of the growth cycle. Exponential phase duration amounted to 48 hours. In case of this cultivation method the strain retained the mycoplasma-typical growth properties: distinct opalescence in the liquid synthetic medium and development of the pronounced fried egg shape when inoculated onto the solid synthetic nutrient medium.

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Inactivation of foot and mouth disease virus for vaccine production

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SUMMARY

Inactivation is the loss by a virus of ability to reproduce and infect susceptible animals while retaining its antigenic properties. In this paper, the effectiveness of aminoethylethanolamine when used as an FMDV inactivant is shown. The inactivation rates under selected parameters, effect of aminoethylethanolamine on virus stability during inactivation and on vaccine immunogenicity after storage at 2–8 °C were determined. The method for calculation of 50% aminoethylethanolamine inactivating concentration (IC_{50}) which enables to determine quality parameters of the virus-containing suspension, to compare the inactivating agent activities and their ability to ensure the vaccine innocuity within the given period of time is presented. It was established that IC_{50} for purified and non-purified virus-containing suspensions was identical (0.0045%), and its safety after 12 hours of inactivation was one $TCID_{50}$ per 10^9–10^{11} L of the virus containing suspension. It was also found that double increase in inactivation time increased the virucidal activity of aminoethylethanolamine by a factor of 1.8 for serotype O and 2.4 for serotype A. At the same time, the removal of cell debris had no significant effect on the inactivation process. Aminoethylethanolamine does not destroy 146S virus particles and it was confirmed by immunogenicity testing of the vaccines during storage. This means that 15% aqueous solution of aminoethylethanolamine, manufactured by Russian Company OOO "Biokhimresurs" (Vladimir) complies with high quality standards. Immunogenicity test of bivalent FMD vaccine for cattle by challenging demonstrated that its potency was 10.08 protective doses per 2 cm^3 of the vaccination dose.

Keywords: foot and mouth disease virus, virucidal activity, inactivating agent, vaccine

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Инактивация вируса ящура для изготовления вакцин

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РЕЗЮМЕ

Инактивация – это потеря вирусом способности к репродукции и инфицированию восприимчивых животных при сохранении антигенных свойств. В работе показана эффективность применения аминоэтилэтиленимина в качестве инактиванта вируса ящура. Определены скорость снижения титра инфекционности при выбранных параметрах процесса инактивации, влияние аминоэтилэтиленимина на стабильность вируса в процессе инактивации и на иммуногенность вакцин после хранения при температуре 2–8 °С. Представлен метод вычисления пятидесятипроцентной инактивирующей концентрации аминоэтилэтиленимина ($К_{50}$), позволяющей определять качественную характеристику вируссодержащей суспензии, сравнивать активность инактивантов и их способность обеспечивать авирулентность препарата в заданный промежуток времени. В результате проведенных исследований было установлено, что значение $К_{50}$ для неочищенной и очищенной вируссодержащих суспензий было одинаковым – 0,0045%, а уровень безопасности после 12 ч инактивации составил одну $ТЦД_{50}$ в 10^9–10^{11} л вируссодержащей суспензии. Также было выявлено, что увеличение времени инактивации в два раза повысило вирулицидную активность аминоэтилэтиленимина: для типа О – в 1,8 раза, для типа А – в 2,4 раза. В то же время очистка от клеточного дебриса на процесс инактивации не оказывала существенного влияния. Аминоэтилэтиленимин не разрушает 146S частицы вируса, что и было подтверждено при исследовании иммуногенной активности вакцин в процессе хранения. Таким образом, аминоэтилэтиленимин, выпускаемый в виде 15%-го водного раствора российской фирмой ООО «Биохимресурс» (г. Владимир), соответствует высоким стандартам качества. При исследовании иммуногенности эмульсионной бивалентной противоящурной вакцины для крупного рогатого скота в остром опыте установили, что активность препарата составила 10,08 защитных доз в прививном объеме 2 $см^3$.

Ключевые слова: вирус ящура, вирулицидная активность, инактивант, вакцина

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INTRODUCTION

The production of whole-virion vaccines against foot and mouth disease is a complex and multi-stage process. One of the mandatory steps in the vaccine technology is the inactivation of the foot and mouth disease virus (FMDV). Inactivation is the loss by a virus of the ability to reproduce and infect susceptible animals while retaining its antigenic properties [1].

The main requirement for the inactivant is that it must change the viral nucleic acid but must not significantly diminish the properties of the protein responsible for immunogenicity. Formaldehyde was the first chemical agent used to inactivate the virus for the vaccine production [2]. An innocuous vaccine suitable for cattle immunization was obtained using this substance.

Formaldehyde as an inactivant has a number of drawbacks: narrow range of concentrations that inactivate viral infectivity and reduce vaccine immunogenicity; the multicomponent nature of the inactivation curve [3–5], which does not allow to determine the endpoint of inactivation and the innocuity of the vaccine [6]; products of formaldehyde reaction with non-functional proteins are allergenic [7]; vaccine shelf life is compromised by higher temperatures. Hydroxylamine, methylglyoxal, and ethylene oxide have been tested as inactivants, but they are not widely used in the manufacture of antiviral vaccines.

Studies by F. Brown et al. [8, 9] have discovered a new class of chemicals (aziridines) for inactivation, which are superior in inactivation rate and in preservation of FMDV immunogenic properties.

Bahnemann H. G. [10, 11] proposed using a mixture prepared by synthesis of ethylenimine from 2-bromoethylamine hydrobromide for inactivation of FMD virus. This drug was called binary ethylenimine, which is widely used in the manufacture of FMD vaccines.

Aziridines inactivate the viral infectivity in a first-order reaction without significant reduction in immunogenicity. Linear inactivation kinetic makes it possible to calculate the innocuity level of the vaccine [12–16].

The interaction rate of the inactivant with the viral protein envelope and the nucleic acid is directly proportional to the concentration of the reacting groups. Beside the concentration of the inactivant, the inactivation rate is influenced by temperature, incubation time, pH of the medium and components of the virus-containing suspension. During vaccine manufacture, special significance is given not to the virus inactivation rate, but to the ability of the chemical agent to ensure its complete inactivation [17].

The complete inactivation of FMD virus is a critical requirement in the production of FMD vaccine to ensure the safety of the product. The vaccine innocuity can be controlled more effectively by the analysis of inactivation kinetics used to determine the theoretical time of inactivation, as well as the procedure of the virus inactivation. The logarithmic graph of the residual infectivity, depending on the inactivation time, should be linear. This straight line allows extrapolation to the endpoint of the inactivation. Validation of the inactivation process is an essential part of quality assurance [18, 19]. Aminoethylethanolamine (AEEA) in the studies of N. A. Ulupov et al. was identified as most suitable inactivant, which is currently used in the manufacture of FMD vaccines [20].

Advantages and prospects of AEEA use in the production of FMD vaccines has been noted by many authors in their works [21–24]. The World Organization for Animal Health recommends using binary ethylenimine to inactivate FMDV for vaccine production [25]. The mechanism of viral inactivation by ethylenimine and its oligomers consists in alkylation of nucleic acid or protein due to the ring opening reaction [6, 20].

This article presents some results of studies of the FMDV infectivity inactivation using AEEA. A method for evaluation of the inactivant virucidal activity and its effect on vaccine immunogenicity and antigen stability during storage is demonstrated.

MATERIALS AND METHODS

Virus. Production strains of FMDV types A, O, C, Asia-1, SAT-1, SAT-2, SAT-3 adapted to newborn rabbits and BHK-21/2-17 cell suspension culture.

Animals: 18–20 g white mice, 3–5 day-old suckling mice, 250–300 kg cattle.

Cell culture: trypsinized primary porcine kidney culture (PK), BHK-21/2-17 suspension cells.

Inactivant: 15% aqueous solution of 1-(2-aminoethyl)-aziridine (OOO "Biokhimresurs", Russia) and anhydrous AEEA stored on dry NaOH.

Determination of IC_{50} of inactivants. The determination of AEEA virucidal activity is based on the determination of inactivant concentration, which reduces the infectivity of the virus-containing suspension to 10^0 LD_{50} or $TCID_{50}$ at the specified parameters of time, temperature and pH. By analogy with potency test by calculation of 50% protective dose, it is possible to calculate the concentration of an inactivant that protects 50% of infected animals or culture flasks from the virus (IC_{50}).

The IC_{50} was determined using Kerber – Ashmarin formula:

$$\lg IC_{50} = \lg D_{max} - \lg d\ (\Sigma L_i - 0.5),$$

where D_{max} is the maximum concentration of the inactivant at which all test targets are not infected with FMD;

d – multiplicity of tested inactivant concentrations;

ΣL_i is the sum of ratios between the number of non-infected suckling mice or PK monolayer flasks free from CPE (cytopathic effect) with the number of infected suckling mice or flasks.

The result of the IC_{50} determination is the percentage concentration of the inactivant in the FMDV suspension, which reduces its infectivity to one LD_{50} or $TCID_{50}$ in the tested suspension volume.

Example:

An inactivant is added to the precisely measured volumes of the tested virus-containing suspension, making two-fold serial dilutions. The same pH and temperature values are adjusted in the samples. After a defined incubation period, the residual infectivity is determined using suckling mice or cell culture. For each concentration of the inactivant, 10 animals or flasks are used. The mice are observed for 7 days, and the cell culture is monitored for 3–4 days. Number of affected and unaffected test targets are recorded.

Concentration of the inactivant	Number of test animals	Number of non-affected animals	Ratio between the numbers of non-infected and infected animals
0.005	10	0	0/10
0.010	10	3	3/10
0.020	10	7	7/10
0.040	10	10	10/10
0.080	10	10	10/10

Calculation of IC_{50} based on the data in the table: $\lg IC_{50} = \lg 0.08 - \lg 2\ (0/10 + 3/10 + 7/10 + 10/10 + 10/10 - 0.5) = 2.9 - 0.3 \times 2.5 = 2.9 - 0.75 = 2.15$.

The antilogarithm of 2.15 is 0.014, hence $IC_{50} = 0.014\%$.

The calculated concentration of the inactivant in the viral preparation, equal to 0.014%, reduces the infectivity to the level of one infectious unit during a specified time, at specified temperature and pH. The advantage of the method is the rapid determination of the viral inactivation mode for vaccine production. The calculated concentration of the inactivant, which protects 50% of test subjects, reflects the qualitative characteristics of any component of the virus-containing suspension, the role of temperature, pH in the AEEA inactivation and its virucidal activity for a different batch and during storage [17].

Determination of the FMDV inactivation rate. The inactivation rate was determined after calculation of IC_{50}. An inactivant was introduced into the viral suspension to obtain a concentration several times higher than IC_{50}, the mixture was incubated at a given temperature, samples of the suspension were taken every hour and stored frozen until testing for infectivity. The constructed graph of the infectivity titer reduction described the inactivation rate and the vaccine innocuity level for a certain duration of inactivation.

Experimental vaccines based on lapinized FMD virus were prepared on acetate, ammonia and phosphate buffer solutions. Viral suspensions containing 10% rabbit tissue were purified with chloroform, AEEA solution was added to a concentration of 0.03%, pH was adjusted within 7.2–7.8 and then incubated at a temperature of 26–27 °C for 24 hours. Vaccines containing 59.5% of the suspension, 30% of aluminum hydroxide with 3% Al_2O_3, 10% of glycerin and 0.5% of 10% saponin solution were formulated from innocuous suspensions.

A bivalent emulsion vaccine was formulated containing using inactivated suspension of cultured FMDV types A and O and adjuvant Montanide™ ISA 206 in a ratio of 1:1 and containing 4 μg of FMDV 146+75S component of each type per inoculation dose.

The immunogenicity of the vaccines was tested on adult white mice, which were vaccinated subcutaneously with 0.4 cm^3 of whole vaccine and of the vaccine diluted 2, 4, 8, 16 times using phosphate buffer. After 21 days, mice were infected with a homologous virus adapted to them at a dose of 10^4 LD_{50}/cm^3. After an 8-day observation, a 50% immunization dose of the vaccine (ImD_{50}) was determined using the Kerber – Ashmarin formula.

A bivalent water-in oil-in water complex emulsion vaccine was tested on cattle using quantitative method. The vaccine was injected intramuscularly to three groups of animals (five animals per each group), each subsequent dose was decreased by a factor of 4 (2.0, 0.5 and 0.12 cm^3). Animals were challenged on Day 28 after immunization by inoculation of 10^4 $ID_{50}/0.2$ cm^3 of homologous virus into the mucous membrane of the tongue. After 7 days, the animals were examined and a protective dose (PD_{50}) per vaccination volume for each type of virus included in the vaccine was established.

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

RESULTS AND DISCUSSION

The results of the studies aimed at determination of the AEEA concentrations in a viral suspension, which reduce the virulence of samples to one LD_{50}, after challenge of 3–5 day suckling mice are shown in Table 1.

As we can see, 0.0025% AEEA concentration in suspensions prepared using acetate and ammonia buffer solutions and 0.0031% AEEA concentration in suspension

Table 1
Effect of medium used for lapinized FMDV type O suspension on AEEA virucidal activity ($n = 7$)

Acetate buffer solution	Ammonia buffer solution	Phosphate buffer solution
IC_{50} value after virus inactivation for 24 hours at 26–27 °C and pH 7.2–7.8		
0.0025 ± 0.00029 $P < 0.005$	0.0025 ± 0.00029 $P < 0.005$	0.0031 ± 0.00031 $P < 0.001$

prepared using phosphate buffer solution reduced infectivity to one LD_{50} after 24 hours incubation at 26 °C.

Taking into account the published data that aziridines inactivate FMDV infectivity in a first-order reaction and the inactivation rate is proportional to the concentration of the inactivant used, graphs were constructed to reduce the infectivity of type O in the phosphate buffer at 26–27 °C for 0.01% and 0.02% AEEA concentrations. These concentrations were 3.22 and 6.45 times higher than the IC_{50} value, which should have accelerated inactivation by a corresponding factor. However, the average inactivation rate after 3 and 6 hours of incubation was 4 and 8 times higher than when 0.0031 ± 0.00031% concentration was used.

The average values obtained by three experiments to study the inactivation kinetics of FMDV types A, O, C, Asia-1, SAT-1, SAT-2, SAT-3 using a phosphate buffer solution, AEEA at the concentration of 0.03% and at 26–27 °C and pH 7.2–7.8, showed that, despite the differences in the infectivity titers before the start of inactivation, infectivity was lost in all 7 serotypes after 2 hours. If inactivation lasted 8 hours, the innocuity level corresponded to one LD_{50} in 0.1 L (3 h) and in 10^9 L (6 h). Extrapolation of the inactivation curves on 8 hour duration suggested that the innocuity level of the suspension corresponded to one LD_{50} in $10^{15.3}$–$10^{15.8}$ L for serotypes A, O, C, Asia-1, SAT-1, SAT-2, SAT-3. The average inactivation rate of all 7 serotypes of the FMD virus was $10^{3.4}$ LD_{50} per hour.

Since the ethylenimine dimer is toxic, it is used as aqueous solutions (for example, a 15% AEEA aqueous solution), and stability of the active substance in the solution decreases during storage. Therefore, the next step was to study the virucidal activity of 15% AEEA solution at 2–8 °C after 0, 2, 4, 6 months of storage against cultural FMDV type O. The results obtained are presented in Table 2.

As we can see, the virucidal activity of the inactivant did not decrease after 6 month storage at 2–8 °C, since the 50% inactivating concentration (IC_{50}) did not change significantly after 2, 4, 6 months compared to the original concentration.

It was also found that 15 and 1% AEEA solutions prepared using demineralized water remained active for 6 months (observation period) at 2–8 °C. Anhydrous AEEA when stored over dry alkali (NaOH) remained active for 20 years (observation period) at a storage temperature of minus 10–20 °C (unpublished data).

The effect of AEEA on the immunogenicity of lapinized FMDV in a vaccine containing aluminum hydroxide, glycerin and saponin was studied for serotype O, which is the most labile one.

After purification with chloroform, AEEA solution was added to the suspension samples of the lapinized virus to a concentration of 0.03%. After 24 hours of the virus inactivation at 26–27 °C and pH 7.2–7.8, innocuity was tested and vaccines were formulated. To assess the immunogenicity of the vaccines, the method of ImD_{50} quantification was used on 18–20 g white mice. The animals were immunized subcutaneously with the vaccine diluted in 1/15 M phosphate buffer solution and challenged 21 days after vaccination. The immunogenicity of the vaccines was determined after manufacture, after a year and 8 years of storage at 2–8 °C. The results of the experiments are presented in Table 3.

Table 2
Effect of storage time on AEEA virucidal activity

Inactivant storage time (months)	IC_{50} (%)
0	0.0036 0.0040 0.0047
$M \pm m$	0.0041 ± 0.0003
2	0.0050 0.0047 0.0043
$M \pm m$	0.0047 ± 0.0002
4	0.0029 0.0038 0.0047
$M \pm m$	0.0038 ± 0.0005
6	0.0050 0.0040 0.0052
$M \pm m$	0.0047 ± 0.0005

Table 3
Dependence of FMDV type O immunogenic component stability on suspension medium and storage time

No.	Virus suspension medium	AEEA concentration in suspension (%)	Immunogenicity of vaccines for mice (ImD_{50} in 1 cm^3)		
			after manufacturing	after 1 year of storage	after 8 years of storage
1	Acetate buffer solution	0.03	0.16 0.17 0.16 0.15	0.15 0.19 0.18 0.15	0.18 0.21 0.16 0.17
$M \pm m$			0.16 ± 0.008	0.17 ± 0.021	0.18 ± 0.022
2	Ammonia buffer solution	0.03	0.19 0.19 0.10 0.11	0.29 0.19 0.23 0.11	0.30 0.25 0.24 0.14
$M \pm m$			0.15 ± 0.049	0.21 ± 0.076	0.23 ± 0.067
3	Phosphate buffer solution	0.03	0.12 0.13 0.13 0.14	0.16 0.18 0.21 0.20	0.21 0.19 0.20 0.23
$M \pm m$			0.13 ± 0.008	0.19 ± 0.022	0.21 ± 0.017

It was found that the AEEA optimal safe concentration made it possible to formulate vaccines identical in immunogenicity, which did not decrease their activity after 8 years of storage. However, this worked with those vaccines in which the virus was suspended in an acetate buffer solution. A tendency to decreased activities were found in the vaccines from the virus suspended in ammonia and phosphate buffer solutions.

In the process of FMDV inactivation, an inactivant obtained by two methods of synthesis was used: the first – anhydrous AEEA was obtained by polymerization of ethylenimine, and AEEA aqueous solution containing piperazine was obtained by cyclization of the AEEA sulfate ester. The latter is still used in the manufacture of FMD vaccines

Table 4
Effect of inactivation time on AEEA virucidal activity

Inactivation time (hours)	IC_{50} (%)	
	FMDV type O	FMDV type A
12	0.0027 0.0030 0.0025	0.0031 0.0030 0.0025
$M \pm m$	0.0027 ± 0.0001	0.0029 ± 0.0001
24	0.0015 0.0008 0.0011	0.0014 0.0009 0.0012
$M \pm m$	0.0011 ± 0.0001	0.0012 ± 0.0001

Table 5
Effect of purification on FMDV type O inactivation

Number of trials	IC_{50} (%)	
	purified suspension	unpurified suspension
1	0.0050	0.0048
2	0.0036	0.0040
3	0.0043	0.0040
4	0.0051	0.0052
$M \pm m$	0.0045 ± 0.0007	0.0045 ± 0.0006

Table 6
Immunogenicity of bivalent emulsion FMD vaccine for cattle

Vaccine description	Vaccination dose (cm^3)	Generalised foot infection		PD_{50}	
		A	O	A	O
Emulsion vaccine based on adjuvant Montanide™ ISA 206; Antigen: Type O – 4 μg, Type A – 4 μg	2.0	– – – – –	– – – – –	10.08	10.08
	0.5	– – – – –	– – – – +		
	0.12	+ + + + +	– + + + +		
Control		+	+		

"–" no podal generalization;
"+" podal generalization.

from a virus reproduced in BHK-21/2-17 cell suspension culture.

At the next stage, the kinetics of FMDV type O infectivity reduction, reproduced in a BHK-21/2-17 cell suspension culture in a 2,000 L bioreactor with 0.02% AEEA concentration and at 37 °C was analyzed. It was found that the inactivation was significantly lower than the inactivation rate of the lapinized virus, despite the fact that the inactivation temperature of the culture virus was 10 °C higher. This can be explained by the fact that the FMDV inactivation rate decreases with an increase in the salt concentration in the suspension and pH increase. In our experiments, the decrease in the inactivation rate is caused by the presence of the entire mass of whole BHK-21 cells and cell debris and a higher concentration of salts and other organic substances in the suspension. However, the innocuity level of the suspension after 12 hours of inactivation was one $TCID_{50}$ in 10^9–10^{11} L of suspension.

Taking into account the fact that an increase in the temperature of the virus-containing suspension contributes to an increase in AEEA virucidal activity, the inactivation process was started immediately after the virus finished its reproduction in BHK-21/2-17 cell suspension culture at 37 °C. Effect of inactivation time at 37 °C on the inactivant virucidal activity was tested after 12 and 24 hours.

The results presented in Table 4 demonstrate that an increase in incubation time at a constant temperature contributed to an increase in AEEA virucidal effect for type O by 1.8 times, for type A by 2.4 times.

Since a finely dispersed matter is formed during inactivation, it is technologically more convenient to perform inactivation before the purification of the virus-containing suspension. It was therefore necessary to prove that such an inactivation mode is sufficient to obtain an innocuous suspension of both purified and unpurefied cultural FMDV (Table 5).

It was found that IC_{50} for unpurified and purified suspensions was the same and amounted to 0.0045%. This fact confirmed that the cellular debris contained in the unpurified suspension did not have a negative effect on the inactivation process.

Complement fixation testing of cultural FMD virus of serotypes A, O, C and Asia-1 before and after inactivation showed that 146S component of the virus was not destroyed.

Using the developed inactivation mode of the FMD culture virus of types O and A, a bivalent emulsion vaccine was formulated, which was tested for immunogenicity by quantitation in cattle (Table 6).

The data in Table 6 demonstrate that the vaccine potency for both types was 10.08 PD_{50} in a vaccination dose of 2 cm^3.

CONCLUSION

FMDV adapted to 2–3 day old rabbits and to BHK-21/2-17 cell suspension culture was used to test the virucidal activity of the ethylenimine oligomer – N-aminoethylethanolamine (AEEA). The first stage of inactivant evaluation was the determination of the concentration that gives a non-virulent viral suspension at the specified parameters of the inactivation process (temperature, virus concentration, duration, pH), and the calculation of IC_{50}. IC_{50} for unpurified and purified suspensions was the same and amounted to 0.0045%. This fact confirmed that the cellular debris and lapinized non-functional proteins contained in the unpurified suspension did not have a negative effect on the inactivation process.

The constructed graph of infectivity titer reduction with the selected inactivation parameters (0.02% AEEA concentration at 37 °C) made it possible to determine the innocuity after 12 hours of inactivation, which amounted

to one $TCID_{50}$ in 10^9–10^{11} L of a virus-containing suspension intended for vaccine production.

The analysis of the dependence of the AEEA concentration and the vaccine storage duration on the FMDV immunogenicity showed that after inactivation 146S antigen did not break down.

Immunogenicity of bivalent FMDV vaccine for types A and O was 10.08 PD_{50} in 2 cm^3 vaccination dose. Virucidal activity, stability, high inactivation rate, minimal damage to 146S antigen indicates that AEEA has a number of advantages over other aziridines, used for FMDV inactivation. Thus, 15% aqueous solution of aminoethylethanolamine, produced by the Russian company OOO "Biokhimresurs" (Vladimir) meets all high quality standards.

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Optimizing a low-temperature preservation technique for *Bacillus anthracis* strains

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SUMMARY

The use of pure microbial cultures is associated with the following key challenges: storage, transportation and resuscitation after a long-term preservation. The currently used anthrax vaccines are produced using various strains of *Bacillus anthracis*. According to the storage passport data, anthrax strains are now stored in 30–40% glycerin solutions, which helps to preserve a sufficient number of viable cells without losses to their pathogenic properties for three years. It is obviously an urgent task to develop a long-term preservation technique for *Bacillus anthracis* strains. The aim of this study was to optimize a low-temperature preservation method for *Bacillus anthracis* strains that ensures viability and no losses to biological properties of the pathogen. Two vaccine strains of *Bacillus anthracis* were selected for the research: i.e. K-STI-79 and 55-VNIIVViM and two cryoprotective media (No. 1 – 15% glycerin solution with 15% glucose solution and No. 2 – 30% neutral glycerin solution in saline solution). At first biological properties of the strains were studied and the number of viable cells was calculated. Later on, the strains were placed into low-temperature preservation facilities, at the temperature of –40 and –70 °C. Six months later, the effect of three thawing cycles on viability and biological properties of the agent was tested: i.e. at room temperature (22 ± 2) °C, in a water bath at a temperature of (37 ± 1) °C and in a household refrigerator at a temperature of (6 ± 2) °C. As demonstrated, the best option is to preserve the cells at –70 °C and thaw them in a water bath at (37 ± 1) °C. Further research will be focused on duration of the low-temperature preservation that will ensure appropriate viability and biological properties of the pathogen.

Keywords: anthrax, *Bacillus anthracis*, strains, low-temperature preservation

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Отработка режима низкотемпературной консервации штаммов *Bacillus anthracis*

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РЕЗЮМЕ

Ключевой проблемой использования чистых культур микроорганизмов является их хранение, транспортировка, восстановление жизнеспособности после длительной консервации с сохранением ценных биологических свойств. Применяемые в настоящее время противосибиреязвенные вакцины создаются с использованием различных штаммов *Bacillus anthracis*. На сегодняшний день штаммы возбудителя сибирской язвы, согласно данным паспортов, консервируют в 30–40-процентных растворах глицерина, позволяющих сохранять достаточное количество жизнеспособных клеток, а также свойства возбудителя в течение трех лет. Очевидно, что разработка способа консервации штаммов *Bacillus anthracis* для более продолжительного хранения возбудителя является актуальной задачей. Целью работы было отработать режим низкотемпературной консервации штаммов *Bacillus anthracis*, обеспечивающий сохранность жизнеспособности и биологических свойств возбудителя. Для проведения исследований были отобраны два вакцинных штамма *Bacillus anthracis*: К-СТИ-79 и 55-ВНИИВВиМ, а также две криопротекторные среды: № 1 – 15%-й раствор глицерина с 15%-м раствором глюкозы и № 2 – 30%-й нейтральный раствор глицерина на физиологическом растворе. На первом этапе были изучены биологические свойства штаммов и подсчитано количество жизнеспособных клеток. После чего штаммы были помещены на низкотемпературную консервацию при минус 40 и минус 70 °С. Через 6 месяцев хранения изучали сохранность их жизнеспособности и биологических свойств при трех режимах разморозки: при комнатной температуре (22 ± 2) °С, на водяной бане при температуре (37 ± 1) °С и в бытовом холодильнике при температуре (6 ± 2) °С. Было установлено, что наиболее подходящим режимом явилось хранение клеток при минус 70 °С и размораживание на водяной бане при (37 ± 1) °С. Дальнейшие исследования будут направлены на установление максимально возможной длительности хранения штаммов при низкотемпературном режиме консервации, при которой сохранятся жизнеспособность и биологические свойства возбудителя.

Ключевые слова: сибирская язва, *Bacillus anthracis*, штаммы, низкотемпературная консервация

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INTRODUCTION

Any research into infectious diseases, basic microbiology, structural and molecular biology is based on the use of pure microbial strains that are also crucial for the purposes of biotechnology and biomanufacturing [1, 2]. The use of pure microbial cultures is associated with the following key challenges: storage, transportation and resuscitation after a long-term preservation without any losses to their valuable phenotypic and genotypic characteristics [3, 4].

The Culture Collection Centres that supply research laboratories and biological establishments with valuable pathological strains face an important task to select an appropriate preservation method and adjust it to a particular microorganism [5]. Optimally selected preservation methods and protocols ensure good viability and stable biological properties of specific microbial strains for a long time. Therefore, collection centers use various preservation methods: subculturing, storage in mineral oil, storage in water and water-salt solutions, drying on solid media, low-temperature preservation (from –10 to –80 °C and below), cryopreservation (storage in liquid nitrogen at –196 °C) and freeze-drying [6]. The most frequently used long-term storage lab methods include low-temperature preservation and freeze-drying.

Low-temperature preservation of microorganisms consists in freezing pure cultures of bacteria or viruses in a cryoprotective medium that protects the microorganisms from ice crystals during storage, freezing and thawing [7, 8]. The storage period depends on the storage temperature and the cooling/thawing rate [9]. Many Collection Centers successfully preserve bacterial cultures in modern freezers that maintain temperatures down to –86 °C [6].

Freeze-drying is a desiccation tool used for frozen biomaterials, when water is evaporated in a frozen state (in a vacuum) and does not pass through the liquid state. Thus, the original structure of the freeze-dried subject is retained [7]. Freeze-drying is now recognized as the method ensuring the longest storage of microorganisms, and convenient transportation of the freeze-dried material [10].

Low-temperature preservation is a more available tool, since it does not require a complex infrastructure (except for a freezer), whereas freeze-drying requires more specific equipment and specially trained engineers and technical staff [11]. This method ensures a 10-year storage of microbial cells without any losses to their properties [5].

There are publications in Russian and foreign literature on low-temperature storage technique mainly used for BSL III and IV pathogens [12–15]. However, development of preservation protocols for BSL I and II pathogens is equally important.

Most highly dangerous diseases were eradicated with the help of various immunobiological drugs produced on pure microbial cultures [16–19]. Anthrax is a highly dangerous zooanthroponosis, widely spread in many countries, the spore-forming microorganism *Bacillus anthracis* is the causative agent [20]. Relative freedom of our country from this disease has been achieved by annual vaccination of all the susceptible animals, as well as humans who may face a risk of infection [21]. Animals and humans are immunized with the vaccines produced from *B. anthracis* living cells: strains 55-VNIIVViM and STI-1, correspondingly [22]. In order to ensure biological safety of our country, it is strategically important to preserve those properties of the pathogen that contribute to a strong anti-anthrax immunity.

Currently, anthrax strains are stored in Collection Centers in a freeze-dried form or in sealed ampoules with 30-40% glycerin solutions. Freeze-drying of *B. anthracis* cells enables to store them for decades, however, recent research done by Russian experts demonstrate that freeze-drying of strains of highly dangerous diseases makes it difficult to achieve a required biosafety level in the course of work. This is explained by the fact that freeze-drying is accompanied by generation of aerosols, which contain cells of the freeze-dried pathogen [23]. Preservation of *B. anthracis* in glycerin solutions is a safer method; however, in this case pathogens may be stored maximum for 3 years (according to the strain data sheet). There are no data in Russian or foreign literature on low-temperature preservation of *B. anthracis* strains. However, based on the data available [5, 6, 8], it can be assumed that, in terms of pathogen handling, preservation method is safer than freeze-drying, and it ensures longer pathogen viability than storage in glycerin solutions.

The above data demonstrate how important it is to search for an optimal low-temperature preservation tool for *B. anthracis* strains. The scientific novelty of the research consists in comparing viability of the *B. anthracis* cells preserved and stability of their biological characteristics under different low-temperature preservation conditions, when two cryoprotective media are used.

Based on the above, the purpose of this work was to optimize low-temperature preservation tool for *B. anthracis* strains that will preserve appropriate viability and biological characteristics of the pathogen.

MATERIALS AND METHODS

Strains. Two anthrax strains from the collection were used for the research: K-STI-79 and 55-VNIIVViM, stored in 30% and 40% glycerin, respectively. These two strains were selected for safety reasons, as they are vaccine strains, which retain all the properties of the pathogen, except for the capsule formation.

Nutrient media: meat-peptone agar (MPA), meat-peptone broth (MPB), 5% blood MPA, 12% gelatin, skimmed milk, GKI medium, Hottinger broth produced by the FGBSI "FCTRBS-ARRVI".

Reagents. For the purposes of this work we used non-specific preservative-free horse serum produced by FKP "Kursk Biofactory – BIOK Company" (Russia); gentian violet (p. a.), basic fuchsin (p. a.), Iodine crystals (p. a.), crystalline glucose (p. a.), glycerin (p. a.) produced by OOO NPO "TatHimProdukt" (Russia).

Equipment. The culture was handled in biosafety cabinet BMB-"Laminar-S"-1,2 PROTECT (LAMSYSTEMS CC, Russia). Cultures were grown in a vertical water thermostat TV-40 (Russia). Smears were examined using MICMED-5 microscope ("LOMO" JSC, Russia). A desktop centrifuge OPn-8 (OAO TNK "Dastan", Kyrgyzstan) was used for centrifugation.

Methods. Before low-temperature preservation, we studied biological properties of the strains given in strain data sheets, according to MUC 4.2.2413-08 "Laboratory diagnostics and detection of anthrax causative agent"[1].

After checking the properties, suspensions of spore-shaped cells were prepared in saline solution. The concentration of viable cells in suspensions was determined after inoculation onto MPA followed by CFU counting[2].

Then the prepared suspension was centrifuged and the supernatant was discarded. The precipitate cells were mixed with 1 cm^3 of cryoprotective medium No. 1 (15% glycerin solution with 15% glucose solution) and cryoprotective medium No. 2 (30% neutral glycerin solution in saline solution) and placed in plastic cryotubes with screw caps. After protective media were added, the tubes with the resulting suspension were carefully rotated along the vertical axis, kept for 30 minutes at room temperature to better intermix the medium with the cells and placed into a low-temperature preservation unit (at –40 °C and –70 °C).

For the purposes of comparison, a cell suspension was also prepared, put into 30% and 40% glycerin solutions (K-STI-79 and 55-VNIIVViM, respectively) and stored at 4 °C in accordance with the recommendations of the strain data sheet.

The preserved cells were thawed after a 6-month preservation, until the ice crystals in test tubes completely disappeared. The following different thawing techniques were used:

– at room temperature (22 ± 2) °C;
– in a water bath at a temperature of (37 ± 1) °C;
– in a household refrigerator at a temperature of (6 ± 2) °C.

After thawing, serial 10-fold dilutions were prepared in 0.9% saline solution, then the resulting dilutions were inoculated into Petri dishes and CFU counted. Each sample was inoculated into 5 Petri dishes. The biological properties of the strains were studied as described above.

The Mann – Whitney U test was used to assess the statistical significance of the obtained results. Differences at $p \leq 0.01$ were considered statistically significant (after recalculating the number of comparisons). Quantitative data in Figure 3 and in Table 2 are represented as $M \pm SD$ (where M is the mean value, SD is the standard deviation) [24].

RESULTS AND DISCUSSION

Biological properties of B. anthracis strains before low-temperature preservation. When assessing cultural and morphological properties, it was found that the strains in the MPB demonstrated a growth typical for anthrax, i.e. they look like a lump of cotton wool at the bottom of a transparent medium (Fig. 1A). When shaking, moiré patterns appeared on the surface of the medium. On day five, the bacteria formed a strong chain along the tube walls on the surface of the medium. On MPA, colonies are grey-white; with a ground-glass surface and prominent wisps (Fig. 1B). Under low magnification, the grown colonies had 'curled hair' appearance – R-shape (Fig. 2A). Microscopy of gram-stained smears demonstrated that the strain cells formed chains of large, spore-forming, gram-positive rod-shaped bacteria (Fig. 2B).

The cultures demonstrated a lack of motility, as observed in the motility test. A 24-hour Incubation of cells on 5% blood agar revealed no hemolytic activity. Five days later, a typical strain growth occurs as "inverted fir tree" appearance in 12% gelatin with typical "stocking-like" liquefaction on the surface. The growth cultures cause coagulation and peptonization of skimmed milk. Cells grown in GKI medium with subsequent Rebiger staining demonstrated no ability to form capsules. The "string pearl" appearance test showed that the strains are sensitive to penicillin.

The study of the biological properties of the vaccine strains K-STI-79 and 55-VNIIVViM *B. anthracis* revealed that all the properties corresponded to strain data sheet and, except for capsule formation, are typical for anthrax pathogen (Table 1).

Counting colony-forming units of B. anthracis strains before low-temperature preservation. The next stage was to count CFU for each strain before cryoprotective media were added. For this purpose, cell suspensions were prepared in saline solution. After that, tenfold cell dilutions

[1] MUC 4.2.2413-08 Laboratory diagnosis and detection of anthrax causative agent: methodical instructions. Moscow: Federal Center of Hygiene and Epidemiology of Rospotrebnadzor; 2009. 69 p. Available at: https://files.stroyinf.ru/Data2/1/4293752/4293752010.pdf. (in Russ.)

[2] Labinskaya A. S. Microbiology and the techniques of microbiological research. 4th ed., revised and supplemented. Moscow: Medicine; 1978. 394 p. (in Russ.)

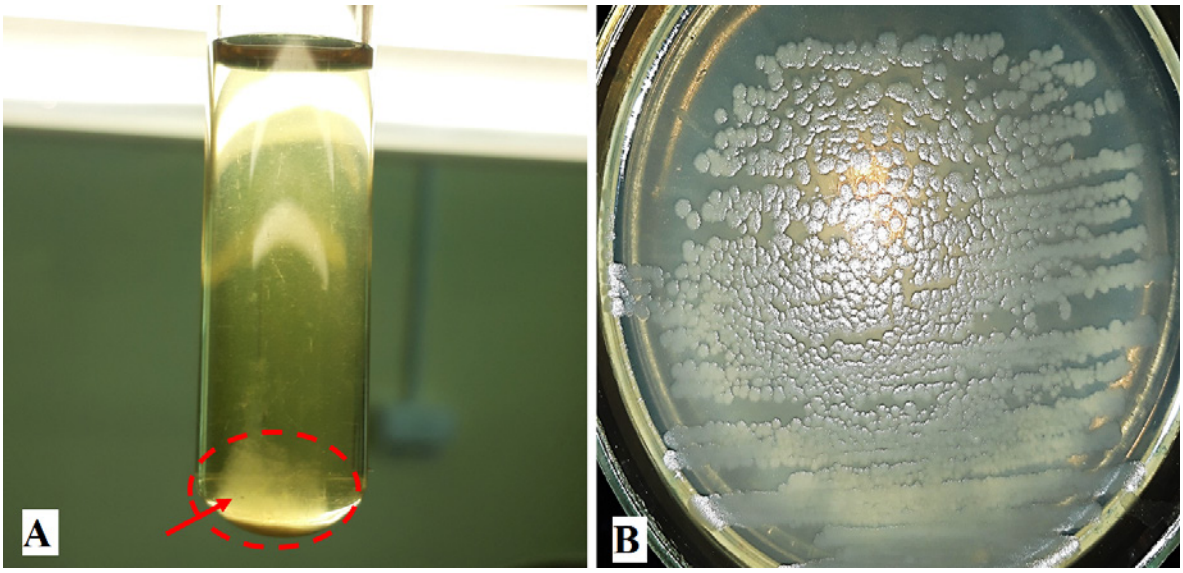

Fig. 1. Culture properties of Bacillus anthracis strain 55-VNIIVViM after 24-hour cultivation:
A – the culture growth looks like a lump of cotton wool (indicated by an arrow) in MPB;
B – the growth of colonies on MPA

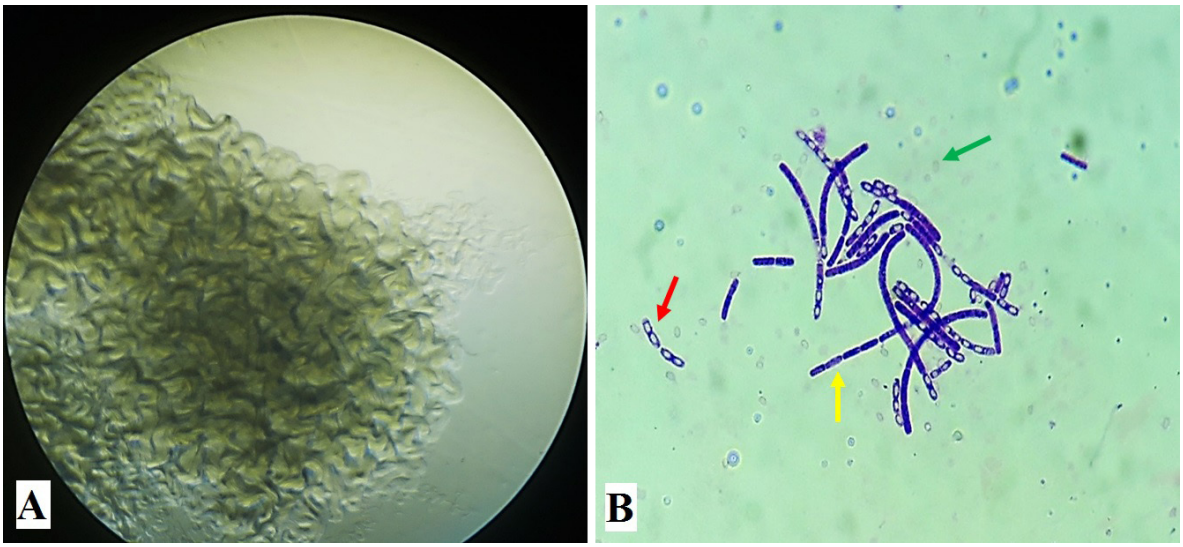

Fig. 2. Colony morphology and shape of B. anthracis bacteria strain 55-VNIIVViM cells after the 24-hour cultivation:
A – R-shaped colonies under low magnification (8 × 40);
B – Gram staining of the strain (yellow arrow – vegetative cell forms; red – emerging spores; green – spores)

were prepared (up to 10^{-5}), inoculated onto Petri dishes with MPA, cultivated at 37 °C for 24 hours and the grown colonies were counted.

The average CFU count for the tested strains is shown in Figure 3.

After the grown colonies were counted, the prepared suspensions were centrifuged at 4,500 rpm for 30 minutes. The precipitated cells were mixed with 1 cm^3 of cryoprotectors, placed for preservation at –40 °C and –70 °C, as well as in 30% and 40% glycerin solutions at (6 ± 2) °C.

Solutions of 15% glycerin with 15% glucose and 30% glycerin were cryoprotective media of choice. The choice of these cryoprotectants resulted from the fact that glycerin is the most widely used protective medium. Glycerol solutions of various concentrations were first used for preservation of pathogenic prokaryotes and viruses as early as the beginning of the 20th century [25]. Currently, it has become the "gold standard" for cryopreservation [26]. As noted by many foreign experts, the glucose solution (1 to 18%) added to the mixture of cryoprotectors improves the survival rate of different microorganisms [8, 25]. Our choice of this combination of solutions as a protective medium was explained by the fact that glucose belongs to protectors that penetrate the cell wall, but do not pass through the cytoplasmic membrane. While glycerin can penetrate through the cytoplasmic membrane of cells [25]. Thus, it can be assumed that the combination of these solutions should ensure greater survival of the cryopreserved cells.

Comparing effectiveness of low-temperature preservation of B. anthracis strains. The work shows that the viability

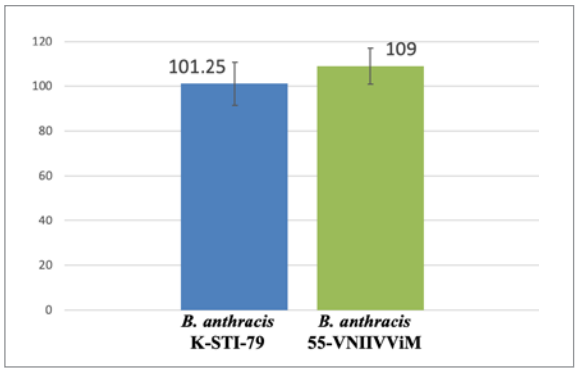

Fig. 3. CFU (M ± SD) of B. anthracis strains K-STI-79 and 55-VNIIVViM before a low-temperature preservation

Table 1
Biological properties of *B. anthracis* vaccine strains K-STI-79 and 55-VNIIVViM

Criterion/property	*B. anthracis* K-STI-79	*B. anthracis* 55-VNIIVViM
Motility	–	–
Hemolytic properties	–	–
Proteolytic properties: 12% gelatin skimmed milk	 + +	 + +
Capsule formation	–	–
Penicillin sensitivity	+	+
Spore formation	+	+

of anthrax strains cells was best preserved when stored at a temperature of –70 °C with further thawing in a 37 °C water bath (Table 2). At the same time, there was no significant difference in viability of cells preserved in different cryoprotectors. The CFU count did not show any statistically significant difference from the CFU count reported during storage in glycerin solutions in a refrigerator. While evaluating the results obtained, it is necessary to take into account the fact that the preparation of cells for low-temperature preservation is associated with huge losses resulting from centrifugation, mixing with a cryoprotector, freezing and thawing, which do not occur during storage in a household refrigerator. It suggests that the selected preservation tool helps to save a larger number of cells than other techniques.

Strain cells stored at –40 °C demonstrated significantly less viability under all thawing schemes. However, thawing at 37 °C helped to save a larger number of viable cells. The results obtained can be explained by the fact that the storage at –40 °C does not help to completely stop recrystallization. Thawing of the stored cells in a refrigerator is a time-consuming process that may result in prolonged ice recrystallization, which is one of the major factors that destroy frozen cells [8, 26].

CFU count after the low-temperature preservation was followed by an assessment of the pathogen biological properties, which demonstrated their full compliance with strain data sheet.

Thus, we can say that the selected method of low-temperature preservation of anthrax pathogen at –70 °C looks promising for further work. Further research will be focused on duration of strain storage using a low-temperature preservation method that will ensure the pathogen viability and preserve its biological properties.

CONCLUSION

The conducted research demonstrate that anthrax cultures stored at a temperature of –40 °C or –70 °C, retained their viability and biological properties for 6 months. Comparison of two preservation methods (at –40 °C and at –70 °C) allowed us to conclude that storage at –70 °C is more preferable.

Table 2
CFU count of *B. anthracis* cells after low temperature preservation for 6 months ($M \pm S_D$)

Cryoprotector	CFU count before freezing	Temperature of storage	CFU count			
			after thawing at a temperature of			after storage in 30/40% glycerin at a temperature of
			(22 ± 2) °C	(37 ± 1) °C	(6 ± 2) °C	(6 ± 2) °C
B. anthracis 55-VNIIVViM						
Medium No. 1	109.00 ± 8.04	–40 °C	93.40 ± 1.81*	94.20 ± 2.58*	87.80 ± 5.71*	101.80 ± 3.96
		–70 °C	100.40 ± 2.96	101.60 ± 3.43	90.80 ± 2.86*	
Medium No. 2		–40 °C	92.20 ± 2.77*	93.80 ± 3.11*	88.40 ± 4.77*	
		–70 °C	101.00 ± 2.12	102.40 ± 2.40	91.40 ± 3.20*	
B. anthracis K-STI-79						
Medium No. 1	101.20 ± 9.50	–40 °C	94.20 ± 4.43	94.60 ± 3.64	88.00 ± 2.73*	101.20 ± 9.55
		–70 °C	100.20 ± 6.26	98.00 ± 4.47	86.40 ± 5.31	
Medium No. 2		–40 °C	92.60 ± 4.82	92.00 ± 1.87*	90.20 ± 3.70	
		–70 °C	101.00 ± 4.30	97.40 ± 5.12	92.80 ± 3.34	

* statistically significant difference ($p \leq 0.01$).

At this stage of research, comparison of pathogen storage in two protective media did not allow us to choose a more effective one. Such comparisons will be made after a longer storage period, and as a result, an optimal cryoprotector will be selected.

Comparison of three thawing methods used for the tested strains revealed that the most gentle way is to thaw the cells in a water bath at +37 °C, because it helps to preserve largest number of viable cells.

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Properties of *Actinobacillus pleuropneumoniae* isolates

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SUMMARY

Results of tests of six *Actinobacillus pleuropneumoniae* isolates recovered from the diseased pigs kept in animal holdings located in the Russian Federation for their biological properties (biochemical, proteomic, antigenic and pathogenic ones) are presented in the paper. Proteomic properties were determined with mass-spectrometry using Autof MS 1000 mass-spectrometer (Autobio Diagnostics Co., Ltd, China): protein profiles were plotted and the peaks characteristic for each isolate were identified. Mass-spectra of tested *Actinobacillus* isolates and reference *Actinobacillus pleuropneumoniae* DSM 13472 strain were found to be in the m/z range of 2,000–12,000 Da. The following peaks (m/z) were common for all *Actinobacillus pleuropneumoniae* isolates and the strain: 2,541 ± 2; 4,267 ± 2; 5,085 ± 2; 6,450 ± 2; 7,207 ± 4; 9,408 ± 3; 11,820 ± 6. Therewith, the highest intensity (100%) was reported for the peak at 5,085 ± 2, that was supposed to be a specific feature of *Actinobacillus pleuropneumoniae*. All isolates were confirmed to belong to *Actinobacillus pleuropneumoniae* species and to 2, 5 and 9 serotypes by real-time polymerase chain reaction using species-specific and serotype-specific primers. *Actinobacillus pleuropneumoniae* isolates were tested for their pathogenic properties by experimental infection of white mice and 2.5–3 month-old piglets. All tested isolates were pathogenic for both white mice and piglets. Isolate No. 4 belonging to serotype 5 was found to be the most virulent out of tested isolates. Thus, LD_{50} was 4.19 lg microbial cells for white mice and 5.49 lg microbial cells for piglets that was consistent to the data of other authors carried out tests of actinobaccilli isolated in the Russian Federation for their pathogenicity. The isolates were deposited to the FGBI "ARRIAH" Collection of Microorganism Strains.

Keywords: porcine (*Actinobacillus*) pleuropneumonia, isolate, properties, polymerase chain reaction, mass-spectrometry, pathogenicity

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Свойства изолятов *Actinobacillus pleuropneumoniae*

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РЕЗЮМЕ

Представлены результаты изучения биологических свойств (биохимические, протеомические, антигенные и патогенные) 6 изолятов *Actinobacillus pleuropneumoniae*, выделенных от больных свиней в животноводческих хозяйствах Российской Федерации. Протеомические свойства определяли посредством масс-спектрометрического анализа с использованием масс-спектрометра Autof MS 1000 (Autobio Diagnostics Co., Ltd, Китай): были построены белковые профили и определены характерные пики для каждого изолята. Установлено, что все масс-спектры изучаемых изолятов актинобацилл и референтного штамма *Actinobacillus pleuropneumoniae* DSM 13472 находятся в диапазоне m/z 2000–12 000 Да. Для всех изолятов и штамма *Actinobacillus pleuropneumoniae* общими были пики m/z: 2541 ± 2; 4267 ± 2; 5085 ± 2; 6450 ± 2; 7207 ± 4; 9408 ± 3; 11 820 ± 6, при этом самая высокая интенсивность (100%) была зарегистрирована для пика 5085 ± 2, который, как предполагаем, можно считать исключительной особенностью *Actinobacillus pleuropneumoniae*. Принадлежность всех изолятов к виду *Actinobacillus pleuropneumoniae* и серотипам 2, 5 и 9 была подтверждена методом полимеразной цепной реакции в реальном времени с использованием видо- и серотип-специфичных праймеров. Патогенные свойства *Actinobacillus pleuropneumoniae* определяли при экспериментальном заражении белых мышей и поросят 2,5–3,0-месячного возраста. Все испытуемые изоляты были патогенны как для белых мышей, так и для свиней. Установлено, что из всех изучаемых изолятов наиболее высокая вирулентность характерна для изолята № 4, который относится к 5-му серотипу. Так, $ЛД_{50}$ для белых мышей составила 4,19 lg м. к., для поросят – 5,49 lg м. к., что согласуется с данными других авторов, проводивших исследования патогенности актинобацилл, выделенных на территории Российской Федерации. Изоляты депонированы в коллекцию штаммов микроорганизмов ФГБУ «ВНИИЗЖ».

Ключевые слова: актинобациллезная плевропневмония, изолят, свойства, полимеразная цепная реакция, масс-спектрометрия, патогенность

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INTRODUCTION

Actinobacillus pleuropneumoniae (APP) causes porcine pleuropneumonia, a disease that is widespread in many countries of the world and in the Russian Federation. This highly contagious disease is characterized by sudden onset, short clinical course, fibrin-hemorrhagic lung lesions in acute form or cough and decreased weight gains in chronic form of the infection. Porcine pleuropneumonia often leads to the animal death. Pigs of all ages are susceptible to the disease but 2–4-month-old piglets are the most susceptible. Persistence is a distinctive feature of the disease associated with long-term carriage of the bacterium by animal [1, 2]. The severity of the disease depends on the several factors the most important of which are APP serotype, infective dose, concomitant infections and animal keeping conditions [3–5].

In recent decades, there has been a trend in the Russian Federation towards an increase in the number of porcine APP-infected holdings, which could be accounted for breeding animal import from Western Europe and Canada, and absence of porcine pleuropneumonia in the list of infections from which the live pigs imported into the Russian Federation shall be free according to veterinary requirements [6–8].

Currently, there are some vaccines for porcine pleuropneumonia prevention classified into bacterin-toxoid, toxoid and bacterin-based (whole-cell bacteria). Bacterins confer protection against homologous serovars but do not protect against infection with heterologous serovars [9–13]. Vaccines based on inactivated Apx toxins are effective for reducing the incidence and clinical manifestations associated with the infection, but they are not able to prevent colonization by the pathogen in the lungs and their use poses a potential threat of infection of the herd by asymptomatic carrier-animals [9, 11, 14]. The development of universal anti-porcine pleuropneumonia vaccine able to protect against all known serovars is a tough task due to the lack of cross-immunity. The best way to eradicate this disease is to isolate and identify the causative agent in a particular APP-infected holding, to test the agent for its biological properties, to manufacture a vaccine and to apply the said autogenic vaccine in the said holding. Autogenic vaccines for porcine pleuropneumonia control are used in such countries as France, USA, Canada, etc. [9, 15, 16]. Considering the above-said, testing of APP isolates recovered from diseased pigs kept in animal holdings of the Russian Federation for their biological properties is of great importance. The novelty of the study was as follows: to recover new APP isolates, to test them for their biological properties, to deposit them into the FGBI "ARRIAH" Collection of Microorganism Strains for further vaccine development.

The study was aimed at isolation of APP agents from diseased pigs in animal holdings of the Russian Federation, to test them for their biochemical, proteomic, antigenic and pathogenic properties.

MATERIALS AND METHODS

Bacteria isolates. The following APP isolates recovered from pigs with respiratory disorders kept in the pig holdings located in the Russian Federation were used:

No. 1 – *A. pleuropneumoniae* "AU-21" isolate of serotype 2 recovered in the Kursk Oblast;

No. 2 – *A. pleuropneumoniae* "N-21" isolate of serotype 2 recovered in the Kursk Oblast;

No. 3 – *A. pleuropneumoniae* "VT-22" isolate of serotype 2 recovered in the Ryazan Oblast;

No. 4 – *A. pleuropneumoniae* "KG-21" isolate of serotype 5 recovered in the Belgorod Oblast;

No. 5 – *A. pleuropneumoniae* "OE-22" isolate of serotype 9 recovered in the Kursk Oblast;

No. 6 – *A. pleuropneumoniae* "DI-22" isolate of serotype 9 recovered in the Kirov Oblast.

Database of MALDI Autoflex III mass-spectrometer (Bruker Daltonik GmbH, Germany). Mass-spectrum of reference *A. pleuropneumoniae* DSM 13472 strain.

Test animals. APP isolates were tested for their pathogenic properties in white mice weighing 16–18 g and in 2.5–3.0 month-old piglets delivered from infectious disease-free holdings.

All tests in animals were carried out in strict compliance with intergovernmental standards on laboratory animal keeping and handling adopted by the Intergovernmental Council for Standardization, Metrology and Certification as well as in accordance with Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

Nutrient media and reagents. Brain-heart infusion agar (Becton, Dickinson and Company, USA) containing 5% of equine serum (AO "NPO "Microgen", Russia), 10% of yeast extract (FGBI "ARRIAH", Russia) was used for isolation

Fig. 1. Proteomic properties (protein profiles) of A. pleuropneumoniae isolates

of APP from pathological material samples. Commercial API NH test kit (bioMerieux, France) was used for testing the isolates for their biochemical properties.

Methods. Samples were collected in accordance with "Methodical guidelines for collection of biological material samples from animals for bacteriological tests"[1]. Bacteria were examined for their morphology by microscopy of Gram-stained smears. Bacteria were cultivated on dense agar media at temperature of (37.0 ± 0.5) °C for 24–48 hours.

APP were identified and tested for their proteomic properties using Autof MS 1000 mass-spectrometer (Autobio Diagnostics Co., Ltd, China). Single colonies of day-old culture were directly applied with sterile plastic loop on metal target plate. Saturated CHCA (*α*-cyano-4-hydroxycinnamic acid) solution prepared with 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid was used as a matrix. The device was calibrated daily using Calibrator Autobio Diagnostics reagent (Autobio Diagnostics Co., Ltd, China). Mass-spectrometric analysis of APP isolates was carried out using linear laser mode [17]. The analysis parameters were optimized for the m/z (mass/charge) range of 2,000 to 20,000 Da, the spectrum obtained by summing 20 single spectra with Auto Acquirer V2.0.130 software was recorded. Analysis of obtained mass-spectra was performed with Autof Analyzer V2.0.14 software (Autobio Diagnostics Co., Ltd, China).

Polymerase chain reaction was performed in accordance with the "Methodical guidelines for *Actinobacillus pleuropneumoniae* detection with polymerase chain reaction"[2].

APP isolates were tested for their pathogenic properties in white mice in accordance with the "Methodical guidelines for testing of the *Actinobaccillus pleuropneumoniae* antigens included in inactivated vaccines for their immunogenicity"[3]. Fifty white mice weighing 16–18 g (10 mice per each dilution) were used for tests of each isolate for

[1] Evgrafova V. A., Kononov A. V., Yashin R. V., Bryantseva M. S., Stepanova I. A., Biryuchenkov D. A. Methodical guidelines for collection of biological material samples from animals for bacteriological tests. No. 03-22. Vladimir: FGBI "ARRIAH", 2022. 11 p.

[2] Scherbakov A. V., Timina A. M., Yakovleva A. S., Kovalishin V. F. Methodical guidelines for *Actinobacillus pleuropneumoniae* detection with polymerase chain reaction. No. 38-05. Vladimir: FGI "ARRIAH"; 2005. 8 p.

[3] Biryuchenkov D. A., Rusaleyev V. S., Frolovtseva A. A., Potekhin A. V. Methodical guidelines for testing of the *Actinobaccillus pleuropneumoniae* antigens included in inactivated vaccines for their immunogenicity. No. 69–08. Vladimir: FGI "ARRIAH"; 2008. 17 p.

its pathogenicity. The mice were infected intraperitoneally with day-old APP culture at the following concentrations: 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4 microbial cells/cm^3 in a volume of 0.5 cm^3. The mice were observed for 5 days.

lg LD_{50} was calculated according to Karber's formula modified by Ashmarin:

$$\lg LD_{50} = \lg D_N - \lg \delta (\Sigma L_i - 0.5),$$

where $\lg D_N$ – logarithm of maximum infective dose;

$\lg \delta$ – logarithm of the agent culture dilution factor;

L_i – dead test animal number/infected test animal number ratio;

ΣL_i – sum of L_i values for all tested doses.

APP isolates were tested for their pathogenic properties in 2.5–3.0 month-old piglets. The piglets were infected intratracheally with bacterium suspension containing 1×10^8 microbial cells in a volume of 1.0 cm^3. The piglets were observed for 10 days.

Microsoft Excel application and standard statistical methods were used for *statistical processing* of the data.

RESULTS AND DISCUSSION

The first stage of the study was the isolation of porcine pleuropneumonia agent from pathological material samples collected from dead and emergency slaughtered animals with respiratory disorders in the APP-infected holdings located in the Belgorod, Kirov, Kursk and Ryazan Oblasts. Six APP isolates were prepared for further study based on the tests results. The list of the said isolated is given above in "Materials and methods" section.

All tested isolates were shown to ferment glucose, sucrose, maltose, fructose and to be active against alkaline phosphatase, urease and *β*-galactosidase. Comparison of the obtained data with reference strain characteristics given in Bergey's Manual of Determinative Bacteriology [18] showed that characteristics of the said isolates determined with all tests were consistent with that ones of the reference strains indicating that the isolates belonged to APP species.

During the next stage of the study proteomic properties of all APP isolates were determined, protein profiles were plotted (Fig. 1) and peak mass lists enabling identification of specific peaks for each isolate were generated (Table 1).

Analysis of obtained results showed that all mass-spectra of tested *Actinobacillus* isolates and reference APP DSM 13472 strain were in the m/z range of 2,000–12,000 Da. The following peaks were common for all isolates and reference APP strain: m/z 2,541 ± 2; 4,267 ± 2; 5,085 ± 2; 6,450 ± 2; 7,207 ± 4; 9,408 ± 3; 11,820 ± 6, therewith, the highest intensity (100%) was recorded for peak of 5,085 ± 2 that was suggested to be a unique feature of APP.

The isolates were confirmed to belong to APP species by testing with real-time polymerase chain reaction (qPCR) with species-specific primers.

Figure 2 shows qPCR DNA amplification curves of tested isolate samples confirming that all samples belong to APP species [19–21].

Then, *A. pleuropneumoniae* isolates were serotyped using serotype-specific primers (Fig. 3–5).

Figure 3 shows qPCR DNA amplification curves of APP isolates No. 1, 2 and 3 confirming that they belong to serotype 2. DNA amplification curves of other isolates coincide with negative control curve indicating that they do not belong to serotype 2.

Table1
Analysis of mass-spectra of *A. pleuropneumoniae* isolates

m/z	Intensity (%) of *A. pleuropneumoniae* isolate and reference DSM 13472 strain spectra						
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	DSM 13472
2,541 ± 2	45	35	41	29	33	18	38
4,267 ± 2	56	53	61	48	49	22	70
5,085 ± 2	**100**	**100**	**100**	**100**	**100**	**100**	**100**
6,450 ± 2	52	51	54	55	51	43	67
7,207 ± 4	22	30	39	66	24	33	24
9,408 ± 3	14	25	29	38	13	22	26
11,820 ± 6	2	8	11	25	6	8	5

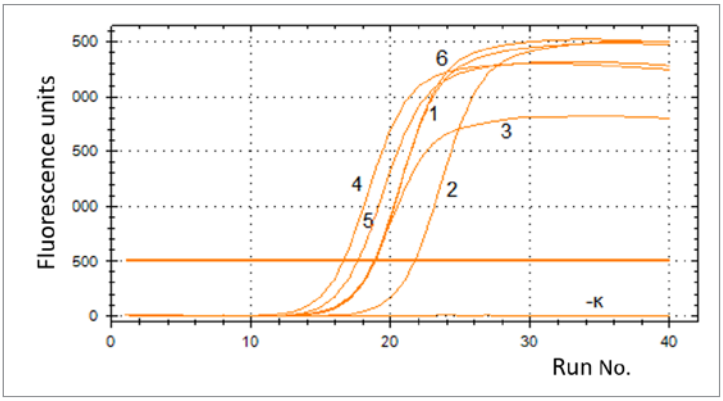

Fig. 2. Real-time PCR confirmation of isolate No. 1, 2, 3, 4, 5, 6 identification as A. pleuropneumoniae species ("-к" – negative control)

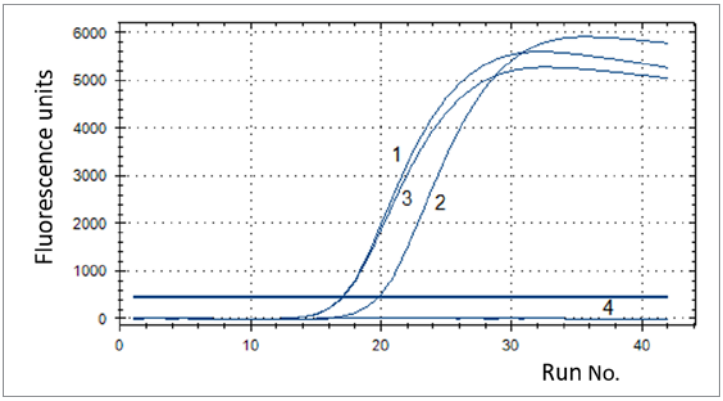

Fig. 3. Real-time PCR identification of A. pleuropneumoniae isolates as serotype 2 ones (1, 2, 3 – isolates No. 1, 2 and 3; 4 – isolate No. 4 and negative control)

Figure 4 shows qPCR DNA amplification curve of APP isolate No. 4 confirming that it belongs to serotype 5. DNA amplification curves of other tested isolates coincide with negative control curve indicating that they do not belong to serotype 5.

DNA amplification curves of serotype 9 APP isolate are shown in Figure 5. Isolates No. 5 and 6 were classified to this serotype based on qPCR results while amplification curves of other isolates coincide with negative control curve.

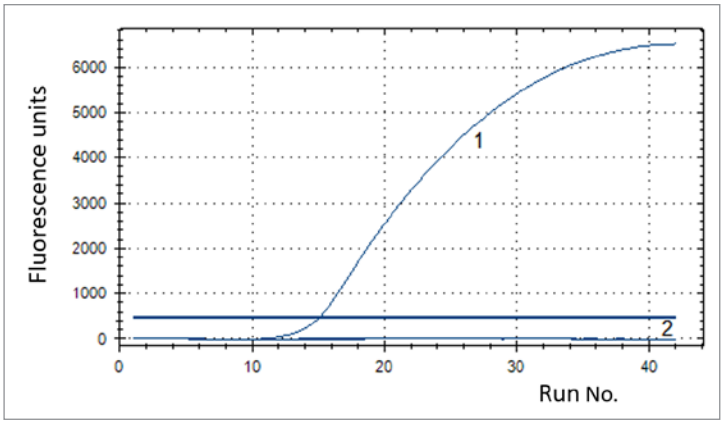

Fig. 4. Real-time PCR identification of A. pleuropneumoniae isolates as serotype 5 ones (1 – isolate No. 4; 2 – isolate No. 5 and negative control)

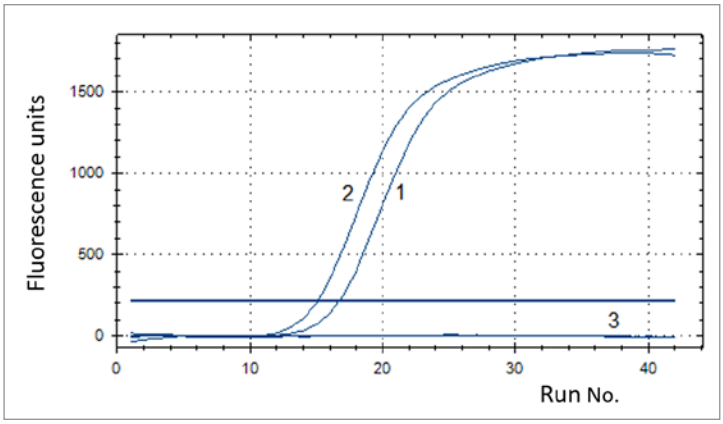

Fig. 5. Real-time PCR amplification of DNAs of serotype 9 A. pleuropneumoniae isolates (1 – isolate No. 5; 2 – isolate No. 6; 3 – isolate negative for serotype 9 and negative control)

Table 2
Pathogenic properties of serotype 2, 5 and 9 *A. pleuropneumoniae* isolates for laboratory and naturally susceptible animals

Animal species	lg LD_{50} microbial cells of isolates					
	serotype 2			serotype 5	serotype 9	
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
white mice	4.99	5.09	4.89	4.19	5.59	5.19
piglets	6.99	7.49	6.69	5.49	7.89	7.29

Thus, qPCR showed that tested APP isolates No. 1, 2 and 3 belonged to serotype 2, isolate No. 4 belonged to serotype 5 and isolates No. 5 and 6 belonged to serotype 9.

The next stage of the study was to test APP isolates for their pathogenic properties in laboratory and naturally susceptible animals. For this purpose, 50 laboratory mice weighing 16–18 g and 2.5–3.0 month-old piglets were experimentally infected in accordance with the above-mentioned method. All tested isolates were pathogenic for white mice and piglets since infected animals of all groups except for animals of control group died. The animal deaths were confirmed to be specific by recovery of pure cultures of tested isolates from brain-heart infusion agar with supplements (serum and yeast extract) inoculated with pathological materials collected from dead animals. White mice were observed for 5 days, piglets were observed for 10 days, observation results were recorded, interpreted. Table 2 shows lg LD_{50} values allowing comparative assessment of APP isolates for their virulence. Obtained data indicate that lg LD_{50} of the isolates for experimentally infected white mice was as follows: 4.99 ± 0.1 lg LD_{50} microbial cells for isolates No. 1, 2, 3 (serotype 2), 4.19 lg LD_{50} microbial cells for isolate No. 4 (serotype 5) and 5.39 ± 0.2 lg LD_{50} microbial cells for isolates No. 5 and 6 (serotype 9). lg LD_{50} of the isolates for experimentally infected 2.5–3.0 month-old piglets was found to be minimum (5.49 lg LD_{50} microbial cells) when isolate No. 4 (serotype 5) was used for inoculation and maximum (7.89 lg LD_{50} microbial cells) when isolate No. 5 (serotype 9) was used for inoculation that was indicative of high virulence of serotype 5 isolate.

Thus, isolate No. 4 belonging to serotype 5 was found to be higher virulent than other tested isolates, that was consistent with the data of other authors [1] carried out tests of actinobacilli isolated in the Russian Federation territory for their pathogenicity.

The isolates were deposited into the FGBI "ARRIAH" Collection of Microorganism Strains under the following names based on results of tests of these isolates for their biochemical, proteomic, serological and pathogenic properties:

– isolate No. 1 – *A. pleuropneumoniae* "AU-21" strain of serotype 2;
– isolate No. 2 – *A. pleuropneumoniae* "N-21" strain of serotype 2;
– isolate No. 3 – *A. pleuropneumoniae* "VT-22" strain of serotype 2;
– isolate No. 4 – *A. pleuropneumoniae* "KG-21" strain of serotype 5;
– isolate No. 5 – *A. pleuropneumoniae* "OE-22" strain of serotype 9;
– isolate No. 6 – *A. pleuropneumoniae* "DI-22" strain of serotype 9.

CONCLUSION

Tests of the bacterial cultures isolated in APP-infected pig holdings located in the Belgorod, Kirov, Kursk and Ryazan Oblast for their biological properties have showed that all tested isolates belong to *A. pleuropneumoniae* species. The following characteristic m/z peaks were determined based on tests of proteomic properties of the isolates: 2,541 ± 2; 4,267 ± 2; 5,085 ± 2; 6,450 ± 2; 7,207 ± 4; 9,408 ± 3; 11,820 ± 6 and were found to be similar to characteristics of reference *A. pleuropneumoniae* strain. The isolates were classified based on their antigenic properties as follows: isolates No. 1, 2 and 3 – to serotype 2; isolate No. 4 – to serotype 5; isolates No. 5 and 6 – to serotype 9. All isolates were pathogenic for laboratory and naturally susceptible animals. lg LD_{50} of the isolates for experimentally infected white mice was as follows: 4.99 ± 0.1 lg LD_{50} microbial cells of serotype 2 isolates, 4.19 lg LD_{50} microbial cells of serotype 5 isolate and 5.39 ± 0.2 lg LD_{50} microbial cells of serotype 9 isolates. The isolates were deposited to the FGBI "ARRIAH" Collection of Microorganism Strains based on the test results to be used for further anti-porcine pleuropneumonia vaccine development.

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