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AIMS AND SCOPE

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

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

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Contribution of Federal Centre for Animal Health to Rosselkhoznadzor's international mandate delivery

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ABSTRACT

The paper covers the history of the Federal Centre for Animal Health, which started 65 years ago by the foundation of the All-Union Foot and Mouth Disease Research Institute. The main research area – FMD prevention and control – was and still remains the leading one for the Centre. The current history of the Federal Centre for Animal Health development is inextricably associated with the public administration reform in agriculture in 2000s, when the Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor) spun off the Ministry of Agriculture of the Russian Federation as an independent executive authority with broad powers in the area of the veterinary and phytosanitary control and surveillance. The grounds for the re-subordination of the Federal Centre for Animal Health to the Rosselkhoznadzor, historically novel executive authority in Russia, included high international prestige of the Centre and nationally and internationally acknowledged qualification of its employees in the field of contagious animal diseases.

Keywords: All-Union Foot and Mouth Disease Research Institute, Federal Centre for Animal Health, Rosselkhoznadzor, foot and mouth disease (FMD), infectious animal diseases

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Вклад ФГБУ «ВНИИЗЖ» в реализацию международных полномочий Россельхознадзора

В. В. Лавровский
Федеральная служба по ветеринарному и фитосанитарному надзору (Россельхознадзор), Орликов переулок, 1/11, г. Москва, 107996, Россия

РЕЗЮМЕ

В статье освещается история Федерального центра охраны здоровья животных, начало которой было положено 65 лет тому назад при создании Всесоюзного научно-исследовательского ящурного института. Основная научная тематика – профилактика и борьба с ящуром – была и остается главенствующей для учреждения. Современная история развития Федерального центра охраны здоровья животных неразрывно связана с реформой государственного управления в области сельского хозяйства в стране в 2000-х гг., когда в июле 2004 г. из структуры Министерства сельского хозяйства Российской Федерации была выделена Федеральная служба по ветеринарному и фитосанитарному надзору (Россельхознадзор) в виде самостоятельного федерального органа исполнительной власти с широкими полномочиями в сфере ветеринарного и фитосанитарного контроля и надзора. Основанием для переподчинения Федерального центра охраны здоровья животных Россельхознадзору, исторически новому для России органу исполнительной власти, явились высокий международный авторитет научного центра и признанная на национальном и международном уровне квалификация его сотрудников в области заразных болезней животных.

Ключевые слова: ВНИИЯИ, ФГБУ «ВНИИЗЖ», Россельхознадзор, ящур, инфекционные болезни животных

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Each anniversary is a good reason to sum up results, outline new plans, honor the memory of tutors and colleagues who created the prestige and glory of the Center as well as to support new generation entering the scientific path in the time of challenges experienced by our country.

Fortunately, we still have talented scientists among us, who in the 60s of the XX century were entrusted with important tasks for the development of domestic livestock production, who made a huge contribution to the study of the nature of foot-and-mouth disease and other infectious animal diseases, methods of their prevention and treatment, who earned the gratitude and recognition of the domestic and foreign colleagues.

It is impossible to cover the entire scale of events and achievements of the Federal Centre for Animal Health (FGBI "ARRIAH") over 65 years in one article, so let's focus on the time period, part of which I witnessed and directly participated as an adviser on agriculture in the Russian Federation Embassy in Canada (2001–2005), then at the Permanent Mission of the Russian Federation to the European Union and Euratom (2006–2013) and as Head of the Rosselkhoznadzor Department for Inspection Work within the Framework of International Cooperation in the Veterinary Field and WTO, Assistant and Adviser to the Head of the Rosselkhoznadzor for International Activities (from 2013 to the present).

The history of the Federal Centre for Animal Health cannot be considered in isolation from the problems, tasks and achievements of the domestic agriculture, formation of industrial livestock production in response to the demand of the country's population for high-quality animal products, and their entry into the global markets.

The stages of the Federal Centre for Animal Health development are inextricably linked with the reform of public administration in the field of agriculture in the early 2000s, with the separation of the new Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor) from the Ministry of Agriculture of the Russian Federation in July 2004 in the form of an independent federal executive authority with broad powers in the field of veterinary and phytosanitary control and surveillance.

In accordance with the Regulations on the Rosselkhoznadzor, the agency received unique powers in the field of international cooperation:

– direct interaction with the veterinary and phytosanitary competent authorities of foreign countries, within which the agency has the right to carry out control and inspection activities in other countries;

– provision of official guarantees (certificates) as for the safety of the imported and exported regulated products through the border control of the commodities, as well as confirmation of the traceability and safety of the commodities of the foreign and Russian origin by means of the laboratory and analytical methods;

– the right to represent the interests of the Russian Federation in international specialized organizations, such as the World Organization for Animal Health (WOAH, founded as OIE), where the Deputy Heads of the Service have been Council members for many years, the International Plant Protection Convention (IPPC); the right to participate in the activities of the Codex Alimentarius Commission, established by the United Nations Food and Agriculture Organization (FAO), and the Committee on Sanitary and Phytosanitary Measures of the World Trade Organization (WTO).

The basic principle, which determines the effectiveness of the Rosselkhoznadzor's activities, is the implementation of the control and surveillance permissive and restrictive measures during the manufacture and movement of regulated commodities exclusively on the basis of modern scientific knowledge and methods based on the international recommendations (codes).

With this approach, the subordinate research institutions (All-Russian State Center for Quality and Standardization of Veterinary Drugs and Feed, All-Russian Plant Quarantine Center and, of course, the Federal Centre for Animal Health, which celebrated its 65th anniversary, became a significant Rosselkhoznadzor's pillar in the implementation of the new tasks.

The reason for the reassignment to the Rosselkhoznadzor was high international prestige of the Federal Centre for Animal Health, nationally and internationally recognized qualifications of its staff, established school for training scientific manpower, serious methodological and infrastructural support for activities, long-term practical experience in the prevention and control of infectious animal diseases in various regions of Russia and the CIS, high effectiveness of the preventive and quarantine measures, history of the veterinary standard-setting activities.

By the start of the public administration reform, the country's leaders had formed a clear understanding of the need for governmental support of the most important sectors of agriculture as well as for regulation of foreign trade in food, which was implemented in the Federal Law "On the Development of Agriculture" of 29 December 2006 No. 264-FZ. In this law the Russian government for the first time declared its direct responsibility for ensuring food security through targeted financing of the leading sectors of the agricultural economics, primarily livestock and crop production.

In the process of preparing these transformations, the experience of the leading agrarian countries of the world (the USA, Canada, Germany, Great Britain, France, the Netherlands, India, and European Union countries) was, of course, actively borrowed.

The reforms were also pushed by the vector to the WTO accession taken back in 1995, the initiators of which promised significant benefits from equitable trade turnover of the Russian commodities on international markets.

One of the reasons why the world's leading food exporters persistently involved Russia in the WTO, promising its free access to global food markets, was annoying allegedly excessive requirements of the national veterinary legislation for imports, which allowed them to subsidize exports and offer dumping prices for the notorious "Bush legs" despite the undeniable competitive advantages of the developed countries.

The Russian Federation accessed the WTO in 2012 as a "developed country" despite the serious economic difficulties caused by the transformation of the state structure and adaptation of the economy to market conditions as well as irrespective of the need to pay off external debts and maintain economic ties within the CIS, which were broken due to the collapse of the Soviet Union. This meant rejection of many trade benefits and preferences that extended to most of the "developing" founders and members of this "trade club", established in 1995, and at the same time a very modest level of governmental support ($9 billion in 2012 and a decrease to 4.4 billion by 2018), as compared, for example, with the developed member countries of the Organization for Economic Cooperation and Development (OECD), and even those funds lacked in the country's budget at that moment.

Russia received modest opportunities for customs and tariff protection in the food sector (the doors were especially wide open for the supply of live slaughter pigs that subsequently put serious pressure on the industry). Meanwhile, the developing countries in Latin America, Middle East, Indochina, India, Turkey, not to mention Africa, still easily impose prohibitive duties of tens or even hundreds percent, and they do not hesitate to declare that this is done to protect their own production and promote rural employment.

It would be fair enough to admit that in the midst of negotiations on the WTO accession, Russia was tightly "on the needle" of food imports, and governmental mechanisms to support agriculture were launched with great difficulty.

Under these conditions, the authority to use sanitary and phytosanitary protection measures (the benefits of the WTO "green" and "amber" boxes allowed investments in food safety) made the Rosselkhoznadzor an important "player" regulating the food turnover on the foreign market, of course, with scientific substantiation of its requirements.

In this regard, it became possible to invest serious funds in development of the skills of veterinary personnel, improvement of the methodological, laboratory and information and analytical activities, in capital construction and purchase of modern equipment to achieve equivalence and confirm scientific validity of the actions aimed at the protection of the domestic market from the subsidized and often substandard imports on the one hand, and to ensure credibility of guarantees as for the safety of domestic exported agricultural products on the other hand, which was accompanied with the harmonization of the national legislation with the international one.

It should be emphasized that at all stages of the WHO accession and membership Russia has been faithfully committed to its obligations, although the "alarm bells" have long been heard, indicating that its concerns in the development of the national market, and even more so the claims to international markets are of no interest for the partners in the West.

Thus, in the year of the Rosselkhoznadzor's formation, ten countries joined the European Union, most of which are in Eastern Europe (Baltic States, Poland, Czech Republic, Slovakia, Slovenia, Hungary) and used to represent an important export market for Russian food and feed. The European Union unceremoniously tore up all existing trade agreements between Russia and these countries, including those in the veterinary and phytosanitary areas, without any conditions or compensation for the loss of the markets (while the United States and Canada received such compensations). In the same year, after Russia's extremely successful export of a large batch of grain to the European Union after a record harvest in 2003–2004, the European Union in collusion with the United States and Canada introduced a tough tariff quota for imports of "Black Sea" (read: Russian) low-protein (it was even called fodder) wheat; after that there were no more records in trade with the European Union.

Despite Russia's heavy dependence on regular meat imports from the European Union, especially in large cities, the "newborn" Rosselkhoznadzor managed to put the European Union in place, forcing, after a three-month ban on the import of all livestock products, to sign a Memorandum on the terms of supply from the European Union, after which it launched a wide-ranging campaign to inspect establishments of the European Union member states and approve new bilateral veterinary certificates.

It was a great disappointment in the fairness of the "free market" and the universality of the international SPS rules, but an important test of the Rosselkhoznadzor's ability to defend the interests of domestic pig farming under the terms of the WTO membership was the dispute in the panel of this organization in 2014 over the legality of the Russian ban on the supply of pig products from the European Union to Russia due to African swine fever (ASF) outbreaks in Lithuania, Poland, Latvia and Estonia, risks of the disease spread over Western Europe and its introduction into Russia.

At that time, the Federal Centre for Animal Health provided expert support at all stages, participated in inspections to the affected areas and in the preparation of the scientific analysis of the risk of ASF spread in the European Union and its entry from the European Union into the Russian Federation. As time has shown, the scientific forecast proving the validity of Russia's concern about the possibility of ASF spread has been fully confirmed. African swine fever spread to half of the European Union member states and caused serious damage to the pig industry in the Baltic States, Poland, Germany, Romania, Hungary, Bulgaria, Czech Republic, Slovakia, and Italy. Unfortunately, at the stage of the proceedings before the WTO panel, the Russian assessments were arrogantly rejected due to "non-compliance" with European criteria for the risk probability.

Faced with the manipulative nature of the *highly likely* principle without sufficient evidence of threats or damage, employees of the Federal Centre for Animal Health, who developed the system of risk assessment and Russian establishments' ranking according to the degree of their veterinary safety in the Rosselkhoznadzor's electronic information systems, subsequently made it a rule to proceed from the actual history of violations committed by the establishment in the course of its activities and keep its personal dossier.

At the same time, with the direct participation of the Federal Centre for Animal Health, the process of strengthening trade relations was developed, and harmonization of the legislation of the countries of the Customs Union, its expansion and transformation into the Eurasian Economic Union with a single market (by the way, the European Union categorically refused the recognition of its establishment in 2011), and therefore, with a unified, as far as it was possible, veterinary and phytosanitary regulation was carried out.

The coup d'etat in the Ukraine, annexation of Crimea to Russia (the new authorities of the country then ignored the year-and-a-half intergovernmental negotiations (2014–2015), which could, among other things, preserve full-fledged relations of the surveillance services and beneficial, primarily for Ukraine, food trade with Russia), imposition of economic sanctions by the United States, European Union, Australia and Norway and imposition of retaliatory "counter-sanctions" on the export of a number of agricultural products from these countries resulted in the reversal to the markets of the friendly countries, and the process of food import substitution was launched. In addition to active operation in the domestic market, this process was accompanied with serious negotiations and inspection activities with developing countries, primarily Asian and Latin American, where timely information support and risk assessments performed by the employees of the Federal Centre for Animal Health allowed to avoid meat shortages without compromising the veterinary safety of Russia.

Being isolated from the pressure of the subsidized food exported from unfriendly countries, Russia took effective measures to develop its own agriculture. The problem of dependence on imported meat was replaced by the task of opening export markets by exporting the surpluses.

The control system well-established by this time, updated laboratories, modern establishments building their business according to the recommendations and with careful inspections by the Rosselkhoznadzor officers, state registration of the establishments capable of being compliant with the requirements of the foreign countries – all these aspects, linked into a single information system "VetIS" moderated from the Federal Centre for Animal Health and ensuring full traceability of products from disease-free zones, make a proper impression on the potential importers.

Suffice it to say that by 2020 Russia became a net food exporter, and in 2023 export revenue exceeded 45 billion US dollars, *inter alia* due to reliable guarantees from the Rosselkhoznadzor regarding the safety of the exported products.

Due to high competencies in the field of prevention and control of animal diseases, the Federal Centre for Animal Health became an essential link in the inspection programs by the representatives of the foreign veterinary services, and the governmental support of the exports of the livestock products made such visits available to specialists from developing countries who see Russia as a worthy competitor to traditional exporters, capable of providing competitive prices and an impeccable level of safety of beef, mutton, poultry meat, table eggs, dairy and fish products.

When summing up, it can be concluded that the choice made by the Rosselkhoznadzor in 2004, when it included the Federal Centre for Animal Health among the organizations providing scientific support to regulatory measures and execution of the new powers of the Service, was fully justified. In any case, at all important stages, where the Rosselkhoznadzor faced the need to change approaches in its work and adapt to changing external conditions, the employees of the institute proved to be on top, worthy of the difficult tasks that befell the industry and the country over the past 20 years.

In the Rosselkhoznadzor system the Federal Centre for Animal Health confirmed its high status as an international center for animal health protection, as evidenced by its diverse and fruitful external relations, recognition of its activities by international organizations such as the WOAH and FAO, the Russian Academy of Sciences, as well as by leading research centers of the world, demand for highly effective author's products (vaccines, diagnostics, test-kits) far beyond the borders of our country.

Over the past 20 years, the Federal Centre for Animal Health not only preserved, but also increased its traditional competencies in the field of dangerous human and animal disease prevention and control (development of control methods and means), while reliably performing the functions of a leading information and analysis center necessary for successful governmental management of the animal husbandry and the market of animal products based on scientifically justified recommendations, new scientific facts, accurate and reliable forecast of the development of the animal disease situation and an immediate response to every case of a disease occurrence.

The Federal Centre for Animal Health became the main base and industry flagship in the development and widespread use of modern biotechnologies and electronic information systems, which made it possible to combine and systematize a huge amount of data on the origin and movement of any regulated animal products in the country.

On this basis, a unique system of regionalization of the country's territory for animal diseases was built, which allows real-time regulation of commodity flows, including those moving across the state border, taking into account information on the animal disease situation, changes in veterinary legislation, departmental instructions and legal veterinary norms, which are constantly updated with the participation of the staff of the Federal Centre for Animal Health.

It was the development of information systems and continuous generation of an objective evidence base on the farm animal disease situation and animal product safety that made it possible to make a breakthrough in the acquisition of a number of international dangerous animal disease-free statuses and, most importantly, to convince during numerous inspections the world's leading importers of the safety of Russian animal products that already in 2023 allowed to increase the animal product exports to 2 billion US dollars, according to the estimates of the National Meat Association.

Knowledge and skills in the Rosselkhoznadzor information systems are mandatory for the organization of the production process and control of the establishments involved in the animal product manufacture and marketing chain, and such systems significantly reduce the burden on the diligent business operators. It is important to emphasize that thanks to the coordinated

work of the Rosselkhoznadzor and its scientific departments, unification and standardization of sampling and processing techniques, improvement of laboratory equipment, state accreditation and international system of proficiency tests, an acceptable level of risk is achieved, confidence in the security system in the country is strengthened, and the risks of introducing counterfeit products into circulation through fraudulent schemes are reduced.

Despite the continuing risks of the occurrence of single outbreaks of ASF, avian influenza, and a number of other diseases, the epizootic situation in the country remains under control. This is evidenced by the increase in the animal product manufacture and consumption, sustained level of governmental support for the agricultural sector, and most importantly, in the continued growth of private investment in dairy and beef cattle breeding, pig farming, poultry farming, industrial processing, storage and retail. The processes of animal waste processing and disposal are developing, which in turn has a beneficial effect on production safety.

People, both consumers and manufacturers, trust the Service and its scientific branch, this is the main result of the activities of the Federal Centre for Animal Health, which celebrated its 65th anniversary.

The report was made on the Scientific and Practical Conference "Veterinary Science for Food and Biological Safety" dedicated to the 65th Anniversary of the Federal Centre for Animal Health (Vladimir, 7–8 December 2023).

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Molecular epidemiology of foot-and-mouth disease (review)

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ABSTRACT

Molecular epidemiological studies are an important tool for regional and global surveillance of foot-and-mouth disease (FMD). These tests are based on constantly progressing technologies of nucleic acid sequencing and phylogenetic analysis. The use of these technologies made it possible to assess the genetic diversity of the FMD virus, to analyze the evolution of the virus in the enzootic regions, and track the pathways of FMD epizootic and panzootic spread. Molecular epidemiological studies have shown that within the long-known seven serotypes of the FMD virus, there are numerous topotypes (geographical types), genetic lineages and sublineages. Usually, the foot-and-mouth disease virus of a certain topotype and genetic lineage evolves within a certain area, periodically causing regional epizootics. However, over the past 30 years, two FMD panzootics have occurred, invloving several continents. The first panzootic occurred in the late 1990s – early 2000s and was caused by O/ME-SA/PanAsia FMD virus, and the second, caused by O/ME-SA/Ind-2001 virus, began in 2013 and continues to the present. The emergence of FMD panzootics is probably a consequence of the economic globalization. FMD is not enzootic in Russia, but sporadic outbreaks of this disease are periodically reported. Molecular epidemiological studies have shown that these outbreaks are caused by the infection introduction from neighboring Asian countries, mainly from China. The FMD virus, which has come to the Russian Federation from other countries, is characterized by great genetic diversity and belongs to three serotypes, five topotypes and eight genetic lineages: O/Cathay, O/ME-SA/PanAsia, O/SEA/Mya-98, O/ME-SA/Ind-2001, O/ME-SA/unnamed, A/Asia/Iran-05, A/Asia/Sea-97, Asia1/V. The results of molecular epidemiological studies are taken into account when vaccine strains are to be selected for preventive vaccination of livestock in FMD high-risk areas. The review is based on the analysis of 68 literature sources.

Keywords: review, foot-and-mouth disease virus, phylogenetic analysis

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Молекулярная эпизоотология ящура (обзор)

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РЕЗЮМЕ

Молекулярно-эпизоотологические исследования являются важным инструментом регионального и глобального надзора за ящуром. Эти исследования базируются на постоянно прогрессирующих технологиях секвенирования нуклеиновых кислот и филогенетического анализа. Применение данных технологий позволило оценить генетическое разнообразие возбудителя ящура, изучить эволюцию вируса в регионах, энзоотичных по заболеванию, и отслеживать пути распространения эпизоотий и панзоотий ящура. Молекулярно-эпизоотологические исследования показали, что в пределах давно известных семи серотипов вируса ящура существуют многочисленные топотипы (географические типы), генетические линии и сублинии. Обычно вирус ящура того или иного топотипа и генетической линии эволюционирует в пределах определенного ареала, периодически вызывая региональные эпизоотии. Однако за последние 30 лет случились две панзоотии ящура, охватившие несколько континентов. Первая панзоотия произошла в конце 1990-х – начале 2000-х гг. и была обусловлена вирусом ящура O/ME-SA/PanAsia, а вторая, вызванная вирусом O/ME-SA/Ind-2001, началась в 2013 г. и продолжается до настоящего времени. Возникновение панзоотий ящура, вероятно, является следствием глобализации мировой экономики. В России ящур не энзоотичен, однако периодически регистрируются спорадические вспышки этой болезни. Молекулярно-эпизоотологические исследования показали, что эти вспышки вызваны заносом инфекции из соседних азиатских стран, главным образом из Китая. Вирус ящура, проникавший на территорию Российской Федерации из других стран, характеризуется большим генетическим разнообразием и относится к трем серотипам, пяти топотипам и восьми генетическим линиям: O/Cathay, O/ME-SA/PanAsia, O/SEA/Mya-98, O/ME-SA/Ind-2001, O/ME-SA/unnamed, A/Asia/Iran-05, A/Asia/Sea-97, Asia1/V. Результаты молекулярно-эпизоотологических исследований учитываются при выборе вакцинных штаммов для профилактической вакцинации скота в зонах с высоким риском заноса ящура. Обзор составлен на основе анализа 68 источников.

Ключевые слова: обзор, вирус ящура, филогенетический анализ

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, that may cause epizootics and inflict dramatic economic damage.

The causative agent of the disease is a non-enveloped RNA virus, which belongs to *Aphthovirus* genus of the *Picornaviridae* family. There are seven viral serotypes: O, A, C, Asia-1, SAT-1, SAT-2 and SAT-3.

Currently, the disease is widespread in Africa, Asia and some parts of South America, in which it causes huge economic losses to the agricultural sector. For the FMD-free countries there is still a danger of the disease introduction from the infected regions.

FMD is characterized by a very dynamic and complex epidemiology. The FMD virus (FMDV) is rapidly evolving, new genetic and antigenic variants of the virus are constantly emerging that can overcome the immunity of vaccinated animals and cause outbreaks of the disease. This significantly complicates the control of the disease and determines the need to constantly monitor the FMDV diversity in nature and to timely develop new vaccines adapted to emerging viral lineages.

Global FMD surveillance is performed by the FMD reference laboratory network of the World Organization for Animal Health and the Food and Agriculture Organization of the United Nations (WOAH/FAO) [1], with the main task to monitor the emergence of new FMDV variants with modified genetic and antigenic properties which render the available vaccines ineffective. An important surveillance tool is the molecular genetic analysis of the FMDV.

THE HISTORY OF THE DEVELOPMENT OF MOLECULAR EPIDEMIOLOGICAL TECHNIQUES

Molecular epidemiology as a scientific field is the combination of molecular biology, epidemiology and population genetics that has become a powerful tool to develop control strategies for infectious diseases [2].

Historically, the first attempts to use laboratory methods in epidemiological studies of FMD were associated with serological tests. More recently, biochemical methods such as FMDV SP polyacrylamide gel electrophoresis and T1 oligonucleotide fingerprinting of the viral genome began to be used [3, 4, 5, 6]. However, the resolution of these methods was low, and they were not widely used.

A revolutionary event in the molecular epidemiology of foot-and-mouth disease was the use of sequencing technologies and phylogenetic analysis of the viral genome sequences. In 1987 E. Beck and K. Strohmaier showed that sequencing and comparative analysis of the VP1 gene of FMDV field isolates from the outbreak reveal their origin [7]. In particular, they established that 14 out of 18 FMD outbreaks in Western European countries between 1964 and 1985 were caused by vaccine strains. In most cases they were induced by improperly inactivated FMD vaccines. Several more outbreaks could occur as a result of the virus escape from the vaccine production plants. The German scientists suggested that nucleotide sequence analysis should be used as a standard method of diagnosis, because when compared with other techniques it more clearly reveals the origin and course of epizootics and offers the possibility of preventing further outbreaks. Beck E. and Strohmaier K. also proposed to prohibit the use of formalin-inactivated FMD vaccines, since they are the main source of FMDV in Europe. After this proposal was implemented, no FMD cases have been reported in Europe.

Since the late 1980s and early 1990s, nucleotide sequence analysis has been widely used to characterize FMDV field isolates [8–28].

Initially, the so-called direct RNA sequencing method was used for these purposes [29]. It included such preparatory steps as FMDV propagation in cell culture, purification and concentration of the virus and viral RNA, and therefore the method was very laborious and time-consuming. In addition, it allowed the determination of only 130–150 nucleotide bases of the FMDV VP1 gene. Since the mid-1990s, polymerase chain reaction (PCR) has been used to amplify genetic material for sequencing [30, 31]. The PCR use has literally revolutionized the FMDV nucleotide analysis technology: the time needed for testing was reduced from 1–2 weeks to 1–2 days, it became possible to produce the full genome sequence of the VP1 gene and even of the FMDV. To sequence the FMDV full genome, E. M. Cottam et al. amplified 24 overlapping viral genome fragments using PCR [32]. Further progress in full genome sequencing was associated with the use of NGS (next-generation sequencing) technology [33].

STUDY OF THE FMDV GENETIC DIVERSITY

An impressive bank of nucleotide sequences of FMDV field isolate VP1 genes from different countries has been created at the World Reference Laboratory for Foot-and-Mouth Disease (Pirbright, UK) and the WOAH/FAO FMD regional reference laboratory network for FMD. The analysis of this database made it possible to evaluate the virus genetic diversity, to determine the viral evolution patterns in the endemic regions, and track the pathways of the FMD epizootic spread.

Molecular biological studies have shown that there are several levels of the FMDV genetic diversity: types, topotypes, genetic lineages and sublineages. The division of the virus into types fully corresponds to its classification into serotypes, that is, at the genetic level, as well as at the antigenic level, seven groups of the virus are distinguished, defined as types: A, O, C, Asia-1, SAT-1, SAT-2 and SAT-3. VP1 varies by 30–50% between the types [34].

Within each serotype, A. R. Samuel and N. J. Knowles [35] identified geographical types, which they called topotypes. VP1 nucleotide differences between the topotypes are up to 15–20%. It is obvious that the topotypes were formed as a result of the relatively independent FMDV evolution in certain geographical regions. The figure shows current FMDV pools. The FMDV division into topotypes generally corresponds to the division of the viral distribution into pools [36].

There are 11 topotypes within type O lineage: Cathay (China), SEA (Southeast Asia), ME-SA (Middle East and South Asia), Euro-SA (Europe and South America), WA (West Africa), EA-1 (East Africa-1), EA-2 (East Africa-2), EA-3 (East Africa-3), EA-4 (East Africa-4) and two extinct Indonesian topotypes ISA-1 and ISA-2 [37, 38, 39].

FMD type A virus is differentiated into 3 topotypes: Euro-SA (Europe and South America), Asia and Africa. Within each of these three topotypes, a very large genetic diversity of the virus has been revealed.

The emergence of the topotypes within serotypes A and O common for Europe and South America is explained by the fact that FMD of these two types was introduced to the American continent from Europe in the 19th century.

Antigenically FMD Asia-1 viruses are less diverse than other serotypes. All known Asia-1 isolates form the only topotype – Asia. Knowles N. J. and Samuel A. R. explain the relatively low genetic diversity of the the serotype by its more recent origin than the others, or a severe 'bottleneck' purification with only a single topotype surviving [34].

FMDV serotype C has not been detected since 2004 and is currently considered to be extinct. FMDV type C, like serotypes A and O, is presumed to have been introduced into South America from Europe. However, the results of molecular biological studies allowed the British scientists from the Pirbright Institute to assume that the serotype C was introduced to South America and evolved from type A in around 1870 [40]. Later, this serotype was introduced to Europe from South America, and from Europe it spread to Africa and Asia. The scientists assume that exactly the occurrence of serotype C viruses in South America is the reason for their lower suitability to the ecological conditions of the other continents, which prevented them from persistence in the Old World.

SAT-1, SAT-2 and SAT-3 viruses are clustered into 13 (from I to XIII), 14 (from I to XIV) and 5 (from I to V) topotypes, respectively [37, 38, 39].

The discovery of the viral topotypes and their distribution pattern formed the basis for global FMD control strategy, which suggests clustering of seven regional alliances (seven regional FMDV pools) of FMD infected countries and coordination of national roadmaps for FMD progressive control pathway [41].

The FMDV is continuously evolving within each topotype, generating new genetic lineages that force out previously existing ones. Some lineages extinct quite quickly after their emergence, while others are able to circulate in their pools for a long time. The viral divergence within such "long-lived" genetic lineages leads to the emergence of sublineages. For example, by 2021, 17 sublineages were distinguished within the genetic lineage A/Iran-05, and 8 sublineages within O/PanAsia-2 [42].

It was found that FMDV genetic lineages evolve at very similar rates, estimated at an average of 1.3×10^{-2} nucleotide substitutions/site/year (min-max range of $1.1 \times 10^{-2} - 1.4 \times 10^{-2}$). These results indicate that FMDV evolves under a strict molecular clock that is largely constant among the different serotypes [42].

STUDY OF FMD PANZOOTIC SPREAD

Usually, FMDV of a certain topotype and genetic lineage evolves within a certain area (pool), periodically causing regional epizootics. However, over the past 30 years, two FMD panzootics have occurred, invloving several continents.

Molecular epidemiological studies made it possible to trace in detail the movements of the most devastating FMD panzootic on record caused by O/ME-SA/PanAsia virus. The virus was first isolated from FMD outbreaks

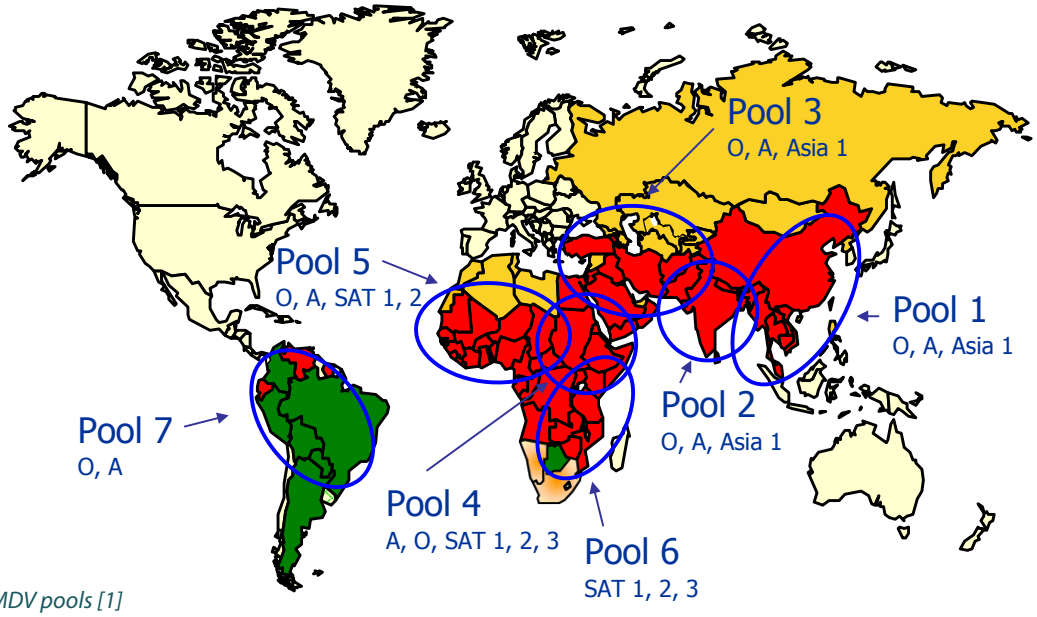

Fig. FMDV pools [1]

in northern India in 1990. From 1991 to 1997 the new lineage appeared to spread to other parts of India and in 1998 it spread to Bahrain, Iran, Jordan, Kuwait, Lebanon, Syria, Saudi Arabia, and Yemen. In 1999, the spread of the PanAsian virus in the Middle East continued (outbreaks in Israel, Turkey and the United Arab Emirates), and it started rapidly spreading eastward from Hindustan: in May 1999 China reported FMD outbreaks caused by this virus, and in June O/ME-SA/PanAsia virus was isolated in Taiwan. Towards the end of 1999, the PanAsia virus moved to Myanmar, Thailand, Vietnam, Laos and by April 2000 all mainland Southeast Asian countries had experienced outbreaks due to the new strain. In March 2000 FMDV type O appeared in South Korea and Japan, and in April in Mongolia and Primorsky Krai of the Russian Federation. It should be noted that these East Asian territories previously had been free from FMD for many decades [34, 43, 44].

FMD panzootic caused by O/ME-SA/PanAsia virus was not limited to Asia. In September 2000 FMD type O was first reported in the Republic of South Africa. The outbreak occurred near the port of Durban. The origin was traced to feeding pigs with uncooked swill from a ship arrived from Asia. In February 2001, PanAsia virus was introduced and caused devastation in the United Kingdom. To eradicate the outbreak 6.5 million animals were destroyed, and direct and indirect losses to the country amounted to about 8 billion pounds. From the UK FMD moved to Ireland, France and the Netherlands [45].

Another FMD epidemic, but less serious, started in 2013. It was caused by O/ME-SA/Ind-2001 genetic lineage and covered the entire Asian continent and North Africa. This virus was first isolated in India in 2001 [45]. By 2009 it had spread widely in the territory of Hindustan and diversified into five sublineages: a, b, c, d, and e. In 2009, the O/ME-SA/Ind-2001 virus moved beyond its original range for the first time and caused an outbreak in Iran. Since 2013, the virus became panzootic and caused extensive outbreaks in North Africa and the Middle East in 2013–2014 [46, 47, 48], in Southeast Asia in 2015 [49] and East Asia in 2016–2017 [50].

Using the nucleotide sequences of VP1 ($n = 424$) full genome ($n = 74$) of O/ME-SA/Ind-2001 isolates from different countries, K. Bachanek-Bankowska et al. [50] used phylodynamic and phylogeographic reconstructions to track the global spread of this virus. In particular, it was shown that FMD outbreaks in the Middle East and North Africa in 2013–2015 resulted from five independent introductions of O/Ind-2001d sublineage virus from Hindustan, and outbreaks in Saudi Arabia in 2016 were caused by two independent introductions of another sublineage – O/Ind-2001e.

In 2015, O/ME-SA/Ind-2001 virus started its spreading eastward from Hindustan: from Bangladesh to Vietnam, and then sublineage d was introduced to Laos, and sublineage e was introduced through Myanmar in 2016 to Thailand and Vietnam. After that O/Ind-2001e sublineage virus caused an FMD epizootic in East Asia, involving China, South Korea, Mongolia and the Zabaikalsky Krai of the Russian Federation.

In December 2021, O/ME-SA/Ind-2001 virus caused an outbreak in the Orenburg Oblast of the Russian Federation, and in January 2022 in the Republic of Kazakhstan [51].

This means that the panzootic caused by O/ME-SA/Ind-2001 virus had much in common with the previous one caused by O/ME-SA/PanAsia virus: both genetic lineages originated from the Indian subcontinent (Bangladesh, Bhutan, India and Nepal), in both cases the disease spread first to the west (to Central and Western Asia), and only then the virus was introduced into Southeast and East Asia. Both viruses demonstrated the ability to spread rapidly in regions where other type O genetic lines were enzootic, as well as to spread to normally FMD-free countries [50]. The emergence of FMD panzootics is probably a consequence of the economic globalization.

REGIONAL FMD EPIZOOTICS IN POOLS 1 AND 3

FMD panzootics, covering the entire Asia and even several continents, are a rather rare event. Most FMD epizootics never expand beyond their regional pools. Regional epizootics occur when antigenically modified FMDV variants appear that break through the existing population immunity [52].

The FMD situation in the Russian Federation is influenced by epizootic in Pools 1 (East and Southeast Asia) and 3 (West and Central Asia).

In Western and Central Asia (Pool 3), the largest regional epizootics were caused by A/Iran-05, O/PanAsia-2 and Asia1/Sindh-08 lineages.

The A/Iran-05 virus was first identified in Iran in 2003. In 2005 it spread widely in this country, and then caused an epizootic that covered Turkey, Afghanistan, Pakistan, Bahrain, Saudi Arabia, Jordan, and Iraq. Sporadic FMD outbreaks caused by this virus were reported in Israel, Lebanon, and Kuwait. A/Iran-05 has forced previously circulating A/Iran-96 and A/Iran-99 lineages out from the Middle East and is dominating in this region to the present. During this period, the virus diverged into many sublineages. Some sublineages (for example, Iran-05^{BAR-08}) became widespread, while others (for example, Iran-05^{ARD-07} and Iran-05^{EZM-07}) were reported only in one country (Turkey). Iran-05^{BAR-08} sublineage virus was introduced to Libya in 2009, and to Egypt in 2010 [53, 54, 55, 56, 57].

O/ME-SA/PanAsia-2 virus is derived from the O/ME-SA/PanAsia lineage, which became widespread in Western and Central Asia in the late 1990s. As an independent lineage, O/ME-SA/PanAsia-2 was first reported in Iran in 2006. In 2007, the virus of this lineage was introduced to Turkey, Afghanistan, Pakistan, Saudi Arabia, Israel, and then to other countries in the Middle East. In 2011, the virus from Turkey was introduced to Bulgaria. O/ME-SA/PanAsia-2 virus has been dominating in Pool 3 since 2007. During this time, it has diverged into several sublineages, among them sublineage PanAsia-2^{SIS-10} is the most widespread [55, 56, 57]. In 2010, the virus of this sublineage spread beyond Western Asia and caused FMD outbreaks in Libya and Bulgaria [56].

Another significant regional FMD epizootic in Western and Central Asia was caused by the Asia1/Sindh-08 virus. It was first identified in 2008 in Pakistan, from where it spread to Afghanistan, Iran, Iraq, Bahrain and Turkey. The Asia1/Sindh-08 virus has forced three other Asia-1 genetic lineages out from the pool and is dominating this region to the present [55, 56, 57].

In 2021, A. Di Nardo et al. [42] reconstructed the evolutionary history and spatial dynamics of FMD in Western and Central Asia (Pool 3) over the last 20 years. Having analyzed the history of A/Iran-05, O/PanAsia-2 and Asia1/Sindh-08 genetic lineages, they showed they highlighted the pivotal role played by virus circulation in Pakistan, Iran, and Afghanistan. These countries represent primary

conveyors of FMDV infection across the region and are important sites for generating genomic diversity in Western and Central Asia. Phylogeographic reconstructions further revealed three main patterns of transboundary movements of viruses in Pool 3: 1) the continuous virus interchange between Pakistan, Afghanistan, and Iran, likely constituting interconnected large metapopulations; 2) the key role of Iran as hub of virus diffusion to the west; and 3) the unidirectional migration of viruses from Iran toward Turkey. This spatial pattern of FMDV spread in the region is common to all its serotypes.

In the 20th century, the region of Western and Central Asia (Pool 3) had an enormous impact on the FMD situation in Russia, however, in the 21st century the main source of FMD for the Russian Federation was Pool 1, which includes East and Southeast Asia. This is explained by the drastic change in the FMD situation in this region. The FMD situation in most of the East Asia countries remained relatively favourable during the 20th century: Japan was free since 1908, Korea since 1934, the Far East of the Russian Federation since 1964, Mongolia since 1973 [43]. To a large extent, this favourable situation was achieved by the buffer role of China, which separated these countries from Southeast Asia, where FMD of types O and A is enzootic. In China itself, probably only the O/Cathay virus has historically been enzootic. This virus has a unique feature: it is capable of causing clinical disease only in pigs [58]. Probably, the absence in China of FMDV capable of infecting cattle ensured the disease freedom in Mongolia, Korea, Japan and the Russian Far East during the 20th century [59].

Globalization and the PRC economic development have changed the situation. In the late 1990s, FMD O/ME-SA/PanAsia virus was introduced and widespread in China, which probably moved from China to South Korea, Japan, Mongolia and the Primorsky Krai of the Russian Federation in 2000.

Numerous Asia-1 FMD outbreaks were reported first in China, and then in Russia (Amur Oblast, Primorsky and Khabarovsk Krais, Chita Oblast), Mongolia and North Korea in 2005–2006. The virus responsible for this epizootic had only 1.11–1.74% VP1 nucleotide difference from viruses from India collected in 1980–1981 and had no close relatives among the isolates circulating at that time [60, 61]. The most probable cause explaining the extremely close relationship of the isolates from 2005–2006 and 1980–1981 is the use of the Indian strain-based vaccine in China [61, 62].

In 2009–2010 East Asia was almost simultaneously covered by two epizootics caused by FMD A/Asia/Sea-97 and O/SEA/Mea-98 viruses. Both genetic lineages are enzootic in Southeast Asian countries. In 2009, the geographic range of A/Asia/Sea-97 expanded and caused outbreaks in six provinces of China, and in January 2010 in South Korea [63]. In 2013, the virus moved from the PRC territory to the RF Zabaikalsky Krai and Amur Oblast and Mongolia [64]. The O/SEA/Mea-98 virus was responsible for a larger epizootic in East Asia in 2010: FMD outbreaks caused by this strain were reported in China, Korea, Japan, Mongolia and the Zabaikalsky Krai of the Russian Federation [63, 65].

Since 2016, O/ME-SA/Ind-2001 virus has probably spread through China to Russia, Mongolia and South Korea [50].

This means, if 25 years ago only FMD O/Cathay virus was enzootic in China, currently four more genetic lineages are circulating in the country: O/ME-SA/PanAsia, O/SEA/Mea-98, O/ME-SA/Ind-2001 and A/Asia/Sea-97. This poses a high risk of FMD introduction from China to Russia.

MOLECULAR EPIDEMIOLOGICAL STUDIES OF FOOT-AND-MOUTH DISEASE IN THE RUSSIAN FEDERATION

In the Russian Federation, FMDV phylogenetic studies are conducted at the Federal Centre for Animal Health (Vladimir), which is the WOAH Regional Reference Laboratory for FMD for Eastern Europe, Transcaucasia and Central Asia and the FAO Reference Center for FMD. Since 2005 the Federal Centre for Animal Health has been involved into the network of WOAH/FAO reference laboratories for FMD responsible for the global FMD surveillance, with the main task to monitor the emergence of new FMDV variants with modified genetic and antigenic properties which render the available vaccines ineffective. The identification of FMDV genetic lineages and variants with modified antigenic properties makes it possible to promptly develop new vaccines against them. Within its international responsibilities the Federal Centre for Animal Health analyzes molecular genetic and antigenic properties of FMDV isolates responsible for the FMD outbreaks in the Russian Federation, Central Asia and Transcaucasia. FMDV isolates that caused outbreaks in Russia, CIS countries and in Mongolia were characterized by molecular genetic methods at the Federal Centre for Animal Health in the period from 1995 to 2022 [51, 59, 62, 64, 65, 66, 67, 68].

Studies showed that FMD outbreaks in post-Soviet Russia were caused by the introduction of the virus belonging to various serotypes, topotypes and genetic lineages from infected Asian countries (Table 1). For example, FMD O/Cathay, O/ME-SA/PanAsia, O/SEA/Mya-98, O/ME-SA/Ind-2001, Asia1, A/Asia/Sea-97 viruses were introduced from China to various regions of the Russian Federation.

Table 1
Characteristics of the virus responsible for FMD outbreaks in the Russian Federation in 1995–2021

Year	Region	Virus (type/topotype/genetic lineage)
1995	Moscow Oblast	O/Cathay
2000	Primorsky Krai	O/ME-SA/PanAsia
2004	Amur Oblast	O/ME-SA/PanAsia
2005–2006	Amur Oblast, Primorsky Krai, Khabarovsk Krai, Chita Oblast	Asia1/V
2010–2011	Zabaikalsky Krai	O/SEA/Mya-98
2012	Primorsky Krai	O/ME-SA/PanAsia
2013	Kabardino-Balkaria, Karachay-Cherkessia, Krasnodar Krai; Zabaikalsky Krai, Amur Oblast	A/Asia/Iran-05 A/Asia/Sea-97
2014	Primorsky Krai Zabaikalsky Krai	O/SEA/Mya-98 O/ME-SA/PanAsia, A/Asia/Sea-97
2016	Zabaikalsky Krai	O/ME-SA/Ind-2001
2017	Republic of Bashkortostan	O/ME-SA/unnamed
2018	Zabaikalsky Krai	O/ME-SA/PanAsia
2019	Primorsky Krai, Khabarovsk Krai; Zabaikalsky Krai	O/SEA/Mya-98 O/ME-SA/Ind-2001
2020	Zabaikalsky Krai	O/SEA/Mya-98
2021	Orenburg Oblast	O/ME-SA/Ind-2001

Table 2
Characteristics of the virus responsible for FMD outbreaks in the CIS countries and Mongolia in 1996–2022

Year	Country	Virus (type/topotype/ genetic lineage)
1996	Armenia	O/ME-SA/Iran-01
1997	Georgia	O/ME-SA/Iran-01
1998	Armenia	A/Asia/Iran-96
1999	Georgia	A/Asia/Iran-96
2000	Armenia, Georgia	Asia-1, O/ME-SA/PanAsia
2000–2002	Mongolia	O/ME-SA/PanAsia
2001	Georgia	Asia1/VI
2001	Kyrgyzstan, Tajikistan	O/ME-SA/PanAsia
2003	Tajikistan, Uzbekistan	Asia1/II
2004	Kyrgyzstan, Tajikistan	Asia1/II
2004	Mongolia	O/SEA/Mya-98
2005	Mongolia	Asia1/V
2006	Armenia	A/Asia/Iran-05
2007	Kyrgyzstan	A/Asia/Iran-05
2007	Kazakhstan, Kyrgyzstan	O/ME-SA/PanAsia-2
2008	Tadjikistan	O/ME-SA/PanAsia-2
2010	Tadjikistan	A/Asia/Iran-05
2010	Kazakhstan	O/ME-SA/PanAsia-2
2010	Mongolia	O/SEA/Mya-98
2011	Kazakhstan, Kyrgyzstan, Tajikistan	O/ME-SA/PanAsia-2
2011	Kyrgyzstan	A/Asia/Iran-05
2011	Tadjikistan	Asia1/Sindh-08
2011	Kazakhstan	O/ME-SA/PanAsia
2012	Kazakhstan	O/ME-SA/PanAsia
2013	Kazakhstan, Mongolia	A/Asia/Sea-97
2014	Tadjikistan	O/ME-SA/PanAsia-2
2014	Mongolia	O/ME-SA/PanAsia
2015	Tadjikistan	O/ME-SA/PanAsia-2
2015	Mongolia	O/SEA/Mya-98, O/PanAsia
2016	Armenia	A/Asia/G-VII
2016	Tadjikistan	O/ME-SA
2016	Mongolia	A/Asia/Sea-97
2017–2018	Mongolia	O/PanAsia, O/Ind-2001
2021	Mongolia	O/ME-SA/Ind-2001
2022	Kazakhstan	O/ME-SA/Ind-2001

FMD outbreaks in the North Caucasus in 2013 were caused by the introduction of A/Asia/Iran-05 virus from Transcaucasia, and the introduction of O/ME-SA virus of a rare non-classified group from Central Asia caused outbreaks in the Republic of Bashkortostan in 2017. In 2021 O/ME-SA/Ind-2001 virus was probably introduced to the territory of the Russian Federation from Kazakhstan. Thus, the results of the studies conducted by the Federal Centre for Animal Health suggest a large genetic diversity of FMDV introduced to the Russian Federation.

Phylogenetic analysis of the virus isolates responsible for FMD outbreaks in Transcaucasia (Armenia and Georgia) and Central Asia (Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan) revealed that they belong to genetic lineages that dominated Pool 3 at different periods: O/ME-SA/Iran-01, A/Asia/Iran-96, O/ME-SA/PanAsia, Asia1/VI, Asia1/II, A/Asia/Iran-05, O/ME-SA/PanAsia-2, Asia1/Sindh-08, A/Asia/G-VII (Table 2). In 2011–2013, FMD outbreaks caused by O/ME-SA/PanAsia and A/Asia/Sea-97 viruses, introduced from China were reported in eastern regions of Kazakhstan. In 2022, O/ME-SA/Ind-2001 virus was introduced from China to Kazakhstan.

FMD outbreaks in Mongolia were caused by the virus responsible FMD epizootics in Pool 1: O/ME-SA/PanAsia, O/SEA/Mya-98, A/Asia/Sea-97, O/ME-SA/Ind-2001.

Within the network of the WOAH/FAO reference laboratories for FMD, the Federal Centre for Animal Health shares information on FMDV field isolates responsible for oubreaks in various regions of the world. The VP1 nucleotide sequences and full genomes of FMDV isolates responsible for the outbreaks in Russia, CIS countries and Mongolia produced by the Federal Centre for Animal Health were used to track the spread of global and regional epizootics caused by O/ME-SA/PanAsia, Asia1/II, Asia1/V, O/SEA/Mya-98, A/Asia/Sea-97, O/ME-SA/Ind-2001 viruses [42, 43, 47, 50, 60, 61].

The results of molecular epidemiological studies performed by the Federal Centre for Animal Health are taken into account for the strain selection to manufacture vaccines and vaccinate livestock in FMD risk areas.

CONCLUSION

Thirty-five years of experience in molecular epidemiology of foot-and-mouth disease proves that phylogenetic analysis of the viral genome nucleotide sequences is an effective tool to monitor FMD both on the regional and global levels. FMD surveillance, which makes it possible to track the emergence of new viral variants with modified genetic and antigenic properties, allows for the rapid development of new vaccines and thus for a significant improvement of FMD control performance.

The use of molecular biological methods has made it possible to progress significantly in understanding of FMD epidemiology, which is important for the development of effective control strategies. The discovery of FMDV topotypes and their distribution pattern formed the basis for global FMD control strategy, which suggests clustering of seven regional alliances (seven regional FMD pools) of infected countries and coordination of national roadmaps for FMD progressive control pathway.

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Influenza D virus in cattle (review)

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ABSTRACT

The influenza D virus was first detected and identified in 2011. The overall amino acid sequence of influenza D virus shares approximately 50% identity with that of influenza C virus, suggesting that both viruses had a common ancestor. Cattle is considered to be the primary natural reservoir for influenza D virus. The involvement of this virus into the bovine respiratory disease complex has been confirmed. The virus causes mild to moderate disease in calves and replicates in both the upper and lower respiratory tracts, promoting bronchopneumonia. The influenza D virus can be transmitted by contact or aerosol over short distances, has a high transmission rate and can potentiate the effects of other respiratory pathogens. There are currently no vaccines or specific treatment for influenza D virus. This virus can replicate and be transmitted by direct contact in ferrets and guinea pigs, which are surrogate models of human influenza infection, as well as in well-differentiated human airway epithelial cells (hAECs). Currently five distinctive lineages of influenza D virus have been identified, co-circulating in worldwide bovine and pig populations that may facilitate genetic reassortment between different viral strains. The virus has a zoonotic potential, and if its pathogenicity for humans changes, its importance for public health will be great. Very high seropositivity rates among persons working with cattle in the USA and Italy have been reported. There is no data in the available literature on the circulation of the influenza D virus in the Russian Federation. Research is needed to study this new virus, as well as monitoring of the virus spread and circulation in our country to understand its role in bovine respiratory disease complex and its zoonotic potential.

Keywords: review, influenza D virus, cattle, respiratory disease complex, genetic lineages, zoonotic potential

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Вирус гриппа D у крупного рогатого скота (обзор)

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РЕЗЮМЕ

Вирус гриппа D впервые был обнаружен и идентифицирован в 2011 г. Его аминокислотная последовательность примерно на 50% идентична аминокислотной последовательности вируса гриппа C, что предполагает наличие общего предка у обоих патогенов. Основной резервуар вируса гриппа D – крупный рогатый скот. Установлено участие данного возбудителя в комплексе респираторных болезней крупного рогатого скота. Вирус вызывает у телят заболевание легкой и умеренной степени тяжести и реплицируется как в верхних, так и в нижних отделах дыхательных путей, способствуя возникновению бронхопневмонии. Возбудитель гриппа D передается контактным и воздушно-капельным путем на короткие расстояния, имеет высокую частоту передачи и может усиливать действие других патогенов. На сегодняшний день вакцин или специфического лечения не существует. Агент способен размножаться и передаваться при прямом контакте в организме хорьков и морских свинок, являющихся суррогатными моделями для изучения человеческого гриппа, а также в культурах высокодифференцированных эпителиальных клеток дыхательных путей человека hAEC. В настоящее время определены пять генетических групп вируса гриппа D, циркулирующих в популяциях крупного рогатого скота и свиней во всем мире, что может способствовать генетической рекомбинации между различными штаммами. Возбудитель обладает зоонозным потенциалом и, если произойдет резкое изменение его патогенности для человека, может явиться серьезной проблемой для общественного здравоохранения. Сообщалось о высоком уровне серопозитивности к вирусу среди персонала животноводческих ферм в США и Италии. В доступной литературе нет данных о циркуляции возбудителя гриппа D на территории Российской Федерации. Необходимы исследования, направленные на изучение этого нового вируса, а также

проведение мониторинга распространения и циркуляции патогена в нашей стране для понимания его роли в комплексе респираторных заболеваний крупного рогатого скота и зоонозного потенциала.

Ключевые слова: обзор, вирус гриппа D, крупный рогатый скот, комплекс респираторных заболеваний, генетические линии, зоонозный потенциал

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INTRODUCTION

Bovine respiratory disease (BRD) complex is one of the most costly multifactorial diseases, affecting predominantly young cattle in the whole world. The pathogens most often responsible for respiratory conditions are infectious rhinotracheitis virus and viral diarrhea virus (mucosal diseases), respiratory syncytial infection, parainfluenza-3, bovine coronavirus infection and *Pasteurella multocida* (*P. multocida*) *Mannheimia haemolytica* (*M. haemolytica*), *Mycoplasma bovis* (*M. bovis*), *Histophilus somni* (*H. somni*) bacteria [1, 2, 3, 4].

Bovines were not considered susceptible to influenza viruses until the discovery of influenza D virus (IDV). This new species has been identified as a new etiological agent involved in BRD [3]. The interest in research on IDV is increasing and this highlights the importance and global impact that this new virus may have, which mainly affects cattle, although there is a wide range of other species that can act as hosts.

Influenza viruses are RNA viruses belonging to the family *Orthomyxoviridae*, which encompasses four monotypic genera, classified on the basis of antigenic differences between their nucleoprotein (NP) and matrix (M) proteins: *Alphainfluenzavirus*, *Betainfluenzavirus*, *Gammainfluenzavirus* and *Deltainfluenzavirus*, each having one species – Influenza A virus (IAV), Influenza B virus (IBV), Influenza C virus (ICV) and Influenza D virus (IDV). Influenza A, B and C viruses are known to cause respiratory diseases in humans [5].

Unlike other influenza virus types that affect a wide range of mammals and birds and cause epidemics and pandemics, cattle serve as the reservoir of IDV, but its circulation among other mammalian species can not be excluded [3, 6, 7, 8, 9].

Influenza D virus was initially isolated from a diseased pig with the severe respiratory symptoms in Oklahoma (USA) in 2011. It shared approximately 50% homology with the human ICV and was initially thought to be its new subtype [10]. Subsequently, genetic, antigenic and biological differences were identified [11]. In August 2016, the International Committee on Taxonomy of Viruses (ICTV) officially classified IDV as a new species that belongs to the genus *Deltainfluenzavirus* of the *Orthomyxoviridae* family. Cattle are thought to be the primary natural reservoir for IDV according to further studies [11, 12]. Specific antibodies were also found in horses, small ruminants, wild pigs, buffaloes, camels and humans, especially in those who had been in contact with cattle [10, 13, 14, 15, 16], this does not exclude a wide range of hosts and transmission from cattle to humans or other animal species.

This review provides information on recent advances in IDV study, the prevalence of the virus and its role in the bovine respiratory disease complex.

INFLUENZA D VIRUS CHARACTERISTICS

Influenza D virus presents an envelope and a segmented genome, composed of seven negative-sense, single-stranded RNA segments. The virus lacks neuraminidases, as does ICV. Virions are 80–120 nanometers in diameter [17]. The segmented IDV genome encodes nine proteins [18]. The three longest segments produce the proteins PB2, PB1 and P3, needed for replication and viral mRNA synthesis. The fourth segment encodes the hemagglutinin-esterase-fusion (HEF), which aids virus entry into host cells, as well as is the main target of neutralizing antibodies. The broader cell tropism of IDV compared to ICV is explained by receptor-binding pocket in the HEF protein of IDV, which allows it to bind to various cell surface molecules [19, 20]. The fifth segment produces the nucleoprotein NP, which constitutes the viral ribonucleoprotein complex [19]. The sixth segment encodes matrix proteins M1 and M2 covering the viral membrane on it's the inside and exhibiting ion channel activity [21]. The seventh segment produces non-structural proteins NS1 and NS2, which are involved in neutralization of the cellular interferon response and mediate the nuclear export of ribonucleoprotein [22].

Influenza viruses have exploited a variety of strategies to increase their genome coding capacities. Splicing has been demonstrated in the NS and/or M segments of influenza viruses. All types of influenza viruses have a similar mechanism for generating NS1 and NS2 proteins. However, each viral type exploits a unique strategy to generate M1 and/or M2 proteins. Herewith IDV also exhibits a new mechanism for generating the M1 protein when compared to ICV. It uses a proteolytic cleavage strategy similar to the ICV strategy to produce M2 protein from P42

protein, while unlike the ICV M segment, which generates M1 protein through splicing, that solely introduces a termination codon, the splicing of the IDV M segment adds an additional 4-amino-acid peptide into the preceding exon [11].

Studies by J. Yu et al. showed that IDV has exceptional stability at high temperatures and acidity due to the role of HEF glycoproteins and it is considered the most stable of the four influenza viruses [17]. Influenza D virus could resist and retain its infectivity even after exposure to a temperature of 53 °C for 120 min. Furthermore, IDV only lost 20% of the original infectivity when subjected to a low pH of 3.0 for 30 min, compared with the rest of influenza viruses that were completely inactive at low pH. The stability of HEF at extremely low pH highlights a new aspect of IDV replication that requires further study [23].

PATHOGENESIS

Influenza D virus has tropism for epithelial cells upper and lower respiratory tracts and can cause mild to moderate interstitial/bronchointerstitial pneumonia. The virus was also detected in the nasal mucosa, trachea, bronchioles and lung tissues 8 days after infection of calves. It was also detected in the tracheobronchial and mediastinal lymph nodes [3]. The highest viral load of IDV was observed in the nasal cavity. High IDV RNA loads were also found in the olfactory bulb and tonsils in sentinel animals that were infected via aerosol, but IDV tropism for these tissues could not be confirmed by immunohistochemistry test or virus isolation [24].

Salem E. et al. [3], Ferguson L. et al. [18] showed that IDV causes mild respiratory disease in calves experimentally infected by direct contact. Influenza D virus infection can alter the structural integrity of the respiratory epithelium and as a result trigger a significant increase in neutrophils in the trachea of infected animals [18]. This pathological effect seems to suggest an etiological role of IDV in bovine respiratory disease complex. Further investigation of IDV pathogenesis and host responses in calves showed that IDV infection resulted in moderate bronchopneumonia with restricted lesions of interstitial pneumonia and significant activations of pathogen recognition receptors and chemokines CCL2, CCL3, and CCL4 [3]. The signaling pathway to activate the type I interferon response was not substantially activated in the lower respiratory tract of IDV-infected calves [25].

The IDV genome was detected in serum samples from seriously sick cattle, which implies that the virus could cause temporary viremia and spread to other organs; IDV was detected by RT-PCR in feces on day five post infection and in the jejunum on day six post infection, which corresponds to the time of greatest viral RNA replication in the respiratory tract. Yu J. et al. suggested that IDV could replicate within the intestinal tract in a similar way to IAV and IBV. This possible enteric tropism of IDV could be due to the high acid stability of this virus [17]. In addition, the high thermal and acid stability of the virus means that IDV has a high resistance potential abroad, which could explain its high transmission efficiency [3].

A number of researchers have demonstrated that infection in guinea pigs or ferrets is asymptomatic [10, 26]. In guinea pigs, the virus has been detected in both the upper and lower respiratory tracts. However, lungs from infected guinea pigs showed severe and extensive inflammatory changes in the alveolar space with inflammatory cell infiltration, perivascular cuffing, and destruction of bronchiolar epithelium with exudation [26]. Animals also demonstrated apoptosis in epithelial cells of the lungs. In ferrets, IDV replicated in nasal turbinates and was not detected in the lungs [10]. Clinical signs were not observed in infected mice. Influenza D virus replication in mice was observed mainly in the upper respiratory tract, less often in the lower respiratory tract. Low titers of IDV were found in mice intestines [27]. Infected mice showed a significant increase of neutrophils and lymphocytes in the lung [28]. Influenza D virus replication in mice led to activate proinflammatory genes including gamma interferon (IFN-*γ*) and chemokine CCL2 [27].

Taken together, cattle infection experiments collectively suggest that IDV is a mild to moderate respiratory disease pathogen of bovines and acts as an important cofactor of clinical bovine respiratory disease complex.

INFLUENZA D VIRUS SPREAD IN THE WORLD

Influenza D virus is widespread among cattle in North and South America [14, 18, 29, 30, 31, 32], Europe [33, 34, 35, 36, 37], Asia [38, 39, 40] and Africa [41, 42].

Serological studies showed that IDV had been already prevalent in cattle in the USA (Mississippi and Nebraska) since 2003 [12, 29]. A serosurveillance across the USA in 2014 and 2015 showed a high overall seropositive rate of 77.5% nationally; regional rates varied from 47.7% to 84.6%. Seropositive samples were found in 41 of the 42 states [30]. A metagenomic virome study conducted in nasal swab samples from the feedlot cattle collected in the USA, Canada and Mexico found a significant association of IDV with other respiratory diseases [43, 44]. Antibodies to IDV were also detected in sheep and goats in North America, in 5.2 and 8.8% of blood serum samples, respectively [13]. Horses also turned out to be IDV carriers – 15.7% (n = 364) of serum samples were positive for specific IDV antibodies [15]. A recent study evaluated the seroprevalence of IDV in feral swine and found that 57 of the 256 (19.1%) tested sera were positive for IDV and may play a certain role in IDV ecology [14].

The presence of IDV in South America has also been demonstrated by serological studies. In a study from Argentina, 85 (73%) of the 116 farms analyzed had at least one positive animal. Of the 165 serum samples from bulls over three years of age that had been collected in 2013, originally to estimate the seroprevalence of reproductive diseases by HI assay, 112 (68%) were seropositive to IDV [31]. Molecular detection of IDV from a case of bovine respiratory disease has also been reported in Brazil [32].

Influenza D virus is widespread in European countries, including France, Italy, Luxembourg, Ireland and the United Kingdom.

Influenza D virus genome was detected by PCR in 6 (4.5%) samples of lung tissue and nasal swab taken from healthy and clinically sick calves in France in 2015. Coinfections with *P. multocida*, *M. haemolytica*, *H. somni*, bovine respiratory syncytial virus and/or bovine herpesvirus 1 were detected in four out of the six IDV positive samples. In the other two samples, no coinfections with the respiratory pathogens analyzed were detected [33]. Additionally, in France, a serological study was performed on bovine sera (n = 3,326) collected from 2014 to 2018,

in five regions. The resulting total seroprevalence was 47.2%, but the results varied depending on the geographical region (31.0–70.0%) [34].

Samples of nasal swabs and lung tissues were tested between the years 2014 and 2016 in Italy. Of the samples taken from cattle with BRD, there was a prevalence of 8.0%, and of the samples taken from cattle without BRD, there was a prevalence of 3.4% Of the 48 IDV positive samples that came from cattle with BRD, in 62.5% of the cases, IDV was the only viral agent detected, which further supports the hypothesis that IDV may play a primary role in the occurrence of BRD. In 37.5% of the remaining samples, IDV was found together with other respiratory viruses, especially bovine coronavirus. Influenza D virus RNA was detected more often in nasal swabs (9.4%) than in lung tissues (3.4%), which reinforces the finding from experimental infection studies that the upper respiratory tract is probably the preferred site of replication for this virus. Serological studies performed in Italy in 2015, demonstrated high prevalence of IDV (92.4%) in dairy farms [2]. In Luxembourg, IDV seroprevalence among animals was 80.2% in 2012–2016 [35]. In Ireland, during 2014–2016, 320 bovine nasal swab samples from 84 farms were tested by RT-PCR. It was determined that 18 calves (5.6%) were positive for IDV from 10 different farms (11.9%) [36].

In the UK, IDV was found in 8.7% of samples from calves exhibiting signs of respiratory infections. In all cases, the causative agent of influenza was detected in combination with bacterial agents and in some cases as part of viral-bacterial associations. Viral RNA was present in both the upper and lower respiratory tract and pathological changes in lung tissues were observed alongside signs of concurrent bacterial infections. Sequencing of one UK isolate revealed that it is similar to viruses from the Ireland and Italy [37].

Influenza D virus was reported for the first time in 2014 in China, where viral genome was detected in 0.7% of samples, collected from clinically health cattle [38]. It is assumed that IDV has been circulating in Asian cattle since 2011. It was found that IDV was widespread among cattle, buffaloes, pigs, sheep and goats in southern China in 2016–2017 [39].

In Japan IDV was detected in cattle in 2016 and was highly contagious [25]. A recent study on the seroprevalence of IDV in sera collected between 2009 and 2018 showed 57% in average. It was proved that the virus of Japanese lineage has been circulating since 2010 [40]. IDV infection of cattle was first reported in Turkey in 2020 [45].

Bailey E. S. et al. [46] reported that IDV was identified in poultry farm bioaerosols in Southeast Asia. Partial genome sequencing of M segment showed that the IDV found in poultry farms was different from virus strains circulating among cattle in North America. In this study, it was not possible to carry out a complete sequencing of the genome, particularly HEF sequence, and identify the lineage of the virus. Further studies on the susceptibility of poultry to infection are needed to investigate IDV infection in this species.

In Africa, IDV has been known to circulate in cattle since 2012. Antibodies to the virus have been detected in cattle, small ruminants and dromedary camels in Morocco, Togo, Cote d'Ivoire, Benin and Kenya [41]. A high level of seropositivity (99.0%) was found in dromedary camels in Kenya, indicating a potential new host for IDV [41]. These results were confirmed by another serological study performed in Ethiopia, where a high seropositivity of dromedary camels to IDV was also observed [42]. Infection among young cattle in African countries is less common, apparently, due to a less intensive animal farming.

The ability of IDV to cause disease in humans has not yet been thoroughly investigated, and it is not clear whether this virus can sustain human-to-human transmission. Viral replication and transmission by direct contact in ferrets and guinea pigs, used for human influenza infection modelling can suggest it [10, 26]. Holwerda M. et al. it has been proved that IDV replicates efficiently in an *in vitro* surrogate model of respiratory epithelium at ambient temperatures that correspond to the human upper and lower respiratory tract. The authors also demonstrated that IDV can be efficiently propagated onto well-differentiated hAEC cultures at both 33 °C and 37 °C [47].

A serological retrospective study conducted in the USA demonstrated the existence of specific antibodies against IDV present in people, finding a prevalence of 1.3% of the human population during 2007–2009 [10]. This means IDV may pose a potential threat as an emerging pathogen to personnel who are in contact with cattle [10]. A very high seroprevalence was observed in the USA (91%) and Italy (46%) in farm personnel. The prevalence of antibodies against IDV in humans implies that the virus can infect humans and pose a potential threat to human health.

The IDV genome was detected by RT-PCR in nasal wash samples of a pig farm worker in Malaysia [49]. In another study conducted in the United States, IDV genome was detected in bioaerosol samples in a hospital emergency room in North Carolina [50] and Raleigh-Durham International Airport [51]. These results suggest that IDV has zoonotic potential. Humans generally have no preexisting immunity against this newly emerging influenza virus.

INFLUENZA D VIRUS GENETIC DIVERSITY

The complete genomes of more than fifty cattle and five pig IDV strains have been sequenced in six countries: USA, France, Italy, Ireland, Japan, and China. According to recent tests IDVs can be classified into five distinct genetic lineages based on HEF [52]:

1) D/OK – detected in Europe (France, Italy and Ireland), America (USA and Mexico) and Asia (China);

2) D/660 – detected in Europe (Italy) and America (USA and Mexico);

3) D/Yama2016 – detected in Asia (Japan);

4) D/Yama2019 – detected in Asia (Japan and China);

5) D/CA2019 – detected in America (USA).

D/OK and D/660 viruses currently circulating in bovine populations in the USA and Europe frequently showed rearrangement events with each other and antigen-antibody cross-reactivity between them which could result in occurring of new antigenic variants that can overcome previously existing herd immunity and pose a threat to livestock health [17]. Chinese D/OK IDV strains differ from strains of the same genetic lineage from the USA and Italy, and are divided into sublineages in each country. D/OK lineage strains isolated from pigs and cattle in the USA and Italy are grouped into one cluster, which suggests a wider distribution of this genetic lineage in the world and virus transmission between these animal species. According to molecular-genetic study the members of IDV D/OK lineage isolated in dairy cows, pigs and goats from Guangdong, PR China showed very low genetic diversity.

In the D/660 lineage, the strains from France and the USA diverged from each other earlier than strains within each country, suggesting that this lineage also evolved into sub-lineages in different countries. Interesting that D/Yama2016, and D/Yama2019 lineages are only present in Japan, and diverge substantially from D/OK and D/660 lineages circulating in other countries. However, in 2022 IDV, identical the D/Yama2019 genetic lineage, was reported from China. During the recent outbreak of bovine respiratory disease in South America, the first case of IDV detection was reported and it was established that the virus circulating in Brazilian cattle herds is divergent from previously described IDV lineages from North America, Europe and Asia according to phylogenetic analysis results [32]. In addition, a new genetic lineage was reported from Turkey (D/Bursa2013) [53] and a new reassorted virus in Namibia [54]. These results highlight the need to monitor the IDV prevalence for better understanding of the viral epidemiology and evolution.

CONCLUSION

The analysis of the publications demonstrates the spread of IDV among animals worldwide. Cattle is considered the main reservoir of the virus and plays a significant role in its spread. Influenza D virus is an important cofactor of clinically BRD complex as it can cause alone a mild to moderate respiratory disease with a high transmission rate, and can potentiate the effects of other pathogens. The increasing number of IDV infection outbreaks in pigs and cattle in recent years may be associated not only with the growing attention to this new pathogen, but also with an increased virulence of the virus. Influenza D virus has the potential for spillover and adaptation to humans, and if there is a drastic change in its pathogenicity to humans, it could be a major public health concern.

The IDV peculiarity is its relative stability in comparison with other influenza viruses, suggesting its evolution is slow. There are currently no vaccines or specific treatment for influenza D virus.

In Russia, studies on IDV spread and its role in the BRD complex have not been conducted. International trade in livestock entails the risks of the virus introduction into our country, which can facilitate the spread of a new infection among animals and pose a potential threat to human health, if no control measures are taken. Therefore, the study of IDV circulation in different regions of the Russian Federation is important to prevent the infection in animals and humans.

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Etiology and epizootology of bovine mastitis (analytical review)

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ABSTRACT

Mastitis is one of the most common global diseases of dairy cattle, it is detected in 5–36% animals in a herd undergoing a single examination, and the incidence reaches 68% within a year, given that some cows get reinfected more than once. The disease causes significant economic losses to dairy industry both in the Russian Federation and globally. Mastitis is an inflammation of the mammary gland that develops in response to the effects of various factors of the external and internal environment, which are classified as mechanical, physical, biological, etc. Based on the clinical symptoms, the disease has two forms: clinical and subclinical. Both the animal and its milk shall be subjected to a thorough examination for the diagnosis establishment. The final stage of the mastitis diagnosis in cows is laboratory testing. In this case, the most informative is the bacteriological method, which helps to isolate a pathogen's pure culture, identify it and determine sensitivity to antimicrobial drugs. The latter plays a specific role in indicating the direction of further therapeutic measures, since the obtained data facilitate selection of effective antibiotics against certain pathogens. Incorrect treatment, incompliances with the prescribed therapy, as well as unnecessary use of antimicrobials can lead to the generation of multi-resistant bacteria. Due to the widespread spread of antibiotic-resistant microorganism strains, despite the large number of drugs currently used, their effectiveness is constantly decreasing. The prospects for the further use of antibiotics as therapeutics are questioned by many researchers and international organizations due to antibiotic resistance rapidly developing in many agents. Vaccination plays a significant role in infectious disease control. The use of vaccines not only reduces mastitis occurrence in cows, but also significantly improves the quality of dairy products.

Keywords: review, bovine mastitis, etiology, epizootology, treatment, prevention, vaccination

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Этиология и эпизоотология мастита коров (аналитический обзор)

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РЕЗЮМЕ

Мастит является одним из самых распространенных заболеваний молочного скота во всем мире, при разовом обследовании стада выявляется у 5–36% животных, а в течение года заболеваемость достигает 68%, при условии что некоторые коровы переболевают два раза и более. В Российской Федерации, как и во всем мире, болезнь наносит значительный экономический ущерб молочному животноводству. Мастит – это воспаление молочной железы, которое развивается в ответ на воздействие различных факторов внешней и внутренней среды, которые классифицируют как механические, физические, биологические и другие. На основании клинических симптомов заболевание можно разделить на две формы: клиническую и субклиническую. Для установления диагноза необходимо проведение всестороннего обследования как животного, так и молока. Завершающим этапом при диагностике мастита коров является выполнение лабораторных исследований, в данном случае преимущество по информативности отдается бактериологическому методу, с помощью которого удается выделить чистую культуру возбудителя, провести его идентификацию и определить чувствительность к антимикробным препаратам. Последнее играет особую роль для определения вектора дальнейших лечебных мероприятий, поскольку полученная

информация позволяет подобрать эффективный антибиотик против конкретного возбудителя. Неправильный выбор, несоблюдение предписанного курса лечения, а также нецелесообразное применение противомикробных средств может привести к генерации мультирезистентных бактерий. Вследствие широкого распространения антибиотикорезистентных штаммов микроорганизмов, несмотря на большое количество используемых в настоящее время препаратов, их эффективность постоянно снижается. Перспективы дальнейшего использования антибиотиков в качестве лекарственных средств многие исследователи и международные организации ставят под сомнение из-за быстро развивающейся к ним резистентности у многих возбудителей. Вакцинопрофилактика занимает значительное место в борьбе с инфекционными болезнями. Применение вакцин позволяет не только снизить количество маститов у коров, но и значительно улучшает качество получаемой молочной продукции.

Ключевые слова: обзор, мастит коров, этиология, эпизоотология, лечение, профилактика, вакцинация

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Конфликт интересов: Прунтова О. В. является членом редколлегии журнала «Ветеринария сегодня» с 2012 г., но не имеет никакого отношения к решению опубликовать эту статью. Рукопись прошла принятую в журнале процедуру рецензирования. Об иных конфликтах интересов авторы не заявляли.

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INTRODUCTION

Mastitis is one of the most common global diseases of dairy cattle, it is detected in 5–36% of the herd population undergoing a single examination. Within a year the incidence reaches 68%, provided that some cows get diseased twice or more [1].

Mastitis is an inflammation of the mammary gland that develops in response to the effects of various factors of the external and internal environment when animal's body resistance decreases and infection becomes complicated. The disease is caused by various microorganism species that penetrate the mammary gland and, as a result of intensive reproduction, induce the infection. The main factors of mastitis are classified into mechanical, physical and biological. Concomitant factors are: animal's resistance and immune status, failures in pre-milking and post-milking udder hygiene procedure, incompliant disinfection of milking devices, etc. Based on the clinical symptoms, mastitis can be of two types: clinical and subclinical. Clinical mastitis is often diagnosed directly by visual assessment of udder inflammation or changes in the organoleptic properties of milk, whereas subclinical mastitis is a latent form of the disease characterized by absence of clinical signs and 3–4-fold rise in milk somatic cells count [2, 3, 4].

Mastitis causes significant economic losses consisting in reduced milk yields and deteriorated milk quality (66%), rejection of products with decreased nutritional and technological properties (6%), premature culling of high-yielding cows due to dysfunction of udder quarters (22%), burden of treatment costs (5%) and increased costs on veterinary staff labour (1%). Drinking colostrum from mastitis-infected cows to calves results in indirect, but significant losses, it generally leads to mass gastrointestinal diseases and is one of the causes for mortality in the early postnatal period. Mastitis is one of the most economically significant cow diseases worldwide. Thus, for example, the damage caused by this disease to the dairy industry in Russia annually amounts to about RUB 1 billion, in England losses reach up to GBP 50 million, and in the United States the annual estimated losses range from USD 3 to 403 per cow [1, 5]. In view of the above, the issues of cow mastitis etiology and epizootology are relevant for veterinary medicine and the developing dairy industry.

The novelty of this study consists in the systematization and detailed review of current data on the factors impacting mastitis occurrence, spread, treatment and prevention methods.

The aim of the review was to analyze and systematize data from current research publications on the etiology and basic patterns of development, spread, treatment and prevention of cow mastitis.

Mastitis is a multi-factorial disease that develops under the influence of mechanical, physical, biological and other factors.

Mechanical factors belong to the most numerous group of negative effects causing injuries to the udder and nipples (microtrauma, bruises, cracks and wounds). The injuries are often the result of incompliances with the milking rules and technology, the most common causes are: excessive vacuum or its fluctuations, high pulsation frequency, use of non-standard teat rubber, unsatisfactory pre-milking udder hygiene, as well as poor-quality preparation of milking equipment.

Physical factors predisposing to the occurrence of mammary gland pathology include non-compliance with the temperature conditions of reared animals. Inflammation can develop under exposure to high and low temperatures, resulting in tissue burns or frostbites which can subsequently lead to an inflammatory process [6, 7].

The biological factors of the disease include different microorganism species. The development of an inflammatory process in the udder often begins with the entry of the pathogen into the animal's body from the environment through the nipple canal. Almost any opportunistic microorganism can cause both clinical and subclinical mastitis when penetrating the mammary gland [8, 9]. The etiopathogenetic study shows that cow mastitis is a polyethiological disease, the most common pathogens are: *Streptococcus* spp. (*S. agalactiae*, *S. dysgalactiae*, *S. uberis*, etc.), *Staphylococcus* spp. (*S. aureus*, *S. hyicus*, *S. xylosus*, *S. epidermidis*, etc.), *Escherichia coli*, *Candida* spp. (*C. krusei*, *C. glabrata*, C. *rugosa*), *Mycoplasma* spp. (*M. bovis*, *M. dispar*, etc.), *Trueperella pyogenes* [10, 11, 12, 13, 14, 15, 16, 17, 18]. Watts J. L. came to the conclusion that 137 microbial species and subspecies may cause inflammation of cow's mammary glands, some of them are part of the normal microflora and, with rare exceptions, do not cause pathological changes in udder tissues [19]. The researcher considers *S. hyicus*, *S. epidermidis*, *Corynebacterium bovis* to be conditionally pathogenic bacterial species. A number of authors studying the problem of cow mastitis indicate that the most severe forms of clinical mastitis in dairy cattle were associated with group B hemolytic streptococcus infection (*S. agalactiae*) [20, 21, 22], *Staphylococcus aureus* (*S. aureus*) [23, 24] and pathogenic *E. coli* [25, 26, 27]. Analyzing the research papers of domestic and foreign authors, it can be concluded that the diversity of microorganisms capable of causing mastitis is very large, in addition, certain statistics on the prevailing pathogens in different countries around the world can be found in the literature [28, 29]. Thus, in the Russian Federation, *Staphylococcus aureus* with detectability ranging from 42.8 to 87.3%, *Streptococcus agalactiae* (9.5–52.0%) and a group of enterobacteria are of predominant importance in the development of mammary gland inflammation (9.6–16.7%) [27]. It should be noted that the mastitis agent detected in biological sample tests can be isolated both in monoculture and in various associations [30, 31].

A number of researchers (S. A. Sheveleva [32], P. N. Gonzalez et al. [33]) have the opposite opinion. They believe that incompliances with the veterinary and zootechnical rules for feeding, keeping and milking cows, injuries and postpartum complications play a major role in the etiology of mastitis. Moreover, the emphasis is made on feeding, and microorganisms are of secondary importance. If an enhanced control over milking, keeping, udder condition maintenance and other preventive measures is carried out in a dairy farm, the udder diseases are detected much less frequently.

The literature data indicate that the season of the year also affects the epizootic process of cow mastitis. Rodin N. V. et al. [34] note in their study that there is a tendency for an increase in cow mastitis cases in the autumn and spring periods, a decrease, in turn, occurs in the winter and summer months. Thus, 14.8% of mastitis cases occur in summer, while 26.9% occur in winter. The percentage of mastitis cases recorded in the autumn-spring period ranges from 19.4 to 40.0%. The largest number of diseased cows is registered in areas where the stall period is the longest [35].

Such factors as gas contamination of premises, the condition and type of flooring material in livestock holdings are also responsible for increased number of cow mastitis cases. According to M. V. Oskolkova and E. V. Kuzmina [36], the incidence of mastitis in winter decreases up to 16.9% when animals are kept in wooden premises with wooden flooring.

Mastitis can occur during lactation, late gestation period, dry period, or immediately after parturition. The transformation of mammary gland tissues during late gestation period, dry-off and before calving results in reduction in its resistance and, in case of infectious onset or a latent inflammatory process, triggers the development of clinical mastitis. Mastitis often occurs after calving due to intoxication as a result of udder edema and other post-parturient diseases [37, 38, 39, 40].

According to the literature, the same infectious agent, depending on its quantity and virulence, as well as local and general resistance of the animal's body, can induce both clinical and subclinical forms of mastitis [1, 2, 3].

Clinical mastitis is characterized by the presence of pronounced signs of udder inflammation and changes in milk properties and composition. A physical examination of the affected mammary gland reveals local symptoms such as hyperemia, redness and pain during palpation, swelling and lumpiness. The disease manifestations can also include such general symptoms as behavioral changes, fever, anorexia, depression, lethargy, inappetence and hyperthermia. Clots and flakes of precipitated casein, transparent or bloody inclusions, as well as pus are found in milk [3, 22, 39].

Subclinical mastitis, occurring without obvious signs of local inflammation or systemic damage, rarely poses an immediate life threat and occurs 15–40 times more frequently than clinical mastitis. But sometimes there are cases of short-term changes in milk composition [3, 23]. Due to the asymptomatic course, farmers often miss the onset of the inflammatory process, are unaware of the deterioration in milk quality and the existing risk of spreading mastitis pathogens to other cows in the herd. The disease becomes chronic if no treatment is administered for more than two months. Depending on the biological properties of the pathogen, the infection can persist throughout lactation or cow's life span [9].

Mastitis became widely known at the end of the nineteenth century. The data accumulated since that time made it possible to describe and classify the types of inflammatory processes occurring in the udder when the clinical form of the disease is manifested. The classification by A. P. Studentsov which identifies 5 main subgroups of clinical mastitis based on the nature of inflammation is most frequently used in practice.

The most common is *serous* mastitis (early stage of the disease) characterized by swelling as the main sign. The affected parts of the udder are hard, enlarged, with pronounced hyperthermia. The general condition of the animals is regarded as normal, without deviations. No abnormalities are found in milk during visual examination at the initial stage of the disease; casein flakes are found and milk becomes more liquid as the inflammatory process progresses [28].

Catarrhal mastitis occurs mainly when inflammation is localized in the teat cistern or in large ducts. In this case, casein flakes are observed only at the beginning of milking; in case of inflammation of the udder alveoli, the milk is heterogeneous and contains flakes throughout milking process. Catarrhal mastitis is typically characterized by gray-colored secretions, as well as a significant decrease in milk fat content due to the formation of protein clots [4].

Hemorrhagic mastitis often develops as a result of serous or catarrhal inflammation. With this type of cow mastitis, all udder quarters are affected. Animals demonstrate intense breathing, inappetence, body temperature can reach 40.0–41.0 °C, pronounced purple spots are found on the surface of the skin. The ejected milk is of pink-red color and has inclusions of casein flakes [41].

Fibrous mastitis is the most painful and one of the most dangerous types of cow mastitis. This form is characterized by a sharp decrease in milk productivity, animal's depression, a painful reaction and a typical sound of crepitation when the affected part of the udder is palpated. Most of the time, the cow lies down, gets up with difficulty, lameness of both hind limbs is frequently observed [4].

When *purulent* mastitis is developed, the general condition of the cow significantly deteriorates. This type is characterized by the formation of dense hard lumps, animals' body temperature increases by 1.0–2.0 °C. Milk contains pus. Abscess may develop in the udder as inflammation progresses. The prognosis is unfavourable in case of abscess or phlegmon. The mammary gland acquires pustules that spread to larger areas and, as a result, merge together. Eventually a part of the gland loses its ability to function. Milk obtained from the infected lobe (lobes) has a gray color and contains multiple clots [3].

When any form of mastitis occurs (clinical or subclinical), there is a deterioration in milk quality with regard to sensory parameters, bacterial count and somatic cell count. In case of mastitis, an increase in somatic cell and bacterial count in raw milk means increased activity of proteolytic and lipolytic enzymes. Plasmin, for example, is a caseinolytic enzyme synthesized from plasminogen, which develops in blood and most likely enters milk due to the destruction of mammary gland epithelium. Casein degradation will generate foul-smelling metabolites that will replace the pleasant odour associated with fresh milk [40, 42].

The regulatory document specifying the requirements imposed on milk and dairy products was substituted in the Russian Federation since January 1, 2016; the Customs Union's Technical Regulation "On safety of milk and dairy products" (CU TR 033/2013) was enforced instead of the Technical Regulation of Russia. The most significant changes related to the quality and safety of milk were the abolishment of milk grading and introduction of stricter standards for antibiotic content. To date, common milk standards have been approved, according to which milk can be placed on the market, or in case of non-compliance, it can be rejected without the possibility of further processing and sale at reduced prices. According to this document, the number of mesophilic aerobic and facultative anaerobic microorganisms shall not exceed 5×10^5 CFU/cm^3; number of pathogenic microorganisms shall be < 25 microbial cells, number of somatic cells shall be $< 7.5 \times 10^5$ in 1.0 cm^3 of the product.

Along with that, the content of antimicrobial drugs (AMDs) shall not exceed: 0.01 mg for levomycetin, 0.01 mg for tetracyclines, 0.2 mg for streptomycin, 0.004 mg for penicillin in 1 kg (litre) of the product. The AMDs admissible levels practically correspond to the analytical limits of methods used for detection of antibacterial drugs, and in general they can be classified as AMDs inadmissible levels.

The above information confirms the relevance of mastitis control issues and the need to prevent financial losses caused by this disease. Preventing the transition of the subclinical form of mastitis into a clinical one is one of the most important links in the complex of measures aimed at preserving udder health. This is possible in case of timely and regular diagnosis (at least once a month) [6].

To establish the diagnosis, it is necessary to examine the animal, measure its body temperature, pulse and respiratory rate. Particular attention should be paid to the inguinal lymph nodes and mammary glands during the examination. Udder examination includes visual examination, palpation and trial milking. Visual examination should be focused on skin integrity, color and udder symmetry. Signs of mastitis during palpation include fever in the udder, pain when pressed and typical lumps in mammary gland tissue. During trial milking the attention is paid to the effort required for milking, amount and sensory parameters of the ejected secretion [43, 44].

Latent (subclinical) mastitis is diagnosed using one of the rapid diagnostic tests (RDTs): dimastin, mastidine, mastotest, etc. The effect of RDTs is based on the detection of exceeding leukocyte levels and pH changes. Milk control plates divided into 4 round sections (one for each udder quarter) are used for testing. Both cisteral and parenchymal milk samples should be used to obtain reliable results [37].

The final stage in the diagnosis of cow mastitis is laboratory testing. In this case, the bacteriological method is preferable as it is the most informative [45], it helps to isolate a pure culture of the pathogen, identify it, perform a comprehensive study if necessary (describe its growth, proteomic and pathogenic properties) and determine susceptibility to antimicrobial drugs. Determination of antimicrobial drug susceptibility plays a specific role in determining further therapeutic measures due to the fact that in this case the data obtained helps to find an effective antibiotic against a specific pathogen. Incorrect treatment, incompliances with the prescription, as well as unnecessary use of antimicrobials can lead to the generation of multidrug-resistant bacteria [46].

Antibiotic therapy is used for all types of mastitis caused by bacterial microflora, or in cases where there is a secondary bacterial infection. The therapy has a number of positive aspects, such as rapid improvement in the animal's clinical condition, a low culling rate in a herd, predictable losses in milk yield, as well as negative ones: rejection of milk within the antibiotic withdrawal period during and after treatment. The presence of residual

amounts of antibiotics in milk is dangerous for human health and reduces the quality of dairy products, most drugs have a long withdrawal period; besides, the cost of AMDs is quite high [47, 48].

Due to the wide spread of antibiotic-resistant microorganism strains, despite the large number of drugs currently used, their effectiveness is constantly decreasing. The prospects for the further use of antibiotics as therapeutics are questioned by many researchers and international organizations due to the rapidly developing resistance to them in many agents. In recent decades, the antimicrobial resistance has become a global problem not only in public health, but also in veterinary medicine [49, 50, 51].

Irrational use of antibiotics, improper selection of therapeutics and non-compliance with the recommendations specified in the instructions, namely dosage, frequency, and duration of treatment, contribute to antibiotic resistance in bacterial populations. The AMR growth has caused serious concerns around the world both from the point of view of public health and food safety, and therefore use of antimicrobials in animal husbandry has been under constant control for many years [2].

It's worth noting that antibiotic therapy should be used only in extreme cases, mainly when the pathological process becomes life-threatening for the animal. In all other cases, it is best to treat animals with mastitis without using etiotropic therapy, and if this is not possible, it is recommended to use synthetic antimicrobials. The most common ones include sulfonamides and nitrofurans [52]. These AMDs have a number of advantages: a wide range of action, low toxicity, and relatively low cost. But there is also a significant drawback – inefficiency in the treatment of purulent mastitis [48].

The pathogenetic, along with etiotropic, therapy has become widespread, it includes methods that affect the nervous system and indirectly the entire body, contributes to an active influence on the course of the pathological process. The most common is novocaine blockade of udder nerves. Its application contributes to the healing of animals with mastitis, not only when used in combination with medicinal products, but also independently. The main novocaine blockades that are recommended for the treatment of cow mastitis are: short novocaine blockade of udder nerves according to D. D. Logvinov, blockade of the external sacral nerve according to B. A. Bashkirov, method of intra-aortic administration of novocaine according to D. D. Logvinov. Pathogenetic therapy is highly effective for acute mastitis before destructive tissue changes have occurred, particularly in case of serous, catarrhal, purulent-catarrhal mastitis [37, 44, 50].

Mastitis infection in cows often has consequences, even if the drug therapy is selected properly. Literature data, mainly foreign publications show evidence on the effectiveness of preventive vaccination of cows with mastitis. Staphylococcal toxoids, bacterintoxoids and their combinations with various adjuvants were used as immunizing agents in different countries [47, 53, 54, 55].

Vaccination plays a significant role in the infectious disease control. The use of vaccines allows not only reducing the number of cows with mastitis, but also significantly improving the quality of dairy products [2, 56, 57].

Vaccination of animals is a recognized way to reduce the cost of veterinary services. Thanks to the development of drugs for the prevention of mastitis, it has become possible to successfully control one of the most common diseases in dairy farming (the effectiveness of drug therapy ranges from 70 to 98%) [55, 56, 58, 59].

Several imported vaccines against cow mastitis are currently available on the global pharmaceutical market: two vaccines manufactured in Spain and one vaccine manufactured in France. However, due to the current global political situation, introduction of expanded sanctions and restrictions on the drugs that are not compliant with the Good Manufacturing Practice (GMP) standards, these vaccines are hardly accessible for the Russian livestock breeders.

This circumstance has become the key aspect for conducting research in order to develop new tools for the specific prevention of cow mastitis.

CONCLUSION

Modern scientific data confirm that mastitis is the main problem of dairy farming in all countries around the world, and its subclinical form (unlike clinical mastitis) is the most common. In addition, the literature data suggest that mastitis is a polyethiological disease. On the one hand, it is caused by infectious agents (bacteria, mycoplasmas, fungi), and on the other hand it is effected by environmental factors that reduce the resistance of the animal's body. Predisposing and concomitant factors have a great influence on the development of this disease. The former factors include animal's resistance and immune status, the latter factors include incompliance with zootechnical, preventive and therapeutic measures, non-performance of pre-milking and post-milking hygiene of the udder, lack of control over the serviceability of milking machines, as well as unsatisfactory disinfection of milking systems. Due to the fact that mastitis remains one of the most common global diseases, it can be assumed that farms engaged in breeding dairy cattle do not fully comply with the required zootechnological, preventive and therapeutic measures. With increased control over the implementation of a set of measures, it becomes possible to reduce the number of cases of clinical and subclinical cow mastitis. Specific prevention is the most effective way to control mastitis, however, a strong and stable immunity can be achieved only with strict observance of specific zoohygienic and technological requirements.

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Avian adenovirus infections: diversity of pathogens, hazard to poultry industry and problems of immunoprophylaxis (review)

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ABSTRACT

The data on diversity of adenovirus pathogens in nature and the role of the main representatives of the *Adenoviridae* family in poultry infectious pathology are presented. Special attention is paid to problematic issues of immunoprophylaxis due to lack of cross-immunity between different virus serotypes. There is no single and effective approach in the global strategy of immunoprophylaxis of avian adenoviruses, therefore, improving the means of avian adenovirus disease control is an urgent and important task. Avian adenovirus infections are represented by different nosological units: egg drop syndrome, hydropericardium syndrome, adenoviral gizzard erosion, marbled spleen disease of pheasants, hemorrhagic enteritis of turkeys, inclusion body hepatitis and many unclassified diseases. The paper provides data on the main nosological forms of adenovirus infections that pose a threat to cost-effective poultry farming, and highlights test results obtained by foreign authors on the effectiveness of some vaccines against adenovirus infection. Most vaccines have been developed to prevent avian hydropericardium syndrome, however, occurrence of many virus serotypes requires effective means of prevention and diagnosis in order to control other infections caused by adenoviruses. There is no registered vaccine against adenovirus infections that cause inclusion body hepatitis and adenoviral gizzard erosion. At the same time, inclusion body hepatitis alone accounts for 2.9% of all recorded avian infectious diseases. Vaccines registered in the Russian Federation are not enough to fully control these infections, and that requires a timely solution to the problem. The variety of avian adenoviruses determines the problems of their differential diagnosis and specific prevention.

Keywords: review, avian adenovirus infections, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*

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Аденовирусные инфекции птиц: многообразие возбудителей, опасность для птицеводства и проблемы иммунопрофилактики (обзор)

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РЕЗЮМЕ

Представлены данные о многообразии возбудителей аденовирусных инфекций в природе и роли основных патогенов семейства *Adenoviridae* в инфекционной патологии сельскохозяйственной птицы. Особое внимание уделено проблемным вопросам иммунопрофилактики ввиду отсутствия перекрестного иммунитета между разными серотипами вируса. В мире нет единого и эффективного подхода в стратегии иммунопрофилактики аденовирозов птиц, поэтому совершенствование средств борьбы с аденовирусными заболеваниями птиц является актуальной и важной задачей. Аденовирусные инфекции птиц представлены разными нозологическими единицами: синдромом снижения яйценоскости, синдромом гидроперикардита кур, эрозией желудка, болезнью мраморной селезенки фазанов, геморрагическим энтеритом индеек, гепатитом с тельцами-включениями и множеством неклассифицированных болезней. Приводится информация об основных нозологических формах аденовирусных инфекций, представляющих угрозу

для рентабельного ведения птицеводства, освещены результаты исследований зарубежных авторов по изучению эффективности некоторых вакцин против аденовирозов. Большинство вакцин разработаны для профилактики синдрома гидроперикардита кур, однако существование множества серотипов возбудителя требует создания эффективных средств профилактики и диагностики в целях контроля и других инфекций, вызываемых аденовирусами. Нет ни одной зарегистрированной вакцины против аденовирусных инфекций, вызывающих гепатит с тельцами-включениями и эрозию желудка. При этом на долю только гепатита с тельцами-включениями приходится 2,9% от всех регистрируемых инфекционных заболеваний птиц. Вакцин, зарегистрированных на территории Российской Федерации, недостаточно для полного контроля данных инфекций, что требует своевременного решения проблемы. Многообразие возбудителей аденовирусных инфекций птиц обусловливает проблематику их дифференциальной диагностики и специфической профилактики.

Ключевые слова: обзор, аденовирусные инфекции птиц, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*

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INTRODUCTION

Food security is a global problem, and poultry farming accounting for about 39% of the global meat production plays a leading role in solving it. In Russia poultry meat accounts for 46% of the total meat production [1]. It should be noted that confessional restrictions do not apply to poultry products – no religion prohibits consumption of chicken meat or eggs [2].

Poultry industry is greatly affected by infectious diseases, not only highly dangerous, but also economically significant, such as adenovirus infections tending to spread widely. Previously researchers did not pay much attention to fowl adenoviruses (FAdV) due to their limited clinical significance, but in recent decades adenoviruses became significant field pathogens causing diseases that have a serious impact on poultry health and industry profitability. In this regard the number of FAdV scientific studies began to grow [3, 4]. In recent years, new data on cross-immunity between different serotypes of adenoviruses were obtained, inactivated, live and recombinant vaccines against certain diseases of adenovirus etiology were developed, and their immunogenic activity was studied.

The aim of this work was to summarize scattered information on fowl adenoviruses with special emphasis on the diversity of pathogens and the problem of effective immunoprophylaxis, as well as to show the role of some adenovirus diseases in avian infectious pathology.

ADENOVIRUSES: TAXONOMY, DESCRIPTION, SUSCEPTIBLE ANIMALS

Adenoviridae is a family of non-enveloped viruses with icosahedral symmetry, 90 nm in diameter. Nucleic acid is represented by double-stranded linear DNA (25–48 kilobase pairs) with inverted terminal repeats and a terminal TP protein (55 kDa) covalently bound to each 5'-end of both chains [5, 6, 7, 8]. The viral capsid has a pseudo-triangulation number of 25 and consists of 252 capsomers: 240 hexones and 12 pentones with fibrous processes ranging in length from 9.0 to 77.5 nm [5, 9].

Adenoviruses were found in many vertebrates from fish to humans and are represented by six genera:

– *Mastadenovirus*: 51 species with a capacity to infect mammals, including humans;

– *Aviadenovirus*: 16 species infecting birds;

– *Atadenovirus*: 10 species with a capacity to infect reptiles, birds, ruminants and marsupial mammals;

– *Siadenovirus*: 8 species infecting frogs and birds;

– *Ichtadenovirus*: 1 species infecting belugas;

– *Testadenovirus*: 1 species infecting red-eared sliders.

The demarcation of the genus is based on phylogenetic, biological characteristics, as well as on the organization of the genome [5, 10, 11].

Birds are affected by representatives of three genera of the family *Adenoviridae*:

– *Aviadenovirus* (FAdV) includes species A, B, C, D, E divided into 12 serotypes using a cross-neutralization test that cause inclusion body hepatitis, hydropericardium syndrome and gizzard erosion in both domestic and synanthropic birds;

– *Siadenovirus* causes hemorrhagic enteritis in turkeys, marble spleen disease in pheasants, adenovirus splenomegaly in chickens;

– *Atadenovirus* is a duck adenovirus that is pathogenic to chickens and causes egg drop syndrome (EDS–76) [5, 12].

Thus, the variety of avian adenovirus infectious agents determines the problematic issues of their differential diagnosis and specific prevention.

MAIN AVIAN ADENOVIRUSES

Egg drop syndrome (EDS-76) is a fowl adenovirus infection affecting layers; it is manifested by decreased egg production, shell thinning (up to its complete loss) and depigmentation. It was first described by

Dutch scientists in 1976 [13]. The causative agent of EDS-76 is duck adenovirus (DAdV-1), contamination of the live vaccine against Marek's disease resulted in primary infection of chickens and adaptation to a new host [14]. The major characteristic of EDS-76 is decreased egg production by 15% or more [15]. Viral carriage is typical of this disease: in the vast majority of cases birds look healthy, the disease is not apparent but develops when a bird reaches reproductive maturity and experiences stress due to egg lay onset. EDS-76 is currently widespread in many countries with developed poultry industry [13].

Hemorrhagic enteritis of turkeys (HE) is a viral disease of poultry caused by adenoviruses belonging to the genus *Siadenovirus*, the species *Turkey siadenovirus A* (TadV-A), and manifested as immunosuppression in turkeys older than 4 weeks of age [16]. The disease has two forms. The first variant implies that hemorrhagic enteritis is caused by highly virulent strains of the pathogen and proceeds with pronounced clinical signs such as depression and gastro-intestinal hemorrhages. The virus causes immunosuppression, so opportunistic bacterial infections are often an intercurrent problem. This disease often results in poultry mortality (up to 80%) due to blood loss and secondary infection [17, 18]. The second form implies that hemorrhagic enteritis develops without visible symptoms and is caused by low-virulent strains of the virus that determine immunosuppression resulting in the development of secondary bacterial complications. This leads to economic losses due to necessity to use antibacterial drugs and perform culling [19, 20]. The disease is widespread in turkey populations all over the world [21].

Marble spleen disease of pheasants (MSD) is an infectious disease affecting 3–8 month-old pheasants which is caused by group II adenovirus of the genus *Siadenovirus*. The pathogen is closely related to the two other representatives of the genus: the causative agent of adenovirus splenomegaly in chickens and the causative agent of hemorrhagic enteritis in turkeys [22]. Typical signs of the disease observed during necropsy include splenomegaly and a marble spleen pattern. Congestion and pulmonary edema are detected. Necrotized lymphoid follicles and focal necroses in the liver are observed. The disease is clinically manifested as respiratory failure, asphyxia and sudden death. The mortality rate can vary from 1–3 to 15% [23], the disease is highly contagious and occurs all over the world [24].

Avian adenovirus splenomegaly (AAC) is a viral disease of chickens which is clinically, pathologically and anatomically similar to the marble spleen disease of pheasants. Necropsy performed in chickens reveals enlarged "marble" liver, degenerative changes in the lymphoid lung tissue, less frequently - pulmonary hemorrhages and pulmonary edema [25].

Adenoviral gizzard erosion (AGE) is a chicken disease caused by group I adenovirus FAdV-1. The capacity of avian adenoviruses to independently cause avian diseases has been disputed for a long time, but the leading role of FAdV-1 in the etiology of adenovirus gizzard erosion was proven in the last decade [26]. FAdV-1 belongs to the genus *Aviadenovirus*, species A (group I avian adenoviruses) [5]. The disease is widespread in Europe and Asia (Iran [27], Japan [28], Sweden [29], Korea [30]). The disease is clinically manifested by depression, anorexia [28], a decrease in weight gain and leads to death. The infection often proceeds without visible clinical signs and is diagnosed post-mortem only. Autopsy shows necroses and inflammation of the gizzard mucosa, and hemorrhagic fluid in the gizzard cavity is observed [26]. The disease spreads both vertically and horizontally (mainly via fecal-oral route) and affects both broilers and layers causing significant economic losses. Thus, during the outbreak of adenovirus gizzard erosion on a farm in Mazandaran province (Iran) in 2019, the mortality rate of broiler chickens was 6%, and the target slaughter weight was gained with a week's delay; besides, lots of affected gizzards were condemned at the slaughterhouse [27].

Avian hydropericardium syndrome (HPS) is a viral disease of poultry caused by fowl adenovirus FAdV-4 group I and leading to large economic losses in poultry industry. It generally affects 3–5 week-old broiler chickens. The mortality rate ranges from 30 to 80% in broilers and from 2.6 to 15.29% in pullets [31]. It was not possible to determine the cause of this disease for a long time. Its occurrence was associated with incompliances in poultry feeding and keeping schemes, mineral and vitamin imbalance, and toxicoinfection [32]. But this association was not further confirmed [33]. The detection of body inclusions in hepatocytes during histological studies gave reason to believe that HPS is caused by the virus [34]. Subsequently, the pathogen was isolated from the liver of chickens that died from HPS and was identified as a group I avian adenovirus [35]. The HPS pathogen is transmitted both vertically from vectors and infected birds, and horizontally through litter, feed, water and inventory contaminated with feces and excretions of infected birds [36, 37]. The hydropericardium syndrome virus is assumed to spread through live vaccines made using adenovirus-infected chicken embryonated eggs. Such cases were described in Pakistan [38]. The disease is often asymptomatic and is associated with sudden death. Diseased birds may demonstrate ruffled feathers, droopy wings, depression, dyspnea, loss of appetite, diarrhea with green to yellow droppings [39]. The disease manifestations depend on a large number of factors and their combination: feeding and keeping conditions, natural resistance of the body, genetics, presence of concomitant diseases. HPS is more often manifested in poultry in a state of immunosuppression due to such factor as infectious bursal disease, feed toxicosis. Pure breed poultry is found to get diseased less often than cross-breds in broiler industry [40]. Postmortem examination of diseased chicks reveals the following pathological and anatomical findings: an enlarged fiable liver with thickened edges of pale brown or yellowish color, sometimes with focal necroses; an overflowing gallbladder with strained walls; swollen kidneys; a deformed flabby heart, often with spot hemorrhages. The pericardial cavity is filled with a clear straw-yellow fluid with a viscous consistency (up to 20 mL) [41]. Anemia, swelling of subcutaneous adipose tissue, jaundice, hemorrhages in organs and

tissues may be observed [42]. Histological studies reveal basophilic and eosinophilic inclusion bodies in hepatocytes [43, 44].

Inclusion body hepatitis (IBH) is an acute viral disease of 2–7 week-old broilers, accompanied by stunting and sudden death, with mortality ranging from 10 to 30%. The inclusion body hepatitis was initially viewed as a secondary disease associated with immunosuppression, but later it was proved that the disease was infectious in nature, the causative agent was the avian adenovirus of group I – FAdV species D (serotypes 2, 11) and E (serotypes 8a, 8b), affecting both layers and broilers. Broilers are most susceptible to the disease, mainly young birds older than 2 weeks get diseased [45, 46, 47, 48]. Thus, 92% of all cases of inclusion body hepatitis were associated with broiler chickens in 2011–2021 in Spain. The pathogen mainly spreads horizontally, but a vertical transmission pathway is also noted [47]. The virus tends to reactivate with the onset of egg production, causing a subclinical course of the disease, providing vertical transmission [45]. Group I adenoviruses are capable of infecting not only chickens, but also other bird species [48], in particular, pigeons, ducks, quails, ostriches [47]. Clinical signs are non-specific, usually observed within 4–5 days and include depression, ruffled feathers, sedentary behavior, birds' maintaining a sitting position. Necropsy reveals extensive or focal necrosis in the liver that increases in size, has a fiable consistency and a pale color [45]. There are cases of pancreatic lesions involving necrosis and atrophy, as well as lesions in kidneys [46] and spleen [49]. Histological examination reveals lymphoid infiltration, focal necroses, cell degeneration [46] and basophilic intranuclear inclusion bodies in hepatocytes [45, 50].

Thus, fowl adenoviruses cause various diseases leading to serious economic losses [49], which are expressed in high mortality (up to 80% for HPS), reduced productivity and the need for forced antibiotic therapy against secondary bacterial infections occurring in the setting of adenovirus immunosuppression. This problem is relevant and urgent for poultry industry due to the abilities of adenoviruses to persist for a long time, reactivate, infect not only livestock, but also synanthropic and wild birds, as well as to spread with their help, to cause infection without visible clinical manifestations, to spillover (EDS-76 virus). Therefore, the development of methods for control of fowl adenovirus infections is an important task and should include not only the improvement of biosafety systems in commercial establishments, but also the development of surveillance programs, as well as specific immunoprophylaxis.

IMMUNOPROPHYLAXIS

Prophylactic immunization against adenovirus infections is of great importance and serves as an effective tool in disease control. A decrease in the body's defenses, immunosuppression and stresses caused by both man-made and biological factors are the reason for sudden manifestation of FAdV-induced diseases [51]. And taking into account the intensification of poultry production [13], control of adenovirus infections is becoming an increasingly urgent problem [26]. Several types of vaccines are used for immunization: subunit, live, autogenic, inactivated. Mainly inactivated vaccines have been developed and used for the prevention of FadV; organ-tissue formolized liver homogenate vaccines are most commonly administered. These vaccines demonstrated high immunogenicity in the field trials. Thus, according to study results, the protectivity level in vaccinated poultry challenged with a field homologous virus isolate ranged within 80.0–98.9%, while the incidence level in unvaccinated birds in the control group was 60–100%. Despite inducing high immunogenicity levels, these vaccines have some disadvantages, such as high cost and impossibility to standardize the antigen dose. A number of inactivated culture virus-based vaccines have been developed. Live vaccines based on attenuated adenovirus are also known around the world. The great advantage of such biologicals over inactivated vaccines is that they can be administered orally. In the field trials these vaccines conferred 94.7–100.0% protection against challenge with homologous virus in the setting of 30.0–100.0% incidence in unvaccinated birds. Trials of subunit vaccines based on recombinant proteins F1, F2, etc. have been conducted in recent years, but the disadvantage of such vaccines is their low immunogenicity [26].

The number of reports on evidence of cross-protection between serotypes belonging not only to one but to different species is increasing, although previously it was believed that group I avian adenoviruses lack or demonstrate weak cross-protection between serotypes [52]. However, this statement is controversial, since some researchers have proven the existence of interspecies cross-protection [45]. A number of studies revealed that vaccination of poultry with combined (FAdV-8a and FAdV-11) inactivated [53] or live [45] vaccines confers cross-protection not only against FAdV-8a and FAdV-11, but also against other serotypes of D and E species [52]. The results of another experiment showed that vaccination against FAdV-4 induced protection not only against the homologous serotype, but also against the FAdV-10 serotype, which, like FAdV-4, is a representative of C species [54]. A study conducted by P. A. Steer-Cope et al. in 2019 demonstrated evidence of interspecies cross-protection between D and E species [52]. It follows from the above that multi-strain vaccines are the most prospective and can protect birds from a wide range of pathogen strains.

Three vaccines against avian adenovirus infections induced by group I adenoviruses have been registered in Russia: inactivated adsorbed vaccine against avian hydropericardium syndrome (ARRIAH, Russia) [55], liquid inactivated vaccine against adenovirus inclusion body hepatitis – avian hydropericardium syndrome based on "T-12" strain (VNIVIP, Russia) and inactivated emulsion vaccine against adenovirus body inclusion hepatitis (AVIVAC) [56]. Unfortunately, there is no registered vaccine against FAdV-induced inclusion body hepatitis and gizzard erosion. At the same time, inclusion body hepatitis alone accounts for 2.9% of all recorded avian infectious diseases [56]. There are several vaccines available in our country to protect poultry from group III adenovirus causing egg drop syndrome: vaccine against EDS-76

based on strain "B8/78" produced by VNIVIP, inactivated mono- and polyvalent vaccines against EDS-76 (strains "BISS" and "B8/78") produced by Federal Centre for Animal Health and some other manufacturers [57].

Unfortunately, there is no single and effective approach in the strategy of preventive immunization against avian adenoviruses in the world, therefore, improving the means of combating avian adenovirus diseases is an urgent and important task [13].

CONCLUSION

Avian adenovirus infections are spread globally, including Russia, and pose a danger to economically effective poultry industry. Diversity of virus serotypes and isolation of individual nosological forms of adenovirosis cause significant challenges in the diagnosis and specific prevention of these infections. Given the absence or insufficiency of cross-protection between different adenovirus serotypes, an urgent problem is the development of specific preventive measures against each serotype of the causative agent of adenoviruses, causing a clinically significant infection. The variability of the adenovirus tropism, the ability to long-term persistence, recorded cases of spillover, a high incidence rate in some nosological forms (HPS, hemorrhagic enteritis of turkeys) require the development of infection control programs in commercial poultry industry, including active and passive monitoring of the pathogen's circulation.

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Testing of diagnostic test-systems for detection of antibodies to foot-and-mouth disease virus structural proteins with enzyme-linked immunosorbent assay for their serotype specificity

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ABSTRACT
A total of 138 serum samples from pigs and cattle vaccinated against foot-and-mouth disease virus (FMDV) of one or two serotypes or infected with FMDV were used for testing of 24 enzyme-linked immunosorbent assay (ELISA) diagnostic tests-systems for detection of antibodies against FMDV structural proteins produced by 6 manufacturers (Federal Centre for Animal Health, Prionics, IZSLER, Innovative Diagnostics, BIONOTE and MEDIAN Diagnostics) for their serotype-specificity. All used test-systems detected apparent serotype-specific activity (homologous reaction) as well as cross-reacting virus-specific antibodies that was accounted for some reasons related to conservative epitopes in amino acid sequence of FMDV virion capsid VP1–VP3 polypeptides, accessibility of internal conservative epitopes of VP4 polypeptide for the animal's immune system during virus replication or vaccine antigen (virus) destruction in the animal's body in the process of immunity development, as well as the pilot anti-FMD vaccine composition, etc. Nevertheless, the analysis of a large data set (about 3,500 tests) showed that the homologous serotype-specific reaction in general was significantly higher and predominant, the proportion of virus-specific non-protective antibodies, including cross-reacting ones, was not significant and did not distort the results of ELISA tests of anti-FMD vaccine for its immunogenicity. Inconclusive test results require confirmation with other serological tests. Complex tests for FMDV using different diagnostic methods such as ELISA with standard and reference test-systems and/or virus neutralization test in cell culture are to be considered as the best option.

Keywords: foot-and-mouth disease, enzyme-linked immunosorbent assay (ELISA), serotype specificity

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Изучение серотипоспецифичности диагностических тест-систем для выявления антител к структурным белкам вируса ящура иммуноферментным анализом

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РЕЗЮМЕ
При изучении серотипоспецифичности 24 диагностических иммуноферментных тест-систем для определения антител к структурным белкам вируса ящура 6 производителей (ФГБУ «ВНИИЗЖ», Prionics, IZSLER, Innovative Diagnostics, BIONOTE и MEDIAN Diagnostics) было исследовано 138 образцов сыворотки крови свиней и крупного рогатого скота, вакцинированных против ящура одного или двух серотипов либо инфицированных вирусом ящура.

Все использованные тест-системы наряду с ярко выраженной серотипоспецифической активностью (гомологичная реакция) выявляли также некоторое количество перекрестно-реагирующих вирусоспецифических антител, что было обусловлено рядом причин, связанных с консервативными эпитопами в аминокислотной последовательности капсидных полипептидов VP1–VP3 вириона возбудителя ящура, с доступностью внутренних консервативных эпитопов полипептида VP4 для иммунной системы животного при репликации вируса или деструкции вакцинного антигена (вируса) в организме животного в ходе иммунного процесса, а также с компонентным составом экспериментальной противоящурной вакцины и др. Тем не менее анализ большого массива данных (около 3500 исследований) показал, что гомологичная серотипоспецифическая реакция в целом была значительно выше и являлась доминирующей, доля вирусоспецифических незащитных антител, в том числе и перекрестно-реагирующих, была не столь значительной и не искажала результаты оценки иммуногенности противоящурной вакцины в иммуноферментном анализе. В сомнительных случаях требуется подтверждение результатов в других серологических реакциях. Оптимальным вариантом следует считать комплексные исследования на ящур с привлечением разных методов диагностики, таких как иммуноферментный анализ с использованием стандартных и референтных тест-систем и/или реакция вирусной нейтрализации в культуре клеток.

Ключевые слова: ящур, иммуноферментный анализ, серотипоспецифичность

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INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most highly contagious viral diseases of domestic and wild cloven-hoofed animals that is well-known since ancient times and can have drastic economic, social, and environmental impact. The disease is caused by aphthovirus of *Picornaviridae* family and is characterized by fever, mucous and epithelial aphthous lesions and other symptoms. There are 7 immunologically distinct serotypes of FMDV: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1 [1, 2].

Specific vaccination is one of important tools used together with other measures to combat the infection. Since the virus of different serotypes practically does not induce any cross-immunity and vaccines based on the virus strains that differ from the field strains of the same virus serotype may confer incomplete protection, thorough selection of the virus strains to be incorporated in the developed vaccines is required to achieve maximum possible protection of animals in the particular region [1, 2].

Two major methods for testing serum samples for antibodies to FMDV structural proteins: virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA), are used for laboratory diagnostics for the purpose of assessment of vaccine effectiveness, immune status and immunity level in susceptible animals [1, 2].

Virus neutralization tests is considered a gold standard for FMDV since its allows direct detection of the virus neutralizing antibodies in serum samples from vaccinated or convalescent animals, thereby determination of the level of protection against FMD caused by the virus of particular serotype.

Enzyme-linked immunosorbent assay based on the inactivated whole virus or recombinant virus antigen covers the whole range of virus-specific antibodies, primarily virus neutralizing ones, owing to the virion structure. This allows ELISA to be used for laboratory FMD diagnosis as an alternative or confirmatory method like VNT.

However, we repeatedly detected cross-reactive antibodies with test-systems for detection of different virus serotypes in serum samples from FMD vaccinated or convalescent animals. Some researchers reported the same phenomenon during performed serological tests [3, 4, 5]. The nature of this phenomenon is unclear and requires further research.

The virion is non-enveloped and is a capsid surrounding RNA-genome. The capsid forms an icosahedral structure with a sedimentation coefficient of 146S and comprises 60 copies of protomer unit consisting of four structural proteins, VP1 to VP4. The virus forms intermediates during its replication: empty 75S capsid lacking nucleic acid, 12S pentamers and 5S protomers. During degradation the capsid appears to dissociate into separate units. The surface proteins VP1–VP3 carry epitopes responsible for FMDV serotype specificity and virus-neutralizing antibody development, while the internal VP4 protein is more conservative in different virus serotypes and antibodies against VP4 epitopes do not confer protection against the infection [3, 6].

Accessibility of internal conservative epitopes for the animal's immune system in the presence of a certain number of 12S and 5S subunits along with an intact antigen (capsid antigen with unchanged structure) contained in the vaccine or during FMDV replication at the time of infection may be one of probable reasons for the

cross-reactivity of antibodies between serotypes. Presence of conservative epitopes in the amino acid sequence of capsid surface proteins (VP1–VP3) may be the other reason. The latter is supported by detection of cross-reactive virus neutralizing antibodies with VNT, which may also be indicative of some cross-serotype protection [4, 5].

In this paper, an attempt was made to explain the nature of the cross-reactions in ELISA, as well as to assess the effect of this phenomenon on the possibility of determination of serotype specificity of post-vaccination and post-infectious antibodies in serum samples from pigs and cattle. For this purpose, mono-specific panel including experimental serum samples collected from infected pigs and cattle or from pigs and cattle single-vaccinated with FMD vaccine based on one FMDV strain. The serum samples were concurrently tested with commercial ELISA test-kits for detection of antibodies against FMDV structural proteins of different manufacturers: Federal Centre for Animal Health (FGBI "ARRIAH"), Russia; Prionics, Netherlands/Switzerland; IZSLER & The Pirbright Institute, Italy/Great Britain; Innovative Diagnostics, France; BIONOTE, South Korea; MEDIAN Diagnostics, South Korea. The used FGBI "ARRIAH" test-kits were strain-specific polyclonal test-systems based on liquid-phase blocking ELISA, used foreign test-kits were serotype-specific monoclonal competitive ELISA test-systems.

MATERIALS AND METHODS

The following samples were used for the test: retained serum samples from pigs and cattle infected with serotype SAT 2 and O FMDV or vaccinated with monovalent and bivalent anti-FMD vaccines. Before testing the samples were kept at minus 20 °C. Monospecific panel contained 81 serum samples including 8 serum samples from convalescent animals and 73 serum samples from animals vaccinated against serotype A (25 samples), serotype O (31 samples), serotype Asia 1 (17 samples) as well as 57 serum samples collected from pigs immunized with pilot bivalent emulsion vaccine against serotype A and O FMD before and after challenge with serotype O FMDV. Serum samples were tested with ELISA and some serum samples were tested with VNT.

Two specimens of pilot emulsion vaccine containing FMDV O 2212/Primorsky/2014 and A 2155/Zabaikalsky/2013 strain antigens at different concentrations were tested in piglets. Challenge tests were carried out in accordance with recommendations of the World Organization for Animal Health (WOAH) [2]. Two groups of animals, 15 pigs per group, were formed for the experiment. The animals were immunized subcutaneously with the vaccine containing different antigen amounts (undiluted, 1:5 and 1:25 diluted antigen, 5 animals per antigen dilution in each group) in a volume of 2.0 cm^3 (1 dose). Another two non-vaccinated animals served as controls. Blood samples were collected from all animals on day 21 after immunization and subjected to serological testing, then the animals were challenged with aphthous FMDV O 2212/Primorsky/2014 strain at a dose of 10^4 ID_{50}/0.2 cm^3. Challenge test results were recorded and blood samples were collected on day 8 after challenge.

All experiments in animals were carried out in strict accordance with GOST 33215-2014, Interstate standard for the 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 Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

The test-kits based on liquid-phase blocking ELISA for detection of antibodies against structural proteins of different strains of serotype A, O, Asia 1 FMDV (Table 1) developed by the FGBI "ARRIAH" in accordance with the instructions on their use as well as the following commercial test-kits for detection of antibodies against serotype A, O, Asia 1 FMDV produced by leading European and Asian manufacturers in according to their manufacturers' instructions were used:

– PrioCHECK® FMDV Type A (Prionics, Netherlands/Switzerland);

– PrioCHECK® FMDV Type O (Prionics, Netherlands/Switzerland);

– PrioCHECK® FMDV Type Asia 1 (Prionics, Netherlands/Switzerland);

– SPCE for antibodies specific to FMDV Serotype A (IZSLER & The Pirbright Institute, Italy/Great Britain);

– SPCE for antibodies specific to FMDV Serotype O (IZSLER & The Pirbright Institute, Italy/Great Britain);

– ID Screen® FMD Type A Competition (Innovative Diagnostics, France);

– ID Screen® FMD Type O Competition (Innovative Diagnostics, France);

– ID Screen® FMD Type Asia1 Competition (Innovative Diagnostics, France);

– FMD Type O Ab ELISA (BIONOTE, South Korea);

– VDPro® FMDV Type O Ab b-ELISA (MEDIAN Diagnostics, South Korea).

Test results were interpreted based on percentage of inhibition (PI) calculated for each tested sample according to the formula and interpretation criteria indicated by the test-kit manufacturer. To easily perceive large data sets, the results obtained with the test-kits as optical density (OD) values to be expressed as S/N ratio (Innovative Diagnostics – IDVet, MEDIAN), were also recalculated to PI values.

Foot-and-mouth disease virus antigen was serotyped with indirect sandwich ELISA using hyperimmune rabbit serum and guinea pig serum prepared against intact capsid antigen of one of FMDV strains (146S-Ag) as described above with some modifications as capture antibody and detection antibody, respectively [2, 7]. For this purpose, 96-well ELISA plates (NUNC, Denmark) were coated with the solution of strain-specific rabbit serum (serotypes A, O, Asia 1 or SAT 2 FMDV) in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Then, successive steps of adding tested or control antigen samples, detection antibodies (strain-specific guinea pig serum for relevant serotype) and anti-species immunoperoxidase conjugate diluted with 0.01 M tris-buffered saline solution supplemented with 0.01% of Tween 20 detergent containing 10% of bovine fetal serum to the plate wells were carried out. All test steps were carried out at 37 °C for 1 hour and the plates were washed after each step to remove non-bound components. The reaction was visualized with ABTS (2,2'-azino-di(3-ethylbenzothiazoline)-6-sulfonic acid) substrate for 20 min at room temperature; the reaction was stopped with 1% sodium dodecyl sulfate.

The intact antigens of FMDV were also serotyped with direct ELISA when the antigen adsorbed in the plate wells

was detected with commercial peroxidase-conjugated serotype-specific monoclonal antibodies produced by Prionics, IZSLER, Innovative Diagnostics, BIONOTE, MEDIAN Diagnostics companies. The reaction conditions were similar to that ones for sandwich ELISA.

Optical density values of tested samples that were 2.5 times greater than conjugate control OD value (background control) in both assays were considered positive.

Foot-and-mouth disease VNT was carried in continuous IB-RS-2 porcine kidney cells in accordance with the WOAH recommendations [2]. The VNT was carried out using 96-flat-bottom well culture microplates. The virus, 10^2 $TCID_{50}$/0.05 cm^3 (accepted range: $10^{1.5}$–$10^{2.5}$ $TCID_{50}$/0.05 cm^3), was added to two-fold dilutions of tested and control serum samples prepared with the nutrient medium (Eagle's MEM). Then, the plates were incubated at 37 °C in 0.5% CO_2 environment for one hour and IB-RS-2 culture cell suspension at concentration of 10^6 cells/cm^3 was added to all wells, 0.05 cm^3 per well, and the plates were incubated under the same conditions for 48–78 hours. The reaction results were read based on cytopathic effect (CPE) developed in the control wells containing infected cell culture without sera from tested animals. Virus-neutralizing antibody (VNA) titre was defined as the last serum dilution showing 50% inhibition of the virus CPE [2].

VP1 amino acid sequences of FMDV of different serotypes were aligned in accordance with the data obtained from the NCBI and publicly accessible sources [6, 8, 9, 10, 11, 12, 13, 14, 15]:

– A (A/TAN/26/2013); GenBank: AXI68858.1;
– A (A_{22}/IRQ/24/64); GenBank: ARO74643.1;
– A (A/TUR/2/2014); GenBank: QWL55674.1;
– A (A/TUR/2006); GenBank: ACC63168.1;
– A (A/PAK/2013); GenBank: APZ88528.1;
– O (wild type of type O FMDV); PDB: 7ENP_1;
– O (O/TUR/33/2011); GenBank: QWL56951.1;
– O (O/PAK/2019); GenBank: UFI08025;
– Asia1 (Asia1/BAN/DH/Sa-319/2018); GenBank: QED10746.1;
– Asia1 (Asia1/284-3/4_ISB/Pak_2012); GenBank: APZ88631.1;
– Asia1 (Asia1/Shamir/89); GenBank: ASV50713.1;
– C1 (C1/Loupogne BEL/53); GenBank: AAA91488.1;
– SAT2 (SAT2/LIB/2012); GenBank: AFU55197.1;
– SAT2 (SAT2/EGY/Ismailia/2018); GenBank: QZE50286.1.

Electrophoretic separation of the proteins in polyacrylamide gel was carried out as described earlier [16, 17].

RESULTS AND DISCUSSION

Twenty-four ELISA test-kits for detection of antibodies against FMDV structural proteins produced by various manufacturers were tested for their serotype-specificity using the panel of monospecific porcine and cattle sera. The test-kits for FMD diagnosis manufactured by leading foreign companies such as Prionics, IZSLER, Innovative Diagnostics, BIONOTE and MEDIAN Diagnostics, are based on direct competitive ELISA. Serum immunoglobulins and horseradish peroxidase-conjugated monoclonal antibodies compete for specific binding to the virus or recombinant antigen. The FGBI "ARRIAH" tests-kits contain specific and nonspecific components for indirect liquid-phase blocking ELISA. This ELISA variant for detection of antibodies against FMDV structural proteins is a competitive ELISA variant and is characterized by preliminary step of FMDV antigen-tested serum interaction ("liquid phase") during which the immunodominant epitopes in the FMDV antigen amino acid sequence are blocked by specific immunoglobulins. The other steps of the assay are generally similar to other ELISA variants. Competing antibodies are homologous strain-specific polyclonal antibodies in guinea pig blood serum (detection antibodies) that bind to free antigenic determinants [2, 18, 19, 20].

Table 1
FMDV strains used in the test-kits produced by the FGBI "ARRIAH" for virus neutralization tests and/or for monospecific serum preparation

FMD virus serotype	Name of production FMDV strain	Short strain designation	Topotype	Genetic lineage
A	A 2155/Zabaikalsky/2013	A/Zab/13	ASIA	Sea-97
	A 2029/Turkey/2006	A/TUR/06	ASIA	Iran-05
	A 2269/ARRIAH/2015	A/ARRIAH/15	ASIA	G-VII
	A_{22}/Iraq/24/64	A_{22}/IRQ/64	ASIA	Iraq-64
	A/Tanzania/2013	A/TAN/13	AFRICA	G-1
O	O 2047/Saudi Arabia/2008	O/SAU/08	ME-SA	PanAsia2
	O 2356/Pakistan/2018	O/PAK/18	ME-SA	PanAsia2
	O 2212/Primorsky/2014	O/Prim/14	SEA	Mya-98
	O_1/Manisa/Turkey/1993	O_1/Manisa/93	SEA	Mya-98
	O_1/Campos/1994	O_1/Campos/94	EURO-SA	–
	O 2344/Mongolia/2017	O/MOG/17	ME-SA	Ind 2001
	O 2311/Zabaikalsky/2016	O/Zab/16	ME-SA	Ind 2001
	O 2620/Orenburg/2021	O/Orenburg/21	ME-SA	Ind 2001
	O/Kenya/2017	O/KEN/17	EA-2	–
Asia 1	Asia-1 1946/Shamir 3/89	Asia1/Shamir/89	ASIA	Shamir
	Asia-1 2145/Tajikistan/2011	Asia1/TAJ/11	ASIA	Singh-08
	Asia-1 2356/14/Pakistan/2018	Asia1/PAK/18	ASIA	Singh-08
SAT 2	SAT2/LIB/39/2012	SAT2/LIB/12	VII	Lib-12
	SAT2/ERI/98	SAT2/ERI/98	VII	–

Figure 1 shows that none of above test-kits used for testing sera collected from animals on day 21–28 after their vaccination with anti-FMD monovalent vaccines based on the virus strains indicated in Table 1 demonstrated 100% serotype-specificity. The reaction to homologous sera was predominant, however, the number of cross-reacting samples turned out to be significant.

The cross-reactivity was also measured during the tests of 6 serum samples from SAT 2 FMD convalescent pigs and cattle (Table 2).

Analysis of the obtained results showed that the majority of used test-systems detected cross-reactive antibodies in sera collected from animals on day 21–31 after their infection with FMDV SAT2/ERI/98 strain. Therewith, the detected cross-reactivity was significantly lower than that one detected by SAT2/LIB/12-ARRIAH test-system: all 6 samples were tested positive with PI_{mean} = 97.6%. Four monoclonal test-systems: A-PrioCHECK, O-PrioCHECK,

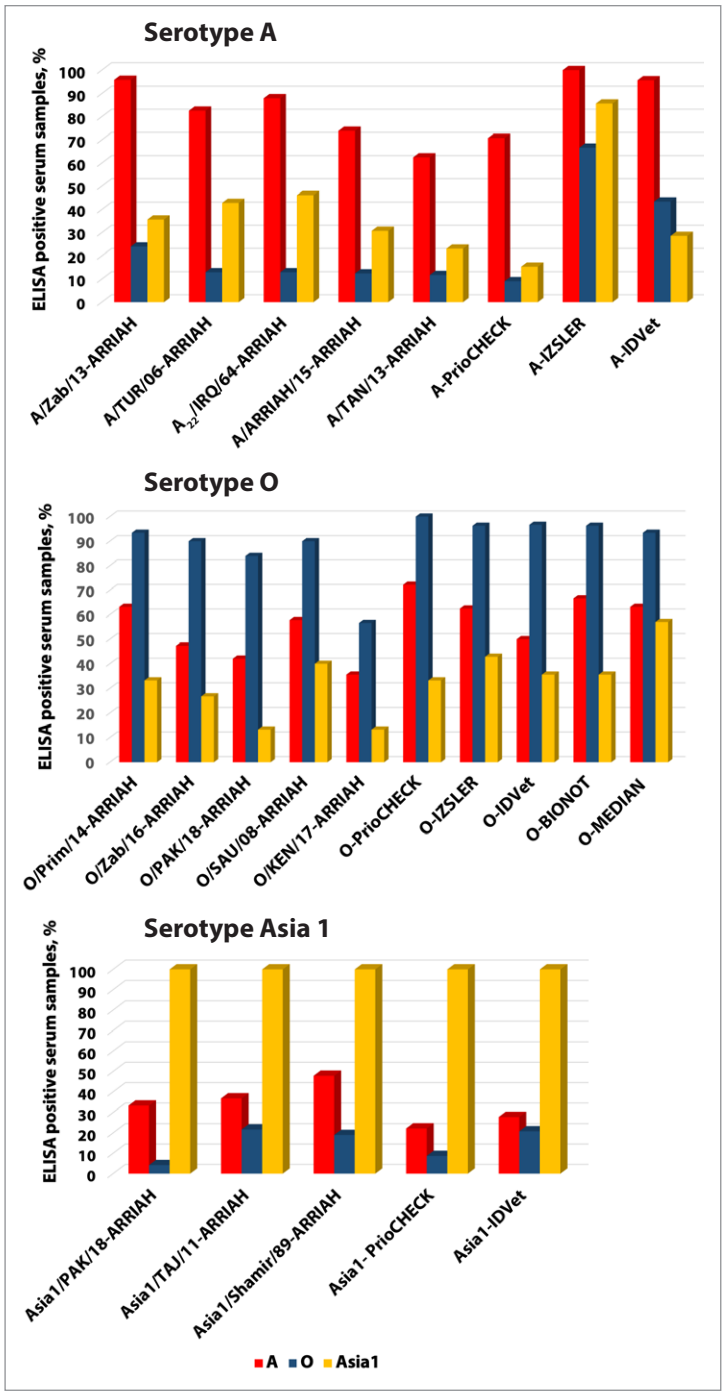

Fig. 1. Results of ELISA tests of serum samples from animals immunized with monovalent anti-FMD vaccine (n = 73)

Table 2
Results of ELISA tests of serum samples from animals infected with SAT 2 FMDV

Diagnostic test-system	Serum samples from cattle and pigs, 21–31 dpi SAT2/ERI/98 (*n* = 6)	
	PI_{mean}, %	pos/n
A/Zab/13-ARRIAH	***64.9***	*5/6*
A/TUR/06-ARRIAH	***66.7***	*5/6*
A_{22}/IRQ/64-ARRIAH	***62.8***	*4/6*
A/ARRIAH/15-ARRIAH	***54.5***	*5/6*
A/TAN/13-ARRIAH	***62.6***	*3/6*
A-PrioCHECK	*36.3*	*0/6*
A-IZSLER	***74.8***	*4/6*
A-IDVet	*45.0*	*3/6*
O/Prim/14-ARRIAH	***60.2***	*5/6*
O/Zab/16-ARRIAH	***51.8***	*4/6*
O/PAK/18-ARRIAH	***52.8***	*4/6*
O/SAU/08-ARRIAH	***64.8***	*5/6*
O/KEN/17-ARRIAH	***61.9***	*3/6*
O-PrioCHECK	*34.8*	*0/6*
O-IZSLER	*45.6*	*0/6*
O-IDVet	*35.8*	*3/6*
O-BIONOT	*15.5*	*1/6*
O-MEDIAN	*37.0*	*3/6*
Asia1/PAK/18-ARRIAH	*37.4*	*2/6*
Asia1/TAJ/11-ARRIAH	***57.8***	*4/6*
Asia1/Shamir/89-ARRIAH	***71.9***	*5/6*
Asia1-PrioCHECK	***52.8***	*4/6*
Asia1-IDVet	*31.4*	*0/6*
SAT2/LIB/12-ARRIAH	**97.6**	6/6

dpi – days post infection; pos/n – number of ELISA positive serum samples to total number of tested serum samples; results of tests with heterologous ELISA test-systems are given in italics, positive PI values are given in bold.

O-IZSLER and Asia1-IDVet did not detect anti-FMDV antibodies in convalescent animals. However, two serum samples from control pigs infected with aphthous serotype O FMD virus-containing suspension during vaccine tests were tested positive with heterologous A-PrioCHECK test-system (Table 3).

The cross reactions were observed in samples from pigs after their challenge with FMDV O 2212/Primorsky/2014 strain during tests of bivalent emulsion anti-FMD vaccine based on A 2155/Zabaikalsky/2013 and O 2212/Primorsky/2014 strains (Table 3). Six heterologous test-systems for serotypes Asia 1 и SAT 2 detected low cross reactivity to serotypes O and A or did not detect any cross reactivity to serotypes O and A, however, only two-test kits (Asia1-PrioCHECK and Asia1-IDVet) did not detect any anti-FMDV antibodies in sera from both vaccinated and control animals after challenge. The following interesting dependence was observed when sera were tested with test-systems for serotype A FMDV: the number of seropositive animals increased and the antibody level increased significantly 8 days after infection with FMD serotype O compared to that ones before infection, however, mean PI value in the test group was lower than that one in control (unvaccinated) animals. This could be the evidence of partial neutralization of serotype O FMDV with antibodies against serotype A FMDV, i.e. the evidence

of cross-protection. This was confirmed by results of VNT of FMDV A 2155/Zabaikalsky/2013 strain. Tables 3 and 4 show that number of animals demonstrating positive VNT reaction to serotype A FMD virus before and after infection remained unchanged but it was not possible to determine the proportion of cross-reactive VNAs since the animals were vaccinated against serotype A FMDV. But virus-specific antibodies of this category were detected in sera from control animals and protective VNA titre was 1:45 or 1.65 lg, similar to VNA titre detected when FMDV Asia-1 2356/14/Pakistan/2018 strain was used.

One animal in each of two groups of pigs vaccinated with the vaccine diluted 1:25 demonstrated systemic disease after challenge with aphthous virus. Therewith, no ELISA antibodies to serotype A FMDV were detected and threshold antibody levels to serotype O FMDV were detected using tested ELISA test-kits for serotype O, except for the FGBI "ARRIAH" test-kits for O 2356/Pakistan/2018, O 2311/Zabaikalsky/2016, O 2047/Saudi Arabia/2008, O/Kenya/2017 strains, in these two pigs before challenge. Virus neutralizing antibody titres against FMDV A 2155/Zabaikalsky/2013 and O 2212/Primorsky/2014 strains in

Table 3
ELISA and VNT results for serum samples from pigs vaccinated with bivalent vaccines against type A and O FMDV before and after challenge test using type O FMDV

Diagnostic test-system	Serum samples collected from pigs on					
	21 dpv (vaccination against serotype A and O FMDV)		29 dpv (vaccination against serotype A and O FMDV), 8 dpi (infection with serotype O FMDV)		8 dpi (infection with serotype O FMDV) non-vaccinated controls	
	PI_{mean}, %	pos/n	PI_{mean}, %	pos/n	PI_{mean}, %	pos/n
A/Zab/13-VNT	n/d	12/29	n/d	12/28	n/d	2/2
A/Zab/13-ARRIAH	39.6	13/28	**59.2**	18/28	***76.0***	*2/2*
A/TUR/06-ARRIAH	36.9	10/28	**57.9**	18/28	***75.1***	*2/2*
A_{22}/IRQ/64-ARRIAH	48.0	15/28	**67.6**	22/28	***80.7***	*2/2*
A/ARRIAH/15-ARRIAH	28.7	4/28	**54.1**	17/28	***73.2***	*2/2*
A/TAN/13-ARRIAH	38.2	9/28	**58.7**	17/28	***65.5***	*2/2*
A-PrioCHECK	36.0	3/28	41.8	9/28	***52.4***	*1/2*
A-IZSLER	**77.3**	23/29	**76.1**	21/28	***84.5***	*2/2*
A-IDVet	**51.54**	15/29	**58.52**	19/28	***78.6***	*2/2*
O/Prim/14-VNT	n/d	10/29	n/d	28/28	n/d	2/2
O/Prim/14-ARRIAH	**74.3**	26/28	**87.8**	28/28	**91.3**	2/2
O/Zab/16-ARRIAH	**53.1**	18/28	**76.8**	24/28	**90.0**	2/2
O/PAK/18-ARRIAH	**50.9**	16/28	**75.6**	23/28	**94.2**	2/2
O/SAU/08-ARRIAH	**51.9**	16/28	**73.4**	23/28	**89.8**	2/2
O/KEN/17-ARRIAH	45.3	12/28	**73.1**	21/28	**93.9**	2/2
O-PrioCHECK	**73.5**	25/28	**82.0**	27/28	**89.9**	2/2
O-IZSLER	58.6	5/29	**71.2**	16/28	**71.4**	2/2
O-IDVet	**63.4**	13/29	**77.9**	21/28	**77.7**	2/2
O-BIONOT	49.4	18/29	**73.1**	25/28	**80.1**	2/2
O-MEDIAN	**52.0**	23/29	**68.0**	27/28	**60.0**	2/2
Asia1/PAK/18-ARRIAH	*23.0*	*0/29*	*39.9*	*6/28*	***78.5***	*2/2*
Asia1/TAJ/11-ARRIAH	*28.7*	*3/29*	*50.3*	*13/28*	***80.3***	*2/2*
Asia1/Shamir/89-ARRIAH	*29.4*	*1/29*	*47.9*	*13/28*	***76.2***	*2/2*
Asia1-PrioCHECK	*21.5*	*0/29*	*24.8*	*0/28*	*35.9*	*0/2*
Asia1-IDVet	*6.6*	*0/29*	*5.8*	*0/28*	*2.0*	*0/2*
SAT2/LIB/12-ARRIAH	*6.8*	*0/29*	*19.0*	*4/28*	***61.6***	*2/2*

dpv – days post vaccination; dpi – days post infection; pos/n – number of ELISA positive serum samples to total number of tested serum samples; n/d – not detected; results of tests with heterologous ELISA test-systems are given in italics, positive PI values are given in bold.

the serum samples were 1.08 and 0.78 lg, respectively, that was lower than protective level (data are not provided). At the same time, serum samples from challenged control pigs were tested positive by 22 out of 24 ELISA test-kits and 7 out of 14 VNT both serotype O-homologous and serotype O-heterologous ones (Tables 3 and 4).

Our studies have detected and repeatedly confirmed the presence of cross-reactive antibodies in sera tested with ELISA and, to a lesser extent, with VNT. In our opinion, this could be accounted for as follows:

– presence of conservative epitopes in VP1–VP3 capsid polypeptide amino acid sequences;

– accessibility of internal conservative epitopes of VP4 polypeptide for the animal's immune system due to presence of 12S and 5S subunits in the vaccine, during FMDV replication or vaccine antigen (virus) degradation in the animal body during immunity development.

The diagnostic kits used in our study had their own distinctive features. First, they differed in competitive antibody origin. Monoclonal antibodies are used as competitive ones in foreign test-systems (Prionics, IZSLER, Innovative Diagnostics, BIONOTE and MEDIAN Diagnostics), the FGBI "ARRIAH" produces the test-kits based on polyclonal antibodies. Serotype-specificity is the main goal of test system developers for any test variant, the serotype specificity is determined by surface capsid epitopes. For this purpose, laboratory animals being antibody donors are immunized with FMDV intact antigen that mainly consists of 146S subunits. In the FGBI "ARRIAH" highly purified FMDV antigen subjected to sucrose density gradient fractionation in the form of the intact capsids not divided into smaller subunits is used for preparation of hyperimmune polyclonal rabbit and guinea pig serum for generating capture and detection antibodies, respectively. Thus, the probability of generating antibodies to internal highly conserved epitopes is low. However, according to the publications of some researchers, surface polypeptides also have conservative antigenic determinants inducing cross-reactive VNAs [4, 5].

Table 4
Results of testing of serum samples from control animals ($n = 2$) infected with FMDV 02122/Primorsky/2014 strain with VNT (8 dpi)

FMDV strain	Virus dose, $TCID_{50}/0.05\ cm^3$	T_{mean} VNA, lg
A 2029/Turkey/2006	$10^{1.58}$	*1.08*
A 2155/Zabaikalsky/2013	$10^{1.46}$	***1.65***
A 2269/ARRIAH/2015	$10^{1.76}$	*0.98*
A_{22}/Iraq/24/64	$10^{1.69}$	*1.34*
A/Tanzania/2013	$10^{1.5}$	*1.34*
O 2356/Pakistan/2018	$10^{1.69}$	**2.56**
O 2311/Zabaikalsky/2016	$10^{1.58}$	**2.03**
O 2212/Primorsky/2014	$10^{1.76}$	**2.26**
O 2047/Saudi Arabia/2008	$10^{2.06}$	**2.10**
O/Kenya/2017	$10^{1.8}$	**2.40**
Asia-1 1946/Shamir/Israel/3/89	$10^{1.5}$	*1.20*
Asia-1 2145/Tajikistan/2011	$10^{1.69}$	*1.08*
Asia-1 2356/14/Pakistan/2018	$10^{1.69}$	***1.65***
SAT2/LIB/39/2012	$10^{1.76}$	*1.08*

T_{mean} VNA – mean titre of virus neutralizing antibodies; results of tests with heterologous VNT are given in italics, protective level of virus neutralizing antibodies is given in bold.

He Y. et al. [4] detected and identified serotype O/A cross-reactive neutralizing antibodies (R50) in cattle with single B cell antibody isolation technique as well as determined virus-NAb complex structures with cryo-electron microscopy. R50 were shown to engage FMDV-O capsids as well as FMDV-A capsids and to bind to BC/EF/GH-loop of VP1 polypeptide and to GH-loop of VP3 polypeptide, revealing a previously unknown antigenic site. The cross-serotype neutralizing epitope recognized by R50 is highly conserved among serotypes O/A.

To illustrate this, 14 amino acid sequences up to 214 a. a. in length, of VP1, one of surface proteins, of FMDV of different serotypes were analyzed in order to determine the probability of conservative epitope presence [9, 10, 11, 12, 13, 14, 15, 16, 17]. The sequences were aligned with FMDV A/TAN/26/2013 strain (GenBank: AXI68858.1). Figure 2 shows that this probability exists, since conservative sites with a minimum number of substitutions are found.

Putative epitopes of surface polypeptides were assessed for their immunogenicity during serotyping the intact antigen of different FMDV strains with ELISA variants: sandwich ELISA with capture and detection polyclonal antibodies, direct ELISA with horseradish peroxidase-conjugated monoclonal antibodies (Tables 5 and 6).

Analysis of obtained data shows that FMDV intact antigen at concentration of 0.025 mg/mL was distinctly serotyped by most of the ELISAs using both polyclonal and monoclonal antibodies. Two direct ELISAs with conjugates detected cross-reaction: A-IZSLER (serotype SAT2/LIB/12 antigen) and A-IDVet (serotype Asia 1 antigen), these findings together with cross-reaction detected with VNT confirmed, in our opinion, the presence of conservative epitopes in surface polypeptides. These epitopes appeared to be less immunogenic as compared to loop-regions (Figure 2) due to their location on capsid surface. Protruding loop regions of amino acid sequence induce primary immune response. The cross-reactive antibody response detected with both ELISA as well as with VNT in convalescent animals was lower than homologous (serotype-specific response) response (Tables 2, 3 and 4) the similar results were obtained when sera from vaccinated animals were tested with the ELISAs (Figure 1).

The performed tests allow us to conclude that parallel tests with the test-systems designed for different FMDV serotypes are required for determination of serotype-specificity of anti-FMDV antibodies in sera from infected animals. The serotype should be determined based on predominant results. Cross-reactive antibody level is not important for post-vaccination immunity level, since this category of antibodies, regardless of the cause of their appearance, is virus-specific and indicates the animal's immune system state. The level of the animal protection from infection is directly proportional to the total number of virus-specific antibodies that are predominantly VNAs due to the structural features of the virion.

Table 7 shows responses of two cattle (animal No. 1 and animal No. 2) to administration of the pilot

A/TAN/26/2013	1	ttatgesadp	vtttvenygg	etqvqrrhht	svefimdrfv	klgvsspthv	idlmqthqhg
A_{22}/IRQ/24/64	1	--t-------	----------	----------	d-t-t-----	-i-nln----	----------
A/TUR/2/2014	1	--ta------	----------	---a------	d-g-------	-inpv-----	---------a
A/TUR/2006	1	--ta------	----------	---a------	d-g------a	-ispv-----	---------a
A/PAK/2013	1	--ta------	----------	---a------	d-g-v-----	-inpv-----	---------a
O, wild type of FMDV	1	-s--------	--a-------	----------	d-s--l----	-vtpkdsin-	l-----ps-t
O/TUR/33/2011	1	--s-------	--a-------	v---------	d-s--l----	-vtpkdqin-	l-----pa-t
O/PAK/2019	1	--s-------	----------	----------	d-s--l----	-vtpkdqin-	l-----pa-t
Asia1/BAN/DH/2018	1	----------	----------	---ta--l--	d-a-vl----	--nepks-q-	l----ipa-t
Asia1/ISB/Pak_2012	1	--tv------	----------	---aa--l--	d-g-vl----	--tnpka-qt	l----ipp-t
Asia1/Shamir/89	1	--t-------	----------	---ta--l--	d-a--l----	--tapkniqt	l----ips-t
C1/Loupogne BEL/53	1	----------	----------	----------	d-a-vl----	-vt--gnq-t	l-v--a-kdn
SAT2/LIB/39/2012	1	--sa--g--v	---dpsth--	nvqeg--k--	e-a-ll--st	hvhtgktsf-	v---n-kka
SAT2/EGY/Ismailia/2018	1	--sa--g--v	---dpsth--	nvqeg--k--	e-a-ll--st	hvhtgktsf-	v---n-keka
A/TAN/26/2013	61	lvgallraat	yyfsdlevvv	rhegnltwvp	ngapeaalan	tsnptayhke	pftrlalpyt
A_{22}/IRQ/24/64	61	----------	-------i--	--d-------	--------s-	-g-----l-a	----------
A/TUR/2/2014	61	----------	-------i--	----------	-----g--v-	----------	----------
A/TUR/2006	61	----------	-------i--	--d-------	----ve----	---------q	----------
A/PAK/2013	61	----------	-------i--	--d-------	----vg----	---------q	----------
O, wild type of FMDV	61	-------t--	---a-----	k-k-d-----	----v---d-	-t-------a	-l--------
O/TUR/33/2011	61	-------t--	---a-----	k---------	--------d-	-t-------a	-l--------
O/PAK/2019	61	-------t--	---a-----	k---------	--------d-	-t-------a	-l--------
Asia1/BAN/DH/2018	61	-------s--	--------al	v-t-pa----	--s-kt--d-	qt-----q-q	-i--------
Asia1/ISB/Pak_2012	61	-------s--	--------al	v-t-pv----	----kt--dc	qt-----q-q	-i--------
Asia1/Shamir/89	61	-------s--	--------al	v-a-pv----	----kd--n-	qt-----q-k	-i--------
C1/Loupogne BEL/53	61	i---------	-------ia-	t-t-k-----	----vs--d-	-t-------g	-l--------
SAT2/LIB/39/2012	61	----i---s-	---c---iac	vgdhtrvfwq	pngaprttqp	gd--mvfa-g	gv--f-i-f-
SAT2/EGY/Ismailia/2018	61	----i---s-	---c---iac	vg-htrvfwq	pngapr-tql	gd--mvfa-g	gv--f-i-f-
A/TAN/26/2013	121	aphrvlatvy	ngtskysaat	sgrrgdlgsl	aarvaaqlpa	sfnygalrat	tihellvrmk
A_{22}/IRQ/24/64	121	----------	--------gg	t-------p-	-----------	---f--iq--	----------
A/TUR/2/2014	121	----------	--v----ttg	g---------	---------s	---f--i---	n---------
A/TUR/2006	121	----------	--v----ttg	n-------p-	---------s	---f--i---	----------
A/PAK/2013	121	----------	--v----tts	g---------	---------g	---f--ik--	----------
O, wild type of FMDV	121	----------	---c--aegs	lpnvrgdlqv	l-qk--rplp	tsfnygaika	-rvte-lyrm
O/TUR/33/2011	121	----------	--nc--gesh	tanvrgdlqv	l-qk--rtlp	tsfnygaika	-rvse-lyrm
O/PAK/2019	121	----------	--nc--geg-	vtnvrgdlqv	l-qk--rtlp	tsfnygaika	-rvte-lyrm
Asia1/BAN/DH/2018	121	----------	--ktt-get-	-r-gdlaala	qrvsrqlpts	fnygavkaen	itel-irmkr
Asia1/ISB/Pak/2012	121	----------	--kta-gqe-	pr-gdlaaia	qrvstslpts	fnygavkadn	itel-irmkr
Asia1/Shamir/89	121	----------	--kta-get-	sr-gdmaala	qrls-rlpts	fnygavkad-	itel-irmkr
C1/Loupogne BEL/53	121	--------a-	t--tt-t-s-	r-dlah-aat	h--hlptsfn	fgavk-eti-	ellvrmk-ae
SAT2/LIB/39/2012	121	----l-s---	--ecd-nktv	tai---raa-	--ky-dntht	lpstfnfgfv	-vdkpvdvyy
SAT2/EGY/Ismailia/2018	121	----l-s---	--ecv-kkpv	asi---raa-	--ky-dstht	lpptfnfgfv	-vdkpvdvyy
A/TAN/26/2013	181	raelycprpl	latevsager	ykqkiiapak	qll		
A_{22}/IRQ/24/64	181	----------	------sqd-	h---------	---		
A/TUR/2/2014	181	----------	------sqd-	h---------	---		
A/TUR/2006	181	----------	-----lsqd-	h---------	---		
A/PAK/2013	181	----------	------sqd-	h--q------	-		
O, wild type of FMDV	181	kraetycprp	-lavhpsaa-	h----v----			
O/TUR/33/2011	181	kraetycprp	-laihpnea-	h----v----	--		
O/PAK/2019	181	kraetycprp	-laihpe-a-	h----v----			
Asia1/BAN/DH/2018	181	aetycprpl-	aldttqdrrk	qeiiapekqv	l		
Asia1/ISB/Pak_2012	181	aetycprpl-	aldttqdrrk	qeiiapekq			
Asia1/Shamir/89	181	aetycprpl-	aldttqdrrk	q-iiapekqv	lnf		
C1/Loupogne BEL/53	181	lycprpil-i	qp-gdrhkqp	lvapakqll			
SAT2/LIB/39/2012	181	rmkraelycp	rpllptydha	grdrfd--ig	verq		
SAT2/EGY/Ismailia/2018	181	rmkraelycp	rpllpaydha	grdrfd--ig	verq		

Fig. 2. Amino acid sequence of surface VP1 polypeptide of FMDV of different serotypes, 214 a. a. (according to the GenBank NCBI data; GH-loop region is indicated in red)

monovalent anti-FMD vaccine based on Asia-1 1946/Shamir/Israel/3/89 strain. Clinically healthy same-aged young bulls kept under similar conditions demonstrated different patterns of immune response development. If sera from both animals tested with ELISA on day 14 after their vaccination reacted only to serotype Asia 1, then on day 23 after vaccination antibodies were detected in serum from animal No. 1 with 7 out of 8 ELISA test-kits for serotype A and with 3 out of 10 ELISA test-kits for serotype O whereas the serum from animal No. 2 remained serotype-specific. Therewith, the level of specific antibodies against serotype Asia 1 FMDV was the same in both animals. This could be accounted for by different rate of the immunity development in the bulls capable of provoking destruction of the injected vaccine antigen. According to our observations, the level of humoral immunity, as a rule, reaches a peak on the 21st day after vaccination and achieves a plateau, over time (observation period: 14–45 days after vaccination), the number of serotype-specific antibodies practically remains unchanged or the changes are insignificant, and cross-reactive antibodies show stable dynamics (data are not provided).

When the antigen with the structure disrupted for various reasons is used for immunization, a large-scale cross-reaction of virus-specific antibodies can occur which makes serotyping with ELISA difficult.

Thus, cattle sera collected on day 21 after their vaccination against FMD with the vaccine based on FMDV O 2620/Orenburg/2021 strain demonstrated high cross

Table 5
Results of serotyping of FMDV intact antigen with sandwich ELISA

146S-FMDV Ag specimens (C ≈ 0.025 mg/mL)	Sandwich ELISA (FGBI "ARRIAH") for detection of FMDV Ag of the virus serotype:							
	A		O				Asia 1	SAT 2
	A/Zab/13	A/TUR/06	O/Zab/16	O/PAK/18	O/SAU/08	O/Prim/14	Asia1/Shamir/89	SAT2/LIB/12
A/Zab/13	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
A/TUR/06	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
A/ARRIAH/15	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
A_{22}/IRQ/64	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
O/Zab/16	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/PAK/18	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/SAU/08	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/Prim/14	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/KEN/17	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
Asia1/Shamir/89	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**	neg.
Asia1/TAJ/11	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**	neg.
Asia1/PAK/18	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**	neg.
SAT2/LIB/12	neg.	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**

FMDV Ag – antigen of FMDV.

Table 6
Results of FMDV intact antigen serotyping with direct ELISA using commercial monoclonal antibody-horse radish peroxidase conjugates

146S-FMDV Ag specimens (C ≈ 0.025 mg/mL)	Direct ELISA with commercial conjugate for detection of FMDV Ag										
	PrioCHECK		IZSLER				IDVet			BIONOTE	MEDIAN
	A	O	A	O	Asia 1	SAT 2	A	O	Asia 1	O	O
A/Zab/13	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
A/TUR/06	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
A/ARRIAH/15	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
A_{22}/IRQ/64	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
O/Zab/16	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	**pos.**
O/PAK/18	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.
O/SAU/08	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	neg.	neg.	**pos.**	**pos.**
O/Prim/14	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.
O/KEN/17	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	**pos.**
Asia1/Shamir/89	neg.	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.	**pos.**	neg.	neg.
Asia1/TAJ/11	neg.	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.	**pos.**	neg.	neg.
Asia1/PAK/18	neg.	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.	**pos.**	neg.	neg.
SAT2/LIB/12	neg.	neg.	**pos.**	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.	neg.

FMDV Ag – antigen of FMDV.

reactivity when tested with 11 out of 13 ELISA test-kits for detection of antibodies to structural proteins of serotype A, Asia 1 and SAT 2 FMDV comparable to homologous reaction and low response when tested with other two test-kits (A-PrioCHECK, Asia1-PrioCHECK), Table 7. In this case, the cross-reactivity was caused by disruption of capsid antigen integrity that resulted in development of large numbers of antibodies against internal conservative epitopes of VP4 polypeptide.

Electrophoretic analysis of the antigen-containing material used for the pilot vaccine preparation showed the cleavage of VP1–VP3 structural polypeptide molecules.

The non-disrupted 146S-Ag of FMDV Asia-1 2145/Tajikistan/2011 strain (intact antigen, band 1) is given for comparison (Figure 3).

However, the VNAs at the amount of 2.70 lg (VNA titre – 1:512), determined by VNT for FMDV O 2620/Orenburg/2021 strain was sufficient to protect from the infection (Table 8). When the same serum sample was tested with VNT for cross virus neutralizing activity, serum neutralization of FMDV A 2155/Zabaikalsky/2013 strain was observed up to final serum dilution of 1:128 (2.10 lg). At the same time, VN testing of the serum against O 2356/Pakistan/2018 showed significant cross-serotype neutralization of FMDV SAT2/LIB/39/2012 strain (1.65 lg).

Thus, the altered vaccine antigen structure did not interfere with the immune response development, however, it could hamper ELISA testing of the vaccine for their protective properties.

Nevertheless, this must be avoided during large-scale vaccine production through strict control of the vaccine components. The above example was used for better understanding of possible causes of cross-reactivity in ELISA.

Our study results are consistent with the data published by the Pirbright Institute (Great Britain) [3]. Testing of 294 monovalent mainly bovine sera collected following infection, vaccination, or vaccination and infection with one of five FMDV serotypes showed that over half of the samples, representing all three immunization categories, were positive to at least one heterologous serotype and some were positive to all serotypes tested. Preliminary studies with stabilized recombinant capsid antigens of serotypes O and A that did not detect internal epitopes showed reduced cross-reactivity of serum samples, supporting the hypothesis that capsid integrity can affect the serotype-specificity of the SP-ELISAs. The residual

Table 7
Results of ELISA tests of serum samples from cattle immunized with monovalent vaccine against type O and Asia 1 FMDV

Diagnostic test-system	Sera from cattle, PI_{mean} (%)				
	21 dpv O/Orenburg/21	animal No. 1 Asia1/Shamir/89		animal No. 2 Asia1/Shamir/89	
		14 dpv	23 dpv	14 dpv	23 dpv
A/Zab/13-ARRIAH	***97.1***	*35.4*	***82.2***	*21.7*	*17.3*
A/TUR/06-ARRIAH	***93.8***	*6.5*	***63.0***	*1.1*	*22.3*
A_{22}/IRQ/64-ARRIAH	***89.2***	*36.4*	***68.8***	*3.8*	*6.56*
A/ARRIAH/15-ARRIAH	***90.1***	*4.4*	*40.5*	*23.3*	*25.9*
A/TAN/13-ARRIAH	n/t	*3.1*	***81.5***	*8.7*	*37.3*
A-PrioCHECK	***58.3***	*23.3*	***63.8***	*17.9*	*35.1*
A-IZSLER	***95.8***	***71.0***	***81.6***	*67.4*	*70.3*
A-IDVet	***97.7***	*35.6*	***81.4***	*36.6*	*34.3*
O/Prim/14-ARRIAH	**96.6**	*48.3*	***58.4***	*31.2*	*39.5*
O/Zab/16-ARRIAH	**95.9**	*16.7*	*38.3*	*6.4*	*14.7*
O/PAK/18-ARRIAH	**96.1**	*35.2*	*33.7*	*12.4*	*18.6*
O/SAU/08-ARRIAH	**96.0**	*34.5*	***68.2***	*22.8*	*25.2*
O/KEN/17-ARRIAH	n/t	*2.1*	*3.3*	*5.3*	*7.1*
O-PrioCHECK	**97.3**	*40.5*	*48.4*	*23.9*	*25.5*
O-IZSLER	**95.8**	*56.1*	*41.5*	*38.6*	*55.6*
O-IDVet	**85.9**	*4.2*	*1.0*	*–26.6*	*–43.6*
O-BIONOT	**98.9**	*32.0*	*39.7*	*21.0*	*22.9*
O-MEDIAN	**90.0**	*40.0*	***54.0***	*30.0*	*20.0*
Asia1/PAK/18-ARRIAH	***92.2***	**67.3**	**84.6**	**63.7**	**84.5**
Asia1/TAJ/11-ARRIAH	***88.7***	**62.3**	**72.1**	**61.6**	**71.5**
Asia1/Shamir/89-ARRIAH	***90.6***	**72.8**	**90.8**	**71.9**	**89.9**
Asia1-PrioCHECK	*47.1*	**74.8**	**80.4**	**74.2**	**81.7**
Asia1-IDVet	***81.9***	**85.2**	**88.9**	**78.9**	**75.9**
SAT2/LIB/12-ARRIAH	***81.5***	n/t	n/t	n/t	n/t

dpv – days post vaccination; n/t – not tested; results of tests with heterologous ELISA test-systems are given in italics, positive PI values are given in bold.

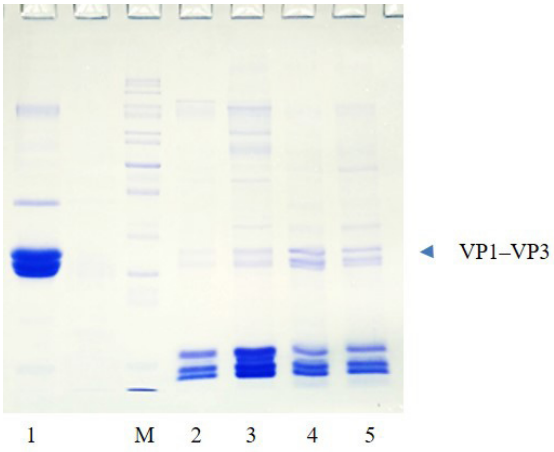

Fig. 3. Polyacrylamide gel (12%) electrophoresis of FMDV antigen specimens:
M – protein marker, 10–200 kDa;
1 – 146S-Ag Asia1/TAJ/11 (intact antigen);
2 – Ag_{UCF35} O/Orenburg/21;
3 – Ag_{PRECIP} O/Orenburg/21;
4 – Ag_{UCF35} Asia1/PAK/18;
5 – Ag_{PRECIP} Asia1/PAK/18.
Ag_{UCF35} – antigen concentrated with 35% sucrose gradient ultracentrifugation (UCF);
Ag_{PRECIP} – antigen precipitated with 8% polyethylene glycol 6,000

Table 8
Results of tests of serum samples from cattle vaccinated with anti-FMD vaccine based on O 2620/Orenburgsky/2021 or O 2356/Pakistan/2018 strain with neutralization test using homologous and heterologous FMDV serotypes

FMDV strain	Virus dose, $TCID_{50}$/0.05 cm^3	T_{mean} VNA, lg	
		O/Orenburg/21	O/PAK/18
A 2155/Zabaikalsky/2013	$10^{1.46}$	***2.10***	*1.38*
O 2356/Pakistan/2018	$10^{1.35}$	**≥ 2.86**	**3.00**
O 2620/Orenburg/2021	$10^{1.88}$	**2.70**	**1.70**
Asia-1 1946/Shamir/Israel/3/89	$10^{2.29}$	*≤ 0.78*	*1.08*
SAT2/LIB/39/2012	$10^{1.16}$	*0.90*	***1.65***

T_{mean} VNA – mean titre of virus neutralizing antibodies; results of tests with heterologous VNT are given in italics, protective level of virus-neutralizing antibodies is given in bold.

cross-reactivity associated with capsid surface epitopes was consistent with the evidence of cross-serotype virus neutralization.

CONCLUSION

Thus, all ELISA diagnostic test-kits for detection of antibodies to FMDV structural proteins produced by different manufacturers: FBGI "ARRIAH" (Russia), Prionics (Netherland/Switzerland), IZSLER & The Pirbright Institute (Italy/Great Britain), Innovative Diagnostics (France) BIONOTE (South Korea), MEDIAN Diagnostics (South Korea) were shown to detect some cross-FMDV serotype reactive antibodies. Such cross-reactive antibodies could develop due to the following: presence of conservative epitopes in FMDV surface polypeptide amino acid sequence, disruption of the capsid integrity provoking development of antibodies against internal highly conservative polypeptide. Nevertheless, the analysis of a large data set (about 3,500 publications) showed that homologous serotype-specific reaction in general was significantly higher and predominant, the proportion of virus-specific non-protective antibodies, including cross-reactive ones, was not significant and did not distort the results of ELISA tests of anti-FMD vaccine for its immunogenicity. In exceptional cases, the test results require confirmation with other serological tests. Complex tests for FMDV using different diagnostic methods such as enzyme-linked immunosorbent assay (ELISA) with standard and reference test-systems and/or virus neutralization test in cell culture are to be considered as the best option.

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Construction of prokaryotic system for expression of porcine circovirus type 2 *ORF-2* gene fragment

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ABSTRACT

Porcine circovirus-associated diseases (PCVDs) are among the most significant challenges for pig farming in developed countries. Porcine circovirus type 2 (PCV-2) is considered the main etiological agent of postweaning multisystemic wasting syndrome in piglets. Mass PCVD occurrence has been reported in most regions of the world, that results in serious economic consequences. Optimal PCVD prevention is known to be achieved through a set of veterinary and sanitary measures in combination with vaccination. High evolutionary virus variability facilitating new genotype and strain emergence requires development of new candidate recombinant vaccines against PCV-2 infection. The study was aimed at construction of prokaryotic system for PCV-2 *ORF-2* gene fragment expression and its functionality assessment. A genetic insert constructed from the most immunogenic type-specific PCV-2 epitopes based on genotype 2a, 2b, 2d strain and isolate consensus sequence was cloned into the expression vector pET-22b(+) that was incorporated into the *Escherichia coli* strain Rosetta 2(DE3). The transformants were selected based on the marker gene of ampicillin resistance on a selective medium. Target gene expression was induced by adding of isopropyl-*β*-D-1-thiogalactopyranoside at different concentrations. As a result, *Escherichia coli* Rosetta 2(DE3)/pET-22b-ORF-2 strain, a producer of capsid protein fragment (92–233 amino acid residues), was constructed. It was found that in the presence of 1 mM isopropyl-*β*-D-1-thiogalactopyranoside, the expression level of soluble truncated rCap was 35–40 mg/L 6 hours after induction. The expression product was tested for its specificity with indirect ELISA using whole-virion PCV-2-hyperimmunized porcine serum. It was shown that the positivity coefficient of producer strain cell lysates averaged to 4.34 ($p < 0.005$). The recombinant rCap protein is suitable for serological diagnosis and is also of interest as a vaccine component, which is the goal of our further studies.

Keywords: porcine circovirus type 2, postweaning multisystemic wasting sindrome, *ORF-2*, prokaryotic expression system

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Конструирование прокариотической системы экспрессии фрагмента гена *ORF-2* цирковируса свиней 2-го типа

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РЕЗЮМЕ

Цирковирусные болезни свиней на сегодняшний день являются одной из наиболее значимых проблем свиноводства в развитых странах. Цирковирус свиней 2-го типа (ЦВС-2) считается основным этиологическим агентом синдрома мультисистемного послеотъемного истощения поросят. Случаи массовой заболеваемости свиней цирковирусными болезнями зарегистрированы в большинстве регионов мира, что влечет за собой серьезные экономические последствия. Известно, что оптимальный профилактический эффект в отношении данных инфекций достигается за счет проведения комплекса ветеринарно-санитарных мероприятий в сочетании с вакцинацией. Учитывая высокую эволюционную изменчивость вируса, способствующую появлению новых генотипов и штаммов, вопрос разработки новых кандидатных рекомбинантных вакцин против цирковирусной инфекции, вызванной ЦВС-2, остается

открытым. Целями настоящего исследования явились конструирование прокариотической системы экспрессии фрагмента гена *ORF-2* ЦВС-2 и оценка ее функциональности. Генетическая вставка, сконструированная из наиболее иммуногенных типоспецифических эпитопов ЦВС-2 на основании консенсусной последовательности штаммов и изолятов генотипов 2a, 2b, 2d, клонирована в экспрессионный вектор pET-22b(+), который был реципиирован в штамм *Escherichia coli* Rosetta 2(DE3). Отбор трансформантов осуществляли на селективной среде по маркерному гену устойчивости к ампициллину. Индукцию экспрессии таргетного гена проводили внесением различных концентраций изопропил-*β*-D-1-тиогалактопиранозида. В результате исследований был сконструирован штамм *Escherichia coli* Rosetta 2(DE3)/pET-22b-ORF-2 – продуцент фрагмента капсидного белка (92–233 а. о.). Установлено, что в присутствии 1 мМ изопропил-*β*-D-1-тиогалактопиранозида уровень экспрессии растворимого укороченного rCap достигает 35–40 мг/л через 6 ч постиндукции. Специфичность продукта экспрессии оценивали в непрямом иммуноферментном анализе с сывороткой крови свиньи, гипериммунизированной цельновирионным ЦВС-2. Было показано, что коэффициент позитивности лизатов клеток штамма-продуцента составлял в среднем 4,34 ($p < 0,005$). Рекомбинантный белок rCap пригоден для целей серологической диагностики, а также представляет интерес в качестве компонента вакцины, что является целью наших дальнейших изысканий.

Ключевые слова: цирковирус свиней 2-го типа, синдром мультисистемного послеотъемного истощения, *ORF-2*, прокариотическая система экспрессии

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INTRODUCTION

Today, porcine circovirus-associated diseases (PCVDs) are among the most significant challenges for pig farming in developed countries. Porcine circovirus type 2 (PCV2) is considered the main etiological agent of post-weaning multisystemic wasting syndrome in piglets and also involved in porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex and infectious congenital tremor of piglets [1]. Porcine circovirus-associated diseases can manifest as subclinical and clinical infections, while subclinical infection is the most common form of the infectious process caused by PCV2, and manifested by reduced average daily weight gain without specific clinical signs [2]. The systemic disease mainly affects piglets aged 6–15 weeks and is characterized by anorexia, dyspeptic disorders and lymphadenopathy [3]. Events of high morbidity of pigs caused by circovirus infections have been reported in most regions of the world that results in serious economic consequences for the pig industry [4, 5, 6, 7].

Porcine circovirus type 2 belonging to the *Circovirus* genus of *Circoviridae* family is a small icosahedral non-enveloped DNA-containing virus with a circular genome that is 1,766–1,768 bp long [8]. There are 4 known types of PCV showing high nucleotide identity (68–76%) and similar genomic organization [9, 10]. The genomic DNA of PCV2 consists of several open reading frames encoding main viral proteins – *ORF-1* (replicative protein), *ORF-2* (capsid protein), *ORF-3* (apoptotic protein), *ORF-4* (apoptosis inhibitor) [11]. *ORF-2* encoding a capsid protein with a molecular weight of ≈30 kDa is of the greatest interest for development of tools for specific prevention and diagnosis of diseases caused by PCV2 [12]. To date, 5 main PCV2 genotypes have been identified: 2a, 2b, 2c, 2d, 2e; over the past 20 years 2a, 2b and 2d genotypes have been predominant in different regions of the world [13].

Optimal PCVD prevention is known to be achieved through a set of veterinary and sanitary measures in combination with vaccination. Currently, two vaccines against circovirus infection (PCV2): "Circostop" whole-virion inactivated vaccine (Shchelkovo Biocombinat, Russia) and "VERRES-CIRCO" recombinant vaccine based on *ORF-2* protein produced in the baculovirus expression system (Vetbiochim-Russia), are available on the Russian market [14]. However, considering high evolutionary PCV2 variability promoting potential emergence of different genotypes and strains [15], it can be stated that development of new candidate recombinant vaccines conferring cross-protection against strains of different virus genotypes requires further discussion. An analysis of commercial vaccines available in the world has shown that vaccines based on the recombinant Cap protein produced in baculovirus expression system are successfully used. However, use of this eukaryotic system is often time-consuming and expensive [16], and therefore it is very important to produce a vaccine protein using other expression tools.

The study was aimed at construction of prokaryotic system for PCV2 *ORF-2* gene fragment expression and its functionality assessment.

MATERIALS AND METHODS

Strains and plasmids. To generate a consensus sequence of *ORF-2* gene fragment, the following nucleotide sequences of PCV2 genotype 2a, 2b and 2d epizootiologically significant strain and isolate genomes deposited in the GenBank database of the National Center for Biotechnological Information (NCBI) were used:

– 2a genotype: IAF2897 (ID AF408635.1), ID AY094619.1, DE99/2014 (ID MW262923.1), AUT-1 (ID AY424401.1), SPA1 (ID AF201308.1), 212 (ID AY256455.1);

– 2b genotype: QZ0401 (ID AY691169.1), NL_Control_1 (ID AY484407.1), 24657 NL (ID AF201897.1), n10eu (ID DQ629116.1), ADDLPP 10069 (ID EU594437.1), DE1054/2014 (ID MW262924.1), AUT5 (ID AY424405.1);

– 2d genotype: 28031_Mantova_32_13/12/2013 (ID KP231140.1), Uy99 (ID KP867050.1), BDH (ID HM038017.1), DE222-13 (ID KP698398.1).

Expression pET-22b(+) vector (Novagen, Germany) was used for the target gene cloning and the following *Escherichia coli* Rosetta 2(DE3) strain was used for the target gene expression: F-*omp*T *hsd* SB (rB- mB-) *gal dcm* (DE3) pRARE2 (CamR) (Novagen, Germany), kindly provided by R. F. Khairullin, Cand. Sci. (Biology).

Antigenic construction design. PCV2 amino acid sequences were analyzed using the Immune Epitope Database (IEDB, USA) resource; density of B-cell epitopes was the main criterion for selection of the fragment for expression. BLAST-analysis was used for searching for homologous amino acid sequences of epizootiologically significant PCV2 strains and isolates. Physical and chemical properties of truncated *ORF-2* gene were predicted using Peptide Property Calculator (Innovagen AB, Sweden), homologous modelling of three-dimensional protein structure was performed with SWISS-MODEL webserver (SIB, Switzerland). Truncated *ORF-2* gene sequence (276–699 bp) was optimized by codons for expression into *E. coli* without changes in its amino acid composition and synthesized by the external institution (Eurogene, Russia) and then cloned in pET-22b vector at BamHI and EcoRI restriction sites. Presence of the whole gene in the vector was confirmed by sequencing. Recipient strain cells were transformed by heat-shock followed by further selection on agar medium supplemented with antibiotics (ampicillin – 200 μg/mL, chloramphenicol – 34 μg/mL).

Expression of rORF-2. To induce target gene expression, *E. coli* Rosetta 2(DE3)/pET-22b/ORF-2 strain cells were cultivated in Luria – Bertani nutrient medium containing ampicillin and chloramphenicol in ES-20 temperature-controlled shaker (Biosan, Latvia) at temperature of +37 °C and 180 rpm to achieve optical density (OD) of 0.7. Gene expression was induced by adding of isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) (Promega, USA) at concentration of 1 мМ, then cells were cultivated for 5–6 hours. Cell lysates were generated by mechanical disintegration of the biomass in L-buffer (50 мМ tris-HCl, pH 8.0; 0.3% KCl; 2 мМ PMSF); the target products were subjected to initial purification using NEBB-10 bacterial protein extraction kit (Diaem, Russia). The protein concentration was determined with M. Bradford method [17]. The cell lysates were tested for mature recombinant rCap protein with analytical disc electrophoresis in 15% polyacrylamide gel with Coomassie G250 stain.

Indirect enzyme-linked immunosorbent assay (iELISA). rCap was examined for its serological activity with iELISA. For this purpose, immunoglobulins G were purified from the serum prepared by hyperimmunization of pigs with PCV2 (Shchelkovo Biocombinat, Russia) by one-step ion exchange chromatography on DEAE cellulose [18], and then used for coating of medium-binding polystyrene plates (Corning, USA), 50 ng per well, and the plates were incubated at a temperature of +4 °C for 16 hours. Then, the plates were washed thrice with Tween-containing phosphate-buffered solution (T-PBS) and cell lysates diluted at 1:10 with PBS were added to the wells and the plates were incubated at temperature of +37 °C for 1 hour. The cell lysate transformed by pET-22b plasmid without insertion was used as negative control, recombinant E2 antigen of classical swine fever virus produced in similar expression system was used as heterologous control. After washing, specific peroxidase conjugate prepared using modified P. K. Nakane and A. Kawaoi method [19]

Table
The main epitopes of Cap protein included in the antigenic composition

Epitope (sequence, name)	Position in proteome, a. a.	Characteristics
RPWLVHPRHRY (external core epitope)	26–36	Conformational epitope that reacted with PCV-positive porcine sera; it is considered one of the predominant domains based on its PCV2 neutralizing capacity [20]
TRLSRTFGYTVK (P100)	47–58	Peptides demonstrated high binding reactivity with sera from PCV2-immune pigs. It is reported that they can be used for differentiation of vaccinated and convalescent animals [21]
PFEYYRIRKVKVEFWP (P102)	92–107	
CSPITQGDRGVGSSAVILDDNFVT KATALTY (C2)	108–137	
VTMYVQFREFNLKDPPLKP (P106)	215–233	
KATALT (EF-region)	134–139	It was identified as type-specific neutralizing epitope. It was shown to be used for differentiation of pathogenic PCV2 and non-pathogenic PCV1 [22]
YHSRYFT	156–162	Conformational epitope fragment was recognized by PCV1- and PCV2-neutralizing monoclonal antibodies; was detected in transfected PK-15 cells [23]
VLDSTIDYFQPNNKR	166–180	The epitope included in the candidate vaccine based on infectious PCV2b clone prevented virus replication in experimentally infected pigs [24]
VDHVGLGTAFENSIY	193–203	It is a potential heparan sulfate binding site responsible for PCV2 attachment to target cells [25]

was added, 20 ng per well, and the plates were incubated as described for the first step. The plates were washed five times and the substrate, tetramethylenbenzidine, was added. The plates were incubated in the dark place at room temperature for 15–20 minutes. Then, stop solution (0.2 M sulfuric acid solution) was added and test results were read at a wavelength of 450 nm using Model 680 plate reader (Bio-Rad, USA). Optical density (OD) values were interpreted semi-quantitatively with an indication of the positivity index – the ratio of test sample OD value to cut-off value calculated based on minimum absorbance of the samples collected at the post-induction stage. The data were analyzed using Statistica 7.0 software (StatSoft, USA) with Mann – Whitney U test adjusted for multiple comparisons. The p-value < 0.05 was considered the threshold value for statistically significant differences.

RESULTS AND DISCUSSION

Bioinformatics analysis has shown that the 92–233 a. a. fragment of Cap protein is of the greatest interest for designing the antigenic composition structure, since it is characterized by the highest density of type-specific epitopes. Moreover, some researchers previously reported the immunogenic potential of different domains of this fragment (Table).

Thus, modified fragment of truncated Cap amino acid sequence comprises the major significant epitopes being the targets for serological diagnosis. Prediction of the physical and chemical properties of this multi-epitope polypeptide demonstrated that its molecular weight was 17.8 kDa, it was highly soluble and had an optimal half-life (more than 20 hours) *in vivo* for *E. coli*.

At the next stage of the study, pET-22b/ORF-2 expression vector was constructed and used for transformation of *E. coli* Rosetta 2(DE3) cells, then the conditions for producer strain cultivation were optimized. Thus, different modes were used for induced cell cultivation: the different ranges of culture OD values before induction (from 0.5 to 1.0 ODU), different concentrations of added IPTG (from 0.2 to 2.0 mM), different time periods (from 2 to 6 hours) and temperatures (from +25 to +37 °C) were tested. Tests of the polypeptide profiles of cell lysates cor-

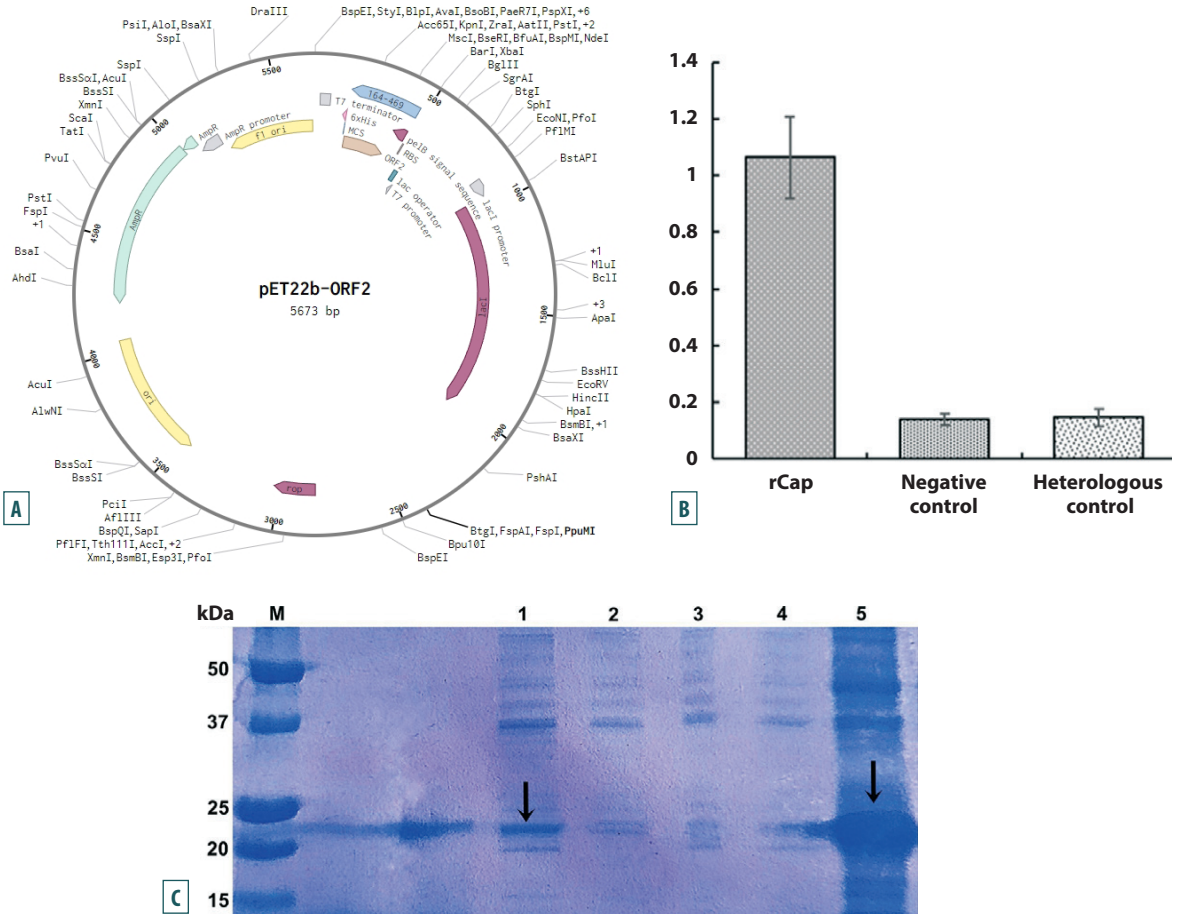

Fig. Characteristics of the prokaryotic PCV-2 rCap expression system:
A – scheme of the pET-22b/ORF-2 expression vector; B – optical density of rCap tested with iELISA using hyperimmune porcine serum, where negative control is lysate of cells transformed with pET-22b plasmid without insertion, and heterologous control is a recombinant E2 protein of classical swine fever virus, produced in the same expression system; C – electrophoregram of expression products, where M band is the molecular weight marker Precision Plus Protein™ Unstained Protein Standards (Bio-Rad, USA), bands: 1 – target protein after primary purification; 2 – negative control (lysate of cells transformed with the pET-22b plasmid without insertion); 3, 4 – lysates of cells transformed with the pET-22b/ORF-2 plasmid before induction; 5 – producer strain cell lysate 6 hours after induction. The target protein fraction is indicated by arrows

responding to each set of cultivation parameters showed that the optimal parameters were as follows: culture OD value before induction – 0.6 ODU, IPTG concentration – 1 mM, post-induction cultivation conditions – 6–7 hours at a temperature of +37 °C. This cultivation mode provides the recombinant product yield at the level of 35–40 mg/L, which is 1.75–2.00% of total biomass of the producer.

rCap serological activity and specificity was confirmed by testing with iELISA. Thus, total protein product OD was 1.065 ± 0.144 ODU, the positivity index was 4.34 ($p < 0.005$). The main characteristics of the prokaryotic rCap expression system are shown in the Figure.

The presented data confirm functionality of the developed expression system that holds great promise for further use of produced rCap for diagnostic and preventive purposes.

Some researchers have been reported successful *coli*-expression for producing anti-PCV2 vaccine components. For example, X. Xi et al. reported that a full-length soluble Cap, expressed in *E. coli,* under neutral pH conditions self-assembles into homogeneous virus-like particle (VLP), corresponding in size to intact PCV2 and providing protection *in vivo* comparable to that one provided by commercial vaccines [26]. Expression of Cap fused with various modified bacterial proteins is of particular interest. In particular, there is evidence that recombinant Cap fused with flagellin has elicited higher virus neutralizing antibody levels in the mouse model thereby promoting humoral and cellular immune responses [27]. However, limitations of prokaryotic expression systems have also been reported. It is known that the full-length Cap can be expressed only in those *E. coli* strains that possess plasmids carrying tRNA genes of rare *E. coli* codons (pRARE). Moreover, the expression product often cannot be purified under native and denaturing conditions so additional optimization of the insertion is required [28].

Despite the acceptable levels of truncated rCap synthesis achieved and the product specificity confirmed during this study, the development of an efficient and effective method of its purification will play a key role in vaccine protein production scaling up.

CONCLUSION

Prokaryotic system for expression of PCV2 *ORF-2* gene fragment containing the most immunogenic capsid protein epitopes was constructed during the study. The expression system functionality was confirmed by presence of mature multi-epitope polypeptide in cell lysates of the producer as well as by serological activity detected by iELISA using anti-PCV2 hyperimmune porcine sera. According to these data, the expressed polypeptide appears to be suitable for serodiagnosis and is also of interest as a component of the recombinant vaccine that will be the goal of our further studies.

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Risk factors for African swine fever spread in wild boar in the Russian Federation

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ABSTRACT

The analysis and assessment of risk factors associated with the occurrence, spread and persistence of African swine fever (ASF) virus in wild boar population are an important tool in determining the strategic measures aimed at eradicating epizootics and mitigating their consequences. A thorough examination of foreign and domestic literature revealed that wild boar population management factors, socio-economic and environmental ones, that mainly account for the density and number of animals were the most significant and associated with the risk of ASF outbreak occurrence in wild animals. In order to identify risk factors for the spread of the disease in wild boar in the Russian Federation subjects, a regression model was built to examine the relationship between the annual number of ASF outbreaks in wild boar at the municipal raion level, wild boar population density and some other factors for the period between 2007 and 2022. Based on the subject-level regression modelling results, a positive association between the intensity of the disease outbreaks and wild boar population density was identified in 42.5% of the model regions of the Russian Federation. Other significant factors were the length of roads, the presence of forest cover and outbreaks in domestic pigs. However, on the whole, for all the infected subjects, the regression model demonstrated the failure of the wild boar population density factor to explain the observed ASF outbreak distribution, and this may be indicative of the existence of other epizootic drivers of the disease spread in the wild. One of such mechanisms may be the persistence of infectious potential in the external environment and in the formed stationary local foci of African swine fever, despite the anti-epizootic measures taken, including the measures aimed at regulating the number of susceptible population – depopulation.

Keywords: African swine fever, risk factors, wild boar, population density, regression analysis, Russian Federation

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Факторы риска распространения африканской чумы свиней среди диких кабанов в Российской Федерации

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РЕЗЮМЕ

Анализ и оценка факторов риска, связанных с возникновением, распространением и персистенцией вируса африканской чумы свиней в популяции диких кабанов, представляет собой важный инструмент в определении стратегических мер, направленных на ликвидацию и смягчение последствий эпизоотий. Детальное изучение зарубежной и отечественной литературы позволило сделать вывод, что факторы управления популяциями диких кабанов, социально-экономические и связанные с внешней средой, в основном определяющие плотность и численность животных, являлись наиболее значимыми и ассоциированными с риском возникновения очагов африканской чумы свиней в дикой фауне. Для выявления факторов риска распространения заболевания среди диких кабанов в субъектах Российской Федерации была построена регрессионная модель, исследующая

взаимосвязь между ежегодным количеством вспышек африканской чумы свиней среди кабанов на уровне муниципальных районов, плотностью популяции кабана и рядом других факторов за период с 2007 по 2022 г. По результатам проведенного регрессионного моделирования на уровне субъектов в 42,5% модельных регионов Российской Федерации была выявлена положительная взаимосвязь интенсивности вспышек заболевания и плотности популяции кабана. Другими значимыми факторами явились протяженность автодорог, наличие лесного покрова и вспышек среди домашних свиней. Однако в целом для всех неблагополучных субъектов регрессионная модель показала несостоятельность фактора плотности популяции кабана для объяснения наблюдаемого распределения вспышек африканской чумы свиней, что может указывать на наличие иных эпизоотических драйверов распространения заболевания в дикой природе. Одним из таких механизмов может являться сохранение инфекционного потенциала во внешней среде и в сформированных стационарных локальных очагах африканской чумы свиней, несмотря на принимаемые противоэпизоотические мероприятия, включающие в себя и меры по регулированию численности восприимчивого поголовья – депопуляцию.

Ключевые слова: африканская чума свиней, факторы риска, кабан, плотность популяции, регрессионный анализ, Российская Федерация

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INTRODUCTION

African swine fever (ASF) is a transboundary viral disease that affects both domestic and wild pigs and causes enormous damage to pig farming [1, 2, 3, 4]. African swine fever may demonstrate unique regional patterns associated with a set of risk factors that should be taken into consideration when choosing appropriate surveillance and control strategies [5]. Multiple studies focus on the elucidation of the role of domestic and wild pigs in the occurrence of outbreaks and the spread of the infection.

Of particular interest is the analysis of risk factors contributing to the spread of the disease in wild boar, including its introduction into the ASF free areas [6]. The ecological cycle with the involvement of the wild boar and ASF virus presence in the environment is the main challenge of the present-day ASF epizootiology, since not all mechanisms of the pathogen persistence in the ASF infected areas have yet been revealed [7, 8].

The analysis of the ASF situation in the Russian Federation shows that the disease is widely spread both in wild boar and domestic pig populations almost throughout the entire territory of the country, including the regions where wild boar population density is reportedly very low [9].

Despite numerous attempts undertaken by researchers in many countries to develop a safe and effective vaccine against African swine fever, the disease eradication strategy is at present based on the principles of risk assessment and identification of the main factors contributing to the infection spread, as well as on compliance with strict biosecurity and biocontainment measures. Most ASF eradication and prevention measures are grounded on the classical principles of disease control, including epizootiological surveillance, investigation and killing of infected herds, establishment of protection and surveillance zones. These measures are coupled with a ban on swill feeding to pigs, strict quarantine and biosecurity/biocontainment measures, as well as with control over the movement of animals and pig products [2, 10, 11].

The importance of assessing the current ASF situation and the spread dynamics of this disease, which is devastating for the pig industry, dictates the need to develop methods for model forecasting of ASF outbreak occurrence in the ASF free areas of the Russian Federation and in the areas where the disease persists.

The remoteness of local foci of the infection in wild boar from initial outbreaks highlights the significance of human economic activities as a major, if not the key, factor associated with the spread of the disease [12, 13, 14]. It should be borne in mind that ASF transmission occurs not only through a direct contact between animals, but also indirectly, for example, through infected carcasses of wild boar killed by the disease and through environmental objects contaminated with the virus [12, 15, 16]. The detection of ASF occurrence in the wild boar habitat during the ongoing epizootic in Europe and its association with environmental factors made it possible to identify and describe the pathogen transmission cycle named "wild boar – habitat" [17, 18]. Thus, it seems justified to conclude that wild boar play an important role in the spread and circulation of ASF virus in the European countries. The complex biology of wild boar and environmental factors having impact on their habitat shall be at the core of efforts aimed at ASF control [19].

Discussions are currently underway regarding the dependence of ASF agent spread rate on wild boar population density. Based on the experience of the European countries, such an association prevails, but it is not always observed [5]. Pejsak Z. et al. suggested that sustained circulation of the virus in wild boar in Poland requires the density that exceeds 2 animals per square kilometer [19]. Due to the peculiarities of the ASF epizootic process, such a trend mainly depends on the structure and social relationships within the susceptible wild boar population and between the age-sex groups.

The threshold density theory does not provide any clear-cut answers regarding the patterns of ASF virus

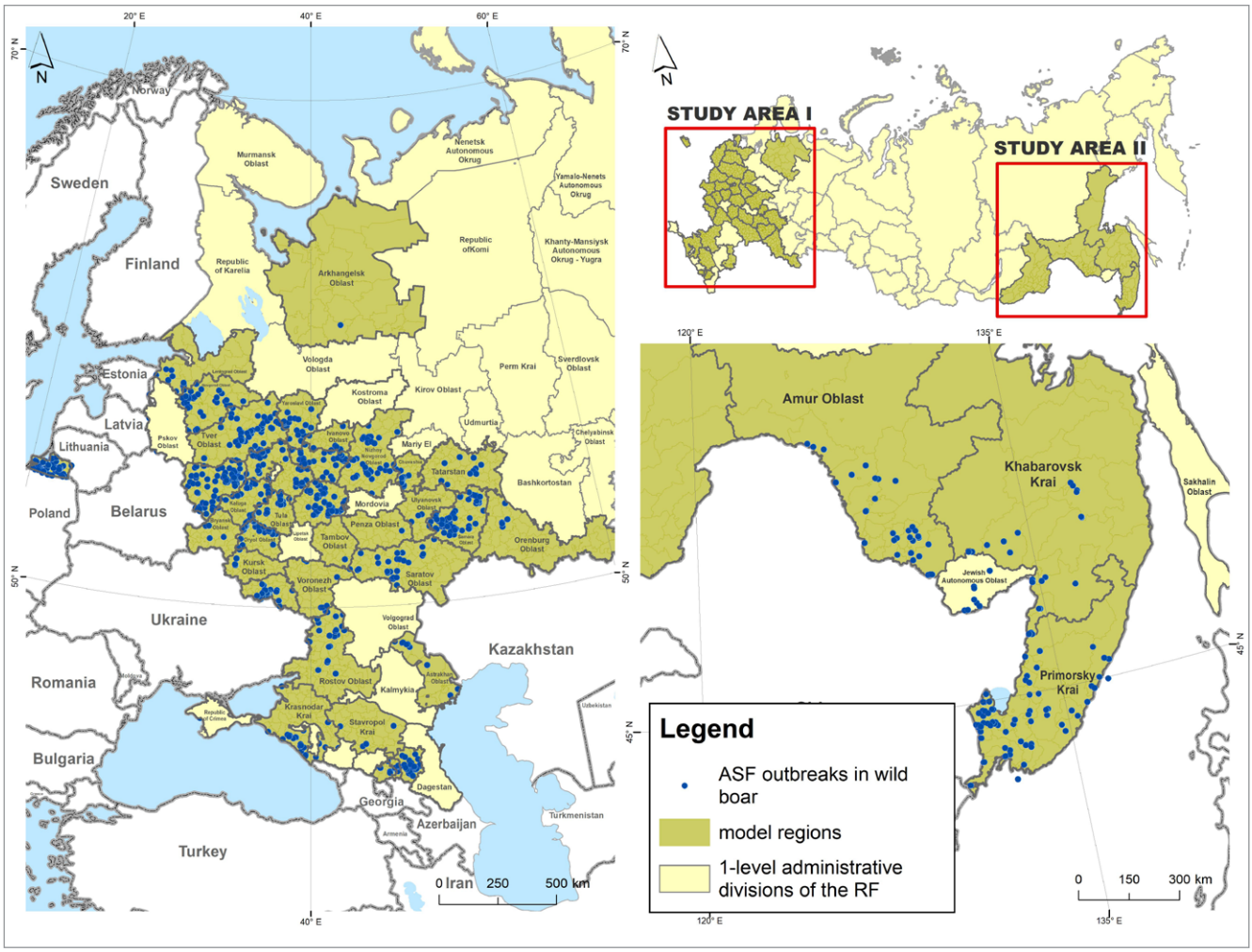

Fig. 1. ASF situation in wild boar population in the Russian Federation subjects (2007–2022)

spread, the persistence of outbreaks in the wild boar population and the disease agent transmission to other susceptible populations, including domestic pigs. Modelling approaches are based on the key assumptions such as homogeneous and random interaction between diseased and healthy animals, which is hardly reproducible in the wild.

The specified urgency of studying the biology of wild boar and ASF virus persistence in the environment highlights ecological, as well as landscape and climatic risk factors for the infection introduction and spread as a special category of predictors that are of priority for consideration [19, 20].

The study was aimed at the identification of the main risk factors for ASF spread in the wild boar population, including the determination of significance of susceptible animal density in the epizootic process in the ASF infected subjects of the Russian Federation.

MATERIALS AND METHODS

Model regions. The subjects of the Russian Federation in which ASF outbreaks were reported in the wild boar population in 2007–2022 and for which the information on the number of wild boar at the raion level was available for the said period were selected for the study. The model regions shown in Figure 1 comprise 40 subjects located in the European (zone I) and Far-Eastern (zone II) parts of Russia. Municipal raions were used as model units for the assessment of the disease occurrence and factors.

African swine fever data. Data on ASF outbreaks reported in the wild boar population in the Russian Federation in 2007–2022 were acquired from the official database of the World Organisation for Animal Health (WOAH)[1]. Annual information on the number and density of the wild boar population for 2007–2022 was acquired through requests submitted to the regional Ministries of Environment and Natural Resources and Committees on Wildlife Protection and Management of the Russian Federation subjects.

Regression analysis. The investigation of dependence between ASF outbreak occurrence and intensity and risk factors, in particular wild boar population density, involved the use of a negative binomial multi-factor regression model for the model subjects with long-term ASF persistence in wildlife. The dependent (response) variable was the number of ASF outbreaks in wild boar in the specified municipal raion in the relevant year, and the explanatory variables were the socio-economic and ecological factors described below.

A negative binomial regression model is a certain type of regression employed for analyzing count data where

[1] WOAH. Disease situation. https://wahis.woah.org/#/dashboards/country-or-disease-dashboard

the variance of the response is greater than its mean (i.e. where overdispersion is observed) [21]. In our case, choosing negative binomial regression was justified by the distribution of the number of outbreaks in wild boar in the municipal raions where the mean value is 0.84, the variance is 38.41.

The significance of the variables was evaluated with Student's *t*-test based on *p*-value ($p \leq 0.05$ is indicative of sufficient statistical significance of the variable as a regression model predictor). The global fit of the models was evaluated using the adjusted coefficient of determination R^2, which is the proportion of the variation in the dependent variable explained by the model.

Modelling was carried out in two stages:

1) for all raions of the model subjects taken as a whole;

2) for the raions of each individual subject.

Selecting potential risk factors for ASF occurrence in wild boar. As a result of literature data analysis, information was collected on potential risk factors for ASF spread in wild boar population, which is presented in Table 1.

The selected factors were the explanatory variables in the regression modelling aimed at the identification of ASF outbreak dependence on population density and other risk factors using negative binomial regression.

Software. The primary processing and evaluation of data were carried using Microsoft Office Excel software (Microsoft Corporation, Redmond, Washington, the USA). The mapping of ASF situation, the number of wild boar and cluster analysis were carried out using ArcMap 10.8.1 software (Esri, Redlands, California, the USA). Regression modelling was performed using R statistically oriented software environment (R Core Team, 2023).

RESULTS

Retrospective assessment of ASF situation in the wild boar population. During the analyzed period (from 2007 to 2022), 1,054 ASF outbreaks were registered in the wild boar population in the model subjects, which accounted for 41.7% of all outbreaks of the disease. The largest numbers of outbreaks in wild boar were reported in 2013 (116 outbreaks), 2016 (118 outbreaks), 2020 (170 outbreaks) and 2021 (104 outbreaks). Geographically, ASF outbreaks were concentrated in the following subjects: the Ryazan, Moscow, Tula, Tver, Vladimir, Smolensk, Samara Oblasts, as well as in the Pskov and Leningrad Oblasts adjacent to the border with Estonia. In the Far East, the long-term persistence and stationary foci of African swine fever were reported in the Primorsky Krai and in the border areas – local territories, in which wild boar population density is currently still rather high.

In some regions, ASF occurrence in the wild boar population is sporadic with a trend towards the virus persistence in the environment, with the disease outbreaks being reported throughout the entire epizootic (for example, in the Nizhny Novgorod Oblast); in other regions, the disease occurs as a mass epizootic and affects a considerable number of animals within a short period. Such an epizootic occurred in the Samara Oblast in 2020, with 60 ASF outbreaks having been reported in wild boar within the year.

The disease occurrence in wild boar was characterized by pronounced seasonality with peaks during summer months (July – August), as well as in November – December and February (Fig. 2).

Identification of dependence of ASF outbreak occurrence in wild boar on population density and other risk factors. Modelling for all the model subjects as a whole showed that wild boar population density is statistically insignificant as an explanatory factor ($p_t = 0.546$) and the coefficient of mutual determination is low ($R^2 = 0.256$). This allows for the conclusion that it is impossible to establish an unequivocal association between wild boar population density and repeated ASF outbreaks for all the model subjects as a whole (Table 2).

At the same time, modelling for individual subjects of the Russian Federation revealed statistically significant ($p_t < 0.05$) positive dependence of ASF outbreak

Table 1
Risk factors for ASF spread in wild boar population (overview)

Risk factors	Measuring units	Information on possible effect	References
Forest cover (proportion of the total area of a raion)	%	The availability of a large forested area, proximity to forest expanses increase the probability of diseased animal detection	[15, 22, 23, 24]
Water bodies (proportion of the total area of a raion)	%	There is an association between the availability of and proximity to surface watercourses and ASF spread	[15, 24]
Fields with shrub vegetation (area)	km^2	There is an association between the availability of meadow and shrub vegetation with a height of more than 1.5 m and infected animal detection	[15]
Height above sea level	m	High probability of infected animal detection under optimal habitat conditions	[25, 26, 27]
Human population density	people/km^2	There is an association between high human population density and infection occurrence	[24]
Concentration of settlements, including rural settlements and villages	settlements/km^2	Changes in the concentration may have an effect on diseased animal detection	[15, 22, 25, 26, 27]
Hunted wild boar	animals	There is an association between the probability of diseased animal detection and the number of hunted animals	[26, 27]
Road length and density	km and km/km^2	An increase in the number of roads may result in increased infected animal detection; it is also an indirect indicator of the economic activity of the human population	[15, 25, 26, 27]
Agriculture, the availability of small pig farms	farms	There is an association between an increase in the number of pig farms, especially small ones, and increased infection occurrence in animals	[26, 27]
ASF outbreaks in domestic pigs	outbreaks	Proximity to outbreaks increases the probability of infection occurrence	[26, 27]
Wild boar population density	animals/km^2	There is an association between high density and the probability of disease occurrence	[22, 25, 26, 27, 28]

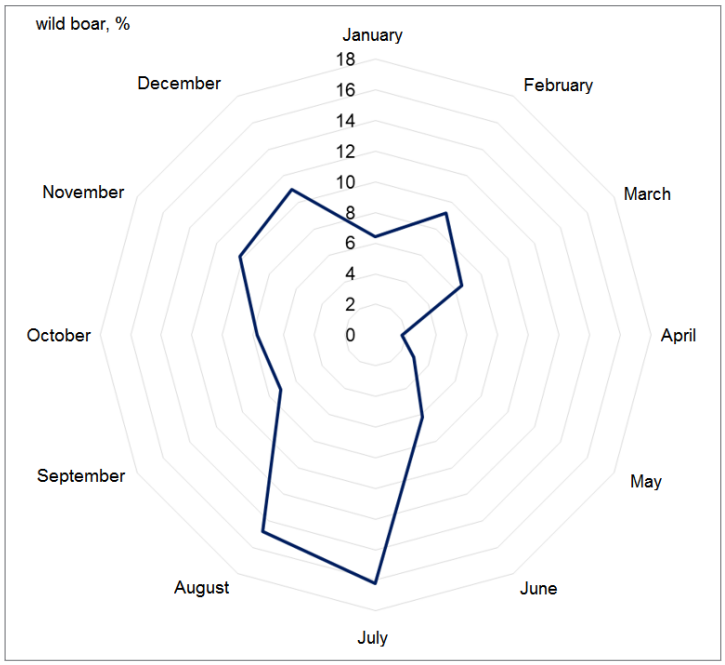

Fig. 2. Seasonality of ASF outbreaks in wild boar in the Russian Federation subjects, 2007–2022

Table 2
Results of regression analysis of ASF outbreak dependence on risk factors in the Russian Federation subjects (2007–2022)

Variables	Regression coefficient	Standard error	*p*-value
Forest cover proportion, %	0.657	0.342	0.0001
Road length, km	0.354	0.235	0.0001
ASF outbreaks in domestic pigs	1.032	0.657	0.003
Wild boar population density, animals/km^2	3.056	3.415	0.546

occurrence on wild boar population density in 17 out of 40 regions (42.5%), which is shown in Table 3.

Despite the revealed dependence of epizootic intensity on wild boar density in certain subjects of the Russian Federation, ASF outbreaks in wild boar in some regions were reported even in those areas where wild boar population density was significantly lower than the recommended value of 0.25 animal per 1,000 ha (0.025 animal/km^2) approved by Order of the Government of the Russian Federation No. 2048-r of 30 September 2016 (as amended on 4 February 2021) on the "Action plan for prevention of African swine fever introduction into and spread in the Russian Federation". This suggests that wild boar population density is not the only risk factor for ASF spread.

Figure 3 shows the results of regression modelling of ASF outbreak occurrence dependence on wild boar population density in the model subjects of the Russian Federation. Also, the distribution of susceptible animal population density in 2022 is shown for the regions in which the reliable dependence of ASF spread on wild boar population density was revealed.

The results of negative binomial regression analysis of reported ASF cases in wild boar taking into account several climatic and socio-demographic factors are presented in Table 2. The proportion of forest cover, the length of roads and outbreaks in domestic pigs were found to be the main predictors of epizootics.

DISCUSSION

Based on the analysis of literature sources describing risk factors for ASF expansion in the countries of the world, human activity is presented as the most significant predictor, especially as regards the movement and import of live pigs and pig products [12, 22, 25]. Many researchers note that wild boar dead of ASF, being a source of the infection spread, act as a no less important factor in the virus transmission both within the wild boar population and to domestic pigs, and this may be indicative of the virus persistence due to the presence of infected wild boar carcasses or their remains [16, 29, 30].

When considering wild boar habitat conditions as part of ASF spread risk analysis, one should take into account both biotic and abiotic factors such as climatic factors (temperature, rainfall amount, humidity, cloud amount, UV radiation level), landscape factors (vegetation type, vegetation cover area, height above sea level, soil type, the presence and accessibility of water bodies), anthropogenic activity and associated changes in the wild boar habitat (human population density, man-made structures, buildings, roads, farm concentration and livestock density), as well as factors related to wild boar population characteristics (wild boar population density, geographical distribution) and the biological properties of ASF virus – the form and stage of the disease (the proportion of seropositive wild boar in the population), viral load, incidence and prevalence levels [31].

Thus, a comprehensive understanding of potential risk factors for ASF spread in wild boar is fundamental and crucial for effective control of the disease. In this respect, it is obvious that in case of single-factor analysis of ASF risk, there is a probability of leaving out essential elements relevant for the infection transmission to wild boar. Therefore, to model the association between ASF occurrence in wild boar and potential predictors, many studies applied multi-factor approaches, thereby emphasizing the significance of the identified risk factors [23, 26, 32, 33].

Environmental factors that directly influence the possibility of the disease occurrence underlie the spatial and temporal patterns of ASF spread. It is known that the condition of forest cover has potential impact on the maintenance of favourable wild boar habitat conditions; another key predictor of ASF occurrence is the presence of water bodies [34].

As regards optimal wild boar habitat conditions, any type of land cover that provides animals with shelter, water and feed should be taken into account as an ecological risk factor for ASF occurrence [28, 35, 36]. It is possible that maintaining the size of wild boar populations at the specified level requires optimal habitat conditions, including forest cover consisting of certain tree species [35]. In accordance with this viewpoint, researchers demonstrated the association between ASF occurrence in wild boar and the area of forest cover in different geographical regions of Europe, including the Baltic countries [25, 26] and Italy [23], which indicates that the probability of detecting the infection agent in this animal species is higher in the regions with large forest expanses [37]. The results of a spatio-temporal study conducted in Czechia in the area affected by ASF in 2017–2018 provided additional

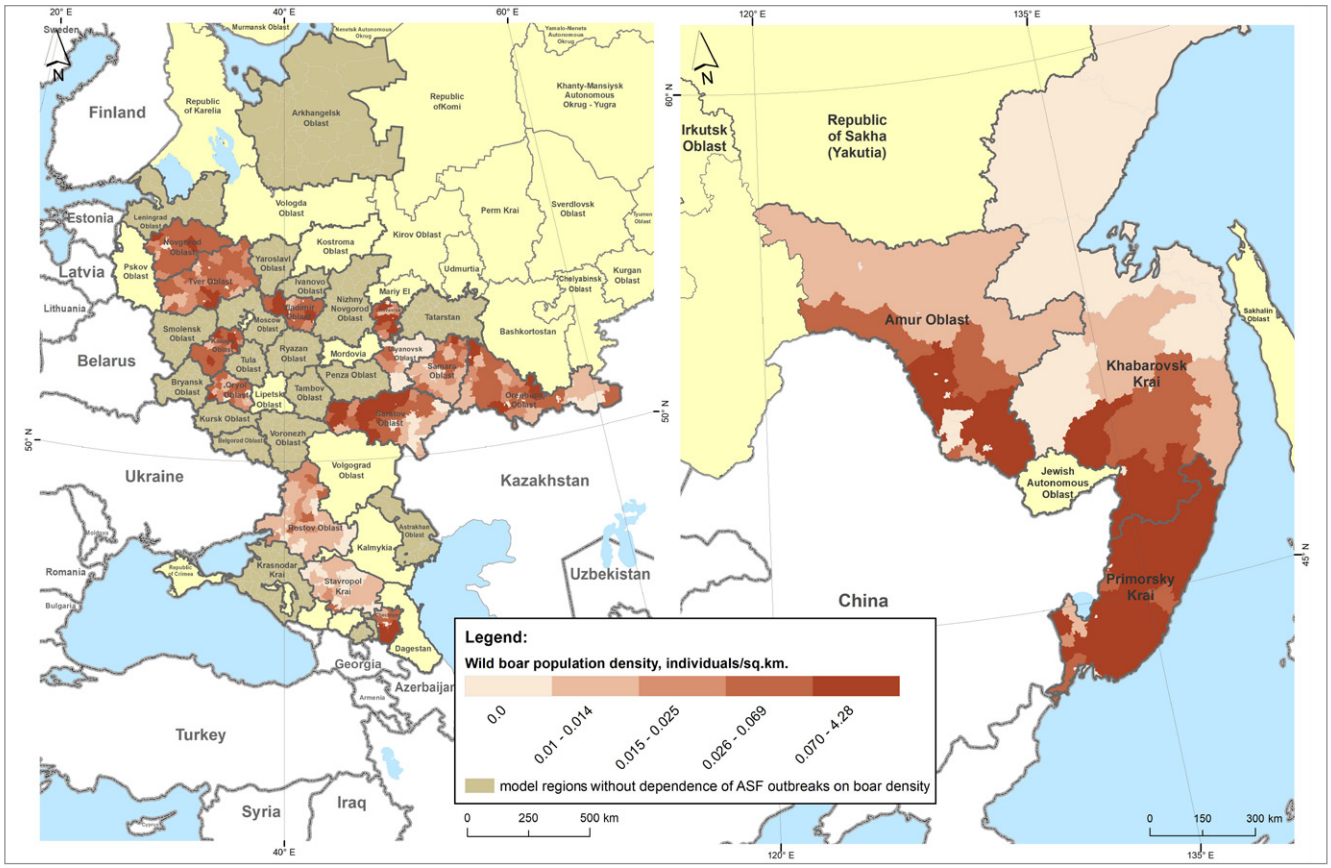

Fig. 3. Dependence of ASF spread in the raions of the Russian Federation subjects on wild boar population density based on regression modelling results. The figure shows the wild boar population density as of 2022

information on the relative effect of forest cover on detection of ASF virus infected wild boar. Though search activities aimed at detecting dead animals were carried out in a relatively small area affected by ASF, more than 70% of all carcasses were found in the forests [15]. The regression modelling of factors underlying the risk of ASF spread in the Russian Federation subjects demonstrated the significance of the forest cover factor, thus confirming the above statement.

Risk factors for ASF occurrence in wild boar associated with the population characteristics of the species include the total number or density, spatial and temporal distribution of animals. The concentration of ASF susceptible animals (domestic pigs, wild boar) plays an important role in the infection transmission chain. Despite the potential significance of the wild boar population density factor for ASF spread [38], it is very difficult, practically impossible, to determine the actual number of these animals and to conduct continuous monitoring due to their constant migrations. Therefore, in many countries, the population density is estimated based on the number of hunted wild boar per size of the area [35, 39] or using other methods such as camera traps [40]. It is important to understand that actual estimates of wild boar density are likely to be rough approximations of the true absolute value and are probably biased depending on the availability of initial data on the number of hunted animals.

The revealed spatio-temporal patterns of ASF outbreak spread in the Russian Federation subjects suggest that even with low wild boar population density (less than 0.25 animal per 1,000 ha of hunting areas), there is still

Table 3
Characteristics of regression indicators of dependence of ASF outbreaks in wild boar on wild boar population density in the Russian Federation subjects (2007–2022)

Russian Federation subject	Regression coefficient	Standard error	95% CI (confidence interval)	*p*-value
Kaluga Oblast	5.565	1.811	1.421–15.634	0.002
Novgorod Oblast	8.834	3.869	0.679–19.679	0.022
Orenburg Oblast	34.163	15.493	5.325–25.456	0.027
Oryol Oblast	13.296	5.807	3.911–28.044	0.022
Chechen Republic	4.345	3.211	1.658–2.213	0.032
Chuvash Republic	39.070	12.047	7.579–91.482	0.001
Rostov Oblast	33.495	0.785	31.944–135.024	0.000
Samara Oblast	27.656	8.844	10.487–50.677	0.001
Saratov Oblast	7.278	2.167	2.269–15.414	0.000
Stavropol Krai	87.722	11.827	64.026–110.587	0.000
Ulyanovsk Oblast	96.345	35.817	12.109–260.338	0.000
Vladimir Oblast	13.059	3.137	1.244–25.423	0.000
Volgograd Oblast	18.234	5.342	5.341–21.231	0.000
Tver Oblast	8.274	1.539	3.661–14.281	0.000
Amur Oblast	21.052	9.438	9.438–34.887	0.000
Primorsky Krai	1.051	0.713	0.123–4.063	0.014
Khabarovsk Krai	6.870	3.398	0.01–10.456	0.043

a possibility of the pathogen transmission from infected objects to susceptible animals and the virus spread in the environment [41, 42]. The overall regression model of ASF outbreak occurrence dependence on wild boar population density in the ASF infected subjects of the Russian Federation in 2007–2022 showed that the intensity of ASF outbreaks generally does not depend on the population density, and this may be attributed to uneven raion-level spatial distribution of this animal species. Despite the said fact, such dependence still exists in some subjects; this confirms the assumption that in some regions, there are raion-level geographical sites with increased susceptible animal densities, and migrations lead to ASF virus spread to new, previously ASF free areas. At the same time, it is important to pay attention to the nature of the revealed dependence: the positive values of regression coefficients confirm that a higher population density contributes to a more intensive spread of the disease.

Our study demonstrated the significance of the road length factor, which is associated with the possibility of people movement during ASF monitoring, especially in summer and autumn; it is also an indirect indicator of the intensity of transport and economic links contributing to the dissemination of products contaminated with the agent throughout the territory. A similar conclusion was made in the studies conducted in Estonia between 2014 and 2017. It was proved that the length of roads in the region is a factor that enhances the chances of detecting infected animals [25].

Hunting is repeatedly mentioned in the literature as a risk factor for ASF spread due to potential wild boar migrations as a result of hunting activities of humans. Therefore, when an ASF virus infected wild boar is detected and quarantine is imposed in the hunting area, it is recommended to stop any kind of hunting within the surveillance zone, except for activities aimed at regulating the number of wild boar to be carried out by specially trained people, while maintaining necessary measures to prevent the movement of animals outside this zone [43, 44].

The analysis of data obtained in Estonia in 2018 and 2019 showed that pig population density on small farms where a maximum of 10 pigs are kept is a risk factor for ASF occurrence in wild boar [6, 27]. This may be due to potential contacts between domestic and wild pigs that are typical for small farms without any biosecurity/biocontainment measures in place. The results of our study also demonstrate that ASF outbreaks in domestic pigs are an important risk factor for the infection spread in wild boar.

CONCLUSION

The literature analysis results allow for the conclusion that main factors for ASF spread in wild boar are environmental factors and human activities, which determine the major directions of the strategy for the infection control in wildlife. Regression modelling was aimed at the identification of main risk factors for ASF spread in the wild boar population in the ASF infected subjects of the Russian Federation. For Russia as a whole, the overall regression model demonstrated the failure of the wild boar population density factor to explain the observed ASF outbreak distribution, and this may be indicative of the persistence of infectious potential in the external environment and in the formed stationary local foci of African swine fever. However, it should be noted that results are heavily dependent on the reliability of available data on the size of the wild boar population. Modelling of an epizootic allowed for the identification of factors for ASF outbreak spread in wild boar such as forest cover percentage, road length and ASF outbreaks in domestic pigs, which confirm the significance of both natural and socio-demographic predictors. The wild boar, being a participant of the ASF transmission chain, may act as a risk factor only in case of high susceptible animal density at the initial stage of an epizootic. Selection of an ASF control strategy should take account of every link in the virus transmission chain and every opportunity for the infection elimination in the area where eradication measures are implemented.

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Scientific justification of *Mycobacterium avium* survival in natural environment of Republic of Dagestan

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ABSTRACT
Contamination of the environment with the infectious animal disease agents is still a pressing problem for the poultry farms. *Mycobacterium avium* can grow and replicate in organic wastes from the poultry farms for a long time thus contaminating vast adjacent areas and being the source of infection not only for wild and domestic animals but also for humans. The studies were aimed at the examination of the duration of *Mycobacterium avium* survival in the natural environment in two geographical regions of the Republic of Dagestan characterized by different soil and climate. Samples of *Mycobacterium avium*-contaminated feces and soil collected from pastures and farmyards (on the surface and from 5 cm depth) were tested. The experiments demonstrated that pathogenic for chickens mycobacteria survived for up to 30 days in the samples collected in the sub-mountainous areas in summertime, when the air temperature ranged from 15.1 to 30.0 °C, land surface temperature – from 17 to 38 °C, air humidity – from 44 to 94% and average monthly precipitation amounted to 1.5 mm. From September to May, with the air temperature ranging from –10.8 to +25.0 °C, land surface temperature from –14 to +30 °C, air humidity 26–100% and average precipitation 0.39 mm, the bacteria survived for up to 213 days on the soil surface on the pastures and farmyards, and for up to 243 days at the depth of 5 cm and in the feces. In the plain area, in the same time period in the slightly saline soil with high humus content and at air temperature from –11.9 to +27.3 °C, soil temperature from –13 to +45 °C, air humidity from 37 to 100% and average precipitation 20.4 mm, *Mycobacterium avium* survived just like in the sub-mountainous area, i.e. for 213 and 243 days, respectively. The post-mortem lesions in the internal organs of the poultry corresponded to the tuberculosis clinical signs in 86 of 171 birds (50.3%). The study results will allow for the development of the optimal algorithm for animal health and management measures aimed at tuberculosis eradication on the poultry farms.

Keywords: *Mycobacterium avium*, tuberculosis, samples, soil, feces, poultry farm, pastures, stability, infection, contamination, source of infections

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Научное обоснование полиадаптогенности микобактерий птичьего вида в объектах внешней среды в условиях Республики Дагестан

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РЕЗЮМЕ
Для птицеводческих хозяйств актуальной проблемой остается загрязнение окружающей среды возбудителями инфекционных болезней. Микобактерии туберкулеза птичьего вида могут длительное время расти и размножаться в органических отходах птицефабрик, загрязняя огромные примыкающие территории и являясь при этом источником заражения не только диких и домашних животных и птицы, но и человека. Целью работы являлось изучение продолжительности жизнеспособности *Mycobacterium avium* в объектах внешней среды в двух географических зонах Республики Дагестан с разными почвенно-климатическими характеристиками. Исследованию подверглись контаминированные микобактериями птичьего вида пробы (поверхностные и с глубины 5 см) почвы пастбищ, выгульного двора и помета. Эксперименты показали, что в образцах из предгорной зоны, отобранных в летнее время, когда температура воздуха колебалась от 15,1 до 30,0 °С, поверхности почвы – от 17 до 38 °С, влажность воздуха – от 44 до 94% и среднемесячное количество осадков составляло 1,5 мм, жизнеспособность патогенных для кур микобактерий сохранялась до 30 дней. С сентября по май при температуре воздуха от –10,8 до +25,0 °С, почвы от –14 до +30 °С, влажности воздуха от 26 до 100% и среднем количестве осадков 0,39 мм на поверхности почвы пастбищ и выгульного двора бактерии оставались жизнеспособными до 213 дней, на глубине 5 см и в помете – до 243 дней. В равнинной зоне

в этот же временной период в слабозасоленной почве со значительным содержанием гумуса при температуре воздуха от –11,9 до +27,3 °С, почвы от –13 до +45 °С, относительной влажности от 37 до 100% и среднемесячных осадках 20,4 мм микобактерии птичьего вида выживали как в условиях предгорной зоны – в течение 213 и 243 дней соответственно. Патолого-анатомические изменения во внутренних органах птиц соответствовали клиническим признакам туберкулеза у 86 (50,3%) из 171 особи. Полученные результаты позволят разработать оптимальный алгоритм проведения комплекса ветеринарно-санитарных и организационно-хозяйственных мероприятий для оздоровления птицеводческих предприятий от туберкулеза.

Ключевые слова: микобактерии птичьего вида, туберкулез, пробы, почва, помет, птицекомплекс, пастбища, устойчивость, заражение, загрязненность, источник инфекций

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INTRODUCTION

One of the distinctive features of mycobacteria is their stability in the body and various ecosystem objects, which creates great problems for the epizootological control and diagnosis of tuberculosis. Despite the apparent freedom of the Republic of Dagestan from tuberculosis, the pathogen circulation still continues, *inter alia* in the environment, thus maintaining a tendency to the infection spread on the previously free farms [1, 2].

Commercial poultry farming and hereto related short life cycle of commercial chickens, their keeping and feeding factored in their exterior and health parameters, undoubtedly made the problem of avian tuberculosis less significant [3, 4, 5].

At the same time, due to the high stability of *Mycobacterium avium* in the environment (according to the published data, it survives in the soil at the depth of up to 60 cm for more than 3 years), the chicken tuberculosis epizootic process has not been interrupted. Moreover, the disease cases are also detected in the private sector, where uncontrolled and unsystematic movement of poultry and slaughter products is often practiced without appropriate veterinary control. The diseased poultry, being the main source of infection, *inter alia* for wild birds, expands and complements the natural reservoir [6, 7, 8].

When eradicating the disease on the farms, timely removal of the diseased animals from the herd is important, but the problem lies in the fact that weakened and emaciated birds do not react to tuberculin, so they remain undetected for some time, while the risk of infection of healthy birds is proportional to the duration of cohabitation with the diseased ones [9, 10].

In this regard, research on the dynamic tracking of the tuberculosis agent, *inter alia* in the environment, using the proposed up-to-date methods, may be essential in terms of control and prevention of the disease in chickens [11, 12].

According to some published data, tuberculosis-diseased chickens often become a source of infection for cattle, causing sensitization to PPD-tuberculin for mammals [13, 14, 15].

One of the reasons aiding to the spread of this infection is known to be contaminated outer environment, which deserves special attention during epizootological examination [16].

The study of the tuberculosis agent stability in the soil of pastures and poultry yards and in feces as well as the related question of the infection sources and transmission routes is of great importance, since disinfection of large pastures infected with the excretions of the diseased chickens is difficult for economic reasons. In this regard, in our opinion, the correct approach to solving the issue is to rely on their self-decontamination in a natural way [17, 18, 19, 20].

Given these circumstances, the task was set to find out the duration of *Mycobacterium avium* stability in the soil of the pastures, poultry yards and in feces in two climatic zones of the Republic of Dagestan.

MATERIALS AND METHODS

The work was carried out in the sub-mountainous and plain areas of the Republic. Certain areas with different topography and soil composition were isolated both on surface and at the depth of 5 cm. Strips of silk contaminated with *M. avium* were introduced into the soil in these sites, as well as into the feces. The material was contaminated with the second generation culture of the mycobacteria isolated from the chicken died of tuberculosis.

Samples were collected from these sites to determine the survival rate of the mycobacteria. Each sample was individually crushed in 2 mL of sterile saline solution. The resulted mass was filtered through ashless filter paper; 1 mL of the suspension was intramuscularly administered to seronegative healthy chickens of 2 months of age. The infected chickens were kept isolated. Every month they were checked by allergy and clinical tools. At the same time, inoculums and smears were prepared from the emulsion after its appropriate treatment.

The chickens that reacted positively to the allergen were subjected to diagnostic slaughter, and post-mortem examination was performed. The lesions were used for inoculation on the culture media for the production of the initial culture and for preparation of the smears for *M. avium* detection.

During the research, the basic ethical principles of experiments in animals were followed, which are set out

in the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, March 18, 1986). Report of the Bioethics Commission of the Caspian Regional Research Veterinary Institute – Branch of Dagestan Agriculture Science Center No. 7 of 16 November 2023.

In the sub-mountainous area, the experiments were conducted in mildly alkaline black soil. The soil samples contained carbonic and sulfuric acid anions, chlorine, calcium, magnesium, potassium and sodium cations. The amount of humus amounted to 5.69%.

Two experiments were carried out in the sub-mountainous area. The first one (June – August 2019) was carried out under the following meteorological conditions: air temperature ranged from 15.1 to 30.0 °C, soil surface temperature – from 17 to 38 °C, air humidity – from 44 to 94%, average monthly precipitation – 1.5 mm.

To determine the mycobacteria stability, the test samples were collected on days 30 and 61.

The second experiment was carried out from September 2019 to May 2020 with air temperature fluctuations from –10.8 to +25.0 °C, soil temperature from –14 to +30 °C, air humidity from 26 to 100%. During the experiment, the average precipitation amounted to 0.39 mm. The test samples were collected on days 61, 91, 122, 153, 182, 213 and 243.

In the plain area, the experiments were conducted from September 2019 to May 2020 in slightly saline soil with a significant humus content under the following meteorological conditions: air temperature ranged from –11.9 to +27.3 °C, soil temperature – from –13 to +45 °C, relative humidity – from 37 to 100%, average monthly precipitation – 20.4 mm. The samples contained large amounts of phosphorus and chloride salts.

The samples were tested in a similar way to the experiment conducted in the sub-mountainous area.

RESULTS AND DISCUSSION

In the first experiment conducted in the sub-mountainous area, on day 30 of the tuberculosis agent presence in the feces and soil of the pastures and poultry yard, viable and pathogenic for chicken mycobacteria were found both on the surface and at 5 cm depth.

M. avium could not be detected in any of the samples collected on day 61 (Table 1).

Results of the second experiment demonstrated that *M. avium* died on day 213 on the soil surface of the pastures and poultry yard, and on day 243 at 5 cm depth and in the feces (Table 2).

In the plain area, *M. avium* introduced to the soil surface of pastures and poultry yard also died within 213 days, and at 5 cm depth and in feces – within 243 days.

Table 1
Parameters of *M. avium* survival in sub-mountainous environment (experiment one)

Samples	Day	Air temperature, °C	Soil temperature, °C	Air humidity, %	Average monthly precipitation, mm	Presence of mycobacteria
Soil	30	15.1–30	17–38	44–94	1.5	+
	61	15.1–30	17–38	44–94	1.5	–
Feces	30	15.1–30	17–38	44–94	1.5	+
	61	15.1–30	17–38	44–94	1.5	–

Table 2
Parameters of *M. avium* survival in sub-mountainous environment (experiment two)

Samples	Day	Air temperature, °C	Soil temperature, °C	Air humidity, %	Average monthly precipitation, mm	Presence of mycobacteria
Soil	61	–10.8…+25	–14…+30	26–100	0.39	+
	91	–10.8…+25	–14…+30	26–100	0.39	+
	122	–10.8…+25	–14…+30	26–100	0.39	+
	153	–10.8…+25	–14…+30	26–100	0.39	+
	182	–10.8…+25	–14…+30	26–100	0.39	+
	213	–10.8…+25	–14…+30	26–100	0.39	–
	243	–10.8…+25	–14…+30	26–100	0.39	–
Soil at 5 cm depth and feces	61	–10.8…+25	–14…+30	26–100	0.39	+
	91	–10.8…+25	–14…+30	26–100	0.39	+
	122	–10.8…+25	–14…+30	26–100	0.39	+
	153	–10.8…+25	–14…+30	26–100	0.39	+
	182	–10.8…+25	–14…+30	26–100	0.39	+
	213	–10.8…+25	–14…+30	26–100	0.39	+
	243	–10.8…+25	–14…+30	26–100	0.39	–

Table 3
Macroscopic post-mortem lesions of internal organs of poultry

Quantity of poultry	Tuberculosis lesions of								
	liver	spleen	intestines	liver and spleen	liver and intestines	liver, spleen and intestines	intestines and mesentery	Generalized process	Total
171	9	10	6	9	16	13	7	16	86
%	5.3	5.8	3.5	5.3	9.4	7.6	4.1	9.4	50.3

In order to identify post-mortem changes in internal organs and to establish the role of emaciated and weakened poultry in tuberculosis infection of the disease susceptible poultry, diagnostic slaughter of such poultry in the amount of 171 birds was carried out on several poultry farms. During pre-slaughter clinical examination, attention was paid to exhaustion, lameness, low mobility, drooping wings, loss of feather shine. Some chickens demonstrated gastrointestinal disorders, anemia of earrings and wattles, significant mortality was also reported (Table 3).

The post-mortem examination of 171 weakened and emaciated poultry demonstrated tuberculosis granulomas, especially in parenchymal organs, in 86 birds, which amounted to 50.3%.

The results of the conducted studies have shown that in the sub-mountainous and plain areas of the Republic of Dagestan the *Mycobacterium avian* died within 61 days on the surface of pastures, poultry yard and at 5 cm depth and in feces in summer; and within 243 days in the autumn-winter-spring period.

Thus, it can be noted that when eradicating the disease on the tuberculosis infected poultry farms, it is advisable to slaughter poultry in summer. When poultry is slaughtered in June, the pastures infected with the tuberculosis agent are self-decontaminated in the shortest possible time, i.e. in 61 days. This period coincides with the time when the poultry farm is repopulated with young birds born in the current year. Egg incubation in the Republic is started in February, and in March poultry farms receive chickens that reach 6 months of age in August.

In view of the established terms for the pastures' self-decontamination, the replacement young poultry can be transferred to the poultry houses, from where the poultry will be delivered for slaughter in June and the premises will be disinfected. In such case, the pastures where the infected poultry grazed until June will not pose any danger of infection for the young birds born in the current year.

CONCLUSIONS

The following conclusions and practical recommendations were made as a result of the research.

1. Quarantine of the pastures contaminated with *Mycobacterium avian* should be imposed for 2.5 months in summer and for 8.5 months in autumn.

2. Slaughter of poultry on the infected farms for tuberculosis infection eradication should be carried out in June, since in summer the pastures are self-decontaminated from the tuberculosis within a shorter period of time. When slaughter is performed in such a time, there is a possibility of using the pastures by the replacement young birds born in the current year and the chance of the poultry infection with tuberculosis is excluded.

3. When carrying out the disease eradication activities, all weakened and emaciated poultry should be periodically culled and destroyed in a specially designated place, and veterinary and sanitary measures should be subsequently carried out.

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Role of ixodid ticks in tick-borne pathogen spread and circulation in the Belarusian Lakeland

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ABSTRACT

Results of ixodid tick analysis for their ecological, epizootological and epidemiological significance for tick-borne pathogen spread across the Belarusian Lakeland are presented. The ticks were collected in publicly accessible areas of the Vitebsk Raion in April – November 2022: 8 routes were tracked, 18 flag-km were passed, 529 tick specimens were collected, including 350 imago ticks and 179 nymph ticks. The ixodid tick genus and species were determined using N. A. Filippova's ixodid tick determinator. All caught ticks were tested for *Borrelia* spp., *Anaplasma* spp. (*Ehrlichia* spp.), *Babesia* spp. and *Tick-borne encephalitis virus* genetic materials with real-time polymerase chain reaction using the reagent kit for nucleic acid extraction from environmental samples in accordance with the manufacturer's instructions. The specimens were grouped in accordance with the MG 3.1.1027-01 "Collection, recording and preparation for laboratory tests of blood-sucking arthropods being vectors of natural focal infections"; therewith, one specimen includes only one tick. Differences in the numbers of ixodid ticks and the occurrence of genetic markers of tick-borne pathogens in them were found to be associated with ecological characteristics of the examined territories. The following epidemically and epizootically significant ticks contributing to transmissible infection and invasion spread were found in the Belarusian Lakeland: ticks of *Ixodes* and *Dermacentor* genera; their frequency index was 70.1 and 29.9%, respectively. Tick-transmitted pathogen prevalence rate in the examined territories of the Vitebsk Raion was as follows: 61.7% for *Borrelia* spp., 25.8% for *Anaplasma* spp. (*Ehrlichia* spp.) and 25% for *Babesia* spp., mixed infections were found in 10.8% of the ticks. No *Tick-borne encephalitis virus* genetic materials were found in the specimens. Total infection rate for ixodid ticks was 22.7%.

Keywords: Belarusian Lakeland, ixodid ticks, transmission infections, borreliosis, anaplasmosis (ehrlichiosis), babesiosis

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Роль иксодовых клещей в распространении и циркуляции возбудителей клещевых инфекций и инвазий на территории Белорусского Поозерья

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РЕЗЮМЕ

Приведены результаты анализа эколого-эпизоотологической и эпидемиологической значимости иксодовых клещей в распространении и циркуляции возбудителей клещевых инфекций на территории Белорусского Поозерья. Сбор образцов проводили с апреля по ноябрь 2022 г. на объектах открытой природы Витебского района, при этом пройдено 8 маршрутов, отработано 18 флаго-километров, собрано 529 экземпляров клещей, в том числе 350 взрослых имаго и 179 нимф. Родовую и видовую принадлежность иксодовых клещей устанавливали с помощью определителя Н. А. Филипповой. Все отловленные особи были исследованы на наличие генетического материала *Borrelia* spp., *Anaplasma* spp. (*Ehrlichia* spp.), *Babesia* spp. и *Tick-borne encephalitis virus* методом полимеразной цепной реакции в режиме реального времени с использованием набора реагентов для экстракции нуклеиновых кислот из объектов окружающей среды в соответствии с инструкцией производителя. Группировку проб осуществляли в соответствии с МУ 3.1.1027-01 «Сбор, учет и подготовка к лабораторному исследованию кровососущих членистоногих – переносчиков возбудителей природно-очаговых инфекций», при этом в одну пробу включали только одного клеща. Определено, что различия в показателях численности иксодовых клещей и встречаемости в них генетических маркеров возбудителей клещевых инфекций имеют определенную связь с экологическими особенностями изучаемых территорий. Установлено, что в Белорусском Поозерье фауна эпидемически и эпизоотически значимых видов клещей, способствующих распространению трансмиссивных инфекций

и инвазий, представлена клещами родов *Ixodes* и *Dermacentor*, индекс встречаемости составил 70,1 и 29,9% соответственно. Показано, что спектр возбудителей инфекционных заболеваний, передаваемых иксодовыми клещами, на исследуемых территориях Витебского района в 61,7% случаев представлен *Borrelia* spp., в 25,8% – *Anaplasma* spp. (*Ehrlichia* spp.) и в 25% – *Babesia* spp., при этом микст-инфицированность переносчиков составила 10,8%. Генетический материал вируса клещевого энцефалита в пробах обнаружен не был. Общая инфицированность иксодовых клещей составляла 22,7%.

Ключевые слова: Белорусское Поозерье, иксодовые клещи, трансмиссивные инфекции, боррелиоз, анаплазмоз (эрлихиоз), бабезиоз

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INTRODUCTION

In the last decade, there has been a steady trend towards increasing the incidence of tick-borne infections in animals and humans, expanding their nosoareals, detecting mixed infections, as well as registering previously unknown pathogens and new nosological disease forms in Europe, including the Republic of Belarus, and in Asia [1, 2, 3, 4, 5].

So far, in the Republic of Belarus, almost all attention has been focused on only two tick-borne infections – viral tick-borne encephalitis and Lyme borreliosis. In fact, the reports on vector-borne diseases non-typical for Belarus – anaplasmosis, tularemia, tick-borne rickettsiosis, have become more often and Crimean hemorrhagic fever, monocytic ehrlichiosis, etc. have become spread intensively in neighboring countries [6]. Detection of human granulocytic anaplasmosis in some countries bordering the Republic is of the greatest interest for practical healthcare. Antibodies to anaplasmas were most often detected in sera from chronic patients with neurological disorders who had a history of tick bite (18.2%), as well as from patients with Lyme borreliosis (13.5%) [1]. Therefore, the attention of epidemiologists and infectious disease specialists has been recently focused on tick-borne mixed infections.

To date, it has been reliably proven that infection of the tick with 2–3 viral as well as bacterial and/or protozoal pathogens is not an exception, but a pattern [7]. According to the literature, the mixed infection can reach 36% in the structure of tick-borne infections in endemic territories [8]. Moreover, up to 5% of ixodid ticks harbor Lyme borreliosis and tick-borne encephalitis agents [9]. Clinical and serological tests have shown that tick-borne encephalitis can develop both in the form of mono- and more severe mixed tick-borne viral and bacterial diseases in individuals who have reported tick bites [10, 11]. Therefore, any disease caused by tick bite should be considered as a potential mixed infection [6].

Analysis of the available literature showed that the largest number of ecological and epizootiological studies of ixodid ticks were carried out in the central and southern regions of Belarus (Gomel, Brest, Minsk Oblasts and city of Minsk); there are also some data for the Grodno and Mogilev Oblasts but sporadic study data – for the Vitebsk region (Belarusian Lakeland) [12].

Geographically, the Belarusian Lakeland is a lake plateau in the north of the country, forming the southeastern part of the Baltic Uplands between the middle part of the Western Dvina River and the middle part of the Neman River basin, and occupies about 19% of the territory of Belarus. The major (larger) part of the Lakeland is located in the Vitebsk Oblast territory, and its small parts are located in the Minsk and Grodno Oblasts [13]. The Lakeland was named after large number of lakes (over 3,000) located in this area. The lakes form the following very picturesque landscape groups: Braslavskaya, Narochanskaya, Lepelskaya, Glubokskaya.

The Lakeland climate is the coldest and wettest in Belarus. The area is a part of the northern moderately warm humid agro-climatic zone.

Forests are the basic natural vegetation in the Lakeland. They occupy about 40% of the territory, which corresponds to the national average. The most wooded (up to 50%) areas are sandy lowlands, covered by coniferous forests that are often marshy. Coniferous species (mainly pine) predominate in the forests, their proportion reaches 60%. The highest proportion of spruce forests (17%) in the Republic is a peculiarity of its vegetation. There are very few oak forests (less than 2%), and other broad-leaved species are almost not found. Birch, alder and aspen forests predominate among small-leaved forests [14].

Meadows occupy a very small area. Drylands predominate among them, and there are significantly fewer lowland and floodplain meadows. The meadows are heavily overgrown.

There are few marshes in the Lakeland, but in some places they form large massifs (Yelnya, Obolsky marshes, Belmont). The widespread upper sphagnum and transitional bogs are a distinctive features of the land.

Thus, all above-described natural and geographical features of the Belarusian Lakeland are similar to subtaiga landscapes that creates unique conditions for ixodid tick habitats.

The study was aimed at analysis of the ecological, epizootiological and epidemiological role of ixodid ticks in tick-borne pathogen occurrence and circulation in the Belarusian Lakeland.

MATERIALS AND METHODS

The numbers and species of sexually mature ixodid ticks were recorded from April to November 2022. Due to the peculiarities of daily activity of ixodid ticks, the recording was performed during the period of maximum ixodid tick activity: on clear days – in the morning, from the moment the dew dries up to the onset of daytime heat, and in the evening after the heat decreases until dusk or evening temperature drop; on cloudy days without midday heat – during the day. At the same time, the night air temperature should have been at least 8 °C [15].

Adult (imago) and nymph ticks were collected in publicly accessible natural environment in the following Vitebsk region territories: Tulovsky botanical reserve, Tulovo agrisettlement (a/s); park named after Soviet Army; beach of "Burevestnik" children's health camp and territories adjacent to it, s. Zui; Pridvinye biological reserve, s. Shevino; Luzhesnyansky dendrological park, s. Luzhesno; "Chertova Boroda" botanical reserve; Ruba Ski Centre territory; forest area near s. Sokolniki. All territories had subtaiga landscapes.

The coordinates of the examined territories were determined using satellite navigators (GLONASS/GPS receivers) in the global positioning system.

In open (clearings, lawns, glades) and forest areas with tall grass and shrubs, flag made of plain light fleecy fabric (waffle, flannel) was used for tick collection. A piece of fabric (60 × 100 cm) is attached with its narrow side to a stick and dragged through vegetation in an unfolded form; the person dragged the flag in front of or at the side of himself/herself and periodically inspected it.

The route length was calculated based on tracked 10-meter distance intervals, the10-meter distance interval was predetermined by counting double steps. Stops was made to take notes and to inspect own clothes after completion of each 10-meter distance interval.

The length of the observation route (1 flag-km of natural biotope) was the main unit for the tick population estimation.

A total of 8 routes were tracked, 18 flag-km were passed, 529 tick specimens were collected, including 350 imago ticks and 179 nymph ticks.

The ixodid tick genus and species were determined using N. A. Filippova's ixodid tick determinator[1]. Tick species were identified *in vivo* with binocular microscope (16×).

Collected ticks were placed in glass tubes with cotton-gauze plug or in plastic tubes with screw caps. Each tube was labeled. The following information was indicated in the label: collection place and time, tick species, sex, life stage and feeding level[2,3].

Caught ticks were tested for animal and human disease agents by detection of disease agent genetic materials with real-time polymerase chain reaction (PCR) using thermocycler. The specimens were grouped in accordance with MG 3.1.1027-01 "Collection, recording and preparation for laboratory tests of blood-sucking arthropods being vectors of natural focal infections"[4], therewith, one tick was considered as one specimen. Genetic materials were extracted from the specimens with the reagent kit for nucleic acid extraction from environmental objects in accordance with the manufacturer's instructions.

Differences in mean values were assessed for their statistical significance with Student's t-test (t).

RESULTS AND DISCUSSION

A total of 529 tick specimens, including 350 imago ticks (66.2%) and 179 (33.8%) nymph ticks were collected in publicly accessible natural environment along the designated routes (Table 1). Therewith, nymph ticks were caught along three out of eight routes.

Species and sex composition of collected ixodid ticks is given in Table 2. Tick frequency index was calculated based on the data given in Table 2: number of specimens in which ticks of particular species were detected expressed as percentage of total number of tested specimens.

It was found that epidemiologically and epizootiologically significant tick species in the Belarusian Lakeland (Vitebsk Raion), responsible for the spread of tick-borne infections and invasions, were represented by ticks of *Ixodes* and *Dermacentor* genera, that was generally consistent with other studies. Thus, the frequency index for *Ixodes* ticks was 70.1%, and for *Dermacentor* ticks was only 29.9%.

Ixodes ricinus (European forest, castor bean, sheep tick) is a temporary ectoparasite with long feeding period and "ambush" technique for attack.

Adult ticks in fasting period are absolutely flat, have a small size, 3–4 mm only, and fasting nymph ticks are about 2 mm in size. During feeding the ticks enlarge significantly in size, especially females (hundreds of times), and their bodies become of ovoid shape. The body of the adult tick is a leathery sac, harder on the back (scutum); ticks lack eyes and wings, but have 4 pairs of legs. The scutum completely covers the body of male ticks, while in female ticks it covers only the anterior third of the body. Females are larger than males and of reddish color; they change their color to gray in engorged state. Nymphs are of light-brown color and change their color to grayish-brown in engorged state. Non-continuous poor-developed marginal groove is a distinctive phenotypic feature of *Ixodes ricinus*.

All active phases of the tick life cycle (egg development and molting) take place in the forest litter.

Ixodes ricinus prefer to live in sparse splashing sites and young small-leaved forests (birch, aspen, alder), where there are clearings with high herbage, and the soil is covered with thick leaf layer. Ticks also like to settle in willow thickets forming along melioration ditches running between fields.

Ixodes ricinus is a carrier of *Tick-borne encephalitis virus* of the European genotype, less pathogenic to humans, *Borrelia burgdorferi sensu lato* complex (ixodid tick-borne borreliosis, or Lyme disease), *Anaplasma phagocytophilum* (granulocytic anaplasmosis), *Babesia* spp. (canine piroplasmosis, babesiosis of cattle) [16].

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[2] MG 3.1.3012-12 Collection, recording and preparation for laboratory tests of blood-sucking arthropods in natural foci of dangerous infectious diseases: Methodical Guidelines. Moscow: Federal Centre of Hygiene and Epidemiology of the Federal Service for the Oversight of Consumer Protection and Welfare of the Russian Federation; 2012. 55 p.
[3] Domonova E. A., Tvorogova M. G., Podkolzin A. T., Shipulina O. Yu., Karan L. S., Yatsyshina S. B. et al. Collection, transportation and storage of biological material for PCR diagnostics: Methodical Guidelines. Moscow: Central Research Institute for Epidemiology; 2021. 112 p.
[4] Collection, recording and preparation for laboratory tests of blood-sucking arthropods being vectors of natural focal infections: Methodical Guidelines. Moscow: Federal Centre for State Epidemiological Surveillance of the Ministry of Health of the Russian Federation; 2002. 55 p.

Table 1
Absolute numbers of ixodid ticks in examined natural biocenoses

Examined territories	Number of imago ticks, absolute units	Number of nymph ticks, absolute units
Tulovsky botanical reserve, Tulovo agrisettlement (a/s)	31	–
Park named after Soviet Army	32	–
Beach of "Burevestnik" children's health camp and territories adjacent to it, s. Zui	11	–
Pridvinye biological reserve, s. Shevino	47	–
Luzhesnyansky dendrological park. s. Luzhesno	78	–
"Chertova Boroda" botanical reserve	26	39
Ruba Ski Centre territory	48	8
Forest area near s. Sokolniki	77	132

Table 2
Species and sex composition of ixodid ticks in the examined natural biocenoses, absolute units

Examined territory	*Dermacentor*			*Ixodes*		
	♀	♂	nymphs	♀	♂	nymphs
Tulovsky botanical reserve, Tulovo agrisettlement (a/s)	18	12	–	1	–	–
Park named after Soviet Army	22	9	–	1	–	–
Beach of "Burevestnik" children's health camp and territories adjacent to it, s. Zui	2	1	–	3	5	–
Pridvinye biological reserve, s. Shevino	23	20	–	2	2	–
Luzhesnyansky dendrological park, s. Luzhesno	4	1	–	34	39	–
"Chertova Boroda" botanical reserve	4	2	8	18	2	31
Ruba Ski Centre territory	5	3	2	32	8	6
Forest area near s. Sokolniki	4	8	10	56	9	122
Total, $n = 529$	82	56	20	147	65	159

Dermacentor reticulatus (meadow tick) can live in open spaces in leafy and mixed forests – forest clearings, forest edges, but prefer meadows and pastures with dense tall grass, can survive under water during flooding, live in flood meadows, can be found on lawns, prefer wet places – ravines, road ditches, springs. So, the tick mass reproduction foci can form in livestock grazing areas [17]. The ticks are highly cold-resistant. The activity of meadow ticks starts almost a month earlier than that one of forest ticks – in April – May.

Ticks of *Dermacentor* genus have special features: all phases of the tick life cycle take place within one year, and imago ticks can survive two to three winter periods without feeding [18].

The characteristic morphological features of *Dermacentor* genus are light enamel pigments in the form of spots of various shapes and sizes, most apparent on the dorsal scutum and less apparent on the legs and proboscis. The shape of enamel spots and their number vary greatly. The male tick is of light color with dark markings. In female tick, only scutum is of light color, but female tick body is dark-brown [19].

Meadow tick is a vector of thick-borne encephalitis, borreliosis, babesiosis (piroplasmosis), anaplasmosis, ehrlichiosis, rickettsiosis, tularemia, listeriosis, erysipeloid, Q fever, tick typhus, Omsk hemorrhagic fever [18, 19].

All collected ticks were tested for genetic materials of *Borrelia* spp., *Anaplasma* spp. (*Ehrlichia* spp.), *Babesia* spp. and *Tick-borne encephalitis virus* with real-time PCR.

It was found that 120 (22.7%) out of 529 ticks were carriers of infectious animal and human disease agents (carrier ticks) and 409 (77.3%) ticks were provisionally "clean" ticks since the range of detectable DNA markers was limited. Therewith, *Borrelia* spp. DNA was detected in the largest number of the carrier ticks: 74 (61.7%) out of 120 ticks; *Anaplasma* spp. (*Ehrlichia* spp.) DNA was detected in 31 (25.8%) out of 120 carrier ticks and *Babesia* spp. DNA was detected in 30 (25.0%) out of 120 carrier ticks (Fig. 1).

Ixodid tick-borne borreliosis is one of the critical health concerns associated with transmissible natural focal infections in many countries, including the Republic of Belarus, in the view of its ever-increasing social, medical and economic importance [20, 21, 22]. In general, borreliosis is a pronounced transmissible tick-borne human and animal disease. For example, according to literature data [23], in Russia the proportion of ixodid tick-borne borrelioses amounts to about 30% out of the total number of natural focal infections and amounts to more than 55% out of infections caused by the ixodid tick-transmitted agents. Other studies results have shown that spontaneous bacteriophoricity (infection of ticks with *Borrelia*) of ixodid ticks in natural foci can reach 70% and even 90% [24].

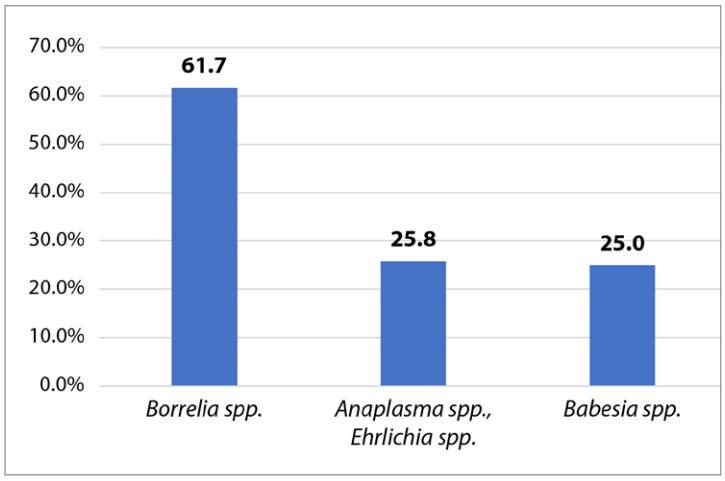

Fig. 1. Detection of tick-borne pathogen DNAs in ixodid ticks-carriers

The natural foci of ixodid tick-borne borreliosis directly depend on natural landscapes: there are especially many of them in mixed forests located in the moderately humid climatic zone, that is highly consistent with that one in the Vitebsk region. Many infected wild and domestic vertebrate species (rodents, deer, moose, goats, cows, dogs, horses, etc.) as well as birds transmitting infected ixodid ticks over long distances during migratory flights serve as *Borrelia* reservoirs and carriers.

According to the data obtained by staff-members of the Group for Arbovirus Infections of the Republican Research and Practical Center for Epidemiology and Microbiology monitoring the transmissible infection pathogen circulation dynamics in the Republic of Belarus, tick bacteriophoricity in natural environment has increased in the Republic on average from 12.4 to 19.1% over the last decade.

Anaplasmas are agents of transmissible seasonal diseases of cattle and humans.

So far, anaplasmas have been known only as animal disease agents, and anaplasmoses have been of veterinary interest, but currently humans are proven to be also susceptible to the infection [25]. PCR amplification method has made it possible to make significant amendments to anaplasmas taxonomy that greatly facilitates the disease diagnosis.

Sick animals and anaplasma carriers are the source of the invasion. The pathogen is transmitted by 11 ixodid tick species, one Argass tick species and insects. Besides cattle, reindeer, zebu, buffaloes, moose, roe deer and antelopes are susceptible to *Anaplasma marginale* [26].

Anaplasmosis is widespread almost everywhere. The disease is usually reported in spring, summer and autumn. However, the disease cases can be reported in winter in case of poor animal management and feeding, as well as violations of aseptic and antiseptic rules during veterinary and zootechnical manipulations (blood collection with one needle, tagging, etc.). Anaplasmosis is often reported in the form of mixed invasion with piroplasmidoses [27].

According to veterinary reports, the Bryansk, Pskov Oblasts bordering to the Republic of Belarus, as well as Ryazan, Kaliningrad, Vladimir Oblast and other regions of the Russian Federation are anaplasmosis-affected [5, 11]. Mass infection and disease in livestock animals result in deaths, reduced growth in young animals and reduced milk yields in cows, inefficient feed conversion, increased costs due to expensive treatment, extensive testings and measures to be taken to combat the disease. All this causes huge economic losses.

Ehrlichiosis as well as anaplasmosis is caused by rickettsia-like bacteria and transmitted by ticks. Both humans are animals (dogs, cats, wild canine and feline animals, etc.) are affected by ehrlichiosis. Different tropism to blood cells is a distinctive feature of these tick-borne rickettsioses. Thus, *Anaplasma* affect leukocyte granulocytes (mainly neutrophils) and *Ehrlichia* have a tropism for monocytes, that was the reason to name the diseases transmitted by them as human granulocytic anaplasmosis and human monocytic ehrlichiosis, respectively. Both anaplasmosis and ehrlichiosis are new infections for the Republic of Belarus and require more detailed study. According to the scientific data published by the Republican Research and Practical Center for Epidemiology and Microbiology, the proportion of human granulocytic anaplasmosis cases among 64 patients with tick-borne infections was 23.4%, and monocytic ehrlichiosis cases in humans were sporadic [1].

Babesiosis (piroplasmosis) is a natural focal ixodid tick-borne disease caused by hemoprotozoan parasites of *Babesia* genus [28]. Ixodid ticks of *Rhipicephalus, Dermacentor, Hyalomma, Ixodes ricinus, Haemaphysalis leachi* genera, as well as *Argas* ticks are main vectors of the infection and main hosts of the parasite [29]. Cattle and canine animals are mainly affected. In humans, the disease is caused by three *Babesia* species: *Babesia microti* – in America, *Babesia divergens* and *Babesia rodhaini* – in Europe. More than 100 cases of human babesiosis have been described in the literature, the most of them were fatal [30].

Western tick-borne encephalitis is a diseases caused by arbovirus, the life cycle of which requires *Ixodes ricinus* ticks as a participant of the virus circulation and as a reservoir of the virus in nature. Tick-borne encephalitis has been studied in Belarus since the early 40s of the last century, when its natural foci were identified in the Belovezhskaya Pushcha territory, and the virus was isolated from *Ixodes ricinus* ticks [12].

According to the Republican Research and Practical Center for Epidemiology and Microbiology, the number of *Ixodes ricinus* ticks infected with *Tick-borne encephalitis virus* has been slowly but steadily increased with periods of stabilization in the Republic of Belarus over the past decade [20].

It should be noted that none of the parasites collected during this study had the *Tick-borne encephalitis virus* genetic markers. At the same time, it is believed that tick-borne encephalitis remains the priority tick infection for the Polesie region, where as in the Republic as a whole, number of ixodid ticks in natural biotopes steadily increases, number of the ticks naturally infected with various pathogens also increases, and infected vector habitats expand [12].

Many cases of mixed infections (from 18 to 32%) in ixodid ticks have been described in the literature [1, 2, 4, 10, 15]; but in our study, only 13 out of 120 ticks were found to be infected with several pathogens (Fig. 2). While, more than two pathogens were detected in two specimens only.

Detection of several pathogens in one tick makes the tick-borne mixed infections an important and priority problem for the Republic of Belarus and requires comprehensive study.

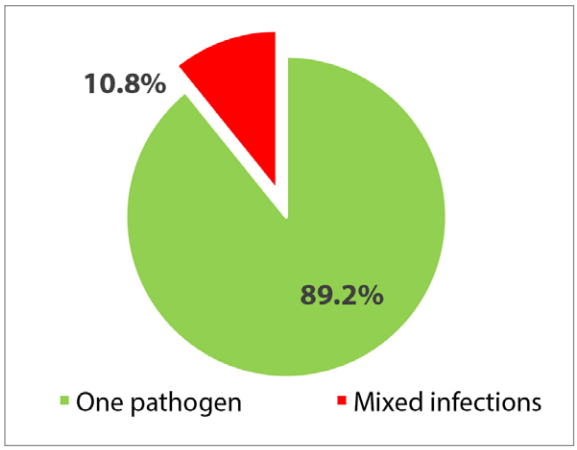

Fig. 2. Mixed infections in examined ticks

The study showed that the largest number of infected ticks (38.5%) was collected on the territory of the "Chertova Boroda" botanic reserve, the smallest number of infected ticks (9.1% at $p = 0.00015$) was collected on the beach of "Burevestnik" children's health camp and in the territories adjacent to it (s. Zui). The proportion of infected parasites was approximately the same in other examined publicly accessible natural environment areas and ranged from 15.6 to 21.5% (Fig. 3).

The results of tests of ixodid ticks-carriers collected in the territory of "Chertova Boroda" botanic reserve and on the beach of "Burevestnik" children's health camp and in the territories adjacent to it (s. Zui) for DNAs of transmissible tick infection pathogens are shown in Figure 4.

Detected differences in ixodid tick numbers and tick-borne pathogen genetic marker occurrence correlate with the ecological characteristics of the examined territories.

The "Chertova Boroda" botanical reserve is a locally important nature reserve located in the Chretova Boroda stow to the south-west of Vitebsk, to west of the Orekhovo microraion, on the right bank of the Western Dvina River. Its terrain is hilly with wet ravines and characteristic mixed-forest landscape with many shrubby thickets and grass deadwood. The territory is ideal for a permanent habitat for ixodid ticks.

The route ran through the territory belonging to "Burevestnik" children's health camp, mixed forest, settlement of Zui (Vitebsk Raion), 6 km from Vitebsk. The landscape is predominantly mixed forest with meadows located closer to the northeastern part of the camp and to the Laucesa

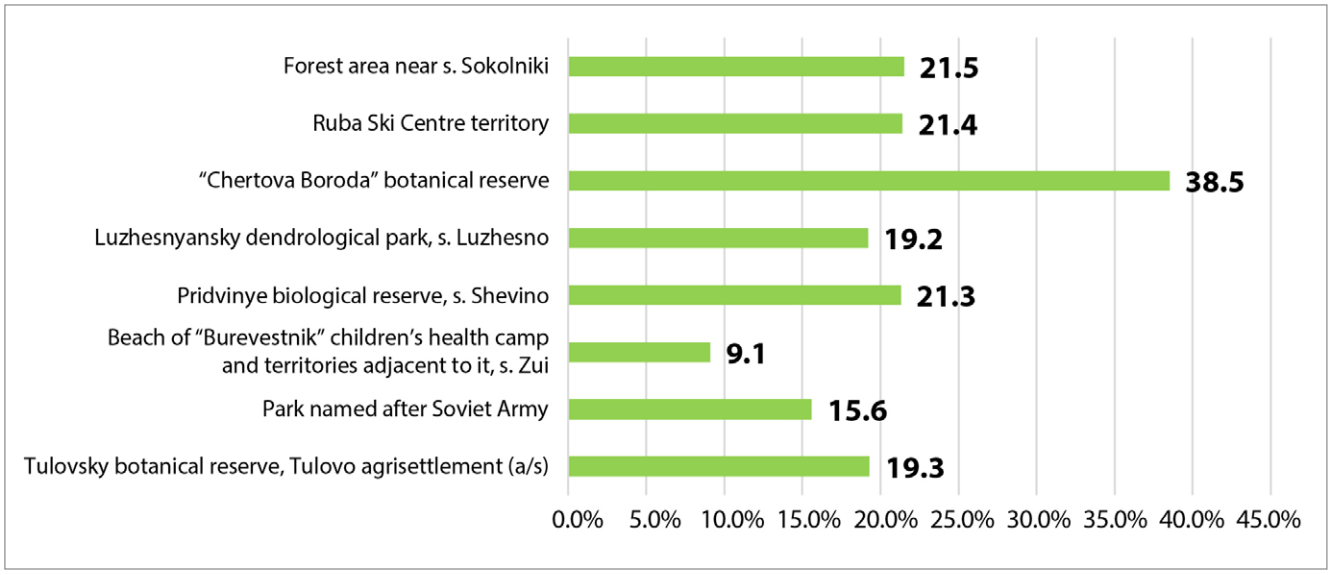

Fig. 3. Proportion of infected ticks in the examined territories

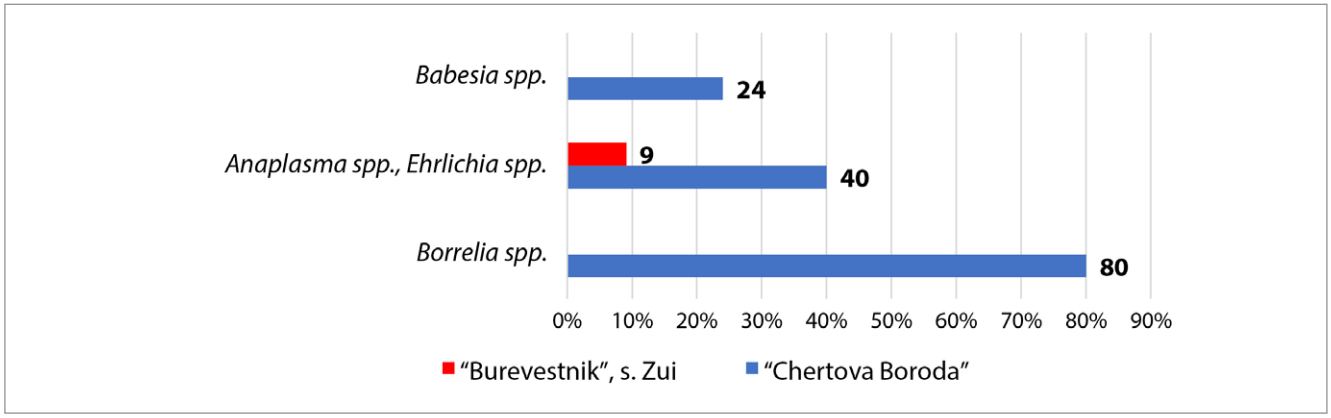

Fig. 4. Results of tests for detection of tick-borne pathogen DNAs in ixodid ticks-carriers in the "Chertova Boroda" botanic reserve as well as on the beach of "Burevestnik" children's health camp and in the territories adjacent to it (s. Zui)

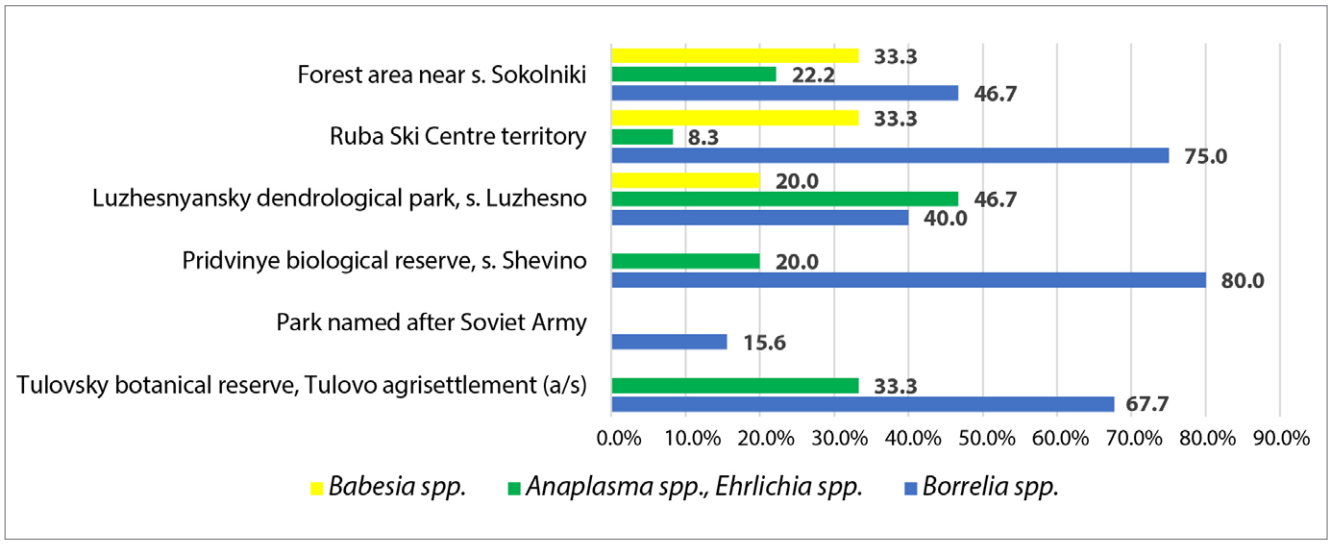

Fig. 5. Detection of tick-borne pathogen DNAs in ixodid ticks-carriers in other six examined territories

River shore. The small number of collected ticks can be accounted for regular measures for destruction of ectoparasites and larvae thereof taken in the territories adjacent to the camp.

The results of tests of ixodid ticks-carriers collected in six other examined territories for DNAs of transmissible tick infection pathogens are shown in Figure 5.

The range of transmissible infection pathogens carried by ixodid ticks was found to be different in examined territories.

Thus, only *Borrelia* spp. DNA was detected in carrier ticks collected in the territory of the Park named after Soviet Army; only *Borrelia* spp. and *Anaplasma* spp. (*Ehrlichia* spp.) DNAs were detected in the ticks collected from the vegetation in the Pridvinye biological reserve (settlement of Shevino) and the Tulovsky botanical reserve (Tulovo agrisettlement). Whereas, *Borrelia* spp. and *Anaplasma* spp. (*Ehrlichia* spp.) as well as *Babesia* spp. genetic materials were detected in the carrier ticks collected along the routes running through the Luzhesnyansky dendrological park (s. Luzhesno), Ruba Ski Centre territory and the forest area near s. Sokolniki.

It is important to note that *Borrelia* spp. was the pathogen most often detected in carrier ticks collected along all routes (15.6 to 80% bacteriophoricity). Thus, tick-borne borreliosis is the predominant transmissible infection in the territory of the Belarusian Lakeland.

CONCLUSION

It was found that epidemiologically and epizootiologically significant tick species in the Belarusian Lakeland responsible for spread of transmissible infections and invasions were represented by ticks of *Ixodes* and *Dermacentor* genera, their frequency index was 70.1 and 29.9%, respectively.

It was shown that 22.7% of ixodid ticks in the Belarusian Lakeland were potential carriers of the following human and animal infectious diseases: tick-borne borreliosis, anaplasmosis (ehrlichiosis), babesiosis.

No *Tick-borne encephalitis virus* genetic materials were found in the specimens tested during the study.

The range of infectious disease agents transmitted by ixodid ticks in the Belarusian Lakeland (Vitebsk region) was found to be as follows: *Borrelia* spp. were detected in 61.7% of tested ticks, *Anaplasma* spp. (*Ehrlichia* spp.) – in 25.8% of tested ticks and *Babesia* spp. – in 25% of tested ticks. Therewith, the proportion of mixed infected vectors was 10.8%.

Numbers of ixodid ticks and presence of tick-borne pathogen genetic markers in them were found to correlate with the ecological features of the examined territories.

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Flow cytometry study of DNA transformation dynamics in BHK-21/SUSP/ARRIAH cell culture during rabies virus reproduction

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ABSTRACT

The study examines the DNA transformation dynamics of BHK-21/SUSP/ARRIAH subline cells during rabies virus reproduction. Cells infected with the virus and control intact cells were cultivated under similar conditions. The identification of dependence of the virus infectivity on reproduction time revealed that the virus infectivity titre increased from (3.2 ± 0.2) lg $CCID_{50}/cm^3$ at the time of inoculation to (7.63 ± 0.3) lg $CCID_{50}/cm^3$ after 48 hours of reproduction, with the most intensive increase having been observed within the first 24 hours. The cell concentration changed from 0.5 to 1.9 million/cm^3, i.e. increased by a factor of 3.8. After 24 hours, the cell growth rate slowed down. Findings from the examination of cell cycle phases during rabies virus reproduction in the host cell allowed for the estimation of duration and predominance of G1, S, G2 + M phases at different stages of cultivation. The dynamics of changes in the apoptotic cell population in the control and test samples was similar within 36 hours of cultivation. After the said period, the proportion of apoptotic infected cells was 28–42% higher than that of apoptotic control cells. After 9 hours, the proportion of cells undergoing G1 phase increased by 11.7% in the test samples, whereas it decreased by 16.6% in the control samples. Subsequently, the number of G1 phase cells in the control and test samples changed in the same way: a 40% decrease was observed after 15–18 hours, it was followed by a 45–46% growth jump, then again a 39–40% decrease and an increase were observed. After 33 hours of reproduction and till the end of cultivation, the proportion of infected cells undergoing G1 phase was significantly higher (by 12–21%) as compared with control cells. The percentage of S phase cells in the test and control samples was the same during the first day of the virus reproduction, with sharp jump-like 3.4- and 2.4-fold increases having been observed after 15 and 24 hours, respectively. After 24 hours, the infected and control cells began to demonstrate differences, which gradually increased from 8 to 137% by the end of reproduction. After 30 hours of reproduction, the proportion of test sample cells undergoing G2 + M phase began to decrease by 17–28% as compared with the control cells. The cell switch-over to the synthesis of complete rabies virus particles occurred after 24 hours of reproduction. This is indicated by changes in the host cell cycle phases, as well as by the slowing down of BHK-21/SUSP/ARRIAH cell population growth.

Keywords: flow cytometry, cell cycle phases, BHK-21 cell suspension, rabies virus

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Исследование методом проточной цитометрии динамики трансформации ДНК в культуре клеток BHK-21/SUSP/ARRIAH при репродукции вируса бешенства

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РЕЗЮМЕ

Исследование посвящено изучению динамики трансформации ДНК клеток сублинии BHK-21/SUSP/ARRIAH при репродукции в них вируса бешенства. Инфицированные возбудителем и контрольные интактные клетки культивировались в аналогичных условиях. При выявлении зависимости

инфекционности вируса от времени репродукции установили, что титр его инфекционной активности увеличивался от (3,2 ± 0,2) lg $ККИД_{50}$/$см^3$ при инокуляции до (7,63 ± 0,3) lg $ККИД_{50}$/$см^3$ через 48 ч репродукции, но более интенсивно он возрастал в первые 24 ч. Концентрация клеток при этом изменилась с 0,5 до 1,9 млн/$см^3$, то есть выросла в 3,8 раза. Спустя 24 ч кратность прироста клеток замедлялась. В процессе изучения фаз клеточного цикла при репродукции в клетке-хозяине вируса бешенства получены результаты, позволяющие оценить продолжительность и доминирование фаз G1, S, G2 + M на разных этапах культивирования. Динамика изменений популяции клеток, находящихся в апоптозе, в контроле и опыте была одинаковой в течение 36 ч культивирования. По прошествии данного времени инфицированных клеток в стадии апоптоза было на 28–42% больше по сравнению с таковыми в контроле. Доля клеток, находящихся в стадии фазы G1, через 9 ч в опытных образцах увеличилась на 11,7%, в контрольных, наоборот, уменьшилась на 16,6%. В дальнейшем количество клеток в G1-фазе и в контроле, и в опыте изменялось одинаково: через 15–18 ч наблюдали уменьшение на 40%, далее – ростовой скачок на 45–46%, потом опять снижение на 39–40%, затем вновь увеличение. После 33 ч репродукции и до окончания культивирования доля инфицированных клеток, находящихся в фазе G1, была значительно больше (на 12–21%), чем контрольных. Количество клеток в фазе S в опыте и контроле в первые сутки репродукции вируса было одинаковым, при этом наблюдали резкое скачкообразное увеличение через 15 и 24 ч в 3,4 и 2,4 раза соответственно. Через 24 ч между инфицированными и контрольными клетками начали проявляться различия, которые постепенно возрастали с 8 до 137% к окончанию репродукции. В фазе G2 + M через 30 ч репродукции количество клеток опытных образцов начинало уменьшаться на 17–28% по сравнению с контрольными. Перестройка клетки на синтез полных частиц вируса бешенства наступала через 24 ч репродукции. Об этом говорят изменения в фазах клеточного цикла клетки-хозяина и замедление прироста самой популяции клеток линии ВНК-21/SUSP/ARRIAH.

Ключевые слова: проточная цитометрия, фазы клеточного цикла, суспензия клеток ВНК-21, вирус бешенства

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INTRODUCTION

Rabies is an acute disease of warm-blooded animals that affects the central nervous system. All domestic and wild animal species, as well as human beings are susceptible to the disease [1, 2, 3, 4, 5].

The virus reproduction cycle includes its adsorption onto the cell surface, penetration into the cell, uncoating, transcription, translation, replication, assembly and budding.

Primary transcription does not require structural protein synthesis. There are 2 types of rabies virus transcription: 1) at first, leader RNA is transcribed with genomic RNA, then 5 monocistronic mRNAs are sequentially transcribed, from which N, P, M, L proteins are translated in the cytoplasm, and G gene mRNA is translated in polysomes associated with the Golgi apparatus membrane; 2) transcription of antigenomic (positive-sense) RNAs, which serve as templates for the synthesis of new generations of negative-sense genomic RNAs, with genomic RNA. Unlike primary transcription, genomic RNA replication by the described mechanism can occur only simultaneously with translation and synthesis of structural proteins, especially N and P ones, necessary for RNA encapsidation by nucleocapsid structures. L, N, and P proteins mainly control RNA transcription and replication, whereas M protein occupies an intermediate position between the nucleocapsid and the virion shell and participates in RNA condensation. Besides, M protein plays a key role in RNA synthesis regulation and budding of the virus [6, 7]. At the final stage of assembly and budding, nucleocapsids "put on" the viral envelope represented by G protein integrated into the cell membrane. Glycoprotein determines the neurovirulence and neuroinvasiveness of rabies virus.

The place of viral particle formation depends on the virus and host cells. For representatives of the genus *Lyssavirus* of the family *Rhabdoviridae*, to which rabies virus belongs, the synthesis and assembly of nucleocapsids occur in the cytoplasm, and budding occurs through the plasma membrane of the cell, from which the virion acquires an additional lipid layer.

Rabies virus does not cause characteristic cytopathic changes in cells. All transcription and replication events occur in the cytoplasm inside Negri bodies. These structures are typical for rabies and, thus, can be used as a pathognomonic symptom of the infection [8].

Flow cytometry is a modern technology that ensures rapid, high-quality and multiparametric analysis of cells. It is widely used in such fields of medicine as immunology, pharmacology, cytology, oncology, hematology, genetics, infectology [9, 10, 11, 12, 13, 14, 15].

Flow cytometry makes it possible to collect a variety of data: to determine DNA and RNA content in a cell, the total number of proteins and the number of specific proteins recognized by monoclonal antibodies, to investigate cell metabolism, to study the transport of calcium ions and the kinetics of enzymatic reactions [15, 16, 17, 18, 19, 20].

Each cell has its life cycle from the moment of its formation by mother cell division to mitosis or death. It is called a cell cycle. The cell cycle consists of two periods: 1) cell growth period (interphase); 2) cell division period called M phase (from the Greek word "mitos", meaning "thread"). In its turn, each of the said periods has several phases. Usually, the interphase takes at least 90% of the time of the entire cell cycle. Most of cell components are synthesized throughout the interphase, and this makes it difficult to identify individual stages within it.

The interphase is divided into G1, S and G2 subphases. A period within the interphase, during which cell nucleus DNA is replicated, is called "S phase" (from the word "synthesis"). It should be noted that not only DNA replication, but also the basic biosynthesis of structural and functional proteins of the cell occurs during the interphase (mainly in S phase). A period between M phase and the beginning of S phase is called G1 phase (from the word "gap"), and a period between the end of S phase and subsequent M phase is called G2 phase [21].

The use of flow cytometers in the studies made it possible to identify important data on the reproduction cycle phases of cells, as well as to obtain the results that allowed for the estimation of duration and predominance of the corresponding cell cycle G1, S, G2 + M phases at different stages of cultivation [18, 19, 20, 22, 23]. In the light of the above, it can be concluded that the nature of changes in the cell cycle during virus replication in a cell is important for understanding the process of virus reproduction, but is understudied.

The aim of the study was to examine the dynamics of DNA transformation in BHK-21/SUSP/ARRIAH cell culture during rabies virus reproduction using flow cytometry.

MATERIALS AND METHODS

Cell line. Suspension continuous culture of newborn Syrian hamster kidney cells BHK-21/SUSP/ARRIAH was used in the work [24]. The cells were grown in metal bioreactors with a working capacity of up to 1,800 dm^3 in accordance with the "Master formula record for production of vaccine against foot-and-mouth disease of different types" approved by the Director of the Federal Centre for Animal Health.

Rabies virus. Production "ARRIAH" strain of rabies virus was used to infect the cells.

Nutrient medium used to grow cells. Eagle's medium supplemented with 5% of fetal bovine serum (Serana, Germany) and 0.25% blood protein hydrolysate (Russia) was used.

Rabies virus cultivation. Rabies virus reproduction was carried out during 48 hours in 0.5 dm^3 flasks using Minisart® RC25 Syringe Filters 17764-ACK Ø 25 mm (Sartorius, Germany).

Growth rate was calculated as the ratio of the final (after 48 hours) and initial cell concentrations within one passage.

Cell infection with rabies virus. Suspension BHK-21/SUSP/ARRIAH cell culture was inoculated with culture rabies virus at a dose of 0.1 $CCID_{50}$/cell. Samples containing infected cells were designated as test samples; cells not inoculated with the virus served as control and were cultivated under similar conditions.

The identification of cell cycle stages was carried out using an Accuri™ C6 flow cytometer and a BD Cycletest™ kit for working with a cytometer, as well as a reagent kit for quantitative DNA content analysis in cells C6 Flow Cytometer Fluid Kit (Becton Dickinson and Company, USA).

The determination of rabies virus infectivity titre was carried out in accordance with "Methodical guidelines for indirect determination of infectivity titre of culture rabies virus "ARRIAH" strain in vaccine production seed with real-time reverse transcription polymerase chain reaction (real-time RT-PCR)"[1].

Samples were collected every 3 hours throughout the entire time of rabies virus reproduction. The concentration of BHK-21/SUSP/ARRIAH cells in the suspension was determined using a Goryaev chamber for counting blood cells, dA0.000.851, compliant with TU 64-1-816-84. The cell suspension in a volume of 1 cm^3 was supplemented with an equal volume of 0.2% trypan blue solution, thoroughly mixed and used to fill the chamber. The number of cells in 1 cm^3 of the suspension was calculated using the formula [21]:

$$X = \frac{A \times B \times 4{,}000}{3{,}600} \times 1{,}000,$$

where X is the number of cells in 1 cm^3; A is the total number of cells in the chamber; B is the suspension dilution.

The counting was performed using a microscope at 10× magnification.

Statistical data processing. Numerical data were statistically processed with generally accepted methods of variation statistics, using a personal computer and Microsoft Excel software.

RESULTS AND DISCUSSION

The virus reproduction dynamics was assessed based on the following indicators:

– dependence of the virus infectivity titre on reproduction time;

– changes in apoptosis and debris;

– changes in cell cycle stages (G1, S, G2 + M).

During cultivation, the hydrogen ion concentration (pH) of the suspension was checked every 3 hours.

Figure 1 shows the dynamics of rabies virus infectivity during its reproduction in BHK-21/SUSP/ARRIAH cell culture.

It was found that the virus infectivity titre increased from (3.2 ± 0.2) lg $CCID_{50}/cm^3$ at the time of inoculation to (7.63 ± 0.3) lg $CCID_{50}/cm^3$ after 48 hours of reproduction, with the most intensive increase having been observed within the first 24 hours (up to 6.76 $CCID_{50}/cm^3$).

Within 48 hours, the cell concentration changed from 0.5 to 1.9 million/cm^3, i.e. increased by a factor of 3.8 (Fig. 2).

At the next stage, experiments were conducted to study changes in rabies virus infectivity titre in BHK-21/SUSP/ARRIAH cells with different initial concentrations: (0.77 ± 0.1); (3.0 ± 0.2) and (1.1 ± 0.1) million/cm^3. It was found that the accumulation of complete viral particles in the infected cells with different concentrations occurred within the first 12 hours of reproduction (Fig. 3).

[1] Doronin M. I., Mikhalishin D. V., Borisov A. V., Balashov A. N., Mudrak N. S., Zakharov V. M. Methodical guidelines for indirect determination of infectivity titre of culture rabies virus "ARRIAH" strain in vaccine production seed with real-time reverse transcription polymerase chain reaction (real-time RT-PCR): approved by FGBI "ARRIAH" 23.12.2021 No. 66–21. Vladimir; 2021. 59 p.

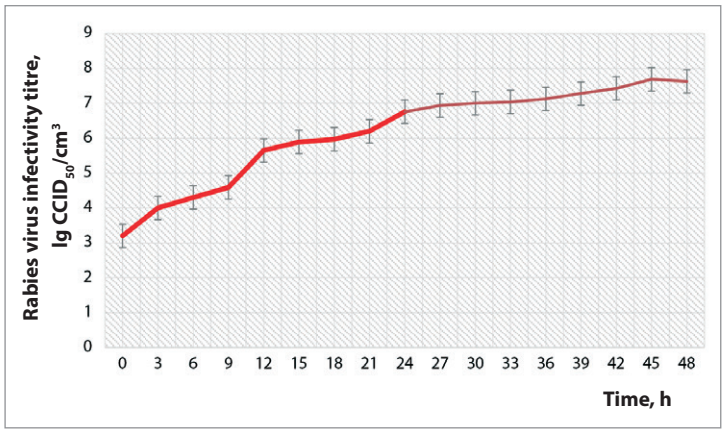

Fig. 1. Rabies virus infectivity dynamics during its reproduction in cell culture (n = 3, p < 0.05)

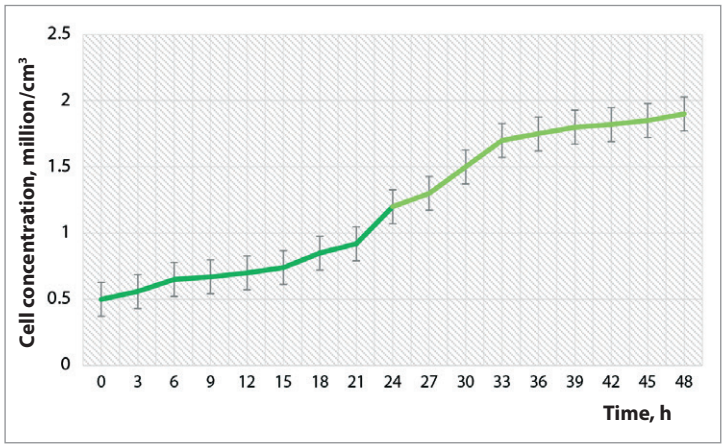

Fig. 2. BHK-21/SUSP/ARRIAH cell concentration dynamics during rabies virus reproduction (n = 3, p < 0.05)

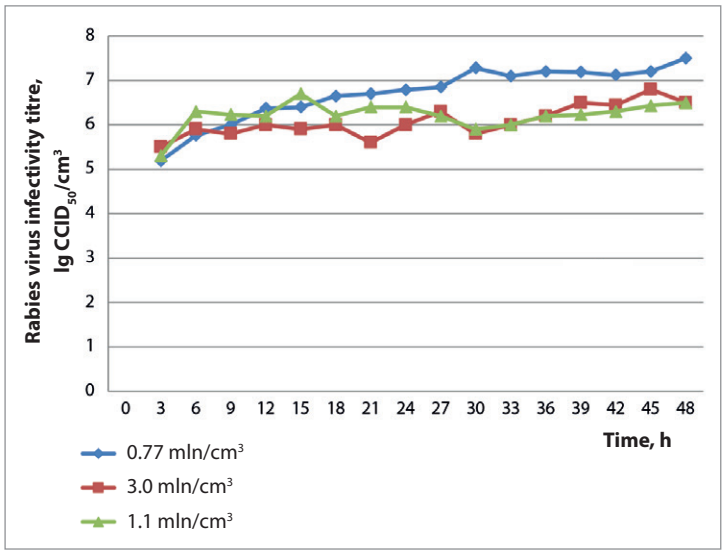

Fig. 3. Rabies virus accumulation dynamics during its reproduction in cells with different initial concentrations (n = 3, p < 0.05)

Subsequently, the virus accumulated 1.1–1.2 times more intensively in the culture with initial cell concentration of (0.77 ± 0.10) million/cm³. This is probably due to the limited amount of nutrients for the reproduction and growth of cells with a higher concentration.

Tests were carried out to study changes in cell cycle phases during rabies virus cultivation in suspension BHK-21/SUSP/ARRIAH cells. Under standard optimal conditions, intact populations are distributed according to cell cycle phases as follows: G1 – 30–75%, G2 + M – 2–18%, S – 2–33%, polyploids – up to 4% and debris – 1–20% [23].

It was found that the dynamics of changes in the apoptotic cell population in the control and test samples was similar within 36 hours of cultivation (a small time shift of 3 hours between the test and control samples was observed after 9 and 12 hours of reproduction). The proportion of apoptotic infected cells was 28–42% higher than that of apoptotic control cells 36 hours after the start of cultivation (Fig. 4).

After 9 hours, the proportion of cells undergoing G1 phase increased by 11.7% in the test samples, whereas it decreased by 16.6% in the control samples. Subsequently, the number of G1 phase cells in the control and test samples changed in the same way: a 40% decrease was observed after 15–18 hours, it was followed by a 45–46% growth jump, then again a 39–40% decrease and an increase were observed. After 33 hours of reproduction and till the end of cultivation, the proportion of infected cells undergoing G1 phase was significantly higher (by 12–21%) as compared with the control cells.

The comparison of the number of cells undergoing apoptosis + debris and G1 phase revealed a certain synchronicity between these phases: after 9 hours of the virus cultivation, G1 phase increased by 10–12%, at the same time apoptosis decreased by 10–11%, then a decrease in the percentage of G1 phase cells and an increase in the proportion of cells in apoptosis and debris were observed. A more pronounced "mirroring" of these cell cycle phases was observed after 24 hours of cultivation. A similar pattern was seen in the study of the cell cycle during foot-and-mouth disease virus reproduction [22].

The diploid phase of the cell cycle (G1), during which the synthesis of mRNA, structural proteins and other cell components began, prevailed in the cell cycle of intact BHK-21/SUSP/ARRIAH population. This phase accounted for 30 to 75% of cells, depending on the cultivation conditions, which is reflected in earlier studies [23].

It is known that during G1 phase, cells start to grow in size, mRNA and enzymes required for subsequent DNA replication are activated [21].

G1 phase cells in the suspension culture were maintained at 37–70% throughout the entire virus reproduction time, i.e. cells infected with the virus served as a source of energy and material for the synthesis of viral components, as well as for the assembly of complete virions.

The transition from diploid (G1) phase to synthetic (S) phase is one of the check points of the cell cycle. Depending on the amount of nutrients and energy, as well as on external factors of cultivation, the cell "decides" whether to enter the cell cycle or to go into a non-dividing quiescent state known as G0 phase that leads to apoptosis. The main event of S phase is DNA replication, which has its specific features [21].

In our tests, the percentage of S phase cells in the test and control samples was the same during the first day of the virus reproduction, with sharp jump-like

3.4- and 2.4-fold increases having been observed after 15 and 24 hours, respectively (Fig. 5). After 24 hours, the infected and control cells began to demonstrate differences, which gradually increased from 8 to 137% by the end of reproduction. It can be assumed that the cells undergoing the said phase also participated in the virus reproduction.

In G2 phase, the last of three successive phases of the cell cycle interphase, and M phase, a tetraploid population was formed. In the intact and rabies virus infected cells of BHK-21/SUSP/ARRIAH line, the phase of preparation for mitosis and mitosis itself accounted for 2 to 20% of the entire population. After 30 hours of reproduction, the proportion of test sample cells undergoing these phases began to decrease by 17–28% as compared with the control cells (Fig. 6). This was probably due to the fact that the overall biosynthesis and functions of the host cell were inhibited as a result of the biosynthesis of rabies virus components; therefore, the percentage of G2 + M phase cells decreased.

The number of polyploid cells in the test samples was approximately equal during 45 hours, with a sharp 2.0–2.5-fold increase having been observed only within the last hours of the virus reproduction. In the control cells, the population ploidy was abrupt: 2.06-, 2.7-, 1.56- and 3.18-fold increases were observed after 9, 21, 42 and 48 hours, respectively. It can be assumed that polyploid cells were partially resistant to the virus. This fact has already been noted earlier in the study of foot-and-mouth disease virus reproduction in BHK-21 cells [22].

The DNA histograms of BHK-21/SUSP/ARRIAH cells during rabies virus reproduction 24 hours after the start of infection and after 42 hours of cultivation (Fig. 7) summarize the graphs shown in Figures 4–6.

CONCLUSION

The phases of the cell cycle during rabies virus reproduction in the host cell were examined, the results obtained allowed for the estimation of duration and predominance of the corresponding cell cycle G1, S, G2 + M phases at different stages of cultivation.

When studying the dynamics of changes in the DNA of BHK-21/SUSP/ARRIAH cells infected with rabies virus, it was found that after 24 hours of the virus cultivation, the proportion of cells in G1 phase increased as compared with the control cells (by 12–21%). Since mRNA, structural proteins, other cell components that are also necessary for rabies virus virion assembly are synthesized during G1 phase, the growth of cells in this phase of the cycle can be considered expectable.

It was found that since the main event of S phase is DNA replication, a linear decrease of S phase during rabies virus cultivation after 24 hours of reproduction may also be indicative of the cell switch-over from cell DNA replication to viral RNA synthesis.

It was revealed that G2 phase and M phase (postsynthetic, or premitotic phase, and mitosis itself) of the test sample cell life cycle were relatively decreased (by 17–28%) as compared with the control cells, i.e. the infected cells spent less time in the division stage than the uninfected ones. This was probably due to the fact that the overall biosynthesis and functions of the host cell were inhibited as a result of the biosynthesis of rabies virus components.

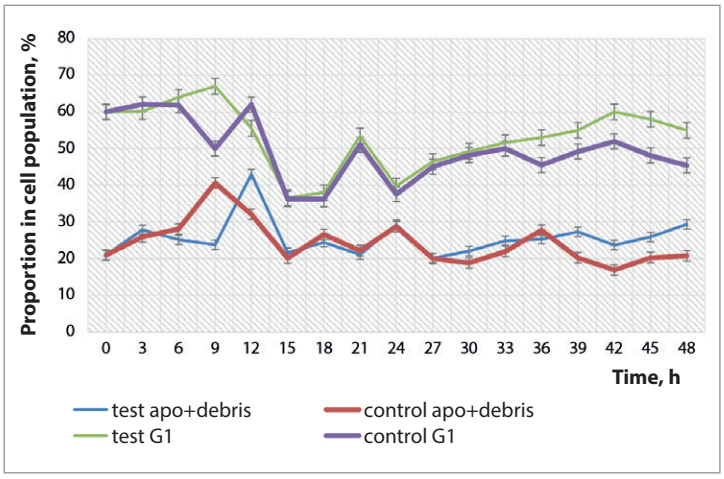

Fig. 4. Changes in percentages of cells undergoing apoptosis + debris and G1 phase in BHK-21/SUSP/ARRIAH culture during rabies virus reproduction (n = 3, p < 0.05)

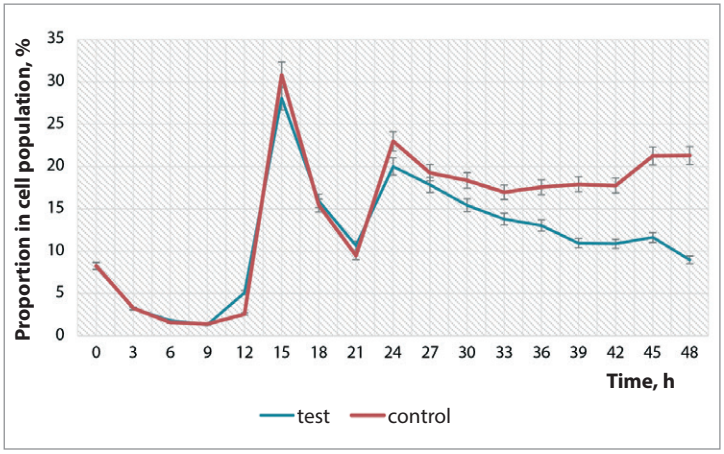

Fig. 5. Changes in percentages of cells undergoing S phase in BHK-21/SUSP/ARRIAH culture during rabies virus reproduction (n = 3, p < 0.05)

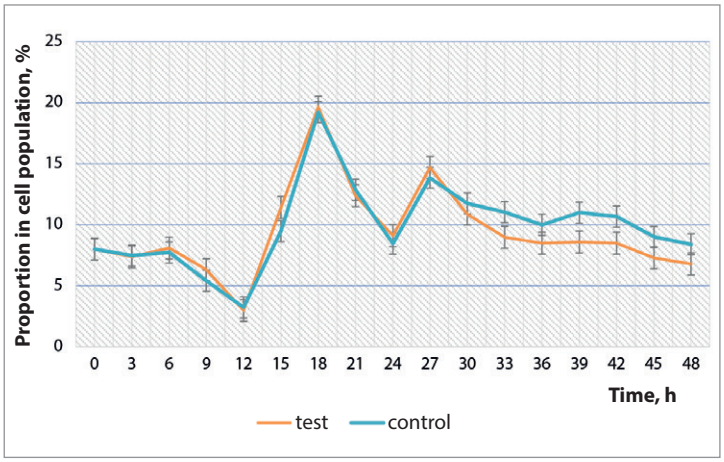

Fig. 6. Changes in percentages of G2 + M phase cells in BHK-21/SUSP/ARRIAH culture during rabies virus reproduction (n = 3, p < 0.05)

The polyploid cells formed during the cell and virus cultivation were found to be partially resistant to rabies virus and probably had the least sensitivity.

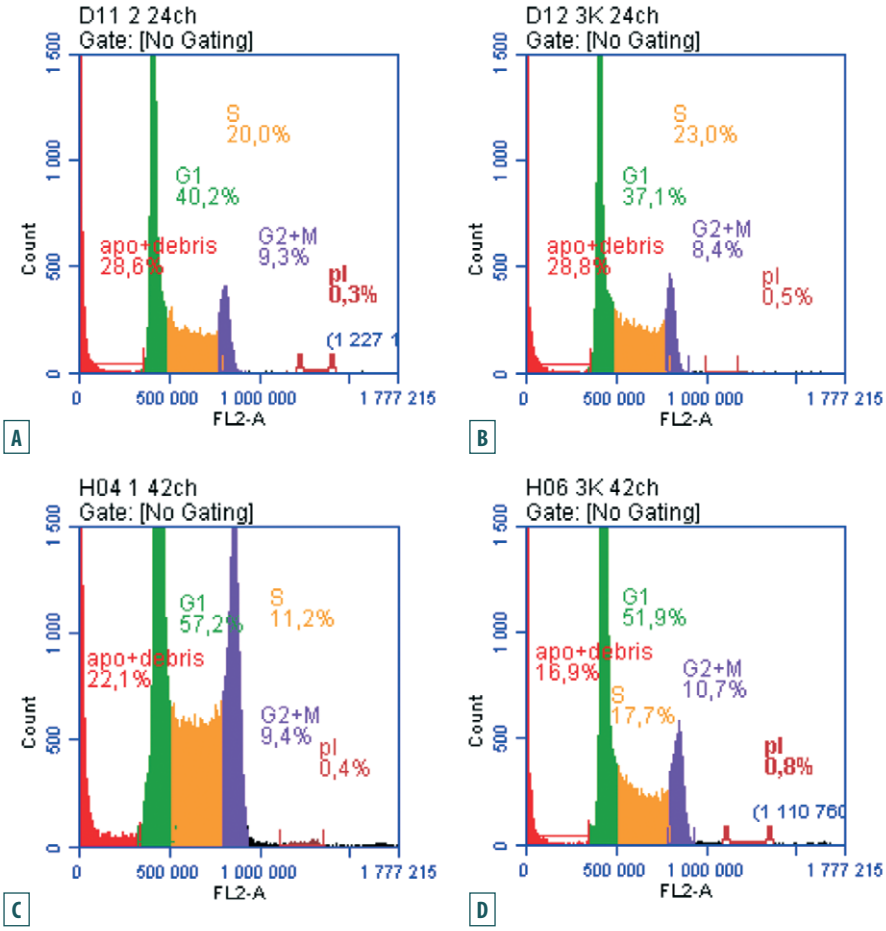

Fig. 7. Comparison of DNA histograms of BHK-21/SUSP/ARRIAH cells during rabies virus reproduction and those of control samples after 24 hours (A – test, B – control) and after 42 hours (C – test, D – control)

It was established that BHK-21/SUSP/ARRIAH cell switch-over to the synthesis of complete rabies virus particles occurred after 24 hours of reproduction, as indicated by changes in the host cell cycle phases, as well as by the slowing down of the cell population growth.

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Improved production strain maintenance technique for *Burkholderia mallei* 5584 (Master seed) used for glander diagnostic agent production

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ABSTRACT

One of the aspects important for strain collection maintenance is the optimization of existing methods and development of new techniques for microbial strain preservation, that is why the improvement of previously developed methods for authentic strain preservation is an urgent task. The article provides information on the maintenance of *Burkholderia mallei* 5584 (Master seed) using previously developed technique, which was supplemented with new stages in accordance with modern requirements for strain collections of highly dangerous disease agents. The previous strain maintenance technique involved its storage in its native state, which facilitated accumulation of genetic mutations and, ultimately modification of bacterial cell properties. To extend the storage time of this strain and to ensure the stability of its biological properties, the freeze-drying method was used. Skimmed milk was used as a cryoprotectant. Freeze-drying was performed under selected conditions. This technique allows for the strain sub-culturing on sensitive models once every 5 years, which is more expedient and safe from an economic and biological point of view. For safe handling of *Burkholderia mallei* 5584 production strain, an inactivation technique using gamma rays at 30 kGy was developed, which allowed to achieve microbial suspension sterility and preserve the bacterial cell structure. When comparing the previously developed and supplemented techniques, it was found that the improved technique of *Burkholderia mallei* 5584 (Master seed) maintenance makes it possible to avoid the loss of its biological properties needed for the production of high-quality laboratory diagnostic agents used for timely disease detection in susceptible animals by diagnostic tests.

Keywords: glanders, *Burkholderia mallei*, passage, freeze-drying, biological properties, gamma irradiation

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Усовершенствование системы поддержания производственного штамма *Burkholderia mallei* 5584 (Master seed), применяемого для изготовления сапных диагностикумов

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РЕЗЮМЕ

Одним из направлений коллекционной деятельности является оптимизация существующих методов и разработка новых технологий консервации штаммов микроорганизмов, поэтому проведение работ по усовершенствованию ранее разработанных методик сохранения аутентичности штаммов

является актуальной задачей. В статье приведена информация по поддержанию производственного штамма *Burkholderia mallei* 5584 (Master seed) с использованием разработанной ранее системы, которая была дополнена новыми этапами согласно современным требованиям, предъявляемым к коллекционным фондам штаммов возбудителей особо опасных болезней. Предыдущая схема поддержания штамма предусматривала его хранение в нативном виде, что способствовало накоплению генетических мутаций и, как следствие, изменению свойств бактериальной клетки. Для увеличения сроков хранения данного штамма и обеспечения стабильности его биологических свойств применен метод лиофилизации. В качестве криопротектора использовали обезжиренное молоко. Сублимационную сушку проводили по выбранному режиму. Данный метод дает возможность пассировать штамм на чувствительных моделях один раз в 5 лет, что более выгодно и безопасно с экономической и биологической точек зрения. Для безопасной работы с производственным штаммом 5584 возбудителя сапа разработан метод его инактивации гамма-лучами при 30 кГр, который позволил добиться стерильности микробной взвеси и сохранить структуру бактериальных клеток. При сравнении ранее разработанной и дополненной схем установлено, что усовершенствованная система поддержания штамма *Burkholderia mallei* 5584 (Master seed) позволяет исключить утрату его биологических свойств, необходимых для производства качественных сапных диагностикумов, используемых для своевременного выявления заболевания у восприимчивых животных при проведении диагностических исследований.

Ключевые слова: сап, *Burkholderia mallei*, пассаж, лиофилизизация, биологические свойства, гамма-облучение

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INTRODUCTION

Freedom from contagious and highly dangerous infectious diseases, in particular from glanders, is ensured by the veterinary service, responsible for anti-epidemic measures aimed at prevention of the pathogen introduction into the Russian Federation using systematic monitoring of the animal health status among horse populations (donkeys, mules), as well as containment and eradication of the disease in case of its occurrence [1].

Glanders is an infectious disease of equine animals, caused by the bacterium *Burkholderia mallei*, which often develops chronic infection. Under natural conditions, feline predators can also be infected (when eating meat of glanderous animals). The infection can be also transmitted to camels and humans [2, 3, 4, 5]. This pathogen is classified as pathogenicity group II (hazard) agent. No specific preventive or therapeutic agents against glanders have been developed yet , and therefore the disease is an exceptional issue for the biological safety of the Russian Federation [6, 7].

Since the late 50s of the last century to the present, glanders has not been reported in Russia. Although there have been cases of glanders suspicion during this period, none of them have been confirmed [8]. To date, a glanders outbreak was reported in the Zabaikalsky Krai followed by the introduction of quarantine (Resolution of the Zabaikalsky Krai Governor of 18.02.2023 No. 8.[1]). The risk of occurrence of new glanders cases can not be excluded due to the infection presence in the countries bordering Russia (Mongolia and China), where the veterinary legal requirements are often not observed, in particular, illegal livestock exchange/movement and import of animal raw materials can occur [1, 9]. Besides modern international contacts, involving glanders-susceptible animals (trade, tours of circuses and animal theaters, equestrian competitions, international auctions, etc.), can create an unpredictable glanders situation.

Currently, glanders is often reported from Mongolia, Turkey, Iran, Iraq, the Arabian Peninsula countries, Brazil, China, India, and the Philippines [9, 10, 11, 12, 13, 14]. According to the World Organization for Animal Health, the Food and Agriculture Organization of the United Nations, and the World Health Organization, there is a tendency in the world of increased glanders cases in humans and animals, which qualifies it as a re-emerging infection.

To prevent the occurrence, importation and introduction of this disease into the country, diagnostic measures are taken using glanders diagnostic agents produced by the Kursk Biofactory – BIOK company (glanders positive serum for complement fixation test – CFT; *B. mallei* antigen for CFT; mallein and *B. mallei* colored antigen for plate agglutination test – AT) using production strain *Burkholderia mallei* 5584. It is provided by the State Strain Collection, containing pathogens of highly dangerous diseases used in the veterinary medicine and livestock industry, where the strain is stored in native and freeze-dried states with its biological properties maintained. A number of authors proved that the storage of microorganisms in their native state does not satisfy the current standards, since mutations accumulate during the sub-culturing process

[1] On the establishment of restrictive measures (quarantine) in the territory of Chita city: Resolution of the Zabaikalsky Krai Governor of 18.02.2023 No. 8. https://media.75.ru/documents/152305/8-ot-18-02-2023.pdf

ultimately resulting in modification of their original biological properties [15, 16, 17, 18]. Taking into account, that one of the aspects important for strain collection maintenance is the optimization of existing methods and development of new techniques for microbial strain preservation, the improvement of previously developed methods for authentic strain preservation is an urgent task [19, 20, 21, 22].

Based on the above, the aim of the work was to improve the production strain maintenance technique for *Burkholderia mallei* 5584 (Master seed), used to preserve its viability and biological properties.

MATERIALS AND METHODS

Activities associated with the maintenance of the *Burkholderia mallei* 5584 (Master seed) were performed in the "State Microbial Collection" of the Federal Center for Toxicological, Radiation and Biological Safety.

The strain is stored in its native form on beef-extract glycerol agar and is subcultured every 30 days followed by testing of its biological properties once a year. The strain stored in freeze-dried state (skimmed milk cryoprotectant) is tested for its viability and compliance with the properties stated in its accession form every 5 years and *in vivo* passaging using sensitive models (golden hamsters) once a year.

The growth properties and cell morphology of the strain stored in native and freeze-dried forms were studied in second-generation cultures grown on beef-extract glycerol agar, beef-extract glycerol broth and Pavlovsky potato agar. The tinctorial properties were studied by microscopy of Gram-stained smears; motility was evaluated by the hanging drop method using light microscopy. The fermentative activities were tested by inoculation in Hiss' medium and skimmed milk, as well as by the formation of hydrogen sulfide in beef-extract glycerol agar and indole in Strogov growth medium inoculated with culture, using test papers (lead acetate papers and oxalic acid papers, respectively), placed above the surface of the medium. The catalase test was performed by adding 3% hydrogen peroxide to the grown culture[2].

The strain pathogenicity was evaluated by inoculation of sensitive laboratory animals (golden hamsters), followed by an assessment of glanders consistent clinical signs, post-mortem lesions in lungs, liver, and spleen and isolation of the pathogen pure culture.

In order to study the agglutination properties of the *Burkholderia mallei* 5584 (Master seed), an antigen and antiserum were prepared, which were tested by plate AT. Serum was prepared by triple immunization of rabbits with inactivated *Burkholderia mallei* 5584 (Master seed) cells at a concentration of 2×10^9 microbial cells/cm^3 by injection into the marginal ear vein at three-day intervals: the first injection was 0.5 cm^3, the second injection was 1.0 cm^3, the third injection was 2.0 cm^3. Total exsanguination of rabbits was performed on day 7 post last injection.

The serum was tested for its antigenic properties (using plate and tube AT and tube CFT).

To determine the specificity, closely related *B. pseudomallei* and *Alcaligenes faecalis* were used [23]. The strains were inoculated on beef broth glycerol agar and meat-peptone agar, incubated at 37 °C for 48 hours, washed with 0.85% NaCl solution and tested by AT and CFT in accordance with SanPiN 3.3686-21[3].

The production strain biological properties were maintained in accordance with the accession form by passaging in golden hamsters, infected subcutaneously in the occipital area.

The experiments with the laboratory animals were performed in accordance with the general ethical principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, March 18, 1986). The experiments were scientifically substantiated and approved by the Bioethics Commission of the Federal Center for Toxicological, Radiation and Biological Safety (Protocol No. 10 of 11.09.2023).

To obtain the antigen, the *Burkholderia mallei* 5584 (Master seed) was inactivated with gamma rays using "Issledovatel" device (Russia) at previously determined radiation dose of 30 kGy in the radiobiology department[4].

When optimizing the inactivation modes, doses of 15, 20, 25, 30, 35 kGy were used. For this purpose, cell suspensions with concentrations of 10^9 microbial cells/cm^3 were prepared using sterile 0.85% NaCl solution. The inactivation was tested by inoculation of cells on growth media (beef-extract glycerol agar and broth) followed by incubation for 10 days at 37 °C.

The freeze-drying conditions were optimized using LZ-9 freeze-dryer (Frigera, Czech Republic). Skimmed milk and sucrose-gelatin medium were used as cryoprotectants.

In this work, the technique of the production strain maintenance for the *Burkholderia mallei* 5584 (Master seed) was used [22], which was supplemented with new stages that meet modern requirements for the storage of collection strains.

RESULTS AND DISCUSSION

To improve the quality and conformity of production, reference and vaccine strains, the comprehensive study of the stability of their biological properties is needed with the focus on their characteristic genetically fixed features: morphological, biochemical, antigenic and others [17, 24, 25]. The viability and biological properties of the *Burkholderia mallei* 5584 (Master seed) were studied using the previously developed and supplemented maintenance technique for the strain used for the production of diagnostic agents, in accordance with the modern requirements for the microorganism storage [8, 22, 26].

The obtained results showed that the strain grew on beef-extract glycerol agar in the form of translucent smooth shiny colonies, becoming confluent on day 3–5 after inoculation on the medium surface (Fig. 1A).

[2] Glanders laboratory diagnosis: guidelines. Moscow: Federal Center for Hygiene and Epidemiology of Rospotrebnadzor; 2011. 22 p.

[3] SanPiN 3.3686-21 Sanitary and epidemiological requirements for the prevention of infectious diseases: approved by Resolution of the Chief Sanitary Inspector of the Russian Federation No. 4 on 28.01.2021. https://docs.cntd.ru/document/573660140 (date of access 15.11.2022).

[4] Shashkarov V. P., Gainutdinov T. R., Idrisov A. M., Guryanova V. A., Vagin K. N., Vasilevsky N. M., etc. Methodological recommendations on the use of ionizing radiation for inactivation of livestock infectious disease pathogens. Kazan: MeDDoK; 2021. 17 p. https://doi.org/10.31016/fctrb-viev-2020-2

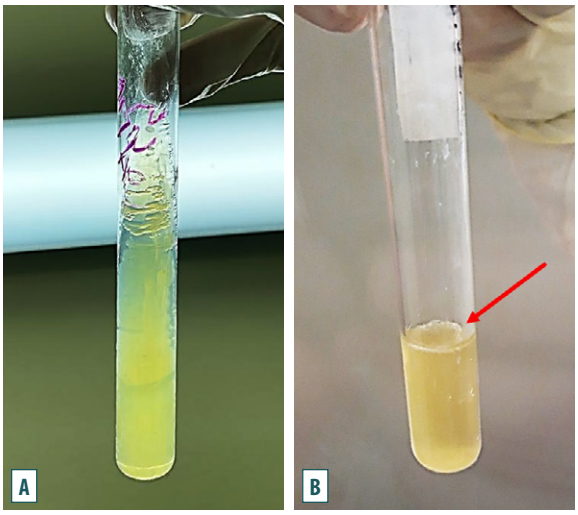

Fig. 1. Growth of Burkholderia mallei strain 5584 (Master seed) on beef-extract glycerol agar (A) and beef-extract glycerol broth (B). The ring and pellicle are indicated with a red arrow on the surface of the growth medium

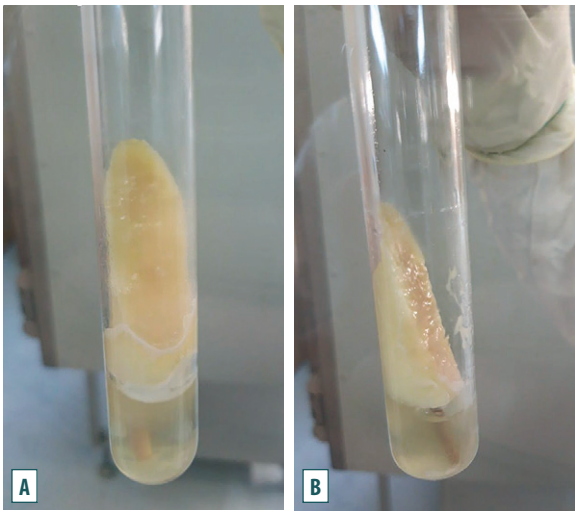

Fig. 2. Growth of Burkholderia mallei 5584 (Master seed) on Pavlovsky potato agar: 2-day culture (A); 5-day culture (B)

The bacterial culture on beef peptone glycerole broth on day 3–5 caused turbidity of the medium, a pellicle and a ring formed on its surface (Fig. 1B). A viscous precipitate was observed at the tube bottom, spinning and breaking when shaken.

When Pavlovsky potato agar was used, amber honey-like growth appeared on day 2 (Fig. 2A), mucous growth of a darker color appeared on day 5 (Fig. 2B).

In the hanging drop, the cells were immotile, but Brownian motion was observed. A smear of the *Burkholderia mallei* 5584 (Master seed) prepared from a 2-day culture and Gram-stained, looked like granular round-end rods when studied by microscopy with oil immersion. The bacteria were gram-negative, colored pink, and caused milk to coagulate without further peptonization (Fig. 3A). During the growth, the test strips changed their color in the broth which suggested the formation of hydrogen sulfide by bacteria (Fig. 3B); 12% gelatin was not liquefied (Fig. 3C), indole and catalase were not formed (the color of the test strip did not change).

The culture of the *Burkholderia mallei* 5584 (Master seed) did not change the color of the Hiss' medium to yellow and did not cause the formation of gas bubbles in floats, since it did not ferment sugars.

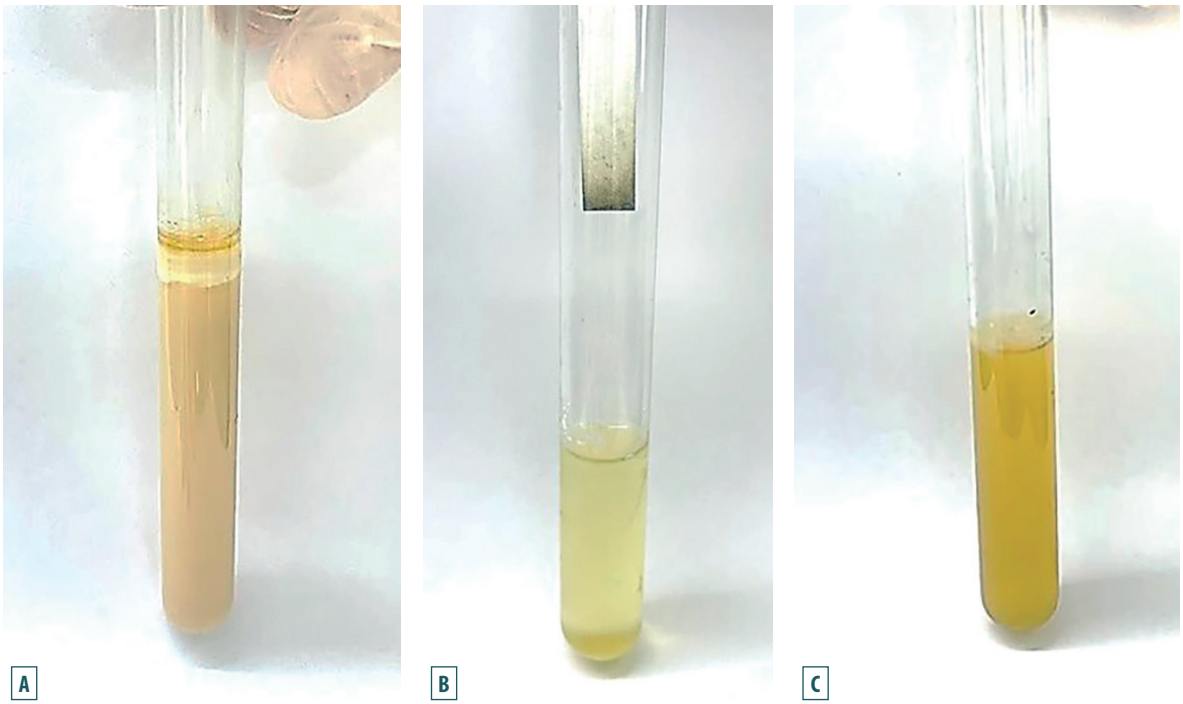

Fig. 3. Milk coagulation without further peptonization (A); formation of hydrogen sulfide (B); no liquefaction of 12% gelatin (C) by Burkholderia mallei 5584 (Master seed)

For safe handling of pathogens of I–II pathogenicity groups (hazard), in particular when preparing antigens and hyperimmune sera, the pathogens must be inactivated by various methods (physical, mechanical, chemical, etc.). The previous technique of strain maintenance included inactivation of cells by autoclaving at a temperature of 120 °C for 15 minutes, which resulted in their complete destruction. Therefore, a gamma radiation dose was selected for the *Burkholderia mallei* 5584 (Master seed). The results are given in Table 1.

It was found that 30 and 35 kGy irradiation completely killed the strain; therefore, a dose of 30 kGy was used in further work.

Plate agglutination test for the agglutination properties of the glanders serum obtained against gamma-irradiated antigen showed positive reaction in the form of small-grained agglutinate formed within 1–2 minutes.

The pathogenicity of the strain was tested by infection of golden hamsters by injecting a 2-day culture grown on beef-extract glycerol agar at 37 °C for 48 hours, washed off with saline solution at a dose of 1×10^9 microbial cells/cm^3 subcutaneously into the occipital area. Animals died on day 5–10. The autopsy revealed the following glanders-consistent post-mortem lesions: a purulent necrotic lesion at the injection site, multiple necrotic 2–3 mm nodules in internal organs (liver, spleen, lungs). A pure bacterial culture was isolated from inoculations made from internal organs, heart blood and injection site.

Table 1
Determination of *Burkholderia mallei* 5584 (Master seed) inactivation dose by gamma irradiation

No.	Gamma radiation dose, kGy	Antigen inactivation
1	15	–
2	20	–
3	25	–
4	30	+
5	35	+

Testing of the antigenic properties of the *Burkholderia mallei* 5584 (Master seed) using sera from inoculated laboratory animals showed that tube AT titre was 1:1600, CFT titre – 1:320, plate AT titre – 1:120.

The resulting serum gives cross-reactions in AT and CFT with *B. pseudomallei* – 1:1600 and *Alcaligenes faecalis* – 1:40.

In order to preserve the purity of unique production and reference microorganism strains, they are passaged *in vivo* using sensitive laboratory animals [15, 27, 28, 29]. In this regard, in order to maintain the biological properties of the production strain *Burkholderia mallei* 5584 (Master seed), as stated in its accession form, it was passaged in golden hamsters. At the same time, a pure strain

Table 2
Original and supplemented techniques of *Burkholderia mallei* 5584 (Master seed) maintenance

No.	Initial technique	Improved technique
1	Regular subculturing of the strain stored in native state on beef-extract glycerol agar (every 30 days), with testing of biological properties once a year	
2	Not done	Testing of viability and biological properties (once every 5 years) of a freeze-dried strain culture
3	Passage in sensitive models (golden hamsters) once a year	Passage in sensitive models (golden hamsters) once 5 years
4	Not done	Freeze-drying of the isolated culture after passage
5	Testing of the cultural properties and colony morphology by inoculation on beef-extract glycerol agar, beef-extract glycerol broth and Pavlovsky potato agar	
6	Testing of tinctorial properties and cell morphology by microscopy of Gram-stained smears	
7	Testing of cell motility and Brownian motion by microscopy of culture in the hanging drop	
8	Indole formation	
9	Testing for catalase	
10	Formation of hydrogen sulfide	
11	Testing of fermentative activities by inoculation in Hiss' medium and skimmed milk	
12	Inactivation of the strain by autoclaving at 120 °C for 15 minutes	Inactivation of the strain by gamma irradiation at 30 kGy for 2 hours
13	Not done	Testing of agglutination properties in plate AT using glanders serum
14	Testing of pathogenicity by inoculation of culture to golden hamsters	
15	Testing of antigenic properties and antigenic specificity by immunization of rabbits and serum testing by plate AT and CFT	

culture was isolated, which was tested for authenticity and put in further storage in native state, with subsequent sub-culturing on the beef-extract glycerol agar every 30 days.

The previous strain maintenance technique of the *Burkholderia mallei* 5584 (Master seed) involved its storage in its native state, which facilitated accumulation of genetic mutations and, ultimately modification of bacterial cell properties [22]. Since the primary task of microbial collections is to preserve strains in an unchanged state for a long time, a freeze-drying stage was added. The freeze-drying step included the following:

– freezing of the culture for 12 hours;

– transfer of the culture to the freezer, plate temperature –52 °C;

– vacuuming;

– freeze-drying in automatic mode for 12 hours;

– heating (p) after 17 hours from the moment of culture loading at the following parameters: plate temperature +10 °C, ambient temperature 0 °C;

– heating (p + 1) after 18 hours with the following parameters: plate temperature +20 °C, ambient temperature +5 °C, vacuum 0.5 trr;

– end of drying after 24 hours: plate temperature +32 °C, ambient temperature +25 °C, vacuum 0.05 trr.

After drying, the bacteria were tested for viability and compliance with the data in the accession form, then put into storage in a freeze-dried state at +4 °C and after 5 years the viability and biological properties were tested [30].

In parallel, the native strain culture was maintained with sub-culturing every 28–30 days on beef-extract glycerol agar and comparative testing of biological properties was performed once a year.

After storage of the *Burkholderia mallei* 5584 (Master seed) for 5 years using an improved maintenance technique, it was found that it retains its viability, without loss or change of its morphological, biochemical, serological and virulent properties.

Thus, the improved maintenance technique for *Burkholderia mallei* 5584 (Master seed) includes the steps shown in Table 2.

The use of the freeze-drying allowed to increase the storage terms of the *Burkholderia mallei* 5584 (Master seed) and ensure the stability of its biological properties throughout the entire storage period.

In addition, freeze-drying allowed passaging of the strain in sensitive laboratory animals once every 5 years, which is more expedient and safe from an economic and biological point of view.

Handling of strains of pathogenicity groups I–II (hazard) poses a threat of potential escape of a biological agent into the production area air, human habitat and personnel infection. Reliable methods for their inactivation must be developed for this purpose. In our case, the gamma irradiation method was used. At the same time, the optimal dose of 30 kGy was determined, which made it possible to inactivate the strain and preserve the structural integrity of bacterial cells, being the antigens for hyperimmune sera preparation and serological reactions.

Thus, the improved technique for the *Burkholderia mallei* 5584 (Master seed) maintenance meets modern requirements for collections of pathogens of highly dangerous diseases for a long periods.

CONCLUSION

Thanks to the work performed, the technique for the *Burkholderia mallei* 5584 (Master seed) maintenance was improved, ensuring the preservation of its biological properties necessary for the production of high-quality laboratory diagnostic agents used during annual diagnostic tests for the timely detection of diseased animals.

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On 75th anniversary of Vladimir A. Mischenko

Vladimir Alexandrovich Mishchenko was born on 4 February 1949 in the village of Staroverovka, Kharkiv Oblast. In 1971, after successfully graduating from the Kharkov Veterinary Institute, he worked as a veterinarian at Staroverovskaya Poultry Farm. But his life was changed by his interest in science, to which he devoted his activities having joined the All-Union Foot-and-Mouth Disease Research Institute as a senior laboratory assistant in 1973. During these 50 years, Vladimir A. Mischenko passed the glorious path of a Russian scientist: in 1977 he defended his Candidate thesis, and in 1991 he became the youngest Doctor of Science at the Institute. In 1994, he was awarded the academic title of Professor. The work records of Vladimir A. Mishchenko in different periods included such entries as "Head of Laboratory for Virus Identification", "Academic Secretary of the Institute", "Head of Laboratory for Bovine Diseases", "Deputy Director for Manufacture", "Head of Department for Bovine and Porcine Disease Monitoring".

The scientific research conducted under the leadership of Vladimir A. Mishchenko dealt with the development of modern tools and methods for the diagnosis of foot and mouth disease and other animal diseases is known to a large number of scholars in Russia and abroad. He was the first to diagnose Aujesky's disease in single-humped camels, and to isolate the causative agent of porcine reproductive and respiratory syndrome in Russia as well as to develop means and methods of the disease diagnosis and specific prevention. Vaccines against rotavirus and coronavirus infection, parainfluenza-3, infectious bovine rhinotracheitis and other immunobiological products were created and introduced into veterinary practice.

The results of Vladimir A. Mishchenko's research were published in more than 260 scientific papers – these are monographs, scientific articles, conference reports, patents and author's certificates. In addition, a large number of methodological guidelines were developed. Vladimir A. Mischenko is a developer of 20 types of vaccines, 15 diagnostic kits, and a co-author of more than 30 regulatory documents in the field of veterinary virology. He was awarded the honorary title of "Honored Inventor of the Russian Federation". For his contribution to the development of the industry, Vladimir A. Mishchenko was awarded 5 medals of the Exhibition of National Economy Achievements.

Possessing professional competence and personal qualities, the Professor is always willing to share his knowledge and vast experience with the younger generation, helping the applicants for academic degrees to prepare theses. His actions, always aimed at results, outstanding mind and desire for constant self-development help to adequately fulfill the responsible role of a mentor. Twenty-two Candidate and three Doctoral dissertations were defended under his scientific supervision. Vladimir A. Mischenko is in demand as a consultant at agricultural establishments, and as a lecturer for the postgraduate students, and at advanced training courses for veterinarians.

Vladimir A. Mishchenko is a member of the Academic Council, Dissertation Councils of the Federal Centre for Animal Health and Federal Scientific Centre VIEV RAS, scientific supervisor of two postgraduate students, and we are sure that Vladimir A. Mischenko is in the prime of his creative powers to solve the tasks set for the next years in the field of veterinary development.

Vladimir Alexandrovich, we believe – you have many more achievements ahead. We wish you good health, patience in solving all issues, cheerfulness and optimism, well-being to you and your loved ones!

Nickolay A. Ulupov
(on 95th anniversary)

Nickolay Alekseevich Ulupov was born on 23 February 1928 in s. Glukhovo, Stavrovsky Raion, Vladimir Oblast. Since childhood he was engaged in agricultural works on a local collective farm. After school he entered the Vladimir Aviation Mechanical College, successfully graduated from it and got a job as an adjuster at a weaving factory in Lakinsk.

From 1949 to 1953 he served in the ranks of the Soviet Army, during 1951–1953 he served as an aircraft technician in the People's Republic of China (PRC) as part of the military contingent of the USSR under the command of three-times Hero of the Soviet Union A. I. Pokryshkin. In February 1951 he was awarded a commemorative medal for assistance to various special forces units of the People's Liberation Army of China by Chairman of the People's Republic of China Mao Zedong.

After demobilization from the Soviet Army he entered the Moscow Veterinary Academy and after graduation in 1959 he was assigned to the All Union Scientific Research Institute of Veterinary Virology and Microbiology (Pokrov) where he worked as a laboratory assistant and junior researcher.

In 1963–1966 he was a postgraduate student at All Union Scientific Research Institute of Veterinary Virology and Microbiology and in 1967 he defended his candidate degree thesis.

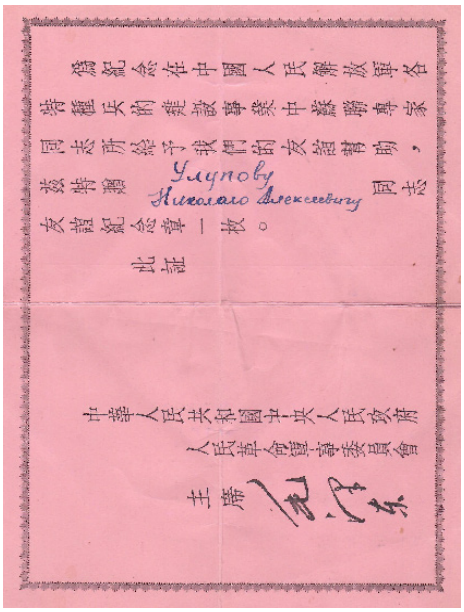
In 1967 he was competitively elected to the position of senior researcher at the All-Union Foot-and-Mouth Research Institute (Vladimir) where he conducted studies on the development of methods for FMD virus cultivation, purification from ballast proteins, inactivation in the manufacture of vaccines based on lapinized virus and biopreparation drying methods. He made an invaluable contribution to the development of production technology for inactivated vaccines based on lapinized FMD virus. He developed a bactericidal method of virus-containing suspension purification for the preparation of sterile vaccines without sterilizing filtration. This manufacturing technology was implemented at the Sumy Biofactory, Tabakhmel Bioplant, Pokrov Biological Plant, Yurievetsvetbiopreparation Plant.

Nickolay A. Ulupov developed an original technique for inactivating the lapinized virus with formaldehyde using ammonium ions, as a result, the vaccine safety level increased significantly. He was the author of the invention on the use of an ethylenimine dimer (aminoethylethylenimine) for inactivation of the FMD virus in the manufacture of viral vaccines. The introduction of this new inactivant made it possible to develop safe, highly effective and competitive biologicals based on lapinized and cultured FMD viruses.

The results of Nickolay A. Ulupov's research were summarized in his doctoral thesis that was successfully defended in 1985. Its main focus was to improve methods of FMD virus reproduction, purification and inactivation. The method of purification from ballast proteins using flocculants developed by Nickolay Alekseevich made it possible to obtain sterile highly active preparations based on lapinized FMD virus of all serotypes.

Nickolay A. Ulupov published more than 100 scientific papers, he was an academic advisor and supervised five candidates of science in getting a degree who later pursued his research studies. He is the author of 25 patents of the Russian Federation.

Nickolay A. Ulupov passed away after a serious and prolonged illness in 2003. We cherish the memory of the outstanding man, innovator in biology, inventor and effective researcher who will remain in our hearts forever.



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